Phytochemical Investigation and Biological Evaluation of Secondary Metabolites from *Asparagus racemosus L* Through *In-vitro and In-silico* Approach

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In

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By

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CERTIFICATE

I declare that the dissertation entitled “Phytochemical investigation and Biological evaluation of Secondary metabolites from *Asparagus racemosus* through *in-vitro* and *in-silico* approach” has been prepared by me under the guidance of Dr. Vikas Jaitak, Assistant Professor, Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Phytochemical Investigation and Biological Evaluation of Secondary Metabolites from *Asparagus racemosus L* Through *In-vitro* and *In-silico* Approach

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Nature has been a source of medicinal products for millennia, and with many useful drugs developed from different natural resources, with majority of drugs are from plant origin. *Asparagus racemosus* belonging to family liliaceae, is one such important medicinal plant. This plant species is used traditionally in India and other parts of the world in epilepsy, *Vaat* disorders, brain tonic, hypertension, hepatoprotection, immunostimulant and antiabortifacient. In this context, the aim of the present study was to explore the roots of *A.racemosus* in terms of its medicinal values for instances antimutagenic, and advanced glycation end-product inhibitor. Antimutagenic activity of different extracts were evaluated using Ames test. *A. racemosus* methanolic extract (RME) and aqueous extract (RAE) have been found to have effective in the inhibition mutation induced by NPD and sodium azide. Among the two extracts, RAE and RME showed maximum inhibition of 49.2%, and 40.63% in Co-incubation mode respectively. The inhibition of BSA-glucose for the determination of antiglycation activity showed that the inhibition varied significantly among different extracts of *A. racemosus.* The highest inhibition measured by BSA-glucose was observed for (Ethyl acetate extract) REE (IC_{50} 37.56 ± 1.65 µg/mL) followed by (methanolic extract) RME (IC_{50} 51.32 ± 1.48 µg/mL). Isolation of molecules from methanol extract led to the characterisation of one molecule

namely nyasol out of total seven isolated molecules. The molecular docking study of isolated molecule Nyasol displayed strong binding affinity with estrogen receptor β and estrogen receptor α, indicating that Nyasol is beneficial in hormone responsive breast cancer. Moreover, *in-silico* study of already reported phytoestrogens from *A.racemosus* was also carried out using Glide docking to investigate interaction pattern with Human placental estrone sulphatases (1P49), human 17β-hydroxysteroid-dehydrogenase type 1 (1FDS), human glucose 6-phosphate dehydrogenase (2BH9) and tubulin protein receptors. The top docking score was obtained for rutin (estrogen receptor β), 3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl (HSP90), 8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside (human placental estrone sulphatase), Shatavarin X (17β-hydroxydehydrogenase`), Racemoside A (Glucose-6-phosphate dehydrogenase), Immunoside (Colchicine binding site of tubulin). The results indicated that phytoestrogens are likely potential candidate for controlling tumor progression with a special emphasis in breast cancer progression.

(Ramit Singla) (Dr. Vikas Jaitak) (Prof. P. Ramarao)

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Ramit Singla

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LIST OF ABBREVATION

Sr.No.	Full Form	Abbrevation
1	5-Hydroxytryptamine	5-HT
2	Advanced Glycation Endproduct	AGE
3	Arginine	ARG
4	Asparagine	ASN
5	Aspartic acid	ASP
6	Bovine serum albumin	BSA
7	Dopamine	DA
8	Distortionless Enhancement by Polarization Transfer	DEPT
9	Dihydroepiandrosterone	DHEA
10	Dimethyl sulphoxide	DMSO
11	Deoxyribo Nuclein Acid	DNA
12	1,1-Diphenyl-2-picrylhydrazyl	DPPH
13	Estrogen response element	ERE
14	Forced swim test	FST
15	Gamma linolenic acid	GLA
16	Glutamic acid	GLU
17	Hydrogen Bond	Hbond
18	Hormone Dependent Breast Cancer	HDBC
19	High Density Lipoprotein-Cholestrol	HDL-C
20	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
21	Histidine	HIS
22	Human Immunodeficinecy Virus	HIV
23	Heteronuclear Multiple Bond Correlation	HMBC
24	3-Hydroxy-3-methylglutaryl-coenzyme A	HMG-CoA
25	Heteronuclear Single Quantum Coherence	HMQC
26	Insulin-like growth factor 1	IGF-1A
27	Interleukin-1	IL-1
28	Isoleucine	ILE
29	InfraRed	IR
30	Leucine	LEU
31	Lipid hydroperoxides	LOOH
32	Lysine	LYS
33	Monoamone oxidase-A	MAO-A
34	Monoamone oxidase-B	MAO-B
35	Mitogen activated protein kinase	MAPK
36	Michigan Cancer Foundation-7 (Breast cancer cell line)	MCF-7
37	Maximal electroshock	MES

39	Nor-epinephrine	NA
40	Nicotinamide adenine dinucleotide phosphate-oxidase	NADPH
41	Sodium Hydrogen Exchanger-1	NHE-1
42	4-Nitro-*o*-phenylenediamine	NPD
43	Serine protease inhibitor of fibrinolysis	PAI-1
44	Phosphate buffered saline	PBS
45	Anti-thrombotic prostacyclin	PG-I_2
46	Phenylalanine	PHE
47	Pregnancy induced hypertension	PIH
48	Protein Kinase-c	PKc
49	Receptor for AGE	RAGE
50	Root mean square deviation	RMSD
51	Ribo Nucleic Acid	RNA
52	Reactive oxygen species	ROS
53	Serine	SER
54	Serum glutamic oxaloacetic transaminase	SGOT
55	Serum glutamic-pyruvic transaminase	SGPT
56	Thiobarbituric acid reactive substances	TBARS
57	Threonine	THR
58	Tumour necrosis factor-β	TNF-β
59	Tryptophan	TRP
60	Tail suspension test	TST
61	Thromboxane-A_2	TXA_2
62	Tyrosine	TYR
63	Valine	VAL
64	Vascular Cell Adhesion Molecule-1	VCAM-1
65	Vascular smooth muscle cell	VSMC

CHAPTER 1.0

INTRODUCTION

“The highest education is that which does not merely give us information but makes our life in harmony with all existence”

....**Tagore**

Chapter 1.0

Introduction

From the primeval times man has used plants to treat and alleviate diseases. Earliest written documentation of knowledge on medicinal properties of plants is found on Assyrian clay tablets dated about 2000 B.C (Potterat & Hamburger, 2008). In India, traditional medicine is well recorded and well-practiced. Plants provide rich source of phytochemicals and phytopharmaceuticals and have been used since antiquity to treat and manage different diseases (Shahidi & Ho, 2000). Recent data suggest that natural products are the key inspiration for the 80% of drug molecules (Harvey, 2008). Studies carried on the sources of new drugs from 1981 to 2007 revealed that as much as half of the drugs approved since 1994 were based on natural products. Natural products continue to be source of high value and potent antitumor drugs. Since 1940, 75% small molecules which were approved for the treatment of cancer were either natural products, semisynthetic derivatives of natural product, or synthetic compounds inspired by natural products pharmacophores (Newman & Cragg, 2012). The active plant constituents developed as a part of their own defence mechanism seems to contribute to man's health. Secondary metabolites are the group of natural compounds that, in contrast to Primary metabolites, are not directly involved in the growth, development or reproduction of organisms. Characteristically, secondary metabolites belong to diverse chemical classes and are regarded as representative of individual species (Chemotaxonomy). A German plant physiologist Kossel, in 1891 introduced the term "Secondary" in context of metabolism (Kossel, 1891). Phytochemicals are secondary metabolites in plants and many of them are incorporated into foods or used as food supplements or nutraceuticals or as pharmaceuticals that can function *in-vivo* to complement or boost the endogenous defense system. Thus functional foods, nutraceuticals or phytoceuticals capable of providing additional physiological benefits such as preventing or delaying the onset of chronic diseases are now as considered alternative health care. More than 5000 phytochemicals have been recognized in food and beverages (Bulku *et al.*, 2010). Secondary metabolites include diverse small molecules classes, such as alkaloids, terpenoids, glycosides or peptides. Over the years therapeutic applications of several medicinal plants have been scientifically proved and their active constituents have been identified. These include *Withania somnifera* which is found to be beneficial in arthritis, and is potent inhibitor of angiogenesis, inflammation and oxidative stress

(Mishra *et al.*, 2000). *Boswellia serrate* has been reported in Indian texts and studies demostrated to possess anti-inflammatory and anti-arthritic activities (Singh & Atal, 1986). *Curcuma longa* has been reported to be source of effective anti-inflammatory and anticancer agents (Bhutani & Gohil, 2010). *Rauwolfia serpentina* was first reported to be anti-hypertensive in India (Vakil, 1949). (Sumitra *et al.*, 2001). Natural products have been used as remedy for the diabetes and similar disorders. Charantin, a steroidal saponin isolated from *Momordica charantia* has been reported to have an insulin-like activity, which is responsible for its hypoglycaemic effect (Krawinkel & Keding, 2006). Gymnemic acid IV, obtained from *Gymnema sylvestre* has been reported to possess strong hypoglycaemic activity in animal models of diabetes comparable to glibenclamide (Sugihara *et al.*, 2000). Andrographolide, isolated from *Andrographis paniculata* exhibit significant hypoglycemic activity (Zhang *et al.*, 2009). *Stevia rebaudiana* has beneficial effects to diabetes patients (Jaitak *et al.*, 2009). Anticancer activity of Indian medicinal plants have been vastly studied. Tagitinin F, isolated from *Tithonia tagitiflora* has been reported to be active against lymphocytic leukaemia (Pal *et al.*, 1976). Combretastatins found in species of Combretaceae family have anti-cancer activity augmented by inhibition of tubulin polymerisation (Pettit *et al.*, 1989). In another important study, Paclitaxel (Taxol) (Figure 1) was isolated from the bark of *Taxus brevifolia*, which was collected from Washington State during a random collection program by the US Department of Agriculture for the NCI (Butler & Newman, 2008). It was the first compound identified that caused the stabilization of microtubules in the mitotic cycle. In 1993 Paclitaxel entered clinical use and in 1995 a semisynthetic derivative, docetaxel (Taxotere®), was approved initially for treatment of refractory ovarian cancer and then breast cancer. Abraxane®, albumin-bound paclitaxel nanoparticles, was approved for the treatment of breast cancer in 2005 (Desai *et al.*, 2006). With the advancement in knowledge, extraction techniques and modern instrumentation facilities, it is possible to extract, separate, isolate and characterise pure bioactive molecules and to understand their structures and therapeutic action.

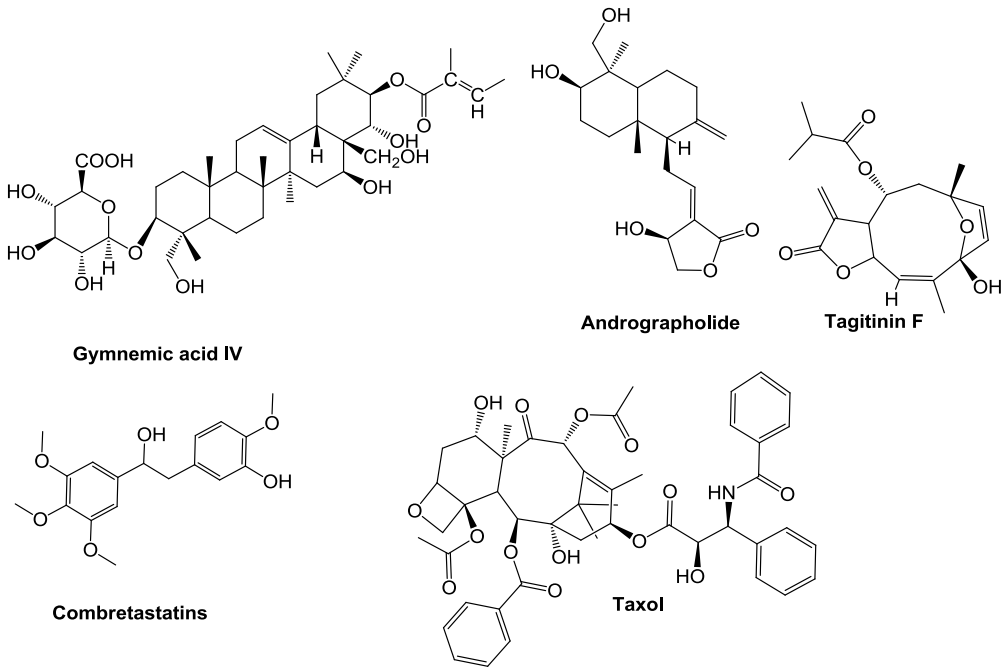

Figure 1: Structures of potent chemical entities of Natural origin

1.1 Process development in natural product

Natural products have proved to be a rich source of therapeutic agents. The process of drug discovery (Figure 2) from natural products involves first identification of the problem or the ailment followed by selection of the source. The source can be selected on the basis of traditional knowledge of the plant species for its therapeutic application. Once the plant species has been identified for a particular ailment, product is extracted concentrated, fractionated and purified to yield libraries of compound. The libraries of compounds are then subjected to in-vitro/in-vivo biological screening for the identification of bioactive compound. Identification of known compounds and avoidance of replication of previous efforts, is greatly supported by directly coupled HPLC-mass spectrometer (LC-MS) systems and natural-product databases (Strege, 1999). Structure determination of compounds which are novel has been modernized by recent advancement in the field of spectroscopic techniques, particularly high-resolution nuclear magnetic resonance technologies. Determination of the molecular formula is very important and is typically done by high-resolution mass spectrometry which required only microgram quantities of material. One of the most powerful technique is Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS), which has a capability of measuring molecular mass with exceptional accuracy (McDonald *et al.*, 2003). Still

the determination of complex structures is technically challenging, but it is no longer a major impasse in the drug discovery process from natural products. The cases in which the bioactivity meets the criteria for potency and selectivity the bioactive compunds are said to be hit and then, preliminary structure–activity relationship (SAR) studies will be carried out and the purification process is scaled up. Once the chances of modification in biological response through synthetic alteration is established, the hit is declared as a lead and then proceeded onward for additional optimization by conventional medicinal chemistry (Koehn & Carter, 2005). The lead enters the phase of drug development where its toxicological profiling and pharmacokinetic parameters are determined. Methods for the delivery system are developed by which it display maximum bioavailability. The drug candidate is then elevated to the level of clinical trials. Another process in the drug discovery is through *in-silico* approach. Facilities of HTS (high-throughput screening) is now available in academic labs as well as in drug companies. The cost of random screening of large libraries of natural product is very large and time consuming, so the use of *in-silico* approach helps in flltering down the number of compounds for real screening. If the 3D structure of the biological target is known, high throughput docking turned out to be a valuable structure-based virtual screening method to be used (Krovat *et al.*, 2005). Pharmacophore concept has proven to be extremely successful in rationalizing structure-activity relationships, but also in developing the appropriate 3D-tools for efficient virtual screening (Langer & Hoffmann, 2006). The pharmacophore models in biological screening of natural product is an resourceful procedure since it quickly excludes molecules that do not possess the required features thus leading to a dramatic increase of enrichment, when compared to a purely random screening experiment (Rollinger *et al.*, 2008).

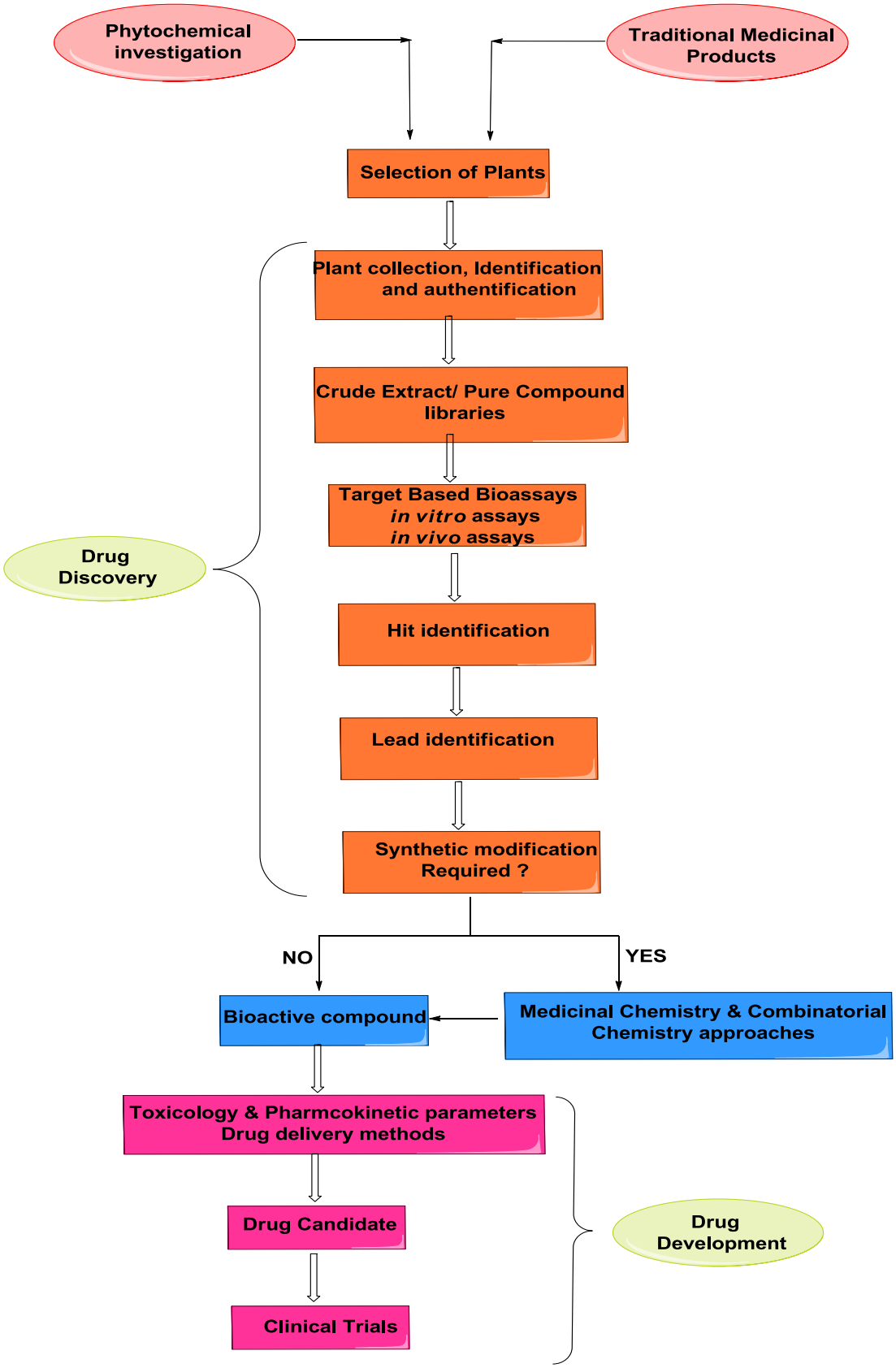

Figure 2: Drug discovery process from plant

1.2 Medicinal attributes

Natural products are diverse source of phytochemicals having an enormous therapeutic potential. Search for new molecules of biological and pharmaceutical importance from plants is an essential study in the field of natural products. These molecules are associated with antidiabetic, antimutagenic, anticancer and many other bioactivities.

Numerous products are marketed as “natural” agents for lowering blood sugar and management of long-term diabetic complications. These include Antibetic, Alphabetic, Diabetics, DB-7, Diabetica, Diabetiks, Dia-Comp, DiaVite, GlucoCare, Glucotize, GlycoNase, SugarMax, and Sugar Loss (Shapiro & Gong, 2002). It has been found that AGE-rich blood vessels show enhanced RAGE immunoreactivity. Evidence suggest that serum TNF-α levels were increased in non-insulin-dependent diabetes mellitus (Hotamisligil *et al.*, 1995; Hotamisligil *et al.*, 1993) and that TNF-α can activate the NF-κB pathway (Beg & Baltimore, 1996; Wang *et al.*, 1996). NF-κB has been reported to play a role in the basal and lipopolysaccharide-induced expression of the RAGE gene (Li & Schmidt, 1997). The AGE upon RAGE induces cellular oxidantive stress, thereby further activating the transcription factor NF-kB. Additionally, it has also been reported that diabetic vasculopathy is often aggravated during pregnancy, probably due to the increased level of serum estrogen (Axer-Siegel *et al.*, 1996; Klein *et al.*, 1990). A recent study demonstrated that that Estradiol act as an alternative inducer of the RAGE gene in human endothelial cells, enhancing its transcription, and this effect was regarded as an indication that estrdiol acts on RAGE gene through an estrogen receptor (Tanaka *et al.*, 2000). Also elevated levels of serum estrogen is associated with inceased incidence of breast cancer (Cauley *et al.*, 1999). Thereby the chronic exposure to AGE, TNF-α, andestradiol and sustained enhancement of RAGE expression causes a further accumulation of AGE in the vasculature, resulting in an exacerbation of AGE-RAGE-mediated vascular dysfunctions like increase VEGF expression in endothelial cells and/or retinal pigment epithelial cells (Lu *et al.*, 1998; Yamagishi *et al.*, 1997), resulting in angiogenesis and high risk of breast cancer. Phytoestrogens like genstein, act as competitive inhibitor of estradiol (Morito *et al.*, 2002). Thereby preventing the estrdiol mediated elevated RAGE overexpression, which in turn

reduces the oxidative stress and prevents oxidative damage like mutagenisis and cancer (Cooke *et al.*, 2003).

1.2.1 Advanced Glycation End-product inhibitor

Hyperglycaemia causes diabetic complications and four hypothesis which are increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. Intracellular and extracellular AGE are initiated by hyperglycaemia is the primary initiating event. Intracellular auto-oxidation of glucose to glyoxal, decomposition of the Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosone, and disintegration of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal. The reactive intracellular dicarbonyls viz. glyoxal, methylglyoxal and 3-deoxyglucosone react with amino groups of intracellular and extracellular proteins and leads to the formation of AGE (Brownlee, 2001; Thornalley, 1990). AGE accumulation causes lipid peroxidation and endothelial dysfunction due to the increased production of the ROS and decreased production of superoxide dismutase. AGE causes vasoconstriction by decreasing the nitric oxide production and increased in endothelin-1. Tissue remodelling and thickening of the basement membrane is resultant of the elevated expression of VCAM-1(Vascular Cell Adhesion Molecule), increased secretion of IL-1, TNF-β, IGF-1A, increased mitogenesis, chemotaxis of mononuclear cells, stimulation of the T-cells and Interferon-γ production. AGE brings about some structural changes which brings about premature ageing in diabetes mellitus. AGE causes irreversible cross-linking between the protein; glomerulosclerueosis in diabetic kidney due to the cell membrane and matrix changes. AGE has also had a profound effect on the thrombosis and fibrinolysis. AGE increases tissue factor, decreases thrombomodulin; increased platelet aggregation and fibrin stabilization due to the elevated PAI-1 (serine protease inhibitor of fibrinolysis) and reduced PGI_2 (anti-thrombotic prostacyclin), decreased platelet survival, increased platelet stickiness due to the glycation of platelet glycoprotein receptor IIB and IIIA(R. Singh *et al.*, 2001). The natural products are extensively studied for their AGE inhibitory activity. The roots of *Averrhoa carambola* L. (Oxalidaceae) is a traditional Chinese medical plant used in treating diabetes and diabetic neuropathy. 2-Dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione isolated from the roots of plant increased the

levels of superoxide dismutase and glutathione peroxidase, which are reduced in the renal injury mediated by AGE in type 2 diabetic KKAy mice (Zheng *et al.*, 2013). Polydatin and resveratrol isolated from the *Polygonum cuspidatum* an Chinese medicine exhibited effective Methyglyoxal trapping capacity indiacting that these could significantly inhibit the formation of AGE, thus can serve as a prospective candidate for postponing and preventing diabetic complications (Tang *et al.*, 2013). *Osmanthus fragrans aurantiacus* extract and acteoside as an active component inhibited formation of AGE and shown to promoted its degradation(Oto & Iwahashi, 2013). *Curcuma longa* L., a traditional herbal medicine, has a preventive role in diabetic vascular complications. The findings suggested that curcumin and demethoxycurcumin was effective in AGE-induced oxidative stress and apoptosis, which were associated with the damage to mesangial cell and concluded that these molecules have an potential preventive role in diabetic neuropathy(Liu *et al.*, 2012). A furanocoumarin Isoimperatorin isolated from the *Angelica dahurica* showed strong inhibitory activity against the formation of AGE (Kim *et al.*, 2012b). 3,4-Seco-lupane type triterpenes chiisanogenin isolated from *Acanthopanax senticosus* exhibited strong inhibitory activity against the formation of AGE (Kim *et al.*, 2012a).

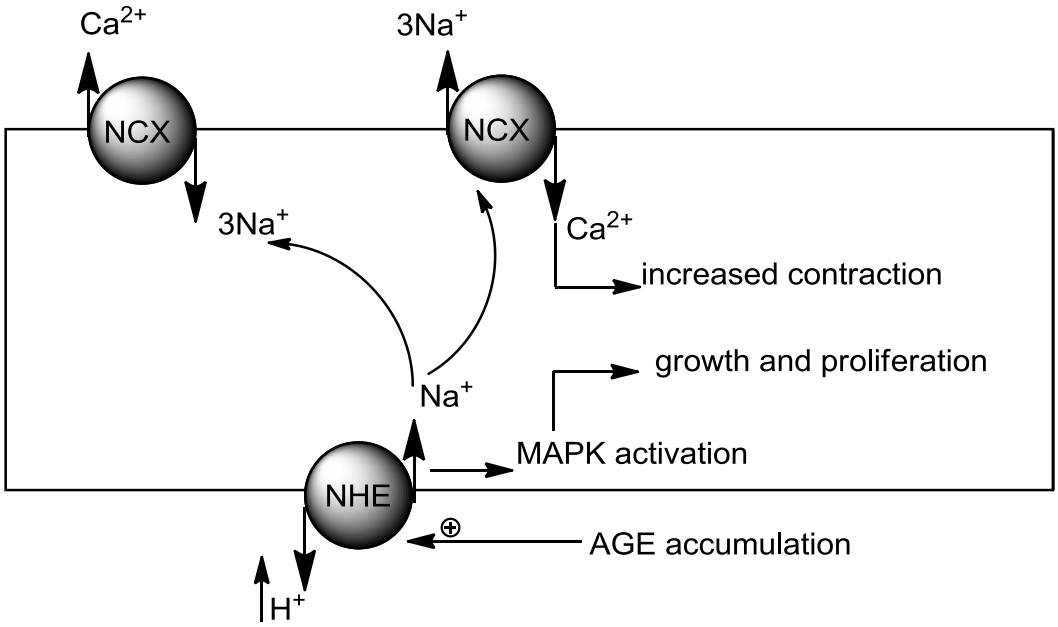

Figure 3: Role of AGE in the hyperglycaemia induced diabetic complication

The data signifies that the natural products are promising source of phytochemicals which have a potential AGE inhibitory activity, thus helping in treating glycation

associated diabetic complications. Hyperglycaemia induces the production and accumulation of advanced glycation end products (AGEs), which induces the MAPK (Mitogen activated protein kinase) activation via binding to RAGE (the receptor for AGEs), thus promoting the VSMC proliferation (Yamamoto *et al.*, 1996). It has been observed that in diabetic cases, activity of NHE-1 is elevated and involved in the signalling pathway of VSMC proliferation (Figure 3). It has been demonstrated that AGE persuades VSMC proliferation by triggering NHE-1 via AGEs/RAGE-mediated MAPK pathway (Wu *et al.*, 2008).

