Chapter 2

Review of Literature

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Cancer has emerged as a global epidemic in the recent decades. In the Middle East countries, there has been a traditional belief suggesting the preventive and therapeutic potential of camel milk against cancer. Now there is more upcoming scientific evidence to lend support to this belief (Korashy et al., 2012b, 2012a; Magjeed, 2005). Camel milk is generally consumed without boiling, thereby retaining its immunological properties that are normally lost in milk by preservation processes like boiling, pasteurization, etc. (Agrawal et al., 2007). Camel is a mammal well adapted to deal with stressful conditions like high temperature and water deficiency. Its proteins are highly thermo-stable and resistant to acid hydrolysis (Atri et al., 2011).

2.1 Milk

Milk has a wide range of immunological molecules that enhance the survival value of infants feeding on mother's milk directly. Many of these molecules are present across mammalian species. Human milk too has numerous compounds with immune modulatory, antimicrobial and anti-neoplastic properties and/or with the combination of the above. It also contains various bioactive components having an anti-cancer action.

2.1.1 Immunological and anti-cancer activity of human milk

Many milk components act not only as nutrients but also have very important immunological and anti-cancer properties. Milk has two main fractions namely, casein and whey. Casein breakdown products have the ability to prevent mutations. The high content of cystine/cysteine and gamma-glutamylcyst(e)ine dipeptides have been implicated for this activity. Dipeptides are efficient substrates for the synthesis of glutathione. Glutathione is a well-known ubiquitous cellular antioxidant that either directly or through its associated enzymes destroys reactive oxygen species (ROS). It also detoxifies carcinogens, maintains proteins in a reduced state and ensures immune

competence. Increased glutathione levels in serum and tissues accompany tumor prevention by dietary whey proteins. Enhanced glutathione level has been found to be associated with an enhanced spleenocyte proliferation and phagocytosis. It is also involved in immune regulation by T Helper cells, cytotoxicity mediated by natural killer cells and CTLs. The whey components, include α-lactalbumin, TRAIL, lactoferrin, etc. are discussed in detail later in this section.

Some vital biologically active molecules present in milk are listed in **Table 1** below.

Table 1: Bioactive components in human milk

Type of molecule	Biomolecule present in milk	Biochemical nature	Significant property	Reference
Immunomodulatory	Fibronectin	Glycoprotein	Increases antimicrobial activity of macrophage Carcinoma development	Goldsby et al., 2003; Han et al., 2006
	Hormones and growth Factors	Peptides and proteins	Development of infants GIT against microbes	Newman, 1995
	Interferons	Proteins	Enhances antimicrobial activity of immune competent cells and signals adjacent cell on being virally infected to take preventive measure	Watanabe, 2004
	Lactoferrin	Globular glycoprotein	Makes Iron unavailable for bacteria thereby inhibiting its growth Enhances lactose tolerance	El-Fakharany, et al., 2008; Cardoso et. al., 2010; Conesa et al., 2008.
Antimicrobial	Secretory Immunoglobulins	Glycoproteins	Prevent microbial infiltration in tissue by blocking entry	Harmsen et al.,2007; Cortez et al.,2002
	B_{12} binding protein	Protein	Retards bacterial growth by reducing the amount of available vitamin B_{12}	Newman, 1995
	Fatty acids	Lipid	Disruption of some viral membrane	Newman, 1995
	Bifidus factor	methyl-N-acetyl D-glucosamine	Promotes growth of harmless *L. Bifidusto* out compete pathogenic strains	Newman, 1995
	Lysozyme	Protein	Kills bacteria by disrupting its wall	El-Agamy et al., 1992
	Mucins	Glycosylated proteins	Prevent bacterial & viral attachment to mucosal surfaces	Newman, 1995
	Oligosaccharides	Carbohydrates	Prevent microbes from binding to mucosal surface by attaching to the microbes	Newman, 1995
Anticancer	Lactoferrin	Globular glycoproteins	Induction of apoptosis, inhibition of angiogenesis and modulation of carcinogen metabolizing enzymes	El-Agamy et al., 1996
	Casein and its peptides	Phosphoproteins	Antimutagenic properties	Parodi et al.,2007
	TRAIL (TNF-related apoptosis inducing ligand)	Cytokine (proteins)	A member of TNF family, kills cancer cells in vitro and in vivo	Horinaka et al., 2010
	XAMLET	Lipoprotein	Induces cell death in certain cancers and cell lines without effecting normal tissue	Zhang et al., 2009
	CLA (conjugated linoleic acids)	Fatty Acid	Antimutagenic activity	Amarù et al., 2009

(Adapted from Dubey et. al. 2016)

The anti-cancer properties of human milk are far better studied than any other species. Detailed immunological properties of Human milk are given in Table 2.

Table 2: Immunological molecules present in human milk

Antibodies of secretory IgA class	Bind to microbes in baby's digestive tract, thereby prevent their attachment to the walls of the gut and their subsequent passage into the body's tissues.
B12 binding protein	Reduces amount of vitamin B12, which bacteria need in order to grow.
Bifidus factor	Promotes growth of Lactobacillus bifidus, a harmless bacterium in the baby's gut. Growth of such nonpathogenic bacteria helps to crowd out dangerous varieties.
Fatty acids	Disrupts membranes surrounding certain viruses and destroy them.
Fibronectin	Increases antimicrobial activity of macrophages. Helps to repair tissues that have been damaged by immune reactions in baby's gut.
Hormones and growth factors	Stimulate baby's digestive tract to mature more quickly. Once the initially leaky membranes lining the gut mature, infants become less vulnerable to microorganisms.
Interferon (INF-γ)	Enhances antimicrobial activity of immune cells.
Lactoferrin	Binds to iron, a mineral many bacteria need to survive. By reducing the available amount of iron, lactoferrin thwarts growth of pathogenic bacteria.
Lysozyme	Kills bacteria by disrupting their cell walls.
Mucins	Adhere to bacteria and viruses, thus keeping such microorganisms from attaching to mucosal surfaces.
Oligosaccharides	Bind to microorganisms and bar them from attaching to mucosal surfaces.

