Anti-infective Synthetic Peptide with Dual Antimicrobial and Immunomodulatory Activities for the Treatment of Sepsis

THESIS

Submitted in partial fulfillment.

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2023

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

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ACKNOWLEDGEMENTS

Gratitude fills my heart as I extend my deepest appreciation to the Almighty for the countless blessings and wisdom that paved the path to completing this thesis. It is with immense gratitude that I acknowledge the unwavering support, guidance, and contributions from a multitude of individuals, without whom this achievement would not have been possible.

Foremost, my heartfelt appreciation goes to Prof. D. Sriram, my supervisor from the Department of Pharmacy at BITS-Pilani Hyderabad Campus. His unwavering guidance, patience, and vast knowledge were instrumental throughout my Ph.D. journey. His encouragement and mentorship steered me through every phase of research and thesis writing, leaving an indelible mark on my life. I could not have imagined having a better advisor and guide for my Ph.D. study. He has always encouraged new thoughts and given constant support throughout the project. The experiences gained under his guidance are memorable and will be cherished throughout my life.

I am equally indebted to Prof. Arti Dhar, my co-supervisor, for her invaluable insights, technical guidance, and continuous encouragement during my research. The guidance and motivational words from Ramakrishna Reddy Isanaka, Issar Pharmaceuticals Pvt. Ltd., and Prof. Venuganti Venkata Vamsi Krishna were pivotal at various junctures of my doctoral pursuit.

The invaluable feedback and encouragement from my DAC members, Prof. Venuganti Venkata Vamsi Krishna and Prof. Onkar Prakash Kulkarni, significantly contributed to shaping my work and refining the thesis. I express my sincere gratitude to the authorities, Prof. V. Ramgopal Rao and Prof. G. Sundar, for their support in allowing me to conduct my doctoral research at BITS-Pilani.

I want to sincerely thank the convener, Dr. Nirmal Jayabalan, Convener, Departmental Research Committee; I would also like to thank the associate dean (AGSRD), for their unwavering administrative support, which streamlined the process.

A special mention to Prof. Sajeli Begum, whose constant simplicity and joy added warmth to the BITS-Pilani Hyderabad campus, and to the Vice Chancellor and Director of BITS-Pilani Hyderabad Campus. The guidance, suggestions, and technical inputs from faculty members Prof. Yogeswari, Dr. Balaram Ghosh, Prof. Swathi Biswas, Prof. Srinivas, and Dr. Nirmal J were invaluable.

I extend my heartfelt appreciation to Issar Pharmaceuticals Pvt. Ltd. for their financial support, Dr. Sesikeran, and Dr. Jerald Mahesh Kumar for easing my access to research facilities and laboratories, enabling my skill development crucial for the research. It wouldn't have been possible to conduct my research without their precious support. They all really mean a lot to me.

Memories shared with fellow lab mates Shiva, Madhu Rekha, Sai, and Nikhila were not only enjoyable but also provided the fortitude needed to meet deadlines. Gratitude extends to friends Deepanjan, Soni Priyanka, Lokesh, Ashutoush, Ganesh, and Deepika for their unwavering support. My friend and animal facility in charge Dr. Mounisha supported me during my research's tough time. We have enjoyed ourselves at our best.

I sincerely thank Suresh, Ashok, Jyothi and Chandrakala, who helped me during animal and lab work, Brahma of CDFD, and my colleagues at Issar Pharmaceuticals Pvt.Ltd., without them my lab work would have not been possible.

I wish to thank the whole non-teaching staff, Ms. Rekha, Ms. Sunitha, Ms.Samreen, Mr.Mousam and Mr. Rajesh for their support.

In closing, my heartfelt thanks go to my parents Rama Gopal Reddy (Father), Thulasamma (Mother), siblings (Sai Praveen Reddy), My In-laws Rama Chandra Reddy (Father-In-law), Shakuntala (Mother-In-law), friends (Sagar Reddy, Sreedhar, and Hima Bindu), cousins and acquaintances whose prayers and support were the bedrock of my journey. Their unwavering support provided the freedom to focus on my studies and goals. My lifelines (parents& my husband) have always supported me morally and financially so that I only pay attention to my studies and achievement objective without any obstacles in my way. I consider myself nothing without them.

My husband, Praveen Vempalli, and mentor, Mr. Ramakrishna Reddy, deserve special mention for their unwavering support, guiding me through challenges and fears, leading to the accomplishment of my goals.

Lastly, a tearful tribute to the animals whose sacrifices in research experiments contribute to the advancement of human well-being. To all those who supported and helped in any capacity, my sincerest gratitude, with apologies if I inadvertently missed mentioning any individual.

Sravani Edula

Abstract:

Background: Sepsis presents a significant challenge in healthcare, disrupting the body's inflammatory balance in response to infection and posing a life-threatening emergency, particularly affecting vulnerable demographics like older individuals, immunocompromised patients, children, and infants. Recent findings revealed sepsis occurrences in 65 out of 200 COVID-19 hospitalizations (32.5%). Among these, 70.8% resulted solely from COVID-19 infection, 3.1% from bacterial infection, and 26.2% from both SARS-CoV-2 and non-SARS-CoV-2 infections. Shockingly, a CDC report indicates that one in three hospital deaths involves sepsis, emphasizing its significance. Over the past 30 years, sepsis incidence has surged, triggering a systemic chain reaction often originating from the lung, urinary tract, skin, or gastrointestinal tract, leading swiftly to tissue damage, organ failure, and fatality. COVID-19 pneumonia stands as a major trigger for sepsis due to its emergence in response to the novel infection.

Despite advancements in diagnosing and understanding sepsis, an effective therapy remains elusive, persisting as the primary cause of ICU fatalities. The surge in antibiotic resistance and limitations of current immunotherapies, failing to substantially improve short-term survival rates, pose critical challenges. Hence, urgently finding a viable therapy to combat or alleviate sepsis is paramount. Recognizing the correlation between sepsis-related immune deficiencies and mortality, the focus has shifted towards immune modulatory therapies with anti-infective actions to enhance clinical outcomes. Antimicrobial peptides (AMPs), particularly Host Defense Peptides (HDPs), are renowned for their ability to eliminate bacteria without triggering pro-inflammatory responses, offering a novel approach to infection treatment.

Consequently, the present study aims to evaluate the antimicrobial and immunomodulatory properties of novel synthetic peptides, Host Defense Peptides (HDPs) from Issar Pharmaceuticals Pvt. Ltd. (Issar Pharma). This evaluation spans in vitro and in vivo models to assess their efficacy comprehensively. The investigation delves into their antimicrobial action, targeting bacterial eradication devoid of pro-inflammatory responses, and their immunomodulatory potential, specifically in RAW 246 cell lines and murine polymicrobial sepsis models. Assessments will encompass cytokine suppression in serum, peritoneal lavage, and mRNA expression alterations in organ tissues. This extensive evaluation seeks to elucidate the peptides' broader range of efficacy in combating sepsis through both antimicrobial action and immune modulation.

Purpose:

The core goal of this study was to unveil the dual capabilities of the lead synthetic peptide. Initially, an assessment encompassed the evaluation of three synthetic peptides (IS 141, IS 111, & IS 217) to gauge their anti-inflammatory properties in vitro. Subsequently, one lead molecule was identified and subjected to further exploration, elucidating its antimicrobial and immunomodulating activities in both in vitro and in vivo environments. The study leveraged polymicrobial septic shock mice models to underscore the groundbreaking demonstration of our lead peptide's robust inhibitory effects against septic shock, representing a significant advancement in its potential therapeutic application.

Methods: This study involved assessing the anti-inflammatory effects of three synthetic peptides—IS 141, IS 111, and IS 217—utilizing LPS-stimulated RAW 264.7 macrophage cells. Furthermore, the impact of these peptides on the MAPK signaling pathways was explored to understand their associated inhibitory mechanisms. Various in vitro assays, such as the MTT assay, The mRNA expression and supernatant levels of pro-inflammatory cytokines, tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6 were evaluated by RT-qPCR and ELISA, respectively and western blot analysis, were conducted to evaluate these effects. Additionally, confirmatory in vivo studies were performed using the carrageenan-induced paw edema model to scrutinize anti-inflammatory activity and polymicrobial sepsis models (*E. coli* Induced peritonitis and Cecal ligation and puncture [CLP] models) to assess the lead peptide's efficacy in treating sepsis. Pathological evaluations were conducted using H&E staining.

Moreover, antibacterial activity assessments were executed, including time-kill profiles against S. *aureus, P. aeruginosa, E. coli*, and *K. pneumoniae*, through microdilution assays and time-kill assays. The study also evaluated the effectiveness of IS 217 against *S. aureus* biofilms.

Results: Our investigation revealed that LPS-induced inflammation led to heightened expression and release of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6. However, our test synthetic peptides (IS 141, IS 111, and IS 217) displayed a notable restorative impact on the depression-like behavior induced by LPS-stimulated murine macrophages (RAW 264.7 cells), demonstrating no cytotoxicity across different dosage ranges. This effect was accompanied by a reduction in various pro-inflammatory cytokines in LPS-stimulated cells alone, Additionally, these peptides downregulated mRNA expression of key pro-inflammatory mediators and triggered anti-

inflammatory signal pathways via the MAPK pathway. These outcomes strongly suggest the potential of these peptides as potent anti-inflammatory agents, primarily operating through the MAPK pathway. Further validation focusing on the lead peptide, IS 217, was conducted using BALB/c peritoneal macrophages. IS 217 notably suppressed the production of NO and pro-inflammatory cytokines while elevating IL-10 levels. It also exhibited reduced paw edema compared to both the standard drug (Dexamethasone) group and control animals across all assessed doses. Furthermore, IS 217 displayed robust antibacterial activity and effectively inhibited *S. aureus* biofilms, showcasing bactericidal effects surpassing ciprofloxacin.

To further confirm the efficacy of the lead peptide IS 217, in vivo studies using animal models of sepsis are conducted to explore the potential of administering IS 217 in reducing organ failure and enhancing survival rates in mice induced with sepsis via cecal ligation and puncture (CLP) and *E. coli*-induced peritonitis. Following the induction of disease, treatment with IS 217 at doses of 0.6 and 1.2 mg/kg (via intravenous and subcutaneous routes) notably decreased pro-inflammatory markers (IL-1β, IL-6, IL-12, and TNF-α) in the blood, lung fluid, and peritoneal fluid. Additionally, it resulted in diminished bacterial count and total leukocyte count in the peritoneal fluid compared to the disease control group. Significantly, peptide IS 217 administration markedly reduced mortality rates and lowered IL-1β and TNF-α levels in the bloodstream. It also lessened macrophage infiltration in the peritoneal fluid and lung tissues of septic mice, along with suppressing VEGF expression in lung tissues. Treatment with IS 217 peptide encouraged the presence of M2 macrophages in isolated peritoneal cells from treated mice, known for their role in controlling excessive inflammation. Moreover, IS 217 inhibited disseminated intravascular coagulation, a condition associated with organ damage and mortality in sepsis.

Furthermore, IS 217 peptide treatment maintained normal levels of AST, ALP, ALT, and creatinine, minimized organ damage, prevented the depletion of monocytes and lymphocytes, and significantly improved survival rates. Consequently, the lead peptide, IS 217, exhibited remarkable capabilities in diminishing inflammation, reducing TNF-α and IL-6 levels in the blood and peritoneal fluid, mitigating neutrophil infiltration, and safeguarding against lung injury in septic mice. Notably, intravenous administration of IS 217 provided superior and rapid protection against sepsis-induced mortality compared to the slower action observed with subcutaneous administration. Hence, our in

vivo efficacy studies using mouse models of sepsis validated the observations made in our in vitro study.

Conclusions: The observations suggest that IS 217, the lead peptide, effectively suppressed the inflammatory response in LPS-induced RAW 264.7 macrophages by regulating the MAPK pathway. In animal trials, administering IS 217 at doses of 0.6 and 1.2 mg/kg notably reduced the generation of proinflammatory cytokines, restrained neutrophil infiltration, and ameliorated lung injury, ultimately enhancing the survival of mice with sepsis. These findings highlight IS 217 as a promising novel therapeutic peptide for managing sepsis. Its antibacterial traits, capability to combat biofilms, and modulation of immune responses position it as a hopeful candidate in addressing inflammatory conditions related to macrophage activation. This research introduces a fresh therapeutic approach for sepsis management, presenting new perspectives on potential therapeutic targets.

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Abbreviations

3D	:	Three-Dimensional
AMP	:	Antimicrobial peptide
ANOVA	:	Analysis of Variance
BALF	:	Bronchoalveolar Lavage Fluid
BITS-Pilani	:	Birla Institute of Technology and Science, Pilani
BSA	:	Bovine serum albumin
CARS	:	Compensatory Anti-Inflammatory Syndrome
CCL2	:	C-C chemokine 2
CDC	:	Centers for Disease Control and Prevention
CFU	:	Colony forming units
DAMPs	:	Damage-Associated Molecular Patterns
DIC	:	Disseminated Intravascular Coagulation
DMSO	:	Dimethyl sulfoxide
cDNA	:	Complementary deoxy ribonucleic acid
CCSEA	:	Committee for Control and Supervision of Experiments on Animals
E. coli	:	*Escherichia coli*
ELISA	:	Enzyme Linked Imuunosorbant Assay
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
HAIs	:	Hospital-Acquired Infections
H&E	:	Hematoxylin and Eosin
HDP	:	Host defense peptides
HEPES	:	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPLC	:	High-Performance Liquid Chromatography
IAEC	:	Institute Animal Ethics Committee
iNOS	:	Induced nitric oxide synthase
IC50	:	Inhibitory concentration
IL-1β	:	Interleukin 1 beta
IL	:	Interleukin
IM		Intramuscular

IP	:	Intraperitoneal
IV	:	Intravenous
JNK	:	Jun N-terminal kinase
K. pneumoniae		*Klebsiella pneumoniae*
L	:	Litre
LPS	:	Lipopolysaccharide
MAPK	:	Mitogen-activated protein kinase
MCOD/MOD	:	Multiple Cause of Death/ Multiple Organ Dysfunction
MCP1	:	monocyte chemotactic/chemoattractant protein 1
MDR	:	Multi drug resistant
Mg	:	milligram
MIC	:	Minimum inhibitory concentration
mL	:	milliliter
mMol	:	millimolar
MPO	:	Myeloperoxidase
mRNA	:	messenger RNA
µg	:	Microgram
mg	:	Milligram
µM	:	Micromolar
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	:	Sodium chloride
N-Fmoc SPSS	:	(*N*-fluorenylmethyloxycarbonyl) solid phase peptide synthesis strategy.
NF-kB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	:	Nitric oxide
OD	:	Optical density
P. aeruginosa	:	*Pseudomonas aeruginosa*
PAMPs	:	Pathogen-Associated Molecular Patterns
PBS	:	Phosphate buffer saline
PCR	:	Polymerase Chain Reaction
PRRs	:	Pattern Recognition Receptors
PLF	:	Peritoneal lavage fluid
PVDF	:	Polyvinylidene difluoride
QIDP	:	Qualified Infectious Disease Product

RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
rpm	:	Revolution per minute
RPMI	:	Roswell Park memorial institute media
RT	:	Reverse transcriptase
RT-qPCR	:	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
S. aureus	:	*Staphylococcus aureus*
SARS-CoV-2	:	Severe Acute Respiratory Syndrome -corona virus
SC	:	Subcutaneous
SDS-PAGE	:	Sodium dodecyl sulfate polymerase agarose gel electrophoresis
SEM	:	Standard error of mean
SIRS	:	Systemic inflammatory syndrome
SVR	:	Systemic vascular resistance
SOFA	:	Sequential Organ Failure Assessment
TLR	:	Toll like receptor
TNF	:	Tumour necrosis factor
TSB	:	Tryptic Soy Broth
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization

Chapter 1.

Introduction and Literature review

Sepsis is a complex disorder of disrupted inflammatory homeostasis that is often initiated by infection, which represents a global healthcare problem concern due to the high mortality rate and its expensive treatment, considered sepsis as a final common pathway to death for many infectious diseases worldwide.

The infection or trauma is caused by the presence of pathogenic microorganisms and/or their toxins (super antigens) in the bloodstream which lead to sepsis or the systemic inflammatory response syndrome respectively and is associated with multiple organ failure and a high risk of death.

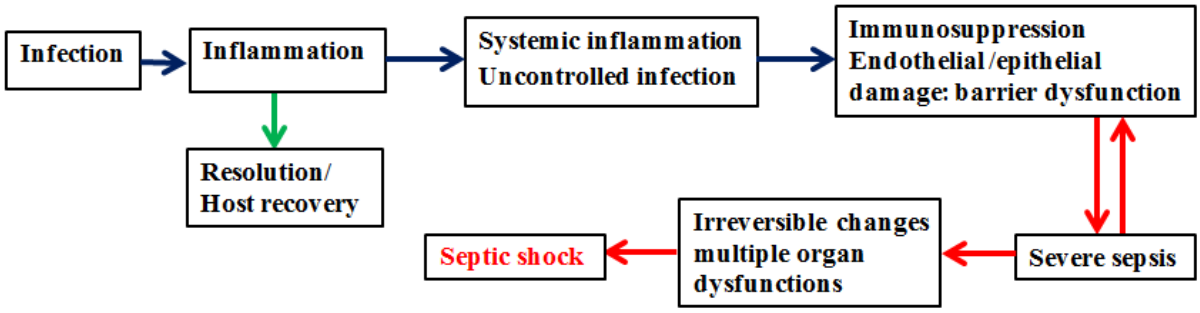


Figure1: Pathogenic mechanisms from infection to septic shock [modified from Jon A Buras et al.,2005]

Sepsis leads following infection, or an inflammatory insult leads to dysregulation of the inflammatory response, with release of inflammatory mediators and activation of the coagulation cascade, thrombosis, and diffuse endothelial disruption following and developing tissue and cellular hypoxia leading to multiple organ dysfunctions and irreversible shock.

"SEPIS IS A MEDICAL EMERGENCY with ANY KIND OF INFECTION."

The early recognition and management of sepsis prevent the development of septic shock, which is associated with a higher mortality rate [Hong Zhu, MD et al., 2016]. The patient will be at more risk of severe sepsis, the timing of clinical intervention is essential to the survival of septic patients. Early and appropriate treatment can significantly increase the chance that the septic patient will survive, as mortality is directly connected to the severity of the sepsis. One of every 2 to 3 in-hospital deaths is caused by this life-threatening clinical syndrome, which requires immediate attention and should be treated within an hour [Centers for Disease Control and Prevention 2022] However, even with adequate and aggressive care, 20–30% of septic shock patients will die if treatment is given too late.

1.1. Definition: Sepsis has been identified by the World Health Organization (WHO) as a global health priority ("Sepsis-3") in 2017 as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" [sepsis alliance,2015; Singer et al,2015 & the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3), 2016]

Sepsis

A life-threatening organ dysfunction caused by a dysregulated host response to infection and/or super antigen.

Septic shock

Subtype of sepsis in which particularly severe metabolic, cellular, and circulatory abnormalities are linked to a higher risk of death than sepsis alone.

Sequential Organ Failure Assessment Score (SOFA) [S.W. van der woude et al.,2018]

The Sequential Organ Failure Assessment (SOFA) score is the standard criteria for sepsis, used to assess the degree of organ dysfunction (Table 1).

Table 1: Sequential Organ Failure Assessment (SOFA) score [Sartelli et al.,2018]

Organ system		**Score**				
		0	**1**	**2**	**3**	**4**
Respiratory	*PaO2/FiO2 (kPa)*	≥ 53.3	< 53.3	< 40	< 26.7	< 13.3
Renal	*Creatinine (µmol/l)*	< 110	110-170	171-299	300-440	> 440
Hepatic	*Bilirubin (µmol/l)*	< 20	20-32	33-101	102-204	> 204
Hematological	*Platelets x10^3/µl*	≥ 150	< 150	< 100	< 50	< 20
Neurological	*Glasgow Coma Score*	15	13-14	10-12	6-9	< 6
Cardiovascular		MAP ≥ 70 mmHg	MAP < 70 mmHg	Dopamine < 5 or dobutamine	Dopamine 5.1-15, epinephrine ≤ 0.1 or norepinephrine ≤ 0.1A	Dopamine > 15 or epinephrine > 0.1 or norepinephrine > 0.1A

The SOFA score is made of 6 variables, each representing an organ system covering abnormalities in respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems. A point value of 0 (normal) to 4 (high degree of dysfunction/failure) is given to each organ system. A SOFA score ≥ 2 in setting of infection strongly supports the diagnosis of sepsis. The correlation of total score and hospital mortality is as follows in table 2.

Table 2: The correlation between SOFA score and percentage of hospital mortality

Maximum SOFA score	Mortality percentage
0 to 6	< 10%
7 to 9	15 - 20%
10 to 12	40 - 50%
13 to 14	50 - 60%
15	> 80%
15 to 24	> 90%

1.2. Epidemiology:

A precise estimate of the global epidemiological burden of sepsis is difficult to ascertain, according to the WHO report of 2018, globally, sepsis affects more than 30 million people and leads to around 6 million deaths in which around 3 million are newborns and 1.2 million children every year, which accounted for almost 20% of all global deaths every year [Global Data. Sepsis—Opportunity Analysis and Forecasts to 2021]. The infection triggers a chain reaction throughout the body; most often start in the lung, urinary tract, skin, or gastrointestinal tract, which can rapidly lead to tissue damage, organ failure, and death [Reinhart K et al., 2017]. According to CDC report, it states 1 in 3 people who die in a hospital had sepsis during that hospitalization. The incidence of sepsis has increased over the past 30 years. “Sepsis kills one person every 2.8 seconds in the world" [*Oktay Demirkiran*, 2022 on world sepsis day]. Almost 85.0% of sepsis cases and sepsis-related mortalities worldwide occurred in low- and middle-income countries, indicating significant regional differences in sepsis incidence and mortality, which is 50% or more, when compared to 15% to 30% of mortality rate in high-income countries for severe sepsis [News and opinions 2022]. The estimated burden of sepsis incident cases and deaths worldwide is twice every year. About half of all sepsis-related deaths result from sepsis worsening an underlying injury or non-communicable condition, which is mostly responsible for this startling rise in prevalence among residents of areas with lower socio-demographic index (SDI) scores. A rise in the number of admissions in acute care hospitals often leads to Hospital-Acquired Infections (HAIs) can trigger sepsis, especially in the geriatric population, newborn babies, and people suffering from chronic diseases, such as diabetes, cancer and leads to Sepsis, sometimes leads to re-hospitalization within 30 days of treatment [Margaret Jean Hall et al., 2011] The prevalence of HAIs is increasing owing to the high incidence of various infections majorly

include Urinary Tract Infections (UTIs) and Surgical-Site Infections (SSIs), which can result in sepsis. According to the WHO, 7 out of every 100 hospitalized patients are likely to develop HAIs in developed countries, whereas this number is likely to increase to 10 in developing countries due to poor healthcare infrastructure. Also, according to the CDC estimates, around 1.7 million patients in the U.S. experience HAIs each year, and approximately 99,000 people pass away because of HAIs [Fleischmann C et al., 2016].

However, The COVID-19 pandemic has significantly altered the landscape of sepsis epidemiology. Among the 200 hospitalizations for COVID-19, sepsis was detected in 65 (32.5%) of these 65 sepsis episodes, 46 (70.8%) were caused by SARS-CoV-2 alone, 17 (26.2%) by both SARS-CoV-2 and non-SARS-CoV-2 infections, and 2 (3.1%) were caused by bacterial infection alone [WHO corona virus disease dash broad ,2020]According to the global sepsis alliance reports (2020 in U.S.), around 75% of COVID-19 patients reported a depressed immune response and 30% had evidence of liver injury and COVID-19 pneumonia is one of the major factors to cause sepsis in patients, that occurs in response to the novel infection[Zhi gang Wang et al.,2020 & Danai PA et al., 2007].

1.2.1. Incidence of sepsis global data:

The true incidence of sepsis in any given country is unknown and has steadily increased over past decades which is expected to continue and remains one of the leading causes of death globally; both in hospital and after discharge. The study discovered there are gross regional and economic disparities, with 85% of sepsis cases and 84.8% of sepsis-related deaths occurring in nations with low, low-middle, or intermediate socio-demographic indices (SDI), especially in sub-Saharan Africa and Southeast Asia [Zarafshan Shiraz,2023]. In 2017, there were 48.9 million incident sepsis cases worldwide. Of these, 11 million led to sepsis-related deaths, which accounted for 19.7% of all deaths among critically ill patients in the intensive care unit (ICU) globally with mortality rates of 41% and 28.3% in Europe and the US respectively [Martin GS ,2000 &Andrew et al., 2022]. As per study by an international research consortium, sepsis claims more lives in India than in other South Asian countries, with an estimated 11 million sepsis cases a year and almost 3 million deaths, where more than half of deaths are in intensive care units and such cases have risen sharply over the past decade in India [Bharath Kumar et al., 2022]. In India, sepsis claims 213 lives per 100,000, compared to 206 in Pakistan, 183 in Nepal, 206 in Afghanistan, 136 in Bangladesh, 109 in Bhutan, 69 in Sri Lanka, and 27 in the Maldives [HINDUSTHAN TIMES, Jan 14, 2023]. The mortality of patients with severe sepsis remains around 40% and represents ~ 30% of overall hospital mortality rate. Mortality reaches

about 40% one year after discharge and up to 80% five years later. Sepsis among hospitalized patients is becoming more common by 8.7% annually in the United States alone [Thai Nguyen et al.,2022]. When it comes to hospital utilization, septic patients are disproportionately burdensome. Compared to patients with most other diseases, sepsis patients' average length of stay (LOS) in US hospitals is about 75% longer, which is 4.5 days for sepsis, 6.5 days for severe sepsis, and 16.5 days for septic shock and dramatically increase with sepsis severity [Carly J. Paoli, et al.2018 & Angus, D. C et al.,2001]. Using the MCOD, researchers found that between 2004 and 2018, the average annual age-adjusted mortality rate per 1,000,000 people from the three most common infection sites (genitourinary, lung, and abdominal infection) increased in both sexes and across all age groups, despite overall steady sepsis mortality rates [Martin GS,2006]. However, there were gender, racial differences and host genetic factors can influence the incidence and the outcome of severe sepsis related mortality rates and increased by 22.4% in the lungs, 28.0% in the abdomen, blood stream (15%) and 53.2% in the renal and genitourinary system [Duncan et al.,2013].

1.3. Aetiology:

Bacterial infections are the most common cause of sepsis and when untreated, can lead to sepsis shock and death. More than 90 % of cases of sepsis are caused by bacteria, in which gram-negative bacterial infections are frequent and more common than gram-positive bacterial infections (gram-negative- 62%; gram-positive- 47% sepsis cases). The major source of sepsis is from community acquired & nosocomial Pathogens and most frequent bacterial sepsis-associated species are *Staphylococcus aureus* (30%, including 14% methicillin resistant), *Pseudomonas* spp. (14%), and *Escherichia coli* (13%) and fungi 19%– candida species. *Escherichia coli* and *Staphylococcus aureus* accounted for most bloodstream infections. In comparison to sepsis brought on by nosocomial pathogens, there is a greater mortality rate, in comparison with community-acquired pathogens, and due to the usage of intravascular devices and rise in pneumonia cases, septic shock is now increasingly prevalent [Itaynara L. et al., 2020 &Abreu A.G. et al.,2015]. The causes of the infection are an important determinant of clinical outcome. More details are listed in (Table 3).

Table 3: The causes of major community acquired & nosocomial pathogenic infections.

[J. Deen et al., 2008]**.**

Cause	**Common Site(s) of Infection**
Gram-negative bacteria	
Escherichia coli	UTIs, prostatitis, meningitis, and peritoneal infections
Klebsiella pneumoniae	UTIs and pneumonia
Pseudomonas aeruginosa	Infected burn wounds and pneumonia in cystic fibrosis patients.
Enterobacter	UTIs
Gram-positive bacteria	
Staphylococcus aureus	Skin and soft tissue infections
Streptococcus pneumoniae	Pneumonia and meningitis
Streptococcus pyogenes	Skin and soft tissue infections
Enterococcus sp.	UTIs
Fungi	
Candida sp.	Intra-abdominal Infections
Others	
Haemophilus influenzae	Pneumonia
The table was adopted from [Neal R. Chamberlain, lecture on Sepsis and Septic Shock,2018]	

***Escherichia coli*, gram-negative bacteria** is one of the most often cited causes of hospitalized patient's infections, both in terms of frequency and severity, which is estimated to result in 20,000 deaths annually in the United States. Worldwide, 60% of mortality is from *E. coli* infections, including sepsis, newborn meningitis, peritonitis, bacteremia, traveler's diarrhea, and pneumonia. *E. coli* pathogenic strains that produce bacteremia have several virulence features, including those involved with adhesion, immune system evasion, iron acquisition, toxin generation, and resistance to the bactericidal action of the serum. They are also reported to be an etiologic agent of urinary tract, central nervous system, and bloodstream infections. These components' presence can ensure the bacteria's persistence in the circulation and spread throughout the body, which can then cause a severe inflammatory response that leads to sepsis [Itaynara L. Dutra et al.,2018 & Abreu A.G et al.,2015].

1.4. Risk factors:

A patient's predisposition to infection is the main focused risk factor for the development of sepsis with pre-existing immune dysfunction but probably includes comorbidities and host genetic factors in addition to pathogen-related factors. The development and progression of sepsis is multi-factorial, and affects the circulatory, immune, and endocrine systems of the body [*Kristina E Rudd* et al.,2020 & Carolin Fleischmann et al.,2021].

Patients are at increased risk of developing sepsis.

Patients of certain ages: men aged ≥50 years, women aged 20–45 years are much more likely to acquire urinary tract infections, and during labor, neonates are more susceptible to infections, mostly common cause is *E coli*.

Patients with underlying diseases: solid tumors, diabetes mellitus, cirrhosis of the liver, acquired immune deficiency syndrome (AIDS), and other serious chronic conditions.

Prior drug therapy: immunosuppressive drugs, especially broad-spectrum antibiotics and alcohol abuse.

Antecedent surgery or instrumentation: including catheters and prosthetic devices.

1.4.1. Co morbid conditions:

Sepsis can be a terminal event contributing to one-third to one-half of all in-hospital deaths in patients who are already dying from other causes/ with chronic diseases (cancer, hypertension, chronic lung & kidney diseases, diabetes and obesity) and the mortality rate increases to 700 cases per 100,000 in such patients.

Chest Infections: It was estimated 90% of worldwide deaths from chest infections and about 70% of the 9 million deaths due to chest infections in neonates and infants are associated with sepsis occur in low-resource settings, and most cases occur in Asia and Africa [Claire N. Shappell et al.,2022].

HIV infection: HIV infection makes sepsis worse, and it was reported that the incidence of sepsis goes up to 1,000 cases per 100,000 persons with HIV infection and the outcome is worse in comparison with patients without HIV/AIDS (150–300 cases per 100,000 persons).

Diabetes: Diabetic patients with confounding factors such as hyperglycemia, obesity, secondary micro- and macrovascular complications, insulin therapy and others are having higher susceptibility to acquire infections. The direct effects of OADs on upcoming sepsis occurrences should be taken into consideration because people with T2D are prone to infection. In addition, the role for intensive insulin treatment in severely ill septic patients remains controversial [Chia-Jen Shih et al.,2015]

AKD & CKD: Up to 60% of patients with sepsis develop acute kidney injury (AKI) and mostly in the intensive care unit (ICU) patients, can be fatal and in which 30% of AKI patients can found to be chronic kidney disease (CKD) patients [Kent Doi, M.D. et al., 2008]. Continuous renal replacement therapy (CRRT) is commonly used for AKI /CKD patients who are hemodynamically unstable, this has the potential to prevent worsening of cerebral edema in patients and associated with electrolyte disturbances and hypotension and leads to sepsis [Srijan Tandukar et al., 2019 & Vinsonneau C et al., 2006].

COVID-19 Pneumonia: Almost 42 million individuals had been infected with the novel coronavirus COVID-19 by 2020, and more than 1.1 million had died as a result of its relentless global pandemic. With an estimated mortality rate of 1-5%, COVID-19 appears to have a lower rate in comparison to the fatality rates of SARS-CoV infection (10%) and MERS-CoV infection (37%), Clinical signs of COVID-19 infection are typically minor. Although there is a very high death rate in the intensive care unit (ICU), roughly 10–20% of all patient's health can deteriorate. Additionally, COVID-19 affects patients with comorbidities (hypertension, diabetes, liver and heart illnesses, obesity, etc.) and older, healthy individuals more severely and leads to sepsis than it does younger, healthy individuals. COVID-19 pneumonia is one of the major factors to cause sepsis in patients, owing to severe lung inflammation that occurs in response to the novel infection, resulting in organ damage and even death [Zhigang Wang et al., 2021 & WHO Coronavirus Disease (COVID-19) Dashboard ,24 October 2020.].

1.5. Pathophysiology: A simplified overview of current notions regarding the pathogenesis of sepsis runs as follows: [The following figure 2 was adopted and modified from Niels C. et al.,2003, Ignacio Rubio, et al.,2019, William C. Aird et al.,2003, Florea Lupu et al., 2014 & Michela Giustozzi a et al.,2021]

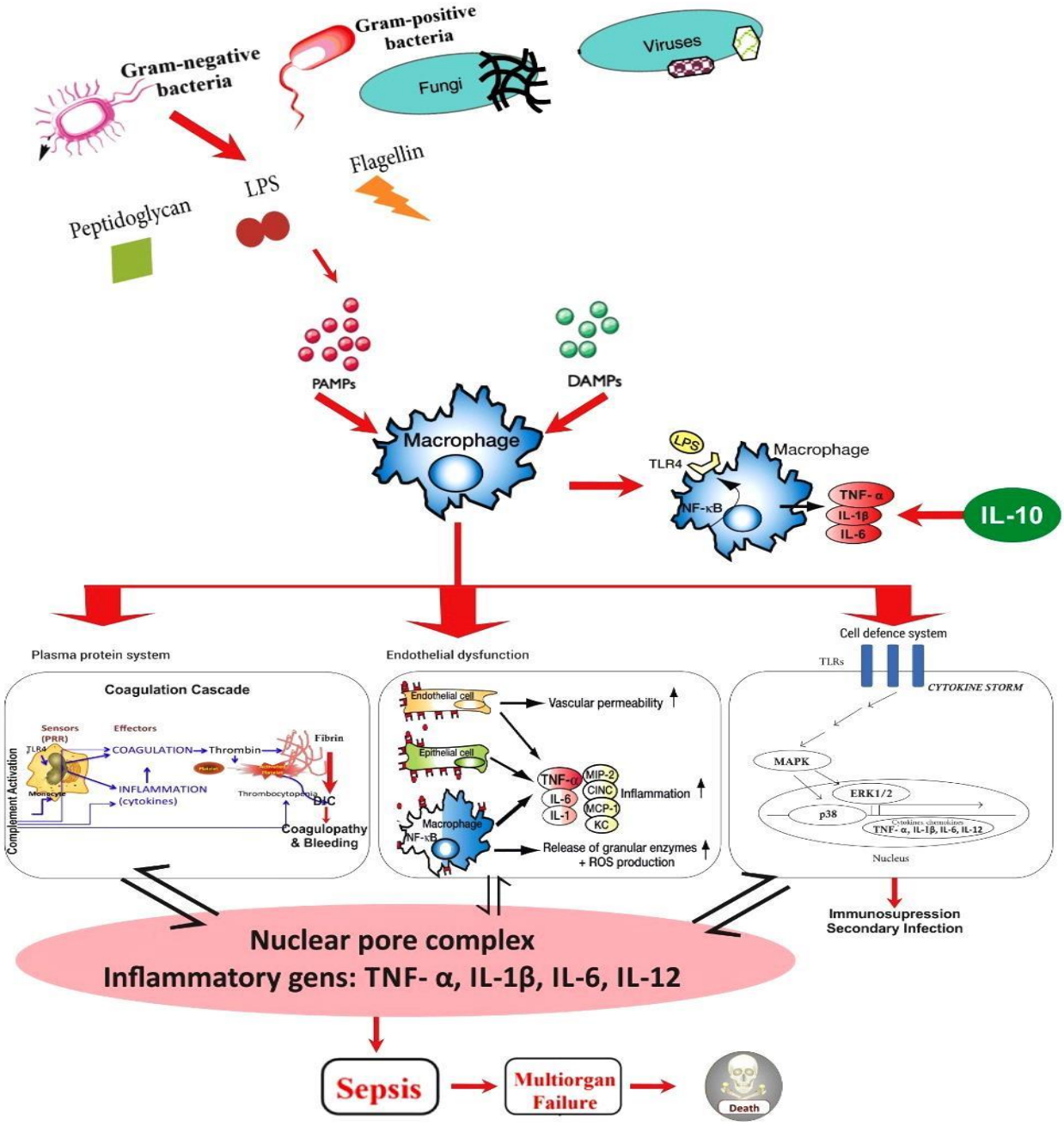


Figure 2: Pathophysiological changes of sepsis due to infection. Release of danger signal molecules (PAMP/ DAMP) activates multiple parallel cascades that contribute to the pathophysiology of sepsis and sepsis shock: cytokine storm, coagulation and complement activation and endothelial dysfunction, which ultimately results in organ failure.

The pathophysiology of sepsis involves complex interactions between the host immune response and pathogen dynamics. Numerous genetic variations in cytokines and other innate immune mediators, coagulation, and fibrinolysis play a role in sepsis but are still not well understood.

Sepsis is a severe infection that triggers an immune response to various components of bacteria and yeast, such as lipopolysaccharide (LPS), flagellin, peptidoglycan, bacterial DNA, and zymosan. Tissue damage during sepsis is not only caused by the invading bacteria but also by an excessive inflammatory response from the immune cells. The immune response consists of the innate and adaptive immune systems [Christina Nedeva et al.,2019]. The innate immune response involves cellular and molecular mechanisms. Epithelial cells in mucosal surfaces and phagocytic cells, including neutrophils, monocytes, and macrophages, play a role at the cellular level. These cells sense microorganisms through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which recognize specific molecular patterns associated with pathogens (PAMPs) and tissue damage (DAMPs). Activation of PRRs leads to the release of pro-inflammatory and anti-inflammatory mediators, including cytokines (TNF-α, IL-1β, IL-6), chemokines (MCP1, IL-8), nitric oxide (NO), and reactive oxygen species (ROS). This results in systemic inflammatory syndrome (SIRS) in the early phase of sepsis. In the late phase, anti-inflammatory mediators (IL-4, IL-10, IL-13, cortisol, etc.) are released, and adaptive immunity mediated by T and B cells comes into play. The transition from Th1 to Th2 in T cells can lead to compensatory anti-inflammatory syndrome (CARS) (Figure 3) [Jonathan Cohen et al.,2002; Benjamin G.et al.,2017].

In a compromised immune state, the body becomes more susceptible to secondary infections and viral reactivation. This leads to upregulated expression of adhesion molecules and activation of the complement and clotting cascades, resulting in disseminated intravascular coagulation (DIC) [J. Hawiger et al.,2015]. These processes attract and migrate leukocytes to the site of infection, aiding in bacterial clearance through phagocytosis [Di Giandomenico A et al.,2014]. Sepsis is also accompanied by hemodynamic and coagulation changes and cellular injuries, leading to cardiovascular, hemodynamic, neurological, and metabolic alterations. Multiple organ dysfunction (MOD) often affects the kidneys (AKI & renal failure), lungs (acute respiratory distress syndrome [ARDS]), and heart (cardiomyopathy). However, dysregulated activation of the innate immune system in severe sepsis or septic shock can have detrimental effects. Excessive production of nitric oxide (NO) in blood vessel walls leads to systemic arterial hypotension, and peripheral inflammation can affect the central nervous system (CNS), resulting in cognitive impairment, delirium, or coma.

This clinical state is known as "severe sepsis." When systemic arterial hypotension is present, leading to cardiovascular collapse unresponsive to fluid resuscitation and vasopressor therapy, it is referred to as "septic shock" and results in systemic failure and death.

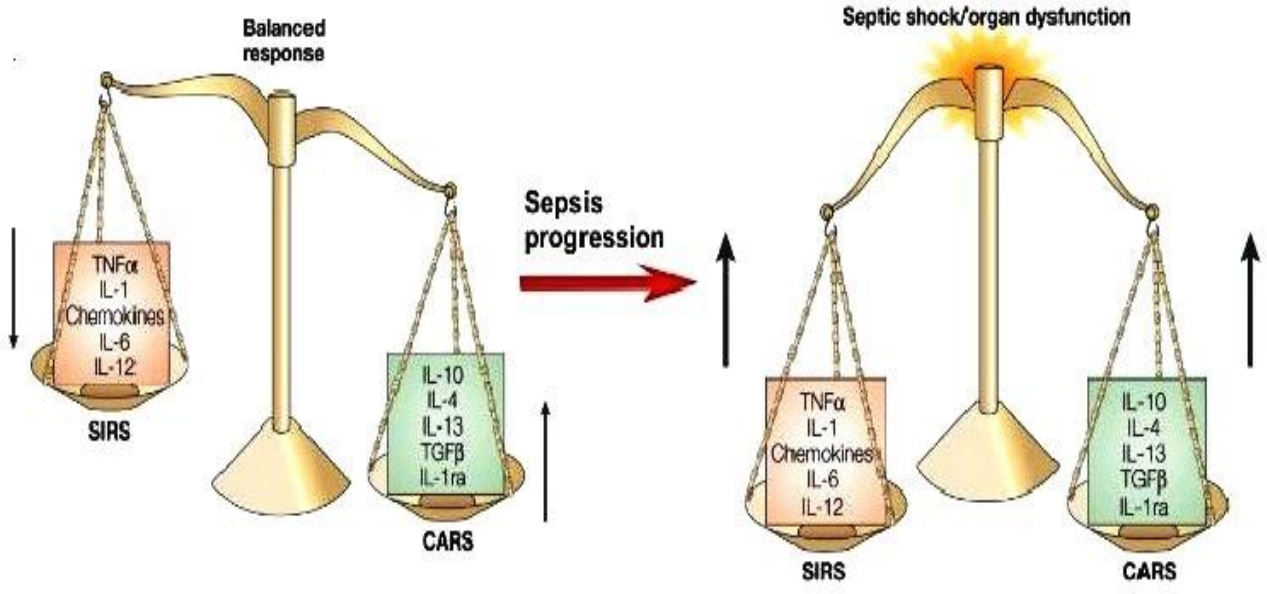


Figure 3: **SIRS and CARS in sepsis.** The host inflammatory response can be viewed as a balanced response between pro-inflammatory mediators (referred to as the systemic inflammatory response (SIRS)) and anti-inflammatory mediators (referred to as the compensatory anti-inflammatory response (CARS)). [Jon A Buras et al.,2002].

1.5.1. Inflammatory Response in pathogenesis of sepsis:

Human sepsis is thought to have at least two stages: an initial hyperinflammatory phase (SIRS) that lasts for several days, followed by a more protracted immunosuppressive phase (CARS) known as immune depression or immune dysfunction; however, these stages can overlap temporally (Figure 4)[Rangel-Frausto MS et al.,1995].These two phases are associated with increased mortality and the current death distribution indicates two peaks; one of which manifests during the early phase albeit to a lesser extent, and another second peak after 2–3 months that continues to increase over the next 3 years [Kent Doi et al., 2016]. The hyper-inflammatory phase has been “Cytokine storm” generates during various systemic acute infections, including causing severe inflammation and tissue damage, plays a crucial role in sepsis, including acute lung injury or ALI, influenza, and severe COVID-19 pathogenesis, which also comprises fever, refractory shock, inadequate resuscitation, and cardiac or pulmonary failure. This promotes an ‘inflammatory state with activation of leucocytes, complement

and coagulation pathways that underpin the endothelial, cellular, and cardiovascular dysfunction that characterizes sepsis. Mortality during the latter period is due to protracted immunosuppression characterized by anergy, lymphopenia, and with secondary infections, culminating in organ injury and/or failure.

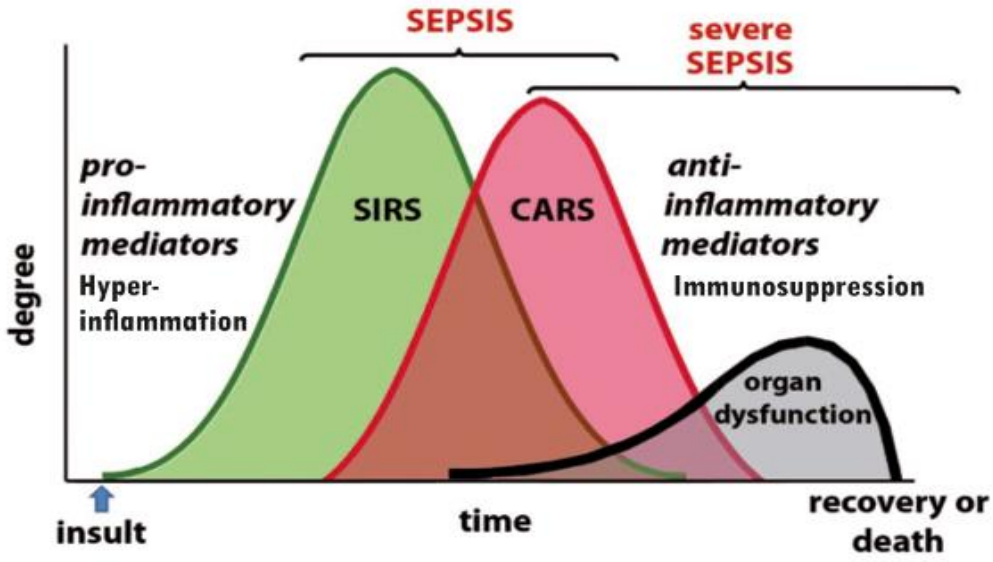


Figure 4: Two stages of sepsis. A hyper-inflammatory phase with immunosuppression and multiple organ dysfunctions occurs after infection. This may also resolve, particularly with appropriate support, but it frequently results in death [James D. Faix et al.,2013].

Overview of the immune system: Cytokine Storm.

PAMPs/ MAMPs and DAMPs activate downstream signaling TLRs pathways, resulting in the release of several pro-inflammatory mediators generating cytokine storm, which cause acute inflammation to control the pathogen and repair the damage [Benjamin G. Chousterman et al., 2017]. Acute viral infection (severe COVID-19)-associated cytokine storm, which is caused by TLR-signaling, is a major factor in sepsis and is responsible for multi-organ failure and patient death [V. Kumar et al., 2020]. Organ failure, tissue damage, and ultimately death are the results of an imbalance in the inflammatory network of pro-inflammatory and anti-inflammatory cytokines [Hina Chaudhry et al., 2013]. Thus, Cytokines plays as important pleiotropic regulators of the immune response, in the process of initiating and escalating the innate immune response as well as the adaptive immune response [Banyer et al., 2000]. However, cytokines may also induce tissue-damaging inflammation and dysregulate the immune response, which can lead to an immunosuppressive state and play a

significant part in the pathophysiology of severe COVID-19, influenza, and sepsis [London NR, et al., 2010]. Thus, understanding and studying endogenous negative regulators of TLR signaling may provide novel therapeutic targets against cytokine storm generated during different diseases, including sepsis and severe COVID-19.

1.5.1.1. Pro inflammatory cytokines: [Wibke Schulte et al., 2013]

It was evident from experimental and human clinical studies; that pro-inflammatory cytokine and chemokine (TNF-α, IL-1β, IL-12, IL-6, IL-8, and IFN-γ) production by the host has been strongly linked to the symptomatology and pathogenesis of sepsis. These responses include but are not limited to enhanced phagocytic activity, vascular endothelial injury with capillary leak, chemotaxis of leukocytes to sites of infection/inflammation, and activation of the coagulation system. Individual pro- and anti-inflammatory cytokines actions are described in more detail, and they are directly related to sepsis pathophysiology (table 4) [Chen L,2018].

TNF*α*, IL-1β and IL-6 [Charalambous A. etal.,2000]

Broadly, TNF and IL-1β levels are both elevated in endotoxin related Gram-negative sepsis within 30 minutes as acute phase response to the infection and those induced by IL-6, All together, activate endothelial cells; enters the circulation, causing fever and induce a shock-like state characterized by vascular permeability, leukocytosis, severe pulmonary edema, and hemorrhage. The former group include the major acute phase response is characterized by the release of hepatic acute phase proteins such as C-reactive protein and serum amyloid A which plays a role in anti-bacterial immunity, while the latter group include complement components, fibrinogen, factor VIII, ferritin, and *α*2 macroglobulin. Recently, this balance was proved in in animals with pneumonia infections, in which it was shown the innate immune response was inhibited by compromising interleukin-1β and tumor necrosis factor- α which facilitates bacterial growth and leads to death. Together, there is existing evidence that blockade of inflammatory pathways, including IL-1β, is beneficial for reducing symptoms of cytokine storm in inflammatory diseases and in COVID-19.

IL-12

IL-12 controls innate immune responses and stimulates the adaptive immunological response, which is characterized by heightened mononuclear phagocyte responses. Upon release, IL-12 stimulates the production of IFN- by T-cells and natural killer (NK) cells, which in turn activates macrophages to increase their bactericidal activity and secrete more T helper 1 (TH1) cytokines. There is still controversy over IL-12's role in sepsis despite years of research. Clinically, it has been shown that a specific deficiency in preoperative monocyte IL-12 production impairs host defense against postoperative infections, raising the risk of fatal sepsis in patients undergoing major visceral surgery. In chimpanzees, IL-12 infusion is associated with a delayed activation of many of the inflammatory pathways that are also activated during sepsis, such as the cytokine network, neutropMs, and coagulation, implying that IL-12 may be involved in maintaining the inflammatory response to a bacterial insult.

Table 4: Summary of the pro- and anti-inflammatory cytokines main features

Cytokine	Main source	Function and interactions with other cytokines	Involvement in sepsis	Reference
Pro inflammatory				
TNF-α	Immune cells, mostly macrophages, lymphocytes, and fibroblasts, of the innate and adaptive immune systems.	Immune cell differentiation and activation; causes fever and coagulation.	↑ Serum levels in sepsis	[E. Choy,2021&M. Schouten,2008]
IL-1β		Helps downstream proinflammatory effector molecules release: Hematopoiesis is stimulated, fever and coagulation are induced, and inflammatory cells are encouraged to extravasate.	↑ Serum levels in sepsis	[J. Cohen 2002, J. M. Rubio-Perez,2012 J. Jawien,2008; & C. A. Dinarello,2004]
IL-6		Released in response to TNF-α and IL-1β, which blocks the release of those molecules; encourages anti-inflammatory responses (sTNFRs, IL-1Ra, and TGF-β): B and T cell activation; hematopoiesis is modulated; and fever results from the acute phase response.	↑ Serum levels following burns, major surgery, in sepsis, RA, and Crohn's disease	[A. M.W. Petersen,2005; J. Scheller,2006 & M. Rincon,2012]
IL-12	Dendritic cells; Monocytes/macrophages; Neutrophils.	Stimulates type 1 adaptive immune response and triggers antitumor immunity causes the generation of IFN-γ production.	↑ Serum levels in acute and chronic inflammatory diseases.	[P. Puccetti,2002 & G. Trinchieri,2003]
Anti-inflammatory				
IL-10	Immune cells of the innate and adaptive immune system.	Inflammatory cytokines are suppressed from being released, sTNFRs and IL-1Ra production is stimulated, and there are other immunosuppressive features.	Dysregulated in autoimmune diseases	[S. Q. Latifi,2002 &M. Seitz.1995]

1.5.1.2. Anti-inflammatory cytokines:

Consequently, patients with SIRS are known to be very sensitive to infection. Alongside the inflammatory response, a robust depletion of both innate and adaptive immune cells through apoptosis occurs to dampen the response. (A) At this stage, patients may experience a controlled anti-inflammatory response, enabling them to restore immune homeostasis. Alternatively, patients may undergo an uncontrolled anti-inflammatory response and enter a hypo inflammatory phase yet survive (B) or succumb. Extracellular anti-inflammatory cytokines IL-4, IL-10, TGF-β and IL-13 counteract the deleterious action of proinflammatory cytokines and chemokines. Their production is a compensatory anti-inflammatory response syndrome (CARS), which is also associated with the 'immunoparalysis' seen in the later stages of sepsis [Bone et al.,2018]. whereas, it has been reported that, among the various mediators circulating in SIRS patients, IL-10 could be a putative actor leading to the deactivation of circulating leukocytes as well as neutrophils this leads to Cellular hypo reactivity [Charalambos A. et al.,200].

A few in vivo observations clearly suggest that IL-10 has some pro-inflammatory activity. The animal sepsis model with trauma or hemorrhagic shock reported that IL-10, as well as transforming growth factor-β (TGF-β), could be associated with the immune suppression and the increased levels of these anti-inflammatory cytokines are found in the circulation of septic patients and following severe surgery are all associated with a hypo reactivity of leukocytes. Knockout animal studies revealed that IL-10 was a powerful anti-inflammatory mediator, resulting in reduced lung neutrophil infiltration and injury after visceral ischemia.

Interestingly, in chronic bronchitis patients, IL-10 can inhibit IL-8 production by activated blood neutrophils whereas it has no effect on sputum derived neutrophils. Human clinical trials are currently underway to elucidate biomarkers to identify patients with increased or decreased inflammation, for example, inhibition of IL-10 in patients with increased inflammation dramatically increased pro-inflammatory cytokines with detrimental effects.

1.5.1.3. VEGF role in onset of sepsis:

In response to systemic inflammation caused by endotoxin, macrophages and lymphocytes produce vascular endothelial growth factor (VEGF), which increases the permeability of the blood vessels increases. And acts as a potent endothelial permeability stimulator, causing "leakiness" in the endothelial cells that line the body's blood vessels. VEGF levels are elevated in severe sepsis and implicated in several inflammatory disease states, such as rheumatoid arthritis, inflammatory bowel disease, tumor angiogenesis and in wound repair. Low circulating VEGF levels are associated with hematological and renal dysfunction, suggesting that VEGF production in severe sepsis may be disturbed. High VEGF concentrations correlate with the severity of organ dysfunction. Even though VEGF levels were lower in non survivors than in survivors, VEGF levels did not predict hospital mortality in patients with severe sepsis [Sari Karlsson et al.,2008].

From several human studies and experimental animal sepsis models, the association between sepsis and circulating levels of VEGF was confirmed. [Aird et al.,2003&2006] hypothesized the importance of endothelium and role of VEGF signaling in sepsis, which contributes to vascular leakage and inflammation. Animal sepsis models demonstrated significant protection against morbidity (as measured by cardiac dysfunction, inflammation, coagulation, and permeability) and mortality, when treated with VEGF inhibitors or neutralizing antibodies against VEGF receptors. Based on their findings, they concluded that VEGF plays a pathophysiological role in sepsis and represents a novel therapeutic agent [Smadja et al., 2012]. [Hauschildt et al., 2020] reported VEGF can distinguish between severe sepsis and non-infectious organ failure.

1.5.1.4. Other Inflammatory mediators in sepsis: Granule-associated mediators –NO.

Nitric oxide plays a role in the pathophysiology of sepsis, has been implicated as a possible causative mechanism for the reduced activity of immune cells in sepsis [Elias Lolis et al., 2003]. There are three isoforms of nitric oxide synthase (NOS), which mediates inflammation: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Among the three isoforms: proinflammatory enzyme inducible nitric-oxide synthase (iNOS) is induced in response to pro-inflammatory cytokines and bacterial LPS [Rockey et al., 1998]. Excessive and prolonged iNOS-mediated NO generation has been linked with inflammation and carcinogenic processes [Sporn and Roberts, 1986; Heiss et al., 2001]. The wave of TNFα and interleukin-1β elicited by both types of bacteria can trigger the production of iNOS, and TLR2 ligands to initiate NO production. These also

serve to activate the vascular endothelium, enhancing adhesion molecule expression and induce local vasodilation. This physiological production of NO is important for blood pressure regulation and blood flow distribution. The hyper production of inducible form of NO synthase (iNOS) may contribute to hypotension, cardio depression, and vascular hyporeactivity in septic shock. In several animal studies of septic models, the treatment with inhibitors of NO synthesis shown to improve hemodynamic variables and survival, whereas in human septic shock, inhibition of NO synthesis has been shown to alter hemodynamic variables for short-term, but it is uncertain whether this treatment has beneficial long-term effects. [Sugitharini et al.,2013] reported higher levels of granule-associated mediators (neutrophils elastase (NE), myeloperoxidase (MPO), and nitric oxide (NO) in neonatal sepsis [Magudumana O et al., 2000] along with proinflammatory cytokines (TNFα, IL-1β, and IL-6), anti-inflammatory cytokines (IL- 10 and IL-13) with early onset sepsis compared with controls [S Sriskandan et al., 2008].

1.6. Diagnosis &Clinical Manifestations:

As there is no gold standard test that confirms the sepsis diagnosis, as further complicated due to nonspecific signs and symptoms and can be challenging, thus early diagnosis and stratification of the severity of sepsis is very important for specific treatment at prompt timing, this reduces the overall burden of sepsis related hospitalization as well as length of stay and cost. As a result, finding a focus of infection that is accompanied by at least two signs of the systemic inflammatory response syndrome is required for a standard clinical diagnosis of sepsis.

The criteria for systemic inflammatory response syndrome & sequential organ failure assessment are as follows:

Table 5: Diagnostic Criteria for the Systemic Inflammatory Response Syndrome (SIRS).

Defined by the presence of two or more of the following clinical findings	
SIRS criteria (≥ 2)	Body temperature > 38.0 °C or < 36.0 °C Heart rate of > 90/min Respiratory rate of > 20 breaths/min or $PaCO_2$ of < 32mm Hg White blood cells count of < 4000 cellsμL^{-1} or > 12,000 cellsμL^{-1} or > 10% immature (bands) forms
qSOFA score (≥ 2)	Respiratory rate ≥ 22 breaths/min Systolic blood pressure ≤ 100 mmHg Altered mental state
SIRS = systemic inflammatory response syndrome; qSOFA = quick sequential organ failure assessment. [S.W. van der woudel, et.al.,2018]	

Patients who are treated early in the course of this disease have fewer complications and a much better chance of survival. Severe sepsis denotes major organ failure in sepsis, whereas septic shock can affect several organs at the same time, resulting in a mixture of signs and symptoms.

The clinical presentation of sepsis is determined by the site of infection (i.e., chest focus versus urinary tract focus) as well as the signs and symptoms of the host response (Figure 5). Human sepsis is characterized by various clinical features, divided into two distinct phases: early and late septic phase. The early phase is referred to as hyper-dynamic phase, characterized by low systemic vascular resistance (SVR) and increased cardiac output (CO). Low CO and SVR are the hallmarks of septic shock and define the late/second hypo-dynamic phase of sepsis. Nonspecific Symptoms of sepsis includes fever (although hypothermia can be present too), chills, and constitutional symptoms of fatigue, malaise, tachycardia, tachypnoea, or altered mental status (anxiety or confusion). Symptoms may be absent in serious infections, especially in elderly patients. Sepsis is multifactorial in its development and progression, affecting the cardiovascular, immune, and endocrine systems of the body. Some of the organ dysfunction complications that results are as follows. [Timothy W. Evans et al.,2002].

Complications associated with septic shock: [Bone, R. C. et al.,1992 & Blanco J, 2008]

- **Brain:** Delirium (Confusion).
- **Liver:** liver dysfunction detoxification, acute hepatitis with elevated levels of serum alkaline phosphatase and bilirubin.
- **Kidney:** Acute Renal Failure (ARF) and Proteinuria.
- **Lung:** Decrease in arterial PO2; Acute Respiratory Distress Syndrome (ARDS) due to leakage of the contents of the capillaries into alveoli; tachypnea.
- **Heart:** Arterial hypotension, cardiomyopathy and organ perfusion can already be impaired even in the context of normal blood pressure.
- **Gastrointestinal tract:** Nausea, vomiting, diarrhea, peritonitis and ileus.
- **Spleen:** splenomegaly.

  Skin: The skin can be mottled and the capillary refill time increased purpuras, erythema and necrosis (ecthyma gangrenosum).

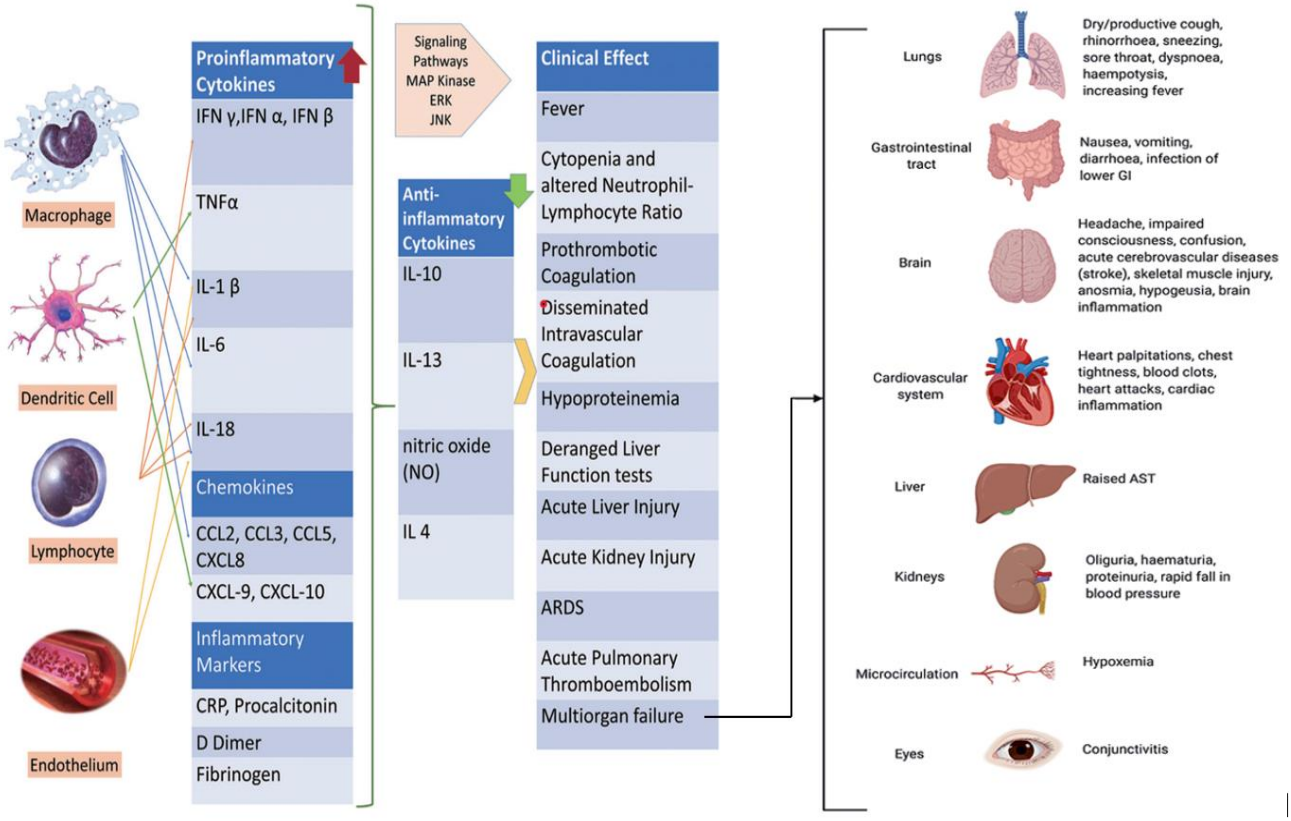


Figure 5: Clinical presentation of sepsis.

1.7. Biomarkers:

Biomarkers used in the diagnosis of sepsis are important for determining whether the patient is in the early hyper inflammatory phase of the disorder or has entered the more protracted immunosuppressive phase, as well as for monitoring treatment and to differentiate source of infection: bacterial from fungal and viral infection and systemic sepsis from local infection. This is important in guiding antibiotic therapy [Savitri Kibe et al., 2011).

Cytokine levels were highest in patients who died from severe sepsis, with marked elevations of both the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 with be associated with the highest risk of death. In critically ill hospitalized patients, there was a similar significant association between early IL-10 elevation and the development of severe sepsis. Plasma levels of IL-6 are stably elevated and correlate with disease severity such as the occurrence of multiple organ failure and septic shock, and the overall mortality [Şerife Kurul, et al.2021]. Although there is an enhanced expression of pro-inflammatory cytokine genes in both early and severe sepsis, enhanced expression of anti-inflammatory cytokine genes, such as IL-10 and TGF-β, was seen only in severe sepsis [Zhengwen Xiao et al.,2015 & Boomer JS, et al.,2014]

Multi-marker approach of cytokine production analysis in the diagnosis of sepsis could identify those patients who are moving from the hyper-inflammatory state to the anti-inflammatory state of sepsis [S.Gibot et al.,2012]. This approach, in which markers of the hyper inflammatory state are combined with markers of the anti-inflammatory state, is the one most likely help to identify patients who would benefit from novel therapies designed to restore immune function and might succeed in predicting the onset of severe sepsis [K. Reinhart et al.,2012]. Various biomarkers that have been used for the diagnosis of sepsis are below mentioned in the table 6.

1.7.1. Laboratory investigations:

Apart From the above-mentioned biomarkers, there are some routine clinical investigations were done on study day 1 and every day thereafter until ICU discharge or death [S. Bhattacharya et al.,2005]. The three commonly done biomarkers are C-reactive protein (CRP), procalcitonin (PCT), and Interleukin-6 (IL-6) and some of the Laboratory tests which are screened routinely are mentioned in the table 7[Guido Marcello M et al., 2016 & K A Kirkebøen et al.,1999].

Table 6: Cytokine biomarkers for sepsis:

Biomarker	Based on experimental studies & clinical studies	Evaluated as a prognostic factor	Comment	References
TNF	✓	-	Distinguished between survivors and non-survivors at 28 days in patients with septic shock.	[S. Kumar,2010]
IL-1b	✓	✓	Increased in septic compared with non-septic individuals.	[R. Berner,1998]
IL-6	✓	✓**	Distinguished between survivors and non-survivors at 28 days.	[A. Engel,1998; N. Chaudhary,2012; A. Engel,1998; R. Berner,1998; & C. S. Calfee,2010]
IL-10	✓	✓**	Higher in septic shock than sepsis, distinguished between survivors and non-survivors at 28 days. Accurate biomarker for neonatal sepsis.	[E. L. Tsalik,2010]
IL-12	✓	✓	Predictive of lethal outcome from postoperative sepsis.	[D. Andaluz-Ojeda,2012]
IL-4	-	✓	Increased levels associated with development of sepsis.	[D. Andaluz-Ojeda,2012]
Total VEGF	✓	-	Estimation of Total VEGF by ELISA on the day of admission (VEGF1) and after 48 h i.e., on the morning of the third day (VEGF2).	[A-ling Tang ,2022; Abo Hamela et al., 2016 & Beth Israel Lahey Health,2006]
*Sensitivity and specificity of less than 90%; **sensitivity of more than 90% but specificity of less than 90%; ***sensitivity and specificity more than 90%; DIC: disseminated intravascular coagulopathy; MOF: multiple organ failure; SOFA: sequential organ failure assessment.				

Table 7: Laboratory clinical and biochemical biomarker investigations:

Biomarkers	Comment	References
CRP: C-reactive protein levels	The most frequently used laboratory test for the diagnosis of neonatal sepsis (LONS). Elevated CRP levels in sepsis have been correlated with increased risk of death and organ failure. sTREM-1 is a more sensitive and specific feasible biomarker rapid and early diagnosis of infectious diseases	[N. Chaudhary,2012 & L. Simon,2004]
PCT: Procalcitonin levels	Detects serious bacterial infections and can be used for guiding duration of antibiotic therapy in early onset sepsis. sTREM-1 is a more sensitive and specific feasible biomarker rapid and early diagnosis of infectious diseases	[M. Limper, M. D et al., 2010; A. E. Jones,2007&C. Wacker,2013.]
Lactate	Biomarker for indicating organ dysfunction is the blood lactate level and useful as a predictor of mortality in sepsis. Serum lactate concentration at time of admission has been recommended by the surviving sepsis campaign guidelines as a marker of hypoperfusion.	[Chen H, Zhao C, & Wei Y et al.,2019]
Complete blood picture: Hemoglobin, Hematocrit, White blood cells and platelet count		
WBC	Biomarkers of activated neutrophils and monocytes in sepsis.	[K. Kofoed et.al., 2007]
Neutrophils	Neutrophils act as key regulators of both acute and chronic sterile inflammatory conditions in regulating vascular endothelial permeability. Fever and hyperthermia are a hallmark symptom are known to increase neutrophil recruitment. Myeloperoxidases (MPOs) Estimation is important for neutrophil recruitment.	[K. Kofoed et.al., 2007 & Thomas Marichal et al.,2020]
ESR	Adjunctive test for inflammation; however, its utility as a biomarker for sepsis is limited.	[Thomas Marichal et al.,2020& A.

		Osei-Bimpong, et al.,2007]
Endothelial cell biomarkers	Identification of Endothelial dysfunction: major contributor to the organ dysfunction observed in severe sepsis and septic shock.	[Aird, W.C et al.,2006 & Kiichiro Yano et al.,2006]
DIC - coagulation system	To monitor hemostatic abnormalities associated with sepsis, which includes platelet count, prothrombin time, fibrinogen level and a marker of fibrin formation.	[Skibsted S et al., 2013]
Liver function tests	Elevated indicate liver dysfunction: Biomarkers of organ dysfunction in sepsis: ALT (Alanine aminotransferase), AST (Aspartate aminotransferase), BIL (bilirubin) and Albumin.	[James D. Faix et al.,2013]
Kidney function tests	Na, K, Creatinine and Urea. Elevated indicate kidney dysfunction: Biomarkers of organ dysfunction in sepsis.	[James D. Faix et al.,2013]
Urinary function tests	Microalbuminuria: Generally expressed as the urinary albumin to creatinine ratio to correct the variations in urinary flow rate, which is Measured using the immunoturbidimetric method and urinary creatinine through a modified kinetic Jaffe reaction (Dimension RxL Max, Dade Behring Inc., U.S.A) on the day of admission.	[James D. Faix et al.,2013]

1.8. Management of sepsis and challenges:

Because sepsis therapy is highly time sensitive, early prediction is critical in reducing mortality. The international medical guidelines, specifies initial and immediate therapy which includes early fluid resuscitation should begin within the first 3 hours of sepsis to stabilize sepsis-induced tissue hypoperfusion, along with intravenous antimicrobial administration as soon as possible, preferably within 1 hour of sepsis [Dellinger RP. et al.,2012 & Rhodes A et al.,2017] Furthermore, each hour of delay in antibiotic delivery increases sepsis mortality. Guidelines for sepsis management focus on

three primary components: (1) hemodynamic stabilization, (2) infection control, and (3) septic response regulation. These three priorities when treating a septic patient are as follows.

1. **Immediate stabilization of the patient:** The first concern while treating patients with severe sepsis in ICUs is the reversal of life-threatening anomalies (ABCs: airway, breathing, circulation), and their vital signs (blood pressure, heart rate, respiratory rate, and temperature) should be monitored.

2. **Infection control:** First, the burden of infection should be promptly reduced by source control measures and early treatments including antibiotics. Second, in addition to symptomatic treatments, the modulation of the host response remains a desired goal in sepsis.

3. **Septic response regulation:** Decreasing coagulation (tissue factor pathway inhibitor, antithrombin, activated protein C, heparin). However, the beneficial impact of anticoagulants in sepsis seems to be widely accepted. The treatment should be provided to improve outcomes, including early goal-directed therapy, early and appropriate antimicrobials, source control, corticosteroids, recombinant human activated protein C, and low tidal volume mechanical ventilation. [Nguyen HB et al.,2006] The treatment of sepsis includes anti-microbial and non-anti-microbial therapy as follows in table 8.

Table 8: Majorly used anti-microbial and non-anti-microbial therapies for the treatment of sepsis.

Antimicrobial therapy	**Non-antimicrobial therapy**
antibiotics: mainly broad-spectrum antibiotics	Steroids Coagulation pathway Non-antibiotic, non-immune drugs: Statins ACE inhibitors Renin-angiotensin-aldosterone system modulators β-Blockers

1.8.1. Anti-microbial therapy:

Anti-microbial therapy is crucial for successful sepsis treatment. The immediate administration of antibiotics for all patients with suspected sepsis and septic shock, ideally within 1 hour of recognition is recommended by the surviving sepsis campaign. The cornerstone of the treatment is the prompt initiation of broad-spectrum antibiotics after sampling of suspected site of infection for cultures, this is recommend based on clinical studies showing an increase in mortality related to the delay to infuse antibiotics in severe sepsis patients [Du B, et al.,2002]. Intravenous antimicrobial therapy should be started within the first hour of recognition of severe sepsis, after appropriate cultures are obtained. Because this treatment is given before culture results are available, it is critical to provide an appropriate treatment. The broad-spectrum antibiotics should be de-escalated as soon as the culture results are obtained [Hong Zhu.et al.,2020]. Delaying antibiotic therapy beyond 3 hours after diagnosis is related to an increase in mortality. It appears that the risk of increased mortality in patients with sepsis without shock increases around the 3- to 6-hour mark until antibiotics are administered and that for patients with septic shock, urgent medications is recommended. [Xiang Zhou et al.,2023] Proposed a 6Rs rule for anti-infection therapy for sepsis, with the goal of establishing a rigorous and scientifically based clinical therapeutic procedure that includes rational decisions regarding treatment timing, pathogen identification, antibiotic selection, formulation of a scientifically based antibiotic dosage regimen, and adequate control of infectious foci. This rule will have a positive impact on improving infection control in patients with sepsis. The severe sepsis and septic shock management bundle (SEP-1) provided the guidelines for the treatment of sepsis, with a multicomponent 3-6 h treatment which includes antibiotic usage, fluid resuscitation, blood culture and lactate measurement, the use of vasopressors for fluid-refractory hypotension and the bedside evaluation of a patient’s response to treatment.SEP-1, recommended appropriate use of antibiotics with monotherapy and combinational therapy for the use of sepsis as follows in table 9. [FDA Drug Shortages. Accessed: Aug 2016] & [Jeremy Weinberger et al.,2013].

Table 9: Recommended antibiotics with monotherapy and combinational therapy:

Monotherapy antibiotics	Combination antibiotic therapy	References
carbapenem doripenem cefepime imipenem/cilastatin meropenem cefotaxime cetazidime ceftriaxone ceftaroline fosamil piperacillin/tazobactam	aminoglycosides + cephalosporins/daptomycin/glycopeptides/ linezolid/penicillins aztreonam + daptomycin/glycopeptides/ linezolid/penicillins/clindamycin iv	Sligl WI, et al.,2015; Dalhoff A.2012; Gasnik LB, et al., 2007; Huh K. et al.,2014; Labby KJ.2013; Cadena J, et al.,2012; Wilson SE, et al.,2008; Solomkin JS ,2010.

1.8.2. Non-antibiotic / adjunctive therapy:

The non-antibiotic measures focus on supportive care, includes: [J. Cohen,2009]

- The administration of intravenous fluids (crystalloid solutions such as 0.9% sodium chloride solution, or colloid solutions such as 5% albumin solution) to restore and maintain adequate intravascular volume [Fang Liu et al.,2021].
- Monitoring lactate levels.
- Removal of unnecessary catheters and daily interruption of sedation remain the best way to improve the outcome of septic patients.

There are three broad approaches to non-antibiotic adjunctive therapy to consider as mentioned in table 10.

Table 10: General approaches to the adjunctive treatment of sepsis: [María Luisa Martínez et al.,2020]

Strategy	Example
Improve supportive care	Oxygenation/ventilation strategies; optimize fluid/vasopressor use; early goal-directed therapy
Target bacterial virulence factors	Anti-endotoxin antibodies, endotoxin-removal columns

Strategy	Example
Target host response factors	Corticosteroids; anti-cytokine drugs; anticoagulants

1.8.2.1. Anti-inflammatory therapy:

Antibiotics are known to treat the primary infection, yet 30 percent of patients with severe sepsis will die despite successful antibiotic therapy because the body's host response is out of control and turns on its bearer [Hosny et al, 2013]. A fundamental theory driving sepsis research in recent years is that this syndrome is caused by excessive inflammation [Antonelli, M et al., 1999]. Depending on the patient's immunological condition, approaches to treating sepsis could involve dampening the rise of inflammatory cytokine levels or neutralizing their activity [Banyer, J. L. et al.,2000]. TNF-inhibitors, nuclear factor-β blockers, neutrophil chemotaxis, platelet activating factor hydrolase inhibitors, and platelet activating factor antagonists are among them. During hyperinflammation the immunomodulatory drugs are used to dampen the inflammatory response, such as hydrocortisone, IVIG, or MSCs. Other promising candidate drugs such as short-acting beta -blockers, selepressin or levosimendan, act on the cardiovascular system. Three broad anti-inflammatory strategies are most used for the treatment of sepsis [Niels C Riedemann et al.,2003&2015].

Corticosteroids:

The commonly used Corticosteroids are hydrocortisone and vasopressors. First, glucocorticoids administered at the onset of sepsis in physiological doses to patients with septic shock requiring vasopressors are effective at restoring hemodynamic stability. The glucocorticoids in high doses appear harmful. Potentially, different dosing regimens or patient selection may render other previously studied anti-inflammatory agents similarly beneficial. The hydrocortisone is a promising adjuvant treatment for community-acquired responsiveness, treatments that stimulate the immune system during immune paralysis such as GMCSF or INF gamma and the hydrocortisone mechanism is not clear if its beneficial effect is from reversal of steroid hypo responsiveness, a purely anti-inflammatory effect, or a combination of both. Corticosteroids at low dose may be beneficial, with lower in-hospital mortality, faster resolution of shock, and reduced organ dysfunction. Corticosteroids' role in sepsis patients has been the subject of much debate and investigation [Chao Ren.et al., 2017].

Anti-endotoxin strategies:

Multiple approaches have been evaluated to disrupt the initial interaction between endotoxin and innate immune cells and currently there are no approved pharmacologic interventions targeting endotoxemia. Studies of anti-endotoxin antibodies suggest that these agents lack efficacy [Sergio L. et al., 2009]. Novel compounds, such as lipopolyamines and BPI, appear very effective at blocking the activity of endotoxin both in vivo and in vitro. The efficacy of these agents has yet to be reported from a human sepsis trial [Elisabeth C. van der Slikke et al., 2021].

Anti-cytokines therapies:

Anti-TNF agents:

[Bradley D freeman et al., 2000] reported the anti-TNF treatments resulted in a slight reduction in mortality, as evidenced by 17 trials. Although anti-TNF medications have become standard of care for a variety of autoimmune inflammatory disorders such as arthritis and inflammatory bowel disease, they are not currently used in the treatment of sepsis. It has been demonstrated that the injection of TNF antagonists improves survival in deadly sepsis animal models. This is not surprising given that the most extensively researched adjuvant treatment of sepsis involves neutralizing TNF-specific antibodies, antibody fragments, or antibody-like fusion proteins [Ali, T., Kaitha, S. et al.,2013].

Table 11: Anti-TNF agents for the treatment of sepsis:

Anti-TNF agents	**Role in treatment of sepsis**	**Reference**
Afelimomab TNF-specific monoclonal antibody	Improve survival with ↓ I IL-6 in sepsis patients with marginal reduction of mortality	[Jeremy Weinberger et al., 2020]
CytoFab polycloncal anti-TNF antibody fragment	Effectively ↓ serum BAL TNF-α and IL-6 levelsin severe sepsis patients and increasing the number of ventilator-free and ICU-free days at day 28 of Phase –II clinical trials	Clinical trial.gov: NCT01144624
Talactoferrin alfa Recombinant human protein	Phase –III trials for safety and efficacy of Talactoferrin Alfa in Patients with severe sepsis (OASIS) was suspended by data safety monitoring board recommendation.	Clinical trial.gov: NCT00854633

IL-1β antagonist: Like TNF, IL-1β also improved survival in a sepsis animal model with recombinant IL-1RA. Dexamethasone is well known to profoundly inhibit IL-1β production and is beneficial for reducing symptoms of cytokine storm in COVID-19. Phase II trial of Anakinra (recombinant human IL-1RA) reported the significant, dose-dependent reduction in 28-day mortality, whereas Phase III studies, however, failed to validate the encouraging findings.

Other pro-inflammatory cytokines:

Monoclonal antibodies directed against the IL-6 receptor – tocilizumab and sarilumab – have not been evaluated for the treatment of sepsis, and improved survival in patients with severe COVID-19 [Sara La Manna et al., 2018] .IL-1ra has also been studied in COVID-19 patients with sepsis. Although there is no convincing evidence of benefit, it may improve survival in individuals with hyperinflammatory states, as seen by elevated plasma levels of soluble urokinase plasminogen activator receptor (SUPAR).

Clinical trials in sepsis have recently concentrated on blocking specific host pro-inflammatory mediators (e.g., TNF, interleukins). While individual trials of inhibitors of these pro-inflammatory mediators failed to show a convincing benefit, pooling the results of these trials suggest that this approach has a marginal effect, supporting a role for excessive inflammation in sepsis. Therefore, it is important to investigate additional inflammatory antagonist-based treatments with the aim of developing a clinically effective antisepsis drug.

Anticoagulant therapy:

After anti-microbial and anti-inflammatory drugs, anticoagulant therapy is most common used drugs to improve the survival of patients with sepsis and sepsis induced DIC. Coagulopathy and DIC are associated with increased mortality in sepsis, which is due to the two factors. First, the immune system closely related to coagulation system, when one of the factors are manipulated, the homeostasis between them is disturbed. A second factor may be failure to identify patients who will benefit from anticoagulation therapy. The most used anti-coagulant is heparin. The only specific pharmacologic agent for the treatment of sepsis that was approved by the FDA: recombinant human activated protein C [drotrecogin alfa, Xigris Eli Lilly, Indianapolis, IN], [Abraham, E., et al.,2005; Amisha Barochia et al.,2011& Anita Marie Hosac et al.,2002] was only an anticoagulant with immunomodulatory and anti-inflammatory properties, has shown to be efficient in severe sepsis

[Bernard, G. R et al.,2001]. The drug was approved in 2001 for patients with severe sepsis, as there was no reduction of mortality in any of the subgroups tested 121, and the efficacy has been questioned by EMA & FDA, drug was withdrawn from the market on October 25, 2011. Selepressin (Ferring Pharmaceuticals), V1a receptor agonist recommended for the treatment of sepsis Phase –III clinical trials was terminated due to futility results [ClinicalTrials.gov identifier: NCT02508649].

Imatinib, an inhibitor of the tyrosine kinase Abl-related gene, attenuated thrombin- induced endothelial barrier dysfunction in a murine polymicrobial sepsis and lung injury mouse model by stabilizing cell–matrix interactions, and reduced organ edema model, while exacerbating ventilator-induced lung injury [Bakhtiari, K et.al.,2004].

Recombinant human thrombomodulin, known as ART 123 (Recomodulin; Asahi Kasei) has been approved for the treatment of DIC for the treatment of patients with severe sepsis and coagulopathy, a phase III RCT of the drug trials was completed and waiting for the approvals [ClinicalTrials.gov identifier: NCT01598831].

Recently, researchers at the royal college of surgeons in Ireland (RCSI), Dublin, Ireland, found Cilengitide, brand name InnovoSep, the first non-antibiotic therapy medication for Sepsis. InnovoSep was used as Prophylactic treatment for patients with sepsis with severe infection to prevent progression to sepsis and its mechanism involves the inhibition of the endothelial cell injury from advancing to septic shock and organ failure. This technology represents a first-in-class, non-antibiotic approach to targeting bacteremia and severe bloodstream infections. This treatment represents a first-in-class, non-antibiotic strategy to treating bacteremia and serious bloodstream infections [Medha Baranwal,2019].

Anti-nitric oxide therapy:

Sepsis-related hypotension implies endothelial instability indicated by hypo responsiveness to catecholamines. Endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) have been hypothesized as the primary causes of hypotension in sepsis. NOS inhibitors, on the other hand, were not uniformly beneficial in alleviating sepsis-induced hypotension. These drugs have a wide range of actions, but in high dosages they behave as long-acting vasoconstrictors. Non-selective NOS inhibitors dramatically increased mortality rates in patients with septic shock in high dosages, according to reported trials. Similarly, 546C88 (l monomethyl arginine, an isoform-unselective nitric

oxide synthase (NOS) inhibitor) considerably and dramatically reduced survival in septic shock patients [Schwartz S et al., 1997].

1.9. Pipeline of drugs for the treatment of sepsis:

Significant progress has been achieved in the knowledge and management of sepsis. Sepsis, on the other hand, has no specific treatment and this may include new antibiotics, anti-inflammatory, and anti-coagulant medicines, however only therapeutic niches are available. Even the new pan-specific vaccines that outperform antigenic drift, as well as diligent precautions to prevent both hospital- and community-acquired infections, in conjunction with new cytoprotective treatments for microvascular endothelium, suggests that the rise in sepsis cases can be reversed. Below table 12 summarizes the proven and experimental antisepsis regimens addressed proposed previously for bacterial, fungal and viral infections. and the table 13 mentions the emerging pipeline drugs of sepsis [J. Hawiger et al.,2015].

Table 12: Compilation of antisepsis regimens used currently as preventive and proven therapeutic measures. In addition, some experimental therapeutic measures and proof-of-concept measures awaiting preclinical testing are listed below.

Recommended strategy	Regimen	Outcome	Ref.
Preventive measures	Vaccination	Reduced incidence	[Sharawy N.2014 & Rauch S,2014]
Proven therapeutic measures (Undetermined)	Empiric /Pathogen-directed antimicrobial therapy + fluid resuscitation/ vasopressors + respiratory therapy	Increased survival	[Dellinger RP,2008; Wong G, Kobinger GP,2015]
Experimental therapeutic measures	The below mentioned regimens are successful in Polymicrobial peritonitis (mice) Recombinant SLIT protein Pepducin (protease-activated receptor 1 peptide agonist) Imatinib (Abl-related gene kinase inhibitor)	Increased survival	[London NR,2010; Veach RA,2012; Kaneider NC,2007]
Proof-of-concept measures	Pathogen-directed antimicrobial therapy + nuclear transport modifier (cSN50) Intracellular protein therapy with recombinant cell-penetrating (SOCS-3 &CRADD)		[Qiao H, Liu Y,2014; Fletcher TC,2010 & Jo D et al.,2005]

Table 13: Summary of the competitive environment for emerging drugs in sepsis [Sepsis - Global Drug Forecast and Market Analysis to 2030]

COMPOUND	Structure	Company	Indication	Mechanism of action	Stage of development
M6229	Non-anticoagulant	Matisse Pharmaceuticals	sepsis	Acts by neutralization of cationic histones.	Phase I clinical studies
HY 209	Gel formulation of taurodeoxycholic acid	Shaperon	Sepsis	G protein-coupled receptor 19 (GPCR19) agonist	phase I clinical trials
Thymosin alpha 1 -Zadaxin	synthetic 28-amino acid peptide	Sci Clone Pharmaceuticals	Hepatitis infections and being developed for the treatment of COVID 2019 infections.	Immune response enhancement	Phase III clinical trials for sepsis
Reltecimod	Peptide	Atox Bio	Necrotizing Soft Tissue Infections	CD28 receptor antagonist	Phase III clinical trial Completed.
Alirocumab	Proprotein convertase subtilisin kexin type 9 (PCSK9) inhibitor monoclonal antibodies	Regeneron/Sanofi	Cardiovascular dysfunction of sepsis.	Blocking the production of LDL cholesterol, therefore decreasing the amount of cholesterol that may build up on the walls of the arteries.	Phase II: Sepsis Phase III: Septic Shock
STC3141	Histone neutralization drug	CGE Healthcare	sepsis	Acts by neutralizing extracellular free histones and neutrophil traps and reverse the organ damage	Phase Ib clinical studies
Rezafungin (formerly	Echinocandin drug	Cidara Therapeutics	candidemia, invasive candidiasis and	Acts by depletion of 1,3-β-D-glucans in the cell	Phase III, FDA has granted

known as CD101)			prophylaxis of invasive fungal infections	wall resulting in osmotic instability and the subsequent cell death and/or inhibition.	QIDP and fast track designations.
VBI-S	Phospholipid nanoparticles	Vivacelle Bio	Hypovolemia treatment due to sepsis/septic shock.	The exact mechanism of action behind this is currently unknown.	Phase II clinical trials
SY 005	Dianexin based therapeutics	Lawson health research institute and Yadao Pharmaceuticals	Sepsis	The exact mechanism of action behind this is currently unknown.	Initiated phase I in 2019.
CYT107	Interleukin-based products Interleukin-7 human recombinant drug.	Revimmune & Amarex clinical research	sepsis	Help restore and maintain lymphocyte counts, reversed the marked loss of $CD4^+$ and $CD8^+$ immune effector cells, a hallmark of sepsis and a likely key mechanism in its morbidity.	Phase II clinical trials Terminated (POOR ACCRUAL) in 2020.

1.10. Market review:

The market size of sepsis is large because, in the US or the EU, severe sepsis affects about 750,000 hospitalized patients, as 15% of patients develop severe sepsis and septic shock in ICU'S. The global sepsis diagnostics market size was valued at over USD 615.4 million in 2021 and is projected to expand at a moderate compound annual growth rate (CAGR) of 9.6% from 2022 to 2030. The US is the largest market of worth 7MM's, accounting for 69.5% of the market in 2020. This is expected to rise to 77% by 2030. The tremendous market potential for sepsis is offset by the failure of several medication candidates for this indication that have not showed clinical benefit in the past. Successful treatment candidates' failures can be attributed in part to a rudimentary understanding of complex sepsis biology, unsophisticated and poor clinical trial design, and an overreliance on preclinical models for proof-of-concept [Kaplan BI et al.,2007].

According to the Global Sepsis Alliance, the outbreak of novel coronavirus (COVID-19) raises the risk of developing sepsis while simultaneously raising demand for diagnostics solutions. Hospitalized COVID-19 patients are more likely to develop coinfections, and among non-survivors, over 50% had a secondary infection, nearly 100% had sepsis, and approximately 70% had septic shock. It was discovered that patients with severe COVID-19 from Seattle experienced severe septic shock, resulting in an increased demand for medications to support heart and blood circulation. Most existing diagnostic tests can currently take up to 72 hours to process and produce results. Thus, there is a significant demand in the market for rapid diagnostic tests for the early detection of sepsis, which could save thousands of lives; an increase in investment by pharmaceutical and biotechnology businesses in medication discovery; and an increase in the launch of new products for the treatment of sepsis [GVR Report: Sepsis Diagnostics Market Size, Share & Trends Analysis Report Report ID: GVR-1-68038-352-2]. For example, Beckman Coulter launched its new DxH 690T hematology analyzer with early sepsis symptoms in 2020. Researchers at the University of Strathclyde discovered a low-cost test for earlier detection of sepsis in 2019. This test uses a microelectrode to evaluate the patient's blood and produces results in around two and a half minutes. In 2018, Sysmex Corp. of Japan announced the release of their new HISCL presepsin Assay Kit to assess presepsin levels, the primary test parameter for bacterial sepsis. As a result, players in the sepsis treatment market have been pushed to assure the availability of medicines and medical supplies to improve patient quality of life [transparency market research Rep ID: TMRGL81989, April 2021].

1.11. Medical need:

The history of therapeutic interventions in sepsis clinical trials has been dubbed the "graveyard for pharmaceutical companies." That is about to change, as new research offers hope for new techniques that will be therapeutically successful in patients suffering from sepsis [Kunj Gohil,2012] Unfortunately, sepsis-specific therapy choices are limited, and Monitoring, pathogen-targeting treatments, supportive care, and symptomatic treatment are used to manage patients with sepsis. The judicious use of current antibiotics can help reduce mortality, but antimicrobial therapy is not enough to improve the survival in large number of patients. Because the variability among patients with sepsis is very large and due to limitations of antimicrobials, combination therapies may be needed to adequately manage an infection or there is a need to have dedicated drugs according to the immune response of each individual [Safiah Mai et al.,2012]. To date, no immunomodulatory drugs have shown better efficacy than placebo in large randomized clinical trials. However, adverse effects related to safety and tolerability towards present prescribed drugs is an ongoing challenge. To overcome this challenge, Research is constantly ongoing at the bench to discover a new candidate target for the treatment of sepsis that has the potential to stop sepsis before the condition reaches major organs and becomes fatal. New licensed antibiotics will probably offer the opportunity to clinicians to refine their treatment choice. Nonetheless, research studies have revealed a significant regulatory role of pro- and anti-inflammatory cytokines in illness progression and have highlighted many diverse pathophysiologic processes implicated in sepsis. These discoveries have resulted in the development of promising anti-cytokine and immunomodulating therapeutic approaches. Peptide anti-infective therapies with antibacterial and immunomodulatory characteristics are a novel approach to infection treatment.

1.12. Therapeutic strategies for sepsis:

The targeted therapeutic strategies for sepsis include anti-microbial or immune response drugs. Antimicrobial de-escalation is considered as one of the therapeutic strategies to replace the broad-spectrum antimicrobial treatment using a narrower antimicrobial therapy. However, this type of therapeutics in sepsis remains unclear [Bingging yang. Et al.,2012]. The targeted therapeutic regimens call for novel antimicrobial pipelines due to increasing resistance crisis, these including narrow-spectrum agents, engineered probiotics, nanotechnology, and phage therapy [Amit Pant et al.,2021]. In addition, antibody strategies were also included. In terms of all strategies mentioned,

they can categorize into combination therapy. The combination therapy is more beneficial due to high delivery efficiency, enough local concentration, synergistic effect, and low resistance frequency than monotherapy.

Key therapies that have led to mortality benefits in severe sepsis/septic shock are directed at reversing these pathogenic mechanisms which involves mainly immune response and cytokines [Brett I Kaplan et al.,2007].

Several additional inflammatory mediators of the SIRS/CARS axis have been found, including the complement C5/C5a protein, high-mobility group box-1 (HMGB1), the adenosine A2 receptor, pro-apoptotic molecules, and TLRs [Meaad A. et al.,2021]. Specific anti-cytokine treatments have not been successful in clinical trials thus far since sepsis complex syndrome involves excessive and suppressed inflammatory and immunological responses and affects varied patient populations with diverse disease aetiologies and comorbidities. Recent advances in understanding of innate immunity, and the interaction between the inflammatory and the haemostatic cascades, are affording new opportunities for therapeutic development. With increased understanding of cytokine effects in recent years, several treatment options targeting cytokines and other immunomodulating drugs for clinical use in septic patients have been proposed [Heming N, et al.,2016]. Thus, it is critical to investigate novel paradigms for anti-infective therapy. One intriguing method is host-directed immunomodulatory treatments, which use natural host mechanisms to increase therapeutic benefit. The goal is to establish or boost antimicrobial immunity while limiting inflammation-induced tissue damage. Innate defence regulator peptides and agonists of innate immunity components such as Toll-like receptors and NOD-like receptors have been postulated as potential immune modulators. An immunomodulatory treatment can be adjunctive to primary treatment with an antibiotic. They are largely designed as adjunctive therapies to support and extend the effectiveness of antibiotics and antivirals. Immunomodulation offers certain advantages by targeting the host rather than the pathogen, thus largely avoids selective pressure for the evolution of microbial resistance [Robert E W Hancock et al.,2016].

