

**Phytochemical and Metabolic Characterization of Pods and
Seeds of *Prosopis cineraria* and *Cyamopsis tetragonoloba* with
respect to Isoflavonoids and Flavonoids for Potential Medicinal
Applications**

THESIS

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By

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CHAPTER 1- Introduction and review of literature

The food you eat can either be the safest and most powerful form of medicine or the slowest form of poison

Ann Wigmore

1.1 Natural products in plants

Natural products or phytochemicals, by definition, include the chemical compounds which are naturally synthesized by plants. In a broader perspective, all the phytochemicals present in plants can be classified as primary and secondary metabolites.¹ Primary metabolites are those compounds which are omnipresent in all the plant species and are responsible for their normal physiological functions such as growth, reproduction, respiration, photosynthesis, etc. Proteins, lipids, amino acids, sugars, and nucleic acids are the essential constituents of the primary metabolites and are the pillars of customary growth and function. The synthesis of primary compounds has been found to be conserved among all the members of the plant kingdom.²

But what stands out among the vast array of the compounds synthesized by plants are the ones which are present in very minute quantities within the system, yet whose functions cannot be undermined. This exciting group of compounds with highly diverse structures, and even more varied presence among plant species are collectively termed as secondary metabolites. These secondary metabolites were formerly regarded as waste products without any physiological function for the plants.³ However, with the emergence of highly advanced analytical techniques in the field of extraction science, proteomics, and metabolomics have facilitated not only the identification of thousands of compounds from plants but also aided in establishing the critical functions these secondary metabolites carry out, which are crucial for interactions between plants and their biotic and abiotic environments. The extreme diversity which is exhibited by these compounds has put them on the research map by scientific communities around the world. The secondary metabolites differ from their primary counterparts not only in their structural diversity but also with respect to their plant-specific presence.⁴ It has been observed that the phytochemical profile of the plants belonging to even the same species shows variation, both temporal and spatial with respect to the secondary metabolites. This variation has also helped in establishing the taxonomic relationships between different plant species.⁵

Plants, being devoid of motility and immune systems akin to that in animals, rely on elaborated and alternative defense strategies which act as tools to fight against the stress constraints and helping them to adapt to changing environment. These strategies involve a vast range of secondary metabolites that are either present constitutively in

plants or synthesized upon receiving the external stimuli. Defense against pest and pathogen, protection from ultra-violet radiation, protection from osmotic, and environmental stresses are some of the crucial mechanisms by which secondary metabolites confer shielding effect in plants.⁶ Besides this, these compounds are also involved in attracting the pollinating organisms and allelopathic interactions with other plants in their surroundings.

The majority of reviews have stated that the secondary metabolites (also referred to as natural products) extracted from plants can be subdivided into three major classes: terpenoids, alkaloids, and phenolics.⁷ Compounds belonging to each of the three categories have roles at a physiological level in the plant system which will be discussed in detail in the next few sections. For the ease of understanding, terpenoids and alkaloids can both be placed in a single category as non-phenolic compounds. This classification emphasizes the difference in the pathways involved in their synthesis (i.e., metabolism), as well as the structural aspects of the compounds categorized. The terpenoids consist mainly of the steroids, carotenoids and gibberellic acids whereas alkaloids define the secondary metabolites which comprise of the nitrogen-containing compounds, for example, morphine, strychnine, quinine, ephedrine, nicotine to name the few. The phenolics class of the compounds consists of hydroxylated aromatic compounds and exhibits a universal presence in a wide range of flora albeit with very high diversity. The structural and biological properties of both non-phenolics and phenolics-based natural products have been extensively researched and documented in the past three decades. The following sections will throw light on some of the prominent compounds and their functions. Such a discussion is crucial towards understanding how the phenolics (major focus of the current thesis) differ in the structural and functional aspects with the non-phenolics (viz. terpenoids and alkaloids).

1.1.1 Non-Phenolic Compounds

Terpenoids are one of the non-phenolic phytoconstituents from the plants, which all together form an extensive family of natural products. The compounds present in this category are chemically derived from 5-carbon isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) units, which are assembled in different ways. The leading members of this class include steroids, carotenoids, and gibberellic acids, which, by and large, do not share considerable similarities on the

structural grounds, but the pathways leading to their synthesis have some likeness. All these compounds are primarily polymeric isoprene derivatives synthesized from acetate via the mevalonic acid pathway, where the linking of isoprene units occur in a head and tail fashion. Hence further classification of terpenoids occurs on the basis of the number of units incorporated into a particular terpene.

Hemiterpenes (single isoprene unit), monoterpenes (two isoprenes), sesquiterpenes (three terpenes), diterpenes (four terpenes), and triterpenes (six terpenes) are the primary classification of terpenoids. The volatile nature of compounds belonging to terpenoids has been found to be beneficial in the defense mechanism of sessile plants. The primary functions of terpenoids include serving as photosynthetic pigments (carotenoids), regulators of growth and development (gibberellins, abscisic acid, cytokinins, and brassinosteroids), electron carriers (side-chains of ubiquinone and plastoquinone), elements of membrane structure and function (phytosterols), and in protein glycosylation (dolichols).⁸ Besides this, a wide range of specialized terpenoid metabolites has also been recognized for an array of biological roles. This includes volatile or partially volatile, low molecular weight terpenoids from classes isoprene, monoterpenoids, diterpenoids, etc. The volatility of terpenoids provides tools for communication in sessile plants with other organisms, such as neighbouring plants, pollinators, air-borne infochemicals, and foes of herbivores. Considering the physiological standpoint, these plant volatiles are involved in three critical processes namely, plant-plant interaction, signalling between symbiotic organisms and attracting the pollinating insects. Since these compounds do have a role in the housekeeping activities, the possible applications could involve looking for suitable and sustainable methods in pest control via regulation of related genes. These compounds are associated with the protection of plants against various biotic and abiotic interactions above or below the ground. It has been reported that when plants undergo thermal stress, significant secretions of isoprene and monoterpenes from multiple vascular and nonvascular plants occur to confer them the protection. This has been presumed that volatile compounds make intercalation with the photosynthetic membranes, hence resulting in an increase in membrane functionality. Studies pertaining to transgenic approaches in poplar and tobacco species have validated the role of terpenoids in oxidative stress protection.⁹ Apart from this, floral scent, which is a resultant of volatile

terpenoids, is also crucial for a mutualistic interaction between plant and pollinating insects.

The biological activities of terpenoids have been extensively investigated; a few of them are mentioned here. Among the commercially available pharmaceuticals, the anticancer drug Taxol® and the antimalarial drug Artemisinin are two of the most renowned terpene-based drugs.¹⁰ Different compounds from subcategories of terpenoids have been reported to have biological activity against cancer, malaria, inflammation, and a variety of viral and bacterial infectious diseases. Limonene, which is the most abundant monocyclic monoterpene present in nature, is a well-established chemopreventive and therapeutic agent against tumor cells.¹¹ The closely related monoterpenes esters, which are represented by pyrethrins, constitute valuable insecticidal components isolated from flowers of *Chrysanthemum cinerariaefolium*, and several other species in the Asteraceae family.¹² A few acyclic monoterpenes, e.g., geraniol, nerol, and citronellol, have been shown to exhibit some antibacterial activity against *Mycobacterium tuberculosis*.¹³ A sesquiterpene lactone called as parthenolide has been found to be a major component of traditional herbal remedy prepared from *Tanacetum parthenium*, which is in use for more than two millennia for the treatment of stomach aches, menstrual difficulties, fever, and headaches.¹⁴ Probably, the most important medicinal sesquiterpene lactone is compound Artemisinin, which has been used as an antimalarial drug in Chinese therapeutic system for more than 200 years. Artemisinin and its derivatives now represent an important class of antimalarial drugs and are now used throughout the world. Diterpenes which represent the largest class of terpenoid family, constitute of taxines that have been thoroughly studied for their anticancerous activities. Taxol is currently one of the most critical anticancer drugs available in the market, which is a cyclic diterpene and has been isolated from *Taxus baccata*.¹⁵ A bromoditerpene, sphaerococcenol A, which was isolated from the red alga *Sphaerococcus coronopifolius*, has antimalarial activity against the chloroquine-resistant *Plasmodium falciparum* FCB1 strain.¹⁶ Structural deduction of the biologically active terpenes has led to their chemical synthesis procedures which have helped in the development of novel drugs that have terpenoids as their backbone structures.

Alkaloids are another class of secondary metabolites that are present in abundance in plants. They are characterized by being low molecular weight nitrogen-containing compounds. The presence of a nitrogen atom on a heterocyclic ring imparts

alkaline nature to them. Besides having carbon, hydrogen, and nitrogen, compounds present in this class may also contain oxygen, sulfur, and other elements, albeit rarely, such as chlorine, bromine, and phosphorus.¹⁷ Despite a faint boundary between alkaloids and other nitrogen-containing natural compounds, amino acids, proteins, peptides, nucleotides, nucleic acids are not usually called alkaloids.¹⁸ When compared with other classes of secondary metabolites, alkaloids are characterized by great structural diversity, and hence there is no uniform classification for them. In general, the classification is based on the similarities between the carbon skeleton present in the alkaloids. Like terpenoids, alkaloids also play a role in defense against various biotic and abiotic stresses which may affect the plants. Some of the strategies may include affecting pollinators, seed disperser visitation, predator repellence by toxicity or bitter taste, or damage repair by antioxidant system.¹⁹²⁰ Stimulant properties of some alkaloids may lead a visit to flowers by pollinators whereas the duration of the visit can be controlled by non-lethal toxicity exhibited by few alkaloids.²¹ There have been reports indicating that consumption of alkaloids by herbivores induce predator-repellence by toxicity or bitterness in taste, disruption of functions in proteins after ingestion or alteration in the central nervous system.²² All these examples of metabolic versatilities lead to substantial improvement in plant survival rate, which is necessary for them, given their immobility and sessile nature. And lastly, being nitrogen-rich secondary metabolites, alkaloids also serve the purpose of nitrogen reservoirs for plants.

Since the alkaloids are primarily present in plants to fight the pathogenic infections, their biological activities in humans are more on the toxic side. Despite this, a few health-promoting properties have been reported for the plant-derived alkaloids. One of the most important alkaloids is morphine, extracted from *Papaver somniferum*, which has been used as an analgesic since as early as 1200 B.C.²³ Berberine, an isoquinoline alkaloid of *Berberis aristata* demonstrated antidepressant-like effect mediated through the noradrenergic, serotonergic, and dopaminergic intervention.²⁴ Piperine isolated from *Piper nigrum* has been shown to inhibit MAO (Monoamine oxidase) enzymes and elevating brain 5-HT (5-hydroxytryptamine), which may affect an antidepressant medication.²⁵ Alkaloid caffeine, which is present in *Theobroma cocoa* and *Thea sinensis* has been used in beverages around the world to act as central nervous system stimulant.²⁶ Vinblastine isolated from *Catharanthus roseus* showed anticancerous

activity in childhood acute lymphoblastic leukemia.²⁷ An opium alkaloid isolated from plant *Papaver somniferum*, narcotine is used to suppress the cough.²⁸ These are just a few of the many examples of alkaloids being used for their medicinal properties towards human health.

1.1.2 Phenolic Compounds

Phenolic compounds derived from plants comprise one of the largest groups of secondary metabolites synthesized by fruits, vegetables, teas, cocoa, and several other plants. The distribution of these compounds in the kingdom Plantae is ubiquitous. It is estimated that out of the 100,000 to 200,000 compounds synthesized by the plants, approximately 20% of the compounds are of the phenolics category.²⁹ Despite the high number of phenolic compounds, the majority of them arise from the specific aromatic amino acids phenylalanine or tyrosine. Both these amino acids, after first getting deaminated to cinnamic acids, eventually enter into the phenylpropanoid pathway. Introducing one or more hydroxyl groups into the phenyl ring is a critical step in the biosynthetic route. This results in the derivation of compounds with a characteristic carbon skeleton of C6-C3 phenylpropanoid. The pathway thus produces a large variety of plant phenolics such as benzoic acids (C6- C1), flavonoids (C6-C3-C6), coumarins (C6-C3), stilbenes (C6-C2-C6), cinnamic acids (C6-C3), proanthocyanidins [(C6-C3-C6)n], and lignins [(C6-C3)n].³⁰ The primary role of phenolics is to serve the defense system of the plants. Many simple and complex phenolics may amass in plant tissues and act as phytoalexins, phytoanticipins, and nematicides against soil-borne pathogens and insects. The presence of multiple hydroxyl groups imparts the virtue of acting as antioxidants agents. The compounds belonging to phenolics show maximum absorption in the range of ultraviolet rays and hence they can confer UV protection in plants. Apart from this, other vital roles played by phenolics include accelerating pollination, coloring for camouflage, antibacterial, antifungal activities and shield against herbivores. The presence of phenolics and tannins on the surface of the aerial parts of plants many times deter birds by affecting their gut microflora and decreasing their digestive ability.³¹ On the other hand, the fragrance and pigmentation conferred by phenyl propanol derivatives of low molecular weight attract pollinators, microbes, and other animals that disperse fruits' seeds, thus aiding plant reproduction.³²

1.2 Natural products in Legumes

What has been discussed until now for the presence, structure, and role of phytochemicals in plants in general, more or less, holds true for the members of the family Leguminosae as well. The family Leguminosae boasts of representing the third largest family in the plant kingdom with 745 genera and over 19,500 species.³³ Therefore, enormous diversity present in secondary metabolites from the Legume family should not come by any surprises. The exclusive ability to fix the atmospheric nitrogen by a significant number of members of the family makes the species the producer of more nitrogen-containing secondary metabolites than any other non-nitrogen fixing family. The detailed description of the different categories of secondary metabolites present in legumes is present in the next few sections.

1.2.1 Alkaloids and amines

Legumes are characterized by the ability to fix the atmospheric nitrogen through the symbiotic association with nitrogen-fixing bacteria. Therefore, there is the synthesis of more nitrogen-containing compounds in legumes than any other family of non-nitrogen fixing flowering plants. This includes alkaloids, amines, NPAA (non-protein amino acids), and glucosinolates. The main storage sites for the nitrogen-containing defense compounds are seeds where they serve a dual function, first being toxic to pathogenic infection, and second, serving as nitrogen reservoir, which can be remobilized during germination and seedling development. The major alkaloids, along with their reported biological activities, are discussed in Table 1.1. Many of the alkaloids are present as neurotoxins or neuromodulators and are likely to be evolved in plants for their defense action against herbivores. These pyrrolizidine alkaloids get activated in the liver of animals upon ingestion and eventually cause severe mutations in DNA via alkylation.⁹ The amines isolated from the legumes are also known to express the psychotropic properties. e.g., β -carboline alkaloids, N, N-dimethyltryptamine, bufotenine, and methylmescaline have a substantial history as being used as hallucinogenic drugs.^{34 35} While a few of the toxins may immediately affect the herbivores; others may lead to long term consequences on the overall physiological health of the herbivores.

Table 1.1 Alkaloids present in Leguminosae, their pharmacological, and toxicological properties.

S.No.	Class of alkaloids	Examples from Legumes	Main occurrence	Biological activity	Reference
1.	Quinolizidine alkaloids (QA)	Sparteine, lupanine, anagyrine, cytisine, matrine, lupinine	Genistoid clade; Ormosia clade; <i>Sophora secundiflora</i> ; Calia, Bolusanthus	Neurotoxins; modulation of nAChR and mAChR; Na ⁺ channel blocker	36-37
2.	Pyrrolizidine alkaloids (PA)	Monocrotaline, senecionine	Crotalaria; Lotononis	Mutagenic and carcinogenic; modulators of several neuroreceptors, including 5HT ₂ , mACh, GABA, D ₂ , and α ₂	38
3.	Pyridine alkaloids	Trigonelline	In all subfamilies, abundant in IRLC clade and Phaseoleae sens. lat.	Antimicrobial	39
4.	Indolizidine alkaloids	Swainsonine, castanospermine	Astragaleae; <i>Castanospermum</i>	Inhibitor of endoplasmic hydrolases	40
5.	Simple indole alkaloids	Physostigmine	<i>Physostigma venenosum</i> , Dioclea spp	AChE inhibitor	41
6.	Simple isoquinoline alkaloids	Salsoline, salsolidine	Desmodium, Alhagi, Cytisus, Dendrolobium	Dopamine antagonist	42
7.	Piperidine alkaloids	2-Piperidine carboxylic acid, 4-hydroxy-2-piperidine carboxylic acid	Many members of all three subfamilies	Causes malformations in embryos	43

1.2.2 Cyanogenic glucosides

Cyanogenic glycosides play crucial roles in establishing the chemical defense system important for plants and plant-insect interactions. These are nitrile-containing plant secondary compounds that undergo the process of cyanogenesis following the enzymatic action and release cyanide and glucose as the main products. Cyanogenic glycosides arise in at least 2000 plant species, the majority of which are edible plants. Despite a great deal of structural difference in cyanogenic glycosides, nearly all of them are said to be derivative of only six different amino acids which are L-leucine, L-phenylalanine, L-valine, L-isoleucine or L-tyrosine. Any external injury to the plant, such as wounding, releases the specific enzyme to act on conjugated cyanogens and thus, HCN is excreted in nearby surroundings. HCN is a respiratory poison that blocks the mitochondrial

respiratory chain. A deadly poison for most of the herbivores, the presence of these compounds has been considered as part of evolutionary trait throughout the plant kingdom. A few compounds belonging to this category have been isolated and characterized from members of legumes as well, which are documented in the following table (Table 1.2).

Table 1.2 Cyanogenic glucosides present in Leguminosae, their pharmacological, and toxicological properties.

S.No	Class of CG	Examples from Leguminosae	Main Occurrence	Biological activity	References
1.	Cyanohydrins	Prunasin, linamarin, lotaustralin, proacacipetalin	Acacia spp.; Holocalyx balansae, Lotononisspp., Lotus spp., Ornithopus spp., Trifolium repens, Phaseolus lunatus	Release HCN; an inhibitor of the respiratory chain; strong animal poison	44 45

1.2.3 Peptides

Legumes, being the most abundant source of proteins, have been sometimes termed as ‘the poor man’s meat.’ Their ability to be able to associate with nitrogen-fixing bacteria leads to the synthesis and accumulation of protein in high quantity as compared to other staple cereals and edible plants. This being said, legume contents are also enriched with bioactive peptides which are protein molecules smaller than 10 kDa and may exist naturally or be derived from enzymatic digestion of native proteins. The hydrolysis of proteins by digestive, microbial or plant proteolytic enzymes release peptides that exhibit a variety of functions, including defense against pathogen infection and regulating growth and development. Interestingly, these bioactive peptides have different biological effects on human health as well, such as antimicrobial properties, cholesterol-lowering ability, blood pressure-lowering (ACE inhibitory) effects, antithrombotic and antioxidant activities. A few important peptides isolated and characterized for their biological activities from legumes have been mentioned in Table 1.3.

Table 1.3 Peptides present in Leguminosae, their pharmacological, and toxicological properties.

S.No	Class of Peptides	Examples from Leguminosae	Main occurrence	Biological activity	References
1.	Lectins	Abrin, robin	Abrus precatorius, Robinia	Inhibitors of ribosomal protein biosynthesis	46
2	Protease inhibitors	Trypsin inhibitors	Several Fabaceae	Inhibition of trypsin in herbivores	47
3	Antimicrobial peptides (AMP)	Cyclotides	Clitoria	Antimicrobial	48
4	Biopeptides	Lunasin	Soybean	Reduce the production of ROS; inhibit the release of pro-inflammatory cytokines (TNF-a) and IL-6	49

1.2.4 Flavonoids and Isoflavonoids

One of the essential categories of the secondary metabolites in legumes is represented by the flavonoids and isoflavonoids. They belong to the phenolics class of secondary metabolites and constitute approximately 50% of the known phenolic compounds in plants. These are generally low molecular weight compounds consisting of two aromatic rings (A & B) joined by a 3-carbon bridge (C6–C3–C6 structure). Ring A is derived from the malonate pathway and ring B is derived from the shikimic acid pathway (Figure 1.1). Malonate pathway is defined by the biosynthesis of maloyl CoA from acetyl-CoA and the shikimic acid pathway is a multistep process of synthesizing the aromatic amino acids from phosphoenol pyruvic acid.

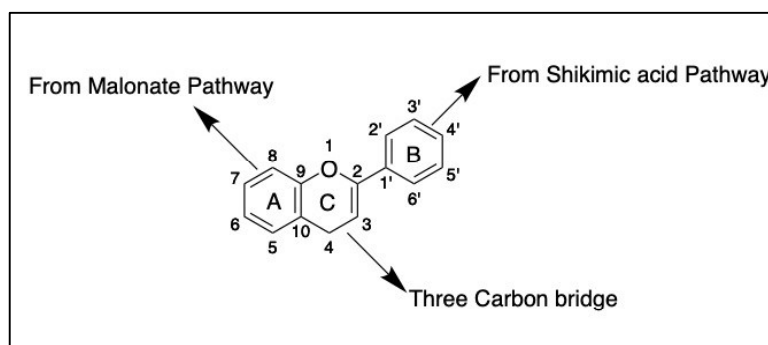


Figure 1. 1 General structure of flavonoid and pathways contributing to rings present in it

On the basis of the coloration, the flavonoids can be further divided into anthocyanins (colored) and anthoxanthins (colorless compounds). The anthoxanthins are further divided into flavones, flavans, flavonols, isoflavones, and their glycosides. Despite the omnipresent nature of flavonoids in the plant kingdom, members of the Leguminosae family are known for the presence of flavanols, flavan-3-ols, flavones and anthocyanidins as the main compounds. A particular class of isoflavonoids has been exclusively reported in the plant members of the Leguminosae family. Functionally, the flavonoids and isoflavonoids in legumes are crucial in protecting against UV damage, pathogenic microbes, modulating auxin distribution, and acting as pigments or copigments in influencing flower coloration for pollination.⁵⁰ Besides this, the flavonoids and isoflavonoids also act as signal molecules to symbiotic microbes which help in root nodulation. The rhizobial symbionts present in the soil environment recognize specific flavonoid signals produced by compatible host legumes and respond by introducing a novel, lipo-chitooligosaccharide signal (Nod signal).⁵¹ The type of flavonoid molecules secreted by the plant and the capability of the rhizobial species to recognize these molecules, and then induce Nod signal biosynthesis marks the earliest step in determining host-specificity.⁵² The significant flavonoids and isoflavonoids isolated and identified from different legumes have been mentioned in Table 1.4.

Table 1.4 Flavonoids/Isoflavonoids present in Leguminosae, their pharmacological, and toxicological properties.

S. No.	Class of flavonoid/ isoflavonoid	Examples from Leguminosae	Main Occurrence	Biological activity	References
1	Simple phenols	Vanillin, syringic acid, ferulic acid, gentisic acid, gallic acid, p-hydroxy benzaldehyde	Widely distributed	Antioxidants, antimicrobial	53 54
2	Flavonols	quercetin, kaempferol, myricetin, rutin, kaempferol 3-O-glucoside, kaempferol 3-O-xylosylglucoside, kaempferol 3-O-rutinosides, myricetin 3-O-glucoside, kaempferol 3-O-(6"-O-malonyl) glucoside, quercetin 3-O-(6"-O-malonyl) glucoside, kaempferol 3-O-(malonyl) glucoside, kaempferol dihexoside-rhamnoside, kaempferol 3-O-acylhexoside, quercetin 3, 4-diglucoside, quercetin 3-O-galactoside,	Widely distributed	Antimicrobial and anti-herbivore activities; antioxidants	55 56 57
3	Anthocyanidins	cyanidin, delphinidin, petunidiin, malvidin, pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside, cyanidin-3,5-diglucoside, pelargonidin 3-O-(6"-O-malonyl) glucoside, cyanidin 3-O-(6"-O-malonyl) glucoside, delphinidin 3-O-glucoside, malvidin-3-O-glucoside, pelargonidin- 3, 5-O-diglocoside, malvidin-3,5-diglucoside, malvidin-3-galactoside, delphinidin	Widely distributed	Antioxidants, antimicrobial	58 59
4	Flavanones	naringenin, eriodictyol glucoside, hesperetin-7-neohesperidoside, hesperetin-7-rutinoside, hesperetin-7-O-glucoside, naringenin 7-O-neohesperidoside, naringenin-7-O-rutinoside, naringenin-7-O-glucoside, naringenin-7-methyl ether 2	Widely distributed	Antioxidants, antimicrobial, anticancerous	60
5	Flavones	apigenin-7-O- glucoside, luteolin-7-O-glucoside	Widely distributed in tribe Crotalariaeae	Anti-inflammatory activity, anticancerous	61
6	Isoflavonoids	genistein, daidzein, glycitein, formononetin, biochanin b 7-O-glucoside, biochanin a 7-O-glucoside,	Widely distributed only in superfamily Papilionoideae	Antioxidants; phytoestrogens; antimicrobial	62
7	Coumarins	Scopolinn psoralen, xanthotoxin	Mostly present in tribe Psoraleae	DNA intercalation and DNA alkylation, mutagenic, antimicrobial	63

1.3 Flavonoid metabolism in plants

Our understanding of the pathways leading to the biosynthesis of flavonoids mainly comes from the studies done on three plants viz. *Arabidopsis thaliana*, *Zea mays*, and *Vitis vinifera*.⁶⁴ The principal enzymes involved in the flavonoid synthesis pathways are present as cytosolic multienzyme complex, also known as flavonoid metabolon. These enzymes are loosely associated with the cytoplasmic face of the ER (endoplasmic reticulum). Interestingly, some of these enzymes belong to the cytochrome-P450 family and hence possess the ability to bind to membranes. Conversely, some of the enzymes involved are loosely associated with membranes of different organelles, such as vacuole, plastids, and nucleus. These findings establish that a multi-branching distribution of the enzymes is involved in flavonoid biosynthesis pathways, thus implying a controlled metabolic channeling within the plant cells. The presence of such complex systems provides mechanisms, not only for removing unstable or toxic intermediates but also for controlling flux among several interlinked branches of related pathways that often function synchronously in the same cell.

Structurally, flavonoids are assembled from a 15-carbon skeleton (Figure 1.2) and composed of two benzene rings (ring A and ring B), which are connected by a 3-carbon linking chain which may or may not give rise to a third ring (C). The absence of ring C corresponds to the chalcones, which are capable of serving as precursors for various other flavonoids.

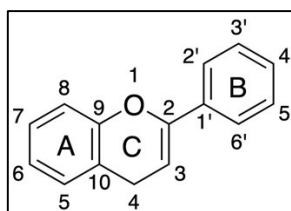


Figure 1. 2 General chemical structure of Flavonoid

The C₆–C₃–C₆ flavonoid structure, which consists of rings A, B and C, is the product of two separate biosynthesis pathways. The three-carbon bridge or C-ring and the aromatic B-ring constitute a phenylpropanoid unit which is synthesized from p-coumaroyl-CoA. The six carbons of ring-A originate from the condensation of three malonyl CoA which are derived from acetyl CoA via the malonic acid pathway. The fusion of these two separate reactions involves the stepwise condensation of three malonyl CoA residues with p-coumaroyl–CoA which is catalyzed by chalcone synthase

(CHS). Each of these molecules donates two carbon atoms to the flavonoid backbone. Naringenin-chalcone is the end product of this reaction. A slight modification in the pathway during the later steps is involved in the biosynthesis of isoflavones, such as daidzein, that are derived from isoliquiritigenin which, lacks a 2-hydroxyl unlike naringenin-chalcone. An NADPH-dependent enzyme, CR (chalcone reductase) which presumably interacts with CHS, catalyzed the formation of isoliquiritigenin. The flavonoid biosynthesis pathway is further carried by the stereospecific conversion of naringenin-chalcone to naringenin by chalcone isomerase (CHI). (Figure 1.3)

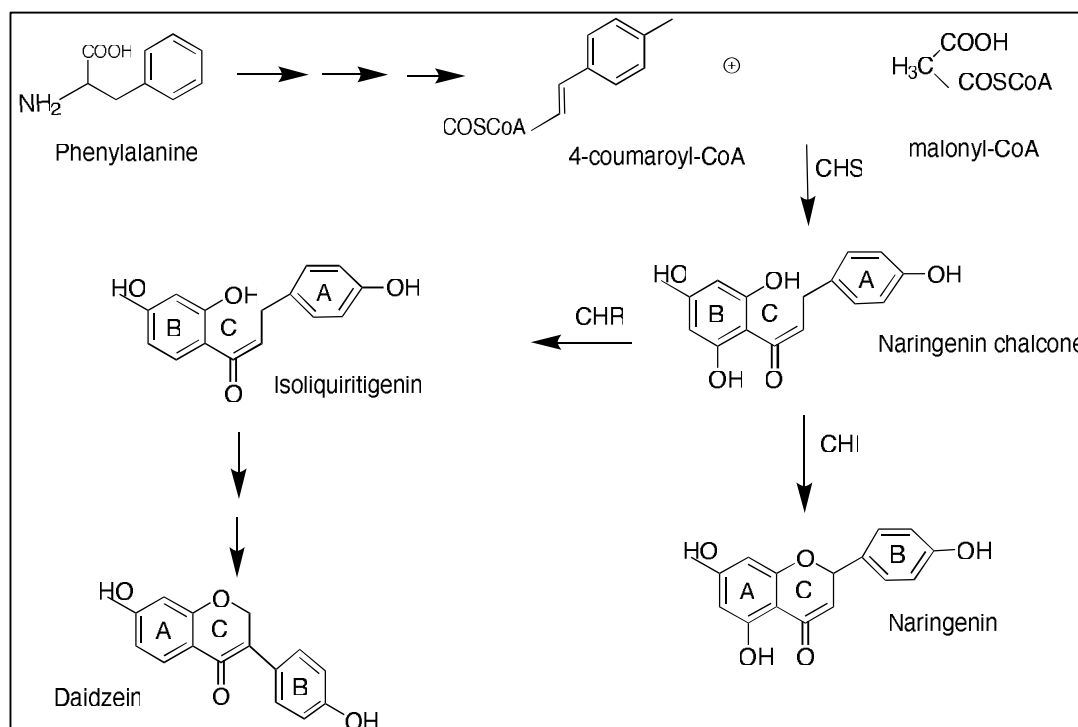


Figure 1.3 Schematic representation showing the derivation of different rings present in flavonoids/isoflavonoids by reactions catalyzed using different enzymes. CHS- chalcone synthase, CHI-chalcone isomerase, CHR-chalcone reductase.

In legumes, the same enzyme CHI also catalyses the conversion of isoliquiritigenin to liquiritigenin, though the rate of isomerization of naringenin-chalcone to naringenin is very high as compared with the isomerization of isoliquiritigenin to liquiritigenin.⁶⁵ This is because of the intramolecular hydrogen bonding present in the substrate molecule. Naringenin is the crucial intermediate in the flavonoid biosynthesis as this compound onwards; the pathway gets diverged into several side branches leading to the production

of different classes of flavonoids such as flavanones, flavones, flavonols, flavan-3-ols, anthocyanins and isoflavonoids.⁶⁶

Since isoflavonoids are principally present in leguminous plants, the enzymes involved in its synthesis are confined to legumes only. The microsomal enzyme isoflavone synthase (IFS) catalyzes the conversion of naringenin and isoliquiritigenin into isoflavones genistein and daidzein, respectively.⁶⁷ Daidzein, when acted upon by isoflavone-O-methyltransferase, yields isoformononetin which can subsequently undergo a series of reactions involving hydroxylation, reduction and dehydration to form medicarpin. Several other less, well-studied steps result in numerable conversions, and eventually yield a range of isoflavonoids viz. rotenoids, pterocarpins and coumarins.⁶⁸ Naringenin holds a key role in synthesizing the various subclasses of flavonoids via reactions catalyzed by multiple enzymes. Figure 1.4 shows a schematic representation of the biosynthesis of multiple flavonoids and isoflavonoids in general.

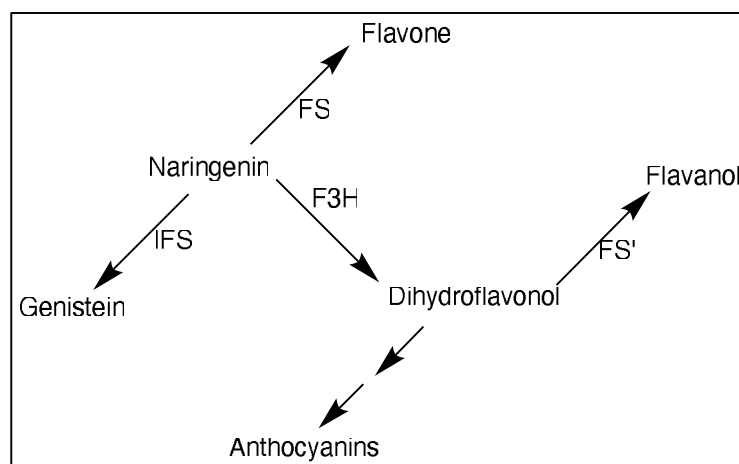


Figure 1.4 General flowchart showing the biosynthesis of flavonoids/isoflavonoids from Naringenin. Enzymes involved FS-Flavone synthase, IFS-Isoflavonoids synthase, FS'-Flavanol synthase and F3H- Flavanone 3-dioxygenase

However, to ease the storage of these compounds within the plant systems, certain chemical modifications are made to the final products of the above-mentioned pathways. Glycosylation is one the most common form of modification which is adapted within the plant environment where the sugar moieties are attached to the compounds via specific glycosyltransferases. This results in the formation of the glycosides conjugates, which, due to increased polarities, are more soluble in the cellular conditions and hence can be easily stored.

1.4 Plant Beta glucosidases

β -glucosidases are one of the ubiquitous enzymes which are present in a wide range of organisms from archaea, eubacteria to eukaryotes. They are involved in diverse cellular functions. The Nomenclature committee appointed by the International Union of Biochemistry in 1984 has classified enzymes on the basis of the type of reaction they catalyse. This puts β -glucosidases in the class which mediates the hydrolysis reactions. The finer nomenclature gives the enzyme commission number of 3.2.1.21 to this enzyme indicating the hydrolysis of β -glucosidic linkages in aryl-, amino-, or alkyl- β -D-glucosides, cyanogenic glucosides, disaccharides and short oligosaccharides. Under certain conditions, they are also able to facilitate the synthesis of a glycosyl-bond between different molecules via reverse hydrolysis or transglycosylation resulting in glycosides as the final products.

The fact that the plants consist of a large number of compounds containing non-reducing terminal glucosides, β -glucosidases have been suggested to have many vital functions in maintaining their normal physiological processes. They are involved in a large number of key processes such as in the synthesis of intermediates required during cell wall lignification⁶⁹, breakdown of the cell wall of endosperm during seed germination⁷⁰, in indole alkaloid biosynthesis⁷¹, activation of plant hormones⁷², the release of toxic cyanide i.e. cyanogenesis⁷³ and in plant defense against biotic stresses.⁷⁴

Plants store defense glycosides in an inactive form which are activated by the action of β -glucosidase enzymes⁷² and released as toxic compounds,^{75,76} In general, there is the compartmentalization of the defense glycosides and the β -glucosidases involved in the former's conversion to release the poisonous compounds.⁷⁷ Vacuole is the primary cell organelle where the defense compounds tend to get stored, whereas their corresponding β -glucosidases are often stored in apoplasts or lysosomes or plastids.⁷⁸ This point is further elaborated in section 1.4.2, particularly with reference to flavonoids and their metabolism.

1.4.1 Types of β -glucosidases

The classification of the β -glucosidases has been done in two different manners. The first is based on the substrate specificity of the enzyme. Under this system, β -glucosidases are divided into three groups; 1) true cellobiases, which hydrolyse cellobiose to glucose, 2) aryl- β -glucosidases, which preferably cleave aryl-glucosides, such as p-nitrophenyl- β -D-glucopyranosides and 3) enzymes with broad substrate specificity, which can act on a wide range of substrates.⁷⁹ Majority of the β -glucosidases which have been characterized at the biochemical and molecular levels belong to the last group. The second type of classification system is based on the similarity of both the nucleotide sequences and the 3D structures of the enzymes. As compared to the first type of classification, this is more recent because of the late arrival of advancement in sequencing technologies. This classification can be accessed through the CAZy website (Carbohydrates Active Enzymes Database).⁸⁰ As per this system, all the glycosyl hydrolases (GH) are currently divided into 153 families. β -glucosidases are primarily found in GH1 and GH3 families with lesser representation in GH families 2,5,9,30,39 and 116. The families are further classified into clans. The basis of subdivision of families is the similarity shared within the catalytic domain structures and conserved catalytic amino acids of the enzymes. This indicates the grouping of enzymes having a common ancestry and catalytic mechanisms. The clans are named from alphabets A to R. The clan containing the largest numbers of families is Clan GH-A which also includes β -glucosidase containing families GH1, GH5 and GH30. Among these three families, GH1 consists of the largest number of characterized β -glucosidases. Family GH1 includes β -glucosidases originating from archaeobacteria, plants and mammals whereas family GH3 comprises β -glucosidases of bacterial, mold and yeast origin.⁸¹

Yet another classification of GH families is done on the basis of the structural features of the enzyme.⁸² This system has been approved by the International Union of Biochemistry and Molecular Biology (IUBMB). Since it is tedious to know about the complete range of substrate specificity for individual enzymes, structural feature-based classification is more informative than their counterparts. Further application of the systems biology approach and some bioinformatics tools, structural features determined for one family can be used to study the structures of other enzymes belonging to the same family.

1.4.2 β -glucosidases in metabolism of flavonoid/isoflavonoid

Flavonoids/isoflavonoids are the crucial secondary metabolites imparting a role in a large number of physiological processes. This includes maintaining the structural integrity, photo protection from ultra violet rays, reproduction and internal regulation of signalling.⁸³ Phenylpropanoids, which are synthesized in the initial steps of the pathways leading to flavonoids as the final products are key components for the chemical modulation of plant communication with insects and microbes, either as repellents or attractants.⁸⁴ They can also act as phytoalexins against herbivores and pathogens or, can facilitate the pollination via flower colors.⁶ Leguminosae family members which are specially known for their nitrogen-fixing abilities, benefit a lot from these flavonoids/isoflavonoids. These compounds have been reported to induce the root nodulation in legumes which eventually help in fixing the atmospheric nitrogen.⁸⁵

Because of a wide range of protective roles flavonoids/isoflavonoids play in the plant systems, it is crucial to maintain the segregation of these compounds within cells so as to avoid their release and activities during the favourable conditions. Now it is well known that these flavonoids/isoflavonoids are stored as 3-O-glycosides or other sugar conjugates, with the exception of flavonols, which are generally in their non-conjugated form.⁸⁶ The conjugation of the compounds serves two purposes simultaneously. First of all, the conjugated forms are more soluble thus making the storage within the cell more convenient; secondly, conjugation decreases the toxicity of the compounds thus preventing cells from auto cell death. The most common form of conjugation is glycosylation and glucose has been observed to be the most preferred glycosidic unit for this purpose.⁸⁷ But other sugar conjugates are also present in plants such as galactose, arabinose, glucorhamnose, and rhamnose. The conjugation can occur in any number of permutations which include mono or di addition of sugar molecules, or more than one type of sugars getting added to the non-conjugated compounds. As a result of such diverse linkages, more than 5000 unique flavonoids have been identified.

So, where do the specific β -glucosidases come in the picture? Well, the flavonoids/isoflavonoids are synthesized via multistep pathways and the resulting free compounds are then glucosylated and further 6''-O-malonylated by the combined activity of enzymes UDP glucosyltransferase and malonyltransferase. These glucosyl and malonyl conjugates accumulate in large amounts in vacuoles. These sugar conjugates are considered to be latent forms and must eventually be converted to aglycones for

interactions with symbiotic or pathogenic microorganisms.⁶⁶ Also, the enzyme(s) involved in this conversion is expected to be spatially separated from those involved in their glycosylation and vacuolar conjugate pools. The release of aglycones in plants has been proposed to involve the hydrolysis of stored conjugates during which specific β -glucosidases hydrolyse the β -glucosidic linkage and thus liberating aglycone and sugar moieties. The aglycone moiety, depending upon its chemical nature, can either be involved directly in the defense activity or can be recruited to enter into the synthesis pathway for other phytoalexins. The sugar released is consumed by cells for energy purposes.⁸⁸

1.4.3 (Iso)flavonoid glycoside specific β -glucosidases (ICHG) characterized from legume sources

As mentioned in the previous sections, β -glucosidases have a key role in the overall defense mechanisms in the plant system. Despite the broad range of activities exhibited by this family of glycosyl hydrolases, many times, it has been observed that the hydrolysis of some sugar conjugates recruits highly specific enzymes which apparently don't cleave other conjugates from related class of secondary metabolites. One such example can be demonstrated by the case of iso(flavonoid) glycoside specific β -glucosidase. As the name indicates, this enzyme is involved in the cleavage of the iso(flavonoid) glycoside conjugates.⁸⁹ Though flavonoids are known to enjoy their ubiquitous presence in the plant kingdom, a particular category of flavonoids has been reported strictly in the members of the Leguminosae family. This category is termed as isoflavonoids which are characterized by the benzenoid substitution at 3rd position instead of the 2nd position.⁹⁰ Genistein, daidzein and glycitein are the most putative isoflavonoids which have been isolated and characterized from various legumes. These are the aglycones with possible glycoside forms as β -D-glycoside, 6''-O-malonyl-glycoside and a 6''-O-acetyl-glycoside.⁹¹ However, in nature, they are primarily present in their β -D-glycoside forms as genistin, daidzin, glycitin. Table 1.5 shows the quantitative analysis of significant isoflavonoids from selected plants from Leguminosae family.⁹²

Table 1.5 Quantitative analysis of isoflavonoids from selected edible legumes

S.No	Common Name	Scientific Name	Daidzein (mg/kg dry weight)	Genistein (mg/kg dry weight)	Total (mg/kg dry weight)
1.	Babchi	<i>Psoralea corylifolia</i>	539.7	1528.0	2067.7
2.	Soybean	<i>Glycine max</i>	37.6	24.1	61.7
3.	Yellow Pea	<i>Pisum sativum</i>	0.4	45.8	46.2
4.	Pinto Bean	<i>Phaseolus vulgaris</i>	23.2	22.3	45.5
5.	Black Turtle Bean	<i>Phaseolus vulgaris</i>	0.4	45.1	45.5
6.	Baby Lima Bean	<i>Phaseolus vulgaris</i>	0.4	40.1	40.5
7.	Anasazi Beans	<i>Phaseolus vulgaris</i>	6.5	29.8	36.3
8.	Mung Bean	<i>Vigna radiata</i>	0.3	21.8	22.1

Despite the fact that the Leguminosae family consists of around 19,500 plant members, only 90 of them have been known to show the presence of compounds belonging to sub-category isoflavonoids. A further survey of the literature indicates that out of these 90, only a few of the legumes are reported for the occurrence of the specific β -glucosidases which can cleave the glycosidic linkages of the isoflavonoids conjugates. The release of the active aglycone part depends on the activity of such specific glucosidases (It is to be noted that aglycones generated by β -glucosidase action are often found to be biologically more active compared to their glycosylated versions, thereby indirectly having implications for human health, and possible metabolic engineering). Interestingly so far, only four legumes are there from which ICHGs have been characterized and these include *Glycine max*⁹², *Dalbergia cochinchinensis*⁹⁴, *Dalbergia nigrescens*⁹⁵ and *Cicer arietinum*.⁹⁶ Table 1.6 sums up the properties of the specific ICHGs isolated and characterized from legumes.