1.2.2 Antimutagenic activity

A mutation is defined as a change of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal genetic element. Mutations is an resultant of unrepaired damage to DNA or to RNA genomes caused by radiation, chemical mutagens, errors in the process of replication, or insertion or deletion of segments of DNA (Bertram, 2000). Antimutagenic properties of the natural plant products is useful in phytotherapy because these compounds serve as an beneficial alternatives to traditional therapeutics that possibly will accomplish clinical goals with reduced adverse events. *Viscum album* L. var. coloratum agglutinin known for its anti-cancer activity (Hong & Lyu, 2012), *Baccharis articulate* used for gastrointestinal and liver disease treatments in Southern Brazil (Rodriguez *et al.*, 2011), *Belamcanda chinensis* plant used as a phyto-estrogenic and chemopreventive agent (Wozniak *et al.*, 2010), *Myrtus communis* used in the treatment of lung disorders (Hayder *et al.*, 2003) are the example of natural plants having antimutagenic activity by capturing the free radicals generated. The Cytochrome-P450 causes the biotransformation and activation of 2- amino fluorine(Steele *et al.*, 1985) induces mutation in the TA98 and TA100 strains of *Salmonella typhimurium*.

1.2.3 Natural product and breast cancer

Natural products are been rapidly accepted by consumers for various remedial or preventive purposes, including breast cancer prevention. Palmatine an alkaloid isolated from *Guatteria friesiana* exhibited a cytostatic effect against on MCF-7 (breast cancer cell line) (Costa *et al.*, 2013). Isobractatin isolated from *Garcinia bracteata* has been shown to have antiproliferative effect on human breast cancer cell lines, the antiproliferatrive effect is exerted via cell cycle arrest and induction of

apoptosis (Shen *et al.*, 2013). Furanodiene, isolated from *Curcuma wenyujin* showed anti-cancer effects in breast cancer cells *in-vitro* as well as *in-vivo* (Zhong *et al.*, 2012).

Natural products consist of important class of category that are called phytoestrogens. Phytoestrogens are the xenoestrogens, which are derived from the plant. These are sometimes referred as “dietary estrogens”. Phytoestrogens can be classified into five types: isoflavones, lignans, stilbenes, ,coumestans and terpenoids. They have wide distribution in diet and herbs. Phytoestrogens exhibit anti-cancer activity via mechanisms including estrogen receptor modulation, aromatase inhibition, and anti-angiogenesis (Liu *et al.*, 2012). Studies carried out indicated that the dietary intake of phytoestrogens reduced the incidence of breast cancer (Kolonel, 1988; Rice & Whitehead, 2008). Genestein, an isolflavone which is regarded as phytoestrogens at higher doses reduced the proliferation of MCF-7 (Breast cancer cell line) cell line (Allred *et al.*, 2001). Phytoestrogens binds preferentially to the estrogen receptor β thereby activating the downregulated receptor (Rice *&* Whitehead, 2008). Geldanamycin, a phytoestrogen act as an inhibitor of HSP90, was observed to decrease the hormone binding to the estrogen receptor (Fliss *et al.*, 2000).

1.3 *Asparagus racemosus*

A. racemosus is a climbing ayurvedic plant, consisting of tuberous root system. The herb have anti-oxidant, diuretic, antidepressant, antiepileptic, antitussive, antileshmanial, anti-HIV, antibacterial, anti-ulcer activity.The plant also used as a immuno-stimulant, hepatoprotective, during pregnancy, cardioprotective and in neurodegenerative disorders. A wide range of seconday metabolites representing steroids, alkaloids, dihydrophenanthrene derivatives, flavonoids, furan derivatives and essential oils have been isolated from this plant. However, the main constituents are steroidal saponins (shatavarins, racemosides) and flavonoids which are considered to be responsible for the pharmacological activities.

With this background and importance of medicinal plants in the development of therapeutic agents, *A. racemosus* was selected for study of following objectives.

Objectives

- Isolation and characterization of secondary metabolites from *A.racemosus* roots
- *In-vitro* biological screening of different extracts of *A.racemosus*
- *In-silico* Study of the interaction pattern of reported Phytoestrogens from the *A.racemosus*

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CHAPTER 2.0

REVIEW OF LITERATURE

“When one door closes, another opens; but we often look so long and so regretfully upon the closed door that we do not see the one that has opened for us”.

…**Alexander Graham Bell**

Chapter 2.0

Review of literature

The genus *Asparagus* includes about 300 species around the world, out of which 22 species are recorded in India. *A. racemosus* belongs to family liliaceae is one such important medicinal plant which is regarded as a 'rasayana' which means plant drugs promoting general well-being by increasing cellular vitality and resistance(Goyal *et al.*, 2003). Use of *A. racemosus* was mentioned in the ancient literature of Ayurveda (Charaka samhita) (Chawla *et al.*, 2011). It is commonly called as *Satavari*, *Shatawar* or *Satmuli* in Hindi; *Satavari* in Sanskrit; *Shatamuli* in Bengali; *Shatavari* or *Shatmuli* in Marathi; *Satawari* in Gujarati, *Toala-gaddalu* or *Pilli-gaddalu* in Telegu; *Shimaishadavari* or *Inli-chedi* in Tamil; *Chatavali* in Malayalam; *Majjigegadde* or *Aheruballi* in Kannada; *Kairuwa* in Kumaon; *Narbodh* or *atmooli* in Madhya Pradesh and *Norkanto* or *Satawar* in Rajasthan(Bopana & Saxena, 2007). Use of *A.racemosus* is mentioned in the ancient literature of Ayurveda (Charaka samhita)(Chawla *et al.*, 2011). Traditionally *A. racemosus* is indicated in epilepsy, Vata disorders (Gomase & Sherkhane, 2010), brain tonic, helps in regulating cardiac disorders and hypertension (Venkatesan *et al.*, 2005). It is extensively used in disorders like male genital dysfunctions, oligospermia, spermatogenic irregularities and other male disorders such as painful micturition (Sahu *et al.*, 2002, Dartsch, 2008). It is extensively used in ayurvedic formulations for digestive discomfort, indigestion, amoebiasis and piles (Sharma *et al.*, 2012). In females, it is prescribed by the doctors in habitual abortions, weakness of the uterus, excessive bleeding during menstruation (Nevrekar *et al.*, 2002). Researchers of modern times have proved through experimental evidences that Shatavari is antidiarrhetic (Venkatesan *et al.*, 2005), antispasmodic, aphrodisiac (Sharma *et al.*, 2012), antidysenteric, demulcent, diuretic (Potduang *et al.*, 2008), galactagogue, nutritive, mucilaginous, refrigerant, stomachic properties and works as a tonic for human beings (Indian Pharmacopoeia, 2007). It improves immunity and protects the heart (Visavadiya & Narasimhacharya, 2009), brain (Goel and Sairam, 2002) and other vital organs of the body. *A.racemosus* is mentioned in Ayurveda for general weakness due to prolong illnesses (Hussain *et al.*, 2011).

2.1 Ethnobotany

In Thailand, traditionally the decorticated roots of the plant have been used as a remedy for diseases of the spleen, liver and other internal organs, including preventing miscarriage (Wiboonpun *et al.*, 2004). In India, conventionally the roots of the plant are used in during internal pain, tumors, fever and as a tonic (Kala, 2009). *A.racemosus* (Shatavari) is a climbing plant consisting of the tuberous roots (Gomase & Sherkhane, 2010). According to Indian pharmacopoeia *A.racemosus* contains not less than 0.1 per cent of shatavarin IV, as calculated on the dried weight basis (Indian Pharmacopoeia, 2007). The taste is initially starchy and then slightly bitter followed by a sweet taste. *A.racemosus* has small pin-needle like phylloclades (photosynthetic branches) that are uniform and shiny green in appearance. The roots are marketed in the form of pieces which are 5-15 cm in length and 2 cm in thickness. These are silvery white or ash-color externally and white internally. The roots are more or less smooth when fresh, and start to develop longitudinal wrinkles upon drying (Hussain *et al.*, 2011). Microscopically the inner parenchymatous zone of cortex is composed of 18-24 layers in the upper portion and 42-47 layers in the middle tuberous portion of the roots. The cells are thin-walled and composed of cellulosic fibers; with circular to oral outlines and distinct inter cellular spaces. In some roots 3-4 layers of cortex immediately adjacent to the endodermis are modified into a sheath of stone cells round the endodermis. The number of vascular bundles ranges from 30-35 in the upper levels and 35-45 in the middle tuberous portions of the roots (Goel & Sairam, 2002). In environmental conditions the plant prefers light (sandy), medium (loamy) and heavy (clay) soil. Black, well drained and fertile soil are brilliant for *A. racemosus* cultivation *(*Chawla *et al.*, 2011) and can also be cultivated in loose and medium black soil. Crops needed tropical, hot climatic conditions and require minimum irrigation with the avoidance of over-watering. Raised beds which are about 3m are harvested in the month of May or June. The time of transplanting is in the month of July-August. In the month of July it produces minute flowers which are white and unisexual in nature (Kundu *et al.*, 2011). In September it begins to bear fruits which are globular or obscurely 3 lobed, pulpy berries which are purplish black when they are ripening (Figure 4), seeds are hard and brittle (Indian Pharmacopoeia, 2007). Weeding operations must be timely carried out. Generally the crops are not affected with pest and diseases. The first harvesting is done after 1.5-2 years of

transplanting, which is continued for 10-15 years. Male and female plants are grown if seed is required (Chawla *et al.*, 2011).

Figure 4: Photographs of *A. racemosus* showing (a) Shoot, leaves and berries (b) tuberous roots adopted from http://tinyurl.com/lrwmv7s on 10/08/2013

2.2 Phytochemicals

A. racemosus consists of a diverse range of molecules in which major constituent is steroidal saponins along with alkaloids, flavonoids, dihydrophenanthrene derivatives, furan derivatives and volatile constituents.

2.2.1 Steroidal saponins

Twenty nine steroidal saponins (**1-29**) were reported from *A. racemosu*s (Figure 5). An oligospirostanoside (**1**) named 3-O-[α-L-rhamnopyranosyl-(1→2)-α-L--rhamnopyranosyl-(1→4)-O-β-D-glucopyranosyl]-25(S)-spirosta-3β-oil from *A. racemosus have* applications in oral administration, potentiated antibody synthesis and enhanced cell-mediated immune response in immune compromised animals (Handa *et al.*, 2003). Racemoside A, B, C (**2-4**) were obtained from the butanolic fraction of defatted fruits (Mandal *et al.*, 2006). Shatavarins (**5-12**) are the major molecules which are named after the vernacular name of *A. racemosus* i.e Shatavari are steroidal in nature. Structure of Shathavarin I (**7**) and IV (**12**) was revised along with the isolation of Asparanin A (**13**) and Immunoside (**14**) by Hayes *et al.*, 2008. (1*S*,2*R*,3*S*,8*S*,9*S*,10*S*,13*S*,14*S*,16*S*,17*R*,22*R*,25*R*)-21-Nor-18β,27α-dimethyl-1β,2β,3β-trihydroxy-25-spirost-4-en-19β-oic acid (**15**) and its acetylated (**16**) and

esterified derivative (**17**) was reported to have immunostimulant property (Sharma *et al.*, 2011). Sarsasapogenin (**18**) and, diosgenin (**19**)

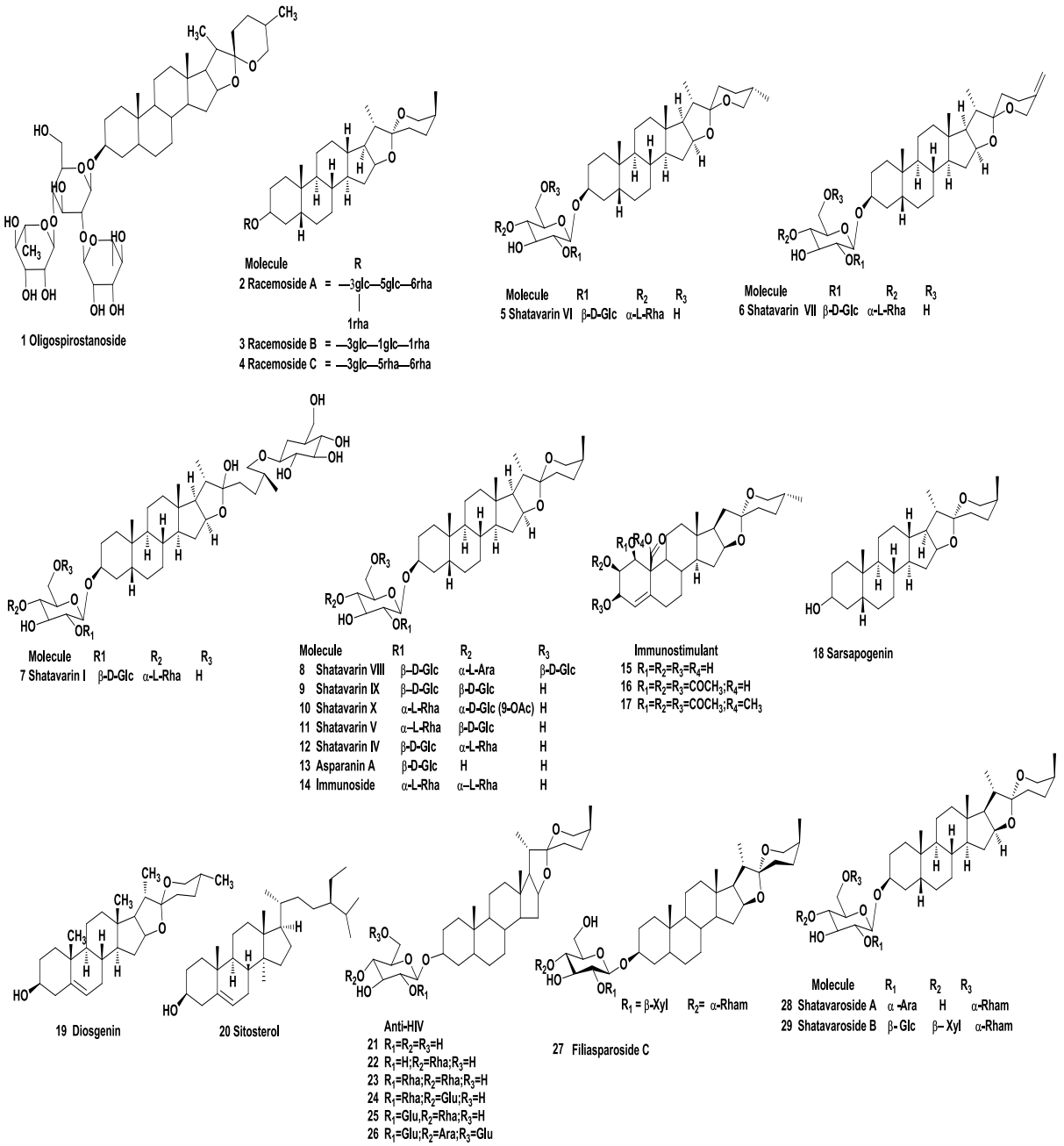


Figure 5: Structures of Steroidal saponins from *A. racemosus*

was isolated from the different parts of the plant (Ahmad *et al.*, 1991). The dried roots yielded sitosterol (**20**) (Khare, 2007, Singh and Tiwari, 1991) which was reported to possess antiprotozoal and spasmolytic property (Bose *et al.*, 2012). Anti-HIV compounds (**21-26**) was isolated from the ethyl acetate, butanol and aqueous root extracts (Sabde *et al.*, 2011). The methanolic root extract of *A. racemosus* yielded filiasparoside C (**27**) (Sharma *et al.*, 2009b). Shatavaroside A (**28**) and Shatavaroside

B (**29**) from the alcoholic root extract of *A. racemosus* which possess an immunomodulatory activity (Sharma *et al.*, 2009a).

2.2.2 Alkaloids

Asparagamine A (**30**) was isolated from the chloroform root extract of *A. racemosus* having antioxytocic property (Sekine *et al.*, 1995). A polycyclic alkaloid (**31**) was found to inhibited the growth of human stomach cancer cells (KATO-III) in cultures with IC_{50} =79. 81µg/mL (Murakoshi *et al.*, 1995) (Figure 6).

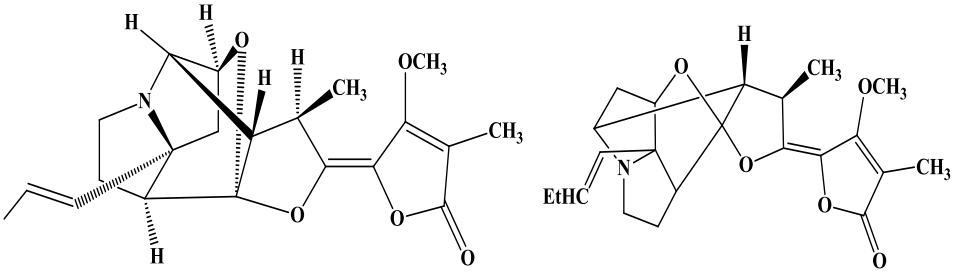


Figure 6: Structures of Alkaloids from *A. racemosus*

2.2.3 Dihydrophenanthrene derivative

A dihydrophenanthrene derivative, racemosol (9, 10-dihydro-1, 5-dimethoxy-8-methyl-2, 7-phenanthrene diol) (**32**) was isolated from the alcoholic root extract of *A. racemosus* (Sekine *et al.*, 1997) (Figure 7).

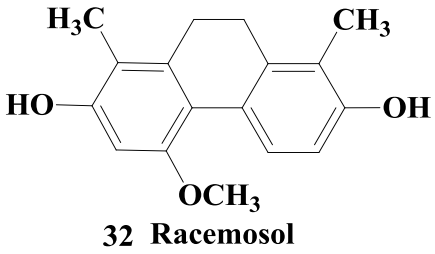
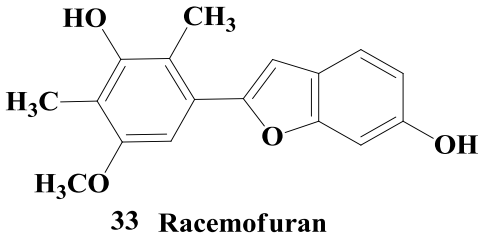


Figure 7: Structures of Dihydrophenanthrene from *A. racemosus*

2.2.4 Furan derivatives

A benzofuran identified as racemofuran (**33**) was isolated from the chloroform root extract, which was reported to possess anti-oxidant activity towards DPPH with an IC_{50} value of 130 µM (Wiboonpun *et al.*, 2004) (Figure 8).



33 Racemofuran

Figure 8: Structure of Furan derivative from *A. racemosus*

2.2.5 Flavanoids

An isoflavone 8-Methoxy-5,6,4-trihydroxyisoflavone-7-*O*-β-D-glucopyranoside (**34**) was isolated from the roots of the plant (Saxena & Chourasia, 2001). Cyanidine-3-galatoside (**35**) and Kaempferol (**36**) was also being isolated from the woody portions of tuberous roots (Ahmad *et al.*, 1991).

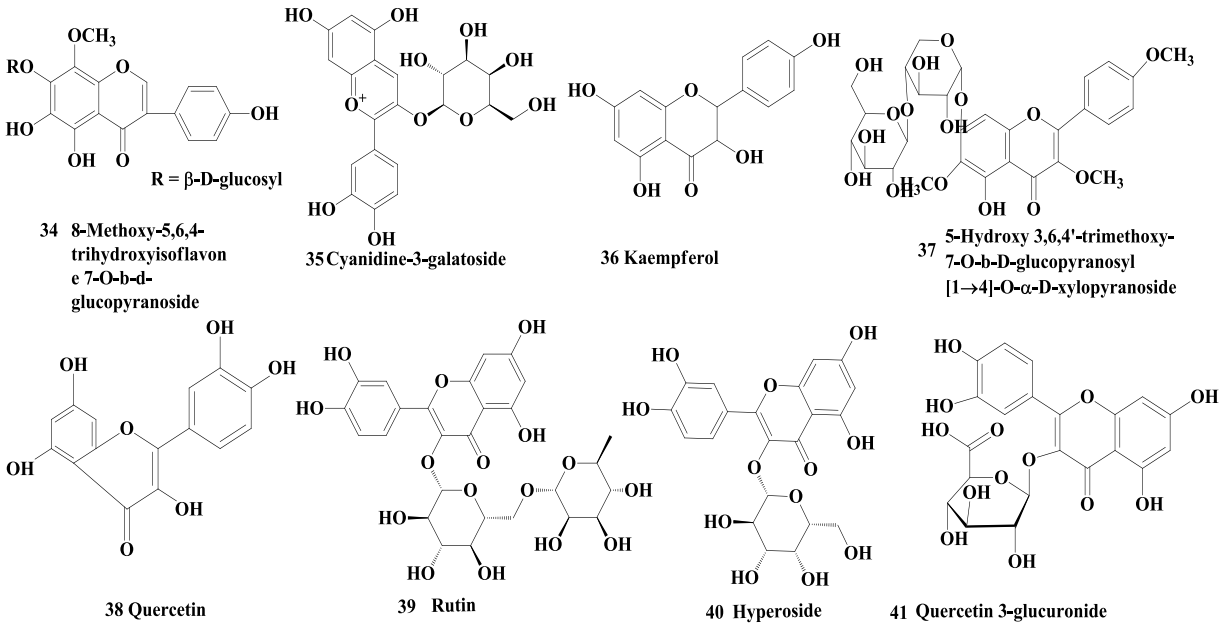


Figure 9: Structure of Flavonoids from *A. racemosus*

In an another study flavone glycoside from the leaves was isolated and identified as 5-hydroxy-3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl-[1→4] -O-α-D-xylopyranoside (**37**) (Saxena *et al.*, 2000). Quercetin (**38**), rutin (**39**) and hyperoside (**40**) were found in the flowers and fruits along with quercetin-3-glucuronide (**41**) which was obtained from the leaves (Bopana & Saxena, 2007) (Figure 9).

2.2.6 Essential oil constituents

Volatile constituents extracted were belonging to a diverse range of chemicalclasses such as acids, alcohol, aldehyde, ester, hydrocarbon, ketone, N-containing compounds (Figure 10). Alcoholic compounds, accounts 49.82% of the total volatile oil content; in which the major ones were borneol (26.40 %) (**42**), myrtanol (13.72 %) (**43**), pinocarveol (2.37%) (**44**) and 2-ethylhexanol (1.76 %) (**45**).

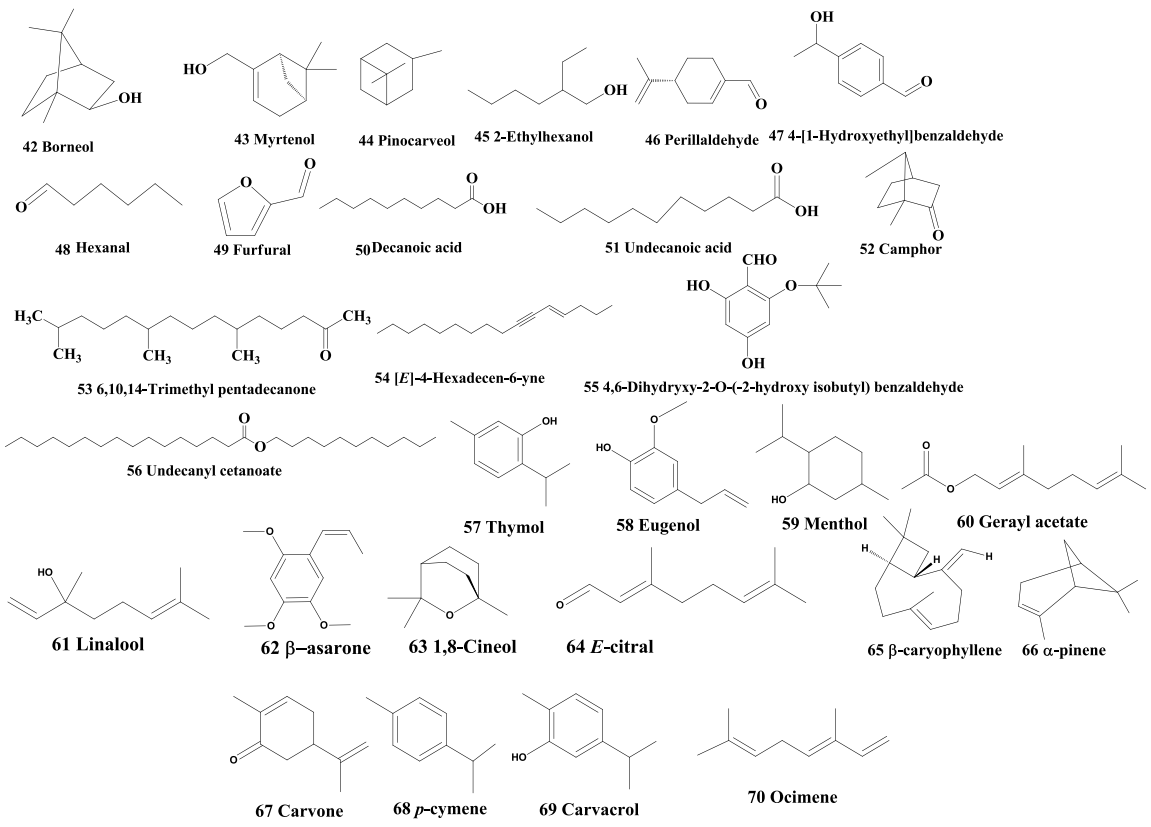


Figure 10: Structure of volatile oils from *A.racemosus*

Aldehydes were the second most abundant chemical group characterized, containing 16.70% of total volatile oil content in which the major one was perillaldehyde (8.97%) (**46**), 4-[1-hydroxyethyl] benzaldehyde (1.55%) (**47**), hexanal (1.34%) (**48**) and

furfural (1.17%) (**49**). Essential oil contained 8.97% of acid and 8.97% of ketone content. The category of acids, consisted of decanoic acid (4.19%) (**50**) andundecanoic acid (2.72%) (**51**). Ketonic essential oil included camphor (3.33%) (**52**) and 6,10,14-trimethyl pentadecanone (1.71%) were major ones (**53**). The hydrocarbons percentage was found to be 5.27%. All the hydrocarbons isolated except [*E*]-4-hexadecen-6-yne (**54**) were monoterpenoidal in nature. Remaining chemical classes which consisted of ester, S-containing compound and N-containing compounds were detected at levels lower than 3%. Only three compounds; borneol (**42**), myrtenol (**43**) and paraldehyde (**46**) could occupy 45.09% of the whole content (Gyawali & Kim, 2011). Roots were also reported to contain (4,6-dihydryxy-2-O(-2-hydroxy isobutyl) benzaldehyde (**55**) and undecanyl cetanoate (**56**) (Ahmad *et al.*, 1991). The fourteen essential oils were obtained from the biodeteriorated *A.racemosus* Thymol (**57**), eugenol (**58**),menthol (**59**),geryl acetate (**60**), linalool (**61**), β-asarone (**62**), 1,8-cineol (**63**), E-citral (**64**), β-caryophyllene (**65**), α-pinene (**66**), carvone (**67**), P-cymene (**68**), carvacrol (**69**), ocimene (**70**) (Mishra *et al.*, 2013).