Reducing costs and increasing chances of success are important barriers to re-engaging industry in sepsis therapeutic development. Also, it is expecting that novel antisepsis strategies have been tested preclinically or clinically for efficacy from in sepsis and will continue to be clinically assessed for the more effective future treatment of sepsis [Angus DC et al., 2001].

An artificial intelligence algorithm was developed, SERA algorithm, which helps to early prediction and diagnose before its onset sepsis in a patient gives clinicians additional lead time to plan and execute treatment plans, thereby lowering mortality [Ivana Pepicetal,2020] and plays a role in designing the small peptide antibiotics against multi resistant pathogens [Cherkasov A, 2009]. These could be developed to augment healthcare workers' knowledge and improve their decision making in the future.

1.12.1. Gaps conclusions in therapy of sepsis:

All the strategies listed above can be classified into two types: monotherapy and combination therapy. Due to high delivery efficiency, sufficient local concentration, synergistic effect, and low resistance frequency, the latter usually outperforms the former. The emergence and fast spread of antibiotic resistance in pathogenic bacteria pose a global hazard to public health. Despite continued efforts to address this dilemma, the discovery of new strong antimicrobials is moving far slower than the emergence of drug resistance. People are clearly entering the "post-antibiotic" era, in which a resistance crisis has emerged, and many antimicrobials are no longer effective. Abuse of broad-spectrum antibiotics not only accelerates resistance formation, but also places a strain on the gut flora, which plays an important role in human homeostasis [Bingqing Yang et al., 2021 & John C. Marshall et al.,2022]. The growing antimicrobial resistance dilemma necessitates the development of innovative antibacterial pipelines, particularly for targeted therapeutic techniques. Narrow-spectrum drugs, engineered probiotics, nanotechnology, phage therapy, and CRISPR-Cas9 technologies are among five promising targeted regimens. Furthermore, antibody generation against pathogens causing infection is another example of several antibodies against Staphylococci, P. aeruginosa, were previously licensed for clinical use or are currently in various stages of clinical efficacy testing.

1.12.1.1. Narrow-spectrum antimicrobial agents: Peptides as therapeutic agents for infectious and inflammatory-related diseases.

The broad-spectrum antimicrobial agents are the first line of treatment followed for years, which is suitable for clinically common mixed infections. However, the indiscriminate killing mode of broad-spectrum agent's results in the death of all bacteria, thus disturbing the microbiota balance and even inducing the superinfection. Along with search of novel antibiotics due to multi drug resistance (MDR), to dampen the inflammatory response, more selective and innovative anti-inflammatory

therapy strategies are also required. Thus, alternatives are in search for anti-microbial with immune modulatory therapeutics; several of them imply the use of peptides. On the contrary, more focused narrow-spectrum antimicrobial medicines, such as narrow-spectrum antibiotics, antimicrobial peptides (AMPs), and lysins, show the potential to overcome this quandary and achieve targeted therapy. In this context, the search for novel antibiotics has become more intense in recent decades, has led to enormous interest in antimicrobial peptides (AMPs) as suitable compounds for developing unusual antibiotics.

1.12.1.1.1. Antimicrobial peptides (AMPs):

These peptides serve as a host defense against microbial infections. Antimicrobial peptides (AMPs) represent a relatively new discovery in the immune system pathway; serve as a potential alternative to available antibiotics, these peptides are cationic due to the presence of lysine and arginine residues and range in size from 12 to 50 amino acids. and amphipathic molecules appear to be ideal candidates with diverse and multiple action modes that, in addition to immunomodulatory activities, may directly lead to bacterial death. Moreover, about 50% of the amino acids in AMPs are hydrophobic and they can adopt amphipathic structures, which enables them to interact with and penetrate cell membranes. Antimicrobial peptides, due to their distinct mode of action, are novel alternatives to standard antibiotics for combating bacterial multidrug resistance. Naturally occurring peptides as well as synthetic derivatives have been shown to exhibit potent antimicrobial, anti-biofilm, and immunomodulatory properties. Their application may be impeded by high toxicity, hemolysis, nephrotoxicity, and neurotoxicity .and so recent designs of synthetically engineered short, specific antimicrobial peptides have demonstrated increased potency and efficacy/tolerability, enhanced specificity, and reduced toxicity in comparison. These peptides, known as designed antimicrobial peptides (dAMP), are resistant to such effects of high solute levels and exhibit even greater action. So, even after extensive design and screening, AMPs with activity must still be tested for protease stability and toxicity [Hancock RE, Rozek et al., ,2000 &2002].

For example, the antimicrobial peptide database (APD) potential peptides F1 and F4 with α-helical symmetrical structure demonstrated short, safe, and stable efficacy against pathogens such as *E. coli* and *P. aeruginosa* [Chou et al., 2019]. ZY4, a cyclic peptide, not only induced membrane permeabilization in bacteria with a low resistance frequency, but it also showed exceptional resistance to persisted cells. It has been shown to be more effective against multidrug-resistant *P. aeruginosa* and infections than *S. aureus* [Mwangi et al., 2019]. These peptides, known as designed

antimicrobial peptides (dAMP), are resistant to these effects and have a stronger antibacterial activity. Bacteria-targeting peptides nanoparticle-aided systems (nanotechnology) have recently outperformed in terms of transport efficacy and plasma half-life. [Hussain and colleagues, 2018] vancomycin-loaded nanoparticles with the cyclic 9-amino-acid peptide CARGGLKSC (CARG) reduce staphylococcal infections more effectively in vivo than untargeted therapy. Due to their unique features, magnetic nanoparticles (MNPs) might also be used as affinity probes to preferentially enrich *S. aureus* with the peptide HHHHHHDEEGLFVD (D) [Kuo et al., 2016]. Lipid-based nanocarriers (NLCs) with combination anti-MRSA and anti-inflammatory properties, such as ciprofloxacin and rolipram, outperformed free medicines in treating MRSA bacteremia [Chia-Chih Liao et al.,2021].

Designed antimicrobial peptides (dAMPs): Host Defense Peptides (HDPs).

Designed antimicrobial peptides (DAPs) are synthesized peptides that demonstrate a wide spectrum of antibacterial activity and synthetic peptides with **Immunomodulatory action are known as Host Defense Peptides (HDPs) [**Bommarius B, Jenssen H et al., 2010 & Nicole J. Afacan et al,2012]. Cationic HDPs are small peptides that typically contain an abundance of positively charged and hydrophobic residues. HDPs and their synthetic derivatives have broad antimicrobial and immunomodulatory activities, exhibiting a broad range of anti-infective activity through both direct and adjunctive (immunomodulatory) action, which has many of the hallmarks of successful anti-infective therapies, particularly rapid action, and broad-spectrum antimicrobial activities. As a result, these peptides have the potential to be a completely new treatment method against bacterial infections. LL-37 (residues 1-13) and defensins affect innate immune cell functions, including the induction and modulation of chemokine and cytokine production, direct chemoattraction of immune cells, angiogenesis promotion, and wound healing and improve the survival of cecal ligation and puncture septic mice [Braff MH, Hawkins MA et al., 2005; Oren Z, Lerman JC et al., 1999 & Tjabringaa GS et al.,2006].A synthetic peptide called clavanin-MO, which exhibits potent antimicrobial and immunomodulatory properties both in vitro and in vivo [Silva, O. N et al., 2016]. The immunomodulatory properties of HDPs have been studied extensively over the last decade and considerable effort has been made to generate synthetic peptides with enhanced immunomodulatory activities. Most studies have reported immunomodulation at the level of innate immunity, refer to the synthetic peptides as **Innate Defense Regulator (IDR)** peptides [Nijnik A, Yang D, Chertov O et al, 2001& Mookherjee N et al.,2007]. The immunomodulatory activities of HDPs and IDR peptides

explain their ability to treat microbial infections. Anti-infective peptides can be biologically active because of their ability to manipulate immune-cell function, direct antimicrobial activities or a combination thereof [Jesse M. Jaynes et al.,2012; &Mohamed F Mohamed et al.,2016].

Physical properties:

The following are the physical characteristics that HDPs share in common.
HDPs typically range in length from 12 to 50 amino acids, with a preference for basic over acidic amino acids, giving them a net positive charge of +2 to +9. Additionally, about 50% of amino acids have hydrophobic properties. When linear HDPs come into contact with lipid bilayers, the presence of basic and hydrophobic amino acids encourages the folding of the molecules into amphipathic secondary structures. The biological characteristics of HDPs are dependent on particular structural features, according to studies on structure-function relationships. According to the secondary structures that HDPs have acquired, there are four structural classes of HDPs, and they are as follows [Yang D et al., 2001].

1. β-sheet (e.g., defensins)
2. α -helical (e.g., cathelicidins, magainin).
3. Looped peptides (e.g., bactenecin) and
4. Extended structures rich in arginine, glycine, histidine, proline, and/or tryptophan.

The first two classes are the most, and the latter two classes are less common HDPs.

Therapeutic potential of HDPs: [Wu M, Maier E, et al,1999]

- **Anti-microbial activity**.

  Synthetic HDPs exhibit a broad spectrum of anti-infective activity, including direct microbial killing, endotoxin neutralization, immune cell recruitment, modulation of pro-cytokine/chemokine production, suppression of potentially harmful inflammation, induction of cell differentiation, enhancement of adaptive immune responses, cell survival, wound healing, and angiogenesis [Hancock RE, et al., 2006].

- **Anti-endotoxin Activity**

  Many antibiotics stimulate the release of endotoxin (LPS), is a classical occurrence of systemic inflammation and deadly syndromes like chronic infections and sepsis. HDPs could

reduce the pro-inflammatory mediator's levels such as tumor necrosis factor alpha (TNF-α) via inhibiting or regulating toll-like receptor (TLR) signaling pathways, as well as in certain circumstances, by direct LPS binding. These activities may be responsible for the potent anti-inflammatory activity.

- **Immunomodulatory Activities**

  Enhancing cell survival and polarizing the adaptive immune system are two aspects of HDPs' immunomodulatory effects (adjuvant activity). The selective immunomodulatory capabilities of HDP against multi-drug resistant infections by acting on the host to boost protective immunity while modulating excessive inflammation, represents the starting point for novel therapies. Mitogen-activated protein kinases (MAPK), p38, JNK, extracellular signal-regulated kinase-1/2 (ERK1/2), Src-family kinases, NF B (transiently), and PI3 kinase pathways are a few of the important signaling pathways that contribute to HDP immunomodulation [Opal SM, 2010].

The synthetic HDPs derivatives possess several attributes as anti-infective therapeutics targeted to MDR pathogens and the small size of HDPs makes them potential prototypes, by enhancing chemokine production, inducing chemotaxis, and block endotoxin responses and acts via a wide acting innate immune system rather than directly on microbe there should not be resistance, the multifunctional nature of these peptides represent excellent candidates to treat infections; especially those caused by MDR pathogens, particularly in combination with other antimicrobial therapies and might be the new approach for the clinical success of antimicrobial HDPs as novel immunomodulatory drugs. The most relevant activities of HDPs are described below. Thus, the following sections explore HDPs in preclinical development or clinical trials, as well as peptides recently identified as potential lead compounds (table 14).

Table 14: Host Defense Peptides in clinical trials and potential lead peptides with described activity:

Drug/peptide	Description	Intended use	Progress	References*
Immunomodulatory/Antimicrobial				
EA-230	β-hCG fragment	Sepsis	Phase II	NCT03145220
AP214 (Action Pharma)	a-MSH derivative	sepsis-induced kidney failure	Phase IIb	NCT 00903604 NCT 01256372
Talactoferrin (Agennix)	Lactoferrin derivative	Non-small cell lung cancer, diabetic ulcers and renal cancer, sepsis, cancer	Phase I/II/III	NCT 00923741 NCT 00630656 NCT 00095186
hLF1-11 (AM-Pharma)	Lactoferrin derivative	Prevention of bacteraemia and fungal infections	Phase II	NCT 00509938
OP-145 (Octoplus)	LL-37 derivative	Treatment of chronic middle ear infection	Phase I/II	http://www.octoplus.nl/index.cfm/octoplus/products/op-145/index.cfm
IMX942 [IDR-1] (Inimex)	Indolicidin derivative	Nosocomial infections, neutro-penia	Phase Ia	www.inimexpharma.com/documents/pressrelease_firstclinicalstudy_apr2709.pdf
Antimicrobial/Immunomodulatory				
Pexiganan acetate [MSI- 78] (Macrochem)	Maganin derivative	Topical antibiotic- diabetic ulcers	Phase III	NCT 00563394
PMX-30063	Defensin	Systemic antibiotic	Phase Ib/II	NCT 01211470

(Polymedix)	structural mimetic	Acute Bacterial Skin and Skin-structure Infection (ABSSSI) Due to Staphylococcus Aureus (MSSA)		
LTX-109 (Lytix Biopharma)	Peptidomimetic	Topical antibiotic	Phase II	NCT01223222
PAC-113 (Pargen bio-pharmaceuticals)	Histatin derivative	antifungal	Phase IIb	NCT 00659971
NZ2114/SAR215500 (Novozyme)	Plectasin Defensins derivative	Systemic antibiotic	Preclinical	http://www.novozymes.com/en/news/news-archive/Pages/45873.aspx
*International clinical trial registration number as indexed on www.clinicaltrial.gov Abbreviations: BPI, bactericidal/permeability-increasing protein; ESBL, extended spectrum beta-lactamase; HLA, human leukocyte antigen; MSH, melanocyte-stimulating hormone; MRSA, methicillin-resistant Staphylococcus aureus, MDR, multidrug resistant; VRE, vancomycin-resistant enterococci; β-hCG, beta-human chorionic gonadotropin.				

Chapter 2.
Gaps in Existing Research

2.1. Gaps in research –potential development issues in sepsis research:

I. In Sepsis Therapy:

1. Multi drug resistance to pathogens.

2. The pharmacologic interventions of existing therapies (Corticosteroids, Anti-endotoxin, pro-inflammatory strategies, and Anticoagulant strategies) are controversy which primarily reflects heterogeneity of treatment effect or biologic redundancy is unclear. As per the literature review, all the above therapies at low dose may be beneficial with lower in-hospital mortality, faster resolution of shock, and reduced organ dysfunction and their roles of pharmacologic interventions are not approved as a therapeutic target of sepsis are controversy. Targeting individual inflammatory mediators has not proven efficacious in sepsis, which primarily reflects patient heterogeneity or biologic redundancy is unclear, with the possibility of harm in septic patients [John C. Marshall et al.,2022].

II. In sepsis Clinical Trials:

1. Early efficacy of anti-sepsis strategies was not replicated in subsequent trials and failed to improve clinical trials, despite numerous clinical trials. Past trials in sepsis have failed due to the inclusion of non-selective, heterogeneous groups of patients. Efforts are needed to help define homogeneous groups of septic patients, through biological or clinical markers.

III. In sepsis Preclinical Trials: Why preclinical studies are unsuccessful in Translational research of sepsis?

1. The sepsis research weaknesses are also related to flaws in preclinical studies. None of the successful preclinical candidates of sepsis drugs have not shown to be efficient in man. This failure might be because of the use of animal models which do not adequately mimic human disease, or for inappropriate experimental design and/or reporting.

2. The animals used are usually young and still too heterogeneous.

3. Differences in the lack of co-morbidities, type of insult, duration, and supportive therapy in the animal models probably contribute to some of the differences in animal studies and clinical trials

in sepsis. For example, Antibiotics are often not administered in animal models, whereas in human sepsis, first line of drugs is antibiotics to fight infection. The ideal host response to fight infection in the absence of antibiotics and life support may be very different from that required in a modern intensive care unit setting [Jean A Nemzek et al.2008].

Though the animal models do not completely and truly mimic the clinical complexity and intrinsic heterogeneity of human sepsis in all the respects but still they provide an insight into specific components of this syndrome. In other words, successful clinical trials can be predicted by the success of pre-clinical trials using several distinct animal models. Thus, the ideal preclinical models should be standardized for the purpose of therapeutic intervention. The standard animal models must mimic a severe and chronic syndrome of sepsis and would accommodate clinical scenario of humans [Marcin F. Osuchowski et.al.,2018]. At the same time, an ideal sepsis model would be low-cost and cause no distress to the animals involved in terms of animal well-being is often a concern in sepsis research. These clinical scenarios and interventions have not been able to mimic this complexity in a single satisfactory pre-clinical model and considering regulatory guidelines, two animal models are to be used in preclinical screening. The common animal models of sepsis are as follows.

(a) Toxaemia models- Injection of an exogenous toxin (e.g., LPS)

(b) Cecal ligation and puncture (CLP) and Colon ascendens stent peritonitis (CASP)- surgical ‘immune barrier disruption models’: alteration of the animal’s endogenous protective barrier, such as intestinal leakage and bacterial infection models

(c) Infusion or instillation of exogenous bacteria.

Using standardized animal models may help to reduce some of the disparities between animal and human investigations, allowing a greater degree of translation [Stevens RD et al, 2009] For instance, LPS animal model is frequently used, its validity as a true sepsis model is debated due to the absence of an actual infection and so it is not considered as standard model, whereas cecal ligation and puncture is more appropriate and mimics human sepsis model and considered as gold standard model.

2.2. Scientific rationale:

The growing concerns of antibiotic resistance constitute a global threat for public health. In parallel, the concept of immunosuppression progressively emerged. This implies that novel antimicrobial strategies and restoring the immune response should be a priority in sepsis. Five promising targeted regimens including narrow-spectrum agents, engineered probiotics, nanotechnology, phage therapy, antibodies against pathogens and CRISPR-Cas9 technology, where the extensive research is ongoing, and some are in different phases of clinical efficacy Testing [Bingqing Yang et al.,2021].
Out of five targeted strategies mentioned above, narrow-spectrum antibacterial peptides (AMPs), show the possibility to cope up and achieve targeted therapy. Narrow-Spectrum Antimicrobial peptides (AMPs), also called host-defense peptides (HDPs), are important components of the innate immune system [Lazzaro et al., 2020] and not prone to induce drug resistance, thus showing broad prospects to perform as ideal antibiotic alternatives in this resistance era [Liu et al., 2021]. Though Narrow-Spectrum Antimicrobial peptides (AMPs) have been put into clinical use for decades, doubts and criticisms constantly exist due to their incomplete success with dose-dependent toxicity and short half-life in vivo. Besides, strategies based on database-filtering technology provided us inspiration to generate ideal, short, specific, and effective synthetic AMPs which are known as HDPS & IDR peptides [Lars Steinstraesser et al., 2009 & Chou et al., 2019]. The synthetic HDPs are developed after sophisticated designing includes structural modification and amino acid type replacement provides protease resistance and screening tests in terms of protease stability and cytotoxicity. (Liu et al., 2017) and (Braunstein et al., 2004). For instance, AMPs like thanatin from Podisus maculiventris and ZY4, a cyclic peptide showed supreme potency and be able to combat with MDR [Ma et al., 2019& Mwangi et al., 2019]. Some synthetic AMPs like Clavanin-MO (FLPIIVFQFLGKIIHHVGNFVHGFSHVF-NH2) [Osmar N. Silva et al.,2015] and vancomycin-loaded nanoparticles with the cyclic 9- amino-acid peptide CARGGLKSC (CARG) is more effective suppression of staphylococcal infections in vivo than untargeted treatment [Hussain et al, 2018], proved potent antimicrobial and immunomodulatory properties both *in vitro* and *in vivo.*

2.2.1. Final thoughts: immune-enhancing therapy.

Because sepsis is an inflammatory condition, much of the research focuses on components of the innate immune response. Both antibacterial and/or immunomodulatory activities are essential components of the innate immunity mechanism, which bears many of the hallmarks of effective anti-

infective therapy. One such emerging novel anti-infective therapy includes Host defense peptides (HDPs). Host defense peptides boost infection-resolving immunity by modulating the innate immune response and, while dampening potentially harmful pro-inflammatory responses both *in vitro* and *in vivo* and further helps resolve infections through its immune modulatory properties. Where the novel synthetic HDPs need to evaluate for their antimicrobial and immunomodulatory properties. Thus, the Peptide anti-infective therapeutics represents a new approach to treat antibiotic-resistant infections with combined antimicrobial and immunomodulatory properties.

2.3. Conclusion:

A complex interplay of host response and pathogen dynamics leads to varying outcomes and therapy responsiveness in sepsis. Therefore, these patients require specific strategies directed to restore the function of immune response beyond the antibiotic therapy and standard supportive treatments, including inhibition of proinflammatory cytokines and immunomodulation activity, presents novel sepsis research. One such emerging novel anti-infective therapy includes Host defense peptides (HDPs). Many candidate treatments for sepsis are currently of particular interest, starting with immunomodulating agents are to be screened with appropriate animal models appear to be crucial to the translational success.

Chapter 3.

Research objectives

Building on the existing literature and identified research gaps, this study aimed to explore the potential antimicrobial and immunomodulatory properties of synthetic HDPs/IDR peptides. The hypothesis proposed that these peptides could serve as effective anti-infective therapeutics for managing sepsis.

3.1. Research objectives: The main objectives of the proposed work are as follows:

1. To study anti –inflammatory activity of synthetic novel peptides.
2. To study anti-microbial activity of synthetic novel peptide.
3. To establish the mechanistic pathway of synthetic peptide for the treatment of sepsis in animal models that mimic human sepsis.

The objectives of the proposed work are categorized as follows:

1. Chemical synthesis and confirmation of mass and purity of three synthetic peptides.
2. In vitro activity of three synthetic peptides for cytotoxicity and anti-inflammatory activity on murine macrophage RAW 264.7 cell lines stimulated with LPS and identification of lead peptide.
3. Development of pre formulation and determination of maximum feasible concentration for Lead peptide.
4. Acute toxicity and immunogenicity of the lead synthetic peptide.
5. In vitro activity of lead synthetic peptide for anti-microbial activity and time kill assays against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae* and the inhibition of biofilm formation against *S. aureus*.
6. Confirmatory studies for In vitro activity and in vivo anti-inflammatory activity of lead synthetic peptide on peritoneal BALB/c mice macrophages stimulated with LPS and carrageenan paw edema model in mice respectively.
7. In vivo efficacy activity of lead synthetic peptide using Caecal Ligation Puncture (CLP) Animal Model & *E. coli* induced peritonitis animal model.

3.2. Plan of work: The plan of work was classified into the following categories.

1. **Synthesis and confirmation of mass and purity of three synthetic peptides,** to accomplish the proposed work objective, following experiments are performed accordingly.
   Synthesis of three synthetic peptides by Fmoc SPSS strategy and characterization of synthesized peptides: The synthesized compounds were fully characterized using modern analytical techniques. HRMS to confirm the mass of test peptides and the purity was evaluated by HPLC.
2. **In vitro activity** of three synthetic peptides for cytotoxicity using MTT assay to evaluate their selectivity index and toxicity profiles and anti-inflammatory activity on murine macrophage RAW 264.7 cell lines stimulated with LPS, establishes the mechanistic pathway by estimation of biomarkers by ELISA, western blotting, and RT qPCR analysis.
3. **Development of pre formulation and determination of maximum feasible concentration** for lead synthetic lead peptide.
4. **Acute toxicity and immunogenicity** of the synthetic lead peptide.
   The identified lead synthetic peptide was screened for its in vivo toxicity profile by following OECD guidelines and the pre formulation studies and the maximum feasible concentrations was established with suitable solvents and Purity by HPLC, and the confirmatory peptide mass done by by HRMS- LCMS and recorded.
5. **The confirmatory studies include anti-microbial and anti-inflammatory studies and were performed as follows:**

1. In vitro activity of lead peptide for anti-microbial activity and time-kill assays against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae*.
   The lead peptide was screened for its in vitro antibacterial activity against above mentioned pathogens by microplate broth dilution method. Time kill kinetic studies are done to know the bactericidal effect of the compounds and evaluated the potency of the identified test peptide for inhibition of biofilm formation against *S. aureus.*
2. In vitro activity confirmatory studies of lead peptide on peritoneal BALB/c mice macrophages stimulated with LPS and in vivo anti-inflammatory activity in carrageenan paw edema mice model evaluating two dose concentrations.

3. In vivo Proof of Concept (PoC) efficacy activity of lead peptide using Cecal Ligation Puncture (CLP) Animal Model & *E. coli* induced bacterial load animal model.

- Induction of sepsis and created infection by injecting non-pathogenic strain of *E. coli* induced peritonitis and in Cecal Ligation Puncture (CLP) animal models, infection and trauma are seen due to alteration of the animal's endogenous protective barrier, such as intestinal leakage.

- Treatment with two doses of lead peptide via two routes of administration i.e., subcutaneous (S.C) & Intravenous (IV) in two animal models mentioned above and observed for the survival rate.

- Therapeutic efficacy was measured by biochemical and clinical biomarker estimations of pro and anti-inflammatory biomarkers &VEGF in serum, peritoneal lavage fluid and BALF of diseased, treated and control animals using ELISA, western blotting, and histology by H& E staining.

Chapter 4.

Materials & Methods

4.1. Materials:

Table15: List of the reagents and antibodies used for conducting this study.

Material	Catalogue	Company
Test Peptides (IS 217, IS 141 & IS 111)	IS 217 -17-10, IS 141-21-01 & IS111-20-02.	Issar Pharmaceuticals PVT. LTD
Macrophage Cell line Raw 264.7 cells	TIB- 71™	American Type Culture Collection, USA.
Staphylococcus aureus subsp. aureus	ATCC® 6538™	American Type Culture Collection, USA.
Pseudomonas aeruginosa	ATCC® 9027™	American Type Culture Collection, USA.
Escherichia coli	ATCC® 8739™	American Type Culture Collection, USA.
Klebsiella pneumoniae subsp. Pneumoniae	ATCC® 700603™	American Type Culture Collection, USA.
Lipopolysaccharide (LPS, Escherichia coli 0111: B4)	LPS25 (665778)	Sigma-Aldrich (St. Louis, MO, USA).
Dulbecco's modified Eagle's medium (DMEM)	30-2002™	American Type Culture Collection, USA.
Fetal Bovine Serum (FBS)	10082147	Gibco BRL Life Technologies (Grand Island, NY, USA)
Antibiotics (streptomycin/ penicillin)	15140122	Gibco BRL Life Technologies (Grand Island, NY, USA)
Ciprofloxacin	R1678-100MG	Fluka.
Cefoxitin	Cefotim (Inj-125 mg)	Zydus cadila, India.
Methyl Thiazolyl Tetrazolium (MTT)	M2128-100MG	Sigma (St Louis, MO, USA).
IL-6	DY406-05 &M6000B	R&D Systems (USA).
TNF-α	DY410-05&MTA00B	R&D Systems (USA).
IL-1β	DY401-05 & MLB00C	R&D Systems (USA).

IL-10	DY417-05	R&D Systems (USA).
IL-12	DY419 & D1200	R&D Systems (USA).
Mouse procalcitonin ELISA kit (Calorimetric)	NBP2- 812212	Novus.
VEGF	SMMV00	R&D Systems (USA)
cDNA synthesis kit	AB1453B	Thermo Fischer Scientific
Sybr mix	RR820A	TAKARA (USA)
Trizol	9109	TAKARA (USA)
PVDF membrane	1620177	Biorad (USA)
TRIS Base	0210313301	MP Biomedicals
RIPA	20-188	Sigma Aldrich (Germany)
Triton X-100	T8787	Sigma chemicals Co., MO, USA
BCA detection kit	23225/ BCA1	Thermo Scientific, Rockford, IL, USA/ Sigma Aldrich (Germany)
Myeloperoxidase levels	DY3667	R&D Systems (USA)
PMSF	10837091001	Sigma Aldrich (Germany)
Protease Inhibitor	P8340-1ML	Sigma Aldrich (Germany)
Protein ladder	PG-PMT2922	Invitrogen (USA)
BSA	MB083-100G	Himedia Laboratories
Non-fat dry milk powder	Sc-2325	Santacruz
RNA iso Plus	9108/9109	Takara
Enhanced chemiluminescence (ECL) Western blotting detection reagent	32109	Thermo Fisher Scientific, Waltham, MA, USA
iNOS	A3774	ABclonal
VEGFA	A12303	ABclonal
TLR4	A5258	ABclonal
TNF-α	ab205587	ABclonal
IL-10	A2171	ABclonal
IL-6	A1115	ABclonal

p38	8690	CST
phosphorylated (p)-38	4511	CST
ERK	4695	CST
p-ERK	4370	CST
GAPDH	5174	CST
β-Actin	4967L	CST
Dimethylsulfoxide (DMSO)	673439	Sigma Chemical Co. (St. Louis, MO, USA).
Phosphate buffered saline (PBS)	10010023	Gibco BRL Life Technologies (Grand Island, NY, USA)
Nitrite Assay Kit (Griess Reagent)	MAK367	Sigma Chemical Co. (St. Louis, MO, USA).
Luria Bertani Agar/ Miller	M1151	(MHA, Mast Diagnostics, U. K.)
Mueller Hinton broth and agar plates	70192 M	HiMedia Laboratories, Mumbai, Maharashtra, India
Thioglycollate broth	LQ007A	HiMedia Laboratories, Mumbai, Maharashtra, India

Table16: List of instruments used in the study.

Instrument Name	Make
96-Well Fast Thermal Cycler	Veriti Applied Biosystems
Automated peptide synthesizer	Applied Biosystems model 433A
Biochemical parameter analyzer	Automated biochemical analyzer (AU2700 Beckman coulter chemistry analyzer (Beckman Coulter, Brea, CA, USA).
Centrifuge Sorvall ST 16R	Thermo Fisher Scientific
Chemiluminescence detection system	Fusion SL, Vilber Lourmat
Digital plethysmometer	Ugo Basile, Comerio VA, Italy
Hematology parameters analyzer	Medonic CA-620 hematology analyzer Medicon Ireland, Newry, Northern Ireland, U.K.
Hematology System	ADVIA 2120. Siemens Healthcare Diagnostics, Forchheim, Germany
HPLC	Shimadzu, Japan.
HRMS	Thermo scientific™ Q orbitrap exploris™ 120 mass spectrometer and couple vanquish™ 3000UHPLC.
Mice rectal probe	Physitemp, Clifton, NJ
Microscope	Olympus
Multi detection Microplate Reader	Biotek
Nanodrop (UV/Vis Spectrometer)	Thermo Fisher Scientific
Real Time PCR System	Step One Plus Applied Biosystems
Spectrophotometer	Multiskan GO Thermo Scientific 51119300, Thermo Fisher Scientific, USA
Sysmex CA-50 Automated Blood Coagulation Analyzer	Sysmex, Kobe, Japan
Weighing Balance	Sartorius
Western-blot apparatus	Bio-rad

4.2. Methods:

4.2.1. Synthesis and characterization of synthetic test peptides:

The test peptides (peptide codes: IS 141, IS 111and IS 217) were synthesized by Issar Pharmaceuticals Pvt. Ltd., (Telangana, India). The peptides were synthesized manually in a stepwise manner at a 0.1 - 10 mmol scale on a using *N*-Fmoc (*N*-fluorenylmethyloxycarbonyl) solid phase peptide synthesis strategy (multichannel peptide synthesizer) and where the peptides of >10 -200 mmol were synthesized by automated peptide synthesizer. Peptides were purified by preparative reversed-phase HPLC (Agilent 1200) using with a C-18 coated, 10-micron bead column (Phenomenex Jupiter C18, 10μm, 300 Å, 250 × 10 mm) using a gradient of 0.1% TFA in water (Mobile phase A) and 100% acetonitrile (ACN) (Mobile phase B) and characterized by RP-HPLC chromatography and MALDI-TOF mass spectrometry at In -house. The peptide IS 217 used in this study was acetylated at the N-terminus. The molecular weights were confirmed by mass spectrometry HRMS –LCMS. The purity was about 93- 95% as determined by analytical HPLC. The peptides used in all biological assays were higher than 90% purity. The schematic protocol of synthetic peptides using solid phase peptide synthesis was described in flow chart format in figure 6. Detailed synthetic protocols are included in **Appendix-I**.

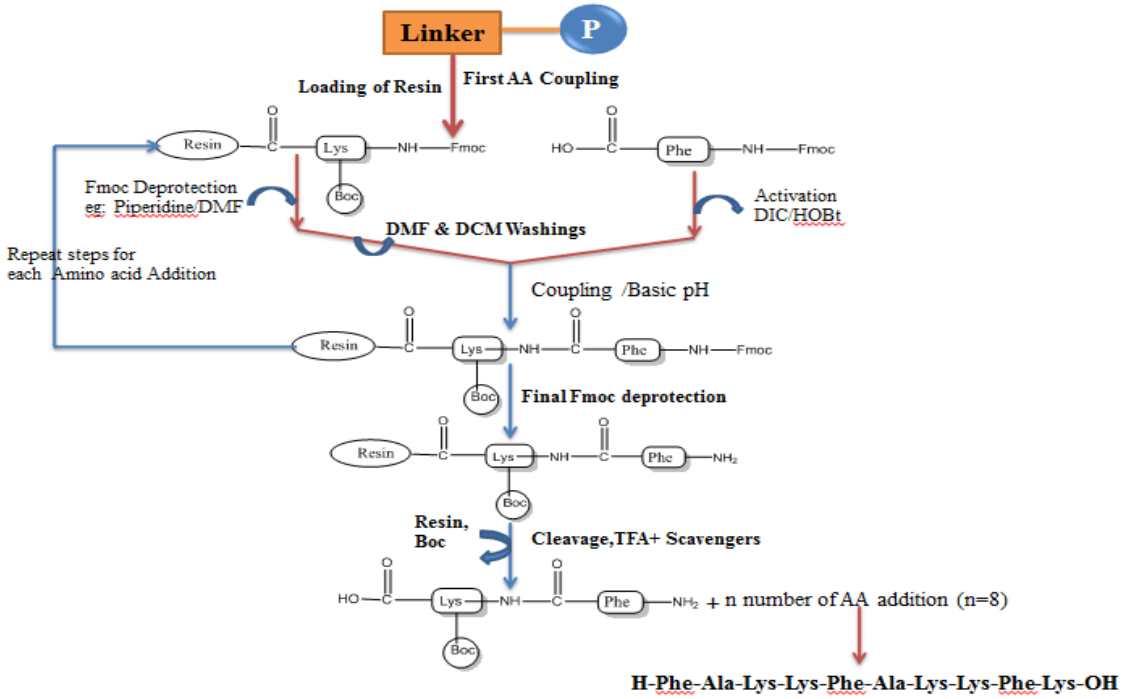


Figure 6: Schematic protocol of synthetic peptides using Solid Phase Peptide Synthesis. (Synthetic pathway basic steps in Solid Phase Peptide Synthesis of IS 217)

All commercially available chemicals and solvents of synthesis grade were used without further purification. The qualitative ninhydrin test was performed for each step to confirm completion of coupling. Purification was done by using reverse phase chromatography. The purity of the final compounds was examined by HPLC, (on Phenomenex C8 (150 * 4.6 mm, 5µm, 100 Å) double end-capped RP-HPLC column and was greater than 95%.

4.2.2. In vitro anti-inflammatory activity of peptides against LPS-induced inflammation in RAW 264.7 cells.

The methods include cytotoxicity and anti-inflammatory assays were performed as described below figure 7.

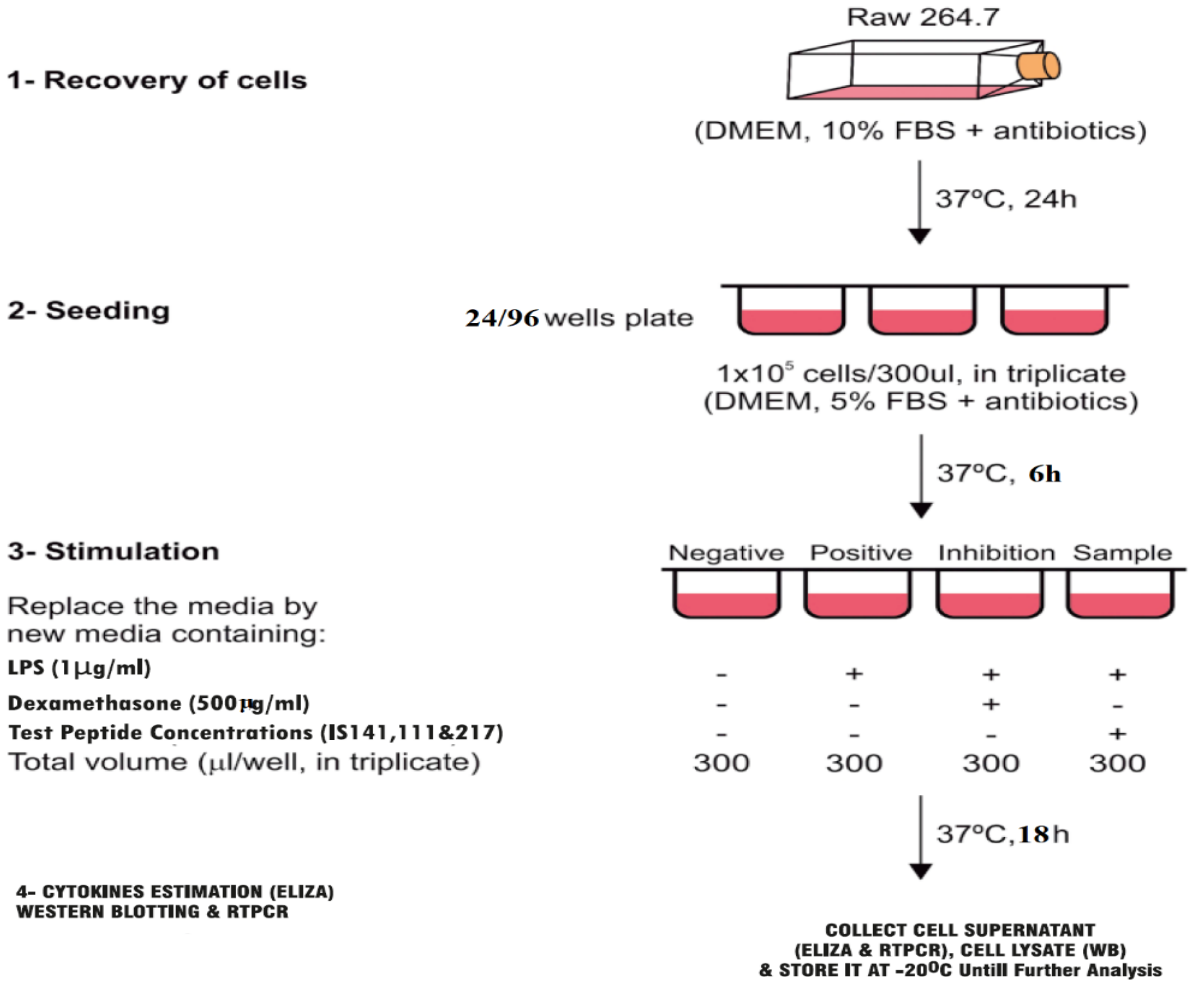


Figure 7: Step wise procedure followed in the In vitro activity.

4.2.2.1. Cell line culture:

The murine macrophage RAW264.7 cells were recovered from the stock and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% low-endotoxin heat-inactivated fetal bovine serum (FBS), 2 μM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured in a humidified atmosphere at 37°C under 5% CO_2 until the cells were confluent around 70–80%. The growth medium was frequently replaced after 2–3 days. The cells were then washed and harvested using trypsin-EDTA. Cell culture has been done according to the methods describe by [Novilla et al. 2017; Rashad A. Al-Salahi et al., 2013 & Amit Kumar et al.,2021].

4.2.2.2. Preparation of synthetic test peptides:

For in vitro experiments, the synthetic test peptides IS 141, IS 111 & IS 217 were dissolved in and diluted in 0.9% sterile normal saline (NS) for further concentrations used in the assay.

4.2.2.3. Study design:

The anti-inflammatory activities of IS 141, IS 111 & IS 217 were evaluated in lipopolysaccharide (LPS) - stimulated RAW 264.7 cells. For establishment of in vitro model, lipopolysaccharide (LPS, 1 μg/ mL) was used to treat the cells for 18 h. RAW 264.7 Cells were seeded at a density of 1 x 10^5 cells/ well ≥ 2,00,000 cells, either into 24/6-well plates for cytokines by ELISA, western blot, and RT-PCR, and 7x10^3 cells/ well in 96-well plates for the cell viability assay. The experimental design and the study parameters are summarized in figure 7& 8.

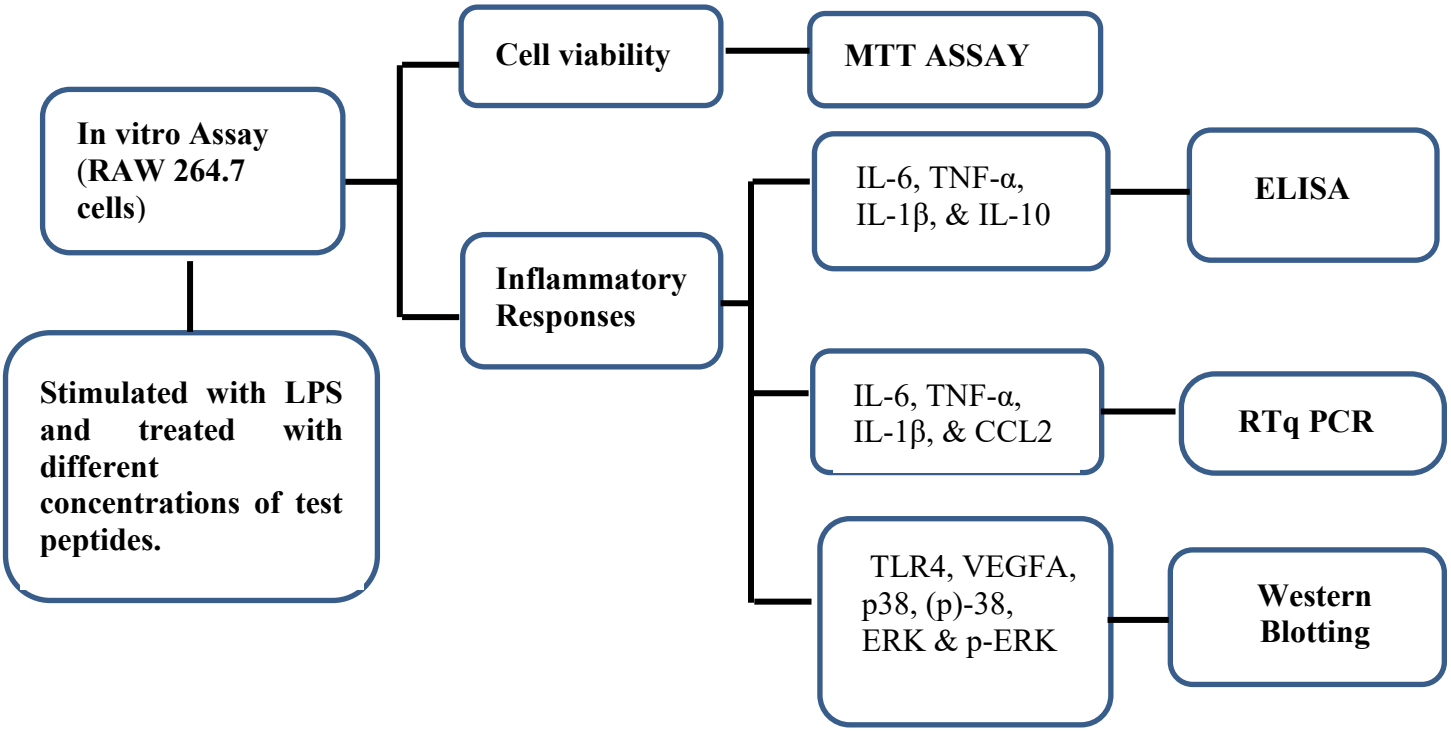


Figure 8: Study design and the study parameters of the In vitro cytotoxicity and anti-inflammatory assay of synthetic test peptides.

4.2.2.3.1. Cytotoxicity test –MTT assay:

The viability of RAW264.7 cells treated with synthetic peptides was assessed using the methyl thiazolyl tetrazolium (MTT) assay after 48 hours. In brief, actively growing RAW264.7 macrophages were mechanically harvested and seeded at a density of $7x10^3$ cells in 100µl per well of a 96-well plate. The cells were then incubated for 12 hours at 37°C under 5% CO_2. Following the initial incubation, the cells were treated with various concentrations of the test peptides (IS 141, IS 111, and IS 217) ranging from 1.5625 to 100 µg/mL, as well as with dexamethasone (at 500 µg/mL) for comparison. The cells were further incubated at 37°C under 5% CO_2 for 42 hours. The cellular morphology was examined using a microscope after the incubation period. After the incubation, MTT solution (20 µL of 5 mg/mL MTT dissolved in 1x PBS) was added to each well and incubated for 4 hours at 37°C under 5% CO_2. The formazan crystals formed by viable cells were dissolved by adding 100 µL of 100% DMSO to each well. The plates were gently swirled for 5 minutes at room temperature to ensure complete dissolution. The absorbance of the solution was measured at 575 nm using a microplate reader. To determine maximum cytotoxicity (100%), cells treated with 1% Triton

X-100 were used, while cells treated with PBS served as the negative control. Untreated cells were considered the control group. The average absorbance for each sample group was used to assess cell viability.

Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data were expressed as the mean percentage of viable cells when compared with untreated cells (control). The half maximal growth inhibitory concentration (IC50) value was calculated from the line equation of the dose-dependent curve of each compound. The results were compared with the cytotoxic activity of dexamethasone, a known anti-inflammatory drug. The concentrations of test peptides that showed significant ($p < 0.001$) cell viability was further selected for in vitro assays. Percentages of cell viability and LC50 of cells were calculated: Percent cell viability was calculated as (O.D. of drug-treated sample/control O.D.) ×100. The data were expressed as percent cell viability compared with control (dimethyl sulfoxide/NS). The experiment was carried out in four samples of each concentration.
The percentages of cell viability and LC50 of cells were calculated by using the formula below.

$$\textbf{\% cell viability} = \frac{\textbf{Absorbance sample}}{\textbf{Absorbance control}} \times 100$$

Where Absorbance control is the absorbance of cells treated with DMSO 0.1% and Absorbance sample is the absorbance of cells treated with test peptides.LC50 values will be determined for dose–response curves. Data are to be expressed as a means of three independent experiments. Statistical analyses will be performed using one-way ANOVA.

4.2.2.3.2. Anti-inflammatory test in LPS-stimulated RAW264.7 cells:

The methods of in vitro anti-inflammatory assay have been done according to the methods describe by [Laksmitawati et al., 2017; Rusmana et al., 2015; Sandhiutami et al., 2017; Widowati et al., 2018].

Cell Culture and LPS Stimulation:

Cells were sub cultured by scraping when plates reached 70-80% confluence with a 1:5 ratio in fresh medium. RAW 264.7 cells were plated at a density of 1×10^5 cells ≥ 2,00,000 cells and allowed for

attachment. The growth medium was then replaced with fresh medium without FBS, and incubated for 6 h, then cells were pre-treated with different concentrations of synthetic peptides: IS 141, IS 111 &IS 217 (3.12, 6.25, 12.5, 25, 50 and100 µg/mL) or dexamethasone (500 µg/mL) as a positive control for 1 h followed by stimulation of LPS (1µg/mL) and then further incubated for 18 h and were allowed to adhere. After 18 h of incubation, plates were centrifuged for 6 min at 400× g and the supernatants and cell lysates were collected and kept frozen at -80 °C until used for further analysis.

At the end of the treatment period, cells were harvested in RNA isoplus reagent for subsequent cellular RNA extraction, for RTqPCR. The pro & anti –inflammatory cytokine production in supernatants was estimated by using ELISA, RTqPCR and western blotting in cell lysates. The experiment was carried out in triplicate. LPS and Dexamethasone were used as the controls.

For analysis, samples of cells or culture supernatant were obtained after 18 h of treatment.

The following treatments were applied for the anti-inflammatory test:

(1) The negative control: RAW 264.7 cells without being induced by lipopolysaccharide; no drug treatment.

(2) The positive control: RAW264.7 cells that were induced by 1µg/mL of lipopolysaccharide (LPS group).

(3) The mixture of Synthetic peptides: IS 141, IS 111 & IS 217 (3.12, 6.25, 12.5, 25, 50 and100 µg/ml), LPS (1 µg/ mL) and RAW 264.7 cells; and

(4) The standard positive control: The mixture of dexamethasone (500 µg/mL), LPS (1 µg/ mL), and RAW 264.7 cells.

Measurement of pro &anti -inflammatory cytokine markers*:*

The levels of IL-6, TNF-α, IL-1β, and IL-10 in the supernatants of RAW 264.7 cell cultures were determined using an ELISA kit, according to the manufacturer's instructions (R&D Systems).

Table 17: Cytokines dilutions used at the time of ELISA.

Cytokines	Dilution
TNF-α	1:300
IL-6	1:25
IL-1β	No dilution
IL-10	1:20

Western blot analysis:

As previously described, RAW264.7 cells were cultured and treated with LPS and synthetic peptides. After 18h, cells were washed with PBS and lysed by incubating with RIPA lysis buffer cocktail (RIPA Lysis buffer, PMSF (1mM), Protease inhibitor (1ug/mL), phosphatase inhibitor (1mM)) at -80°C for 30 minutes. Post incubation, cell lysate was collected by scraping cells and centrifuged at 12000rpm for 15min at 4^0c.

The protein concentration was determined using the Bicinchoninic acid kit method with bovine serum albumin as the standard (provided in the kit), according to the manufacturer's instructions. Protein bands were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane via semi-dry transfer blotting, Membranes were blocked with 5% BSA in PBST (1% Tween 20 in PBS) at 20–24 °C for 1 h, then incubated with primary antibodies against IL-10, TLR 4, VEGF, P38 p-p38, ERK and p-ERK, the dilutions are listed in table 18., at 4°C overnight. GAPDH was used as a protein-loading control. The membrane was washed 3 times (10min. each) with Tris-buffered saline/Tween 20 (TBST) and then treated with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 1000) for 1 h. The membrane was washed again with TBST. The membrane-binding antibodies were visualized later with the ECL detection reagent and images were acquired in a Fusion SL Imaging System. Band density was analyzed by densitometry using Image J software.

Table 18: Antibody dilutions used for western blotting.

Primary antibody	**Dilution**
IL-10	1:1000
VEGFA	1:1000
TLR4	1:2000
p38	1:1000
phosphorylated (p)-38	1:1000
ERK	1:1000
p-ERK	1:1000
GAPDH	1:1000

The Buffer compositions are mentioned as below.

Table 19: Resolving gel composition.

Resolving gel(10ml/gel)	8%	10%	12%	15%
H2O	4.6ml	3.8ml	3.2ml	1.2ml
30% Acrylamide	2.6ml	3.4ml	4ml	6ml
1.5M Tris (pH 8.8)	2.6ml	2.6ml	2.6ml	2.6ml
10%SDS	0.1ml	0.1ml	0.1ml	0.1ml
10%APS	0.1ml	0.1ml	0.1ml	0.1ml
TEMED	10µl	10µl	10µl	10µl

Table 20: Stacking gel composition

Stacking gel(5ml/gel)	4%
H2O	2.975ml
30% Acrylamide	0.67ml
0.5M Tris (pH 6.8)	1.25ml
10% SDS	0.05ml
10% APS	0.05ml
TEMED	5µl

Running buffer composition (1x)-1Litre

1. Tris base- 3.02gm
2. Glycine-14.4gm
3. SDS-1gm,
4. Water-1000ml

Transfer buffer composition (1x)-1 Litre

1. Tris base- 3.02gm
2. Glycine-14.4gm
3. Methanol-200ml,
4. Water-800ml

Table 21: Loading dye/laemmli buffer composition

Laemmli buffer	2x dye(10ml)
10% SDS	4ml
1M Tris	1.2ml

Bromophenol blue	2mg
β-mercapto ethanol	1ml
Glycerol	2ml
Water	1.8ml

ECL Buffer

1. 1M Tris buffer (pH7.2)- 10ml
2. 30%H2O2- 5-10ul (Depends on how many times it exposes to light)
3. P-Coumaric acid (90mM)-22µl
4. Luminol (250mM)-50µl

   Mix all the reagents just before membrane incubation.

1x PBS preparation: 1Litre

1. 8 g of NaCl.
2. 0.2 g of KCl.
3. 1.44g of Na2HPO4.
4. 0.24 g of KH2PO4.
5. Adjust the pH to 7.4 with HCl

   Add distilled water to a total volume of 1 litre.

Reverse transcription–polymerase chain reaction:

Total RNA extraction, purification, and cDNA synthesis:

Total cellular RNA was isolated using the RNA isoplus reagent (Takara Bioscience, India) following the manufacturer's instructions.

Quantification of RNA: RNA was quantified spectrophotometrically by absorption measurements at 260 and 280 nm using the nano drop system. RNA samples with A260/280 >1.8, A260/230 >1.8 were used for qPCR experiments.

cDNA synthesis steps: As per table 22, RNA and the reaction mixture were mixed in PCR a tube and incubated in a thermal cycler for 5 min at 65^0c in and then cooled immediately on ice. Further,

in the same tube, the step 2 reaction mixture was added and further incubated in the thermal cycler as mentioned below.

Table 22: cDNA synthesis procedure Step-1

S.no	Reagent	Volume for 1 reaction from the kit
1.	Random6mers(50µM)	1µL
2.	dNTPMixture (10 mMeach)	1µL
3.	RNase Free dH2O	3 µL
4.	Template RNA	5 µL
	Total	10 µL

Table 23: cDNA synthesis procedure Step-2

S.no	Reagent	Volume for 1 reaction from the kit
1.	Template RNA Primer Mixture (From step 1)	10µL
2.	5X PrimeScript Buffer	4µL
3.	RNase Inhibitor (40 U/µl)	0.5 µL
4.	PrimeScript RTase (200 U/µl)	1 µL
5.	Water	4.5 µL
	Total	20 µL

- Mixed gently.
- And incubated the reaction mixture using the following conditions.

  30^0c - 10 min (required when using Random 6 mers)

  42^0c (50^0c) - 30-60min

Inactivated the enzyme by incubating at 95^0c for 5 min and then cooled on ice.

After cDNA synthesis, samples were stored at -80^0c till used for RT-qPCR.

RT-qPCR: Primers were designed using the Primer3 online tool. GAPDH was used as a normalizing control. The sequence of primers used is provided in below table 24. Real-Time PCR was performed according to the manufacturer's protocol using Takara biosystem's real-time PCR mix (Takara, Bio-India) with ROX as a passive reference dye using applied Biosystem's step-one Real-Time PCR system. The following PCR program was used for all real-time PCR-based experiments: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 60°C for 15 seconds. Real-time PCR was performed with step one plus Real-Time PCR system (Thermo Scientific). PCR reactions were performed in 20-µL reactions with SYBR Green Real time PCR Master Mix (Toybo, Osaka, Japan). **The following primer sequences used for PCR amplification are:**

Table 24: Primer Sequences used in the RT-qPCR.

Gene	**Primer sequence**	
Mouse -TNF-α	Forward	5'-AAGCCTGTAGCCCACGTCGTA-3'
	Reverse	5'-GGCACCACTAGTTGGTTGTCTTTG-3'
Mouse-IL-1β	Forward	5'-CCAAGCAACGACAAAATACC-3'
	Reverse	5'-GTTGAAGACAAACCGTTTTTCC-3'
Mouse- IL-6	Forward	5'-GAACAACGATGATGCACTTGC-3'
	Reverse	5'-CTTCATGTACTCCAGGTAGCTATGGT-3'
Mouse- IL-10	Forward	5'-AAATTCATTCATGGCCTTGTA-3'
	Reverse	5'-TGCCTTCAGCCAGGTGAAGACT-3'
Mouse- IL-12p70	Forward	5'-TCCATGTTTCTTTGCACCAGCC-3'
	Reverse	5'-TCCTCAGAAGCTAACCATCTCC-3'
Mouse-CCL2	Forward	5'-GCTACAAGAGGATCACCAGCAG-3'
	Reverse	5'- GTCTGGACCCATTCCTTCTTGG-3'
GAPDH	Forward	5'-AGG CCG GTG CTG AGT ATG TC-3'
	Reverse	5'-TGC CTG CTT CAC CAC CTT CT-3'

Gene expression analysis: For quantification of gene expression, CT values of each gene were normalized to GAPDH and calibrated to the appropriate control sample using the SYBR Green-based comparative CT method (2-ΔΔCt). Fold change was calculated using the formula 2-ΔΔct.

4.2.2.4. Statistical analysis:

The experiments were repeated three times, and the results were presented as the mean ± error of means (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test for comparing two independent groups. A significance level of $P<0.001$, $P<0.01$, and $P<0.05$ was considered statistically significant and denoted as ****, ***, and ** respectively. Graphs were generated using GraphPad Prism version 8.2/9.0.

4.2.3. Development of pre formulation and determination of maximum feasible concentration for test synthetic peptide IS 217: [Strickley, R.G. et al. 2004; & Gad SC et al., 2006].

The pre-formulation assessment is a first step to ensure that all the required information is in place to begin the pre-formulation development studies. Pre formulation in early product development of R&D is important to cut back the risks of last stage attrition and to attenuate expensive issues. This helps to observe and distinguish the different physical and chemicals factors of a new molecule to develop a safe and stable drug delivery system (DDS). The different factors that are evaluated are.

1. Solubility and Stability analysis of peptide IS 217 in different suitable aqueous vehicles.

4.2.3.1. Methods for pre-formulation studies:

Selection of the suitable aqueous vehicle for test synthetic peptide IS 217:

Four diluents were selected for solution stability studies. The diluents are as follows.

1. 0.9% NaCl
2. Water
3. Phosphate buffer pH 5.0 &
4. Phosphate buffer pH 7.4

4.2.3.2. Preparation of system suitability solution:

50µl of l0mg/ml stock of the lead peptide (IS 217) was diluted to 1mL with different diluents as follows in the table 25.

Table 25: The diluents and dilutions followed at the time of the experiment.

Diluent	Stock solution volume (µL)	Diluted to volume (µL)	Final Concentration (µg/mL))
0.9% NaCl	50	1000	500
Water	50	1000	500
Phosphate Buffer pH 5.0	50	1000	500
Phosphate Buffer pH 7.4	50	1000	500

Three aliquots of above samples were kept at three different temperatures (2-8^{0}c, Room temperature, and 37^{0}c). Samples will be withdrawn from each aliquot at time points Initial and 24 h. These

samples will be analyzed using chromatographic conditions mentioned below.

Table 26: Chromatographic conditions:

Instrument	Shimadzu LC2010C-IT
Column	Inertsil OD3,150X4.6mm,5µm
Mobile phase Composition	0.1% TFA in Acetonitrile-0.1%TFA in Water
Flow rate	1.0mL/Min
Column oven temperature	35^0c
Injection Volume	5µL
Run Time	12 min
Wavelength	205 nm
Auto sample temperature	8^0c

4.2.3.3. Development of pre-formulation of test synthetic peptide IS 217:

The following excipients were assessed for pre formulation preparation of IS 217. These are follows:

1. 10%v/v propylene glycol in 0.9%Nacl
2. 10%v/v Tween 20 in 0.9%Nacl
3. 0.9%Nacl solution

The stock solution of 25mg /ml of peptide IS 217 was prepared by dissolving 10mg (total content present in the vial) in 400µL of 0.9% NaCl solution. 80µL of stock solution will be diluted to 200µl with the above-mentioned excipients. In separate Eppendorf tubes to obtain a concentration of 10mg/ml. Samples were kept at room temperature. The concentration of peptide IS 217 in Initial and 24h samples were determined using chromatography as mentioned above.

4.2.3.4. Determination of maximum feasible concentration of test synthetic peptide IS 217 in Pre formulation:

Pre-formulation of concentration 25mg/ mL of peptide IS 217 was prepared by adding 400µL of excipient in a vial containing peptide. Three pre-formulations were prepared by using excipients mentioned above. Initial and 24 h assay of pre-formulations kept at room temperature will be performed using HPLC.

Pre-formulation of concentration 50mg/ mL of peptide IS 217 was prepared by adding 200µl of excipient in a vial containing peptide. Three pre-formulations were prepared by using excipients mentioned above. Initial and 24 h assay of pre-formulations kept at room temperature will be performed using HPLC.

4.2.3.5. Stability of test synthetic peptide IS 217 in 0.9% NaCl at Different conditions:

Stability testing of the peptide IS 217 was conducted by dissolving 1mg of IS 217 in 1 mL of 0.9% NaCl (Normal saline) freshly prepared and was kept under refrigerated (2–8^{0}C) and room temperature under light-resistant conditions. Stability testing of a 50% solution was determined that the solution was stable for at least 24h. The exact mass of the pre-formulation IS 217 of Initial and 24 h assay kept at room temperature and refrigerated (2–8^{0}C) will be performed using HRMS as mentioned in table 27.

Table 27: Test sampling details:

Peptide	**Sampling time points (h) with 0.9% NaCl at different temperatures.**		
IS 217	**2-8^{0}c**		**Room temperature**
	0 h	24 h	24 h

4.2.4. Acute toxicity and Immunogenicity of test synthetic peptide IS 217:

While developing novel therapeutic proteins/peptides, preventing immunogenicity, and establishing the acute toxicity profile are important issues to consider and the animal models are used to study immunogenicity prediction and acute toxicity of therapeutic proteins. The acute toxicity studies were conducted in compliance with the guideline of Organization for Economic Cooperation and Development (OECD) and schedule Y Guidelines for acute oral toxicity and previous work [OECD 401,402,423&425; SCHEDULE Y, DCGI -CDSCO document, whereas OECD 401 was replaced by TGs 420, 423 and 425, introducing refinement and reduction)] with some modifications.

Details of synthetic peptide (IS 217): The peptide IS 217 with a peptide content of 95% was supplied by Issar Pharmaceuticals Pvt. Ltd and was stored at −20°C until usage, if stored for long term. During the study period, IS 217 was stored in a refrigerator (2-8^{0}C) under light-resistant conditions. For the acute toxicity studies, the required amount of peptide IS 217 was weighed and dissolved and diluted in 0.9% sodium chloride as a vehicle. A 100% (wt/vol) solution was prepared for the acute toxicity study.

Animals and maintenance: A total of 40 BALB/c and 40 C57BL/6 male & female mice (6−8 weeks old at the beginning of the experiments) were used for toxicity and an additional 15 BALB/c male mice are used for immunogenicity assessments. Animals were weighing between 20 and 35 g aged 6–8 weeks, were obtained from Jeeva Life Sciences (Registered breeder: 1757/PO/RcBiBt/S/14/CPCSEA), and the studies are carried and approved by Birla Institute of Technology and Science–Pillai (BITS–Hyderabad), IAEC Protocol number BITS-Hyd/IAEC/2020/17), as specified by the Committee for Control and Supervision of Experiments on Animals (CCSEA). The animals were randomized and grouped and identified by marking by ear-punching after group assignment. The animals were acclimated to the laboratory conditions for 7 days prior to dosing, during which body weights and general conditions were observed. During the acclimation period, 4-5 mice were housed per cage, whereas during the study period, 2 animals were caged (acute study). The mice were housed in polypropylene cages and allowed free access to food and water, ad libitum and were maintained under specifically regulated environmental conditions (rearing conditions): 12-hour light/dark cycle, temperature 24^{0}C + 2^{0}C, humidity 55% + 10%, and ventilation 11 to 15 air exchanges per hour (all-fresh-air system). All the waste materials were disposed of in a safe and sanitary manner after performing the experiments.

4.2.4.1. Experimental procedure of acute toxicity study:

Table 28: Test system

IAEC Protocol No.	BITS-Hyd/IAEC/2020/17
Animal Model used	Mice
Strain	C57BL/6 & BALB/c mice
Gender	Males & Females
Age & Body Weight	6-8 weeks & 25-30 g
Test Doses	0.6, 2.4, & 4.8 mg/kg
Test Routes	Subcutaneous &Intravenous
Volumes	0.1 ml

Once after acclimation period, a total of eighty mice for two routes of administration, forty mice (half male and half female)/route were randomized based on bodyweight divided into four groups (10 in each group/per route, in each group 5 per sex) per strain via. two route of administration (SC & IV); prior to dosing initiation (table 29). On the day of dosing (designated as Day 0 of the study), the mice were 6 weeks old, and their body weights ranged from 20 to 25 g. The dosing volume was 0.1ml per mice and the actual volume of peptide IS 217 solution administered was calculated based on body weight measurements obtained on the day of administration and was administered SC & IV in increasing doses of the synthetic peptide IS 217 0.6, 2.4, & 4.8 mg/kg; (administration once). The routes of administration were the same proposed for use in humans [Food and Drug Administration, 1988]. Prior to dosing the animals were deprived of food for 12 hours but had free access to drinking water. Food was provided 3 hours after dose administration and was available ad libitum thereafter.

Table 29: Group Allocation of BALB/c for IV administration & C57BL/6 mice for SC administration of peptide IS 217

Test groups	**Dose (mg/kg b. wt.)**	**IV No. of Animals (M+F)**	**SC No. of Animals (M+F)**
Control	0	5+5	5+5
IS 217 –Low dose (LD).	0.6 mg/kg	5+5	5+5
IS 217–Medium dose (MD).	2.4 mg/kg	5+5	5+5
IS 217–High dose (HD).	4.8 mg/kg	5+5	5+5
The dose calculations are mentioned in the Appendix -III.			

After a single dose administration, mortality and clinical signs associated with toxicity were observed and recorded daily for consecutive two weeks; body weight changes were measured before and after administration on the 14th day. The animals were observed frequently during the first 4- 6-hour period following dose administration and once daily, thereafter, for 7 days, during which animals were evaluated for mortality, clinical signs of toxicity, and behavior. Each animal was weighed on Day 0 (prior to dosing) and on Day 7 during the observation period. At the end of the observation period, on day 14, after being weighed, animals were fasted for 12 h (free access to water) and anesthetized with ketamine (80-100mg/kg BW) and xylazine in a dose (5-10mg/kg) intraperitoneally (for 20 g mice ketamine of 0.2ml and xylazine of 0.1ml) as in combination the anesthetized with 0.2 ml via, IP. Blood samples were collected from orbit into nonheparinized Eppendorf tubes for the determination of serum biochemical parameters. Then animals were euthanized with co_2 Inhalation /isoflurane, and a complete necropsy was performed. The main organs of the thoracic and abdominal cavities and the brain were examined macroscopically for gross abnormalities. The number of animals killed for each of the doses was noted and the LD50 calculated by the Up and Down method, which is one of the most used to reduce the number of animals used [P.A. Botham, 2004]. Some vital organs comprising brain, heart, lungs, liver, spleen, and kidney were harvested and weighed. Organs collected from animals were preserved in formalin solution (10%, pH7.4) for the further histopathologic examination [P.A. Botham. 2004].

Relative organ weight was calculated according to the following formula:

Relative organ weight (g) = organ weight/body weight × 100

Functional Observation: The mortality, general health status, and toxic reaction symptoms of the experimental groups were documented. Before the first day of IS 217 administration and during the experiments, a detailed clinical observation was carried out and recorded the changes in skin, eyes and mucous membrane, respiratory system, nervous system, activity, and behaviors of the rats. The body weight was measured and recorded before the first day of administration.

Biochemical analysis: Laboratory analysis of biochemical parameters was performed on serum samples. Serum aspartate transaminase (AST), alanine transaminase (ALT), urea, creatinine and BUN were performed in automated biochemical analyzer (AU2700 Beckman coulter chemistry analyzer (Beckman Coulter, Brea, CA, USA).

4.2.4.2. In Vivo Immunogenicity: In acute toxicity, as a part of immunogenicity activity investigation, total 15 mice are randomized into three groups of each 5/ group which received 0.6 mg.kg, 1.2 mg.kg respectively as treatment groups and mice (n=5) received 10 µg LPS were used as a positive control. The blood was collected after 48 h via retro orbital plexus in non-heparinized tubes using ketamine and xylene in saline solution anesthesia via IP injection and the sera was separated as mentioned above in the acute toxicity and stored at -80^{0}c until for further cytokines analysis (TNF-α& IL-1β) [Julia Suhorutsenko et al.,2011]

4.2.4.3. Statistical analysis: For the acute toxicity study, the body weight data measured on days 1, 7 and 14 were subjected to statistical assessment using one-way analysis of variance was performed. All parameters measured were analyzed statistically, apart from the general condition of the mice and findings of the macroscopic and histopathological examinations. The results were expressed as mean ± SEM of the groups and significance difference between groups was evaluated by using ANOVA. If ANOVA shows significant differences, post hoc analysis was performed with Dunnett's test. The differences were considered statistically significant when $p < 0.01$ and denoted *** as data were analyzed using Graph Pad Prism 8.2/9.0 software.

4.2.5. In vitro confirmatory studies for anti-microbial and anti-inflammatory activity of synthetic peptide IS 217:

4.2.5.1. In vitro evaluation of anti-microbial activity of synthetic peptide IS 217:

The aim of this section was to determine the spectrum of antimicrobial activity of synthetic peptide IS 217. The anti-microbial activity was assessed by broth microdilution time-kill viability assays and inhibition of bio film assays.

Microbial strains:

The following were used as test bacteria: *Staphylococcus aureus subsp. aureus* (ATCC® 6538™), *Pseudomonas aeruginosa* (ATCC® 9027™), *Escherichia coli* (ATCC® 8739™) and *Klebsiella pneumoniae subsp. Pneumoniae* (ATCC® 700603™).

Growth media:

The bacteria cultures were maintained in cryotubes at -80^{0}C in 15% glycerol. A single colony of bacteria were refreshed in Mueller Hinton broth and incubated for 12 h at 37^{0}C and inoculated on Mueller Hinton agar plates for purity check. Overnight-cultured bacterial cells were transferred to MH medium and cultured to the exponential phase (optical density at 600 nm OD600 = 1.0). The culture was centrifuged and resuspended in sterile phosphate buffered saline (PBS) and adjusted to a final amount of 1X10^5 CFU ml^{-1} by using the equation CFU ml^{-1} _ OD600 1-2.5x 10^8 [Evelien Gerits et al.,2016]. The number of colony-forming units was determined, and the bacterial cultures were adjusted to 5 X 10^4 CFU/mL. Plates were prepared under aseptic conditions.

Peptide Details:

The peptide used here (IS 217) was synthesized by Issar Pharmaceuticals Pvt. Ltd., (India) and the lyophilized peptide of 10mg vials was supplied and stored at -20^{0}C until usage, if stored for long term. During the study period, IS 217 was stored in a refrigerator (2-8^{0}C) under light-resistant conditions. For in vitro and in vivo experiments, IS 217 was dissolved in 0.9% normal saline while preparing stock and working solutions. The peptide purity used in biologic assays was higher than 90-95%.

4.2.5.1.1. Determination of the minimal inhibitory concentration (MIC): Microdilution assay.

The minimum inhibitory concentration (MIC) of the peptide and antibiotic was determined using the broth microdilution technique. The assay was performed in Mueller Hinton broth (MHB) medium with an initial inoculum of 1-2.5 × 10^8 bacterial cells in untreated polystyrene microtiter plates (Corning, USA), following the methodology described by [Wiegand et al. and Banfi et al. 2003]. The MIC was defined as the lowest concentration of the peptide or antibiotic that completely prevented visible bacterial growth after 24 hours of incubation at 37 °C. The microdilution assay setup is visually depicted in Figure 9.

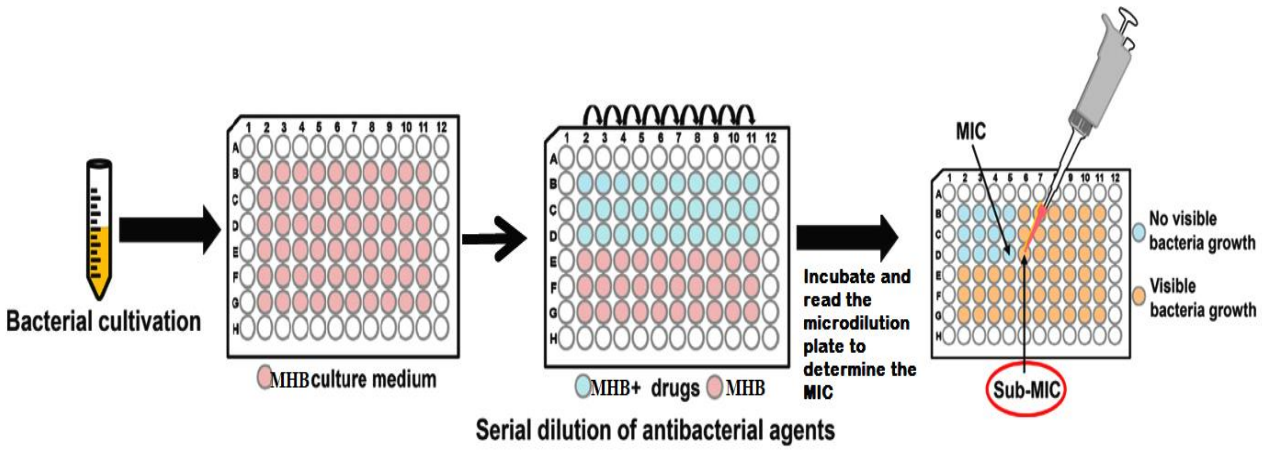


Figure 9: The picture representation of microdilution assay.

The minimum inhibitory concentrations (MICs) of peptide IS 217 against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and *Klebsiella pneumoniae* ATCC 700603 were determined using a standardized broth microdilution assay based on the reference methods provided by the Clinical Laboratory Standards Institute (CLSI) for bacteria [Clinical Laboratory Standards Institute 2006]. All bacterial cultures were grown, propagated, and preserved following the guidelines outlined in the ATCC bacterial culture guidebook. Stock solutions of the test compound IS 217 at a concentration of 10 mg/ml were prepared, and dilution series ranging from 0.195 to 25 µg/ml were prepared in 0.9% NaCl solution in micro-test tubes. These dilutions were then transferred to 96-well microtiter plates. Each well of the microplate received 100 µL of each dilution, and 100 µL of bacterial suspension containing 1-2.5 × 10^8 CFU/mL of all test organisms was inoculated into each well, resulting in a final concentration of 2.5 × 10^8 CFU/mL and a final volume of 200 µL per well. The first column of the microplate contained

the inoculum (positive control) and culture medium (negative control), while the final column contained the ciprofloxacin antibiotic control with concentrations ranging from 0.5 to 1.0 µg/ml.

Finally, the microplate was incubated with a sterile film cover for 24 h at 37^0C. Bacterial growth was detected by optical density using ELISA reader, Spectra Max M5 multi-detection reader and checked the OD600. The results were expressed in micrograms per milliliters. The MIC was defined as the lowest concentration of antibacterial agent that resulted in the complete inhibition of visible growth [49]. The bacterial growth was indicated by the presence of turbidity meaning. Three independent experiments were performed, each with three biological replicates. Ciproflaxin was used as a standard antibacterial agent. Finally, microplate was incubated with a sterile film cover for 18–24 h at 37°C Subsequently, 20 µL of 4% resazurin bacterial growth indicator was added to wells, which were then incubated for 30 min at 37 °C. The lowest concentration of test drug concentration that visually showed no growth was determined as MIC. The MIC was determined as the lowest tested concentration that leads to complete inhibition (100%) in comparison to the negative- control group. Antibacterial activity was expressed as the concentration of extract inhibiting bacterial growth by 50% (IC50) [Mounyr Balouiri et al.,2016].

4.2.5.1.2. Time-Kill assay:

The survival of bacteria, time kill efficacy assay of IS 217 was further evaluated for activity against bacterial strain mentioned above according to CLSI reference method, with slightly modification. MICs were determined by broth microdilution assay as described above. The test compound IS 217 was incorporated into 4.9 ml Mueller Hinton Broth (MHB) at concentrations of 0.5 x MIC, 1 x MIC and 5 x MIC for each bacterial species. Test tubes of MHB without test compound IS 217 were used as vehicle controls. Overnight cultures of the bacterial strains at cell densities of approximately 1 x 10^8 CFU/ml were used to inoculate both test and control tubes. The standard tube dilution method was opted to evaluate the time-kill efficacy of bacteria. Bacterial inoculum (1 mL) was diluted by adding 9 mL sterile saline solution and serially diluted up to 10^{-3}. On testing the bacterial dilution (10^{-3}), bacterial colonies were observed in a discrete form which was easily countable. One mL of 10^{-3} bacterial cultures were then incubated with an equal amount of IS 217 in a shaker at 37^0C for 1, 2, 3, 4, 5, 12 and 24 h.100 µL incubated suspension was transferred on the agar plates and spread through the spreader. Colony counts were performed after 24 h incubation at 37^0C. Plates with 10–300 colonies were used for these counts, and the kill rate was determined by plotting log10 viable

counts (CFU/ml) against time. Bactericidal activity was defined as a ≥ 3 log10 decreases in CFU/ml of the initial microbial population, while bacteriostatic activity was defined was defined as a < 3 log10 decrease in CFU/ml. The assay was performed in triplicate [Olufunmiso O Olajuyigbe et al.,2015; Sutthiwan Thammawat et al.,2017].

4.2.5.1.3. Efficacy of test synthetic peptide IS 217on *Staphylococcus aureus* biofilms:

The efficacy of IS 217 to disrupt biofilms was conducted as follows, Briefly, the Isolates of *S. aureus* (ATCC 6538) was grown overnight and diluted 1:100 in TSB + 1% glucose were inoculated with approximately 1×10^8 CFU/mL of *S. aureus* and incubated in 96-well plates at 37 °C for 48 hours. After removing media, wells were rinsed with PBS to remove planktonic bacteria before re-filling wells with fresh MHB daily. On the fourth day, Peptide and antibiotic are added at desired concentrations to the matured biofilms and the plates are incubated at 37 °C for 48 hours. After incubation, the wells are washed, and biofilms are stained with 0.5% (w/v) crystal violet for 30 minutes. The dye was solubilized with ethanol (95%) and the optical density (OD) of biofilms was measured. Bacterial growth was detected by optical density. The results were expressed in micrograms per milliliters. The reaction mixture was read spectrophotometrically at 595nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula. [Monroe D. 2007; 43. Hall-Stoodley et al.,2004; Stewart, P.S. et al.,2001]

$$\% \text{ of inhibition} = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100$$

4.2.5.1.4. Statistical analysis:

The experimental data were conducted in triplicate and presented as the mean ± SEM. The percentages of cells surviving at different time points were calculated and expressed as the mean and standard error of the mean. Statistical comparisons of the proportions of surviving cells were performed using Student's two-tailed t-test, assuming unequal variance and differences were considered significant where $P < 0.005$, $P < 0.05$ and denoted as *** &** and $^{###}$ & $^{##}$ when compared with standard drug and vehicle control groups respectively. The data were analyzed following Analysis of Variance (ANOVA) with the assistance of graph pad prism 8.2/9.0.

4.2.5.2. Invitro anti-inflammatory activity of synthetic peptide IS 217 in BALB/c mice peritoneal macrophages:

Male BALB/c mice aged 6-8 weeks were intraperitoneally injected with 1 ml of 4% sterile thioglycollate broth dissolved in PBS. After a period of 4 days, the mice were euthanized, and peritoneal macrophages were collected following the described procedure [Meurer SK, 2016]. This particular model is commonly referred to as the "Thioglycollate-induced peritonitis model" (see figure 10).

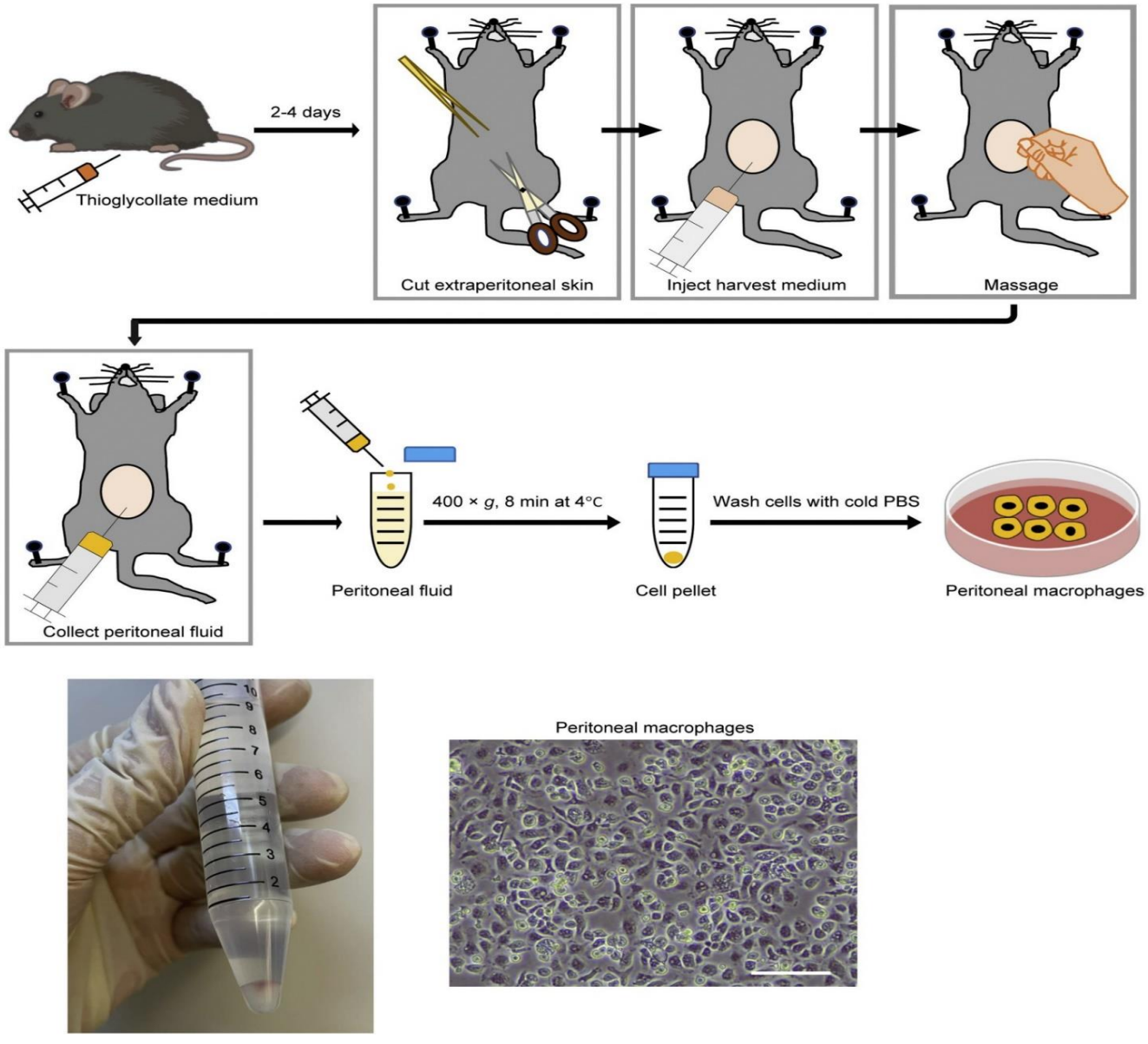


Figure 10: Collection of peritoneal macrophages from BALB/c by using thioglycollate-induced peritonitis model

Preparation of peritoneal macrophages:

After a period of 4 days, the mice were euthanized, and the peritoneal macrophages were collected by lavaging the peritoneal cavity with 5-6 mL of harvest medium consisting of 5 mM EDTA and PBS. The isolated peritoneal exudate cells were then washed twice with RPMI and centrifuged at 1000 rpm/400xg for 8-10 min at 4 °C. Subsequently, the cell suspension was placed in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and allowed to adhere to the surface of a 6 cm culture plate at 37 °C for 4 hours in the presence of 5% CO_2. Non-adherent cells were removed by washing the plates with warm PBS, while the attached cells were identified as macrophages with a purity of approximately 90% [Meurer SK, 2016]. The study design is depicted in the figure 11 provided below.

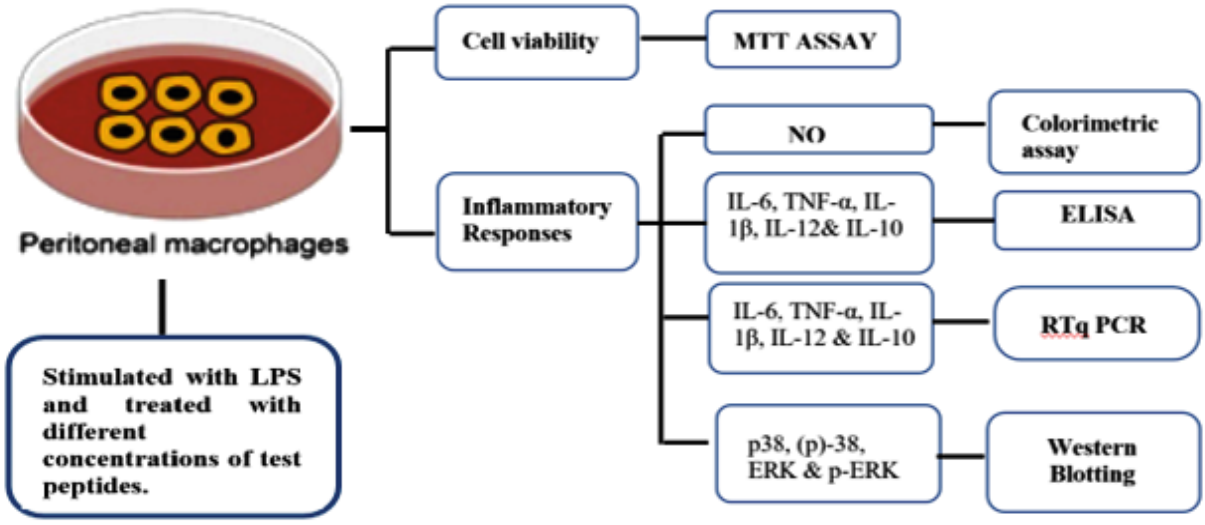


Figure 11: Study design of the In vitro cytotoxicity and anti- inflammatory assay of synthetic peptide IS 217 in peritoneal BALB/c mice macrophages.

Cell culture and treatments:

Macrophages were cultured either in 96-well plates at a density of 0.2 X 10^6 cells per well or in 24-well plates at a density of 0.8 X 10^6 cells per well. They were treated with various concentrations (3.125, 6.25, and 12.5 µg/mL) of the peptide IS 217 for 1 hour. Subsequently, the macrophages were stimulated with 1 mg/mL of bacterial lipopolysaccharide (LPS) from Sigma-Aldrich. The cells were

cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and maintained under sterile conditions at 37°C, 5% CO_2, and 85% humidity. After 24 hours, the culture supernatants were collected for cytokine analysis using ELISA and RT-qPCR, while the cells were harvested for western blotting. The procedures for ELISA, RT-qPCR, and other experimental conditions were the same as described in section 4.2.2.3 [Min Jee Kim et al., 2014].

Western Blot Analysis:

The cells were trypsinized, washed twice with phosphate buffered saline (PBS), and lysed with lysis buffer (modified RIPA buffer) at 4◦C. The pellet cellular debris was removed by centrifugation at 12500 rpm for 30min and the supernatants were then either analyzed immediately or stored at −80◦C. Protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL, USA). Lysates in sample buffer (2% SDS, 10%glycerol, 80mM Tris-base, 720mM DL-dithiothreitol, and 0.001% bromophenol blue) were denatured at 95◦C for 5min. Equivalent amounts of protein (25 μg) from total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane. Nonspecific binding was blocked by soaking the membrane in Tris-buffered saline (TBS, 20mM Tris-base, and 300mM NaCl) containing 5% fat-free milk for 1 h. The membrane was incubated with primary antibodies (antip38, and anti-ERK, at 1: 5000 in TBS; anti-actin at 1: 7500 in TBS) overnight at 4◦C. The membrane was then incubated with a secondary antibody, a goat antirabbit IgG, or goat antimouse IgG conjugated to horseradish peroxidase. The protein levels were determined by using enhanced chemiluminescence (ECL) plus western blotting detection reagents (Amersham Bioscience, Uppsala, Sweden) and the bands intensities were scanned. Densitometric analyses were conducted using the Quantity One software (Bio-Rad). Incubation with polyclonal mouse antihuman β-actin antibody was performed for comparative control.

Measurement of nitric oxide (NO):

The nitrite concentration was measured in supernatants of cultured RAW 264.7 cells according to the griess reaction as an indicator of NO production by using NO colorimetric test [Min Jee Kim et al., 2014; Widowati et al. 2018]. Aliquots (100 µl) of the culture supernatant were transferred in triplicates to a 96-well microplate, mixed with 100µL of griess reagent, and incubated at room temperature for 10 min in the dark, followed by the measurement of absorbance at 540 nm using an

enzyme-linked immunosorbent assay (ELISA) reader. The inhibition activity toward NO was calculated between the percentages (%) of NO concentration in each treatment compared to the positive control. Nitrite concentrations were calculated by referencing a standard curve plotted by known concentrations of sodium nitrite (NaNO2) (17) [Kiemer and Vollmar, 2001].

4.2.5.3. In vivo anti-inflammatory activity of synthetic peptide IS 217 by using Carrageenan induced paw edema model:

In vivo anti-inflammatory activity of peptide IS 217 was evaluated based on inhibition of paw edema volume by using carrageenan-induced mouse hind paw edema [Sravani et al., 2014], which is highly reproducible model of inflammation as previously described by [yonathan et al. 2006]. Carrageenan is a sulfated polysaccharide, that promotes acute inflammation by activating proinflammatory cells. The inflammation was quantitated in terms of ml i.e., displacement of water by edema using a digital plethysmometer immediately before and after Carrageenan injection at different time points.

In the present study, A total of 27 BALB/c female animals (6−8 weeks old weighing between 25 g at the beginning of the experiments) were used for carrageenan-induced mice paw edema model, were obtained from Jeeva Life Sciences (1757/PO/RcBiBt/S/14/CPCSEA), and the studies are carried and approved by Birla Institute of Technology And Science–Pilani (BITS–Hyderabad) IAEC Protocol number BITS-Hyd/IAEC/2020/17) and the animals were maintained and acclimatized as mentioned in the section 4.2.4.2.

Once after the acclimatization of Animals was fasted 12 hours before the experiment; however, they were given access to water ad libitum. Mice were divided into five groups of six animals each (n=6) except the normal control group (n=3), weighed and numbered. In vivo anti-inflammatory activity of two dose levels of IS 217 (i.e., 0.6 and 1.2 mg/kg body weight,) suspended in 0.9% Normal saline and a positive control group given dexamethasone (5 mg/kg body weight), while control group given with 0.9% Normal saline (Disease control) [Sharma Rakesh et al., 2011].

Table 30: Allocation of treatment groups in carrageenan-induced mice paw edema model

Test Group	Dose (mg/kg b.wt) & Volume (mL)	No. of Animals (M/F)
Control	0	3
Disease control	0.9% Normal saline	6
IS 217–0.6mg/kg	0.6mg/kg &0.1mL	6
IS 217–1.2mg/kg	1.2mg/kg &0.1mL	6
Dexamethasone	5mg/kg &0.1mL	6
The dose calculations are mentioned in the Appendix -III.		

The treatments of IS 217 (0.1ml) were administered Intravenously or a similar volume of vehicle (10 ml/kg) 30 min prior to sub plantar injection of 0.1 ml of 1% (w/v λ carrageenan) freshly prepared carrageenan in saline into the sub-plantar region of left hind paw. An equal volume of the vehicle (0.9% Normal Saline) was given to the control group. Initial paw sizes (basal volume) were measured by a volume displacement method using a digital plethysmometer [Kou et al., 2005].

All mice were marked on the tail with an indelible pen for identification. The left paw is marked with ink at the level of lateral malleolus; basal paw volume is measured plethysmographically by volume displacement method using plethysmometer by immersing the paw till the level of lateral malleolus. The increase in volume and the percent change caused by the irritant were measured before, and 1, 2, 3, 4, 5 and 18 h after injection of carrageenan injection. The percentage inhibition of edema was calculated for each group with a vehicle-treated control group [Winter et al., 1962]. Paw volume was measured immediately prior to the injection of carrageenan and thereafter at an interval of 1 h for a period of 5 h and then after 18 h. Edema inhibitory activity, the increase in paw volume, i.e., inflammation expressed in percentage was calculated according to the following formula [Gupta AK et al., 2015]:

$$\textbf{Percentage inhibition} = \frac{(C_t - C_0)\ \text{control} - (C_t - C_0)\ \text{treated.}}{(C_t - C_0)\ \text{control}} \times 100$$

Where, C_t=mean paw volume for each group at time t, and C_0=mean paw volume for each group before carrageenan injection.

4.2.5.4. Statistical analysis:

The data were presented as the mean ± SEM of at least three independent experiments. Statistical analysis was conducted using one-way analysis of variance (ANOVA). Multiple comparisons between the mean values of the two groups were performed using Dunnett's test. Column charts were generated using Prism 8.2/9.0 software, where $P < 0.005$, $P< 0.05$ and denoted as *** &** when compared with LPS (Disease control) group respectively in invitro assay and whereas $P < 0.005$, $P< 0.05$ and denoted as *** &** when compared with standard drug control group.

4.2.6. Proof of concept efficacy studies by using in vivo animal models:

How animals are currently used in sepsis research –Translational and Ethical issues:

The central goal of animal models is to create reproducible systems of clinically relevant sepsis pathogenesis that mimic human disease and used for preliminary testing of potential therapeutic agents [Safiah Mai et al., 2012] Animal models are used for two main categories in the in the context of sepsis research:

1) To identify involvement and changes in physiological, molecular and/or cellular pathways, and to understand the mechanism of sepsis syndrome progression.

2) To study the efficacy/safety/pharmacokinetics of therapeutic targets for proof of concept or regulatory studies.

Requirements for animal models of sepsis to mimic human sepsis:

An optimal animal model for sepsis should accurately simulate the progression and severity of human sepsis, which is a severe and prolonged syndrome typically treated in the intensive care unit (ICU). To achieve this, it is essential to replicate the pathophysiology of sepsis, focusing on critical aspects such as patterns of inflammation and cardiovascular parameters. These include hemodynamic stages characterized by warm shock followed by cold shock, as well as immunologic stages involving proinflammatory stimulation and subsequent counter-regulation with anti-inflammatory responses, often resulting in immune depression. Additionally, the animal model should strive to mimic histological findings observed in key organs (such as the lungs, liver, spleen, and kidneys) that are commonly affected in sepsis, although the severity of these changes may vary among individual animals. This variability, rather than being counterintuitive, is an important aspect to consider in animal modeling of sepsis.