Table 1.6 ICHG purified from legumes along with their essential properties

S.No	Source	Substrates	Molecular weight	pH/ Temperature optima	References
1	<i>Glycine max</i>	Daidzin, Genistin	100 kDa	5.5/30°C	93
2	<i>Dalbergia nigrescens</i>	Daidzin, Genistin	240 kDa	5.0–6.0/ 65°C	95
3	<i>Cicer arietinum</i>	Daidzin, Genistin, Formononetin, Biochanin A	135 kDa	7-7.5/45°C	96
4	<i>Dalbergia cochinchinensis</i>	Phloridzin, Glycitin	330 kDa	5.0/30°C	97

As observed from Table 1.6, the enzymes isolated, purified, and characterized from these four legumes show a lot of variation in their biochemical properties. The primary substrates that the enzymes were capable of acting upon were daidzin and genistin which are the isoflavonoid glycosides found in abundance. ICHG purified from *Cicer arietinum* was also subjected to cleave glycosides of formononetin and biochanin A. It is interesting to note that similar enzymes from other sources have not been reported yet. ICHG from *Dalbergia cochinchinensis* was also able to hydrolyze phloridzin and glycitin, other isoflavonoids. In all the four cases, it was reported that the purified enzyme was inactive or showed insignificant enzymatic activities against other flavonoids indicating the high specificity of these enzymes. The variation in other biochemical properties was also observed which can be seen from the above table. The molecular weight of ICHG from *Glycine max* was observed as low as 100 kDa whereas that from *Dalbergia cochinchinensis* was 330 kDa. The difference in the number of enzyme subunits was also reported. ICHG from *Glycine max* and *Cicer arietinum* were dimeric, from *Dalbergia nigrescens* was tetrameric and from *Dalbergia cochinchinensis* was pentameric. Similarly, surprisingly high temperature optima were observed for the ICHG isolated from *Dalbergia nigrescens*. Therefore, these comparative data reinforce the point that the enzymes catalyzing identical reactions may have different biochemical characteristics. In one instance, ICHG purified from one particular source has been

employed for biotransformation of isoflavonoid conjugated glycosides from other plants. More specifically the enzymatic hydrolysis of daidzin, genistin and glycitin isolated from soybean was catalyzed by ICHG from *Dalbergia cochinchinensis*.⁹⁸

The need to identify enzymes with higher substrate specificity and catalytic efficiency has increased the opportunity to explore novel sources. Since it is well understood that the biological activities of the isoflavonoids depend upon the released aglycone product after enzymatic hydrolysis, the role played by ICHG becomes significant. Understanding of the enzymes involved in the metabolism of the isoflavonoids will not only assist in illuminating the metabolic pathway responsible for the catabolism of these isoflavonoids within the plant system but will also help in elucidating the strategies for the genetic manipulation of the edible legumes so as to achieve an increased quantity of active compounds (aglycones) in the naturally obtained pod/seeds itself.

The diverse physiological roles played by this enzyme across the living system include glucoside ceramide catabolism in human tissues, cell wall, pigment, and cyanoglucoside metabolism, defense against pathogens in plants, and utilization of oligosaccharide substrates by many fungi and bacteria. Besides this, there are several other commercial applications e.g. production of biofuel and ethanol from agricultural waste, hydrolysis of bitter compounds during the extraction of juices, aroma liberation from wine grapes, because of which this enzyme is continuously sought after from novel resources. So far, the enzyme has been isolated from a vast number of living organisms ranging from lower prokaryotes to that of the higher plants and animals, both in native forms and via heterologous expression in host organisms. However, the recombinant enzymes impose certain limitations which raises the need to purify the enzymes from the native sources. One such limitation includes glucose induced inhibition of the recombinant enzymes. It was mentioned earlier that the reactions where the synergistic effect of multiple cellulases is required with glucose being one of the end products, one or more enzymatic component gets inhibited by the glucose. This drastically inhibits the overall efficiency of the enzyme. These reactions are usually characterized by the rapid release of glucose in the beginning, and subsequently inhibiting the reaction. Although most studies carried out on β -glucosidases are focused on their industrial applications, it is important to identify and characterize the enzyme from a plant source in its native

form. This will throw light on the pathways underlined in the metabolism of many putative compounds.

1.5 Biological activities of plant-derived flavonoids and isoflavonoids

Far-reaching studies over the past few decades have shown that flavonoids and isoflavonoids, both in vitro and in vivo experimental systems, exhibit several biological activities, like antioxidant, antimicrobial, anticancerous and antifungal. As mentioned before also, the role of these metabolites in the plant system is to combat biotic and abiotic stresses and also to act as growth regulators. But human beings have primarily benefitted from the extended impact flavonoids and isoflavonoids execute related to health promoting properties. Some of the biological activities of this class of secondary metabolites are discussed briefly in the following sections.

1.5.1 Antioxidant activities

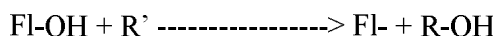
Antioxidants have been scientifically defined as “any substance that delays, prevents or removes oxidative damage to a target molecule”.⁹⁹ Hence the biological role of these compounds, as the definition implies, is to avert damage to cellular mechanisms arising as a consequence of chemical reactions involving free radicals.¹⁰⁰ Reactive oxygen species (ROS) are the free radicals which are generated as a result of the abiotic stress in cells followed by the oxidation of chemical substance. The most common examples of ROS include superoxide anion (O_2^-), hydrogen peroxide H_2O_2 , hydroxyl radical (OH), singlet oxygen, and nitric oxide (NO), which cause cellular damage by reacting with cellular molecules such as fats, proteins, or DNA. Superoxide anion is known as primary ROS, which reacts with a new molecule to form secondary ROS ions. Low levels of ROS production are essential to maintain physiological functions, including proliferation, host defense, signal transduction, and gene expression.¹⁰¹ However, an excessive build-up of ROS causes oxidative damage to deoxyribonucleic acid (DNA), proteins, and lipids. ROS can readily react with the nucleic acids attacking the nitrogenous bases and the sugar-phosphate backbone and can evoke single- and double-stranded DNA breaks.¹⁰² ROS can also attack structural and enzymatic proteins by the oxidation of residual amino acids, prosthetic groups, the formation of cross-links, protein aggregates, and proteolysis. The inactivation of the critical proteins can lead to severe consequences in the vital metabolic pathways.¹⁰³

Evolution has led to the integration of anti-oxidation systems within humans to protect against free radicals as a result of which, under physiological conditions, there is a cellular balance between ROS generation and its clearance. These integrated systems comprise two different pathways. Firstly, endogenous mechanisms that refer to some antioxidants produced in the body and, secondly, exogenous mechanisms where antioxidants are obtained from the diet. The first include (a) enzymatic defenses, viz. catalase, Se-glutathione peroxidase, and superoxide dismutase, which metabolize hydrogen peroxide, superoxide, and lipid peroxides, thus thwarting most of the formation of the toxic HO•, and (b) nonenzymatic defenses, such as histidine-peptides, glutathione, the iron-binding proteins ferritin, and transferrin and melatonin, dihydrolipoic acid, urate, and plasma protein thiols, with the last two making substantial contributions to the radical-trapping capacity of the plasma membrane. All these defenses act in a synergistic manner against different oxidative species at cellular levels. However, in spite of these innate defense strategies, a few ROS still manage to evade the scavenging which eventually leads to cell damage. Owing to the inadequate efficiency of our endogenous defense systems and the presence of some environmental factors e.g., air pollutants, cigarette smoke, UV radiation, inflammation, high polyunsaturated fatty acid diet, etc. in which ROS are generated in excess, dietary antioxidants are needed for subduing the accumulative effects of oxidative damage over the life span of humans.

Flavonoids/isoflavonoids boast of many biological properties, but the best-described property of almost every group of flavonoids is their ability to act as antioxidants. The arrangement of the functional groups around the nuclear structure affects the antioxidant activity of the flavonoids. The presence of multiple hydroxylation sites ensures that there is a possibility of significant variations pertaining to configuration, substitution, and the total number of hydroxyl groups on the flavonoid backbone.¹⁰⁴ As mentioned previously, the flavonoids/isoflavonoids have two rings C6-C3-C6 carbon skeleton or A and B rings connected by 3 carbons containing chain namely C. The hydroxyl groups present on the B ring are the prime determinant of the scavenging ability of ROS and RNS (Reactive Nitrogen Species) since they can donate hydrogen and an electron to peroxy, hydroxyl and peroxy nitrite radicals, which further stabilize them and generate relatively stable flavonoid radicals. The different mechanisms via which flavonoids can decrease the cellular damage caused by the free radicals are mentioned as following on next page:

- direct scavenging of reactive oxygen species (ROS),
- metal ion chelation
- activation of antioxidant enzymes,
- inhibition of oxidases,
- decreasing α -tocopheryl radicals,
- promotion of antioxidant properties of low molecular antioxidants.

Over the years, several flavonoids/isoflavonoids isolated from plants have been tested for their antioxidant properties and mechanisms through which they act. Due to their lower redox potential, flavonoids (FI-OH) are thermodynamically efficient to reduce the harmful oxidizing free radicals such as peroxy, superoxide, alkoxy, and hydroxyl radicals by hydrogen atom donation:



where R^\bullet represents peroxy, superoxide, alkoxy, and hydroxyl radicals. The peroxy radical (FI-O $^\bullet$) may then react with a second radical, forming a stable quinone structure (Figure 1.5)

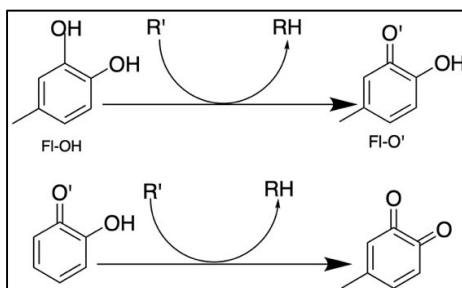


Figure 1.5 Formation of stable quinone structure after oxidation of flavonoid

There is now significant evidence depicting the correlation between the structure of flavonoids and their radical scavenging capacities. The primary determinants include the substituents of the heterocyclic and B rings.¹⁰⁵ Among the isoflavonoids, 8-hydroxy daidzein and genistein were found to be the most potential radical scavengers.¹⁰⁶ Interestingly, daidzein which is the parent compound of 8-hydroxy daidzein, was found to be a weak radical scavenger.¹⁰⁷ This is in corroboration with the fact that substitution with hydroxyl groups in ring B affects the cumulative antioxidant effect. Besides this, several studies have shown that glycosylation of flavonoids/isoflavonoids considerably decreases the antioxidant potential as compared to their aglycone counterparts. e.g., the (iso)flavonoid glycosides daidzin, genistin, rutin are poor radical scavengers as against their aglycones daidzein, genistein, and quercetin, respectively.⁶² (The role of β -

glucosidases in bringing about this conversion i.e. glycoside to aglycone *in planta* was discussed in the previous sections).

Flavonoids are known to chelate iron and copper ions, which are causal factors in inducing oxidative stress. The 4-oxo and 5-hydroxyl groups between the rings C and A, and catechol moiety in the ring B have been reported to be the primary contributors to metal ion chelation capacity of flavonoids. Quercetin and rutin both act as potent iron chelators by stabilizing the free iron radicals indicating that glycosylation doesn't affect the metal-binding efficacy.¹⁰⁸ Morin and quercetin were also shown to form complexes with Cd (II) and exhibit antioxidant activity in *in vitro* conditions. The isoflavonoid genistein has been reported to chelate copper ion via binding through hydroxyl groups present in the C ring.

Flavonoids can also activate the enzymes involved in antioxidant activities. Many times, there is a combined effect of the radical scavenging and regulation of enzyme functions. Flavonoids are able to stimulate phase II detoxifying enzymes (e.g., glutathione S transferase, NAD(P)H-quinone oxidoreductase, and UDP-glucuronosyl transferase), which are the primary defense enzymes against electrophilic toxicants and oxidative stress.^{109 110} Quercetin and catechin showed a cytoprotective effect against hydrogen peroxide cytotoxicity in cultured rat hepatocytes BL-9 by activating cytosolic glutathione peroxidases.¹¹¹ Daidzin and daidzein exhibited antioxidant activity by stimulating catalase and total superoxide dismutase in human liver carcinoma cells HepG2.¹¹²

Flavonoids inhibit the enzymes in control for superoxide (O_2^*) production, such as protein kinase C and xanthine oxidase.¹¹³ Quercetin and silybin inhibited xanthine oxidase activity which resulted in decreased oxidative injury.¹¹⁴ Flavonoids have also been shown to inhibit cyclooxygenase, lipoxygenase, microsomal succinoxidase, and NADH oxidase. Quercetin was found to inhibit NADPH oxidase by inhibiting the protein kinase C.¹¹³ Apart from this; flavonoids also inhibit microsomal monooxygenase and glutathione activating antioxidant enzymes.¹¹⁵

The flavonoids can act as hydrogen donors to α -tocopheryl radical, which otherwise is potential prooxidant molecule present in cell membranes and in low-density lipoproteins (LDL). Flavonoids, by interacting with α -tocopheryl radicals, help in delaying the oxidation of LDL. Flavonols kaempferol, myricetin, quercetin, and morin

have shown a variable protective activity against the reduction of α -tocopherol in LDL.¹¹⁶ In another study, the addition of green tea extract, which is rich in catechins like epigallocatechin, epigallocatechin gallate, etc. epicatechin, and epicatechin gallate demonstrated a gradual regeneration of α -tocopherol in human LDL.¹¹⁷

Accelerating the antioxidant properties of low molecular weight antioxidants is another mechanism of action for flavonoids. It has been reported that β -carotene shows prooxidative in synergism with an antioxidant under certain conditions.¹¹⁸ Another study carried out to investigate the interaction between β -carotene and flavonoids naringin, rutin, and quercetin, showed a significant decrease in DNA damage incurred by UV radiations in mouse embryo fibroblasts C3H10T1/2 cell lines.^{115 120} These studies suggest that the incorporation of flavonoids with other antioxidants may increase the safety and efficacy of the latter.

1.5.2 Antimicrobial activities

One of the crucial roles of secondary metabolites is to act as antimicrobial agents in response to microbial infections in plants.¹²¹ Hence it should not be startling that these compounds can be explored for antimicrobial properties against the wide range of microbes that cause diseases in humans. Moreover, the drastic rise in the resistant bacterial population in the past few decades has prompted scientists to look for novel compounds that can offer a solution to treat infections caused by resistant bacteria. It has been reported that of 2 million people who acquire bacterial infections in US hospitals each year, 70% of the cases involved bacterial strains that are resistant to at least one drug.¹²² Phytochemical extracts have a long history of being used as antibacterial agents in folk medicine. So far, 100,000 plants have been tested for their medicinal properties, with antibacterial potential being one of them. As per a report published by WHO in 2007, 25% of the available drugs are derived from the plants which are used in folk medicine.¹²³ Between 1981-2006, 109 novel antibacterial drugs entered the market out of which 69% were originated from the plant. Flavonoids being one of the most diverse and abundant classes of plant secondary metabolites have garnered substantial interest from the scientific community and it has been found to be present in ethnobotanical preparations commonly used as therapeutic formulas.¹²⁴

Several mechanisms have been proposed and stated by which flavonoids can act as antibacterial agents. Like their antioxidant potential, structure-based activity plays a significant role in determining any flavonoid to act as an antibacterial agent. The following list includes the mechanism of antimicrobial action of flavonoids:

- a. Inhibition of nucleic acid synthesis
- b. Inhibition of electron transport chain and ATP synthesis
- c. Alteration of membrane structure
- d. Reduction in biofilm formation

Inhibition of nucleic acid synthesis has been an attractive target for several commercially available drugs. Blocking the enzymatic activity of enzymes decisive in DNA and RNA synthesis is one of the mechanisms of action which has been shown by flavonoids/isoflavonoids. DNA gyrase from *E. coli* was shown to be inhibited by flavanones apigenin, quercetin, and 3,6,7,3',4'-pentahydroxyflavone.¹²⁵ *In silico* study carried out analyzing the interaction between DNA gyrase from *Mycobacterium smegmatis* and *M. tuberculosis* showed that a subunit of DNA gyrase could be targeted by quercetin¹²⁶ via blocking the ATP binding pocket, thus terminating the action of the enzyme.¹²⁷ Other flavonoids chrysin and kaempferol significantly inhibited DNA gyrase from *E. coli* whereas tangeritin and myricetin inhibited DNA gyrase to a lesser extent.¹²⁸ Isoflavone genistein was shown to inhibit the growth of *Vibrio harveyi* with an intermediate effect on *Bacillus subtilis* and negligible impact on *E. coli*, in a dose-dependent manner.¹²⁹ Intercalation of DNA with flavonoids myricetin, robinetin, and EGCG resulted in decreased DNA, RNA, and protein synthesis of *Proteus* and *S. aureus* which was proposed to be mediated by a free hydroxyl group at C-3 of ring A and multiple hydroxyl groups present at Ring B of the flavonoid/isoflavonoid.¹³⁰

An investigation carried out into the antibacterial mechanism of action of two chalcones viz licochalcone A and C, isolated from the roots of *Glycyrrhiza inflata*, showed the inhibitory activity against *S. aureus* and *Micrococcus luteus*. A hypothesis was proposed that these compounds are interacting in a similar way as that of the respiratory-inhibiting antibiotics.¹³¹ Two flavonoids extracted from *Dorstenia* species resulted in membrane depolarization of *S. aureus*.¹³² Recently it has been reported that

polyphenols isolated from plants can bind to a distinct binding site on ATP synthase, which may lead to complete inhibition of the reaction.¹³³

Cellular membrane disruption in any organism can lead to malfunctioning of cellular respiration, osmoregulation, and transport processes, biosynthesis, and cross-linking of peptidoglycan, as well as biosynthesis of lipids. Catechins have been reported to alter the lipid bilayer in both gram-positive and Gram-negative bacteria. Flavonoid 2,4,6'-trihydroxy-3'-methylchalcone was found to create leakage of intercellular proteins and ions in *Streptococcus mutans*.¹³⁴ Quercetin isolated from *Propolis* was found to decrease proton-motive force in *S. aureus*, which increased the permeability of membrane leading to the augmented effect of the antibiotic treatment.¹³⁵ Flavones apigenin and acacetin; flavonols rhamnetin and morin, have been shown to initiate destabilization of the cellular membrane structure by inducing leakage of ions from vesicles in membranes. Similarly, flavanones naringenin and sophoraflavanone G showed antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) by reducing the mobility in hydrophobic and hydrophilic regions of both inner and outer cellular membrane.¹³⁶

Bacterial biofilm formation forms the basis of a significant amount of all bacterial infections in humans and animals.¹³⁷ When bacteria grow as biofilm, they become approximately 1000 times more resistant to antibacterial treatment as compared to their planktonic cells counterpart.¹³⁸ Interestingly, flavonoids have been reported to support the bacterial aggregate formation, which is present in the biofilms. Flavonoids EGCG (epigallocatechin gallate) and 3-O-octanoyl-epicatechin (EC) were found to form pseudo multicellular aggregates in *S. aureus* culture post incubation. Galangin, which is a flavonol, was found to cause bacterial cell aggregation in *S. aureus*.¹³⁹ However, it was observed that bacterial growth was subsequently repressed after aggregation. It was proposed that flavonoids induce bacterial aggregation by partially lysing the cells which lead to membrane fusion. This eventually results in reduced uptake of nutrients via a smaller membrane surface area. Another study showed that flavonoids isovitexin and 5,7,4'-trihydroxyflavanol significantly inhibit the biofilm formation against *S. aureus*. A 2-15% EC exposure to *S. mutants* was able to decrease biofilm formation by 55-66%.¹⁴⁰

1.5.3 Anti-cancerous activities

According to the World Health Organization, cancer is the leading cause of death worldwide, with 9.6 million deaths in 2018 alone. The newest report published by the International Agency for Research on Cancer (IARC) mentioned that the total cancer burden has risen to 18.1 million new cases (Figure 1.6). When speaking of the statistics with respect Indian sub-continent, a total of 7,84,821 deaths due to cancer were reported in the year 2018 and over 11,57,294 new cases were registered which is approximately 6 % of global data. The agency <http://cancerindia.org.in> which monitors cancer-related statistics for India also states that at present, around 2.25 million people are living with this disease in our nation. Between the years 2012 and 2014, there has been a jump of 6% in cancer-related deaths. However, early diagnosis along with thorough treatment which includes surgery, radiotherapy, and drugs, has led to an increased average life span among many patients. Nevertheless, finding a cure for cancer is not only the foremost objective of innumerable drug companies, but it will be considered as finding the holy grail of medical science itself.

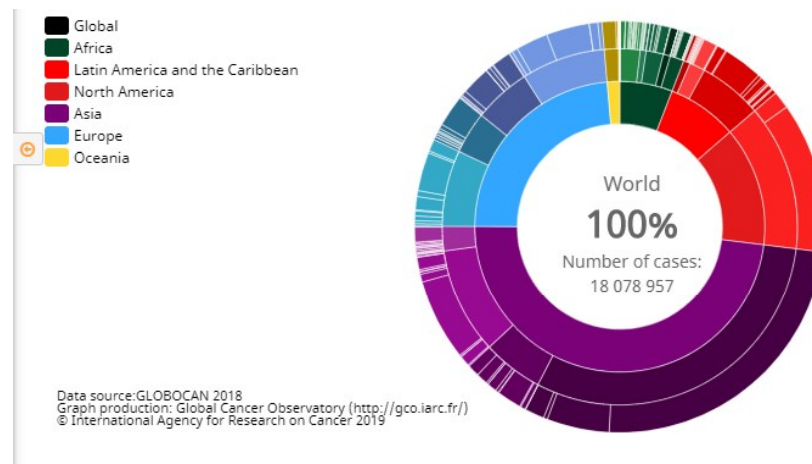


Figure 1. 6 Global distribution of cancer cases as observed in year 2018; Image source: Globocan 2018

Because of the complexities linked with the cancer disease, the treatments associated with it pose even more significant challenges. Immense efforts have been made to minimize the harmful side effects accompanying the treatment of the cancer patients because one of the critical aspects of any medical care is not only to increase the life span of the patient but to give equal focus for its quality as well. The need of the hour

is to find novel ways to decrease the side effects on the nearby cells and tissues, increasing the accumulation of the drug and its efficacy in the lesion and establishing unique drug delivery and targeting systems. The primary methods for cancer therapy involve surgical removal of the tumor, immunotherapy, radiotherapy, chemotherapy, photodynamic therapy, stem cell transformation or combination of two or more from the above-mentioned procedures.^{141 142} This is frequently accompanied by severe side effects, which include limited bioavailability of the drug, non-specificity, toxicity, and inability to restrict metastasis. The most commonly used chemotherapeutic agents for treating patients can be based on the mechanisms involved in targeting the tumor cells. For instance, drugs acting as topoisomerase inhibitors {e.g. irinotecan (side effects include: vomiting, deep vein thrombosis, and diarrhea)}¹⁴³ and doxorubicin (side effects include: cardiomyopathy), alkylating agents {e.g. melphalan, oxaliplatin, cisplatin, carboplatin and cyclophosphamide (side effects include: nephrotoxicity, low blood count, gastrointestinal toxicity, cardiovascular toxicity, pulmonary and ototoxicity), microtubules depolymerizing/inhibition agents {e.g. vincristine, vinblastine, docetaxel and paclitaxel (side effects: peripheral neuropathy, nausea, vomiting, low blood count, arthralgia etc)} comprise of the list of the drugs which are highly effective against a wide range of tumor cells.¹⁴⁴ Apart from the discussed issues arising from the treatment, one uncontrollable factor is mutating of the cancer cells into resistant cancer cells thus making the usage of the drugs eventually ineffective. One example of such resistant cancer cells is the overexpression of drug-resistant genes (ABCA4 and ABCA12) in human MCF-7 breast cancer cells when treated with docetaxel drugs.¹⁴⁵ However, there have been reports suggesting that incorporation of phytochemical curcumin in association with therapeutic drugs induced the downregulation of the drug resistance genes. This indicates that in order to effectively treat cancer, it is crucial to look beyond mono-target chemical agents and explore the compounds which are effectively acting as multi-target drugs during cancer therapy.

The previous sections 1.5.1 and 1.5.2 have extensively presented the antioxidant and anti-microbial activities of the flavonoids and isoflavonoids. In continuation of these, the anti-cancerous activities of these compounds shall be discussed in this section. It is not novel news that dietary factors play an important role in the prevention of cancer. Many fruits and vegetables are known to have flavonoids and isoflavonoids as one of their many phytochemical constituents. There has been substantial evidence indicating

that consumption of onions and/or apples, which are two key sources of flavonol quercetin, inversely affects the incidence of cancers of breast, lung, prostate, and stomach.¹⁴⁶ Additionally, moderate wine drinking also seems to decrease the risk of developing tumors in the colon, oesophagus, endometrium, lung, and stomach. The thorough and detailed documentation of the correlation between dietary intake of fruits and vegetables and cancer prevention suggests that major public health benefits could be attained by considerably increasing the consumption of these foods.¹⁴⁷

Quite a few mechanisms have been proposed and studied by which flavonoids and isoflavonoids are able to exhibit the initiation and promotion of their anticancerous activities on the tumor cells. The primary molecular mode of action of these compounds are as below:

- a. Regulation of AR (Androgen Receptor) and ER β (F Receptor) Pathway,
- b. Protein tyrosine kinases (PTKs) inhibitor,
- c. Inhibition of cell cycle progression,
- d. Downregulation of mutant p53 protein,
- e. Inhibition of expression of Ras proteins,
- f. Inducing apoptosis by up-regulating Bax and down-regulation of Bcl-2.

AR signalling pathway is involved in the development and progression of prostate cancer through the regulation of transcription of prostate-specific antigen (PSA). Monitoring the treatment response, prognosis and progress in prostate cancer patients involve measuring the level of PSA protein in response to the expression of androgen-responsive genes. The isoflavonoid genistein has been shown to down-regulate the expression of androgen receptor in the human prostate cancer-derived LNCaP cell.¹⁴⁸ Soy phytochemical concentrates have also been reported to substantially decrease the growth of LNCaP tumors in nude mice. The impact of oral ingestion of genistein on early-stage prostate cancer has been analysed by clinical studies that have shown the reduced levels of PSA proteins. Similarly, because of the structural similarity of soy isoflavones with that of estrogen hormones, these compounds pose a potent agonist for the Estrogen Receptors (ER β). The proposed hypothesis that ER β exerts an antiproliferative effect on tumor cells is consistency with the fact that cancer progression marks the decrease in expression of ER β receptors. Soy derived isoflavonoids, which are being termed as “weak-estrogens,” mimics the endogenous estrogens with respect to their structure as well as function.¹⁴⁷ These isoflavonoids bind and activate the ER α and ER β which are

the primary receptors for the estrogen hormones in humans. The estrogen-like activities of soy isoflavonoids are thought to play an essential role in imparting their health-enhancing properties.

Tyrosine kinases are a family of proteins sited either inside the cell membrane or in its proximity, which are directly involved in signal transduction of growth factors to the nucleus. The role of the expression of tyrosine kinases in oncogenesis has been thought to be evolved via their abilities to override the normal growth of the cells. A part of the conventional chemotherapy includes drugs which are capable of inhibiting tyrosine kinase activity without minimal side effects. Genistein was the first isoflavonoid which was identified as an inhibitor of tyrosine specific protein kinases.¹⁴⁹ Quercetin flavonoid was the first to be tested in the human phase I trial.¹⁵⁰

Induction of cytotoxicity in cancer cells via inhibition of cell cycle progression has also been a reported mechanism of action of flavonoids/isoflavonoids. This could be achieved by targeting different checkpoints of the cell cycle. There is considerable evidence that shows that genistein halts the cell cycle at the G2/M phases and induces apoptosis in prostate cancer cells, breast cancer cells, gastric cancer cells, and lung cancer cells. Additionally, genistein also arrests the cell cycle at G0/G1 in fibroblasts and melanoma cells.¹⁵¹ Luteolin and apigenin arrest the cell cycle at either the G1 or G2 phases in different cell types. Quercetin has been reported to induce S phase arrest in breast cancer cells and leukemic cells.¹⁵² Naringin and hesperidin prompted cell cycle arrest by targeting the G2/M checkpoint in lung cancer cells A549.¹⁵³

The role of the p53 tumor suppressor gene has been thoroughly established in preventing the onset of cancer. The occurrence of mutations in p53 is the most common genetic abnormalities in human cancers. The mediation of cell cycle arrest in cancer cells by aiming at the G2-M phase checkpoint is initiated because of the inhibition of expression of p53 protein.¹⁵⁴ Flavonoids and isoflavonoids have been found to downregulate the expression of mutant p53 protein to nearly untraceable levels in human breast cancer cell lines. Apigenin has been reported to activate the p53 gene expression in mouse keratocyte 308 cell lines and fibroblasts.¹⁵⁵

Ras is another protooncogene, mutation in which leads to uncontrolled cell growth and tumor proliferation. Ras proteins are implicated in the transduction of the external effector molecule Raf serine/threonine kinase. This is executed by its capability to bind with GDP/GTP and also to express intrinsic GTPase activity allowing

inactivation of the external stimuli in the healthy cellular environment. A point mutation in the Ras gene is associated with abnormal cell growth in various cancers related to humans. Since the mutated Ras protein, Ras-p²¹ loses its GTPase, this eventually creates an imbalance inside the cell and thus leading to malignant phenotyping. The mutated Ras protein, therefore, has been perceived as an essential target for cancer drugs. Formononetin, an important isoflavonoid isolated from red clover induced apoptosis in estrogen receptor-positive human breast cancer cell MCF-7 by activating Ras/p38 pathway.¹⁵⁶ An *in-silico* analysis for testing the efficiency of different plant-derived flavonoids against the function of mutant Ras-p²¹ protein showed that naringenin, daidzein, and hesperetin are potential drug target for cancer treatment.¹⁵⁷

The inception of cancer is primarily marked by the anomalies associated with the apoptosis. The pathways involved in the normal processes leading to cell death are complex in nature and are facilitated by different proteins. Bcl-2 family protein is the best-characterized protein involved in the regulation of apoptotic cell death and it mainly consists of anti-apoptotic or pro-apoptotic members. There have been reports of flavonoids/isoflavonoids inducing apoptosis in human cancer cell lines by regulating the genes of the Bcl-2 family. Morin, a flavonoid isolated from *Machura pomifera*, prompts apoptosis in the human leukemia cell line U937.¹⁵⁸ Morin upregulates the BAX protein and downregulates the Bcl-2 protein. Hesperetin, which is an abundantly found flavonoid in citrus fruits, cherries, and tomatoes, reduced the Bax to Bcl-2 in gastric cancer cells. It was also found to reduce the Bcl-2 transcription in prostate cancer cell line PC-3.¹⁵⁹ Naringenin, which is mostly present in oranges and grapefruits, is capable of decreasing the expression of Bcl-2 and thereby inducing the apoptosis in human gastric cancer cell line SGC-7901. Quercetin, the most abundant flavonoid among plants, is capable of exhibiting anticancerous activities via an array of mechanisms and inducing apoptosis by upregulation of Bax protein is one of them. This property was observed in MCF-7 and HL-60 cell lines.

In the previous sections, it was mentioned that flavonoids and isoflavonoids are abundant in their antioxidant potential. Since oxidative stress contributes to cancer initiation, the plant-derived compounds show promising prospects for combating the progression of carcinogenesis via significantly reducing the oxidative stress within the cells. The likeliness of antioxidants as an anticancer agent depends on their competence as an oxygen radical inhibitor and inactivator. Therefore, the incorporation of diets that

are rich in radical scavengers would lower the cancer-promoting action of some free radicals.



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CHAPTER 2: Gaps in research and objectives

2.1 Gaps in research

Rajasthan is the largest state of the Indian sub-continent covering 10.5 % of the geographical area. With 5.5 % of the total country population, it is the 7th most populated country. 53% of the entire geographic area is under agriculture indicating that the majority of the population i.e. 2/3rd depends on the agricultural productivity for their living. The cultivated area in Rajasthan is 17.4 million hectares which constitute almost half of the total area of the state. The soil of the Rajasthan region has a light texture and low water retention capacity. The agro-climate is of extreme temperatures, high evapotranspiration, erratic, uncertain and scanty rainfall. The rain-fed area is over 70% with an average precipitation of 575 mm. Despite the limited rain in the semiarid region, Rajasthan is home to some of the most important agricultural crops in the country. The primary crops of the state are pearl millet, maize, chickpea, mustard, clusterbean and spices. The climate and the soil conditions extensively promote the cultivation of plant members of Leguminosae family such as *Cyamopsis tetragonoloba* (Guar), *Vigna radiata* (Moong dal), *Vigna aconitifolia* (Moth dal) and *Prosopis cineraria* (Sangri), making Rajasthan the largest producer of certain members of the pulse family. Among these, *C. tetragonoloba*, *V. radiata* and *V. aconitifolia* are the legume grains whereas the *P. cineraria* grow as a tree. In the previous chapter, it was discussed that in depth phytochemical characterization and metabolic profiling of only a few legumes such as Soybean and chickpea have been completed till now. But these edible legumes may not always be the primary food source in a given geographical location (such as in Rajasthan).

Among legume plants of the Indian desert, *Prosopis cineraria* (L.) Druce (Leguminosae/Fabaceae), locally known as janti or khejri, holds a unique position as all parts of the plant have some utility to the local populations. Its wood has been utilized for fuel and timber.¹⁶⁰ It has been labelled as the lifeline of the agroforestry system of the Indian desert and is well-adapted to the stresses that arid conditions impose.¹⁶¹ Members of the *Prosopis* genus have been extensively used in the indigenous medicinal system for treatment of a number of diseases such as leprosy, leukoderma, dysentery and also as anthelmintic, antibacterial, antifungal, antiviral and anticancer agents.¹⁶² Leaf paste has been used to treat boils, blisters, and sores on the skin. Recently, the bark of *P. cineraria* was shown to possess antihyperglycemic, antihyperlipidemic, antidiabetic

and antioxidative potential.¹⁶³ Of particular interest is the fact that the local populations most relish the delicious fruits of this tree.¹⁶⁴ Fruiting in *P. cineraria* usually occurs in the period between March to June and the seeds are contained within cylindrical light green pods. The edible pods, locally known as “Sangri,” are composed of seeds within a pulp that is sweet in taste. These pods are consumed raw, or as curry and in pickles after processing through boiling and/or drying. They are known to be rich sources of nutrients particularly vitamin C, calcium and iron.¹⁶⁵ Dry pods have been known to be effective in the prevention of protein-calorie malnutrition and iron-calcium deficiency in human blood. The seeds of *Prosopis* genus have also been recommended as a potential candidate for development into an economical food for humans and animals.

Cyamopsis tetragonoloba (L.) Taub. commonly known as Guar is another major edible legume that is grown in semi-arid and subtropical areas of North and North-West India predominantly in Rajasthan and East and South-East Pakistan. It is well adapted to the hot climate and is drought tolerant. Its beans are consumed as food in household and dry forage is used as livestock feed. Guar gum, which is obtained from the endosperm of the guar seeds, holds extreme importance in industries as a natural thickener, bonding agent, soil stabiliser, hydrocolloid, gelling agent, natural fiber, emulsifier, flocculants and fracturing agent. Apart from the industrial applications, guar has been reported to have anti-ulcer, hypolipidemic, cell-protective, hypoglycemic, and anti-secretory bioactivities. Many of these health benefitting activities have been accredited to the presence of secondary metabolites of polyphenolic class such as daidzein, naringenin, caffeic acid, gallotannins, genistein, gallic acid, catechol, luteolin, chlorogenic acid, ellagic acid, myricetin-7-glucoside-3-glycoside, quercetin, catechin, 2,4,3,-trihydroxybenzoic acid, kaempferol, rutin, hydroxycinnamic acid, texasin-7-O-glucoside, and p-coumaroylquinic acid.¹⁶⁶ The almost exclusive presence of isoflavonoids in legume seeds is another critical point that makes them striking candidates for development as pharmaceutical and nutraceutical additives in foods. However, metabolic studies vis-à-vis the same, particularly w.r.t. biotransformation of the inactive metabolites into active ones are lacking for many of the edible plants, including *P. cineraria* and *C. tetragonoloba*.

With increasing population and food demands, it would be highly desirable to investigate *P. cineraria* and *C. tetragonoloba* scientifically as economically important species of Indian desert, considering the utility of pods/seeds in treating malnutrition

among local people or as a famine food. As discussed above, there is meagre scientific information linking the phytochemical and metabolic profiles of *P. cineraria* and *C. tetragonoloba* pods/seeds. Further, most of the biological activity-based studies have been carried out with non-edible portions of these plants, using simple crude extracts (which may contain some activity-inhibiting substance); finally, the metabolic studies are almost non-existent. In the same context, it would be worthwhile to identify and isolate nutraceutical flavonoids/isoflavonoids from the pods, which are the edible portions of these plants. (It goes without saying that designing a suitable extraction method is also of utmost significance here. Therefore, the phytochemical examination of the *P. cineraria* and *C. tetragonoloba* pods (including seeds) vis-à-vis the content of flavonoids/isoflavonoids, along with identification and biochemical/ molecular characterization of the relevant isoflavonoid conjugate-hydrolyzing β -glucosidase activity, merits consideration as a primary area of focus for possible metabolic engineering to enhance their nutraceutical potential.¹⁶⁷ Furthermore, the biological activities of the compounds isolated from the pods/seeds of experimental plants could also be studied. To address the gaps present in the research related to these two important legumes of Rajasthan, the following objectives were proposed.

2.2 Objectives of the proposed research:

1. Phytochemical characterization of edible pods and seeds of *Prosopis cineraria* and *Cyamopsis tetragonoloba* for medicinally important flavonoid and/or isoflavonoid along with their glycosides
2. Purification and biochemical characterization of flavonoid/isoflavonoid conjugate hydrolyzing β -glucosidase from *P. cineraria* pods and *C. tetragonoloba* seeds
3. Investigation into the biological activities of purified, flavonoid-enriched extracts from *P. cineraria* and *C. tetragonoloba*



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Chapter 3: Phytochemical characterization of edible pods and seeds of *Prosopis cineraria* and *Cyamopsis tetragonoloba* for medicinally important flavonoid and/or isoflavonoid along with their glycosides

3.1 Introduction

An effective strategy to establish the medicinal importance of any plant material is to first identify the novel compounds present in them or to validate the biological activity of known compounds. This is initiated by developing methodologies to extract and purify the compounds from the crude extracts in such a way that their structure and biological activities remain unhindered. Despite the extensive development of separation techniques in the past decades, geographical restrictions have led to the unavailability of local plants to be studied for their comprehensive phytochemical composition. Validating the phytochemical (and subsequent health-benefiting) potential of locally available edible plants or their parts is of great significance for any region/country since it reduces its over-dependence upon non-native plants obtained from other states/countries. Most of the folk medicine involves the usage of either the plant extracts or the isolated active components. The ever-increasing resistance towards antibiotics in the pathogenic microorganisms have posed a need to look for more and more novel compounds which can be employed as drugs. Many a time when plants are subjected to phytochemical screening, researchers often target a single compound, or a limited number of compounds, which leads to loss of many crucial metabolites during purification. Hence carrying out the phytochemical analysis of the crude plant preparations is as important as identifying the novel compounds from the plant species. Desert legumes are important plants that accumulate nitrogen-based natural products such as flavonoids in response to many different kinds of environmental stress. As discussed in section 2.1, despite the knowledge of usage of different parts of the *Prosopis cineraria* (khejdi) tree in folk medicine, only isolated reports exist with respect to the flavonoids/isoflavonoids in the edible pods.^{164 168} Also, the literature survey informs only about the usage of preliminary techniques to investigate the phytochemical analysis. Most of the time, the biological activities have been determined only with the crude extracts. Similarly, pods of *Cyamopsis tetragonoloba* (guar) have not been subjected to extensive research with respect to their flavonoid/isoflavonoid profiles.

A few critical questions in the above context are 1) How does the nature of the extraction technique influence the overall metabolite profile of a given plant extract? 2) Does the ripeness stage of the edible plant part affect its metabolite profile? 3) Does purification improve or modify the biological activity of a particular plant extract? The present study was undertaken to derive answers to these questions (with a more detailed

response to the third question dealt with in chapter 5), besides also aiming to isolate new flavonoids and/or flavonoid combinations in terms of their biological activity.

3.2 Materials and Methods

3.2.1 Collection of experimental plants

The pods/seeds of *Prosopis cineraria* and *Cyamopsis tetragonoloba* were purchased from different regions viz. Pilani, Bikaner, Jodhpur, and Jaipur markets of Rajasthan. The flowering of *P. cineraria* starts in the month of January and reaches in full bloom during February - March. The fruit setting starts from April onwards, which attains full maturity in May. Since handpicking the pods is the most common practice for harvesting, only green, and healthy pods are harvested. The pods of *P. Cineraria* were collected in two forms, the ones which are unprocessed (i.e., raw) and were sold directly in the market after getting plucked from the trees and another one (i.e., processed) which is sold after heating the pods in boiling water followed by shade drying. The later type of processed pods is the standard mode of selling them, which will eventually be purchased for the purpose of cooking it in households as a relished vegetable (sangri). Once purchased from the market, the pods were again shade dried and were ground to a fine powder in a mixer-grinder. The powder was then stored in airtight container at room temperature until further use. The unprocessed fresh pods which were obtained from local farms of Pilani were frozen in liquid nitrogen and subsequently stored at -80 °C until further use. For *C. tetragonoloba*, the seeds and pods were air dried again at room temperature and then were ground to fine powder followed by storage in airtight container until further use. Figure 3.1 shows the morphology of the tree/plant, pods, cooked form of these two legumes.

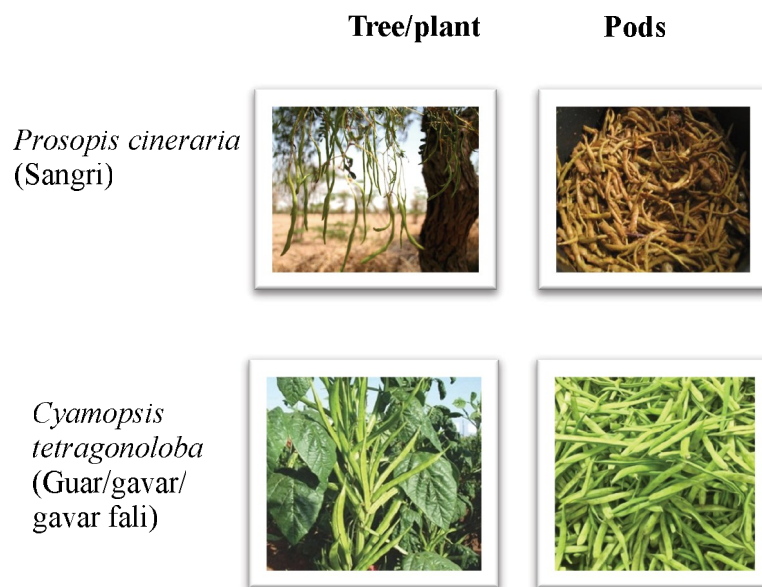


Figure 3.1 *P. cineraria* and *C. tetragonoloba* in the different forms; tree and pods
(Image courtesy: personal collection and online database)

3.2.2 Reagents and chemicals used

All the organic solvents hexane, toluene, chloroform, ethyl acetate, methanol, acetone, formic acid and glacial acetic acid used for the experiments were of analytical or High-Performance Liquid Chromatography (HPLC) grade and were supplied by SRL Laboratories and HiMedia. The standards of the flavonoids/isoflavonoids daidzein, daidzin, genistin, genistein, hesperidin, hesperetin, naringin, naringenin, rutin hydrate, and quercetin used were obtained from Sigma Chemical Company. The Thin Layer Chromatography (TLC) plates (precoated with aluminum-backed silica gel 60 F₂₅₄) were procured from Merck (Darmstadt, Germany).