(Adapted from J Newman, 1995)

In context of the present study it is important to understand two evolutionarily conserved relevant molecules from human milk with anti-cancer property, namely α – lactalbumin and TRAIL. These immunological molecules are shared amongst many species including human and camel milk.

2.1.1.1 α-lactalbumin

α-lactalbumin performs many vital functions such as facilitating mineral absorption, being a component of lactose synthetase (thus role in milk formation), protein synthesis in developing infants, etc. Multimeric α-lactalbumin induced apotosis in transformed cells. HAMLET is a complex derived from α-lactalbumin and oleic acid. HAMLET is a very promising anti-cancer agent derived from human milk. It induces massive cell death in various types of cancers without causing any damage to normal tissues. Given below are details regarding the same.

2.1.1.2 HAMLET (Human α–lactalbumin made lethal to tumors)

HAMLET treated cells underwent shrinkage, nuclear condensation, caspase activation and DNA fragmentation, the characteristic features of programmed cell death (Håkansson et al., 1995). HAMLET-induced cell death is p53-independent and it also triggers an autophagic response (Aits et al., 2009). HAMLET not only translocates into tumor cells, activates cell death pathways but also induces adherent cell detachment *in vitro* (Trulsson et al., 2011). It also triggers tumor cell detachment *in vivo* in bladder cancer patients (Mossberg et al., 2007).

This molecule *in vitro* was able to induce programmed cell death in many human and murine cell lines such as Jurkat, L1210 (a leukemia cell line), A549 (a lung carcinoma line), and A498 (kidney carcinoma cell line). It also effectively destroys a great variety of tumor cells ranging from carcinomas of lung, throat, kidney, colon, bladder, prostate, ovaries; melanomas, to glioblastomas of the brain and leukemia (Spolaore et al., 2010; Svensson et al., 2000). Mossberg *et al*., (2007)

examined the effect of HAMLET on plasma membrane vesicles (PMVs) obtained from tumor cells. The treatment of bladder tumors by intravesicle HAMLET delivery resulted in a significant reduction. in eight out of nine treated patients. HAMLET has shown striking effects in a rat model xenografted with human glioblastomas (Fischer et al., 2004). The interaction of tumor cell plasma membrane with HAMLET resulted in change in the fluidity of lipid bilayer leading to membrane elongation (Mossberg et al., 2010). Membranes of tumor cells have altered lipid composition and fluidity. This may alter membrane-bound receptors and endocytic pathways and thereby affecting the activation of cell death in a manner that discriminates tumor cells from normal cells. After passing through the first barrier of plasma membrane, HAMLET entered cytoplasm and translocated into the nucleus (Düringer et al., 2003). HAMLET, being a partially unfolded protein, triggers endoplasmic reticulum stress signal in the cytoplasm, and is directed to 20S proteosome for degradation. Here it triggers a change in structure of proteosome and resists its own degradation by proteolytic enzymes (Gustafsson et al., 2009). In the nucleus histone proteins, mainly H3 and H4 act as nuclear receptors for HAMLET. In their study in 2007, Brest *et al.*, have shown that histone deacytylase inhibitors enhance the tumoricidal effects of HAMLET. They acted by enhancing the hyperacetylation response which disrupts chromatin structure. α-lactalbumin upon combination with oleic acid can be converted to the complex, HAMLET, which kills tumor cells selectively. The thermal stability of camel milk α-lactalbumin and its complexes with oleic acid and linoleic acid were studied at 60°C. Both these complexes were observed to have a stable structure. They were able to exhibit a cytotoxic effect on DU145, a human prostate cancer cell line, even after exposure to temperatures as high as 60°C (Atri et al., 2011).

2.1.1.3 TNF - Related Apoptosis Inducing Ligand (TRAIL)

TRAIL is a member of the tumor necrosis factor (TNF) super family of cytokines. It may be present either in a trans-membrane form or occur in the cytoplasm as a soluble protein. Extremely high levels of TRAIL have been detected in human colostrum and milk (Davanzo et al., 2013). TRAIL is also a pleiotropic cytokine with important functions in regulating immune response and inflammation. It plays a key role in controlling cell death and cell proliferation in various organs and tissues. The best characterized activity of TRAIL is its ability to kill cancer cells both *in vitro* and *in vivo*. It is currently being used as a recombinant protein in many clinical trials against a wide variety of human cancers. Moreover, it has recently been shown that certain *Lactobacillus* strains induce the production of TRAIL. This further facilitates natural killer activity against cancer cells (Horinaka et al., 2010). Endogenous soluble TRAIL itself represents a strong candidate to explain the overall biological effect of breastfeeding against cancer.