For these mechanistic models of inflammatory and immune pathways and enhancing the translational gap are in need. Furthermore, an ideal sepsis model would be low-cost and would not cause any distress to the animals involved [Marcin F. Osuchowski et al, 2018]. The perfect model of sepsis does not exist based on these characteristics. Thus, in order to mimic human sepsis for the purpose of therapeutic intervention, considering the points mentioned above, the screening has to be done in two or more distinct animal models [S. Manoj Kumar et al.,2016; Wendy E. Walker ,2021 & Charles T Esmon et al.,2004], this helps in clinical trial prediction to be success.

A full harm: benefit impact assessment is important while designing the animal model. Harm relates to welfare experience of animal whilst benefit relates to value of scientific data, considering (3Rs) principles [Sudhir Verma ,2016; Manasi Nandi,2020; Jean-Marc Cavaillon et al., 2020].
To date, most used models based on initiating agent, include administration of an inflammatory trigger (e.g., endotoxin -LPS), amicrobial trigger (e.g., infusion or instillation of exogenous bacteria or peritonitis) or co-morbidity models (e.g., surgical 'immune barrier disruption models': trauma plus infection). Considering regulatory guidelines, the present work involves the use of two Standard animal models of sepsis. The Comparison of two rodent sepsis models is summarized in the table 31.

Justification and significance of C57BL/6 and BALB/c mice in sepsis studies:

The animal models of sepsis should be immunological and metabolic while designing the animal study, moreover in the in vivo environment, local humoral and cellular factors probably alter the way that cells respond to pro-inflammatory stimuli. The key information we gathered from the animal studies are efficacy and safety of the lead molecules (as determined by standard regulatory tests). As a part of preclinical research for sepsis, mice are most popularly used.

At present, the most used inbred mouse strains in the laboratory are C57BL/6 and BALB/c, as they can improve the reproducibility and reliability of study results and majorly used in immunology and antitumor activity studies involves inflammation and macrophage function.
C57BL/6 mice, in particular, are notable for their immunogenicity due to the initial strain development goal being their use in the study of cancer and immune responses, in addition due to their cell mediated immunity and NK cell activity are relatively higher in C57BL/6 mice than in other mice, in respect to immunological characteristics and , mortality rates, liver MPO activity, metallothione mRNA, leptin as well as IL-10 levels are significantly higher in C57BL/6J compared to other strains used for sepsis[Hyun Keun Song et al., 2017]. Whereas BALB/c mice tend to produce a stronger humoral response than C57BL/6 mice; it is easier to induce Th2 immune response in BALB/c mice, which is very common in infectious diseases and allergic reactions.

In the present study, the two gold standard models which mimic human sepsis are bacterial (*E. coli*) infection induced peritonitis model and Cecal ligation and puncture (CLP) models are used in C57BL/6 and BALB/c mice of aged 10-12 weeks (figure 12).

Table 31: The comparison of two in vivo animal sepsis models used in the present study.
[Sudhir Verma 2016].

Sepsis model	Type	Variability factors	Advantages
Bacterial Infection Model	Injectable	• Bacterial load • Route of administration • Time of infusion • Antibiotic/fluid resuscitation	Presence of bacterium allows insights into mechanisms of host response to pathogens
Cecal Ligation and Puncture (CLP)	Surgical	• Needle size and number of punctures. • Amount of cecum ligated/ necrosis induced. • Antibiotic/ fluid resuscitation • Sex, age and strain	• Mimics human sepsis most • Simple procedure • Polymicrobial • Prolonged and lower cytokine elevation as in humans

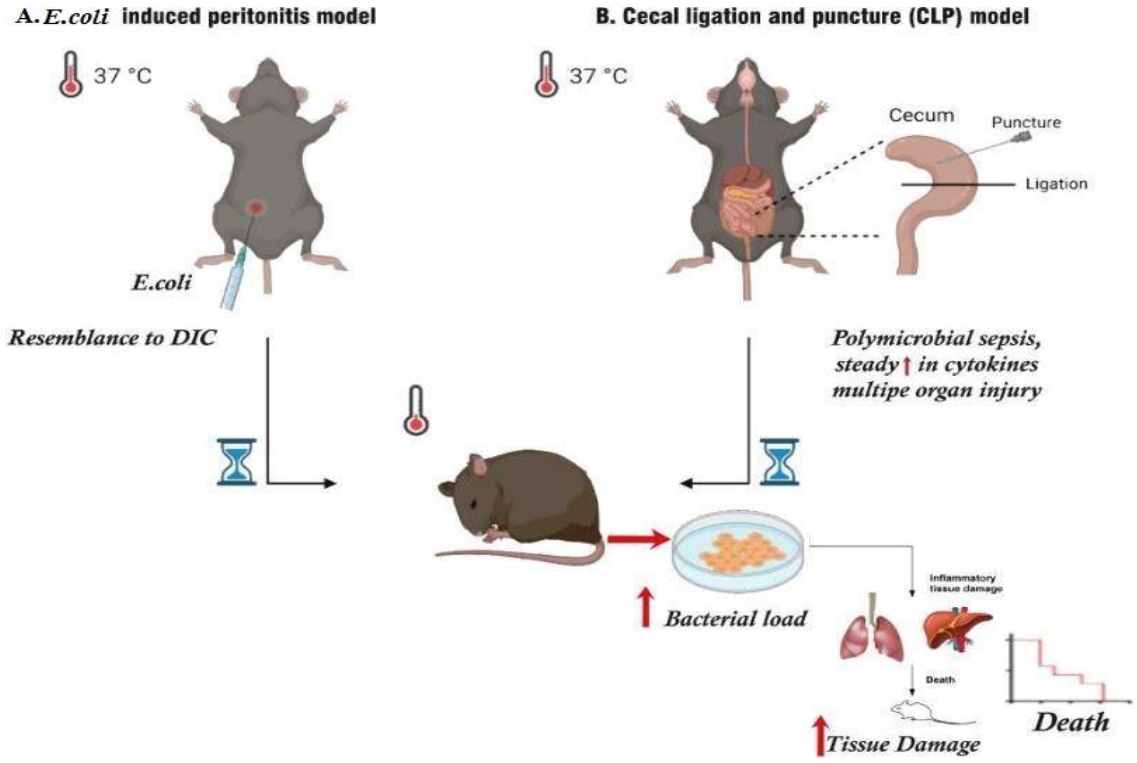


Figure 12: Schematic representation of two most used sepsis models.
(**A**) The injection of live bacteria *E.Coli* (8739™) (Intraperitoneally). (**B**) The ceacal ligation and puncture (CLP) model by puncturing and ligating the cecum, faeces can reach the peritoneal cavity was established. Hypothermia is one of the illness signs in both models, and it can be evaluated by measuring body temperature. The figure was adopted from BioRender.com and modified.

Cecal Ligation and Puncture (CLP) model: Polymicrobial sepsis:

The CLP model is one of the most stringent clinically applicable models of sepsis, involve a localized infection, such as surgically induced polymicrobial sepsis, that gradually propagates a systemic immune response, compared to other models, CLP provides a better representation of the complexity of human sepsis and is the crucial pre-clinical test for any new treatment to human sepsis. CLP involves a combination of three insults: tissue trauma from laparotomy, necrosis from cecal ligation, and endogenous infection from microbial leaking. In this model, feces are leaking into the abdominal cavity, introducing peritonitis and further followed by translocation of bacteria into bloodstream which activates inflammatory response [Wichterman, Baue, and Chaudry, 1980].

The advantage of CLP is that the pathogens are endogenous, simulating severe damage that leads to peritonitis in humans. Furthermore, the course of sepsis is strikingly comparable to that of human sepsis, with both hyper- and hypo-inflammatory responses [Rittirsch D ,2009; Wendy E. Walker, 2021].

Being one of the best representatives of human sepsis, it has been recognized as the gold standard for sepsis research [Dejager L, 2011].

Bacterial infection model: *E. coli* Induced peritonitis sepsis model.

Bacteremia is suggested to play a significant role in the progression of sepsis, as the prevalence of positive blood cultures increases with the severity of sepsis (17% in sepsis, 25% in severe sepsis, and 69% in septic shock). Numerous studies have investigated the ability of various aerobic bacterial species to induce sepsis and septic shock, with *Escherichia coli* being the most commonly found pathogen. In experimental models of bacterial infection, live bacteria are introduced into the body to elicit an immune response and study the processes involved in sepsis. Animal subjects have been inoculated with either pure or mixed bacterial populations for an extended period to investigate septic conditions. In these models, the dosage and duration of bacterial infusion can vary greatly. Small animals exposed to low doses of *E. coli* over several hours exhibit minimal early physiological changes, while higher doses often lead to a biphasic response. This response is characterized by an initial increase in cardiovascular function followed by a subsequent decline, leading to cardiovascular collapse, organ dysfunction, and eventual mortality [Wendy E. Walker et al., 2021].

Peptide details:

Peptide IS 217 (Batch No: IS 217-17-10) with 90% purity was supplied by Issar Pharmaceuticals Pvt. Ltd and was stored at −20°C until usage, if stored for long term. During the study period, IS 217 was stored in a refrigerator (2-8^{0}C) under light-resistant conditions. For In vivo animal studies, the solution was prepared freshly by dissolving the required amount of peptide IS 217 in 0.9% normal saline. 0.9% normal saline was used for dilution and delivery media. The appropriate concentrations in 0.1 ml volume were administered to mice by S.C & I.V injections. The standard drugs and other materials used for in vivo animal studies were mentioned in the section 4.1.

Rationale behind the chosen route of administrations in the in vivo animal studies:

The success of peptide products hinges on advancements in drug delivery methods. Presently, the primary choice for administering peptide-based drugs is through injections due to limitations in oral delivery's reliability and effectiveness. Emerging trends prioritize patient-centered approaches to ease the burden on patients and healthcare systems.

Currently, the preference leans heavily towards injections, with a shift from intravenous (IV) to subcutaneous (SC) options, demonstrating a commitment to improving patient experiences. SC extended-release forms provide a competitive advantage and a practical shift from daily oral treatments. Weekly SC products, driven by patient demand, stand out as strong alternatives to daily oral doses.

In our initial studies, routes like intraperitoneal (IP) and intramuscular (IM) showed less effectiveness. The urgency of conditions like sepsis necessitates swift action, prompting consideration for the intravenous route. Later, a strategic move towards subcutaneous delivery, in line with broader trends in peptide drug development focusing on patients, follows the immediate treatment phase for stability.

Both intravenous and subcutaneous routes are the chosen modes of administration in this study. This addresses the urgent need for swift sepsis treatment and reflects the industry's evolving focus on patient-centered drug delivery solutions.

Animals and maintenance:

10-12 weeks old male BALB/c & C57BL/6 mice weighing 25 -30g, were used in the study. The animals were randomized and grouped and identified by marking by ear-punching after group

assignment. BALB/c mice are used for Intravenous (IV) route of administration, whereas C57BL/6 mice are used for Subcutaneous (SC) route of administration in both in vivo animal studies.

The animals were acclimated to the laboratory conditions for 7 days prior to dosing, during which time body weights and general conditions were observed. During the acclimation period, 5 mice were housed per cage, whereas during the study period, 3 mice per cage were limited. The mice were housed in polypropylene cages and allowed free access to food and water, ad libitum and were maintained under specifically regulated environmental conditions (rearing conditions): 12-hour light/dark cycle, temperature 24^0C + 2^0C, humidity 55% + 10%, and ventilation 11 to 15 air exchanges per hour (all-fresh-air system).

To reduce the number of mice used considering 3R principle (Reduction, Replacement and Refinement), the same control BALB/c mice strain were used for experiments involving BALB/c strain and the same applies to C57BL/6 mice involved animal experiments.

Ethics approval:

The present study was approved by the Ethics Committee of Birla Institute of Technology and Science–Pilani, Hyderabad campus (BITS–Hyderabad) (IAEC approval numbers are mentioned in the below table 32) and all procedures, care, and handling of the animals were performed according to the guide for the care and use of laboratory animals published by the CCSEA. The Animals were obtained from Jeeva Life Sciences provided registered breeder: 1757/PO/RcBiBt/S/14/CPCSEA), The experiments were conducted in an animal room equipped with Biosafety Level 2 (BSL2) facilities. Throughout the study, strict adherence to hygienic conditions and aseptic procedures was maintained. All the techniques/procedures have been refined to provide for maximum comfort/minimal stress to the animals. The male mice are used in PoC in vivo animal studies.

Table 32: IAEC numbers approved for the In vivo animal studies are mentioned as follows:

Study details	**IAEC Approval number**
Preclinical efficacy study of a synthetic peptide for the treatment of sepsis.	BITS-Hyd/IAEC/2020/17
Preclinical efficacy study of a synthetic peptide for the treatment of sepsis.	BITS-Hyd-IAEC-2022-43

4.2.6.1. Cecal Ligation and Puncture (CLP) model: induction of poly microbial sepsis:

Polymicrobial sepsis was induced using the CLP method described by [Wendy E. Walker, 2021; Benjamim et al. 2000], with minor modifications and the anesthetic used (figures 13 &14). Initially, the mice were fasted for 12 hours before to the CLP process, and then, in accordance with [Machado et al. 2012], they were anesthetized using intraperitoneal injections of ketamine (80-100mg/kg BW) and xylazine in a dose (5-10mg/kg) (for 20 g mice, ketamine of 0.2ml and xylazine of 0.1ml). A 10mm/1 cm midline abdominal incision was made under sterile conditions, followed by a laparotomy, in which the cecum was mobilized, ligated below the cecal valve at the distal end of the cecum, and punctured twice with a 20-gauge needle at the center of the distal end of the occlusal cecum and squeezed gently to extrude a small amount of intestinal contents through the perforation site to induce sepsis. The cecum was placed back into the peritoneal cavity, and the abdominal incision was then closed in two layers with absorbable ethilon suture 5.0, and the animals were resuscitated by subcutaneous injection of 0.1–0.2 mL of normal saline after the cecum was closed with sutures. The sham group of mice had the same procedure as the treated and disease control groups, which included exposing the bowel and opening the peritoneum, but without having their cecum ligated or punctured with a needle. After surgery, mice were given betadine/tramadol to treat their post-operative pain. To avoid eye dryness during surgery, an atropine solution was rubbed into the eyes. After being allowed to obtain food and water, all of the animals were placed back in their cages. After 2h of CLP, the treatment groups were administered with IS 217 peptide (0.6 &1.2 mg/kg) and the standard drug through the subcutaneous &intravenous route.

To reduce variability between studies, the CLP method was always carried out by the same investigators. [Zingarelli B et al., Libert C et al., Hellman J et al., 2019], the mice were subjected to the CLP in the sequence demonstrated in the figure 13 and figure 14 provides a pictorial representation detailing the CLP suturing surgical procedure.

Figure 13: Step wise procedure of the Cecum Ligation and Puncture (CLP) model.

(a) Setup of the surgery table. (b) IP injection of anesthesia. (c) Confirmation of anesthesia by touch reflex. (d) Shaving the surgical portion of the mice. (e) Placing the mice on the surgery table and disinfecting the surgical part of the mice. (f) Skin incision. (g) Locating and exposing the cecum. (h&i) Ligated the cecum. (j) Puncturing the cecum with a 20-gauge needle. (k) Extruding the cecal matter /suspension. (l) Replacing the cecum back into peritoneal sac of the mice. (m&n) Skin closing suturing steps. (o) After the suturing. (p) Applying the betadine to the surgical part of the mice. (q&r) Placing the mice on the heating pad for the maintained of the body temperature and recovery of the anesthesia. (s) Post operated surgical mice after recovery from anesthesia.

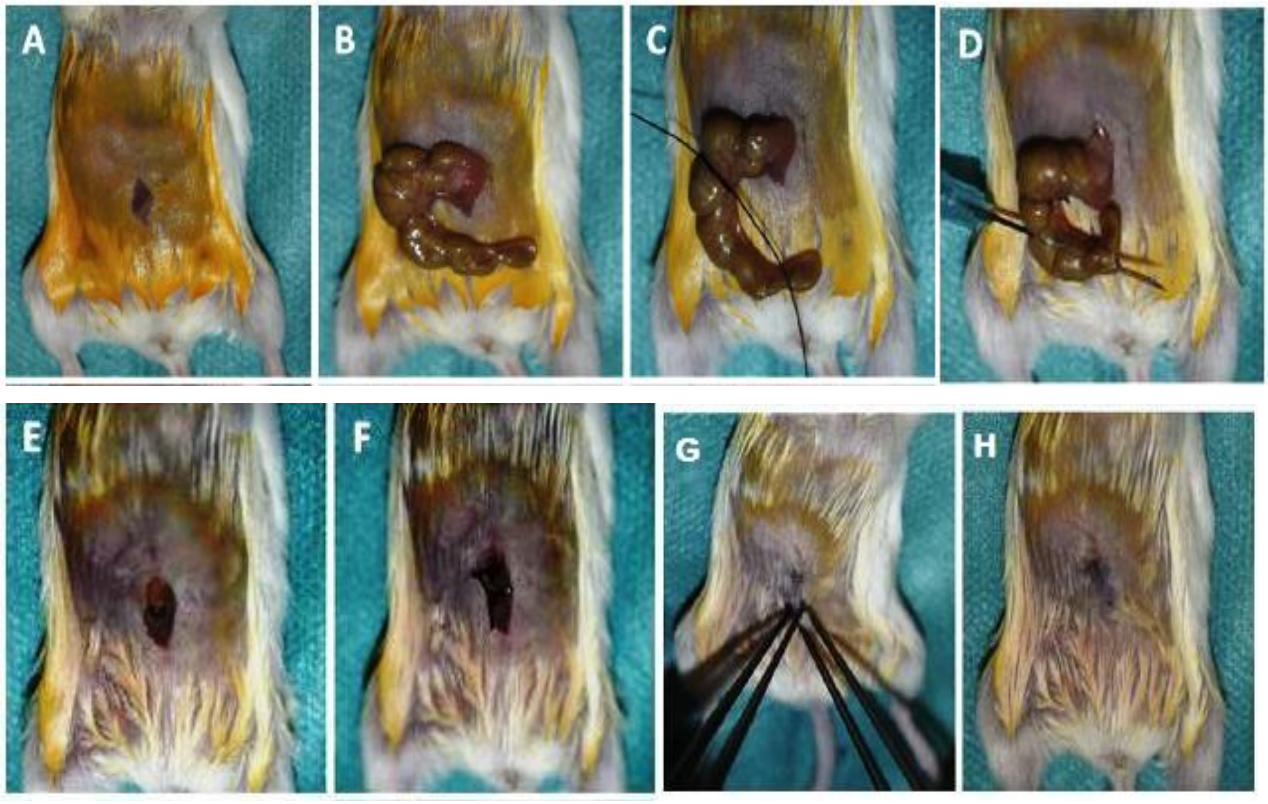


Figure 14: Detailed CLP skin suturing surgical procedure: (a) incision of skin, (b) cecum exteriorization, (c) cecum ligation, (d) cecum puncture, (e) repositioning of the cecum, (f +g) abdominal wall closure with single button sutures, (h) closed skin, postoperatively.

4.2.6.1.1. Pilot study: standardization of CLP model and peptide IS 217 doses:

In standardization model, The CLP procedure was done as described above. The animals of 8-10 weeks of age, are grouped into normal control, sham control, disease control and treatment groups and where the sham control group were considered as negative control and disease control group was considered as positive control. The peptide IS 217 at different doses were administered to treatment groups via intravenously and the animals were observed for 24 h for survival rate and physical activity. After the 24 h of post infection, the mice are bled retro-orbitally and left to coagulate at room temperature for ~2 h. Sera was then separated by centrifugation and the Inflammatory biomarkers IL-10, TNF-α, IL-1β and IL-6 levels were determined by using ELISA kits. The standardization of this experiment was required to fix the doses of peptide IS 217 and CLP procedure for disease induction.

4.2.6.1.2. Main CLP study: Experimental design and treatment groups.

Mice were subjected to CLP surgery and were discarded if they died during the procedure. In all the animal experiments, the peptide IS 217 was administered by subcutaneous and intravenous injections respectively; according to the clinical dosage regimen. IS 217 at two doses (0.6 &1.2 mg/kg) was administered at 2 h after CLP surgery and recovery of animals from anesthesia in the treatment groups as mentioned in the table 33. The mice were divided into the following treatment groups at random: BALB/c mice are used for Intravenous (IV) route of administration, whereas C57BL/6 mice are used for the Subcutaneous (S.C) route of administration. In each group the half of the animals were killed after 4 and 18 hours, [Hubbard, W. J 2005; Rittirsch D 2009] and another half of the animals are continued for survival analysis and observed for 10 days, in this subset of survival analysis animals, the peptide IS 217 at two doses 0.6 & 1.2 mg/kg were injected SC & IV daily for 5 days following post-CLP surgery. 0.9% Normal saline was administered in the sham group. The survival of mice was monitored for up to 18 h and the other half of the animals are continued for the survival monitored upto 10 days. The study design was described as below figure 15.

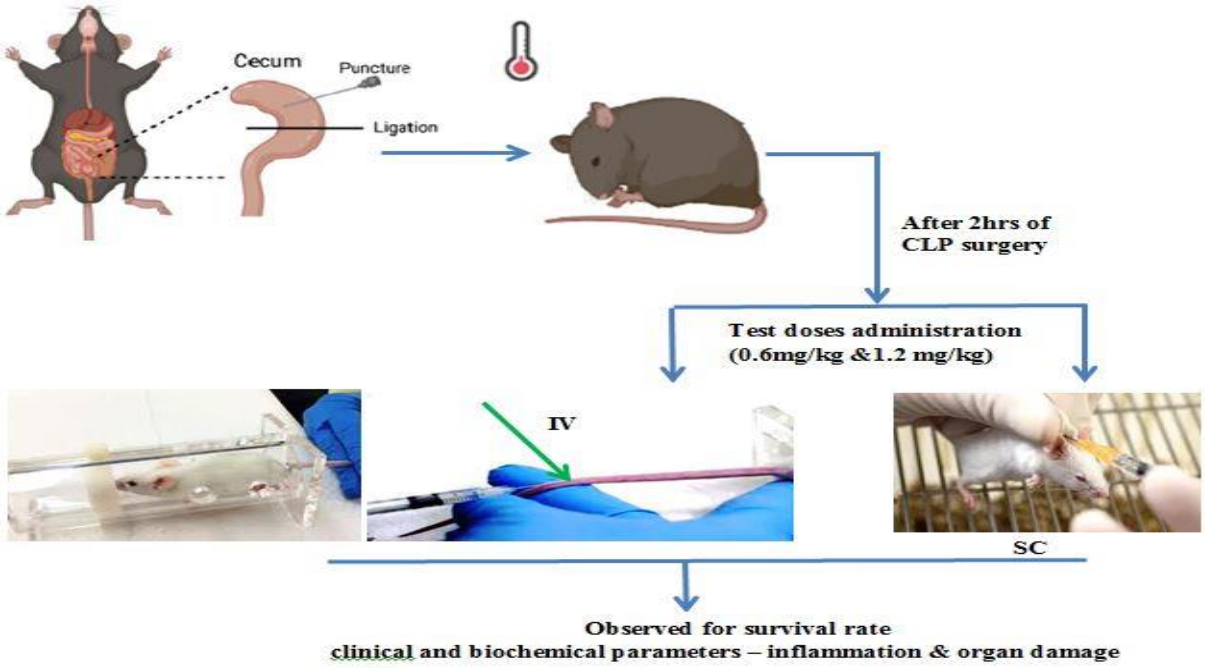


Figure 15: Study design and treatment regimen in CLP model

The figure was modified from [Jon A Buras et al.,2005].

Table 33: Group allocation of animals & dosing paradigms[@] per group and the study parameters of the study -CLP model.

S.no	Group	Dose	No. of animals[#]	Study Parameters
1	Normal control	NA	06	• Survival rate • Physical activity: By scoring system • Blood parameters: Coagulation tests, & Biochemical parameters (ALP, AST &ALT) levels • Estimation of cytokine levels (Pro inflammatory and Anti-inflammatory): TNF-α, IL-6, IL-1β & IL-10 in serum and peritoneal Lavage Fluid and lungs. • Histopathology
2	Sham control	0.9%NS	24	
3	Disease control (CLP)	NA	30[*]	
4	CLP + IS 217	0.6mg/Kg	24	
5	CLP + IS 217	1.2mg/Kg	24	

#: The no. of animals was divided for three time points: 1) at 4 h; 2) 18 h and 3) 10 days, except the control group. The control group animals are considered the same for all three time points.

@: The above table for one route (IV) and the same type of allocation was followed to another route (SC). The dose calculations are mentioned in the Appendix -III.

*: To match the minimum number of animals for statistical analysis, and considering the mortality rate of animals in the disease control group, the number of animals is higher compared to other groups.

4.2.6.1.2.1. post-surgical procedures:

To prevent post-surgical infection, the mice were placed in the cage supine position until the anesthetic effect wore off. To prevent hypothermia after surgery, the cage was warmed with a heater.

4.2.6.1.2.2. Behavioral test:

The behavioral assessments were carried out on the tenth day following CLP on all animals [Liu Y et al., 2017].

Novel object recognition test (NORT):

One of the most well-known tests for measuring recognition memory in rodents is the novel object recognition (NOR) test. The apparatus is made up of a transparent Plexiglas box that measures 40 cm× 40 cm× 40 cm. Objects are around 4 cm× 4 cm× 4 cm in size. Objects, like building blocks, have simple shapes. Wooden, metal, and hard plastic objects are preferred, since they are resistant to biting damage [Bharathi Hattiangady et al., 2014] (figure 16).

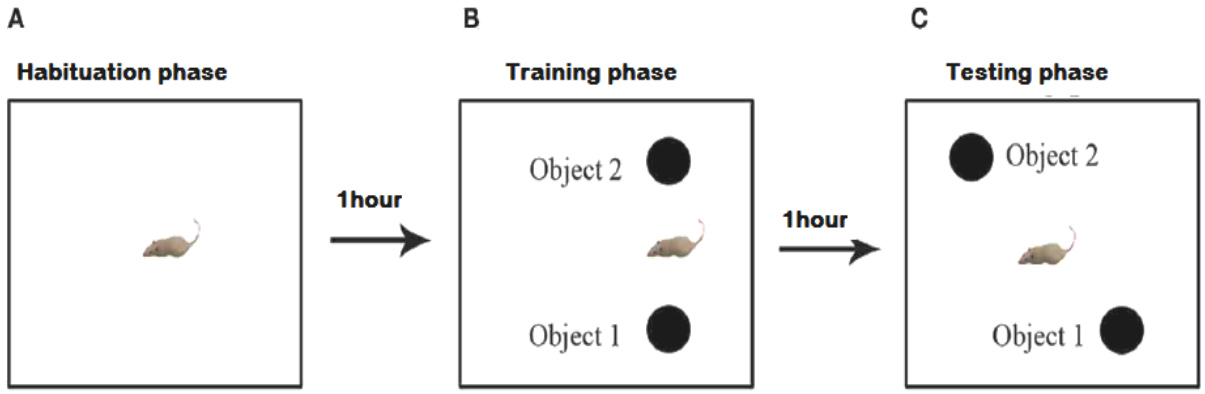


Figure 16: (A–C) shows as schematic representation of three trials and inter-trial intervals in NOR test.

Exposure of CLP post operated diseases control and IS 217 treated groups to NORT test for the evaluation of stress conditions and memory learning process.

Procedure:

Grouping and treatment were carried out in accordance with the procedure. To reduce stress, the mice were treated in a controlled environment. Mice were first moved for 10 minutes to a preparation area near the operative space. The mice are then placed in the apparatus for analysis for 10 minutes to acclimatize. This test also had several trials for each mouse, with a 60-minute break between each trial [Hattiangady and Shetty, 2012].

As described, the first two trials involved placing the mice in the middle of an empty open field box and letting them freely explore the box for 5 minutes (first trial, the habituation phase, Figure 4A), and then placing the mice in the middle of an open field box with two identical objects on opposite sides of the box and letting them freely explore the objects for 5 minutes (second trial, the training phase, Figure 4B).The third trial (the objection recognition memory testing phase) began 60 minutes

after the second trial. The mice were given 5 minutes to explore the objects in the same open field box, which had one object from the second trial (the familiar object) and a new object in place of the second object from the second trial (the novel object, Figure 4C). A mouse is exploring an object when its nose is within 2cmof the object. The movement of mice in the third trial was continuously tracked and noted the time(sec) and the apparatus was cleaned with 70% alcohol and air-dried prior to the commencement of each trial for every mouse. The total amount of time spent exploring the new object, the known object, and both objects (i.e., the total object exploration time) were recorded. Furthermore, novel object discrimination index was calculated by using the formula, to calculate the object preference which is: **The preference % =**

Time to explore the individual novel object/ Total exploration time to objects X 100

Then, within each group, the amount of time spent exploring the new object versus the familiar item was measured in percentages (%). The preference rates were calculated by dividing overall exploration time by item exploration time. This demonstrates the ability of each group of animals to investigate new item recognition under stressful conditions.

The novel object discrimination index was also directly compared with control groups of mice. The mice in the control group were also tested on how well they could tell the difference between new and old objects. The speed and total distance moved during the third trial (test phase) were also recorded and compared between the two groups to see if depression (or lack of motivation) affected the object recognition memory test. The fact that the new object was looked at more than the known object reflects learning and (recognition) memory processes are used.

4.2.6.1.2.3. Experiment 1: Survival study:

In the survival study, Survival rates were determined by setting the day 0 from 0 to 18 h after surgery and continuing observation until day 10. In one set of animals/group, survival was monitored every 4 h up to 18 h and in another half subset of animals was followed for 10 days for survival assessment every 12 h after sepsis induction to verify mortality rates. The time of death was recorded as accurately as possible.

4.2.6.1.2.4. Disease parameters: The following parameters are in common in both in vivo animal studies [Shrum B, 2014].

Vital clinical symptoms evaluation:

The physical activity of the mice was recorded independently by two independent observers who were blinded to treatment before sampling as previously described. In this study predetermined grading system was used with a scale of 1 (healthy) to 5 (agony). This scoring system is based on grading physical activity and food intake (table 34) using spontaneous activity of mice, reactivity to external stimuli, and spontaneous food intake to differentiate between grades 1 and 5. Animals were closely observed for first 4- 18 h for the development of symptoms, and where appropriate, time to death was recorded. Mice were observed at least every 6 h and for next over a 5-day period for the development of symptoms, and where appropriate, time to death was recorded. The mice that were moribund and those with a body temperature less than 27^0C were euthanized and counted as dead at each time point indicated. Body temperature was measured by an electronic thermometer with a mice rectal probe. Mice were manually restrained, the probe was lubricated with sterile glycerol, inserted for 5 seconds (or until the temperature reading stabilized), and the data were recorded. Malaise, immobility, and ruffled coat was noted in some animals. Humane endpoints were strictly observed (immobility, dyspnea, paralysis) so that no animal became distressed. Clinical signs, including conjunctivitis, fur coat ruffling, and responsiveness to external stimulation, were noted in all experimental groups and evaluated using the established grading system. Based on the severity of these symptoms observed in the majority of animals, an overall grade was assigned to each group.

The clinical score was determined using a 0–4 grade scoring system, with grades 0 being normal, 1 being mildly lethargic, 2 being lethargic and hunched, 3 being extremely lethargic, hunched, and shaky, and 4 being dead. To compare body temperatures for physiological analysis.

Table 34: Scoring system for measuring mice physical activity:

Grade	Quality	Criteria
1	**Very active**	Strong, curious, quick movements, normal food intake
2	**Active**	Strong, curious, occasional interruptions in movements, normal food intake
3	**Less active**	Adequate response to environment, frequent interruptions in movements, slight decreased food intake
4	**Slow**	Sleepy, slow activity, severely decreased food intake
5	**Lethargic**	No activity, motionless, no food intake

4.2.6.1.2.5. Experiment 2: Acute hyperinflammation study: In this study, we measured pro and anti-inflammatory cytokines TNF-α, IL-6, IL-1β, IL-12 and IL-10 in the serum and Peritoneal lavage.

1. Quantification of cytokine levels:

For the quantitative measurement of cytokines (IL-1β, TNF-α, IL-12 p70, IL-6, IL-10, and VEGF), ELISA kits from R&D Systems (Minneapolis, MN) were utilized. These kits were employed to measure the cytokine levels in various samples, including mouse lung (BALF supernatants), sera, and peritoneal exudate cell (PEC) supernatants, following the manufacturer's instructions. The results were reported as picograms per milliliter (pg/mL) of the analyzed samples.

4.2.6.1.2.6. Experiment 3: Late immune paralysis studies:

In the late immune paralysis study, cytokine levels were assessed along with the estimation of colony-forming units (CFUs) in the blood, peritoneal fluid (PLF), and organs. [Kieslichova E, et al., 2013].

Sampling time points:

The animals were observed for 18 h and 10 days after the CLP surgery and the remaining animals are euthanized under humane conditions, the mice were anesthetized with ketamine (80-100mg/kg BW) and xylazine in a dose (5-10mg/kg) intraperitoneally for blood and peritoneal lavage fluid collection and euthanized with an overdose of (150 mg/kg ketamine hydrochloride and 120 mg/kg xylazine hydrochloride) /isoflurane for organ collection. For clinical observations, the time points and parameters are listed as follows (table 35).

Table 35: List of the parameters screened for biochemical and clinical analysis.

Time point	Parameter	Technique
4th hour	Estimation of cytokines in lungs, spleen, blood, and peritoneal fluid. Estimation of MPO in lungs and VEGF levels in lungs and sera.	ELISA
18th hour	Estimation of cytokines in lungs, spleen, blood and peritoneal fluid.	ELISA, western blotting in lungs (BALF).

	Biochemical parameters (AST, ALP & ALT). Coagulation tests (PT, aPTT) Bacterial count evaluation Histopathology	
Day 7/10	Estimation of cytokines in blood and peritoneal fluid.	ELISA

Experimental outcomes:

1. Blood sampling

At 4 h, 18 hours, and the last day following the cecal ligation and puncture (CLP) procedure, whole blood and tissues were collected from the surviving animals for hematologic and biochemical analysis. The mice were anesthetized using a combination of ketamine (80-100mg/kg BW) and xylazine (5-10mg/kg) administered via intraperitoneal injection at a ratio of 2:1. For blood collection, mice were bled from the retro-orbital region at specific time points using either 4% EDTA or 3.2% sodium citrate as anticoagulants for whole blood and plasma collection, respectively. The blood was allowed to clot at room temperature for approximately 2 hours and then centrifuged at 2,000 rpm for 10 minutes to separate the plasma. Serum was obtained by centrifuging at 1500 rpm for 10-15 minutes. The collected serum and plasma were aliquoted and stored at -20 °C until further use in ELISA assays.

2.Assessment of clinical &biochemical parameters:

Coagulation tests & Hematology: Blood collected in sodium citrate tubes was promptly centrifuged to obtain plasma for the assessment of prothrombin time (PT), fibrinogen (FBG), and activated partial thromboplastin time (aPTT) using an automated blood coagulation analyzer. Whole blood was used to determine the white blood cell (WBC) and platelet (PLT) counts using a hematology analyzer.

Liver and kidney function tests: The levels of liver function markers, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as the renal function marker serum creatinine (SCr), were routinely measured using automated analyzers and hemagglutination analyzers.

Differential leukocyte counts: For the assessment of differential leukocyte count, blood was collected in tubes containing EDTA and analyzed using an automatic cell analyzer (ADVIA 2120 Hematology System).

Serum procalcitonin (PCT), lactate and CRP levels: The quantification of serum PCT levels was performed using an enzyme-linked immunosorbent assay (ELISA) Kit, following the manufacturer's instructions (R&D Systems).

Peritoneal fluid collection:

In certain experiments, the peritoneal cavity of euthanized mice was rinsed with 5 ml of sterile ice-cold phosphate-buffered saline (PBS) using an 18-gauge needle. The peritoneal lavage fluid was collected in sterile tubes and immediately placed on ice. Sequential centrifugation was then carried out, first at 2500 rpm to remove the peritoneal cells from the sample. A portion (10-100 µL) of the resulting supernatant was used for the bacterial count, followed by a second centrifugation at 5000 rpm to eliminate *E. coli* bacteria and obtain a clear lavage. The peritoneal cell-free clear supernatant was used for cell counting and ELISA, while the cell pellets were utilized for Western blotting. Both sets of samples were stored at -80°C until further analysis (Figure 17).

Our objectives were twofold: (1) to investigate the direct impact of pro-inflammatory and anti-inflammatory cytokines (IL-1β, TNF-α, IL-12 p70, IL-6, and IL-10) in the peritoneal lavage fluid and (2) to assess bacterial growth and cell counts in the peritoneal lavage fluid of anesthetized mice[Stefan Wirtz et al., 2006].

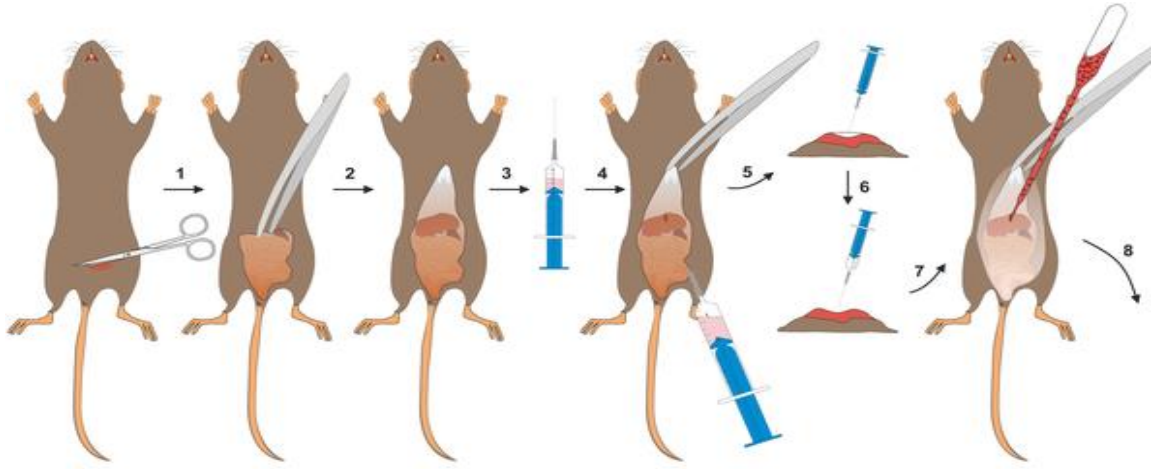


Figure 17: Collection of peritoneal lavages from the mice. [Meurer SK,2016]

1. Mice bacterial clearance in PLF after treated with IS 217: CFU assay.

Peritoneal lavage fluid samples were used to count bacterial CFUs, the collection of peritoneal lavage fluid was as mentioned above.

For bacterial clearance, serial dilution in peritoneal lavage fluid (1: 10, 1: 100, 1: 1000, 1: 10,000, and 1: 100,000). A 100µL aliquot of each dilution was spread on a tryptic soy agar plate/Solid Luria-Bertani (LB) culture plates. All plates were incubated at 37^0C for 48 h. Colonies were counted and expressed as colony forming units CFU per milliliter for all samples.

2. Isolation of leukocytes from the peritoneal cavity of mice:

To collect cells, present in the peritoneal cavity, a solution of 3.0 ml of phosphate-buffered saline (PBS) containing 1 mM EDTA was injected. The total number of cells was determined by counting them using a hemocytometer. The peritoneal lavage fluid was then subjected to centrifugation at 1,000 rpm for 10 minutes at room temperature. The resulting pellet was collected and suspended in 200 µl of PBS containing 0.6 mM EDTA. Differential cell counts were obtained using cytospin preparations. After cytospinning, the cells were stained with Wright-Giemsa stain and examined under a magnification of 600x using an oil immersion lens to distinguish between different cell types.

Preparation and processing of spleen tissue and cell harvesting:

The spleen tissue was collected from all mice in each experimental group. A portion of the tissue was immediately fixed in 10% neutral buffered formalin for further processing, while the remaining tissue was homogenized and suspended in phosphate-buffered solution (PBS) containing 10% Triton X-100. The cells obtained from each spleen were suspended in RPMI 1640 containing antibiotics. The cells were then placed in flat-bottomed wells of 96-well plates at a cell density of 5x105 cells in a final volume of 150 µl. Spleen cells from uninfected mice were used as a control. After 72 hours of incubation, the plates were centrifuged at 1200 rpm for 10 minutes, and the supernatants were collected and stored at -80°C for cytokine analysis. Cytokine levels in the spleen tissue were determined in the supernatant using ELISA and commercial assay kits from R&D Systems. Briefly, the cytokine assay plate layout consisted of a standard series in duplicate, four blank wells and 20 µl duplicates of tissue supernatant samples and diluted to 50 µl with mouse serum diluent provided in

the kit. The quantification of the assay was performed using a standard curve following the manufacturer's instructions, and the results were presented in pg/ml. [Iqbal J et al.,2016]

Preparation of lung samples:

To examine histological changes in lungs, to estimate the lung edema and estimate myeloperoxidase (MPO) activity, the lung tissue from the mice was collected. The lungs were harvested, weighed, and homogenized in ice-cold PBS after being placed on ice. The resulting homogenates were centrifuged at 8000 x g for 10 minutes at 4°C, and the supernatants were stored at -80°C until analysis.

1. **Measurement of the lung wet/dry weight ratio:**

The wet-to-dry weight ratio of lung samples from the upper and middle lobes of the right lung in each mouse was measured. The surface water on the lung was absorbed with filter paper to assess lung tissue edema. After euthanization, the mice underwent whole left lung pneumonectomy, and the lungs were immediately weighed to obtain the wet weight. Subsequently, the samples were wrapped in aluminum foil and desiccated in an oven at 60-80°C for 48 hours until a stable dry weight was achieved. The ratio of wet weight to dry weight was calculated by dividing the wet weight by the dry weight, providing an evaluation of pulmonary edema. Additionally, the lung index (lung index % = wet lung weight/body weight × 100) was calculated to further confirm the degree of pulmonary edema.

2. BALF collection and analysis of total protein concentration and cell count of the BALF:

BALF was extracted as described [Van Hoecke L, et al. 2017] and prepared as previously described [Kuo et al. 2011]. Bronchoalveolar lavage fluid (BALF) was collected by inserting a catheter into the trachea of terminally anesthetized mice, through which a saline solution was instilled into the bronchioles and then gently retracted to retrieve the BALF. This process was repeated three times with a total of 1.5 mL sterile saline. The collected BALF was centrifuged at 1500 rpm for 10 minutes at 4°C, and the supernatant was stored at -20°C for the assessment of inflammatory cytokines and VEGF levels using commercially available ELISA kits. The protein concentration in the supernatant was measured using a BCA detection kit (Thermo Scientific, Rockford, IL, USA). The OD value was read at 540 nm according to the kit's instructions, and protein concentrations were expressed in milligrams per milliliter of BALF.

3. Measurement of MPO activity:

The activity of myeloperoxidase (MPO) was assessed using the following procedure: Lung tissue was perfused with sterile PBS to remove MPO activity from any blood contamination. After 18 hours of inducing sepsis, the mice were anesthetized and their lung tissues were dissected, weighed, and homogenized in 0.5% HTAB buffer (hexa decyl tri methyl ammonium bromide in 50 mM potassium phosphate buffer) to obtain a 10% homogenate. The homogenized tissues were then stored at -80°C to rupture the cells. Upon thawing, the homogenates were centrifuged at 10,000 g for 2-5 minutes at 4°C. The resulting supernatants were used to determine MPO levels (in U/mL) using ELISA kits according to the manufacturer's instructions. To measure MPO, the supernatant samples were diluted in phosphate citrate buffer (pH 5.0), and their absorbance was measured at 460 nm using a microplate reader. The amount of MPO in the lung was quantified as units per milligram of lung tissue.

4. Western blot:

Western blot analysis was performed to detect the phosphorylation of MAPK. The protein expression of total ERK1/2, p38, and phosphorylated ERK1/2, p38 in lung tissue was examined. Initially, lung tissue lysate was prepared as follows: 50 mg of lung tissue was homogenized in 200 µL of cold RIPA lysis buffer, followed by centrifugation at 10,000 rpm for 5 minutes at 4°C. Subsequently, 5× SDS loading buffer was added to the resulting supernatant, and the mixture was boiled at 95°C for 5 minutes. Next, 20 µg of protein samples were loaded onto a 10-12% SDS/PAGE electrophoresis gel for separation, which was then transferred to a polyvinylidene fluoride (PVDF) membrane at 110 V for 1.5 hours on an ice bath. The membrane was then blocked with 5% milk for 1 hour at room temperature. For the primary antibody incubation, the PVDF membrane was incubated overnight (~12 hours) at 4°C with the primary antibodies diluted in a 5% FBS solution. The primary antibodies used were p-ERK (1:1000), ERK (1:1000), p-P38 (1:2000), and P38 (1:2000) (abclonal). Subsequently, the membrane was washed three times with 1× TBST solution for 10 minutes at room temperature and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody anti-mouse (1:5000) diluted in 1× TBST solution at room temperature for 1 hour (1:10000). After three additional washes (10 minutes each) with Tris-buffered saline/Tween 20 (TBST), the membrane-bound antibodies were visualized using the ECL detection reagent (ECL, Advansta), and the images were captured using a Fusion SL Imaging System (VILBER LOURMAT, France). The density of the bands was analyzed by densitometry using Image J software.

4.2.6.1.2.7. Histopathological evaluation: macro- and microscopic assessment of the organs:

Animals that remained until the end of the study (at least n = 3 for each group) underwent histopathological evaluation. Vital organs, including the heart, brain, lungs, liver, kidneys, and spleen, were harvested and weighed immediately after collecting blood samples. The macroscopic analysis of the organs considered parameters such as size, weight, integrity, and the presence of visually detectable changes.

The relative organ weight was calculated using the following formula:

Relative organ weight % = (organ weight/body weight) × 100

For microscopic assessment, the organs were fixed in a 10% formalin solution (pH 7.4) for subsequent histopathological examination. One representative animal per group was selected for further analysis.

Hematoxylin-eosin (H&E) staining:

The organs were removed, fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm using a microtome. The sections were then stained with hematoxylin and eosin. To prepare the sections for analysis, they were dewaxed and rehydrated by sequentially immersing them in xylene I and II (each for 20 minutes), absolute ethanol I and II (each for 5 minutes), and 75% alcohol (5 minutes). The sections were then rinsed with tap water, stained with hematoxylin for 3-5 minutes, blued, dehydrated in increasing concentrations of alcohol (85% and 95%) for 5 minutes each, and counterstained with eosin for 5 minutes. Afterward, the sections were cleared with absolute ethanol I, II, and III (each for 5 minutes), followed by xylene I and II (each for 5 minutes). The sections were mounted with neutral gum and observed under a light microscope using 100X/200X lenses. At least 10 different fields were analyzed for each mouse. Pathologists who were unaware of the experiment observed and scored damage to the spleen, lungs, liver, kidneys, heart, and brain.

Histopathologic Observation:

A pathologist, blinded to the group information, evaluated and scored the entire slide surface. The criteria for scoring lung inflammation were based on Murakami's technique, which assessed pulmonary edema by evaluating alveolar wall thickening, vascular congestion, and interstitial and alveolar leukocyte infiltration. Lung parenchyma was graded on a scale of 0-4 (0 = absent and

appears as normal tissue; 1 = mild; 2 = moderate; 3 = severe; 4 = intense) for congestion, edema, inflammation, and hemorrhage. Mean scores for each parameter were calculated, and lung injury scores were determined by assessing neutrophil infiltration, hemorrhage, necrosis, congestion, and edema, using the following scale: 0 = normal, 1 ≤ 25%, 2 = 25-50%, 3 = 50-75%, and 4 ≥ 75%.

To score liver injury, the following parameters were analyzed (16): interstitial inflammation, formation of thrombi, hepatocellular necrosis, and portal inflammation.

4.2.6.2. Bacterial infection model: *E. coli*–Induced Septic Peritonitis Model.

The bacterial infection model used in this study was *E. coli*–induced septic peritonitis, which is Murine lethal infection by intraperitoneal injection of live bacteria *E. coli.* [Asma Ahmed et al., 2018]

Preparation of bacterial suspension:

The laboratory-maintained E. coli ATCC 8739™ strain, intended for the mouse sepsis model, underwent identity confirmation using a UTI chrome plate (see Figure 18). This strain was stored previously at -80˚C in Luria-Bertani (LB) broth supplemented with 15-20% glycerol. To initiate growth, individual colonies from viable Luria-Bertani (LB) agar plates were transferred to sterile liquid LB medium containing 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. Cultures were aerobically incubated in 50-ml volumes on an orbital shaker at 37 °C for 24 hours in a 5% CO_2 environment. Subsequently, these cultures were transferred to 500 ml of fresh LB medium and allowed to grow for an additional 12 hours. To reach the desired bacterial concentration during the exponential growth phase, the suspension's optical density (OD) was adjusted to 0.3 using a spectrophotometer. This OD value corresponds to roughly 1×10^8 colony-forming units (CFU)/mL. After centrifugation at 1500 ×g for 5 minutes at 4 °C, the supernatant was discarded, and the bacterial pellet underwent three washes and resuspensions in phosphate-buffered saline (PBS) at pH 7.4. Vortex mixing ensured a concentration of about 1×10^8 CFU/mL. To render the cells inactive, the bacterial suspensions were heat-treated in a water bath at 100 °C for 30 minutes. Portions of the suspension (100 µL) were serially diluted with a sterile saline solution and plated onto LB agar plates for incubation over 18 hours to determine bacterial concentration. The remaining culture suspension was stored at 4 °C for future use. Just prior to the intraperitoneal injection, the bacterial suspension was adjusted to a concentration of 1×10^8 colony-forming units (CFU) per 0.1 mL using a normal saline solution/PBS.

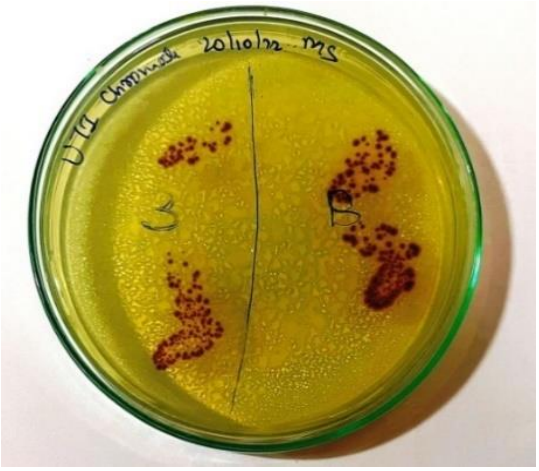


Figure 18: Confirmation of *E. coli* strain used in the study on UTI chrome plate.

Infection of peritonitis in animals:

This study was carried out within an animal containment BSL2 facility. The animals were infected with suspensions containing 2.5/ $5X10^8$ CFU/mL *E. coli* 8739™ (200µL) inoculated intraperitoneally. The experiment designated the day of the challenge as day 1, considering it as 0 hour. [Wendy E. Walker,2021; Komal Dolasia et al.,2018]

4.2.6.2.1. Pilot study: standardization of peritonitis in animals.

To standardize peritonitis in animals, the pilot study was carried out by using male C57BL/6 mice that were matched in terms of age and weight. Log phase *E. coli* 8739™ cells were suspended in 0.1 ml of sterile PBS and injected intraperitoneally (IP) into the mice. Two different doses were administered: 2.5 x 10^8 CFU per mouse and 5.0 x 10^8 CFU per mouse. The aim was to induce septic peritonitis and observe the survival rate and physical activity of the mice. After 24 hours post-infection, blood samples were collected from the mice by retro-orbital bleeding and allowed to coagulate at room temperature for approximately 2 hours. The serum was then separated through centrifugation, and the levels of inflammatory biomarkers IL-10, TNF-α, IL-1β, and IL-6 were determined using ELISA kits. Standardizing this experiment was necessary to determine the appropriate dose of *E. coli* cells for inducing infection/disease and to fix the doses of peptide IS 217. The study design is described in detail in Figure 19.

4.2.6.2.2. Main study: *E. coli*–induced septic peritonitis model:

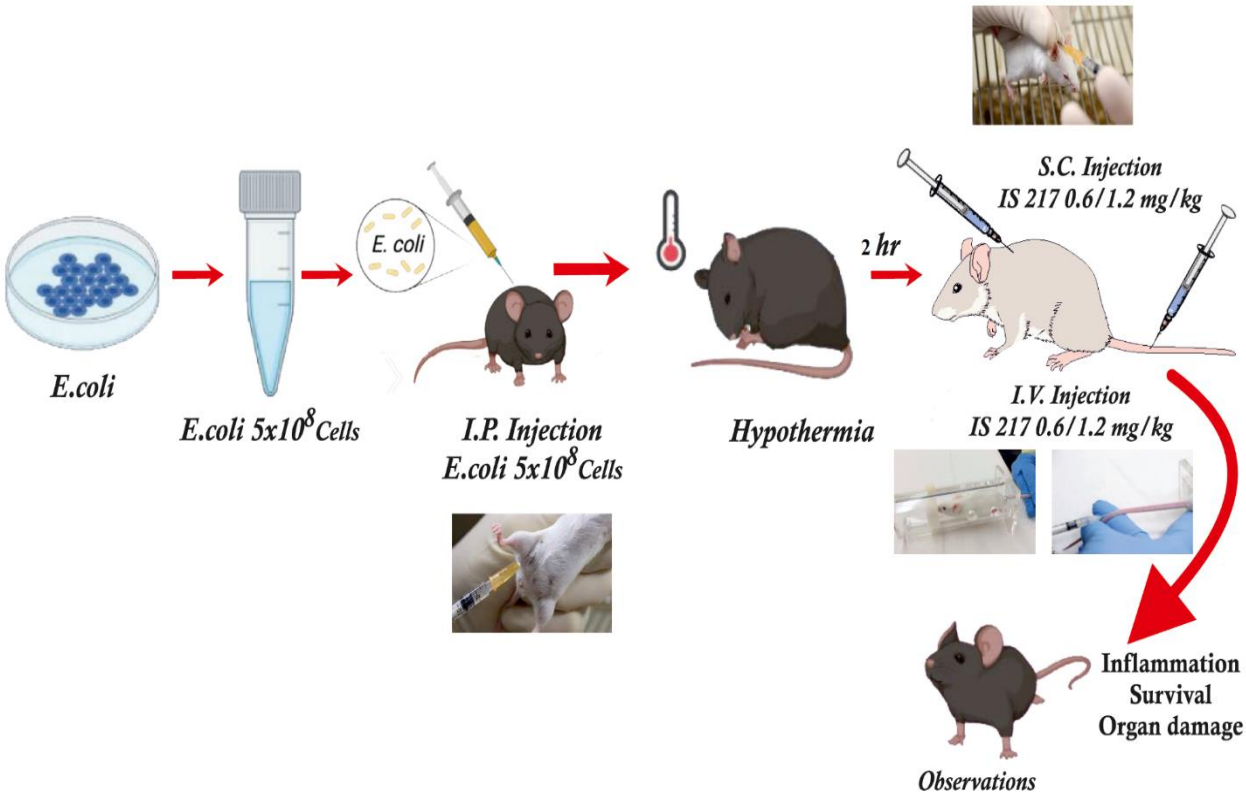


Figure 19: Study design and treatment regimen in *E. coli*–induced septic peritonitis model.
The figure was adopted and modified according to the present study. [Henrique G et al.,2020].

Following the intraperitoneal injection of *E. coli* ATCC 8739 at a dosage of 5.0 x 10^8 CFU per mouse in a volume of 200µL, septic peritonitis was induced in the mice as described earlier. After 2 hours of *E. coli* infection, the treatment groups mentioned in table 36, were administered treatment with two test doses of IS 217 (0.6mg/kg and 1.2 mg/kg) subcutaneously and intravenously. Control mice received 200 µL of normal saline.

Experimental design:

In all the animal experiments, IS 217 were administered by subcutaneous and intravenous injections C57BL/6 mice and BALB/c mice respectively; According to the clinical dosage regimen.

Table 36: Group allocation of animals & dosing paradigms[@] and the study parameters of the study - study -*E. coli* induced peritonitis model.

S.no	Group	Dose	No. of animals[#]	Study Parameters
1	Normal control	NA	06	• Survival Rate • Physical activity: By scoring system • Blood Parameters: Coagulation tests, & Biochemical parameters (ALP, AST &ALT) levels • Estimation of cytokine Levels (Pro inflammatory and Anti-inflammatory): TNF-α, IL-6, IL-1β & IL-10 in serum and Peritoneal Lavage Fluid and lungs. • Histopathology
2	Disease control Injection of heat-killed E. coli ($5X10^4$ CFU per mice)	0.9%NS	30*	
3	Disease Induction + IS 217 -0.6mg/kg	0.6mg/Kg	24	
4	Disease Induction + IS 217-1.2smg/kg	1.2mg/Kg	24	

#: The no. of animals was divided into three time points: 1) at 4 h; 2) 18 h and 3) 7 days, except the control group. The control group animals are considered the same for all three time points.

@: The above table for one route and the same type of allocation was followed to another route. The dose calculations are mentioned in the Appendix -III.

*: To match the minimum number of animals for statistical analysis, and considering the mortality rate of animals in the disease control group, the number of animals is higher compared to other groups.

IS 217 at two doses (0.6 &1.2 mg/kg) were administered at 2 h after the intraperitoneal injection of heat-killed *E. coli*. The mice were divided into the following treatment groups at random: BALB/c mice are used for Intravenous (IV) route of administration, whereas C57BL/6 mice are used for Subcutaneous (S.C) route of administration. In each group the half of the animals were killed after 4 and 18 hours, [Hubbard, W. J 2005; Rittirsch D 2009] and another half of the animals are continued for survival analysis and observed for 7 days, in this subset of survival analysis animals, the test synthetic peptide IS 217 at two doses 0.6 & 1.2 mg/kg were injected SC & IV daily for 5 days following post *E. coli* infection. The survival of mice was monitored for up to 18 h and the other half of the animals are continued for the survival monitored up to7 days.

4.2.6.2.2.1. Experiment 1: survival study.

In the survival study, the survival rates of the mice were assessed over a period of 7 days, with evaluations conducted every 12 hours. The observation period was set from day 0, starting from 0 to 18 hours after surgery, and continued until day 7. In each group, half of the animals were euthanized after 4h ,18 hours, while the remaining subset of animals was followed for 7 days to determine the survival rate after sepsis induction and validate mortality rates. IS 217, administered at two doses (0.6 mg/kg and 1.2 mg/kg), was injected subcutaneously and intravenously once daily for 5 days following the *E. coli* infection.

During the study, the animals were observed for 18 hours and 7 days after the *E. coli* infection. The remaining animals were euthanized under humane conditions. Prior to euthanasia, the mice were anesthetized with intraperitoneal administration of ketamine (80-100mg/kg BW) and xylazine (5-10mg/kg). Blood and peritoneal lavage fluid were collected, and the animals were then euthanized with an overdose of ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (120 mg/kg) to obtain organ samples. The disease parameters and procedures followed in this study were similar to those mentioned in the CLP animal model described in sections **4.2.6.1.2.4.** to **4.2.6.1.2.7.**

4.2.6.3. Statistical analysis:

Statistical analysis and data management were performed following recommendations on experimental design and analysis in pharmacology and all experiments were blind and based on randomly assigned groups. Data were presented as the mean ± SEM of n = 5 to 6 animals in each group.

For statistical analysis, the data were managed and analyzed following recommended guidelines for experimental design and analysis in pharmacology. All experiments were conducted blindly, with randomly assigned groups. The data were presented as the mean ± SEM of n = 5 to 6 animals in each group. Survival analysis of the groups was performed using Kaplan-Meier analysis, and comparisons were made using the log-rank test. Other experiments were analyzed using one-way ANOVA to compare groups under univariate conditions. For multi-group analysis, intergroup comparisons were conducted using Dunett's t-test and Bonferroni multiple comparison test for in between groups for some parameters. GraphPad Prism 8.2/9.0 (GraphPad Software, La Jolla, CA, USA) was used for data analysis. A significance level of $P < 0.001$, $P < 0.01$, and $P < 0.05$ was considered statistically significant and denoted as ***, **, * and ###, ##, compared to the disease control and sham control groups, respectively in CLP study and a significance level of $P < 0.001$, $P < 0.01$, and $P < 0.05$ was considered statistically significant and denoted as ***, **, * compared to the disease control group.

Chapter 5.

Results and Discussion

Section 1.
Synthesis and confirmation of mass and purity of synthetic test peptides

5.1. Results of Synthesis and confirmation of mass and purity of synthetic test peptides:

The three synthetic peptides which are synthesized, and their mass and purity were confirmed by HRMS and HPLC details are as follows: The purity and the mass of the peptides confirmed at In - house and the details are mentioned in table 37.

Table 37: The details of the peptides used in the present study.

S.no	Peptide code	No. of residues	Molecular weight (m/z)	Purity (%)
1	IS 141	10	1259.67	93.7
2	IS 217	10	1242.58	96.5
3	IS 111	07	839.04	99.2
The certificate of analysis of all three-test peptide generated by In-house are enclosed in the Appendix -II.				

5.1.1. HRMS Results:

The goal of the analysis was to confirm the peptide molecular weight. However, MS confirms that only m/z =2 belong to the peptides. The details of the instrument and mobile phase used are as mentioned in table 38 and the obtained chromatograms of the peptides are shown in figures 20-22.

Table 38: Conditions used for HRMS analysis.

Instrument name	Thermo scientific™ Q orbitrap exploris™ 120 mass spectrometer and couple vanquish™ 3000UHPLC
Source	Electro Spray Ionization ESI with full scan
Mobile phase	Water +Methanol (30&:70%)
Flow rate	0.6 flowrate o.6ml/min

Figure20: HRMS chromatogram of peptide: IS 141.

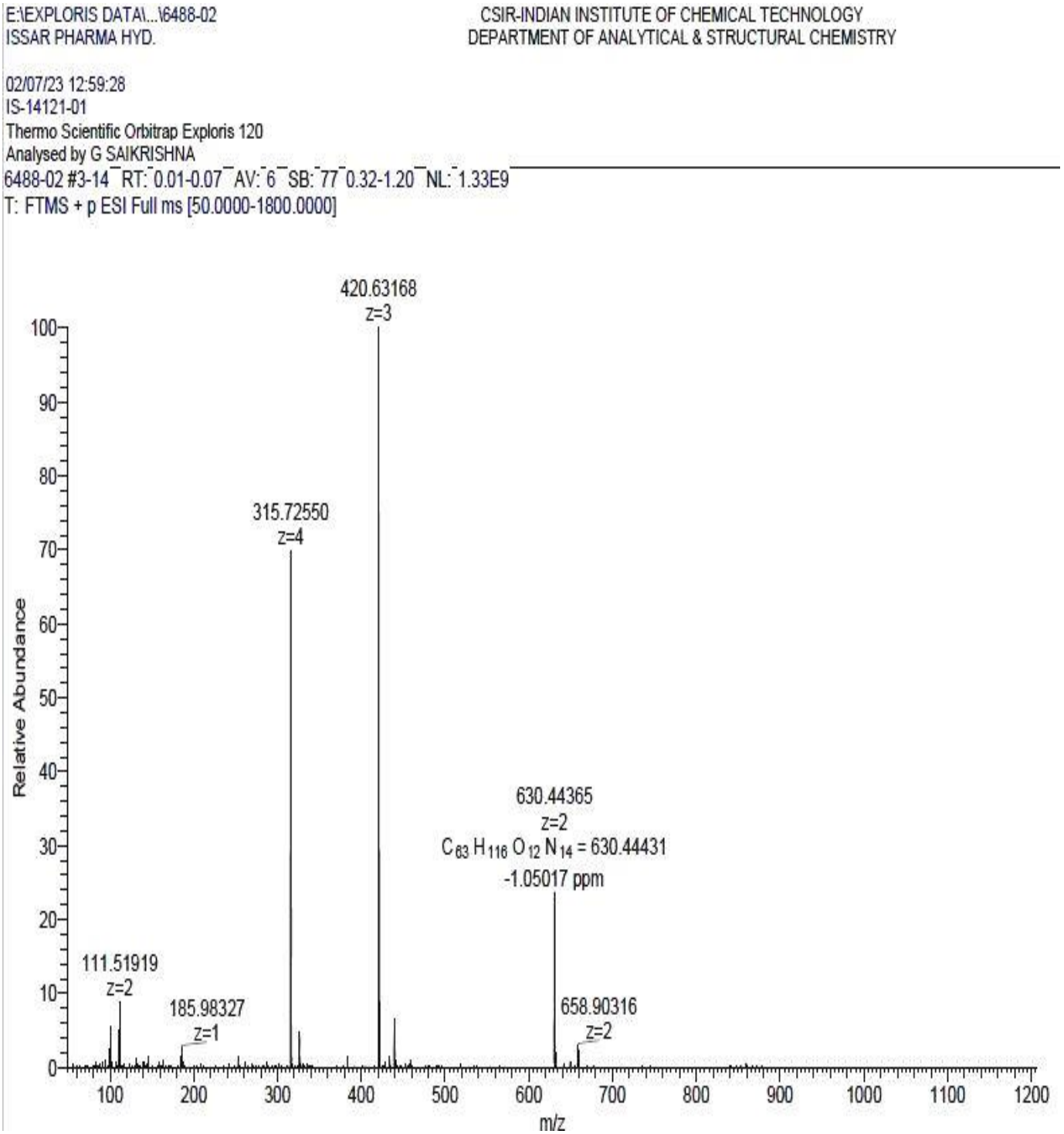


Figure 21: HRMS chromatogram of peptide: IS 111.

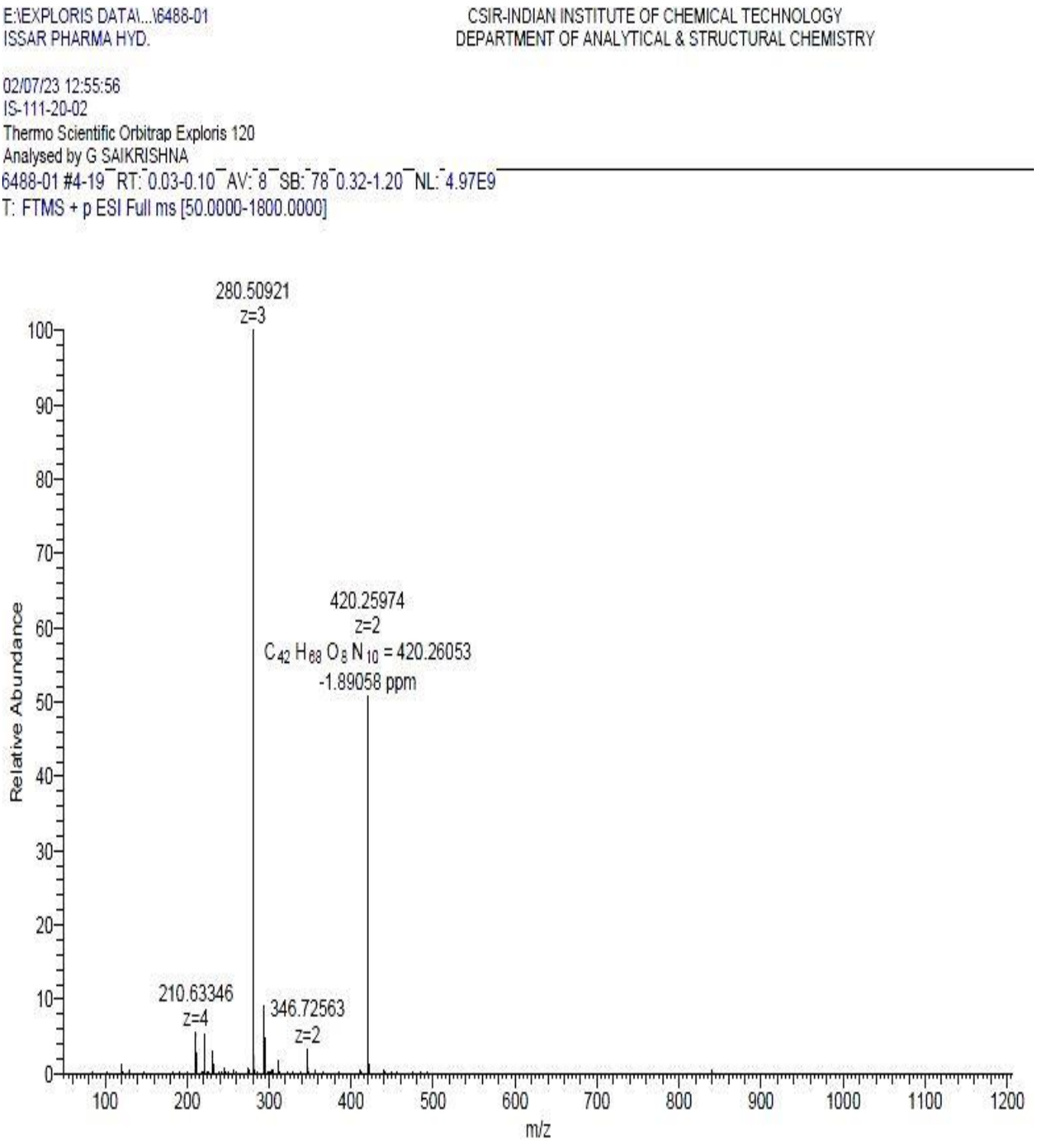


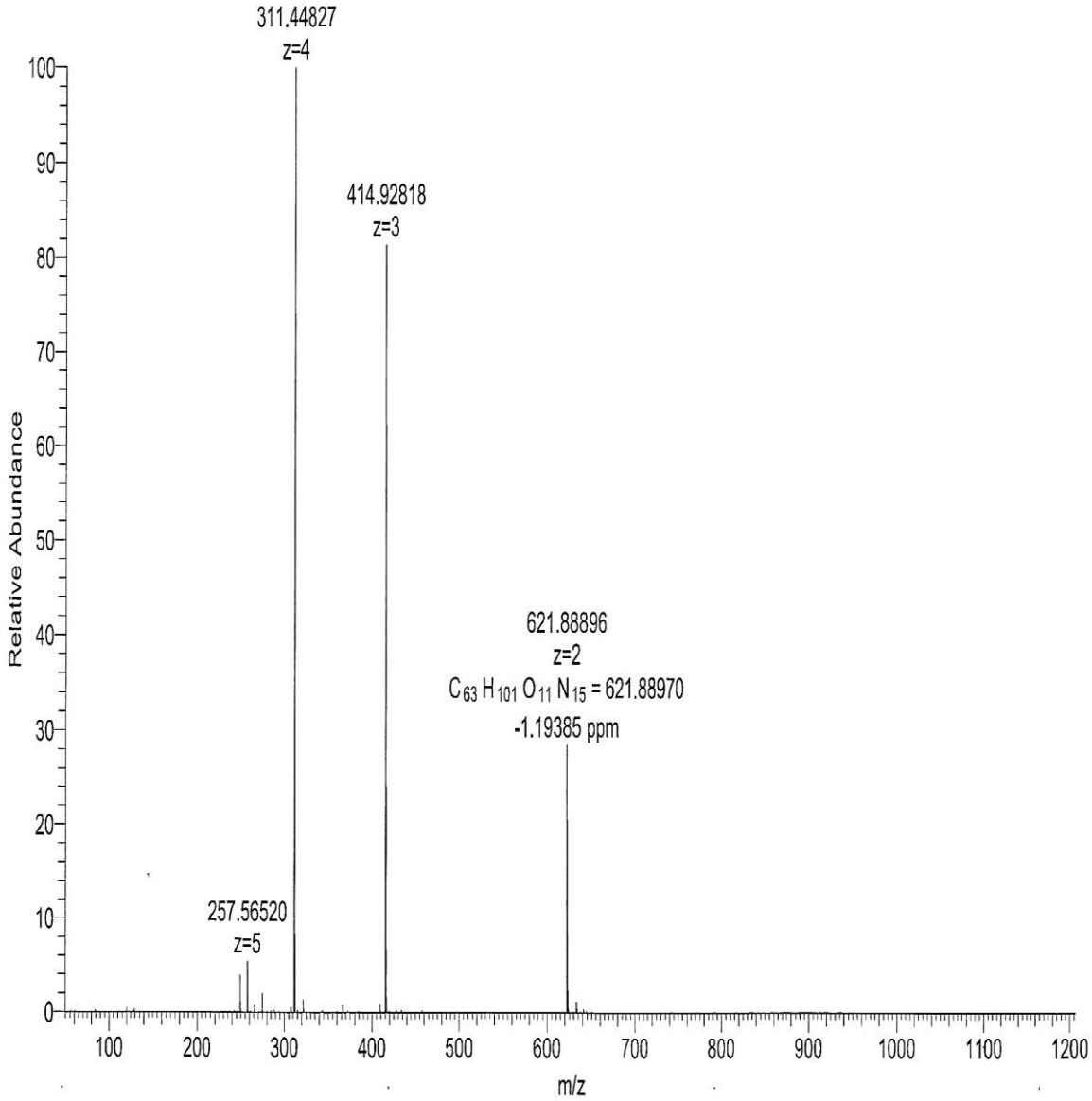
Figure 22: HRMS chromatogram of peptide: IS 217.

E:\EXPLORIS DATA\...\6486-01
ISSAR PHARMA HYD.

CSIR-INDIAN INSTITUTE OF CHEMICAL TECHNOLOGY
DEPARTMENT OF ANALYTICAL & STRUCTURAL CHEMISTRY

02/07/23 13:13:39
IS21 717-10
Thermo Scientific Orbitrap Exploris 120
Analysed by G SAIKRISHNA

6486-01 #4-16 RT: 0.03-0.08 AV: 6 SB: 77 0.32-1.20 NL: 2.84E9
T: FTMS + p ESI Full ms [50.0000-1800.0000]



5.1.2. HPLC analysis results:

The goal of the analysis was to confirm the purity of the peptides used in the present study. The details of the instrument and mobile phase used are as mentioned in table 39 and the obtained chromatograms of the peptides are shown in figures 23-25.

Table 39: The Chromatographic conditions used for the peptides IS 141, IS 111& IS 217 are as follows.

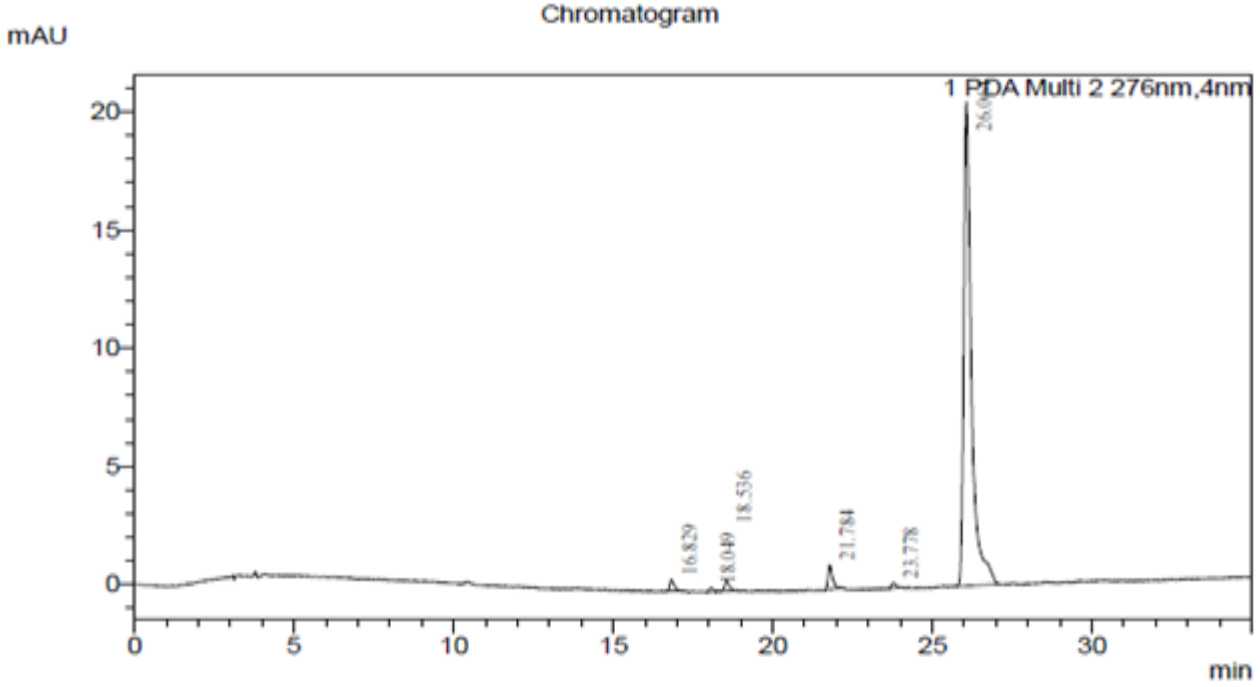
<table>
<tr><td>Instrument Name</td><td>HPLC Shimadzu with Lab solutions software (SPD-M20 Detector)</td></tr>
<tr><td>Column</td><td>Puro sphere Star RP-18 End capped (250 x4.6 mm, 5 µm)</td></tr>
<tr><td>Column Oven Temperature</td><td>ambient</td></tr>
<tr><td>Flow rate</td><td>1 mL / min</td></tr>
<tr><td>Wavelength / Detector</td><td>276 nm / PDA;215 nm –IS 217</td></tr>
<tr><td>Injection Volume</td><td>10 µL</td></tr>
<tr><td>Mobile phase</td><td>MP-A: - 0.1%TFA in Milli-Q water or equivalent grade water
MP: -B: - 0.1%TFA in ACN
Gradient programme
<table>
<tr><td>Time</td><td>MP-A (%)</td><td>MP-B (%)</td></tr>
<tr><td>0.01</td><td>90</td><td>10</td></tr>
<tr><td>17.00</td><td>72</td><td>28</td></tr>
<tr><td>30.00</td><td>72</td><td>28</td></tr>
<tr><td>32.00</td><td>90</td><td>10</td></tr>
<tr><td>35.00</td><td>90</td><td>10</td></tr>
</table></td></tr>
<tr><td>Diluent</td><td>Acetonitrile + water (50:50)</td></tr>
<tr><td>Run Time</td><td>35 minutes ;31 minutes –IS 217</td></tr>
</table>

Figure 23: HPLC chromatogram of peptide: IS 141.

==== Shimadzu LabSolutions Analysis Report ====

Sample Information	
Acquired by	: System Administrator
Sample Name	: IS-14121
Sample ID	: IS-14121
Tray#	:
Vial#	: 2
Injection Volume	: 10
Data File	: IS-14121.lcd
Method File	: ISSAR PHARMA_HPLC.lcm
Batch File	: ISSAR PHARMA_HPLC.lcb
Report Format File	: LSS Data Comparison.lsr
Date Acquired	: 07/03/2023 12:06:25
Date Processed	: 07/03/2023 17:09:03

Chromatogram



Peak Table

PDA Ch2 276nm

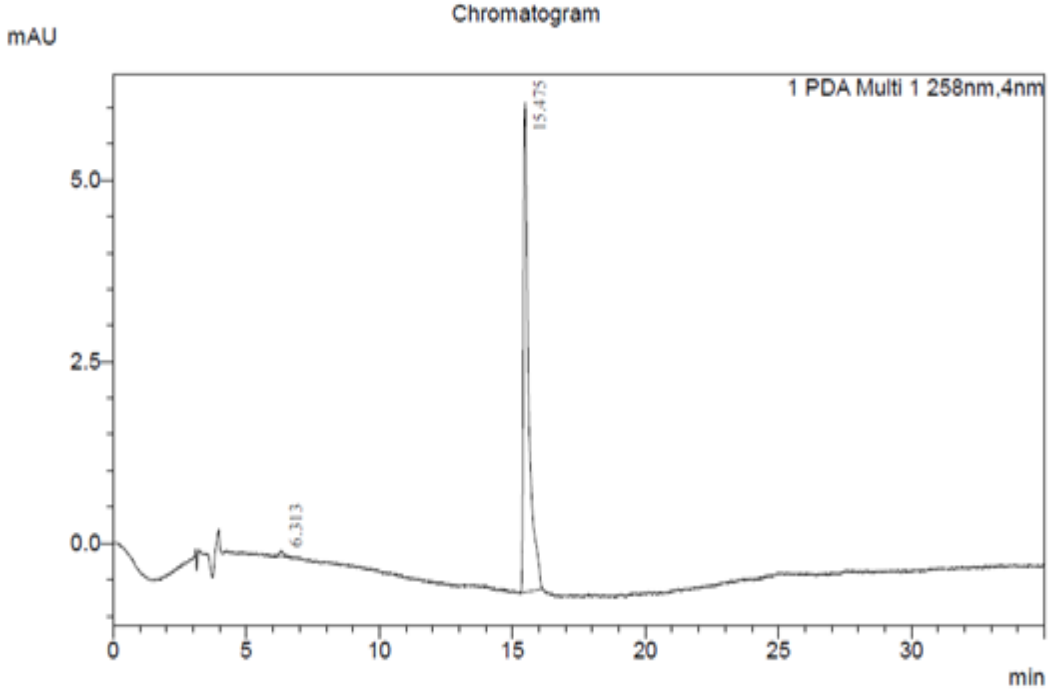
Peak#	Ret. Time	Area	Height	Height%	Area%
1	16.829	4982	514	2.245	1.359
2	18.049	1597	176	0.767	0.436
3	18.536	4479	464	2.024	1.222
4	21.784	9762	1052	4.591	2.663
5	23.778	2177	227	0.992	0.594
6	26.064	343542	20474	89.380	93.726
Total		366540	22906	100.000	100.000

Figure 24: HPLC chromatogram of peptide: IS 111.

==== Shimadzu LabSolutions Analysis Report ====

Sample Information	
Acquired by	: System Administrator
Sample Name	: IS-111-20-02
Sample ID	: IS-111-20-02
Tray#	:
Vial#	: 2
Injection Volume	: 10
Data File	: IS-111-20-02.lcd
Method File	: ISSAR PHARMA_HPLC.lcm
Batch File	: ISSAR PHARMA_HPLC.lcb
Report Format File	: LSS Data Comparison.lsr
Date Acquired	: 07/03/2023 15:13:28
Date Processed	: 07/03/2023 17:15:35

Chromatogram



Peak Table

PDA Ch1 258nm

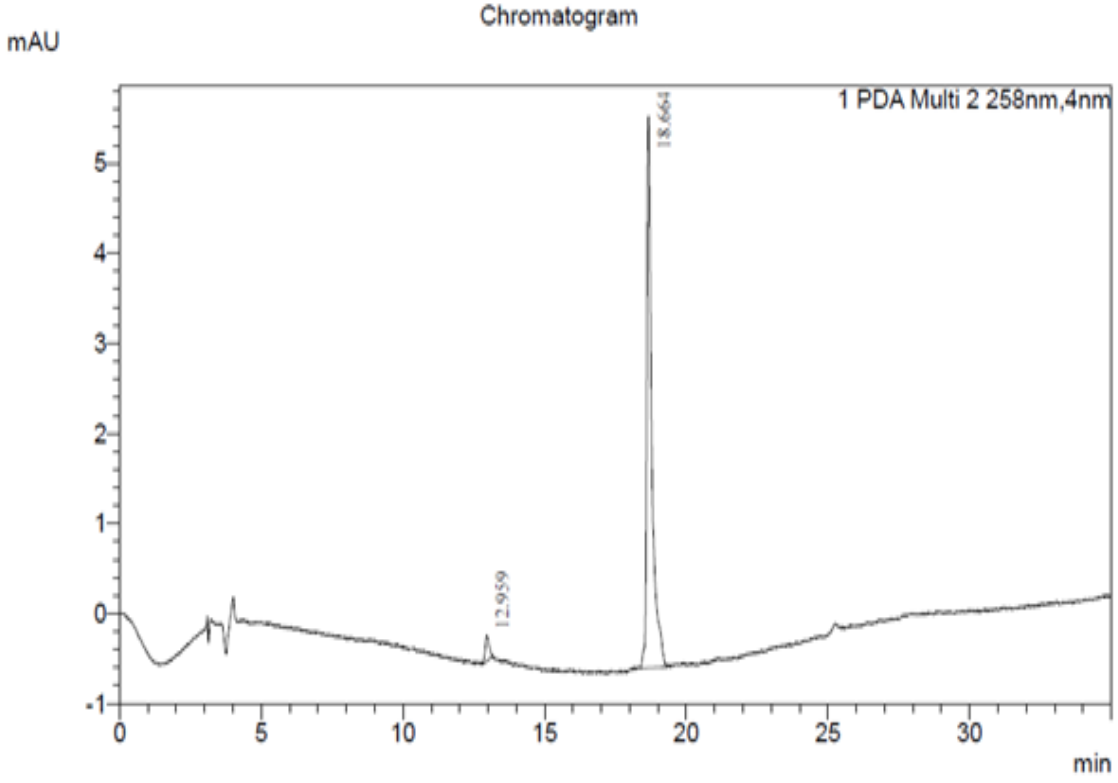
Peak#	Ret. Time	Area	Height	Height%	Area%
1	6.313	715	73	1.072	0.779
2	15.475	91100	6744	98.928	99.221
Total		91815	6817	100.000	100.000

Figure 25: HPLC chromatogram of peptide: IS 217.

==== Shimadzu LabSolutions Analysis Report ====

Sample Information	
Acquired by	: System Administrator
Sample Name	: IS-21717
Sample ID	: IS-21717
Tray#	:
Vial#	: 5
Injection Volume	: 10
Data File	: IS-21717.lcd
Method File	: ISSAR PHARMA_HPLC.lcm
Batch File	: ISSAR PHARMA_HPLC.lcb
Report Format File	: LSS Data Comparison.lsr
Date Acquired	: 07/03/2023 13:57:28
Date Processed	: 07/03/2023 17:12:41

Chromatogram



Peak Table

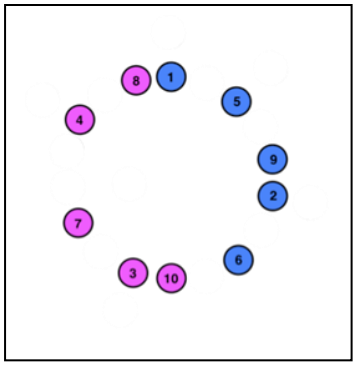
PDA Ch2 258nm

Peak#	Ret. Time	Area	Height	Height%	Area%
1	12.959	2680	291	4.531	3.453
2	18.664	74932	6128	95.469	96.547
Total		77612	6419	100.000	100.000

Table 40: The Physical characteristics of lead peptide IS 217 are as follows:

S.no.	PROPERTIES	SPECIFICATION
01	Peptide Name/Code	IS 217
02	Number of Residues	10
03	Amino Acid sequence (1 letter code)	FAKKFAKKFK
04	Aminoacid séquence (3 letter code)	Phe-Ala-Lys-Lys-Phe-Ala-Lys-Lys-Phe-Lys-NH_2
05	Molecular Formula	$C_{63}O_{11}N_{15}H_{99}$
06	Molecular Weight	1242.58
07	Molecular Weight (Acetate-salt form)	1619.58
08	Molecular primary structure	linear peptide
09	Molecular secondary structure	β- sheet
10	Color	it is a white flocculent powder
11	Solubility	soluble in water
12	Charge at pH 7.0	5.0
13	Isoelectric Point pI	11.41
14	Attribute	Basic
15	% of Hydrophilic	50
16	% of Hydrophobic	30
17	Average Hydrophilicity	0.7
18	Ratio of Hydrophilic residues/ Total number of residues	50%
19	Storage Condition	-20^{0}C for long term storage and while experimentations, store at 2-8^{0}C

Chemical Properties of lead peptide IS 217 are as follows:



a) Sequence of Peptide--FAKKFAKKFK
b) Molecular weight of the Peptide (MALDI) --1619.58; acetate salt
c) No. of charged amino acids in sequence--5.
d) No. of Hydrophobic and Hydrophilic amino acids—5 hydrophobic; 5 hydrophilic
e) PI / PK of the Peptide---pI is 11.4.
f) No. of Acidic amino acids---0
g) No. of Basic amino acids---5

h) No. of Polar amino acids---5
i) No of Non – Polar amino acids---5
j) Helical wheel
k) Amphipathicity of Peptide—100%

The chemical structure and 3D structures of IS 217 are as follows:

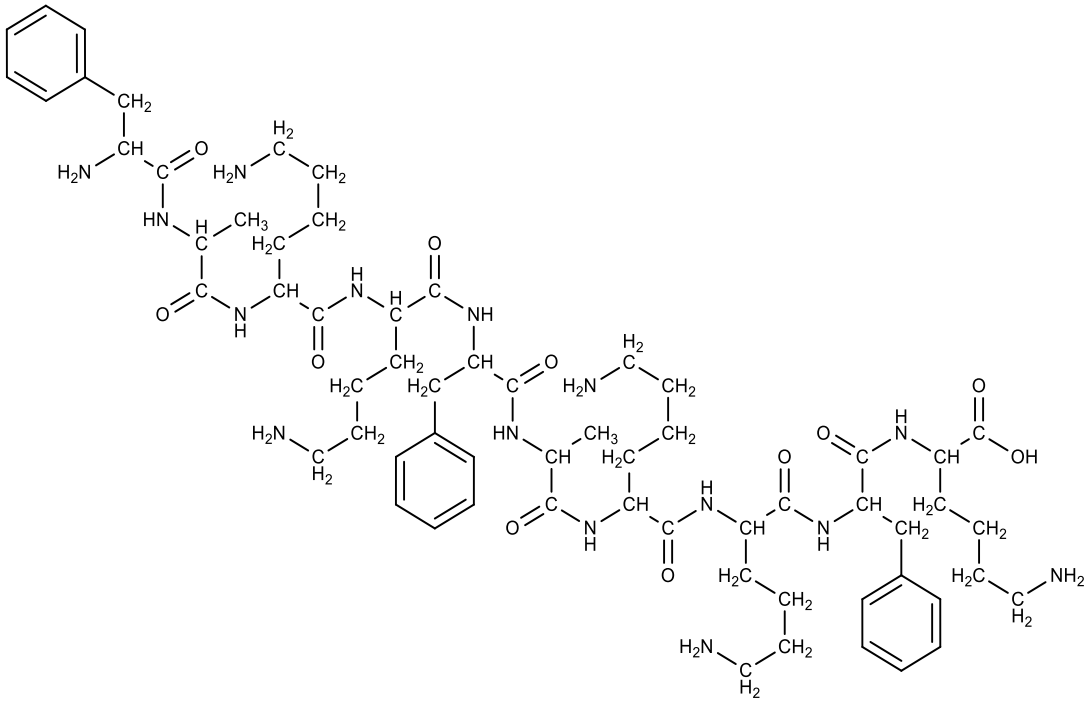


Figure 26: The chemical structure of lead peptide IS 217.

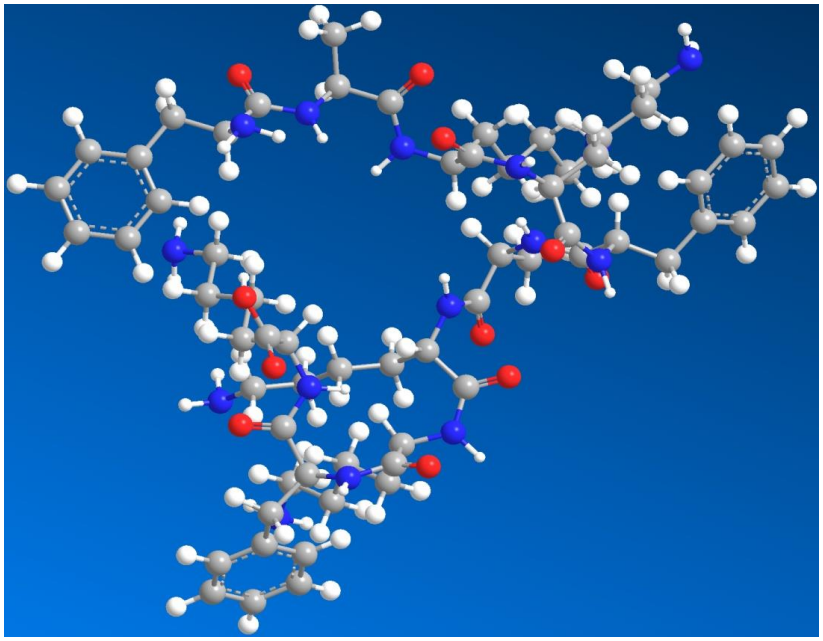


Figure 27: 3D Structure of lead peptide IS 217

Section 2.

In vitro anti-inflammatory data of test peptides against LPS-induced inflammation in RAW 264.7 cells

5.2. Results of Invitro anti-inflammatory activity of test peptides against LPS-induced inflammation in RAW 264.7 cells:

5.2.1. Effect of synthetic test peptides (IS 141, IS 111 & IS 217) on the cell viability of RAW 264.7 macrophages:

The cytotoxic effects of synthetic test peptides (IS 141, IS 111, and IS 217) on RAW 264.7 mouse primary cells were assessed using the MTT assay. RAW 264.7 cells were treated with various concentrations of the test peptides (1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL) for 48 hours. The MTT assay measures cell viability by converting a yellow tetrazolium salt into a purple formazan product. The viability of RAW 264.7 cells was determined and recorded in table 41. Results from the MTT assay indicated that the different concentrations of the test peptides (ranging from 1.56 to 100 µg/mL) did not exhibit significant cytotoxicity in RAW 264.7 macrophage cells. The cells remained viable, by at least 80%. Based on these findings, concentrations of the test peptides ranging from 3.12 to 100 µg/mL were selected for the subsequent assays. The cell viability results demonstrated that treatment with the peptides did not result in cell viability exceeding 80% at the tested concentrations.

Table 41: The cell viability of the synthetic test peptides (IS 141, IS 111 & IS 217) in comparison with dexamethasone.

Test peptides concentrations	% of Cell viability of test peptides				Dexamethasone concentrations
	IS141	IS111	IS 217	Dexamethasone	
100	104.64	91.37	81.36	84.61	**500**
50	113.19	94.98	87.30	90.795	**250**
25	109.85	96.39	92.27	95.96	**125**
12.5	104.95	99.72	87.92	91.44	**62.5**
6.25	116.35	90.21	79.622	82.80	**31.25**
3.125	84.487	91.55	86.53	90.00	**15.625**
1.5625	88.49	84.54	98.42	102.36	**7.8125**

5.2.2. In vitro anti-inflammatory activity of synthetic test peptides (IS 141, IS 111 &IS 217):

5.2.2.1. For analysis of anti-inflammatory effects, the cells were pretreated with test peptides of different concentrations (3.12, 6.25, 12.5,25,50 and100 µg/mL) before incubation with LPS for 18 hours. The Morphology of cells were visualized by optical microscopy (400×) at scale bar 60µm for 18 hours; see (Figures 28-30).