3.2.3 Crude extract preparation from plant material

For the crude extract preparation from seeds/pods of *Prosopis cineraria* and *Cyamopsis tetragonoloba*, 250 gm of the ground dry powder of pods/seeds were taken and initially defatted using 3-5 volumes of hexane. The powder was made in a beaker and kept for stirring for 3 hours after the addition of hexane. This was followed by centrifugation at 3000 g for 5 minutes. The supernatant was decanted, and the pellet was extracted again with hexane and centrifuged, as mentioned above. The lipophilic (hydrophobic) compounds were obtained in hexane, and the pellet was dried thoroughly at room

temperature. This dried pellet was then extracted using a polar solvent such as methanol. For the preliminary studies, different concentrations of methanol in water were used for extraction. A range of 10%- 100% Methanol in water was used for extraction. Briefly, 1 gm of defatted powder was subjected to methanolic extraction with the addition of 5 volumes of solvent. Continuous stirring was done for 1 hour, followed by centrifugation at 3000 rpm for 5 minutes. The procedure was repeated two more times. All the supernatants were pooled and concentrated to dryness. After drying, the extracts were dissolved in 500µl methanol and analysed by TLC.

3.2.4 Solvent systems for TLC development

Throughout the entire work, thin layer chromatography was used for preliminary detection of the compounds isolated from crude or partially purified extracts as well as for the enzymatic product analysis (Section 5.4.7). This required the standardization of solvent systems for the detection of different standards as well as for the proper separation of the compounds present in the plant extracts. For this purpose, an extensive literature survey was carried out in our lab, and multiple trials run for various solvent systems were executed for analysing putative flavonoids/isoflavonoids and their respective glycoside conjugates. The difference in the polarities of the aglycones and glycosides pose a challenge while standardizing the solvent system for TLC based chromatographic separation since a single solvent system many times fails to induce migration/separation of all the compounds spotted on the TLC plate. Table 3.1 indicates a few of the solvent systems in various combinations which were subject to trial runs for flavonoids/isoflavonoids. All the TLC runs mentioned below were of the single ascent nature (i.e., one dimensional or 1D). Also, post standardization, minute modifications were made from time to time as per the requirement and quality of the plant extract.

Table 3. 1 Optimization of solvent systems for TLC of isoflavonoids/flavonoids and corresponding glycosides.

Flavonoids analysed	Solvents	Ratio	Solvent System
Daidzein, Genistein, Quercetin	Toluene: Ethyl acetate: Acetone: Formic Acid	22:4:3:1	1
		20:4:3:1	2
		20:4:2:2	3
		20:4:2:1	4
Daidzin, Genistin, Rutin Hydrate	n-Butanol: Acetic acid: Water	7:2:2	5
		10: 0.5: 0.5	6
		10:0.5: 0	7
Hesperetin, Hydroquinone, 6,7 – dihydroxy coumarin, Naringenin,	Chloroform: Methanol	9:1	8
		9:4	9
		7:2	10
		8:2	11
Hesperedin, Naringinin	Ethyl acetate: Methanol: Water	15:3:2	12
		12:3:5	13
		14:2:1	14
		14:4:2	15

3.2.5 Purification of flavonoids from pods/seeds of *P. cineraria* and *C. tetragonoloba*

In the current work, secondary metabolites (particularly flavonoids/phenolics) from the *P. cineraria* and *C. tetragonoloba* have been isolated using different column chromatography techniques. The initial steps included the collection of the experimental material and preparing the crude extracts. This has been explained in detail in section 3.2.3. The preliminary phytochemical analysis was done using the crude extract to observe the different classes of the phenolics present in the seeds/pods of the *P. cineraria* and *C. tetragonoloba*. To isolate the compounds further, crude extract preparation was subjected to column chromatography, which included sequential usage of matrix Amberlite XAD7HP and Sephadex LH-20. This flavonoid enrichment protocol was a modified version of that reported by Ferres *et al.*¹⁶⁹ The Amberlite XAD7HP matrix was packed in a glass column with dimensions

1.8 cm (internal diameter) and 30 cm height. The matrix procured was washed thoroughly using distilled water and then soaked in excess of methanol. The matrix was then washed with 100% methanol and then was packed into the glass column. The packed column was equilibrated again using 100% methanol and 10 gm of the crude extract of the *P. cineraria* was dissolved in absolute methanol and then loaded on the column. The flow through fraction containing the unbound compounds was collected and a wash using water was given to the column. This eluted out the unbound compounds thus avoiding the interference with the elution of the phenolics. The final elution was done using absolute methanol until the discoloration of the matrix was observed. The eluent was collected in a flask and stored at 4°C until further use. (The column was washed, equilibrated and regenerated for further experimentation, viz. the isolation of flavonoids from the *C. tetragonoloba* crude extracts). The eluent obtained post-Amberlite column chromatography was concentrated to dryness using a rotary evaporator. The dried extract was divided into two parts. One part was subjected to gel filtration chromatography using Sephadex LH20 matrix and the second part was analysed for its biological activities. From 200 g of seeds, approximately 25 g of the dried extract was obtained post-Amberlite column chromatography.

Sephadex LH20 matrix, which was procured from the Sigma Aldrich, was thoroughly washed using distilled water and then packed into a column of height 50 cm and 1.8 cm internal diameter. The column was equilibrated using two volumes of 100% methanol. 5 g of the post-Amberlite flavonoid-enriched fraction was dissolved in methanol and was loaded on to the column. The column was first eluted using 50 % methanol, and 20 fractions of 5 mL each were collected. A second elution was performed using 100 % methanol and again, 20 fractions of 5 mL each were collected. All the fractions were subjected to analytical thin layer chromatography (TLC). The fractions showing the maximum number of spots/bands were pooled and used for further studies pertaining to their structure elucidation.

3.2.6 Determination of UV-Visible spectra for the purified plant extracts

The different fractions collected were also subjected to UV-Visible spectra analysis to identify flavonoids. The three ring systems present in the flavonoids/isoflavonoids contribute to multiple absorption maxima for each compound. All the extracts were dissolved in absolute methanol at a concentration of 1mg/mL, and absorption spectra

were recorded for a wavelength range of 200-800 nm with an interval of 2nm. Methanol solvent was used as a sample blank.

3.2.7 TLC fingerprint profile for the purified plant extracts

The different fractions which were collected after the experimental material obtained from various kind of extraction procedures were analysed using thin layer chromatography. All the extracts to be tested using TLC were dissolved in 100% methanol at a concentration of 1mg/mL and were filtered using 0.45µm filters. For developing the TLC plates, a cylindrical glass chamber (10 cm x 11 cm; D x H) was used; its temperature was kept at 30± 1°C. 5-10 µg of the methanolic extract was spotted near the bottom of the TLC plate. The samples were subjected to analysis using different solvent systems. Post development, the TLC plates were immediately dried at 30-40°C and were visualized under UV light at 254 nm and 366 nm. Images of the developed TLC plates were captured either using a simple camera or TLC Visualizer (CAMAG). The plates were also analysed via spraying the flavonoid-specific NPR (natural product reagent), which is 1% methanol solution of 2-Aminoethyl diphenylborinate, and 5 % ethanolic solution of the PEG-4000, one after the other onto the developed TLC plate. Intense fluorescent colours appeared immediately or after 15 min in UV-365 nm.

3.2.8 Total flavonoid content in plant extracts

Total flavonoid content was measured by the aluminium chloride colorimetric assay.¹⁷⁰ In brief, an aliquot (1mL) of extracts or standard solutions of rutin hydrate (200, 400, 600, 800, and 1000µg/mL) was added to 10 mL volumetric flask containing 4 mL of distilled water. To the flask was added 0.30 mL 5% NaNO₂; after five minutes 0.3 mL 10 % AlCl₃ was added. After five minutes, 2 mL 1M NaOH was added, and the volume was made up to 10 mL with distilled water. The solution was mixed, and absorbance was measured against the blank at 510 nm. The total flavonoid content was expressed as mg Rutin Hydrate equivalents (RH).

3.2.9 HPLC analysis of the isolated flavonoids/isoflavonoids

The methanolic fractions of the extracts obtained from previous steps were dried thoroughly and dissolved in HPLC grade methanol and subjected to chromatographic separation. The liquid chromatography system employed was LC-

2010 CHT (Shimadzu Corporation, Tokyo, Japan) with a solvent delivery system of two intelligent pumps, an intelligent auto injector, and UV-VIS detector. Data collection and integration was carried out using LabSolutions software.

Prior to this, the standardization of the methods to be employed in the analytical separation of the compounds was done by a combined modification of previously reported methods.^{91 171} The chromatographic separation was achieved on an end-capped C₁₈ reverse-phase column (Spherisorb®, 250 mm long and 4.6 mm internal diameter, particle size 5 µm, Waters). The optimized mobile phase consisted of an aqueous phase A (Milli Q water, pH adjusted to 3.0 using ortho-phosphoric acid), and an organic solvent B (HPLC grade Acetonitrile). The buffer was filtered through a 0.22 µm Millipore® filtration membrane. The HPLC system was equilibrated for a minimum of 1 h by passing the mobile phase through the system at a flow rate of 1 mL/min, prior to actual analysis. For the separation run, low pressure gradient flow mode was followed. Starting a concentration of 10% aqueous phase A and 90% acetonitrile were taken. In 33 minutes, the proportion of solvent B reached 35% and then brought back to 10% in the 40th minute. This concentration was maintained for 5 minutes, and the run was terminated. A flow rate of 0.7 mL/min was selected. The analysis was carried out at ambient temperature (25 ± 2°C).

HPLC method was optimized for four isoflavonoid standards viz. daidzein, daidzin, genstein, and genistin. Each of these isoflavonoid glycosides and their aglycones (standards) were detectable at a wavelength of 254 nm. This method with varying wavelengths for detection was used for other standards like arbutin, naringin, naringenin, hesperetin, but no peaks were observed.

Partially purified methanolic fractions of the *P. cineraria* and *C. tetragonoloba* obtained via different extraction procedures were filtered using 0.45µm puradisc filters and then subjected to HPLC analysis using the above-mentioned procedure. The low-pressure gradient method was designed in such a manner that the more polar compounds would elute out first, followed by relatively less polar compounds. The retention time of the test samples was compared with that of the standard samples, and identities were assigned to them. Throughout the HPLC analysis, suitable changes in the standard run methods were incorporated as per the requirement and separation profile of the test sample.

3.2.10 Mass spectrometric analysis of the purified fractions

To further identify the structure and chemical nature of the purified fractions from *P. cineraria* and *C. tetragonoloba*, LC-MS (Liquid chromatography-mass spectrometry) analysis was carried out. A gradient flow separation was performed using acetonitrile and milli Q water (pH=3) for a total run time of 50 minutes. The flow rate for the run was 0.7 mL/minute. Absorbance was recorded at 254 nm and 365 nm. The eluted peaks were then subjected to MS analysis using Waters, Micromass Q-TOF micro via the electrospray ionisation (ESI) technique (positive ion mode) in methanol. The products formed from LC-MS were analysed using the Agilent database library MassHunter associated with the instrument. The fragments obtained on the mass spectrum were again analysed using RIKEN-RESPECT software.¹⁷²

3.3 Results and Discussion

3.3.1 Optimization of solvent systems for TLC of flavonoid/isoflavonoid

The procedures which are employed for the extraction of different phytochemicals from the plant samples vary according to the part of the plant involved and the nature of the compounds to be extracted. The work presented here dealt with the isolation of the flavonoids/isoflavonoid compounds, which are relatively polar in nature. But the chemical nature of all the compounds present in the plants varies from highly non-polar to highly polar one. This calls for selective strategies for the extraction of the metabolites from plants. A general norm is to start with a highly non-polar solvent and further proceed with the solvents of increasing polarity. This ensures the step wise separation of compounds according to their polarities, which enables the compounds of our interest to get fractionated out in one type of solvent. The first step is to separate the hydrophobic or non-polar compounds present in the pods which are mainly pigments and fatty acids. This is generally done by using hexane or chloroform or step wise usage of both the solvents. The polar compounds are extracted with the usage of solvents with relatively higher polarity, such as methanol or ethanol. The overall purpose in the current work was to extract the maximum number of compounds with the minimum usage of the organic solvents, thereby also aiming towards an environmental-friendly approach.

TLC offers a simple, rapid, and inexpensive technique to perform the separation of different natural product classes obtained after extraction from plants. Despite the development of more advanced separation techniques over the past few decades, TLC is still one of the most common techniques being employed in identifying the compounds at the preliminary stages. Therefore, throughout the entire study, TLC was carried out to a) identify the presence of putative flavonoids/isoflavonoids in the extracts obtained from seeds/pods of *P. cineraria* and *C. tetragonoloba*, and b) to analyse the enzymatic products obtained upon the reaction of flavonoid/isoflavonoid glycosides with the purified isoflavonoid specific β -glucosidase. However, the versatile nature of the metabolites in the extracts (specifically in terms of polarity) obtained poses certain limitations while developing the TLC method. As discussed previously, the flavonoids/isoflavonoids can be present in two forms inside the cellular environment in the plant at any given stage: the aglycone form and the conjugated glycosylated form. This difference in the structural configuration leads to a significant difference in the polarities of the two types of compounds. The separation of the compounds on the TLC plate is possible by virtue of the difference in polarities of compounds undergoing the separation. Hence, the nature of the compounds in the extract is the deciding factor in the choice of the mobile phase. The crude and enriched fractions which were obtained from *P. cineraria* and *C. tetragonoloba* when loaded onto the TLC plates, it was observed that a single solvent system was not capable of separating all the constituents (flavonoids and their glycosides) present in the extracts. After an extensive literature survey followed by experimentation in our lab, several mobile phases were chosen to optimize the pattern of the bands observed in TLC (See Table 3.1). Initial standardization was done using the commercial standards available for the flavonoids/isoflavonoids. It was noted that the toluene: ethyl acetate: acetone: formic acid::20:4:2:1 (solvent system A) offers a proper separation of the aglycones of isoflavonoids daidzein and genistein, but the glycosylated forms daidzin and genistin didn't move from the point of origin on the plate. This indicated that the solvent system A was not an appropriate choice for the relatively more polar compounds like glycosides. This issue was addressed by selecting a separate solvent system for the polar compounds. Butanol: Acetic Acid: Water::7:2:2 (solvent system B) has been used extensively by other researchers to obtain separation of relatively polar natural products. Similarly, for other flavonoids such as hesperetin, naringenin, etc., other solvent systems were optimized.

In brief, preliminary detection of different flavonoids and their glycosides from the extracts of *P. cineraria* and *C. tetragonoloba* were performed by thin layer chromatography. For this, solvent systems for various standards were optimized. Table 3.2 represents the solvent systems selected for optimal TLC based chromatographic separation and identification of the compounds. The development of an appropriate TLC method also enabled us to analyse enzymatic biotransformation pertaining to flavonoid glycosides (discussed in Chapter 4).

Table 3. 2 Solvent systems optimized for chromatographic separation of flavonoids and their glycosides using Thin Layer Chromatography

Compounds separated	Solvents	Ratio	Solvent System
Daidzein, Genistein, Quercetin	Toluene: Ethyl acetate: Acetone: Formic Acid	20:4:2:1	4 (Solvent system A)
Daidzin, Genistin, Rutin Hydrate	n-Butanol: Acetic acid: Water	7:2:2	5 (Solvent system B)
Hesperetin, Hydroquinone, 6,7 –dihydroxy coumarin, Naringenin,	Chloroform: Methanol	9:1	8
Hesperedin, Naringinin	Ethyl acetate: MeOh: Water	15:3:2	12

3.3.2 TLC profiling of the plant-derived phytochemicals (specifically flavonoids)

TLC analysis provides a simple, effective, and quick procedure to separate and identify the compounds present in a mixture. The extracts obtained from both the experimental plants (i.e., *P. cineraria* and *C. tetragonoloba*) were subjected to TLC analysis using the commercially procured analytical TLC plates. The visualization of flavonoids was done by illuminating the developed TLC plates (using solvent systems, as mentioned in the preceding section) under UV illumination at 254 nm and 365 nm wavelengths.¹⁷³ The plates were also analysed using NPR and 5 % ethanolic solution of the PEG-4000, one after the other onto the developed TLC plate. Intense fluorescent colors appeared immediately or after 15 min in UV-365 nm. The

flavonoids/isoflavonoids showed distinctive colours when visualized under 254 nm and 365 nm, either without spraying or post-spraying. Table 3.3 shows the general colour properties of the different flavonoids under the UV illumination, pre and post spraying with natural product reagent. The flavonoids/flavonoid classes indicated by the different colours are derived from the TLC atlas prepared by Sabine Bladt (2009).

Table 3.3 Colorimetric analysis for different flavonoids as observed under 254 nm and 365 nm post spraying with Natural Product Reagent.

S.No.	Visible color under 254 nm illumination	Visible colour under 365 nm illumination post spraying with NPR	Flavonoids indicated
1.	Orange	Blue/Yellow green	Anthocyanidin-3-glycosides
2.	Bright yellow	Dark green	6-hydroxylated flavonoids, flavones, chalcones glycosides
3.	Very pale yellow	Yellow-green	Most flavonol glycosides
4.	Dark mauve	Faint orange	Most isoflavones and flavanonols
5.	Faint blue	Orange	5-deoxyisoflavones and 7,8- deoxyisoflavones
6.	Dark mauve	Orange, Yellow green	Flavones and their glycosides
7.	Dark mauve	Orange-yellow, Yellow-green	Flavonols and their glycosides

On the basis of Table 3.3, phytochemical profiling of the methanolic extracts of the edible portions of *P. cineraria* and *C. tetragonoloba* was carried out using TLC, which has been discussed in the following sections. One of the essential criteria to be kept in mind wrt TLC is that the best solvent system has to be selected so as to efficiently separate the constituents present in a given mixture.

3.3.2.1. TLC analysis of flavonoids in the extracts of *Prosopis cineraria* from selected regions of Rajasthan

The methanolic crude extracts after TLC analysis showed the presence of different bands on the TLC plates when observed at 254 and 365 nm. The extracts that were obtained from the pods collected from different regions showed variations in the phytochemical

profile wrt the flavonoids present. Table 3.4 indicates the abbreviations used for samples collected from different regions.

Table 3. 4 Abbreviations designated for the samples of *P. cineraria* collected from different regions of Rajasthan

S. No	Region	Abbreviation
1	Bikaner 1*	PC-B1
2	Bikaner 2*	PC-B2
3	Bikaner 3*	PC-B3
4	Jodhpur	PC-Jo
5	Jaipur	PC-Ja
6	Pilani Market	PC-M
7	Pilani Farms	PC-F

(* PC-B1 young, PC-B2 senescent, PC-B3 mature; pods collected from three different stages)

The R_f values were calculated for all the compounds present in individual fractions and were compared with the standards. Daidzein, genistein, and quercetin, which are a few of the most important aglycones among flavonoids/isoflavonoids, were run as standards. Figure 3.2 shows the resultant TLC as obtained after separation was carried out in the solvent system A, i.e. Toluene: Ethyl acetate: Acetone: Formic acid::20:4:2:1, which facilitates the separation of relatively less polar flavonoids upon the polar stationary phase. 10 μ g of the sample was loaded on the TLC plate and it can be clearly observed from the following images that not the entire sample loaded onto the plate was able to migrate in the mobile phase, hence implying the presence of highly polar compounds (possibly glycosylated versions of flavonoids) in the methanolic extracts. However, significant numbers of dark-colored and well-separated bands against the fluorescent background were obtained under 254 nm and orangish, and bluish-colored bands against the fluorescent background at 365 nm. Considering the information given in Table 3.3, these colours indicate the presence of the following compounds: 6-hydroxylated flavonoids, flavones, chalcones glycosides, flavonols and their glycosides. Multiple bands were observed in all the methanolic extracts of different samples. Therefore, the phytochemical richness of *P. cineraria* pods wrt flavonoids is immediately apparent from the TLC analysis.

Pods collected from the Pilani region showed bands at R_f values of 0.1, 0.31, and 0.37; all of them were dark colored under 254 nm. Three samples from Bikaner regions

included three different stages of the pods: young (7 days old), mature (15 days old), and senescent (25 days old), respectively. The young pods showed two dark bands at R_f values of 0.20 and 0.312, whereas the mature pods showed the presence of the most bands at R_f values of 0.20, 0.25, 0.312, and 0.33. The senescent pods showed only one major band at R_f value of 0.312. The UV active compounds were also observed when the TLC plate was illuminated at 365nm. The change in the pattern of bands at different stages of the pods indicates that plants synthesize different phytochemicals at different stages of growth. In general, the flavonoids start becoming more glycosylated as the plant/plant part matures. Methanolic extract of the pods collected from the Jodhpur region showed two dark bands at R_f values of 0.20 and 0.312. Sample from the Jaipur region showed a single major band at R_f value of 0.12. It is evident that a band at R_f value of 0.312 is prominently present in extracts obtained from all the regions. When the R_f values were calculated for the bands visualized under 365 nm, a different separation pattern was observed. Blue colored bands were found predominantly in samples from Bikaner pods (young)*, Bikaner pods (senescent)*, Bikaner pods (mature)*, Jodhpur pods, and Jaipur pods indicating the presence of flavonols (sub-class of flavonoids) and their conjugates since not all the compounds showed migration in the solvent system used for separation.

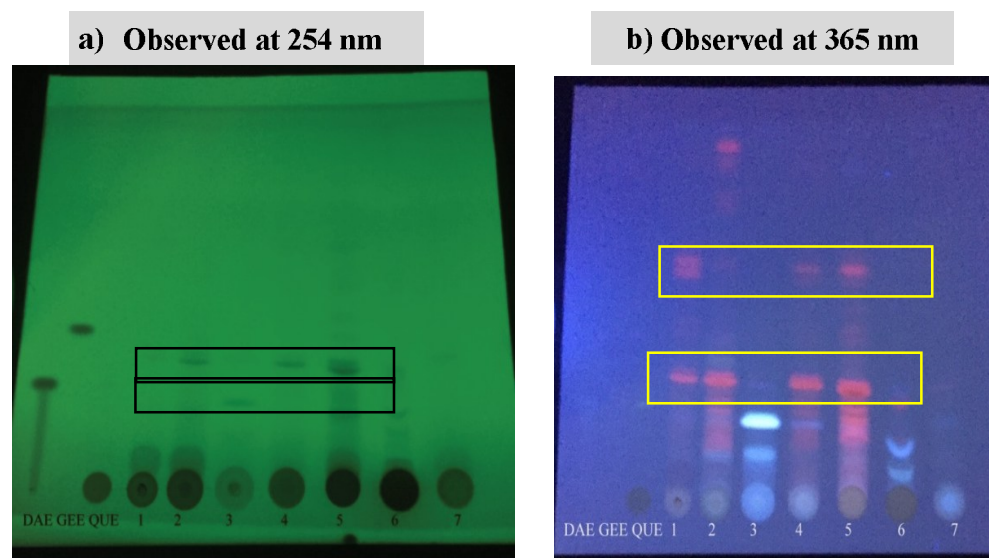


Figure 3.2 TLC images of defatted methanolic extracts obtained from pods of *P. cineraria* from selected regions of Rajasthan a) observed at 254 nm and b) observed at 365 nm. Solvent system used is as follows: Toluene: Ethyl Acetate: Acetone: Formic Acid:: 20:4:2:1. Immediately apparent variations in intensity of a few flavonoids are enclosed within rectangles.

Samples : Daidzein, Genistein, Quercetin, 1-PC-M, 2- PC-F, 3-PC-B1, 4-PC-B2, 5-PC-B3, 6-PC-Jo, 7-PC-Ja **Sample loaded:** 10 μ g of each extract

As it is evident from the images of TLC run in solvent system A, the majority of the sample was localized on to the origin spot and could not get separated thoroughly post run. This indicates that the solvent system A was not sufficiently polar to mobilize the compounds present in the methanolic extract of the pods. However, we observed the movement of the standards loaded on to the TLC plates, which are the aglycones of the flavonoids/isoflavonoids. On the basis of the colour of the different bands obtained in the TLC plates, it is evident that multiple flavonoids/isoflavonoids might be present in the defatted methanolic extracts of the pod samples obtained for *P. cineraria* (from different regions). Also, the non-migration of compounds in the extract from the origin indicated the highly polar nature of the compounds, which may be attributed to the fact that they could be conjugated with sugar molecules and making the molecule more polar, thus restricting their movement. For this, the extracts were loaded on another TLC plate and solvent system B, which comprises of Butan-1-ol: Acetic Acid: Water in the ratio of 7:2:2, was used as a mobile phase.

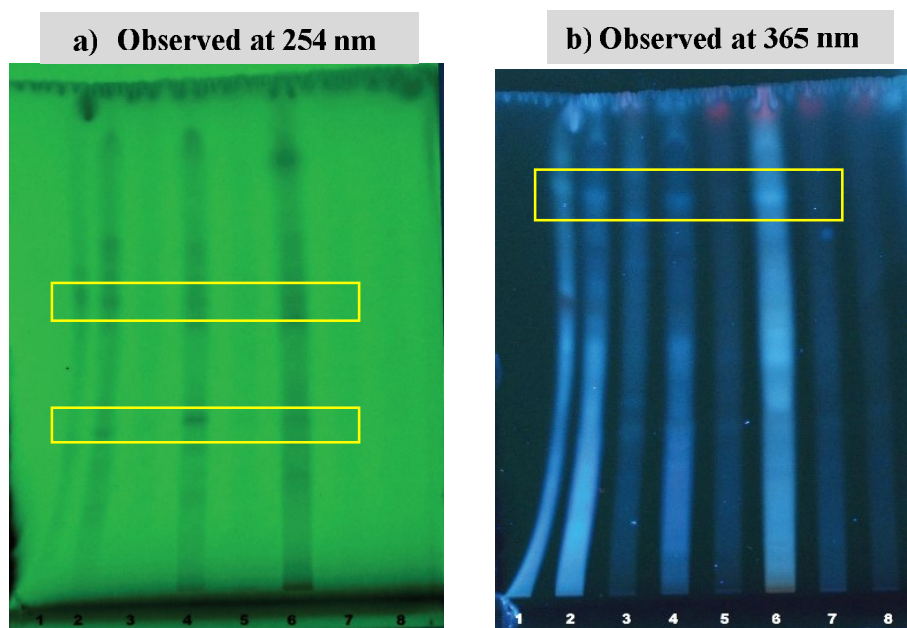


Figure 3.3 TLC images of defatted methanolic extracts obtained from pods of *P. cineraria* from selected regions of Rajasthan. Solvent system used is as follows: n-Butanol:Acetic Acid: Water::7:2:2. Immediately apparent variations in intensity of a few flavonoids are enclosed within rectangles.

1-PC-Ja, 2-PC-Jo, 3- PC-F, 4- PC-M, 5- PC-M', 6- PC-B1, 7- PC-B2, 8-PC-B3. Sample loaded: 10 μ g of each extract

The solvent system B imparts a substantial amount of polarity to the mobile phase, thus enhancing the separation of the compounds present in the extracts. It is evident from Figure 3.3 that majority of the compounds were able to migrate on the TLC plate under

the solvent system B, thus implying that they could be present in their conjugated forms. The band pattern observed after the separation is varying among the samples obtained from different regions, thus presenting a fingerprint of all the UV-active phytochemicals (flavonoids) present in the samples. When visualized under 254 nm, faint mauve-colored bands at R_f values of 0.33, 0.58, and 0.66 were observed in the majority of the examples of the *P. cineraria* indicating the presence of flavonols/flavonones glycosides. A dark unified band at the R_f value of 1.0 indicates that the possibly non-conjugated compounds present in the extracts moved along with the solvent front only because of the significantly higher polarity of the mobile phase. Similarly, visualization under 365 nm indicated that compounds with R_f values 0.73 and 0.9 showed the presence in the majority of the samples. Orange colored unified bands alongside the solvent front stated the presence of aglycones of flavonoids which showed higher mobility as compared to their conjugated counterparts due to differences in their polarities.

To conclude this section, it was seen that when the solvent system was made more polar, better separation of metabolites was observed; in this case, possible flavonoid aglycones and their glycosides. Glycosides, being more polar, tend to have a lower R_f value in comparison to aglycones, when normal phase TLC is carried out. The above experiments also helped us to optimize the TLC solvent system further and to observe, at least qualitatively, the flavonoid richness and region-wise phytochemical variation in the *P. cineraria* pods. Deriving such as TLC-based phytochemical fingerprints, even without knowing the exact identities of individual components, is often enough for a scientist to understand chemotypic or seasonal variations in plants.¹⁷⁴ To the best of our knowledge, such detailed TLC analysis for flavonoids has not been done before for *P. cineraria* pods. Liu et al. reported a number of metabolites in methanolic extracts of *P. cineraria* pods using TLC, but they have not highlighted flavonoids.¹⁷⁵ In the current work, the subsequent identification and quantitative analysis of the specific flavonoids in the pods was carried out using LC-MS analysis (results discussed in a later section).

It must be mentioned in passing that the procurement of *P. cineraria* pod samples from year-to-year was often limited by seasonal changes. It was observed that if the month of April was not hot enough, and/or frequent dust storms occurred, the edible pods turned into inedible parts. For both phytochemical and metabolic characterization, we restricted our studies to the edible pods only.

3.3.2.2. TLC analysis of flavonoids in the extracts of *Cyamopsis tetragonoloba* seeds/pods from different regions

The TLC analysis was also carried out from the samples of *C. tetragonoloba* collected from different locations of Rajasthan. The abbreviation used for different samples is indicated in Table 3.5.

Table 3.5 Abbreviations designated for the samples of *C. tetragonoloba* collected from different regions of Rajasthan

S.No.	Region	Abbreviation
1.	Pilani (Green Pods)	CT-G
2.	Pilani	CT-P
3.	Jhunjhunu	CT-Jh
4.	Mukundgarh	CT-Mu
5.	Jaipur	CT-Ja
6.	Sikar	CT-S

Figure 3.4 represents the TLC image of the extracts run using solvent system A, which is a relatively less polar solvent system. Daidzein, genistein, and quercetin were used as the standard samples. It is evident from the following figure that defatted methanolic extracts showed the presence of flavonoids/isoflavonoids on the basis of the color of the bands observed under illumination at 254 nm and 365 nm.¹⁷³ Two prominent compounds were found under visualization at 254 nm with R_f values of 0.1 and 0.363. The colour of the bands is faint mauve against the green fluorescent background. Both the bands were present in all the samples of *C. tetragonoloba* obtained from different regions of Rajasthan. Here it was also noted that a significant quantity of the extract loaded was concentrated at the origin, implying that conjugated (glycosylated) polar compounds are possibly present in high amounts in the extract obtained from the seeds of *C. tetragonoloba*. When the same TLC plate was visualized under 365 nm, the bands visualized at R_f values of 0.18 and 0.312 appeared bluish-coloured indicating the presence of the flavonoids (anthocyanidin-3-glycosides). The extract collected from the CT-P region showed some variation when compared with the other samples. An orangish colored band at the R_f value of 0.36 was observed exclusively in the pods. This indicated

that there exists a phytochemical variation between the green pods and the seeds obtained from the pods upon drying. The orangish color visualized under the 365 nm shows the presence of flavonoid (5-deoxyisoflavones and 7,8- deoxyisoflavones) in the whole pods.

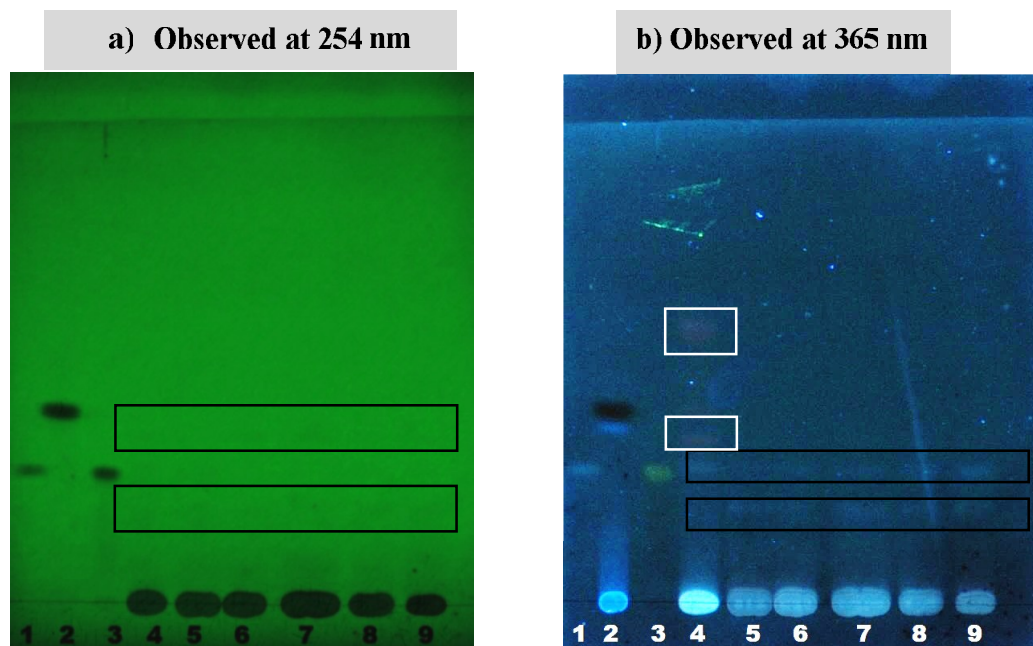


Figure 3.4 TLC images of defatted methanolic extracts obtained from pods of *C. tetragonoloba* from selected regions of Rajasthan, observed under a) 254 nm and b) 365 nm. Solvent system used is as follows: Toluene: Ethyl Acetate: Acetone: Formic Acid:: 20:4:2:1. Immediately apparent variations in intensity of a few flavonoids are enclosed within rectangles. Samples 1- Daidzein, 2- Genistein, 3- Quercetin, 4- CT-G, 5- CT-Jh, 6-CT-Mu, 7-CT-S, 8-CT-Ja, 9-CT-P. Sample loaded: 10 μ g of each extract

In order to obtain the improvised separation of the compounds (specially for those possibly present in their conjugated forms in the methanolic extracts), solvent system B was also employed to carry out the TLC analysis. Figure 3.5 represents the phytochemical separation under a more polar mobile phase (i.e., solvent system B), which facilitated the migration of even conjugated glycosides present in the extracts. It is evident from the images that the extracts isolated were rich in multiple compounds. The phytochemical profile of the extracts from different sources poses a comparable analysis since the band pattern as visualized under 254 nm and 365 nm is identical to a great extent. The TLC plate was also sprayed with Natural product reagent followed by observation at 365nm. The presence of bluish-colored, orange colored, yellowish-green colored bands at R_f values of 0.416, 0.433, and 0.58 respectively indicated the presence of flavonoids in the CT-G sample. It is known that different classes of flavonoids show distinguish colors when reacted with Natural product reagent. Thus, the bands observed at R_f values of 0.416, 0.433 and

0.58 indicated the categories of hydroxycinnamics, deoxyisoflavones, and flavonol glycosides (Table 3.3). The phytochemical analysis of all extracts carried out using TLC with a solvent system. B shows the presence of the flavonoids in them, which paved a way to perform further separation and identification of the compounds. As mentioned for *Prosopis cineraria*, such a simplistic and quick TLC analysis quickly has an excellent potential to inform the researcher about phytochemical variations existent in different chemotypes of a plant, even though the exact identities of individual components are not known.

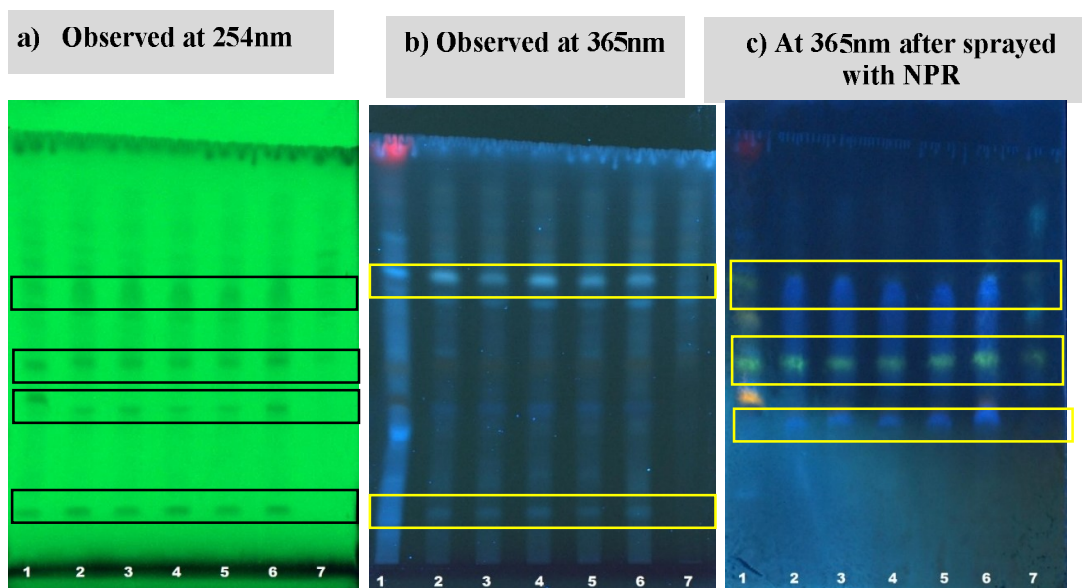
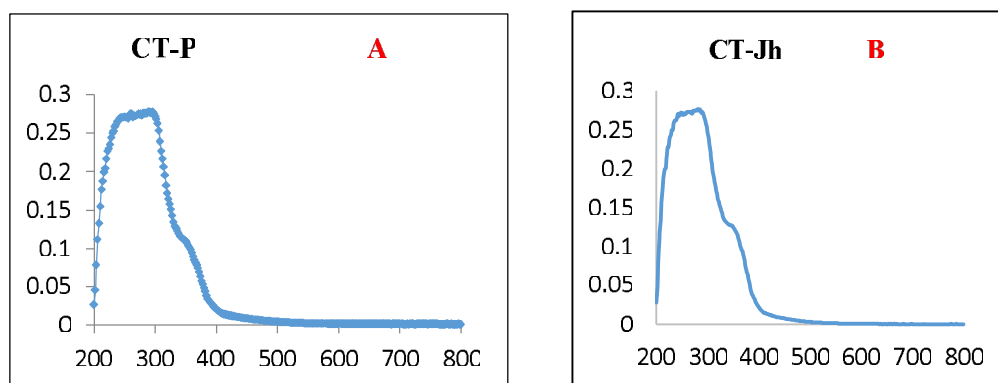


Figure 3.5 TLC images of defatted methanolic extracts obtained from pods/seeds of *C. tetragonoloba* from selected regions of Rajasthan, observed under a) 254 nm and b) 365 nm, and c) 365 nm post NPR spraying. Immediately apparent variations in intensity of a few flavonoids are enclosed within rectangles. Solvent system used is as follows: n butanol: Acetic Acid: Water:: 7:2:2. Samples; 1-CT-G, 2-CT-Ja, 3-CT-Jh, 4-CT-Mu, 5-CT-S , 6-CT-S' ,7-CT-P. Sample loaded: 10 μ g of each extract.

3.3.3 UV-Visible spectra profiling of the selected plant extracts

Throughout this work, our focus has been to prepare phytochemical fingerprints of the selected plants using so-called simple techniques such as TLC. These not only reduce the overall cost of analysis for the researcher but also give preliminary and swift information about the regional and seasonal phytochemical variations. Therefore, such techniques become all the more remarkable because the considerable diversity in natural products that exist in the plant kingdom and practical considerations pertaining to cost, location, and time does not always mandate the use of high-end techniques such as HPLC. Further, in this league, UV-

Visible spectroscopy has been used, which offers a simple and efficient technique to gain structural insights into the flavonoids/isoflavonoids present in any given sample. This is because of the multiple absorption maxima shown by different compounds as a result of the conjugation present between A, B, and C rings (Figure 1.1). In general, all the flavonoids show absorption in UV range only with the exception of anthocyanin which shows absorption in the visible range of 500-550 nm as well.¹⁷⁶ The typical UV-Vis spectra of flavonoids include two absorbance bands. The absorbance contributes band I showed by ring B, and Band II is because of the ring A. Band II lies in the 210-285 nm range whereas Band I lies in the range of 300-400 nm.¹⁷⁷ Moreover, different subclasses of flavonoids also exhibit differences in their respective absorbance maxima for both the bands. For example, Band I of flavones occurs in the range 304 - 350 nm whereas Band I of 3-hydroxyflavones appears at a slightly longer wavelength (352 - 385 nm). However, in 3-hydroxyl substituted flavonols with methyl or glycosyl group, Band I (328 - 357 nm) overlaps the region for Band I in flavones, and the general shape of absorption spectral curve approach those of flavones. Hence the UV-visible spectral analysis was carried out to observe the phytochemical fingerprinting of different methanolic extracts obtained from pods of *C. tetragonoloba* and *P. cineraria*. Figure 3.6 (A-F) represents the UV-Spectra of different extracts of *C. tetragonoloba* measured from 200-800 nm.



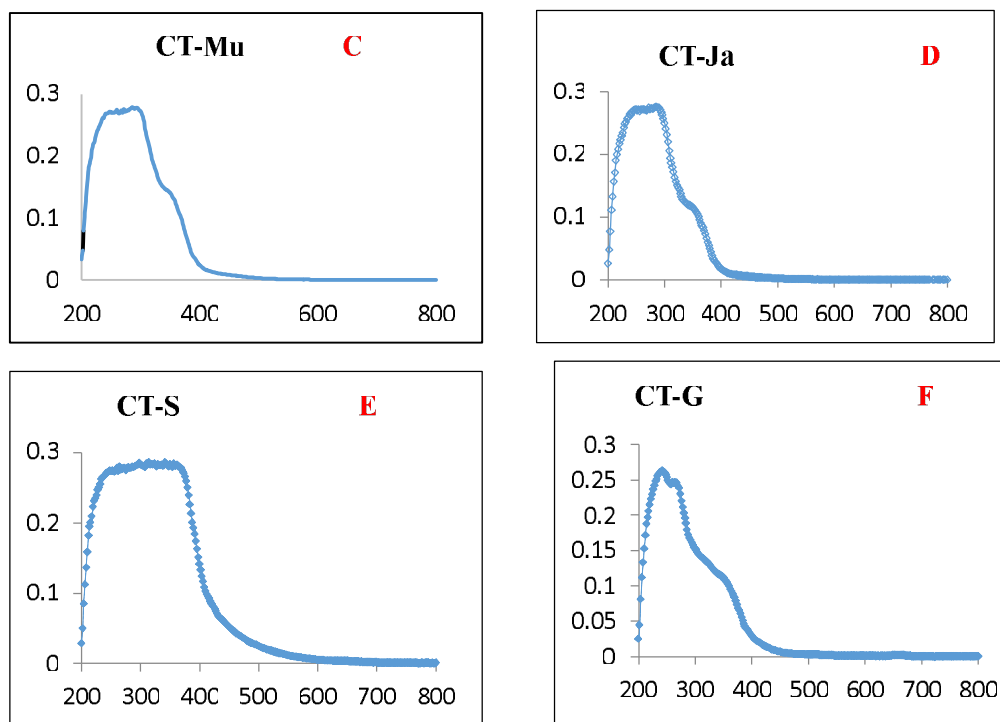


Figure 3.6 A-F represents the UV spectrum of the methanolic extracts of the samples of *C. tetragonoloba* collected from selected regions of Rajasthan

The distinct absorption maxima obtained for pods obtained from different regions indicated the differences in the nature of the compounds present in the methanolic extracts. This is in accordance with the data obtained from the TLC fingerprinting (Figure 3.4). This indicates that despite all the pods collected from the different regions of semi-arid Rajasthan, variations at the phytochemical level are present in constituents among the plants belonging even to the same species. Table 3.6 shows the absorption maxima observed in the various samples of *C. tetragonoloba*, and it can be seen that slight variations in the wavelengths were there which is a result of the substitution groups present in different flavonoids/isoflavonoids (as mentioned earlier).