2.2 Epidemiological evidences of anti-cancer properties of Human milk

Epidemiological evidences strongly suggest benefits of mother's milk against cancer in humans. A qualitative review assessing the association between infant feeding and childhood cancer was undertaken by Davis et. al. in 1998. The results suggested that children who were never breast-fed or fed for short-term (< 6 months) were at a higher risk of developing Hodgkin's lymphoma. In another study, the total duration of breastfeeding and of exclusive breastfeeding was compared in 99 childhood cancer cases and 90 controls. The outcome suggested that breast feeding had a protective effect against childhood cancer. This study also indicated that exclusive breast feeding provided more beneficial immunological effects than breast feeding supplemented by artificial feeding (Mathur et al., 1993). The high rate of breast feeding in India may explain the low

incidence of childhood cancer in India as compared to many countries such as Israel (6/100,000 in India vs. 18/100,000 in Israel). There exists no substitute for mother's milk in any mammalian species other than humans. In animal's infants deprived of mother milk are likely to simply die away due malnutrition and disease. The research conducted in human milk shows presence of vital immunological factors (Goldsby *et al*., 2003). Similar benefits are likely to be present in other mammalian species too, although not many studies have been done. Benefits of maternal milk may be presumed because there is an evolutionary preservation of vital molecules, required for survival, across diverse species. Molecules present in maternal milk may help to optimize the function of the immune system and regulate cell proliferation to avoid tumor development in the rapidly growing neonatal cells.

2.3 Camel milk

Camel milk is recognized for its therapeutic potential against many diseases. It is reported to have microbicidal and immuno stimulatory properties as it contains immunoactive proteins like lysozyme, lactoperoxidase and lactoferrin. Camelid antibodies have a unique structure. They possess the heavy chains but are devoid of the usual light chains. This special feature enhances their penetration. Camelid proteins have a very high degree of thermal stability and are resistant to acid hydrolysis. Camel milk components act like a ligand to the aryl hydrocarbon receptor. They significantly inhibit the induction of some cancer-activating genes and also induce tumor suppressor genes. Modulation of aryl hydrocarbon receptors is now recognized to have a vital role in cancer therapy.

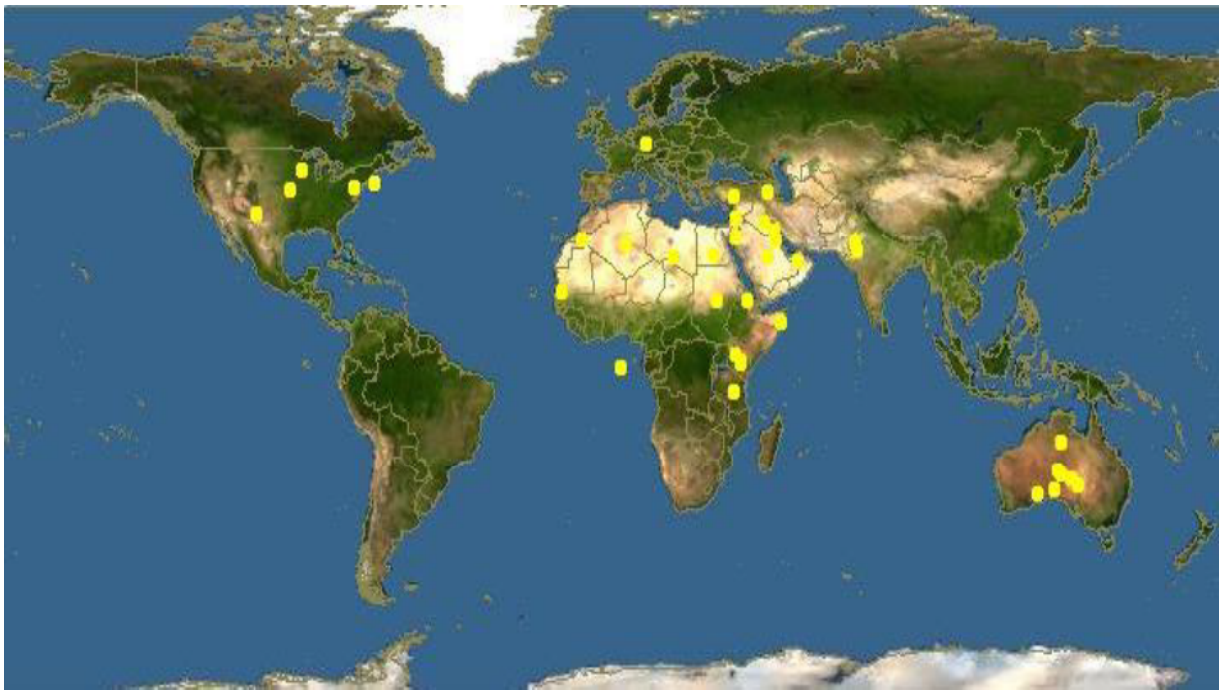
Camel is a mammal well adapted to deal to stressful conditions like high temperature and water deficiency (Faye, 2014). Its proteins are highly thermo-stable and resistant to acid hydrolysis (Atri

et al., 2011). The ability of camel to withstand elevated temperatures may be useful in killing pathogenic thermosensitive infectious agents. This may be the speculated cause behind the so called pathogen free state of its milk. Therefore, camel milk rarely ever causes any disease even though it is normally consumed raw. Consumption of camel milk without boiling helps in retaining its various biological and immunological properties that are normally lost in milk preservation processes (Agrawal et al., 2007). The details of its classification, habits and habitat are listed in **Table 3** below. Also given alongside is the distribution of camels in the world in Figure 3.