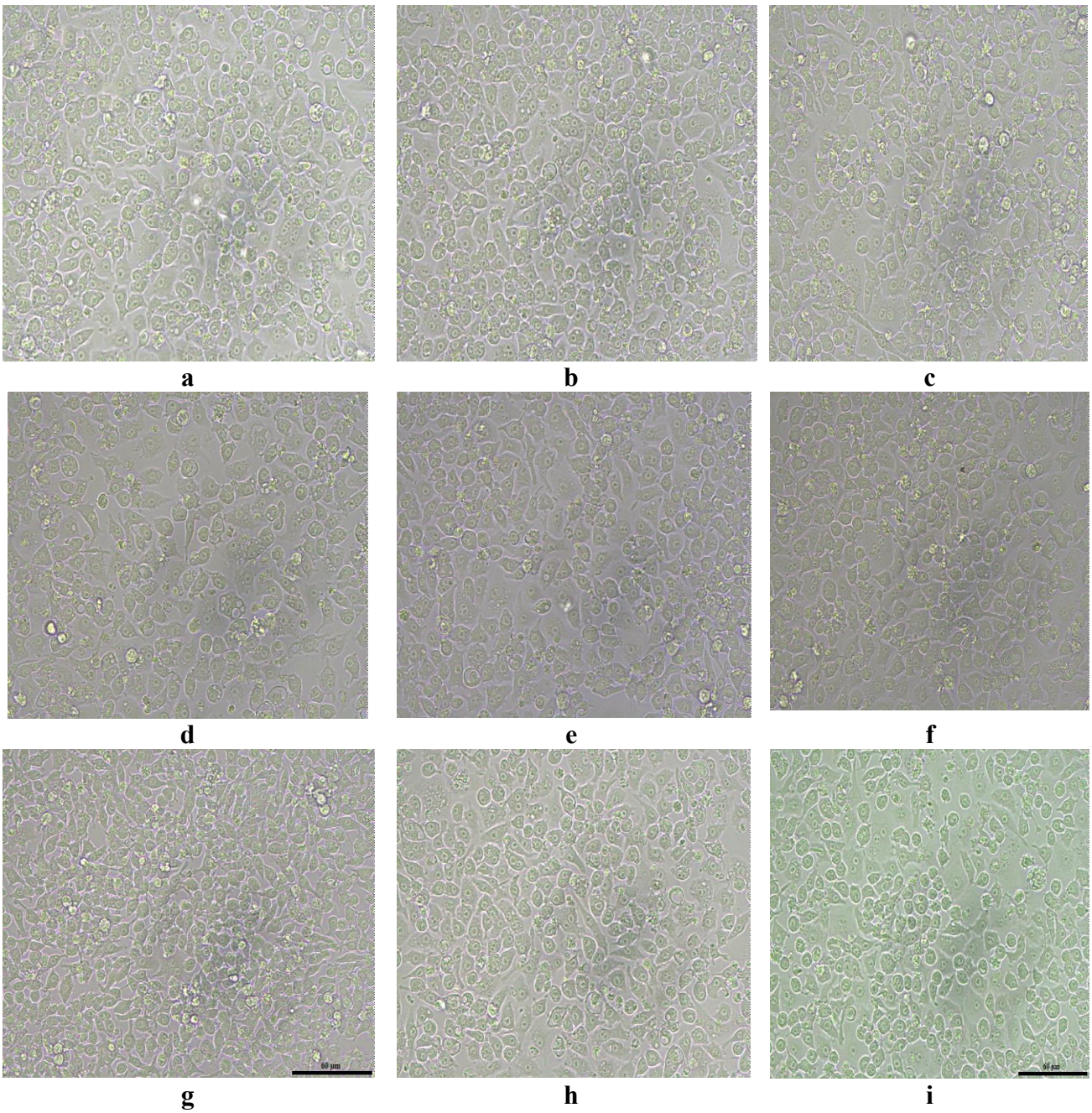


Figure 28: Morphological change in macrophage RAW 264.7 cells. (a -f) LPS-treated with test peptide **IS 141**(3.12, 6.25, 12.5,25,50 and100 µg/mL respectively, (g) Control/untreated, (h) LPS-(1 µg/mL) treated only and (i) LPS-treated with dexamethasone (500 µg/mL).

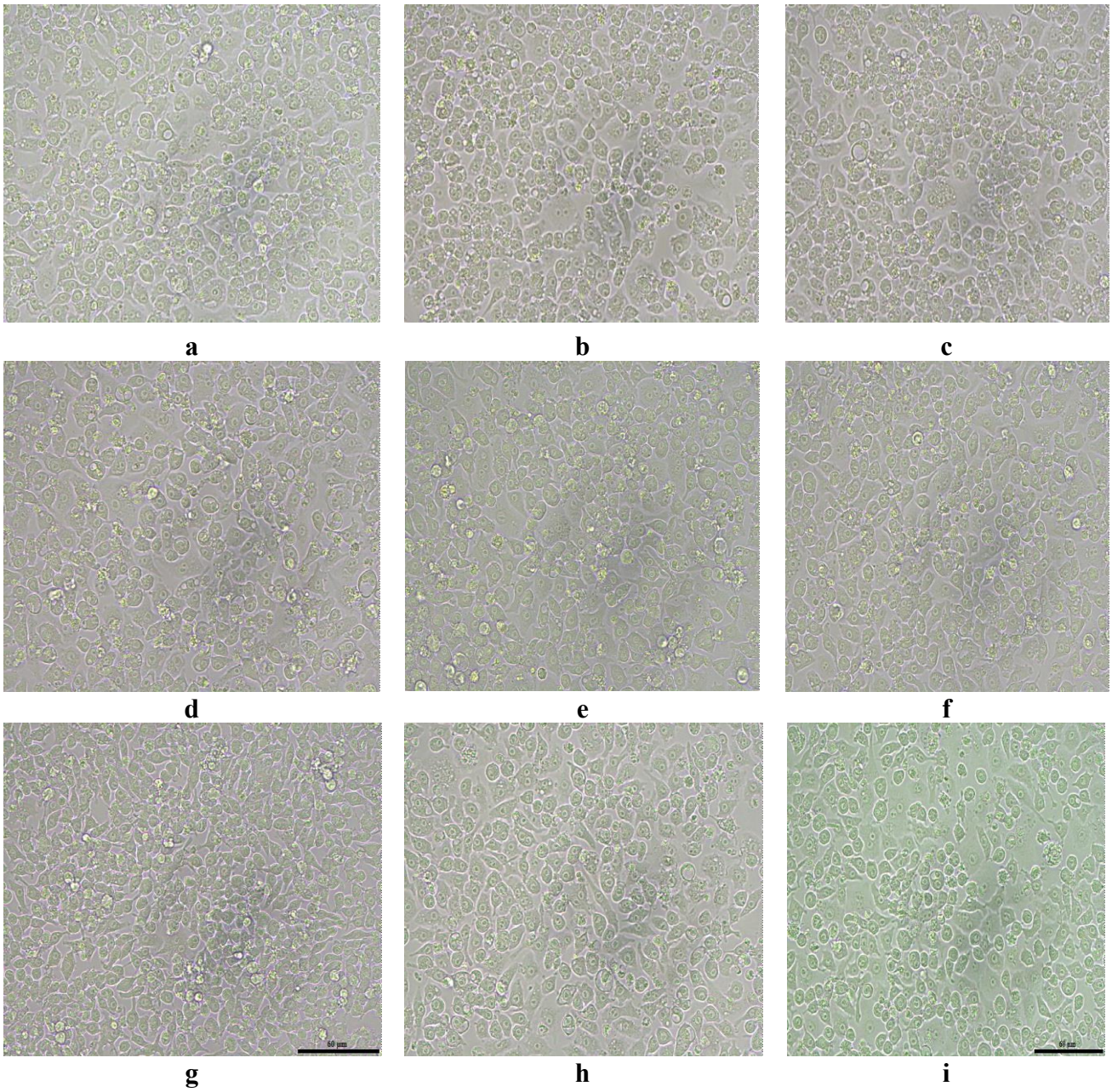


Figure 29: Morphological change in macrophage RAW 264.7 cells. (a -f) LPS-treated with test peptide **IS 111**(3.12, 6.25, 12.5,25,50 and100 µg/mL respectively, (g) Control/untreated, (h) LPS- (1 μg/mL) treated only, and (i) LPS-treated with dexamethasone (500 μg/mL).

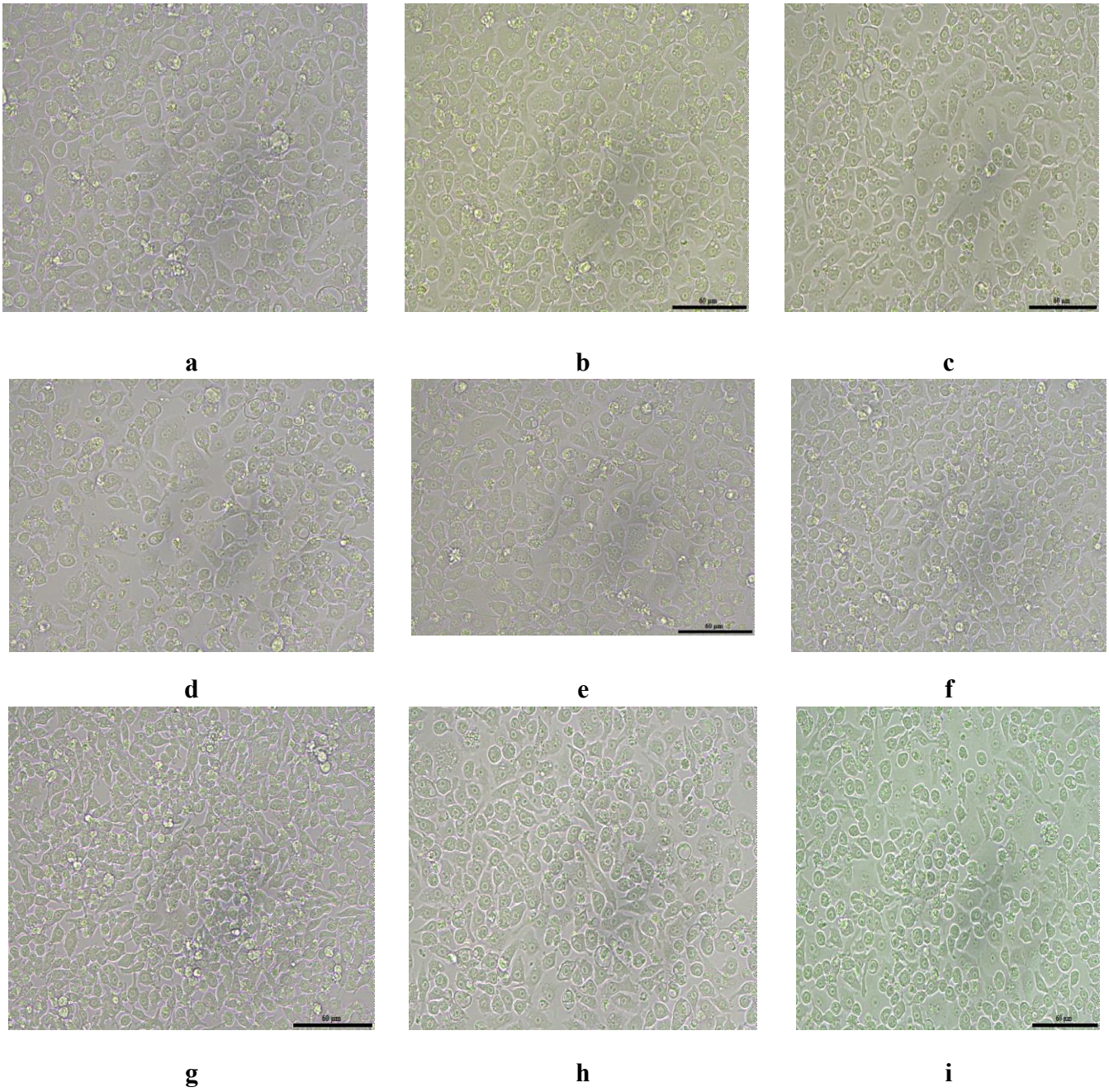


Figure 30: Morphological change in macrophage RAW 264.7 cells. (a -f) LPS-treated with test peptide **IS 217** (3.12, 6.25, 12.5,25,50 and100 µg/mL respectively, (g) Control/untreated, (h) LPS- (1 μg/mL) treated only, and (i) LPS-treated with dexamethasone (500 μg/mL).

5.2.2.2. Effect of early phase cytokines on the LPS-stimulated RAW 264.7 macrophages:

The synthetic test peptides (IS 141, IS 111 & IS 217) reduce the production of pro-inflammatory cytokines.

Certain HDPs can modulate the innate immune response due to their ability to stimulate the induction of chemokines while suppressing potentially harmful pro-inflammatory cytokines. The study examined the effects of three test peptides (IS 141, IS 111, and IS 217) on the production of pro-inflammatory cytokines. These peptides have the ability to stimulate the production of chemokines while suppressing harmful pro-inflammatory cytokines. Murine macrophage-like RAW 264.7 cells were stimulated with 1 µg/mL LPS, and the levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and the anti-inflammatory cytokine IL-10 were measured in the cell culture supernatants using an ELISA kit. The anti-inflammatory activities of the test peptides were compared to a standard drug called dexamethasone (500 µg/mL) (Fig. 31-33).

Based on the literature, in general the changes of inflammatory factors in LPS-treated RAW264.7 cells and different treatments at different intervention time (0 h, 4 h, 6 h, 8 h, 12 h, 16 h,18 h and 24 h) were analyzed by ELISA, RT-qPCR western blot etc. Where in the Present Study, the invitro assay protocol was standardized which was adapted from [Bartosh et al., 2013; Ylostalo et al., 2012]. where the routinely incubation of the macrophages with LPS for approximately 16–18 h. However, an abundance of macrophage-derived cytokines can be detected in the conditioned medium as early as 4–6 h after LPS stimulation.

When the RAW 264.7 cells were stimulated with LPS alone, there was an increase in cytokine production compared to the control group. Specifically, IL-6, IL-1β, and TNF-α levels were strongly induced, while IL-10 levels were low. However, when the cells were treated with the test peptides (IS 141, IS 111, and IS 217), there was a significant decrease in the release of pro-inflammatory cytokines and a notable increase in IL-10 production. The reduction in TNF-α levels was particularly significant compared to IL-6. These effects were observed in all three test peptide groups and were almost as effective as the standard drug dexamethasone. The ELISA results confirmed that LPS stimulation increased the levels of TNF-α (figure. 32b), IL-1β (figure. 31), and IL-6(figure. 32a) while decreasing IL-10 levels (figure. 33). Treatment with IS 111 and IS 217 reduced IL-6 levels, although not in a dose-dependent manner. On the other hand, treatment with IS 141, IS 111 or IS

217 significantly increased IL-10 levels, indicating their anti-inflammatory properties. These peptides also effectively reduced IL-1β levels. However, they did not show dose-dependent inhibition. Dexamethasone, like the test peptides, demonstrated significant anti-inflammatory effects by inhibiting the expression of all markers. Important observations made in this study are where in the IL-6 assay, the results of the LPS group were not consistent with the expected values. In contrast, other assays demonstrated that the LPS group results were consistent when compared to the control group. Notably, in the IL-6 assay (figure 32a), the focus of the comparison was specifically on evaluating the effects of dexamethasone relative to the treatment groups.

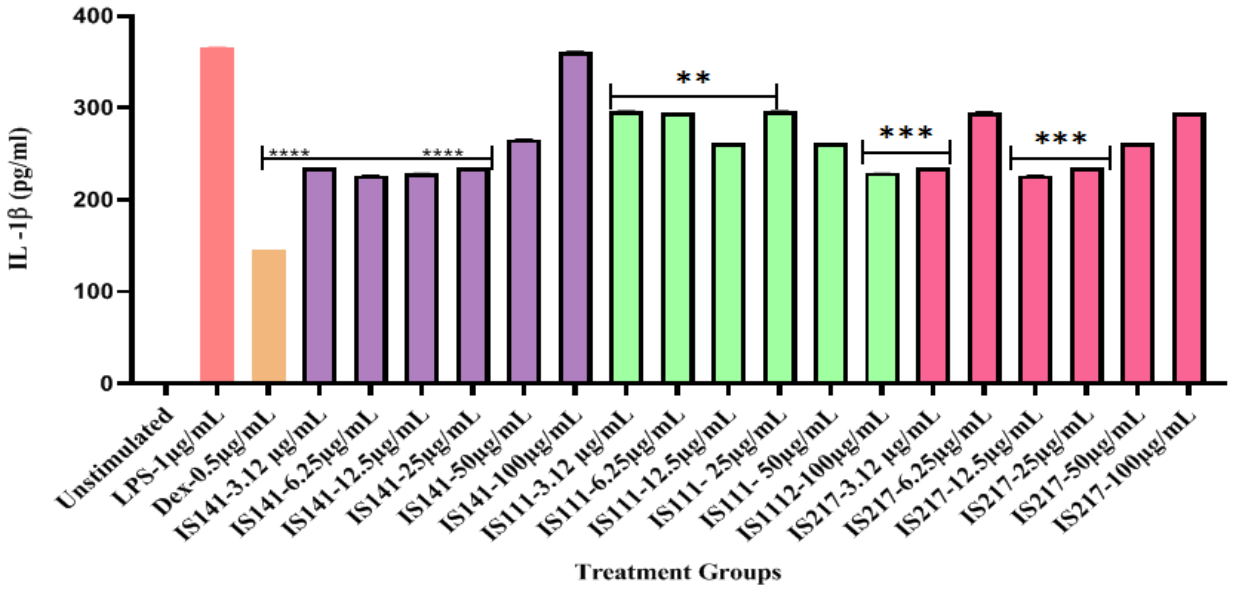
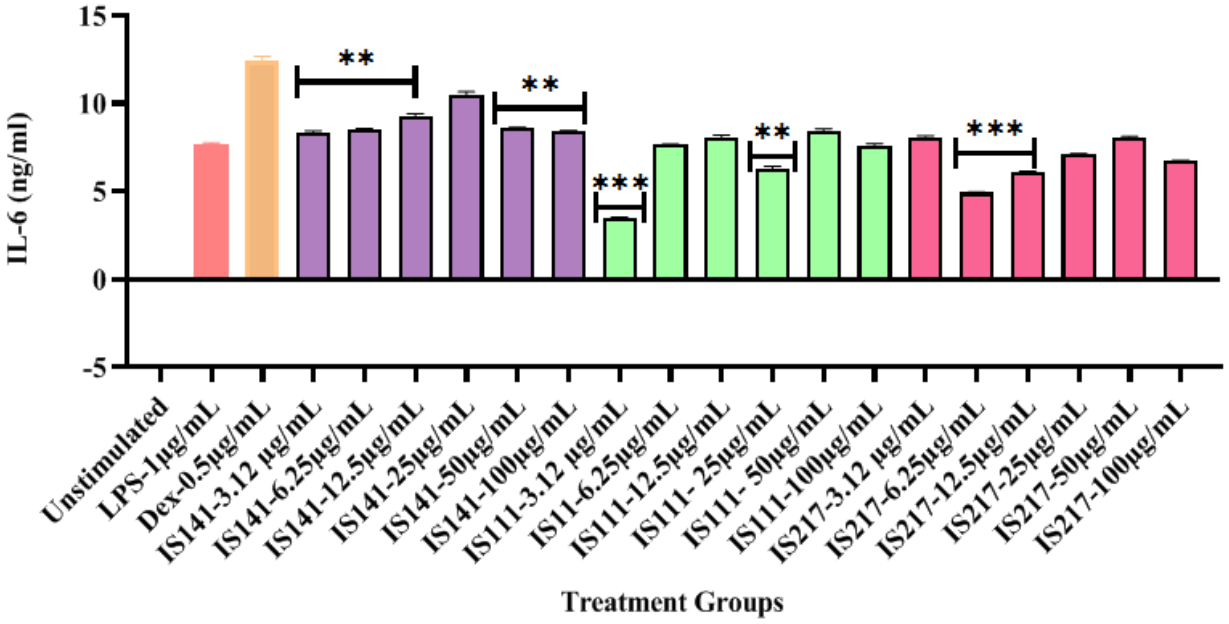
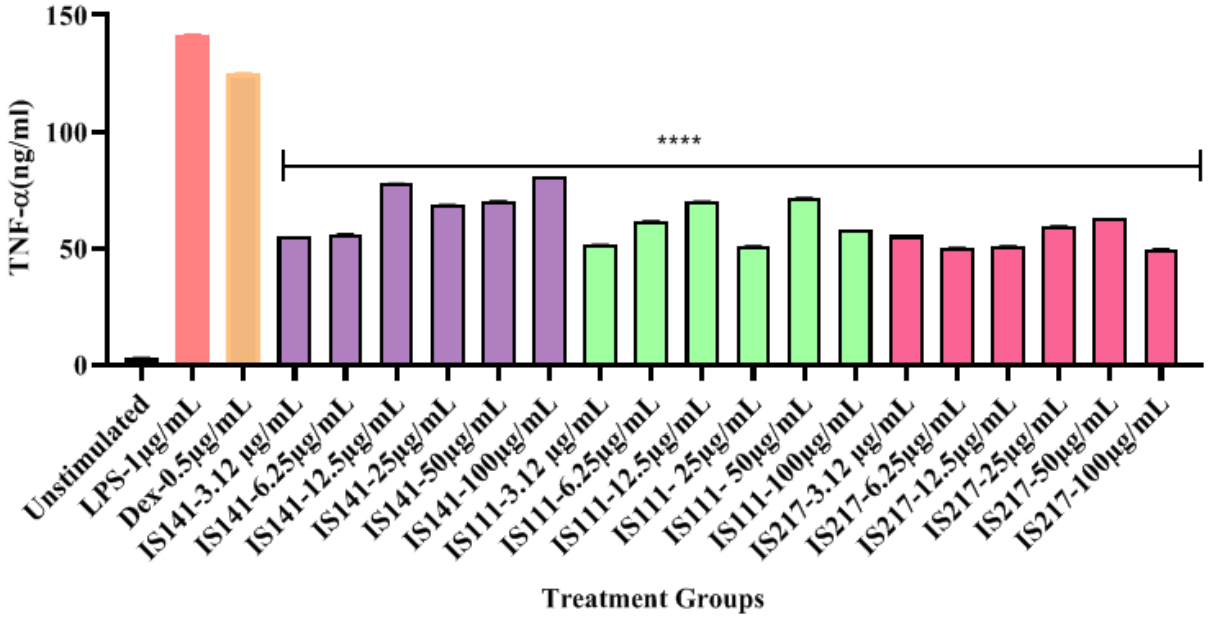


Figure 31: IL-1β secretion was measured using ELISA.

The data presented in this study were shown as fold change relative to the LPS-stimulated cells and the unstimulated (control) cells. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate.



(a)



(b)

Figure 32 (a.b): IL-6 and TNF-α secretion was measured using ELISA.

The data presented in this study were shown as fold change relative to the LPS-stimulated cells and the unstimulated (control) cells. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate.

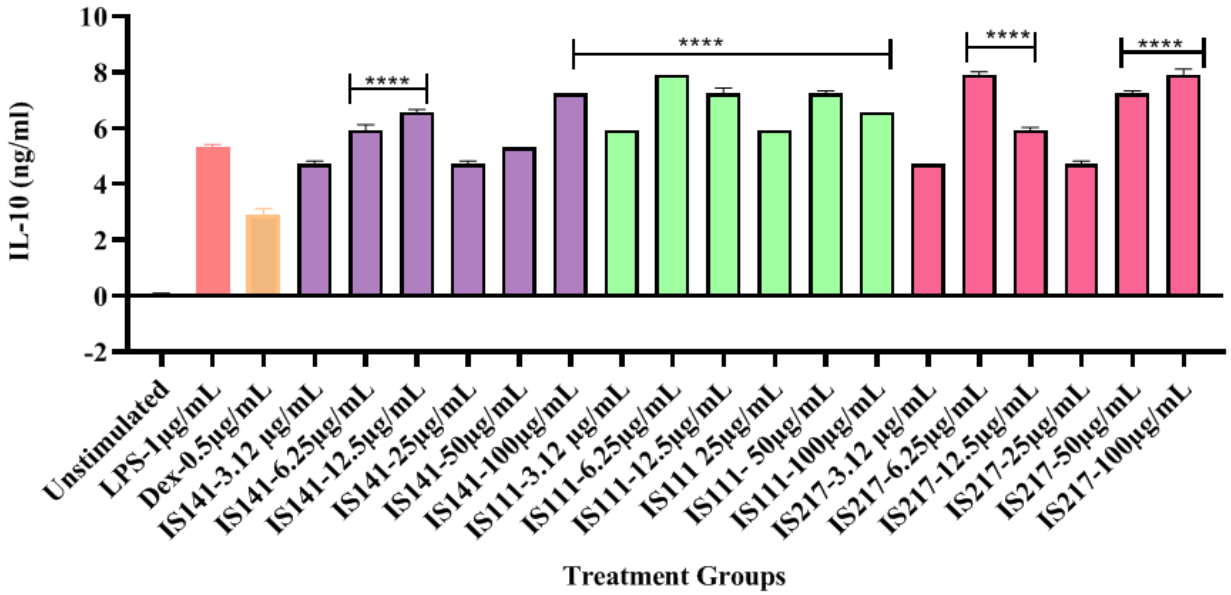


Figure 33: IL-10 secretion was measured using ELISA.

The data presented in this study were shown as fold change relative to the LPS-stimulated cells and the unstimulated (control) cells. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate.

5.2.2.3. The effect of synthetic test peptides ((IS 141, IS 111& IS 217)) on LPS-induced MAPK pathways activation:

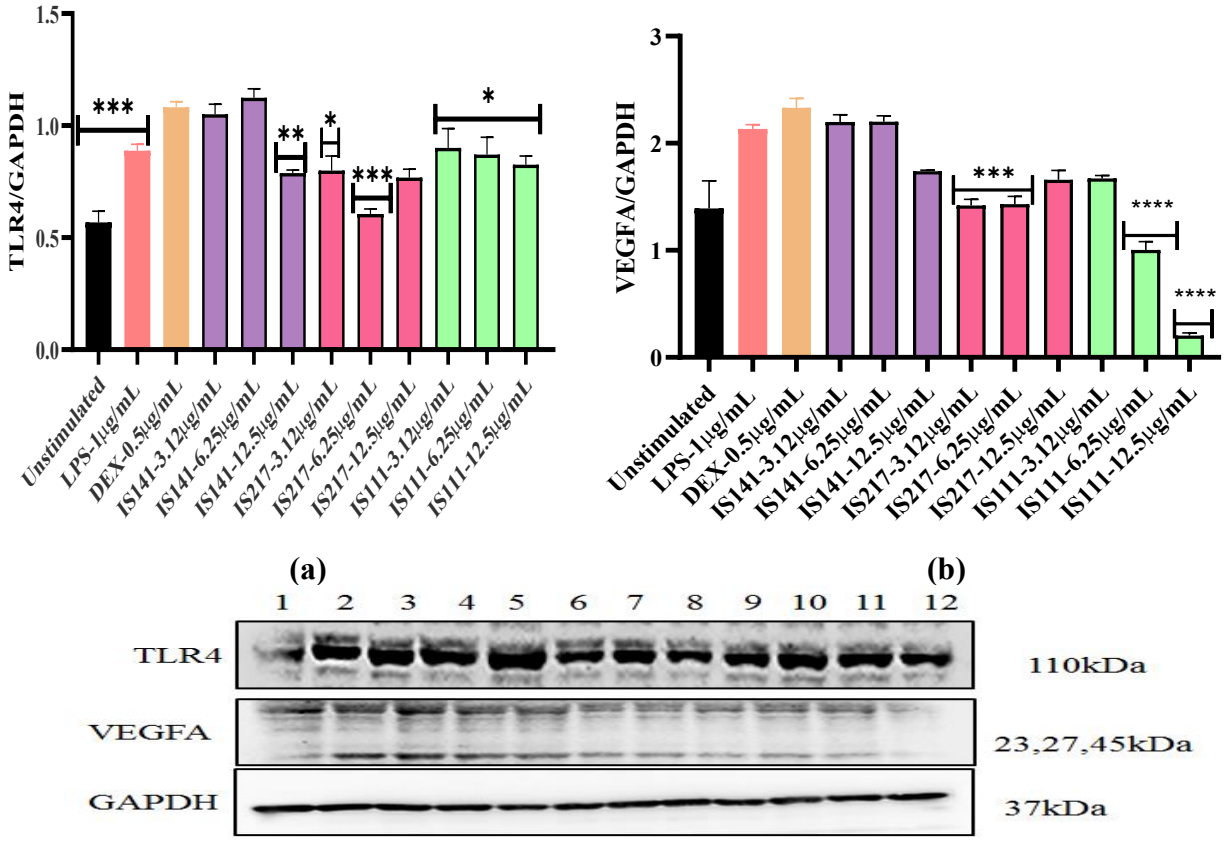
It is well known that the modulation of inflammatory cytokines and chemokines involves various signaling pathways, among which the Mitogen-activated protein kinase (MAPK) pathway, comprising extracellular signal-regulated kinases (ERKs) and p38, plays a pivotal role. To elucidate how specific test peptides—IS 141, IS 111, and IS 217—exert their anti-inflammatory effects, their impact on the production of pro-inflammatory cytokines induced by LPS was examined through western blot analysis. The analysis aimed to investigate how these peptides influence the phosphorylation of ERK and p38 in RAW 264.7 cells. The findings from western blot analysis demonstrated that LPS stimulation led to a notable increase in ERK and pp38 levels (Fig. 35-36).

However, cells treated with the test peptides (IS 141, IS 111, and IS 217) exhibited a significant reduction in LPS-induced phosphorylation of ERK and p38 levels (Fig. 35-36).

In this assay, the impact of the test peptides on ERK1/2 and p38 MAPK phosphorylation, integral to the MAPK signaling pathway involved in pro-inflammatory gene expression in LPS-treated RAW 264.7 cells, was assessed. MAPKs are pivotal targets in inflammatory responses. Figures 35 and 36 illustrate a substantial increase in ERK1/2 and p38 MAPK phosphorylation post-LPS stimulation. Notably, IS 217 showed a concentration-dependent attenuation of p-ERK1/2 and MAPKs induced by LPS, especially at lower concentrations, while the total protein expression of unphosphorylated MAPKs remained unaffected by LPS or test peptide treatment, IS 111 effectively inhibited the phosphorylation of ERK and prevented the LPS-induced increase in p38 phosphorylation (Figures 35-36). These findings suggest that pretreatment with these peptides can impede pro-inflammatory responses by inhibiting the MAPK signaling pathway.

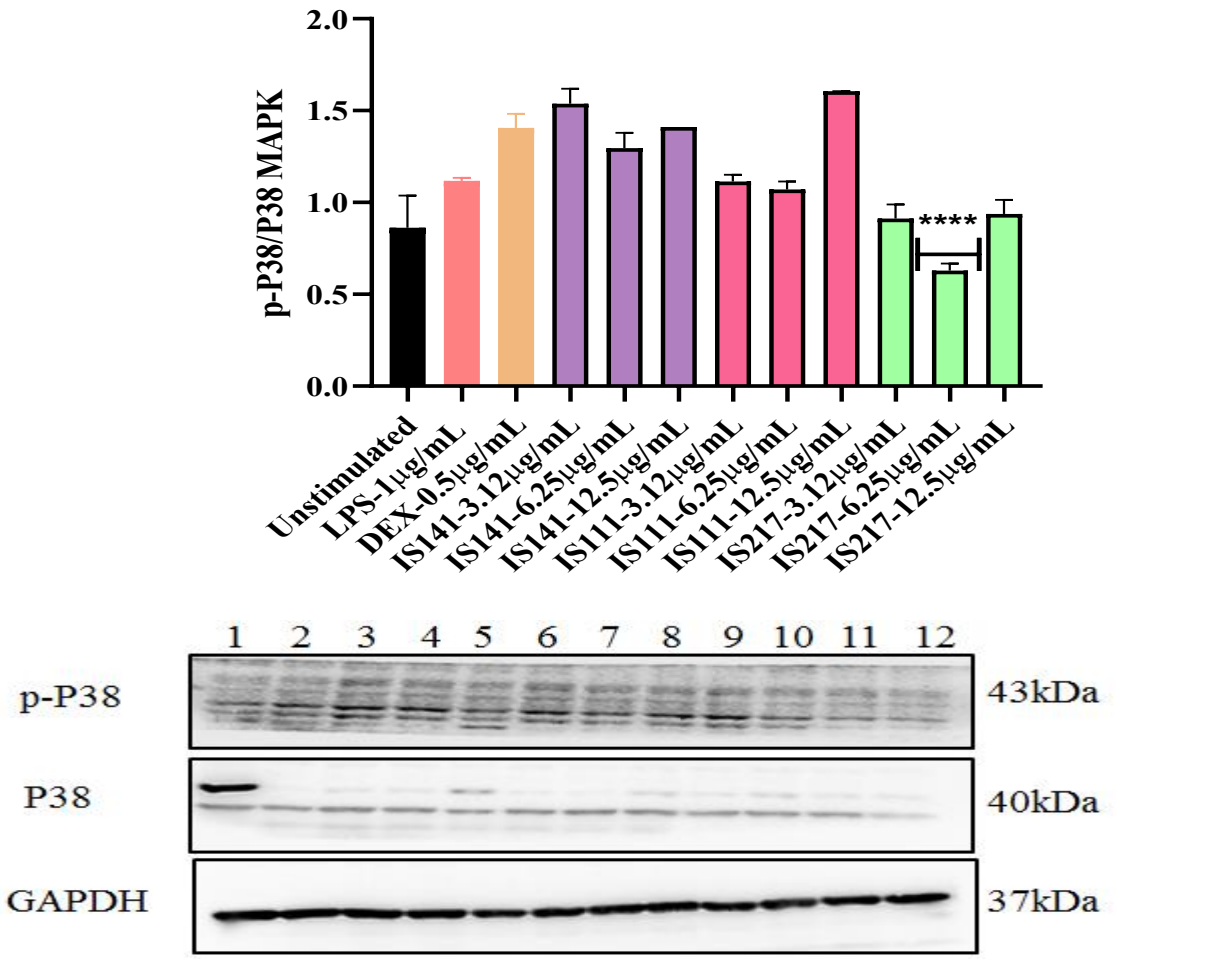
Additionally, considering that both TLR-2 and TLR-4 pathways converge on the common target NF-kB, crucial for full expression of TNF-α and IL-6 in macrophages, the present study explored the impact of the test peptides on TLR-4. Results indicated heightened TLR-4 levels post-LPS stimulation compared to the control group, yet treatment with IS141 (12.5 µg/mL) marginally reduced TLR-4 levels, and IS 217 peptide treatment notably reduced TLR4 levels (Figure 34a). Furthermore, although VEGF levels did not rise in the LPS control group, they were significantly diminished in IS141 (12.5 µg/mL), IS217, and IS111-treated samples (Figure 34b).

The three syntetic peptides (IS141, IS 217, and IS111) treatment reduces inflammatory pathway through modulating the MAPK pathway.



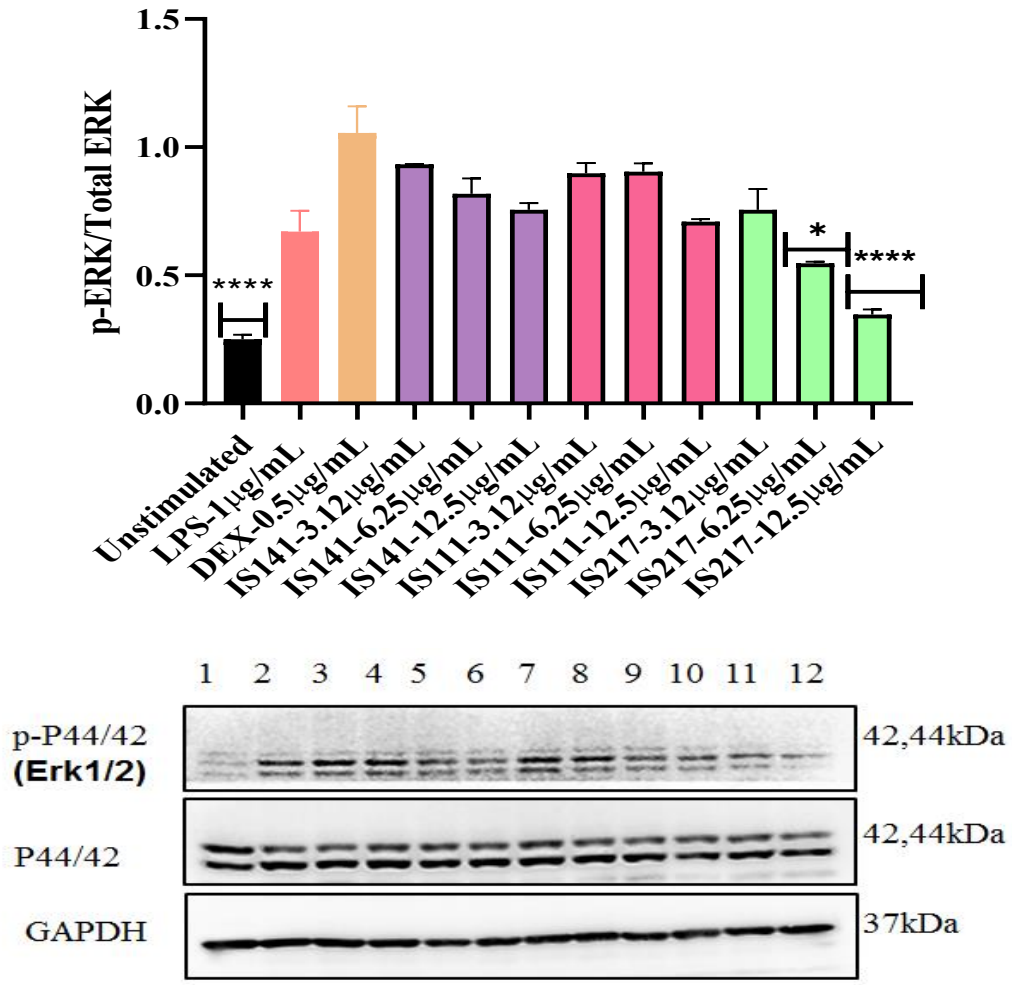
1.Unstimulated	4.IS141-3.12µg/mL	7.IS 217-3.12µg/mL	10.IS111-3.12µg/mL
2. LPS-1µg/mL	5.IS141-6.25µg/mL	8.IS 217-6.25µg/mL	11.IS111-6.25µg/mL
3. DEX-0.5µg/mL	6.IS141-12.5µg/mL	9.IS 217-12.5µg/mL	12.IS111-12.5µg/mL

Figure 34 (a &b): A representative western blot images showed the expression of TLR4 and VEGF. TLR4 and VEGF expression was detected by western blot. GAPDH was used as an internal control. Protein expressions were quantified using image J software and graphs were plotted against each specified protein markers. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate. One way ANOVA was performed for statistical analysis.



1.Unstimulated	4.IS141-3.12µg/mL	7.IS 217-3.12µg/mL	10.IS111-3.12µg/mL
2. LPS-1µg/mL	5.IS141-6.25µg/mL	8.IS 217-6.25µg/mL	11.IS111-6.25µg/mL
3. DEX-0.5µg/mL	6.IS141-12.5µg/mL	9.IS 217-12.5µg/mL	12.IS111-12.5µg/mL

Figure 35: Effect of test peptides (IS141, IS 217 and IS111) on LPS-induced phosphorylation of p38 MAPK.p38 MAPK expression was detected by western blot. GAPDH was used as an internal control. Protein expressions were quantified using image J software and graphs were plotted against each specified protein markers. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate. One way ANOVA was performed for statistical analysis.



1.Unstimulated	4.IS141-3.12µg/mL	7.IS 217-3.12µg/mL	10.IS111-3.12µg/mL
2. LPS-1µg/mL	5.IS141-6.25µg/mL	8.IS 217-6.25µg/mL	11.IS111-6.25µg/mL
3. DEX-0.5µg/mL	6.IS141-12.5µg/mL	9.IS 217-12.5µg/mL	12.IS111-12.5µg/mL

Figure 36: Effect of test peptides (IS141, IS 217 and IS111) on LPS-induced phosphorylation of ERK1/2.ERK1/2 expression was detected by western blot. GAPDH was used as an internal control. Protein expressions were quantified using image J software and graphs were plotted against each specified protein markers. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate. One way ANOVA was performed for statistical analysis.

5.2.2.4. The effect of test peptides (IS 141, IS111 &IS 217) on mRNA expressions of cytokines:

To assess the gene expression levels of IL-β, IL-6, CCL2, and TNF-α, RT-qPCR analyses were performed. The mRNA expression levels of these genes were measured to estimate their relative expressions. The results of gene expression analysis revealed that in the LPS-treated groups, the mRNA expression levels of IL-β, IL-6, CCL2, and TNF-α were increased compared to the control groups. However, in the groups treated with the test peptides, the mRNA expression levels of IL-β, IL-6, CCL2, and TNF-α were decreased compared to the LPS-treated groups (Figures 37-39). Treatment with IS141 and IS111 demonstrated a dose-dependent reduction in the expression of CCL2 and IL-1β. Moreover, treatment with IS111 significantly reduced the expression of TNF-α and IL-6 compared to the LPS control. IS 217 peptide treatment also showed a dose-dependent decrease in the expression of IL-1β compared to the LPS control.

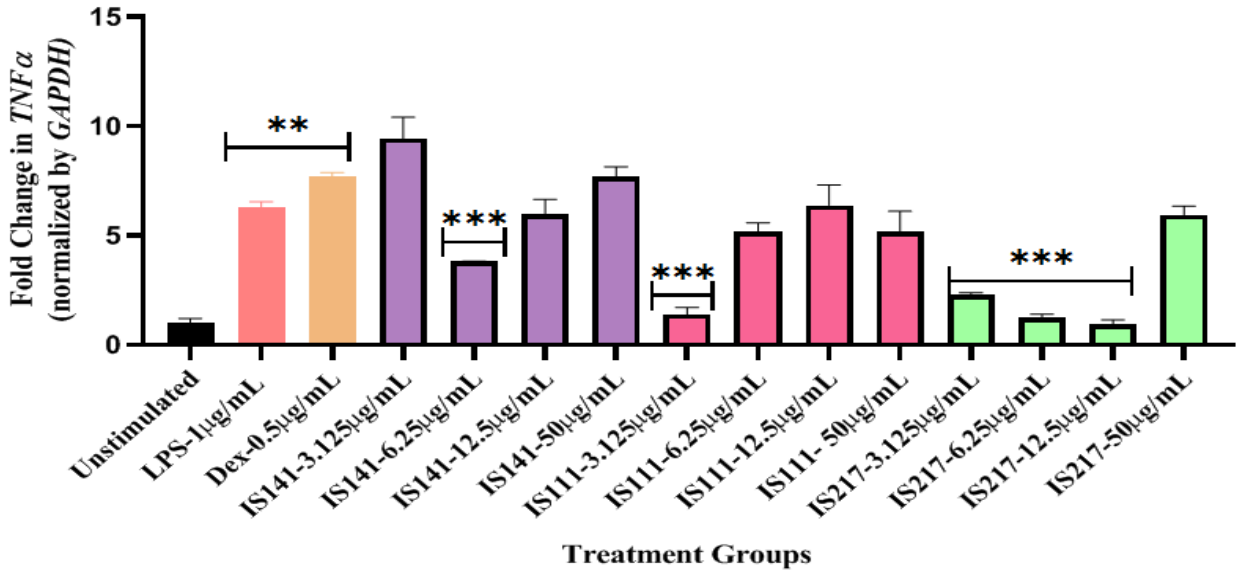
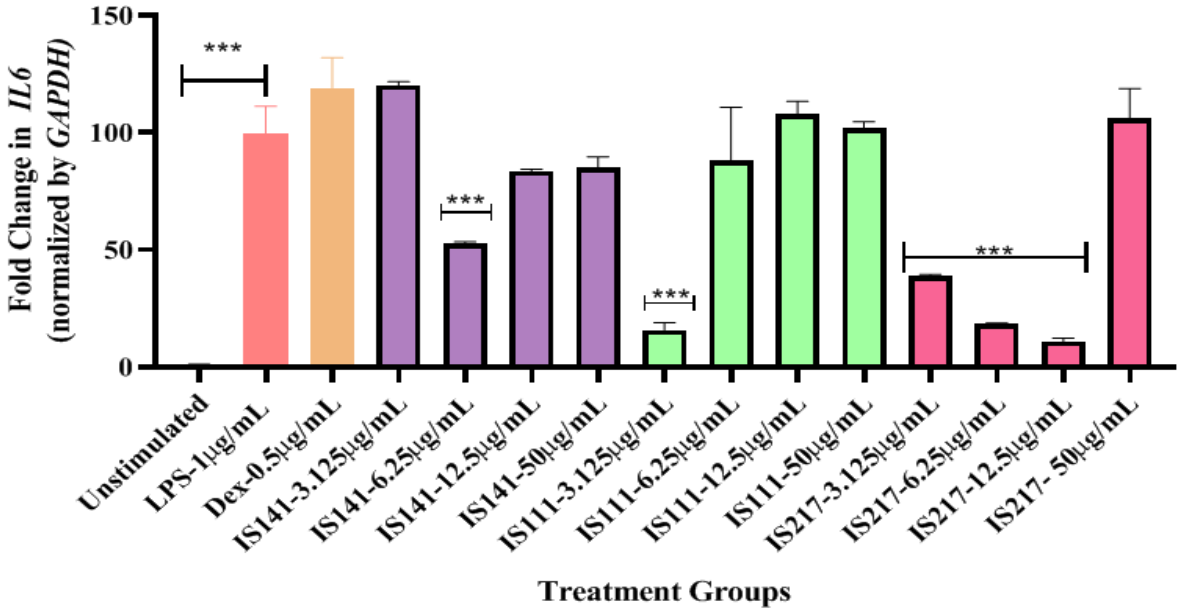
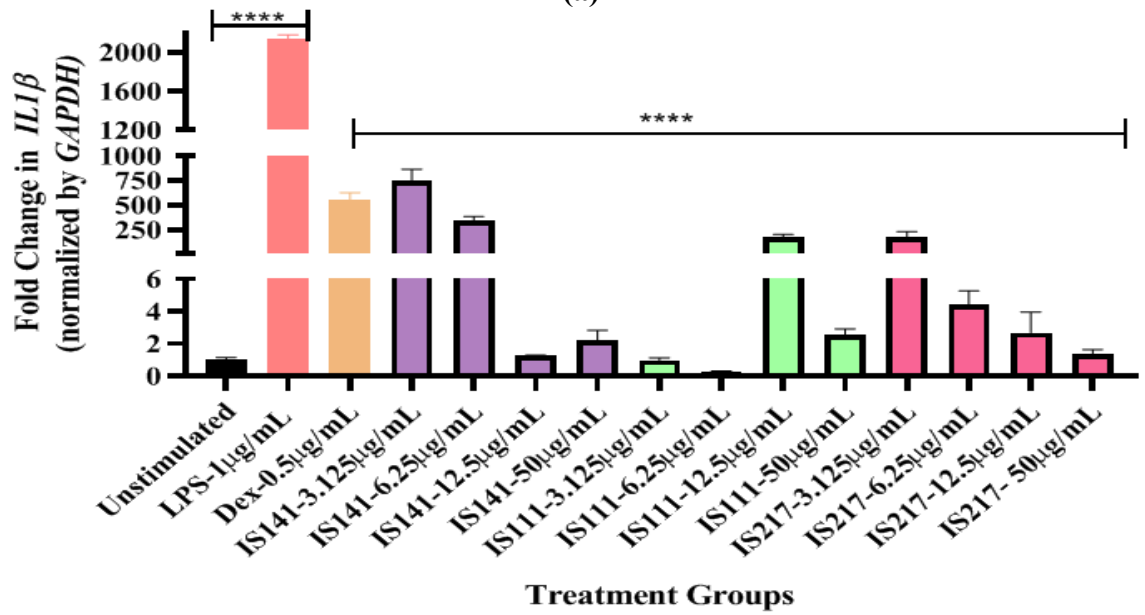


Figure 37: Effect of test peptides (IS 141, IS 111 & IS 217) on TNF- α mRNA expression was detected by RT-qPCR. The data presented in this study were shown as fold change relative to the LPS-stimulated cells and the unstimulated (control) cells. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate.



(a)



(b)

Figure 38(a,b): Effect of test peptides (IS 141, IS 111 & IS 217) on IL-6 &IL-1β mRNA expression was detected by RT-qPCR. The data presented in this study were shown as fold change relative to the LPS-stimulated cells and the unstimulated (control) cells. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate.

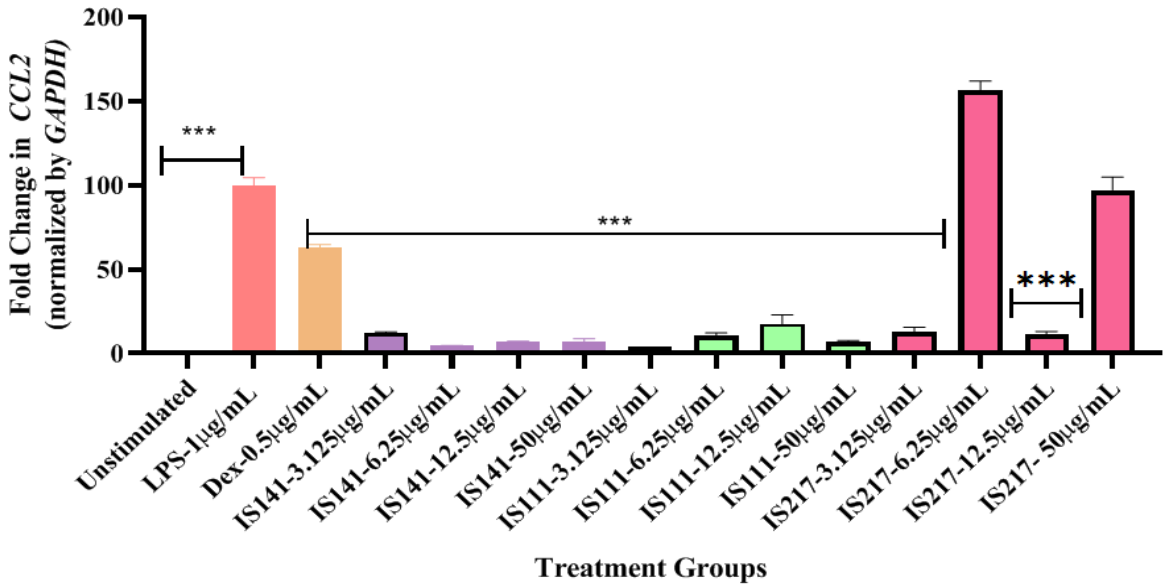


Figure 39: Effect of test peptides (IS 141, IS 111 & IS 217) on CCL2 mRNA expression was detected by RT-qPCR. The test peptides inhibit LPS-induced pro-inflammatory cytokine expression at the mRNA level. The data presented in this study were shown as fold change relative to the LPS-stimulated cells and the unstimulated (control) cells. The means ± SEM was used to represent the data. Each experiment was repeated three times to ensure reliability. Statistical significance was indicated as ****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$ when comparing the results to the LPS group. The bars in the figures represent the means, while the vertical lines represent the standard error of the mean, based on three independent experiments analyzed in duplicate.

5.2.3. Discussion:

The process of designing in vitro, cell-based assays hold crucial significance in the early stages of screening potential new compounds. Key considerations involve the choice of primary cell cultures over immortalized cell lines due to their relevance and physiological representation. LPS, a component found in the outer membrane of gram-negative bacteria, acts as a potent trigger for releasing various proinflammatory mediators like TNF-α, IL-6, IL-1β, and IL-12 [Jiang Z et al., 2004]. Its impact extends beyond direct infection by gram-negative microorganisms, contributing to systemic inflammatory responses and bacteremia, including those caused by gram-positive bacteria via bacterial translocation from the gut.

Certain Host defense peptides (HDPs) possess the remarkable ability to modulate the innate immune response by inducing chemokines and suppressing harmful pro-inflammatory cytokines [Jiang et al., 2004]. In this particular study, the focus was on evaluating the anti-inflammatory activity of peptides IS141, IS111, and IS217 on murine macrophage-like RAW 264.7 cells stimulated with 1μg/mL LPS (as indicated in figures 31–39). These peptides were compared to the standard drug dexamethasone. Crucially, the observed inhibitory effect of IS141, IS111, and IS217 on pro-inflammatory cytokines did not stem from any cytotoxic activity, as cell viability remained unaffected. However, it's noteworthy that the tested concentrations of peptides did not exceed 80% viability.

Results from quantifying TNF-α, IL-1β, and IL-6 showcased that the tested peptides exhibited inhibitory activity on the production of these inflammatory mediators in LPS-induced RAW 264.7 cells. Interestingly, the lower concentration of 12.5 μg/mL demonstrated better activity compared to the higher concentration of 100 μg/mL. This nuanced dosage-dependent response highlights the potential for these peptides to effectively modulate the inflammatory response at specific concentrations without compromising cell viability.

TNF-α, a pivotal cytokine in inflammation, is primarily induced by activated macrophages and orchestrates various inflammatory processes, including adhesion molecule induction and NF-κB activation (An H et al., 2002). TNF-α, an endogenous pyrogen, holds the capacity to induce fever, apoptotic cell death, and cachexia while influencing tumor genesis, viral replication, and sepsis response through IL-1β and IL-6 production. Its dysregulation is implicated in diseases like Alzheimer's, cancer, major depression, psoriasis, and inflammatory bowel disease (IBD). Given its role in initiating an inflammatory cascade involving various mediators and cell recruitment, assessing TNF-α inhibitory activity is crucial when evaluating potential anti-inflammatory agents [McCoy SL et al.,2004]. Its involvement in numerous human diseases emphasizes the significance of assessing its inhibitory activity when screening potential anti-inflammatory agents. Treatment of macrophages with LPS led to significant increase in the levels of both TNF-α and nitrites in the culture supernatants relative to control levels IL-6 has a wide range of effects on immune system cells and is involved in acute inflammatory responses. Its increased levels have been reported in various inflammatory conditions. Inhibition of IL-1β synthesis indirectly benefits autoimmune disease and inflammation treatment. Moreover, IL-6, with its broad effects on immune cells, is involved in acute inflammatory responses and is elevated in various inflammatory conditions. Inhibiting IL-1β synthesis indirectly aids in autoimmune disease and inflammation treatment. In our

study, IS141, IS111, and IS217 demonstrated the ability to curb TNF-α production in RAW 264.7 cells, indicating their anti-inflammatory impact via downregulating pro-inflammatory cytokines.

Additionally, IS111 and IS217 notably reduced IL-6 levels, while all tested peptides exhibited significant reduction in TNF-α levels. Our findings demonstrated that IS141, IS111, and IS217 reduced IL-6 and IL-1β production in LPS-stimulated RAW 264.7 cells [Hong et al., 2004]. IL-1β's significance lies in initiating and amplifying the inflammatory response during microbial infections by inducing the secretion of proinflammatory cytokines like IL-6 and IL-8 [Liao JC et al., 2012]. Interestingly, in our study, LPS-stimulated RAW 264.7 cells exhibited low IL-10 levels while significantly inducing TNF-α, IL-1β, and IL-6. However, treatment with IS141, IS111, and IS217 markedly increased IL-10 production, particularly IS111 and IS217, showing a substantial and nearly complete reduction in TNF-α, IL-1β, and IL-6 release in LPS-stimulated cells (Figure 30-32, respectively). Additionally, dexamethasone exhibited significant anti-inflammatory action by inhibiting the expression of all these markers [Eicher SD et al., 2004].

The western blot analysis revealed a significant increase in TLR-4 levels following LPS stimulation compared to the control. However, treatment with IS141 (12.5 ug/mL) showed a slight reduction in TLR-4 levels, as evidenced in studies by [Eicher SD et al. 2004, Schmalz G et al. 2011, and Bhattacharyya S et al.2007]. Additionally, VEGF levels were notably decreased in samples treated with IS141 (12.5 ug/mL), IS217, and IS111. In the case of pERK levels, LPS treatment increased their levels, whereas IS111 treatment significantly decreased pERK levels. Our investigation delved into the underlying mechanisms behind these observed effects.

The induction of pro-inflammatory mediators and cytokines by LPS relies on MAPK signaling pathways, crucial for regulating cellular responses to various stimuli such as cytokines, stressors, and cell growth. Studies indicate that the peak expression of MAPK occurs around 20-30 minutes post-LPS exposure in human and murine macrophages. To impede the induction of TNF-α by LPS, inhibition of one of the three MAPK pathways (JNK, p38 MAPK, and ERK) is necessary. Our findings highlight that IS141, IS111, and IS217 significantly diminish the phosphorylation of ERK1/2 induced by LPS in activated macrophage cells. This suggests the involvement of p38 and ERK1/2 in the inhibitory effects of these peptides in RAW 264.7 cells, regulating the expression of

cytokines and other mediators involved in the inflammatory response. These outcomes align with prior research findings.

The gene expression analysis further supported our findings, indicating that LPS stimulation markedly increased the expression of CCL2, TNF-α, IL-6, and IL-1β compared to the control. Treatment with IS141 and IS111 resulted in dose-dependent reductions in CCL2 and IL-1β expression. IS111 notably decreased TNF-α and IL-6 compared to the LPS control, while IS217 dose-dependently reduced IL-1β expression. An interesting observation was the increase in TNF-α expression with escalating peptide doses, particularly at 50 μg/kg. This dose-dependent response between the administered peptide and the immune response might explain the amplified TNF-α expression. TNF-α, a crucial pro-inflammatory cytokine, plays a significant role in immune responses. In certain experimental or therapeutic scenarios, peptide administration can modulate immune functions, and varying doses may influence cytokine expression. At 50 μg/kg, the peptide might interact with immune cells or signaling pathways, potentially enhancing TNF-α production. This dose-dependent effect is vital in understanding the peptide's impact on the immune system, suggesting that different concentrations of the peptide induce varying levels of TNF-α expression.

5.2.4. Summary:

In summary, the synthetic test peptides—IS141, IS111, and IS217—exhibited substantial anti-inflammatory effects against LPS-induced inflammation in RAW 264.7 cells. These peptides showcased their efficacy by inhibiting the expression of pro-inflammatory cytokines (CCL2, TNF-α, IL-6, and IL-β) and by suppressing the phosphorylation of p38 MAPK, a crucial signaling molecule in the inflammatory cascade.

Given the promising results from synthesis and in vitro studies, IS217 stood out and was chosen for further investigation in proof-of-concept studies. This selection likely stems from its demonstrated potency and efficacy in modulating the inflammatory response, making it a prime candidate for subsequent in-depth research and potential therapeutic development.

Section 3.

Development of pre formulation and determination of maximum feasible concentration for peptide IS 217

5.3. Results of pre formulation studies:

5.3.1. Results of stability of peptide IS 217 with the solvents used for pre formulation:

In the present study, the pre formulation of peptide IS 217 was developed and determined maximum feasible concentration of developed formulation, which was used further through various routes in non-clinical efficacy studies. Four different aqueous based pre formulations of peptide IS 217 were prepared. All the pre formulations were first subjected to stability study at intended use of three temperature's for 24h. During the period of 24 h pre formulations showed no visual changes in the physical appearance from initial sample. Out of these four aqueous based preformulations.0.9% NaCl solution was selected for intravenous (IV) and 10%v/v propylene glycol in 0.9%Nacl for subcutaneous (SC) route as co-solvent, based on its stability and suitability at physiological conditions. The maximum feasible concentration for IS 217 was greater than 50 mg/ml.

Physical Description: white fluffy powder is not affected with solvents used.

Table 42: Percentage of peptide IS 217 remaining in respective solvents after 24 h

Test Conditions	**% remaining peptide IS 217 after 24 h**			
	Phosphate buffer pH 5.0	**Phosphate buffer pH 7.4**	**0.9% NaCl solution**	**water**
2-8^{0}c	112.98	107.53	99.89	101.85
RT	92.37	99.18	100.42	81.23
37^{0}C	109.51	97.43	90.02	84.96

Stability of IS 217 in four different diluents was performed at 2-8^{0}c, room temperature (RT) & 37^{0}C.It was observed that after 24 h the percentage of peptide IS 217 remaining in different solutions was in the range of 81.23 to 112.98%.

5.3.1.1. Results of Pre formulations development is summarized in table below:

Table 43: Percent change from initial after 24 h in different solvents for the peptide IS 217.

Sample	**Initial Assay**	**%Change from Initial after 24 h**
10%v/v propylene glycol in 0.9%Nacl	102.23	-1.57
10%v/v Tween 20 in 0.9%Nacl	104.96	0.04
0.9%Nacl solution	96.29	-6.65

For pre formulation preparation, the peptide IS 217 was dissolved in different vehicles, as listed in table 42. The assay was performed initially and after 24 hours at room temperature. Percent change from Initial was determined and listed in table 43.

5.3.1.2. Results of Maximum feasible concentration is summarized in table below:

Table 44: Percent change from initial after 24 &48 h at 25 mg/ml of peptide IS 217.

Sample	Initial Assay	%Change from Initial after 24 h	%Change from Initial after 48 h
10%v/v propylene glycol in 0.9%Nacl	101.34	9.03	5.65
0.9%Nacl solution	103.64	-6.53	-2.96

Selected vehicles were used to dissolve peptide IS 217 at concentration 25 mg/ml. Assay was performed initially and after 24 and 48 hours at room temperature. Percent change from initial was determined and listed in table 44.

Table 45: Percent change from initial after 24 h at 50 mg/ml of peptide IS 217.

Sample	Initial Assay	%Change from Initial after 24h
10%v/v propylene glycol in 0.9%Nacl	106.62	6.79
0.9%Nacl solution	113.02	2.28

IS 217 was dissolved in selected vehicle at concentration 50 mg/ml. Assay was performed initially and after 24 hours at room temperature. Percent change from initial was determined and listed in table 45.

5.3.1.3. Results of Confirmation of mass (m/z) of peptide IS 217 in 0.9% NaCl solution after 24 h:

The mass (m/z) of peptide IS 217 in 0.9% NaCl solution was confirmed by HRMS, at two different temperatures (2-8^{0}c and at room temperature) after 24 h. the obtained spectra are shown in figures 40-42.

Figure 40: HRMS chromatogram of peptide: IS 217 +0.9% NaCl solution at 0 h.

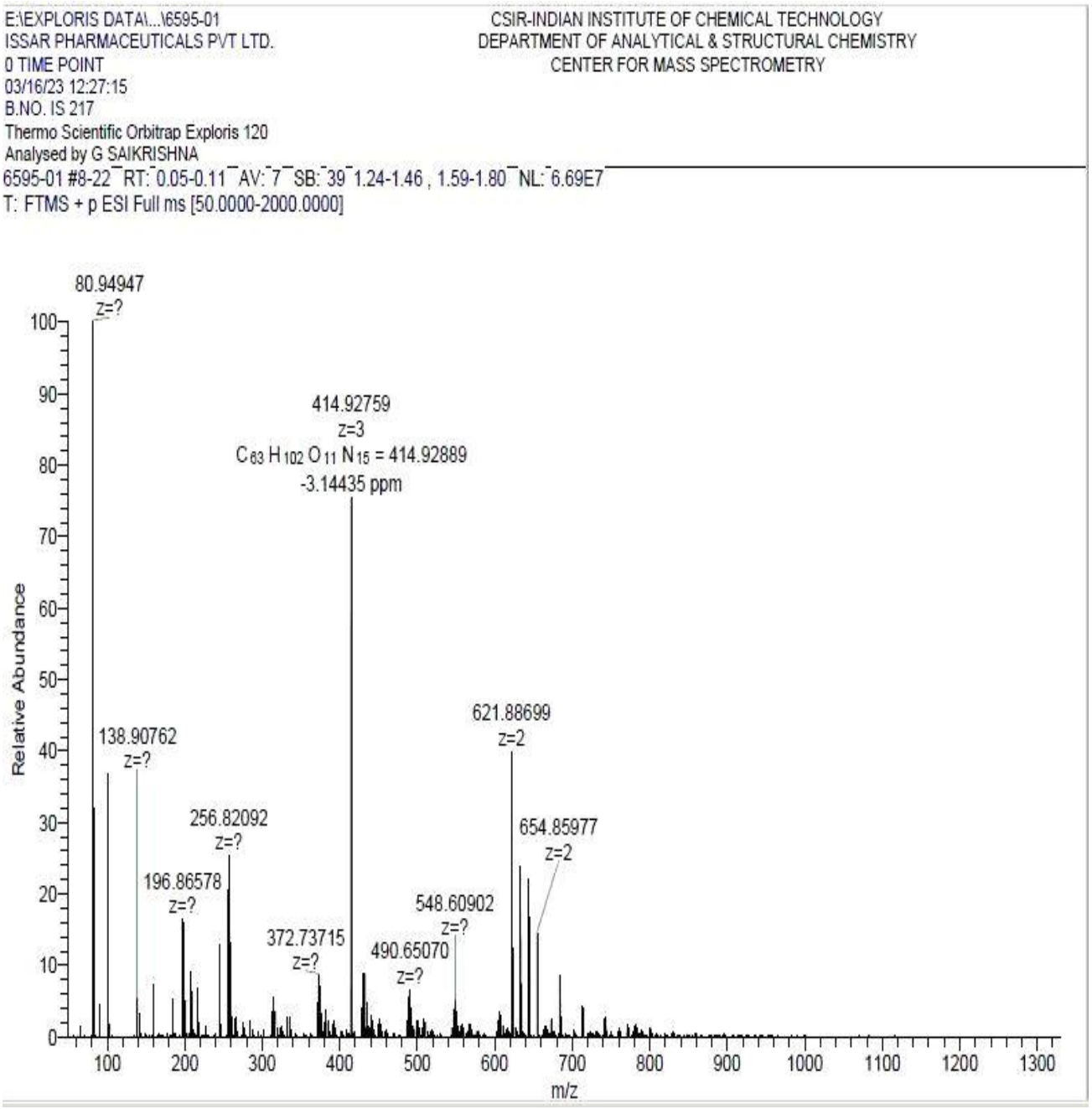


Figure 41: HRMS chromatogram of peptide: IS 217 +0.9% NaCl solution at 2-8^{0}c after 24h.

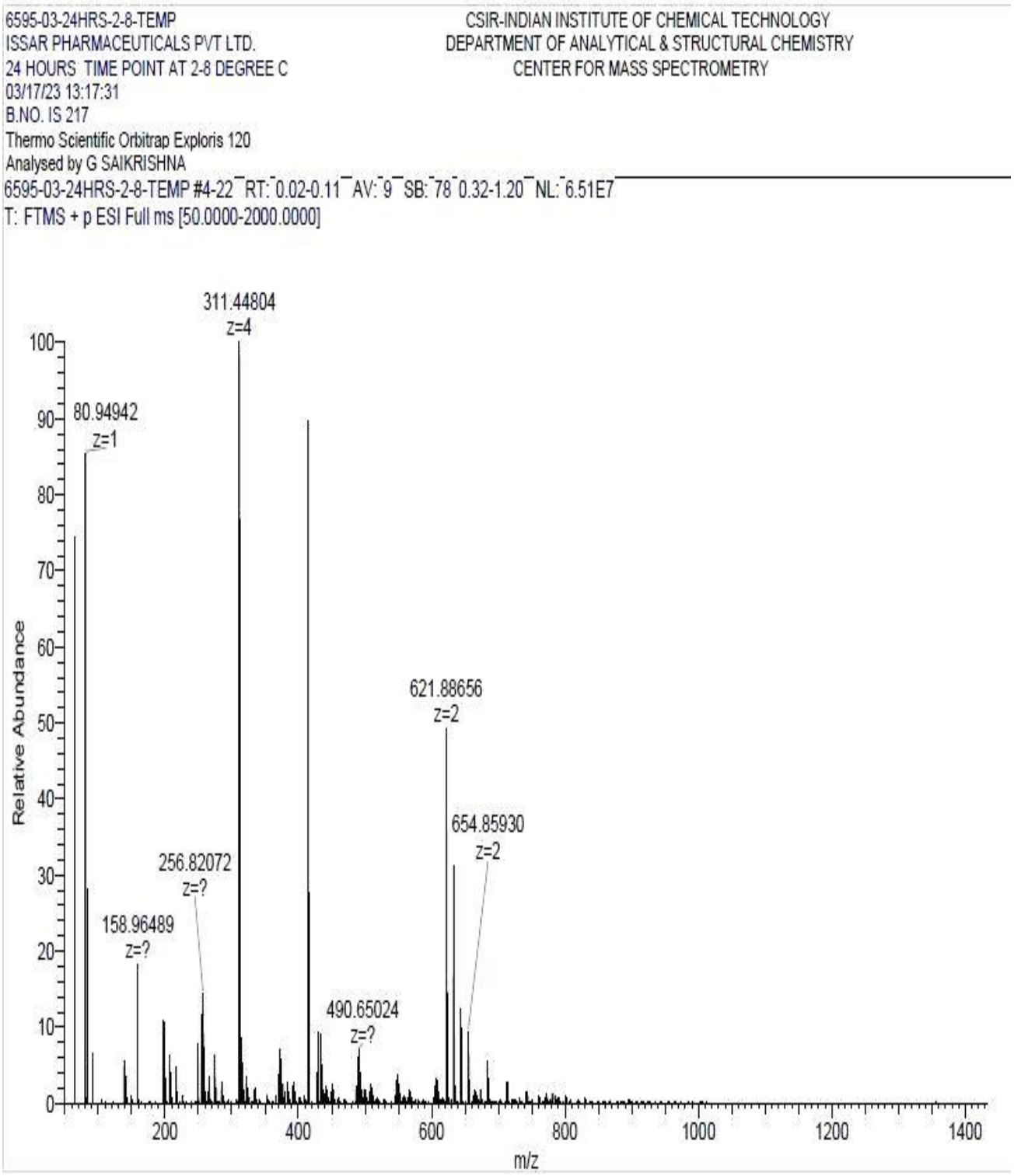
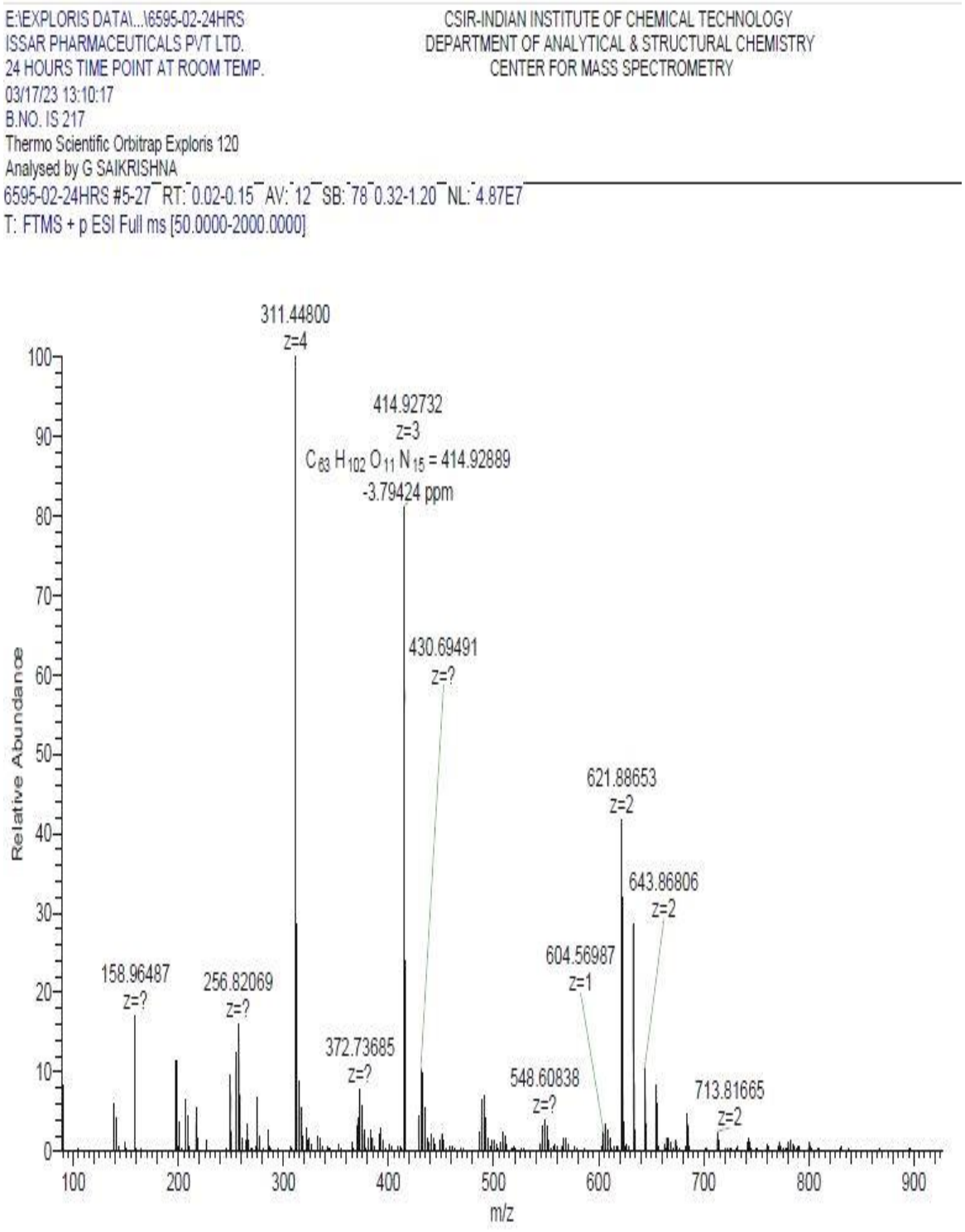


Figure 42: HRMS chromatogram of peptide: IS 217 +0.9% NaCl solution at room temperature after 24h.



5.3.2. Discussion:

In the present study, it was proved that the lead peptide IS 217 has good anti-inflammatory activity from in vitro assays. Further to investigate the in vivo efficacy of peptide IS 217 through four selected routes i.e., Intravenous, intraperitoneal, intramuscular, and subcutaneous pre formulations of IS 217 were required.

The present study was conducted to develop suitable pre formulation of peptide IS 217 and to determine the maximum concentration of the peptide which was feasible in the pre formulation i.e., Maximum feasible concentration (MFC) for the four routes of administration for further In vivo studies. This was achieved in this study by first, developing a suitable pre formulation in the selected aqueous solutions at various high concentrations like 25 mg/ml and 50 mg/ml. Since the peptides have good aqueous solubility, total aqueous based vehicles seem more suitable for intravenous and intraperitoneal routes. Subcutaneous and intramuscular route requires combination of hydrophobic vehicles (co-solvents), as the drug must pass through the tissues. A good hydrophilic and lipophilic balance (HLB) value was provided by these co-solvents which help drug to penetrate through the tissues to reach the systemic circulation. Considering the above approach, first four different aqueous vehicles were tested and then one of the aqueous vehicles was selected based on stability and its suitability in physiological conditions. Aqueous vehicles tested were phosphate buffer pH 5.0, Phosphate buffer pH 7.4, 0.9% Na l solution and water. The stability was performed at 2-8^{0}c, RT and 37^{0}C at 500 ppm concentration. A HPLC based method was developed to evaluate the stability. After collection of initial samples, the remaining sample was kept at three different temperatures as mentioned above 24 h. The % change in chromatographic peak areas from the initial sample area were used to calculate the stability of the peptides (Figures 40-42).

Percentage changes in the peak areas of peptide IS 217 from initial were found in range of 80-110% across the various temperature conditions (table 42-43). While the stability values of the peptide showed acceptable range for all the four aqueous vehicles, normal saline (0.9% NaCl solution) was selected as aqueous vehicle for peptide IS 217 based on suitable pH and osmolarity at physiological conditions. Based on the above experiments 0.9% NaCl solution was finalized for IV and IP preparations and was also used as aqueous vehicle for co-solvent based pre formulation development studies of SC and IM.

For developing the pre formulation for subcutaneous and intramuscular route, a suitable co-solvent was selected from various aqueous based co-solvents. Propylene glycol (PG), and Tween 80 are selected as co solvent for the pre formulation development as these are water soluble organic compounds and are widely used for SC/IM preparations. Here again the stability of peptides in various co-solvent based pre formulation were checked. HPLC based assay method was used to evaluate the stability of these peptides in the developed pre formulation. Based on the stability, viscosity, and suitability to the physiological conditions of these pre formulations, a suitable co-solvent based pre formulation was selected.

Peptide (IS 217) were formulated separately in 10% Tween 80 v/v, and 10% PG v/v in 0.9% NaCl solution at concentration 10mg/ml. Initially on visual inspection, all the pre formulations solutions were found clear (peptides solubilizes in co-solvent). Initial assay was performed using suitable HPLC method. Pre formulations were kept at room temperature and assay was performed after 24 h. All the pre formulations tested were found stable till 24 h, 10% v/v PG and 0.9% NaCl were further tested for stability at higher concentration, as these have excipients more suitable for intended use. Tween 80 based pre formulation was not further tested based on its less suitability as compared to the other two pre formulation in physiological conditions (slightly more toxic than other two)
[Robert G. Strickley et al., 2004; Shayne C. Gad et al., 2006].

A further similar experiment as discussed above was performed with selected pre formulations (10% v/v PG and 0.9% NaCl) at higher concentration i.e., 25mg/ml. All the pre formulations in 0.9% NaCl (1mg/mL) at 2-8^{0}c and at room temperature were found stable for 24 h from initial 0 h (Figure 40-42).

Hence based on solubility and stability of peptides in co-solvents two pre formulation i.e., 10% propylene glycol in 0.9% NaCl solution was selected for Intramuscular (IM) and Subcutaneous (SC) routes and normal saline (0.9% NaCl solution) is selected for Intravenous (IV) and Intraperitoneal (IP) routes. Since the peptides have good solubility in water and addition of co-solvent is done only to facilitate the drug administration through IM/SC route (these routes require formulation to be both hydrophilic and lipophilic), 10% v/v addition of co solvent provides good HLB value with appropriate viscosity.

Pre formulations of peptide IS 217 in developed pre formulations were prepared at concentration of 50mg/ml for determination of maximum feasible concentration and the stability was evaluated (Figure 45). Initial Assay of IS 217 pre formulations were 106.62% in 10% propylene glycol in 0.9%

NaCl solution and 113.02% in normal saline (0.9% NaCl solution). IS 217 pre formulations were stable till 24 h. Visual inspection also showed a clear solution. This reflects that MFC of peptide IS 217 in both the pre formulations is greater than 50mg/ml.

The study demonstrated that 10% propylene glycol in 0.9% NaCl solution is suitable for IM and SC route and Normal Saline (0.9% NaCl solution) is suitable for IV and IP route for the peptide IS 217. The maximum feasible concentration of IS 217 in both the pre formulations was greater than 50mg/ml.

5.3.3. Summary:

This study's summary suggests that a solution of 10% propylene glycol in 0.9% NaCl is suitable for administration via intramuscular (IM) and subcutaneous (SC) routes, while Normal Saline (0.9% NaCl solution) is appropriate for intravenous (IV) and intraperitoneal (IP) routes for delivering the IS217 peptide. **Based on these findings, the conclusion drawn recommends the utilization of 10% propylene glycol in 0.9% NaCl solution for SC administration and 0.9% NaCl solution for IV administration in subsequent in vivo animal studies involving the IS 217 peptide**.

Section 4.

Acute toxicity studies and Immunogenicity

5.4.1. Results of acute toxicity study of peptide IS 217 in mice:

The acute toxicity of peptide IS 217 was then examined with single dose of subcutaneous and intravenous injections in C57 BL/6 & BALB/c mice respectively. 0.9% Normal saline, single doses of IS 217 (0.6, 2.4, and 4.8 mg/kg) were administered subcutaneously and intravenously respectively in C57 BL/6 & BALB/c mice and were observed twice daily for clinical signs of toxicity and mortality and morbidity for 14 days of the study. As described in the method blood and tissue samples were collected after 14 days. The results of acute safety investigation of peptide IS 217 showed that subcutaneous & intravenous injection of IS 217 at 4.8 mg/kg are safe and does not cause mortality or organ damage.

No deaths occurred throughout the 14-day observation period following single administration of IS 217 and higher dose of 4.8 mg/kg b.wt appeared to be safe. Normal gain in body weight was observed in both the control and treatment groups. No macroscopic abnormalities were observed upon necropsy in any of the animals treated with IS 217. Based on these results, subcutaneous & Intravenous administration of IS 217 was not associated with any toxic effects, and the LD50 was determined to be greater than the dose level tested in this study (i.e., 4.8 mg/kg BW). The summary of the results is as follows.

5.4.1.1. Functional Observational Battery:

In any of the cases, no mice treated with peptide IS 217 were found dead or dying by cage observation, and all mice were in a healthy mental state with actively free moments. The changes in the mice fur, eyes, and mucosa, along with their respiratory system, circulatory system, nervous system, as well as the behaviors were meticulously documented (data not shown). All subjects showed no abnormalities after receiving a single subcutaneous and intravenous injection of peptide IS 217 at a high dose of 4.8 mg/kg b.wt.

5.4.1.2. Mortality:

During the period of 14 days, no death and noticeable clinical signs associated with toxicity were found in NC and all IS 217-treated groups in both routes of administration (IV & SC) (table 46).

Table 46: Summary of Mortality of BALB/c-IV & C57BL/6 –SC administration of peptide IS 217

Test Group	Treatment Dose (mg/kg b.wt)	No. of Animals* (M+F)	No. of mortality	
			IV	SC
Control (NS)	10 ml/kg	5+5	0	0
IS 217 – LD	0.6 mg/kg	5+5	0	0
IS 217– MD	2.4 mg/kg	5+5	0	0
IS 217– HD	4.8 mg/kg	5+5	0	0
Low dose (LD); Medium dose (MD); High dose (HD); b.wt: Body Weight; M:Male;F:Female ;NS: Normal Saline. *: the dose and animals allocated are same number of IV & SC routes and the studies are conducted separately in BALB/c & C57BL/6 mice respectively.				

5.4.1.3. Bodyweight and Food Intake:

A single subcutaneous and intravenous injection of peptide IS 217 had no effect on food intake, body weight, and weight change in mice. On the 14th day, the mice's body weight was also measured after an overnight fast. During the study period, the body weights of both male and female mice increase proportionately and found no significant differences in body weight or food intake among the experimental groups and the control group. The changes of body weight after 14 days of subcutaneous and intravenous injection of peptide IS 217 are shown in table 47 & 48.

Table 47: Effect of peptide IS 217 (0.6, 2.4, and 4.8 mg/kg) on body weight of BALB/c mice, IV administration.

Sex	Test Groups	Experimental Days (Mean ± SEM) (g)		
		0	7	14
Male	**Control (NS)**	25.6± 2.20	26.3 ± 2.30	26.5 ±2.61
	IS 217 – LD -0.6 mg/kg	25.8 ±2.58	25.9 ±2.49	26.5± 2.52
	IS 217– MD-2.4 mg/kg.	26.9± 2.19	27.5± 2.30	28.5 ±2.33
	IS 217– HD -4.8 mg/kg.	25.7 ±1.12	26.1 ±1.59	27.5± 1.13
Female	**Control (NS)**	23.8± 2.20	23.9 ±2.23	24.1 ±2.19
	IS 217 – LD -0.6 mg/kg	21.4 ±2.67	22.5 ±2.63	22.7± 2.93
	IS 217– MD-2.4 mg/kg.	21.6± 1.63	21.4± 1.48	21.4± 1.67
	IS 217– HD -4.8 mg/kg.	20.9±1.84	20.7± 1.53	20.8 ±1.42
Low dose (LD); Medium dose (MD); High dose (HD); b.wt: Body Weight; M: Male;F:Female ;NS: Normal Saline.				

Table 48: Effect of peptide IS 217 (0.6, 2.4, and 4.8 mg/kg) on body weight of C57BL/6 mice, SC administration.

Sex	Test Groups	Experimental Days (Mean ± SEM) (g)		
		0	7	14
Male	**Control (NS)**	20.4 ± 1.20	23.3 ± 3.02	31.3 ±2.27
	IS 217 – LD -0.6 mg/kg	22.4 ±1.29	24.6 ±2. 94	30.8± 2.07
	IS 217– MD-2.4 mg/kg.	23.1± 0.91	26.1± 2.30	30.9 ±1.93
	IS 217– HD -4.8 mg/kg.	22.6 ±1.42	27.0 ±1. 95	31.1± 2.38
Female	**Control (0.9%NS)**	19.2± 0.72	22.1±2.32	28.7 ±1.87
	IS 217 – LD -0.6 mg/kg	19.7 ±0.94	23.4 ±2.31	28.4± 1.39
	IS 217– MD-2.4 mg/kg.	20.7± 0.62	24.4± 1.68	28.8± 2.12
	IS 217– HD -4.8 mg/kg.	21.0±0.79	25.0± 1.83	29.2 ±1.82
Low dose (LD); Medium dose (MD); High dose (HD); b.wt: Body Weight; M:Male;F:Female ;NS: Normal Saline.				

5.4.1.4. Clinical biochemistry analysis: Biochemical examination on day 14 after single-dose administration revealed no abnormal parameters in male or female mice that received a single subcutaneous and intravenous injection of different doses of peptide IS 217, reflecting the pathological changes of vital organs were determined, and results were shown in (Table: 49 and 50), including hepatic and renal functions as well as other important parameters. In comparison to the control group, female mice had significantly lower levels of alanine aminotransferase (AST) in their blood. However, because this difference was only found in one sex and there was no evidence of a dose-effect relationship of peptide IS 217, as this difference was observed in the control animals. Statistical analysis of these parameters indicated that there were no significant differences between NC and IS 217-treated groups. Other blood biochemical indicators in the blood were within the normal limits.

Table 49: Effect of peptide IS 217 (0.6, 2.4, and 4.8 mg/kg) on biochemical parameters of BALB/c mice, IV administration.

Sex	Test Groups	AST (U/l)	ALT (U/l)	GLU mmol/l	TG mmol/l	CRE µmol/l	BUN mmol/l)
Male	**Control (0.9%NS)**	143.0 ± 12.10	31.9 ± 4.08	4.53 ± 0.58	2.13 ± 0.08	45.2 ± 6.13	7.18 ± 0.55
	IS 217 – LD -0.6 mg/kg	142.1 ± 11.62	33.0 ± 3.34	4.41 ± 0.51	2.04 ± 0.17	47.6 ± 7.47	6.79 ± 0.46
	IS 217– MD-2.4 mg/kg.	141.2 ± 12.31	32.7 ± 3.93	4.49 ± 0.53	2.05 ± 0.09	43.9 ± 6.08	7.06 ± 0.68
	IS 217– HD - 4.8 mg/kg.	142.1 ± 11.34	32.3 ± 4.22	4.51 ± 0.64	2.03 ± 0.07	45.8 ± 5.66	7.36 ± 0.71
Female	**Control (0.9%NS)**	118.0 ± 10.71	26.9 ± 3.52	4.38 ± 0.69	1.84 ± 0.12	39.6 ± 5.73	7.73 ± 0.91
	IS 217 – LD -0.6 mg/kg	117.5 ± 9.14	28.1 ± 3.83	4.75 ± 0.53	1.87 ± 0.28	37.2 ± 5.48	8.06 ± 0.54
	IS 217– MD-2.4 mg/kg.	121.3 ± 8.76	27.1 ± 2.89	4.63 ± 0.62	2.06 ± 0.15	36.8 ± 7.61	7.94 ± 0.62
	IS 217– HD - 4.8 mg/kg.	118.2 ± 10.21	27.7 ± 3.23	5.06 ± 0.68	1.83 ± 0.26	38.4 ± 6.33	8.15 ± 1.23

Low dose (LD); Medium dose (MD); High dose (HD); b.wt: Body Weight; M:Male;F:Female ;NS: Normal Saline.

Table 50: Effect of peptide IS 217 (0.6, 2.4, and 4.8 mg/kg) on biochemical parameters of C57BL/6 mice, SC administration.

Sex	Test Groups	AST (U/l)	ALT (U/l)	GLU mmol/l	TG mmol/l	CRE µmol/l	BUN mmol/l)
Male	**Control (0.9%NS)**	135.3 ± 7.47	28.3± 3.83	3.35 ± 0.58	2.09± 0. 19	40.2± 0.91	5.01± 1.19
	IS 217 – LD - 0.6 mg/kg	131.0± 5.66	23.2± 2.89	3.82 ± 0.15	2.10± 0.15	41.9± 3.23	4.9± 2.03
	IS 217– MD- 2.4 mg/kg.	128.4± 6.08	21.4± 3.52	3. 94 ± 0.35	2.00± 0.26	42.0± 5.73	5.21± 1.23
	IS 217– HD - 4.8 mg/kg.	123.1± 6.13	32.5± 3.23	4.54 ± 0.46	1.9± 0.91	1.89± 0.91	42.1± 0.23
Female	**Control (0.9%NS)**	105.4 ± 9.71	19.1± 3.52	2.83 ± 0.76	1.6 ± 0.91	34.84 ± 0.12	5.61 ± 5.73
	IS 217 – LD - 0.6 mg/kg	98.5 ± 7.24	16.7 ± 3.83	4.57 ± 0.35	2.06 ± 0.54	39.87 ± 0.28	5.22 ± 5.48
	IS 217– MD- 2.4 mg/kg.	101.2 ± 6.87	22.0 ± 2.89	3.63 ± 0.44	2.94 ± 0.62	32.06 ± 0.15	6.80 ± 7.61
	IS 217– HD - 4.8 mg/kg.	109.0 ± 11.30	21.4 ± 3.23	4.06 ± 0. 8 6	1.65 ± 1.23	31.83 ± 0.26	4.41 ± 6.33
Low dose (LD); Medium dose (MD); High dose (HD); b.wt: Body Weight; M:Male;F:Female ;NS: Normal Saline.							

5.4.1.5. Gross necropsy and relative organ weight of vital organs:

Gross necropsy showed that the subcutaneous and intravenous injection of different doses of IS 217 (0.6, 2.4, and 4.8 mg/kg) showed no effect on the weight of the organs, the ratio of viscera to the body. The effects of IS 217 on relative weight of vital organs including Brain, heart, lungs, liver, kidney, and spleen, were demonstrated in Table 52 & 53. No significant differences in relative organ weight were noted between NC and IS 217 -treated groups. Internal(visceral) as well as external

gross pathological examination did not reveal any peptide IS 217 related abnormalities in the treated and control groups of both the sexes.

Table 52: Effect of peptide IS 217 (0.6, 2.4, and 4.8 mg/kg) on relative organ weights of BALB/c mice, IV administration.

Sex	Test Groups	Relative organ weight (g)					
		Brain	**Heart**	**Lungs**	**Liver**	**Kidneys**	**Spleen**
Male	**Control (0.9%NS)**	1.5± 0.05	0.6± 0.38	0.9± 0.04	6.6± 0.38	1.7± 0.05	0.8± 0.18
	IS 217 – LD - 0.6 mg/kg	1.5± 0.09	0.6± 0.08	0.7± 0.05	6.0± 0.05	1.6± 0.32	0.7± 0.04
	IS 217– MD- 2.4 mg/kg.	1.5± 0.04	0.6± 0.05	0.8± 0.18	5.9± 0.08	1.7± 0.18	0.7± 0.05
	IS 217– HD - 4.8 mg/kg.	1.5± 0.09	0.6± 0.04	0.8± 0.39	5.7± 0.09	1.5± 0.28	0. 7± 0.05
Female	**Control (0.9%NS)**	1.7± 0.38	0.8± 0.18	0.9± 0.05	6.8± 0.05	2.1± 0.09	0.8± 0.18
	IS 217 – LD - 0.6 mg/kg	1.8± 0.28	0.9± 0.09	1.1± 0.04	6.8± 0.15	2.0± 0.15	0.9± 0.15
	IS 217– MD- 2.4 mg/kg.	2.0± 0.37	0.9± 0.38	1.3± 0.18	8.5± 0.43	2.2± 0.43	0.9± 0.09
	IS 217– HD - 4.8 mg/kg.	2.0± 0.18	1.01± 0.08	1.2± 0.09	7.4± 0.15	2.2± 0.37	0.9± 0.14
Low dose (LD); Medium dose (MD); High dose (HD); M: Male; F: Female; NS: Normal Saline.							

Table 53: Effect of peptide IS 217 (0.6, 2.4, and 4.8 mg/kg) on relative organ weights of C57BL/6 mice, SC administration.

Sex	Test Groups	Relative organ weight (g)					
		Brain	Heart	Lungs	Liver	Kidneys	Spleen
Male	Control (0.9%NS)	1.3± 0.04	0.5±0.05	0.8± 0.05	5.6± 0.15	1.4± 0.09	0.7± 0.05
	IS 217 – LD - 0.6 mg/kg	1.3± 0.18	0.6±0.09	0.7± 0.04	5.1± 0.05	1.4± 0.15	0.6± 0.05
	IS 217– MD- 2.4 mg/kg.	1.4± 0.39	0.6± 0.04	0.7± 0.09	5.4± 0.08	1.6± 0.43	0.7± 0.15
	IS 217– HD - 4.8 mg/kg.	1.3± 0.05	0.5±0.08	0.7± 0.09	5.1± 0.05	1.4± 0.38	0.6± 0.18
Female	Control (0.9%NS)	1.5± 0.04	0.6±0.08	0.8± 0.38	5.7± 0.18	1.7± 0.28	0.7± 0.18
	IS 217 – LD - 0.6 mg/kg	1.5± 0.05	0.7±0.05	0.9± 0.18	5.4± 0.15	1.6± 0.18	0.7± 0.04
	IS 217– MD- 2.4 mg/kg.	1.5± 0.09	0.7±0.09	0.9± 0.37	6.2± 0.38	1.6± 0.37	0.7± 0.14
	IS 217– HD - 4.8 mg/kg.	1.4± 0.18	0.7±0.38	0.9± 0.28	5.3± 0.43	1.6± 0.32	0.7± 0.09
Low dose (LD); Medium dose (MD); High dose (HD); M: Male; F: Female; NS: Normal Saline.							