Table 3. 6 Absorption maxima of the compounds in defatted methanol extracted fractions of *C. tetragonoloba*

S.No.	Sample	λ_{\max} (nm)
1.	CT-P	260, 290, 304
2.	CT-Jh	242, 268, 280
3.	CT-Mu	248, 284
4.	CT-Ja	248, 286
5.	CT-S	260, 356, 314, 342, 398
6.	CT-G	242

Similarly, UV-Visible spectra of the methanolic fractions of the samples collected for *P. cineraria* were taken and are shown in Figure 3.7 A-F. The concentration of the samples was chosen as 1mg/mL in absolute methanol, and characteristic peaks were determined. All the defatted methanolic extracts showed the peaks in the range of 206-210 nm and between 250-270 nm. Three samples collected from the Bikaner region were of different stages of the *P. cineraria* pods, viz. young (7 days), mature (15 days) and senescent (25 days). The young pods showed the maximum absorbance peak at 212 and 274 nm with an absorbance of 0.5907 and 0.1709 respectively. The mature pods showed three peaks at 206, 216, and 268 nm with an absorbance of 0.3049, 0.2445, and 0.848 respectively. In mature pods, the O.D. was recorded at lower values as compared to that of the young pods; however, a unique peak at 216 nm was observed. In senescent pods, peaks were observed at 210 and 270 nm having absorbance of 0.3778 and 0.1289 respectively. In pods obtained from the Jodhpur region, peaks were obtained at 208 nm and 268 nm with an absorbance of 0.2849 and 0.0871 respectively. PC-M showed peaks of 210 and 272 nm with an absorbance of 0.392 and 0.11 respectively, whereas methanolic extracts from PC-F showed absorption maxima at 210 and 268 nm with an absorbance of 0.7847 and 0.2868 respectively. As per the values obtained for the absorbance, it indicates that PC-F showed increased flavonoids/isoflavonoid content as compared to that of the PC-M. This is in coherence with the TLC image in Figure 3.3, where a methanolic extract of PC-F showed a new band of faint mauve colour at a R_f value of 0.714.

Table 3.7 shows the absorption maxima observed in the various samples of *P. cineraria*. (next page)

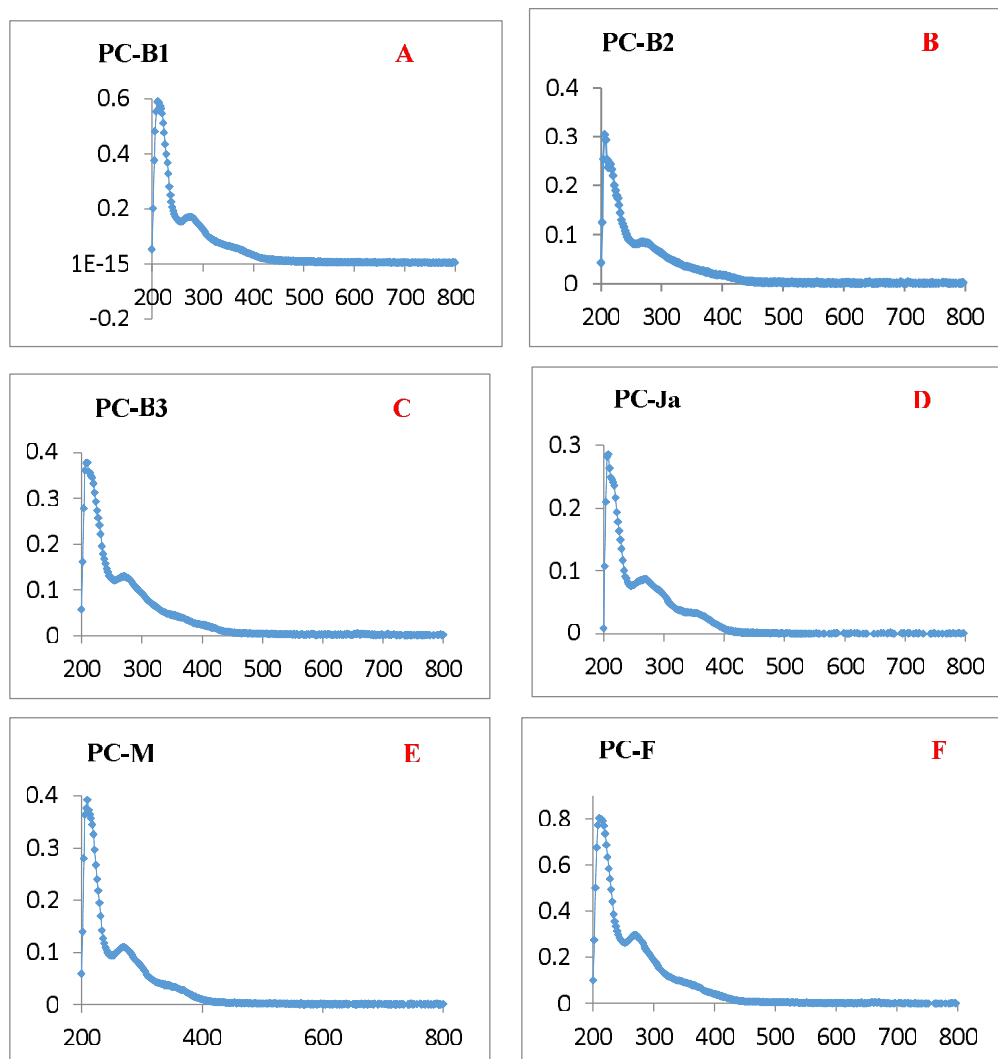


Figure 3.7 A-F represents the UV spectrum of the methanolic extracts of the samples of *P. cineraria* pods collected from selected regions of Rajasthan

Table 3.7 Absorption maxima of the compounds in defatted methanol extracted fractions of *P. cineraria*

S.No.	Region	λ_{\max} (nm)
1.	PC-B1	212, 274
2.	PC-B2	206, 216
3.	PC-B3	210, 270
4.	PC-Jo	208, 268
5.	PC-M	210, 272
6.	PC-F	210, 270

To sum up the previous two sections and as a preamble to the next section, it is important to note here that the fingerprint approach using chromatography has become one of the most effective tools for quality assessment of phytochemicals from different sources of plant and food supplements. The complexity of the chromatographic fingerprint and reproducibility of the chromatographic data under similar experimental conditions offers an ergonomic chemometric approach to deal with multiple plant samples simultaneously. The phytochemical profile of the different plant samples also helps in establishing the taxonomic identities of the flora belonging to related species. Distinguishing the different genotypes and assessing the quantitative and qualitative values of the botanical samples in a rapid manner will of immense advantage in novel drug discovery. In this regard, TLC analysis and UV-Visible spectra methods could be of immeasurable benefit for carrying out the preliminary investigation in the novel samples. There are many reports about different techniques to address the identity and quality of botanicals, which are mainly chromatographic analysis, including HPLC, gas chromatography (GC)¹⁷⁸, ultra-performance liquid chromatography (UPLC), and capillary electrophoresis (CE)¹⁷⁹. However, the acquisition of a fingerprint and quantitative analysis by these methods is a laborious operation as it generally requires expensive instruments and about one or more hours for a single run.¹⁸⁰ The chromatographic fingerprinting profile based on TLC analysis, thus together with the spectroscopic method, can be employed in obtaining the specific botanical profiles and in quantifying the main bioactive compounds present in the samples. Furthermore, differential profiling also indicates that geographical locations can have an impact on natural products and hence screening.

3.3.4 Total flavonoid content in pods/seeds of the *P. cineraria* and *C. tetragonoloba*

In continuation of the region wise phytochemical fingerprint profiling, total flavonoid content analysis was carried out in different defatted methanolic extracts obtained from the pods of *P. cineraria* and *C. tetragonoloba* using aluminium colorimetric assay as described in Section 3.10. The standard curve was plotted using rutin hydrate solution in methanol at a concentration range of 200-1000 $\mu\text{g mL}^{-1}$. Figure 3.8 represents the standard curve plotted using Rutin hydrate. The R^2 was observed as 0.9855 for the given graph. The flavonoid content in the test samples was expressed as mg RH equivalent /g dry weight of the extract.

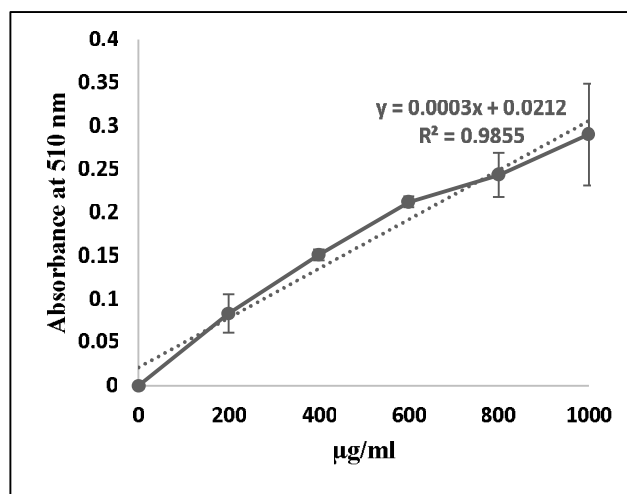


Figure 3.8 Standard curve plotted using Rutin Hydrate for total flavonoid content analysis. $y = 0.0003x + 0.0212$, $R^2 = 0.9855$

Figure 3.9 shows the total flavonoid content in pods of *Prosopis cineraria* collected from selected regions of Rajasthan. From the figure, it is evident that pods collected from Pilani local farms had the highest flavonoid content of 432 mg RH/g of methanolic extract, whereas Bikaner 2 sample, which represents mature pods, showed the lowest flavonoid concentration as 44.44 ± 7.6 mg RH/g of extract. Pods purchased from Pilani market had 400 ± 29.56 mg RH/gm of extract. Interestingly, pods collected from the Bikaner region in young and senescent stages consisted of flavonoid content higher than their mature counterpart. Young pods had 166.66 ± 26.7 mg RH/gm whereas senescent pods showed 288.88 ± 59.8 mg RH/gm of methanolic extract. Pods collected from Jaipur, Jodhpur and markets of Pilani had 152.88 ± 80.95 , 232 ± 40.56 , and 400 ± 29.56 mg RH/gm of methanolic extract of flavonoid respectively. Pods from Pilani local farms and markets consisted of comparable flavonoid contents. The TLC analysis of the same

samples (Figure 3.1) showed results in correlation with the total flavonoid content examination.

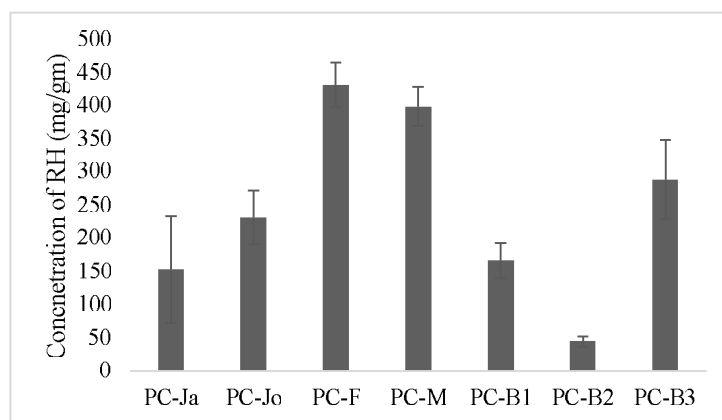


Figure 3.9 A comparison of total flavonoid content (mg RH/g methanolic extract) in different samples of *P. cineraria* collected from selected regions in Rajasthan; data represent mean \pm SD, n = 3.

Similarly, the total flavonoid estimation in the different samples of *C. tetragonoloba* is shown in Figure 3.10. The pods collected from the Pilani region showed the highest flavonoid concentration as 556 ± 47.14 mg RH/g of methanolic extract followed by Jaipur region as 376 ± 14.14 mg RH/g, Mukundgarh region as 363.77 ± 25.01 mg RH/g of defatted methanolic extract. The pods collected from the Sikar region had 328.22 ± 18.19 mg RH/g, and Jhunjhunu region pods showed 260.44 ± 5.19 mg RH/g. The CT-G sample showed the lowest concentration of flavonoids at a value of 129.33 ± 4.714 mg RH/g of methanolic extract. When compared with the TLC images of the different extracts ran for phytochemical profiling, it is again in correlation with Figure 3.5. As per the TLC analysis, the defatted methanolic extract showed the presence of the highest number of bands as compared to the other samples. The TLC band patterns also showed that the samples collected from different regions had comparatively identical band patterns with a few exceptions. This reflects the constitutive presence of the major flavonoids in *C. tetragonoloba*, albeit with a difference in total flavonoid content associated with geographical variations.

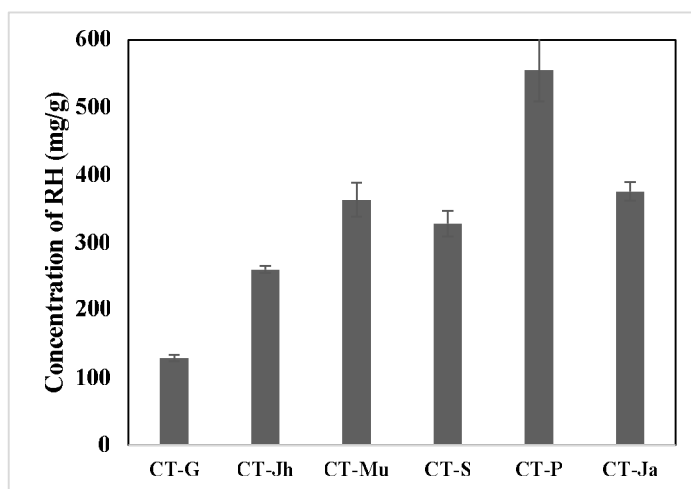


Figure 3.10 A comparison of total flavonoid content (mg RH/g methanolic extract) in pods/seeds samples of *C. tetragonoloba* collected from selected regions in Rajasthan; data represent mean \pm SD, n = 3.

To the best of our knowledge, this is the first report where region wise comparative analysis of the flavonoid concentration in defatted methanolic extracts of the pods of the edible legumes *P. cineraria* and *C. tetragonoloba* found in Rajasthan region has been carried out. The extensive literature survey revealed that the previous phytochemical investigation on these two legumes available was limited either to other (non-edible) aerial parts of the plants or was carried out using a plant sample from a single geographical location. *Prosopis* genus has distribution in semi-arid regions of the other countries as well, such as Iran, Pakistan, and Afghanistan. Thus, examining the secondary metabolite content in species localized to the Indian subcontinent will prove beneficial to the local population. *C. tetragonoloba* is primarily cultivated for the production of resin, guar-gum, which can be used in bath products, hair conditioners, hair dyes, other hair care products and skin care products. However, not much emphasis has been given to the pods obtained from this legume (except as a vegetable dish in Rajasthani cuisine) despite the fact that India is the largest exporter of guar gum in the world. From the TLC analysis and total flavonoid content estimation, it is clear that the environment and geography (even within the same state) have a significant impact on the phytochemical (flavonoid) content in the pods. The identical band pattern in the case of TLC images of *C. tetragonoloba* but a relatively different flavonoid concentration indicates the presence of other flavonoids as well which might have gone undetected in the TLC imaging because of the limitation in the sensitivity of the technique.

Flavonoids are one of the most critical classes of the phenolics present in the plant kingdom. The determination of flavonoid concentration would add value to the

various plant accessions for their use as a functional food vegetable. When compared with the other species of the Leguminosae family, *P. cineraria* and *C. tetragonoloba* showed very high flavonoid content. Even in the bark and other aerial parts, *P. cineraria* shows a relatively less flavonoid content.¹⁸¹ *Gleditsia Triacanthos* L pods, which are native to the Middle East Region, were found to have 25.160 ± 0.016 and 22.023 ± 0.660 mg RH/g of ethyl acetate and chloroform fractions respectively.¹⁸² Ethanolic extracts of leaves of *Tephrosia purpurea* Linn (sarpankh) had 2.54 ± 0.12 % w/w RH in them.¹⁸³ Flavonoid concentration in methanolic extract of *Trifolium pratense* L. (Red Clover) was found to be 26.61 ± 0.92 mg RH/ g of dry extract.¹⁸⁴ *Glycine max* (soybean) is one of an essential member of the Leguminosae family. Extensive research has been done on the phytochemical constituents present in various extracts obtained from *Glycine max*. Ana Josipović et al. (mention year) reported that per gm dry weight of soybean contains 0.433 to 0.659 mg RH depending upon the season.¹⁸⁵ The investigation included 33 genotypes of *Glycine max*, and the maximum flavonoid concentration was 0.659 mg RH/g dry weight. An interesting observation was reported by Dajanta et al. (2013) that cooking and fermentation significantly increase total phenolic and total flavonoid content in yellow and black soybean seeds (probably as a result of conjugates breaking down at high temperature) which additionally supports the idea of using legumes as a highly functional foodstuff.¹⁸⁶ The higher concentration of flavonoids in *P. cineraria* and *C. tetragonoloba* definitely fuel the purpose of establishing these legumes as a functional food with high nutraceutical value at the regional level.

3.3.5 HPLC method development for the characterization of flavonoids/isoflavonoids from *P. cineraria* and *C. tetragonoloba*

HPLC gives an advantage over other analytical techniques viz TLC in terms of sensitivity. The compounds which might be otherwise left unidentified by TLC methods can be easily detected with the help of HPLC. Among the analytical methods for plant extracts fingerprinting, high performance liquid chromatography, hyphenated to UV – visible diode array detector (HPLC-DAD), is still the most popular to be applied in order to ensure product quality and for discriminating related genotypes or adulterated samples. In the present work, separation of the different isoflavonoids was carried out using the mobile phase consisting of absolute methanol and distilled water of pH=4 using orthophosphoric acid under isocratic elution mode. However, the mobile phase,

as well as the isocratic conditions, led to the inadequate separation of the standard mixture. The chromatogram showed the absence of expected peaks corresponding to the number of compounds present in the mixture (about which we already had obtained an idea by performing the TLC based studies, as highlighted in the previous sections). The isocratic method, when utilized for separating the flavonoids/isoflavonoids from defatted methanolic extracts of the experimental plants (i.e., *Prosopis cineraria* and *Cyamopsis tetragonoloba*), did not show proper separation and peaks in the chromatogram. Hence, considering the significant difference in the polarities of the compounds present in the methanolic extracts of *P. cineraria* and *C. tetragonoloba*, low pressure gradient mode of elution was selected for the HPLC chromatographic runs. It was a reverse phase chromatography, where the C18 column was used. The mobile phase consisted of solvent A (Milli Q water, pH=3) and solvent B (Acetonitrile). The low- pressure gradient mode of chromatographic separation facilitates the continuous variation in the polarity of the mobile phase. This ensures the complete elution of the more than one compound present in the samples (when dealing with mixtures) subjected to separation.

Figure 3.11 shows the HPLC chromatogram for the separation of putative isoflavonoids from a mixture of commercially procured standards. (We targeted isoflavonoids, as these are the characteristic flavonoid class present in the legume plant family.) The mixture consisted of equal concentrations of standards daidzin, genistin, daidzein, and genistein. The glycosides daidzin and genistin eluted out at lower retention time as compared to their aglycone counterparts. The retention time for daidzin was 24.47 minutes; for genistin, 25.95 minutes; for daidzein, 33.39 minutes, and for genistein 38.9 minutes. It can also be inferred from Figure 3.11 that peaks corresponding to standards showed a difference in their areas. This is interesting to observe since the equal concentration of the standards was loaded onto the column. This indicates that different compounds exhibit variable sensitivity and absorbance in an HPLC run, which could be of advantage while performing the quantitative estimation of the compounds in the unknown samples. The separation of the peaks at the baseline is one of the crucial factors in establishing the validation of the method. Therefore, the above HPLC method was further used for characterizing the compounds present in the experimental plant extracts. (It is to be noted here that the structural determination of the unknown compounds was done using LC-MS technique, as explained in the subsequent sections. The chromatographic method used during LC-MS was based on the low-pressure

gradient elution method consisting of Acetonitrile and Water (pH=3, using phosphoric acid) and was modified as per the experimental requirements.

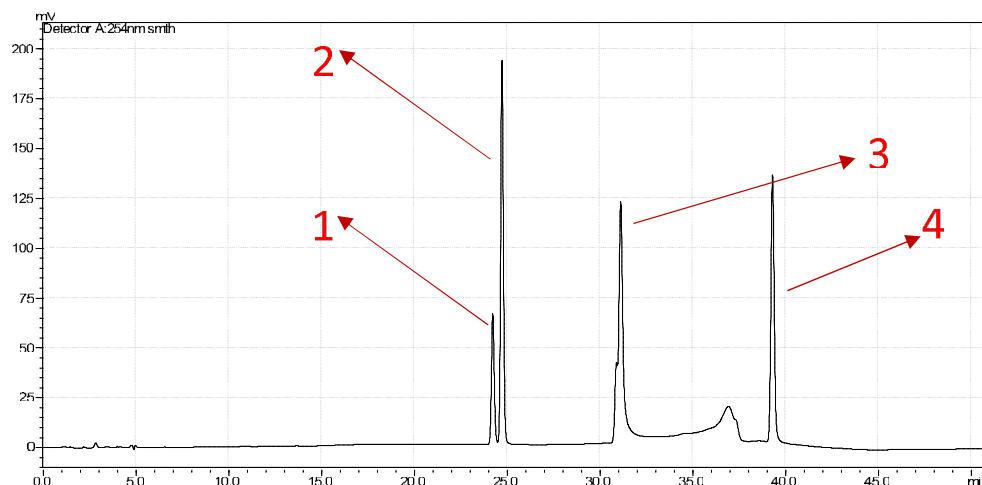


Figure 3.11 HPLC chromatogram of mixture of standards for isoflavonoids. Mobile phase: solvent A (Milli Q water, pH=3) and solvent B (Acetonitrile). Solvent B \rightarrow 35% in 33 minutes ; \rightarrow 10 % in 40 minutes, maintained for 5 minutes. The flow rate was 0.7 ml/minute. Detection at 254 nm. 1: Daidzin, 2: Genistin, 3: Daidzein, and 4: Genistein.

The retention times and the chromatographic pattern for separation of the isoflavonoids in the mixture of standards loaded onto the reverse phase C18 column was also compared with the literature available. Zican Huang and Ming Xia (2019) reported the simultaneous detection of six putative isoflavones and their glycosidic conjugates in soybean yogurt using a low-pressure gradient elution method.¹⁸⁷ Another group developed a method for detection of isoflavonoids in homogenous and heterogenous systems which are generally involved in the formulations present in capsules and beauty products.¹⁸⁸

The selection of the total runtime depends on various factors such as column dimension and sensitivity of the instrument. These parameters define the flow rate selected for a particular run method, which in turn is the limiting factor for the runtime. In the method standardized for the current study, a flow rate of 0.7 mL/min was found to be most optimal.

3.3.6 Purification of flavonoids from the selected plants using column chromatography:

Isolation of pure compounds (as possible active components) from plant sources has always been of immense interest to the researchers because of the possibilities of

isolation of potential novel and better drugs. The recent advances in the separation processes have significantly decreased the difficulties faced during the extraction procedures, but still, there are many species of plants that are entirely unexplored (or relatively less explored) with respect to their secondary metabolite content. One of the many reasons behind this is their geographical (and endemic at times) distribution which limits the probability of local plants being chosen as the experimental material. Purification of natural products is an essential area of research in phytochemistry as it is a prerequisite for detailed insights into structural aspects and biological activities. In the present work, in order to purify flavonoids/isoflavonoids from the resource material, the defatted crude extracts obtained from the pods/seeds of *P. cineraria* and *C. tetragonoloba* were subjected to the column chromatography using the Amberlite XAD7HP matrix. This matrix is specialized in retaining the phenolics/flavonoids, which can be eluted out using the polar solvent methanol. The usage of the Amberlite XAD 7HP matrix has an advantage over other conventional separation techniques like liquid-liquid chromatography, which offers relatively less degree of purification of the defatted extract. The usage of the Amberlite extraction aided recovery values of 80–90% for flavonoids, allowing a more effective elimination of sugars, acids, pigments, and other interfering compounds. The application of Amberlite resin was first applied to isolate the flavonoids from honey samples by Ferreres et al. in 1991.¹⁶⁹ This technique allowed them to identify 16 flavonoids from the honey samples, some of them include quercetin, kaempferol, 8-methoxykaempferol, quercetin 3-methyl ether, isorhaninetin, kaempferol 3-methyl ether, galangin, luteolin, and apigenin. Since then, it has been adapted as the most widely used method for polyphenol extraction from samples obtained from different sources. Albeit, modifications may be incorporated in the methodology as per the requirement of the experiments.

Figures 3.12 and 3.13 show the TLC analysis of the purification profile of the extracts obtained at different steps from the edible portions of *P. cineraria* and *C. tetragonoloba*. Since the solvent system containing Toluene: Ethyl Acetate: Acetone: Formic Acid:: 20:4:2:1 didn't show any migration of the compounds of the extract on the silica plate, the purification profile was attempted in a polar solvent system which consists of n-Butanol: Acetic Acid: Water::7:2:2 ratio (The optimization aspect has already been discussed in a previous section). It can be observed from Figure 3.8 where purification profile of *P. cineraria* indicates that when the equal concentration of all the

samples was loaded on to the plate, the defatted methanolic crude fraction (CE) and the unbound fraction (UB; unbound meaning the fractions that eluted out of the column after the phenolics/flavonoids had adsorbed onto the column matrix) showed significantly less intense bands as compared to that of the flavonoid enriched fraction (FEF). The illumination under 254 nm exhibited clear lanes for the CE and UB fractions, whereas FEF fractions showed the presence of multiple dark bands against the light background. The flavonoids being UV active compounds can be detected in high quantity in the FEF fraction eluted post-Amberlite column run. The same TLC, when illuminated at 365 nm, revealed that the bands observed in FEF fractions are of flavonoids nature since the characteristic property of the flavonoids compounds is to get illuminated as yellow, orange, or bluish colors under 365 nm. Referencing from Table 3.3 indicates the presence of flavones, chalcones glycosides, and deoxyidoflavones in the purified FEF. Hence the isolation of the flavonoids enriched fraction was obtained through 2 steps purification procedure.

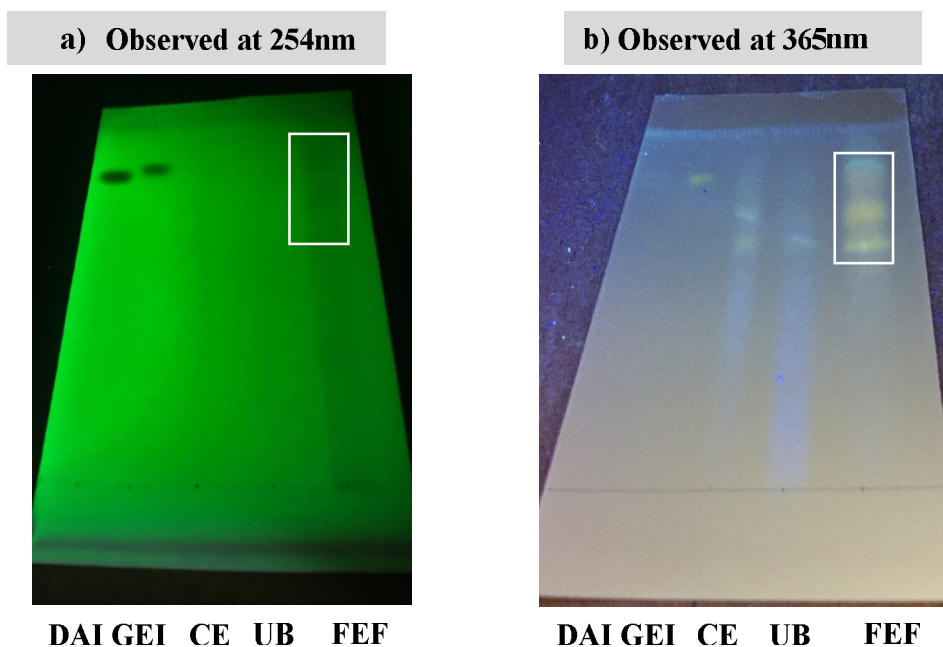


Figure 3. 12 TLC analysis of flavonoids purification from pods/seeds of *P. cineraria*. Solvent system used is as follows: n Butanol: Acetic Acid: Water:: 7:2:2. 2.5 μg of standards and 25 μg the purified samples loaded. DAI- Daidzein, GEI-Genistein, CE- Crude extract, UB- unbound, FEF- eluted sample in 100% Methanol

Similarly, when extracts from different purification steps obtained for *C. tetragonoloba* were analyzed using TLC, illumination at 254 nm showed that the band patterns of CE and UB fractions were identical to each other, and in the lane, for FEF fraction, novel bands at R_f value 0.58 and 0.67, different from the previous ones were

observed(Figure 3.13). This depicts the usage of the Amberlite XAD7HP matrix has resulted in partial purification of the defatted methanolic crude extract and fractions enriched in flavonoids/isoflavonoids were obtained.

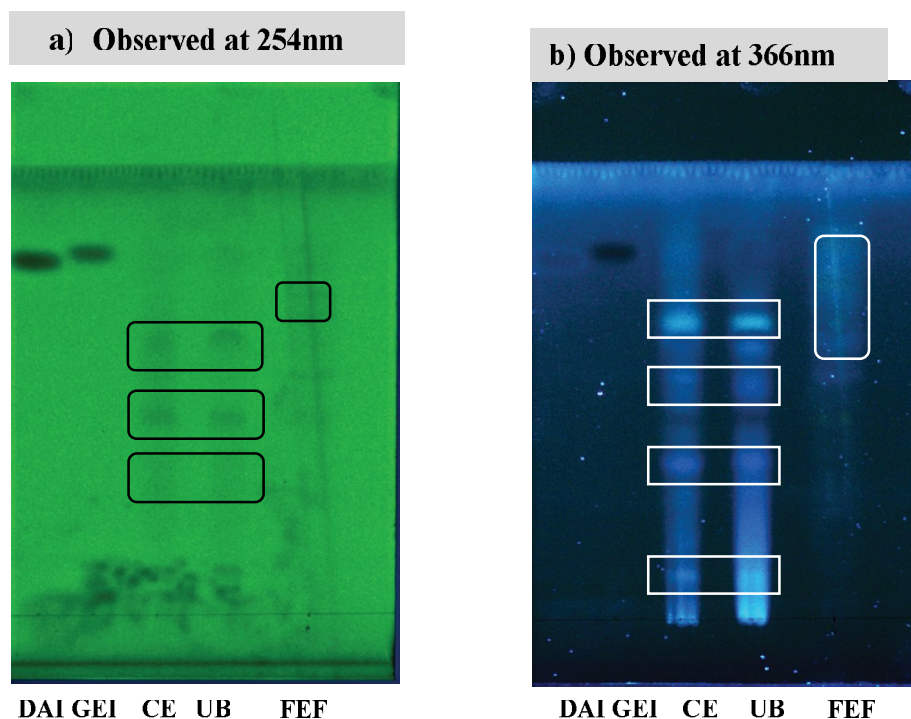


Figure 3. 13 TLC analysis of flavonoids purification from pods/seeds of *C. tetragonoloba*, Solvent system used is as follows: n Butanol: Acetic Acid: Water:: 7:2:2. 2.5 µg of standards and 25 µg of the purified samples loaded. DAI- Daidzein, GEI-Genistein, CE- Crude extract ,UB- Unbound, FEF- eluted sample in 100% Methanol

Flavonoids/isoflavonoids in their conjugated forms are highly water-soluble. This has been proved beneficial for the plants where they can be stored in the plant vacuoles at higher concentrations because of the increased polarity. However, when the flavonoids/isoflavonoids have to be separated from the crude extracts which contain other water-soluble compounds as well, it becomes difficult to employ an economical and less tedious separation procedure. Here, Amberlite XAD- 7HP offers an exceptional ability to distinguish between the adsorption of the flavonoids/isoflavonoids on its matrix while at the same time letting the other water-soluble compounds such as sugars, proteins, and other polar metabolites to go as an unbound fraction. A comparative analysis of using different Amberlite matrices such as XAD-2HP, XAD-4HP, XAD-16HP , XAD-7HP, and XAD-8HP for purifying flavonoids showed that XAD-7HP offers a higher recovery rate of flavonoids and

their conjugates.¹⁸⁹ For further purification of the flavonoids/isoflavonoids, fractions obtained post -Amberlite XAD7HP column was used.

The flavonoid-enriched fractions obtained post Amberlite column run were subjected to Sephadex LH-20 column chromatography. The purification profile of different fractions obtained was analysed using spectrophotometric readings at 254 nm, and TLC profiling using solvent system Toluene: Acetic Acid:Acetone:Formic Acid::20:4:2:1 as mobile phase. Figure 3.14 shows the TLC analysis of the different fractions collected after elution from the *P. cineraria* and *C. tetragonoloba*, respectively.

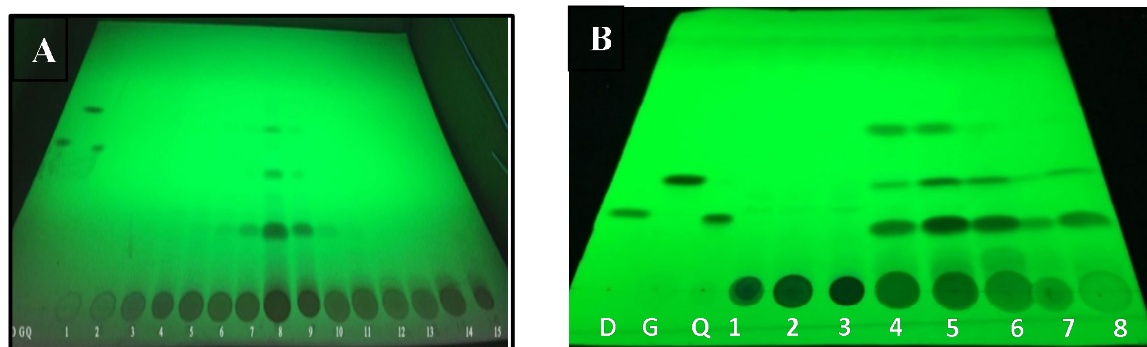


Figure 3.14 TLC run for post Sephadex LH-20 fractions obtained from A) *P. cineraria* and B) *C. tetragonoloba*. D, G, Q represents Daidzein, Genistein and Quercetin. Images represent illumination at 254 nm.

As observed from the figures above, the eluted fraction no. 8 for *P. cineraria* (P8) and eluted fraction no. 5 for *C. tetragonoloba* (C5) showed the presence of a maximum number of bands as observed under illumination at 254 nm. The intense bands seen at 254 nm indicates that the compounds are UV active which is an inherent property of the compounds belonging to flavonoids/isoflavonoids. The fractions thus eluted out contained 5-6 putative compounds, which were further identified using LC-MS analysis. Another conclusion that can be inferred from the TLC images is that despite the pattern of the bands detected, some compounds still remained localized to the origin and were not able to migrate in the given mobile phase. Thus, it indicates that the compounds present in the eluted fractions varied in their polarities and were of aglycones as well as glycosidic nature. Sephadex LH-20 has been used previously for isolating flavonoids from other plant sources as well. However, the purification step including Sephadex LH-20 was preceded

by other purification steps. For e.g., Lin *et al.* (2016) isolated kaempferol, quercetin, kaempferol-3-O-d-glucopyranoside, and rutin from *Pteris vittata* L. using silica gel chromatography and Sephadex LH-20 matrix.¹⁹⁰ *Olea europaea* L. commonly called olives are a crucial part of the Mediterranean diet. Quercetin, apigenin, luteolin, and rutin were isolated from olives using Sephadex LH-20 and preparative paper chromatography.¹⁹¹

These flavonoid-enriched fractions, which have been obtained from the edible portions of both *P. cineraria* and *C. tetragonoloba* using sequential chromatography on Amberlite XAD7 and Sephadex LH-20, contain a unique mix of flavonoids (final validation done by LC-MS, discussed in the next section) and can serve as crucial sources of individual flavonoids or themselves can be used for establishing biological activity (assuming synergism). It has also been simultaneously shown how TLC can be used to track the flavonoids during purification.

3.3.7. LC-MS analysis of the fractions obtained post Sephadex LH-20 from *P. cineraria* and *C. tetragonoloba*

3.3.7.1 LC-MS analysis of enriched fraction P8 obtained from *P. cineraria*

The chemical composition of the purified and flavonoid-enriched fractions obtained from Sephadex LH 20 was also analysed using the HPLC-MS-UV technique. LC-MS analysis of the P8 fraction was carried out by slightly modifying the mobile phase and running conditions. Figure 3.15 represents the chromatogram obtained under low pressure gradient flow method, and it depicts the peaks obtained at 254nm. The significant peaks were then subjected to ESI-MS full scan mode analyses in order to identify the protonated ions. A constant collision energy value was applied to each constituent in order to obtain mass spectra with various fragmentation patterns so as to retain as much structural information as it was possible. The different m/z values were analysed using the RIKEN ReSPect database which is extensively designed to study the mass spectra of the plant based secondary metabolites.

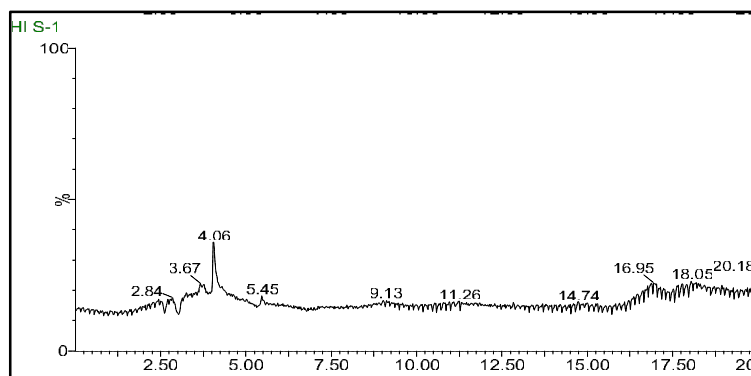


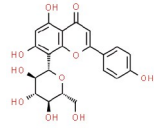
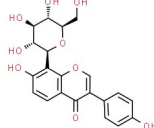
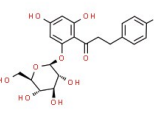
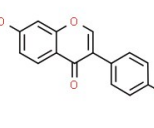
Figure 3.15 LC-MS chromatogram of purified fraction (P8) obtained after Sephadex LH-20 run for *P. cineraria*. Column used: 150×3.9 mm I.D. 5 mm C18 RP column; elution done with a 20 min gradient of 15-35% acetonitrile in water adjusted to pH 3 with PFA. Flow-rate: 0.7 mL/min.

Table 3.8 shows the most frequent ions which characterize the fragmentation of the compounds present in the fraction eluted out during Sephadex LH20 column chromatography. As a technique, ESI-MS provides sufficient information regarding the aglycone structure and the glycan sequence. The protonated flavonol glycosides generate the protonated ions upon fragmentation whose pattern depends on the type of the glycosyl bonds present in the parent flavonoids. The neutral losses of the sugar units prompt in identifying the glycosides present in the extracts. The literature shows that a difference of 162 amu indicates a hexose, 146 amu denotes a desoxyhexose, supposedly rhamnose, and 132 amu points to a pentose, presumably xylose moiety. The relative abundance of the fragmented ion generated, as compared to that of the parent ion, indicates the type of the glycosidic linkage present between the aglycone moiety and the conjugated sugar. There are reports indicating that relative abundance of the aglycone ion deriving from a homolytic and heterolytic cleavage, respectively, is correlated with the position of the glycosylation of the flavonoids/isoflavonoids conjugates.¹⁹² An in-depth analysis of the fragmentation pattern followed by comparison with the reported data for mass spectrometry analysis of flavonoids/isoflavonoids helped to gain an insight into the type of compounds present in the enriched fraction of the plant extracts.

However, recently a few studies have been published where the novel isoflavonoids have been reported from species belonging to other families as well, which includes Amaranthaceae^{193, 194}, Rosaceae¹⁹⁵, and Poaceae.¹⁹⁶ The inverse association of consumption of an isoflavonoid-enriched diet with the risk of breast cancer has gathered significant attention in identifying the edible plants where the

presence of these compounds could be elucidated. A relatively less abundance (as observed from the MS count ion) of Vitexin, which is a flavone, was detected in the extract purified from *P. cineraria* pods. The neutral ion losses from the different compounds indicated that the phytochemical constituents in the pods were present in glycosylated as were their aglycone form. This is coherent with the results observed in TLC analysis where mobile phases of different polarities were used to depict the separation of compounds on stationary phase, since the sugar conjugates of the flavonoids tend to show increased affinity towards the polar silica.

Table 3.8 The [M+H]⁺ molecules of the flavonoid/isoflavonoids detected in the *P. cineraria* pods, their retention time, and structures. All the structures are derived from the ChemSpider database.

S.No.	Retention time (min)	[Typical MS Ions] (m/z)	Formula	Identification	Structure	Category
1.	4.06	323.98, 297.01	C ₂₁ H ₂₀ O ₁₀	Vitexin (Apigenin-8-C-glycoside)		Flavone
2.		350.952, 297.01, 281.01	C ₂₁ H ₂₀ O ₉	Puerarin (daidzein-8-C-glycoside)		Isoflavonoid
3.	5.453	437.988	C ₂₁ H ₂₄ O ₁₀	Phloridzin (phloretin-2'-O-glucoside)		Bicyclic flavonoids
4.	16.95	199.06, 151.07, 126.01	C ₁₅ H ₁₀ O ₄	Daidzein		Isoflavonoids

The TLC analysis of the fractions eluted out showed the presence of the multiple bands which although didn't match with the standards loaded simultaneously but were designated as flavonoids/isoflavonoids on the basis of the absorption spectra and illumination under 254nm. Being a relatively sensitive technique, Mass Spectrometric analysis further enriched the understanding of the presence of the nature of flavonoids/isoflavonoids. The fragmentation carried out at

positive ionization depicts the tentative occurrence of flavonoids/isoflavonoids in their aglycone as well as glycoside conjugated forms.

MS data indicated the identities of the flavonoids/isoflavonoids present in the *P. cineraria* pods (Table 3.8). Major peak 1 (with R_t of 4.06 minutes) showed fragments with m/z values of 323.98, 297.01 corresponding to flavonoid Vitexin, and 350.95, 297.01, and 281.01 corresponding to C-glycosylated isoflavonoid Puerarin. The second major peak (with R_t of 5.453 minutes) showed the presence of Phloridzin, an O-glucoside of phloretin, which belongs to a family of bicyclic flavonoid, dihydrochalcone. m/z was observed as 437.988, which represents the $[M+2H]^+$ since the molecular weight of Phloridzin is 436.13. The third major peak at 16.95 minutes elucidated the fragments of m/z corresponding to 199.06, 151.07, and 126.01 which are characteristic of Daidzein, an isoflavonoid aglycone. These three ions are generated by a parental ion with m/z of 254.25.