2.2.7 Miscellaneous

The variation in the content of trace element was done (Table 1).

Table 1: Summary of the trace elements from the *A. racemosus* collected different regions of Uttranchal,India.

S.No	Metal	Root (mg/kg)	Leaves (mg/mg)
1	Zinc	44.0±0.2 to 148.0± 1.2	53.0±0.2 to 165.0±3.2
2	Copper	14.0±0.1 to 23.0±0.3	15.0±0.6 to 34.0±0.5
3	Manganese	5.0±1.4 to 62.0±2.5	14.0±0.4 to 84.0±0.7
4	Iron	211.0±0.5 to 1493.0±0.2	505.0±0.2 to 2040.0±0.3
5	Cobalt	84.0±0.3 to 122.0±1.5	85.0±0.3 to 88.0±0.2
6	Potassium	2652.0±0.4 to 13260.0±3.5	5460.0±0.2 to 10842.0±2.5
7	Calcium	961.0±0.6 to 2115.0±3.2	1346.0±0.3 to 6153.0±1.6
8	Lithium	18.0±0.2 to 58.0±3.8	28.0±0.6 to 48.0±1.6

In the study *A.racemosus* from the different regions, varying in altitudes from the state Uttranchal, India (Negi *et al.*, 2010). Their study indicated that there is much variation in the trace element content with the changing attitudes and the best altitude for the cultivation is 2250 meters.

2.3 Biological activities

The second century physician Galen described *Asparagus* as "cleansing and healing". Nutritional studies demonstrated that *Asparagus* is a low-calorie source of folate and potassium. In the first Century, Pliny wrote, '*Asparagus*, of all the plants of the garden, receives the most praiseworthy care'. The plant is widely used in about 64 Ayurvedic formulations which include traditional formulations such as '*Shatavari Kalpa*', '*Phalaghrita*', '*Vishnu taila*'(Bopana & Saxena, 2007). The plant has numerous traditional practices and these traditional practices were verified by the experimental studies.

2.3.1 Antioxidant property

A.racemosus has been reported to have antioxidant effects of the crude extract and purified aqueous fraction (Kamat *et al.*, 2000). The activity were tested in the mitochondrial membrane damage induced free radicals generated in rat liver mitochondria. The lipid peroxidation induced was assessed by the formation of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) (Kamat & Devasagayam, 1995, Kamat and Devasagayam, 1996). The extract exhibited an antioxidant effect against oxidative damage in terms of protection from lipid peroxidation, protein oxidation and depletion in the levels of protein thiols and antioxidant enzyme, superoxide dismutase. The purified aqueous fraction which consisted of polysaccharides was found to be effective even at a low concentration. It was observed that the antioxidant effect of purified aquous fraction was more effective against lipid peroxidation, which was assessed by TBARS formation, whereas the antioxidant effect of the crude extract was more effective in inhibiting protein oxidation. The crude and purified aquous extracts indicated protection against radiation induced loss of protein thiols and inactivation of superoxide dismutase (Kamat *et al.*, 2000). Isolated racemofuran which is a benzofuran, (**33**) and Asparagamine A (**30**) from chloroform extract which showed antioxidant activity against DPPH (Wiboonpun *et al.*, 2004). Other study indicated that there was an increase in antioxidant defense due to the significant increase in enzymes superoxide dismutase, catalase, and ascorbic acid and significant decrease in lipid peroxidation upon treatment with *A. racemosus* root extract (Bhatnagar *et al.*, 2005).

2.3.2 Diuretic activity

A.racemosus is used as diuretic in Ayurveda and has been validated by a suitable experimental model. The study was carried using an aqueous extract of the roots utilizing three dose vials 800 mg/kg, 1600 mg/kg and 3200 mg/kg for its diuretic activity in comparison with standard drug (furosemide) and control (normal saline) rats after doing acute toxicity study. The extract demonstrated diuretic activity at a 3200 mg/kg dose without any acute toxicity (Kumar *et al.*, 2010).

2.3.3 Antidepressant activity

Dhingra and Kumar, 2007 conducted a study on mice using tail suspension test (TST) and forced swim test (FST) to determine the antidepressant activity. The methanolic extract decreased immobility periods significantly in TST, FST, which indicated significant antidepressant activity. The efficiency of the extracts was comparable to fluoxetine and imipramine which were used as reference drugs in the study. Methanolic extract administered to mice significantly decreased brain MAO-A (Monoamine Oxidase A) and MAO-B (Monoamine Oxidase B) activity levels and found that methanolic extract possesses antidepressant activity probably by inhibiting MAO-A and MAO-B; through interaction with adrenergic, dopaminergic, serotonergic and GABAergic (Gamma aminobutyric acid) systems. Experiments have been performed on rats using the methanolic extract and subjected to forced swim test (FST), learned helplessness test (LH) and has been found that extract decreases immobility in the FST and increases avoidance response in LH indicating antidepressant activity. Further behavioral experiments were carried out in which by administering extract, number of head twitches produced by 5-HT (5-hydroxy tryptamine) increased, increased clonidine-induced aggressive behavior and concluded that the methanolic extract has a significant antidepressant activity mediated via serotonergic, noradrenergic systems and precipitation of antioxidant defenses (Singh *et al.*, 2009) and evaluated inhibitory activities of different extracts on the enzyme kinetics of acetyl and butyryl cholinesterases, and monoamine oxidase (Meena *et al.*, 2011). It has been found that the methanolic extract significantly inhibited cholinesterase and MAO activities and act as a non-selective competitive inhibitor as compared the extracts of hexane and chloroform, a direct possible correlation between the spinning content in methanolic extracts and

cholinesterase, monoamine inhibitory activities because hexane and chloroform extract showed no measurable saponin content was draw out.

2.3.4 Antiepileptic effect

The anticonvulsant activity was evaluated using the chloroform, methanol and aqueous extracts of the plant species on seizures. The seizures were induced in the rat models by maximal electroshock (MES) and pentylenetetrazole. In the test carried out, the methanolic extract has shown most significant anticonvulsant effect which was anticipated by the observation of a decrease in the duration of the hind limb extension, clones and also the duration of stupor phase. There was a prolonged onset of the tonic clonic seizure induced by pentylenetetrazole in the groups treated with methanolic and aqueous extracts. It was conclude that the mechanism behind the activity is probably GABAergic (Jalalpure *et al.*, 2009).

2.3.5 Antitussive effect

The methanolic extract of roots has been reported to possess antitussive. The activity was tested against sulfur dioxide (SO_2) -induced cough in the mouse model (Mandal *et al.*, 2000). The methanolic root extract administered at at the concentration of 200, 400 mg/kg, and codeine phosphate was taken as a standard antitussive reference drug. Upon oral administration of methanol extract displayed 40% and 58.5% inhibition of SO_2-induced cough at a dose of 200 and 400 mg/kg respectively. Thus, concluded that the antitussive effect produced is dose dependent for both extracts as well as standard drug which further supported the claims put forward by traditional medicine practitioners about the usefulness of *A.racemosus* in the treatment of cough.

2.3.6 Antileshmanial activity

Leishmaniasis can occur in diverse clinical forms such as cutaneous, mucosal, visceral leishmaniasis (VL, the most severe) and remain a major health problem in the tropical and subtropical areas, threatening almost 350 million people in 88 countries (Chava *et al.*, 2005, Murray *et al.*, 2005). The viability of promastigotes after treatment with Racemoside A (**2**) as evaluated using a modified MTT assay

(Dutta *et al.*, 2007). There has been decreasing in formazan production in the promastigotes indicating Racemoside A (**2**) decreases the viability of the cells. Treatment with Racemoside A (**2**) also demonstrated a dose-dependent removal of phagocytosed amastigotes. Racemoside A treated *L. donovani* promastigotes have shown signs of programmed cell death, i.e. the flagellated promastigotes shrank and became aflagellated,oval or round with an increase in vacuoles. There was alsotranslocation of phosphatidylserine from the inner side of the outer layer of the plasma membrane which is an observation of cell death (Koonin & Aravind, 2002).

2.3.7 Anti-plasmodial activity

The ethyl acetate extract of the roots of *A. racemosus* has been tested for anti-plasmodial activity. The extract with yield value of 7.9% per 100g have shown dose dependent inhibition of chloroquine resistant strain of *Plasmodium falciparum* (3D7) with an IC_{50} value of 29µg/mL (Kaushik *et al.*, 2013).

2.3.8 Anti-HIV activity

A.racemosus has been shown immunomodulatory activity. The ethyl acetate, butanolic, and aqueous extracts of roots were found to be active. Steroidal saponin glycosides (**21-26**) have been reported from these extracts. Compound **21** isolated from the ethanolic extract exhibited the highest anti-HIV activity as compared to other saponin glycosides (Sabde *et al.*, 2011). Glycoside **22** with two sugars exhibited weak anti-HIV activity and saponins with three sugar units **(23-26)** showed weak to no activity. Structurally similar compounds have been reported to have anti-HIV protease activity (Figure 11) (Yang *et al.*, 1999).

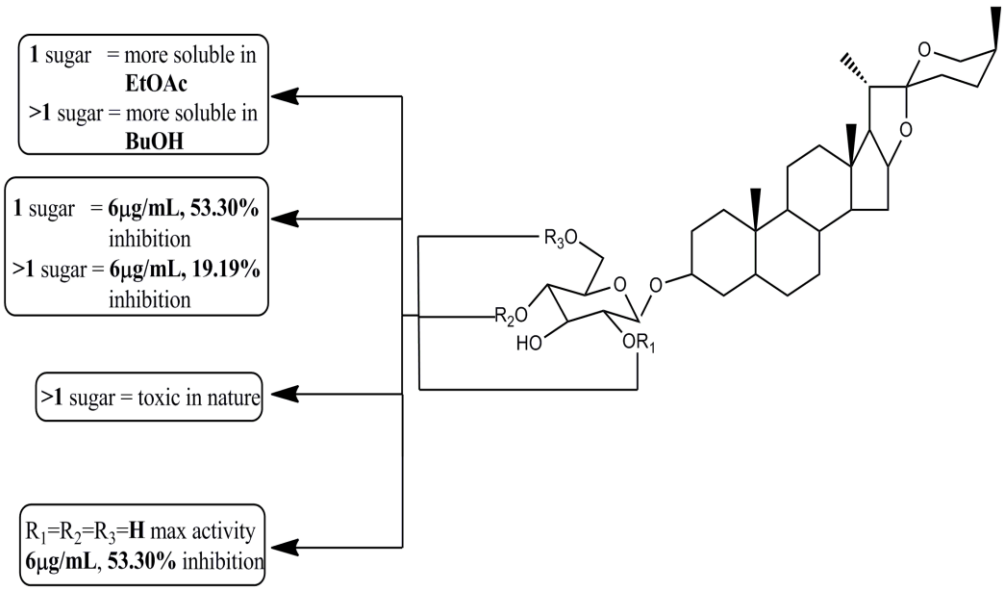


Figure 11: SAR study of Anti-HIV compounds isolated from *A. racemosus*

2.3.9 Immunostimulant

Immunodeficiency disorders are the group of disorders in which the body's defense system is compromised, making it to be less effective against foreign invaders. Consequently, the body's ability to fight infections is impaired. As a result, the person with an immunodeficiency disorder will have frequent infections that are generally more severe and remain longer than usual. Isolated polyhydroxylated steroidal sapogenin acids (**15-17**) were studied on the immune system of normal and cyclosporine-A induced immune-suppressed animals and it has been found that compound is a potent immune system stimulator (Sharma *et al.*, 2011). The study mainly focused on the lymphocytes and cytokines, since T and B lymphocytes are the backbone of the immune system and modulation of Th1/Th2 immunity are important biological targets for immunostimulant (Glavin *et al.*, 1994). Upon oral administration of the compounds there has been significant and dose dependent increased CD3 & CD19 count and Th1/Th2 cytokines. The results obtained were comparable to levamisole, indicating that the compounds were potent immune system stimulator. Steroidal saponins, shatavaroside A (**28**) and shatavaroside B (**29**), isolated from the methanolic extract of *A. racemosus*, and their immunomodulatory activity have been evaluated using poly-morphonuclear leukocyte function test and some more sensitive assays such as nitroblue tetrazolium, nitrous oxide and chemiluminescence assays were used as a confirmatory test for the

activity. The steroidal saponins isolated were found to be active at nano concentrations (5ng/mL) and can act as a potent immunostimulant (Sharma *et al.*, 2009a).

2.3.10 Hepatoprotective activity

There have been an investigation of the hepatoprotective activity of *A. racemosus* against isoniazid-induced hepatotoxicity in male albino rats. Animals exposed to isoniazide showed necrotic changes resulted in the release of hepatic enzymes aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and γ-glutamyl transpeptidase that mark liver injury. The increased level of aspatate aminotransferase and alanine amino transferase indicated increased permeability and hepatic cell damage. Upon administration of extract they observed restoration of glutathione levels in cases with isoniazide toxicity (Ergul *et al.*, 2010). *A.racemosus* extract exerts its hepatoprotective activity by inhibiting the production of free radicals, acting as a scavenger and reducing the free radical generation via inhibition of hepatic CYP2E1 activity (Palanisamy & Manian, 2011). There was a reported hepatoprotective activity of *A. racemosus* extract in paracetamol induced liver injury in rats. There has been observed increased levels of SGOT, SGPT, serum bilirubin and serum alkaline phosphatase, upon treatment with the ethanolic roots extract and reversal in their levels indicating the hepatoprotective activity. There were depleted levels of catalase and superoxide dismutase which act as antioxidants and upon treatment with the extract there was an improvement in their levels (Rahiman *et al.*, 2011).

2.3.11 Antibacterial activity

The root extracts of *A. racemosus* have been studied for antibacterial activity employing standard cylinder method. Microbes used were *Bacillus subtilis, Staphylococcus aureus, Staphylococcus werneri, Pseudomonas aeruginosa* and *Escherichia coli, Proteus mirabilis, Kleibsella pneumonia*, *Pseudomonas putida*. Both gram-positive and gram-negative bacteria were sensitive to the extract. Ethanolic extract of concentration100mg/mL, 300mg/mL, 500mg/mL were prepared and there antibacterial activity was comparable to reference standard drug Gentamycin . The gram positive bacteria were most affected by *S. aureus* (Ravishankar *et al.*, 2012).

2.3.12 Pregnancy

2.3.12.1 Antiabortifacient

The preparations containing *A. racemosus* roots (eg. *Shatavari sidh ghrit*) were prescribed in the cases of threatened abortions (Garg *et al.*, 1971). The observed activity was due to the Shatavarin-I (**7**) (Dev, 1999). It was also confirmed that the *in vivo* effect of shatavarin IV (**12**) i.e. Saponin A4 on the uterine muscles is similar to the estrogen (Gaitonde & Jetmalani, 1969). The polycyclic alkaloid Asparagamine A (**30**) have been reported to possess an anti-oxytocic action (Sekine *et al.*, 1997) and showing an antiabortifacient affect.

2.3.12.2 Antenatal tonic

A capsule Sujat containing *A. racemosus* extract, in a clinical trial containing a group of 450 patients, reported that regular use of this capsule during antenatal period increases the fetal weight and decreases the occurrence of perinatal deaths (Bhasale *et al.*, 1994). The occurrence of pregnancy induced hypertension (PIH) have also been observed to decreased. PGI2 and NO (nitric oxide) are the important vasodilators; a deficiency of these can lead to PIH. Essential fatty acid GLA (Gamma linolenic acid) obtained from *A. racemosus* known to mediate the produce PGI_2 in preference to TXA_2 (Bhasale *et al.*, 1994).

2.3.13 Anti-Ulcer

Acute gastric ulcers were induced in rat model by cold restraint stress, pyloric ligation, aspirin plus pyloric ligation, and duodenal ulcers induced by cysteamine. There has been significant protectective activity of the extract which was due to the increase in mucosal defensive factors like mucus secretion, cellular mucus, life span of cells and anti-oxidant effect. Satavari mandur an Ayurvedic preparation that contains *A. racemosus* when given in the dose of 1.5 g, twice daily for a month there was a noteworthy improvement in symptoms of peptic ulcer and healed peptic ulcers which was endoscopically verified (Sairam *et al.*, 2003). A marked decrease in cell shedding and the increase in mucin secretion indicated its predominant effect on mucosal defensive factors have been reported (Goel & Sairam, 2002). The gastric

ulcer induced by indomethacin in rats, upon treatment with crude extract significant reduction in ulcer index was observed. It was observed that the reduction in gastric lesions was comparable to standard Ranitidine. There have been significant reductions in the volume of gastric secretion and concluded that *A. racemosus* had antiulcerogenic activity. The activity was the result of inhibitory effect on release of gastric hydrochloric acid and protects gastric mucosal damage (Bhatnagar & Sisodia, 2006). Study conducted on humans found that the *A. racemosus* root powder is effective in chronic peptic ulcers. There was an increase in the lifespan of gastric mucosal epithelial cells, secretion and viscosity of gastric mucus (Mangal *et al.*, 2006).

2.3.14 Anti-diarrheal activity

In the developing countries, diarrhea is the reason for quarter of infant and childhood mortality (Jousilahti *et al.*, 1997). The peak mortality rates were reported in children of less than five years of age. The use of oral dehydration therapy reduced mortality due to acute diarrheal disease, but chronic diarrhea is still a life-threatening problem in the regions where malnutrition is a regular co-existing and complication factor. Ethanolic and aqueous extracts of *A. racemosus* were evaluated for antidiarrheal activity in castor oil-induced diarrhoealrats. Evaluation was done for determining the effect of ethanolic and aqueous extracts on gastrointestinal tract motility after charcoal meal administration and PGE_2 induced enteropooling by taking Loperamide as a reference drug. The extracts have shown to have inhibition against castor oil induced diarrhea and PGE_2 induced enteropooling. Both the extracts also displayed a reduction in gastrointestinal motility in charcoal meal test in rats (Venkatesan *et al.*, 2005).

2.3.15 Anticandidal activity

The experimental results suggested, that methanol extracts had high anticandidal activity against different *Candida* species. The disc diffusion method was chosen for antifungal susceptibility tests by taking fluconazole as a reference drug. *Candida* strains were isolated from vaginal thrush patients, and the species were identified using their conventional tests. Zone of inhibition observed ranged from 13 to 16 mm. The MIC (Minimum inhibitory concentration) values were between 2.5 to 0.312

mg/mL, while MFC (Minimum fungicidal concentration) values were between 5 to 0.625 mg/mL (Uma *et al.*, 2009).

2.3.16 Anti-aflatoxigenic activity

There have been fourteen essential oils obtained from the biodeteriorated *A. racemosus* (Figure 10) which as individual component and in combination were tested for the Anti-aflatoxigenic activity. The fourteen essential oils obtained were Thymol (**57**), eugenol (**58**), menthol (**59**), gerayl acetate (**60**), linalool (**61**), β-asarone (**62**), 1,8-cineol (**63**), *E*-citral (**64**),β-caryophyllene (**65**), α-pinene (**66**), carvone (**67**), P-cymene (**68**), carvacrol (**69**), ocimene (**70**). Among the 14 essential oil, thymol and eugenol have shown fungicidal activity as both blocked the growth of spores and the rest of essential oil components showed moderate antifungal activity (P. K. Mishra *et al.*, 2013).

2.3.17 Cardio protective effects

A formulation named “Abana”, herbomineral drug manufactured by Himalayan drugs, have been found useful in controlling hypercholesterolemia, prevention and management of coronary heart disease. Abana was given in normal as well as in cases of essential hypertension and angina pectoris and was found to reduce the total cholesterol and triglyceride levels. There was an observed significant increase in high-density lipoprotein cholesterol levels (Tiwari *et al.*, 1990). The lipid-lowering effects of *A. racemosus* root extract in hypercholesteremic rats was demonstrated, the investigation revealed, the primary reason of antihypercholestrolemic effect is increased excretion of cholesterol, neutral sterols, bile acid and an increase in hepatic bile acid content. They observed increased HMG-CoA reductase activity in hypercholesteremic rats upon treatment with *A. racemosus* root powder. Interestingly they found normocholesteremic animals under *A. racemosus* treatment, exhibited no significant variations either in excretion of cholesterol, neutral sterols, bile acid, hepatic cholesterol and bile acid content. A significant increase in plasma HDL-C levels with a concurrent decline in the plasma cholesterol level and an improvement in the atherogenic index of hypercholestrolemic test animals clearly indicated the beneficial role of root administration in hypercholesteremic animals. The reduction in the levels of HDL-C is an indicative of high risk of cardiovascular disease, so

improvement in its levels gives cardioprotective activity (Visavadiya & Narasimhacharya, 2009).

2.3.18 Neurodegenerative disorders

“EuMil”, polyherbal formulation which contains standardized extracts of *A.racemosus*. The formulation is used as an anti-stress agent to ease the various aspects of stress related disorders. Rat brain monoamine neurotransmitter levels and tribulin activity was evaluated. Upon Eumil administration there was normalization in the elevated levels of NA (nor-epinephrine), DA (dopamine), 5HT (5-hydroxy tryptamine) concentrations, which were increased by chronic electroshock stress. Such a decrease in the neurochemical levels in the brain indicates the effectiveness of the formulation in neurological disorders (Bhattacharya *et al.*, 2002). *A. racemosus* have been found to be effective in neurodegenerative disorders like Alzheimer’s and Parkinson’s disease. The potential of methanolic root extract roots against kainic acid induced hippocampal and striatal neuronal damage in mice have been evaluated (Parihar & Hemnani, 2004). Upon injection of kainic acid in intra-hippocampal and intra-striatal region in anesthetized mice leads to the production of excitotoxic lesions in the brain. After the injection of kainic acid, there was an observed impairment of hippocampus and striatal regions of the brain with an increased lipid peroxidation, increased protein carbonyl content, decreased glutathione peroxidase activity and reduction in the glutathione content. Glutathione is an important antioxidant which is a nucleophilic scavenger of toxic compounds and also act as substrate in the glutathione peroxidase mediated destruction of hydro-peroxides which would otherwise accumulate to toxic levels in brain tissues. The mice treated with methanolic root extract showed an enhancement in glutathione peroxidase activity, glutathione content, reduction in membrane lipid peroxidation and protein carbonyl. It was concluded that plant extract plays the role of an antioxidant by attenuating free radical induced oxidative damage. The oxidative damage protection of the hippocampal and striatal regions of the brain is useful in the neurodegnerative disease (Parihar & Hemnani, 2004).

2.3.19 Anti-cancer property

The *A. racemosus* root extract was shown to have a protective effect in the mammary cell carcinoma (Rao, 1981). The steroidal components of the *A.racemosus* were investigated for the apoptotic activity and inferred to have the capacity to tumor cell death (Bhutani *et al.*, 2010). The anticancer activity of shatavarins (containing shatavarin IV) which was isolated from the roots of *A.racemosus* have been evaluated by MTT assay using MCF-7 (human breast cancer), HT-29 (human colon adenocarcinoma), and A-498 (human kidney carcinoma) cell lines and *in vivo* experimental model of Ehrlich ascites carcinoma (EAC) tumor bearing mice. The experimental results suggested that the extract (containing Shatavarin IV) possess potent anti-cancer activity (Mitra *et al.*, 2012).

2.4 Drugs under marketing

According to the National Medicinal Plant Board, 2003 the demand for *A. racemosus* was 10,924.7 tonnes in 2001–2002 which risen up to the level of 16,658.5 tonnes in 2004–2005 which suggest that the annual growth rate of its demand is 15% (Rath, 2005).There are a number of formulations which are prepared using *A. racemosus* extract (Table 2). Himalayan herbal health care, currently manufactures formulations containing significant amount of extract of the plant. *A. racemosus* is not only a research oriented plant; the marketing status displays its economic importance worldwide.

Table 2: *A.racemosus* containing formulations

S.No	Drug	Content of *A.racemosus*	Medicinal property	Reference
1	Abana®	10 mg Shatavari root extract per tablet	Hyperlipidemic conditions	(Venkataramaiah, 2002)
			Mild to moderate hypertension	(Verma & Bordia, 1992) (Dubey *et al.*, 1985)
			Adjuvant in the angina with cardiac risk factor	(Verma & Bordia, 1991)
			Inhibition of platelet aggregation	
2	Diabecon®	20 mg Shatavari root extract per tablet	Monotherapy in non-insulin-dependent diabetes mellitus	(Ganguly *et al.*, 1995)
			Adjuvant to other oral antidiabetic drugs	(Kohli *et al.*, 2004)
			NIDDM with hyperlipidemia	(Mitra *et al.*, 1996)
			Early retinopathy	(Kant *et al.*, 2002)
			Microalbuminuria	(Yajnik *et al.*, 1995)
			Promotes β-cell repair/ regeneration and increases the C-peptide level	(Maji & Singh, 1996)
3	EveCare®	32 mg Shatavari root extract per 5 ml syrup	Dysmenorrhea	(Swarup & Umadevi, 1998)
			Menorrhagia	(Sarda *et al.*, 2007)
			Metrorrhagia	(Mitra *et al.*, 1998)
			Oligomenorrhea	(Venugopal, 1998)
4	Geriforte®	20 mg Shatavari root powder per tablet	Geriatric stress	(Ghosh & Mitra, 1985) (Shah *et al.*, 1990)
			Generalized anxiety disorders	(Boral *et al.*, 1989)
			Stress related anxiety	(Vaidya, 1979)
			Prolonged illness and convalescence	
5	Himplasia®	80 mg Shatavari root powder per tablet	Benign prostatic hyperplasia	(Sahu & Kulkarni, 2003)
6	Lukol®	40 mg Satavari root extract per tablet	Leukorrhea	(Bhatnagar & Bhatnagar, 1984)
			Malaise	(Dabak *et al.*, 1982)
			Backache associated with leukorrhea and Pelvic inflammatory disease	(Tewiri & Kab Suliw Kulkiroi, 2001)
7	Menosan®	110 mg Satavari root extract per tablet	Natural menopause	(Sarkar *et al.*, 2004)
			Surgical menopause	(Singh & Kulkarni, 2002)
8	Renalka®	50mg shatavari root extract per 5mL of syrup	Burning micturition	(Sahu *et al.*, 2002)
			Cystitis, Recurrent Urinary Tract Infection,Dysuria, Hematuria	(Prakash, 2001)

CHAPTER 3.0

MATERIAL AND METHODS

“I have not failed. I've just found 10,000 ways that won't work”

.....**Edison**

Chapter 3.0

Material and methods

3.1 Chemicals and Instruments

S. typhimurium strains TA98, TA100 were procured from IMTEC (Institute of Microbial Technology), Chandigarh. Sodium azide for TA100, 4-Nitro-*o*-phenylenediamine (NPD) for TA98, Dimethyl sulfoxide (DMSO) was procured from M/S Sigma Chemicals Co. (St Louis, MO, USA). Solvent methanol was procured from SRL, India, HEPES buffer, Sodium propionate, EIPA were purchased from Sigma-Aldrich (St. Louis, MO, USA), Disodium hydrogen orthophosphate (Thermo Fisher Scientific India Pvt. Ltd., Mumbai), potassium dihydrogen phosphate (LOBA Chemicals Pvt. Ltd., Mumbai), glucose (SD FChemL), bovine serum albumin (BSA) (S.D.Fine-Chem Limited, Mumbai), sodium chloride (S.D.Fine-Chem Limited, Mumbai),potassium chloride (Merck Specialities Private Limited, Mumbai), aminoguanidine (Lancaster, White Lund, Morecambe, England), sodium hydroxide (LOBA Chemicals Pvt. Ltd., Mumbai), trichloroacetic acid (Qualigens Fine Chemicals, Mumbai) were employed for the present study. Petroleum ether, chloroform, ethyl acetate. Laboratory grade Reverse osmosis water from Rions R.O system was used. Silica gel 60/120 for column chromatography was procured from SDFCL. Glassware of fine quality were used and procured from Borosil, Perfit, JSGW. Readymade TLC plates F_{254} from Merck were used.