5.4.1.6. Discussion:

In the present study the acute toxicity profile of peptide IS 217 was established as it was necessary in identifying and characterizing the adverse effects associated with a peptide molecule in laboratory animals (mainly rodents) is used for several purposes, which includes: clinical diagnosis, treatment and prognosis of acute human poisoning cases; and design (e.g., dose-setting, identification of potential target organs) of longer-term (e.g., 28-day) toxicity studies. Acute toxicity test is typically the first step to determine the LD50 and lethality estimated by the LD50 test has been a primary

toxicological endpoint in acute toxicity tests, these studies are conducted following guidelines established by the Organization for Economic Cooperation and Development [OECD, 1992, 1996, 1998b,2001]. e.g., protocols that use new sequential dosing methods such as the Acute Toxic Class method [ATC, OECD TG 423; OECD, 2001] and the Up-and-Down Procedure [UDP, OECD TG 425; OECD, 2001].

In present study, only a single-dose of SC & IV injections with an observation of 14-day interval was conducted to evaluate the safety of IS 217, and 4.8 mg/kg was taken as an upper limit dose. During the observation period, no death and noticeable clinical signs associated with toxicity were found in NC and IS 217-treated groups; there were also no significant changes in body weights (Table 48 & 49), suggesting that the LD50 of IS 217 may be higher than 4.8mg/kg in mice [ICCVAM document, 2001; Directive 67/548/EEC; Annex V. Revision. European Commission, 1997]. Then a complete necropsy and serum biochemical and histopathological examinations were performed to assess the toxicity effects of IS 217 on inner organs [Xiaodan Yang,1 Junjie Li , et al., 2022] During necropsy, no noticeable abnormalities were not noticed, and there were no significant differences in relative weights of vital organs including brain, heart, lungs, liver, kidney, and spleen between NC and IS 217 -treated groups (Table 52&53). Another key profile to determine the in vivo injury degree of organs is serum biochemical parameters, such as ALT and AST, which are strongly associated to liver function, while CRE and BUN are important biomarkers of renal toxicity [Xiaodan Yang,1 Junjie Li, et al.,2022]. When compared with NC, no significant differences in AST, ALT, GLU, TG, BUN, and CRE were found in IS 217-treated groups (Table 50 &51), indicating that SC & IV administration of IS 217 has no harm on absolute and relative organ weights of Brain, heart, lungs, liver, kidney, spleen and testis/ ovary and for future histopathological examination, in the Brain, heart, lungs, liver, kidney, and spleen , the organs are fixed in 10% Formalin solution and stored at specific storage conditions. Slight weight changes in several organs, such as the liver and kidney, were not considered treatment-relevant because neither gender showed such a similar appearance [Xiaodan Yang,1 Junjie Li, et al., 2022; Katsuhiro Osajima et al.,2009]. **The results confirmed the peptide IS 217 possesses high-safety property, even in the maximum dose of 4.8mg/kg.**

5.4.2. Results of immunogenicity of peptide IS 217:

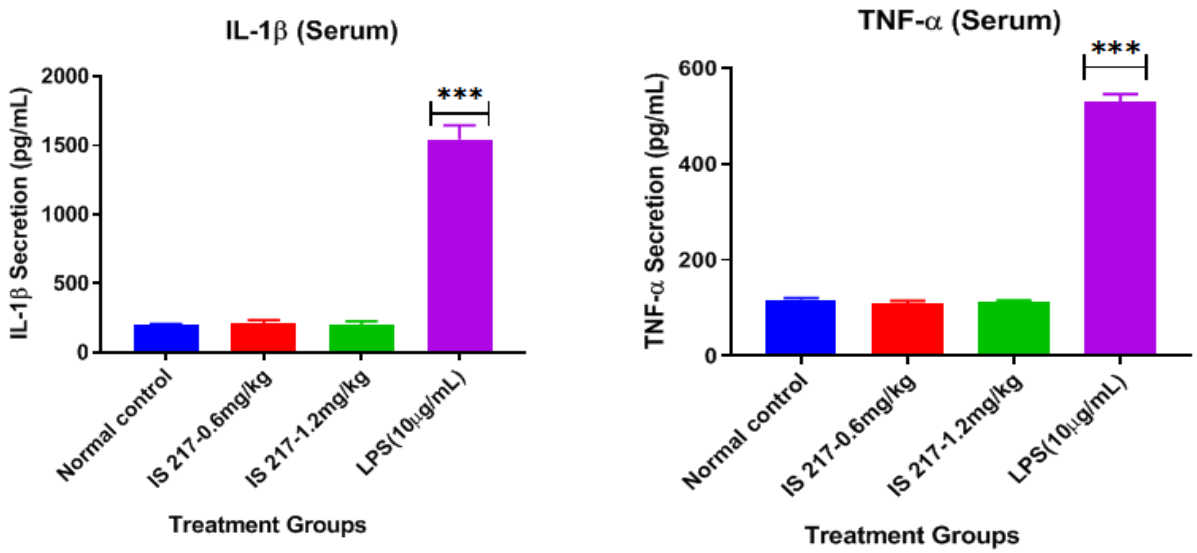
Peptide molecules can sometimes elicit an immune response in the body and so an investigation into the immunogenic profile of the test molecule was conducted specifically at the intended efficacy doses. The intended route of administration for the peptide molecule is intravenous (IV). Additionally, the choice of intravenous administration as the intended route could be influenced by factors such as the molecule's properties and the desired pharmacokinetics, and so intravenous was selected for route of administration and the doses of 0.6 and 1.2 mg/kg were chosen based on the results of a pilot efficacy study.

The results of ELISA assay of the separated serum samples also showed no evidence of enhanced IL-1β or TNF-α cytokine production after 48 h of treatment with peptide IS 217 at doses of 0.01 and 0.06 μmol per animal (corresponding to 0.6 mg/kg and 1.2 mg/kg, respectively, as it is shown in Figure 43 a,b). Indicated release of IL-1β and TNF-α cytokines was insignificant if compared to lipopolysaccharide (LPS). Possible toxic and immunogenic effects of peptide IS 217 was compared with the immunogenic activities of positive control (LPS).

Table53: The IL- 1β and TNF- α levels detection of in vivo immunogenicity study

Groups	**IL-1β (pg/mL)**	**TNF- α(pg/mL)**
Normal Control	198 ±9.39	117 ± 3.74
LPS (10μg)	1543±102.55	530± 15.54
IS 217 -0.6mg/Kg	203±25.40	119 ± 5.61
IS 217 -1.2mg/Kg	200±27.39	112± 3.74

The results expressed as mean ±SEM, (n=6)



(a) **(b)**

Figure 43: IL-1β (a) **and TNF-α** (b) **cytokines level detection in vivo Immunogenicity study**. BALB/c mice were treated with peptide IS 217 at the dose (0.6 &1.2 mg/kg) of 0.1ml per animal. Serum samples were collected 48 h after IV injections and applied to the ELISA assay. $p < 0.01$ and denoted *** as data were considered statistically significant when compared to normal control.

5.4.2.1. Discussion:

Although therapeutic protein agents and peptides used in clinical settings are generally considered nontoxic, anti-therapeutic protein antibodies can develop during the treatment and thus immunogenicity is a major disadvantage of these drugs. Almost all therapeutic proteins induce an antibody response and antibody formation may lead to a loss of efficacy, neutralization of the endogenous counterpart or cause infusion reactions, anaphylaxis, and anaphylactic reactions in response to immune response. To prevent these and thereby warrant better patient well-being and lower therapeutic costs, it is important to treat patients with therapeutic proteins that have no or minimal immunogenicity [Immunogenicity Assessment for therapeutic protein products: guidance for industry: by FDA,2014].

In the present study, the immunogenicity of the peptide was established, as immunogenicity of therapeutic peptide is important for novel peptides as immunogenicity lowers patient well-being, which drastically increases therapeutic costs and considered as important issue to prevent immunogenicity while developing novel therapeutic proteins and applying them in the clinic. As peptides do not possess intrinsic toxicity due to harmful metabolites; they are broken down to amino

acids, their use as drugs is under development for inflammatory diseases diagnosis and for many other applications in molecular biology. Thus, in vitro and in vivo assessments of therapeutic protein products including proliferation and cytokine release are recommended. Thus, the animal models are used as predictive tools during drug development as considered low, they should be considered for selected immunogenicity studies [European Medicine Agency (EMA) Directive 67/548/EEC, Annex V. Revision. European Commission, 1997.)]. C57BL/6 and BALB/c mice are inbred strains and being small and easy to real, little or no harm to laboratory personnel. The screening of diagnostic/immunological assay kits prefers mice over large animals at early drug developmental stages. The size, surface charge, surface hydrophilicity, surface morphology, and the amino acid sequence of the peptides also influence the immunogenicity of the peptide and the toxicity.

The d-amino acids, particularly d-alanine (d-Ala) and d-glutamic acid (d-Glu), peptides and the peptides which are less than 15 amino acids are not immunogenic and less toxic [M. H. V. Van Regenmor et al., 2011]. The current study, also investigated possible toxic and immunogenic activity of the peptide IS 217, which consist of alanine, lysine and phenylalanine amino acids and 10 amino acids sequence, trying to bring out the differences or possible enhanced influence of peptide IS 217 on the activation of the immune system. Our results demonstrated the lack of viability reduction in RAW 246 cell line, as well as no indication of primary inflammatory effects after 48 h of incubation with IS 217 peptides. Peptides showed no toxic influences on RAW 246 cell viability even at concentration of 100μg/mL Moreover, LPS exhibited strong cytotoxic effect, reducing cells viability up to 55%. The peptide IS 217 was not toxic at tested concentrations *in vitro*, and we proceeded with testing peptide IS 217 in vivo [Vera Brinks et al., 2011; Julia Suhorutsenko et.al.,2011].

Similar to the results from in vitro studies, none of the two doses of peptide IS 217 induced an increase of the cytokine levels in blood serum (Figure 43 a,b), showing no influence on the host system and conclude that the peptides have no evidence of inflammatory effects in vivo. ELISA analyses of the separated serum samples also showed no evidence of enhanced IL-1β or TNF-α cytokine production after 48 h of treatment with peptide IS 217 at doses 0.6 mg/kg and 1.2mg/kg, respectively, as it is shown in Figure 43a, b). Indicated release of IL-1β and TNF-α cytokines was insignificant if compared to lipopolysaccharide (LPS) [Rodrigo de Almeida Vauchera et al., 2011]. Additionally, no pathological deviations were observed in liver, kidney, lung, and spleen dissected from animals, which received 0.6 mg/kg/2.4 mg/kg /4.8 mg/kg of peptide IS 217, in addition the

biochemical parameters AST & ALT are in normal levels in treated groups compared to the normal control group.

5.4.3. Summary:

The findings from this study indicate that the IS217 peptide exhibited non-toxic and non-immunogenic properties, suggesting promising prospects for its future therapeutic applications devoid of any inflammation risks.

Section 5.

In vitro anti-microbial study

5.5.1. Results of In vitro anti-microbial study:

In the context of global health, multi-drug resistance poses a significant challenge, contributing to millions of deaths annually [WHO, Geneva; 2014]. Various "in vitro" methods exist to assess the antibacterial potential of novel compounds, with the broth dilution method serving as the gold standard for determining the minimum inhibitory concentration (MIC). In this study, the antibacterial activity was evaluated using broth microdilution and time-kill viability assays against specific pathogenic bacteria outlined in the methods section.

The bacterial strains chosen—S. aureus, P. aeruginosa, E. coli, and K. pneumoniae—were selected for specific reasons. These strains represent the most common causes of adult sepsis and prevalent sites of infections leading to septic shock, including pneumonia, intra-abdominal, and urinary tract infections. They also encompass major community-acquired and nosocomial pathogens known to cause infections in various areas such as skin, soft tissues, the intestinal tract, central nervous system, oropharynx, instrumentation sites, contaminated equipment, and intravenous fluids [Neal R. et al., 2018].

Staphylococcus aureus and *Streptococcus pneumoniae* are frequently encountered gram-positive isolates, while *Escherichia coli, Klebsiella sp.,* and *Pseudomonas aeruginosa* predominate among gram-negative isolates. The rationale behind selecting these specific strains lies in their prevalence, approval, and documented relevance in infectious disease and antimicrobial resistance research. Additionally, previous literature supporting the antimicrobial efficacy of synthetic peptides has influenced the choice of these strains for the preliminary screening of antimicrobial activity for new chemical entities or test molecules [Osmar N. Silva et al., 2015, Attila Farkas, et al.,2018, Rasheed JK, et al.,2000 & ATCC product library].

Our study results confirmed that the peptide IS 217 at concentrations did not exhibit cytotoxicity at the physiologically effective antibacterial concentrations.

5.5.1.1. Minimum Inhibitory Concentration (MIC) Assay:

In the present study, MIC values were determined by the micro broth dilution method in Mueller Hinton broth procedure and MIC values calculated in the present study are listed in Table 54. Peptide IS 217 displays rapid killing activity against pathogenic bacteria mentioned above. The assay

demonstrated that peptide IS 217 exhibits antibacterial effects against different bacterial pathogens (MIC between 0.39 and 1.56 µg/ml).

Table 54: Minimum inhibitory concentration (MIC) of peptide IS 217 and Antibiotic (Ciprofloxacin) against *S. aureus, P. aeruginosa, E. coli* & *K. Pneumoniae.*

S.no	Species	IS 217 (µg/mL)	Positive control Ciprofloxacin (µg/mL)
1	***Staphylococcus aureus***	1.56	1
2	***Pseudomonas aeruginosa***	0.78	0.5
3	***Escherichia coli***	0.39	0.5
4	***Klebsiella pneumoniae***	1.56	1

Results shown in the table are Mean ‘s obtained from triplicate experiments.

5.5.1.2. Time‑killing assay/survival of microbial species: Time-kill kinetics of peptide IS 217 against *S. aureus, P.aeruginosa , E.coli and K.pneumoniae.*

Table 55: The MIC concentration of Peptide IS 217 and the standard antibiotic against test species are tabulated as follows:

Groups	MIC concentrations of peptide IS 217 & Ciprofloxacin used against test species			
	S.aureus	***P.aeruginosa***	***E. coli***	***K.pneumoniae***
Vehicle Control	0	0	0	0
IS 217 -0.5 x MIC	0.78µg/mL	0.39µg/mL	0.195µg/mL	0.78µg/mL
IS 217 -1 x MIC	1.56µg/mL	0.78µg/mL	0.39µg/mL	1.56µg/mL
IS 217 -5 x MIC	7.8µg/mL	3.9µg/mL	1.95µg/mL	7.8µg/mL
Ciprofloxacin -1 x MIC	1µg/mL	0.5µg/mL	0.5µg/mL	1µg/mL

The time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents [Olajuyigbe & Afolayan, 2012] and can be classified as bacteriostatic or bactericidal, based on the characterization of the relationship between agent concentration and activity over time. The peptide IS 217 displays rapid killing activity against pathogenic bacteria. In the present study, time-kill assays were performed to analyze the killing rate of peptide IS 217 and to compare it with that of conventional antibiotic "Ciprofloxacin" which is frequently used in clinical settings. MIC values for peptide IS 217 and selected antibiotic (Ciprofloxacin) were determined and are listed in Table 55. The increase in viable count of bacteria in the control group shows these bacteria were actively growing from 1 to 24 h. Figures: 44 -47 shows the killing curves of peptide IS 217 and Ciprofloxacin for *S. aureus, P.aeruginosa , E.coli* and *K.pneumoniae* [Boorn KL,et al.,2010; Pradhan, S., et al., 2020].

5.5.1.2.1. Time-kill kinetics of peptide IS 217against *S. aureus:*

The result obtained with the peptide IS 217 and *S. aureus* was shown in Figure 44. After 24 h incubation with 0.5x MIC (0.78µg/mL) of IS 217, a 1 log10 CFU/mL reduction in viability of *S. aureus* occurred, indicating the IS 217 was bacteriostatic against this strain. At a concentration of 1x MIC (1.56µg/mL), however, the IS 217 was bacteriostatic against S. *aureus* by 5 h. Also, increasing the concentration to 5xMIC (7.8µg/mL) resulted in rapid bacteriostatic activity against *S. aureus* by 1 h (Figure 44& Table 56) and the Complete Reduction of Initial inoculum of *S. aureus* was achieved by 2h. These results indicate that anti-bacterial activity of IS 217 was both time- and concentration-dependent. In comparison, the killing activity of Ciprofloxacin at 1xMIC was slower and Complete Reduction of Initial inoculum of *S. aureus* was achieved by 3 h treatment. At 3hr the IS 217 and ciprofloxacin display similar killing activities.

Table 56: Time Kill Kinetics of peptide IS 217 against *S.aureus.*

Groups	Viable count at different time of exposure (1X10^{5}CFU/mL)							
	0hr	1 hr	2 hr	3 hr	4 hr	5 hr	12 hr	24 hr
Vehicle Control	6.5±0.35	6.8±0.08	7.1±0.10	7.4±0.05	7.2±0.08	6.9±0.18	6.2±0.15	5.9±0.08
IS 217 -0.5x MIC	6.3±0.25	5.3±0.10	4.9±0.25	3.9±0.25	3.3±0.08	2.5±0.05	2.1±0.18	1.2±0.18
IS 217 -1 x MIC	6.5±0.33	4.0±0.15	1.9±0.33	1.0±0.15	0.5±0.08	0.3±0.03	0	0
IS 217 -5 x MIC	6.4±0.15	0.5±0.05	0	0	0	0	0	0
Ciprofloxacin – 1 x MIC	6.4±0.25	3.2±0.08	1.4±0.18	0	0	0	0	0

Results shown in the table are Mean ± SEM obtained from triplicate experiments.

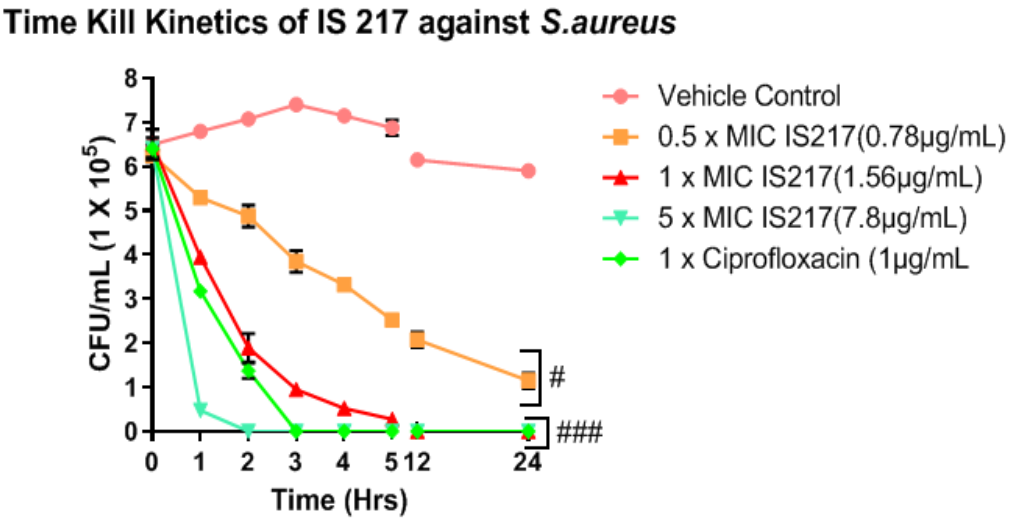


Figure 44: Time-kill kinetics of peptide IS 217 against *S. aureus.*

Results shown in the table are Mean ± SEM obtained from triplicate experiments where $P < 0.005$, $P< 0.05$ and denoted as *** &** and $^{###}$ & $^{##}$ when compared with standard drug and vehicle control groups respectively.

5.5.1.2.2. Time Kill Kinetics of peptide IS 217 against *P. aeruginosa:*

The result obtained with the peptide IS 217 and *P. aeruginosa* was shown in Figure 45. After 24 h incubation with 0.5x MIC (0.39µg/ml) of IS 217, Complete reduction in viability of *P. aeruginosa* occurred, indicating the IS 217 was bacteriostatic against this strain. At a concentration of 1x MIC

(0.78µg/ml), however, the IS 217 was bacteriostatic against *P. aeruginosa* by 3h. Also, increasing the concentration to 5x MIC (3.9µg/ml) resulted in bacteriostatic activity against *P. aeruginosa* by 1h (Figure 45& Table 57) and the complete reduction of Initial inoculum of *P. aeruginosa* was achieved by 2 h treatment with 5x MIC. These results indicate that anti-bacterial activity of IS 217 was both time- and concentration-dependent. In comparison, the killing activity of Ciprofloxacin at 1x MIC was Similar to 5 x MIC of IS 217 and Complete Reduction of Initial inoculum of *P. aeruginosa* was achieved by 2 h treatment. At 2h, the IS 217 and ciprofloxacin display similar killing activities.

Table 57: Time Kill Kinetics of peptide IS 217 against *P. aeruginosa.*

Groups	**Viable count at different time of exposure ($1X10^5$CFU/mL)**							
	0hr	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**12 hr**	**24 hr**
Vehicle Control	5.9±0.18	6.3±0.18	6.7±0.20	7.0±0.10	7.1±0.15	6.5±0.40	5.9±0.23	5.6±0.15
IS 217 – 0.5 x MIC	5.8±0.28	4.0±0.23	3.3±0.33	2.8±0.33	2.2±0.33	1.0±0.10	0.7±0.20	0.4±0.15
IS 217 – 1 x MIC	5.6±0.25	3.4±0.25	1.4±0.18	0.7±0.20	0.3±0.08	0	0	0
IS 217 – 5 x MIC	5.9±0.15	0.5±0.10	0	0	0	0	0	0
Ciprofloxacin – 1 x MIC	5.8±0.23	0.9±0.08	0.4±0.13	0	0	0	0	0

Results shown in the table are Mean ± SEM obtained from triplicate experiments.

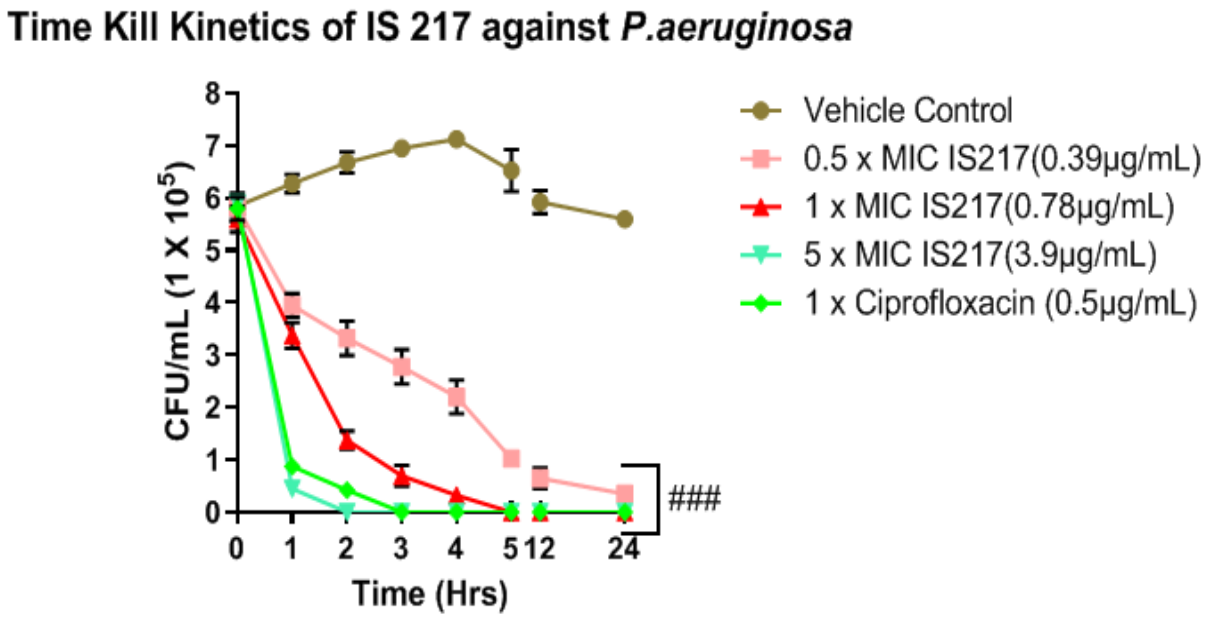


Figure 45: Time-kill kinetics of peptide IS 217 against *P.aeruginosa* .

Results shown in the table are Mean ± SEM obtained from triplicate experiments where $P < 0.005$, $P< 0.05$ and denoted as *** &** and ### & ## when compared with standard drug and vehicle control groups respectively.

5.5.1.2.3. Time Kill Kinetics of peptide IS 217 against *E. coli:*

The result obtained with the peptide IS 217 and *E. coli* was shown in Figure 46. After 24 h incubation with 0.5 x MIC (0.39µg/ml) of IS 217, a 1 log10 CFU/ml reduction in viability of *E. coli* occurred, indicating the peptide IS 217 was bacteriostatic against this strain. At a concentration of 1x MIC (0.78µg/ml), however, the peptide IS 217 was bacteriostatic against *E. coli* by 6h. Also, increasing the concentration to 5x MIC (3.9µg/ml) resulted in bacteriostatic activity against *E. coli* by 2h (Figure 46& Table 58) and the complete reduction of Initial inoculum of *E. coli* as achieved by 3h treatment with 5x MIC. These results indicate that anti-bacterial activity of peptide IS 217 was both time- and concentration-dependent. In comparison, the killing activity of Ciprofloxacin at 1x MIC was Similar to 5 x MIC of IS 217 and Complete Reduction of Initial inoculum of *E. coli* was achieved by 3 h treatment. At 3h, the IS 217 and ciprofloxacin display similar killing activities.

Table 58: Time Kill Kinetics of peptide IS 217 against *E. coli.*

Groups	Viable count at different time of exposure (1X10^{5}CFU/mL)							
	0hr	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**12 hr**	**24 hr**
Vehicle Control	6.5±0.18	6.3±0.20	6.5±0.30	7.0±0.28	7.4±0.20	7.2±0.13	6.8±0.30	6.2±0.25
IS 217 – 0.5 x MIC	6.5±0.30	5.9±0.23	5.3±0.18	4.3±0.13	3.7±0.15	3.0±0.25	2.5±0.23	1.6±0.30
IS 217 – 1 x MIC	6.5±0.10	4.0±0.23	2.2±0.28	1.2±0.15	0.8±0.20	0.4±0.10	0	0
IS 217 – 5 x MIC	6.6±0.23	0.9±0.15	0.4±0.10	0	0	0	0	0
Ciprofloxacin – 1 x MIC	6.2±0.30	1.2±0.23	0.6±0.15	0	0	0	0	0

Results shown in the table are Mean ± SEM obtained from triplicate experiments.

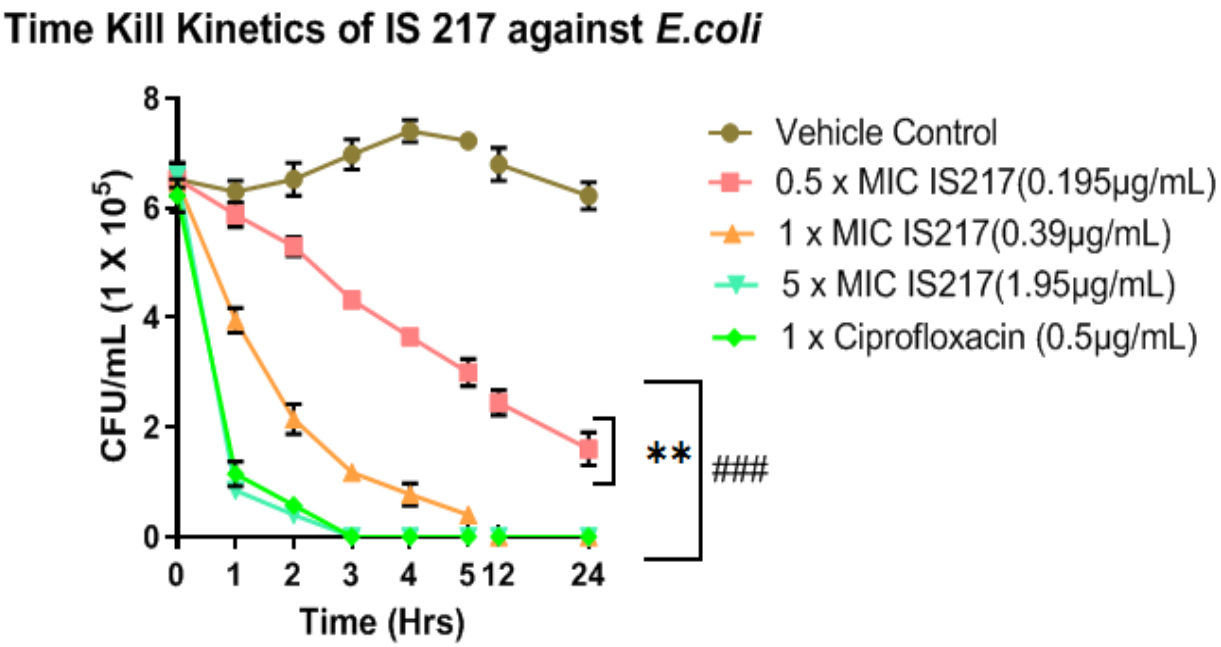


Figure 46: Time-kill kinetics of peptide IS 217 against *E. coli.*

Results shown in the table are Mean ± SEM obtained from triplicate experiments where $P < 0.005$, $P< 0.05$ and denoted as *** &** and $^{###}$ & $^{##}$ when compared with standard drug and vehicle control groups respectively.

5.5.1.2.4. Time Kill Kinetics of peptide IS 217 against *K.pneumoniae:*

The result obtained with the peptide IS 217 and *K. pneumoniae* was shown in Figure 47. After 24 h incubation with 0.5 x MIC (0.78µg/ml) of IIS Complete reduction in viability of *P. Aeruginosa* occurred,indicating the IS 217 was bacteriostatic against this strain. At a concentration of 1x MIC (1.56µg/ml), however, the IS 217 was bacteriostatic against *K.pneumoniae* by 2 h. Also, increasing the concentration to 5 x MIC (7.8µg/ml) resulted in rapid bacteriostatic activity against *K.pneumoniae* by 1 h (Figure 47& Table 59). Complete Reduction of Initial inoculum of *K.pneumoniae* was achieved by 4 h treatment with 5x MIC. These results indicate that anti-bacterial activity of IS 217 was both time- and concentration-dependent. In comparison, the killing activity of Ciprofloxacin at 1x MIC was slower and Complete Reduction of Initial inoculum of *K.pneumoniae* was achieved by 12 h treatment. At 12 h, the IS 217 and ciprofloxacin display similar killing activities.

Table 59: Time Kill Kinetics of peptide IS 217 against *K.pneumoniae.*

Groups	**Viable count at different time of exposure (1X10^{5}CFU/ml)**							
	0hr	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**12 hr**	**24 hr**
Vehicle Control	5.3±0.25	5.9±0.23	6.3±0.30	6.6±0.15	6.2±0.25	5.6±0.23	5.2±0.18	5.0±0.53
IS 217 – 0.5 x MIC	5.2±0.38	4.4±0.35	3.5±0.25	2.8±0.45	2.0±0.30	1.2±0.30	0.9±0.33	0.5±0.13
IS 217 – 1 x MIC	5.3±0.20	4.4±0.18	2.0±0.25	1.1±0.10	0.7±0.05	0.4±0.05	0	0
IS 217 – 5 x MIC	5.3±0.18	1.7±0.15	0.9±0.18	0.4±0.13	0	0	0	0
Ciprofloxacin – 1 x MIC	5.3±0.15	3.4±0.25	1.9±0.10	1.0±0.05	0.7±0.13	0.4±0.05	0	0

Results shown in the table are Mean ± SEM obtained from triplicate experiments.

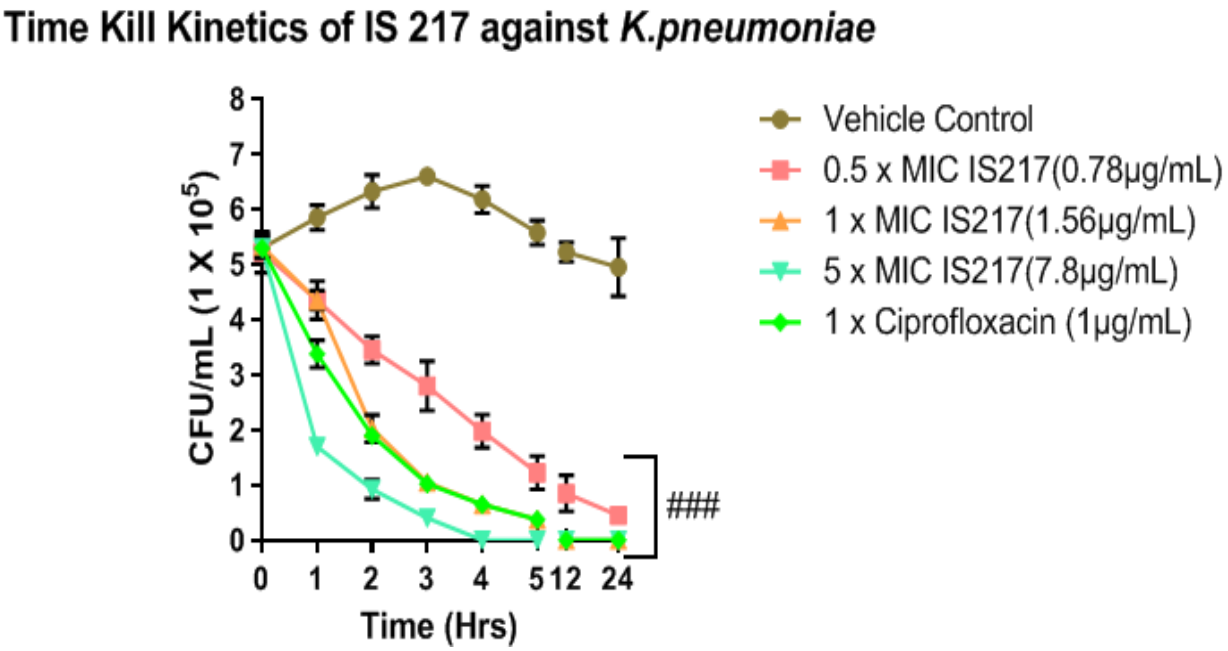


Figure 47: Time-kill kinetics of peptide IS 217 against *K.pneumoniae*

Results shown in the table are Mean ± SEM obtained from triplicate experiments where $P < 0.005$, $P< 0.05$ and denoted as *** &** and $^{###}$ & $^{##}$ when compared with standard drug and vehicle control groups respectively.

5.5.1.2.4. Conclusion of time kill assays:

Time-killing assay was performed treatment of organisms with peptide IS 217 for 24 h and IS 217 has showed good antimicrobial activity at the concentration of 1x MIC (1.56µg/ml) against *S. aureus,* 1x MIC (0.78µg/ml) against *P. aeruginosa,* and at 2h,5x MIC of IS 217 showed the completed reduction of initial inoculum. 5x MIC of IS 217 and 1x MIC of ciprofloxacin display similar killing activities, whereas IS 217 has showed good antimicrobial activity at the concentration of 1x MIC (0.39µg/ml) against *E. coli* and at 3h,5x MIC of IS 217 and 1x MIC of ciprofloxacin display similar killing activities. IS 217 has showed good antimicrobial activity at the concentration of 1x MIC (1.56 µg/ml) against *K.pneumoniae* and at 12 h,5x MIC of IS 217 and 1x MIC of ciprofloxacin display similar killing activities .Qualitative analysis of bacteria survival/time-killing assay, at different time intervals.

5.5.1.3. Effect of peptide IS 217 on 48 h old biofilms of *S.aureus:*

Since IS 217 presented a lower MIC and comparable to ciprofloxacin and in the present study, IS 217 was investigated whether the peptide IS 217 was able to inhibit the viability of *S. aureus* biofilms. A significant reduction in CFU/mL was observed after 48 h exposure of a biofilm to IS 217 (Figure 48).

Table -60: The MIC concentration of peptide IS 217 and the standard antibiotic against *S.aureus* are tabulated as follows.

Groups	**MIC Concentrations of IS 217 & Ciprofloxacin used against *S.aureus***
Vehicle Control	0
IS 217 -0.5 x MIC	0.78µg/ml
IS 217 -1 x MIC	1.56µg/ml
IS 217 -5 x MIC	7.8µg/ml
Ciprofloxacin -1 x MIC	1µg/ml

Biofilm formation is one of the major virulence factors of *S. aureus* [Mah, T. F.et al.,2001; Lowy FD 1998]. The polysaccharide matrix of biofilm shields microscopic organisms from host immune defenses and obstructs the capacity of anti-microbial to target profound situated microorganisms living inside the biofilm. Moreover, biofilms go about as an irresistible specialty with continued arrival of microbes inside the host, which prompts unending contamination, backslides, hazardous circulation system diseases, and treatment failure. Given the genuine difficulties related with staphylococcal biofilms and their job in advancing repeating diseases in the host, we next moved to survey whether our peptide is equipped for disturbing full-grown biofilms (formed after 48 hours) of *S. aureus* to compare it with that of conventional antibiotic "Ciprofloxacin" which is frequently used in clinical settings. MIC values for IS 217 and selected antibiotic (Ciprofloxacin) were determined. The results in Table 61 clearly indicate the enhanced anti-biofilm effect of IS 217 with 0.5 x MIC,1 x MIC and 5 x MIC concentrations recorded 65, 43.7 and 25.7% of biofilm formation. The result obtained with the test compound IS 217 and *S. aureus* was shown in figure 48. The adherent biofilm stained by crystal violet, then the dye was extracted with ethanol, measured at 595 nm absorbance,

and presented as percentage of biofilm reduction compared to untreated wells “control” and standard drug.

Although some conventional antibiotics might be capable of disrupting 24 hour-mature bacterial biofilms, most antibiotics are not effective against 48 hour-mature biofilms due to the dormant state of growth of the bacterial cells present within the mature-biofilms. To examine whether the potential therapeutic application of IS 217 is having the ability to disrupt 48 hours-mature staphylococcal biofilm was tested. As expected, and observed in figure 48, IS 217 (at $1 \times$ MIC) significantly disrupted the 48-hour mature biofilms of S. aureus, resulting in a 50% reduction in biofilm mass. Conversely, Ciprofloxacin at 1 X MIC was able to decrease only 40% of the biomass ($p < 0.05$).

Table 61: Effect of peptide IS 217 on 48 h old biofilms of *S.aureus.*

S.no	**Groups**	**MIC Concentration**	**Biofilm Mass percentage**
1	Vehicle Control	-	100
2	IS 217 -0.5 x MIC	0.78µg/ml	65.0
3	IS 217 -1 x MIC	1.56µg/ml	43.7
4	IS 217 -5 x MIC	7.8µg/ml	25.7
5	Ciprofloxacin -1 x MIC	1µg/ml	61.0

Results shown in the table are Mean values of percentage obtained from triplicate experiments.

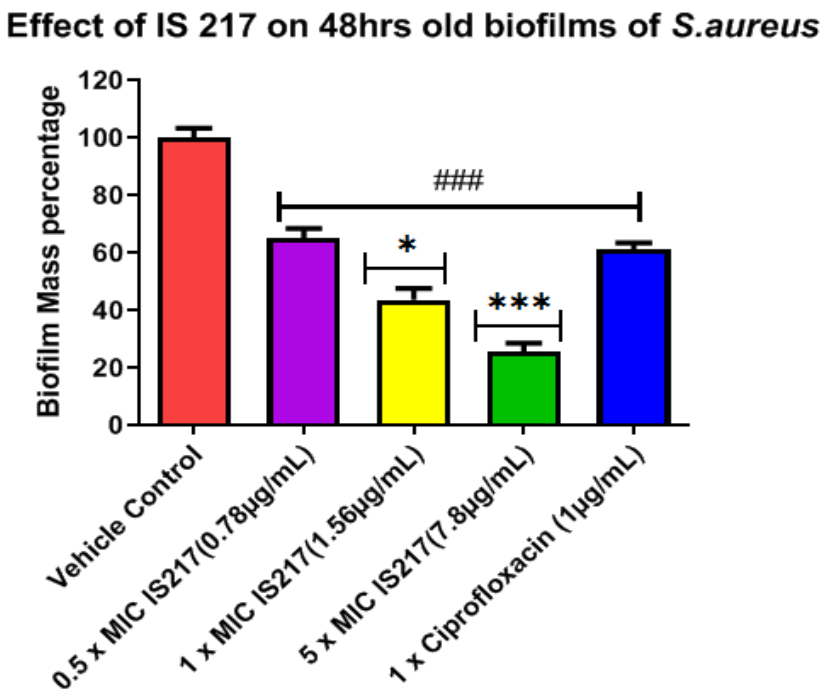


Figure 48: Effect of IS 217 on 48 h old biofilms of *S.aureus.*

The effect of peptide IS 217and antibiotic (Ciprofloxacin) on 48 h old biofilms of *S. aureus*. Results shown in the table are Mean ± SEM obtained from triplicate experiments where $P < 0.005$, $P < 0.05$ and denoted as *** &** and $^{###}$ & $^{##}$ when compared with standard drug and vehicle control groups respectively.

5.5.1.4. Discussion:

It is well known that the incidence of pathogen resistance to antibiotics is increasing day by day and is the most serious issue in today's world[Infectious Diseases Society of America, 2004; Peters NK et al. 2008].Bacterial pathogens (ethological agents of human illnesses) are a severe hazard to human health and the antibiotic abuse and misuse generate major environmental concerns, as well as the evolution and rapid spread of antibiotic resistance bacteria [Gums JG 2002 ; Jorgensen and Ferraro, 2009], resulting in an increasing number of deaths each year [Nathan and Cars, 2014].Most of the current antimicrobials have natural origin deriving from microbes, plants or animals [Bérdy, 2005] like Host defense peptides (HDPs) and/or antimicrobial peptides (AMPs) [Steinstraesser et al., 2009]. Antimicrobial peptides are classified based on their mode of action, which can include interfering with cell wall synthesis, protein, DNA, or RNA synthesis, as well as inhibiting various metabolic pathways

or the cell cycle [Hancock and Sahl, 2006; Hale and Hancock, 2007; Hilpert et al., 2010; Maróti and Kondorosi, 2014]. Due to some toxicity profile, the synthetic HDPs are the focused area of research to combat the resistance to the various pathogens.

In the present study, the IS 217, one of the synthetic peptides known as Host Defense Peptide (HDP/AMP) was investigated for its anti-bacterial profile by estimating MIC and time kill survival rates. Our study results showed that IS 217 was significant inhibitory properties against *S. aureus (*ATCC® 6538™) 1.56(µg/ml), *P. aeruginosa* (ATCC® 9027™)0.78(µg/ml), *E. coli (*ATCC® 8739™)0.39(µg/ml) and *K.pneumoniae*(ATCC® 700603™)1.56(µg/ml). Based on the MIC results, further studies are continued to investigate the time kill survival to determine how long all bacteria are necessary for peptide IS 217 to eliminate above mentioned pathogens. Unlike an MBC/MIC assay, this assay enables the measurement of the compound's rate of cidal action [Aiyegoro, Afolayan, & Okoh, 2009]. The curves were determined to assess the correlation between MIC and bactericidal activity of IS 217 at concentrations ranging from 0.5-fold MIC to 5-fold MIC [Mohamed F. et al. ,2016].

The compound was rapidly bactericidal at 1 × MIC for all four pathogens after 1 h incubation. Meanwhile *S. aureus* and *P. aeruginosa* were completely eliminate after 2h incubation, whereas *E.coli* after 3h incubation and *K.pneumoniae* after 4h incubation at concentration of 5 × MIC. The 5x MIC concentration of IS 217 are comparable with 1x MIC of ciprofloxacin and display similar killing activities. The results of the time-kill assay are presented in Figure 42-46 and the data showed that the response of the bacteria to the tested compound IS 217 varied among the strains, concentration, and time dependent. The differences in susceptibility may be due to the differences in cell wall composition and/or genetic content of their bacteria. The antibacterial activity is most likely due to the adsorption of compounds causing membrane disruption, subsequent leakage of cellular contents and cell death.

Based on literature study, Peptide IS 217 is designed antimicrobial host defense peptide (HDP). In general, the anti-microbial activity of AMP’s might be as follows, where the AMPs must interact with membranes as part of their direct antibacterial mechanism (or mechanisms) of action, leading to membrane perturbation, disruption of membrane-associated physiological events such as cell wall biosynthesis or cell division, and/or translocation across the membrane to interact with cytoplasmic targets and destroy the cell by changing membrane conductance and altering intracellular function and alterations in membrane structure results in the reorientation of peptide molecules in the membrane culminating in eventual pore formation and lysis of the target microbe. The concentrations of the

peptides also play an important role which promotes the cell lysis, and capability of channel formation [Jaynes, J. M. Drug News & Perspectives 3: 69 [1990]; and Reed, W. A. et al. Molecular Reproduction and Development 31: 106 1992]. Thus, at a certain concentration, these peptides stimulate or create channels that can be advantageous to the normal mammalian cell in a benign environment where it is not necessary to exclude toxic chemical compounds.

The test compound at a concentration equal to 5 x MIC was rapidly bactericidal, achieving complete elimination of both test bacterial strains within 3h. All the time-kill data obtained with the test compound IS 217 showed its antibacterial activity to be time – and concentration-dependent. In follow-up studies, the peptide IS 217 will be examined to see if the in vitro time-kill statistics are predictive of in vivo efficacy.

After confirming the excellent antimicrobial activity of IS 217 against *Staphylococcus aureus*, the efficacy of IS 217 on 48-hour-old *S. aureus* biofilms was assessed. Biofilms serve as an infectious niche, allowing bacteria to be released continuously inside the host, resulting in chronic infection, relapses, life-threatening bloodstream infections, and treatment failure. *S. aureus* is the key virulence factors in biofilm development. Additionally, considering the significant challenges posed by staphylococcal biofilms and their contribution to recurrent infections in hosts, the assessment shifted towards investigating the potential of our peptide, IS 217, in disrupting mature *S. aureus* biofilms formed after 48 hours. This evaluation aimed to compare IS 217's efficacy with that of the conventional antibiotic 'Ciprofloxacin,' commonly utilized in clinical settings [Archer, N. K. et al.,2011; Nickel, J.C. et al., 1994; Hojo, K. et al. 2009; Hojo, K. et al., 2009].

In the present study, anti-biofilm effect of IS 217 against *Staphylococcus aureus* has been studied adopting biofilm inhibition spectrophotometric assay. All the concentrations tested inhibited biofilm in a dose dependent manner and the result was shown in Figure 47. Although some standard antibiotics may be capable of disrupting 24 hour-mature bacterial biofilms, most antibiotics are ineffective against 48 hour-mature biofilms due to the dormant state of growth of the bacterial cells present within mature-biofilms. As appeared in Fig. 06, IS 217 (at 1 × MIC) significantly disrupted the 48 h matured biofilms of S. aureus, reducing biofilm mass by 50%. Ciprofloxacin at 1 X MIC were able to decrease only 40% of the biomass [iberio SA et al.,2011; Soerya Dewi Marliyana et al.,2017]. These findings suggest the potential utility of synthetic peptides, like IS 217, as effective antibacterial agents, particularly in combating biofilm-related infections. The characteristics demonstrated by IS 217 peptide holds promise for diverse clinical applications, including addressing chronic infections and dental biofilms. Notably,

the formation of biofilms is closely linked to the pathogenesis of caries, indicating a potential role for IS 217 in this context as well. However, further research is necessary to comprehensively understand the underlying mechanisms involved.

5.5.5. Summary:

IS 217 displayed notable antimicrobial activity comparable to the standard antibiotic Ciprofloxacin. Additionally, it exhibited a more substantial disruption of mature S. aureus biofilms at 48 hours compared to commonly used antibiotics. In summary, peptide IS 217 exhibited rapid bactericidal activity and proved effective against 48-hour-old biofilms of S. aureus. Considering these findings collectively, IS 217 holds promise as a backbone molecule for developing novel anti-infective therapies, potentially extending to applications as coatings for implants. Further investigations encompassing physicochemical studies, toxicity assessments, animal efficacy evaluations, and pharmacokinetic/pharmacodynamic studies are imperative to comprehensively unveil the therapeutic potential of this class of molecules.

Section 6.

Confirmatory studies for anti-inflammatory activity (In vitro & In vivo)

5.6.1. Results of Confirmatory studies for anti-inflammatory activity in BALB/c mice peritoneal macrophages:

Effect of peptide IS 217 on viability of BALB/c mice peritoneal macrophages:

The viability and cytotoxicity of peptide IS 217 on BALB/c mice peritoneal macrophages were evaluated using the MTT assay. Peritoneal macrophages were exposed to various doses of IS 217 (ranging from 1.565 to 100 g/mL), and cell viability was assessed 48 hours later. The results showed that IS 217 had no cytotoxic effects on mouse peritoneal macrophages at concentrations between 1.56 and 100 g/mL.Therefore, subsequent experiments were conducted using concentrations below 100 μg/mL.

5.6.1.1. Effect of peptide IS 217 on the levels of cytokines in LPS-stimulated BALB/c mice peritoneal macrophages: Immunomodulatory activity.

In order to examine the immunomodulatory activity of peptide IS 217, and its effects on the levels of cytokines in LPS-stimulated BALB/c mice peritoneal macrophages are evaluated. LPS-induced inflammatory response and macrophage activation were inhibited by peptide IS 217. Macrophage proliferation increases in response to LPS stimulation, leading to an excessive immune response. The cytotoxicity of IS 217 on peritoneal macrophages was assessed, and concentrations lower than 100 g/mL showed no significant cytotoxicity. Hence, peptide IS 217 concentrations below 100 μg/mL were used in subsequent experiments. To further confirm the anti-inflammatory effects of IS 217 on macrophages, the production of cytokines IL-6, IL-1, IL-12P70, and TNF-α levels are measured in mouse peritoneal macrophages. The results demonstrated inhibitory effects on the production of pro-inflammatory cytokines (as shown in Figure 49), while IL-10 levels were increased (as shown in Figure 50).

TNF-α and IL-6 are well-known pro-inflammatory mediators in inflammatory diseases. To investigate the regulation of TNF-α and IL-6 production by peptide IS 217, peritoneal macrophages were treated with different concentrations of IS 217 (3.12, 6.25, and 12.5 μg/mL) in the presence of LPS stimulation. Figure 49 shows that LPS significantly increased the levels of pro-inflammatory cytokines IL-6, IL-1β, IL-12P70, and TNF-α in peritoneal macrophages compared to the normal control group ($P < 0.001$). However, pre-treatment with IS 217 resulted in a dose-dependent reduction in LPS-induced production of IL-6, IL-1β, IL-12P70, and TNF-α levels. This confirms the anti-inflammatory effect of peptide IS 217 on the secretion of IL-10 levels (Figure 50) as well as the

pro-inflammatory cytokines IL-6, IL-1β, IL-12P70, and TNF-α in LPS-stimulated murine peritoneal macrophages.

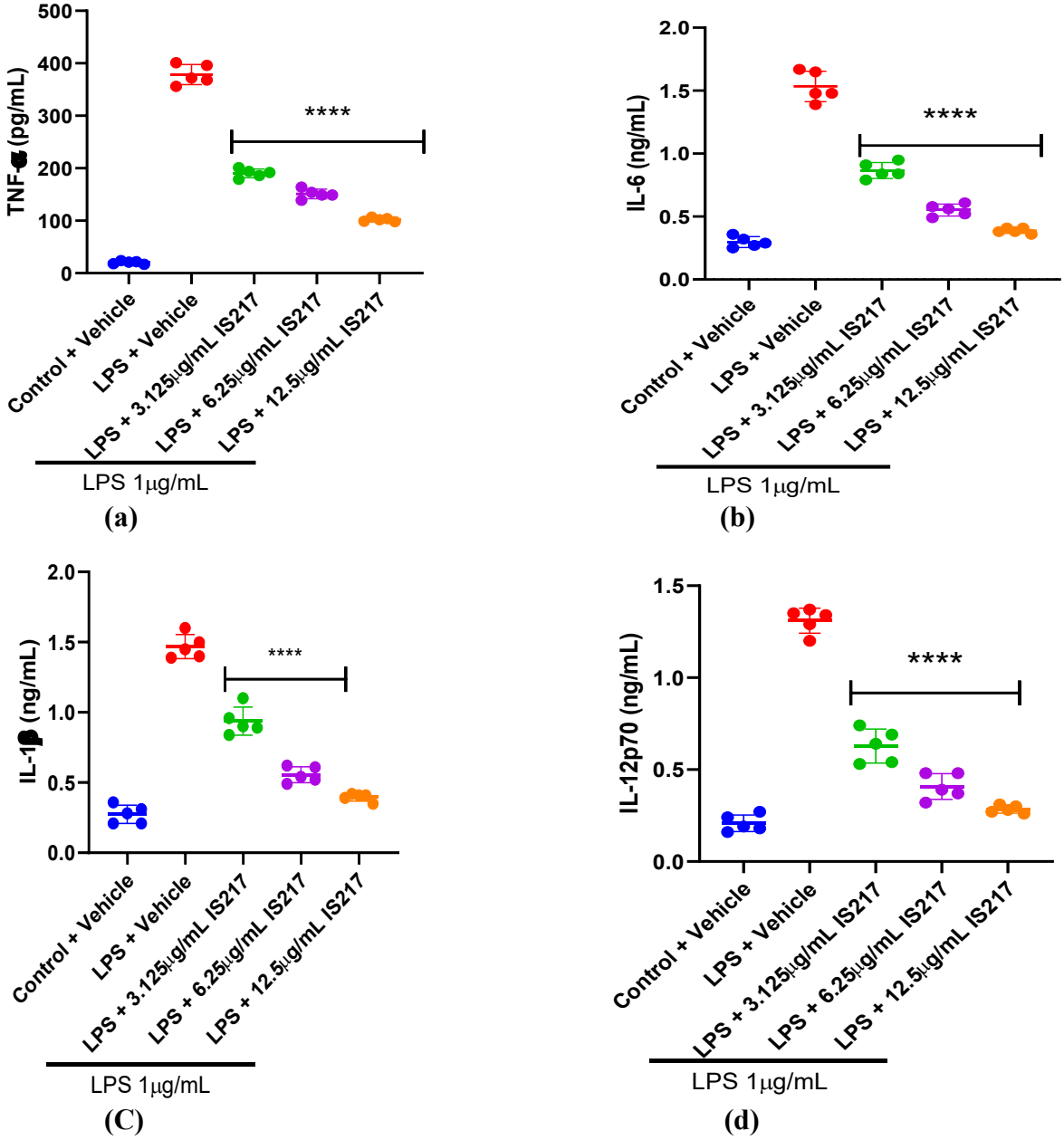


Figure 49: TNF-α (a), **IL-6** (b), **IL-1β** (c) **and IL-12p70** (d) **cytokines level detection in vitro BALB/c mice peritoneal macrophages**.

The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "*".

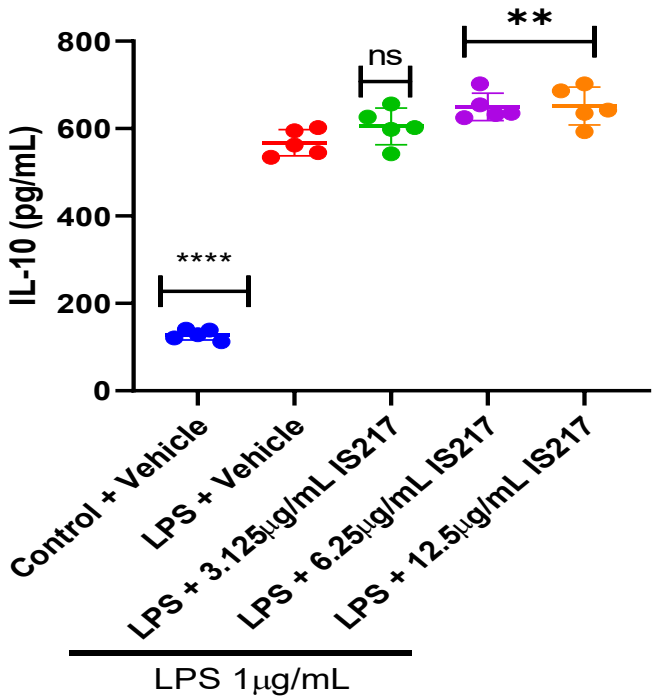


Figure 50: IL-10 cytokines level detection in vitro BALB/c mice peritoneal macrophages.

The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "*".

5.6.1.2. Effect of IS 217 on the levels of NO in LPS-stimulated peritoneal macrophages:

In murine macrophage RAW 264.7 cells, LPS stimulation alone has been demonstrated to induce iNOS transcription and its protein synthesis, with a corresponding increase in NO production [Xie *et al*., 1994; Henkel *et al*., 1993]. The Griess reaction, a spectrophotometric determination for nitrite, was carried out to quantify the nitrite levels in the conditioned medium of RAW 264.7 cells treated with LPS. Figure 51 shows the inhibitory activity by peptide IS 217 towards NO production by LPS-activated macrophages.

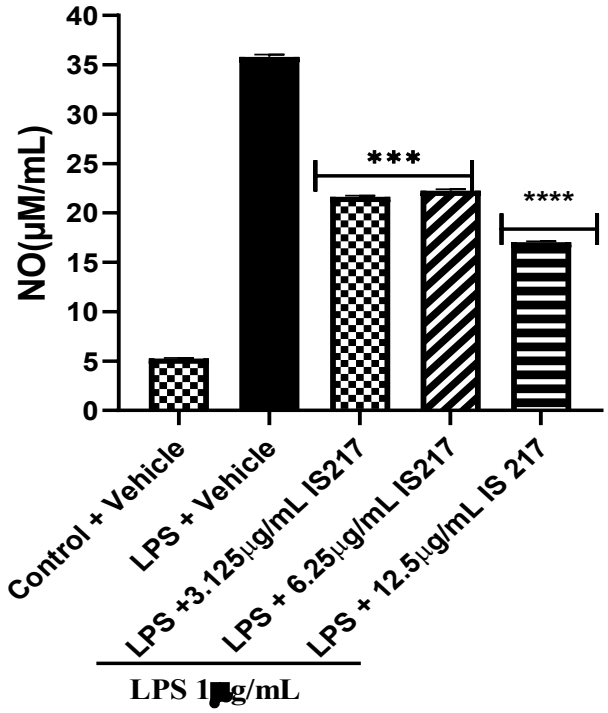


Figure 51: The production of NO level's in vitro BALB/c mice macrophages.

Effect of IS 217 on NO levels in LPS-stimulated RAW264.7 macrophages. The nitrite concentration was analysed using griess reagent method. The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "**".

5.6.1.3. Effect of peptide IS 217 on mRNA expressions of cytokines:

Next, the possible effects of IS 217 on the expression of proinflammatory cytokines in macrophages exposed to LPS are also evaluated. RT-qPCR analysis revealed that LPS alone significantly enhanced the mRNA expression of IL-12, IL-β, IL-6, and TNF-α (Figure 52) compared to the control groups, which were suppressed by pretreatment with IS 217 peptide in macrophages and IL-10 expression was depicted (Figure 53). Then, further confirmed that pretreatment with IS 217 peptide also inhibited LPS-induced production of TNF-α and IL-β protein in macrophages by ELISA (Figure 49). These results indicated that IS 217 peptide suppressed LPS-induced proinflammatory cytokine expression.

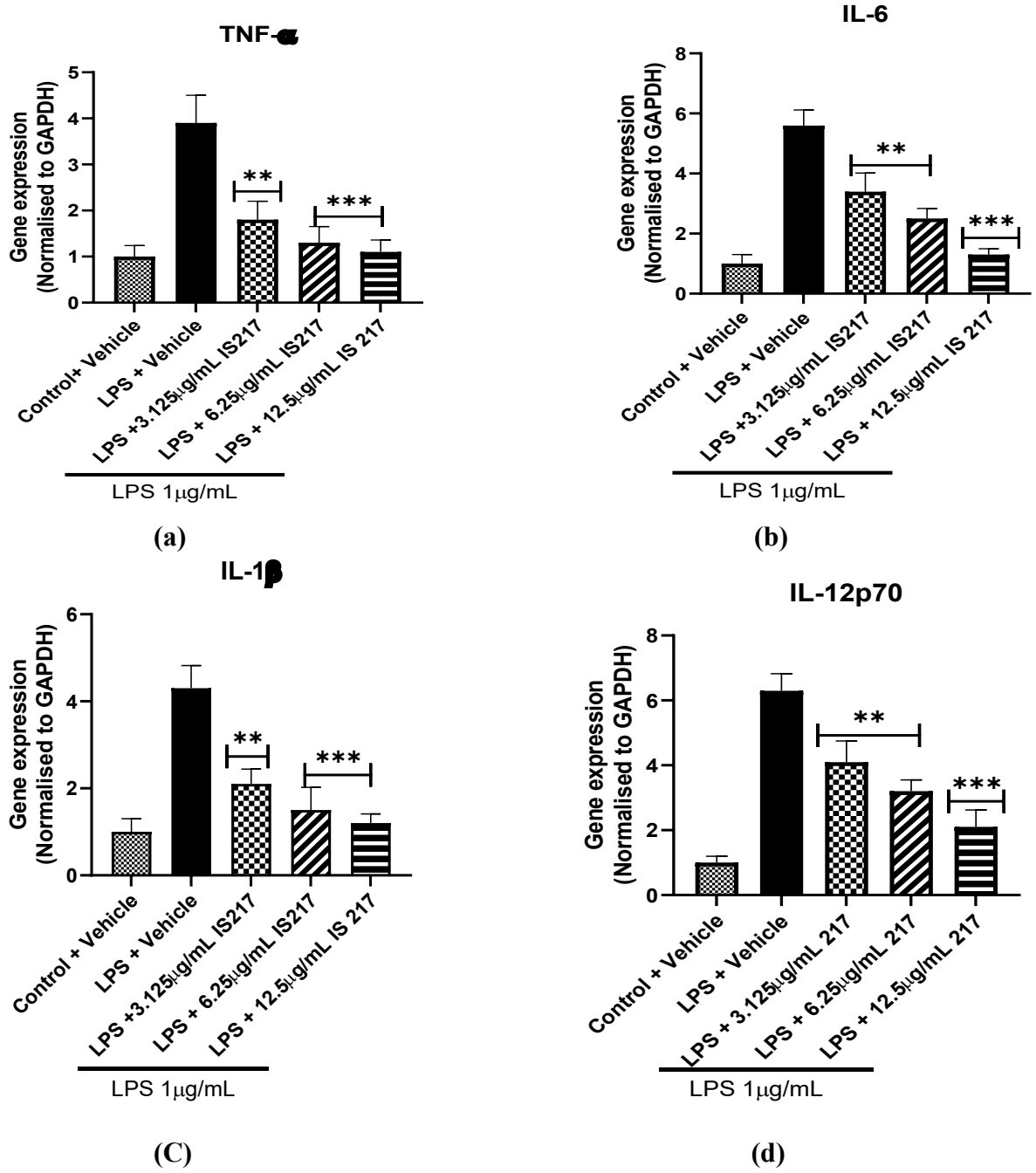


Figure 52: Effect of peptide IS 217 on TNF-α (a), IL-6 (b), IL-1β (c) and IL-12p70 (d) cytokines expression was detected by RT-qPCR. The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "**".

Since IS 217 peptide suppressed LPS-induced expression of representative proinflammatory cytokines, attempt was made whether IS 217 peptide had any effects on anti-inflammatory cytokine expression. First, examined IL-10 expression in LPS-induced macrophages pretreated with IS 217 peptide. The results showed that pretreatment with IS 217 peptide could enhance the LPS-induced mRNA expression (Figure 52) in peritoneal macrophages. Taken together, these results implied that IS 217 peptide was also involved in regulating the expression of anti-inflammatory cytokines.

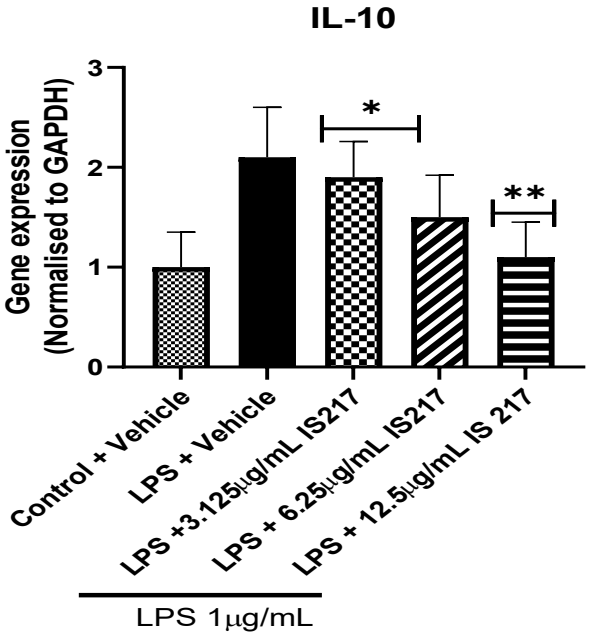


Figure 53: Effect of peptide IS 217 on IL-10 cytokine expression was detected by RT-qPCR.
The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "**".

5.6.1.4. Inhibitory effect of IS 217 peptide on iNOS protein and mRNA expression in LPS-stimulated peritoneal macrophages:

In this study, the investigation focused on the impact of IS 217 on inflammatory mediators, particularly NO (nitric oxide), which plays a significant role in inflammation. The expression levels of iNOS (inducible nitric oxide synthase), the enzyme responsible for NO production, were assessed.

The findings revealed that when macrophages were exposed to LPS alone, there was a significant increase in iNOS mRNA levels across three different macrophage sources (depicted in Figure 54 using RT-qPCR analysis). However, this enhancement was notably diminished when the macrophages were pretreated with IS 217. Concurrently, the levels of NO in the supernatant of peritoneal macrophages were elevated upon LPS induction, yet this increase was suppressed in the presence of IS 217 at a concentration of 12.5 µg/mL (as shown in Figure 54).

Thus, the study demonstrated that IS 217 exhibited a similar inhibitory effect on the production of the identified pro-inflammatory mediator in response to LPS-induced inflammation. RT-qPCR and Western blot analyses revealed that iNOS expression by IS 217 peptide was in parallel with comparable inhibition of NO production.

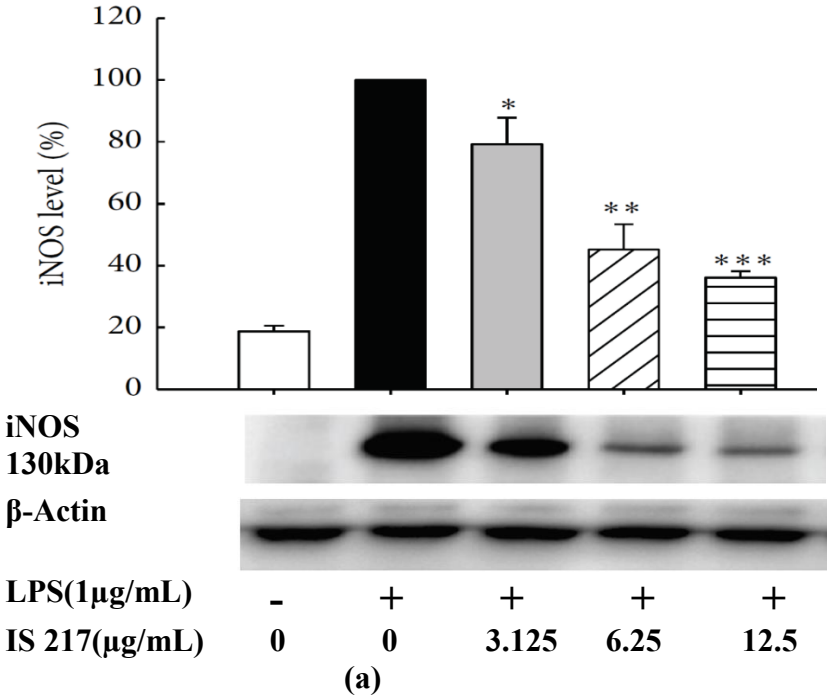


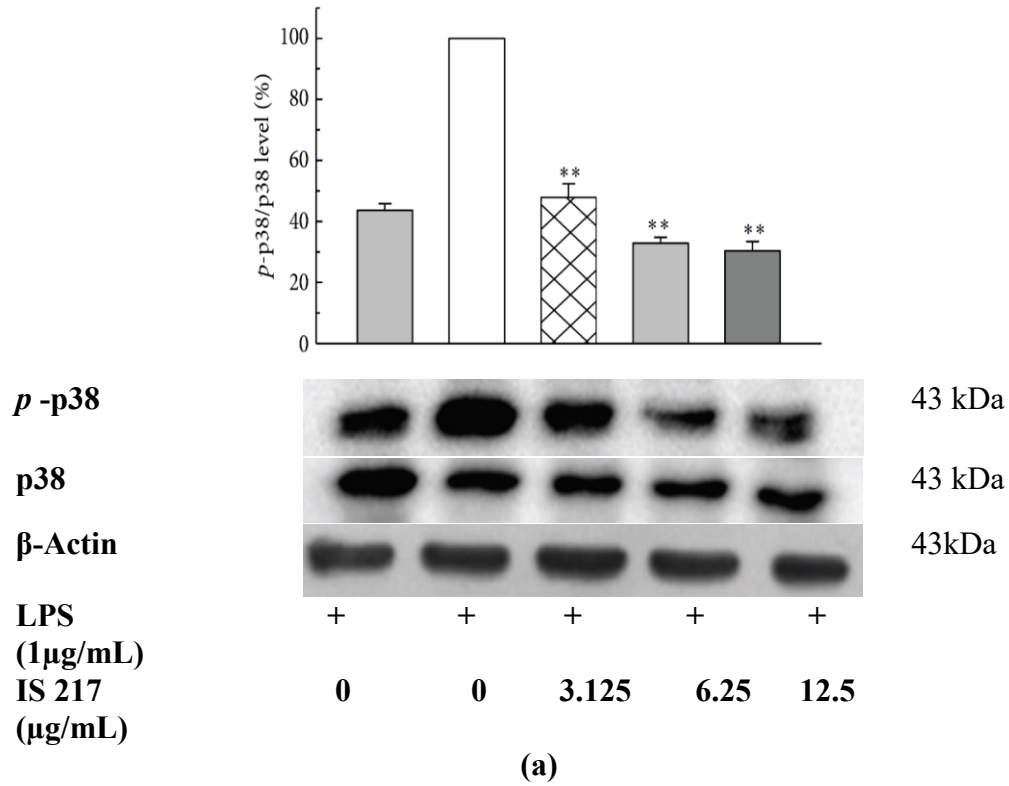
Figure 54: Effect of peptide IS 217 on iNOS expression, and the expression of the iNOS protein by western blotting analysis. The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "**".

5.6.1.5. Effect of IS 217 peptide on the phosphorylation of MAPKs in LPS-stimulated peritoneal macrophages:

The mitogen-activated protein (MAP) kinases are pivotal in regulating cell growth, differentiation, and responses to cytokines and stressors. Additionally, they are crucial for activating NF-kB, a key transcription factor involved in various cellular processes.

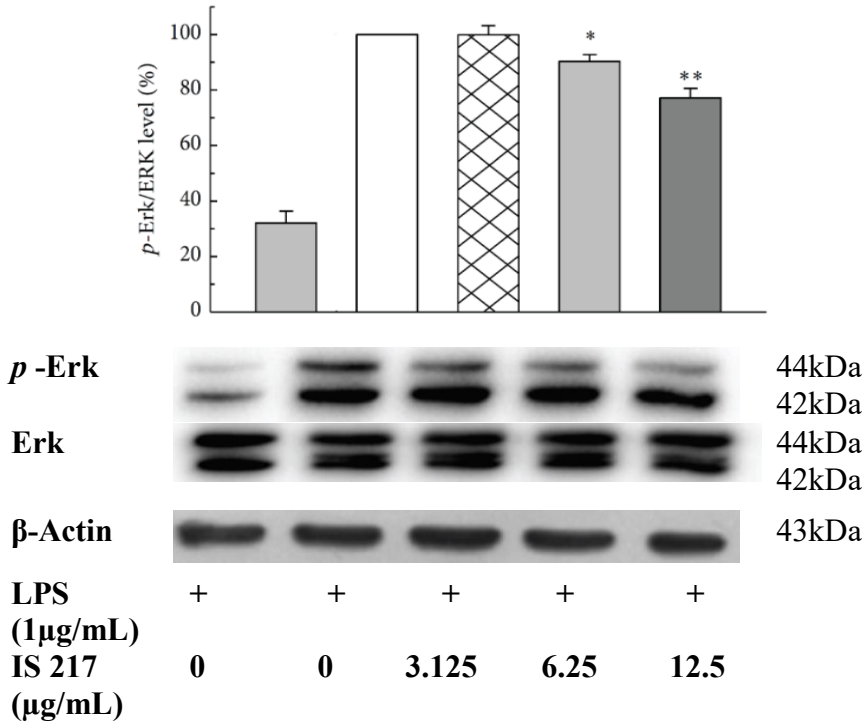
To explore whether IS 217's suppression of NF-kB activation is linked to the MAP kinase pathway, this study investigated IS 217's effect on the phosphorylation of ERK 1/2 and p38 MAP kinase induced by LPS in peritoneal macrophages. Western blot analyses, as depicted in Figure 55, revealed that IS 217 peptide inhibited the LPS-induced activation of p38 MAP kinase and ERK 1/2 in a dose-dependent manner.

These findings strongly suggest that the phosphorylation of p38 MAP kinase and ERK 1/2 plays a role in mediating the inhibitory impact of IS 217 peptide on the LPS-induced NF-kB binding in murine peritoneal macrophages.



(a)

Figure 55 (a): Effect of peptide IS 217 on LPS-induced phosphorylation of p38 MAPK.



(b)

Figure 55(b): Effects of peptide IS 217 on LPS-induced phosphorylation of *p*-Erk.

The reported values represent the means of at least three independent experiments conducted in triplicate, and the values are expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by comparing the results to the control group (LPS alone). A significant difference from the control at a p-value of less than 0.001 was denoted as "**", while a significant difference at a p-value of less than 0.01 was denoted as "**".

5.6.2. Effect of peptide IS 217 on paw volume and percentage inhibition in carrageenan-induced paw edema:

In this study, acute paw inflammation was induced in mice by sub-plantar injection of carrageenan, resulting in paw swelling due to edema. Figure 57b illustrates the changes in paw volume thickness in different groups of mice treated with varying doses of the peptide IS217 and dexamethasone (DEX). Following carrageenan injection, paw edema progressively increased and reached its peak after 3 hours (Figure 57a). The highest percentage reduction in paw edema, reported as 90.2%, was observed after 5 hours of treatment with IS 217 at a dose of 1.2 mg/kg body weight, as shown in Table 62. According to Table 62, both the standard (dexamethasone) and IS 217 exhibited similar anti-inflammatory effects at a dose of 1.2 mg/kg after 18 hours. The anti-inflammatory effects of dexamethasone (88.9%) and IS217 (88.9%) were observed 18 hours after carrageenan injection.

When compared to the control groups, pre-treatment with DEX (5 mg/kg) and IS217 (0.6 mg/kg and 1.2 mg/kg) significantly reduced edema after 3 hours of injection ($P<0.05$, $P<0.05$, $P<0.01$, respectively). Figure 60a & b demonstrate that after 18 hours, IS217 (1.2 mg/kg) reduced swelling by 88.9% ($P<0.01$), effectively resolving the edema. Photographs of the paws from different groups of mice, including the normal control and carrageenan-induced paw edema mice treated with placebo, IS217, or dexamethasone, are shown in Figure 55. These images clearly demonstrate a significant reduction in paw thickness in the IS217 and dexamethasone-treated groups compared to the control group (Figure 56).

Table 62: The Percentage Inhibition (%) of paw edema at mentioned time intervals.

Group	Carrageenan	Time interval (h) inhibition (%)				
		1	2	4	5	18
Disease Control (PBS)	+	0	0	0	0	0
IS 217-0.6 mg/kg	+	37.5	60.0	64.3	54.5	55.6
IS 217 –1.2mg/kg	+	50.0	60.0	80.0	90.9	88.9
Dexamethasone – 5 mg/kg	+	62.5	60.0	64.3	63.6	88.9

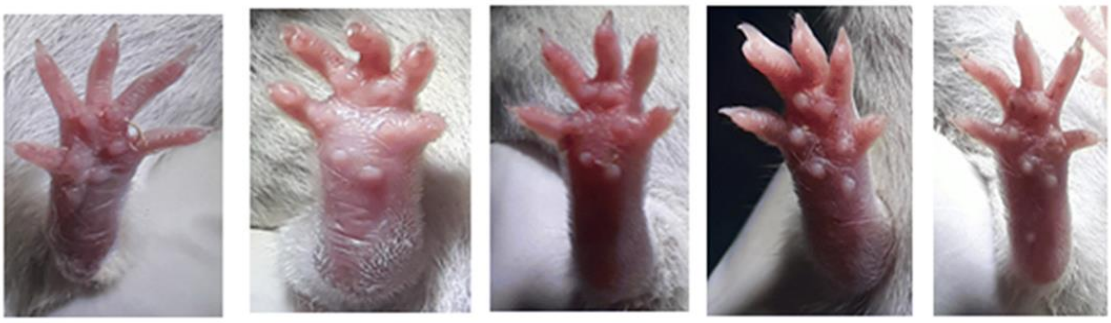


Figure 56: Representative photographs of carrageenan-induced inflamed mice paws treated with peptide IS 217 of two doses 0.6 &1.2 mg/kg and with dexamethasone (5 mg/kg body weight) at 18 h: (a) Control, (b) Disease control, (c) IS 217 -0.6mg/kg, (d) IS 217 -1.2 mg/kg, (e) Dex-5mg/kg.

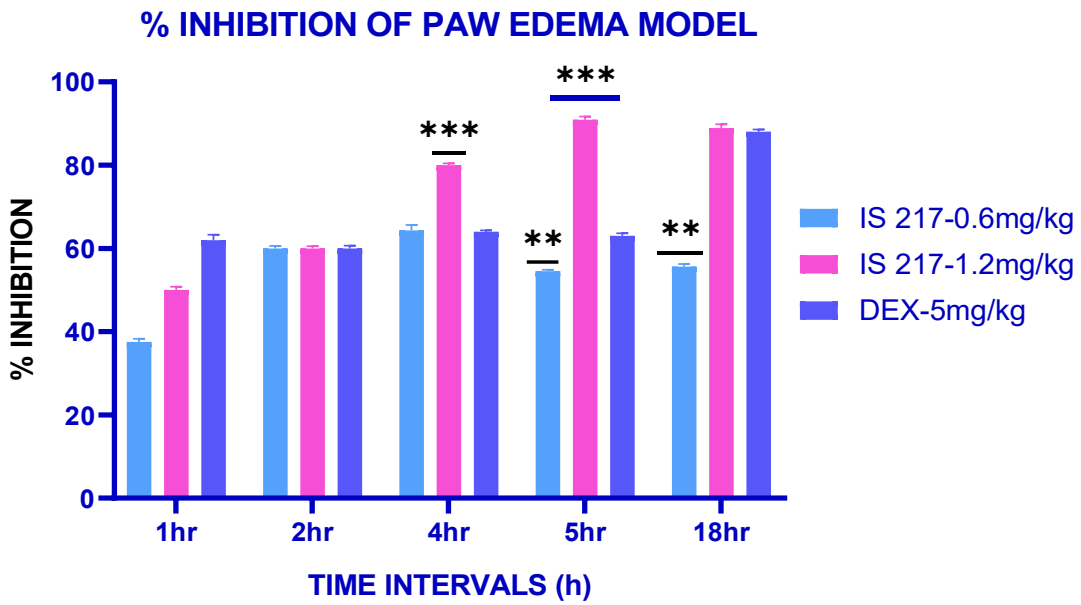


Figure 57(a): % inhibition of paw edema measured by the volume displacement in carrageenan-induced inflamed mice paws.

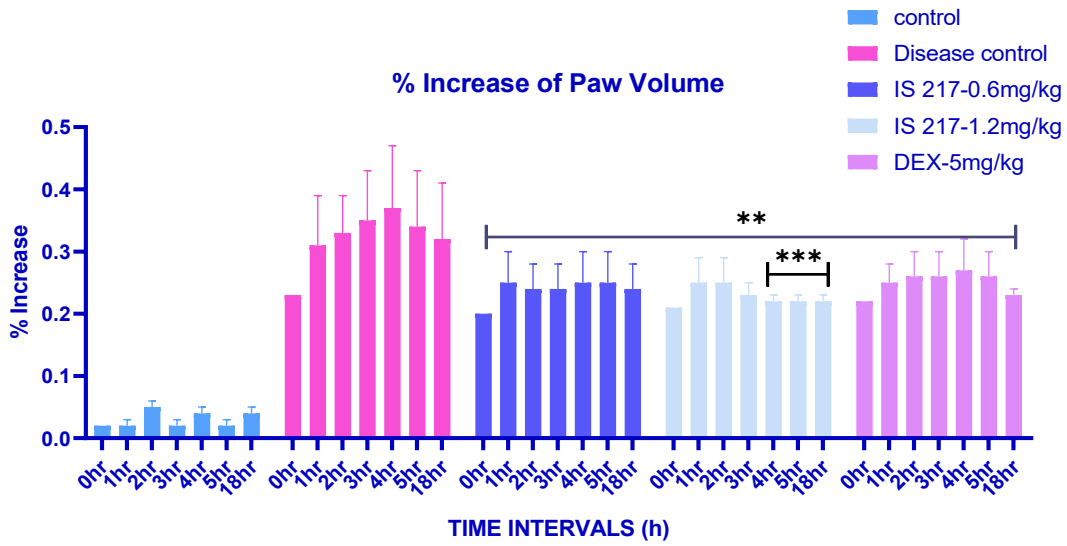


Figure 57(b): % increase in paw volume in carrageenan-induced inflamed mice paws treated with peptide IS 217 of two doses 0.6 &1.2 mg/kg and with dexamethasone (5 mg/kg body weight).

Data was expressed as mean ± SEM (n=6) in both the figures 57 a&b. A significant difference was indicated as $P < 0.01$, $P < 0.05$ and denoted as *** &** respectively, when compared with standard drug dexamethasone at different time intervals.

5.6.3. Discussion:

The primary objective of this study was to assess IS 217's impact on acute inflammation, both in in vivo and in vitro, while examining its potential mechanisms. The findings demonstrated that pretreatment with IS 217 significantly diminished the secretion of IL-12, IL-β, IL-6, and TNF-α levels in peritoneal macrophages stimulated by LPS. Moreover, RT-qPCR analysis indicated that IS 217 lowered the mRNA expression levels of IL-12, IL-β, IL-6, and TNF-α in LPS-exposed groups compared to control groups. Additionally, the study showed that pretreating mice with IS 217 effectively reduced paw edema induced by carrageenan, further supporting its anti-inflammatory properties both in vivo and in vitro.

Considering the detrimental effects of excessive nitric oxide (NO) production in inflammatory diseases [Min Jee Kim et al.,2014], the study investigated the role of IS 217 in inhibiting NO and pro-inflammatory cytokines (IL-12, IL-β, IL-6, and TNF-α). IS 217 notably suppressed the expression of iNOS and subsequent NO production in a dose-dependent manner, potentially by inhibiting iNOS mRNA transcription, as assessed by western bolt analyses and RT-qPCR.

Furthermore, the study highlighted IS 217's impact on MAP kinases, demonstrating its inhibition of p38 MAP kinase and ERK1/2 phosphorylation induced by LPS. Mammalian cells possess three distinct families of MAP kinases (ERK, JNK, and p38 MAPK) that play crucial roles in regulating cell growth and differentiation, especially in response to cytokines and stress [Johnson GL et al.,2002]. Various research studies have highlighted the involvement of MAPKs in LPS-triggered iNOS expression and the activation of NF-kB. When cells are stimulated, a signaling cascade initiates the activation of MAPKs through phosphorylation of tyrosine and threonine residues. This activation leads to a conformational change exposing the active site for substrate binding. Consequently, IS 217 demonstrated a dose-dependent inhibition of LPS-induced p38 MAP kinase activation. Moreover, IS 217 inhibited the phosphorylation of ERK1/2 and p38, both integral members of the MAPK pathway crucial for cellular processes, including inflammation [Xiaofeng Niu, et al., 2015]. In essence, this study showcases IS 217's ability to hinder LPS-induced production of IL-12, IL-β, IL-6, and TNF-α, as well as iNOS gene expression in macrophages. These effects are believed to be mediated through the inhibition of NF-kB activity and the phosphorylation of p38 & ERK1/2. This inhibition correlated with reduced production of IL-12, IL-β, IL-6, and TNF-α in macrophages, suggesting a potential regulation of NF-kB activity by IS 217.

The carrageenan-induced paw edema model is a well-established method for assessing anti-inflammatory drugs and is characterized by edema formation [Xiaofeng Niu et al., 2012]. In this study, mice injected with carrageenan exhibited increased paw volume, which peaked at 3 hours. However, pretreatment with IS 217 effectively prevented paw edema at all time points, indicating its anti-inflammatory effects on carrageenan-induced inflammation. The acute inflammatory response induced by carrageenan involves the release of different mediators in two distinct phases edema [Inmaculada Posadas et al.,2004]. The early phase, occurring within the first hour, is associated with the release of histamine, serotonin, bradykinin, and prostaglandins. The delayed phase, starting after 1 hour, involves the infiltration of polymorphonuclear (PMN) leukocytes and continued prostaglandin production, along with the involvement of pro-inflammatory cytokines like TNF-α and IL-1β. LPS, a component of Gram-negative bacteria, induces the release of various inflammatory cytokines, including TNF-α and IL-6[Xiaofeng Niu et al., 2012]. Macrophages play a crucial role in the immune response and can produce TNF-α and IL-6 in response to LPS. Overproduction of these cytokines by macrophages is associated with chronic inflammatory conditions. The present study assessed the impact of IS 217 on TNF-α and IL-6 production in LPS-stimulated peritoneal macrophages using ELISA and western blotting [Stephen B. et al., 2010]. The results demonstrated that IS 217 significantly reduced the levels of TNF-α and IL-6, indicating its potential as an anti-inflammatory agent. In the carrageenan-induced paw edema model, IS 217 effectively mitigated inflammation at various time points. The peptide displayed potential in reducing inflammatory mediators associated with both early and delayed phases of inflammation, supporting its anti-inflammatory properties.

Overall, the study showcased IS 217's anti-inflammatory effects through various pathways, including the suppression of pro-inflammatory mediators and modulation of MAP kinase signaling. Despite promising findings, further investigations are required to fully comprehend the mechanisms behind IS 217's anti-inflammatory actions.

The study emphasizes the reliability of using the LPS-activated macrophage cell model and the carrageenan-induced paw edema model for cost-effective screening of anti-inflammatory agents. The results suggest that IS 217 holds therapeutic promise in managing immunopathological conditions characterized by heightened macrophage activity, positioning it as a potential candidate for anti-inflammatory therapy.

5.6.4. Summary:

This study provides a concise overview of two cost-effective models for screening anti-inflammatory activity: the LPS-activated mouse macrophage cell model and the carrageenan-induced mice paw edema model. **It demonstrated that IS 217, a peptide, effectively inhibited IL-12, IL-β, IL-6, and TNF-α production and suppressed the MAPK pathway activation in peritoneal macrophages exposed to LPS, reaffirming previous in vitro findings using RAW 246.7 cells**.

Moreover, **IS 217 showcased a protective effect against carrageenan-induced paw edema in mice, a crucial test for evaluating agents targeting acute inflammation mediators**. The study's outcomes suggest that IS 217 possesses anti-inflammatory and immunomodulatory properties, positioning it as a potential treatment for conditions marked by excessive macrophage activation. These promising results indicate IS 217's potential as a novel therapeutic agent for preventing inflammation.

Section 7.

In vivo Proof of Concept (PoC) Efficacy studies by using Cecal Ligated and Puncture (CLP) model and *E. coli* induced peritonitis model in mice.

5.7.1. Results of In vivo Proof of Concept (PoC) efficacy study by using Cecal Ligated and Puncture (CLP) model.

5.7.1.1. Pilot study of CLP:

After 18h of observation, the surviving animals are euthanized in a humane manner and collected blood for cytokines estimation. The survival rate of disease control is > 50% when compared to treated groups and sham control, whereas the treated groups with IS 217 -0.6 mg/kg & 1.2 mg/kg survival rate was ≥ 60 & 80% respectively (table 63).

Table 63: The Percent survival rate of all the groups of post -CLP after 5 days.

Days (%) death	Groups					
	Sham + Vehicle	Sham + IS 217-0.6mg/Kg	Sham +IS 217-1.2mg/Kg	CLP + Vehicle	CLP + IS 217-0.6mg/Kg	CLP + IS 217-1.2mg/Kg
0	100	100	100	100	100	100
1(18h)	100	100	100	66.66	80	80
5	100	100	100	33.33	60	80
No.of deaths	0	0	0	3	2	1

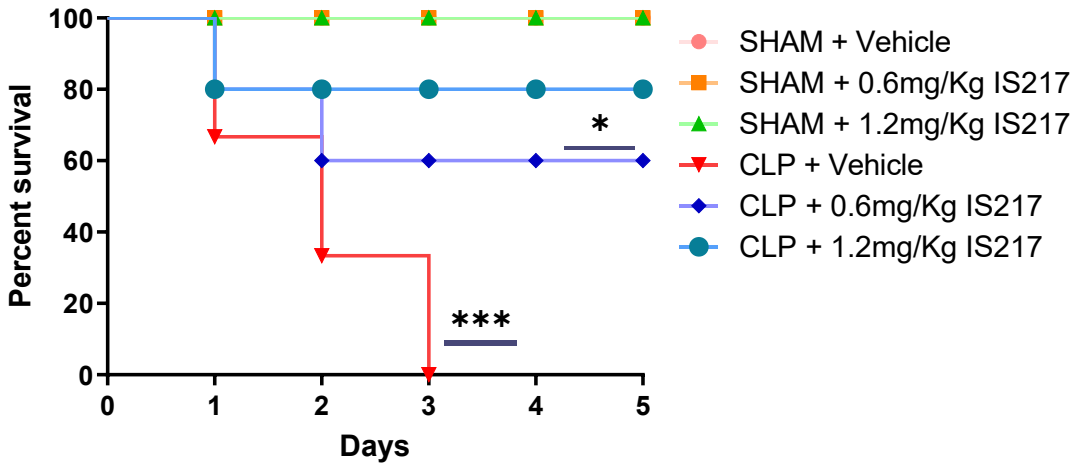


Figure 58: The Percent survival rate of all the groups of post -CLP after 5 days.IS 217 administrations protect against established CLP sepsis. BALB/c mice were subjected to sepsis (CLP) and 2 h later treated with IS 217 0.6 mg/kg and 1.2 mg/kg respectively. Survival was monitored for up to 5 days. The results are the Mean ± SEM of (n=5) animals *P < 0.02 and *** P < 0.05 compared to sham control group.

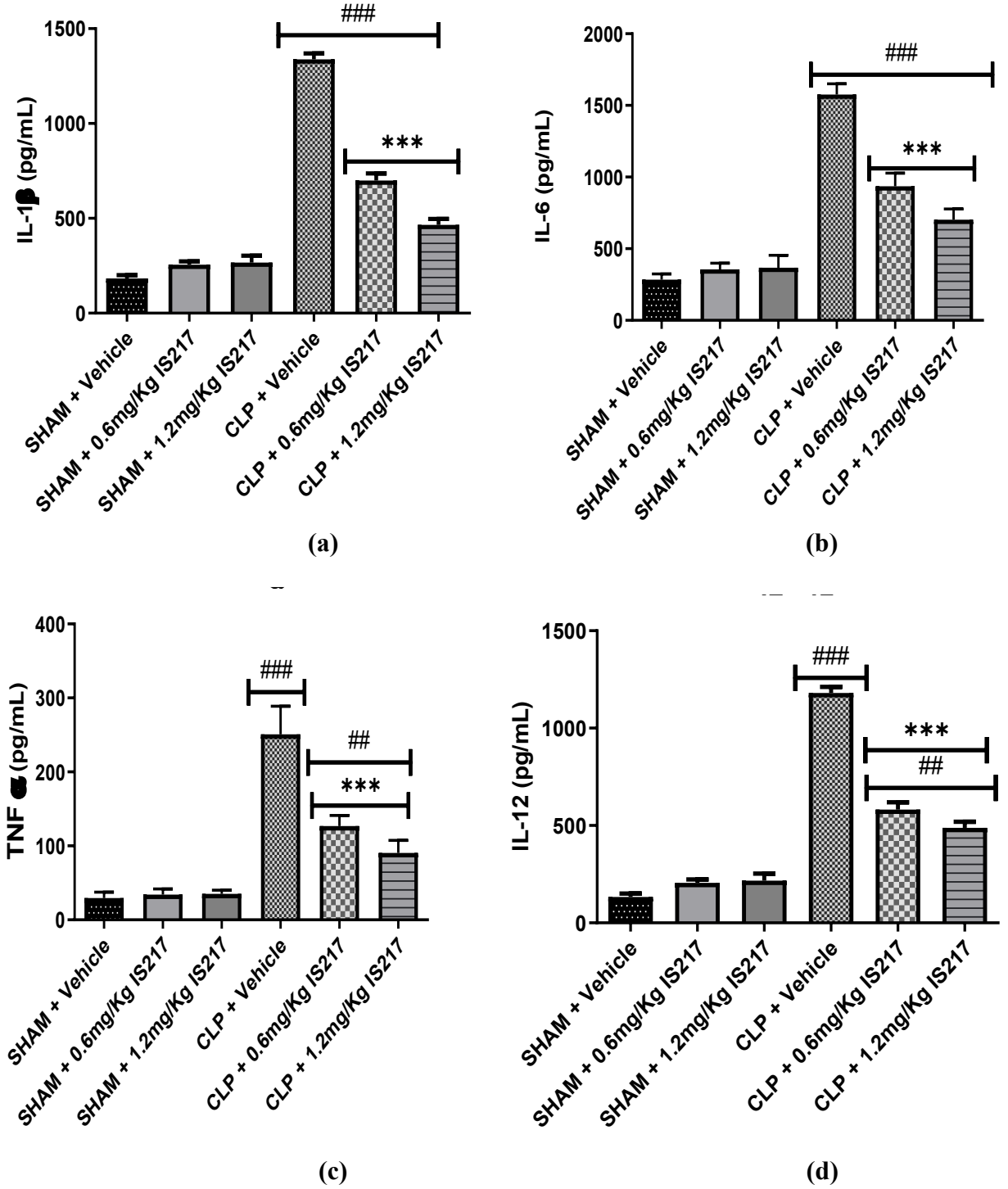


Figure 59(a,b,c&d): Quantification of IL-1β (a) , IL-6 (b) ,TNF-α (c) & IL-12 (d) in serum was performed 24 h after the CLP.