This is the first time where LC-MS/MS based techniques were employed to study in-depth the phytochemical constituents (flavonoids in particular) of the pods of *P. cineraria*. The obtained chromatogram was in according to the hydrophobicity of the compounds eventually detected by MS analysis. Firstly, flavanols glycosides, followed by flavanones glycosides and lastly, isoflavones reached the detector, which was expected in the case of gradient elution during liquid chromatography. The analysis showed that the methanolic extract contains a putative isoflavonoid daidzein. The daidzein was present in its aglycone form. Isoflavonoid is the particular category of flavonoids which are the characteristic feature of Leguminosae family. The flavonoids/isoflavonoids reported from other members of this family were thoroughly discussed in table 1.4.

3.3.7.2 LC-MS analysis of the enriched fraction C5 obtained from *C. tetragonoloba*

Similarly, the C5 fraction (the *C. tetragonoloba* fraction selected on account of showing maximum number of flavonoids on TLC analysis) obtained from the *C. tetragonoloba* purified extract, the fractions eluted out from Sephadex LH-20 column chromatography, were subjected to LC-MS analysis. The results obtained from the LC-MS analysis of the flavonoid enriched fraction of the same are presented in Table 3.9. Similar usage of software and databases as mentioned previously, were

employed while deducing the tentative presence of flavonoids/isoflavonoids and their fragmentation pattern. The low-pressure gradient flow method was chosen to separate the flavonoids in liquid chromatography. The significant peaks eluted out had the retention times of 3.98 min, 18.0 min, and 19.65 min respectively (Figure 3.16). These peaks were then subjected to ESI-MS full scan mode analyses in order to identify the protonated ions.

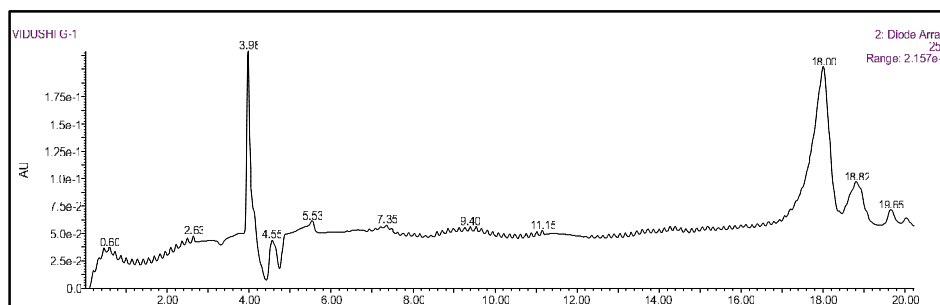
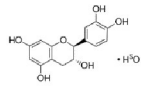
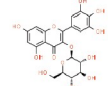
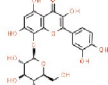
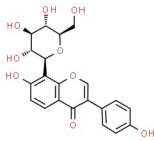


Figure 3.16 LC-MS chromatogram of purified fraction (C5) obtained after Sephadex LH-20 run from *C. tetragonoloba*. Column used: 150×3.9 mm I.D. 5 mm C18 RP column; elution done with a 20 min gradient of 15-35% acetonitrile in water adjusted to pH 3 with PFA. Flow-rate: 0.7 ml/min. Absorbance was recorded at 254 nm

Table 3.9 shows the MS data for establishing the identities of the flavonoids/isoflavonoids present in the purified fractions of the *C. tetragonoloba* pods. Major peak 1 eluted out at 3.98 minutes showed fragments with m/z of values fragment of m/z 305.1310 indicating the presence of dihydroflavonol catechin hydrate. The second major peak, which was eluted out at 18.00 minutes showed the fragment with m/z observed at 481.472 indicated the presence of flavonols Myricetin-3-Galactoside and Gossypetin-8-glucoside. The third major peak at 19.65 minutes indicated the presence of Puerarin which is daidzein 8-C-glucoside belonging to isoflavonoid class and was identified via the m/z fragments of 268.0568 and 297.0857.

Table 3. 9 The [M+H]⁺ molecules of the flavonoids/isoflavonoids detected in the purified extract of *C. tetragonoloba* pods, their retention time and structures. All the structures are derived from ChemSpider database.

S.No.	Retention time (min)	[Typical MS/MS Ion]	Formula	Identification	Structure	Category
1.	3.98	305.1310	C ₁₅ H ₁₂ O ₇	Catechin hydrate		Dihydroflavonol
2.	18.00	481.472	C ₂₁ H ₂₀ O ₁₃	Myricetin-3-Galactoside		Flavonol
3.		481.472	C ₂₁ H ₂₀ O ₁₃	Gossypetin-8-glucoside		Flavonol
4.	19.65	268.0568 297.0857	C ₂₁ H ₂₀ O ₉	Puerarin (daidzein-8-C-glucoside)		Isoflavonoid

Like in the case of the *P. cineraria*, a novel attempt in purifying flavonoids and carrying out detailed phytochemical analysis of *C. tetragonoloba* using LC-MS technique was made. Here also, the chromatogram obtained was according to the polarity of the compounds eventually detected by MS analysis. The analysis showed that the methanolic extract contains only one isoflavonoid viz. puerarin, which was present in its glycosylated form. A relatively less abundance, as observed from the MS count ion, of Vitexin, which is a flavone, was also detected in the extract. The neutral ion losses from the different compounds indicated that the phytochemical constituents in the pods were present in glycosylated as were their aglycone form. Kobeasy *et al.*(2011) investigated the major flavonoids present in the *C. tetragonoloba* collected from the Egypt region and showed the presence of luteolin and quercetin in aqueous extracts of seeds.¹⁹⁷ Additionally, Wang and Morris (2007) explored the potential of *C. tetragonoloba* L. beans as a good source of kaempferol and quercetin in different cultivars grown in Georgia, USA under laboratory conditions.¹⁹⁸

3.4 Conclusions:

Concluding this chapter, it is noted that the phytochemical profiling of the indigenous plants which have not been explored opens up the possibilities in discovering the novel compounds that can be used as different health promoting agents. Previous studies carried out on *P. cineraria* and *C. tetragonoloba* involved the phytochemical characterization mainly from the crude extracts where organic solvents used in extract preparation included polar solvents viz. Methanol, Acetonitrile or Ethyl acetate. The major drawback while working with crude preparations is that the compounds present in minute quantities may go undetected during qualitative or quantitative estimation. Also, inhibitors of biological activity are often present in crude extracts, which get removed upon purification. Moreover, the lack of detailed studies pertaining to the metabolism of the specific secondary metabolites such as flavonoids/isoflavonoids was addressed in the current study (the metabolic studies are dealt with in a later chapter). The flavonoids were often seen to be primarily present as glycosidic conjugates with glucose being the most abundant sugar in the conjugated form. As discussed in Section 1.4.2, the glycosidic conjugates are beneficial for plants since they facilitate the ease of storage inside plant vacuoles, release of the stored flavonoids/isoflavonoids from their glycosides (rather than de novo synthesis) during stress and releasing sugars as a source of energy.

TLC and UV-based analytical methods offered a time efficient protocol to establish the phytochemical fingerprinting of the plant samples collected from selected regions of Rajasthan. It was observed that qualitative variation with respect to the presence of flavonoid/isoflavonoids was present in defatted methanolic extracts. This technique, hence, could be used for a simultaneous preliminary detection of the compounds from multiple samples in an economic and time-friendly manner. Moreover, different stages of the pods tend to have different expression of flavonoid/isoflavonoid which was observed for the pods collected from Bikaner region. Total flavonoid content analysis depicted that despite the fact that samples were collected from semi-arid region only, quantitatively the flavonoid content was different among them. Since, the structural elucidation and identification of the flavonoid/isoflavonoid is preceded by their purification, a two steps purification protocol was established for the pods/seed of *P. cineraria* and *C. tetragonoloba*.

Amberlite XAD-7HP was found to be an efficient matrix for separating the flavonoid and their conjugates from other polar biochemical compounds present in the pods/seeds.

Through the subsequent LC-MS analysis, Vitexin, Puerarin, Phloridzin, and Daidzein were found to be significant flavonoids/isoflavonoids present in conjugated form in the pods of *P. cineraria*. Similarly, *C. tetragonoloba* pods showed the presence of glycosides such as Catechin hydrate, Myricetin-3-Galactoside, Gossypetin-8-glucoside, and Puerarin (daidzein-8-C-glucoside). Since the functional role of these compounds towards the plant system significantly depends on the aglycones (released from their respective conjugates), specific glucosidases play a crucial role in metabolizing them. Hence, it is vital to characterize the glucosidases involved in the metabolism of the conjugated metabolites within the plant system. In light of this, Chapter 4 focuses on the purification and biochemical characterization of the specific glucosidase(s) present in the pods of *P. cineraria* and *C. tetragonoloba*.



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CHAPTER 4: Purification, and biochemical characterization of flavonoid/isoflavonoid conjugates hydrolyzing β -glucosidase from *Prosopis cineraria* pods and *Cyamopsis tetragonoloba* seeds

***This chapter has been published in parts in following manuscripts**

1. Purification and characterization of an isoflavones conjugate hydrolyzing β -glucosidase (ICHG) from *Cyamopsis tetragonoloba* (guar), V Asati, PK Sharma - Biochemistry and Biophysics Reports, 2019.
2. Identification of an isoflavone glycoside hydrolyzing β -glucosidase in the seeds of the desert legume *Prosopis cineraria*. V Asati, PK Sharma - Legume Research: An International Journal, 2018.
3. Using thin-layer chromatography for the metabolic characterization of an isoflavone glycoside-specific β -glucosidase in *Cyamopsis tetragonoloba*. V Asati, PK Sharma - JPC-Journal of Planar Chromatography-Modern TLC, 2018.

4.1 Introduction

Flavonoids and isoflavonoids represent one of the largest and most studied classes of plant-based secondary metabolites with an estimate of more than 10,000 different compounds. These compounds, although they don't directly participate in the essential life processes, such as growth, respiration, storage, and reproduction, significantly help plants in adapting to abiotic and biotic stresses.^{199 3} Flavonoids are reported to be ubiquitously present in all plants, whereas isoflavonoids are more specifically present in large quantities in legumes. Recent interest in these plant-based metabolites has actually kindled because of the presence of many health-promoting properties such as antioxidant, anticancerous, antibacterial, antifungal, etc. as discussed in chapter 1. The functional attributes of these compounds lie in their structural properties. The core backbone consists of two phenyl groups joined by a 3-carbon bridge and then further substitution at different positions of the overall fused rings. The hydroxyl groups present at the different substitution positions of the phenyl backbone also affect the activity of the enzymes, which are associated with multiple pathways involved in conferring protection to plants as well as to human beings. However, it is essential to understand that these compounds many times exist as two different forms; the one glycosylated, which is water-soluble, relatively inactive, and preferred by plants for the storage purpose; another one (aglycone) is less soluble in water and the active form. Different organelles of the plant cell are involved in the compartmentalization of the secondary metabolites where their precursors for the active form can be stored via certain chemical modifications. These chemical modifications include glycosylation, prenylation, methylation, acetylation, etc. of the compounds, which make the latter more soluble so that they are easily stored and are relatively less toxic and inactive in nature.

These different forms of the secondary metabolites play a crucial role in the biological activities they carry out eventually, either within the plants themselves or inside the human system once consumed via food. Because of the biological activities flavonoids/isoflavonoids hold, it is exceptionally crucial for plants that the spatial arrangement of the metabolites within their cells exists in such a way that these compounds should not hinder normal physiological processes of the cells. The absence of such an arrangement will lead to the suppression of the normal cellular processes and thus inhibiting the growth of the cell.⁶⁶

Glycosylation is one of the critical reactions by which a large number of secondary metabolites get modified to be eventually stored in plant compartments (such as vacuoles), which later on can be acted upon by specific hydrolytic enzymes to release their active forms. This modification is carried out mainly by the enzymes having transglycosylation activity so as to attach the sugar moieties to the aglycone molecules and thus making them suitable for storage purposes.²⁰⁰ When the plant cells undergo stress, highly specific β -glucosidases cleave the isoflavonoid/flavonoid glycosides conjugate, and therefore the active aglycones are released along with sugars. The aglycones then act as antioxidant, antibacterial, or antifungal compounds, whereas sugars are generally used for energy purposes.

Although scattered reports do occur in literature vis-à-vis the natural product categories (flavonoids, terpenoids, alkaloids) in different parts of *Cyamopsis tetragonoloba* and *Prosopis cineraria*, studies pertaining to specific active metabolites and their in planta biotransformation are few.²⁰¹ The specificity of the enzymes with respect to the substrates present at the biochemical level within the plant environment makes them interesting biomolecules to be isolated and further explored for their commercial applications such as ethanol and biofuel production, food and beverage industry, and cosmetic industry. The current chapter focuses on identification, partial/complete purification, and characterization of isoflavonoid conjugates hydrolyzing β -glucosidase from seeds/pods of *Prosopis cineraria* and *Cyamopsis tetragonoloba*.

4.2 Materials and Methods

4.2.1 Chemical and Reagents

Plant material: The dried seeds /pods of *Cyamopsis tetragonoloba* and *Prosopis cineraria* were purchased from local vendors in Pilani belonging to Jhunjhunu district of Rajasthan. The same was verified by Prof. Jitendra Panwar (Botanist), Department of Biological Sciences, BITS, Pilani – Pilani Campus. The acquired material was stored at room temperature until further use.

Chemical and Reagents: All the reagents used in enzyme purification were procured from HiMedia Laboratories except the matrices for column chromatography viz Sephadex G-25, Sephadex G-75 and DEAE-Sepharose which were obtained from

Sigma-Aldrich (St. Louis, MO, USA). Flavonoid and isoflavonoid standards daidzein, daidzin, genistin, genistein, hesperidin, hesperetin, naringin, naringenin, rutin hydrate and quercetin used were also obtained from Sigma-Aldrich, while the organic solvents hexane, toluene, chloroform, ethyl acetate, methanol, acetone, formic acid and glacial acetic acid (AR grade), from HiMedia. The TLC plates (precoated with aluminum-backed silica gel 60 F254) were procured from Merck (Darmstadt, Germany).

The preliminary studies for identifying the presence of the enzyme beta-glucosidase was done using the crude preparation from the seeds/pods of both the plants. The complete purification of the ICHG (Isoflavonoid conjugates hydrolysing β -glucosidase) was carried out from the seeds/pods of the *C. tetragonoloba* and *P. cineraria*. For both the procedures, the initial steps involved are similar in their principles. However, a difference in the quantity of the starting materials of the plant samples is evident. For the ease of the explanation, the common steps have been accounted in the same subsections together, whereas the specific steps employed for the partial and complete purification of enzyme, respectively, have been explained under separated subheadings. Unless mentioned otherwise, all the steps of purification were performed at 4 °C.

4.2.2 Crude extract preparation

Powder of dried seeds of *P. cineraria* (30 gm) and *C. tetragonoloba* (200 gm) were extracted with approximately four volumes of chilled 0.1M sodium phosphate buffer (pH 6.0) in a beaker with continuous stirring for 30 minutes. Crude preparation was collected after centrifugation at 10000 rpm for 30 minutes at 4°C. 1% w/v of PVPP (polyvinylpyrrolidone) was added to this crude extract and kept for stirring for 3 h. The role of PVPP is to remove phenolic compounds present in the seeds so as to decrease interference with the enzyme activity. The extract was centrifuged again for 10 minutes at 10,000 rpm. The supernatant was then collected and further subjected to ammonium sulfate precipitation.

4.2.3 Ammonium sulfate precipitation

The crude extract was slowly brought to 30% ammonium sulfate saturation and was stirred for 3 h. The suspension was centrifuged at 10,000 rpm for 30 minutes. The supernatant was collected and then further brought down to 80% ammonium sulfate saturation. Pellet was collected after centrifugation at 10,000 rpm for 30 minutes. The

pellet obtained was dissolved in a minimum amount of extraction buffer containing 0.02 M sodium phosphate buffer (pH 6) and then subjected to further purification steps.

4.2.4 Size exclusion chromatography for PC-ICHG (*P. cineraria* ICHG)

3 mL of the dissolved suspension obtained from the previous steps for both the plant samples were taken and subjected to the Sephadex G-25 column chromatography separately. A column of dimensions 1 cm x 28 cm was packed using the elution buffer, which is 0.02 M Sodium Phosphate buffer (pH 6) and equilibrated with three column volumes. The enzyme preparation was loaded onto the column, and fractions of 2 mL each were collected at a flow rate of 2 mL/ min upon elution. The protein-rich fractions as verified by OD₂₈₀ obtained from UV-Visible spectrophotometer (JASCO-V630), were pooled together. This step resulted in the partially purified enzyme preparation which was further used for enzymatic assays.

4.2.5. Size exclusion chromatography for CT-ICHG (*C. tetragonoloba* ICHG)

For the complete purification of the enzyme, which was continued only for the *Cyamopsis tetragonoloba* plant sample, the suspension obtained at the end of the step 6.2.3 was loaded on to Sephadex G-75 column (1.8 cm x 50 cm) pre-equilibrated with 0.02 M sodium phosphate buffer (pH 6). Post sample loading, 2 mL fractions were collected at a flow rate of 1 mL/min. O.D at 280 nm was measured, followed by checking for beta-glucosidase enzyme activity as described in section 4.2.7.1. Fractions with maximum enzyme activity were pooled for further purification.

4.2.6 Ion exchange chromatography

The pooled enzyme fraction from 6.2.5 was loaded onto a DEAE-sepharose column (1.8 x 10 cm) previously equilibrated with 0.02 M sodium phosphate buffer (pH 6). The column was eluted with a linear gradient of NaCl solution from 0 to 1.0 M at a flow rate of 0.5 mL/minute. Fractions of 2 mL each were collected and checked for absorbance at 280 nm, followed by beta-glucosidase enzyme activity. Protein estimation at each step involved in complete purification of the enzyme from *Cyamopsis tetragonoloba* was performed using the Lowry method.²⁰²

4.2.7 Enzyme assay

The β -glucosidase activity was routinely checked at each step of purification using the synthetic substrate para nitrophenyl - β -D- glucopyranoside (PNPG). A reaction mixture of 200 μ l was set up consisting of 80 μ l of assay buffer (0.1 M Citrate phosphate buffer; pH 4.5), 50 μ l 10 mM PNPG, 50 μ l distilled water and 20 μ l enzyme preparation and was incubated at 37°C for 30 minutes. The reaction was terminated by adding 800 μ l of 1 M Na_2CO_3 . The absorbance was measured at 405 nm using a UV-Visible spectrophotometer (JASCO-V630). The enzyme activity is represented in IU (International Units).

4.2.8 Biochemical characterization of ICHG

Biochemical characterization studies of the enzyme were performed using PNPG with respect to pH optima, temperature optima, metal ion dependence, and enzyme kinetics (for completely purified enzyme only). All the assays were performed in accordance with the protocol mentioned in the above section, with necessary changes incorporated as per the experimental requirements. For determining the pH optima for enzyme activity, the assay buffer was prepared by varying pH from 3.5 to 10 {pH range of 3.5 to 6.5 (Citrate Phosphate Buffer, 0.1M); pH 7 to 8 (Tris-HCl buffer,0.1M) and pH 9-10 (Carbonate-Bicarbonate buffer, 0.1M)}. The data points were plotted to yield the pH profile for the activity. The temperature optimum of ICHG was determined by incubating the enzyme-substrate mixtures over a temperature range of 28°C to 70°C for 30 minutes and terminating the reaction by adding 1 M Na_2CO_3 . The effects of selected cationic salts on enzyme activity were determined by adding the following metal ion salts at a concentration of 5 mM final concentration: HgCl_2 , CoCl_2 , NaCl , ZnSO_4 , MnSO_4 , MgCl_2 , CaCl_2 , and CuSO_4 . Each assay buffer was prepared with the addition of these ions, and the enzyme-substrate mix was incubated for 30 minutes. For enzyme kinetics studies, PNPG was used at different substrate concentrations in the range of 0.1 mM to 5 mM were taken, and V_{max} and K_m were calculated using the Lineweaver-Burk plot.

4.2.9 Molecular weight determination using Gel filtration chromatography

The molecular weight of the purified enzyme of *Cyamopsis tetragonoloba* was determined by size exclusion chromatography using a Sephadex G-75 column with dimensions of 1.8 x 50 cm. Elution was performed using 0.02 M sodium phosphate buffer (pH 6). The standard proteins of molecular weights ranging from 66kDa to 124 kDa were used. The graph was

plotted between \log_{10} of molecular weight and the elution volume, and the molecular weight of the enzyme was determined using this straight-line graph.

4.2.10 SDS-PAGE

Proteins obtained at each step of purification were subjected to SDS-PAGE analysis as per standard procedure with adequate modifications. Samples were denatured in 2X sample buffer containing 60 mM Tris (pH 6.8), 25% glycerol, 2 % SDS, 14.4 mM 2-mercaptoethanol, 0.1 % bromophenol blue and boiled for 5 min. Electrophoresis was performed on a Mini Protean® Tetra-Cell instrument (BioRad Laboratories, USA) at a constant voltage of 80 kV. After electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250 stain and was observed in a BioRad Gel Doc™ XR imaging system. The molecular mass of the protein bands was determined by interpolation from a semi-logarithmic plot of relative molecular weight versus the R_f value (relative mobility).

4.2.11 Peptide Mass Fingerprinting

The purified ICHG band obtained for *C. tetragonoloba* was excised from the SDS –PAGE gel, and it was diced to small pieces. These pieces were further destained for 10-minute intervals (3-4 times) until they became translucent white. Dehydration of gel was performed using acetonitrile and concentrated till complete dryness. Rehydration of gel was carried out with DTT, followed by incubation with iodoacetamide. Two subsequent incubations with ammonium bicarbonate solution and acetonitrile for 10 minutes were then done, and the gel was thoroughly dried under vacuum. To this dried gel, the trypsin solution was added and incubated overnight at 37°C. The digest solution was then transferred to fresh eppendorf tubes. The gel pieces were extracted thrice with extraction buffer, and the supernatant was collected each time into the single Eppendorf tube and then wholly dried using Speedvac. The dried pepmix was suspended in the Tris-Acetate buffer. The peptides obtained were mixed with α -Cyano-4-hydroxycinnamic acid (HCCA) matrix in a 1:1 ratio and the resulting 2 μ l was spotted onto the MALDI plate. After air-drying the sample, it was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument, and further analysis was done with FLEX ANALYSIS SOFTWARE for obtaining the peptide mass fingerprint. The masses obtained in the peptide mass fingerprint were submitted for the MASCOT search in the “CONCERNED” database for identification of the protein.

4.2.12 Prediction of accessory proteins for ICHG purified from *C. tetragonoloba*

The sequences of the internal peptides obtained from the peptide mass fingerprinting were analysed using the MASCOT server against the NCBI database. The sequence of the closest possible match protein was retrieved from the Protein Database. This sequence was used to draw a confidence interval map of the accessory proteins with undefined functions and structures were analysed from STRING Version 11.0.²⁰³ (<https://string-db.org/>)

4.2.13 Enzyme activity with natural substrates

The PC-ICHG and CT-ICHG were subjected to enzyme activity with natural substrates. Here natural substrates indicate the sugar conjugates of the selected flavonoids/isoflavonoids. For testing the ability of the purified β -glucosidase to hydrolyze natural substrates, commercially obtained glycoside standards viz. genistin and daidzin (isoflavonoids); rutin, naringin and hesperidin (flavonoids) were used. The assay mixture contained 155 μ l Citrate Phosphate Buffer (0.1 M, pH 4.5), 25 μ l isoflavonoid, or flavonoid substrate (dissolved in DMSO: Citrate Phosphate buffer::1:1, 10 mM) and 20 μ l of the purified enzyme. After incubating the reaction mixture for 3 h at 50°C, the reaction was terminated using 200 μ l Methanol. Negative control in the form of reaction terminated at zero time was used where methanol was added prior to enzyme followed by incubation, as mentioned above. The reaction mixture was then centrifuged at ambient temperature for 1 minute at 3000 rpm. The supernatant was collected and subsequently extracted thrice using chloroform. The resultant extracts were dried entirely and dissolved in 50 μ l of absolute methanol. The enzymatic product formation was checked using Thin Layer Chromatography (TLC) and High-Performance TLC (HPTLC).

TLC plates with 0.2mm pre-coated silica gel 60 F₂₅₄ (Merck, Germany) of 8 x 10cm were taken. The mobile phase Toluene: ethyl acetate: acetone: formic acid::20:4:2:1 was used to obtain the separation in either of the cases. For TLC analysis, about 2.5 μ L of standards and 10 μ L of enzymatic products were loaded manually onto a 10-cm long TLC plate using a micropipette. In the case of the HPTLC analysis, the enzymatic products were spotted using Linomat 5 automated sample spotter (CAMAG) using 100 μ l syringe (Hamilton, Switzerland). The sample solution (2.5 μ l) was spotted as bands of 6mm width with the syringe with a 5mm distance between each spot. The spotted plate was developed in a glass twin trough chamber (10 x 10 cm, CAMAG). The length of the chromatogram was 80 mm from the application position. The standard solution of the different compounds was prepared by dissolving them in pure methanol at a concentration of 1 mg/mL. After

development, the TLC/HPTLC plates were dried using a hot air oven at 50°C. The simple TLC plates were visualized using UV-Lamp (Thermoscientific), and images were captured manually at 254 nm. The HPTLC analysed plates were scanned densitometrically at 254 nm, and images were captured using TLC Visualizer (CAMAG) at 254 nm. The following scan conditions were applied: Slit width: 4 x 0.10mm, distance from Y-position: 10mm, and distance from X-position: 10 mm.

4.2.14 High Resolution Mass Spectrometry (HRMS) analysis of the enzymatic product

In continuation of the analysis of the enzymatic products obtained from the reaction of ICHG from *C. tetragonoloba*, the reaction mixtures obtained at step 6.2.9 were also subjected to HRMS on an Agilent 6545 Q-TOF LC/MS platform at positive Dual ESI ionization mode. The HRMS data were acquired at a full scan mode, with a mass range of 100–1500 m/z. ESI conditions: capillary temperature 350 °C; capillary voltage 3000 V; tube lens –43.46 V. Nitrogen was used as sheath gas (30 arb) and auxiliary gas (10 arb). The products formed were analysed using the Agilent database library MassHunter associated with the instrument. Pure standards which were commercially obtained from Sigma-Aldrich (USA) also ran along with the samples, and the spectrum was obtained for them for the comparative analysis.

4.3 Results and Discussion:

4.3.1 Purification and biochemical characterization of ICHG from pods/seeds of *P. cineraria* (PC-ICHG)

The isoflavonoid glycoside hydrolyzing β -glucosidase was (partially) purified from the pods of the *P. cineraria* using two steps purification procedure. The preliminary tests of β -glucosidase activity using synthetic substrate PNPG revealed that pericarp of the pods didn't have any enzymatic activity. On the other hand, significant activity was present in the seeds; hence the partial purification proceeded with the latter only. Figure 4.1 represents the biochemical characterization of the partially purified enzyme. pH and temperature optima were found to be 4.5 and 50 °C, respectively. A pH range from 3.0–6.5 was chosen, and it was observed that at acidic pH 4.5, the enzyme showed the maximum activity. At pH 5.0 and 5.5, approximately 85 % of activity was retained. Post that, there was a sharp decline of about 80% in the enzymatic activity at pH 6.0 and 6.5, and only 20 % of the maximum activity was retained by the enzyme (Figure 4.1.a). This implies that the β -glucosidase purified acts on the substrate under acidic conditions. The

temperature optima were on the higher side, and it was 50 °C. A range of temperatures from 28 °C to 60 °C was chosen. The enzyme showed a steady increase in activity from 28 °C to 50 °C, and after that, there was a steep decrease in the activity (Figure 4.1.b). The high temperature denatures the protein making it inactive. A comparative analysis with respect to the biochemical parameters of the similar enzyme isolated from other legumes indicated the significant variation. For e.g., the soybean root and chickpea seed enzymes have pH optima of 6.0 and 7.0, with temperature optima of 30°C and 45°C, respectively (Hsieh and Graham 2001; Hosel and Barz 1975). The time-dependent assay showed that there was a linear increase in enzymatic activity until 120 minutes (Figure 4.1.c). The coefficient of determination R^2 was observed as 0.9941 for the graph.

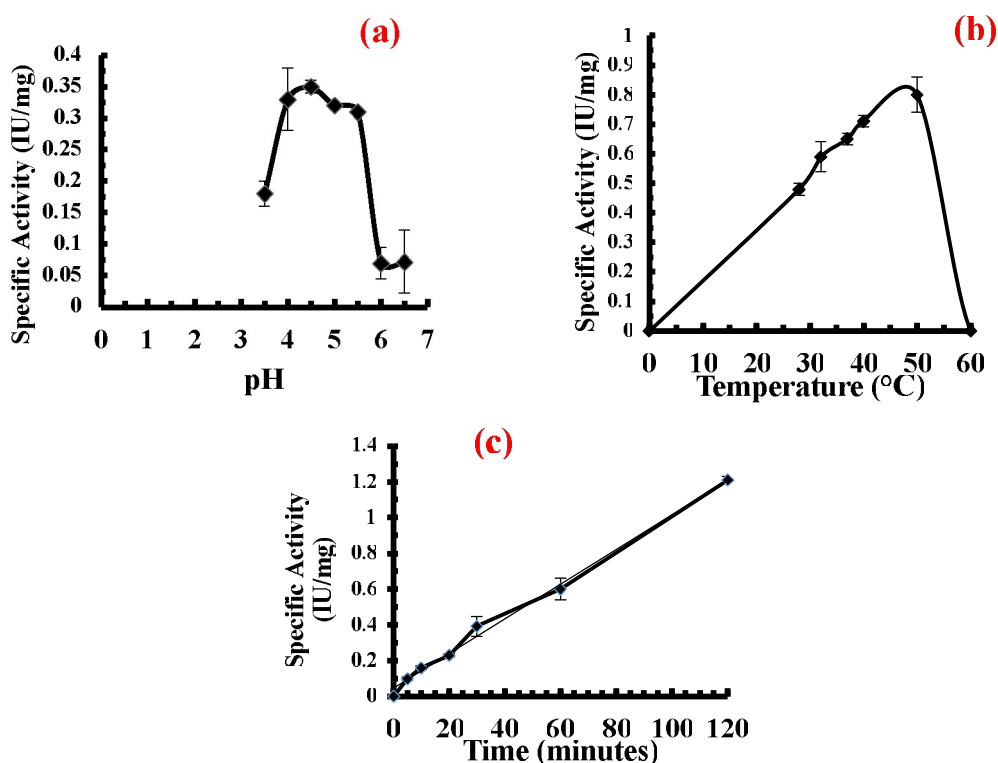


Figure 4.1 a) pH dependent assay; pH range of 3.5 to 6.5 (Citrate Phosphate Buffer, 0.1M), b) Temperature dependent assay, and c) Time-dependent assay of PC-ICHG.

The enzymatic activity was then checked with the natural isoflavonoid glycosides substrates daidzin and genistin. These two compounds are the putative isoflavonoid glycosylated conjugates present in legumes. The enzymatic action on the glycosides will release the aglycone daidzein and genistein, respectively. Figure 4.2 represents the TLC analysis of the experimental samples and control. Standards were also spotted on the same TLC since spots corresponding to the putative aglycone products (daidzein and genistein,

respectively) could be detected in the appropriate TLC lanes under UV 254 illumination. It can be observed from Figure 4.2 that the experimental samples with daidzin and genistin upon enzymatic treatment are showing bands corresponding to standards daidzein and genistein, respectively. The R_f values of Daidzein (D) and Genistein (G) are 0.246 and 0.392, respectively. The R_f values calculated for the enzymatic product Daidzein in experimental lane D-E and Genistein in experimental lane G-E are 0.217 and 0.362 respectively which are comparable with those of the pure standards Daidzein and Genistein. The minor variations observed in the R_f values could be because of the residual particles present in the enzymatic product. The experimental controls of Daidzin (D-E) and Genistin (G-E) did not show any bands post TLC run however intense illumination can be observed at the spot of loading the samples on plates indicating the glycoside substrates. The solvent system chosen to execute the TLC run was Toluene: Ethyl Acetate: Acetone: Formic Acid:: 20:4:2:1 ratio. This solvent system facilitates the separation of relatively less polar isoflavonoids (aglycones in this case), and hence, the zero-time control samples, as well as the isoflavonoid glycosides, were not expected to migrate from the point of origin. From Figure 4.2, it is evident that the observations are in concurrence with the expected results. Apart from this in the experimental lanes, no bands other than the aglycones of daidzin (i.e., daidzein) and genistin (i.e., genistein) were observed. This leads us to infer that the enzyme is highly specific for the isoflavonoids glycosides conjugates, and no other product was released as the result of the enzymatic reaction.

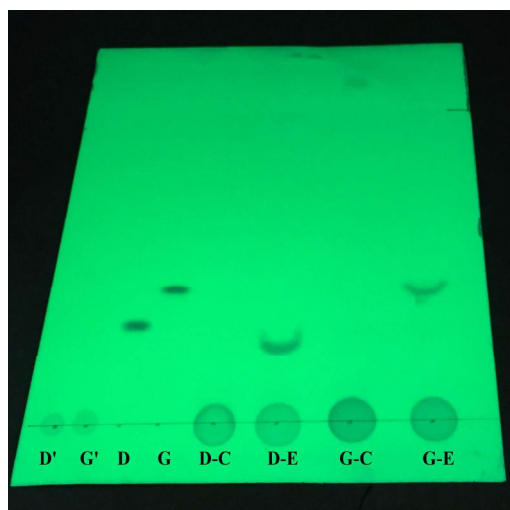


Figure 4. 2 TLC for PC-ICHG activity with natural substrates (**D'**- Daidzin, **G'**- Genistin, **D**- Daidzein, **G**- Genistein, **D-C** - Daidzin zero time control, **D-E** - Daidzin experimental, **G-C**- Genistin zero time control, **G-E** – Genistin experimental. G-254 labeled Silica gel plates were used.

PC-ICHG could not be subjected to complete purification due to low/loss of activity during purification, which is not an unprecedented case as far as enzymes of secondary metabolism are concerned. Secondly, it was observed that PC-ICHG showed temporal variation: activity seen in one specific year was missing in subsequent years. These observations could explain the fact that a very a smaller number of native ICHGs have been purofoed/reported from plants to date. The present result is of extreme significance since, to date, such specific β -glucosidases have been characterized by only a few legumes such as soybean, chickpea, and Thai rosewood.^{204 205 206} The current finding points out to the fact that in *P. cineraria* seeds, the glycoside-to-aglycone metabolism could be mediated by these enzymes, which might serve the seed during germination in hostile environments. The fact that the plant recruits such enzymes for isoflavonoid glycoside breakdown, underline essential roles played by these metabolites for the plant. The released aglycone could protect the seed from both biotic and abiotic stresses while the released sugars could provide energy to the developing seedling. Nonetheless, the commercial utility of such enzymes in enhancing isoflavonoid bioavailability in soy and similar ‘healthy flour’ extracts on account of releasing the more bioactive aglycone forms⁹⁸ merits further purification and detailed characterization of the ICHG enzyme from *P. cineraria*.

4.3.2 Purification and characterization of ICHG from seeds of *Cyamopsis tetragonoloba* (CT-ICHG)

4.3.1.2 Purification of CT-ICHG:

The preliminary studies which were carried out with the seeds of the *C. tetragonoloba* showed significant activity of the ICHG; hence, large scale purification was subjected using 200 gm of the dried seeds powder. The aim was to purify, characterize and sequence the ICHG so as to obtain insight regarding its properties and to understand how much similarity there exists with other such enzymes as reported in the literature. ICHG from *C. tetragonoloba* seeds was purified from crude extract by means of ammonium sulfate precipitation, size exclusion chromatography, and anion exchange chromatography. Protein precipitation by 30-80% ammonium sulfate proved to be an elementary but efficient step to separate the unwanted proteins from the ICHG. Size exclusion chromatography was performed using the Sephadex G-75 matrix which further separated proteins on the basis of their molecular weight. The presence of a single band on SDS-PAGE analysis (Figure 4.7) in the fraction isolated from the last purification step indicates that purification of ICHG has been achieved, which was also confirmed by the peptide mass fingerprinting. The low yield of the purified ICHG after the final step (Table 4.1) implies that the enzyme has a tendency to lose the activity quickly with time. But it is essential to mention that only two chromatography steps were involved in achieving the purification viz size exclusion and ion exchange chromatography as against the usage of multiple columns followed in similar studies from other legumes.

93 207 208

Table 4. 1 Purification of Isoflavone conjugates hydrolyzing β -glucosidases from seeds of *Cyamopsis tetragonoloba*

Fraction	Total protein (mg)	Total activity (IU)	Specific activity (IU * 10 ⁻³ /mg)	Yield (%)	Purification fold
Crude extract	3440	10.32	3.0	100	-
Ammonium sulphate	530	1.06	2.0	9.6	0.66
Sephadex G-75	256	0.768	3.0	7.4	1
DEAE-Sepharose	9.28	0.0074	0.8	0.07*	-

*Low purification yield of ICHG could be accounted for the loss of significant enzyme activity over time taken to complete the purification.

4.3.2.2 pH and temperature optima of CT-ICHG:

The biochemical characterization of the purified enzyme showed a sharp optimum pH curve with maximum activity at pH 4.5 (Figure 4.3.a). which is in accordance with the preliminary studies done using the crude extract (data not shown). The ICHG activity kept increasing from pH 3.0, reached maximum at 4.5 and then decreased, and finally, after pH 8.5, declined drastically. At pH values of 4, 5 and 5.5, around 85% of maximum activity was retained, whereas, at pH 6, this value came down to 62%. After this, the enzyme showed very little activity in a range of pH 7-8.5, but after that, complete loss of activity was observed. When compared with the enzymes isolated from other plants with similar activity, individual variations were seen. The pH optima of ICHG from soybean and chickpea are 6.5 and 6.0 respectively.^{93 205} ICHG from another non-edible legume Thai rosewood has a pH optimum of 5.0.⁹⁷ This shows that the same enzyme, when isolated from different sources, exhibits variation with respect to the biochemical parameters. Temperature optima for the ICHG were observed as 37°C (Figure 4.3 b). A very crucial point that must be mentioned here is that at slightly higher temperatures, the enzyme was not completely inactive. As observed in Figure 4.4.b, at 42°C, 85% of maximum activity was still retained by the enzyme. As the incubation temperature was further increased to 50°C, 50% of activity was lost, primarily because of the denaturation of enzyme caused by the heat. At 80 °C, total loss of activity was seen. When compared with β -glucosidase (ICHG) from other putative legumes viz chickpea and soybean, it can be concluded that the purified enzyme exhibits a broad range of optimum temperature since in the latter mentioned plants, the maximum enzyme activity was obtained at 45°C and 30°C respectively.^{204 205} Similarly, β -glucosidase from Thai rosewood showed temperature optima of 30°C. It is also evident that ICHG purified from *P. cineraria* and *C. tetragonoloba* both have a pH optimum of 4.5, but there exists variation in their temperature optima. It is interesting to observe that both the enzymes were significantly active at higher temperatures. This could be attributed to the geographical location of both the plants (which are the sources of these enzymes) which is dry to semi-arid regions. The higher temperature of the surroundings poses a need for specific physiological adaptations in the plant (in terms of high temperature enzyme activity) in these cases at the cellular and biochemical levels to enhance the survivability under the external stress factors.

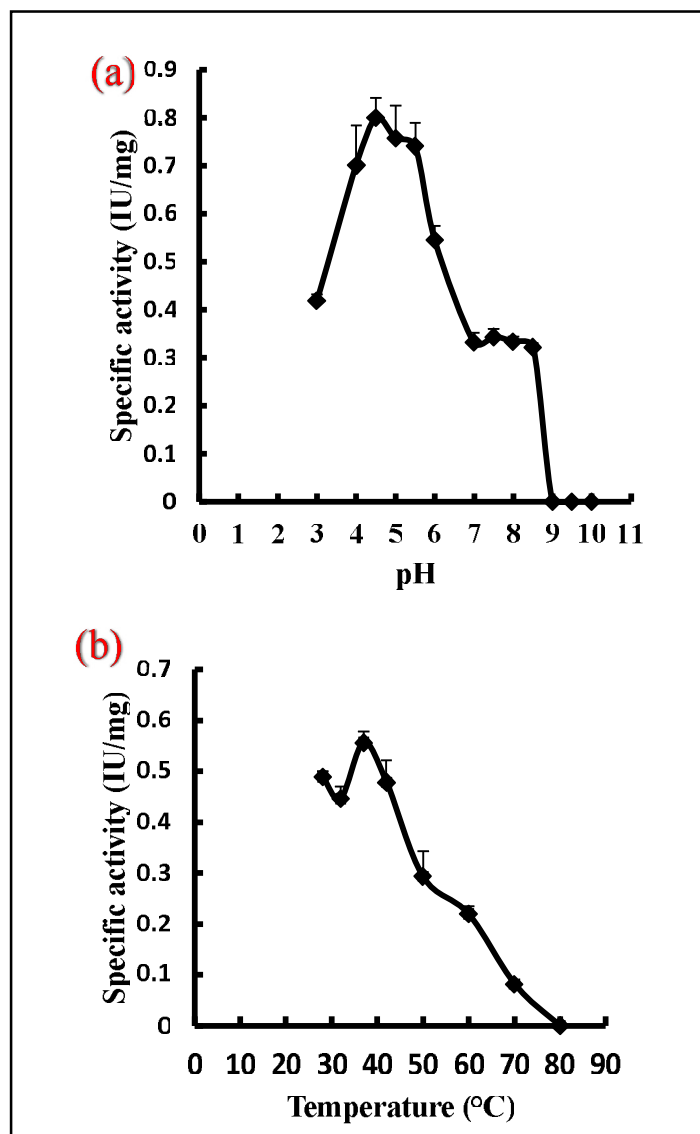


Figure 4.3 Effect of pH (a) and temperature (b) on the *C. tetragonoloba* ICHG activity. For calculating pH optima, 0.1 M assay buffers from 3.5-10 were used {3.5 to 6.5 (Citrate Phosphate Buffer, 0.1M); pH 7 to 8 (Tris-HCl buffer) and pH 9-10 (Carbonate-Bicarbonate buffer)}.

4.3.2.3 Effect of Metal ions on CT-ICHG activity:

The effect of metal ions in the form of cationic salts on the activity of CT-ICHG was studied, and it was observed that all the divalent metal ions affected the enzyme activity differently (Figure 4.4). There was almost complete inhibition by divalent cations Hg^{2+} and Co^{2+} . The enzyme sensitivity to inhibition by Hg^{2+} is consistent with the reports that it interacts with the cysteine residues present in the sulphhydryl linkages, thus indicating the presence of

sulfhydryl linkages at the active site in the ICHG structure.²⁰⁹ Surprisingly, Co^{2+} ions, which did not inhibit beta-glucosidase activity from other sources, showed 100% inhibition in this case. Divalent cobalt ions have been reported to form the complexes with amino acids, such as aspartic acid, lysine, histidine, and asparagine.²¹⁰ The total loss of enzyme activity in the presence of the cobalt ions could indicate that the amino acids present in the active sites of the ICHG bound to cobalt ions irreversibly and hence completely inhibiting its activity. Other ions like Mg^{2+} , Mn^{2+} , Ca^{2+} , Na^+ , Cu^{2+} , and Fe^{3+} also inhibited the enzyme activity, albeit to moderate levels. These metals also tend to form the metallo complex with the proteins and hereby altering their structure, which eventually deactivates them. Inhibitory effect of these ions, and the absence of enhancement of the activity in their presence when compared to control also imply that CT-ICHG is not a metal ion-dependent protein.