Table 3: Classification, habits and habitats of *Camelus dromedarius*

Kingdom	Animalia
Phylum	Chordata
Class	Mammalia
Order	Artiodactyla
Family	Camelidae
Genus	Camelus
Scientific Name	*Camelus dromedaries*
Habitat	Arid desert and scrubland
Colour	Cream, Tan, Brown, Black
Skin Type	Hair
Size (L)	2.2m - 3.5m (7.25ft - 11.5ft)
Weight	300kg - 690kg (660lbs - 1,500lbs)
Top Speed	64kph (40mph)
Diet	Herbivore (Thorny and Salty Plants, Grass, Grain)
Lifestyle	Diurnal
Group Behaviour	Herd
Life Span	40 - 50 years
Age Of Sexual Maturity	3 - 5 years
Gestation Period	390 - 410 days

Average Litter Size	1
Name Of Young	Calf
Age Of Weaning	4 months
Most Distinctive Feature	Long, curved neck and large hump



(URL:https://www.discoverlife.org/nh/maps/Vertebrata/Mammalia/Camelidae/Camelus/map_of_Camelus_dromedarius.jpg)

Figure 3: *Camelus dromedarius* population across the globe.

2.3.1 Nutritional value of camel milk

Camel milk is given to the sick, elderly and very young because of the belief that it is not only healthier, but works especially well in bone formation. A high content of unsaturated fatty acids (such as oleic acid) further improves its overall dietary quality (El-Agamy et al., 2009;

Konuspayeva et al., 2009; Nikkhah, 2011; Shabo et al., 2005; Shamsia, 2009). Camel milk is mostly consumed fresh and raw by some native communities in India. In this way its medicinal properties are not destroyed by preservation techniques like boiling, pasteurization, etc., Also it does not form coagulum in acidic environment (J et al., 1998). This lack of coagulum formation allows the camel milk to pass rapidly through stomach with proteins available for absorption in intestine. The pH of camel milk is between 6.5 - 6.7. Camel milk is different from other ruminant milk because it has reasonable content of cholesterol, high minerals (sodium, potassium, iron, copper, zinc and magnesium), high vitamin A, B2, C and E, low protein and high concentrations of an insulin-like protein (Konuspayeva et al., 2009; Shamsia, 2009). One liter of camel milk has ample amount of minerals to meet 100% of the daily human requirements for calcium and phosphorus, 57.6% for potassium, 40% for iron, copper, zinc and magnesium, and 24% for sodium. It helps treat liver problems, lowers bilirubin output, and lowers vitamin inadequacy and nutrient deficiency besides boosting immunity. As compared to cow milk, camel milk contains substantially less vitamins A and B2, similar vitamin E content and about 3-10 times greater vitamin C (Konuspayeva et al., 2009; Nikkhah, 2011).

A comparative lipid profile indicates that the short chain fatty acids (C4 –C12) although were present in small amounts but they were higher than in human milk fat. On the contrary the concentration of higher chain fatty acids such as C14:0, C16:1 and C18:0 were relatively high in camel's milk fat as compared to human milk fat. Appreciable amounts of essential fatty acids were also present in camel milk (Konuspayeva et al., 2009; Shamsia, 2009).

In the Middle Eastern countries fresh butter derived from camel milk is often used as a base for many medicines. Not only is it totally devoid of any allergic properties but it also heals food allergies and gut problems. It can be conveniently consumed by lactase deficient people

and even by those who have a weak immune system. The hypo-allergic effect of camel milk is associated with a low level of β-casein and lack of β-lactoglobulin. This milk also apparently has slimming properties attributed to camel milk owing to its cholesterol level (El-Agamy et al., 2009).

2.3.2 Medicinal properties and therapeutic potential of camel milk

Similar to human milk, camel milk too possess an astonishing array of immunologically important molecules like lactoferrin, lysozymes, peptidoglycan recognition proteins (PRPs), lactoperoxidase, N- acetyle glucosaminidase and only heavy chain antibodies. Medicinal properties present in camel milk have been attributed to a wide array of immunologically significant protective proteins present in it. The level of peptidoglycan recognition protein (PRP) is very high in camel milk. PRP not only stimulates the host's immune response but also has an antimicrobial activity (Assaf and Ruppanneb, 1992). It acts by preventing studies have microbial overgrowth and inhibiting pathogenic invasion.

The functions of these immunologically relevant molecules have been shown in the Table 4 below.

Table 4: Molecules with immune benefit in camel milk

S. no.	Name of molecule	Function	Reference
1.	Heavy chain antibodies (HCAb) or variable heavy antibodies (VHH) or nanobodies	Able to interact with less immune dominant parts of antigens More tissue penetration but similar specificity Equivalent specificity Rapid renal clearance in human	Hamers-Castermanet al. (1993); Muydermans, 2013
2.	Peptidoglycan recognition protein (PRP)	Stimulates immune response and has antimicrobial activity	El Agamy et al., 1992
3	Lactoferrin	Prevents pathogenic invasion and microbial overgrowth	El Agamy et al., 1992; Konuspayeva et al., 2006
4	Lactoperoxidase	Antitumor activity Antibacterial against gram negative bacteria like *E. Coli, Salmonella* and *Pseudomonas* Bacteriostatic against gram positive	El Agamy et al., 1996
5	Lysozyme	Targets Gram positive bacteria	El Agamy et al., 1992
6.	N-acetyl-glucosamineidase (NAGase)	Antibacterial and antiviral activity	Jassim and Naji, 2001

(Adapted from Dubey et. al., 2016)