The results were expressed as the mean ±SEM (n=4-5 animals/group). $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* and $^{###}$,$^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

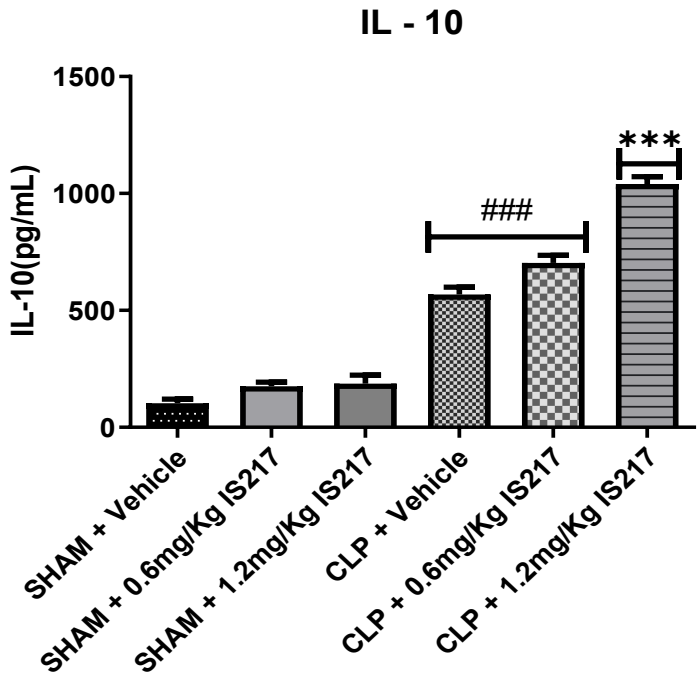


Figure 60: Quantification of IL-10 in serum was performed 24 h after the CLP.

The results were expressed as the mean ±SEM (n=4-5 animals/group). $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* and $^{###}$, $^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

Discussion:

After the observation that treatment with peptide IS 217 activates phagocytes at the site of infection and considering that the evolution of sepsis occurs in parallel to systemic inflammation, a pilot study investigated key cytokines associated with sepsis progression. This included pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) and the anti-inflammatory cytokine IL-10. The observations revealed that IS 217 peptide treatment inhibited the production of IL-6, IL-1β, and TNF-α (depicted in figures 59). Conversely, IS 217 peptide treatment increased IL-10 levels (shown in figure 60). Additionally, IL-12, known for initiating IFN-gamma production and contributing to phagocytic cell activation and inflammation, was reduced by IS 217 peptide treatment, aligning with the inhibition of other pro-inflammatory cytokines (figure 59). These observations indicated that IS 217 negatively regulates inflammatory cytokines and enhances survival during sepsis. However, it remains unclear whether improved survival is solely due to the inhibition of TNF-α, IL-1β, and IL-6 together.

Considering the rapid induction of TNF-α and IL-1β within hours [Arash Karimi et al., 2019; Cai J, Cui X et al., 2021], treatments with IS 217 were administered within 2 hours after CLP surgery. Notably, in the sham control groups treated with IS 217, no effect on the pro-inflammatory cytokines was observed. This demonstrated that the IS 217 peptide was non-immunogenic, aligning with previously reported immunogenicity results (figure 43). Consequently, for the main experiment, sham control groups were excluded due to the lack of IS 217 peptide effect on inflammatory cytokines.

In the initial in vivo pilot study, doses ranging from 0.15 to 1.2 mg/kg (0.15, 0.3, 0.6, 0.9, and 1.2 mg/kg) were administered. However, due to high mortality rates at 0.15 and 0.3 mg/kg and disturbances at 0.9 mg/kg (data not shown), these observations were considered. Considering acute toxicity data and doses used for other disease targets involving the same molecule, the main efficacy study proceeded with doses of 0.6 and 1.2 mg/kg.

Administration of peptide IS 217 protected animals from death due to CLP-induced sepsis and effectively inhibited systemic inflammatory cytokines.

5.7.1.2. Main CLP study:

The primary objective of this study was to explore the immunomodulatory properties of peptide IS 217 at two different doses (0.6 and 1.2 mg/kg) in managing the systemic inflammatory response, phagocyte activation, and bacterial growth control within a sepsis experimental model. Male C57BL/6 and BALB/c mice, aged 10-12 weeks, were subjected to the sepsis model through cecal ligation and perforation (CLP group) or laparotomy alone (sham group). Two hours post-CLP surgery, the mice received IS 217 (0.6 and 1.2 mg/kg) intravenously (IV) in BALB/c mice and subcutaneously (SC) in C57BL/6 mice. The sham control group received saline. Blood samples were collected at 4 hours, 18 hours, and 10 days after CLP for cytokine analysis and other biochemical marker assessments. A subset of animals was monitored for survival over 10 days, and behavioral test was conducted on the surviving animals on the 10th day. The administration of peptide IS 217 effectively counteracted the increase in IL-1β, TNF-α, IL-6, and IL-10 cytokine levels in the bloodstream even after 10 days following CLP.

At 4 hours and 18 hours post-cecal ligation and puncture (CLP), the lungs, spleen, and blood were collected to measure serum cytokine levels, and colony-forming unit (CFU) assessment and the animals were euthanized for histopathological evaluation after 18 h of CLP and treatment with peptide. The findings revealed that treatment with peptide IS 217 significantly inhibited bacterial growth in the peritoneum and reduced the influx of inflammatory cells, particularly macrophages, and neutrophils. Moreover, the administration of peptide IS 217 led to a decrease in pro-inflammatory cytokines present in the bloodstream and organs (lungs & spleen), indicating a systemic anti-inflammatory effect.

In this study, our objectives were twofold: (1) to investigate the direct impact of proinflammatory cytokines (IL-1β, IL-6, IL-12and TNF-α) in abdominal lavage fluid, lungs, spleen, and serum, and (2) to measure bacterial growth in the abdominal lavage fluid of mice.

1. Activity and vital parameters:

The activity of mice in the sham, peptide IS 217 treated groups was significantly higher (each $P < 0.05$ and demoted as ***, Table 64) compared to the sepsis-control group 18 hours after CLP. The general activity and body temperature results at 18 h after CLP surgery are shown in Table 64. Both the CLP animals exhibited tachypnea and hypothermia. The peptide IS 217 treated animals body temperatures are in range of normal values. [Asma Ahmed, et al., 2018]

Table 64: Activity Index and body temperature after 18 h of sepsis.

Group	Activity index (Grade points)	Body temperature
Control	1.1 ± 0.3	37.1 ± 0.05
Sham control	1.8 ± 0.3	37.5± 0.76
Disease control (CLP)	4.4 ± 0.8	34.5±1.09
CLP + IS 217-0.6mg/Kg	2.8 ± 0.5***	35.8±0.43
CLP + IS 217-1.2mg/Kg	2.2 ± 0.7***	36.7±0.67

2. Results of Behavioral test:

2.1. Results of Novel object recognition test (NORT):

In NORT, naive mice displayed a clear propensity for exploring the novel object in comparison to the familiar object, as these mice spent 80% of their object exploration time in the test phase with the novel object **(**table 65). On the other hand, diseased control showed no preference for the novel object, as their total object exploration time was more or less proportionately split between the novel object (57%) and the familiar object (Table 65). indicating the effect on memory impairment due to CLP surgery and infection. The mice treated with peptide IS 217 at 0.6& 1.2 mg/kg, showed normal and increased tendency to recognize the new object (69.61 & 75.97 % respectively) and which is evident by increase in tendency to recognize the new object in a dose dependent manner in comparison to control group. These observations imply that any underlying depression likely did not interfere with the novel object recognition memory testing in IS 217 treated groups. Thus, exposure to peptide IS 217 treated groups has the normal tendency of novel object recognition memory function.

Table 65: Effect of peptide IS 217 (0.6 &1.2 mg/kg) injected IV, on percentage preference for new object recognition at 10th day.

Group	**% Preference for New object recognition**
Sham control	80.00
Disease control (CLP)	58.90
CLP + IS 217-0.6mg/Kg	69.61***
CLP + IS 217-1.2mg/Kg	75.97***

Data are expressed as means ±SEM. $p<0.05$ was considered as significant and denoted as *** in comparison with disease control group.

Discussion:

The sepsis survivors present acute and long-term cognitive impairment and the pathophysiology of neurological dysfunction in sepsis involves microglial activation leading to memory impairment in

sepsis-surviving rats and performed two behavioral tests performed at 10 days after sepsis [Lucineia Gainski Det.al.,2020]. Wu and colleagues revealed a reduction for long-term memory in the freezing time of animals submitted to the fear conditioning test at 7 days after sepsis and treatment with peptide SS-31, and the same pattern was observed in another study that tested animals at 2 weeks after inducing sepsis and treating with MCC950 or a caspase-1 inhibitor. In 2018, Zarbato and colleagues exhibited the protective effect of dimethyl fumarate on the short-term object recognition memory of rats submitted to the CLP model [Wu J, 2014]. In contrast, Della Giustina and colleagues demonstrated that fish oil–treated rats had positive effects only on long-term memory [Danielski LG, Della Giustina A et al., 2017].

As previously documented in existing literature, the current study aimed to assess memory impairment and activity levels in mice that underwent CLP. A behavioral test, namely the Novel Object Recognition test, was conducted. The outcomes from this test suggest the need for expanding this study by incorporating a series of assessments involving various stress-inducing stimuli, such as novelty and openness. Increasing the number and range of stress-related tests and behavioral tasks could significantly contribute to comprehensively understanding the underlying mechanisms of rodents' emotional behavior [Lucineia Gainski Danielski et al., 2020].

To assess memory impairment in IS 217 treated groups post CLP surgery, mice from all groups were examined after a 10-day post-surgery period using a stress-free test known as the Novel Object Recognition Test (NORT). This test involved the exploration of two identical objects during the acquisition phase and later, an assessment of the exploration duration between a familiar and a novel object in the test phase [Bussey et al., 2000; Langston et al., 2010].

Naive mice typically spent more time exploring the novel object than the familiar one, indicating intact object recognition memory. Conversely, disease control animals, which underwent CLP surgery, showed no preference between the novel and familiar objects, suggesting impaired object recognition memory. However, the IS 217 treated groups, particularly those administered with IS 217 at a dosage of 1.2 mg/kg, demonstrated restored object recognition memory function when maintaining a minimal inter-trial interval (5 minutes) between the acquisition and test phases. This indicates that mice treated with IS 217 at doses of 0.6 & 1.2 mg/kg displayed deficits in long-term object recognition memory function.

In summary, this test revealed that **IS 217 treated groups exhibited deficits in long-term object recognition memory function following post CLP surgery, while maintaining normal activity levels comparable to the control groups**.

3. Experiment 1: Survival study:

Peptide IS 217 treatment enhanced survival of mice after induction of sepsis.

The Kaplan–Meier curve for survival analysis of mice subjected to polymicrobial sepsis and treated with peptide IS 217 (0.6 &1.2mg/kg) showed lower mortality after sepsis when compared with non-treated septic mice (figure 61 & 62). Peritonitis induced by CLP with a 21-gauge needle results in 100% lethality at day 8(figure 62) [Yona Kalechman, Uzi Gafter, et al., 2002 & Konstantin Tsoyi, et al., 2009; Kim, Y. K., 2017] CLP was performed as described in the methods section and animals were Intravenously (IV) &subcutaneously (SC) injected with various doses of IS 217 or vehicle at 2h after CLP procedure and then mice were observed for 18 h (figure 61a & 62 a) and subset of animals are continued for observation for 10 days (figure 61b, 62 b). No significant difference was observed when peptide IS 217 was injected at the time of injury (time zero). Notably, peptide IS 217 treatments at 18 h shown decreased survival, compared with disease control group mice.

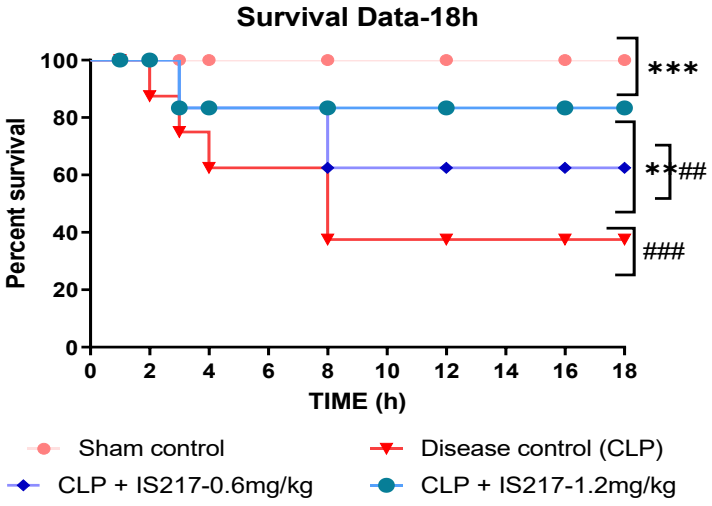
Table 66: The Percent survival rate of all the groups of post CLP surgery for 18 h & 10 days with IV administration of IS 217.

Time/days	**No. of deaths in groups**			
	Sham control	**Disease control (CLP)**	**CLP + IS 217-0.6mg/Kg**	**CLP + IS 217-1.2mg/Kg**
Till 18h	0	5	2	1
From 24 h to 10 days	0	4	2	0

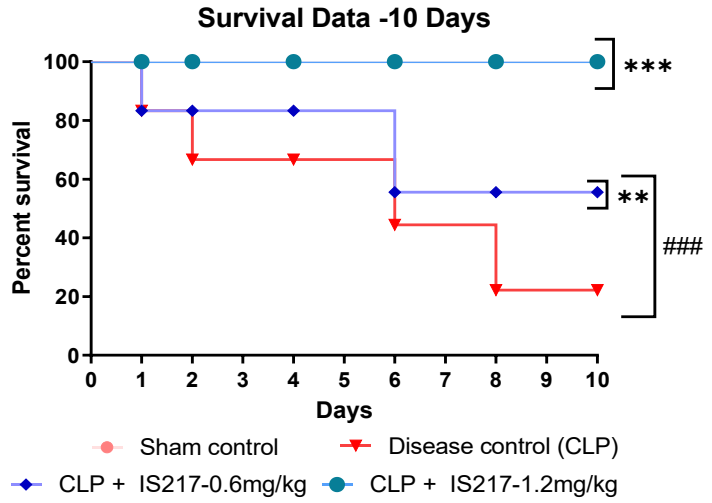
Table 67: The Percent survival rate of all the groups of post CLP surgery for 18 h & 10 days with SC administration of IS 217.

Time/days	No. of deaths in groups			
	Sham control	Disease control (CLP)	CLP + IS 217-0.6mg/Kg	CLP + IS 217-1.2mg/Kg
Till 18h	0	5	4	3
From 24 h to 10 days	0	4	2	1

Mice treated Intravenously with IS 217 after 2 h of CLP showed improved survival rates (62.5%) in 0.6 mg/kg and (83.3%) in 1.2 mg/kg, compared with CLP mice (28.5%; P,0.001) or sham control (0%; P,0.001) (figure 61a &62 a) at18h. whereas the 18h survival rates of mice subcutaneously treated with IS 217 after 2 h of CLP showed were 30% lower compared with that of mice treated intravenously, showed survival rates (33.3%) in 0.6 mg/kg and (50%) in 1.2 mg/kg, after 18h compared with CLP mice (37.5%) or sham control (0%; (figure 61a). Simultaneously, Mice treated Intravenously with IS 217, after 2 h of CLP showed improved 10-day survival rates (55.5%) in 0.6 mg/kg and (100%) in 1.2 mg/kg, (figure 61b) compared with CLP mice (22.3%;) or sham control (0%;) (figure 61a). whereas, the 10-day survival rates of mice subcutaneously treated with IS 217 after 2 h of showed survival rates (55.5%) in 0.6 mg/kg and (83.3%) in 1.2 mg/kg, (figure 62b) compared with CLP mice (22.2%) or sham control (0%). In intravenous administration, the first sign of the death started from 2h and continued for 8 days in disease control and whereas in treatment groups it was observed first 4hrs and last death observed at 3rd day. Analysis of the survival rates showed that peptide IS 217 at 1.2 mg/kg, (IV& SC) protected mice from death caused by CLP-induced sepsis. Compared with the CLP control group, the IS 217 (0.6 &1.2mg/kg) treated group showed a better disease score. Thus, it appears that IS 217 could protect mice from sepsis-induced death only if injected after induction of sepsis.

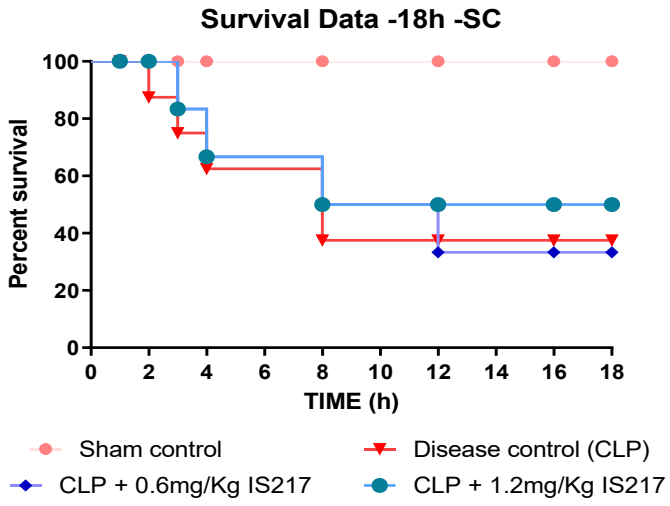


(a)

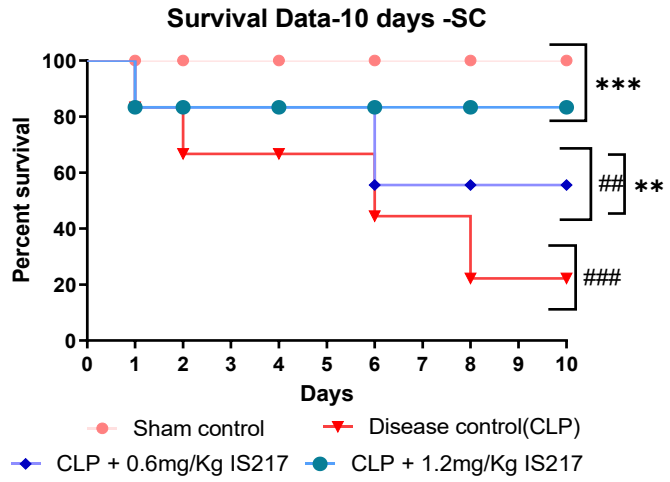


(b)

Figure 61(a,b): The therapeutic administration of peptide IS 217, Intravenously (IV), improves survival in septic mice. (a) Survival was monitored at regular intervals until 18 h, and (b) survival curves were plotted at 10 d, which was a representative graph of the remaining animals from 24 h up to 10 d. Data are shown as mean ± SEM of 5-6 mice in each group. Significance in differences of survival percentages were assessed by a log rank test for trend. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* and $^{###}$,$^{##}$&$^{#}$ compared to disease control and sham control groups respectively.



(a)



(b)

Figure 62(a,b): The therapeutic administration of peptide IS 217, subcutaneously (SC), improves survival in septic mice.

Survival was monitored at regular intervals until 18 h (a), and survival curves were plotted at 10 d (b), which was a representative graph of the remaining animals from 24 h up to 10 d. Data are shown as mean ± SEM of 5-6 mice in each group. Significance in differences of survival percentages were assessed by a log rank test for trend. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* and $^{###}$, $^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

4. Biochemical markers, Serum lactate and DIC:

Liver & kidney function tests: The administration of peptide IS 217 proved beneficial in reducing CLP-induced organ injury in mice. Sepsis often leads to multiple organ failure, which is a major cause of mortality. In order to investigate the mechanism behind the resistance of IS 217-treated mice to CLP and to evaluate the extent of organ damage caused by CLP, liver damage markers (AST, ALT) and indicators of renal failure (creatinine, BUN) at 18 hours after CLP are measured, following the administration of IS 217 via IV and SC routes. Table 68 and 69, along with figures 63 and 64, present the relevant parameters. The results clearly demonstrate that control mice subjected to CLP ~ disease control, exhibited more severe multiple organ damage, as evidenced by significantly higher concentrations of ALT, AST, creatinine, and BUN (all $p < 0.05$). On the other hand, the levels of these markers in IS 217-treated mice were comparable to those in mice that were not subjected to CLP ~normal control, indicating that organ injury was prevented in the IS 217-treated group. The observed reduction in liver damage (AST, ALT) and renal failure (creatinine, BUN) aligns with previous studies [Asma Ahmed et al., 2018 and Ajaz Ahmad et al., 2020].

Table 68: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) administrations on serum liver & kidney function tests in cecal ligation and puncture (CLP)-induced sepsis in mice. IS 217 shows protective effect on liver injury in CLP mice.

Groups	ALP(U/L)	AST (U/L)	ALT(U/L)	Cr(mg/dL)	BUN (mg/dL)
Control	171.6 ±1.6	52.19 ±2.99	39.41 ±2.39	0.37 ±0.05	52.23 ±1.84
Sham control	172 .3 ±8.7	92.1 ±6.8	78.4 ±5.3	0.2±0.00	129.78±1.16
Disease control (CLP)	218.2 ±14.2	153.1 ±11.8	116.4± 6.7	0.17 ±0.05	392.22±2.81
CLP + IS 217-0.6mg/Kg	174.3± 8.66***	108.3± 7.5***	85.4 ±7.1***	0.15 ±0.06	257.58±1.75***
CLP + IS 217-1.2mg/Kg	171 .5± 14.8***	104.1 ±10.6***	81.2 ±3.8***	0.21 ±0.01***	191.16±1.33***

The results represent means ± SEM of 5-6 mice/group. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as *** compared to disease control group.

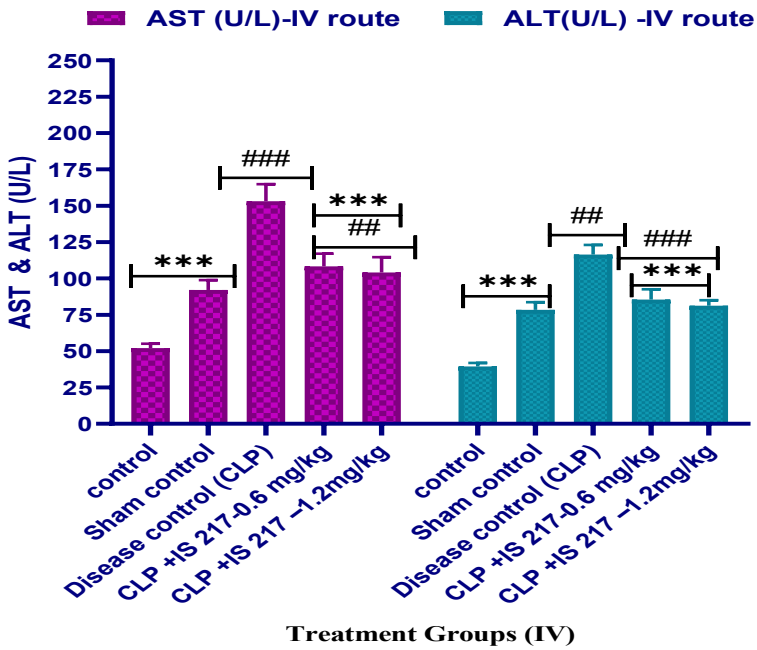


Figure 63: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) administration on liver function of mice after CLP surgery.

The results represent means ± SEM of 5-6 mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* and $^{###}$, $^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

Treatment with IS 217 ameliorates organ injury induced by CLP. IS 217-0.6 & 1.2 mg/kg, SC was injected into mice 2 h after CLP. At 18 h after CLP, mice were killed, and the amounts of AST, ALT, BUN, and creatinine, in sera were measured (figure 64).

Table 69: Effects of peptide IS 217 (0.6 & 1.2 mg/kg -SC) administrations on serum liver function tests in cecal ligation and puncture (CLP)-induced sepsis in mice.

Groups	**AST (U/L)**	**ALT(U/L)**
Control	121.6 ±1.6	99.64 ±18.04
Sham control	141.4± 68.8	54.1± 9.20
Disease control (CLP)	523.8± 47.0	158.4± 32.88
CLP + IS 217-0.6mg/Kg	471.4± 122.2**	121.2±28.59**
CLP + IS 217-1.2mg/Kg	309.0± 118.2***	90.8±24.71***

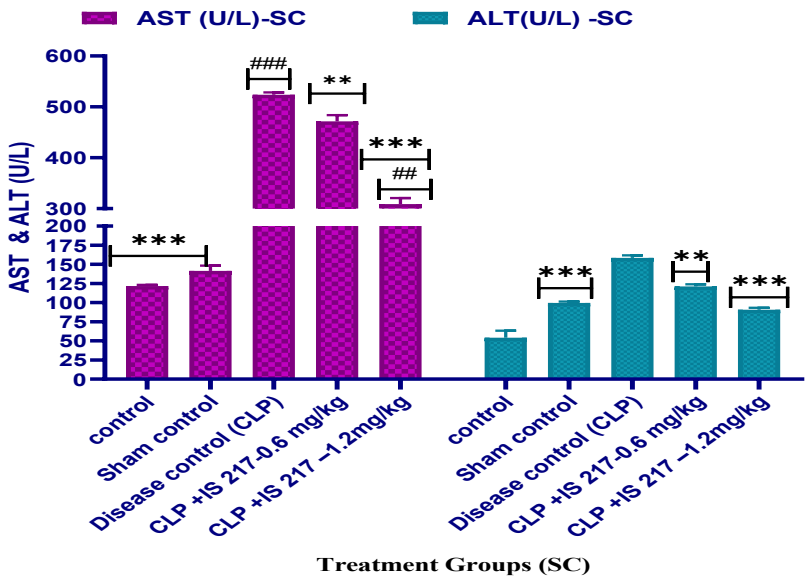


Figure 64: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -SC) administrations on liver function of mice after CLP surgery.

Coagulation tests: To assess IS 217's potential as a sepsis treatment, its impact on inflammation and DIC suppression was studied. Therapeutic injections of IS 217 were explored for their ability to mitigate DIC symptoms. Post CLP surgery, after 2 h, IS 217 was administered intravenously to BALB/c mice and blood was collected for assessing DIC coagulation parameters at 18h. Results (table 70) revealed lower PT and aPTT values in the IS 217 treated group compared to PBS-treated septic mice. IV administration of peptide IS 217 effectively reduced clotting time, indicating its potential to prevent DIC and inflammation in septic mice.

Table 70: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) administrations on Coagulation tests in cecal ligation and puncture (CLP)-induced sepsis in mice.

Groups	**DIC – time in sec – 18 h**	
	PT	**aPTT**
Control	9.53	55.92
Sham control	10.66	59.5
Disease control (CLP)	13.33	57.54
IS 217-0.6 mg/kg	12.62**	56.14**
IS 217 –1.2mg/kg	11.71***	56.26**

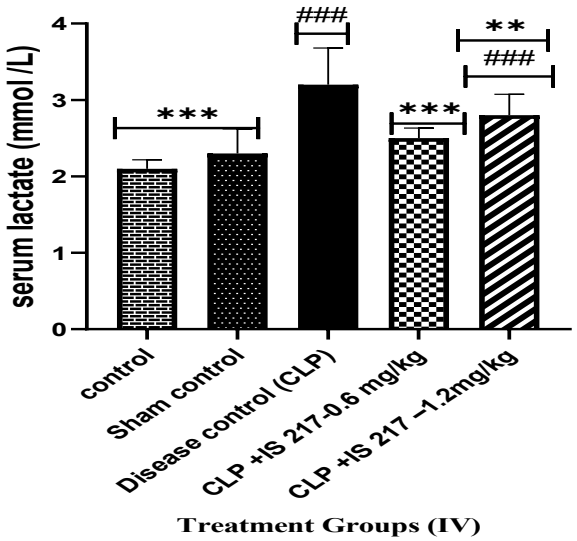
The results represent means ± SEM of 5-6 mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as *** compared to disease control group.

Serum lactate levels:

The estimation of serum lactate concentration serves as an indicator of hypoperfusion and organ dysfunction, being a valuable biomarker for assessing mortality prediction in sepsis [Chen H, Zhao C,2019].

In this study, serum lactate levels were measured across various groups, revealing higher concentrations in the CLP group. However, detailed examination in (table 71 & figure 65) [Chen H, Zhao C, 2019] indicates that the sham control and treatment groups, receiving IV administration of peptide IS 217 at 18 hours post CLP, show a restoration of serum lactate levels.

Table 71: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) administrations on serum lactate in cecal ligation and puncture (CLP)-induced sepsis in mice.



Groups	Serum lactate (mmol/L) -18h
Control	2.1 ±0.118
Sham control	2.3±0.321
Disease control (CLP)	3.2 ±0.481
CLP + IS 217-0.6mg/Kg	2.5±0.132***
CLP + IS 217-0.6mg/Kg	2.8± 0.275**

Figure 65: The serum lactate levels after the treatment with IS 217 -0.6 & 1.2 mg/kg -IV at 18h of post CLP.

Data are shown as mean ± SEM of 5-6 mice in each group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* and $^{###}$,$^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

5. Experiment 2: Acute hyper inflammation study:

5.1. Cytokine estimation in Serum:

To examine the potential underlying mechanism behind the protective effect of IS 217, the key proinflammatory cytokines (TNF-α, IL-1β, IL-12, IL-6) levels are measured in the peritoneal cavity,

lungs, spleen, and blood sera of mice with severe CLP. Both intravenous (IV)and subcutaneous (SC) administration of IS 217 resulted in a significant reduction of TNF-α, IL-1β, IL-12, and IL-6 levels in the serum 18 hours after CLP surgery, compared to CLP mice. Notably, the levels of TNF-α, IL-1β, IL-12, and IL-6 are lower following IS 217 peptide treatment than after IV administration, compared to SC administration. Only at a high dose of IS 217 (1.2 mg/kg), there was a slight increase in IL-10 levels. Pro-inflammatory macrophages, characterized by an M1 phenotype, play a critical role in driving inflammation. The same pattern of cytokine levels was observed in samples collected after 10 days of daily IS 217 treatment for 5 days [Burgelman, M et.al, 2021].

Effect of IS 217 (0.6 & 1.2 mg/kg -IV & SC) administrations on proinflammatory cytokines levels after CLP surgery in sera:

In terms of the effect of IS 217 administration (0.6 and 1.2 mg/kg, IV & SC) on proinflammatory cytokine levels in sera after CLP surgery, it is important to note that organ injury observed in sepsis is primarily caused by the excessive release of cytokines into the bloodstream. Therefore, the present study was aimed to determine the serum levels of cytokines following CLP and their response to IS 217 peptide treatments. The initial inflammatory insult triggers the production of classic cytokines, including TNF-α, IL-1β, IL-6, and IL-12, followed by an overproduction of IL-10. During the first hour after CLP, there were no detectable amounts of TNF-α or IL-1β in the serum. However, at 6 and 12 hours, the levels of these cytokines reached their peak. In this study, samples were collected at 4 hours and 18 hours, as the inflammatory reaction rapidly diminished and became barely detectable within 24-48 hours after CLP [Wendy E. Walker, 2021]. The results are as follows: TNF-α, IL-1β, IL-6, and IL-12 levels were elevated in the sepsis-control (CLP) group but significantly reduced in the IS 217-treated groups (0.6 and 1.2 mg/kg) compared to the sepsis-control group ($p < 0.001$). The trend of TNF-α levels at 4 hours, 18 hours, and 10 days in the treatment groups can be observed in figure 66, particularly for intravenous administration.

TNF-α, IL-1β, IL-6 and IL-12 were increased in the sepsis-control (CLP)group and significantly decreased in the IS 217 -0.6& 1.2 mg/kg compared to the sepsis-control group ($P < 0.001$) and the trend of TNF-α levels at 18 h and 10 days in treatment groups (figure 67) subcutaneously.

IV treatment:

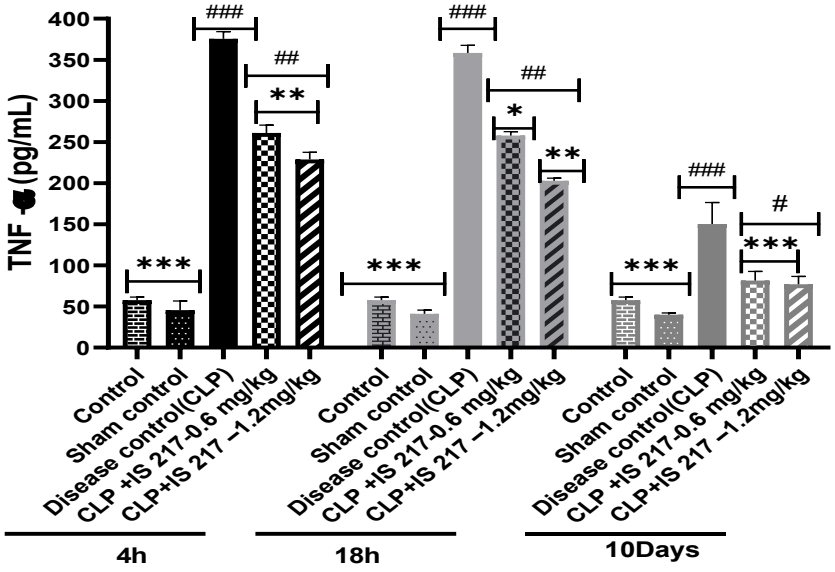


Figure 66: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on serum TNF-α levels in CLP-induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$,compared to disease control and sham control groups respectively.

SC treatment:

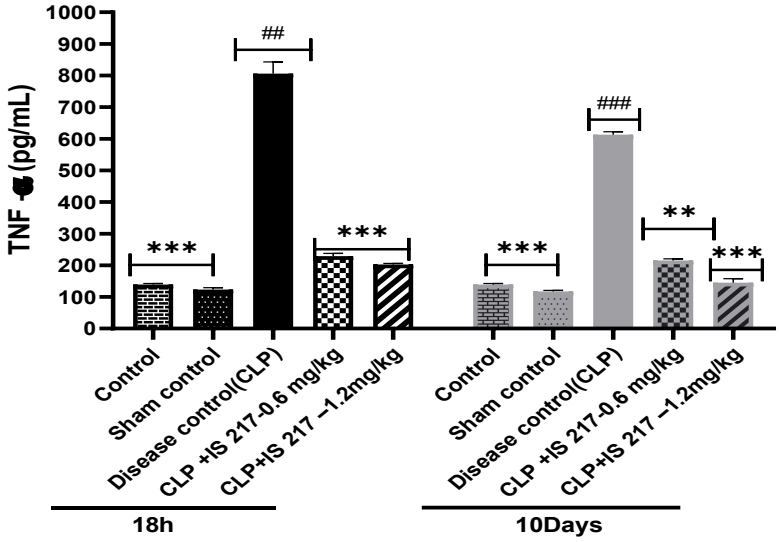


Figure 67: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -SC) treatment on serum TNF-α levels in CLP- induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured

at 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

IV treatment:

Treatment with IS 217 -0.6 mg/kg did not reduce **IL-6** release as compared to IS 217-1.2 mg/kg and the trend of IL-6 levels at 4h, 18 h, and 10 days in treatment groups (figure 68) intravenously.

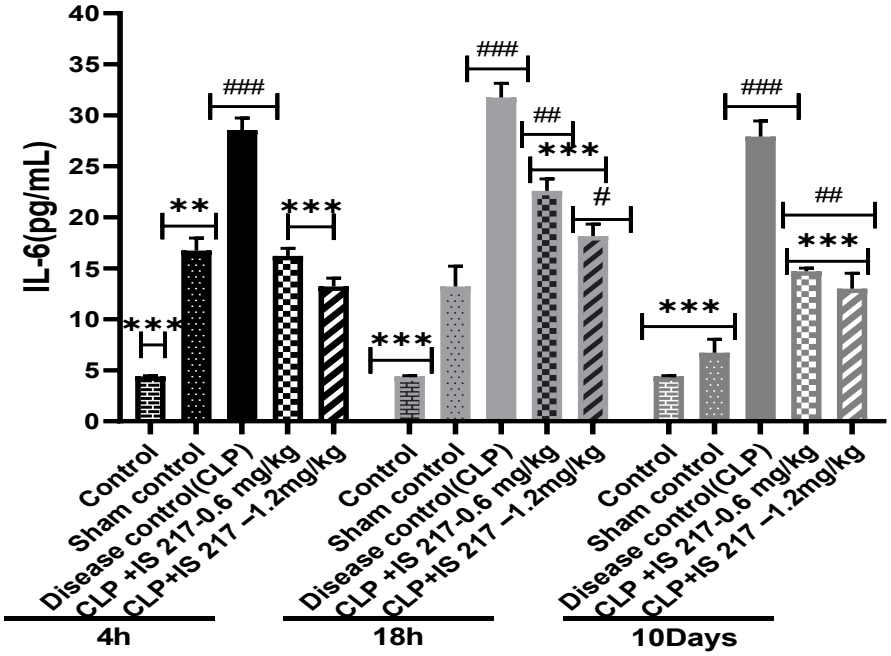


Figure 68: Effect of IS 217 (0.6 & 1.2 mg/kg -IV) treatment on serum IL-6 levels in CLP-induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

SC treatment:

Treatment with IS 217 -0.6 mg/kg did not reduce **IL-6** release as compared to IS 217-1.2 mg/kg and the trend of IL-6 levels at 18 h and 10 days in treatment groups (figure 69) subcutaneously.

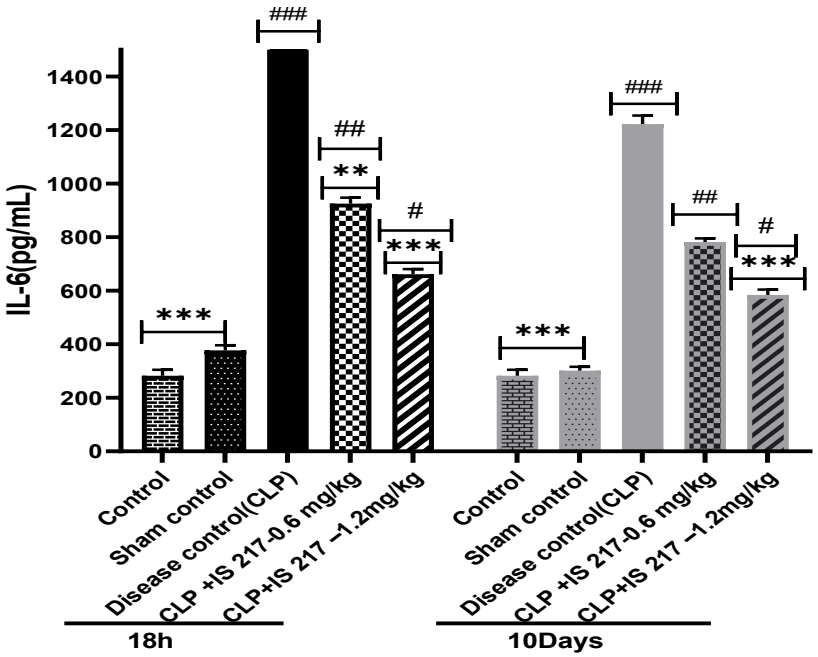


Figure 69: Effect of IS 217 (0.6 & 1.2 mg/kg -SC) treatment on serum IL-6 levels in CLP-induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

The trend of **IL-1β levels** at 4h, 18 h and 10 days in treatment groups (figure 70) intravenously and the trend of IL-1β levels at 18 h and 10 days in treatment groups (figure 71) subcutaneously.

IV treatment:

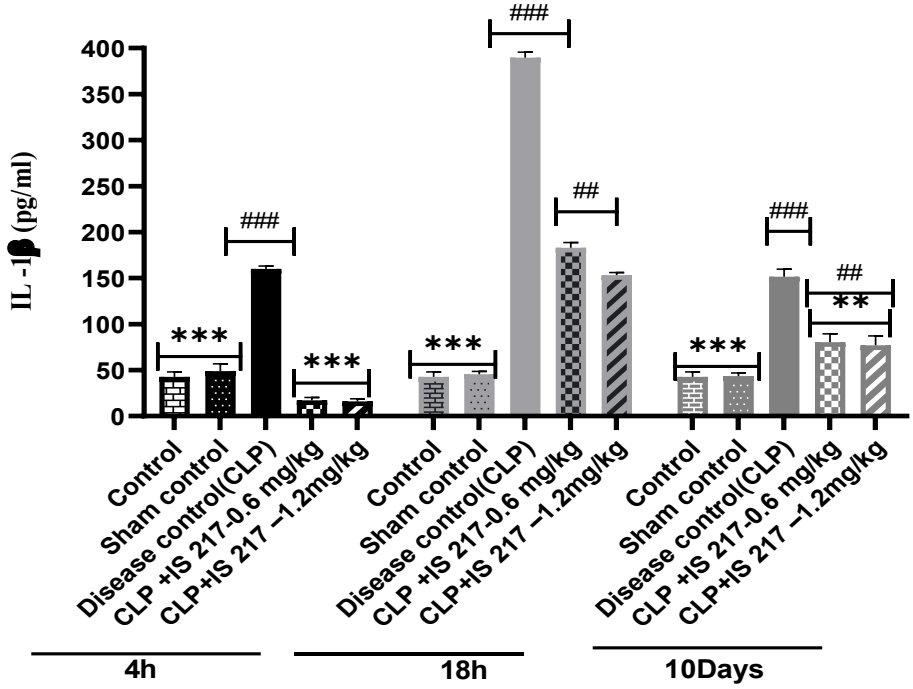


Figure 70: Effect of IS 217 (0.6 & 1.2 mg/kg -IV) treatment on serum IL-1β levels in CLP-induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

SC treatment:

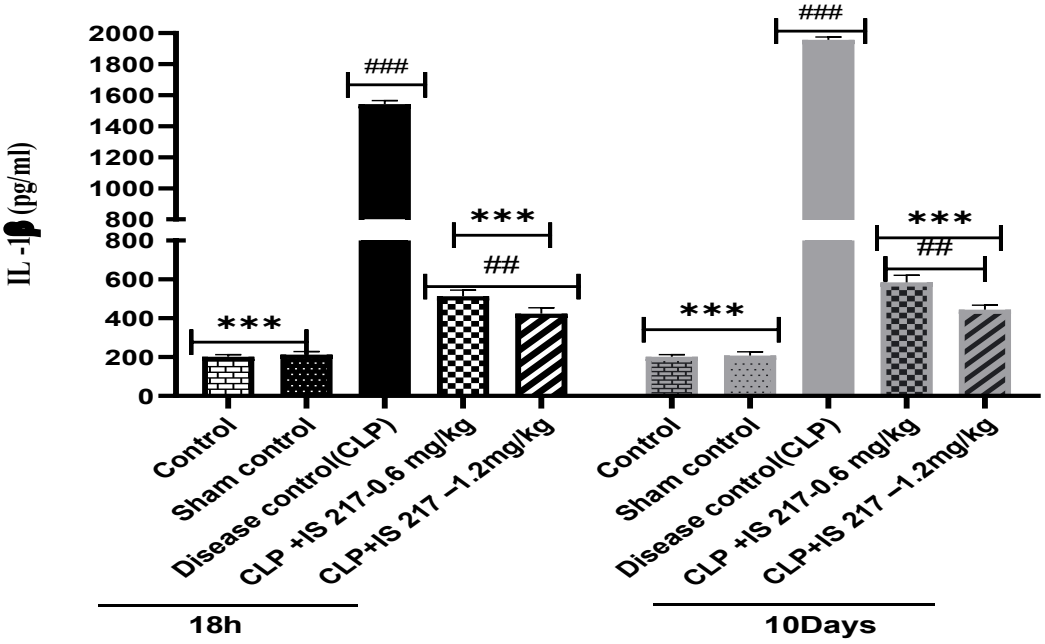


Figure 71: Effect of peptide IS 217 (0.6 & 1.2 mg/kg - SC) treatment on serum IL-1β levels in CLP-induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

Likewise, the **IL-12 level**s were significantly reduced in the IS 217 -1.2 mg/kg ($P < 0.001$), but not much in the IS 217 -0.6 mg/kg groups compared to the sepsis-a disease control group and the trend of IL-12 levels at 4h, 18 h and 10 days in treatment groups (figure 72) intravenously and the trend of IL-12 levels at 18 h and 10 days in treatment groups (figure 73) subcutaneously.

IV treatment:

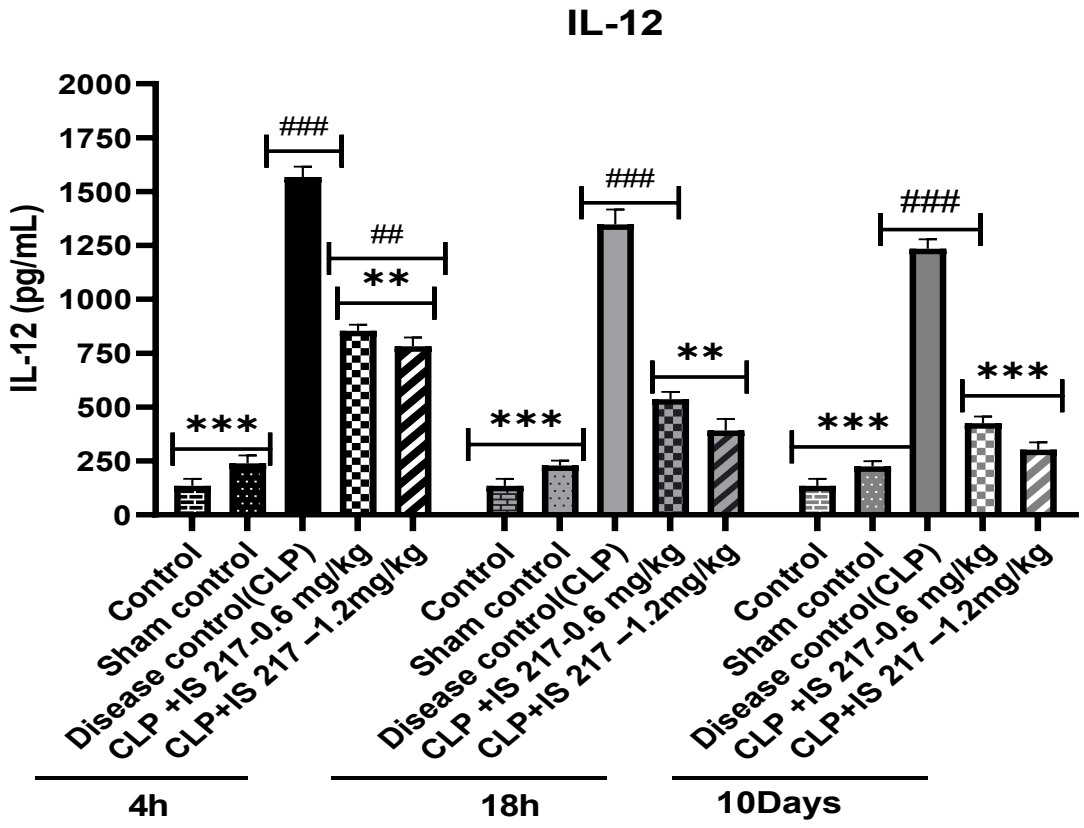


Figure 72: Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV) treatment on serum IL-12 levels in CLP-induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

SC treatment:

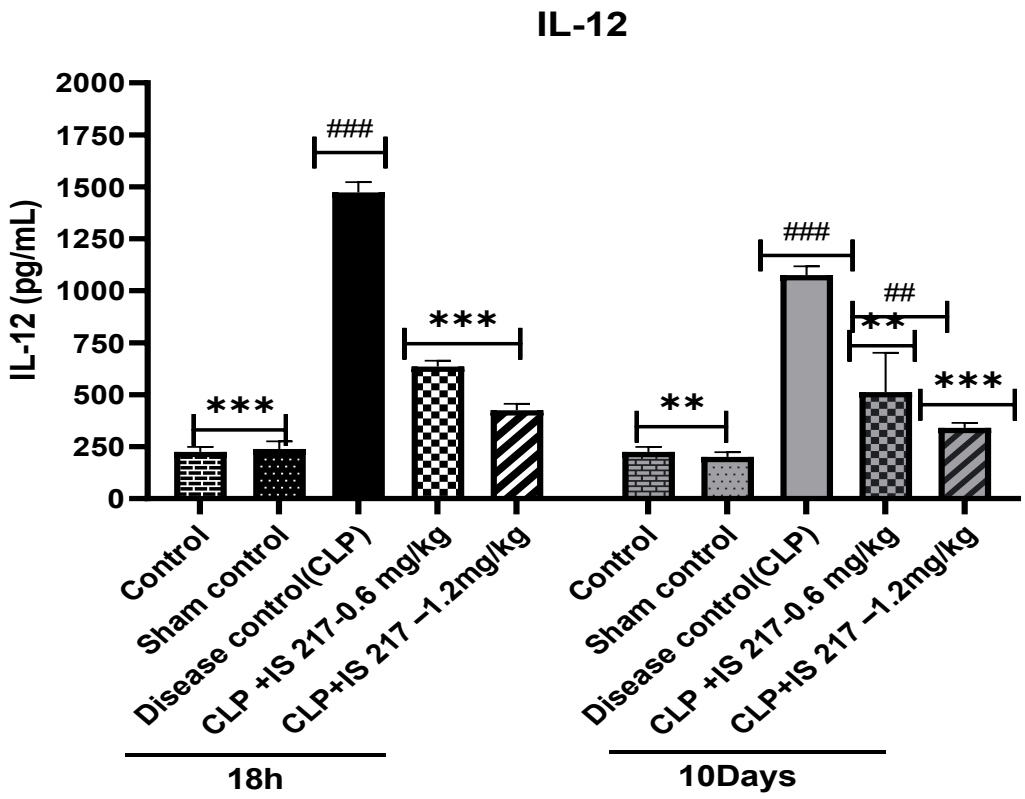


Figure 73: Effect of peptide IS 217 (0.6 & 1.2 mg/kg-SC) treatment on serum IL-12 levels in CLP- induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

The levels of **IL-10** were notably increased in the IS 217-treated groups compared to the sepsis-control group, as depicted in Figures 73 and 74. Analysis of IL-10 levels demonstrated a gradual increase in the serum concentration of this anti-inflammatory cytokine, reaching its peak at 18 hours following CLP. However, the administration of IS 217, given 2 hours after CLP, promptly and significantly increases IL-10 levels at both 18 hours and 4 hours, as shown in figure 74 & 75. In the samples collected after 10 days, IL-10 levels in the IS 217-treated group were increased. This trend was consistently observed across the treatment groups. Specifically, figure 74 represents the trend of IL-10 levels at 4 hours, 18 hours, and 10 days for the intravenous administration group, while figure 75 illustrates the trend of IL-10 levels at 18 hours and 10 days for the subcutaneous administration group.

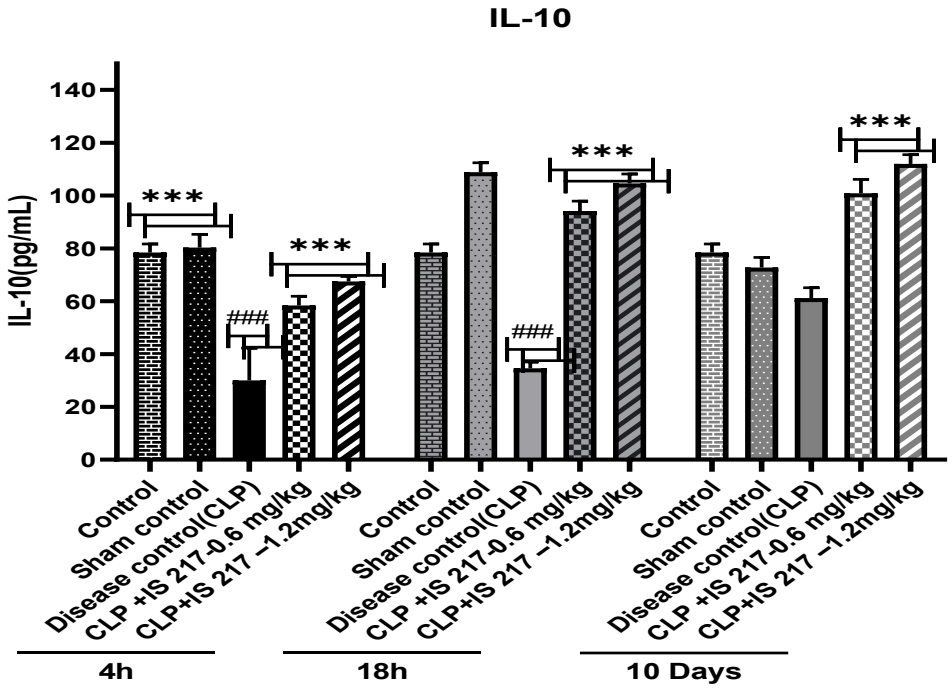


Figure 74: Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV) treatment on serum IL-10 levels in CLP- induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

SC treatment:

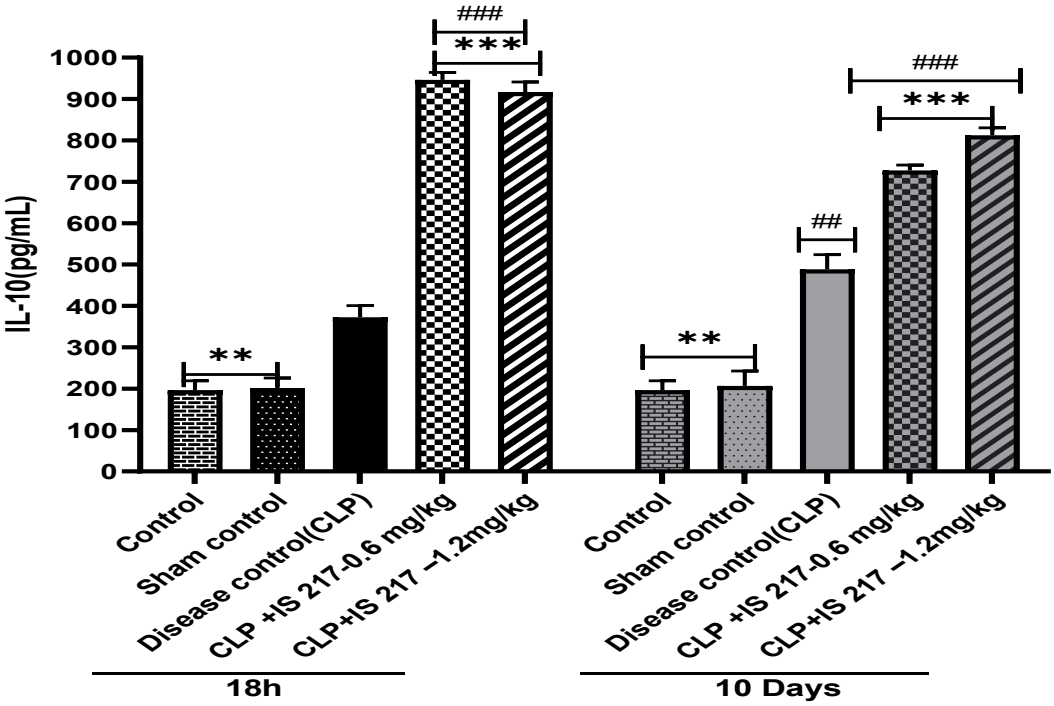
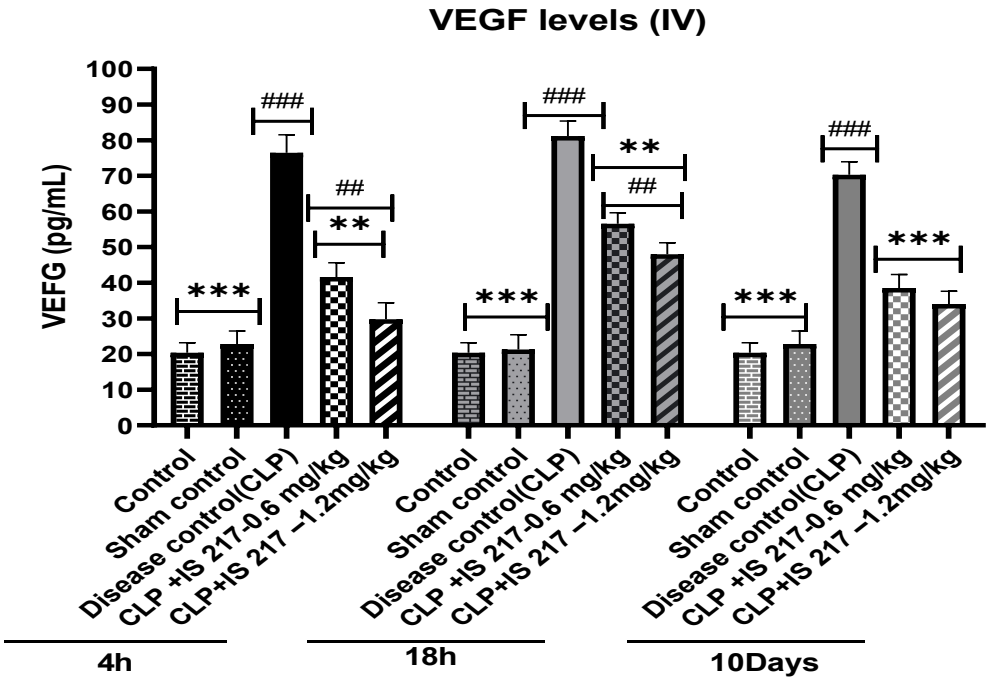


Figure 75: Effect of peptide IS 217 (0.6 & 1.2 mg/kg-SC) treatment on serum IL-10 levels in CLP- induced sepsis.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

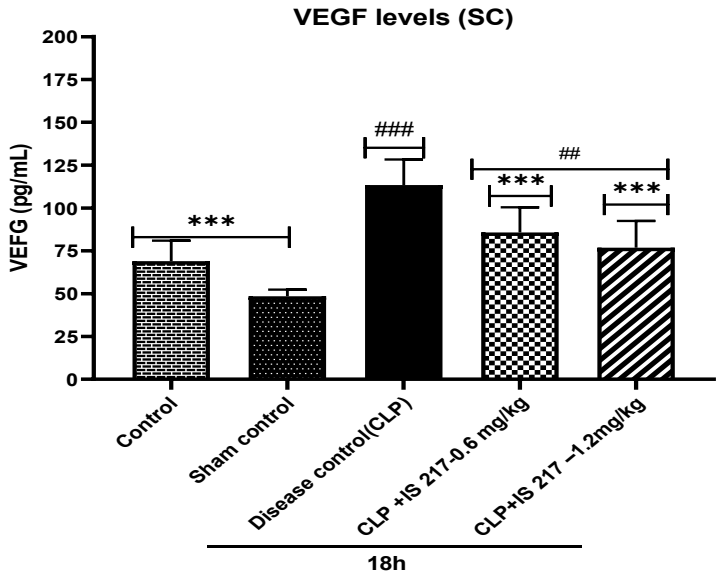
VEGF concentrations, with peak levels occurring at 18 h. In contrast, circulating levels of IL-6 and TNF-α were maximal at the earliest time point measured (6 h). In a cecal ligation puncture (CLP) model of sepsis, peak levels of VEGF occurred at 18 h, respectively. The trend of VEGF levels at 4h, 18 h and 10 days in treatment groups (Figure 76a) intravenously and the trend of VEGF levels at 18 h in treatment groups of subcutaneous was shown (figure 76 b).

IV treatment:



(a)

SC treatment:



(b)

Figure 76(a,b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV &SC) treatment on serum VEGF levels in CLP- induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route (a)and at 18h via subcutaneous route (b). The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

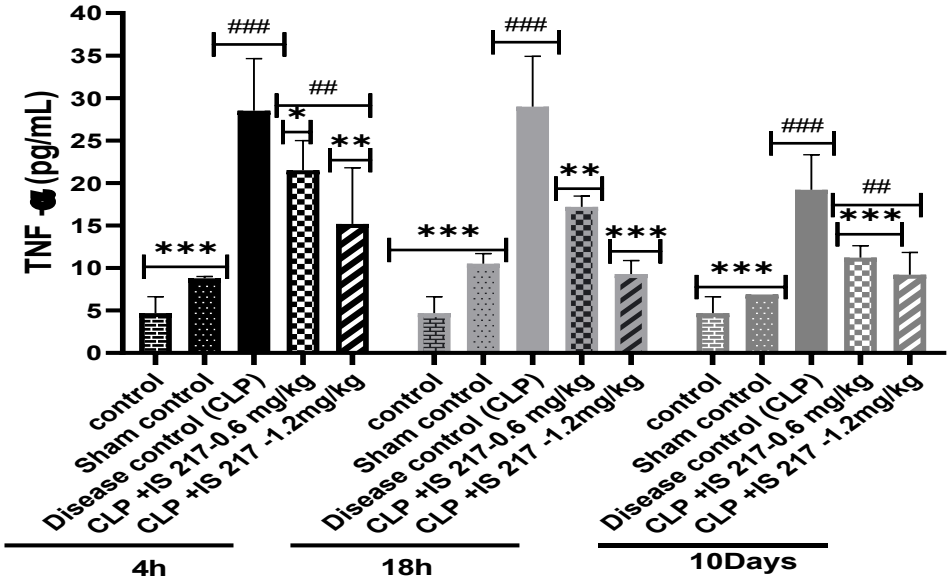
5.2. Cytokine estimation in abdominal peritoneal lavage fluid (PLF):

Numerous preclinical and clinical investigations have highlighted that intra-abdominal injury or ischemia, especially when followed by infection, triggers an excessive production of inflammatory and protein mediators. This leads to uncontrolled inflammation in the peritoneum, primarily through the lymphatic pathway, which can subsequently impact the systemic circulation, potentially exacerbating the adverse effects of sepsis and multiple organ dysfunction (Stefan Wirtz et al., 2006; Yoon Ju Cho1, et al., 2011). In this study, the levels of cytokines, bacterial count, and total cell count were assessed in peritoneal lavage fluid (PLF). The concentration of secreted cytokines in the abdominal lavage fluid was quantified as pg of protein per milliliter (pg protein/mL).

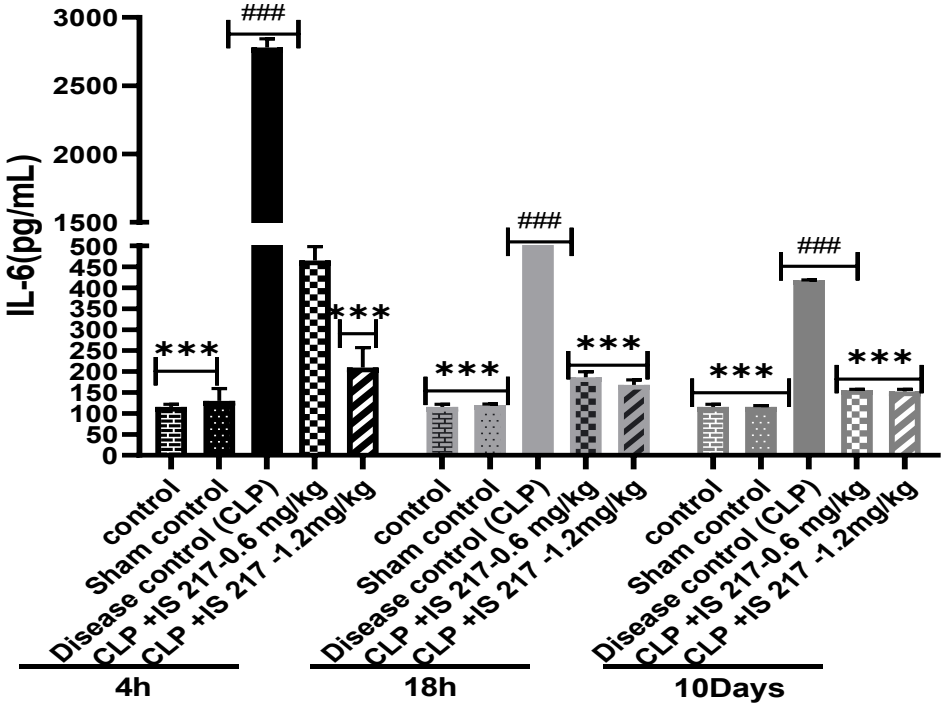
Pro-inflammatory cytokines were all increased sharply from 4h to 18 h after CLP surgery in all groups. Mice in the present study showed a significant increase in TNF-α, (figure 77a), IL-6 expression (figure 77b), and IL-1β (figure 78) in CLP mice than treated and sham groups and IS 217 treatment groups at 4h, 18h and 10 days post CLP. The IL-6 levels of treatment groups showed a similar trend with sham control group when treated intravenously, whereas the expression of TNF-α, and IL-6 levels are increased in CLP group when compared to the IS 217 SC treatment at 18 h (figure 79), but not as observed at 10-day samples.

However, treatment of IS 217 (0.6, and 1.2 mg/kg) significantly decreased the levels of serum TNF-a and IL-6 in a dose-dependent manner. In addition, the serum level of IL-1b can also be decreased by treating with IS 217 (0.6, and 1.2 mg/kg).

IV treatment:



(a)



(b)

Figure 77 (a,b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on TNF -α & IL-6 levels in the peritoneal fluid of CLP- induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

IV treatment:

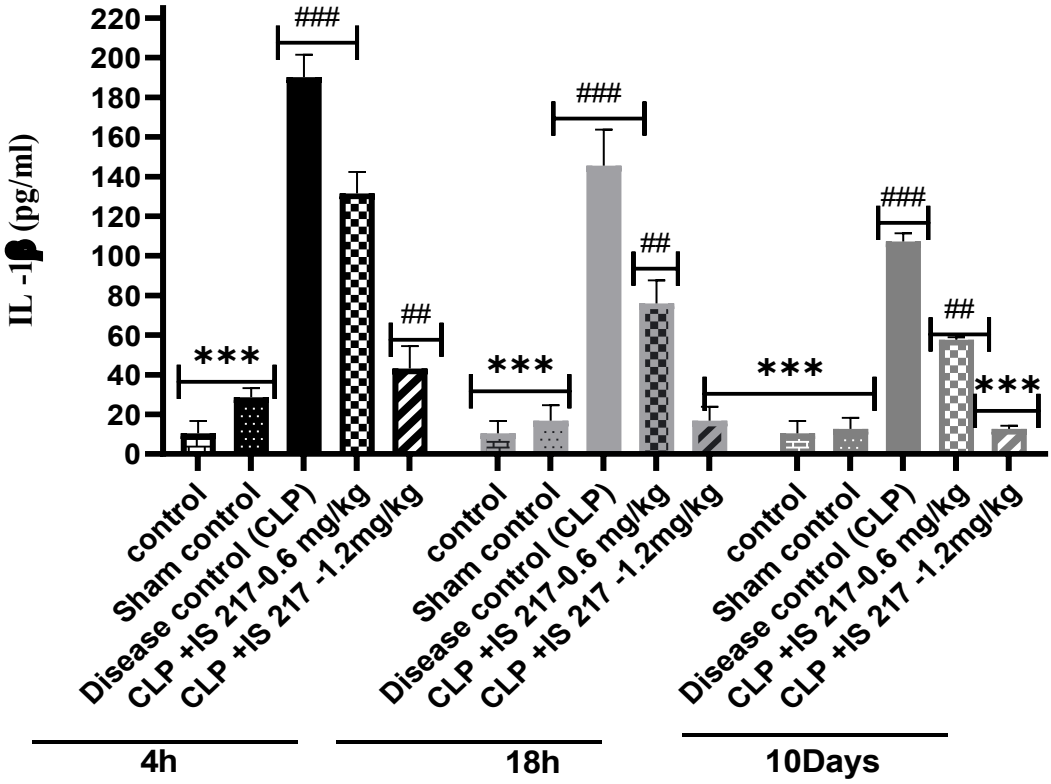


Figure 78: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on IL-1β levels in the peritoneal fluid of CLP- induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

SC treatment at 18 h:

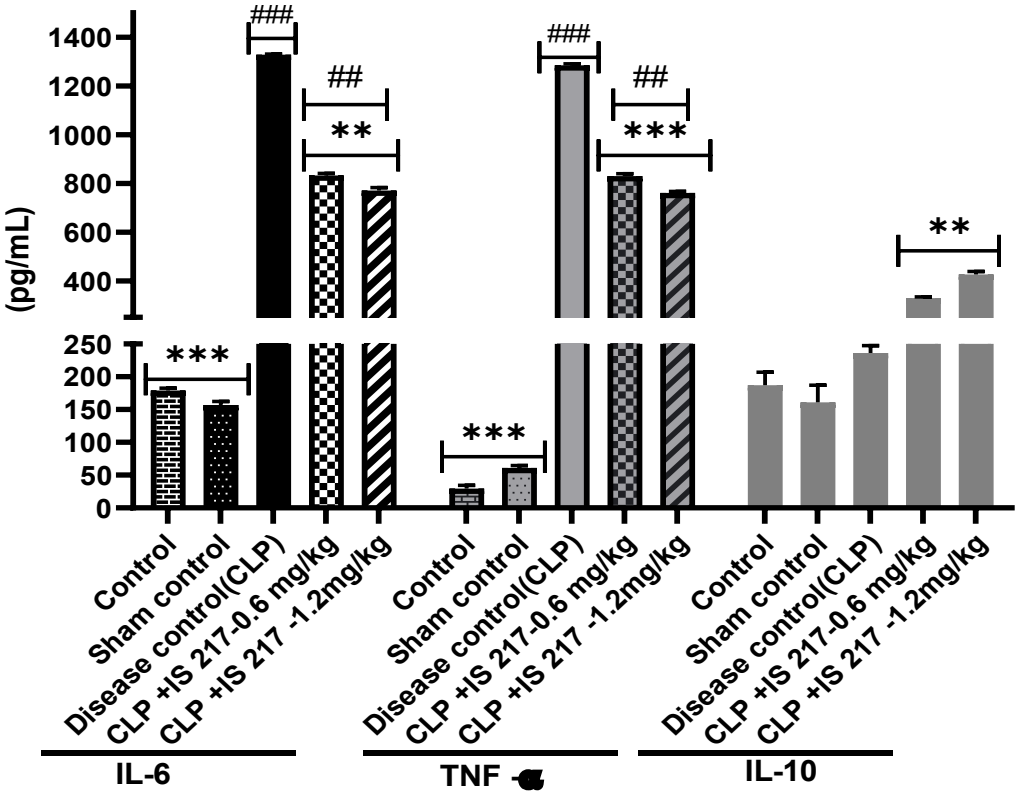


Figure 79: Effect of peptide IS 217 (0.6 & 1.2 mg/kg-SC) treatment on IL-6, TNF -α and IL-10 levels in the peritoneal fluid of CLP- induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

Recently, it has been suggested that septic patients may enter a state where the capacity of monocytic cells to secrete TNF is significantly reduced, while their ability to produce IL-10 remains at least temporarily intact (Donghong Yan et al., 2002). This largely anti-inflammatory state, in contrast to the assumption of persistent hyperinflammation, has been associated with a substantial risk of death due to persistent infection and multiple organ failure.

In the context of mice induced with CLP, peritoneal macrophages gradually lose their ability to release TNF-α, IL-6, and IL-1β, as illustrated in Figures 77-78. This decline in function becomes notably evident at 24-48 hours post CLP. Peritoneal macrophages cease secretion of these pro-

inflammatory cytokines entirely after 18 hours. However, in mice treated with IS 217, this function significantly recovers ($p<0.01$) at all time points following IS 217 injections. While macrophages lose their capability to release TNF-α and IL-1β, PEC cells (peritoneal exudate cells) maintain this ability at 24- and 48-hour intervals. Yet, when IS 217 is administered subcutaneously, PEC cells from IS 217-treated mice notably reduce their release of IL-10 at 18 hours post CLP (figure 79; $p<0.001$).

5.3. Measurement of procalcitonin in peritoneal lavage fluid:

Table 72: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV & SC) treatments on procalcitonin levels in peritoneal lavage fluid after 18h of CLP surgery.

Groups	**Procalcitonin – PCT levels (pg/ml)**	
	IV -route	**SC -route**
Control	191.13 ±51.75	163.11 ±17.15
Sham control	182± 14.7	182.2± 21.71
Disease control (CLP)	256 ±23.0	5369 ±11.20
CLP + IS 217-0.6mg/Kg	193 ±23.0***	401.1 ±109.11***
CLP + IS 217-1.2mg/Kg	157 ±19.0***	312.5 ±106.23***

Procalcitonin levels are directly reflected in the severity of the sepsis. The higher levels of PCT levels are observed in CLP mice at 18 h post CLP and whereas the treatment groups also showed higher PCT levels when compared to sham control, and considered as significant in comparison to disease control CLP mice and the PCT levels of peptide IS 217 IV treatment was mentioned in (table 72 & figure 80 a) and whereas the PCT levels of peptide IS 217 SC treatment was mentioned in (table 72& figure 80 b)

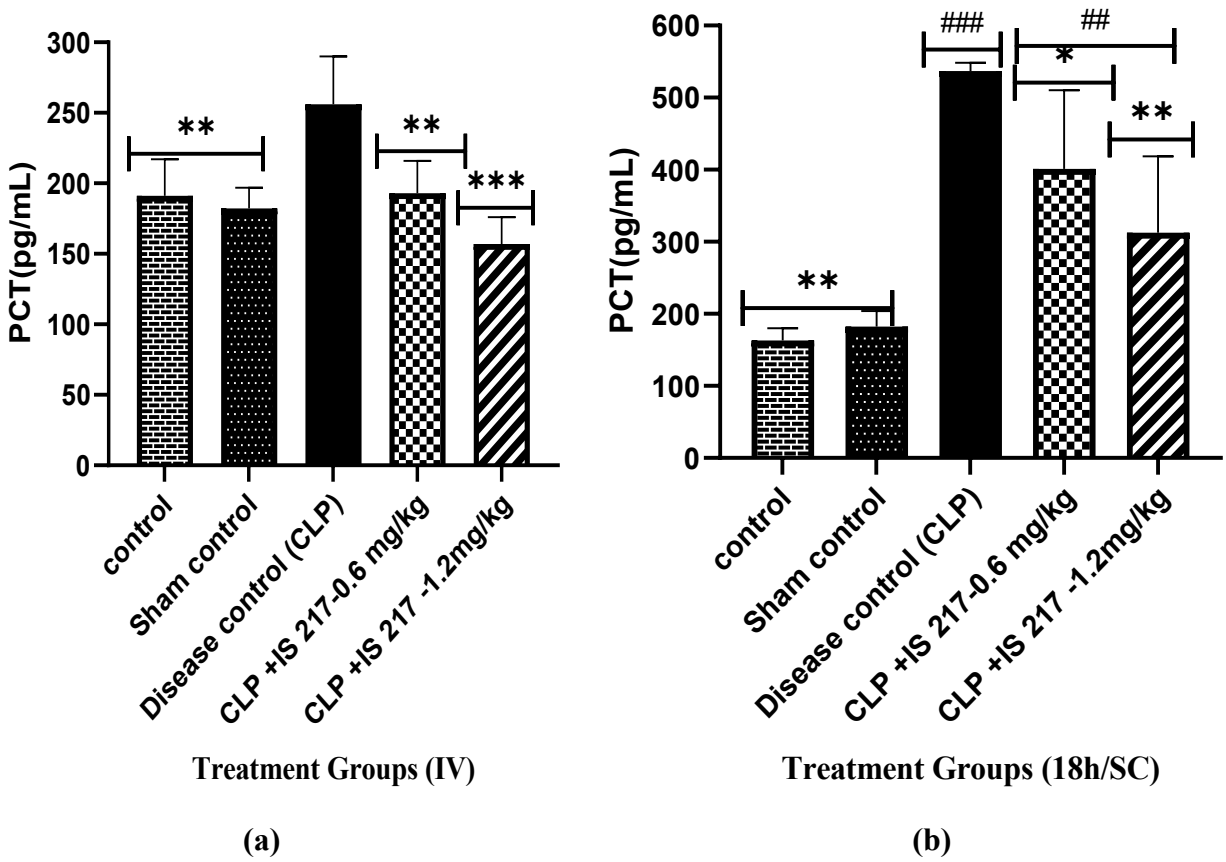


Figure 80 (a,b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV&SC) treatment on procalcitonin levels in the peritoneal fluid of CLP- induced sepsis. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

5.4. Measurement of peritoneal leukocytes in PLF:

Based on the effects of CLP (cecal ligation and puncture) and its impact on the control of bacterial growth, it was noticed that CLP caused a noticeable increase in the number of inflammatory cells, primarily neutrophils. Thus, in the present study, the total and differential cell count were analyzed and also assessed the bacterial load in the peritoneal fluids of mice treated with IS 217 and all other groups.

Treatment with peptide IS 217 resulted in a decrease in the influx of total cells. The response pattern concerning macrophages and neutrophils was similar to that observed in the total cell count, although it did not reach statistical significance (figure 81) when administered intravenously.

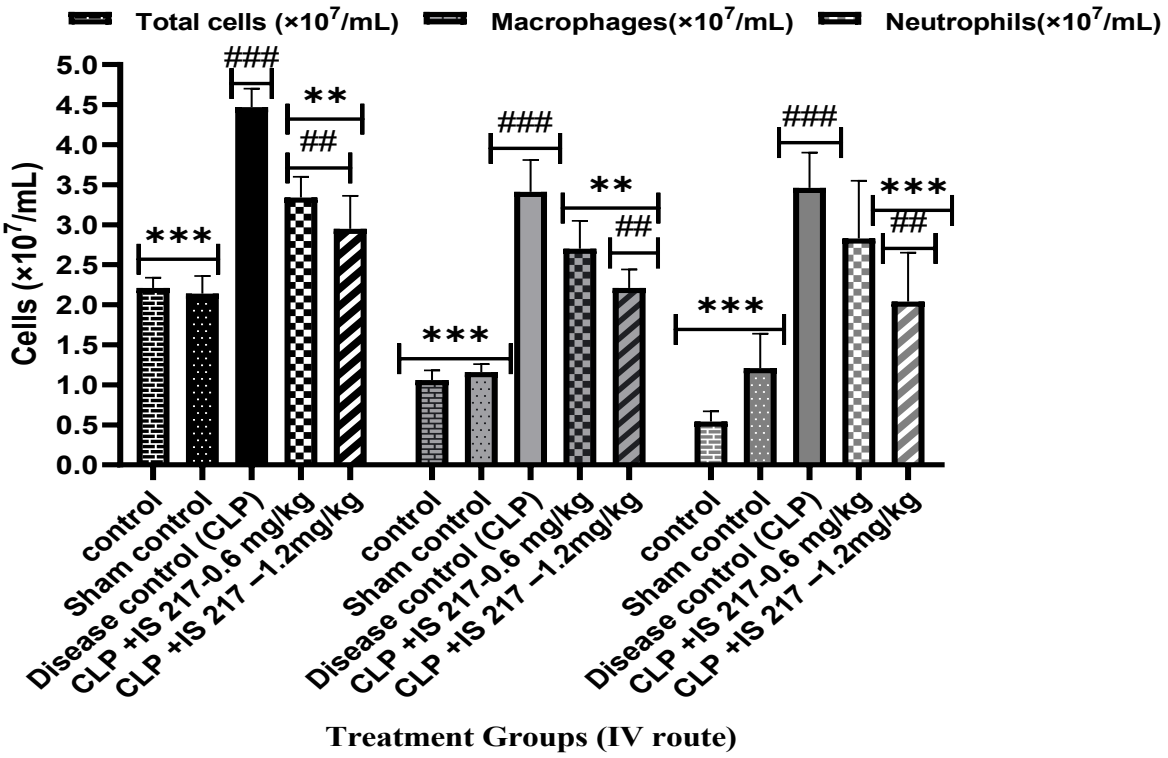


Figure 81: Total and differential blood cell counts from mice treated with IS 217 (0.6 & 1.2 mg/kg-IV) of CLP- induced sepsis and from sham and CLP induced mice.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

6. Experiment 3: Late immune paralysis studies:

6.1. Measurement of peritoneal bacterial load and leukocytes in PLF:

Recent studies have demonstrated that IL-10, a cytokine, hampers the elimination of bacteria from the peritoneal cavity and promotes the spread of bacteria to distant organs. The significant changes in IL-10 levels in mice treated with IS 217 peptide treatment after CLP, along with the improved survival of these mice, prompted us to investigate the role of IS 217 in the clearance of bacteria in CLP-induced mice. No detectable bacterial growth was observed in the abdominal lavage fluid of mice after 48 hours of culturing. However, in the CLP model, there were animals with either negative cultures or very few bacteria, and there were significant individual variations (data not presented). The peritoneal fluids collected 18 hours after CLP in mice contained a significant number of bacteria within the peritoneum (table 73). At this specific time point, the bacterial load in the peritoneum of IS 217-treated mice showed a significant decrease ($p < 0.01$). Among the IS 217-treated mice, approximately 20% exhibited clinical symptoms of active sepsis, such as shivering and bristled hair, and had an increased peritoneal bacterial load (>300 CFUs). However, in the remaining mice, the bacterial load recovered from the peritoneum was significantly lower in IS 217-treated mice compared to diseased control mice treated with Normal Saline.

The administration of IS 217 leads to a reduction in the number of colony-forming units (CFUs) present in the peritoneal cavity fluid after 18 hours of culturing. The intravenous (IV) treatment was specifically mentioned in table 73.

Table 73: Bacterial CFU counts from peritoneal lavage from mice treated with IS 217 (0.6 & 1.2 mg/kg -IV) of CLP -induced sepsis at 18 h of sham and CLP-induced mice.

Groups	**Bacterial count (CFU)**
Control	22.65 ± 15.42
Sham control	46.18± 15.98
Disease control (CLP)	>300
CLP + IS 217-0.6mg/Kg	87.63 ±39.85***
CLP + IS 217-1.2mg/Kg	56.10 ±116.4**

The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, compared to the disease control group.

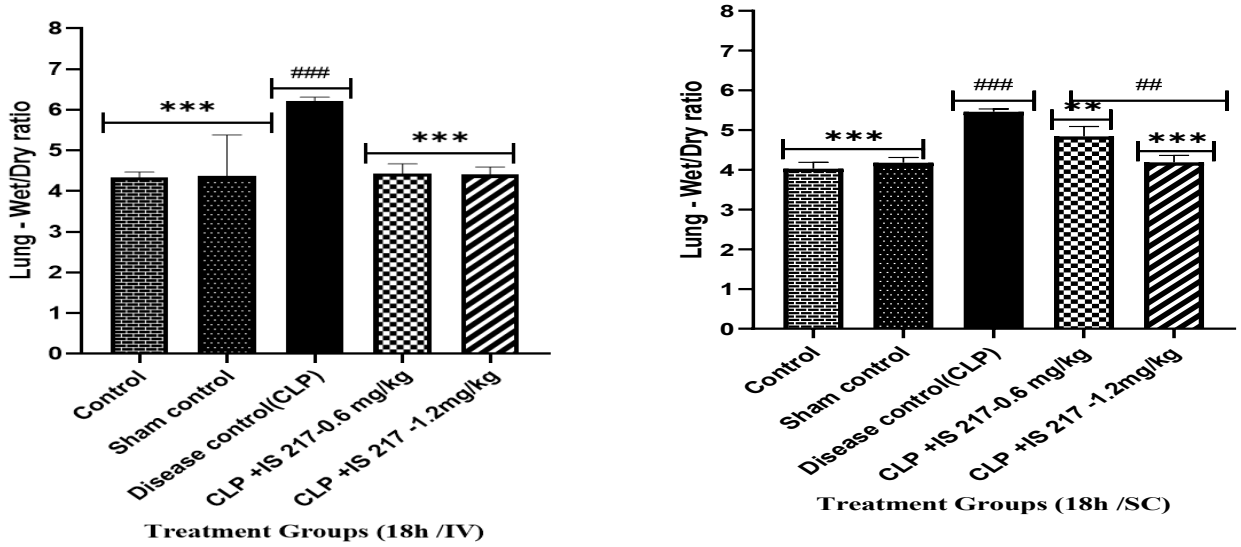
6.2. Changes in the Lung wet/dry Ratio, of lungs after the treatment with peptide IS 217:

The lungs were collected, and the wet/dry weights were measured to determine lung oedema, which can partly indicate the effects of intravenous treatment of IS 217 treatments. The wet/dry ratio of the lungs in the IS 217 administration mice was significantly lower than that in the CLP group or isotype-treated animals (P,0.01, respectively) by intravenously and by subcutaneously (table 74& figure 82a, b).

Table 74. Effects of IS 217 (0.6 & 1.2 mg/kg -IV & SC) treatments on Lung w/d ratio changes.

Groups	Lung w/d ratio	
	IV -route	SC -route
Control	4.34±0.19	4.03 ± 0.16
Sham control	4.38±0.10	4.18±0.13
Disease control (CLP)	6.22±0.09	5.46 ± 0.07
CLP + IS 217-0.6mg/Kg	4.43±0.24***	4.84 ± 0.25**
CLP + IS 217-1.2mg/Kg	4.41±0.18***	4.19 ± 0.17 ***

The results represent means ± SEM of 5-6 mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as *** compared to disease control group.



(a) **(b)**

Figure 82(a,b): Peptide IS 217 (0.6 & 1.2 mg/kg via IV(a) & SC(b) treatment on the lung wet-to-dry weight ratio and protein concentration in the BALF of CLP -induced sepsis mice. Data

are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* and ###, ##&#compared to disease control and sham control groups respectively.

6.3. Evaluation of MPO levels in Lungs:

Neutrophil extravasation serves as a significant histological indicator of both inflammatory and immune responses within lung tissue injuries. In cases of acute lung injury (ALI), the extent of tissue damage often correlates with the activity of pulmonary myeloperoxidase (MPO), which is commonly linked to the infiltration of neutrophils in the lungs [Abraham 2003; Zhou et al., 2012]. To estimate neutrophil migration to affected organs, researchers frequently rely on assessing MPO levels [Yung-Yang Liu, 2008]. Studies conducted by [McCabe et al. 2001] have affirmed pulmonary MPO activity as a dependable marker for evaluating lung neutrophil infiltration.

Hence, the evaluation of MPO activity in lung tissue homogenates of mice across various treatment groups was estimated after 18 h of post CLP sepsis induction. The outcomes highlighted a significant rise in pulmonary MPO activity within the sepsis-induced CLP group compared to the sham group, which did not undergo sepsis treatment. This elevation was vividly depicted in Figure 83. Notably, mice subjected to IS 217 peptide treatment exhibited markedly lower pulmonary MPO activity in comparison to the sepsis group (refer to Table 75). These findings found support in data related to neutrophil infiltration within the bronchoalveolar lavage fluid (BALF). The lung MPO levels were notably higher in CLP mice than in those treated with IS 217, 18 hours post sepsis induction, as illustrated in Figure 83. The elevation in MPO levels within the lungs was statistically significant ($p < 0.001$; $p < 0.01$). The amount of MPO in the lungs of CLP mice was approximately tenfold higher compared to untreated, normal mice. Conversely, when IS 217 was administered intravenously, it resulted in only a marginal increase in lung MPO levels (around 4-7-fold) compared to untreated mice (Figure 83a). Similarly, subcutaneous injections of IS 217 led to a twofold reduction in lung MPO levels compared to CLP mice (Figure 83b).

Table75. Effects of IS 217 (0.6 & 1.2 mg/kg -IV & SC) treatments on MPO levels.

Groups	MPO Levels (U/mg tissue)	
	IV -route	**SC-route**
Control	5.1 ±1.1	5.67± 3.1
Sham control	16.09 ±1.34	19.01 ±4.31
Disease control (CLP)	57.53 ±5.82	24.1± 14.1
CLP + IS 217-0.6mg/Kg	37.01 ±3.68***	11.2±3.1***
CLP + IS 217- 1.2mg/Kg	20.16 ±1.23***	8.5 ±2.2***

The results represent means ± SEM of 5-6 mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as *** compared to disease control group.

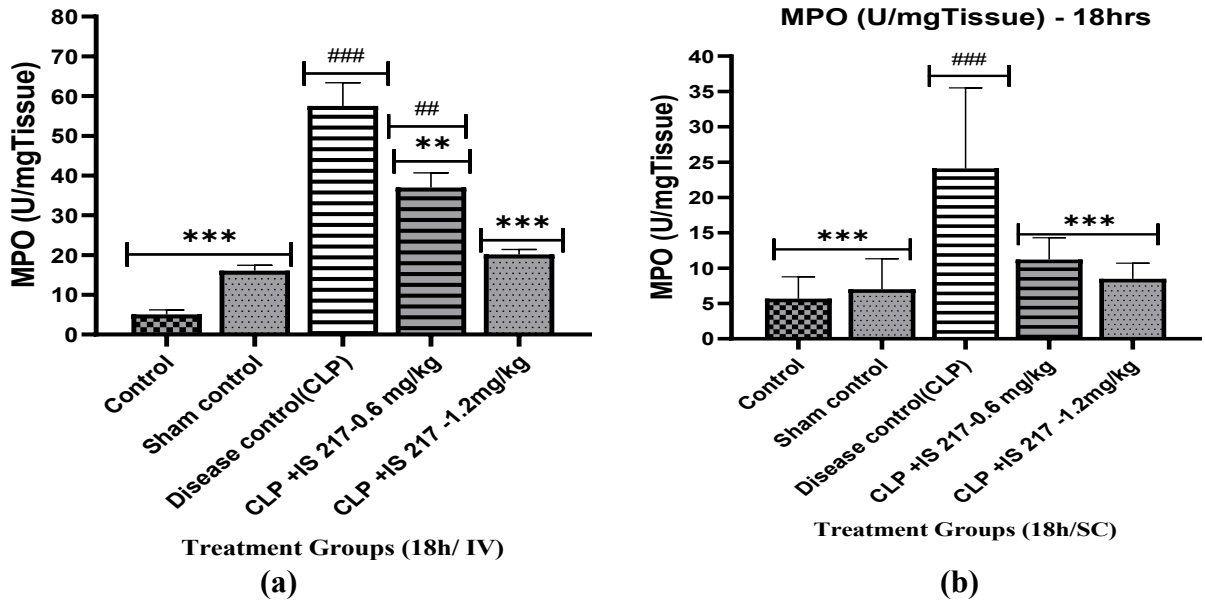


Figure 83(a,b): Peptide IS 217 (0.6 & 1.2 mg/kg) via IV(a) & SC(b) treatment on sepsis induced pulmonary MPO activity. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* and $^{###}$, $^{##}$&$^{#}$compared to disease control and sham control groups respectively.

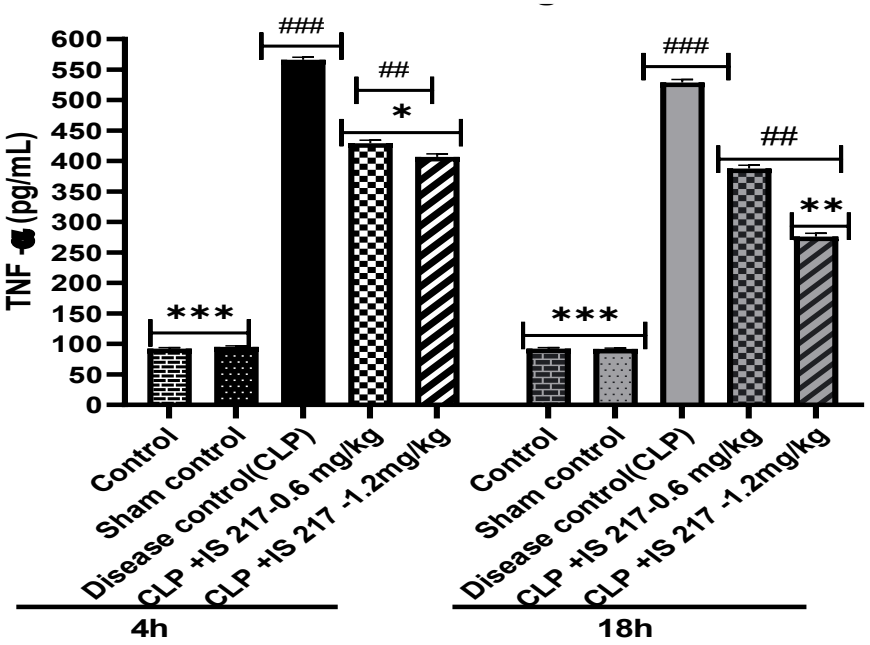
6.4. The effect of IS 217 on the inflammatory cytokine concentration in the BALF of sepsis mice:

In this present study, one of the objectives was to evaluate the levels of pro-inflammatory factors, namely TNF-α, IL-6, and VEGF, as well as the anti-inflammatory factor IL-10, in the context of sepsis-induced acute lung injury (ALI) [Eun Jung Park et al.,2014 & Kengo Tomita et al., 2020]. The concentrations of these cytokines were measured using an ELISA assay in the bronchoalveolar lavage fluid (BALF) of mice treated with different regimens post-cecal ligation and puncture (CLP), either intravenously (IV) after 4 and 18 hours or subcutaneously (SC) after 18 hours.

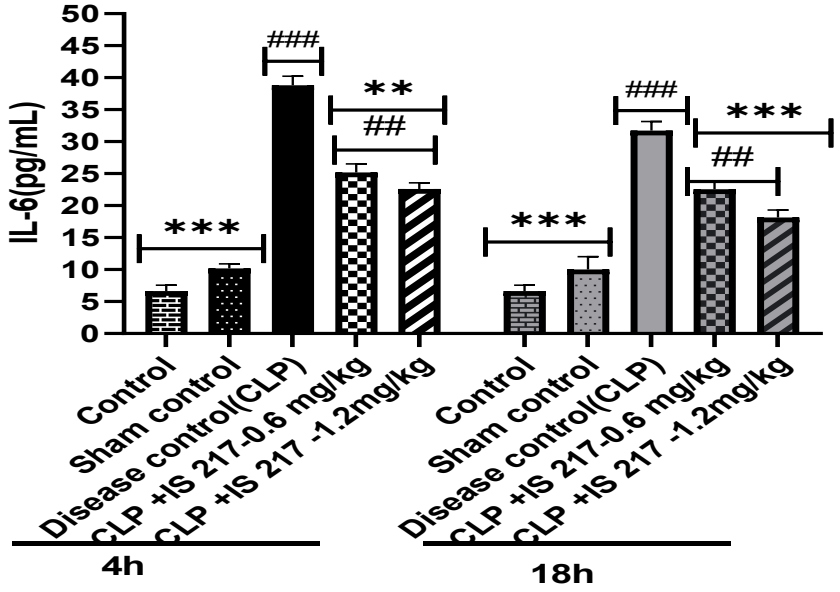
The figures demonstrate a notable increase in pro-inflammatory cytokine concentrations—TNF-α (figures 84a, 87), IL-6 (figures 84b, 87), VEGF (figure 85)—and considerable decline in IL-10(figures 86, 87) levels in the bronchoalveolar lavage fluid (BALF) of sepsis-induced mice in comparison to the sham group. Upon treatment with peptide IS 217 (at doses of 0.6 and 1.2 mg/kg), a noteworthy reduction in TNF-α and IL-6 levels in the BALF was evident, while IL-10 levels displayed an increase compared to the CLP group. These outcomes suggest the anti-inflammatory impact of IS 217 treatments in mice experiencing acute lung injury (ALI), as indicated by the decrease in VEGF, TNF-α, and IL-6 levels and the elevation of IL-10 levels in the BALF.