3.2 Procurement and Preparation of Plant material

The roots of *A. racemosus* were collected in the month of September, 2012 from Palampur, Himachal Pradesh. The roots of *A .racemosus* were cleaned to remove the major of dust and soil. The roots were chopped into small fragments to increase the surface area for the drying purpose. The small fragments were dried under shade, and the dried roots were grinded to the powder form (Figure 12).

Figure 12: Powdered *A. racemosus* roots

3.3 *A. racemosus* extract preparation and isolation

The powdered material (3.1kg) was subjected to successive extractions (Figure 13) with petroleum ether (RPE), chloroform (RCE), ethylacetate (REE), methanol (RME) and aqueous methanol (20:80) (RAE) 22g, 37g, 50g, 115g, 135g, respectively.

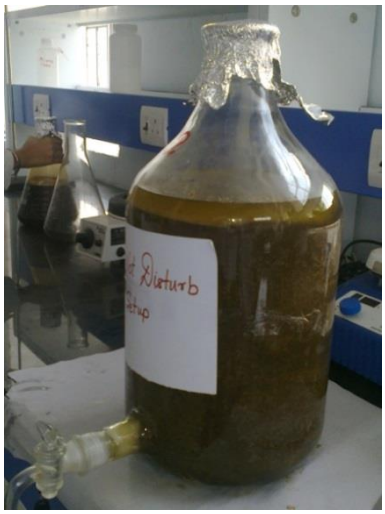


Figure 13: Powdered root of *A. racemosus* packed in Aspirator

The RME (100g) was subjected to CC over silica gel (60-120 mesh, 1800 g) using a gradient elution of 5%, 10 %, 25%, 50%, 75 %, 100% petroleum ether : ethyl acetate to give 60 fractions. Fraction 31 to 33 were combined and dried on the rotavapour to yield 200 mg of reddish brown solid mixture, which was further chromatographed over silica gel (60-120, 4g) and eluted with 15% ethyl acetate in petroleum ether.

Sub-fractions 5-22 (25 mL each) were combined together on the basis of a single spot on precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on a rotavapor yielding RVA-1 (Nyasol) (109.1 mg) as a brown amorphous solid. Fraction 34 to 35 were combined and dried on rotavapour to give 228mg of brown mixture which was further chromatographed over silica gel (60-120, 4.5 g) and eluted with 12.5 % ethyl acetate in petroleum ether. The fractions 11-19 (10mL each) were combined together on the basis of a single spot on precoated silica gel $60F_{254}$ and dried *in vacuo* to give 21.2 mg of RVA-2**.**

The methanolic extract RME was then chromatographed using gradient elution of 5%, 10%, 25%, 50%. 75%,100% methanol in ethyl acetate to give 60 fractions. Fractions 30 to 40 were combined and dried on the rotavapour to give 2.5 g of brown mixture, which was further chromatographed to give over silica gel (60-120, 50 g) eluted at 25 % ethyl acetate in petroleum ether, 65:1:1 and 65:2:1 solvent system of chloroform : methanol: water, to give 600 fractions. Sub-fractions 82 to 116 (10mL each) were mixed together on the basis of a single spot on precoated silica gel $60F_{254}$ and dried *in vacuo* to give 25mg of RVA-3. The fraction 256-347 were combined together on the basis of a single spot on precoated silica gel $60F_{254}$ and then dried *in vacuo* to give brown amorphous solid RVA-4 (43.3 mg) Fractions 410 to 425 (10mL each) were combined on the basis of a single point on the precoated silica gel $60F_{254}$ TLC plate and dried *in vacuo* yielding 102.9 mg of RVA-5. Fractions 478 to 526 (10mL each) were combined together on the basis of a single spot on precoated silica gel $60F_{254}$ TLC plate. The combined fractions were dried *in vacuo* to yield RVA-6 (102.6 mg). The fractions 535 to 555 (10mL each) were combined on the basis of a single spot on precoated silica gel $60F_{254}$ TLC plate and then dried *in vacuo* to yield 50.2 mg RVA-7.

3.4 Spectral details of the isolated compound

^{1}H NMR (400MHz,$CDCl_3$): 4.38(m,1H, H-3), 5.03-5.09(t, 2H, H-5a, 5b), 5.54-5.56 (m, 1H, H-2), 5.87-5.95 (m, 1H, H-4), 6.63-6.75 (m, 4H, H-3',5',3",5"), 6.980-6.987 (d, 2 H, H-2', 2"), 7.06-7.094 (d, 3H, H-1, 2", 6"), ^{13}C NMR (100MHz,$CDCl_3$): 45.80 (C-3), 113.94 (C-5), 114.16 (C-5'), 114.38 (C-3'), 114.43 (C-3''), 127.58 (C-6', 1'), 127.81 (C-2'), 128.57 (C-1, 2"), 128.96 (C-6''), 130.58 (C-2), 134.34 (C-1''), 139.75 (C-4), 153.21 (C-4'), 153.76 (C-4''). Some impurities peaks corresponding for carbonyl and methyl carbon of ethyl acetate were observed in the NMR spectra.

ESI-QTOF-MS: m/z $[M-H]^-$ calculated for $(C_{17}H_{16}O_2 - H)^+$: 251.12 ; Found: 251.0 corresponding to molecular formula $C_{17}H_{16}O_2$.

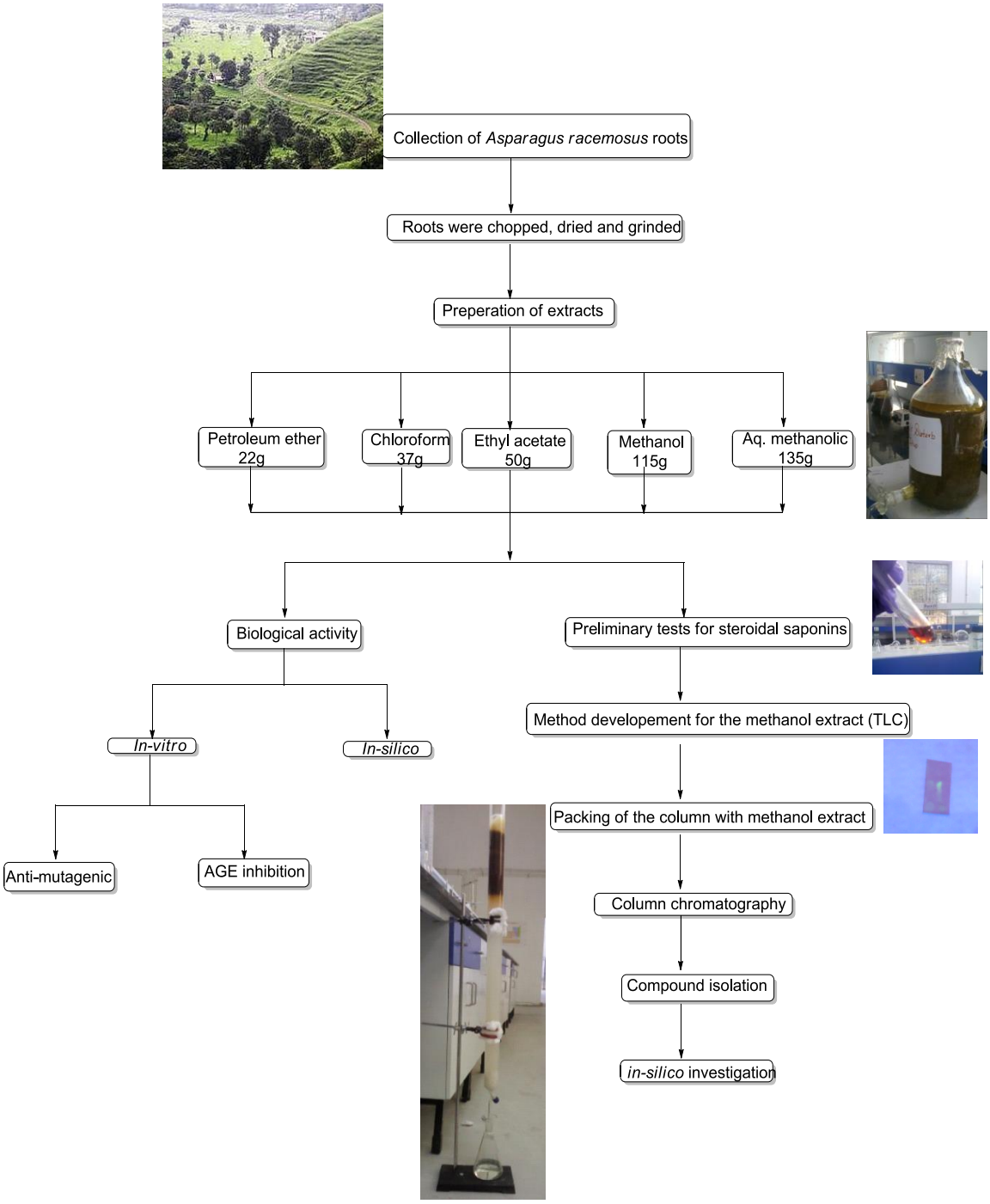


Figure 14: Overview of the work done

3.5 Preliminary Phytochemical Investigation of Extracts

Qualitative chemical test of petroleum ether, ethyl acetate, chloroform, methanol, and aqueous extracts of *A. racemosus* were subjected to tests detect the presence of various phytoconstituents. Salkowski'sTest, Froth test for saponins, Keller Kilani test was performed for the preliminary phytochemical investigation (Table 3).

Table 3: Preliminary phytochemical investigation of Extracts of *A.racemosus*

S.No	Test	Fraction	Result	Inference
1.	**Salkowski'sTest:** Treat extract in chloroform with few drops of concentrated sulphuric acid, shaken well and allow to stand for some time, red colour appears in the lower layer indicate presence of sterols	Methanolic extract	positive	Steroidal present
		Aquous extract	positive	Steroidal present
		Ethyl acetate extract	positive	Steroidal present
		Chloroform	positive	Steroidal present
		Petroleum ether	positive	Steroidal present
2.	**Froth test for saponins**-0.5g of each plant extract was shaken with water in a test tuber. Frothing which persists on warming was taken as preliminary evidence for the presence of saponins	Methanolic	positive	Saponins present
		Aquous extract	positive	Saponins present
		Ethyl acetate	negative	Saponins absent
		Chloroform	negative	Saponins absent
		Petroleum ether	negative	Saponins absent
3.	**Keller kiliani test-** 0.5g of extract will be dissolved in 2mL of glacial acetic acid containing one drop of ferric chloride solution. This will then be underlayed with 1mL of concentrated sulphuric acid. A brown ring obtained at the interface will indicate the presence of a deoxy-sugar characteristic of cardenolides.	Methanolic extract	positive	Cardenolides present
		Aquous extract	positive	Cardenolides present
		Ethyl acetate	positive	Cardenolides present
		Choloroform	negative	Cardenolides absent
		Petroleum ether	negative	Cardenolides absent

3.6 *In-silico* study of Nyasol

The molecule isolated from the methanolic extract RVA-1 or nyasol was docked over estrogen receptor beta (1OLS) taking estradiol as standard, and estrogen receptor alpha (3ERT) tamoxifen as standard drug using Autodock 4.2.

3.6.1 Protein preparation

The PDB for the crystal structure of estrogen receptor beta ligand binding domain (3OLS) (Mocklinghoff *et al.*, 2010), estrogen receptor alpha ligand-binding domain (3ERT) were retrieved from protein data bank. During the protein preparation only polar hydrogens and kollman charges were added. The atoms in the protein were assigned for Autodock 4 type. Now the PDBQT file of the protein was saved in which its different pdb records from “ATOM” select till **“**END” were added.

3.6.2 Ligand preparation

The 3D structure of the ligand was created using ChemBio Draw 3D, and saved in the .pdb format. The ligand was selected from the ligand menu of autodock and from the torsion tree, its roots were first detected and then selected. Now it will show number of rotatable bonds in the ligand. The resultant ligand file is saved in standard autodock .pdbqt file format.

3.6.3 Grid generation

From the GRID menu of autodock, the macromolecule (targeting protein) was selected and the protein is initialized in the autodock for the grid generation. Herein this step additional charges and bond orders are assigned. The PDBQT file generated in this step overwrites the previously created PDBQT file. The Grid was generated by taking the bound ligand as the centre of grid. The grid maps representing the proteins will be calculated using auto grid and grid size was set to 60x60x60 points with grid spacing of 0.375 Å.

3.6.4 Docking

Docking of protein to ligands were carried out using LGA (Lamarckian Genetic Algorithm)(Morris *et al.*, 1998) with standard docking protocol on the basis a

population size of 150 randomly placed individuals; a maximum number of 2.5 x107 energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80 and an elitism value of 1. Ten independent docking runs will be carried out for each ligand and results will be clustered according to the 1.0 Á rmsd criteria.

3.7 Determination of protein glycation inhibitory activity

The extent of protein glycation inhibitory activity was assessed by measuring the AGE formation using the spectrofluorometric method (Deepralard *et al.*, 2009; Jedsadayanmata, 2005; Mahomoodally *et al.*, 2012; Matsuura *et al.*, 2002; Suzuki *et al.*, 2003; Tang *et al.*, 2004; Yang *et al.*, 2009). Briefly, 1 mg/ml of bovine serum albumin (BSA) was incubated with D-glucose (200 mM) with or without plant extracts or aminoguanidine in 50 mM potassium phosphate buffered saline (pH 7.4). The reaction was allowed to proceed at 60^{o}C for 24 hours. The control group was free of any plant extract and aminoguanidine. The reaction was stopped by adding 100% (w/v) tricholoroacetic acid and after ten minutes the mixtures were centrifuged at 15,000 rpm, 4^{o}C for 4 min. The precipitate were re-dissolved in alkaline phosphate buffered saline (PBS). The content of advanced glycated end products was determined spectrofluorimetrically at excitation wavelength of 370nm and emission wavelength of 440nm. In protein glycation inhibition studies, the concentrated stocks of hydroalcoholic and alkaloidal extracts of all the plants along with standard drug (aminoguanidine) were prepared in DMSO. The various concentrations of extracts and standard drug were added to the assay mixture. Any sample giving fluorescence equal to the fluorescence of BSA/glucose implied that there was no inhibition of glycation; whereas, any sample giving fluorescence lower than that of BSA/glucose indicated that there was inhibition of glycation by the extract. The percentage of anti-glycated activity was calculated as

$$\frac{(A_{control} - A_{sample})}{(A_{control})} \times 100$$

where,

$A_{control}$ represents the fluorescence of control group,

A_{sample} represents the fluorescence of sample group.

Measurements were performed in triplicate, and the concentration required for a 50% inhibition (IC_{50}) of the fluorescence intensity was determined graphically.

3.8 Antimutagenic assay

The *Salmonella* histidine point mutation assay proposed by (Maron & Ames, 1983) was followed. *S. typhimurium* strains TA98 (frameshift mutation test) and TA100 (base pair substitution test). The fresh cultures of tester strains TA98 and TA100 of *S. typhimurium* having density of 1-2 x 10^9 CFU/mL were used to investigate the antimutagenic activity of different extracts of *A. racemosus*. The minimal agar plates were prepared one day before experiment. Top agar was autoclaved and stored at 4°C, before the initiation of the experiment. It was melted and kept at 45°C. Co-incubation and pre-incubation two modes of experimentation were followed. The concentrations of the test sample used for investigating the antimutagenicity were 2.5x10^3,1.0x10^3, 0.5x10^3, 0.25x10^3, 0.10x10^3 and 0.01x10^3 µg 0.1mL/plate. All these concentrations of test samples were dissolved in DMSO under sterile condition. The negative control was run with different concentration of extracts, to verify its toxicity. The concentrations were considered non-toxic if the number and size of the revertant colonies in the negative control were equivalent to that of spontaneous revertant colonies. The fraction will be non-toxic if the intensity of background lawn is equivalent to the control having bacteria culture only. For the determination of toxicity of test sample, 0.1mL of sample with 0.1mL of freshly grown culture was added to the top agar which was maintained at 45 °C. The mixture was then spread over the minimal agar plates which were then incubated at 37 °C for 48 hrs. The antimutagenic effect of the different concentration of the extracts was determined against the known mutagen which is the characteristic of each strain depending on the reversion event. The mutagenicity of mutagen was also checked on the tester strains, in order to ensure the responsiveness of tester strain and efficacy of promutagen. The confirmation of the effect of mutagen on tester strain was determined by taking 0.1mL of freshly grown culture along with 0.1mL of the mutagen specific for the tester strain were added to the soft agar, then poured on to the minimal glucose agar plates after thorough mixing. Then plates were incubated at 37 °C for 48 hrs and effect of mutagen was accessed by counting the number of revertant colonies. In order to determine the antimutagenic potential of the different concentrations of the extracts co-incubation mode of experiment was followed. In co-incubation experimentation,

0.1 mL of bacterial culture, 0.1 mL of NPD or 0.1 mL of sodium azide and 0.1 mL of non-toxic concentrations of the extracts were added to 2mL of top agar containing 0.5 mm histidine/biotin. The antimutagenicity potential of extract was also determined in pre-incubation experiment mode. In the pre-incubation mode of experimentation, equal volumes of the mutagens and the extracts were mixed in sterile capped tubes and allowed to stand for 30 min at 37^{o}C under continuous shaking and 0.2ml of this was added to 2 mL of soft agar with 0.1 mL of fresh *Salmonella* culture. The dose inhibiting 50% of mutagenicity (IbD50) was inferred from the dose–response curve and used as an indication of antimutagenicity potency (De *et al.*, 1992). All the test samples were assayed using triplicate plates per run and each experiment was conducted twice in order to make estimation of variation. The activity of each extract was expressed as the percentage decrease of reverse mutation.

$$\text{Activity (\%)} = \frac{a-b}{a-c} \text{ X } 100$$

$$\text{Percent of Control} = \frac{b}{a} \text{ X100}$$

Where

a = No. of histidine revertants induced by mutagen (NPD, sodium azide)

b = No. of histidine revertants induced by mutagen in the presence of extract

c = No. of revertants in the negative control

3.9 *In-silico* study of Phytoestrogens

In the present study with the aid of molecular docking environment, using Maestro software 9.3 (Maestro version 9.3.,2012). the mechanism behind the anti-proliferative activity of thirty phytoestrogens (Figure 15) from *A. racemosus* was determined (Handa *et al.*, 2003; Hayes *et al.*, 2008; Kumeta *et al.*, 2012; Mandal *et al.*, 2006; Sharma *et al.*, 2009). The targets selected for the study were estrogen receptor β, HSP90 (Heat shock protein) protein, steroid sulphatase/human placental estrone sulpatase, glucose-6-phosphate dehydrogenase, 17β-hydroxy dehydrogenase and tubulin.

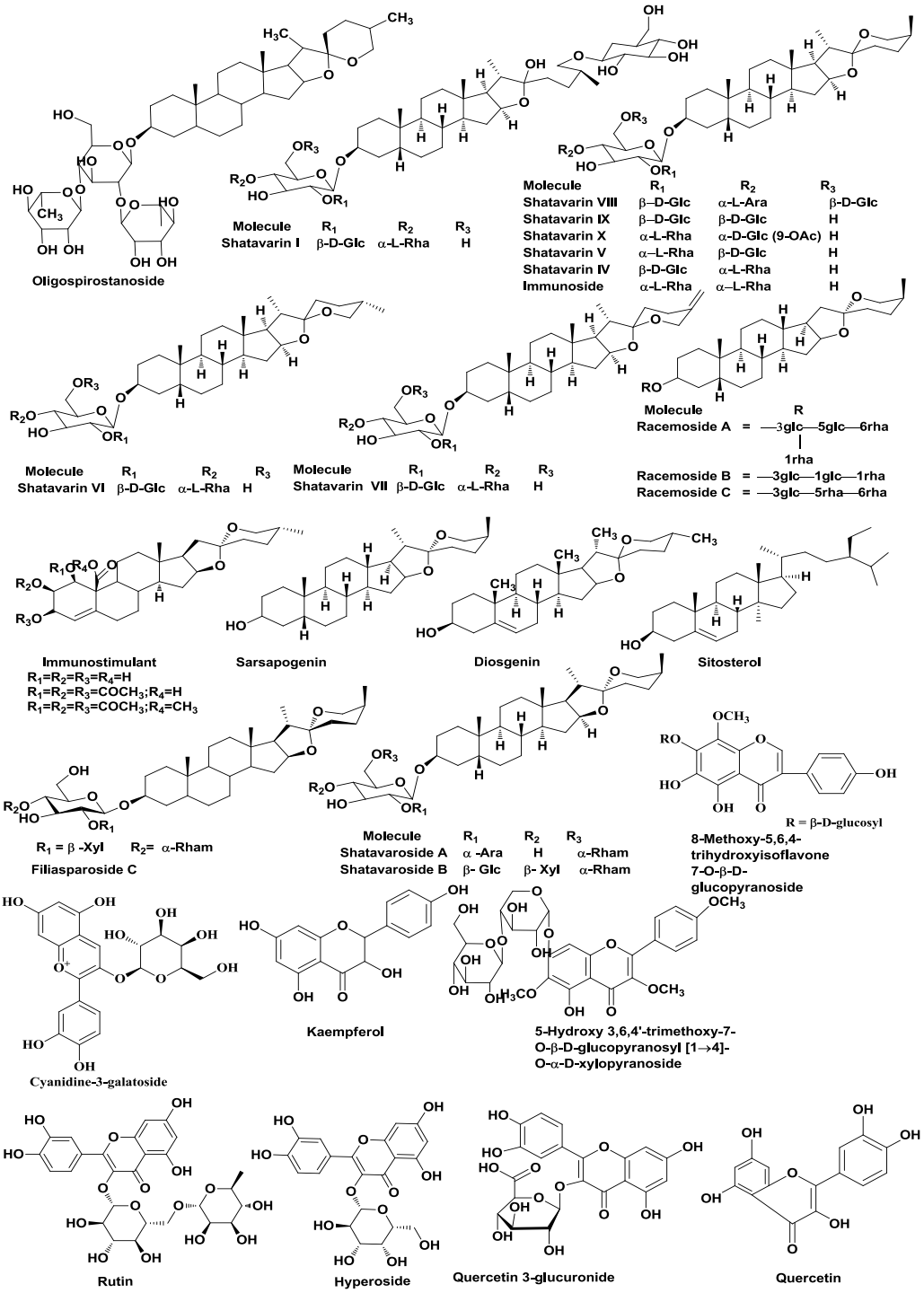


Figure 15: Phytoestrogens reported from plant *A.racemosus*

3.9.1 Protein preparation

The PDB for the crystal structure of estrogen receptor beta ligand binding domain (3OLS) (Mocklinghoff *et al.*, 2010), HSP90 (1YET) (Stebbins *et al.*, 1997), Human placental estrone sulphatases (1P49) (Hernandez-Guzman *et al.*, 2003), human 17β-

hydroxysteroid-dehydrogenase type 1 (1FDS) (Breton *et al.*, 1996), human glucose 6-phosphate dehydrogenase (2BH9) (Kotaka *et al.*, 2005), tubulin protein (1SA0) (Ravelli *et al.*, 2004) was retrieved from the RCSB. The protein structure with polar hydrogen was prepared using the protein preparation wizard in Maestro 9.3 (Maestro version 9.3., 2012). In this step, bond orders were assigned, all hydrogens were added, and bonds to metals were deleted and formal charges were set on the metal and the neighboring atoms and water molecules were deleted that were more than the 5 Å specific distance. Any missing disulphide bonds were added. The H-bonds were optimized using protassingn at pH 7.0. With generated Het states options, prediction of ionization, and tautomeric states of the het group at pH 7.0 was achieved. In protein preparation, reorienting hydroxyl group, water molecules, and amino acids lead to the optimization of hydrogen bond network. The refinement of the structure was the final step in the protein preparation, with the help of restrained minimization. It was initiated in the imperfect minimization with the 0.3 Å RMSD for the minimization OPLS 2005 force field All bound ligands (small molecules and BH3 peptides), waters beyond 5 Å and ions, molecules and heteroatoms were removed from the complexes (Sastry *et al.*, 2013).

3.9.2 Ligand preparation

The ligand used were sketched by using ChemBioDraw Ultra 12.0 (Kerwin, 2010) and saved in SDF format. The molecules were converted to 3D structure from the 2D using Lig Prep version 2.5 (Maestro version 9.3., 2012).The Lig Prep produces a single, low energy, 3D structure with correct chiralities for each input structure. During the performance of this step, chiralities were determined from 3D structure and original states of ionization were retained. Ligprep application of the maestro 9.3 utilizes OPLS 2005 force field.

3.9.3 Docking

SiteMap was used to determine the potential top ranked binding site of the protein human glucose 6-phosphate dehydrogenase, Human placental estrone sulphatases which the ligand is not available in the co-crystal structure. In the parameters we went for the default parameters in the sitemap, which included use of more restrictive definition of hydrophobicity and the use of standard grid. OPLS-2005 force field was

used. Five sites were generated the top ranked site was selected on the basis of Sitescore, size, D Score and volume for grid generation (Maestro version 9.3., 2012). The grid was generated in which Van der Waals scalling was reduced to 0.20 for the Estrogen receptor β and 0.50 for the HSP90, human placental estrone sulphatase, human 17-β-hydroxysteroid-dehydrogenase type 1, human glucose 6-phosphate dehydrogenase, tubulin to soften the potential for non-polar parts of the receptor with partial atomic charge cutoff of 0.25. The length of the ligands to be docked was increased to 36 Å. The X, Y, Z co-ordinates were 46, 46, 46 respectively. Docking of the Reported phytoestrogens and the inhibitor was done with XP (extra precision) , XP descriptors were written. Ligand was taken as flexible. Sample nitrogen inversions and sample ring conformations were taken into account. Bias sampling of torsions was one only for the amides and non-polar conformations were penalized. Epik penalties were added to the docking score. Vander Waals scalling was taken as 0.20 for estrogen receptor β; 0.50 for HSP90, human placental estrone sulphate, glucose-6-phosphate dehydrogenase and tubulin; 0.80 for 17-β-hydroxydehydrogenase type I and the partial charge cutoff was taken 0.15 to soften the non-polar parts of the ligand. 10000 poses per docking run were allowed to be run and 1 pose per ligand was allowed to be written. In the post docking minimization number of poses per ligand to be included was taken to be 10. The threshold energy below which the pose to be rejected was 0.5 kcal/mol (Maestro version 9.3., 2012).