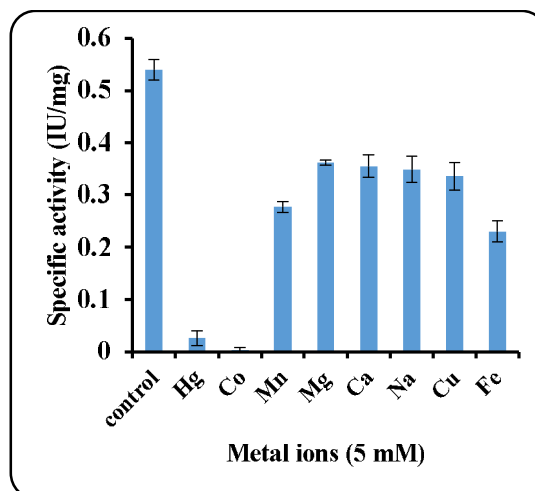


Figure 4. 4 Effect of divalent metal ions on the activity of CT-ICHG

4.3.2.4 Enzyme Kinetic Studies:

The V_{\max} and K_m for CT-ICHG were calculated using β -PNPG as substrate and carrying out the reaction at optimum pH and temperature as determined earlier. V_{\max} and K_m were found to be 6.6 IU/mg glucosidase and 0.86mM, respectively, as observed from the Lineweaver-Burk plot (Figure 4.4). The initial reaction rate versus substrate concentration plot for the purified β -glucosidase isolated followed Michaelis–Menten kinetics. V_{\max} and K_m of similar ICHG enzymes isolated from legumes like chickpea and soybean were 18.6 IU/mg ; 0.0015mM and 20.0 IU/mg ; 0.0013 mM, respectively.²⁰⁴ These kinetic studies of ICHG isolated from the soy and chickpea involved the substrate concentration

dependent assay with respect to natural compounds like daidzin and genistin. The relatively high value of K_m of guar enzyme denotes that it has a low affinity for the synthetic substrate. Nevertheless, the HPTLC profile where enzyme activity was carried out with natural substrates showed that the enzyme is able to cleave the isoflavonoid glycosides specifically. (Figure 4.11)

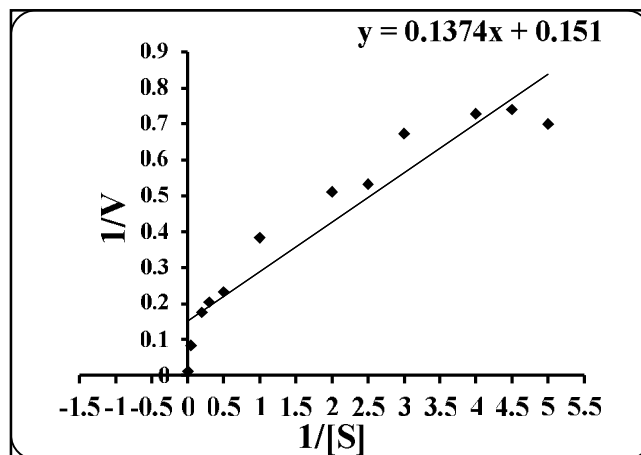


Figure 4. 5 Lineweaver-Burk plot for the purified CT-ICHG ($R^2 = 0.9031$)

4.3.2.5 Molecular weight determination:

The molecular weight of purified native CT-ICHG, as determined by size exclusion column chromatography was approximately 150 kDa (Figure 4.6). Under denaturing conditions, the purified ICHG yielded a single protein band on SDS-PAGE and migrated with an apparent molecular mass of approx. 50 kDa, as determined by relative mobility (Figure 4.7). The molecular weight determined by size exclusion column chromatography (150 kDa) and SDS-PAGE analysis (50 kDa) indicated that the enzyme possibly consists of three identical subunits of 50 kDa each.

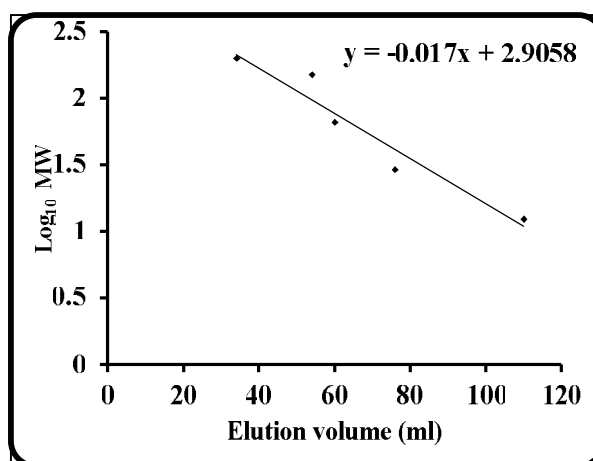


Figure 4. 6 Plot of Log₁₀ MW vs. elution volume to calculate the molecular weight of CT-CHG. The ICHG was eluted out at 40 mL, and thus the corresponding Log₁₀ MW was equal to 2.17 giving a native molecular weight of ~ 150kDa.

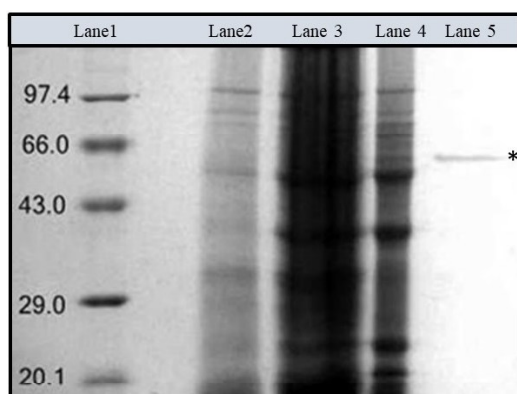


Figure 4. 7 SDS-PAGE analysis of different steps in CT-ICHG purification

Lane 1: Molecular marker
 Lane 2: Crude extract
 Lane 3: Ammonium Sulphate Precipitate(30-80%)
 Lane 4: Size exclusion chromatography fraction
 Lane 5: Ion exchange chromatography fraction
 *Purified CT-ICHG enzyme

Despite the relative similarity in the functions exhibited by this enzyme in different organisms, there is a considerable variation in terms of the molecular weight of the same protein when purified from various sources. β -glucosidases, in general, are known to show significant variation in their molecular weights. the β -glucosidase enzyme from bacteria *Thermus flavus* has a molecular weight as low as 49 kDa²¹¹, whereas functionally similar enzyme purified from *Aspergillus niger* had a molecular weight of 200 kDa²¹². When the purified ICHG was compared with similar ICHG from other plant sources, it was found that ICHG from soybean is homodimeric in nature with 58 kDa as its subunit²¹³, and ICHG from chickpea is also homodimeric with 68 kDa as its subunit.²⁰⁵ β -glucosidase isolated from Thai Rosewood is that of pentameric nature having 66 kDa as the subunit weight and a total molecular weight of 330 kDa.

4.3.2.6 Peptide mass fingerprinting:

The MALDI-TOF analysis of purified CT-ICHG was done, and sequences of five internal peptides were obtained whose amino acid composition is given in Figure 4.8 (a). Furthermore, the sequences of the internal peptides were analysed using the MASCOT server against the NCBI protein database to find out the closest β -glucosidases. The sequence matches with more than the score of 78 were considered significant events. Around 20 matches with a MASCOT score greater than 78 were observed during analysis. Though the complete protein sequence for the CT-ICHG is not available so far considering the top twenty sequence matches from Figure 4.8 (b) on next page, the enzyme purified belongs to the Family 1 glycoside hydrolases (GH). Functional and structural studies for enzymes belonging to Family 1 have shown that these proteins have $(\alpha/\beta)_8$ -barrel shape structure hereby, implying that the CT-ICHG could also consist of this highly conserved structure at its active site. The MALDI-TOF analysis also showed sequences of the five resultant peptide sequences are closest to those of β -glucosidase from *Cicer arietinum* (Figure 4.8. a). The isoflavonoid specific β -glucosidase from the *C. arietinum* has also been reported to exhibit the isoflavone conjugates hydrolyzing properties⁹⁶ suggesting the functional nature of the enzyme purified from the Guar seeds.

4.8 (a)

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
gi1502163136 Mass: 59412 Score: 176 Expect: 8.4e-12 Matches: 5 PREDICTED: beta-glucosidase 44-like [Cicer arietinum]								
1654.0340	1653.0267	1652.8311	118	210	- 225	0	75	R.VVAALGYDNGFFAPGR.C
1835.2213	1834.2140	1834.0029	115	272	- 286	0	---	R.IGILLDFVWYEPLTR.S
2232.3509	2231.3437	2231.0953	111	299	- 316	0	---	R.DFHVGWFIHPLVYGEYPR.T
2332.4595	2331.4522	2331.2263	96.9	152	- 171	0	55	K.GITPYANLYHYDLPLALELR.Y
2459.4795	2458.4723	2458.2335	97.1	297	- 316	1	---	R.ARDFHVGWFIHPLVYGEYPR.T

4.8 (b)

	Accession	Mass	Score	Description
1.	gi 502163136	59412	176	PREDICTED: beta-glucosidase 44-like [<i>Cicer arietinum</i>]
2.	gi 590665214	54093	108	B-S glucosidase 44 isoform 2 [<i>Theobroma cacao</i>]
3.	gi 590665235	60742	108	B-S glucosidase 44 isoform 3 [<i>Theobroma cacao</i>]
4.	gi 590665211	58727	108	B-S glucosidase 44 isoform 1 [<i>Theobroma cacao</i>]
5.	gi 659066821	58623	107	PREDICTED: beta-glucosidase 44-like [<i>Cucumis melo</i>]
6.	gi 629119488	58767	106	hypothetical protein EUGRSUZ_B00859 [<i>Eucalyptus grandis</i>]
7.	gi 629119495	43983	101	hypothetical protein EUGRSUZ_B00859 [<i>Eucalyptus grandis</i>]
8.	gi 449439441	58095	98	PREDICTED: beta-glucosidase 44-like [<i>Cucumis sativus</i>]
9.	gi 629119491	58572	98	hypothetical protein EUGRSUZ_B00859 [<i>Eucalyptus grandis</i>]
10.	gi 702259801	58599	98	PREDICTED: beta-glucosidase 44-like [<i>Eucalyptus grandis</i>]
11.	gi 629119492	58644	98	hypothetical protein EUGRSUZ_B00859 [<i>Eucalyptus grandis</i>]
12.	gi 629119489	58608	97	hypothetical protein EUGRSUZ_B00859 [<i>Eucalyptus grandis</i>]
13.	gi 823169519	58555	97	PREDICTED: beta-glucosidase 44-like [<i>Gossypium raimondii</i>]
14.	gi 902186387	57690	97	hypothetical protein SOVF_142210 [<i>Spinacia oleracea</i>]
15.	gi 225456104	58602	95	PREDICTED: beta-glucosidase 44 [<i>Vitis vinifera</i>]
16.	gi 147811579	58482	95	hypothetical protein VITISV_040977 [<i>Vitis vinifera</i>]
17.	gi 567919310	46261	89	hypothetical protein CICLE_v10008010mg [<i>Citrus clementina</i>]
18.	gi 641843986	46292	89	hypothetical protein CISIN_lq010049mg [<i>Citrus sinensis</i>]
19.	gi 641843983	47350	89	hypothetical protein CISIN_lq010049mg [<i>Citrus sinensis</i>]
20.	gi 734428106	47194	89	Beta-glucosidase 44 [<i>Glycine soja</i>]

Figure 4. 8 a) Peptide sequence matches for CT-ICHG as observed using MASCOT software. b) MALDI-TOF and MS analysis of CT-ICHG showed maximum similarity with isoflavone-7-O-glucoside-specific beta-glucosidases from *Cicer arietinum*. (Score 176)

4.3.2.7 Identification of accessory proteins

STRING results for the anticipated functional partners of the investigated CT-ICHG are given in Figure 4.9. STRING projects the functional association between different proteins based on the genomic association of their genes. For the query gene, the software retrieves all the genes which are present in the near vicinity and which encode all those proteins which are part of the same protein complex or metabolic pathways. This is crucial since the identification of accessory proteins in the metabolic pathways helps in understanding the targets and co-targets of the enzyme at the cellular level. The internal peptides sequencing of the purified CT-ICHG showed the maximum identity with the β -glucosidase (ICHG) from *Cicer arietinum* (Chickpea). Hence, the protein sequence of the latter was chosen as a template to study the protein-protein interaction using the STRING V 11.0 software and to deduce the possible interaction for CT-ICHG. The interaction of the CT-ICHG with other proteins have been indicated in the Figure 4.9.

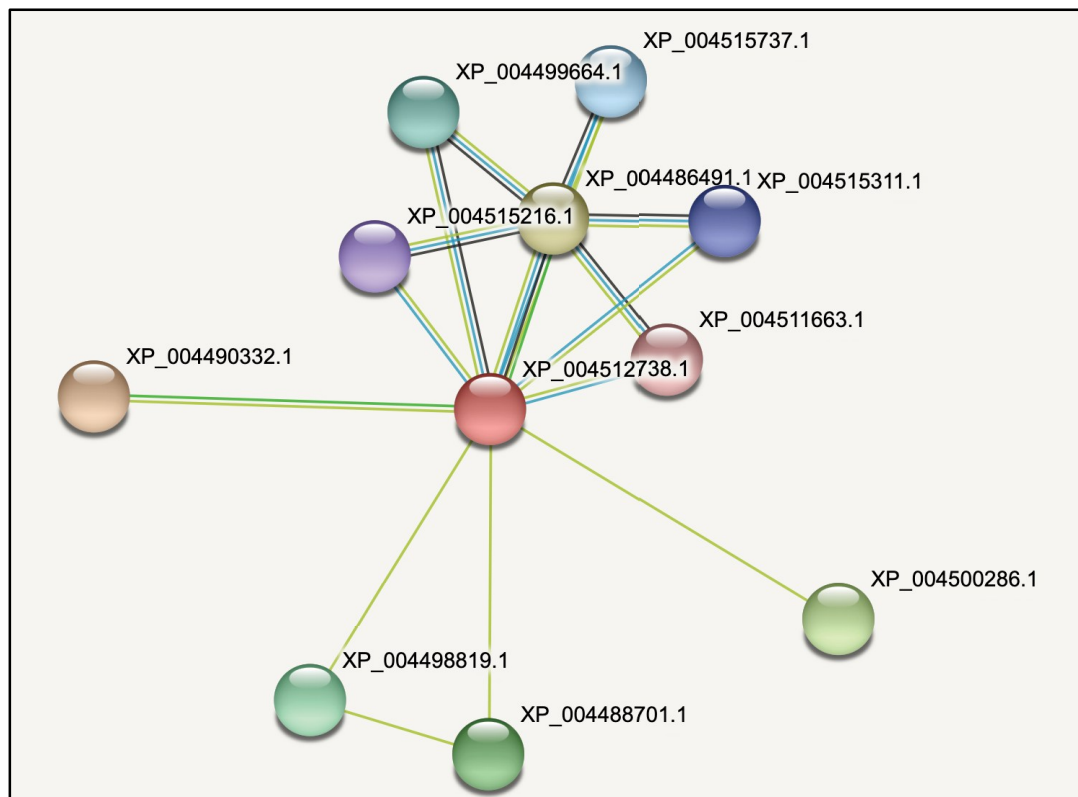


Figure 4. 9 Interactive network view of CT-ICHG with predicted proteins using the STRING V 11 tool

XP_004512738, which is the NCBI reference id for the *Cicer arietinum* beta-glucosidase 44-like, was given as input query for the STRING analysis. The concerned sequence was chosen on the basis of the N-terminal sequence analysis carried out previously, which showed the purified protein exhibited maximum similarity with the *Cicer arietinum* beta-glucosidase 44-like. The protein interactions obtained from the STRING analysis are presented in Figure 4.10. Interacting partners that had high confidence interaction scores, i.e., higher than 0.700, was chosen for the analysis. From the table, it can be concluded that the enzyme beta-glucosidase from *Cicer arietinum* is showing strong interaction with an uncharacterized protein but relatively weaker interaction with glycosidases belonging to glycosyl hydrolases family GH 31 and 32. The families GH 31 and GH 32, both comprise of the glucosidases, which are capable of cleaving glycosides having α and β glycosidic bonds, respectively. It was also observed that the enzyme in consideration was showing co-expression and neighbourhood presence with an alpha-glucosidase. In the plants, the flavonoids and isoflavonoids are stored as their sugar conjugates, which are mainly in their glycosylated form. Since glycosylation is not only limited to the β -glucoside conjugates, it is vital for the plant system to express

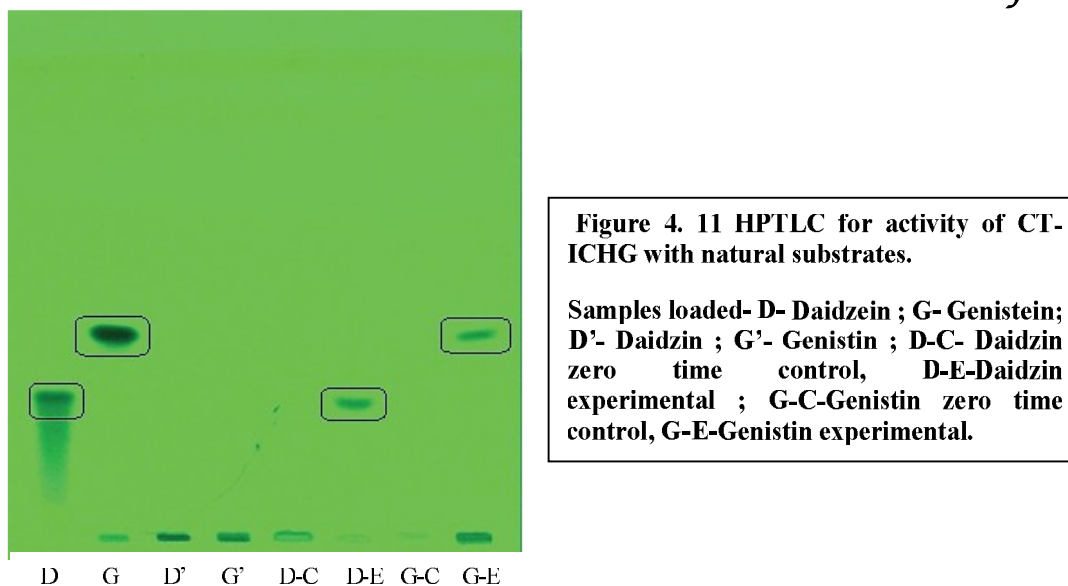
the enzymes which can cleave the other conjugate forms as well. Despite the absence of any experimental data, from the above analysis, it can be predicted that apart from the specific β -glucosidases, there is a possibility of the simultaneous expression of alpha-glucosidases during the catabolism of the stored glycosyl conjugates.

Your Input:		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
XP_004512738.1 Beta-glucosidase 44-like; Belongs to the glycosyl hydrolase 1 family (512 aa)										
Predicted Functional Partners:										
XP_004490332.1	Uncharacterized protein LOC101509357; Derived by automated computational analysis using gene prediction metho...									0.702
XP_004486491.1	Alpha-glucosidase-like isoform X1; Belongs to the glycosyl hydrolase 31 family (908 aa)									0.671
XP_004500286.1	SAGA-associated factor 11; Ataxin-7-like protein 3-like isoform X1 (195 aa)									0.663
XP_004488701.1	Probable caffeoyl-CoA O-methyltransferase At4g26220; Tricin synthase 1-like (297 aa)									0.662
XP_004498819.1	ER membrane protein complex subunit 4-like (178 aa)									0.659
XP_004499664.1	Beta-fructofuranosidase, soluble isoenzyme I-like; Belongs to the glycosyl hydrolase 32 family (636 aa)									0.653
XP_004515737.1	Acid beta-fructofuranosidase-like; Belongs to the glycosyl hydrolase 32 family (658 aa)									0.652
XP_004515311.1	Beta-fructofuranosidase, cell wall isozyme-like isoform X1; Belongs to the glycosyl hydrolase 32 family (603 aa)									0.652
XP_004515216.1	Beta-fructofuranosidase, insoluble isoenzyme 1-like; Belongs to the glycosyl hydrolase 32 family (573 aa)									0.652
XP_004511663.1	Acid beta-fructofuranosidase-like; Belongs to the glycosyl hydrolase 32 family (646 aa)									0.652

Figure 4. 10 Predicted functional partners of *Cicer arietinum* beta-glucosidase 44-like

4.3.2.8 CT-ICHG activity with natural substrates of flavonoid and isoflavonoid:

The purified CT-ICHG was tested with different natural flavonoid and isoflavonoid glycoside substrates. The HPTLC results obtained from the assay for isoflavonoid glycoside hydrolyzing β -glucosidase showed that the seeds of *Cyamopsis tetragonoloba* contain β -glucosidase activity specific towards the conjugated glycosides of isoflavonoids. The R_f values of the products formed, when ran on HPTLC and observed under the scanner matched with that of the corresponding standards for aglycones (daidzein; $R_f=0.3$) and (genistein $R_f=0.41$), respectively (Figure 4.11). The enzyme activity with conjugated glycosides of flavonoid substrates like rutin, naringin, and hesperedin did not show any conversion to their aglycone forms. Thus, a β -glucosidase, which is highly specific towards the isoflavonoid glycosyl conjugates (but not towards flavonoids), has been isolated from the seeds of *C. tetragonoloba*. ICHG reported in other legumes like soybean has been known to be capable of cleaving the malonylated and acylated conjugates as well. In fact, the benzenoid substituent on the condensed ring of isoflavonoid structure is present at 3-position as compared to that of 2-position in case of flavonoids. This structural difference between the isoflavonoids and other flavonoids could account for the ability of the native enzyme's active site to bind the former but not the latter.²⁰⁰



4.3.2.9 Analysis of the enzymatic product via HRMS

To further confirm the identity of the product released upon the enzymatic reaction, HRMS was performed, and the mass spectrum obtained from the product was matched against that of the standards. Figures 4.12-4.14 represent the HRMS spectra for the standard Daidzein(D), Daidzin zero-time control (D-C), and Daidzin experimental (D-E), respectively. As observed in Figure 4.12, the standard daidzein showed a significant peak at m/z value of 255.05. Figure 4.13 represents the HRMS of the Daidzin control where the reaction was stopped at zero time, and hence the enzymatic conversion of daidzin to its aglycone daidzein was not expected. So, a mass spectrum peak for the glycoside daidzin was expected at 417.186 which can be seen in Figure 4.12. This is in accordance with the HPTLC results from Figure 4.10 as well. The Daidzin experimental sample showed the conversion of the glycoside to aglycone, as seen in Figure 4.10, and hence the mass spectrum of the same should give the majority of a peak for daidzein. A look at Figure 4.14 indicates that the product released post ionization gave m/z similar to that of the standard daidzein($m/z=254.241$) from Figure 4.12 hence concluding that the daidzein was the sole resulting product of the enzymatic reaction, therefore, establishing the specificity of the purified CT-ICHG. Similar results were obtained for the samples containing genistin as a substrate where the mass spectra of product released matched with that of the pure standard as represented in Figures 4.15-4.17.

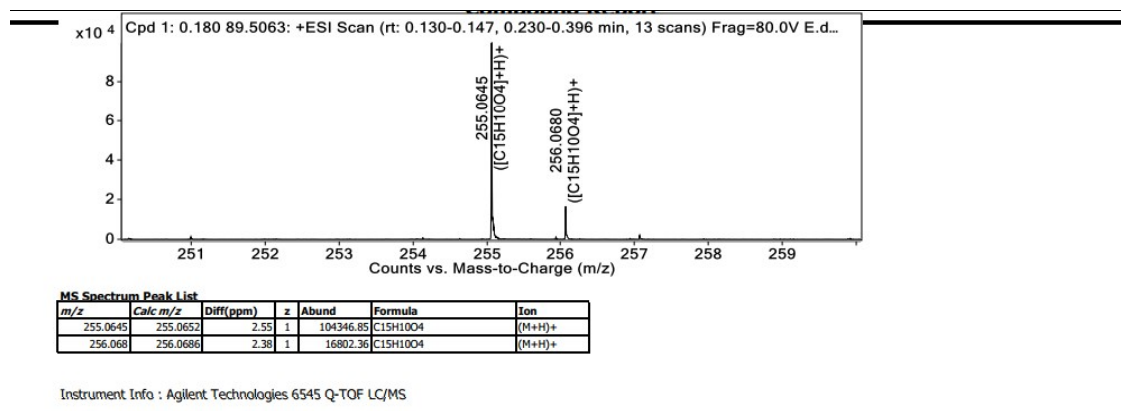


Figure 4. 12 Mass spectra of standard Daidzin. m/z of Daidzin is 254.241

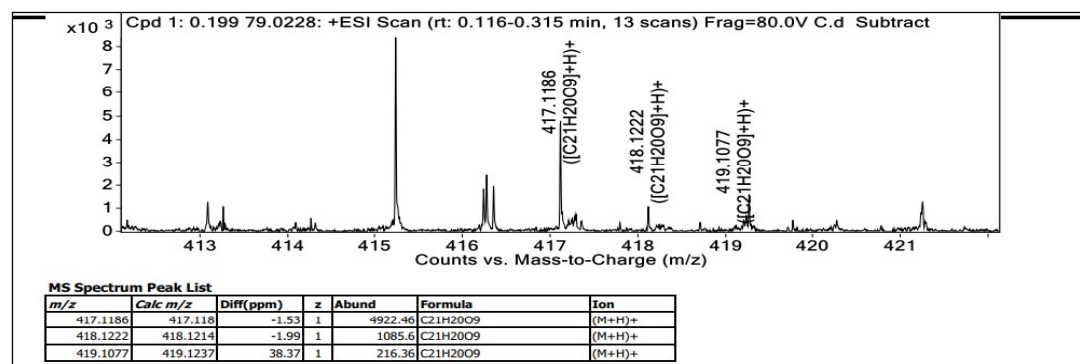


Figure 4. 13 Mass spectra of Daidzin control, which consisted of Daidzin of m/z 416.38 as substrate

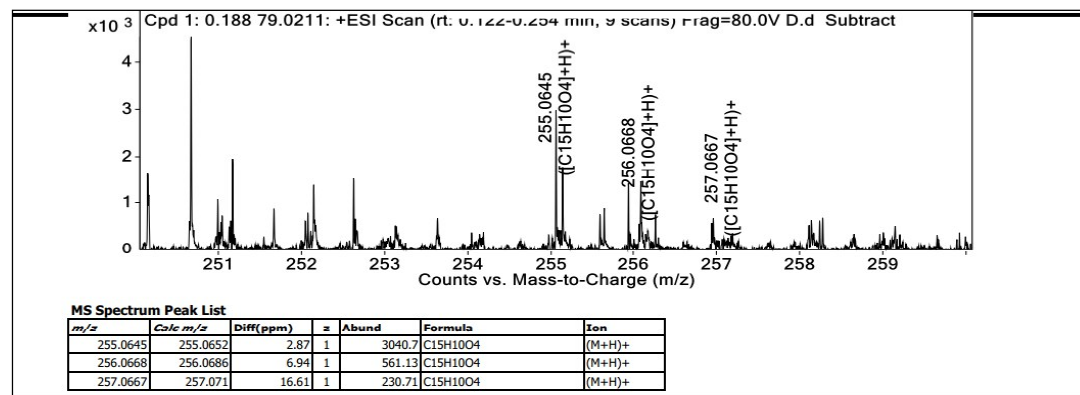


Figure 4. 14 Mass spectra of Daidzin experimental, which consisted of Daidzin as product released upon the enzymatic activity. The labeled peaks represent the parent peaks and further ionized peaks by different isotopes of hydrogen

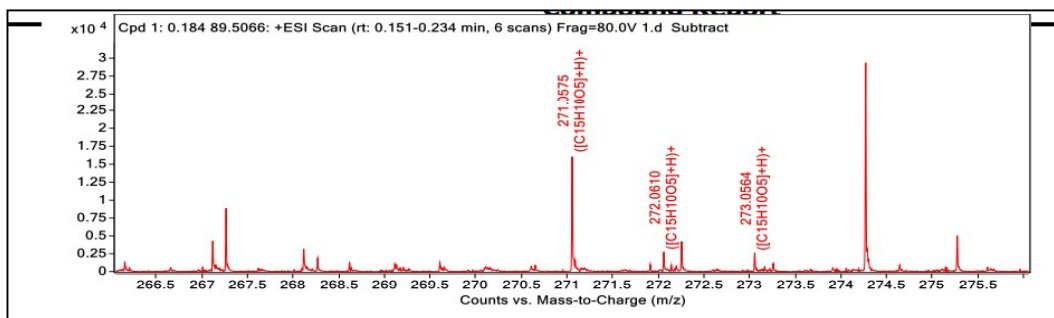


Figure 4. 15 Mass spectra of standard Genistein. m/z of Genistein is 271.0575

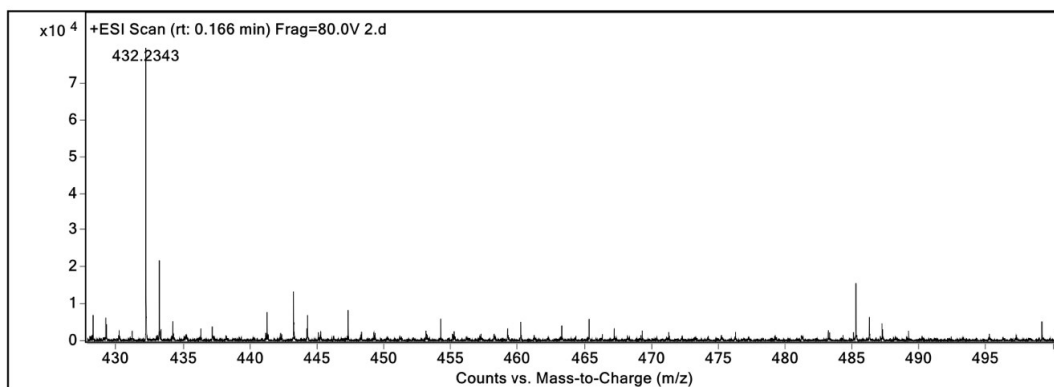


Figure 4. 16 Mass spectra of experimental control which consisted of Genistin of m/z 432.2343 as a substrate

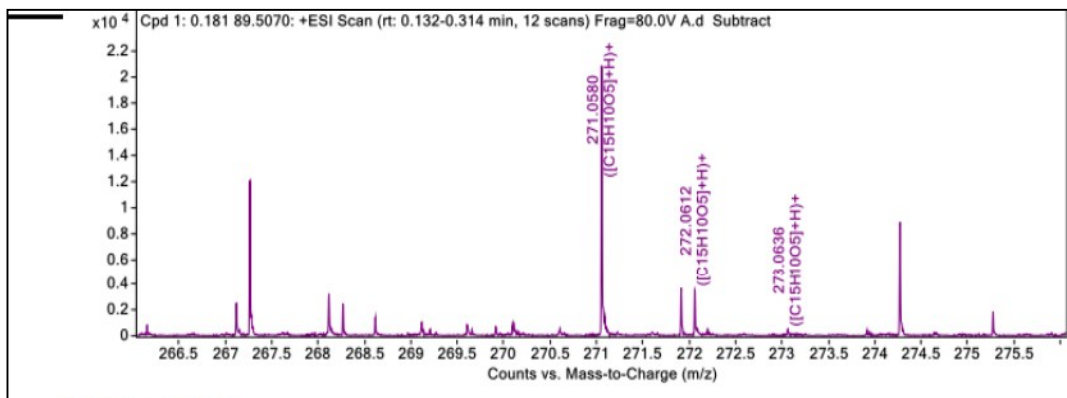


Figure 4. 17 Mass spectra of experimental test which consisted of Genistin as substrate released aglycone Genistein upon enzymatic reaction. The m/z 271.0580 indicates the presence of Genistein

4.4 Conclusions

In conclusion, this is the first time that an attempt has been made to establish the presence of an isoflavone specific isoflavone conjugate hydrolyzing β -glucosidase (ICHG) from seeds/pods of *P. cineraria* and *C. tetragonoloba*. Furthermore, these enzymes were

purified from the seeds of the *P. cineraria* and *C. tetragonoloba*. The purified enzyme from both the sources was biochemically characterized, and the properties were found to be comparable with the already reported enzymes from related members of the Leguminosae family. The ICHG in both cases was specific and shown to be active towards isoflavonoids glycosides (but not flavonoid glycosides). However, as discussed in Chapter 3, the isoflavonoids aglycones and glycosides viz daidzein, genistein, daidzin, and genistin could not be detected in the concerned tissues. This observation suggests that in both the cultivated plants, the ICHG is a part of ‘silent metabolism’, a term coined by Lewinsohn and Gijzen²¹⁴ which means that plants have been known to show the presence of enzymes which at times don’t observe any endogenous substrate or function, implying that a reservoir of metabolic capabilities remains either hidden or unused within the plant system. These enzymatic activities could be a result of the artefacts of the enzymes from the recent past which have not been entirely eradicated throughout the course of evolution. This indicates that the plant system may show activities against the particular substrate under conditions where a threshold level of substrate is present for the product (aglycone) to appear. Otherwise, the enzyme system is merely existing as a result of molecular evolution, irrespective of the presence/absence/low concentration of the metabolites in question.

Here we have seen that PC-ICHG and CT-ICHG were active against the isoflavonoids conjugates only, and the identity of CT-ICHG confirmed by the peptide mass fingerprinting where it showed the maximum similarity with a functionally similar β -glucosidase from *Cicer arietinum*. The hydrolysis of glucosyl isoflavone conjugates is biologically relevant for plants since the aglycone part released upon the action of enzymes is considered functionally more active and participates in imparting the antibacterial, antioxidant, antifungal activities (Figure 4.18). The sugar moiety freed as a by-product can be used for energy requirements. Isoflavonoids are the metabolites that are restricted mainly to the legume family of plants, and since most of the legumes are consumed by human beings, the isolation of the ICHG can be of high significance to them as well. It has been reported that when the biological activities of these compounds on the human population are considered, the aglycone part of the conjugates is the one that exhibits health-promoting properties and importance.²¹⁵

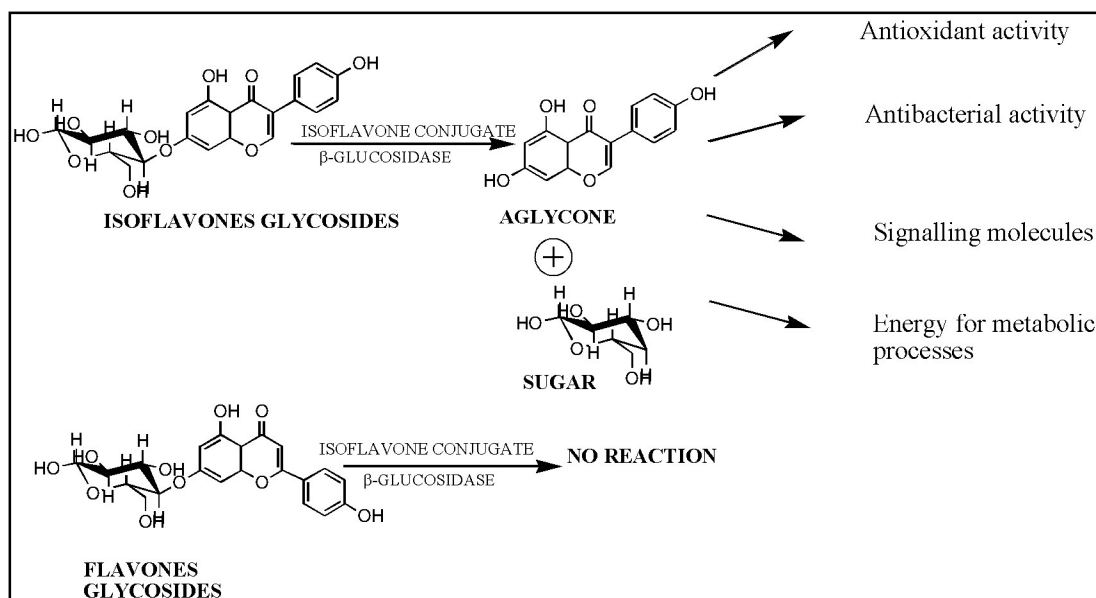


Figure 4. 18 ICHG acts on Isoflavonone conjugated glycosides to release active aglycones along with glucose

Because of the highly stable nature of the conjugated glycosylated compounds, the preparation of the food products doesn't impact much on their chemical nature, and the conversion from the conjugated glycosides to aglycones occurs at a minimal level. Hence, the bioavailability of the isoflavones depends mainly on the action of the specific glucosidases present in the human gut. This, again, is restricted by the inadequate presence of the specific glucosidases in our system resulting in the non-absorption of many valuable compounds. The ICHG from *P. cineraria* and *C. tetragonoloba* can be a potential candidate in the biotechnology industry to achieve the enzyme-mediated conversion of stored glycosides conjugates of isoflavonoids to their active form. Similarly, it also opens up the possibility of using a native enzyme from this source to achieve the bioconversion of the isoflavonoid conjugates in other edible products, e.g., tofu, soymilk. Several reports are there citing the biotransformation of the glycosides using the enzymes of microbial origin.^{216 217} However, their usage is often limited by glucose inhibition. In continuation of this, ICHG purified from the above-mentioned plants presents a compelling case where enzymes from novel sources may be used. Several attempts have been made to genetically engineer the legumes leading to increased availability of beneficiary aglycones for the consumption of the human population.²¹⁸ But such genetic manipulation cannot be achieved without the identification and characterization of native ICHG activity in the concerned plant. With the ever-increasing demand for enriched food in the market, the bioengineering of the edible guar pods can

be explored so as to cultivate them with increased content of health-promoting isoflavonoid aglycones. Such kind of research will be of high significance in terms of dealing with problems related to food for local populations.



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**CHAPTER 5 Investigation into the biological activities
of purified, flavonoid-enriched extracts
from *P. cineraria* and *C. tetragonoloba***

5.1 Introduction

Plants have not only been the primary provider of nutrition to the living world, but their biological/therapeutic activities towards human health have also been thoroughly documented. An array of compounds especially, secondary metabolites that are synthesized in plants as a response to various biotic and abiotic stress, are also reported to show antibacterial, anticancerous, antiviral, antifungal, anti-inflammatory and antioxidants activities in humans.²¹⁹ Plants being an inexhaustible source of novel compounds, present numerous possibilities of their usage in medicinal and other applications. The different plant parts such as leaves, bark, seeds, fruits, root, flowers, etc. are enriched with many active compounds viz. phenolics, flavonoids, alkaloids, steroids, tannins, glycosides, volatile oils, and resins. The health-promoting medicinal effect of plants is a resultant effect of either one or the combination of different secondary products. These compounds have a wide range of biological targets, which have been discussed in detail in section 1.5. Natural products or their derivatives and structural analogs still represent over 50% of all the clinical drugs in use with natural products derived from higher plants constituting 25% of the total.²²⁰ As per World Health Organization (WHO), 80 % of the people in developing countries, which account for about 3.5 to 4 billion people in the world, still bank on traditional medicine for their primary health care, and about 85 % of conventional medicine involves the use of plant extracts. Regardless of the availability of different tactics for the discovery of therapeutic agents, plant-based secondary metabolites still remain as the most versatile reservoirs of new structural types.²²¹ In the constant effort of improving the effectiveness and integrity of current medicinal practice, scientists have increased their attention to folk medicine as a source of new drugs.²²² The harmful side effects which are generated by the extensive usage of many artificial therapeutic agents have also initiated the interest in synergistic effects of the natural products when given along with the drugs, especially for cancer treatment and an anti-bacterial agent.

Since its inception in 1928, antibiotics have become the ‘wonder drugs’ to fight bacterial infections in humans as well as animals. Increased demand for antibiotics across the health sector has led to inexpensive and off-label usage of the drugs. Previously, the development of novel antibiotics was correlated with the development of resistant strains. However, the conventional approach in fending off the bacterial

diseases is now focused on the modification of the combating strategies, which are no longer effective in keeping a check on the emerging and re-emerging resistance of pathogens across the globe. In lieu of this, exploring the natural products for novel antibacterial agents and studying the antimicrobial properties of the plants yet to be studied is the need of the hour.

Another field of research where the plant-based natural products have contributed immensely is in the area of anticancerous therapeutic agents. More than one-third of the FDA sanctioned drugs are derived from natural products.²²³ These drugs include either pure phytochemicals or their semi-synthetic derivatives based on the active compounds extracted from plants. Investigation of plants capable of surviving in extremely hostile environments may be considered as a vital strategy in screening for the speedy development of new anticancer drugs. Cancer is a disease that is marked by the tuning out of the signals of the proliferation and dedifferentiation, which make the cells to sustain proliferation, overcome apoptosis, and giving them the power of angiogenesis and invasion. The existing treatment for cancer is associated with certain limitations, which have resulted in extensive research to look for alternatives to drugs having harmful side effects.¹⁴⁵ They affect not only the growth of cancerous cells but also the healthy cells which are rapidly dividing, such as bone marrow cells, digestive tract cells, and hair follicle cells. Additionally, widespread distribution of the drug to the non-targeted organs and rapid elimination by the human system requires a substantial dose of the drug which increases the aforementioned side effects and is also not economical. Besides this, cancer cells can undergo mutations and become resistant to the drugs which eventually make the treatment ineffective. Therefore, given the actuality of inadequate chemotherapy, novelties in treating cancer with fewer side effects are the trending research direction. An anticancer drug that is highly specific with respect to the cytotoxicity toward the cancer cells along with fewer side effects, shall be considered as an ideal drug. Synergistic effect between the plant-derived compounds and the conventional chemotherapeutic drugs could help in achieving a decreased dosage of the drugs given during treatment and hereby, lowering the harmful side effects caused on the healthy cells in the body. In this regard, studying the anticancerous properties of the extracts obtained from edible plants goes a step further since the dietary intake of the putative compounds can significantly affect the overall execution of the treatment. Studies have reported that nutrition can

potentially enhance the overall immune response of the body against cancer. The broad mechanisms that link nutritional principles to immune functions, usually involve the systemic or local effects of food nutrients and/or nutraceuticals within the tumor microenvironment.⁵⁸ Thus consumption of diet enriched with the phytochemicals which have biological activities such as anticancerous or antibacterial will be able to affect the conventional treatment in a synergistic manner. Therefore, unexplored edible plants with respect to their phytochemical profiles as well as the biological activities, open up the possibilities of finding new plant-based drugs and extrapolating the benefits of the study to the population which consumes the plants undertaken for the study.