Recent studies have shown that the amount of lactoferrin is almost similar to that in cow milk however its bioactivity is higher (Conesa et al., 2008; Narmuratova et al., 2006). The camel milk has the same quantity of sugar (lactose) but the only difference is the better lactose tolerance for consumers. Lactoperoxidase present in camel milk has bactericidal activity on gram-negative bacteria. Its anti-tumor activity has also been suggested. Lysozyme is a part of the soluble components of the innate immune system and targets gram-positive bacteria. N-acetyl-beta-D-glucosamidase is found in similar quantities as in human milk where it has antibacterial activity. Lactoferrin, lactoperoxidase, lysozyme, immunoglobulin G and secretory immunoglobulin A were extracted from camel milk (Assaf and Ruppanneb, 1992). The activity of these protective proteins was assayed against *Lactococcus lactis*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* & rotavirus and compared with egg white lysozyme, bovine lysozyme and bovine lactoferrin. The spectrum of antibacterial activity of camel milk lysozyme was similar to that of egg white lysozyme, but differed from bovine milk lysozyme. The camel milk lactoperoxidase was bacteriostatic against the gram-positive strains and was bactericidal against gram-negative cultures. The immunoglobulins had little effect against the bacteria but high titers of antibodies against rotavirus were found in camel milk. The lactoperoxidase system was ineffective against rotavirus (Elagamy et al., 1996).

As compared to human milk, camel milk has a higher ratio of immunoglobulins but a lower ratio of both lysozyme and lactoferrin. Ahmed *et al*., in 2011 studied the role of camel milk in the reversal of liver damage caused by Sudanese liquor, popularly known as *Aragi*. Their study showed a statistically significant increase in the liver enzyme markers in the group given liquor as compared to the controls. Camel milk was observed to reduce the level of vital liver enzymes. It

was thus suggested that camel milk can be used as a remedy for treatment of alcoholism and other liver diseases.

Camel milk is emerging as a potent therapeutic alternative which can help in reducing insulin doses in diabetic patients. Its well established role in management of diabetes has rendered it the title of "white gold of desert". Epidemiological surveys strongly indicate low prevalence of diabetes in communities consuming camel milk (Agrawal et al., 2013). Global market potential for camel milk needs to be further exploited.

A study was conducted to see if the consumption of camel milk was beneficial for diabetes. A significant improvement in glycosylated hemoglobin (HbA1c) and fasting blood sugar levels was observed. Also a significant reduction in insulin requirement in patients receiving camel milk was noted. Although there was a 30% reduction in doses of insulin in 92% of patients but there were no statistically significant change in lipid profile, plasma insulin and c peptide (Agrawal et al., 2007, 2003). It has been reported that one of its milk protein has many characteristics similar to insulin (Beg et al., 1986).

Camel milk has been successfully used in the treatment of various diseases such as diabetes (Agrawal et al., 2005), hepatitis (El-Fakharany et al., 2008), allergy (El-Agamy et al., 2009) and to counter alcoholism (Ahmed et al., 2011) (Table 4). Similar to human milk as described in section 3.2, camel milk also contains protective proteins like lysozyme, lactoperoxidase, lactoferrin, polypeptide recognition protein and N-acetyl-beta-D-glucosamidase (Elagamy et al., 1996; Shamsia, 2009). Kamal et al., (2018) have reported enhanced anti-proliferative, anti-diabetic, and anti-inflammatory activities upon hydrolysis of camel whey proteins, indicating their potential utilization as bioactive and functional ingredients.

As mentioned earlier, camel milk lactoferrin also has anti-viral and anti-bacterial properties (Elagamy et al., 1996; Narmuratova et al., 2006). Camel proteins are able to maintain a high degree of thermal stability and remain functional even at elevated temperatures. Their extraordinary stability is attributed mainly to their efficient refolding after chemical or thermal denaturation. To a lesser extent, an increased resistance against denaturation may be responsible for it (Atri et al., 2011).

According to Khatoon et al., (2015), anti-convulsant activity of camel milk could be due to potentiation of both glycinergic and GABAergic activities. Antioxidant activity can also amplify its antiepileptic activity. Mohamed Hamzawy et al., (2018) suggests that camel milk is a potential therapeutic candidate for autism via regulation of inflammatory and apoptotic pathways when studied in rat model of pregnant female rats. Leptin plays an essential role in alleviation of autistic behavior through antioxidant effects. (Al-Ayadhi and Elamin, 2013) These findings suggest that camel milk could play an important role in decreasing oxidative stress by alteration of antioxidant enzymes and non-enzymatic antioxidant molecule levels, as well as the improvement of autistic behavior as demonstrated by the improved Childhood Autism Rating Scale (CARS).

Given below are applications of camel milk in various diseases (Table 5)

Table 5: Application of camel milk in various diseases.

S. no.	Treatment against disease conditions	Molecule implicated	Reference
1.	Diabetes	Insulin like molecule	Agrawal, et al., 2013; Agrawal, et al., 2002; Beg et al., 1986; Singh, 2001
2.	Hepatitis C Virus	Amylase & lactoferrin	El-Fakharany, 2008
3.	Allergy	Low levels of β-Casein & lack of β-lactalbumin	El Agamy et al., 2009; Shabo et al., 2005
4.	Liver and kidney function	Alanine amino transferase and aspartate aminotransferase	Hamad et al., 2011; Jadambaa et al., 2000; Sharmanov et al., 1978
5.	Slimming properties	Low protein content and reasonable cholesterol content	Yasin and Wahid, 1957 Faye et al., 2015
6.	Bacterial infection	Lysozyme, lactoperoxidase	El Agamy et al., 1992
7.	Nutritional supplements	Unsaturated fatty acids	Konuspayeva et al., 2009
8.	Immuno enhancer	Peptidoglycan recognition protein	El Agamy et al., 1992
9.	Lactase deficiency and easy assimilation	L-lactate	Cardoso et al., 2010; Baubekova et al., 2015
10.	Bone formation	High level of calcium	Riad et al., 1994
11.	Diarrhea	High level of sodium and potassium	Yagil, 2013; El Agamy et al., 2009