However, the administration of peptide IS 217 at doses of 0.6 and 1.2 mg/kg—whether through IV or SC routes—effectively suppressed the rise of these pro-inflammatory cytokines in sepsis-treated mice. These findings highlight the potential of peptide IS 217 to inhibit sepsis-induced inflammatory responses in ALI mice.

IV treatment



(a)



(b)

Figure 84(a,b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on TNF-α & IL-6 levels in the bronchoalveolar lavage fluid. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

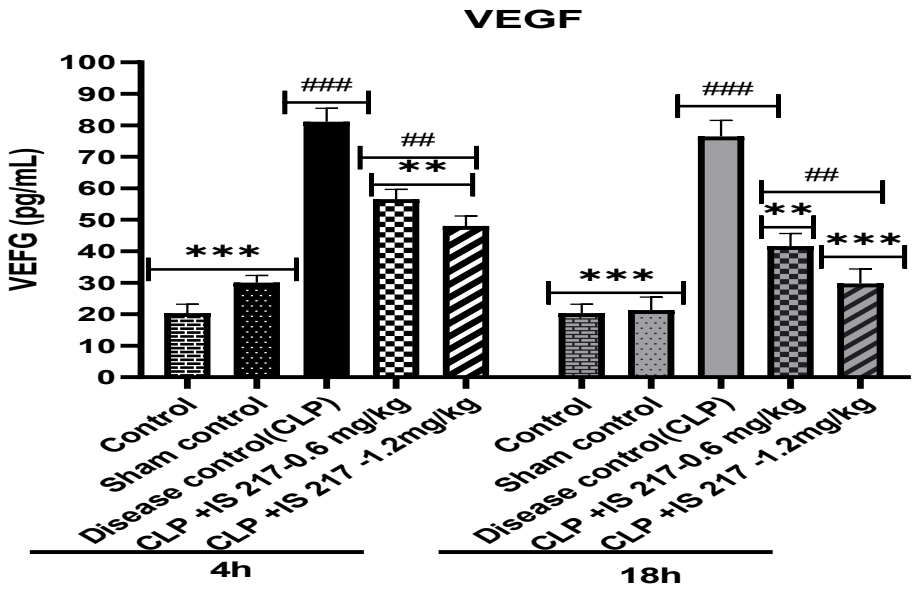


Figure 85: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on VEGF levels in the bronchoalveolar lavage fluid.

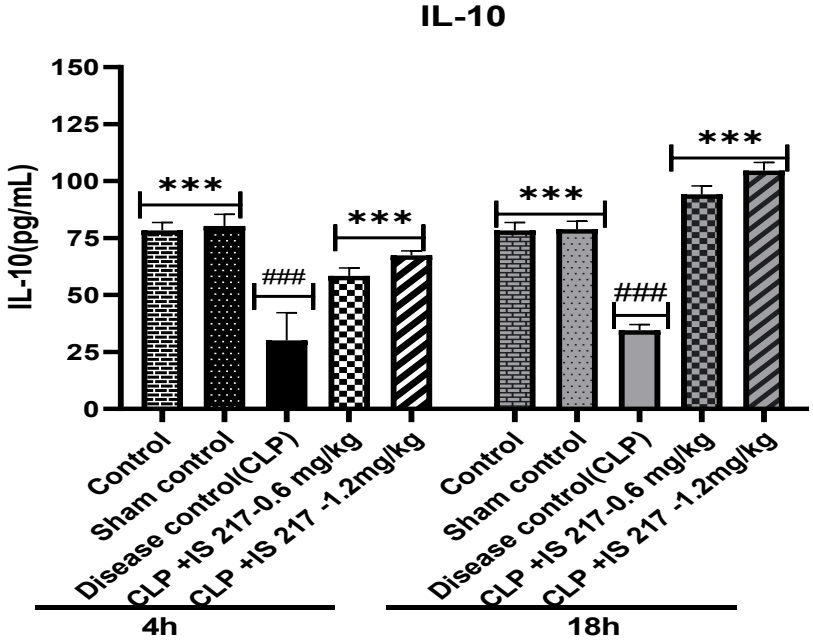


Figure 86: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on IL-10 levels in the bronchoalveolar lavage fluid. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

SC treatment -18 h:

As shown in figure 85, sepsis induction significantly increased the pro inflammatory cytokines concentration of TNF-α (figure 85), IL-16 (figure 85), and IL-10 (figure 85), (figure 76, 78) in the BALF of sepsis-induced mice when compared to the sham group mice via SC administration of IS 217 at 0.6 &.2 mg/kg. The peptide IS 217 treatments inhibited the elevation of these pro-inflammatory cytokines, as observed in the IS 217- treated sepsis mice.

SC treatment at 18 h:

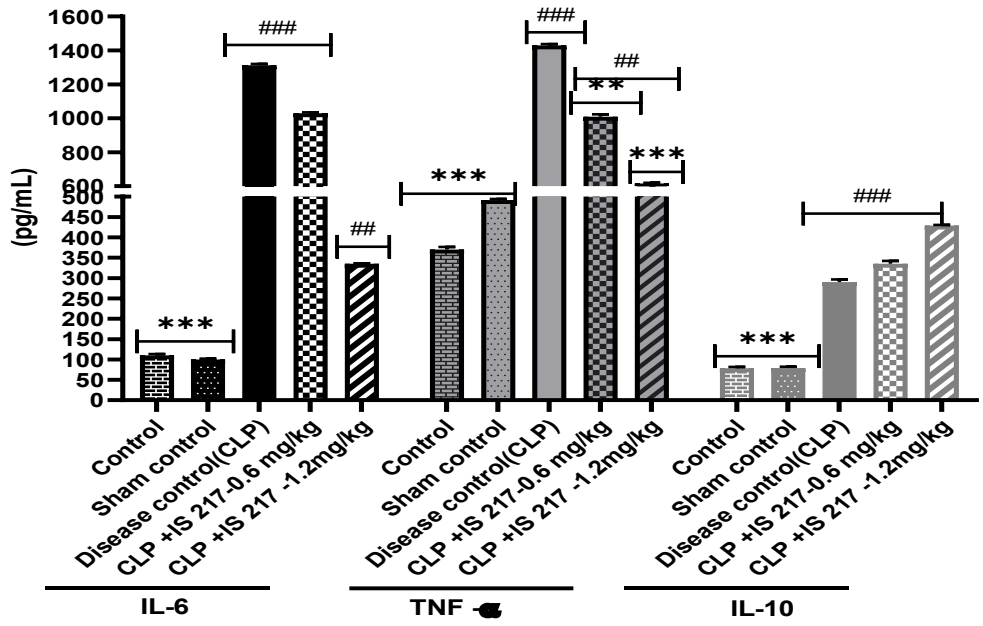


Figure 87: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -SC) treatment on IL-6, TNF-α and IL-10 levels in the bronchoalveolar lavage fluid. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$,$^{##}$&$^{#}$, compared to disease control and sham control groups respectively.

6.5. Effect of peptide IS 217 (0.6 & 1.2 mg/kg) via IV treatment on mitogen-activated protein kinases signaling pathway in CLP induced sepsis mice:

Furthermore, the mitogen-activated protein kinases (MAPKs) pathway alongside the estimation of pro-inflammatory cytokines (Figure 88) are also investigated. Specifically, the levels of phosphorylated ERK1/2 and phosphorylated p38 in lung tissue of sepsis-induced mice were estiamted. The results demonstrated a significant increase in the levels of phosphorylated ERK1/2 and phosphorylated p38 in the lung tissue of the CLP group compared to the control group (Figure 88, $p < 0.01$). However, the IS 217-treated group (at doses of 0.6 and 1.2 mg/kg) via the intravenous (IV) route exhibited a decrease in the expression of phosphorylated ERK1/2 and phosphorylated p38 compared to the CLP group, with the IS 217-1.2 mg/kg-IV route group showing particularly noteworthy results (Figure 88, $p < 0.01$ and $p < 0.05$). These findings suggest that IS 217 effectively alleviated sepsis-induced acute lung injury (ALI) in mice by modulating the MAPKs signaling pathway [Eun Jung Park et al., 2014].

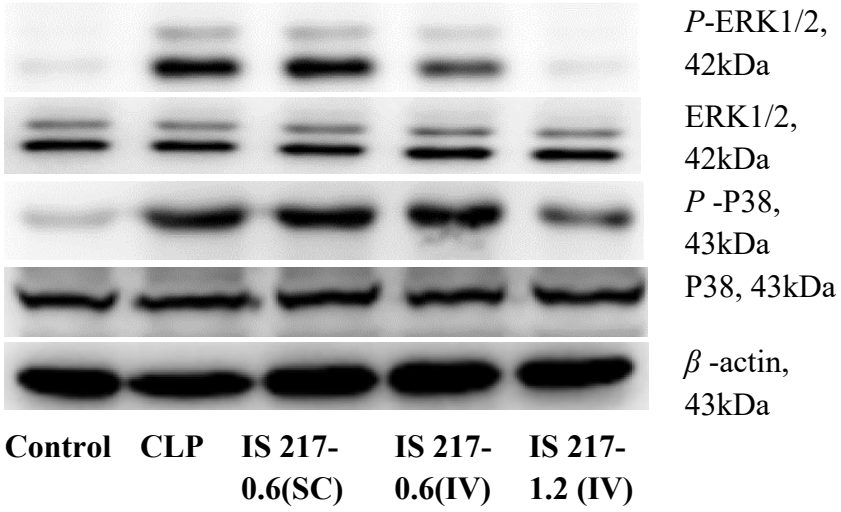


Figure 88. Representative bands showed the levels of, p-ERK, ERK, p-P38, and P38 protein in lung tissue at 18h after surgery.

The data was repeated by duplicate independent experiments. The results are pooled samples of each group.

IS 217 peptide treatment Inhibited the Activation of the Inflammatory Signaling Pathway: mitogen-activated protein kinases (MAPKs) pathway.

Summary:

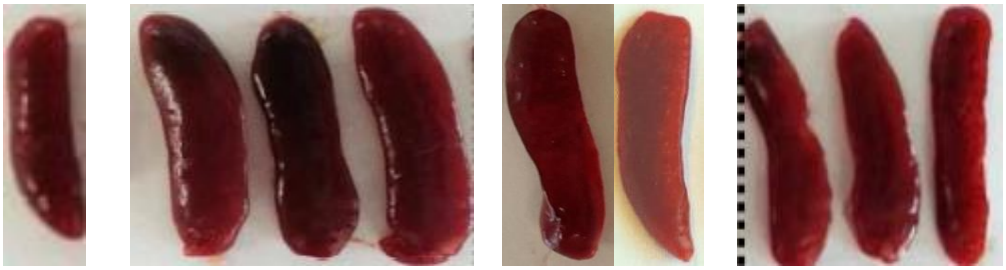
In summary, in vitro studies demonstrated that the IS 217 peptide displayed potent activity against the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 induced by LPS. This peptide exhibited its anti-inflammatory properties in a dose-dependent manner. Mechanistically, IS 217 peptide dose-dependently hindered LPS-induced ERK phosphorylation in macrophages.

Moreover, in mice models subjected to CLP-induced sepsis resulting in acute lung injury (ALI), treatment with **IS 217 significantly mitigated several aspects associated with ALI**. This included the reduction of CLP-induced pulmonary edema, pathological alterations, inflammatory cytokines in both serum and BALF, inflammatory cell infiltration, mRNA expression of inflammatory cytokines, and ERK phosphorylation.

These collective findings suggest that the IS 217 peptide holds considerable promise as a potential therapeutic agent for treating ALI. Although the precise anti-inflammatory mechanisms and specific targets of IS 217 remain undisclosed, its beneficial effects in countering LPS-induced inflammation highlight IS 217 as a noteworthy candidate for ongoing drug development and further research endeavors.

6.6. Cytokines estimation in the spleen:

The spleen functions as a crucial reticuloendothelial organ responsible for opsonization, phagocytosis of pathogens, and cellular maintenance [Iqbal J et al., 2016]. Despite its importance, there has been limited research focusing on determining cytokine levels within the spleen. In the current study, efforts were made to estimate cytokine levels in spleen tissue. The production of cytokines was quantified and expressed as pg/mL supernatant of spleen. The differences in relative organ weight of the spleen among various groups are illustrated in Table 76, and a graphical representation of these differences is depicted in Figure 90. Additionally, Figure 89 provides a macroscopic view of the spleens from all the experimental groups.



Sham control | **CLP** | **IS 217 -0.6 mg/kg** | **IS 217 -1.2 mg/kg**

Figure 89: Macroscopic pictures of spleen of all groups of mice -18h after CLP surgery.

Table 76: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on spleen relative organ weight.

Groups	Relative organ weight (g)
Control	0.73± 0.04
Sham control	0.87± 0.11
Disease control (CLP)	1.53± 0.32
CLP + IS 217-0.6mg/Kg	0.89 0.15***
CLP + IS 217-1.2mg/Kg	0.79± 0.03***

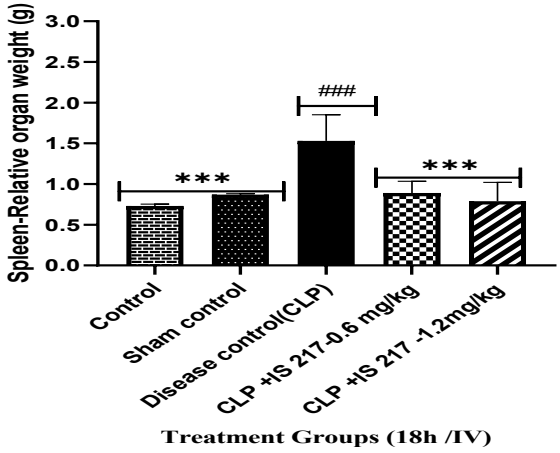


Figure 90: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on spleen relative organ weight changes of mice-18h after CLP surgery. The results represent means ± SEM of 5-6

mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* and ###, ##&# compared to disease control and sham control groups respectively.

IV treatment: In the spleen of sham controls (371.1±26.0 pg/mL) at 4 h post CLP, **TNF-α levels** were significantly higher than in IS 217-0.6 & 1.2 mg/kg treated mice (272.0±13.0, 249.1±15.7 respectively). However, TNF-α levels of CLP group are higher than IS 217 treated groups at 18 h and 10 days post CLP and not high as at 4h (figure 91).

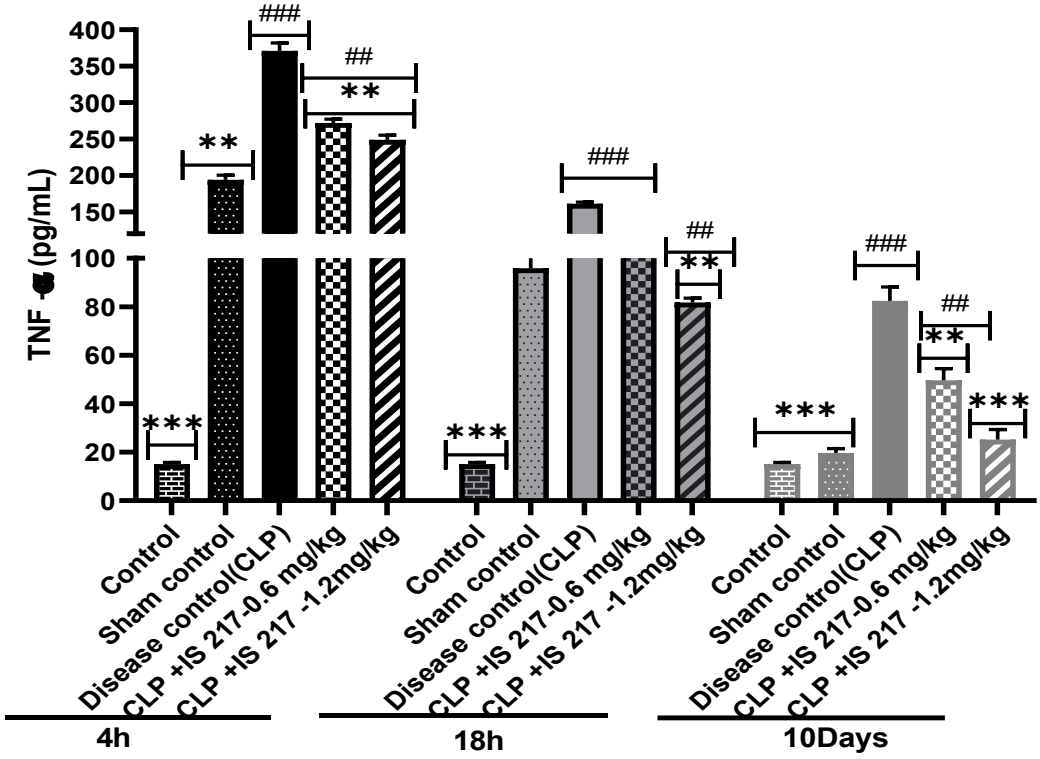


Figure 91: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on TNF-α levels in the spleen homogenate. Mice (n =5- 6/ group) and estimated at 4 h,18 h and 10 days post-CLP challenge via IV route. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* and ###, ##&#compared to disease control and sham control groups respectively.

A significant difference was found in **IL- 1β expression** between disease control and IS 217 treated group mice spleen at 4h,18h and 10 days post-CLP. IL-1β production in CLP mice at 4h, 18 h (402.1±21.3 and 310±13.5pg/mL) was less (208.7±4.2 pg/mL) at 10 days (figure 92). Post CLP Mice in treatment groups showed a significant increase in IL-1β levels in comparison to sham control at 4h,18h and 10 days post CLP, whereas at 18 h, the levels are decreased in comparison to 4h.

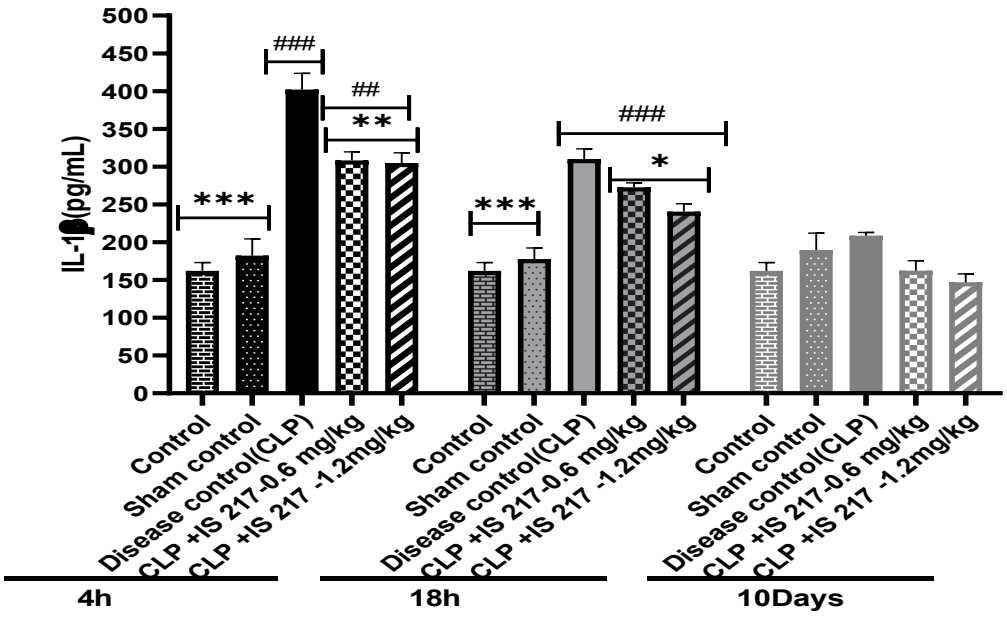


Figure 92: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on IL-1β levels in the spleen homogenate. The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$, $^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

The **IL-6 expression** in controls was not expressed and the levels of **IL-6 expression** was high in disease control CLP mice when compared to IS 217 treated groups and in treatment groups IS 217 -1.2 mg/kg showed better results when compared to IS 217 -0.6 mg/kg, when treated intravenously (figure 93).

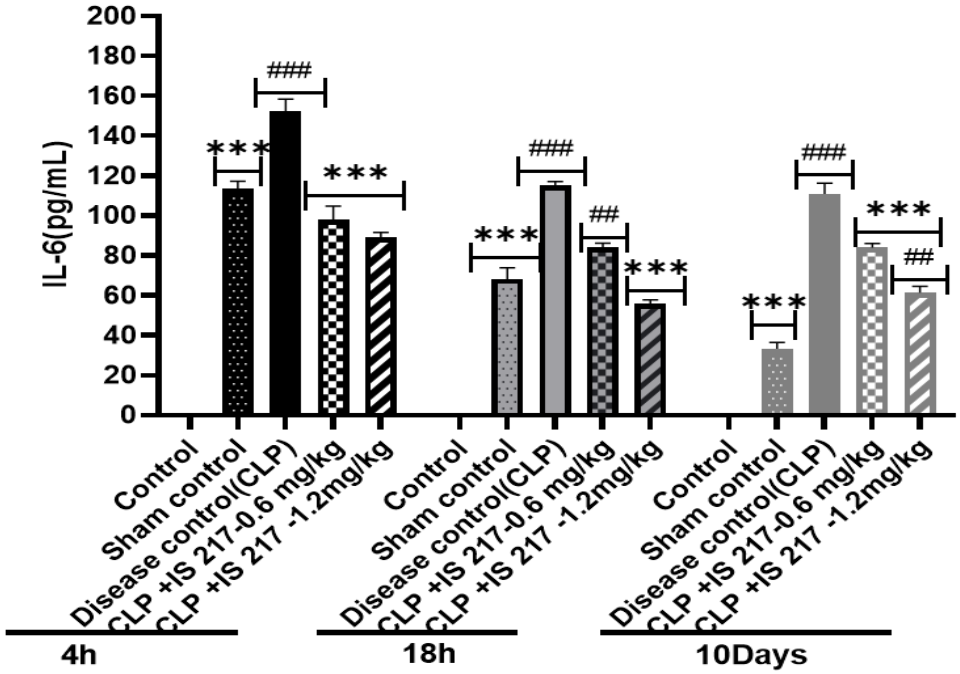


Figure 93: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on IL-6 levels in the spleen homogenate.

The study involved mice (n = 5-6/group), and cytokine levels were measured at 4 h, 18 h, and 10 days after the CLP challenge via the intravenous route. The data is presented as mean ± SEM. Statistical significance was considered at $p < 0.001$, $p < 0.01$, and $p < 0.05$, denoted as ***, **&*, and $^{###}$, $^{##}$&$^{#}$ compared to disease control and sham control groups respectively.

7. Histopathological changes of Lung &Liver tissues: IS 217 treatement ameliorated organ injury in CLP mice:

Organ damage is a leading cause of death in patients with sepsis. Thus, the organ protection afforded by IS 217in CLP mice was investigated. No significant changes were observed in the body weight of the animals and in relation to the organs weight, besides, no macro- or microscopic alteration was detected in the brain, heart, lungs, liver, kidney, and spleen. All the tissues from different experimental groups were harvested after 18 h, considering the early phase of immunosuppression and most of the animals of the CLP group are not survived while the other treated groups (IS 217 -0.6 mg/kg and IS 217 -1.2 mg/kg groups) lived longer, histological examinations were carried out on

lung and liver to assess lung and liver damage. The presence of congestion in the groups analyzed was due to the euthanasia procedure. Necrosis was not observed in any analyzed organs.

Briefly, lung and liver tissues were fixed in buffered 10% formaldehyde and then embedded in paraffin. The embedded tissue samples were sectioned (5 μm) and stained with haematoxylin and eosin to examine general histological features. These investigations showed that CLP-induced sepsis in mice caused hepatic inflammatory cellular infiltration, hepatic steatosis, and hepatic fibroplasia in the portal tract. A semi-quantitative scoring system was used. For liver tissue evaluation, hepatocyte degeneration and portal/lobular inflammation were scored (each 0–3), Lung injury scores were determined by assessing neutrophil infiltration, hemorrhage, necrosis, congestion and edema as previously described. The score of each tissue sample represented the mean score of ten different fields. The stained tissue sections were evaluated under a light microscope (Eclipse E200-LED; Nikon, Kawasaki, Japan) at ×200 magnification.

The general architectures of the lung and liver in the sham and normal control groups were of normal histological structure (figure 94). There was also no statistically significant difference between both the sham and Normal control groups ($P<0.01$). However, lung tissue in the CLP-control showed histopathological changes in the alveolar walls (figure 94). Also, interstitial edema, infiltration of polymorphnuclear leukocytes and monocytes, hemorrhage, vascular congestion, and cellular hyperplasia were observed, and the tissue damage was more prominent in the disease control group. Inflammatory cell types were generally neutrophils and macrophages. In the lungs, congestion and neutrophil infiltration were observed in both groups (table 77). Neutrophil infiltration into the alveolar space was not observed in any case, and no traces of pneumonia were noted in any of the lung samples (figure 94). The morphologic study showed that the lungs of CLP mice were damaged. Severe oedema, wider interalveolar septa, severe alveolar haemorrhage, and extensive inflammatory cell infiltration was observed. But the lungs and livers of the IS 217 -0.6 mg/kg group and IS 217 -1.2 mg/kg group had normal histological structure, when compared with the CLP group (figure 94). Mild lung oedema, haemorrhage, and inflammatory cell infiltration were seen in the IS 217 -0.6 mg/kg treatment group. Furthermore, it was evident that IS 217 (1.2mg/kg) reduced these injuries by reducing edema and macrophage infiltration. Histological evaluation of lung tissue revealed that IS 217 reduced macrophage infiltration and alleviated lung tissue damage (figure 94).

Table 77: Histopathology evaluation of lungs and livers from all the groups of 18h post -CLP surgery.

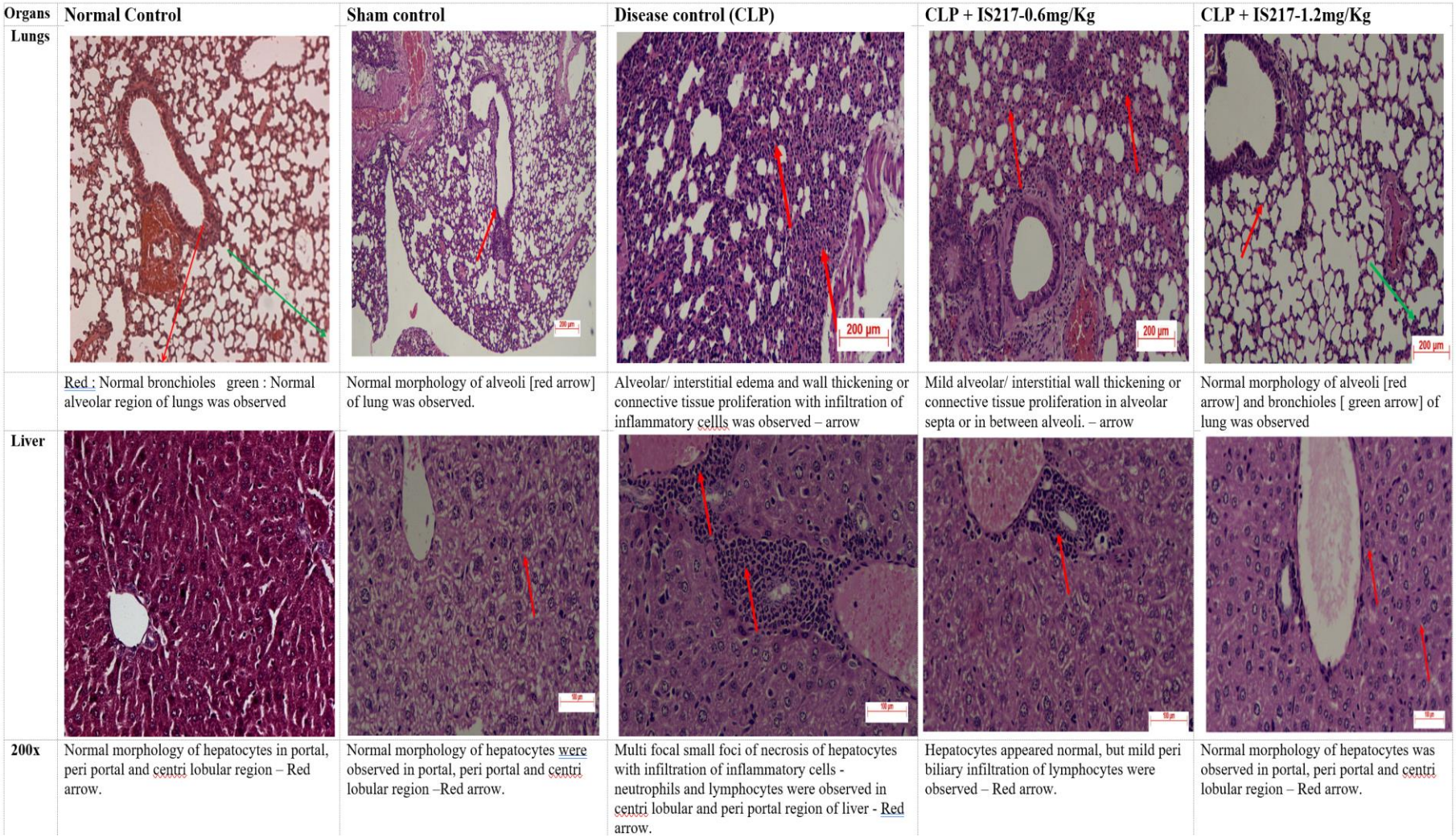
Parameters	Normal Control	Sham control	Disease control (CLP)	CLP + IS 217-0.6mg/Kg	CLP + IS 217-1.2mg/Kg
Lungs					
Inflammatory infiltration	0.0 ±0.00	0.0 ±0.00	1.8±0.40	0.7 ±0.50	0.4 ±0.20
Hemorrhage	0.0 ±0.00	0.0 ±0.00	1.3±0.70	0.0 ±0.00	0.0 ±0.00
Fibers accumulation	0.0 ±0.00	0.2 ±0.00	2.4±0.55	1.8±0.55	1.2±0.47
Tissue damage	0.0 ±0.00	0.0 ±0.00	2.2±0.53	0.0 ±0.00	0.0 ±0.00
Edema	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Necrosis	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Liver					
Inflammatory infiltration	0.0 ±0.00	0.0 ±0.00	2.8±0.70	0.5 ±0.22	0.2 ±0.00
Hemorrhage	0.0 ±0.00	0.0 ±0.00	2.2±0.40	0.0 ±0.00	0.0 ±0.00
Fibers accumulation	0.0 ±0.00	0.0 ±0.00	2.5±0.50	1.2±0.43	0.8±0.19
Tissue damage	0.0 ±0.00	0.0 ±0.00	2.6±0.35	0.0 ±0.00	0.0 ±0.00
Edema	0.0 ±0.00	0.0 ±0.00	0.4 ±0.70	0.0 ±0.00	0.0 ±0.00
Necrosis	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00

The mean ± SEM scores of the study were reported as follows: 0 represented the absence of injury, 1 indicated mild injury, 2 indicated moderate injury, and 3 indicated intense injury. The study involved groups of 3-4 mice in each group.

In the study, mice were administered either 0.6 mg/kg or 1.2 mg/kg of IS 217 after 2 hours of post-CLP surgery. The control group, which was uninfected, received an equivalent volume of normal saline alone. The levels of TNF-α, IL-6, and IL-1β in serum (refer to figures 75-78) and bronchoalveolar lavage fluid (BALF) (refer to figures 82-84) were measured using ELISA at 4 hours, 18 hours, and 10 days post-infection. At 18 hours post-infection, blood samples were collected from the mice via retro-orbital bleeding to measure AST & ALT levels. Additionally, the

mice were sacrificed, and histopathological changes in the lung and liver tissues of CLP mice were observed (figure 94) after 18 h of post CLP. The results demonstrated that the administration of IS 217 at a dose of 1.2 mg/kg significantly reduced lung and liver injury caused by sepsis, as indicated by the total histology score. The group receiving IS 217 at a dose of 0.6 mg/kg exhibited mild lung injury induced by sepsis (figure 94). The groups treated with IS 217 at doses of 0.6 mg/kg and 1.2 mg/kg showed a significant decrease in septic effects compared to the CLP-sepsis group ($P<0.01$). Notably, there was no statistically significant difference in lung histological structures between the sham group and the IS 217 -0.6 mg/kg and 1.2 mg/kg treated animals ($P<0.01$). Therefore, the study demonstrated that IS 217 effectively reduced pro inflammatory cytokines such as IL-1β, IL-6, and TNF-α, and improved the negative changes in MPO tissue levels and histopathological alterations in lung sections under the given conditions. Additionally, IS 217 inhibited macrophage infiltration into the liver in a concentration-dependent manner. The results presented in Table 70 indicated that CLP increased the levels of liver damage markers ALT and AST, while IS 217 significantly and dose-dependently reduced these levels (figure 62). The IS 217 -1.2 mg/kg treatment group exhibited a few neutrophil infiltrates in the portal veins, central veins of the liver, and renal small vessels (figure 94). The results indicated that IS 217 at a dose of 1.2 mg/kg reduced these injuries by decreasing edema and macrophage infiltration (figure 94), thereby minimizing CLP-induced lung and liver damage. Moreover, peptide IS 217 at a dose of 0.6 mg/kg moderately reduced the injuries. Furthermore, the findings presented in Table 68 showed that CLP led to increased levels of liver damage markers ALT and AST, whereas the IS 217 peptide significantly and dose-dependently reduced these levels. **Thus, the peptide IS 217 effectively inhibited TNF-α, IL-6, and IL-1β levels and reduced organ damage in septic mice.**

Figure 94: Histological sections of lungs (a) and liver (b) of IV treatment groups after 18h of post CLP.



Histological sections of lungs (a) and liver (b) of IV treatment groups after 18h of post CLP and stained with H & E, visualized at 200X magnification and the scale bar represents 200 µm for lungs and 100 µm for livers. Data shown in mean ±SEM from 3-4 mice of all groups of CLP induced sepsis, treatments, and control animals-IV route -18 h.

5.7.1.3. Discussion:

The cecal ligation and puncture (CLP) model was utilized to induce polymicrobial infection, resulting in positive blood cultures for *Escherichia coli*, *Streptococcus bovis, Proteus mirabilis, Enterococcus*, and *Bacteroides fragilis*. Bacteremia was observed in the peritoneal cavity fluid, which tested positive for the aforementioned microbes, as well as *Streptococcus viridians* and *Clostridium sporogenes*. This model also exhibited a mortality rate of 70% [Konstantin Tsoyi et al., 2009]. Mice that were mildly ill were sacrificed 10 hours after CLP, demonstrating the early hyperdynamic phase of sepsis, characterized by increased blood flow to organs, hyperinsulinemia, and hyperglycemia [Burgelman, M et al., 2021; Hotchkiss, R. S., et al., 2013]. The pro inflammatory cytokines TNFα, IL-1β and IL-6 levels that increase and remain elevated over an 8-hour period and peak levels from 5 to 18 hr and so in the present study, peritoneal lavage fluid (PLF), lungs, and sera were collected after 4 h of CLP surgery to investigate the effects of IS 217 treatments on the early hyperdynamic phase of sepsis. Mice sacrificed 18hours post-operatively represented a hypodynamic late septic state, characterized by decreased blood flow to organs, hypoinsulinemia, hypoglycemia, and high serum lactate levels. Therefore, in the present study, PLF, lungs, and sera were collected after 18 hours of CLP surgery to investigate the effects of IS 217 treatments on the late hypodynamic phase of sepsis. For survival and post-surgery observations to reflect the clinical time course of the sepsis, 10 days was kept as sampling point and also estimated the cytokines levels at 10^{th} day samples also. The CLP procedure encompasses various aspects that mimic the clinical course of sepsis, including polymicrobial infection of the peritoneum, release of bacteria and pathogen-associated molecular patterns (PAMPs) into sterile areas, and translocation of enteric bacteria into the bloodstream. This model the stages of intraabdominal clinical sepsis [Fourrier, F., C. et al., 1992].

The pro-inflammatory response during sepsis is characterized by significant increases in cytokines such as TNF-α, IL-1β, IL-6, and IL-12, which remain elevated for an 8-hour period [Schulte, W., J. Bernhagen et al., 2013; Matsukawa, A., 2003; KYUNG JUN JANG et al., 2016 & Gil, M., 2016]. Several studies have highlighted the crucial role of an early pro-inflammatory response in the progression of sepsis [Schuerholz et al., 2013; Singleton K, et al., 2003; Silva, O. N., 2016]. As part of the screening of the host response to CLP, the following estimations were performed in PLF, lungs, spleen, and sera, and the results are detailed in the results section [Stefan Wirtz et al., 2006;

Yung-Yang Liu1, 2008]. Consequently, in the present study, peritoneal lavage fluid was examined for cytokine levels and bacterial colony-forming units (CFUs) to evaluate the efficacy of IS 217 treatments.

Given that, only a subset of patients with severe sepsis and septic shock display signs of disseminated intravascular coagulation (DIC), the observed level of effectiveness is not surprising [Vincent, J. L. et al., 2005; Levi, M., 2010 & 1999]. In this study, a strategy involving the use of IS 217 peptide treatments to control bacterial multiplication, inhibit excessive proinflammatory cytokines and prevent DIC may be more effective in managing sepsis in humans. Recent research has demonstrated a direct correlation between systemic levels of IL-10 and the severity of the illness [Yona Kalechman, Uzi Gafter et al., 2002]. However, neutralizing IL-10 has been associated with higher mortality rates in septic peritonitis induced by cecal ligation and puncture (CLP) [Grace Y., 1999]. The study resuls showed that administering IS 217 peptide treatment two hours after CLP significantly prolonged the survival of septic mice. This was accompanied by a significant change in serum IL-10 levels and IL-10 release from peritoneal macrophages 24-48 hours after CLP. Treatment with IS 217 restored the ability of these cells to release TNF-α, IL-6, IL-12, and IL-1β for a period of 10 days. This improvement in cytokine release was reflected in the 10-day survival rate and tissue damage. Recent findings from [Grace Y. Song, et al., 1999] suggest that IL-10 may have a negative impact on host antimicrobial clearance systems during later stages of infection. However, when administered two hours after CLP in a cecal ligation model, IS 217 peptide therapy demonstrated a protective effect. These results emphasize the critical importance of timing in modulating IL-10 activity in sepsis.

Additionally, IS 217 peptide treatment improved bacterial clearance in the peritoneum and blood and reduced severe multiple-organ damage, as indicated by clinical chemistry. Furthermore, peptide IS 217-treated mice showed a significant decrease in lung myeloperoxidase levels, which indirectly reflects the recruitment of neutrophils. This study proposed that the non-immunogenic peptide IS 217, with its ability to inhibit IL-10 and enhance macrophage functions, may have clinical potential in sepsis treatment, particularly when administered during the early phase characterized by immune suppression.

Sepsis survivors often experience acute and long-term cognitive impairments, and microglial activation plays a role in the pathophysiology of neurological dysfunction in sepsis. Therefore, in CLP model, the memory and learning abilities of mice post-CLP surgery using the novel object

recognition test are investigated. The groups treated with IS 217 peptide displayed deficits in long-term object recognition memory function but showed normal activity comparable to the control groups after post-CLP surgery.

Acute lung injury (ALI) is a common complication of sepsis. Sepsis-induced ALI is believed to be dependent on polymorphonuclear neutrophils, resulting in a cytokine/chemokine storm in the lungs that leads to severe ALI and acute respiratory distress syndrome [Yibin Zeng et al., 2022; Yali Zhang et al., 2015]. Sepsis initially triggers systemic inflammation mediated by pro-inflammatory cytokines/chemokines such as TNF-α, IL-1β, IL-6, IL-12, and VEGF, as well as anti-inflammatory cytokines like IL-10 [Stefan Wirtz et al., 2006; Jinbao Li et al., 2012]. These proinflammatory cytokines induce the recruitment and activation of neutrophils, NK cells, and monocytes/macrophages, which produce harmful reactive oxygen species and lysosomal enzymes [Anasuya Patel et al., 2020]. Studies have demonstrated that the stress induced by cecal ligation and puncture (CLP) can activate the MAPK signaling pathway, regulate cytokines TNF-α, IL-1β, and IL-6, and recruit/activate white blood cells through the production and activation of inflammatory mediators. These inflammatory cascades triggered by the activation of inflammatory signaling pathways contribute to the development of ALI [Eun Jung Park et al., 2014]. In this study, the results showed that the treatment with peptide IS 217 can reduce the protein levels of phosphorylated ERK1/2 and p38 in lung tissue, indicating that the effect of IS 217 peptide is involved in the MAPK signaling pathway.

Overall, our study explored the effects and potential mechanisms of peptide IS 217 in CLP induced sepsis and sepsis-induced ALI mice in vivo. The results demonstrated that peptide IS 217 significantly reduces the expression of phosphorylated ERK1/2 and p38 in lung tissue. Additionally, peptide IS 217 dramatically decreases the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and VEGF, while increasing the levels of the anti-inflammatory cytokine IL-10. Furthermore, IS 217 peptide alleviating the histopathological injury in the lungs & livers. These findings indicate that the protective effect of peptide IS 217 on ALI may be associated with the MAPK signaling pathway.

5.7.1.4. Summary:

In summary, **IS 217 peptide showcases notable protective effects in CLP-induced mice by mitigating inflammation, improving lung histopathology, and reducing white blood cell count,**

primarily by modulating the MAPK signaling pathway. These findings highlight that administering IS 217 peptide diminishes bacterial growth, likely by activating phagocytes, while also curbing the release of inflammatory cytokines and limiting tissue damage in septic mice.

Furthermore, the peptide IS 217 serves as a shield against sepsis-related mortality, underscoring its potential as a targeted therapeutic using host defense peptides against sepsis. Consequently, IS 217 emerges as a promising therapeutic agent against lethal sepsis, as evidenced by its antibacterial and anti-inflammatory effects observed in CLP-induced sepsis.

Moreover, our study indicates that IS 217 peptide treatment may offer protective effects against sepsis-induced acute lung injury (ALI) by dampening inflammatory responses in lung tissue. This suggests its potential as a potent safeguarding agent against pulmonary injury, especially in cases of COVID-19 pneumonia.

5.7.2. Results of In vivo Proof of Concept (PoC) efficacy study by using *E. coli* induced peritonitis model in mice:

5.7.2.1. Results of pilot study -Standardization of *E. coli* induced peritonitis:

In the in vivo proof of concept (PoC) efficacy study using an *Escherichia coli*-induced peritonitis model in mice, the following results were obtained: The standardization of *E. coli*-induced peritonitis was performed in the pilot study. After isolating, identifying, and counting the *E. coli* bacteria (strain 8739™), the lethal dose (LD-50) was determined for infection. Mice, being less sensitive than humans, rats, or mice, can effectively clear a large number of nonpathogenic *E. coli* bacteria without mortality. In acute *E. coli* infection, the inflammatory response is predominantly harmful, so the most effective anti-inflammatory agents generally improve organ function and/or survival.

However, a dose of 1.5x 10^8 per mouse typically results in 10-20% mortality. Therefore, the model was standardized using doses of 2.5x 10^8 and 5x 10^8 per mouse, as these doses were sufficient to identify decreased resistance to sepsis, which would lead to higher mortality. This model is intended to represent sepsis in humans, where the loss of gastrointestinal barrier function (due to factors like trauma, appendicitis, impaired liver function, or other conditions) initiates the disease. In human peritonitis, a single species of bacteria often dominates, with *E. coli* being isolated from blood cultures in approximately half of the cases. By administering a single strain of indigenous *E. coli* in this model, attempt was made to create more controlled conditions compared to cecal ligation and puncture, while still inducing peritonitis and sepsis similar to what is observed in humans.

In this study, *E. coli*-induced infection was standardized using doses of 2.5x 10^8 and 5x 10^8 CFU in eighteen mice aged 8-10 weeks. After 18 hours of infection, cytokine levels were measured in the sera using ELISA kits (R&D Biosystems) to assess the extent of infection caused by the 2.5x 10^8 and 5x 10^8 CFU doses and to establish the standardized infection dose (Figure 95).

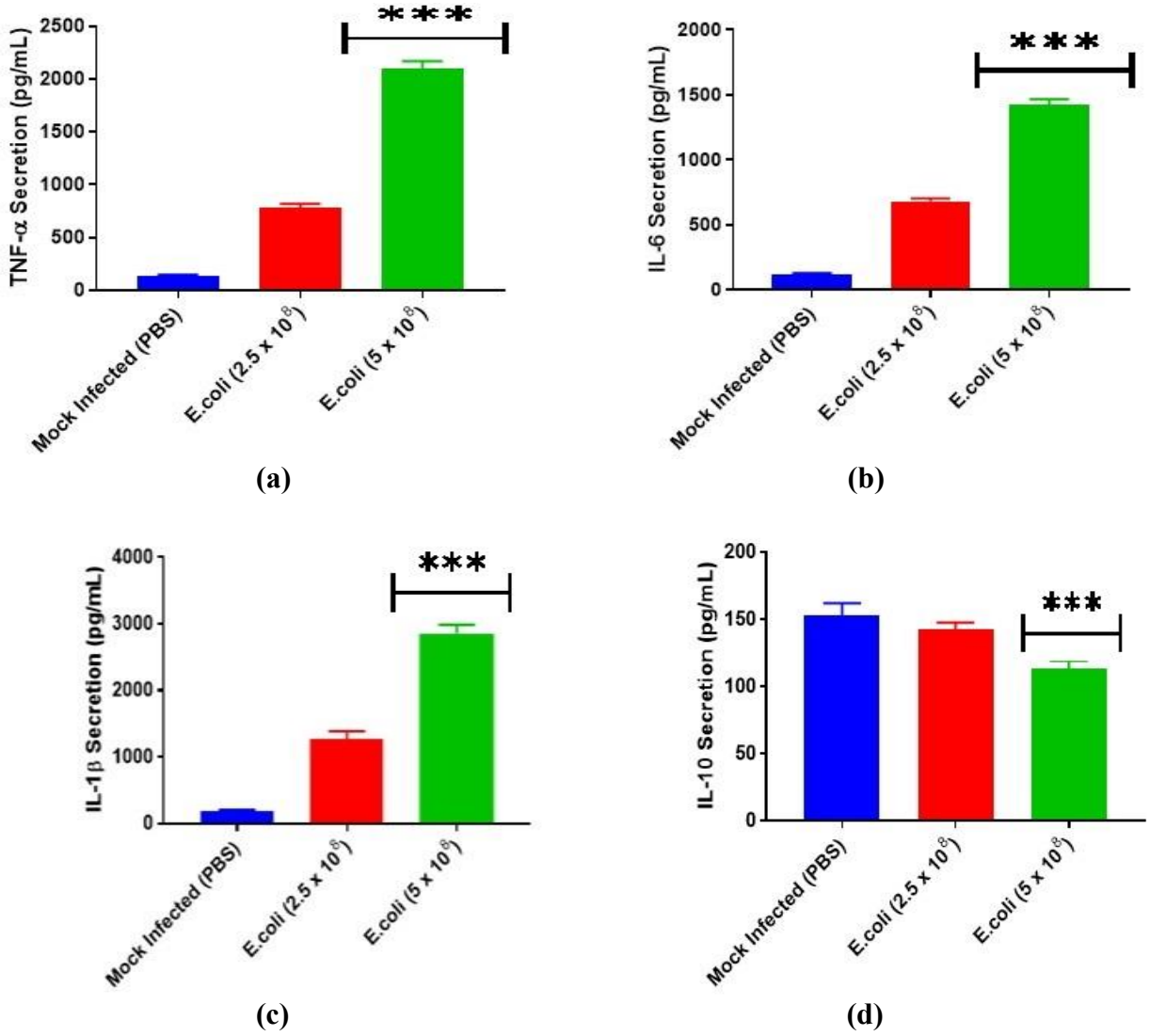


Figure 95 (a,b,c&d): Production of cytokines. Quantification of TNF-α (a) , IL-6 (b), IL-1β (c) & IL-10 (d) in serum was performed 18 h after the *E. coli* infection. The results were expressed as the mean ±SEM (n=4-5 animals/group).

5.7.2.2. Results of main *E. coli* induced infection model study:

Synthetic peptide IS 217 provided broad-spectrum protection against lethal infections caused by E. coli in mice.

Considering the potent antimicrobial properties and immunomodulatory activity of peptide IS 217 observed in the CLP model, further the efficacy of IS 217 peptide was assessed in an *E. coli*-induced infection model. In this study, a highly aggressive bacterial infection mouse model was established by intraperitoneal injection of *E. coli* ATCC 8739™ bacteria. Two hours later, the mice were treated

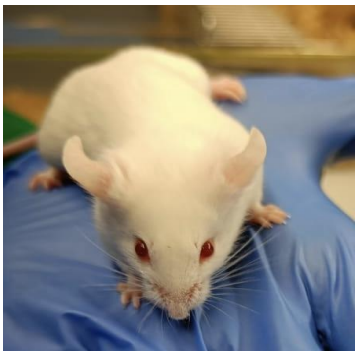
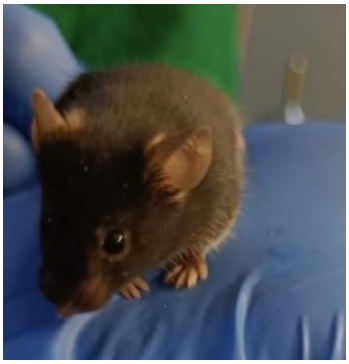
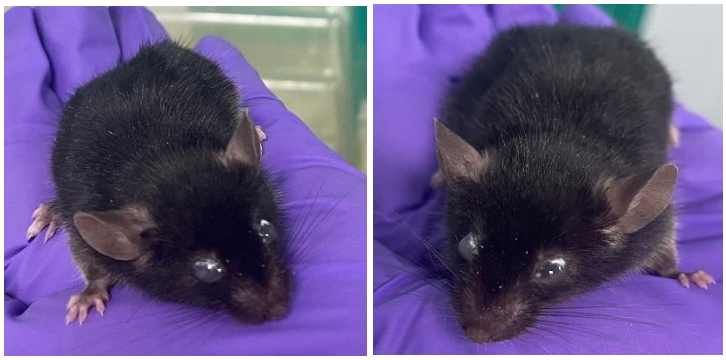
with 0.6 mg/kg and 1.2 mg/kg of peptide IS 217 suspended in sterile saline via subcutaneous (SC) and intravenous (IV) administration. The in vivo protective activity of IS 217 peptide was evaluated in this invasive infection model of *E. coli*. Daily treatments were administered for 5 days. After 18 hours of infection, half of the surviving animals from each group were anesthetized for blood collection and euthanized to collect organs, while the other subset was monitored for survival rate over 7 days. Mice treated with peptide IS 217 appeared to be in better clinical condition compared to infected animals after the induction of septic shock. The mice infected with 5.0 x 10^8 CFU of *E. coli* were given either normal saline or treated with 0.6 mg/kg and 1.2 mg/kg of IS 217 via IV & SC administration 2 hours after infection. After 18 hours of infection, the activity index was measured, and the parameters listed in Table 78 were observed. Each group consisted of 5-6 mice, and photographic representations of the diseased mice are shown in Figure 96. This confirms the development of *E. coli* infection with 5.0 x 10^8 CFU, and the severity of symptoms was graded as normal, mild, marked, or severe. Similar observations were made for SC treatment of peptide IS 217, but the results were not as significant as those observed for IV treatment. Mice treated with peptide IS 217 appeared to be in better clinical condition after the induction of septic shock.

Mice treated with IS 217 appear clinically healthier after induction of septic shock.

Table 78: Activity index of mice after 18hr of post *E. coli* Infection induction of septic shock.

Parameter	**Disease Control** (*E. coli* Infection)	**IS 217-0.6mg/kg**	**IS 217 –1.2 mg/kg**
Conjunctivitis	Extreme (Both eyes closed with heavy serous discharge) (severe)	Mild (Usually serous discharge present in one eye, eyes were open)	Mild (both eyes are open with moderate discharge)
Hair coat	Extremely ruffled coat, ruffling evident as early as 3 h and at 18 h.	Moderately ruffled coats at 20 h. No ruffling seen at 3 h.	Mild ruffling in most animals. Some animals had shiny unruffled coats.
Activity upon stimulation	Moribund, extremely sluggish, animals exhibited huddling behavior, did not respond or move to stimulation.	Sluggish activity. Animals exhibited huddling behavior but upon stimulation responded and broke out of huddle.	Animals remained normally active.

Figure 96: Experimental mice show the signs of infection at 18 h after post *E. coli* ATCC 8739™ (5.0 X10^8 *E. coli* CFU/per mouse).

Mice	**Normal mice**	**Diseased mice**: Infection with *E. coli* ATCC 8739™ (5.0 X10^8 *E. coli* CFU/per mouse)
BALB/c mice -at 18 h post infection.		
C57BL/6 mice -at 18 h post infection.		

1. Body temperature:

Mice were observed at least every 2 h after the challenge, and mice that were moribund and as hypothermia / fever is the first symptom of sepsis/ infection, the body temperatures are measured and noted (table 79), those with a body temperature less than 33^{0}C were euthanized and counted as dead at each time point indicated. In this model of sepsis (as in some cases of sepsis in humans), profound hypothermia is observed. However, in mice that will ultimately survive, hypothermia is less severe. It should be noted that severe illness typically proceeded to death very quickly, such that most deaths occurred between the 6-h observation points, and only a few cases of moribund mice or mice with very low body temperatures were removed from the study.

Table 79: Body temperatures of mice treated with IS 217 at 18 h after induction of septic shock.

Groups	Body temperature(^{0}C)18h	
	IV treatment	**SC treatment**
Control	37.1 ±0.05	37.5 ±0.42
Disease Control	34.5±1.09	33.9±1.02
IS 217-0.6 mg/kg	35.8±0.43*	34.5±1.34*
IS 217 –1.2mg/kg	36.7±0.67**	36.4±0.67**

Data are expressed as mean ± SEM. Mice (n =5- 6/ group) and estimated at 18 h post-CLP challenge via IV route. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

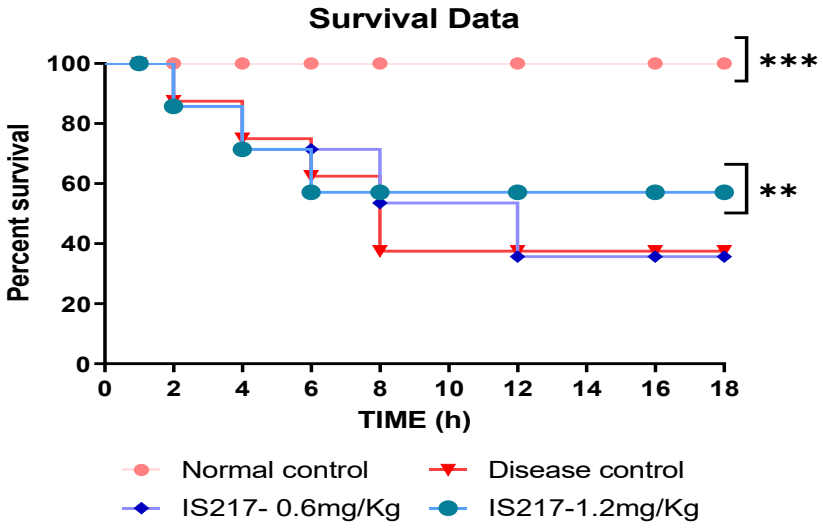
2. Experiment 1 Survival study:

IS 217 treatment enhanced Survival of mice after induction of sepsis:

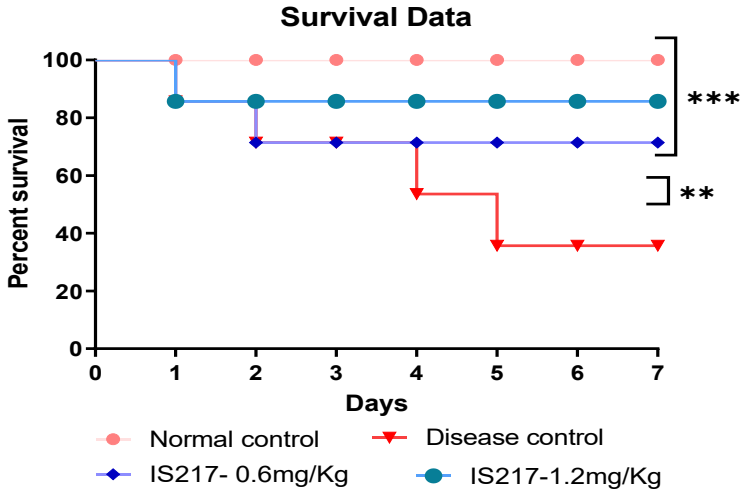
The Kaplan–Meier curve for survival analysis of mice subjected to polymicrobial sepsis and treated with peptide IS 217 (0.6 &1.2mg/kg) showed lower mortality after sepsis when compared with non-treated septic mice (Fig. 97 &98). Peritonitis induced by *E. coli* infection results in 100% lethality at day 8(Fig. 97 ,98) Induction of infection was described in the methods section and animals were Intravenously (IV) & subcutaneously (SC) injected with various doses of IS 217 or vehicle at 2h after post infection and then mice were observed for 18 h (Table 80a,81 &Figure 97a ,98a) and subset of animals are continued for observation for 7 days (Table 80a,81 &Figure 97b ,98b). No significant difference was observed when IS 217 was injected at the time of injury (time zero). Notably, treatment with IS 217 18 h decreased survival, compared with disease control mice.

Table 80a: The Percent survival rate of all the groups of post-*E. coli* infection for 18 h & 7 days with IV administration of IS 217.

Time/days	No. of deaths in groups			
	Normal control	**Disease Control** (Infection)	**IS 217-0.6mg/kg**	**IS 217 –1.2 mg/kg**
18h	0	5	4	3
From 24 h to 7 days	0	4	2	1



(a)



(b)

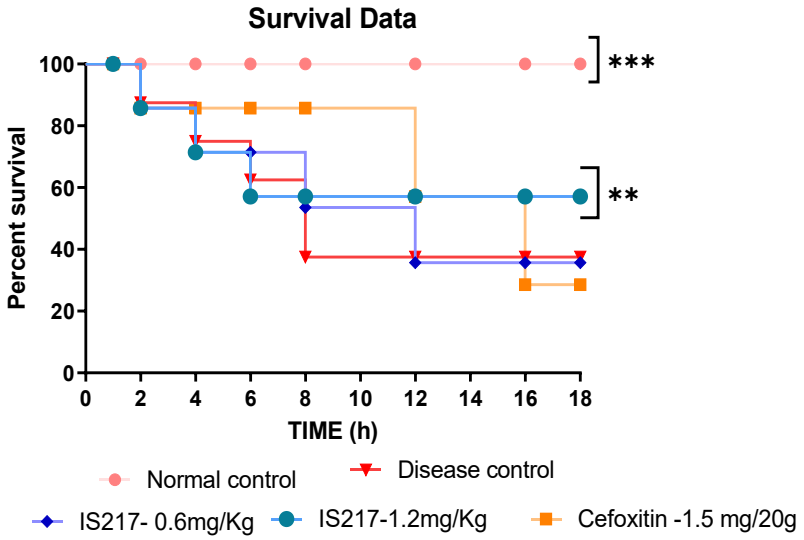
Figure 97(a,b): The therapeutic administration of peptide IS 217, Intravenously (IV), improves survival in septic mice. Survival was monitored at regular intervals until 18 h (a), and survival curves were plotted at 7 d (b), which was a representative graph of the remaining animals from 24 h up to7 d. Data are shown as mean ± SEM of 5-6 mice in each group. Significance in differences of survival percentages were assessed by a log rank test for trend. A significant difference from the disease control (*E. coli:* 5X10^8 CFU/per mouse) was indicated as $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

As shown in figures 97 a & b, both IS 217 0.6 and 1.2 mg/kg of IV treatment prolonged the survival of infected mice in the treatments (IV). After 7 days of infection, 37.5% survival was observed for

animals infected with *E. coli* ATCC 8739™ (table 80a, b &figure 97a,b&c) and treated with 0.6 and 1.2 mg/kg of IS 217 was 53.5 % and 68.71 % respectively. *E. coli* infected mice treated with IS 217 at a dose of 1.2mg/kg showed a survival rate of 50 % after 18h of Infection (figure 97a,). In this case, the lethality rate in the PBS control group was 100%. All groups treated immediately after infection with IS 217 (0.6 and 1.2 mg/kg) showed a better outcome than disease control groups ($P < 0.05$). The survival rates in mice were 71 % in both groups treated with IS 217 (0.6 and 1.2 mg/kg). The IS 217-IV treatment shows similar results to the standard drug Cefoxitin (table 80 b &figure 97c).

Table 80b: The Percent survival rate of all the groups of post *E. coli* infection for 18 h with IV administration of IS 217, compared with standard marketed drug (Cefoxitin).

Time/days	**No. of deaths in groups**				
	Normal control	**Disease Control** (Infection)	**IS 217-0.6mg/kg**	**IS 217 –1.2 mg/kg**	**Cefoxitin (1.5 mg/20g)**
18h	0	5	4	3	3

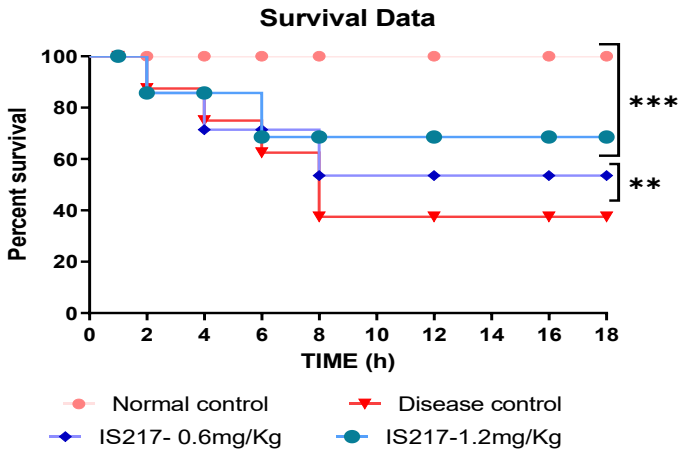


(c)

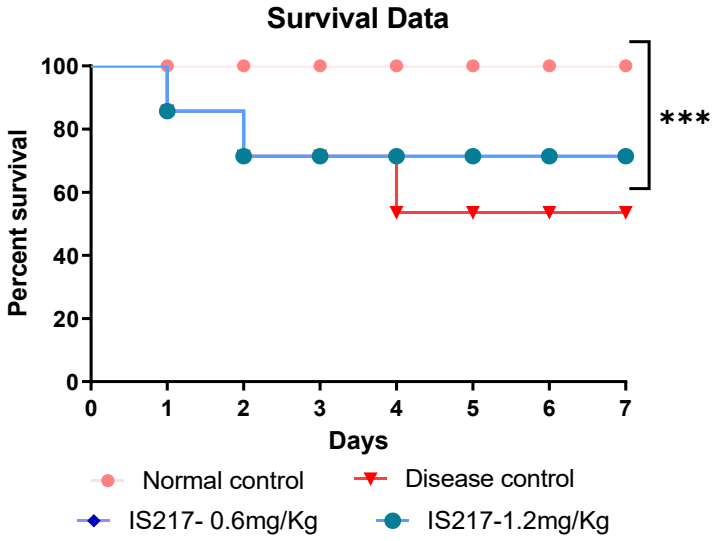
Figure 97c: The therapeutic administration of IS 217, Intravenously (IV), improves survival in septic mice in comparison with standard drug (Cefoxitin). Survival was monitored at regular intervals until 18h and survival curves were plotted. Data are shown as mean ± SEM of 5-6 mice in each group. A significant difference from the disease control (*E. coli:* $5X10^8$ CFU/per mouse) was indicated as $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

Table 81: The Percent survival rate of all the groups of post *E. coli* infection for 18 h & 7 days with SC administration of IS 217.

Time/days	No. of deaths in groups			
	Normal control	Disease Control (*E. coli* Infection)	IS 217- 0.6mg/kg	IS 217 –1.2 mg/kg
18h	0	5	3	2
From 24 h to 7 days	0	4	1	1



(a)



(b)

Figure 98 (a,b): The therapeutic administration of peptide IS 217, Subcutaneously (SC), improves survival in septic mice. Survival was monitored at regular intervals until 18 h (a), and survival curves were plotted at 7 d (b), which was a representative graph of the remaining animals from 24 h up to7 d.

Data are shown as mean ± SEM of 5-6 mice in each group. Significance in differences of survival percentages were assessed by a log rank test for trend. A significant difference from the disease control (*E. coli:* 5X10^8 CFU/per mouse) was indicated as $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

As shown in Fig. 98, both IS 217 0.6 and 1.2 mg/kg of SC treatment prolonged the survival of infected mice in the treatments (SC). After 7 days of infection, 37.5% survival was observed for animals infected with *E. coli* ATCC 8739™ (table 81& figure 93) and treated with 0.6 and 1.2 mg/kg of IS 217 was 71.4 % and 85.71 % respectively (figure 93). *E. coli* infected mice treated with IS 217 at a dose of 1.2mg/kg showed a survival rate of 60 % after 18h of Infection (figure 98 a). In this case, the lethality rate in the disease control group was 100%. All groups treated immediately after infection with IS 217 (0.6 and 1.2 mg/kg) showed a better outcome than disease control groups ($P < 0.05$). The survival rates in mice were 40, and 60 % in the groups treated with IS 217 (0.6 and 1.2 mg/kg, respectively).

3. Biochemical markers, CRP levels and DIC:

3.1. Liver & kidney function tests: To better understand how IS 217-treated mice developed resistance to *E. coli* infection, experiments were conducted to examine the extent of organ damage caused by the infection and its response to IS 217. To assess this, we measured certain indicators of liver damage (AST, ALT) and kidney function (creatinine, BUN) after administering IS 217 intravenously (IV) and subcutaneously (SC) to the infected mice at 18 hours post-infection (Table 82 &83 and Figure 99a, b).

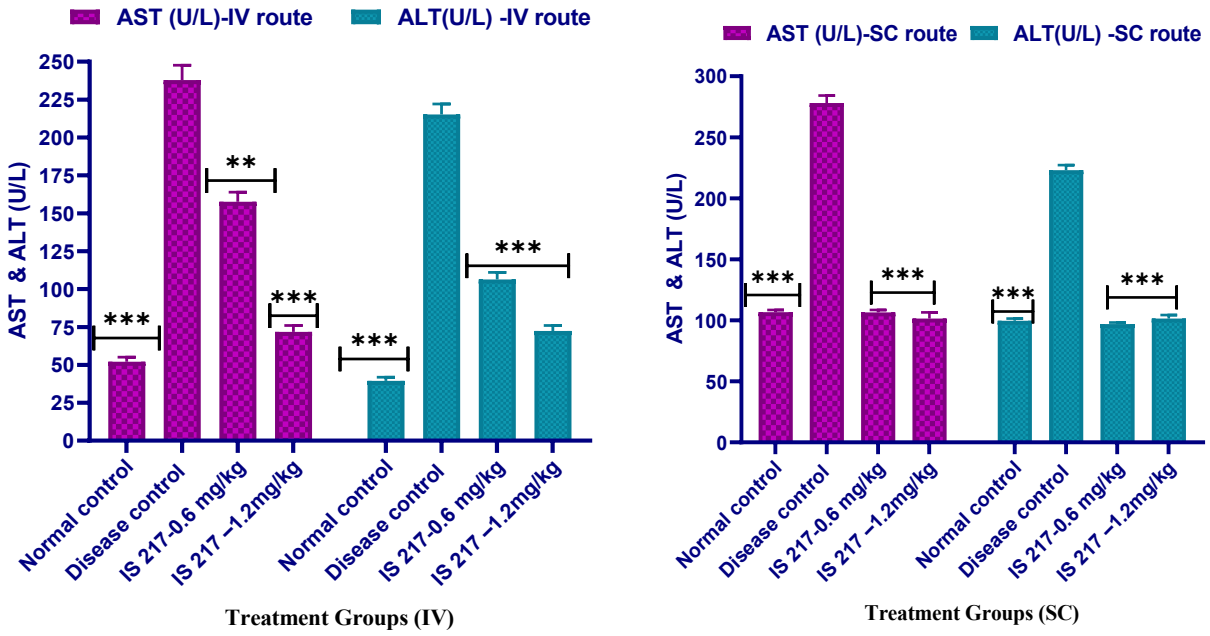
The results demonstrated that the infected mice exhibited more severe damage to multiple organs, as indicated by higher concentrations of ALT, AST, creatinine and BUN. Conversely, peptide IS 217-treated mice showed levels of these indicators comparable to those of normal control mice. This suggests that peptide IS 217 treatments effectively prevented organ injury in the treated mice.

Table 82: Effects of peptide IS 217 (0.6 & 1.2 mg/kg -IV) administrations on serum liver & kidney function tests in *E. coli*- induced sepsis in mice.

Groups	ALP(U/L)	AST (U/L)	ALT(U/L)	Cr (mg/dL)	BUN (mg/dL)	BIL (µmol/L)
Control	171.6 ±1.60	52.2 ±2.99	39.4 ±2.39	0.37 ±0.05	52.2 ±1.84	1.55 ±0.42
Disease Control	321.1 ±3.88	237.9 ±9.86	215.3 ± 6.91	0.66 ±0.03	155.5 ±6.44	7.7 ±1.11
IS 217-0.6 mg/kg	210.4± 2.09	157.7 ±6.31	106.4 ± 4.59	0.50 ±0.04	95.4 ± 4.83	5.4 ± 0.95
IS 217 – 1.2mg/kg	179.8±2.05	71.8 ± 4.19	72.4 ±3.69	0.42 ±0.03	71.9 ± 3.61	2.7 ±0.81

Table 83: Effects of peptide IS 217 (0.6 & 1.2 mg/kg -SC) administrations on serum liver & kidney function tests in *E. coli*- induced sepsis in mice.

Groups	ALP(U/L)	AST (U/L)	ALT(U/L)	Cr (mg/dL)	BUN (mg/dL)	BIL (µmol/L)
Control	121.6 ±1.60	106.7± 1.70	99.64±18.04	0.37±0.05	52.2±1.84	0.43± 0.05
Disease Control	245.1 ±2.33	278.10±6.07	223.1± 4.12	0.66±0.03	155.5 ±6.44	0.67 ±0.07
IS 217-0.6 mg/kg	156.6± 3.90	106.4 ±2.02	97.0 ±1.15	0.50±0.04	95.4± 4.83	0.89 ±0.04
IS 217 – 1.2mg/kg	143.5±5.02	101.4± 5.13	101.4± 2.98	0.42±0.03	71.9± 3.61	0.47 ±0.09



(a) **(b)**

Figure 99 (a&b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV & SC) administration on liver and kidney functions of mice after *E.coli* infection. At 18 h after infection induction, mice were killed, and the amounts of AST and ALT in sera were measured. The results represent means ± SEM of 5-6 mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically 5significant and denoted as ***, **&* compared to disease control groups respectively.

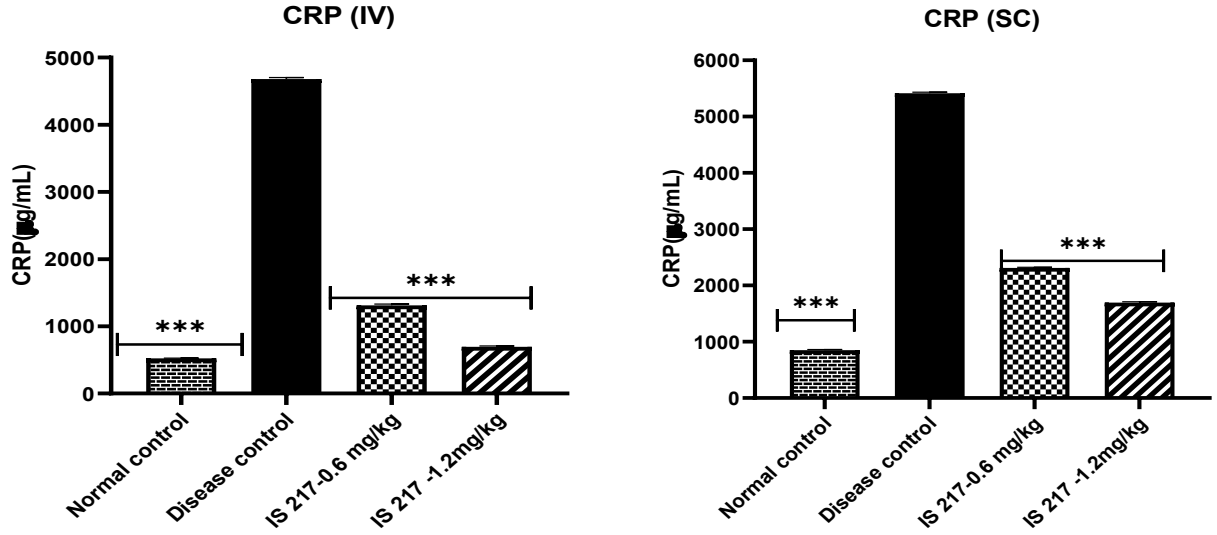
3.2. Effect of peptide IS 217 treatments on serum CRP levels:

Cytokines play a crucial role in either inhibiting or promoting inflammation by activating or deactivating genes involved in cellular responses. Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 can stimulate the liver to produce C-reactive proteins (CRP), which are significantly elevated during acute inflammation. In the current study, the levels of CRP were measured to assess the inflammatory response. The disease control animals, due to the infection, exhibited increased CRP levels compared to the groups treated with IS 217 at doses of 0.6 mg/kg and 1.2 mg/kg, administered via both intravenous (IV) and subcutaneous (SC) routes. These findings are presented in Table 84 and Figure 100, indicating that IS 217 treatment effectively reduced CRP levels in the treated groups compared to the disease control group.

Table 84: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV & SC) administrations on serum CRP levels in *E. coli*- induced sepsis in mice.

Groups	CRP(µg/mL) -18h	
	IV treatment	SC treatment
Control	524 ±2.80	852 ±8.29
Disease Control	4680±22.30	5415±17.60
IS 217-0.6 mg/kg	1313±17.90***	2313±10.56***
IS 217 –1.2mg/kg	694±12.65***	1694±15.09****

Data are expressed as mean ± SEM. Mice (n =5- 6/ group) and estimated at 18 h post- *E. coli*- infection via IV route. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.



(a) **(b)**

Figure 100 (a,b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV & SC) administration on serum CRP levels of mice after *E.coli* infection.

The results represent means ± SE of 5-6 mice/group. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group respectively.

3.3. DIC -Coagulation tests: To evaluate the potential of peptide IS 217 as a therapeutic tool for sepsis treatment, its ability to reduce inflammation and disseminated intravascular coagulation (DIC)

was investigated. Therapeutic administration of IS 217 was examined to determine its inhibitory effect on DIC.

BALB/c mice were infected with 5 x 10^8 CFU of E. coli ATCC 8739 and then injected with IS 217 two hours post-infection. After 18 hours, blood samples were collected and coagulation parameters associated with DIC were assessed. The results showed that therapeutic administration of IS 217 (IV) led to a reduction in clotting time, as indicated by decreased prothrombin time (PT) and activated partial thromboplastin time (aPTT) values (Table 85). These findings demonstrate that peptide IS 217 can effectively prevent inflammation and DIC in septic mice, even when administered therapeutically.

Table 85: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) administrations on Coagulation tests at 18 h in *E. coli*- induced sepsis in mice.

Groups	**PT** (sec.)	**aPTT**(sec.)
Control	9.53	55.92
Disease Control	13.26	66.28
IS 217-0.6 mg/kg	10.68***	60.82***
IS 217 –1.2mg/kg	10.16***	56.94***

Data are expressed as mean ± SEM. Mice (n =5- 6/ group) and estimated at 18 h post- *E. coli*-infection via IV route. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

4.Experiment 2: Acute hyper inflammation study: Cytokine estimation in serum:

4.1. The peptide IS 217 prevents *E. coli*-induced septic death and inhibits TNF-α, IL-1β, IL-12 and IL-6 release in mice serum:

In the current study, two doses of IS 217 were evaluated using a mouse model of *E. coli*-induced peritonitis sepsis. The mice challenged with an intraperitoneal injection of *E. coli* strain exhibited significantly elevated levels of TNF-α and IL-1β in their serum, indicating an induction of the pro-inflammatory response. However, treatment with a single dose of IS 217 (0.6 or 1.2 mg/kg) administered intravenously (IV) at 18 hours and 7 days, and subcutaneously (SC) at 18 hours post-

infection, provided significant protection against lethal septic shock (Figure 101-104). Measurement of pro-inflammatory cytokines (IL-6, IL-12, IL-1β, TNF-α) and an anti-inflammatory cytokine (IL-10) in the serum revealed that IS 217 treatments via IV route significantly attenuated the serum levels of TNF-α (Figure 101), IL-6(Figure 102a), IL-1β(Figure 102b), IL-12(Figure 103a)and IL-10 (Figure 103b) at 18 hours compared to *E. coli*-treated mice and SC route results are depicted in the figure104(a,b). Moreover, most mice receiving IS 217 treatment doses for 5 days survived up to 7 days after the injection, indicating the protective effect of IS 217 against *E. coli*-induced peritonitis. These results demonstrate that acute administration of IS 217 significantly improves the survival rate of mice at risk of septic death caused by *E. coli*, exerting its effects through anti-inflammatory modulation of cytokine production.

IV treatment:

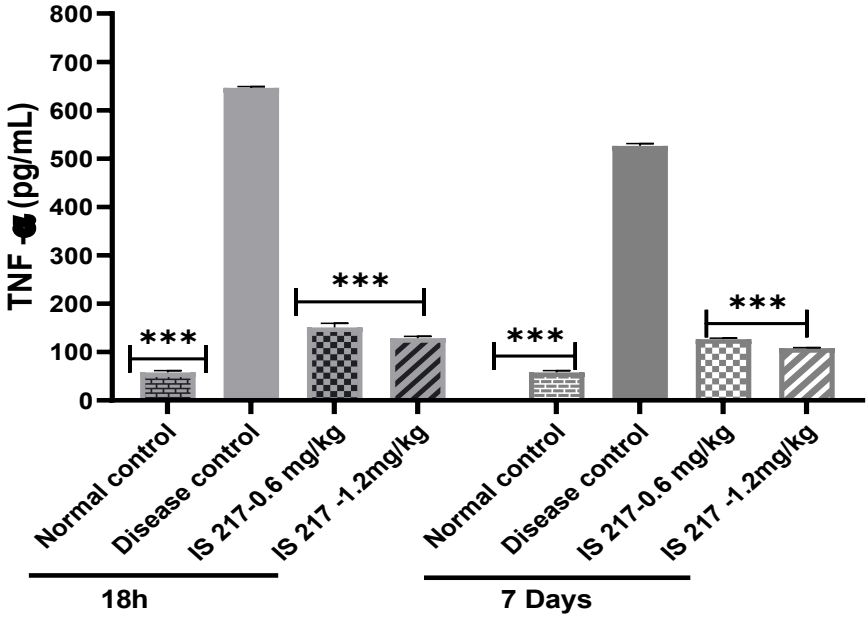
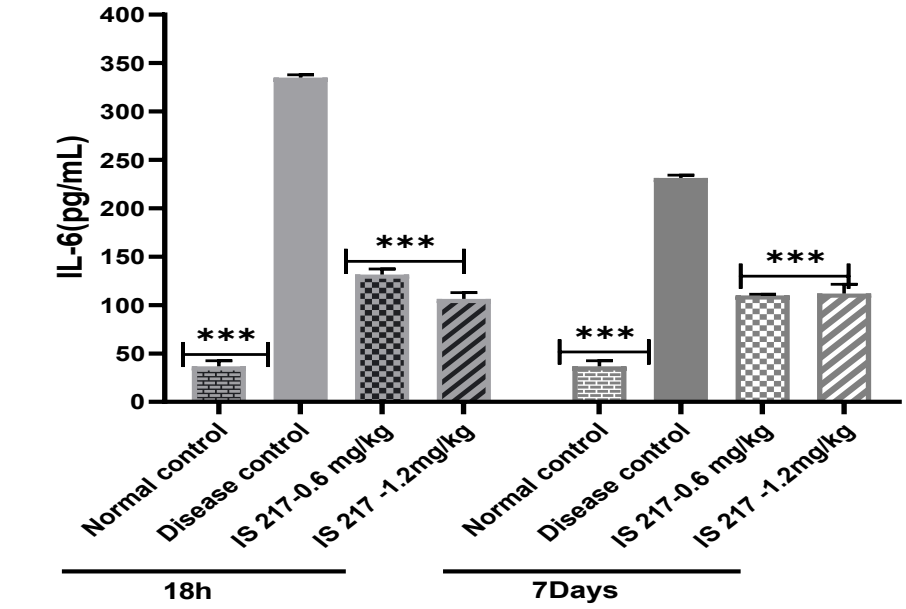
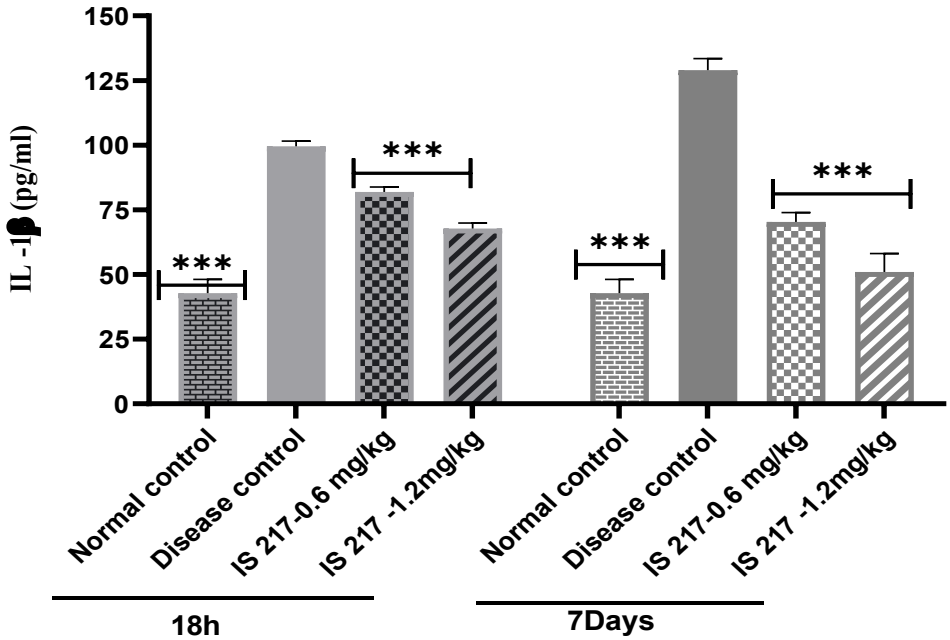


Figure 101: Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV) treatment on serum TNF-α levels in *E. coli* induced sepsis. Mice (n =5- 6/ group) and cytokine levels were estimated at 18 h and 7 days post-*E. coli* infection via IV route and Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

IV treatment:



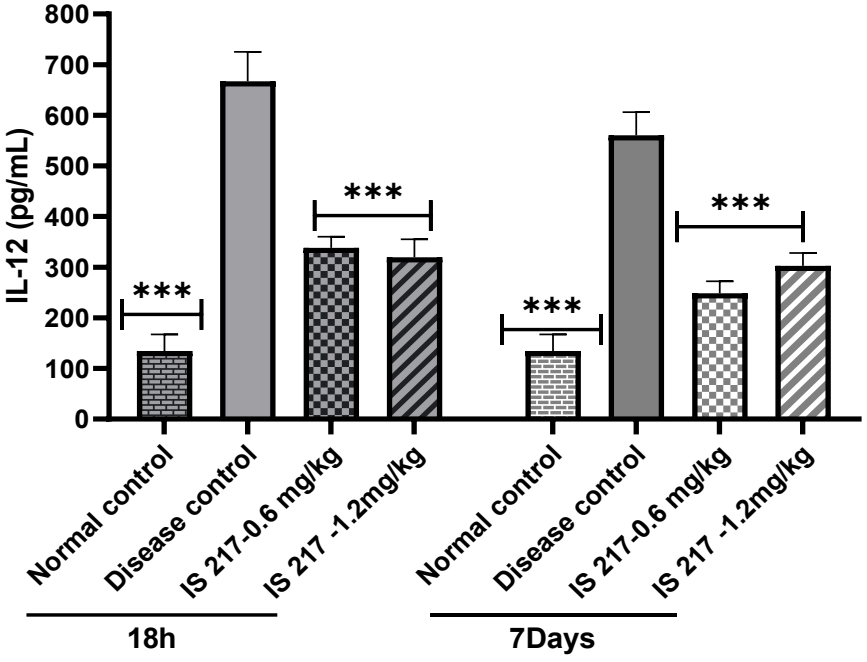
(a)



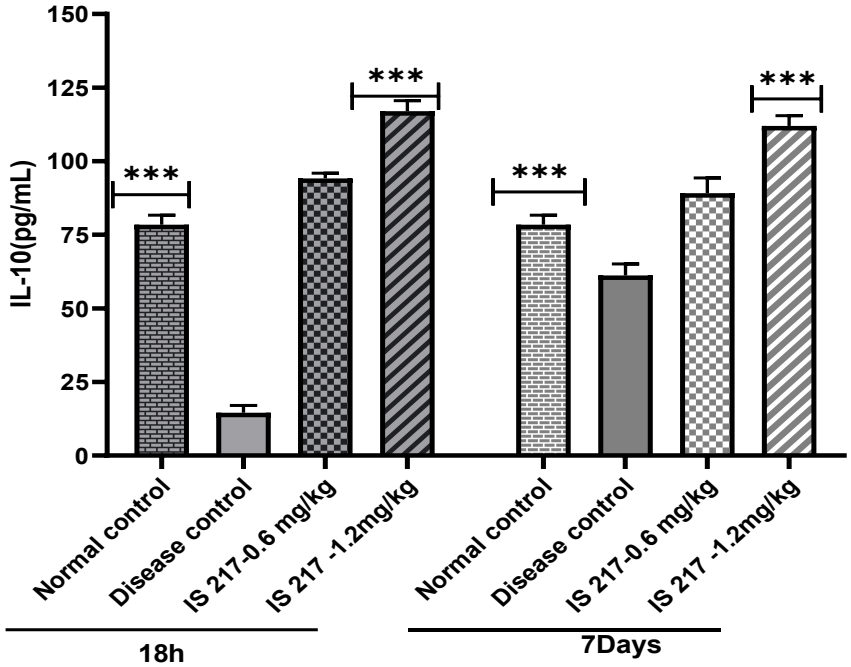
(b)

Figure 102(a,b) : Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV) treatment on serum IL-6 &IL-1β levels in *E.coli* induced sepsis. Mice (n =5- 6/ group) and cytokine levels were estimated at 18 h and 7 days post-*E. coli* infection via IV route and Data are expressed as mean ± SEM. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* compared to disease control group.

IV treatment:



(a)



(b)

Figure 103(a,b) : Effect of peptide IS 217 (0.6 & 1.2 mg/kg-IV) treatment on serum IL-12 &IL-10 levels in *E.coli* induced sepsis. Mice (n =5- 6/ group) and cytokine levels were estimated at 18 h and 7 days post-*E. coli* infection via IV route and Data are expressed as mean ± SEM. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* compared to disease control group.

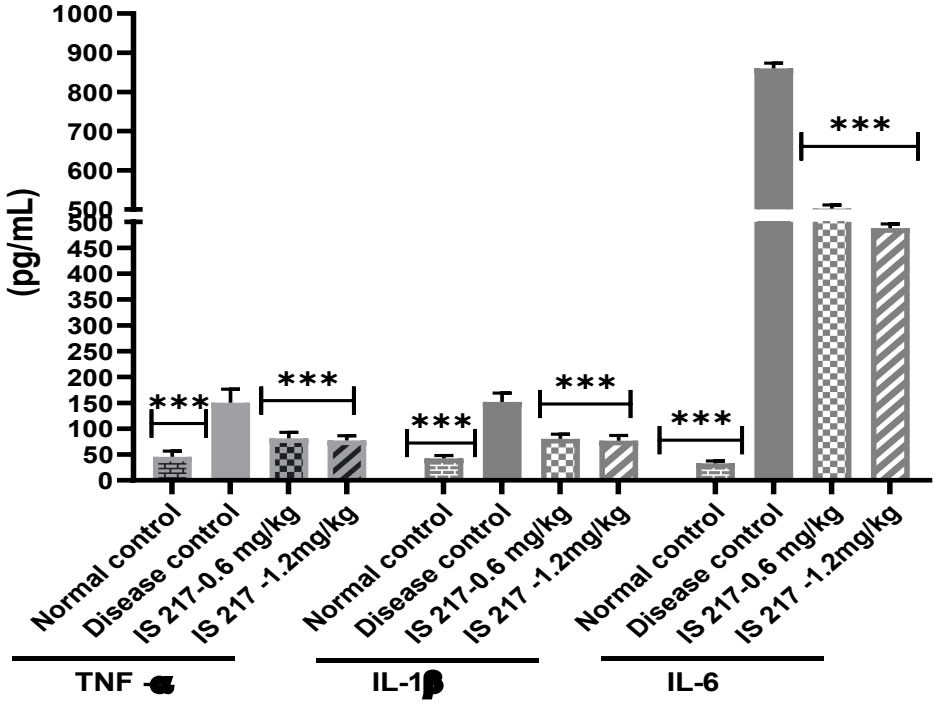
Similar findings were observed in the cytokine analysis conducted 7 days after bacterial injection. Mice infected with *E. coli* and treated with IS 217 via the IV route exhibited reduced levels of pro-inflammatory cytokines TNF-α (2.5-fold and 2-fold reduction in the IS 0.6 mg/kg and 1.2 mg/kg groups, respectively) (Figure 101), IL-6 (3-fold reduction in both IS 0.6 mg/kg and 1.2 mg/kg groups) (Figure 102a), IL-1β (1.5-fold reduction in the IS 0.6 mg/kg group, similar to control in the IS 1.2 mg/kg group) (Figure 102b), and IL-12p70 (1.85-fold and 2.5-fold reduction in the IS 0.6 mg/kg and 1.2 mg/kg groups, respectively) (Figure 103a). Additionally, an increase in IL-10 levels was observed (Figure 103b), indicating a shift towards an anti-inflammatory response. In contrast, the disease control group showed substantial increases in TNF-α (10-fold), IL-6 (6.5-fold), IL-1β (4-fold), IL-12p70 (4-fold), and IL-10 (68%).

SC treatment at 18 h:

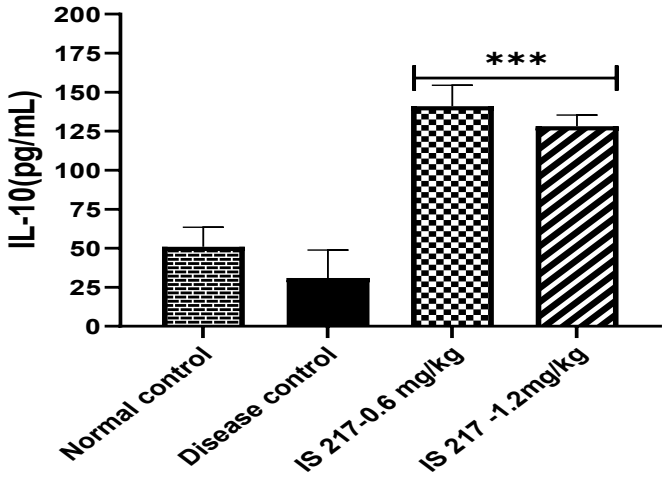
Similar trends were noted in the cytokine analysis conducted 18 hours after bacterial infection. Treatment with IS 217 via the SC route resulted in decreased levels of pro-inflammatory cytokines TNF-α (Figure 104a), IL-6 (Figure 104a), IL-1β (Figure 104a), and IL-12p70 (Figure 104a). Correspondingly, there was an elevation in IL-10 levels (Figure 104b), indicating a shift towards an anti-inflammatory response. In contrast, the disease control group exhibited significant increases in TNF-α, IL-6, IL-1β, IL-12p70, and a decrease in IL-10 levels.

Overall, these findings highlight the ability of IS 217 to modulate cytokine production, suppress pro-inflammatory responses, and improve survival rates in mice at risk of septic death induced by *E. coli* infection.

SC treatment at 18 h:



(a)



(b)

Figure 104(a,b): Effect of peptide IS 217 (0.6 & 1.2 mg/kg -SC) treatment on serum IL-6, IL-1β ,TNF-α & IL-10 levels in *E.coli* induced sepsis. Mice (n =5- 6/ group) and cytokine levels were estimated at 18 h post-*E. coli* infection via IV route and Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

4.2. Peptide IS 217 promotes a decrease in cytokine levels in peritoneal lavage fluid:

The intra-abdominal injury/ischemia, especially with subsequent infection, induces an excessive inflammatory/protein mediator production and uncontrolled inflammation in the peritoneum mostly via the lymphatic pathway) into the systemic circulation may precipitate the deleterious effects of sepsis and multiple organ dysfunction [Stefan Wirtz et al., 2006; Yoon Ju Cho1, et al., 2011]. In the present study, the cytokines, bacterial count, and total no of cells are measured in PLF. The amount of secreted cytokines levels in the abdominal lavage fluid was expressed as pg protein/mL.

The IS 217 treatment by IV route at 0.6 & 1.2 mg/kg doses significantly reduced the levels of all proinflammatory cytokines (TNF-α, IL-6, and IL-1β), and anti-inflammatory cytokines (IL-10), were detected in peritoneal lavage (figure 105 -106) from the *E. coli* infected group and IS 217 via SC treatment also reduced the levels of all proinflammatory cytokines (TNF-α, IL-6, and IL-1β) were detected in peritoneal lavage (figure 107) from the *E. coli* infected group and the IL-10 values are not estimated in SC treatment due to lack of sample size.