CHAPTER 4.0

RESULTS AND DISCUSSION

“A positive attitude causes a chain reaction of positive thoughts, events and outcomes. It is a catalyst and it sparks extraordinary results”

…**Wade Boggs**

Chapter 4.0
Results and Discussion

4.1 Spectroscopic analysis of isolated compounds

Compound RVA-1, was isolated as reddish brown amorphous powder from methanolic extract (Figure 16). In the IR spectrum, a band was observed for hydroxyl group at 3400cm^{-1} (br). Absorption at 1511cm^{-1} and 1609cm^{-1} indicated the presence of aromatic ring. A negative ESI-MS showed ion peak at m/z 251.0 $[M-H]^-$ (calculated for $[C_{17}H_{16}O_2-H]$, 251.12) corresponding to their molecular formula $C_{17}H_{16}O_2$, with eight degree of unsaturation. The DEPT spectrum showed thirteen well resolved peaks assigned to 1-CH_2 and 12-CH signals. Four signals were assigned to quaternary carbons. Evidence of the presence of alkylated moiety at C-3 position was confirmed by long range HMBC correlations between H-3(δ 4.38) and δ113.94(C-5), 139.75(C-4), 130.58(C-2). The presence of hydroxyl group on benzene ring was confirmed by the ^{13}C NMR values δ153.71 (C-4') and δ153(C-4''). Another methlene proton δ_H 5.03 (t, 2H, H-5) showed HMBC correlation with δ_C 139.75 (C-4), 45.8 (C-3). Methine proton at δ 5.54 (m, 1H, H-2) showed correlation with δ 139.75 (C-4), 134.34 (C-1''). On the basis of NMR data, HMBC and HMQC correlations and other spectral assignments, the structure of RVA-1 was confirmed Nyasol (Tsui & Brown, 1996).

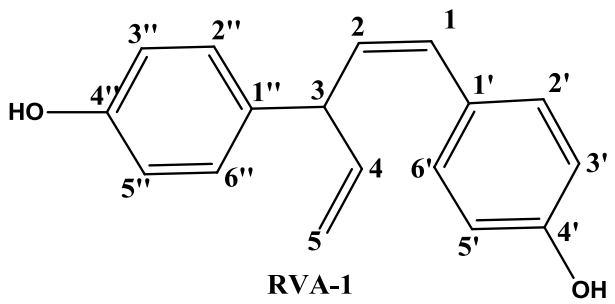


Figure 16: Structure of isolated molecule Nyasol

4.2 *In-silico* study of Nyasol

In-silico study of Nyasol, isolated from the methanolic extract was done on estrogen receptor alpha (3ERT) and estrogen receptor beta (3OLS) (Figure 17). The docking score obtained for the respective receptor along with the score of their standard are given in Table 4. Result indicated that the nyasol has a strong binding affinity for the

estrogen receptor beta as compared to its standard oestradiol. It was reported that nyasol and its derivatives are used in the estrogen receptor β mediated disorders and hormone responsive breast cancer (Cohen, 2009). It has been evidently found that, in breast cancer there is an overexpression of estrogen receptor α and in around 70% cases the treatment with anti-estrogen therapy like tamoxifen have noticeable success (Ali & Coombes., 2000)

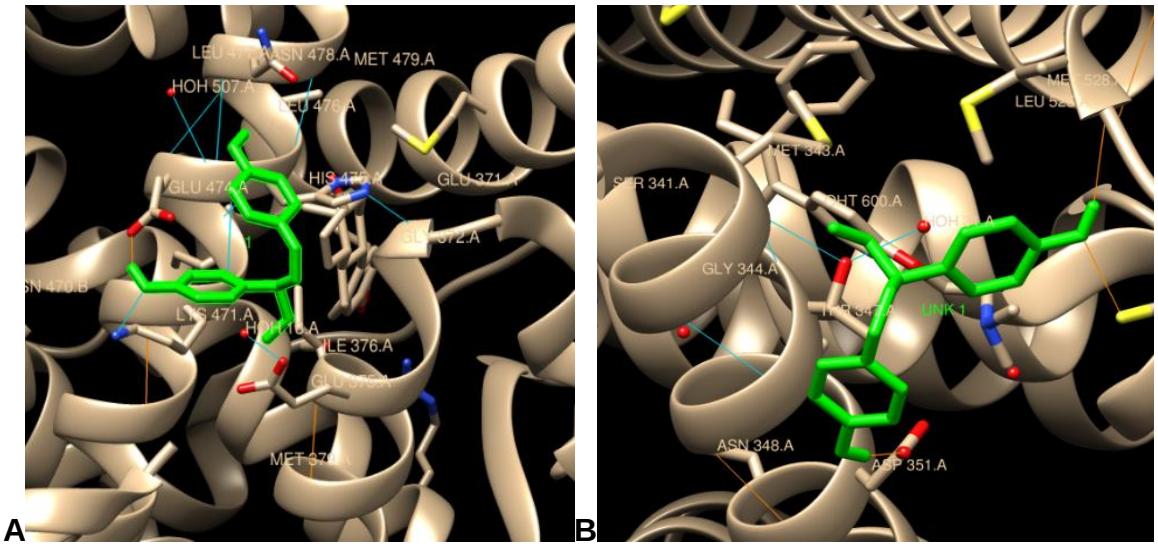


Figure 17: Docking pose of Nyasol on (**A**) estrogen receptor beta (3OLS),(**B**) estrogen receptor alpha (3ERT)

The docking study revealed that nyasol have an good binding affinity for the estrogen receptor α which Indicates the importance of nyasol as a potent inhibitor of estrogen receptor α and preventing the proliferative effect of estrogen receptor α.

Table 4: Docking energy of Nyasol and standard with their respective receptor

S.No	Receptor	Ligand	Binding Energy (kcal/mol)
1.	Estrogen receptor β	Nyasol oestradiol	-3.95 -3.09
2.	Estrogen receptor α	Nyasol tamoxifen	-4.30 -5.09

4.3 *In-vitro* antiglycation activity

Advanced glycation end products (AGEs) are part of a major pathogenic process in diabetic complications including neuropathy, nephropathy, retinopathy, atherosclerosis, hypertension, platelet aggregation, reduced platelet survival and cataracts(Peng *et al.*, 2008; Singh *et al.*, 2001). BSA-glucose models were used for the evaluation of inhibitory effect on the formation of AGEs (Hori *et al.*, 2012). The inhibition measured by BSA-glucose varied significantly among different extracts of *A. racemosus* (Figure 18).

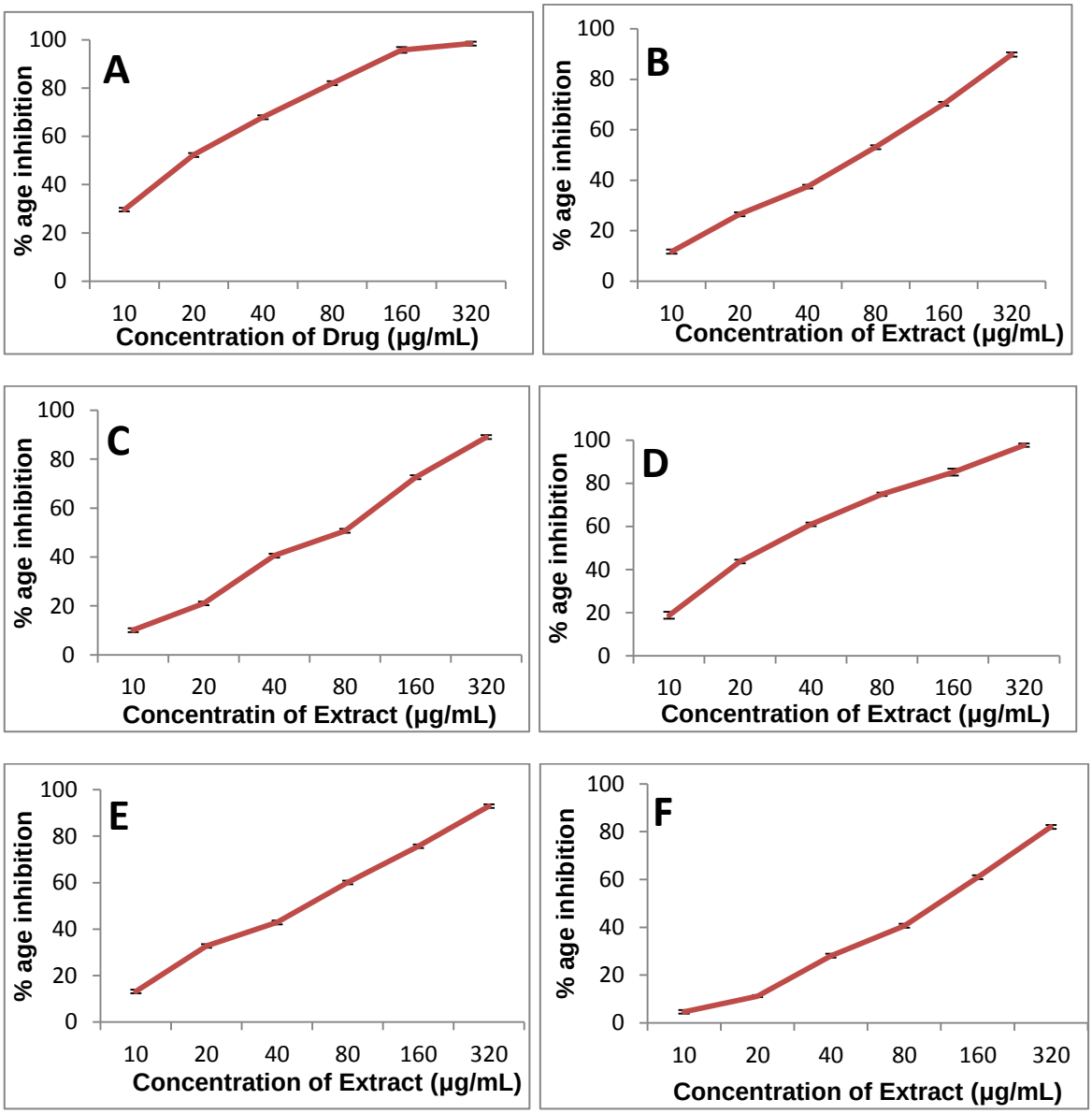


Figure 18: Anti-glycation activity (in % age) of different concentrations of the standard drug, (A) Aminoguanidine, (B) RPE, (C) RCE, (D) REE, (E) RME, (F) RAE. The values are expressed as mean ± SD in triplicate.

The highest inhibition measured by BSA-glucose was observed for REE (IC_{50} 37.56 ± 1.65µg/mL) followed by RME (IC_{50} 51.32 ± 1.48) (Table 5). Recent studies indicated that the *A. racemosus* is beneficial in ameliorating diabetic nephropathy, the treatment of alcoholic extract and aminoguanidine, reversed microalbuminuria, kidney hypertrophy (Somania *et al.*, 2012). These effects are augmented by the accumulation of AGEs in the body (Fukami *et al.*, 2008).

Table 5: IC_{50} (µg/mL) values of different extracts showing anti-glycation activity.

Sr. No.	**Extracts**	**IC_{50} Value (µg/mL)**
1.	Aminoguanidine	22.45 ± 1.89
2.	RPE	64.50 ± 1.73
3.	RCE	68.36 ± 1.57
4.	REE	37.56 ± 1.65
5.	RME	51.32 ± 1.48
6.	RAE	103.66 ± 2.09

4.4 *In-vitro* antimutagenic activity

The mutagenic and antimutagenic effects of *A. racemous* extracts RME and RAE induced by NPD in TA98; Sodium azide in TA100, respectively (Table 6 and Table 7). The extract of *A. racemosus* at a concentration of 0.1mL did not show any effect on spontaneous revertants of both TA98 and TA100 *S. typhimurium* tester strain. In this study, antimutagenic activities of RME and RAE were evaluated by determining reductions in the number of Histidine revertant mutations induced by selecting positive mutagens in *S. typhimurium* strains TA98, TA100. Only the positive control showed significantly higher number of revertants than the treatment groups. The result of negative control experiment in which RME and RAE extracts were added but no mutagen have shown an insignificant number of histidine revertants in both the strains. RME and RAE reduced the frameshift mutation induced by NPD in TA98 and sodium azide induced mutation in TA100 strains of *S. typhimurium*. RME have shown a 40.63 % inhibition in the formation of revertants in TA98 strain at co-incubation mode and 45.7% inhibition at pre-incubation mode. The extract has shown 40.1% and 42.89% inhibition at co-incubation and pre-incubation

mode in TA100 tester strain. RAE has shown 49.2% and 16.3% inhibition during co-incubation and pre-incubation mode in TA98 tester strain. 45.4% and 27.2% inhibition was observed during the co-incubation and pre-incubation mode respectively in TA100 tester strain

Table 6: Effect of RME on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S. typhimurium.*

Treatment	Dose(µg/0.1mL)	TA98		TA100	
		Mean±SE	%inhibition	Mean±SE	%inhibition
Spontaneous		24.66±1.85		75.33±6.33	
Positive control NPD					
		961.00±5.68		-	
Sodium azide		-		1194±39.52	
	2.5x10³	10.66±2.72	-	84.33±5.60	-
	1.0x10³	8.66±0.33	-	64±2.51	-
	0.50x10³	9.33±0.88	-	81.33±1.20	-
	0.25x10³	10.33±1.45	-	87±0.57	-
Negative control	0.10x10³	10±0.57	-	85.33±2.02	-
	2.5x10³	575±31.97	40.63	748.66±39.70	40.10
	1.0x10³	632.66±12.67	34.45	726.66±15.93	41.35
	0.50x10³	633.66±3.84	34.36	730.66±36.79	41.63
	0.25x10³	853±4.58	11.35	877.33±8.19	28.62
Co-incubation	0.10x10³	855.33±6.69	11.12	885±17.57	27.86
	2.5x10³	525.66±11.79	45.7	718±58.04	42.89
	1.0x10³	598±75.97	38.11	789±2.08	35.84
	0.50x10³	593±41.50	38.66	775.33±59.58	37.56
	0.25x10³	601.33±32.82	37.84	951±20.50	21.95
	0.10x10³	649±37.11	32.8	923±58.00	24.43
Pre-incubation					
One-way ANOVA					
Positive control and co-incubation		F(5,12)=114.93	HSD=88.18	F(5,12)=37.33	HSD=175.31
Positive control and pre-incubation		F(5,12)=14.27	HSD=246.42	F(5,12)=14.68	HSD=271.33
Two-way ANOVA					
Treatment		F(1,20)=29.90		F(1,20)=2.47	
Dose		F(4,20)=12.71		F(4,20)=11.04	
Treatment*Dose		F(4,20)=4.76		F(4,20)=0.58	

Among the two extracts, RAE showed maximum inhibition of 49.2% (Table 7, Figure 19). On the contrary, RME showed inhibition of 40.63% in Co-incubation mode (Table 6, Figure 18). The results demonstrated that RME and RAE extracts inhibited the mutagenicity induced by NPD in TA98 and Sodium azide in TA100 tester strains of *S. typhimurium.* There are numerous pathways through which natural products show their antimutagenic effects. Natural products have been projected to be potential therapies that reduce the genotoxicity related to the exposure certain therapeutic drugs, free radical damage and environmental contaminants.

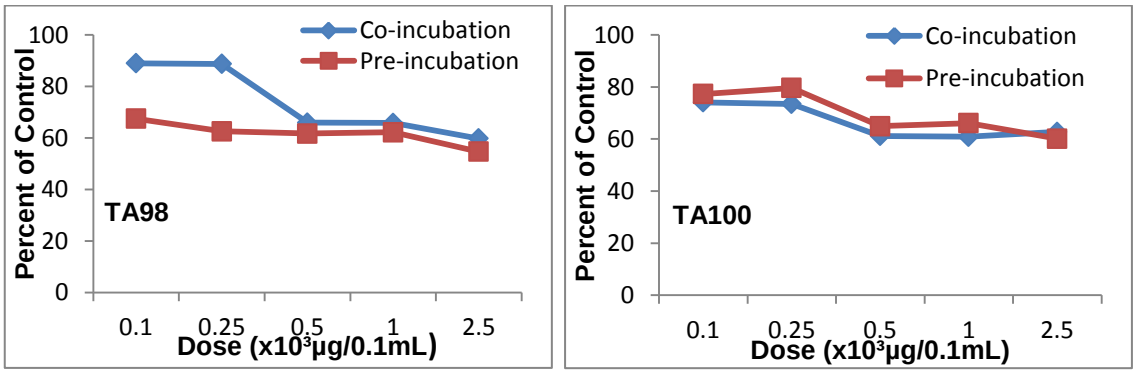


Figure 19: Effect of RME on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium*

Vitamins and natural compounds are the major sources of antimutagenic compounds which are isolated from plants (Ames, 1983 and 1984; Bandyopadhyay *et al.*, 2013). The primary use of these compounds is to reduce the risk of mutation and cancer. The protection mechanism of these natural products is complex and multifactorial (Newmark, 1996). The consumption of natural products helps in the enhancement of the enzymes involved in the metabolism of carcinogens. The effect of heat treated homogenized *Asparagus* on the mutagenicity using *S. typhimurium* TA100 strain was studied and found there was considerable increase in activity upon heat treatment (Yamaguchi, 1992). In another study, acetone, *n*-hexane, dicholoromethane, 2-propanol extracts of *Asparagus* were examined for the mutagenicity in TA98 strain of *S. typhimurium* induced by 2-amino-3-methylimidazo [4,5-f] quinolone and 2-amino-3,4-dimethylimidazo [4,5-f] quinoxaline and it was observed that *n*-hexane extract have maximum protective activity (Edenharder *et al.*, 1995). *A. offcinalis* juice has been shown to inhibit the mutagenic effects of cyclophosphamide in mice and rats (Asita *et al.*, 2008). Antimutagenic potential of the RME and RAE extract of *A.*

racemosus agrees with the literature in which the extracts possess free radical scavenging property (Kamat *et al.*, 2000) and due to their ability to decrease cytochrome P450 activity and affecting the process of enzymatic activation (Palanisamy *et al.*, 2011).

Table 7: Effect of RAE on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S. typhimurium.*

Treatment	Dose(µg/0.1mL)	TA98		TA100	
		Mean±SE	%inhibition	Mean±SE	%inhibition
Spontaneous		18.33±2.02		74±4.04	
positive control NPD		1273±55.18		-	
Sodium azide		-		1090.66±94.06	
Negative control	2.5x10³	10±1.52	-	66.66±4.17	-
	1.0x10³	11±1.00	-	66.66±2.72	-
	0.50x10³	10.66±2.60	-	64.33±6.17	-
	0.25x10³	14.33±1.85	-	60±4.93	-
	0.10x10³	13±0.57	-	58.66±2.18	-
Co-incubation	2.5x10³	651.66±22.22	49.2	626±55.24	45.4
	1.0x10³	668.66±12.83	47.9	659±11.78	42.1
	0.50x10³	828.33±72.11	35.2	698.66±47.92	38.2
	0.25x10³	836.33±23.02	34.6	724±9.01	35.6
	0.10x10³	962.33±25.36	24.6	840.66±11.83	24.2
Pre-incubation	2.5x10³	1067.33±51.36	16.3	811.66±9.83	27.2
	1.0x10³	1136±18.03	10.8	866.33±15.85	21.9
	0.50x10³	1174±11.13	7.8	848±39.20	23.6
	0.25x10³	1211±57.53	4.9	911.33±26.29	17.4
	0.10x10³	1215±25.15	4.5	894±20.51	19
One-way ANOVA					
Positive control and co-incubation		F(5,12)=31.19	HSD=245.90	F(5,12)=12.20	HSD=295.54
Positive control and pre-incubation		F(5,12)=6.19	HSD=245.87	F(5,12)=4.87	HSD=266.60
Two-way ANOVA					
Co-incubation and pre-incubation					
Treatment		F(1,20)=283.38		F(1,20)=14.70	
Dose		F(4,20)=19.34		F(4,20)=12.55	
Treatment*Dose		F(4,20)=0.74		F(4,20)=5.71	

A. racemosus was found to have a significant antidepressant activity and this effect is probably mediated through the serotonergic and the noradrenergic systems and augmentation of antioxidant defences mediated by scavenging of oxidative free radical increased during the depression (Singh *et al.*, 2009).

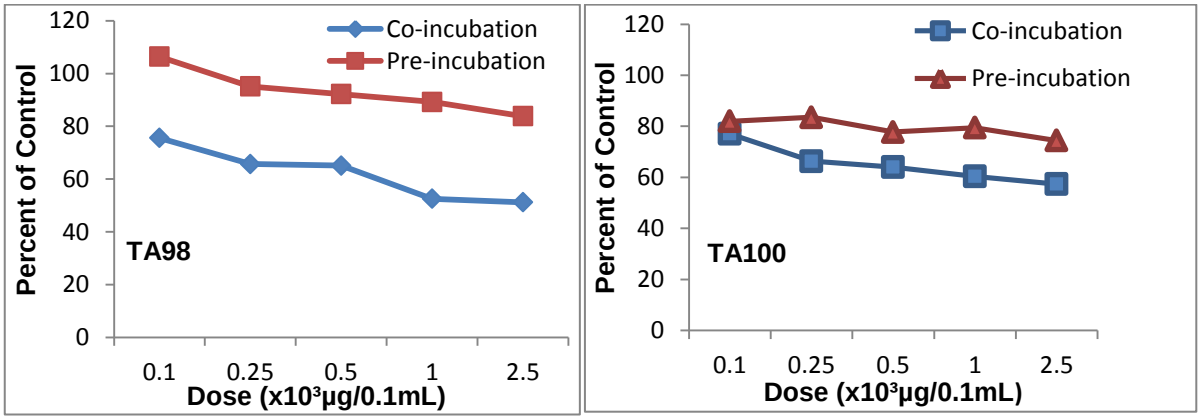


Figure 20: Effect of RAE on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium*

4.5 *In-silico* activity of phytoestrogens from *A.racemosus*

Experimental evidences suggest that the extract of *A. racemosus* possesses

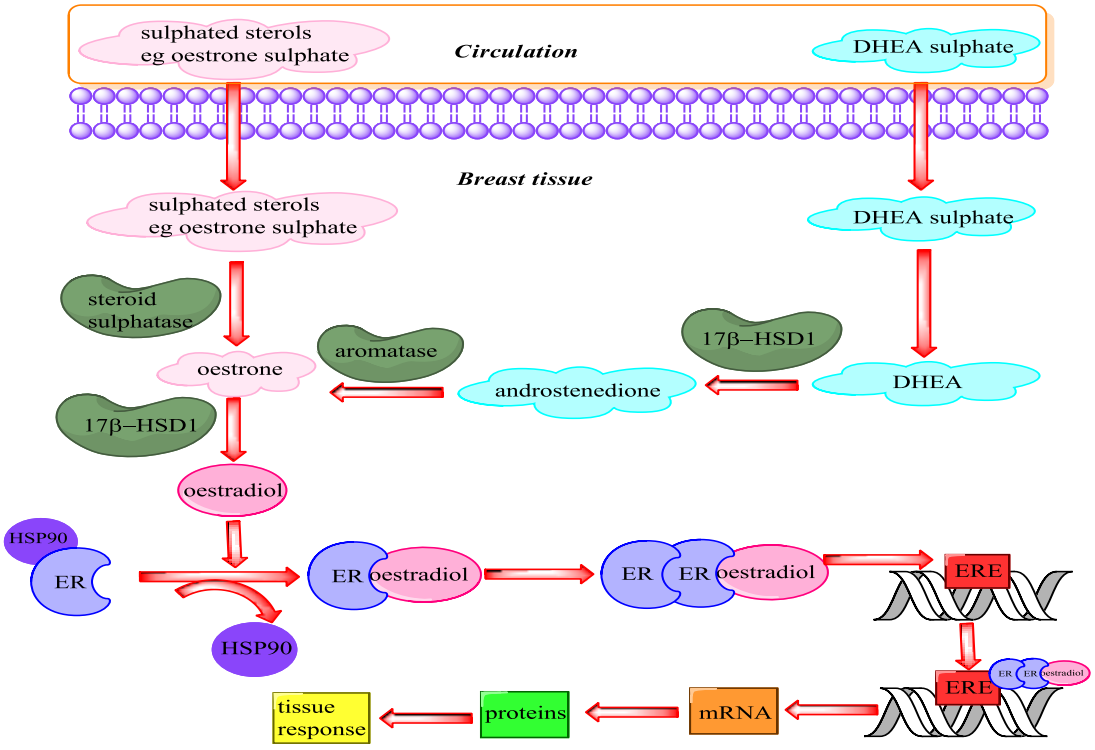


Figure 21: Oestradiol biosynthesis pathway and its binding to the estrogen receptor

anticancer activity particulary, breast cancer (Rao, 1981). But the mechanism and the receptor involved in the impressive anti-cancer activity were unknown. Docking experiment bring to light that phytoestrogens prevents the binding of oestradiol with its receptor, thereby down regulating the signalling pathway and these phytoestrogens also acts as inhibitor of enzymes involved in the biosynthesis of endogenous oestradiol (Figure 21). The targets selected for the study are estrogen receptor β, HSP90 (Heat shock protein) protein, steroid sulphatase/human placental estrone sulpatase, glucose-6-phosphate dehydrogenase, 17β-hydroxy dehydrogenase and tubulin these potential targets selected in the study were having an imperative role in the breast cancer proliferation.

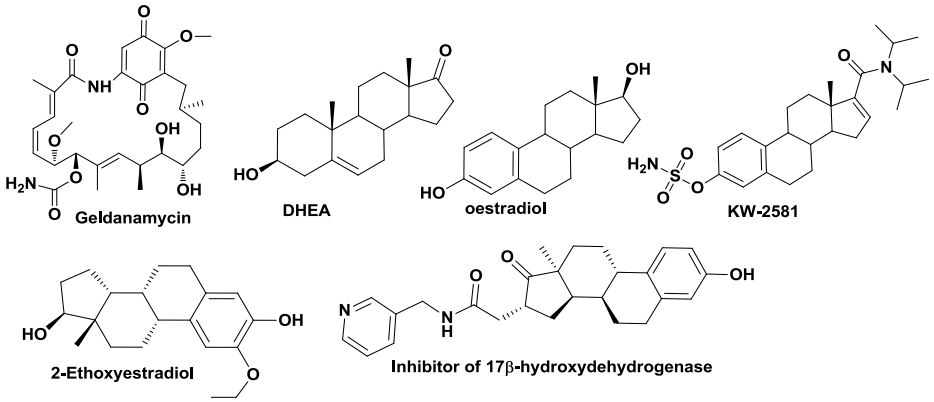


Figure 22: Inhibitors and Standard drugs used as reference in docking experiment

Phytoestrogens were docked over estrogen receptor β, HSP90, human steroid sulphates (human placental estrone sulphatase), 17β-hydroxydehydrogenase, glucose-6-phosphate dehydrogenase, colchicine binding site of the tubulin and standard chosen oestradiol, geldanamycin (Fliss *et al.*, 2000), KW-2581 (Mostafa & Taylor, 2013), Estra-1,3,5(10)-triene-16-acetamide, 3-hydroxy-17-oxo-*N*-(3-pyridinylmethyl)-, (16β)-methyl (Allan, Bubert*, et al.*, 2006), DHEA and 2-methoxy estradiol respectively (Figure 22) (Gordon *et al.*, 1995; Schwartz & Pashko, 2004; Z. Wang *et al.*, 2000). The top scoring phytoestrogens and their LipophilicEvdW, Hbond value and the residues involved in H-bonding and π-π stacking obtained upon docking over estrogen receptor β, HSP90, human steroid sulphates (human placental estrone sulphatase), 17β-hydroxydehydrogenase, glucose-6-phosphate

Table 8: Dockscore, LipophilicEvdW, HBond value, Residues in H-bonding and π-π stacking of Phytoestrogens and standards upon docking over respective receptors.