(Adapted from Dubey et.al., 2016)

2.4 Camelid antibodies

Camel milk has an amazing immunological profile. It contains very special types of Camelid Antibodies besides the normal types of antibodies. Camels unlike all other species (except shark) produce very special antibodies devoid of any light chains yet fully capable of antigen binding (De Genst et al., 2006; Desmyter et al., 2001; Hamers-Casterman et al., 1993; Harmsen et al., 2000; Harmsen and De Haard, 2007; Muyldermans et al., 1994; Nguyen et al., 2000; Spinelli et al., 1996; Van Der Linden et al., 1999; Vu et al., 1997; Yoo et al., 1967). Heavy chain antibodies can be expressed in microorganisms and have a high stability and solubility. Furthermore, camel milk IgGs are well suited for construction of larger molecules. It can also be used for selection systems such as phage, yeast, or ribosome display (Harmsen and De Haard, 2007). Therefore, the special features of these antibodies and the presence of other biologically important molecules and/or their derivatives confer camel milk with unique medicinal properties.

Structurally, a classical antibody present in mammals consist of two heavy and two light chains interconnected by disulfide bonds. The antibodies present in humans and most other mammalian species are essentially Y-shaped proteins which have a vital role in various antigen-elimination mechanisms and also act against cancer. The classical antibody contains an N-linked oligosaccharide attached to the second heavy-chain constant domain (CH2) that is essential for antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytolysis (CDC), and for retaining a long serum half-life. Even after isolation, the heavy (Utsumi and Karush, 1964) and light chains (Yoo et al., 1967) of an antibody can retain their antigen binding specificity; however, reduction in their affinity and solubility is often observed (Ward et al., 1989). Camelids (Bactrian and dromedaries' *camels, as well as llamas) generate antibodies devoid of light chains* (Hamers-Casterman et al., 1993). *The* single N-terminal domain of these heavy chain antibodies is fully capable of binding with the antigen. These single domain antibody fragments are called VHHs (standing for Variable - Heavy - Heavy) or Nanobodies. Another specialty of these antibodies is that they lack the CH1 domain, which remains associated with the light chain as well as to some extent with the VH domain, in case of classical antibodies. Besides these special antibodies, camelids also have the normal type of mammalian antibodies. The affinities of VHHs are generally comparable to those of conventional antibody fragments (Atri et al., 2011). Dedicated Immunoglobulin Heavy chain genes are used to produce heavy chain antibodies in camels. Similar to conventional antibody genes, the dromedary genes have identity in the organization of relevant regions such as exons and introns of the immunoglobulins (Muyldermans, 2013). The sequence study (Hamers-Casterman et al., 1993) and revelation of crystal structure (Desmyter et al., 2001; Spinelli et al., 1996) have exposed various structural characteristics of VHH domains of camelid antibodies. The

smaller size of VHHs gives them an added advantage to access and recognize hidden antigenic sites. Even their extended CDR3 loop has the ability to penetrate and access such sites (De Genst et al., 2006; Desmyter et al., 1996). The smaller size of VHHs (12-15 kDa) as compared to common antibodies (150-160kDa), allows them to rapidly pass the renal filter, which has cut-off of around 60kDa, thereby facilitating their rapid blood clearance. In addition to this the small size also aids in quick tissue penetration which can be used for targeting tumors by VHHs coupled to cytotoxic drugs (Cortez-Retamozo et al., 2004). VHH coupled tumor targeting can be used for *in vivo* diagnosis in association with imaging techniques. Furthermore, it can even be used in the treatment of snake bites (Harrison et al., 2006).

2.5 Anti-cancer properties of camel milk

Very recently some researchers stated that camel milk can inhibit the tumor and malignant cells of a number of cancers such as hepatocellular carcinoma, colon carcinoma, human glioma cells, lung cancer cells and leukaemic cells (Badawy et al., 2018; Dubey et al., 2016) . The anti-carcinogenic properties of camel milk could be due to a number of reasons. Firstly, the antibodies of camel milk are very active and able to bind to tumor cells and kill them while keeping the healthy cells undamaged. Secondly, possession of strong anti-oxidant and antimicrobial activity by camel milk, enables it to reduce the inflammation of liver and healthy functioning of liver. Additionally, camel milk proved to have thrombolytic activity which inhibits the coagulation and formation of fibrin consequently hinders the metastatic tumor cells to growth and spread.

2.5.1 Evidences supporting anti-cancer properties of camel milk

Middle Eastern countries have traditionally used camel milk for therapeutic benefits against cancer. There are upcoming clear cut indications suggesting the potential role of camel milk against cancer.

Recently, Korashy et al., in 2012 have reported that camel milk has the ability to significantly inhibit the induction of the Cyp1A1, a cancer-activating gene. Further it also induces NQO1, a cancer chemo-preventive gene in murine hepatoma Hepa 1c1c7 cells. Both these functions were studied at the transcriptional and posttranscriptional level. It was observed that the survival of HepG2 cells was significantly reduced upon incubation with camel milk. Further, they observed that camel milk significantly induced caspase-3 and DR4 mRNA expression levels. The induction of Casepase-3 was blocked by the action of Act-D. This indicates that camel milk increased the caspase-3 mRNA level by the *de novo* synthesis of RNA. It was further observed that pretreatment of cells with MAPK inhibitors alone, slightly, but not significantly decreased the basal expression level of caspase-3 mRNA. Furthermore, it was reported that the induction of caspase-3 mRNA by camel milk in HepG2 cells was significantly decreased by both the JNK and p38 MAPKs inhibitors and was potentiated by an ERK inhibitor.