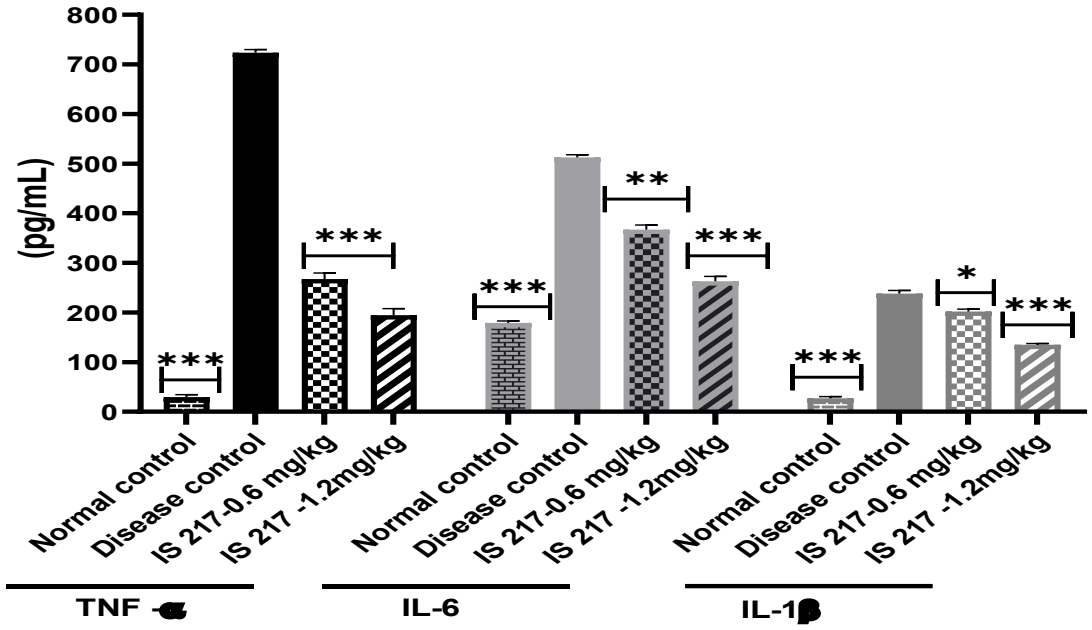


Figure 105: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on TNF -α, IL-6 and IL-1β levels in the peritoneal fluid of *E. coli* infection induced sepsis. Mice (n =5- 6/ group) and cytokine levels were estimated at 18 h post-*E. coli* infection via IV route and Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

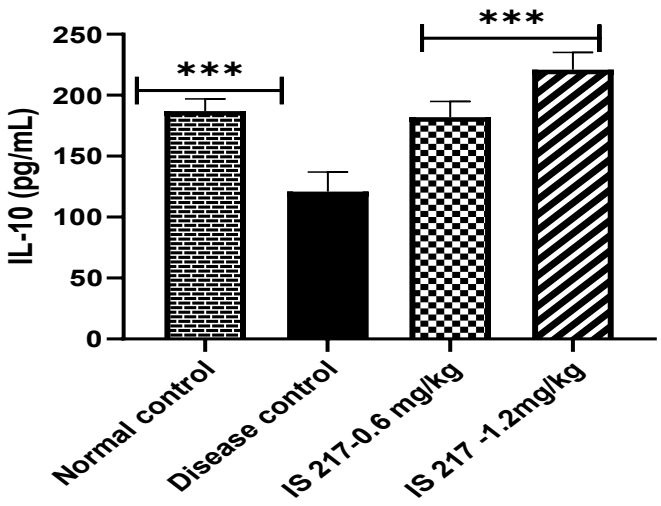


Figure 106: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on IL-10 levels in the peritoneal fluid of *E. coli* infection induced sepsis. Mice (n =5- 6/ group) and estimated at 18 h post-infection challenge) via IV route. Data are expressed as mean ± SEM. P < 0.001, P <0.01 & p < 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

SC treatment at 18 h:

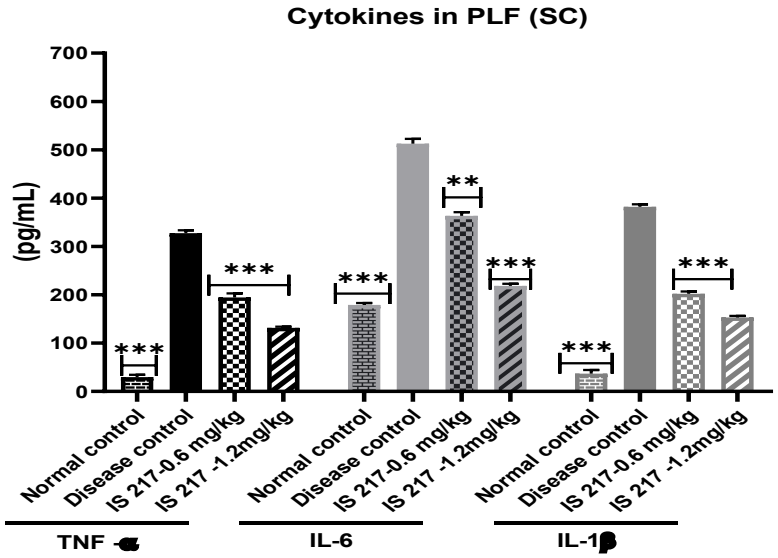


Figure 107: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -SC) treatment on TNF -α, IL-6 and IL-βlevels in the peritoneal fluid of *E. coli* induced sepsis. Mice (n =5- 6/ group) and estimated at 18 h post-CLP challenge via SC route. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

4.3. IS 217 promotes a decrease in PCT levels in peritoneal lavage fluid:

Procalcitonin levels are directly reflected in the severity of the sepsis. The higher levels of PCT levels are observed in *E. coli* infected mice at 18 h post infected groups and whereas PCT levels of the IS 217 treatment groups shows decreased PCT levels when compared to disease control CLP mice, considered as significant and was mentioned in (table 86 & figure108)

Table 86: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on procalcitonin levels in peritoneal lavage fluid after 18h of *E.coli* Infection.

Groups	Procalcitonin -PCT (pg/ml)
Control	191.1± 26.00
Disease Control	699.4± 2.17
IS 217-0.6 mg/kg	338.1 ±12.19***
IS 217 –1.2mg/kg	287.5 ±10.62***

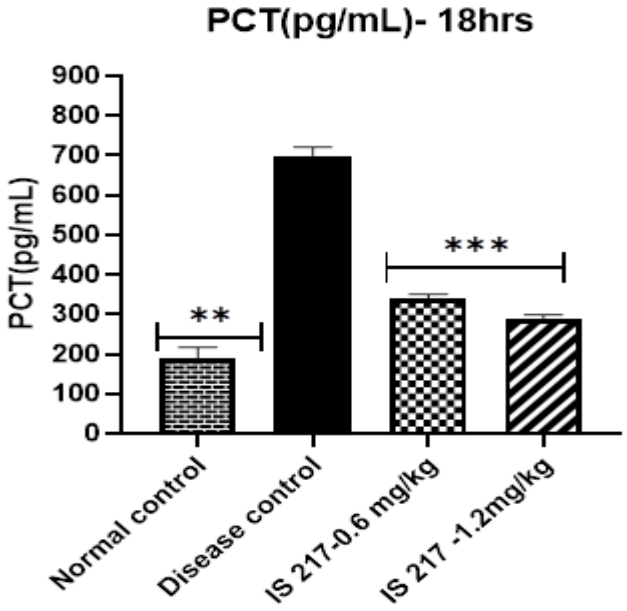


Figure 108: Effect of IS 217 (0.6 & 1.2 mg/kg -IV) on procalcitonin levels in the peritoneal fluid of *E. coli* induced sepsis. Mice (n =5- 6/ group) and estimated at 18 h post-*E. coli* infection challenge via IV route. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

4.4. The peptide IS 217 treatment promotes an increase leukocyte in the peritoneal cavity:

In comparison to the control and peptide IS 217 treated groups, the infection caused a decrease in the total blood cell count, which was associated with a reduction in lymphocytes. After infection, the number of neutrophils and monocytes did not decrease (figure 109). Similarly, fewer peritoneal cells were seen in the infected animals than in the control and IS-217-treated groups. Compared to the infected group that was not treated, the administration of peptide IS 217 led to an increase in the

number of peritoneal cells (table 87). The non-infected group's cell count can be seen to have slightly decreased. This decrease is not statistically significant, though. Conversely, the administration of peptide IS 217 to infected animals leads to a significant increase in the number of total cells in peritoneal (figure 109) as compared with the non-treated infected group. [Donghong Yan et al., 2002].

Table 87: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatmens on total cell counts and leucocytes in peritoneal lavage fluid after 18h of *E.coli* infection.

Groups	TNCC ($\times10^3/\mu L$)	Lymphocyte ($\times10^3/\mu L$)	Macrophages ($\times10^3/\mu L$)	Neutrophil ($\times10^3/\mu L$)	Eosinophil ($\times10^3/\mu L$)	Basophil ($\times10^3/\mu L$)
Control	0.47±0.02	0.09±0.01	0.02±0.00	0.02±0.00	0.00±0.00	0.00±0.00
Disease Control	1.88±0.08	0.18±0.03	0.09±0.02	0.75±0.04	0.01±0.00	0.01±0.00
IS 217-0.6 mg/kg	0.72±0.03	0.19±0.04	0.02±0.00	0.02±0.00	0.00±0.00	0.00±0.00
IS 217 – 1.2mg/kg	0.86±0.02	0.10±0.02	0.02±0.00	0.02±0.00	0.00±0.00	0.00±0.00

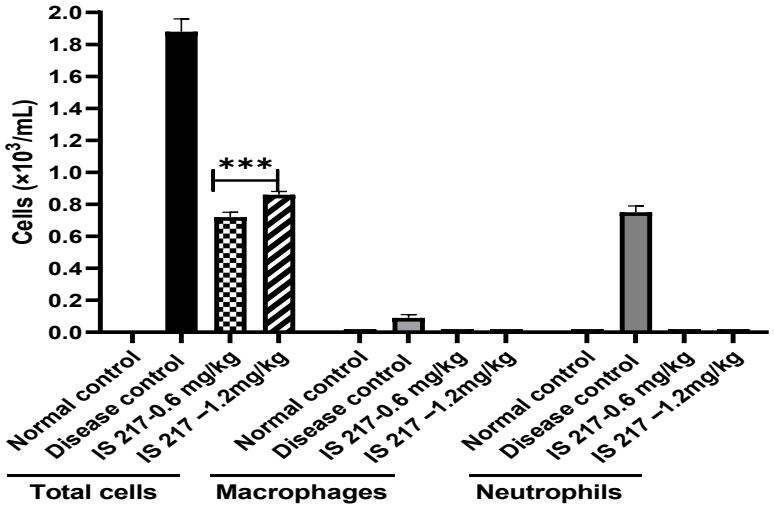


Figure 109: Total and differential blood cell counts from mice treated with peptide IS 217 (0.6 & 1.2 mg/kg) of *E. coli* - induced sepsis. Mice (n =5- 6/ group) and estimated at 18 h post-*E. coli* infection challenge via IV route. Data are expressed as mean ± SEM. P < 0.001, P,0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

5. Experiment 3: Late immune paralysis studies:

5.1. Measurement of peritoneal bacterial load in PLF:

To assess the efficacy of intravenous (IV) administration of IS 217 in combating *E. coli*-induced infection, a mouse model was utilized. The mice were euthanized 18 hours after infection, and bacterial colony-forming unit (CFU) counts were evaluated in the peritoneal fluid to determine the extent of bacterial presence. The results of this assay demonstrated a significant decrease in the number of viable bacterial counts in the groups treated with IS 217 (Table 88). This reduction indicates that the highly protective activity of IS 217 is attributed to its ability to kill bacteria. The peptide treatment resulted in approximately a ten-fold reduction in bacterial counts compared to the disease control group. These findings highlight the potent bacterial killing activity of IS 217.

Overall, the treatment with peptide IS 217 effectively reduced both the bacterial load and the associated mortality caused by the infection. This suggests that IS 217 possesses strong antimicrobial properties, leading to its efficacy in combating *E. coli*-induced infection in the mouse model.

Table 88: Bacterial CFU counts from peritoneal lavage from mice treated with IS 217 (0.6 & 1.2 mg/kg -IV) after 18h of *E.coli* infection.

Groups	**Bacterial count (CFU)**
Control	26.25± 21.54
Disease Control	>300
IS 217-0.6 mg/kg	148.38 ±58.93***
IS 217 –1.2mg/kg	34.25 ±14.61***

Data are expressed as mean ± SEM. Mice (n =5- 6/ group) and estimated at 18 h post-CLP challenge via IV route. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group.

5.2. Changes in the Lung Wet/dry Ratio, of lungs after the treatment with peptide IS 217:

The lungs were collected, and the wet/dry weights were measured to determine lung oedema, which can partly indicate the effects of intravenous treatment of IS 217 treatments. The wet/dry ratio of the lungs in the IS 217 administration mice was significantly lower than that in the *E. coli* infected group or isotype-treated animals (P,0.01, respectively) by intravenously (table 89 & Figure 110).

Table 89: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatment on Lung w/d ratio changes.

Groups	Lung W/D ratio -18 h IV -route
Control	4.34 ±0.130
Disease Control	5.22±0.070
IS 217-0.6 mg/kg	4.44±0.250***
IS 217 –1.2mg/kg	4.29±0.170***

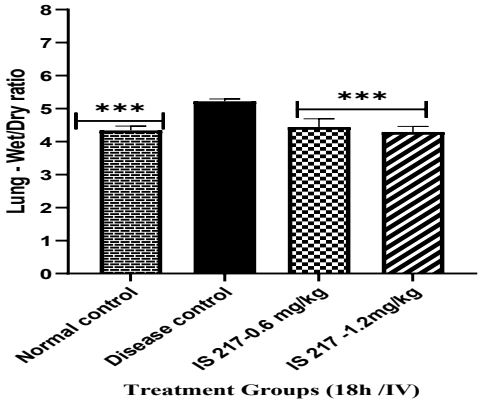


Figure 110: Peptide IS 217 (0.6 & 1.2 mg/kg) via IV treatment on the lung wet-to-dry weight ratio and protein concentration in the BALF of *E. coli* Infection -induced sepsis mice. Mice (n =5- 6/ group) and estimated at 18 h post-*E. coli* infection. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group respectively.

5.3. Evaluation of MPO levels in lungs:

Neutrophil extravasation, a process in which neutrophils migrate out of blood vessels and into the injured lung tissue, is a key histological marker of inflammatory and immunological responses in lung injuries such as acute lung injury (ALI). Tissue damage in ALI is closely associated with the activity of myeloperoxidase (MPO) in the lungs, as indicated by studies conducted by Abraham in 2003 and Zhou et al., 2012. Moreover, according to McCabe et al., 2001, pulmonary MPO activity serves as a reliable indicator of neutrophil infiltration in the lungs. In our study, we measured the MPO activity in lung tissue homogenates of mice from different treatment groups, 18 hours after sepsis induction.

As shown in Table 87 and Figure 111, sepsis induction significantly increased the pulmonary MPO activity in the disease control group of mice compared to the sham group mice that were not induced with sepsis and were not treated. Notably, the mice treated with the IS 217 peptide exhibited significantly lower pulmonary MPO activity than the mice in the sepsis group (Figure 111). These findings were further supported by the examination of neutrophil infiltrates in the bronchoalveolar lavage fluid (BALF). MPO levels indirectly indicate the recruitment of neutrophils to infected organs. Figure 111 illustrates that 18 hour after infection, MPO levels in the lungs were elevated in the disease control mice compared to the IS 217-treated mice. This increase was particularly

significant in the lungs ($p<0.001$; $p<0.01$). In fact, MPO levels in the lungs of disease control mice were three times higher than those in normal untreated mice. However, the MPO levels in the lungs of IS 217-treated mice showed only a slight increase compared to untreated mice in the case of intravenous treatment (Table 90). Furthermore, IS 217 peptide subcutaneous treatments led to a two-fold decrease in MPO levels in the lungs compared to the disease control mice (Figure 111).

Table 90: Effect of peptide IS 217 (0.6 & 1.2 mg/kg -IV) treatments on MPO levels.

Groups	**MPO (U/mg tissue) - 18hrs**
	IV treatment
Control	5.1±1.10
Disease Control	13.4±3.10
IS 217-0.6 mg/kg	6.6±1.00
IS 217 –1.2mg/kg	5.2±1.20

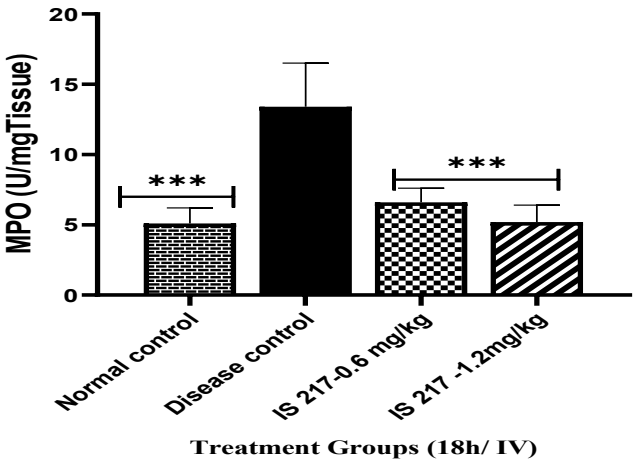


Figure 111: Effect of Peptide IS 217 (0.6 & 1.2 mg/kg) via IV treatment on sepsis induced pulmonary MPO activity. Mice (n =5- 6/ group) and estimated at 18 h post-*E. coli* infection. The myeloperoxidase (MPO) activity in the lung homogenates from sepsis mice and in the different treatment groups. Data are expressed as mean ± SEM. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* compared to disease control group.

5.2. The effect of IS 217 on the inflammatory cytokine concentration in the BALF of sepsis mice:

In the present study, the levels of pro-inflammatory factors, namely TNF-α and IL-6, as well as the levels of VEGF, were estimated using an ELISA assay. Additionally, the levels of the anti-inflammatory factor IL-10 were also measured. Analyzing the inflammatory response was deemed highly relevant in this study [Eun Jung Park et al., 2014].

To assess the impact of IS 217 on the production of inflammatory cytokines in sepsis-induced acute lung injury (ALI), the concentrations of TNF-α, IL-6, and IL-10 were determined in the bronchoalveolar lavage fluid (BALF) of mice belonging to different treatment groups. These mice were infected with E. coli either intravenously at 4h and 18 hours post-infection (Figure 112-113)

and subcutaneously at 18 hours post-infection (Figure 114). The ELISA assay was employed for these measurements.

The figures clearly demonstrate that sepsis induction led to a significant increase in the concentration of TNF-α (Figure 112, 114), IL-6 (Figure 113a, 114), and IL-10 (Figure 113b, 114) in the BALF of mice with sepsis-induced ALI, as compared to the mice in the sham group. However, treatment with the peptide IS 217 showed inhibitions of the elevated levels of these pro-inflammatory cytokines, as evidenced in the sepsis mice that received IS 217 treatments by both IV & SC route of administrations (Figure 112-114). These findings indicate that IS 217 has the potential to suppress the inflammatory responses induced by sepsis in mice with acute lung injury (ALI).

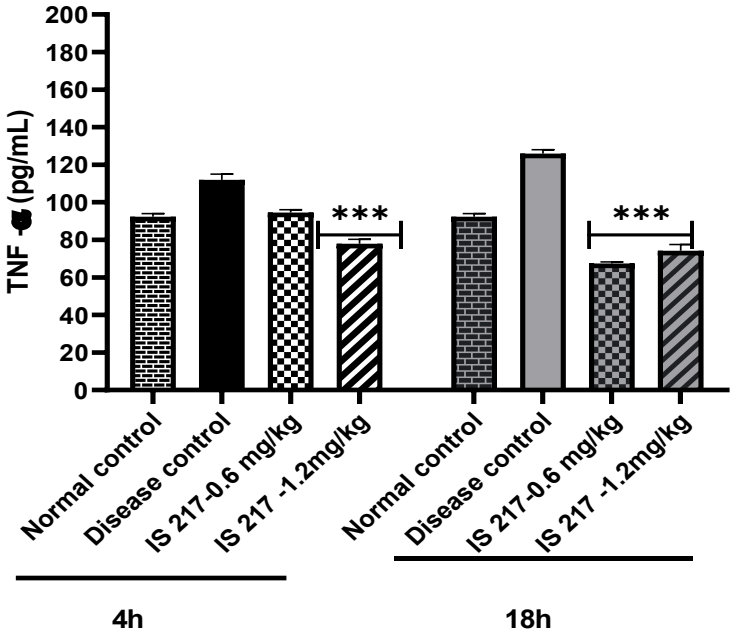


Figure 112: Effect of peptide IS 217 (0.6 & 1.2 mg/kg) via IV route on TNF-α levels in the bronchoalveolar lavage fluid. Mice (n =5- 6/ group) and estimated at 4 and 18 h post-*E. coli* infection. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group respectively.

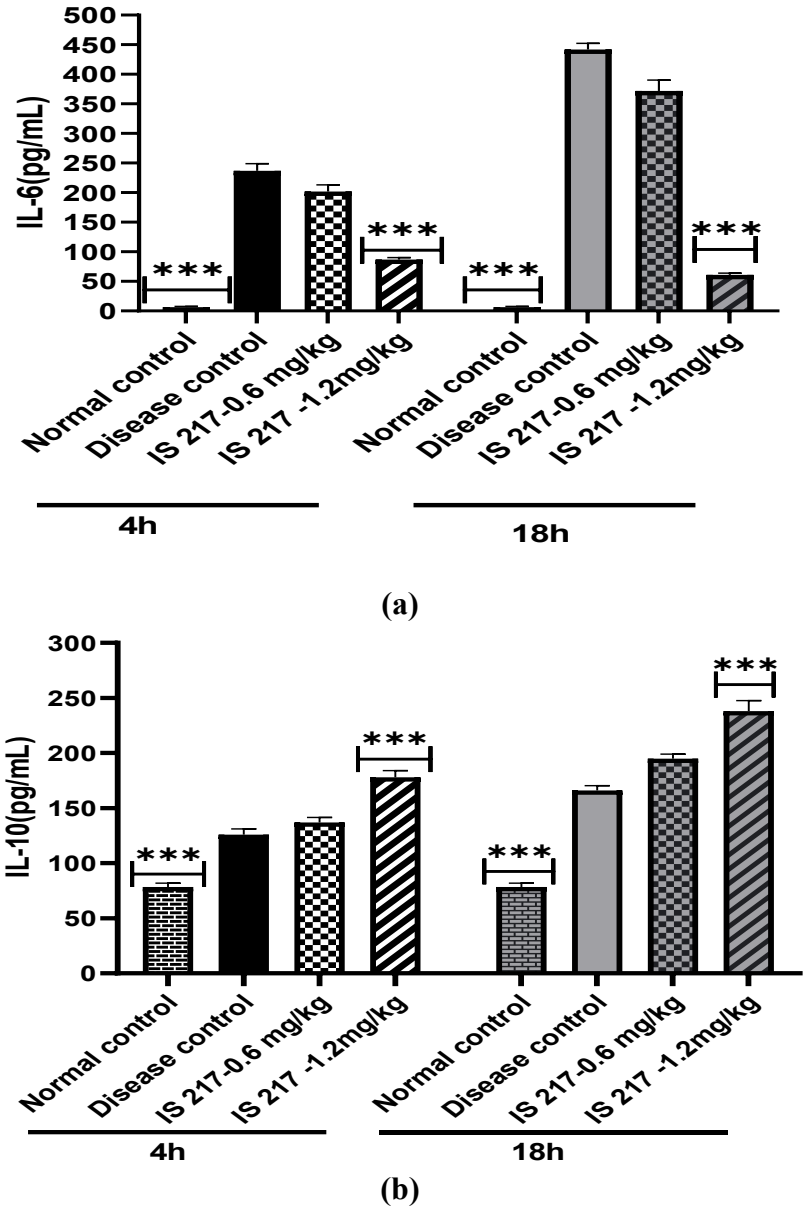


Figure 113: Effect of IS 217 (0.6 & 1.2 mg/kg) via IV route on IL-6& IL-10 levels in the bronchoalveolar lavage fluid. Mice (n =5- 6/ group) and estimated at 4 and 18 h post-*E. coli* infection. Data are expressed as mean ± SEM. $P < 0.001$, $P<0.01$ & $p< 0.05$ was considered as statistically significant and denoted as ***, **&* compared to disease control group respectively.

SC treatment at 18 h

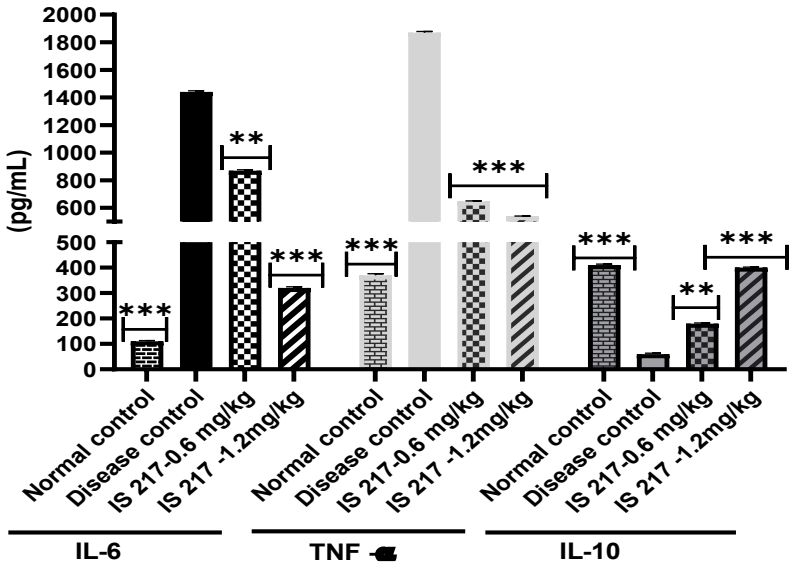


Figure 114: Effect of peptide IS 217 (0.6 & 1.2 mg/kg) via SC route on TNF-α, IL-6 &IL-10 levels in the bronchoalveolar lavage fluid. Mice (n =5- 6/ group) and estimated at 18 h post-*E. coli* infection. Data are expressed as mean ± SEM. P < 0.001, P<0.01 & p< 0.05 was considered as statistically significant and denoted as ***, **&* compared to disease control group respectively.

6. Histopathological Changes of Lung &Liver Tissues: IS 217 restores organ damage following polymicrobial infection:

The high bacterial load or virulence can cause an exaggerated inflammatory response, resulting in tissue damage and organ dysfunction, which is mainly seen in sepsis. Organ damage is a leading cause of death in patients with sepsis. Thus, we investigated whether the organ protection afforded by peptide IS 217 in *E.coli* indcued infection. No significant changes were observed in the body weight of the animals and in relation to the organs weight, besides, no macro- or microscopic alteration was detected in the brain, heart, lungs, liver, kidney, and spleen.

The disease control animals infected with *E. coli (*ATCC 8739™ ($5X10^8$ CFU/per mouse) were lethargic and presented histopathological changes characteristic of inflammation in the lungs and liver as compared with the control and IS 217- treated groups, with the presence of edema, hemorrhage, and cellular infiltrate, but the presence of necrosis was not evidence. However, it was

possible to observe a moderate reduction in the hemorrhagic process, as well as a reduction in the inflammatory infiltrate in animals infected and treated with peptide IS 217 in relation to the non-treated infected group (Table 91 and figure 115). The histological damage and clinical signs were rarely observed in treatment groups. The presence of congestion in the groups also analyzed if any euthanasia procedure causes any congestion. Necrosis was not observed in any analyzed organs; however, the presence of mild periportal edema was detected in the liver of most animals in the non-treated infected group. In summary, the peptide IS 217 treatment was able to reduce tissue damage, especially in the lungs, contributing to animal survival.

Table 91: Histopathology evaluation of lungs and livers from all the groups of post -infection of *E.coli* (IP).

Parameters	**Normal Control**	**Disease control (*E. coli* Infection)**	**IS 217 - 0.6mg/kg**	**IS 217 - 1.2mg/kg**	**Standard drug: cefoxitin**
Lungs					
Inflammatory infiltration	0.0 ±0.00	2.5±0.54	1.6±0.83	0.0 ±0.00	1.20 ±0.34
Hemorrhage	0.0 ±0.00	1.3±0.70	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Fibers accumulation	0.0 ±0.00	2.4±0.55	1.0±0.00	1.2 ±0.47	1.60 ±0.54
Tissue damage	0.0 ±0.00	2.2±0.53	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Edema	0.0 ±0.00	0.3 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Necrosis	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Liver					
Inflammatory infiltration	0.0 ±0.00	2.8±0.70	2.50±0.54		
Hemorrhage	0.0 ±0.00	2.2±0.40	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Fibers accumulation	0.0 ±0.00	2.5±0.50	0.0 ±0.00		
Tissue damage	0.0 ±0.00	2.6±0.35	0.5 ±0.20	0.0 ±0.00	0.0 ±0.00
Edema	0.0 ±0.00	0.7 ±0.50	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Necrosis	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00

The results are expressed as mean ± SEM of the scores :0-absent,1-mild,2-moderate,3-intense of n=3-4 mice /group.

To test whether peptide IS 217 treatment restored organ damage against sepsis-induced injury, we harvested tissues from vital organs that easily succumb to infection such as the lungs and liver, from all experimental groups to study histopathological changes. All the tissues from different experimental groups were harvested after 18 h, considering the early phase of immunosuppression and most of the animals of the *E. coli* infection (disease control) group are not survived while the other treated groups (IS 217 -0.6 mg/kg and IS 217 -1.2 mg/kg groups) lived longer. The methodology used for scoring was adapted from [Kubiak *et al*. and Klopfleisch]. A semi-quantitative scoring system was used. For liver tissue evaluation, hepatocyte degeneration and portal/lobular inflammation were scored (each 0–3), Lung injury scores were determined by assessing neutrophil infiltration, hemorrhage, necrosis, congestion and edema as previously described (table 93). These criteria were scored as follows;0 = Normal,1 ≤ 25%, 2 ═ 25—50%, 3 ═ 50—75%, and 4 ≥ 75%.

Scoring and assessment were conducted by an investigator unaware of treatment details. The score of each tissue sample represented the mean score of ten different fields. The stained tissue sections were evaluated under a light microscope (Eclipse E200-LED; Nikon, Kawasaki, Japan) at ×200 magnification.

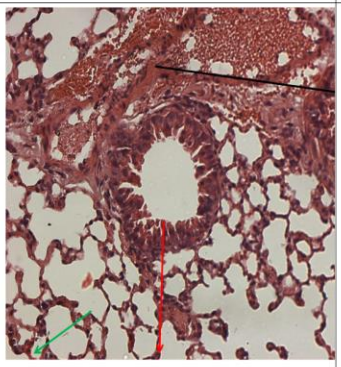
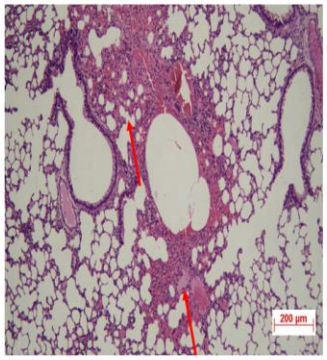
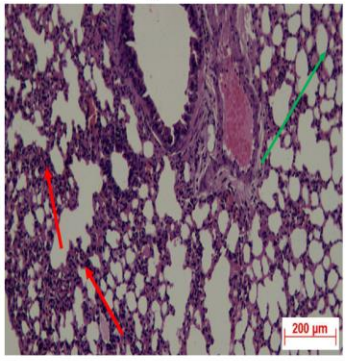
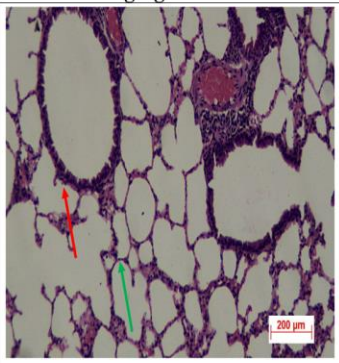
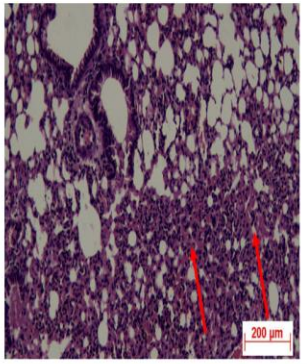
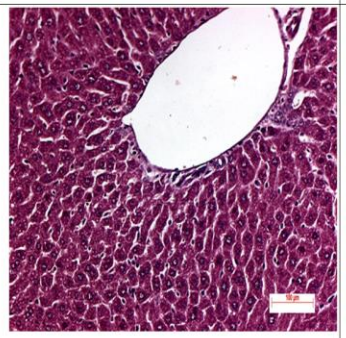
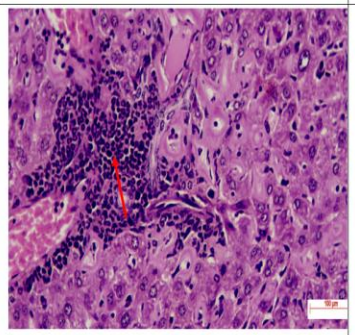
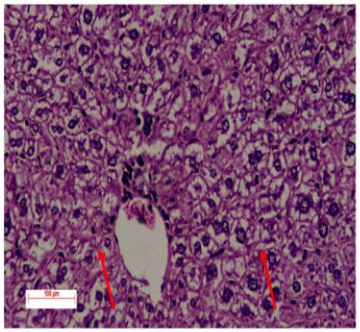
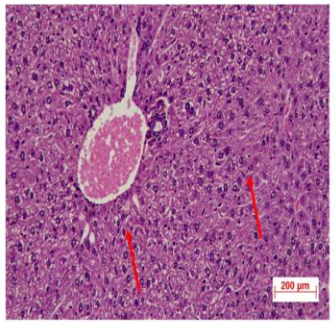
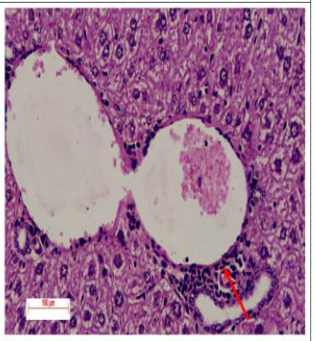
Peptide IS 217 treatments inhibiting TNF-α, IL-6 & IL-1β and organ damage in vivo in septic mice. BALB/c mice were given either 0.6 mg/1.2 m,g/kg of IS 217 via IV injection after 2h of post infection with 5X 10^8 CFU/ *E. coli* ATCC 8739™ via the IP route. The uninfected control group received an equivalent volume of normal saline alone. TNF-α, IL-6 & IL-1β levels in sera were measured by ELISA at 4h,18h and 7d post infection. At 18 h post infection, mice were bled retro-orbitally, and sera were separated to measure ALT levels. Also, after 18 h mice were sacrificed and observed for the histopathological changes indicated lung and liver tissue in *E. coli* infected group (figure 116). Furthermore, it was evident that IS 217 (1.2mg/kg) reduced these injuries by reducing edema and macrophage infiltration (figure 115) and showed that minimized *E. coli* infection induced lung &liver damage. In addition, IS 217 (0.6 mg/kg) reduces the injuries to moderate extent. Furthermore, the results in (Table 82) show that increased the levels of ALT and AST and that IS 217 peptide concentration-dependently and significantly reduced liver damage marker levels.

The criteria of scoring used in this study is summarized in (Table 91) and highlighted in (Figure 115). Tissues from the *E. coli* infected group showed interstitial edema, infiltration of polymorphnuclear leukocytes and monocytes, hemorrhage, vascular congestion, and cellular in the

lungs and liver. Tissue damage was more prominent in the disease control group (figure 115); treatment with IS 217 -1.2 mg/kg alone reversed these changes in all organs studied, (figure 115) towards a normal phenotype to resemble the normal control group; there was no trace of hemorrhage. Table 91 shows the scoring summary of tissue damage after *E. coli* infection and recovery following treatment with IS 217 in 1.2 mg/kg and 0.6 mg/kg. Similar changes were also observed in mice treated with standard broad spectrum antibiotic cefoxitin (figure 115).

Hence, the present study provides evidence for the anti-bacterial and anti-inflammatory effects of IS 217 in *E. coli*-induced sepsis.

Figure 115: Histological sections of lungs (a) and liver (b) of IV treatment groups after 18h of post *E. coli* Infection.

Organs	Normal Control	Disease control (*E. coli* Infection)	IS 217 -0.6mg/kg	IS 217 -1.2mg/kg	Standard drug: cefoxitin
Lungs					
	Red arrow: Normal bronchioles; green: Normal alveolar region of lungs; black: Normal pulmonary artery.	Alveolar/ interstitial inflammation with infiltration inflammatory cells [lymphocytes] was observed – arrow alveolar hemorrhages with edema of alveoli was observed – Red arrow.	Mild alveolar/ interstitial wall thickening, or connective tissue proliferation noticed in alveolar septa or in between alveoli – Red arrow with Normal morphology of alveoli- green arrow.	Normal morphology of alveoli [green arrow] and bronchioles [red arrow] of lung was observed.	Mild alveolar/ interstitial oedema with thickening of the wall of alveoli – alveolar septa - red arrow
Liver					
	Normal morphology of hepatocytes in portal, peri portal and centri lobular region – Red arrow.	Peri portal/ peri biliary inflammation with infiltration of lymphocytes / neutrophils were observed in multi focal region – Red arrow.	Hepatocytes appeared normal, but few foci of infiltration of lymphocytes were observed in peri portal/ peri biliary region - red arrow.	Normal morphology of hepatocytes was observed in portal, peri portal and centri lobular region – Red arrow.	Hepatocytes are appeared normal, but few foci of infiltration of lymphocytes were observed in peri portal/ peri biliary region – Red arrow.

Histological sections of lungs (a) and liver (b) of IV treatment groups after 18h of *E. coli* Infection and stained with H & E, visualized at 200X magnification and the scale bar represents 200 µm for lungs and 100 µm for livers. Data shown in mean ±SEM from 3-4 mice of all groups of *E. coli* induced sepsis, treatments, and control animals-IV route -18 h.

5.7.2.3. Discussion:

The rise of resistant bacterial strains has diminished the effectiveness of numerous antibiotics and triggered the urgent requirement for alternative antimicrobial medications to address infectious diseases [Dellinger RP, 2013; Du B, et al., 2002; Gasnik LB, et al., 2007].

This research aimed to evaluate IS 217's efficacy in treating sepsis induced by extraintestinal pathogenic E. coli in an animal model. Initially, IS 217 was incubated with *E. coli* ATCC 8739™ to ascertain its minimum inhibitory concentration. In the animal experiment, septicemia was induced in mice by intraperitoneally injecting a high concentration of Gram-negative *E. coli* (*E. coli*: $5X10^8$ CFU/per mouse). This elevated bacterial dosage triggered the production of proinflammatory cytokines (TNF-α, IL-1β, IL-12, and IL-6) and caused organ damage, which are characteristic of septicemia. Moreover, this high concentration of *E. coli* ATCC 8739™ led to 100% mortality within 72 hours. Thus, this model effectively represents excessive inflammation and septicemia, consistent with previous reports [Burgelman, M et al., 2021].

The impact of IS 217, an antimicrobial peptide from the host defense peptide (HDP) family encompassing various immunomodulatory proteins, was assessed [Jesse M. Jaynes et.al., 2012]. Mice received injections of *E. coli* ATCC 8739™ (*E. coli*: $5X10^8$ CFU/per mouse), and their survival was monitored for 18 hours, with a subset observed for 7 days. Following 18 hours, a subset of mice was euthanized to evaluate immunological, biochemical, and histological parameters, as well as the presence of bacteria in the peritoneal fluid. Inoculation with the bacteria resulted in the mortality of 90-100% of the animals within 18 hours. However, treatment with IS 217 (0.6 & 1.2 mg/kg) led to 50% and 80% survival after 18 hours of infection, respectively. Even after seven days, an 80% survival rate was observed in the IS 217 treatment groups.

A significant decrease in TNF-α, IL-1β, IL-12, and IL-6 levels was observed upon IS 217 peptide administration. Elevated IL-1β and IL-6 are typically associated with septicemia [Matsukawa, A., 2003]. Administering a single dose of IS 217 two hours post-infection attenuated *E. coli*-induced inflammatory cytokine expression and lowered mortality rates. However, the role of IL-6 in experimental sepsis models is debated due to its dual anti- and pro-inflammatory nature [Wang J et al., 2006; Bin Li a, et al., 2008]. Blocking IL-6 has shown benefits in sepsis and inflammatory

conditions [Riedemann, N. C., T et al., 2003; ishimoto, T. et al., 2010], indicating a link between elevated IL-6 levels and sepsis severity [Stefan Wirtz et al., 2006].

IS 217-treated mice during E. coli infection exhibited reduced coagulation time, evident from decreased PT and aPTT, suggesting its potential to diminish inflammation, lower sepsis toxicity by inhibiting TNF-α, IL-1β, and IL-6 production, and prevent disseminated intravascular coagulation [Fourrier, F., C et al., 1992; Vincent, J. L., 2005]. Excessive TNF-α and IL-1β production leads to tissue damage, organ failure, and fatal sepsis, but IS 217 treatment reduced sepsis-induced organ damage, as shown by decreased AST & ALT levels and histopathological analysis. Furthermore, IS 217-treated mice displayed enhanced clinical health and increased survival rates.

Macrophages are pivotal in septicemia due to their microbicidal functions and involvement in the inflammatory response [Wegiel B et al., 2015]. The balance between M1 and M2 macrophages has been linked to sepsis severity and the survival of infected animals [Renckens R, et al., 2006]. Animal models have shown that an increased proportion of M2 macrophages correlates with protection against sepsis [Isabella F. S. 2022]. In earlier studies using a cecal ligation and puncture (CLP) model, IS 217, functioning as an immunomodulator, notably stimulated IL-10 production. IL-10 plays a role in shifting macrophages toward the M2 phenotype and is secreted by M2 macrophages. Additionally, IS 217 peptide treatment reduced levels of IL-12 and TNF-α induced by CLP surgery, characteristic of M2 macrophages [Yea-Chen Liu et al., 2018]. Same observations were observed in *E. coli*-induced sepsis model and the Continued administration of IS 217 was observed to effectively suppress *E. coli*-induced inflammation across a 5-day period [A Brauner, et al., 2001] [Tjabringaa GS et al., 2006 & Silva, O. N., C. et al., 2016]. TNF-α and IL-1β are rapidly released during systemic inflammatory responses [Hotchkiss, R. S., 2013; Schulte, W., 2013], creating a small window for therapeutic intervention. This swift response underscores why acute administration of IS 217 is more efficient at suppressing inflammation development compared to chronic treatment. Acute IS 217 treatment notably decreased cytokine levels in the lungs, serum, and peritoneum, while boosting cell production in the peritoneum and lymphocytes at the infection site. IS 217 peptide treatment demonstrated its capacity to limit tissue damage, thereby mitigating detrimental effects on the organism, contributing to sepsis control, and improving animal survival. Consequently, IS 217 holds promise as a potential candidate for novel drug development. Agents that dampen proinflammatory cytokine expression show potential as effective treatments for preventing lethal sepsis [Tobias Schuerholz et al., 2013].

5.7.2.4. Summary:

In summary, administering the IS 217 peptide at a 1.2 mg/kg dosage led to decreased mortality rates and lowered levels of circulating proinflammatory cytokines—TNF-α, IL-1β, IL-12, and IL-6—in C57BL/6J and BALB/c mice affected by sepsis induced by gram-negative bacteria E. coli ATCC 8739™ (E. coli: $5X10^8$ CFU/per mouse). The peptide IS 217 acted as a protective shield against sepsis-related mortality, highlighting its potential as a targeted therapeutic using host defense peptides against this condition. These findings underline IS 217's promise as a therapeutic agent against lethal sepsis, supported by its antibacterial and anti-inflammatory effects observed in *E.coli*-induced sepsis.

5.8. Highlights of the study:

- IS 217, a peptide, has demonstrated its potential as both an antimicrobial agent and an immune modulator based on reported research findings. Its effectiveness in combating bacteria and reducing inflammation has been observed in laboratory settings (in vitro) as well as in animals (in vivo). Studies specifically conducted on macrophage cell lines and peritoneal macrophages confirmed its ability to alleviate inflammation.
- Acute toxicity and immunogenicity profile of peptide IS 217 has been established.
- Treatment with IS 217 showcased its capacity to inhibit inflammation, notably in the context of paw edema induced by carrageenan.
- IS 217 can either suppress or restore the levels of pro-inflammatory cytokines and increase the levels of anti-inflammatory cytokines in early immune suppression during sepsis. This has been observed in models of lethal sepsis such as cecal ligation and puncture, as well as *E. coli*-induced peritonitis.
- Further studies indicated that IS 217 significantly mitigated organ damage, particularly in the lungs and liver, caused by sepsis in mice. In instances of sepsis-induced acute lung injury (ALI), IS 217 exhibited notable protective effects by reducing inflammatory cytokine levels and improving lung tissue health based on histopathological assessments.

Chapter 6.

Proposed Mechanism of action of peptide IS 217

6.0. Mechanism of action of peptide IS 217:

Further elaboration on one hypothesis regarding the mechanism of action of IS 217 is provided below.

The peptide IS 217 is a designed antimicrobial peptide (dAMP) as Host Defense Peptide (HDP). When one explores its mechanism of action one must consider at least two separate mechanisms of action.

1. Direct antimicrobial activity.
2. Immune modulation activity.

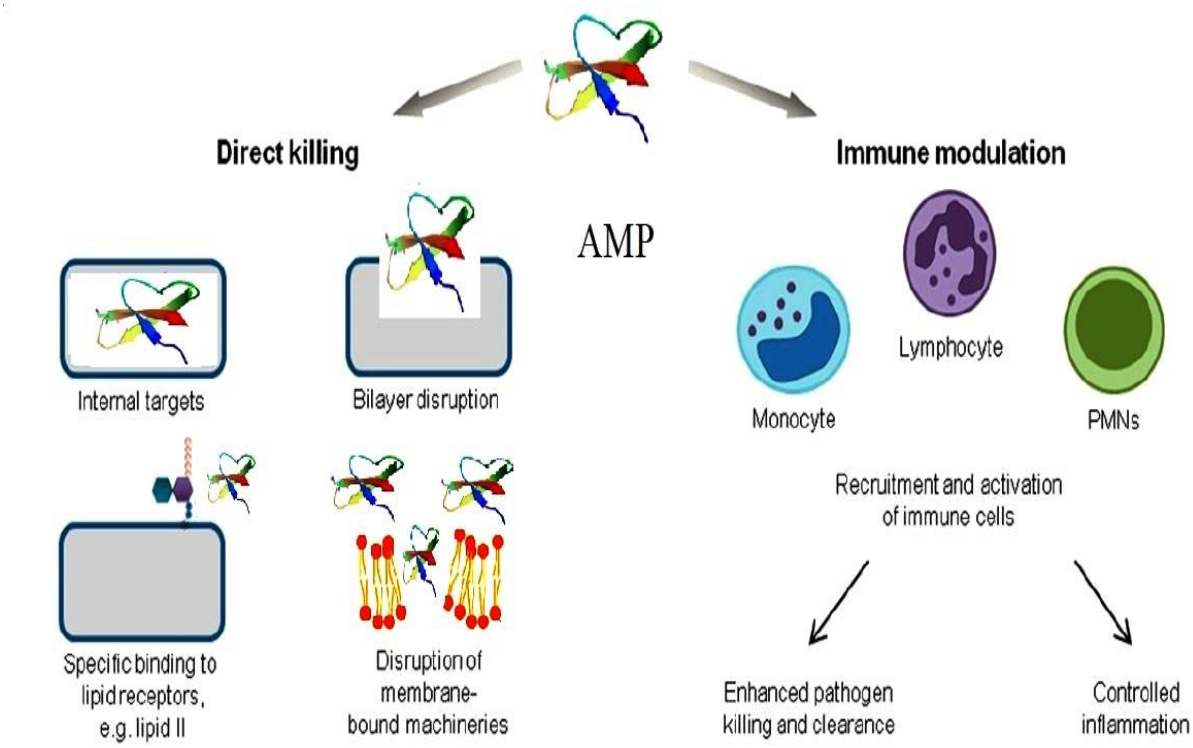
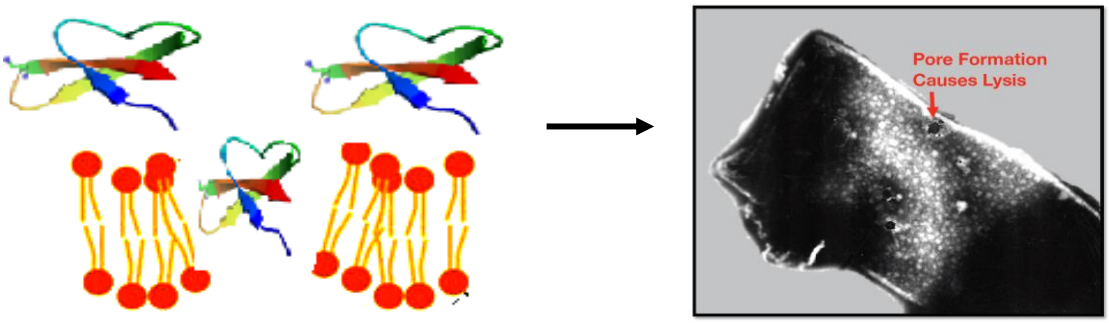


Figure 116(a). Various mechanisms of action of antimicrobial peptides. Adapted with permission from [Ulm, H et al,2012]. MN: polymorphonuclear neutrophils; ADP: adenoside diphosphate; ATP: adenoside triphosphate.

The antibacterial action of HDPs is initiated through their interaction with cellular membranes as part of their direct antibacterial mechanism (or mechanisms) of action, leading to membrane perturbation, disruption of membrane---associated physiological events such as cell wall biosynthesis or cell division, and/or translocation across the membrane to interact with cytoplasmic targets. First, they bind to the negatively charged surface of bacterial membranes then insert into

the membrane, through hydrophobic and electrostatic interactions. Thus, the charge, hydrophobicity and amphipathicity are important to HDPs biological function. The peptide is then inserted, in an approximately parallel orientation to the bilayer, into the outer leaflet of the cytoplasmic membrane lipid bilayer, leading to the displacement of lipids. (figure 116 a). Models illustrating the membrane interactions of antimicrobial peptides.



(b) **(c)**

Figure 116(b,c): The proposed mechanism of action of anti-microbial activity of peptide IS 217.

The antimicrobial peptide (multi color) is illustrated as a stack of arrows (beta-sheet aggregate) first interacts with the phospholipid heads of the outer leaflet of the bacterial membrane. As the peptide is drawn into the membrane the hydrophobic face of the AMP begins to interact with the hydrophobic alkyl chains of the fatty acids composing the lipid bilayer. Continued disaggregation occurs as the peptide is drawn further into the membrane with reorientation of the hydrophilic face of the peptide with the phospholipid heads of the bilayer. To find thermodynamic equilibrium, more peptide molecules are drawn into the reaction and reorientation occurs to form a pore with the hydrophilic faces of the peptide internal to the water channel and the hydrophobic faces orientated to the hydrophobic core of the membrane. The resultant loss of osmotic integrity causes lysis of the target microbe (figure 116c). Alterations in membrane structure result in the reorientation of peptide molecules in the membrane culminating in eventual pore formation and lysis of the target microbe.

The interactions of an AMP with the membrane cannot be explained by a particular sequence of amino acids. They originate from a combination of physicochemical and structural features including size, residue composition, overall charge, secondary structure peptides hydrophobicity

and amphipathy. All these design features were utilized to fabricate IS 217 and other novel bioactive peptides.

In this study, the antimicrobial activity of the peptide IS 217 was confirmed. The peptide displayed notable comparable Minimum Inhibitory Concentration (MIC) results when compared to established research peptides—clavanin A and LL-37—commonly used in pipeline research. Clavanin A and LL-37 exhibited MIC values against *S. aureus* at 45.0 µM & 12.5 µM, respectively, and against *E. coli* at 24.0 µM & 1.5 µM, respectively. In contrast, IS 217 peptide demonstrated much lower MIC values against *S. aureus* (0.013µM) and *E. coli* (0.0003µM), and also compared to standard broad-spectrum antibiotic ciprofloxacin against *S. aureus* (0.003µM) & *E. coli* (0.0015µM).

Moreover, IS 217 peptide showcased significant results both in vitro when compared with ciprofloxacin (as indicated in Table 54) and in vivo animal studies. These in vivo assessments involved observing survival rates (Table 80b & Figure 97c) and evaluating organ damage in mice induced with *E. coli*-induced sepsis, were compared with the effects of a broad-spectrum antibiotic, cefoxitin (Figure 115).

Immune modulation mechanism:

In several other in vitro and in vivo experiments of synthetic peptides, we have observed distinct and reproducible modulation of many signaling pathways mainly in wound healing, currently, it is believed that the peptide directly interacts with epidermal growth factor receptor (EGFR) and modulates its wound-healing pathway. EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity. This auto-phosphorylation elicits downstream activation and signaling by other proteins that are associated with the phosphorylated receptor.

These downstream signaling proteins start several signal transduction cascades, primarily the MAPK, Akt, and JNK pathways, which result in DNA synthesis and cell proliferation. These proteins influence the phenotypes of cell migration, adhesion, and proliferation. All processes play roles in a number of normal cellular processes including wound healing.

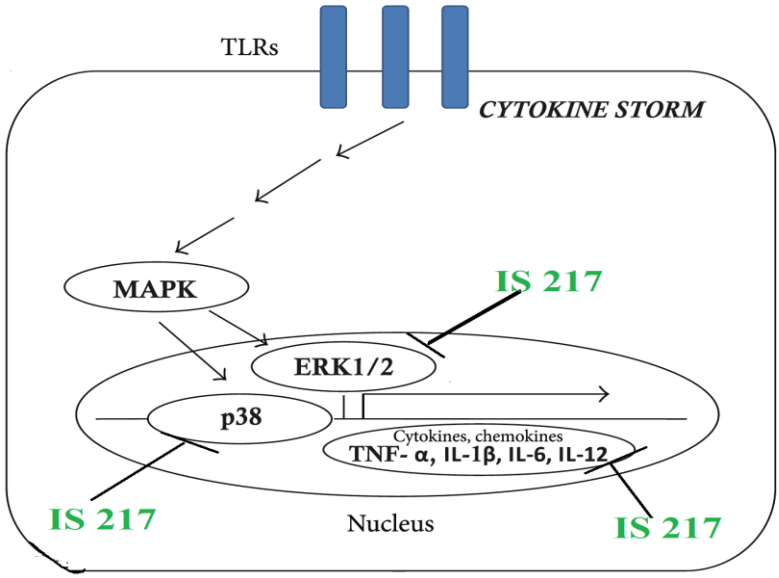


Figure 116d: Proposed mechanisms of peptide IS 217 by activating the MAPK signaling pathway, decreasing/ inhibiting TNF-α, IL-1β and IL-6 production. TLR, toll like receptor; ERK, extracellular signal-related kinases; p38, p38 mitogen-activated protein kinase.

In the present study, IS 217 demonstrated consistent and notable modulation of the immune response in both laboratory (in vitro) and living organism (in vivo) experiments. This modulation was evident through the inhibition of LPS-induced phosphorylation of ERK1/2 and p38 in various settings: the murine macrophage cell line RAW 264.7 (as shown in Figures 35 & 36), peritoneal macrophages (depicted in Figures 55a and b), and in the bronchoalveolar lavage fluid (BALF) of mice experiencing sepsis-induced acute lung injury (demonstrated in Figure 88) and based on the in vitro and in vivo evaluations, the immunomodulation of IS 217 by activating the MAPK signaling pathway, decreasing/ inhibiting TNF-α, IL-1β and IL-6 production as illustrated in figure 116d.

Based on the data provided in the present study experiments, there is evidence to suggest the remarkable antimicrobial and immunomodulatory activities of peptide IS 217. Further experiments will continue to investigate the mechanism of action and other relevant aspects.

Chapter 7.

Summary of all the studies

&

Conclusion

7.1. Summary of the study:

Table 92: The protective effects and mechanism of peptide IS 217 in sepsis -related animal or cell model:

In vivo/ vitro	Animal or cell model	Therapeutic dose	Effects and Mechanisms.
In vivo	CLP-induced sepsis in male BALB/c mice septic mouse model (8-10 weeks)	0.6 mg/kg/1.2 mg/kg via., Intravenous route -4h & 18h.	↑Survival rate; restores liver (ALT&AST,) and kidney (creatinine, bilirubin and BUN) function tests; ↓serum lactate levels and DIC ; ↓TNF-α, IL-1β, IL-6 and IL-12,VEGF &↑IL-10 in serum; ↓TNF-α ,IL-6, and VEGF &↑IL-10 in BALF of lungs ; ↓Lung damage and injury scores; ↓injury of pulmonary permeability;↓MPO activity; Normal Lung Wet/dry Ratio; Normal spleen weight; ↓ IL-6/-1β, and TNF-α in spleen tissue; ↓ IL-6 /-1β, and TNF-α in peritoneal fluid (PLF);↓Procalcitonin (PCT);↓ TNCC, macrophages & neutrophils ; ↓bacterial counts ;↓liver tissue injury and injury score.
		0.6 mg/kg/1.2 mg/kg via., Intravenous route -10 Days.	↑Survival rate; ↓TNF-α, IL-1β, IL-6 and IL-12, VEGF &↑IL-10 in serum; ↓TNF-α, IL-6, and VEGF &↑IL-10 in BALF of lungs; ↓ IL-6/-1β, and TNF-α in spleen tissue; ↓ IL-6 /-1β, and TNF-α in peritoneal fluid (PLF); ↓ Procalcitonin (PCT).
In vivo	Behavioral studies in CLP-induced sepsis mice.	0.6 mg/kg/1.2 mg/kg via., Intravenous route	Normal activity and long-term object recognition memory function after the post CLP surgery.

In vivo	CLP-induced sepsis in male BALB/c mice septic mouse model (8-10 weeks)	0.6 mg/kg/1.2 mg/kg via., Subcutaneous route -18h.	↑Survival rate; restores liver (ALT&AST,) function tests; ↓serum lactate levels; ↓TNF-α, IL-1β, IL-6 and IL-12,VEGF &↑IL-10 in serum; ↓TNF-α ,IL-6, and VEGF &↑IL-10 in BALF of lungs ; ↓MPO activity; Normal Lung Wet/dry Ratio; Normal spleen weight; ↓ IL-6/-1β, and TNF-α in spleen tissue; ↓ IL-6 /-1β, and TNF-α in peritoneal fluid (PLF);↓Procalcitonin (PCT);↓ TNCC, macrophages & neutrophils ; ↓bacterial counts ;↓liver tissue injury.
		0.6 mg/kg/1.2 mg/kg via., Subcutaneous route -10 Days.	↑Survival rate; ↓TNF-α, IL-1β, IL-6 and IL-12, VEGF &↑IL-10 in serum.
In vivo	*E. coli*-induced peritonitis sepsis in male BALB/c mice septic mouse model (10-12 weeks)	0.6 mg/kg/1.2 mg/kg via., Intravenous route -4 &18h.	↑Survival rate; restores liver (ALT&AST,) and kidney (creatinine, bilirubin and BUN) function tests; ↓serum CRP levels and DIC ; ↓TNF-α, IL-1β, IL-6 and IL-12,VEGF &↑IL-10 in serum; ↓TNF-α ,IL-6 and ↑IL-10 in BALF of lungs ; ↓Lung damage and injury scores; ↓injury of pulmonary permeability;↓MPO activity; Normal Lung Wet/dry Ratio; Normal spleen weight; ↓ IL-6/-1β, and TNF-α in spleen tissue; ↓ IL-6 /-1β, and TNF-α in peritoneal fluid (PLF);↓Procalcitonin (PCT);↓ TNCC, macrophages & neutrophils ; ↓bacterial counts ;↓liver tissue injury.
		0.6 mg/kg/1.2 mg/kg via., Intravenous route -10 Days.	↑Survival rate; ↓TNF-α, IL-1β, IL-6 and ↑IL-10 in serum; ↓ IL-6, IL-1β, and TNF-α in lungs; ↓Lung damage and injury

			scores; ↓injury of pulmonary permeability; ↓ IL-6/-1β, and TNF-α; ↑IL-10 in PLF; ↓MPO activity.
In vivo	*E. coli*-induced peritonitis sepsis in male BALB/c mice septic mouse model (10-12 weeks)	0.6 mg/kg/1.2 mg/kg via., Subcutaneous route -18h.	↑Survival rate; ↓ serum CRP levels; ↓TNF-α, IL-1β, IL-6 and ↑IL-10 in serum; ↓ TNF-α, IL-1β, IL-6 in serum; ↓TNF-α, IL-6, and ↑IL-10 in BALF of lungs; ↓Lung damage and injury scores; ↓injury of pulmonary permeability; ↓ MPO activity; Normal Lung Wet/dry Ratio; ↓Procalcitonin (PCT);↓ TNCC, macrophages & neutrophils ;↓ IL-6/-1β, and TNF-α; ↑IL-10; In PLF; ↓bacterial counts; ↓ liver tissue injury; (injury score, edema and inflammation infiltration).
		0.6 mg/kg/1.2 mg/kg via., Subcutaneous route -10 Days.	↑Survival rate; ↓IL-6, IL-1β, and TNF-α in lungs; ↓ IL-6/-1β, and TNF-α; ↑IL-10 in PLF.
In vivo	Carrageenan Paw edema model	0.6 mg/kg/1.2 mg/kg via., Intravenous route	↑ % inhibition of paw edema at 5h and comparable inhibition of paw edema even after 24 h.
In vivo	Acute toxicity	Single dose of 0.6 /2.4 /4.8 mg/kg via., Intravenous & Subcutaneous routes	No mortality; no changes on body weight and organ weights in treated groups.
In vivo	Immunogenicity test	0.6 mg/kg/1.2 mg/kg via., Intravenous route	No evidence of enhanced IL-1β or TNF-α cytokine production in sera at 48 h administration of peptide IS 217.
In vitro	LPS -stimulated RAW 246.7 cell lines of murine	3.125/6.25/12.5µg/mL	↓Cytotoxicity; ↓ NO production; ↓ IL-12, IL-β, IL-6, and TNF-α and ↑ IL-10 levels;); ↓ mRNA expressions of IL-12, IL-β, IL-

	macrophages		6, and TNF-α. block activation of MAPKs, Inhibiting the induction of iNOS expression.
In vitro	LPS -stimulated BALB/c mice macrophages	1.56/3.12/ 6.25/12.5 /25/50 /100 µg/mL	↓Cytotoxicity; ↓IL-6, IL-1β, and TNF-α and ↑ IL-10 levels; could inhibit the activation MAPK pathway (ERK1/2, and p38 MAPK); ↓ mRNA expressions of IL-β, IL-6, CCL2 and TNF-α.
In vitro	Anti-microbial studies: Minimum inhibitory concentration (MIC) & Time-killing assay of peptide IS 217 against *S. aureus, P. aeruginosa, E. coli* & *K. Pneumoniae.*	1.56/0.78/0.39/1.56 µg/mL	MIC; IS 217 has showed good antimicrobial activity at the concentration of 1x MIC (1.56µg/ml) against *S. aureus,* 5x MIC (0.78µg/ml) against *P. aeruginosa,* at 2h ,1x MIC (0.39µg/ml) against *E. coli* at 3h and 1x MIC (1.56 µg/ml) against *K.pneumoniae* and at 12 h showed the completed reduction of initial inoculum at 24 h incubation.
In vitro	Bio film inhibition assay against *S.aureus.*	5x MIC(0.78µg/ml)	Inhibited 48 hours-mature staphylococcal biofilms.

Note: In the present in vivo animal studies, IS 217 peptide was extensively examined via intravenous (IV) administration in both CLP and *E. coli*-induced sepsis models, chosen for its rapid action in addressing the urgency of sepsis treatment. Subsequently, the efficacy of IS 217 peptide via subcutaneous (SC) route was also assessed, albeit to a lesser extent due to limited sample availability in the SC treatment. This limitation is due to the nature of sepsis treatment, where IV administration is often prioritized. After stabilizing the immediate treatment phase, the transition to subcutaneous delivery aligns with the current trend in peptide-based drug development, emphasizing patient-centered approaches.

7.2. Conclusion of the study:

According to the Centers for Disease Control and Prevention (CDC) in the United States, sepsis is a severe condition marked by high mortality rates, stemming from widespread inflammation due to infection. In light of the escalating prevalence of multidrug-resistant diseases, there's a growing interest in antimicrobial peptides (AMPs) as potential therapies to create new medicines with immune-regulating capabilities. Consequently, developing treatment strategies that modulate the immune system has become a crucial area of focus in managing sepsis. Host defense antimicrobial peptides, known for their abilities to combat microbes and regulate the immune response, are highly regarded as promising molecules for therapeutic use.

In this research, three synthetic peptides (IS 141, IS 111 & IS 217) were specifically chosen based on their secondary and parent sequence structures and then evaluated for their ability to reduce LPS-induced inflammation in RAW 264.7 cells. These peptides demonstrated effectiveness by inhibiting the expression of pro-inflammatory cytokines (CCL2, TNF-α, IL-6, and IL-β) and by suppressing the activation of p38 MAPK, a pivotal signaling pathway in the inflammatory process. Among these peptides, IS 217 displayed remarkable potential, prompting its selection for further investigation in proof-of-concept studies. This choice likely stems from its evident potency and effectiveness in modulating the inflammatory response, positioning it as a strong candidate for subsequent in-depth research and potential therapeutic development. The synthetic peptide IS 217 is a derivative of designed antimicrobial peptides (dAMPs) that falls within the host defense antimicrobial peptide (HDP) family. It comprises a sequence of 10 amino acids and has been studied as a promising lead molecule for infection control purposes.

In this study, IS 217 demonstrated robust antibacterial and immunomodulatory effects through both in vitro and in vivo experiments. In vitro assessments showcased its capacity to efficiently eradicate several prevalent bacterial strains, such as *S. aureus, E. coli, P. aeruginosa* and *k.pneumonia*, alongside the Raw 246.7 mouse macrophage cell line. Notably, IS 217 didn't exhibit any cytotoxic effects on primary macrophage cells, despite effectively combating both Gram-positive and Gram-negative bacteria. Additionally, acute toxicity studies indicated the absence of any adverse reactions across all tested doses.

Additionally, this study further assessed the effectiveness of IS 217 in treating sepsis using an in vivo animal model. Initial experiments using male and female mice revealed activity level differences, often

seen in gender-specific responses to stimuli like septic insult. Due to concerns about variations in responses related to the estrus cycle in females, male mice are commonly favored for sepsis studies [Northern AL et al.,1994, Wichmann MW et al.,1996 & Zellweger R et al.,1997]. Following Marshall et al.,2005's approach, this study prioritizes male mice for early proof-of-concept studies to establish the efficacy of IS 217, while future experiments in both male and female rats aim to broaden our understanding of its effects in higher species. This focus allows us to follow a two-step methodology and consider potential adverse effects, alternative targets, and clinical relevance in preclinical trials. Considering the lack of prior studies on the test molecule (peptide is 217), which is a new chemical entity in the present study, and following the suggested approach by Marshall et al., the primary focus of the main study is on male mice to establish the efficacy of the test molecule (peptide is 217).

The findings showed that IS 217 notably decreased mortality in mice infected with Gram-negative *E. coli* and those with induced peritonitis/sepsis via cecal ligation and puncture (CLP). Compared to diseased control animals receiving normal saline (NS), the peptide led to an 80% reduction in mortality, indicating its potential to prevent sepsis onset and improve survival rates. Furthermore, IS 217 demonstrated antimicrobial effects in animal models by reducing bacterial counts and aiding in infection clearance. Additionally, IS 217, the peptide, demonstrated immunomodulatory effects by impacting innate immunity. It prompted the recruitment of leukocytes to the infection site while reducing levels of pro-inflammatory cytokines—such as IL-12, IL-1β, IL-6, and TNF-α—in peritoneal macrophages, bronchoalveolar lavage fluid (BALF), and serum. Furthermore, IS 217 elevated the production of anti-inflammatory cytokines like IL-10, aiding in curbing an excessive and potentially harmful inflammatory response. The peptide also hindered disseminated intravascular coagulation (DIC), restored procalcitonin (PCT) levels, and decreased C-reactive protein (CRP) levels in mouse models of *E. coli*-induced and CLP-induced septicemia. IS 217 peptide treatment displayed protective effects against organ injury by restoring liver and kidney enzyme levels. IS 217 displayed significant effects on sepsis-induced acute lung injury (ALI) by reducing lung edema (measured by lung weight-to-dry weight ratio), lowering myeloperoxidase (MPO) and vascular endothelial growth factor (VEGF) levels, and suppressing the production of pro-inflammatory cytokines linked to ALI. These effects were achieved by modulating the MAPK signaling pathways.

Additionally, a short-term treatment with a single dose of IS 217 suppressed the expression of pro-inflammatory cytokines, effectively protecting mice from systemic inflammation and sepsis-related mortality. These findings indicate that IS 217, functioning as an HDP-dAMP, possesses both direct

antimicrobial properties and immune-modulatory capabilities. It can directly eliminate bacteria while assisting in infection resolution by reducing harmful immune responses and enhancing protective responses in sepsis.

The present study research work represents an initial exploration into the efficacy of the lead peptide, IS 217, serving as an early proof of concept for its potential in treating sepsis. This lays the foundation for further investigation into the intricate mechanisms underlying the IS 217 peptide's effectiveness against sepsis. **The IS 217 peptide presents a fresh perspective in anti-infective treatments, holding encouraging prospects as a candidate for antisepsis therapy, as highlighted in figure 117.**

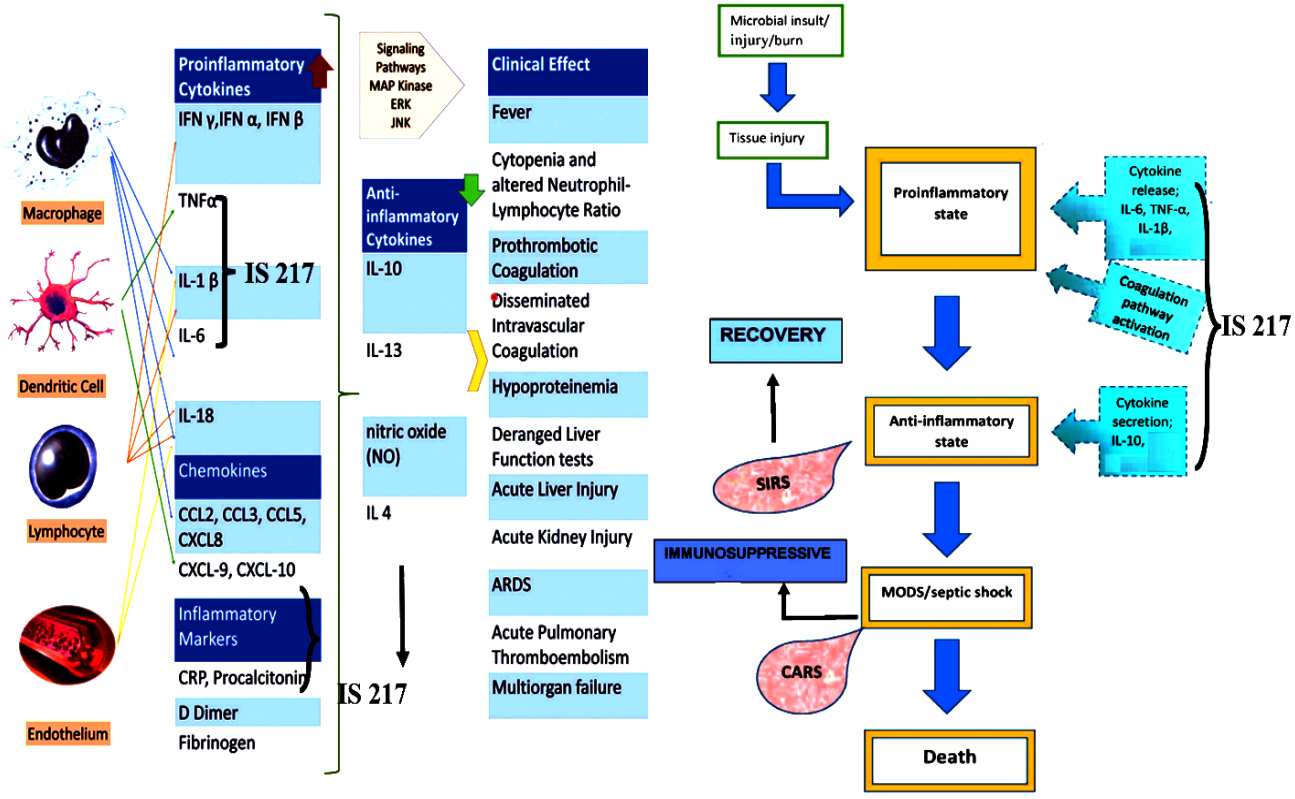


Figure 117: Proposed mechanism /effect of peptide IS 217 on cytokine storm and role of IS 217 in the treatment of sepsis.

Chapter 8.

Future perspectives

The use of peptides with dual antimicrobial and immunomodulatory properties represents a promising approach for addressing antibiotic-resistant infections. The findings of this study support the effectiveness of IS 217, an HDP-dAMP peptide, in not only kills bacteria but also possesses anti-inflammatory and immunomodulatory capabilities. IS 217 exerts its action by directly eliminating bacterial pathogens and exerting effects on the host's immune cells, leading to the suppression of potentially harmful inflammation that could result in sepsis.

The administration of peptide IS 217 demonstrated its ability to protect mice from lethal infections caused by gram-negative *E. coli* strain and sepsis induced by CLP. These results highlight the potential of IS 217 as an innovative anti-infective therapy, by combining its antimicrobial and immunomodulatory properties.

Overall, the findings of this study showcases IS 217 as a peptide-based approach against antibiotic resistance, emphasizing its dual action against pathogens and the host immune system. This peptide presents a novel strategy in the battle against antibiotic resistance and offers promise for better outcomes in treating infectious diseases.

- Further exploration is essential to gauge **IS 217's efficacy against various multi-resistant strains of gram-positive and gram-negative bacteria**. Delving deeper into the **mechanisms underlying IS 217's action** warrants attention, possibly through studies in higher animal species, to elucidate its efficacy and clinical potential.

- The study revealed IS 217's ability to alleviate lung injury induced by sepsis-associated ALI, suggesting its potential in treating acute lung injury, pneumonia, and even alleviating COVID-19 symptoms. Future studies on IS 217 should explore its effectiveness in conjunction with other **co morbid conditions with sepsis**.

- The goal of discovering a new lead molecule to treat human disease is not only that the molecules need to be efficacious, but they also need to have appropriate bioavailability, absorption distribution, metabolism, excretion, pharmacokinetic properties, and a lack of toxicity. While the acute toxicity profile of IS 217 was established in the present study, further research is imperative **to assess its pharmacokinetics, toxicity, and enhance**

stability. Strategies like incorporating D-amino acid residues into the peptide sequence could bolster its therapeutic potential.

- Considering IS 217 as a synthetic peptide, preventing immunogenicity is crucial when developing and applying such therapeutics in clinical settings. Although its immunogenicity in animals has been established, further studies are necessary to deepen our understanding of the **mechanisms behind IS 217's immunogenicity.**

- Formulation development and stability evaluation are critical aspects in ensuring the practicality and efficacy of peptide-based therapeutics. Future studies involve **formulation development, will center on refining a user-friendly injection formulation, which includes the drug substance and its vehicle in a vial for direct administration. Further exploration will specifically target long-term storage stability**.

Chapter 9.
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APPENDIX (es)

Appendix-I

Manufacturing procedure of Synthetic peptide IS 217 (Fmoc Synthesis)

Synthesis:

The Fmoc process synthesizes the decapeptide, one amino acid at a time, starting from C-terminus amino acid which is attached to a solid resin via a linker group. The coupling procedure was a continuous process during which a series of wash steps were performed before and after addition of each amino acid derivative. The process was mainly controlled by de-protection time and reagents used for the reaction. For each cycle, de-protection was performed twice; first in about 20% piperidine in DMF for about five minutes. The solution was then drained out and a second de-protection was conducted for about an additional 25 minutes to ensure the amino group is free for the coupling reaction. Coupling time was also a critical step for the Fmoc synthesis. Per Fmoc synthesis protocol, the coupling reaction with a specific Fmoc protected amino acid was controlled by the reaction time and the reaction time was at least 2 hours. The peptide requires protection of their side chains during the Fmoc synthesis. The side chains of these amino acids were pre-protected by tBu, Boc, Trt, or Pbf groups. The cleavage of these side chain protection groups was completed using trifluoroacetic acid (TFA) solution, but not using piperidine which was used to remove the Fmoc group. As an example of a 300-mmol synthesis, the solid phase synthesis is performed on a peptide synthesizer and about 3 times excess of Fmoc protected amino acids were used for each coupling. The qualitative ninhydrin test was performed for each step to confirm completion of coupling. The ninhydrin test was performed by adding several drops of each of the ninhydrin test reagents to the sample of the peptide resin. The presence of free amino groups was indicated visually by presence of blue color beads, which represented a positive result. If the testing result was positive, a double coupling process was then performed. If it was determined that free amino groups are still present after the double coupling, the sequence was then acetylated using acetic anhydride to avoid undesirable reactions in the next cycle. After coupling the last amino acid (completion of the sequence) the peptide resin was dried and weighed. The weight of the peptide resin was an initial indicator of quality of the synthesis at that stage. The final peptidyl resin was obtained after final wash with methanol and dried under vacuum. The weight gain of the solid phase synthesis matched the synthetic scale as a fully protected peptide on the resin. With the substitution of 0.4-0.5 mmol/g of rink amide resin, a 300-mmol synthesis gives about 1.2 Kg of resin.

The resin-bound peptide was then cleaved off the resin with trifluoroacetic acid (TFA/TIPS/water (95/2.5/2.5%). The side chain was also removed during this cleavage process. After 3-4 hours of reaction, the crude peptide was cut off, precipitated out, and washed with ethyl ether. Further vacuum dry gives an off-white powder (crude peptide). After cleavage, the crude peptide undergoes a series of processing steps, including purification by reverse phase chromatography and ion exchange. The resulting solution of purified peptide was then lyophilized to ensure complete removal of residual solvents.

Upon completion of the synthesis, the peptide acetate bulk drug substance was analyzed by gas chromatography for traces of residual solvents that may remain from the process. The specification of acetonitrile, dichloromethane and dimethylformamide in the final peptide acetate drug substance was ≤ 410 ppm, ≤ 600 ppm and ≤ 880 ppm, respectively. These established specifications provide assurance that residual solvents were removed during purification and were not present in the bulk drug substance with quantities over the specifications.

Cleavage and precipitation:

Cleavage of rink amide linker and AM (Amino Methyl) resin also requires acidic conditions. TFA is the primary reagent for such cleavage; however, since the cleavage involves multiple types of protection groups, the effective cleavage of these protection groups was accomplished by mixing TFA (95%) with triisopropylsilane (TIPS) (2.5%) and Water (2.5%). This mixture, of TFA, TIPS and water, is called Cleavage Cocktail (it is 10 times of weight of peptidyl resin). After completion of the last coupling de-protection was done twice with 20% Piperidine in Dimethyl formamide (DMF), 3 DMF washing, 2 DCM washing, 2 methanol washing, 2 diethyl ether washing and vacuum drying of peptidyl resin. Add cleavage cocktail reagents, one by one, in round bottom flask (RBF) and stir on magnetic stirrer. Take pre weighed peptidyl resin in another RBF and add cleavage cocktail into RBF containing peptidyl resin. Stir in a magnetic stirrer for about 4 hours. The resulting crude peptide (peptide solution) was then filtered through G2 sintered funnel. Collected filtrates evaporate in Rota-evaporation, at 40°C water bath temperature, until the volume gets reduced to 1/3 of its original volume. The resulting reduced volume of peptide solution transfers (drop by drop) into RBF containing chilled diethyl ether. The solution is left for mixing under stirring on a magnetic stirrer and after stirring time elapsed, the solution is left for settling down the precipitate in the bottom of the flask. Once precipitate is settled, the supernatant is decanted into an empty container. This process

(precipitation of peptide) repeated 4 more times. Then isolation of solid crude peptide was done by centrifugation. The solid crude peptide is left for air drying overnight under fume hood. Powdery crude peptide kept in self-sealed covers, which are placed in PVC container and then stored at 2-8°C.

Purification by reverse phase chromatography:

The purification equipment used was based on the principle of axial compression in which the chromatographic support is packed in a stainless-steel compression module. The stationary phase material was dedicated to the peptide, eliminating the possibility of cross-contamination. The choice of module used was based on the amount of material to be processed. Solvents were delivered through pumps and the necessary gradients can be created with the pump integrated with an automatic gradient maker. The column can be used for purification of several syntheses of peptide until the column no longer meets the performance requirements.

The dried crude peptide (dissolved in 1000 ml of 20% Acetonitrile solution) was purified using reverse phase chromatography with a C-18 coated, 10-micron bead column. The first purification buffer system included about 0.1% TFA in water (Mobile phase A) and 100% acetonitrile (ACN) (Mobile phase B). The flow rate was maintained with a linear gradient. All collected fractions are sent for analytical analysis. Pooled all fractions with purity ≥ 98% and evaporate it in rota-evaporation at 37°C of water bath temperature, until the volume gets reduced to 1/3 of its original volume.

Counter ion exchange by reverse phase chromatography:

The second in-process peptide solution was further purified, and ion exchanged on the C-18 reverse phase high performance liquid chromatography (RP-HPLC) column. Mobile phases utilized for pre-wash were about 50 mM ammonium acetate buffer (pH 6, buffer A) and about 100% acetonitrile (ACN) (Buffer B). The mobile phases utilized for final ion exchange and purification were about 0.1% acetic acid (AcOH) in water (pH 3.5, Buffer A) and about 0.1% acetic acid (AcOH) in acetonitrile (ACN) (Buffer B). The flow rate was maintained at about 100 mL/min with a linear gradient of 10% to 70% Buffer B in about 60 minutes. Fractions with ≥98% purity were collected. The peptide solution was then lyophilized with a 12-hour (minimum) lyophilization cycle. This last purification process simultaneously converted the peptide drug substance into the acetate salt form.

Final lyophilization:

The collected reduced volume after roto evaporation was lyophilized under vacuum 50-100 millitorr and -70 to -50°C (condenser temperature) for 48 hours to complete the peptide manufacturing process.

Disposition of final bulk drug substance:

Upon completion of the manufacturing process, the final bulk drug substance was held in the production until all analytical testing has been completed by quality control department, and quality assurance has reviewed the batch production record (including its related supporting documentation) and has released the drug substance. The characterization of synthesized peptides was fully characterized using modern analytical techniques: HRMS- LCMS, 1H-NMR and 13C NMR to confirm the structure of the compounds. The purity of the compounds was evaluated by HPLC. After receiving the report from the quality control department, production handover the drug substance to the stores department for storage and further use in future.

Appendix-II –COA's of the test peptides
(IS 141, IS 111 &IS 217)

Issar Pharmaceuticals Pvt. Ltd. Sy.No.230 to 243, Plot No. 3 Alexandria Knowledge Park, Turkapally Village Shameerpet Mandal Ranga Reddy Distt. (T.S.)-500 078	
Title	**Certificate of Analysis**

Quality Control Department

Name of the product: IS 217			
Batch No:	IS 217-17-10	**B. Size**	200m.mol
Mfg. Date:	June 2019	**Exp. Date**	NA
Qty. received for analysis	500 mg	**Report date:**	02/07/2019

S.no	Test	Specification	Result
1.0	**Description**	White to off white colored	white colored powder
2.0	**Solubility**	Soluble in water.	Complies
3.0	**Identification (By HPLC)**	The retention time of the major peak in the chromatogram of the sample preparation corresponds to that in the chromatogram of the standard preparation as obtained in the purity.	Complies
4.0	**Identification (By LCMS)**	Should be 1242.6 ±2 M. W	1242.6
5.0	**Water content (By KF-Titrator)**	Not more than 5.0%	4.43%
6.0	**Purity (By HPLC)**	Not less than 99.0%	99.03%
7.0	**Related Substance (By HPLC)** **a) Any Max. Impurity** **b) Total Impurities**	To be reported	 a) 0.72% b) 0.97%
Remarks: The product complies/does not comply			

Prepared By	Reviewed By	Approved By
Quality control Head	Quality Assurance -Manager	Quality Assurance -Head

Note: *This is a computer-generated statement and does not need any signature*

ISSAR PHARMACEUTICALS PRIVATE LIMITED ★ Hyderabad

	Issar Pharmaceuticals Pvt. Ltd. Sy.No.230 to 243, Plot No. 3 Alexandria Knowledge Park, Turkapally Village Shameerpet Mandal Ranga Reddy Distt. (T.S.)-500 078
Title	**Certificate of Analysis**

Quality Control Department

Name of the product: IS 141			
Batch No:	IS 141-21-01	**B.Size**	200m.mol
Mfg.Date:	May 2021	**Exp.Date**	NA
Qty received for analysis	500 mg	**Report date:**	23/05/2021

S.No	Test	Specification	Result
1.0	**Description**	White to off white colored	white colored powder
2.0	**Solubility**	Soluble in water.	Complies
3.0	**Identification (By HPLC)**	The retention time of the major peak in the chromatogram of the sample preparation corresponds to that in the chromatogram of the standard preparation as obtained in the purity.	Complies
4.0	**Identification (By LCMS)**	Should be 1259.6± 2 M. W	1260.5
5.0	**Water content (By KF-Titrator)**	Not more than 5.0%	99.17%
6.0	**Purity (By HPLC)**	Not less than 99.0%	99.03%
7.0	**Related Substance (By HPLC)** **a) Any Max. Impurity** **b) Total Impurities**	To be reported	 a) 0.34% b) 0.86%
Remarks: The product complies/does not comply			

Prepared By	Reviewed By	Approved By
Quality control Head	Quality Assurance -Manager	Quality Assurance -Head

Note: *This is a computer-generated statement and does not need any signature*

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	Issar Pharmaceuticals Pvt. Ltd. Sy.No.230 to 243, Plot No. 3 Alexandria Knowledge Park, Turkapally Village Shameerpet Mandal Ranga Reddy Distt. (T.S.)-500 078
Title	**Certificate of Analysis**

Quality Control Department

Name of the product: IS 111			
Batch No:	IS 111-20-02	**B.Size**	200m.mol
Mfg.Date:	June 2020	**Exp.Date**	NA
Qty received for analysis	500 mg	**Report date:**	20/07/2020

S.No	**Test**	**Specification**	**Result**
1.0	**Description**	White to off white colored	white colored powder
2.0	**Solubility**	Soluble in water.	Complies
3.0	**Identification (By HPLC)**	The retention time of the major peak in the chromatogram of the sample preparation corresponds to that in the chromatogram of the standard preparation as obtained in the purity.	Complies
4.0	**Identification (By LCMS)**	Should be 839.04 ±2 M. W	839.04
5.0	**Water content (By KF-Titrator)**	Not more than 5.0%	4.45%
6.0	**Purity (By HPLC)**	Not less than 99.0%	99.76%
7.0	**Related Substance (By HPLC)** **a) Any Max. Impurity** **b) Total Impurities**	To be reported	 a) 0.46% b) 0.97%
Remarks: The product complies/does not comply			

Prepared By	**Reviewed By**	**Approved By**
Quality control Head	Quality Assurance -Manager	Quality Assurance -Head

Note: *This is a computer-generated statement and does not need any signature*

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Appendix-III

Dose calculations of Lead peptide (IS 217) and standard drug used in the In vivo animal studies.

1.Preparation and administration of Lead peptide IS 217 doses – In vivo studies:

- The test compound is provided in a 10 mg quantity in a vial and prepare the stock solution.
- Prepare the stock solution containing 2mg / 1ml (10 mg is diluted in 5 ml of saline) and name it as **STOCK SOLUTION-I.**
- From the above stock solution, the three working solutions are calculated and prepared to the required dose for mice as per group according to the body weight.
- The acute toxicity and In vivo test dose preparation are as follows.

Table 93: The preparation IS 217 - test doses from the stock solution.

Test dose	Preparation:
0.6 mg/kg	0.06 mg/mL was prepared form the stock solution (8.61mg/mL) by diluted 0.035µL of stock in 4.965mL of 0.9%NaCl.
1.2 mg/kg	0.24 mg/mL was prepared form the stock solution (8.61mg/mL) by diluted 139µL of stock in 4.861mL of 0.9%NaCl.
4.8mg/kg	0.48 mg/mL was prepared form the stock solution (8.61mg/mL) by diluted 279µL of stock in 4.721mL of 0.9%NaCl.

1.2. Preparation of standard drug doses – In vivo studies:

The standard drugs dose is prepared by using the following formula: [Jang-Woo Shin et al., 2010 &Anroop B. Nair, et al., 2016]

Animal dose (mg/kg) = HED (mg/kg) X conversion factor.

HED: Human equivalent dose; Conversion factor from human to mouse is 12.33.

All the animals will receive the same volume of the test dose 0.1 mL by subcutaneously and intravenously.

Repeat the procedure for preparation of test drug daily for fourteen days in acute study and in vivo efficacy studies the test drug was prepared freshly at the time of administration based on the requirement.

List of patents applied from thesis work:

Patent -01:

Title of the patent: A novel synthetic peptides with immunomodulatory activities.

Patent number: 202341000025, dated :01.01.2023.

Applicants: 1. Edula sravani; 2. Isanaka Ramakrishna Reddy and Dharmarajan Sriram.

Patent -02:

Title of the patent: A short synthetic anti-infective peptide with dual antimicrobial and immunomodulatory activities.

Patent number: 202341005211, dated :25.01.2023.

Applicants: 1. Edula sravani; 2. Isanaka Ramakrishna Reddy and Dharmarajan Sriram.

List of the conferences attended:

Presented a Poster on "Antimicrobial activity of synthetic peptides against the ESKAPE pathogens and addressing Methicillin-Resistant Staphylococcus aureus infection" at 62nd Annual International Conference of Association of Microbiologists of India (AMI), September 21-23,2022.

Brief Biography of Sravani E

Mrs. Sravani Edula is currently employed as a research scientist specializing in new drug development at Issar Pharmaceuticals Pvt. Ltd., and concurrently pursuing a Ph.D. in the Department of Pharmacy under the guidance of D. Sriram at BITS Pilani, Hyderabad campus. Her professional background includes roles as a technical officer at CDFD, Hyderabad, and NIN-ICMR, Hyderabad. Mrs. Edula holds a Master's degree in pharmacy with a focus on pharmacology from Sri Krishnadevaraya University, Anantapur, and a Bachelor's degree in pharmacy from Nirmala College of Pharmacy (JNTU). She has notable experience participating in two pre-IND USFDA filings for psoriasis and burn wound molecules and contributing to two regulatory submissions related to burns and wound healing at DCGI-CDSCO, India. Moreover, she has contributed to four scientific publications in esteemed international journals and delivered presentations at national and international conferences. She holds one patent, alongside applications pending for two additional patents based on her thesis research work.

Previous publications:

- **Sravani Edula,** Chaitanya Mannava, et.al., Preclinical bioavailability–bioequivalence and toxicokinetic profile of stable succinic acid cocrystal of temozolomide. CURRENT SCIENCE, 2015; 108(6):1097-1106.

- **Edula S**, Nimgulkar C, Racha V, et.al., Role of in- silico COX2 molecular docking bioinformatics tool and in-vivo carrageenan-induced v various inflammatory phase in mouse and rat models, in the evaluation of the anti-inflammatory activity of molecules. *Int J Pharma Res & Rev*, 2014; 3(4):57-65.

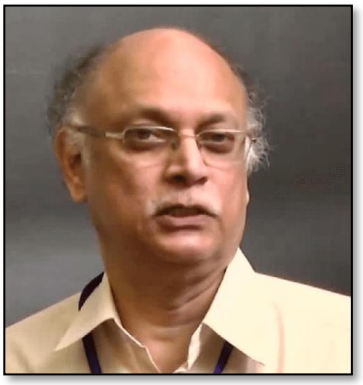
Biography of Prof. D. Sriram

D. Sriram is presently working in the capacity of Senior Professor at Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Hyderabad campus. He received his Ph.D. in 2000 from Banaras Hindu University, Varanasi. He has been involved in teaching and research for last 20 years. He has more than 465 peer-reviewed research publications, 5 patents and 1 book to his credit. He has collaborations with various national and international organizations such as Karolinska institute, Sweden, the Indian Institute of Science, Bangalore and National Institute of Immunology, New Delhi. He was awarded the Young Pharmacy Teacher of the year award of 2006 by the Association of Pharmacy Teachers of India. He received ICMR Centenary year award in 2011. Prof. D. Sriram has been selected for prestigious Tata Innovation Fellowship of the Department of Biotechnology, Government of India for the year 2015-2016. He has guided 27 Ph.D. students. His research is funded by agencies like the UGC, CSIR, ICMR, DBT and DST.

Biography of Prof. Arti Dhar

Dr Arti Dhar is currently working as a Professor in the Department of Pharmacy at Birla Institute of Technology and Science (BITS), Pilani, Hyderabad campus. Dr. Dhar received her PhD from College of Medicine, University of Saskatchewan, Canada in the year 2010. During her PhD she received scholarships from Heart and Stroke Foundation of Canada (HSFC) and Arthur Smith Memorial Scholarship from University of Saskatchewan, Canada. Dr. Dhar also won numerous travel awards from Canadian Physiological Society and Canadian Hypertension Society. Her PhD thesis was nominated for Governor General's Gold medal and her thesis work was presented on CBC channel Canada in March 2011. She did her postdoctoral trainings from Lakehead University, Ontario, Canada and University of Saskatchewan, Canada from the year 2010 to 2013. After joining BITS Pilani, Hyderabad Campus in 2014, she has received research funding from DST-SERB, CSIR, ICMR and from BITS under additional competitive grant. She has published more than 42 research publications in peer-reviewed international journals. She has guided four doctoral students, six master's students and six undergraduate students in fulfilment of their dissertation work. Currently 5 students are pursuing PhD under her supervision. Her main research interests are centered on novel therapeutic targets for cardiovascular, metabolic disorders and cancer. She has won numerous awards at national and international level.

Biography of Dr. B. Sesikeran



Dr. B. Sesikeran is presently working in the capacity of Scientific Advisory Member to Issar Pharmaceuticals Pvt. Ltd. He is a former Director of the National Institute of Nutrition, Indian Council of Medical Research, and has been a nutritional pathologist of repute for over 30 years. He has an MBBS degree from Stanley Medical College, Chennai, and an MD in Pathology from Gandhi Medical College, Hyderabad. He nurtured an interest in Nutrition Sciences right in his student days. He has to his credit over 120 publications in national and international journals, 7 chapters in books and monographs, several technical reports, papers in proceedings of conferences, and popular articles He is presently holding numerous positions as chairman of the review committee for Genetic Manipulation – Govt of India, President-Nutrition Society of India; Vice President- Microbiota and Probiotic Science Foundation of India and many. He has guided many Ph.D., MD, and MDS students of NTR University of Health Sciences.