S.No	Receptor	Molecule	Dockscore	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)	Residues in H-bonding and π-π stacking
1	Estrogen receptor β	Rutin	-11.01	-1.52	-4.59	LEU_{301},GLU_{305} (Figure 23A)
		Oestradiol	-0.14	-0.68	0	*PHE_{356}* (Figure 23B)
2	HSP90	3,6,4'-Trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-12.01	-2.7	-4.21	ASP_{93}, THR_{115} , *TYR_{139}* (Figure 24A)
		Geldanamycin	-3.74	-1.19	-1.05	ASP_{102} , LYS_{112} (Figure 24B)
3	Human placental Estrone sulphatase	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-11.06	-3.3	-4.81	ARG_{98}, VAL_{101}, TRP_{555}, *PHE_{233}*, *PHE_{553}* (Figure 25A)
		KW-2581	-3.45	-1.84	-0.7	ARG_{98} (Figure 25B)
4	17β-Hydroxydehydrogenase`	Shatavarin X	-14.15	-5.34	-6.7	ILE_{14}, ASN_{90}, TYR_{155}, THR_{190}, VAL_{196} (Figure 26A)
		Estra-1,3,5(10)-triene-16-acetamide, 3-hydroxy-17-oxo-*N*-(3-pyridinylmethyl)-, (16β)-methyl	-6.74	-3.73	-1.87	SER 142, TYR 155, HIS_{221}, *PHE_{259}* (Figure 26B)
5	Glucose-6-phosphate dehydrogenase	Racemoside A	-11.79	-3.32	-6.6	ASP_{42} , LYS_{47}, LYS_{171}, GLU_{244}, ASP_{258}, LYS_{360} (Figure 27A)
		DHEA	-3.25	-1.51	-0.68	LYS_{171}, ASP_{258} (Figure 27B)
6	Colchicine binding site of tubulin	Immunoside	-10.92	-2.06	-6.84	VAL_{238}, VAL_{315}, ALA_{317}, LYS_{352} (Figure 28A)
		2-Ethoxyestradiol	-6.17	-2.49	-1.18	VAL_{315} (Figure 28B)

The subscript refers to the residue number. Residues involved in hydrogen bond interactions are shown in boldface, and those involved in π-π stacking are shown with italics.

dehydrogenase, colchinine binding site of tubulin and the score of their inhibitor in their respective sites are given in Table 8.

This docking study of the reported phytoestrogen revealed that these phytoestrogens from *A. racemosus* have a multiple approach for protection against breast cancer. Estrogen receptor comprised of two subtypes, estrogen receptor α and estrogen receptor β. Estrogen receptor β, antagonize the growth promoting effect of estrogen therefore its activation is beneficial in estrogen sensitive tumors cells (Leclercq & Jacquot, 2012). It has been discovered that estrogen receptor β is significant down regulated in the breast cancer epithelium as compared to the epithelium of the normal breast (Hartman *et al.*, 2009) thereby activating specifically this receptor will be the beneficiary in tumor suppression. Phytoestrogens have an ability to bind preferentially to the estrogen receptor β as compared to estrogen receptor α, thus is the reason behind their protective effect in breast cancer (Kuiper *et al.*, 1998; Limer & Speirs, 2004; Rice *et al.*, 2008).

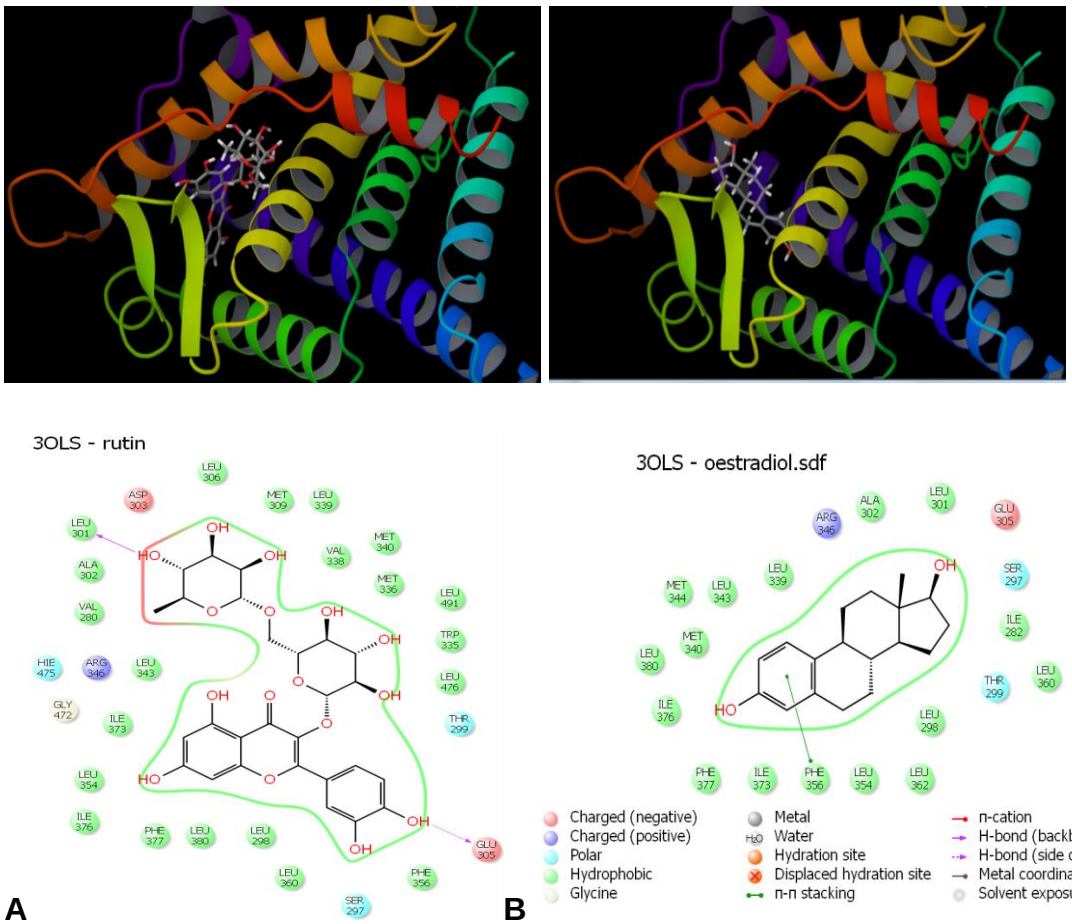


Figure 23: Ligand interaction diagram of rutin (**A**) and ostradiol (**B**) with the Estrogen receptor β (PDP id- 3OLS)

S-(-)-equol is known to have a much stronger affinity for ER β compared to R-(+)-equol, and inhibited the growth of the breast cancer cell line MDA-MB-231(Minatoya *et al.*, 2013).The high dockscore of the phytoestrogen in comparison to the oestradiol signifies strong binding affinity of the phytoestrogens for the estrogen receptor β. The dockscore of rutin was -11.01 kcal/mol and dockscore of oestradiol was -0.14 kcal/mol and the interaction profile are displayed in Figure 23A, 2B. Phytoestrogens bind to the estrogen receptor with greater affinity and activate these receptors thereby help in tumor suppression.Estrogen receptor when in the inactive state, remains in association with HSP90 (Knoblauch & Garabedian, 1999) a chaperone protein. Once the Ligand oestradiol binds with the estrogen receptor, HSP90 gets dissociated from the estrogen receptor, gets dimerised and recognizes a DNA stretch known as ERE (Estrogen response element). Thereafter upon association of the ligand receptor complex with ERE it causes the target gene activation leading to the organization of structural and functional protein essential for the cellular proliferation. The HSP90 is found to be up regulated in tumor cells (Neckers, 2002). HSP90 inhibitor Geldamycin which is a natural product isolated from fermentation of Streptomyces hygroscopicus (Amolins & Blagg, 2009) was noted to decrease the hormone binding to the estrogen receptor (Fliss *et al.*, 2000). Therefore inhibitors of HSP90 have potential for decreasing breast cancer proliferation. Docking score of the majority of phytoestrogen from the *A. racemosus* shows that these molecules act as a potent inhibitor of HSP90, thus decreasing the downstream signalling initiates upon binding of the estrogen to estrogen receptors. 3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4] -O-α-D-xylopyranoside glucopyranpsyl a phytoestrogen have a docking score of -12.01 kcal/mol and Geldanamycin have a docking score of -3.74 kcal/mol. Their interaction profile can be visualised in Figure 23A, 3B.

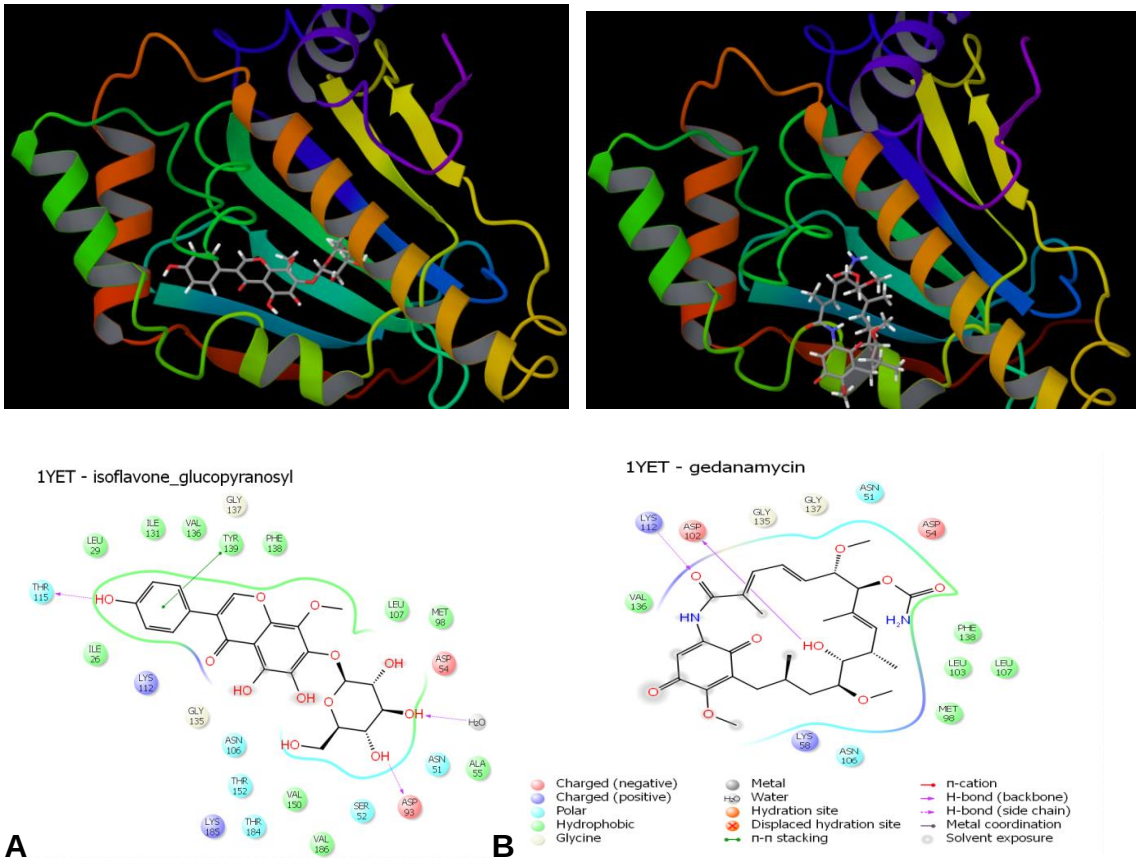


Figure 24: Ligand interaction diagram of isoflavone glucopyranpsyl (**A**) and geldanamycin (**B**) with the HSP90 (PDP id- 1YET)

Estrone sulphatase is the enzyme that catalyses the estrone sulphate into estrone which is subsequently converted to estradiol by 17- β-hydroxy-dehydrogenase type 1 (Phan *et al.*, 2011). Estrone sulphatase is an important factor in the steroid dependent breast cancer. There is an elevated expression of the steroid sulphatases in the breast cancer cells (Utsumi *et al.*, 2000).Steroidal derivative KW-2581 an inhibitor of the steroid sulphatases (Mandal *et al.*, 2006), decreases the availability of the estrogen to the cancer cells and there is reduced proliferation (Utsumi *et al.*, 2000). Quercetin and natural product derivative is found to be an inhibitor of estrone sulfatase (Huang *et al.*, 2013). Impressive dockscore for steroid sulphatase of the phytoestrogen 8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside (-11.06 kcal/mol) as compared to the dockscore of the standard drug KW-2581(-3.45 kcal/mol) indicate that phytoestrogens have an inhibitory activity against steroid sulphatase (interaction diagram depicted in Figure 24A, B and thereby decreasing endogenous availability of estradiol.

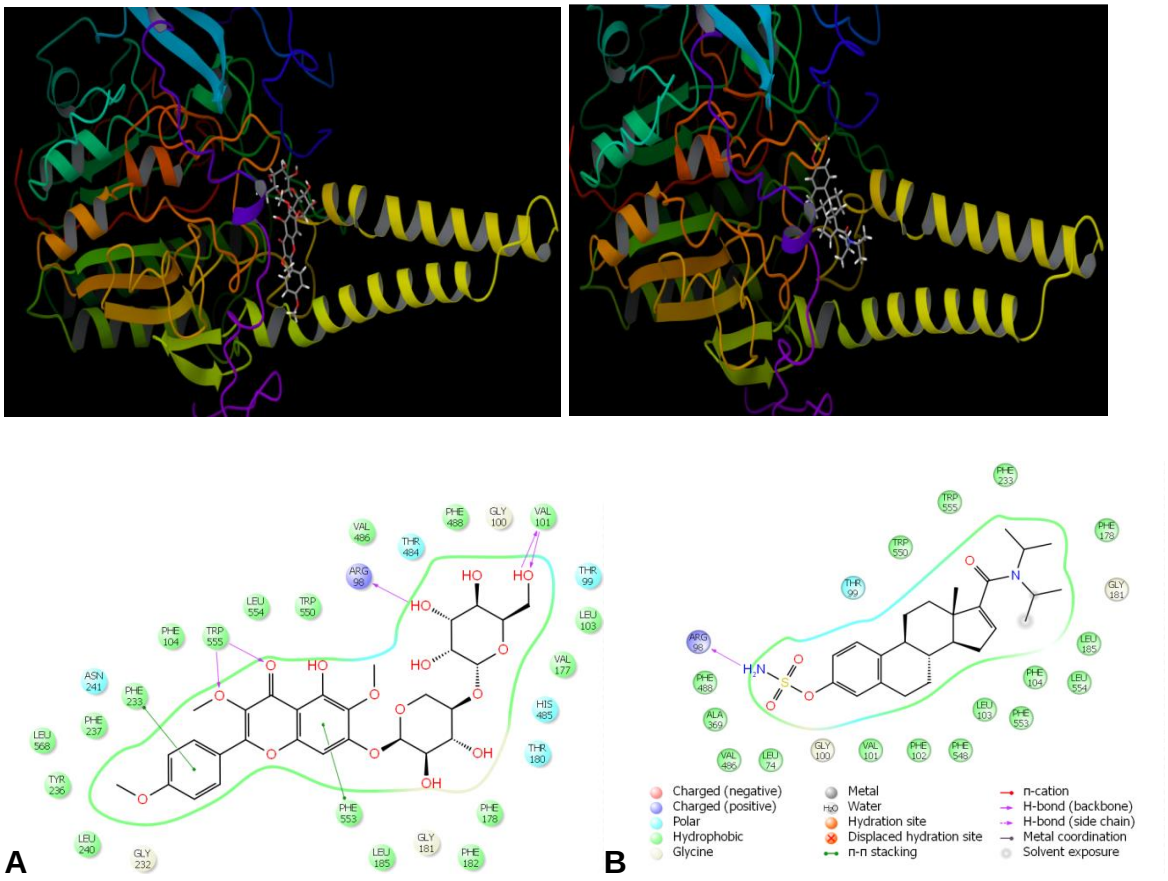


Figure 25: Ligand interaction diagram of 8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside (**A**) and KW-2581 (**B**) with the Human placental estrone sulphatase (PDP id- 1P49)

The inactive estrone is converted to the active oestrdiol by the action of the 17-β-hydroxy-dehydrogenase type I (Nguyen *et al.*, 1995). There is a positive regulator of NM23 anti-metastatic gene on the breast cancer, but 17-β-hydroxydehydrogenase increases the migration and stimulated breast cancer growth (Aka *et al.*, 2012). Therefore designing of the 17-β-Hydroxydehydrogenase inhibitors is a striking target for treatment of HDBC. Abietic acid, Flavanone, 2'-hydroxyflavanone have been shown to have an inhibitory activity on 17-β-Hydroxy-dehydrogenase type I (Deluca *et al.*, 2005). The novel inhibitors were designed on the foundation of the structure of estrone, thus blocking the biosynthesis of estrdiol and beneficial in HDBC (hormone dependent breast cancer) (Lawrence *et al.*, 2006) Strong binding energy of phytoestrogens (top scorer Shatavarin X -14.15 kcal/mol) as reflected by the dockscore in comparison to the inhibitor (Estra-1,3,5(10)-triene-16-acetamide, 3-hydroxy-17-oxo-*N*-(3-pyridinylmethyl)-, (16β)-

methyl ; dockscore -6.74 kcal/mol) signifies their greater binding affinity for the 17β-hydroxydehydrogenase, thereby decreasing the endogenous availability of the oestradiol by inhibiting its biosynthesis. The interaction profile of Shatavarin X and standard is displayed in Figure 25.

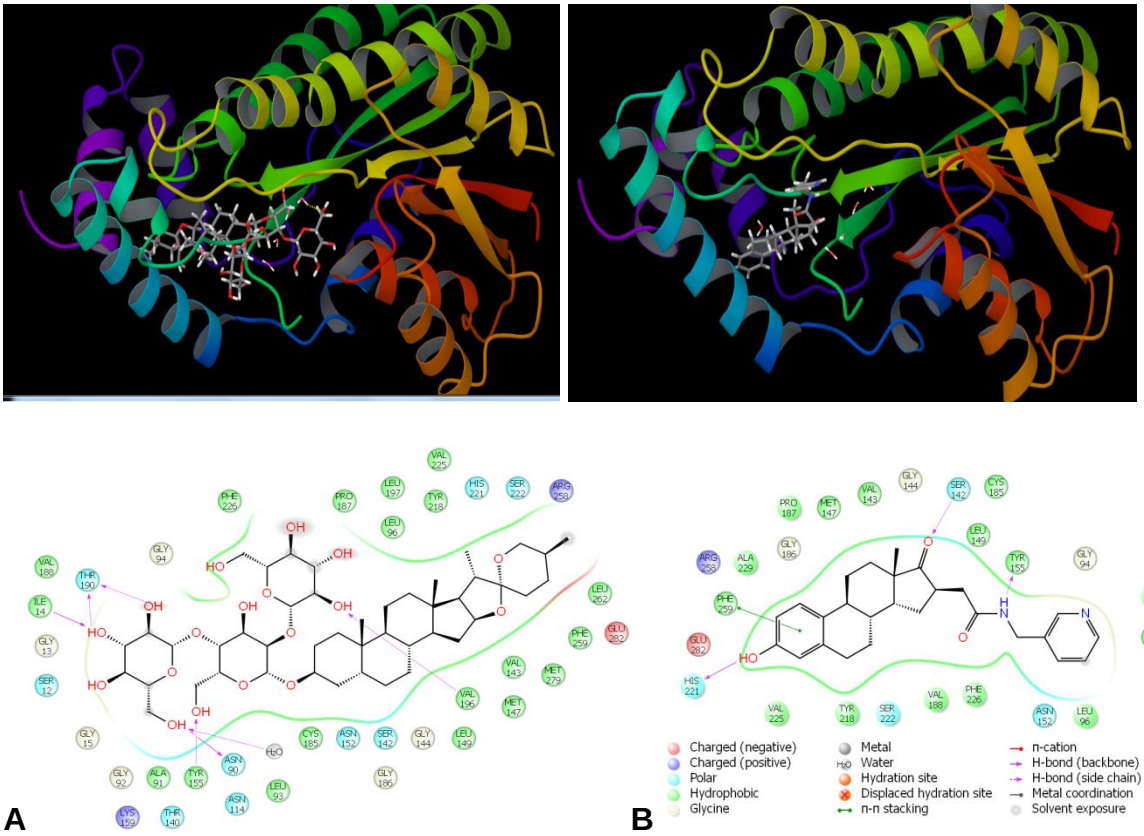


Figure 26: Ligand interaction diagram of Shatavarin IX (**A**) and inhibitor (**B**) with the 17β-Hydroxydehydrogenase (PDP id- 1FDS)

Glucose-6-phosphate dehydrogenase an enzyme involved in the Pentose phosphate pathway, which provides the NADPH. The tissues like mammary cells, liver cells, fat and adrenal gland are actively involved in the production of NADPH in the isoprenoid pathway or in the biosynthesis of fatty acids (Robbins *et al.*, 2010). Genistein and praziquantel from *Flemingia vestita* were shown to possess inhibitory activity on Glucose-6-phosphate dehydrogenase (Das *et al.*, 2004). DHEA a potent inhibitor of Glucose-6-phosphate dehydrogenase is protective in breast cancer (Boccuzzi *et al.*, 1993; Monaco *et al.*, 1997). In addition, it was found that there is reduced risk of breast cancer in Glucose-6-phosphate deficient women (Feo *et al.*, 1984). The dockscore of Racemoside A (-11.79 kcal/mol) was found to be much greater that the dockscore of the standard DHEA (-3.25

kcal/mol) suggesting that these phytoestrogens strongly binds and inhibits G6PD, similarly to the DHEA. Interaction profile of Racemoside A and DHEA is depicted in Figure 26.

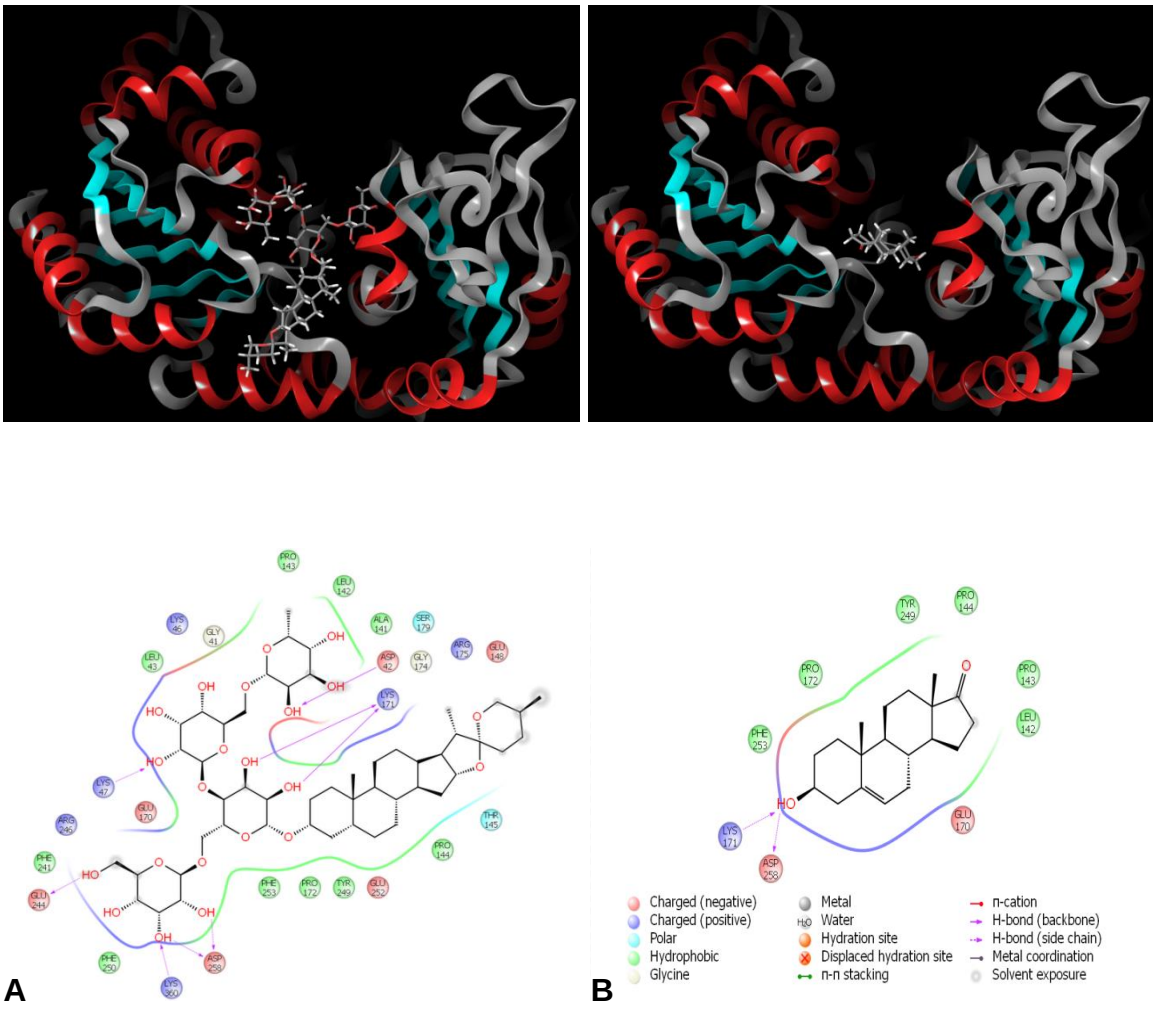


Figure 27: Ligand interaction diagram of Racemoside A (**A**) and DHEA (**B**) with the glucose-6-phosphate dehydrogenase (PDP id- 2BH9)

Tubulin is the building blocks of the microfilaments (Verdier-Pinard *et al.*, 2000) required for the chromosome separation during the metaphase of the cell cycle (Murray & Hunt, 1993). 2-methoxy estradiol which is formed upon hydroxylation and methylation of ostradiol endogenously, have a potent anti-proliferative and anti-angiogenic activity, which has been demonstrated in both *in-vivo* and *in-vitro* experiments (Mooberry, 2003; Pribluda *et al.*, 2000). It has an IC_{50} value of 1.4 µM±0.2 (Wang *et al.*, 2000) and causes the depolymerisation of the microtubules by binding to the colchicine binding site of the tubulin (D'Amato *et al.*, 1994). A glycoside of genistein, ITB-301 has been shown to inhibit the proliferation of

SKOv3 ovarian cancer cells, by acting as a anti-tubulin agent (Ahmed *et al.*, 2011). Docking results suggested that phytoestrogens binds to the collagen binding site with a much greater affinity as compared to the standard which is supported by impressive dockscore of phytoestrogens (Immunoside; dockscore -10.92 kcal/mol) in comparison to the standard 2-ethoxyestradiol. Their interaction diagram is exhibited in Figure 27A and 7B. The molecular docking simulation results clearly indicated that Rutin, 5-hydroxy 3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4] -O-α-D-xylopyranoside glucopyranpsyl, 8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside, Shatavarin X, Racemoside A, Immunoside, we're having more binding affinity as compared to their respective standards.