The detailed phytochemical studies of the desert legumes *P. cineraria* and *C. tetragonoloba* in Chapter 3 showed the presence of the putative flavonoids and isoflavonoids in the crude as well as purified plant extracts. From the current perspective, i.e. biological (antioxidant, anti-bacterial and anti-cancer) activities of purified and flavonoid enriched fractions of both these legumes, which are localized to a great extent in the arid/semi-arid north western regions of India, have not been researched in depth, unlike other legumes which are the predominant part of the human diet. Chickpeas (*Cicer arietinum*), Peas (*Pisum sativum*), Pigeon pea (*Cajanus cajan*), Mung bean (*Vigna radiata*), Soya (*Glycine max*), Peanut (*Arachis hypogaea*), etc. are few of the species belonging to Leguminosae family about which several reports reflecting their antioxidant, antibacterial and anticancerous effects are present.²²⁴ A comparative analysis of the antioxidant potential of different variety of legumes commonly consumed in India was done by Marathe *et al.* (2011) which includes as many as fifteen legumes. Several other reports are present where the antioxidant activities from the crude extracts of the legume's seeds/pods were calculated but studies with purified extracts are rare. To the best of our knowledge, no information regarding the antioxidant potential of flavonoids enriched methanolic extracts of the seeds/pods of the *P. cineraria* and *C. tetragonoloba* is present till now. Biological activity studies with the enriched/purified natural products are required because of the possible interference from inhibitory compounds often present in the crude plant extracts. Also, the identification of novel biologically active compounds requires structural elucidation, which is dependent on purification and/or enrichment. However, the possibility of reduction in biological activity after purification cannot

be ruled out as activity could also be a result of synergistic effects of natural products. Similarly, the antibacterial and anti-cancerous activities reported so far for these two plants have either employed different parts of the plants or different organic solvent(s) to isolate the extract.^{226 227} The current chapter focuses on exploring the biological activities of the crude and purified extracts of *P. cineraria* and *C. tetragonoloba*.

5.2 Material and methods

5.2.1 Antioxidant activities of the purified extracts of pods/seeds of *P. cineraria* and *C. tetragonoloba*

The antioxidant potential of the plant extracts was calculated using the α , α -diphenyl- β -picrylhydrazyl (DPPH) assay method. This method was established by Blois in 1958 with the viewpoint to determine the antioxidant activity easily using a stable free radical DPPH ($C_{18}H_{12}N_5O_6$, $M = 394.33$). The DPPH assay is one of the most popular and routinely used methods to check the ability of the compounds to act as free radical scavengers and to estimate the antioxidant potential of foods. The reduction of the electron of a nitrogen atom in DPPH is carried out by the free hydrogen atom from the antioxidants which leads to the synthesis of hydrazine. The DPPH radical is stable organic nitrogen radical with a deep purple colour. The virtue of delocalization of spare electron over the molecule as a whole makes the DPPH highly stable free radical which allows it to avoid the dimerization unlike other free radicals.²²⁸ This delocalization also gives rise to the signature purple color with absorption at 517 nm when dissolved in ethanol solution.²²⁹ Since it is commercially procured, it does not need to be generated before starting the assay, unlike other scavenging methods. The formation of hydrazine upon mixing of the DPPH radical with an antioxidant/reducing compound results in the changing of the deep purple color of DPPH to a yellow color (hydrazine). The spectrophotometric measurement of the reduction in the color intensity of DPPH at 517 nm will indicate the antioxidant potential of the compounds of interest. The mechanism of the reaction between DPPH and reducing agent depends on the ability to abstract the H-atoms from the A-H bonds.

For the experiment, DPPH was purchased from Sigma Aldrich, methanol, and ascorbic acid were purchased from HiMedia Laboratories. Ascorbic acid is the most common naturally occurring organic compound which can act as an antioxidant in both animals and plants. The presence of multiple OH groups makes it an excellent

reducing agent and hence ascorbic acid is the gold standard to be used in comparing the antioxidant potential of other compounds. The modified method proposed by G. Sakthidevi et al. was employed to calculate the effect of *C. tetragonoloba* and *P. cineraria* extracts on DPPH radical.²³⁰ A 0.1 mM solution of DPPH was prepared in 95% Methanol and stored in the dark at -20°C until further use. For performing the reaction, 1 mL of the DPPH solution was added to 3 mL of a solution of extracts in methanol at different concentrations (25,50,75 &100µg/mL). The reaction was incubated at room temperature for 30 minutes in the dark. The absorbance was then measured at 517 nm using a UV-VIS spectrophotometer (Jasco). A standard curve was plotted using ascorbic acid at a concentration of (5,10,15,20 µg/mL). 95 % of methanol was used as a blank solution. While plotting the standard curve, a time-dependent assay was also set up using an incubation time of 10, 20, and 30 minutes. Post completion of the reaction, the absorbance of yellow color was measured. Lower values of absorbance of reaction mixture indicate higher activity of radical scavenging. The scavenging potential towards DPPH radical was calculated by using the formula as given below:

$$\text{DPPH scavenging effect (\% inhibition)} = \{(A_0 - A_1)/A_0\} * 100\}$$

Here, A_0 indicates absorbance of the control reaction, and A_1 is the absorbance in the presence of all of the extract samples and ascorbic acid. All the tests were executed in triplicates, and the values were averaged to give a standard deviation. The IC_{50} value which is defined as the concentration of the sample resulting in 50% reduction of the initial DPPH concentration, was obtained from the linear regression of plots of mean percentage of the antioxidant activity against the concentration of the test extracts and ascorbic acid (µg/mL) obtained from three replicate assays.

5.2.2 Anticancerous activities of the purified extracts of pods/seeds of *P. cineraria* and *C. tetragonoloba*.

5.2.2.1 Cancer cell lines and their maintenance

For investigating the anti-cancerous effect of the extracts, Human Hepatocellular carcinoma cell lines (Huh 7) were used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% Fetal bovine serum (FBS; Gibco Life Technologies) and were maintained at 37° C and 5% CO₂. 100 U/mL penicillin and 100 µg/ mL streptomycin (Invitrogen) were added to the culture medium. Cells were typically grown to 60–70% confluence, rinsed in phosphate-

buffered saline (PBS; Invitrogen) and placed in fresh medium prior to treatments. Regular monitoring of the cell lines and change of growth medium whenever required was performed to ensure the overall physiology of the cells.

5.2.2.2 *In vitro* cytotoxicity assay

In vitro Cytotoxicity assay was performed according to the methods previously mentioned.²³¹ Briefly, cells were seeded at a density of 8×10^4 /well in a 96 well plate. After overnight incubation at 37°C and 5% CO₂, cells were treated with different doses of extracts obtained from the seeds/pods of the *C. tetragonoloba* and *P. cineraria*. Chapter 4 mentioned the purification of the flavonoids enriched fraction from both these plants. For the current study, two different types of fractions from each plant was chosen; one is the crude extract which was obtained after defatting the powder and then preparing an extract with 80% Methanol, and another fraction was obtained after subjecting the crude extract to column chromatography using Amberlite-XAD7HP matrix which is exclusively used in purifying the flavonoids from the plant. The extracts were prepared by dissolving 1 mg of dried powder in 30 µl of organic solvent DMSO and making up the final volume to 1 mL using Phosphate Buffer Saline. The concentration of DMSO reaching the cells was non-toxic.

The treatment of the extracts was given for 48 hours. Thereafter, MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] (SRL) was added equally in all the wells and incubated for 4 hours, following which the formazan crystals formed by the mitochondrial activity of live cells were dissolved in DMSO. Absorbance was measured at wavelength 570nm with a differential filter of 630nm using the Multiskan Micro plate Spectrophotometer (Thermo Scientific). Percentage of viable cells was calculated using the formula:

Viability (%) = (mean absorbance value of treated cells)/(mean absorbance value of control)*100.

5.2.2.3 Bright field imaging and Dye exclusion assay

For bright field microscopic imaging, cells were cultured at density of 2×10^4 cells/plate in 6 cm culture dishes, treated with flavonoids enriched fractions from both the plants and then images were captured using Olympus (CKX41) microscope at

20 X magnification. To assess the number of dead cells versus the number of live cells, dye exclusion assay using trypan blue was performed. This assay is built on the principle that living cells possess intact cell membranes that exclude certain dyes such as Trypan Blue, whereas dead cells lose this capacity and hence retain the dye within the cell.²³² 0.4% of the concentration of Trypan Blue (procured from HiMedia) was prepared using molecular grade water. Equal volumes of cell suspension (diluted) and 0.4% of trypan blue solution were mixed in a tube and incubated at room temperature for 5-10 minutes. 20 µl of this mixture was loaded onto the Biorad Chamber slide and the number of live and dead cells were analysed using Biorad TC20 Automated cell counter.

5.2.2.4 Flow cytometric analysis of apoptotic cells

To calculate the percentage of apoptotic cells post-treatment by different extracts, flow cytometric analysis was done. HuH7 cells were seeded in 6 wells plate at a seeding density of 1×10^5 . Treatments with the flavonoid enriched plant extracts of concentration 200 µg/mL were given to cells for 24 hours. Cells were then harvested and sequential washes with 1X PBS were given. Cells were further resuspended in 500 µl of 1X Binding Buffer (BD BioSciences). Thereafter, 4µl of Annexin-V-FITC and 10µl of Propidium Iodide (PI) was added to cells and incubated in the dark for 20 minutes at room temperature. The samples were then acquired to a flow cytometer (Cytotflex, Beckmann Coulter) and analysis of acquired data was executed using CytExpert software. For the detection of early and late apoptotic cells, the lower (LR) quadrant cells which are representative of only Annexin, and upper right (UR) quadrant which is representative of Annexin V and Annexin V-PI positive cells both, were considered. The percentage of apoptotic cells was represented via a bar diagram.

5.2.3 Antibacterial activities of the purified extracts from pods/seeds of *P. cineraria* and *C. tetragonoloba*

5.2.3.1 Selection of the bacterial strains

The antibacterial activities of the extracts were checked in four different strains of bacteria belonging to non-pathogenic and pathogenic classes. For the non-pathogenic quality, *Escherichia coli* K-12 was selected and for pathogenic class, three different

strains were selected which are *Salmonella enterica subsp. enterica serotype Typhimurium* (ATCC 14028), *Shigella flexneri* (MTCC 1457), and *Klebsiella pneumoniae* (MTCC 618). All the bacterial strains were kindly gifted by Dr. Sandhya A. Marathe, BITS-Pilani, India. All the cultures were grown in LB-Agar media (Hi-Media Laboratories Pvt. Limited, Mumbai, India), and stocks were stored at -20°C in glycerol. For the experimental design, all the four bacterial strains were streaked from the glycerol stock on LB agar plates. The plates were then incubated at 37°C for 8-9 h. Special care was taken to ensure that experiments were performed under Biosafety hood only because of the usage of pathogenic strains. After the incubation, a single colony was picked from each strain plate and then inoculated in 5 mL of LB Broth and was grown overnight at 37 °C at shaking of 150 rpm to acquire an O.D. of 0.6 for starting the assay. The overnight culture was diluted to 1:100 using LB broth immediately before adding to 96 well plates.

5.2.3.2 Selection and preparation of purified extracts from pods/seeds of *P. cineraria* and *C. tetragonoloba*

The selection of the extracts was made on the basis of the phytochemical profiling of *P. cineraria* and *C. tetragonoloba* seeds/pods extracts carried out using TLC, HPLC, and LC-MS (Detailed discussion in Chapter 3). The combined results observed from all the analysis suggested that the fraction obtained after the post amberlite XAD7HP column chromatography found out to be most enriched with the compounds which have been already reported to exhibit antibacterial activities. Albeit, there does not exist any reports to best of our knowledge that antibacterial activities were checked in the flavonoid enriched methanolic extracts obtained from the seeds/pods of the *P. cineraria* and *C. tetragonoloba*. Also, the earlier studies which were done before using another kind of extract or part of the plant involved the research related to entirely different strains of pathogenic and non-pathogenic bacteria.

The enriched methanolic extract was thoroughly dried and stored in the falcons tubes. The high polarity of the compounds present in the enriched fractions eases the process of dissolving them in autoclaved water. Concentration between the range of 50-800 µg/mL of the extract was chosen and to achieve this, a stock of 1.6 mg/mL of solution was prepared. During the assay, appropriate dilutions were made to reach the final working concentrations.

5.2.3.3 Minimum inhibitory concentration

The recent years have seen a growing interest in investigating and developing new microbial agents from different sources, whether biological or synthetic ways, to combat the microbial resistance which has currently reached its peak in the microorganisms. This warrants the need to develop screening assays for testing the efficiency of chemicals and drugs in a short amount of time. In this regard, over the past few decades, several methods have been modified and many more have been invented to perform the antibacterial activity assays. A few of them include, disk-diffusion, well diffusion, broth or agar dilution, Thin-layer chromatography (TLC)-bioautography, Time-kill test, ATP bioluminescence assay, Flow cytometric method, etc. The last two methods are time consuming and involve the usage of specialized expertise and instruments to perform the experiments, whereas the ease of execution and obtaining results in less time make the other methods as most routinely used antibacterial activity assays in the laboratories around the world.²³³

In the current study, the broth dilution method was used to determine the MIC of the flavonoid enriched fractions obtained from the plants against four different bacterial strains. Briefly, 100 µl of two-fold dilutions of PC-FEF (*P. cineraria* flavonoid enriched fraction) and CT-FEF (*C. tetragonoloba* flavonoid enriched fraction) were dispensed in 96-well microtitration plate. Then, each well was inoculated with a microbial inoculum after adjusting its O.D to 0.6. After gentle mixing, the 96-well microtitration plate was incubated (without agitation) at 37 °C. Each treatment was done in technical triplicates. O.D at 600 nm were taken at different time points of 4h, 8h, 12h, and 24 h. Antibiotics Ampicillin and Kanamycin were considered as positive controls. The concentrations of the antibiotics to be added to different bacterial strains were designed based on the literature available. The table below states the concentration of the antibiotics chosen for the assay.

Table 5.1 Concentration of antibiotics chosen as positive control

S.No.	Bacterial strain	The concentration of Ampicillin ($\mu\text{g/mL}$)	The concentration of Kanamycin ($\mu\text{g/mL}$)	Reference
1	<i>Escherichia coli</i> K-12	16	32	234
2	<i>Shigella flexineri</i>	32	64	235
3	<i>Klebsiella pneumoniae</i>	16	16	236
4	<i>Salmonella Typhimurium</i>	16	16	237

5.3 Results and discussion

5.3.1 Antioxidant activities of extracts purified from pods/seeds of *P. cineraria* and *C. tetragonoloba*

Free radical scavenging potentials of test extracts, which include crude and enriched fractions of *P. cineraria* and *C. tetragonoloba*, at different concentrations (25, 50, 100, and 200 $\mu\text{g/mL}$) was tested by the DPPH method and the results are shown in Figures 5.2-5.5. The standard curve was plotted using ascorbic acid and the graph is shown in Figure 5.1. IC_{50} values of the standard and plant extracts which indicate the concentration of the extract at which 50% radical scavenging activity is observed, is shown in Table 5.2. The IC_{50} value for ascorbic acid was observed as 18.64 $\mu\text{g/mL}$.

Table 5.2 IC_{50} values of ascorbic acid and different purified extracts

S. No.	Name of the Extract	IC_{50} Value ($\mu\text{g/mL}$)
1	Ascorbic Acid	18.64
2	<i>P. cineraria</i> crude extract	30.0
3	<i>P. cineraria</i> flavonoid enriched fraction	40.63
4	<i>C. tetragonoloba</i> crude extract	7.25
5	<i>C. tetragonoloba</i> flavonoid enriched fraction	111.75

From the results obtained (Figures 5.2-5.5), it is clear that the crude as well as enriched fractions, both showed considerable radical scavenging activities. The crude extract of *P. cineraria* showed high radical scavenging activity as compared to that of

the enriched fraction. The IC_{50} value for crude extract was observed as 30.0 $\mu\text{g/mL}$. The enriched fraction, which was obtained after the column chromatography step, showed an increased IC_{50} value of 40.63 $\mu\text{g/mL}$. The activity observed in the case of the enriched fraction was in a dose dependent manner. At the highest concentration (200 $\mu\text{g/mL}$), 86.36% of scavenging potential was observed. The difference observed in the scavenging potential of crude and enriched fractions could be accounted to the fact that the crude extracts consist of a vast number of secondary compounds that were exhibiting antioxidant properties. The purification step which was specific for the separation of flavonoids/isoflavonoids from the crude extracts might have steered the elimination of potential scavengers. A similar pattern of results was observed with the DPPH assay carried out using crude and enriched fractions of *C. tetragonoloba*. The crude extracts showed a maximum of 78.85% of radical scavenging at a concentration of 200 $\mu\text{g/mL}$ whereas the enriched fraction showed a maximum of 75.21% radical scavenging potential. However, the IC_{50} values showed that the crude extract was significantly more efficient as a scavenger as compared to the enriched fraction. The IC_{50} of crude extract for *C. tetragonoloba* seeds was obtained as 7.25 $\mu\text{g/mL}$, whereas for enriched fraction, 111.75 $\mu\text{g/mL}$ was observed as the concentration to express 50 % radical scavenging activity. This indicates the presence of potent scavengers in the crude extracts as against in the enriched fraction. A differentiation expression of phytochemicals in both the plants fostered the different antioxidant potential in the extracts which is evident from Table 5.2.

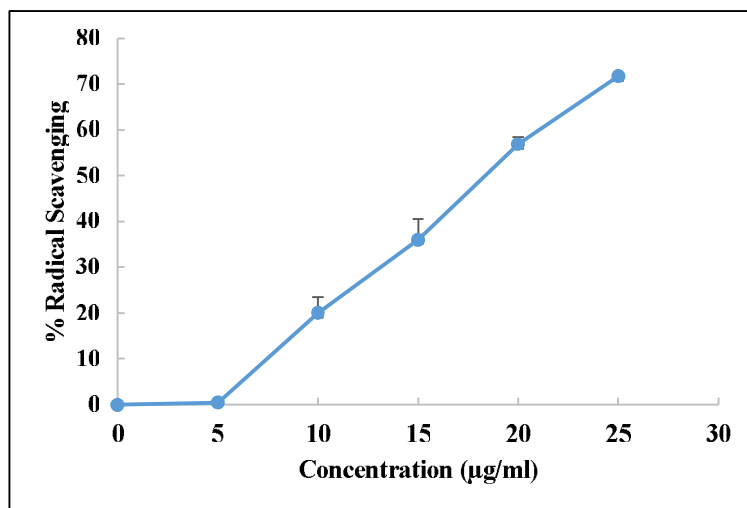


Figure 5.1 DPPH radical scavenging activity of standard ascorbic acid. The results are expressed as mean \pm SD (n=3)

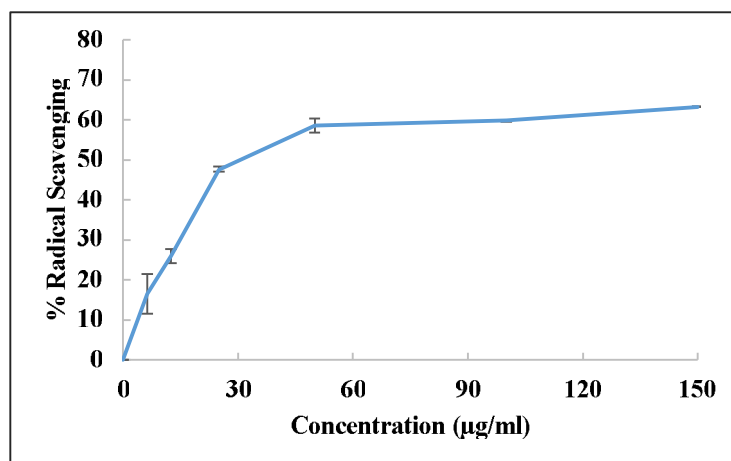


Figure 5.2 DPPH radical scavenging activity of *P. cineraria* pods crude extracts. The results are expressed as mean \pm SD (n=3)

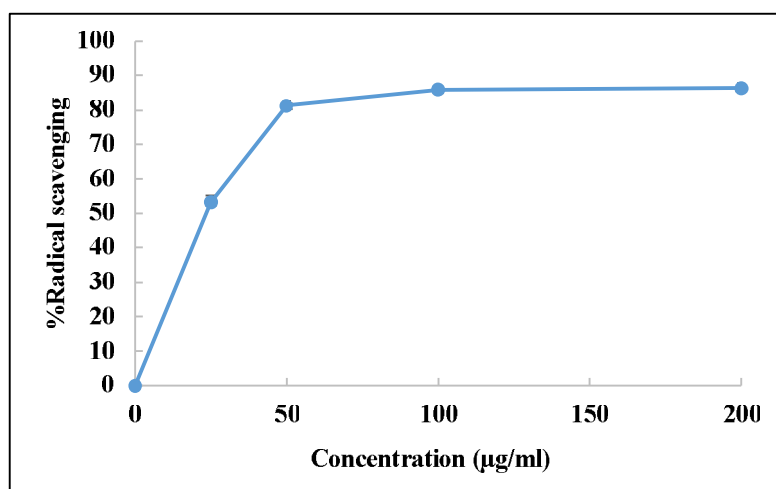


Figure 5.3 DPPH radical scavenging activity of *P. cineraria* pods flavonoids enriched fraction. The results are expressed as mean \pm SD (n=3)

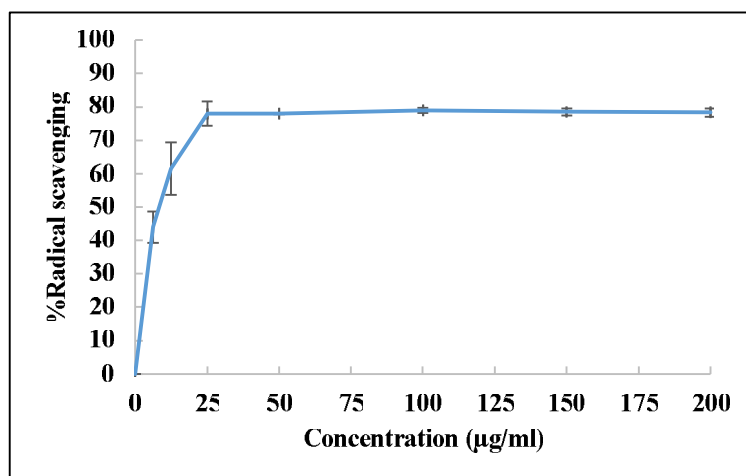


Figure 5.4 DPPH radical scavenging activity of *C. tetragonoloba* seeds flavonoids crude extracts. The results are expressed as mean \pm SD (n=3)

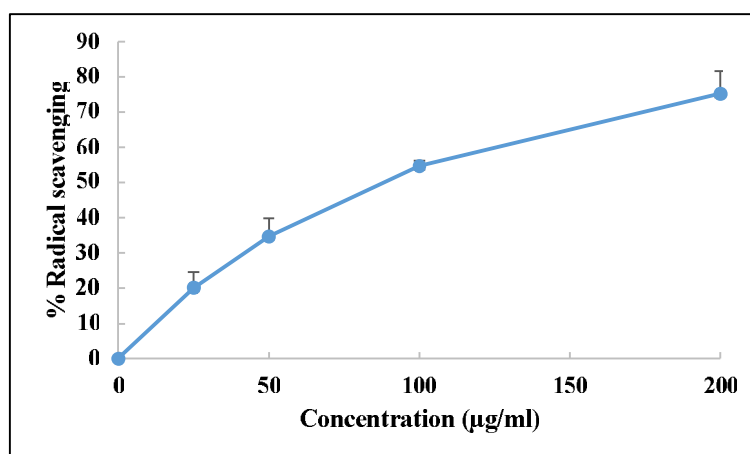


Figure 5.5 DPPH radical scavenging activity of *C. tetragonoloba* seeds flavonoids enriched fraction. The results are expressed as mean \pm SD (n=3)

The association between the role of diet and some chronic human diseases have been studied thoroughly for a very long time. Several epidemiologic and related experimental investigations have established that the antioxidants present in diet effectively neutralize the harmful free radicals.²³⁸ These free radicals are detrimental to cells and initiating diseases. Antioxidants, therefore, offer a protective mechanism for cells against a wide range of radicals and in the long term, can significantly decrease the disease occurrence.²³⁹ This is the reason that current markets are now filled with antioxidants supplements that have found an enormous consumer base and people are taking the supplements as a preventive measure. Besides this, antioxidants are also crucial in the food industry since they are added as natural or synthetic preservatives to weigh down the effect of oxidative deterioration on storage and

processing. Because of their low volatility and highly stable chemical nature, the antioxidants assist in preserving the level of nutrients, the color, freshness, taste, aroma texture, and functionality of the food. But there have been reports indicating that some of the commonly used synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene (BHA and BHT, respectively) need to be swapped with natural antioxidants because of their impending health threats and harmfulness.²⁴⁰ Considering the vast applications of antioxidants in general, the quest for isolating them from natural resources has garnered much attention, and apparent efforts have been made to identify new natural resources for active antioxidant compounds.

The antioxidant efficacy of two edible legumes *C. tetragonoloba* and *P. cineraria* of semi-arid regions was studied using DPPH assay. Despite the convenience in its execution and quick analysis, which it provides, the DPPH assay holds certain limitations regarding the reproducibility of the results. It has been suggested by many researchers that the DPPH assay has to be standardized accordingly by individual laboratory since the results vary profoundly from experiment to experiment. Most of the previously published protocols have followed an incubation time of 30 minutes for the reaction. In the current study, before analyzing the radical scavenging activity of the extracts, we standardized the protocol using ascorbic acid as a standard antioxidant, and 30 minutes as the incubation time for the reaction.²⁴¹ Amorati and Valgimigli (2015) reported that the stoichiometric nature of the reaction with antioxidants should be considered while setting up the experiment.²⁴² The reaction between the antioxidants and DPPH follows either first-order or pseudo-first order reaction depending upon the chemical nature of the reducing agents. As observed in Table 5.2, the IC₅₀ values for the crude defatted extract were found to be lower as compared to that of the enriched fractions for both the plants. This could be because of the loss of some critical compounds during the purification process which is present in their active form in the crude defatted extract but couldn't be obtained in the enriched fractions.

The antioxidant potential of the aerial parts such as barks, leaves (mostly inedible for humans) of *P. cineraria*, and *C. tetragonoloba* has been studied previously by many researchers. Soni *et al.* investigated the antioxidant potential in the chloroform, ethyl acetate and methanolic crude extracts of the bark of *P. cineraria*. There the IC₅₀ values were observed as 28.87 µg/mL, 26.20 µg/mL, and 25.50 µg/mL,

respectively.²⁴³ Napar *et al.*(2012) reported that methanolic crude extract of *P. cineraria* leaves was capable of exhibiting 60% radical scavenging activity for DPPH.²⁴⁴ Hydroethanolic extract of bark of *P. cineraria* was also found to increase the activity of catalase and glutathione peroxidase enzyme in diabetic mice, indicating that the phytochemicals present in the plant extracts can also induce antioxidant activity via inducing these enzymes.²⁴⁵ A comparative analysis of the radical scavenging activity of the leaf extracts of *P. cineraria* obtained in different solvents like petroleum ether, benzene, chloroform, ethyl acetate, methanol and water showed that the methanolic and ethyl extract showed the highest values viz 84.06% and 84.63 % respectively.²⁴⁶ A preliminary analysis that was done at the beginning of the current study to deduce the extraction capacity of various solvents showed that 80 % of methanol offers the best solvent for crude preparations. The values of the antioxidant potential obtained for the crude and flavonoid enriched fraction of pods of *P. cineraria* were found to be significant compared with those of the previous reports. It is evident that methanol is the best choice of solvents to extract the antioxidants from different aerial parts of the plant. One interesting observation that was perceived during the review studies related to biological activities is the lack of in-depth studies carried out with the seeds/pods of *P. cineraria* which are its edible portion for humans. Nevertheless, the current study showed that the seeds/pods of this edible legume are prominently rich in antioxidants properties which could be eventually probed for isolating the more purified, individual natural radical scavengers.

Similarly, the crude and enriched fractions of *C. tetragonoloba* also showed the presence of radical scavenging properties. *C. tetragonoloba* has a long tradition of being used in folklore medicine and has been known to act as a cooling agent, appetizer, laxative, and digestive aid.⁸⁷ Besides this, it is a commercially important plant with respect to guar gum production. Most of the biological activity studies have focused on crude extracts (mainly ethanolic) for e.g., Kobeasy and El-sala (2011) investigated the antioxidant activity of the crude ethanolic extract obtained from seeds where maximum scavenging of 30% was observed at a concentration of 100 ppm.¹⁹⁷ Another study involved the usage of ethanolic extract of fresh, young and tender pods of *C. tetragonoloba*, where it showed radical scavenging activity of 51.6%.²⁴⁷ Dietary intervention of tender pods were observed in high-cholesterol-fed rats where elevated ascorbic acid and glutathione concentrations and increased activities of antioxidant

enzymes, both in blood and liver were observed.²⁴⁸ The level of antioxidants activities was found more in crude extracts as compared to that of the enriched fraction suggesting a synergistic and additive effect of all the compounds present in the crude extracts.

5.3.2 Anticancerous activities of extracts purified from pods/seeds of *P. cineraria* and *C. tetragonoloba*

5.3.2.1 In vitro cytotoxicity assay of purified extracts against Huh7 cell line

The detailed phytochemical analysis of *P. cineraria* and *C. tetragonoloba* was discussed in Chapter 4. The results obtained after TLC and MS analysis revealed the presence of putative flavonoids in the purified extract. The lack of any published data regarding the effect of these compounds on the cell viability of the Hepatocellular carcinoma (Huh7) cell line generated our interest in performing the current study. Despite the fact that *P. cineraria* and *C. tetragonoloba* have been an integral part of folk medicine for a long time, negligible reports are present where a comparative analysis of the effect of crude as well as flavonoid enriched fraction on the cancer cell line was studied. Throughout this work, 80% Methanol has been the primary organic solvent used to perform the phytochemical extraction studies. The extract thus obtained was found to be more abundant in total phenolic and flavonoid content as compared to the extracts obtained via other organic solvents such as n-hexane, ethyl acetate, chloroform, and water. Hence the methanolic crude extract and the flavonoids enriched fraction eluted in methanol obtained after subjecting the crude extract to Amberlite-XAD7HP column chromatography were the ideal choice to proceed further with the cytotoxicity assays.

Figures 5.6-5.7 show the effect of different extracts on the cell viability of Huh7 cells. Different doses of PC-CE (*P. cineraria* Crude Extract), PC-FEF (*P. cineraria* Flavonoids Enriched Fraction), CT-CE (*C. tetragonoloba* Crude Extract), and CT-FEF (*C. tetragonoloba* Flavonoids Enriched Fraction), were used to treat the cancerous cells. This concentration was optimized between 50-200 µg/mL. All four extracts viz. PC-CE, PC-FEF, CT-CE, and CT-FEF were found to inhibit the proliferation of Huh7 cells in a dose-dependent manner. At the initial concentration, which was taken as 50 µg/mL, PC-CE, as well as PC-FEF, showed the absence of any cytotoxicity on Huh7 cells (Figure 5.6). Whereas at 50 µg/mL of CT-CC, there was a

5 % decrease in cell viability and for CT-FEF, there occurred a 10 % decrease (Figure 5.7). The highest cytotoxicity was observed at 200 μ g/mL of PC-CC treatment to cells where a reduction in 60% of cell viability was recorded. At 200 μ g/mL of PC-FEF treatment, 63 % of cell death was seen. Treatment is given using CT-CE and CT-FEF at a concentration of 200 μ g/mL resulted in a decrease of 52% and 63% of cell viability respectively. This is a significant decrease in the cell viability obtained for the Huh7 cell line. The IC₅₀, which is defined as the concentration of the drug or treatment at which 50% of the maximum inhibition occurs under *in vitro* conditions, was calculated for all the extracts. IC₅₀ is an indication of the effectiveness of a drug or compound in inhibiting a specific biological function. The IC₅₀ values for the extracts obtained from the experimental plants are as follows:

Table 5.3 IC₅₀ of the purified extracts of pods/seeds of *P. cineraria* and *C. tetragonoloba* against Huh7 cell line

Name of the extract	IC ₅₀ value in μ g/mL
<i>C. tetragonoloba</i> Crude (CT-CE)	>200
<i>C. tetragonoloba</i> flavonoids enriched (CT-FEF)	150
<i>P. cineraria</i> Crude (PC-CE)	175
<i>P. cineraria</i> flavonoids enriched (PC-FEF)	150

It can be seen from the above table that the IC₅₀ value of the extracts after 48 hours of drug exposure was found to be between 150-200 μ g/mL. The values were lower for the flavonoid enriched fraction as compared to that of the crude fractions indicating that some of the inhibitors of biological activity were removed during the purification process. The decrease in the cell viability obtained for the crude and flavonoids enriched fractions for *P. cineraria* and *C. tetragonoloba* are comparable with each other.

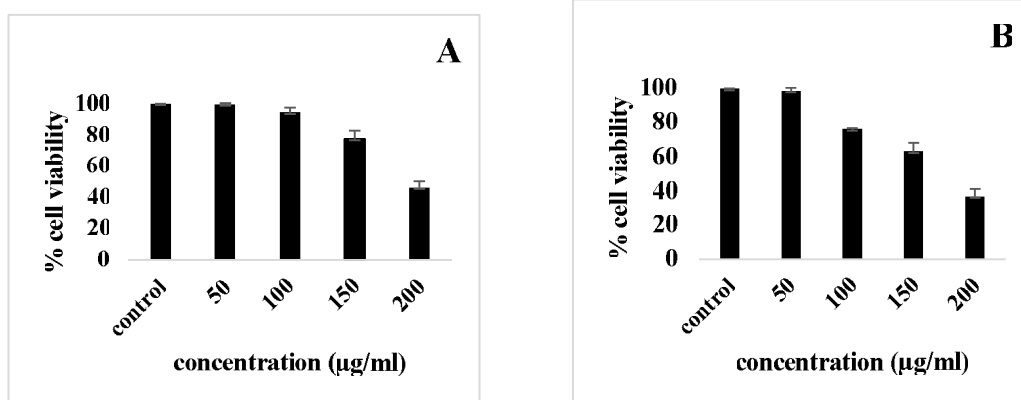


Figure 5. 6 Cytotoxic effect of *P. cineraria* extracts (A) crude , (B) flavonoid enriched fractions against Hepato cellular carcinoma cell line Huh7. Percentage growth inhibition of Huh7 cells was assayed at different concentrations of extracts (50-200 µg/mL). Data represent mean±SD of three replicates (p<0.05)

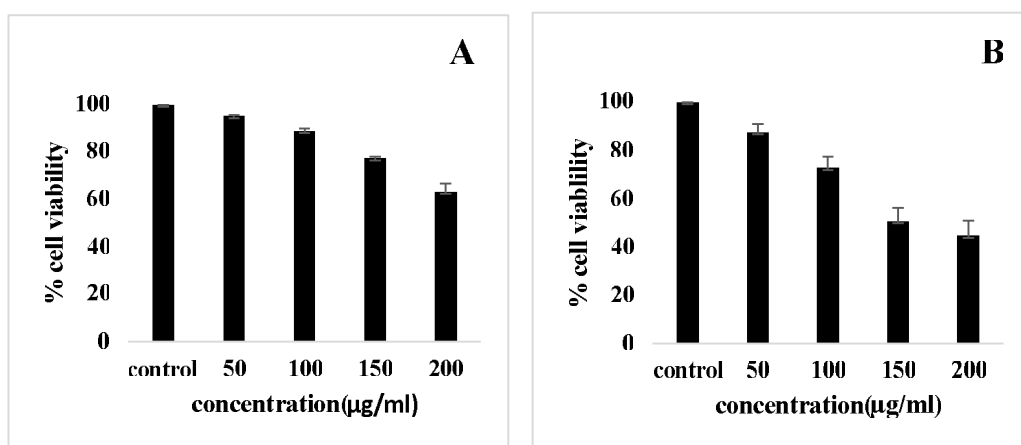


Figure 5. 7 Cytotoxic effect of *C. tetragonoloba* extracts (A) crude, (B) flavonoid enriched fractions against Hepato cellular carcinoma cell line Huh7. Percentage growth inhibition of Huh7 cells was assayed at different concentrations of extracts (50-200 µg/mL). Data represent mean±SD of three replicates (p<0.05)

5.3.2.2 Bright field imaging and dye exclusion assay

The MTT results indicated lower IC₅₀ values for the flavonoid enriched fractions; hence for bright field imaging and dye exclusion assays, Huh7 cells were treated for 48 h with the enriched fraction using a concentration of 200 µg/mL. Only a single dose was chosen to conduct the studies because the dose-dependent study of MTT analysis showed that maximum cell death occurred at a concentration of 200 µg/ mL. Phase contrast images of cells treated with the flavonoid enriched fractions showed

distinct rounding up of cells posttreatment, indicative of apoptotic cells (Figure 5.8 A-D). The decrease in cell viability observed during MTT assay and bright field imaging indicate that exposure of Huh7 cells to flavonoids enriched fractions might have triggered the pathways which result in apoptotic death. To confirm whether the cell death occurred because of apoptosis or necrosis, flow cytometric analysis was carried out with the treated and untreated samples which have been discussed in later sections. The number of the live cells and dead cells obtained after the treatment, as assessed by the trypan blue staining are shown in Figure 5.8 E. The graphical representation clearly indicates that there is a significant decrease in the viability of the Huh7 cells after 48 h treatment of the flavonoids enriched extracts. This shows that the compounds which have been extracted out from the experimental plants *P. cineraria* and *C. tetragonoloba* are capable of inhibiting proliferation in the cancer cells.

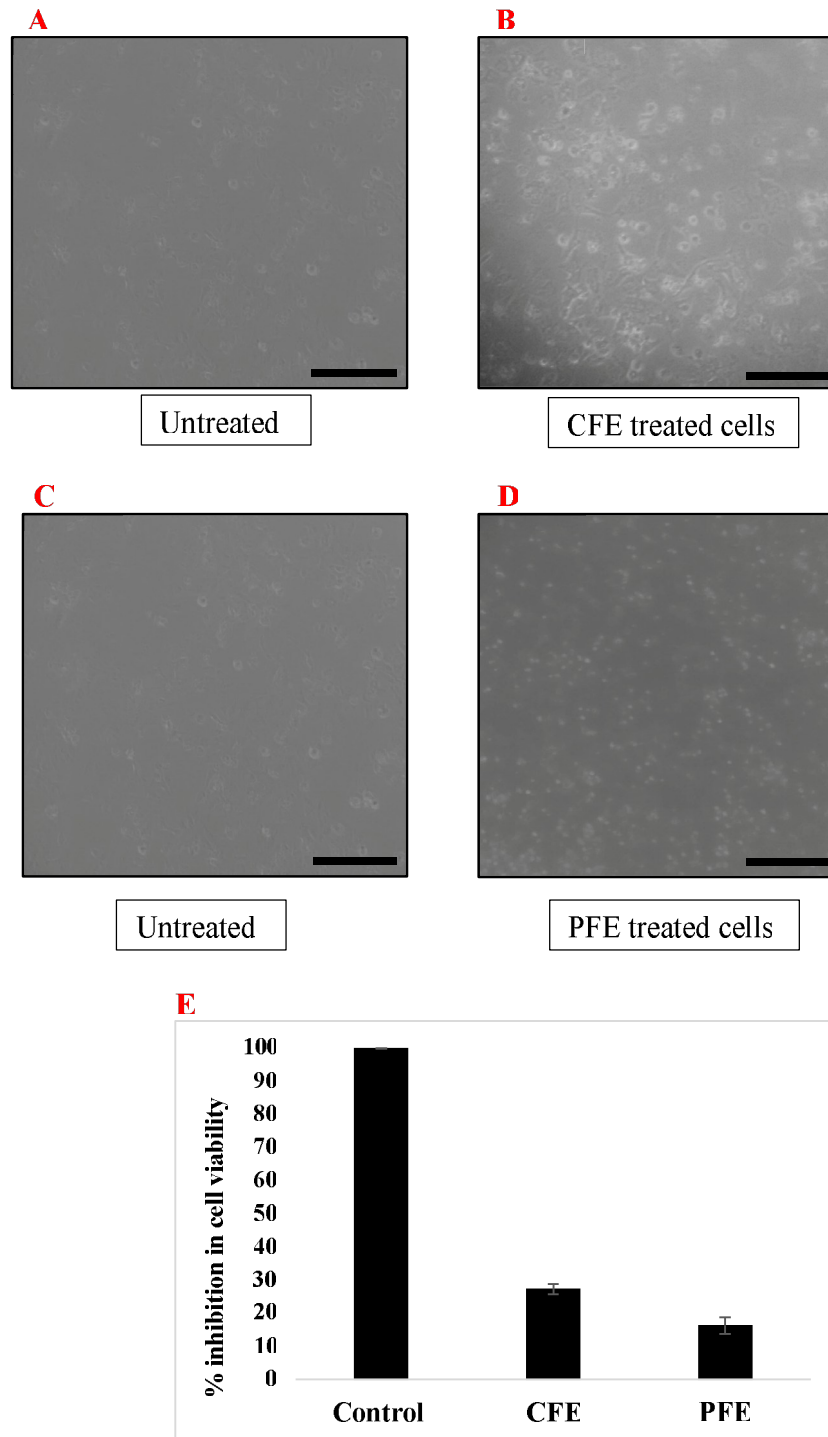


Figure 5.8 A-D) Phase contrast images of Huh7 cells were captured after treatment with flavonoids enriched fraction of *P. cineraria* (PFE) and *C. tetragonoloba* (CFE) for 48 h. Scale bar = 100 μ m. **E)** % inhibition in cell viability of Huh7 cells in untreated (control) vs treated cells using flavonoids enriched fraction of *P. cineraria* (PFE) and *C. tetragonoloba* (CFE) was checked using Trypan blue dye exclusion assay. The concentration of extracts was chosen at 200 μ g/mL. Data represent mean \pm SD of three replicates ($p < 0.05$)

5.3.2.3 Flow cytometric analysis of apoptotic cells

We got interested in analysing the occurrence of the apoptosis in Huh7 cells by flow cytometric analysis. Figure 5.9 represents the results obtained after the AnnexinV-PI staining assay carried out to study the apoptosis in the selected cells. The four sections of the quadrant in each sub figure of Figure 5.9 from the upper left in a clockwise direction represent necrotic cells, late apoptotic cells, early apoptotic cells, and live cells, respectively. Of these four kinds of cell modes, necrosis and late apoptosis are generally considered as cell death and thus, their values were omitted during calculating apoptotic cells.

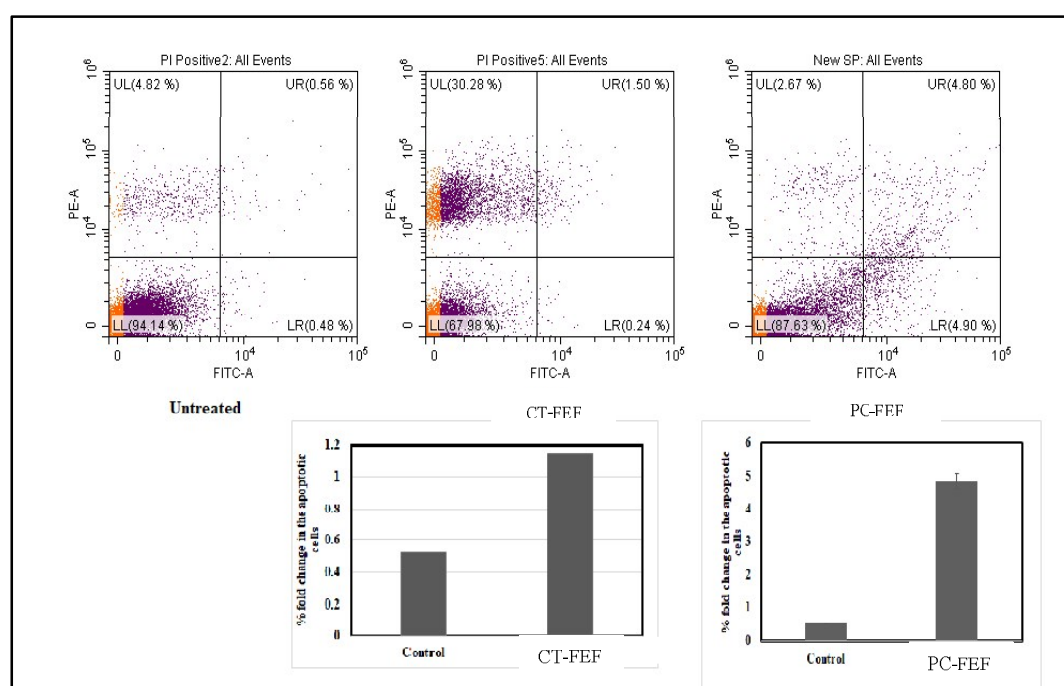


Figure 5.9 The Presence of apoptotic cells was evaluated by AnnexinV-PI staining after treatment of Hepato cellular carcinoma (Huh7) cells with CT-FEF and PC-FEF at 200 $\mu\text{g}/\text{mL}$ dose for 48 h through flow cytometry. In the figure, cells in the lower right and upper right quadrant represent early and late apoptotic cells, respectively. A fold increase in apoptotic cells was calculated with respect to untreated control (taken as an arbitrary unit “1”) and is represented through the bar diagram.