Mohamed E. M. Afifi (2010) studied the protective effect of camel milk against cisplatin-induced renal oxidative stress in mice. Cisplatin induced stress was indicated by increased level of liver tissue metabolites such as malondialdehyde, serum creatinine and urea. This stress also led to a decrease in the concentration of glutathione, vitamin C and E. At an enzymatic level cisplatin decreased both, the activity and gene expression of superoxide-dismutase, catalase glutathione reductase and glutathione peroxidase. In their study, treatment of these animals significantly

increased the level of malondialdehyde as well as the enzymes viz. superoxidedismutase, catalase, glutathione reductase and glutathione peroxidase. The increase caused in catalase was not significant. The camel milk caused reduction in all these biochemical alterations and counteracted the deleterious effects of cisplatin. This study demonstrated the renoprotective potential of camel's milk against cisplatin-induced oxidative stress.

M. Ehlayel et al. (2011) suggested that Camel milk is a safer choice than goat milk for feeding children with cow milk allergy. In cow milk allergy, the skin prick test indicated low cross-reactivity between camel milk and cow milk, and camel milk was found to be a safer alternative than goat milk.

Talarico et al., (2019) concluded from their studies that small wheels at the skin prick test towards cow milk antigens together with low IgE titers against these allergins could work as predictors in selecting patients that are expected to have negative camel milk skin prick test and then could be fed with camel milk with lower risks of allergic reactions.

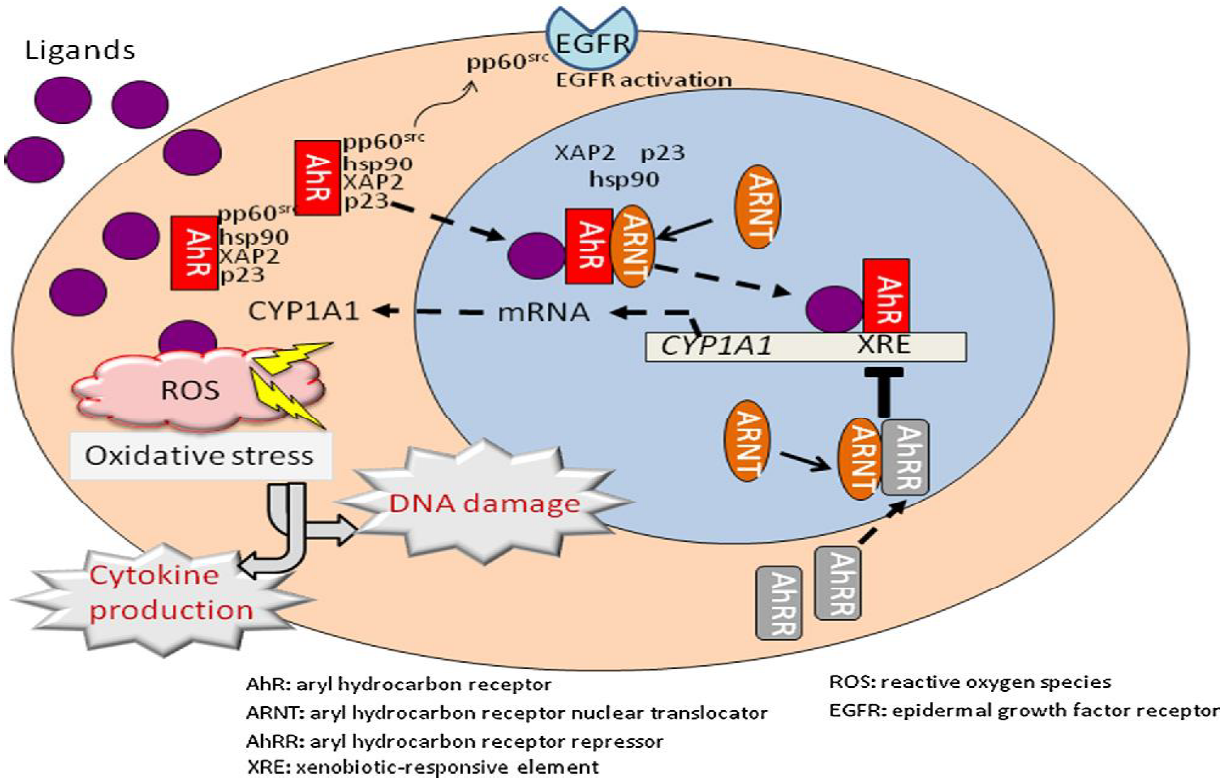
2.6 Mechanism of action of camel milk proteins

Camel milk components act as a ligand to the aryl hydrocarbon receptor (AhR). Signaling via aryl hydrocarbon receptor has a definite anti-cancer action. This receptor is an endogenous transcription factor known to have a preventive as well as therapeutic benefit to patients with cancers of breast, liver, prostate, etc. (Zhang et al., 2009).