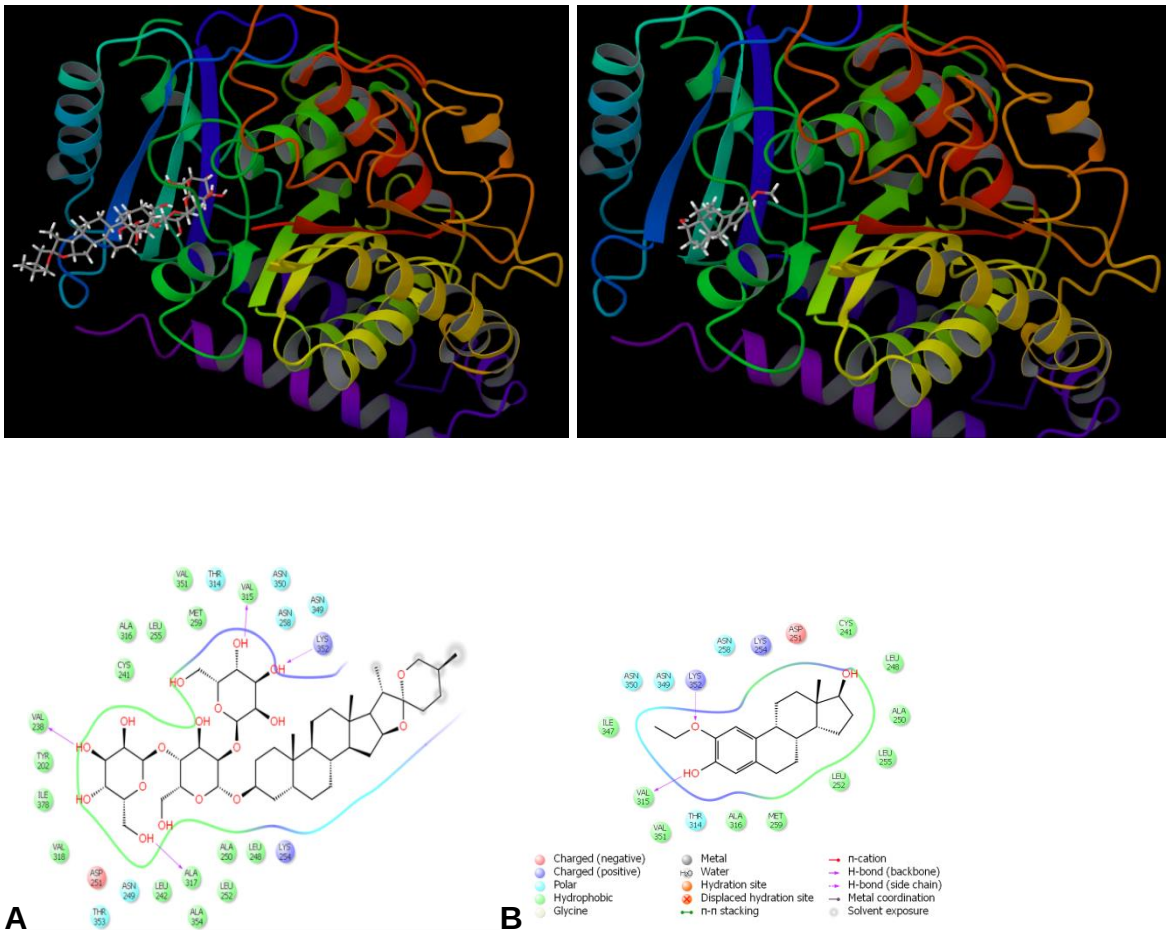


Figure 28: Ligand interaction diagram of Immunoside (**A**) and 2-ethoxy oestradiol (**B**) with the colchicine binding site of tubulin (PDP id- 1SA0)

Chapter 5.0

Summary

“Once you replace negative thoughts with positive ones, you'll start having positive results”

….. **Willie Nelson**

Chapter 5.0

Summary

The experimental finding through the anti-glycation assay suggests that *A. racemosus* extract has a potent antiglycation activity, thus preventing the hyperglycaemia induced increased vascular contraction leading to hypertension. In diabetes due to elevated levels of VSMC proliferation there is increased incidence of atherosclerosis and the AGE is beneficial in atherosclerotic cardiovascular disease. The maximum antiglycation activity measured by BSA-glucose was observed for (Ethyl acetate extract) REE (IC_{50} 37.56 ± 1.65 µg/mL) followed by (methanolic extract) RME (IC_{50} 51.32 ± 1.48 µg/mL). RME and RAE extracts from *A. racemosus*, demonstrated antimutagenic activity, with RAE showed maximum inhibition of 49.2%. Nyasol isolated from methanolic extract was docked over estrogen receptor β and estrogen receptor α. The molecular docking study revealed that strong binding affinity with both the receptors, indicating that nyasol was beneficial for hormone responsive breast cancer. The molecular docking simulation of reported phytoestrogens from *A. racemosus* have shown that the endogenous binding of the oestradiol decreased with its receptor by acting as an inhibitor of HSP90. It prevents the association of ligand receptor complex with the ERE which is required for the target gene activation. It was found that phytoestrogens have a strong affinity for estrogen receptor β indicated by docking score thereby activating the receptor will be a choice for the tumor suppression. Phytoestrogens decrease the endogenous availability of the oestrdiol by inhibiting its biosynthesis. These are known to inhibit 17β-hydroxy dehydrogenase, Glucose-6-phosphate dehydrogenase and docking study reflected that phytoestrogens from *A. racemosus* act as inhibitors of the enzyme, thereby decreasing the endogenous availability of oestradiol. There is a good relation between activity of the sulphotransferase and steroid sulphatase which regulate the availability of the endogenous oetradiol. The study reckoned that phytoestrogens inhibit steroid sulphatase so that estrone sulphate is not converted to the estrone therefore, decreased availability of the oestrone for the further biosynthesis of oestrdiol. Aside from the inhibition of the biosynthesis of oestradiol and its binding to the receptor, additionally phytoestrogens causes the depolymerisation of the microfilaments in the tubulin by binding to the colchicine binding site, similar to the 2-methoxyestradiol and thus helping in tumor suppression. Docking results

suggested that phytoestrogens can be a potential candidate for controlling tumor progression with a special emphasis in breast cancer progression. There is further need to perform *in-vitro* and *in-vivo* bioassays for the establishment of phytoestrogens from the A. *racemosus* in search of the lead in the evolution of cancer chemotherapy.

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APPENDIX A:

Spectroscopic Data of RVA-1 (Nyasol)

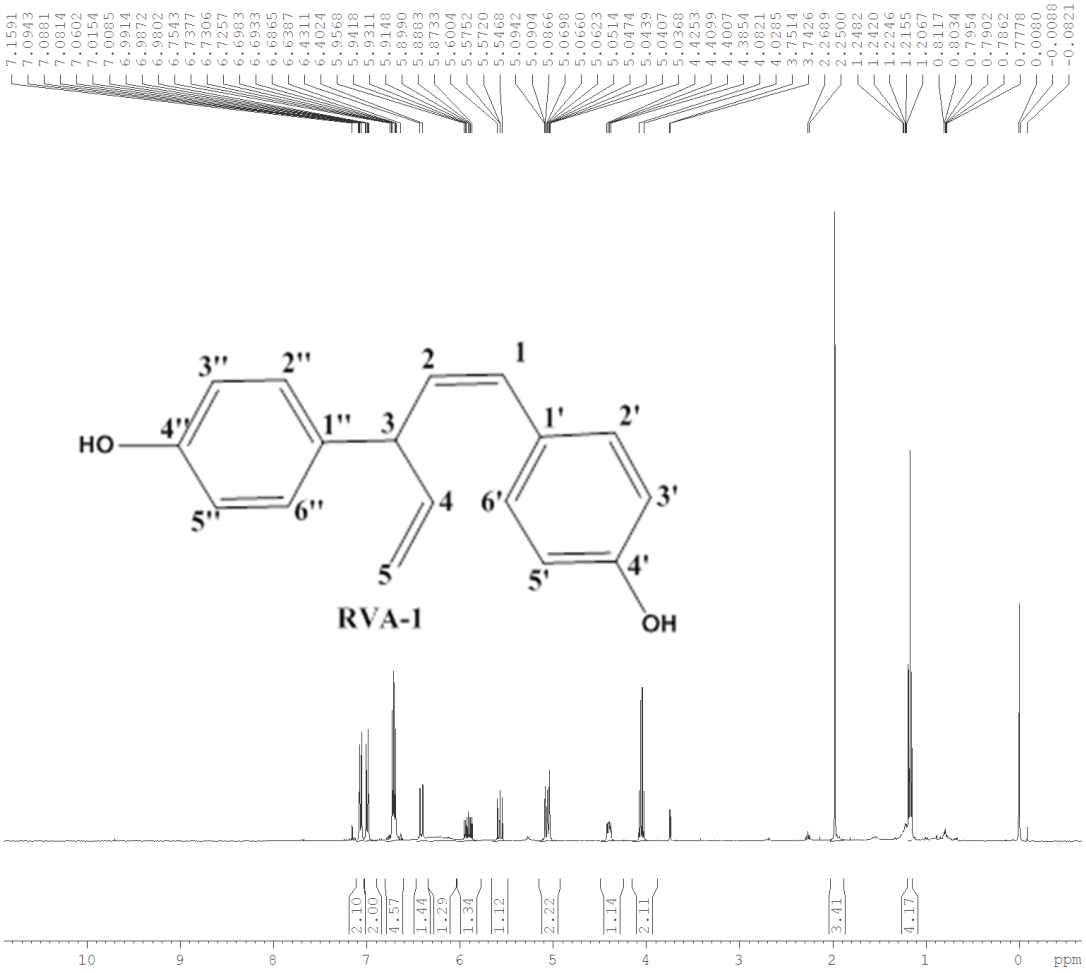

Figure 1: ^{1}H NMR of compound RVA-1

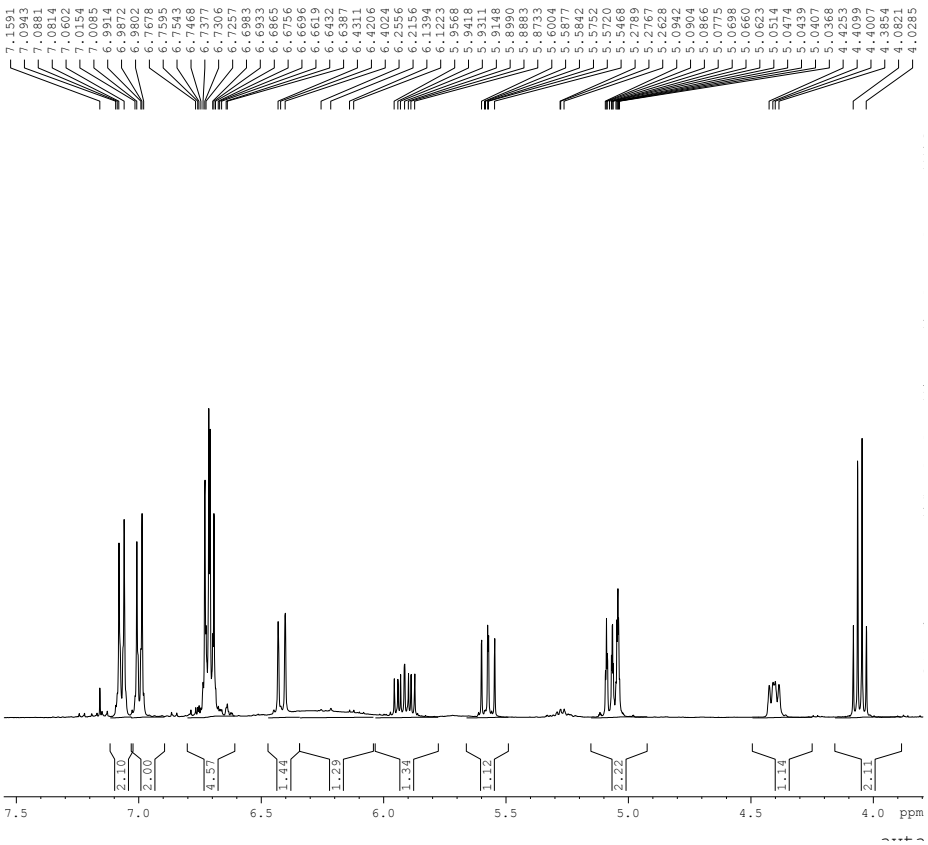

Figure 2: ^{1}H NMR of compound RVA-1 (Expanded)

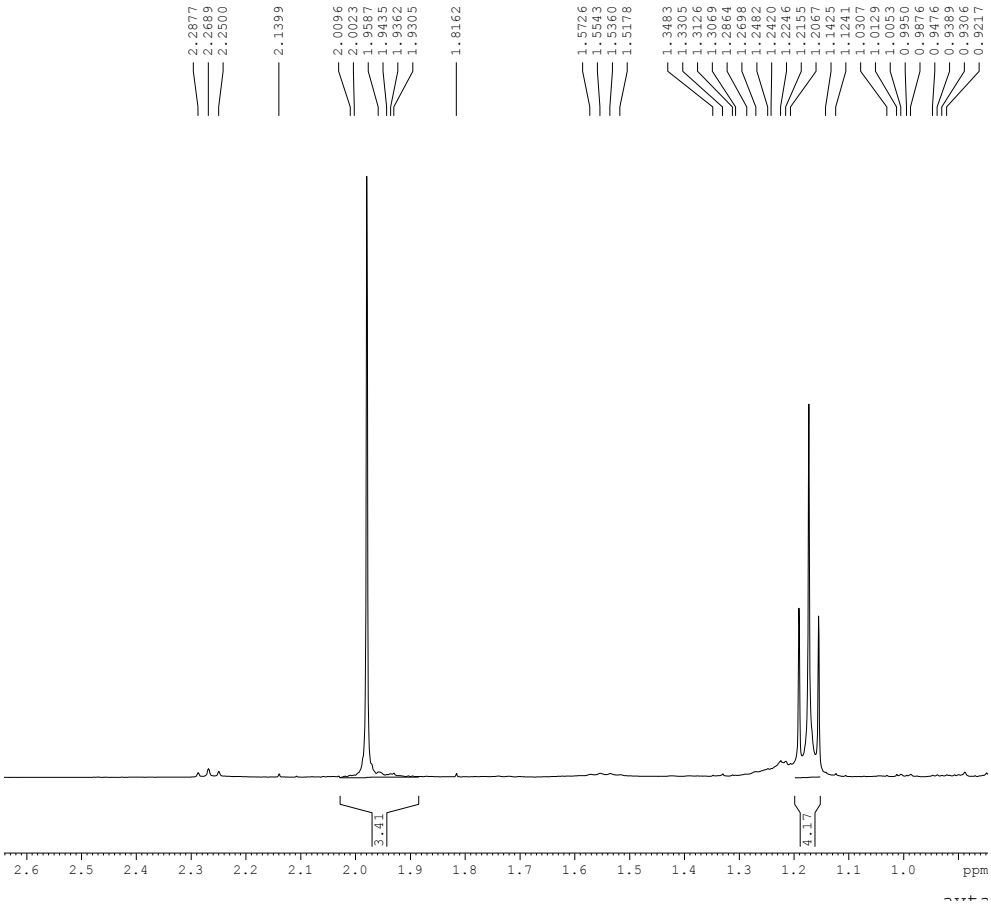

Figure 3: ^{1}H NMR of compound RVA-1 (Expanded)

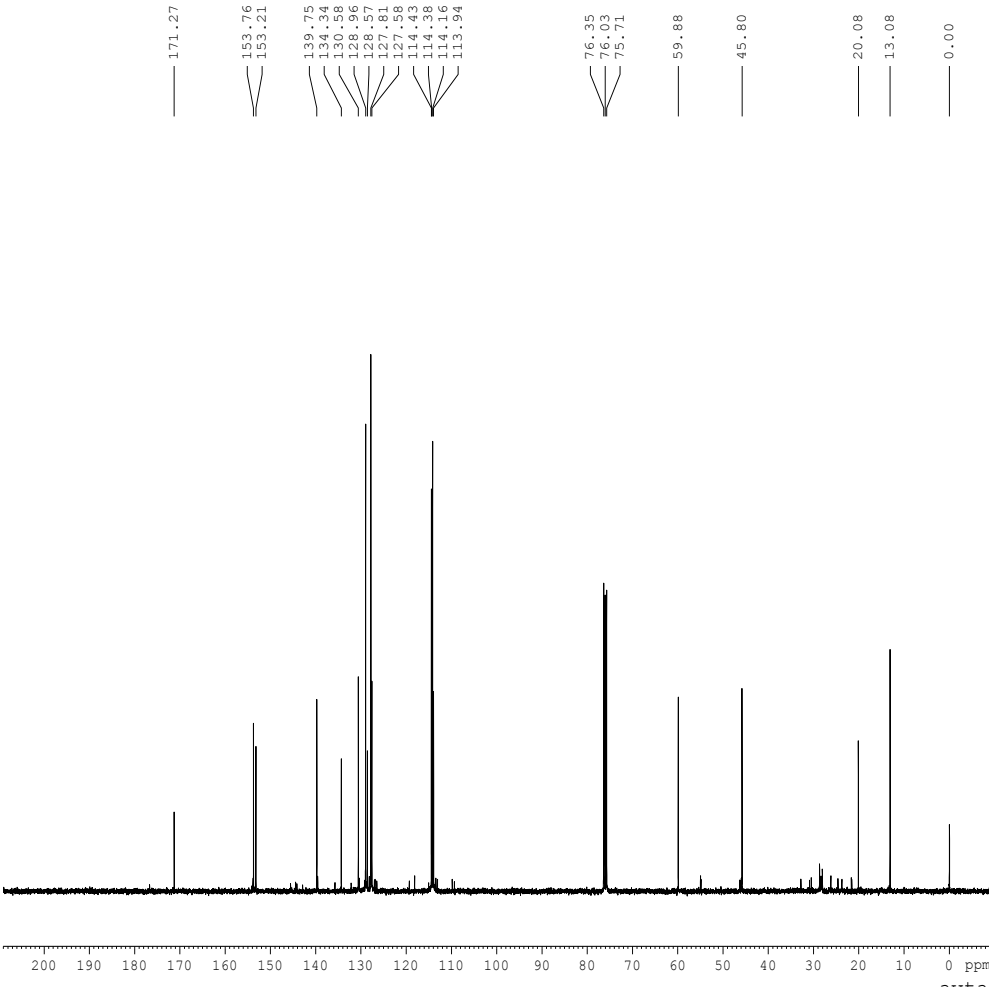

Figure 4: ^{13}C NMR of compound RVA-1

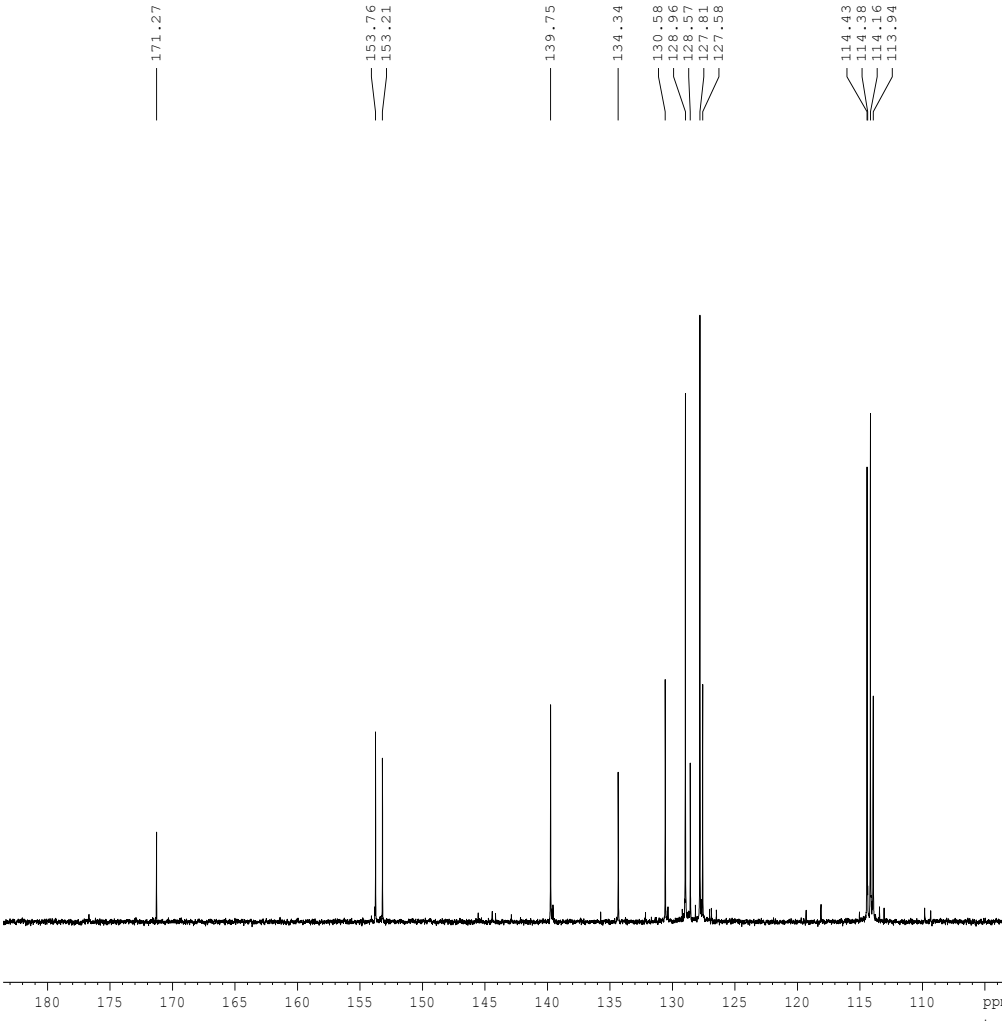

Figure 5: ^{13}C NMR of Compound RVA-1 (Expanded)

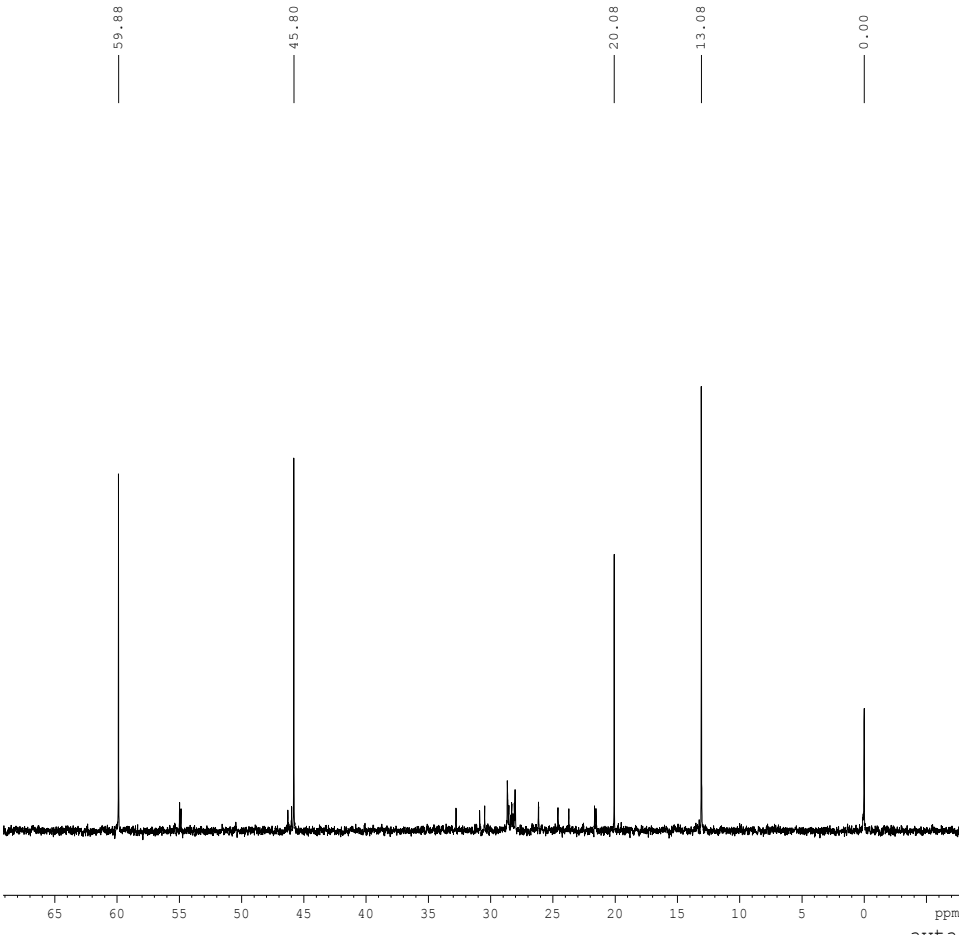

Figure 6: ^{13}C NMR of Compound RVA-1 (Expanded)

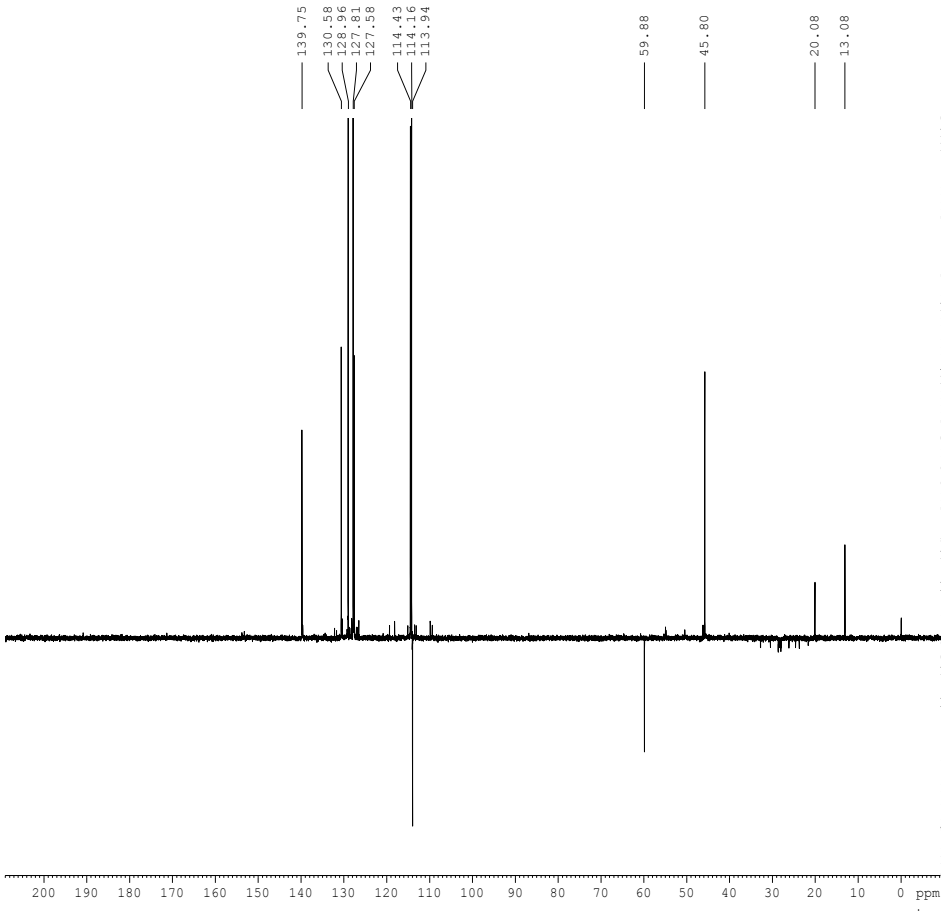

Figure 7: DEPT NMR of Compound RVA-1

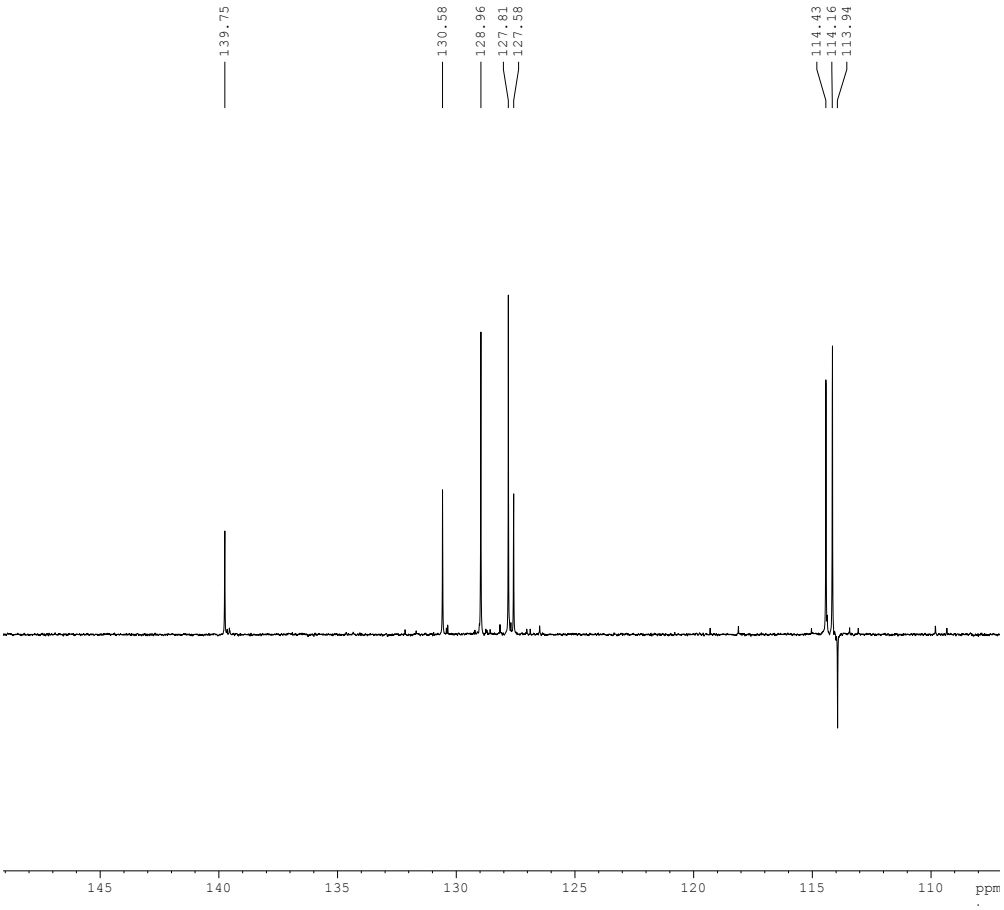

Figure 8: DEPT NMR of Compound RVA-1 (Expanded)