The number of the live cells in untreated samples, as observed from the lower left quadrant, accounted for 94.4% of the total count. The total cells that underwent necrosis are 4.82% whereas, as expected, only 1.04% of the cells comprised of the early and late apoptotic cells. When the number of the live cells was evaluated in case of the Huh7 cells treated with flavonoid enriched fraction, CT-FEF led to a drastic decrease to 67.98% in live cells whereas PC-FEF led to a decrease (87.63%, lower left

quadrants). Similarly, when the number of necrotic cells was compared in all the three samples, it was found to be 4.82%, 30.28%, and 2.67 % in Untreated, CT-FEF, and PC-FEF respectively. The right upper and right lower quadrants represent the early and late apoptotic cells correspondingly. The CT-FEF treated cells showed that 1.74% of cells were undergoing apoptosis post treatment for 48h, whereas the PC-FEF treated cells accounted for a total of 9.7% apoptotic cells. This was a significant increase in the total fold increase in the apoptotic cells as compared to the untreated cells. Taking into consideration into only the early apoptotic cells from the above data, the CT-FEF showed a 2.2-fold increase and PC-FEF showed a substantial 9.3-fold increase as against that of the untreated cells. The data obtained here implies that the compounds (mainly flavonoids) in the CFE were causing more necrosis whereas those of the PC-FEF were able to cause cell death by inducing apoptosis.

Evaluating the apoptosis inducing potential of the extracts showed that the PC-FEF was more efficient in inhibiting the Huh7 cells by apoptosis as compared to that of CT-FEF. Hepato cellular carcinoma is the fifth most common cancer in the world, causing more than 1 million casualties in world in a year.²⁴⁹ The conventional therapeutic regime is associated with an array of side effects, which has generated the interest to look for alternative treatments. A remarkable number of epidemiological investigations suggest that consumption of a diet rich in fruits and vegetables can reduce the risk of various cancers, and Hepato cellular carcinoma is no exception to this.²⁵⁰ An intensive review on the role of phytochemicals in the prevention and treatment of liver cancer by Bishayee *et al.*(2013) states that phenolics especially flavonoids, decrease tumor cell viability by inducing apoptosis.²⁵¹ The phytochemical profiling of the crude and enriched fractions of *P. cineraria* showed the presence of putative flavonoids such as Myricetin, Myricetin-8-Rhamnoside, Quercetin glycoside, Quercetin-3-Galactoside-Rhamnoside, Quercetin glycoside, and Isorhamnetin-glycoside. These compounds are reported to induce apoptosis in not only hepatocarcinoma but also other cancer cells by one of the several mechanisms, as discussed in section 1.5.3, which include inhibition of expression of Ras proteins, inhibition of cell cycle progression, regulation of cyclin D1 expression, as inhibition of STAT1 etc.

The cytotoxicity effect of the extracts obtained from different aerial parts (excluding pods) of the *P. cineraria* plant has been studied previously and reported in

the literature. Sumathi *et al.* (2013) showed that the crude methanolic extract of leaves of *P. cineraria* was showing a 70 % decrease in cell viability of MCF-7 cells as compared to untreated cells.²⁵² Interestingly, the cytotoxicity of the plant extract was in the range of that with the commercial drug tamoxifen, implying that the leaves are a good source of the phytochemicals which are able to induce cytotoxicity. Another study revealed that hydroethanolic extract of leaves was able to reduce tumor cell viability in N-nitrosodiethylamine induced Phenobarbital promoted experimental liver tumors in male Wistar rats.²⁵³ When the efficacy of hydroalcoholic extract of leaf and bark was tested in Swiss albino mice against an Ehrlich ascites carcinoma tumor model, the haematological parameters of the treated mice were comparable with that of the healthy mice.²²⁷ The investigation of anti-cancerous properties of *P. cineraria* was also extended to the green synthesis of silver and copper nanoparticles using the phytoextracts and it was observed that the phytochemicals bound nanoparticles were showing 10 % more cell death as compared to that of cell death induced by crude phytoextract indicating that there is an enhancement of the nanoparticles bioactivity via the compounds present in the *P. cineraria*.²⁵⁴

Similarly, the anti-proliferative potential of the crude ethanolic extracts of seeds of *C. tetragonoloba* has been previously tested against Acute Myeloblastic Leukemia and at a concentration of 100 µg/mL of the extract, approximately 40 % of cell death was observed.¹⁹⁷ Another study showed that hydroethanolic extract of guar seeds collected from the Egypt region, was inducing cytotoxicity on intestinal carcinoma (CACO-2) cell line, colon carcinoma cell line (HCT116) and human prostate carcinoma cell line (PC3) with IC₅₀ values of 101.1, 41.0, and 40.5 µg/ml respectively.²²⁶

The phytochemical investigation of the purified extracts of the *P. cineraria* and *C. tetragonoloba* had shown the presence of the compounds Vitexin, Peurarin, Phloridzin, Daidzein, Catechin hydrate, Myricetin-3-Galactoside, and Gossypetin-8-glucoside; all of which belong to the different sub-categories of flavonoids as mentioned in table 3.8 and table 3.9. All these compounds have been reported to induce cytotoxicity in other tumor cells via several mechanisms. Myricetin, which is an aglycone form of myricetin-3-Galactoside, is known to be of inhibiting JAK1/STAT3 pathways, thereby hindering cell transformation in EGF-activated mouse JB6 P+ cells.²⁵⁵ The compound was also found to inhibit DNA-binding and

transcriptional activity of STAT3, and thus stalling the phosphorylation of STAT3 at Tyr705 and Ser727. The constitutive activation of the STAT3 is associated with the cell survival of the healthy cells. The presence of the 3-, 3'- and 4'-hydroxy groups, C2-C3 double bond, and the phenylchromone skeleton were found to be crucial for the activity. Similarly, daidzein was shown to inhibit cell proliferation in choriocarcinoma cell proliferation by arresting the cell cycle, which could be facilitated by the suppression of expression of the ERK pathway. Hence, the presence of these flavonoids in the phytoextracts of the *P. cineraria* and *C. tetragonoloba* could confer the anticancerous properties against the hepato cellular carcinoma Huh7 cell lines by any of the discussed mechanisms which need to be explored further.

5.3.3 Antibacterial activities

The antibacterial activities of the PC-FEF and CT-FEF were checked against the bacterial strains *Escherichia coli*, *Salmonella enterica subsp. enterica serotype Typhimurium*, *Shigella flexneri*, and *Klebsiella pneumonia*. The absence of any previous reports regarding the efficiency of the flavonoids enriched extracts of the *P. cineraria* and *C. tetragonoloba* interested us in undertaking the current study. However, the enriched fractions did not show any inhibitory effect on the growth of the bacteria. The experiment had the technical replicates, and the entire experiment was performed three times; still, no antibacterial activity was observed. Time points of 4h, 8h, and 12 h were chosen, but even at 12 h, the growth of the bacterial cells remained unaffected for both plant extracts. 800 µg/mL was the highest concentration selected which is a significantly high value, but the absence of a decrease in bacterial cell viability even at this concentration indicates that the compounds which have been extracted in the enriched fractions through Amberlite XAD 7HP were lacking the antibacterial activities against the bacterial strains taken up for study.

Though the plant derived compounds have been reported extensively in the literature to possess the antibacterial activities against a wide range of pathogenic and non-pathogenic microorganisms, no reports were available for the antibacterial activities of the *P. cineraria* and *C. tetragonoloba* extracts against the bacterial strains undertaken for the current study. The absence of bioactivity could be attributed to the fact that the compounds present in the extracts were not able to act against the growth and regulatory mechanisms of the bacteria which is indicated by the unchanged color

intensity of the culture when compared between treated and control. A time dependent assay was also performed, and no activity was obtained even at 12 h time point.

5.4 Conclusions

From the multitude of assays performed and the studies documented previously, it could be said that the *P. cineraria* and *C. tetragonoloba* extracts obtained from seeds/pods are rich sources of antioxidants and anticancerous potential. When compared with the previously published data regarding the biological activities of other aerial parts of these plants, the activities present in edible seeds/pods were found significantly high. The increased resistance in the cancerous cells against a wide range of conventional treatments and the harmful side effect of the drugs has led the research for new substitutions. The dietary intervention of the phytochemicals on the disease prognosis and treatment has also been demonstrated extensively in diverse reports which makes establishing the phytochemical profile of edible plants much relevant. If investigating non-edible parts of the plants have facilitated in discovering the potential drug molecules against diseases, phytochemical profiling of the edible parts, moreover for the local plant species, plays a crucial role in recognizing their importance and extended utility for the local population. The vast flora and fauna of the Indian subcontinent offer a congregation of plant species which are yet to be explored. Thus, the present study provides substantial evidence on the biological potential of two regional legumes viz *P. cineraria* and *C. tetragonoloba*. Besides this, we have tried to see if the purification process carried out to obtain the flavonoids enriched fraction increases or decreases the biological activities pertaining to antioxidants and anticancerous strategies. We have observed that flavonoid enriched fraction had higher IC₅₀ values as compared to their crude counterpart for antioxidant activities indicating that the purification process might have caused the removal of some non-flavonoids compounds which were exhibiting a compounded antioxidant effect whereas an inverse observation was recorded while comparing the anticancerous activities of crude and flavonoid enriched fractions isolated from both the plants implying that significant flavonoids present in them have a significant anti-cancerous potential against the Huh7 cell lines. Further studies will be required to through light on the underlying mechanisms via which the flavonoids present in *P. cineraria* and *C. tetragonoloba* induce apoptosis in Huh7 hepatocellular carcinoma cell lines.



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Abstract

The family Leguminosae boasts of representing the third largest family in the plant kingdom with 745 genera and over 19,500 species and hence serves as a large reservoir of the secondary metabolites. One of the essential categories of the secondary metabolites in legumes is represented by the flavonoids and isoflavonoids. They belong to the phenolics class of secondary metabolites and constitute approximately 50% of the known phenolic compounds in plants. The lack of in-depth phytochemical and metabolic characterization of the two edible legumes *Prosopis cineraria* (L.) Druce and *Cyamopsis tetragonoloba* (L.) Taub., which is cultivated in the semi-arid region of Rajasthan, was addressed in the current study. We established a thin layer chromatography and UV-Visible spectrometry based fingerprinting profile for the samples collected from selected regions of Rajasthan. This helped in understanding the effect of geographical locations on the phytochemical profiling of the plants. Besides this, we also observed the effect of the ripeness stage of the edible plant part on its metabolite profile. The purification of flavonoid/isoflavonoid was then carried out using column chromatography techniques, which included the usage of Amberlite XAD7HP and Sephadex LH-20 matrixes. The purification process resulted in the isolation of the flavonoid/isoflavonoids from pods/seeds in their aglycone as well as glycoside conjugated which was showed first by the TLC analysis and then by liquid chromatography mass spectrometry. We concluded from this that flavonoids/isoflavonoids are present in two different forms within the plant system; one in aglycones form and the other being glycosides conjugated form. Vitexin, Puerarin, Phloridzin, and Daidzein were found to be significant flavonoids/isoflavonoids present in the pods of *P. cineraria*. Similarly, *C. tetragonoloba* pods showed the presence of flavonoid/isoflavonoid such as Catechin hydrate, Myricetin-3-Galactoside, Gossypetin-8-glucoside, and Puerarin (daidzein-8-C-glucoside). The *in-planta* functions of these metabolites include the protection against UV damage, pathogenic microbes, modulating auxin distribution, and acting as pigments or co-pigments in influencing flower coloration. However, when plants are not under stress, the spatial and temporal localization of these metabolites within the plant system is crucial to protect from self-damage. Also, it is to be noted that the nature of the metabolites should be polar enough to ease their storage within the plant vacuoles. This is facilitated by the glycosyl modification of the end products of the flavonoid synthesis pathway. When the plant cells experience the biotic or abiotic stress, specific glucosidases act on the stored forms of glycosylated flavonoid/isoflavonoids and release

the active aglycone form along with the sugar. This mechanism helps the plant cells in enforcing a prompt response against the adversities via bypassing the de novo synthesis of flavonoid/isoflavonoids. Since, out of the large diversity of plant kingdom, only members of the Leguminosae family have been reported to show the presence of isoflavonoids, we were interested in isolating and purifying the isoflavonoid conjugates hydrolyzing β -glucosidase (ICHG) from the unexplored edible legume of Rajasthan. In regard to this, we were able to purify ICHG from pods/seeds of both *P. cineraria* and *C. tetragonoloba* which was highly specific towards isoflavonoids conjugates Daidzin and Genistin, since activity against the other flavonoids was absent. The ICHG from *P. cineraria* was found to have pH optima of 4.5 and temperature optima of 50°C. The higher temperature optima could be attributed to an adaptation strategy by the plant cell for withstanding the higher temperature of the Rajasthan region. However, the enzyme could not be subjected to complete purification which is not an unprecedented case as far as enzymes of secondary metabolism are concerned. We also purified and biochemically characterized ICHG from *C. tetragonoloba* and its temperature and pH optima were found to be 37°C and 4.5 respectively. The effect of metal ions on the ICHG activity indicated that the enzyme was not metal ion dependent. Further N-terminal sequencing of the enzyme indicated that peptide sequences matched closely with the β -glucosidase from *Cicer arietinum* which has been reported to be an ICHG. Lastly, when the enzyme activity was tested with the natural substrate of flavonoid and isoflavonoid glycosides, the enzyme was found to be highly active and specific towards the isoflavonoids glycosides conjugates Daidzin and Genistin and didn't show enzymatic activity towards other flavonoids conjugates. This was validated by mass spectrometric analysis as well. Finally, we investigated the biological activities of the purified flavonoid enriched fractions (FEF) from the *P. cineraria* and *C. tetragonoloba* and checked their antioxidants, anti-cancerous, anti-bacterial properties. The antioxidant potential of FEF for these plants was found to be significantly higher than the values reported for other legumes. Moreover, the FEF(s) were found to exhibit anti-cancerous activity against the Hepatocellular carcinoma Huh7 cell line by inducing the apoptosis in cells. The isolated fractions were also checked for the antibacterial activities, but no activity was observed. The vast flora and fauna of the Indian subcontinent offer a congregation of plant species which are yet to be explored. Thus, the present study provides substantial evidence on the biological potential of two regional legumes viz *P. cineraria* and *C. tetragonoloba*.

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List of Abbreviations

AR -Androgen Receptor
CE -Capillary electrophoresis
CR-Chalcone reductase
CHI -Chalcone isomerase
CHS-Chalcone synthase
CT-CE -*C. tetragonoloba* Crude
CT-FEF -*C. tetragonoloba* flavonoid enriched fraction
CT-ICHG - *C. tetragonoloba* Isoflavonoid conjugates hydrolysing β -glucosidase
DPPH - α , α -diphenyl- β -picrylhydrazyl
ER-Endoplasmic reticulum
EGCG -Epigallocatechin gallate
EC- 3-O-octanoyl-epicatechin
ESI- Electrospray ionisation
FEF -flavonoid enriched fraction
GC-Gas chromatography
GH-Glycosyl hydrolases
5-HT- 5-hydroxytryptamine
HPLC -High Performance Liquid Chromatography
HRMS- High Resolution Mass Spectrometry
ICHG- Isoflavonoid conjugates hydrolysing β -glucosidases
IFS- Isoflavone synthase
LC-MS -Liquid chromatography-mass spectrometry
LDL -Low-density lipoproteins
MAO -Monoamine oxidase
MTT - [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide]
MRSA- Methicillin-resistant *Staphylococcus aureus*
NPR -natural product reagent
PNPG -para nitrophenyl - β -D- glucopyranoside
PC-ICHG- *P. cineraria* Isoflavonoid conjugates hydrolysing β -glucosidase
PC-CE -*P. cineraria* Crude extract
PC-FEF -*P. cineraria* flavonoid enriched fraction
PTKs -Protein tyrosine kinases

PSA -prostate-specific antigen

PVPP- polyvinylpyrrolidone

ROS -Reactive oxygen species

TLC -Thin Layer Chromatography

UPLC -Ultra-performance liquid chromatography



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CHAPTER 6 Thesis conclusions and future perspectives

6.1 Thesis conclusions

To the best of our knowledge, the current study is a first attempt made to carry out the region wise comparative analysis of flavonoid concentration in defatted methanolic extracts of pods of the edible legumes *P. cineraria* and *C. tetragonoloba* found in Rajasthan region using thin layer chromatography and uv-visible spectroscopy. Moreover, total flavonoid content was also evaluated in samples collected from different regions of Rajasthan. We found out that *P. cineraria* from the Pilani region (PC-F) i.e. 432 mg RH/gm of methanolic extract, and *C. tetragonoloba* also from Pilani region (CT-P) with a value of 556 ± 47.14 mg RH/g of the methanolic extract showed the highest concentration of the flavonoids. The previous phytochemical investigation reports available on these two legumes were limited either to other (non-edible) aerial parts of the plants or were carried out using a plant sample from a single geographical location. The values observed for *P. cineraria* and *C. tetragonoloba* were found to be higher than other members of Leguminosae which shall definitely fuel the purpose of establishing these legumes as a functional food with high nutraceutical value at the regional level.

The TLC fingerprinting analysis indicated that samples from the Pilani region had the highest concentration of flavonoids hence for further purification, defatted methanolic extracts from this region were chosen. A two steps purification process using Amberlite-XAD7HP and Sephadex LH-20 was established which was validated using the tlc analysis which showed that the enriched fractions obtained after elution from Amberlite-XAD7HP column had a significantly higher concentration of flavonoids. Similarly, the Sephadex-LH20 matrix which separates the compounds on the basis of molecular weight further facilitated the purification process. The identification of the putative flavonoids was then carried out using Liquid chromatography mass spectrometry technique. The purified extract from *P. cineraria* showed the presence of Vitexin (Apigenin- 8 C-glycoside), Puerarin (daidzein-8-C-glucoside), Phloridzin (phloretin-2'-O-glucoside), and Daidzein, which belong to different subcategories of flavonoids such as flavones, bicyclic flavonoids, and isoflavonoids. Similarly, *C. tetragonoloba* showed the presence of Catechin hydrate, Myricetin-3-Galactoside, Gossypetin-8-glucoside, and Puerarin (daidzein-8-C-glucoside) which fall under the dihydroflavonol, flavonol, and isoflavonoids subcategories of the flavonoids.

The ease of storage of the flavonoids/isoflavonoids within the plant system results from the biochemical modifications catalyzed by different enzymes. One such modification is the addition of sugar moiety in the newly synthesized flavonoids/isoflavonoids. This means that the primary form of the flavonoids/isoflavonoids storage in plant vacuoles is in their specific sugar conjugated form. Under stress conditions, specific β -glucosidases are released from the lysosomes which convert the flavonoid/isoflavonoid glycoside conjugates into aglycones and glucose as a byproduct. So far, the isoflavonoid conjugates hydrolyzing β -glucosidases (ICHG) have been isolated and characterized only from four legumes and hence the gaps present with respect to the biochemical characterization of novel glucosidases in the legumes of Rajasthan were addressed. The specific ICHG from *P. cineraria* (PC-ICHG) was purified and biochemically characterized. It was observed that the temperature optima for PC-ICHG were 50°C which was towards the higher range. pH optima were observed at an acidic pH of 4.5. The enzyme is usually localized in the lysosomes, tend to have an acidic environment for the optimal activity. When the activity of the PC-ICHG was checked against the natural flavonoids and isoflavonoids substrates, it was observed that the PC-ICHG was active against glycosidic conjugates of isoflavonoids; Daidzin and Genistin only and activity with other flavonoids conjugates was not observed. The purification and biochemical characterization of ICHG from *C. tetragonoloba* (CT-ICHG) showed that the pH optima were observed at acidic pH of 4.5 and temperature optima was found to be 37°C. The CT-ICHG was found to retain 50 % of activity at a temperature as high as 50 °C, post which total loss of activity was there at 80°C. The study of effect of metal ions on CT-ICHG showed that cationic ions of metal Hg, Co, Mn, Mg etc had an inhibitory effect on the enzyme activity and since none of the metal ions showed an increased enzyme activity as compared to control, it was deduced that the enzyme CT-ICHG was not metal ion dependent. V_{max} and K_m were found to be 6.6 IU/mg glucosidase and 0.86mM, respectively. The molecular weight of the CT-ICHG was determined using gel filtration chromatography and SDS-PAGE which revealed that the protein weighs 150 kDa and is trimeric with subunits weighing 50 kDa each.

The purified CT-ICHG was subjected to peptide mass fingerprinting, and the N-terminal amino acid sequencing revealed that it belongs to the Family 1 glycoside hydrolases (GH). The sequences of the five resultant peptides were found to be closest

to those of specific ICHG from *Cicer arietinum*; hence it established the identity of the purified CT-ICHG. Furthermore, enzymatic activity with natural substrates showed that the CT-ICHG was able to cleave the isoflavonoid conjugates Daidzin and Genistin but was inactive towards the other flavonoid conjugates such as Naringin, hesperetin and rutin hydrate. Hence, we were able to isolate and purify the novel ICHG from the local legumes of Rajasthan.

The biological activities pertaining to anti-oxidant, anti-cancerous, and anti-bacterial potential were investigated in the defatted crude methanolic extracts (CE) and flavonoid enriched fractions (FEF) of *P. cineraria* and *C. tetragonoloba*. IC_{50} values for the PC-CE and PC-FEF were observed as 30.0 and 40.63 $\mu\text{g/mL}$ respectively. For CT-CE and CT-FEF, IC_{50} values were found to be 7.25 and 111.75 $\mu\text{g/mL}$. The lower values of IC_{50} for crude extracts indicated the compounds which might have been removed during the purification process were showing synergistic effects for the antioxidant activity. However, examining the anti-cancerous activity of different extracts on hepatocellular carcinoma cell lines Huh7, revealed that PC-CE and CT-CE had higher IC_{50} as compared to the FEF. PC-FEF and CT-FEF showed IC_{50} of 150 $\mu\text{g/mL}$ which was lower than the PC-CE (>200 $\mu\text{g/mL}$) and CT-CE (175 $\mu\text{g/mL}$) respectively. This indicated that some of the inhibitors of biological activity were removed during the purification process. Dye exclusion assay showed that as compared to the control treatment of Huh7 cells, the treated cell with PC-FEF and CT-FEF at a concentration of 200 $\mu\text{g/mL}$ showed inhibition of 20% and 25 % respectively. To analyze the occurrence of apoptosis in the treated Huh7 cells, flow cytometric analysis was carried out which showed that CT-FEF led to a drastic decrease to 67.98% in live cells, whereas PC-FEF led to a reduction of 87.63 in cell viability. However, further analysis indicated that 1.74% of CT-FEF treated cells were undergoing apoptosis and in the case of PC-FEF treated cells, 9.7% were undergoing apoptosis. Taking into consideration into only the early apoptotic cells from the above data, the CT-FEF showed a 2.2-fold increase and PC-FEF showed a substantial 9.3 times fold increase as against that of the untreated cells. The data obtained here implies that the compounds (mainly flavonoids) in the CFE were causing more necrosis whereas those of the PC-FEF were able to cause cell death by inducing apoptosis.

Figure 6.1 represents the schematic diagram of the correlation between the enzymatic and metabolic pathways involved in the flavonoids' metabolism with their industrial applications and biological activities towards humans. In summary, we concluded that flavonoids/isoflavonoids were present in conjugated as well as non-conjugated forms in the *P. cineraria* and *C. tetragonoloba* and specific ICHG were purified from them. However, the presence of the enzyme and substrate is mutually exclusive, and a plant may have the enzyme activity but not the suitable substrate. This is a resultant strategy of the phenomenon termed as Silent Metabolism. The novel compounds isolated from the plants can open up the possibility of novel molecules for drug discovery.

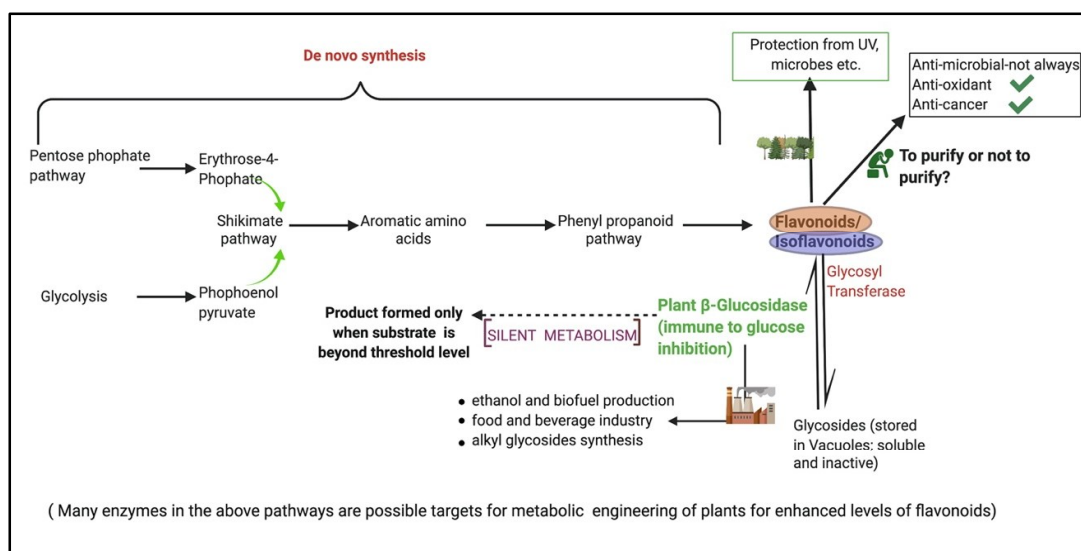


Figure 6.1 Schematic representation showing the pathways involved in flavonoids/isoflavonoids metabolism and their applications. Flavonoids/isoflavonoids are derived from aromatic amino acids via the phenyl propanoid pathway and get eventually stored in vacuoles after converting into glycosides. β -glucosidases act on the conjugates glycosides and release back aglycones form which confers protection in plants under stress conditions. The enzyme can often manifest silent metabolism which implies that enzymatic activity and subsequent product formation depends upon the threshold level of the substrate within the plant system. Besides this β -glucosidases purified have several industrial applications as illustrated above

6.2 Future perspective

1. Further purification and isolation of individual flavonoids using preparative high-performance liquid chromatography or preparative thin layer chromatography will result in obtaining flavonoids in higher quantity, which can be used in studying the biological activities.
2. The insights into mechanisms via which the purified flavonoids/isoflavonoids show the antioxidant and anticancerous activities can be explored.
3. Metabolic engineering of the edible pods with an aim to increase the flavonoid content by targeting one/more enzymes of the multiple pathways, as mentioned in Figure 6.1, will be of immense nutraceutical value to the human population.



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APPENDIX

A.1. List of publications

Publications from thesis

- Asati V, Sharma PK. Purification and characterization of an isoflavones conjugate hydrolyzing β -glucosidase (ICHG) from *Cyamopsis tetragonoloba* (guar). *Biochem Biophys Rep.* 2019; 20:100669
- Asati V, Sharma PK. Using thin layer chromatography for metabolic characterization of an isoflavone glycoside-specific β -glucosidase in the edible legume *Cyamopsis tetragonoloba*. *Journal of Planar Chromatography-Modern TLC.* 2018;2: 169-172
- Asati V, Sharma PK. Identification of an isoflavone glycoside hydrolyzing β -glucosidase in the seeds of the desert legume *Prosopis cineraria*. *Legume Research.* 2018;6: 837-841

Other Publications

- Vidushi Asati and Badri Narain Pandey (October 2016) “Studies on level of cytokines and expression of connexin43 in tumor and normal cells in culture conditions”. **Journal of Radiation and Cancer Research**

Publications in Pipeline

- Vidushi Asati and Pankaj Kumar Sharma “Development and validation of HPTLC method for simultaneous detection and identification of putative flavonoids/isoflavonoids and their glycosides conjugates from legumes”
- Vidushi Asati, Abhilasha Srivastava, Sudeshna Mukherjee and Pankaj Kumar Sharma. “Comparative analysis of antioxidant and antiproliferative activities of crude and purified flavonoid enriched fractions of pods/seeds of two desert legumes *P. cineraria* and *C. tetragonoloba* [SEP]” (Communicated in Food Chemistry)

Publications in Conferences

- “Scripting success tales for Indian biodiversity by validating phytochemical and metabolic potential of local edible plants”, Plants of The Future, 13th -14th June 2019 at New York University, New York, USA. **(DST-SERB Travel Grant Award)**
- “It's never too late: Phytochemical and metabolic studies of local edible legumes with a vision to arrive at their medicinal potential”, 1st National Biomedical Research Competition, 15th October 2018 at All India Institute of Medical Sciences, Rishikesh. **(Best paper presentation Award)**
- “Phytochemical fingerprinting of the pods of edible legume *Cyamopsis tetragonoloba* (Guar) of different regions of Rajasthan”, 24th ISCB International Conference (ISCBC-2018), 11th -13th January 2018 at Manipal University, Jaipur

- “Extraction and identification of secondary metabolites from seeds of *Cordia dichotoma* (Lassora)”, 5th Biennial International Conference on New Developments in Drug Discovery from Natural Products and Traditional Medicines, 18th-20th November 2016, NIPER, Mohali (presented as Co-author)
- “Studies on level of cytokines and expression of connexin43 in tumour and normal cells in culture conditions”. International Conference on Radiation Research: Impact on Human Health and Environment (ICRR-HHE), 11th-13th February 2016 at BARC, Mumbai
- “Purification and characterization of isoflavone conjugate-hydrolyzing β -glucosidase (ICHG) from *Cyamopsis tetragonoloba*”, 11th National Research Scholars Meet in Life Sciences, 17th-18th December, 2015 at ACTREC, Mumbai
- “Reporting a novel isoflavones glycoside hydrolyzing β -glucosidase from *Prosopis cineraria*”. International Conference on Frontier Discoveries and Emerging Opportunities in Life Sciences, 13th-14th February, 2014 at Dr. Harisingh Gour University, Sagar, M.P

A.2. List of workshops

- 7 days short term course on “Bioactive Compounds from Natural Sources and their Healthcare Applications” during 8th-14th January 2018 at National Institute of Technology, Durgapur
- 5 days International workshop on Modern Biophysical Tools and Techniques during 11th-15th December 2017 at IIT Bombay, Mumbai
- 2 days training course on Fundamentals of HPLC with Lab solution workstation during 12th-13th October 2017 at Shimadzu Analytical India Pvt Ltd, New Delhi
- 5 days National workshop on Cheminformatics during 1st-5th July 2017 at Department of Mathematics, MANIT-Bhopal
- 3 days Workshop on the applications of HRMS and LC-HRMS/MS instruments for the analysis of natural products (small molecules) during 27th-29th March 2017 at CSIR-CDRI, Lucknow
- 2 days Seminar and Workshop on Liquid Chromatography and Tandem Mass spectrometry during 23rd-24th August 2016 at Department of Pharmacy, BITS-Pilani, Pilani
- 2 days Workshop on Analytical Instruments for Chemical and Environmental Engineers (WAICEE-2015) during 27th-28th February 2015 at Department of Chemical Engineering, BITS-Pilani, Pilani
- 5 days Hands-on Workshop on Molecular Biotechnology and Bioinformatics during 24th-28th November 2014 at ICSSB, Pune

A.3. Biographies

Brief biography of the supervisor



Dr. Pankaj Kumar Sharma is currently working as Assistant Professor in the Department of Biological Sciences at BITS-Pilani since 2008. He did his M.Sc. (Hons.) Biological Sciences and M.E. Biotechnology from BITS, Pilani in the years 2000 and 2002, respectively. He received CSIR-NET(JRF) in year 2001 and completed his Ph.D in Biotechnology from Central Institute of Medicinal and Aromatic Plants, CSIR, Lucknow under the supervision of Dr. R.S. Sangwan; the degree was awarded by Uttar Pradesh Technical University, Lucknow in 2008. The title of the thesis was "Biochemical and Molecular Investigation of Biotransformation of Terpenoids in Selected Medicinal-Aromatic Plants". His research area includes enzymology and plant based secondary metabolites discovery. Besides this he is a prolific teacher and has done some teaching-based research, whereby he has developed some class activities to engage students in a proactive manner. Findings from his works on all these research areas have been published in reputed international scientific journals. At present he is guiding 2 Ph.D. students.

Brief biography of the student



Ms. Vidushi Asati has graduated in Biotechnology from Holkar College, Indore in 2010 and has completed her Masters in Biochemistry from Devi Ahilya Vishwavidyalaya, Indore in 2012. In year 2013, she joined under Dr. Pankaj Kumar Sharma, BITS-Pilani, Pilani Campus to pursue her doctoral research work. Her area of interest is enzymology, natural products discovery and metabolomics. She has presented poster in seven international conferences and has received travel award from DST and CSIR for presenting her research work in Plants of The Future, 13th -14th June 2019 at New York University, New York, USA. She has published 4 research articles in peer-reviewed journals. Along with research, she is passionate about teaching and interacting with undergraduate students. She was the Director appointed member for the Internal Complaints Committee, BITS-Pilani from 2017-2020. She is Editor of the annually published departmental magazine Biologue. When not doing science, she can be found binge watching F.R.I.E.N.D.S, browsing her kindle, daydreaming, reading poetry, or cooking herself a full course meal.



Identification of an isoflavone glycoside hydrolyzing β -glucosidase in the seeds of the desert legume *Prosopis cineraria*

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ABSTRACT

Legumes, in general, are rich in glycosidic isoflavone conjugates, whereby specific β -glucosidases act to release the free aglycones that could serve in plant-microbe interactions and defense. *Prosopis cineraria* is an important legume tree, bearing medicinally useful and edible pods, generally growing in extreme arid/semi-arid zones of Rajasthan. However, specific phytochemical-cum-metabolic profiles are lacking for the same. Therefore, the present investigation undertook phytochemical and metabolic screening of the pods/seeds of *P. cineraria* for the presence of putative isoflavonoids viz genistein and daidzein, their glycosides and β -glucosidase(s) capable of catalyzing the glycoside hydrolysis. Extraction and identification of these two aglycone forms of the above isoflavonoids were performed with solvent partition chromatography and Fluorescent/High Performance Thin Layer Chromatography, respectively. Furthermore, optimization of the isoflavone conjugate-specific β -glucosidase activity with respect to pH optima, temperature and time were carried out. The partially purified enzyme showed a temperature optima of 50°C and pH optima of 4.5. The enzyme also demonstrated activity towards natural substrates daidzin and genistin which are glycosides of isoflavonoids daidzein and genistein respectively. The methanolic extracts of the seeds of *P. cineraria* indicated the presence of related isoflavonoids which needs to be further validated.

Key words: Glycoside, β -Glucosidase, Isoflavonoid, *Prosopis cineraria*

INTRODUCTION

Prosopis cineraria (khejri) is an important legume tree growing in Rajasthan (India). The tree has immense historical significance as Bishnoi community near Jodhpur sacrificed themselves for its protection (Jain, 2011). Although medicinal properties viz. anticancer, antidiabetic, antioxidant etc have been attributed to its leaves, bark, flowers, roots as well as related agro-waste byproducts (Sharma *et al.*, 2010; Beniwal and Jood, 2014), the pods (known as *sangri*) are the real edible parts for humans. Nutrition-wise, these contain significant amounts of vitamin C (Rathore, 2009). However, today many farmers tend to cut down the tree for firewood and short term monetary gains (Narayan and Kumar, 2015). It is high time the validation of the phytochemical and biochemical aspects of the tree is undertaken by the scientific community to further its categorization as a nutraceutical food source.

Although scattered reports do occur in literature *vis-à-vis* the natural product categories (flavonoids, terpenoids, alkaloids) for different parts of khejri (Purohit *et al.*, 1979; Sharma *et al.*, 2010), studies pertaining to specific active metabolites and their *in planta* biotransformation are few. An important medicinally valuable class of (phenolic) compounds present in legumes is the

isoflavonoids. These can act as phytoestrogenic, anticancer, antibacterial, etc. (Chaukhayan *et al.*, 2007). Some well-studied legumes in this regard are *Glycine max* (soybean), whose seeds possess therapeutically important (anticancerous and anti-oxidant) isoflavonoids like daidzein, genistein and glycitein (Zhu *et al.*, 2005; Sinha *et al.*, 2013), and *Vicia faba* to name a few (Sinha *et al.*, 2013). However, since such phytoanticipins may be toxic to the synthesizing tissue itself, these are converted to 7-O-glycoside conjugates and stored in the embryo/seed coat/vacuoles (Lepiniec *et al.*, 2006). When the plant needs the active metabolite, indigenous β -glucosidases release both the aglycone and the sugar.

Besides soybean, the isoflavone conjugate hydrolyzing β -glucosidases have been reported from chickpea (Hosel and Barz, 1975) and Thai rosewood (Srisomsap *et al.*, 1996). In both cases, detailed characterization has been done and implications in plant defense aptly established. The present study was undertaken with a view to screen the pods/seeds of *Prosopis cineraria* for soybean-like isoflavonoids and corresponding enzymes involved in their metabolism. As per our knowledge, this is the first time an isoflavonoid conjugate hydrolyzing β -glucosidase is being reported from *P. cineraria*.

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Using Thin-Layer Chromatography for the Metabolic Characterization of an Isoflavone Glycoside-Specific β -Glucosidase in *Cyamopsis tetragonoloba*

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Key Words:

Cyamopsis tetragonoloba

Legume

Isoflavonoid

β -Glucosidase

Substrate specificity

Thin-layer chromatography

1 Introduction

Cyamopsis tetragonoloba (Taub), which is commonly known as cluster bean or "guar", is a drought-resistant annual leguminous herb which is cultivated throughout India, particularly in semi-arid regions. This plant is commercially grown for obtaining natural polysaccharide (galactomannan) also known as guar gum, from the endosperm of its seeds [1]. The pods including beans or seeds of this plant are edible and used as vegetable food by humans, both raw and in dried form. Another very important use of guar is in traditional medicine. It also acts as appetite-improving agent, laxative, digestive support, and cooling means and has also been reported to be useful in dyspepsia and anorexia. Apart from this, in-vitro studies on rats have demonstrated its anti-ulcer, hypolipidemic, cell-protective, hypoglycemic, and anti-secretory activities as well [2]. Some of these characteristics have been attributed to the presence of natural products of the polyphenolic class in the guar plant, e.g., daidzein, naringenin, caffeic acid, gallotannins, genistein, gallic acid, catechol, luteolin, chlorogenic acid, ellagic acid, myricetin-7-glucoside-3-glycoside, quercetin, catechin, 2,4,3-trihydroxybenzoic acid, kaempferol, rutin, hydroxycinnamic acid, texasin-7-O-glucoside, and *p*-coumaroylquinic acid, as reported in many studies [3–6]. The almost exclusive presence of isoflavonoids in legume seeds is another important point making them attractive candidates for

development as pharmaceutical and nutraceutical additives in foods [7, 8]. However, metabolic studies vis-à-vis the same, particularly with respect to biotransformation of the inactive metabolites into active ones, are lacking for many of the edible plants, including guar.

It is now well established that most of the bioactive metabolites are stored in the form of their conjugated glycosides in vacuoles where, at the time of their requirement, they can be acted upon by glycoside hydrolases which mainly includes specific β -glucosidases. Because of the involvement of these enzymes in plant defense/communication and cell wall metabolism, there has been a major focus on their research recently. The biotechnological applications of these enzymes extend to various industries like food, biofuel, surfactant, pharmaceutical, and agriculture [9]. The crucial point in the planta function of these enzymes lies in the hydrolysis of the conjugated glycosylated compounds into relatively more bioactive aglycones and sugar molecules. The aglycone part is significant to plant defense/communication, while the sugar moiety serves as an energy source for the growing seedling. The enzymatic action of these β -glucosidases could be general or specific because of the substrate preferences. Till date, considering the legumes, the enzymes specific for the cleaving of isoflavonoid glycosides have been isolated and characterized only from soybean, chick pea, and Thai rosewood [10, 11]. The characterization of such specific enzymes has highlighted their importance in maintaining metabolic homeostasis in plants. The current study was carried out with a view to (1) understanding the type of glycoside hydrolases present in the guar seed and (2) using thin-layer chromatography (TLC) for analyzing the substrate specificity for the β -glucosidase activity present in the same. It is worth mentioning here that putative isoflavonoids such as daidzein and genistein, as well as certain flavonoids, have already been reported in *Cyamopsis tetragonoloba* (guar) [3]. As per our knowledge, this is the first report of the presence of an isoflavonoid conjugate hydrolyzing β -glucosidase activity from *C. tetragonoloba*. Although thin-layer chromatography is extensively used for phytochemical characterization, the present work also highlights how it could be used for a preliminary metabolic characterization of plants.

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Purification and characterization of an isoflavones conjugate hydrolyzing β -glucosidase (ICHG) from *Cyamopsis tetragonoloba* (guar)

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Highlights

- A specific Isoflavones conjugate hydrolyzing β -glucosidase (ICHG) from *Cyamopsis tetragonoloba* (guar) was purified and characterized for the first time.
- The purified enzyme is characterized as a trimer with a molecular weight of 150 kDa
- The isolated enzyme can be further used in the bioengineering of the edible pods for the human consumption.

Abstract

A β -glucosidase with high specific activity towards isoflavone glycosidic conjugates was purified from seeds of Guar (*Cyamopsis tetragonoloba*) by ammonium sulphate precipitation followed by size exclusion and ion exchange chromatography. The pH and temperature optima of the purified Isoflavones conjugate hydrolyzing β -glucosidase (ICHG) were found to be pH 4.5 and 37 °C, respectively. The enzyme was relatively stable at higher temperatures. Effect of different divalent metal ions was studied and it was found that Cobalt and Mercury ions completely inhibited the enzyme activity. K_m and V_{max} of the purified isoflavones conjugates hydrolyzing β -glucosidases (ICHG) was 0.86 mM and 6.6 IU/mg respectively. The enzyme was most likely a trimer (approximate Mr 150 kDa) with potential subunits of 50 kDa. The purified enzyme showed activity against isoflavone conjugate glycosides viz daidzin and genistin but was inactive towards other flavonoid conjugates. The product conversion was confirmed by HPTLC and HRMS analysis. The MALDI-TOF analysis of the ICHG showed a score greater than 78 with 20 matches in MASCOT software. The five resultant peptides obtained had highest similarity in sequence with β -glucosidase from *Cicer arietinum*. The β -glucosidase from the *C. arietinum* has also been reported to exhibit the isoflavone conjugate hydrolyzing properties thus confirming the nature of the enzyme purified from the Guar seeds.



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