2.6.1 Role of aryl hydrocarbon receptors

Aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription factors which is found in the cytosol and is activated after binding with its ligand. This family of transcription factors regulate the pathways involved in cellular proliferation and differentiation (Kerzee and Ramos, 2001; Whitelaw et al., 1993). In the cytosol, AhR remains as an inactive complex when bound with Heat Shock Protein-90 (HSP90) and AhR interacting protein (AIP). The interaction of AhR with its ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a polycyclic aromatic hydrocarbon (PAH), results in the dissociation of bound HSP90 and AIP and hence its activation. This subsequently leads to its translocation into the nucleus. After entering the nucleus it forms a dimer by interacting with the AhR nuclear translocator (ARNT) and then proceeds to bind with xenobiotic-responsive element (XRE) found in the promoter region of the so called AhR regulated genes. It's binding results in the transcription and translation of these genes (Nebert et al., 2000; Whitlock, 1999). The group of genes regulated by AhR includes both phase I xenobiotic metabolizing enzymes such as the cytochrome P4501A1 (C-YP1A1), CYP1A2, CYP1B1 and phase II enzymes such as NAD (P) H: quinone-oxidoreductase 1 (NQO1), Glutathione S-transferase A1 (GSTA1), Uridinediphosphateglucuronosyltransferase 1A6, and Aldehyde dehydrogenase-3 (Nebert et al., 2000; Whitlock, 1999). Among the above mentioned genes, CYP1A1 is considered a cancer activating gene as it plays an important role in the bioactivation of procarcinogens into carcinogens and other toxic metabolites (Nebert et al., 2004). On the other hand, NQO1 and GSTA1 catalyze the reduction of numerous environmental contaminants and also maintain the optimum level of endogenous antioxidants like vitamin E and ubiquinone. This helps in protecting the tissues against

mutagens, carcinogens and damages induced by oxidative stress (Ross, 2004; Vasiliou et al., 2006).



(Adapted from Masutaka et. al., 2014)

Figure 4: Schematic representation of the AhR/ARNT signaling system.

2.6.2 Modulation of aryl hydrocarbon receptors in cancer therapy

Several lines of evidence have shown that the induction of CYP1A1 strongly correlates with increased incidences of human colon, rectal, and lung cancers (Shah et al., 2009; Slattery et al., 2004). In addition, studies on carcinogenicity and mutagenicity of PAHs have demonstrated a significant role in induction of CYP1A1 in bioactivating these environmental toxicants into their ultimate carcinogenic forms (Shimada and Fujii-Kuriyama, 2004). Thus, CYP1A1 induction is considered a useful biomarker of exposure to carcinogenic substances (Williams et al., 2000).

Accordingly, one of the strategies for protecting human cells and tissues from the toxic effects of carcinogenic and cytotoxic metabolites is to attenuate the carcinogen-activating genes, CYP1A1 signaling pathways, and/or enhancing the adaptive mechanisms by increasing the expression of detoxification and antioxidant genes, such as NQO1 and GSTA1. Therefore, camel milk can be postulated to protect against or decrease the deleterious effects of many environmental toxicants and carcinogens such as PAHs, probably through modulation of AhR-regulated genes, such as CYP1A1, NQO1, and GSTA1 at the transcriptional and post-transcriptional level. Aberrant aryl hydrocarbon receptor expression and the activation of AhR pathway is involved in carcinogenesis. Exogenous AhR agonists promote differentiation in a putative mammary cancer stem cell line. Moreover, activation of the AhR is known to inhibit invasive and metastatic features of human breast cancer cells (Zhang et al., 2009). It has been shown that the aryl hydrocarbon receptor functions as a suppressor of liver carcinogenesis. It has also been observed that AhR pathway activation enhances gastric cancer cell invasiveness through a c-Jun-dependent induction of matrix metalloproteinase-9. New anti-tumor drugs like aminoflavone and benzothiazoles, require AhR-mediated signaling to expedite DNA damage.

2.6.3 Signaling mechanisms

Experiments conducted by Badr et al., (2017) suggested that camel whey protein protects lymphocytes from apoptosis via the PI3K-AKT, NF-κB, ATF-3, and HSP-70 signaling pathways in heat-stressed male mice. Heat stressed mice treated with camel whey protein presented significantly restored levels of reactive oxygen species and pro-inflammatory cytokines near the levels observed in the control mice. This study suggests the immunomodulatory role of camel whey protein. Very recently, Gamal Badr et al. (Badr et al., 2018) found that treatment of AML

cells with CWP mediated significant reduction in the phosphorylation of AKT, mTOR and STAT3. Additionally, they demonstrated that blockade of PI3K/AKT signaling pathway by wortmannin (WM) impaired the expression of Bcl-2 and Bcl_{XL} in the primary AML cells, suggesting an essential cross-talk between PI3K and Bcl-2 that maintains the survival of AML cells. In this context, treatment of AML cells with CWP disrupted the PI3K/Bcl-2 cross-talk; significantly downregulated the expression of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl_{XL}; markedly upregulated the expression of the pro-apoptotic Bcl-2 family members Bak and Bax; and subsequently sensitized tumor cells to growth arrest. Their study indicated the therapeutic potential of CWP and the underlying mechanisms against leukemia.

2.7 Gaps in Research

Our extensive literature survey (Chapter 3) of the work related to camel milk and its derived proteins published till date has identified gaps in certain areas which needs to be explored further. For instance, no study has been done on the comparative computational analysis of camel milk's antineoplastic proteins with milk from other species. To be more specific, α-lactalbumin, a component found in the whey fraction of milk, is shown to have anti-cancer properties in case of human milk, when complexed with Oleic acid (HAMLET). But no such studies have been reported in case of camel milk, although it contains ample amounts of α-lactalbumin. The anti-cancer properties of whole camel milk have been studied using murine cancer cell lines. Such studies have not been done using human cell lines. Moreover, the component that could be associated with it has not been studied in camel milk. Molecular mechanisms responsible for this activity yet remain unexplored.