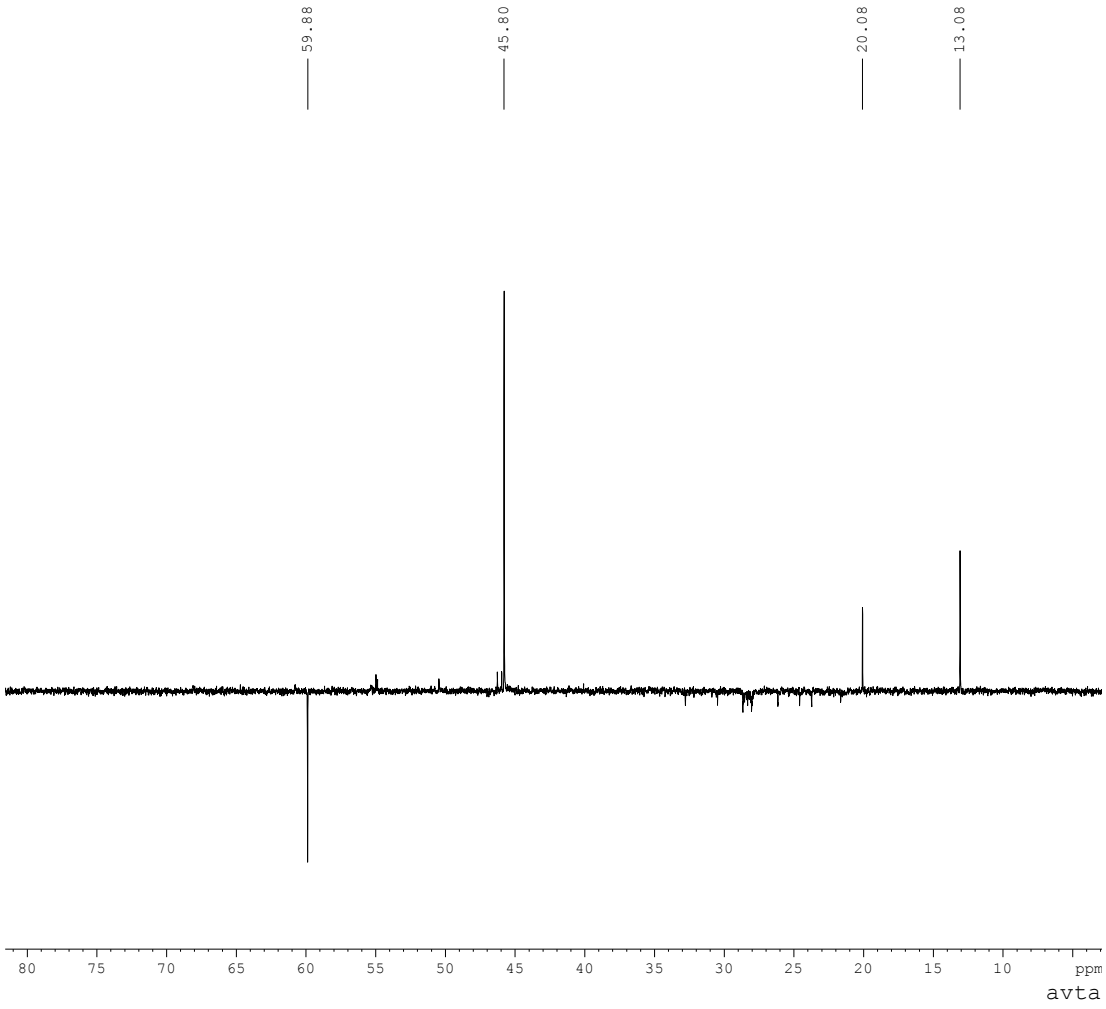

Figure 7: DEPT NMR of Compound RVA-1 (Expanded)

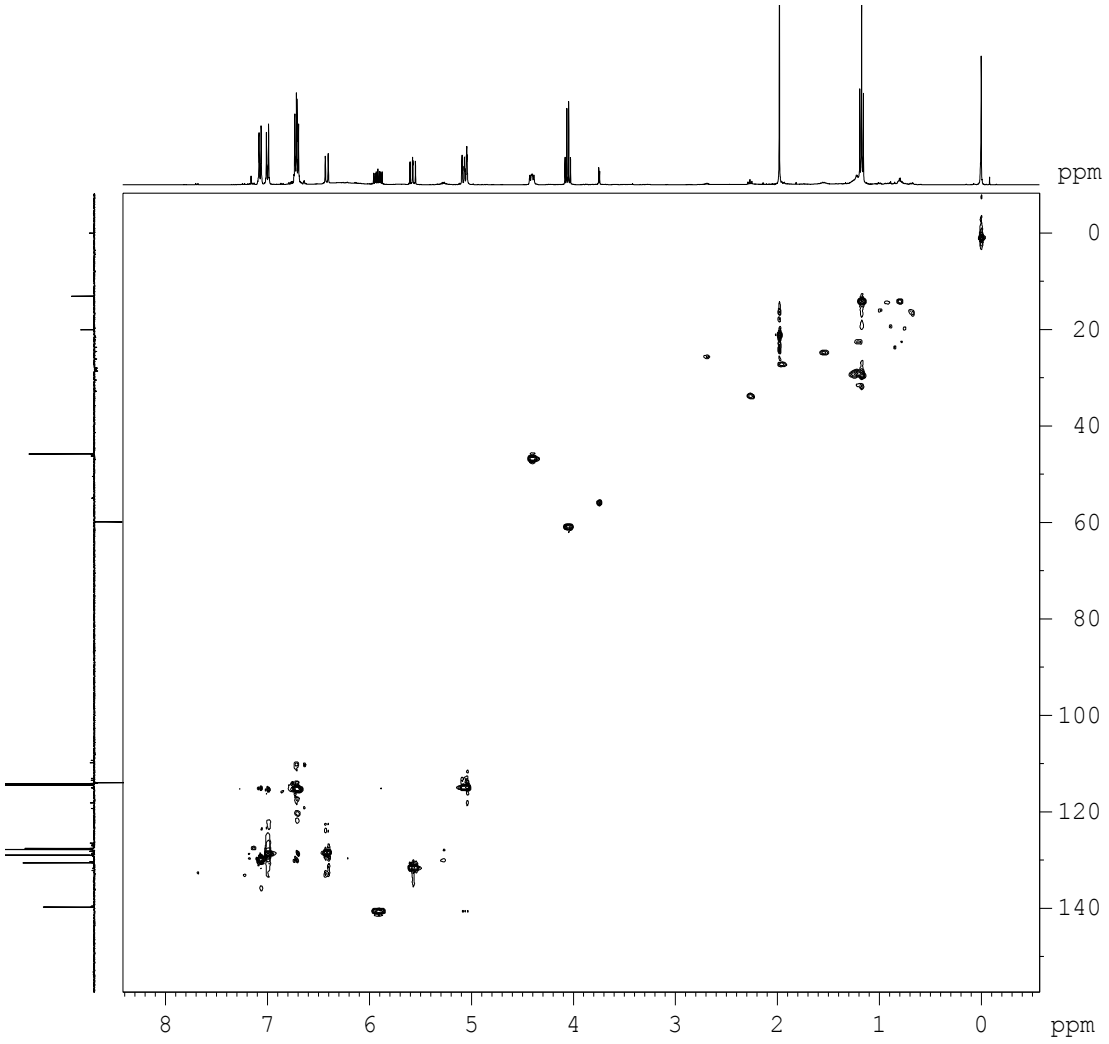

Figure 8: HSQC NMR of Compound RVA-1

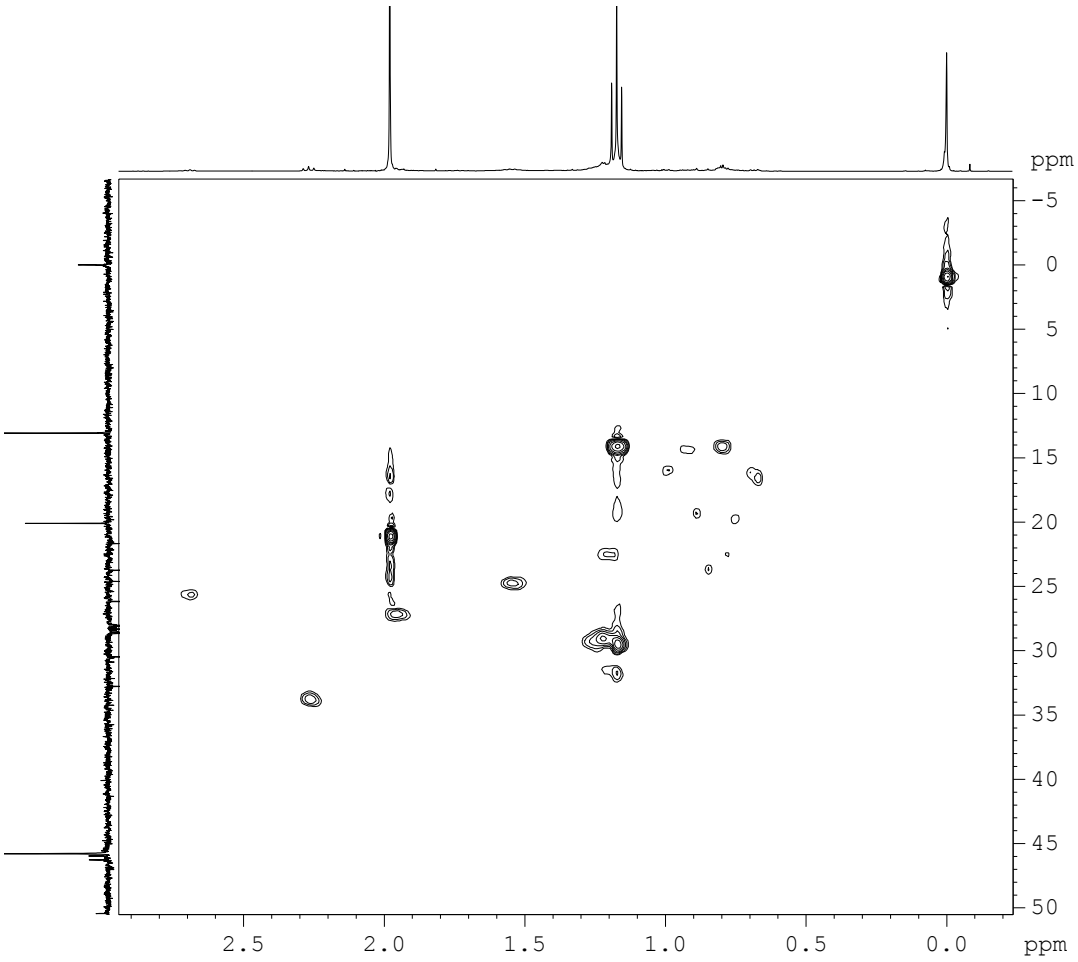

Figure 8: HSQC NMR of Compound RVA-1(Expanded)

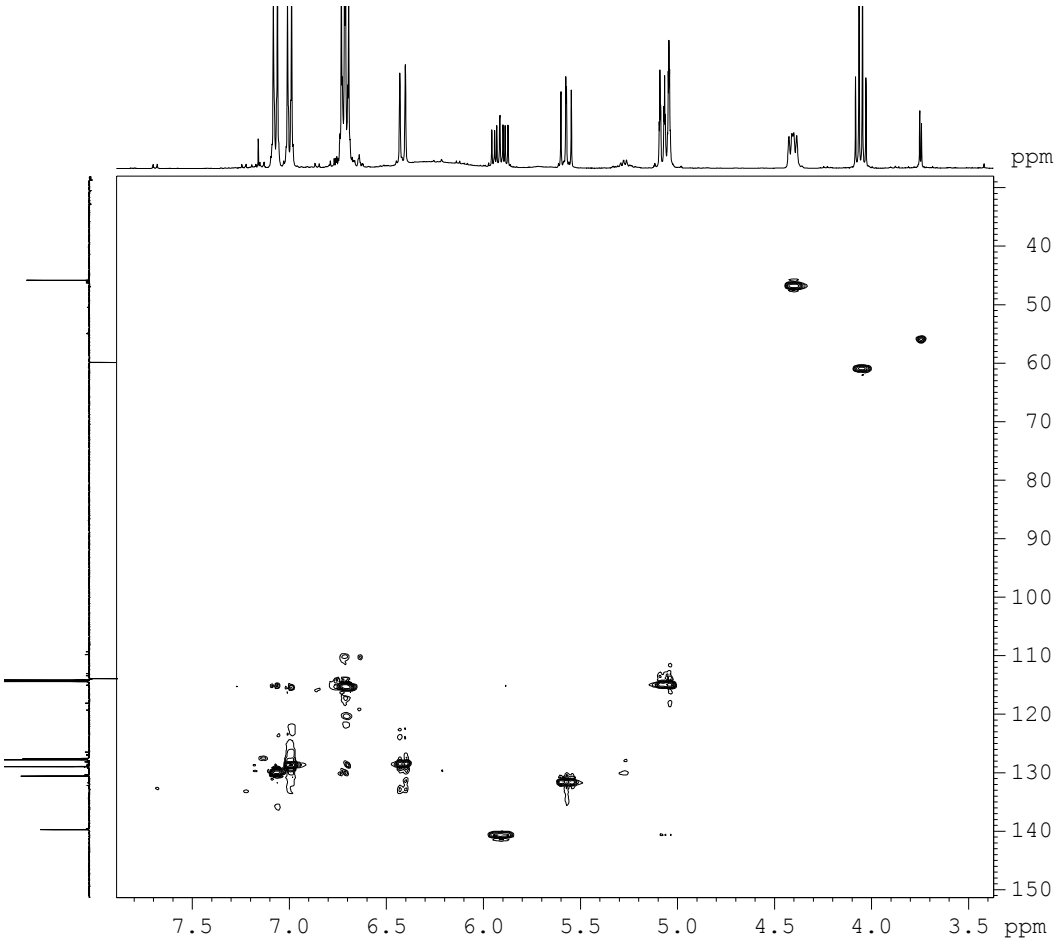

Figure 9: HSQC NMR of Compound RVA-1(Expanded)

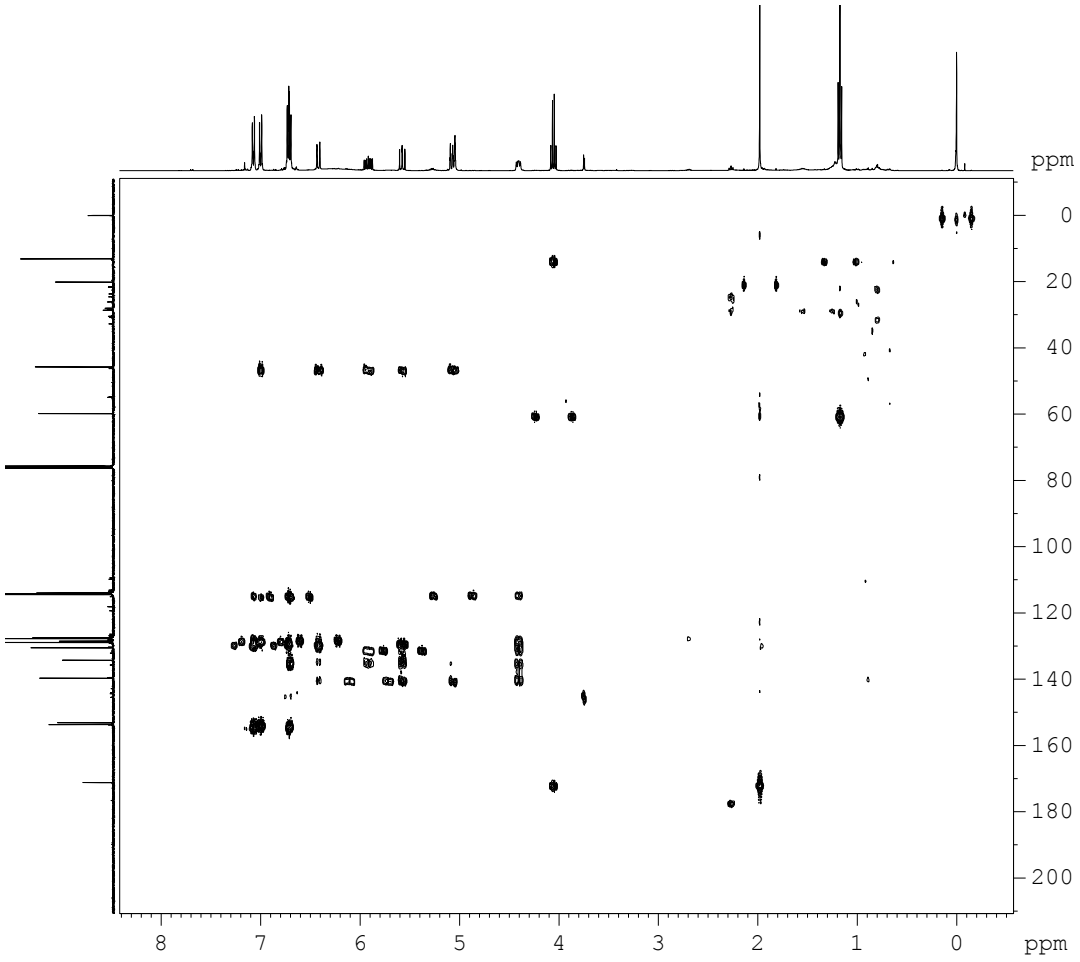

Figure 10: HMBC NMR of Compound RVA-1

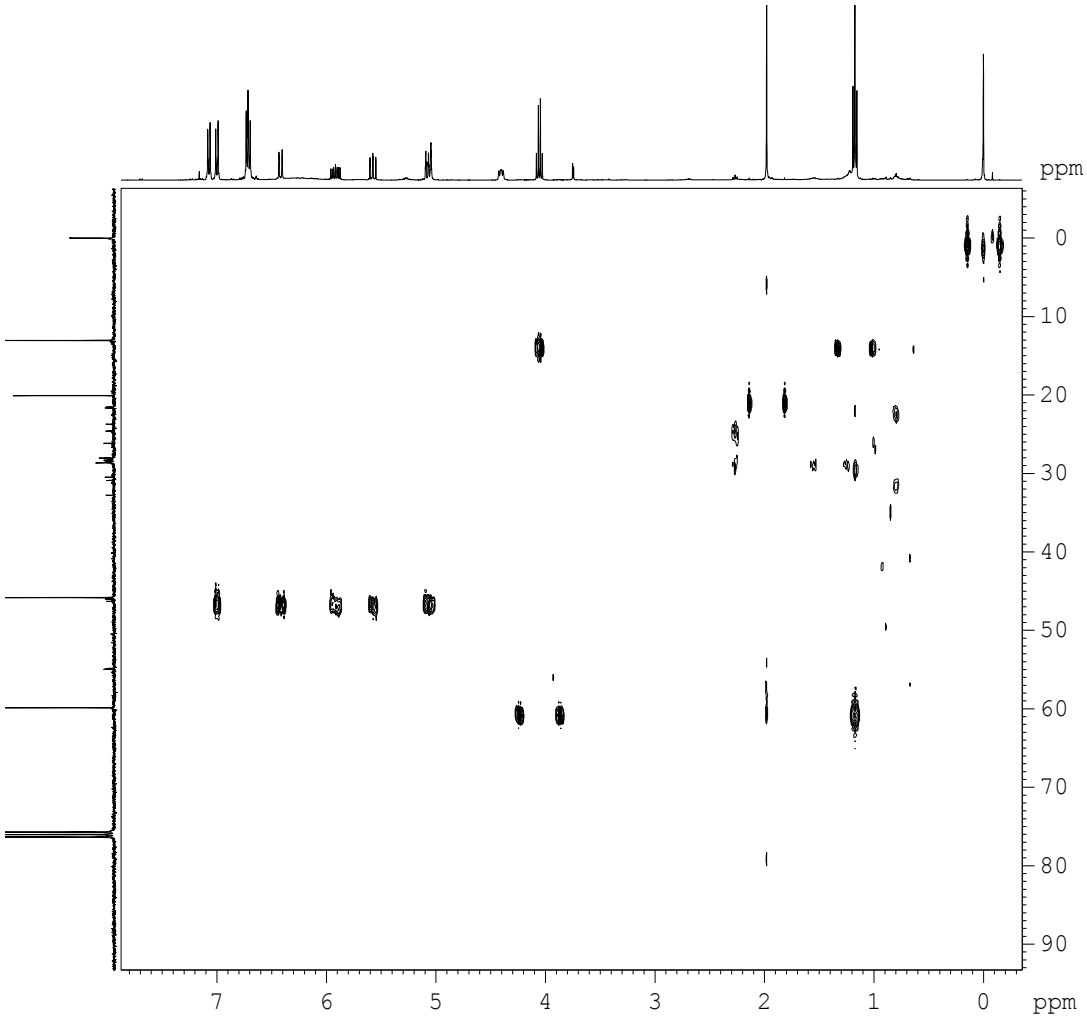

Figure 11: HMBC NMR of Compound RVA-1(Expanded)

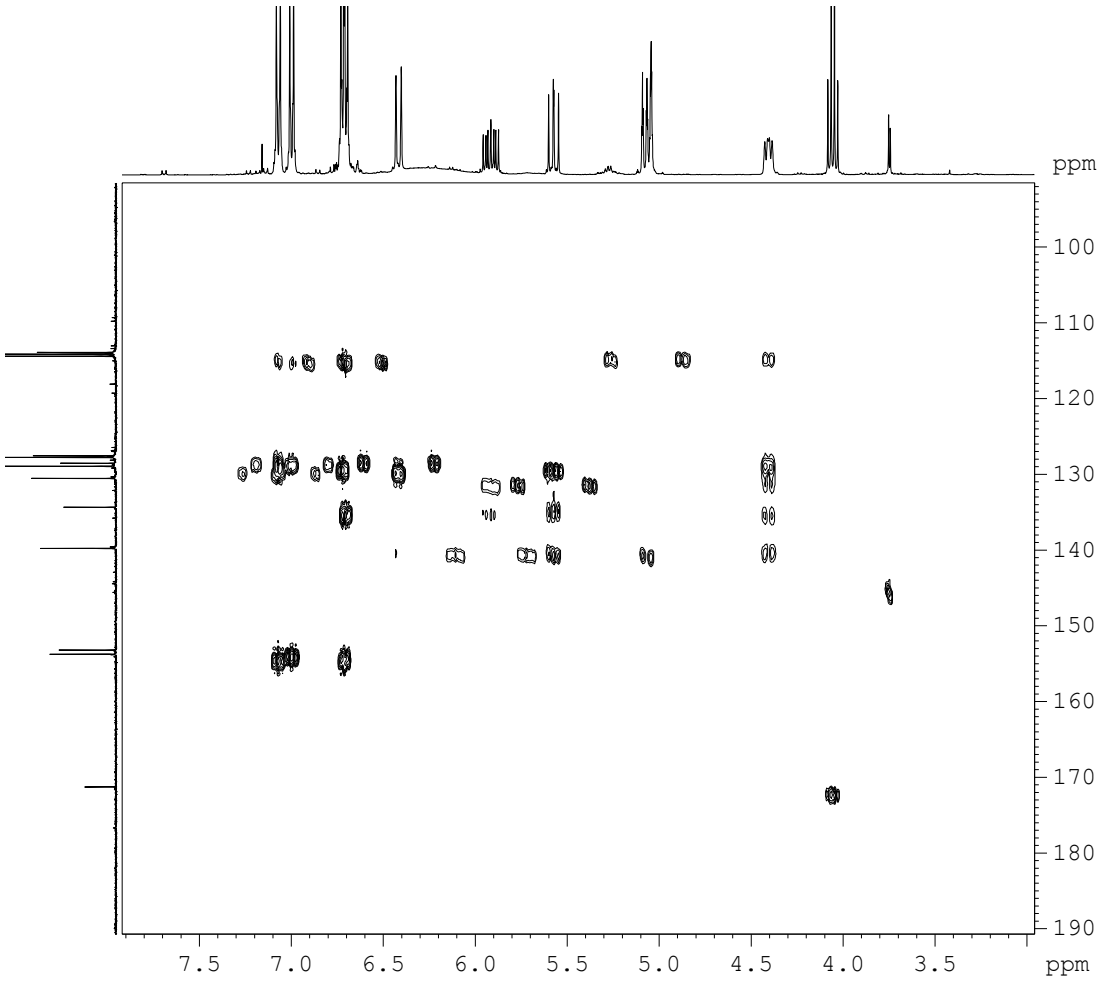

Figure 12: HMBC NMR of Compound RVA-1(Expanded)

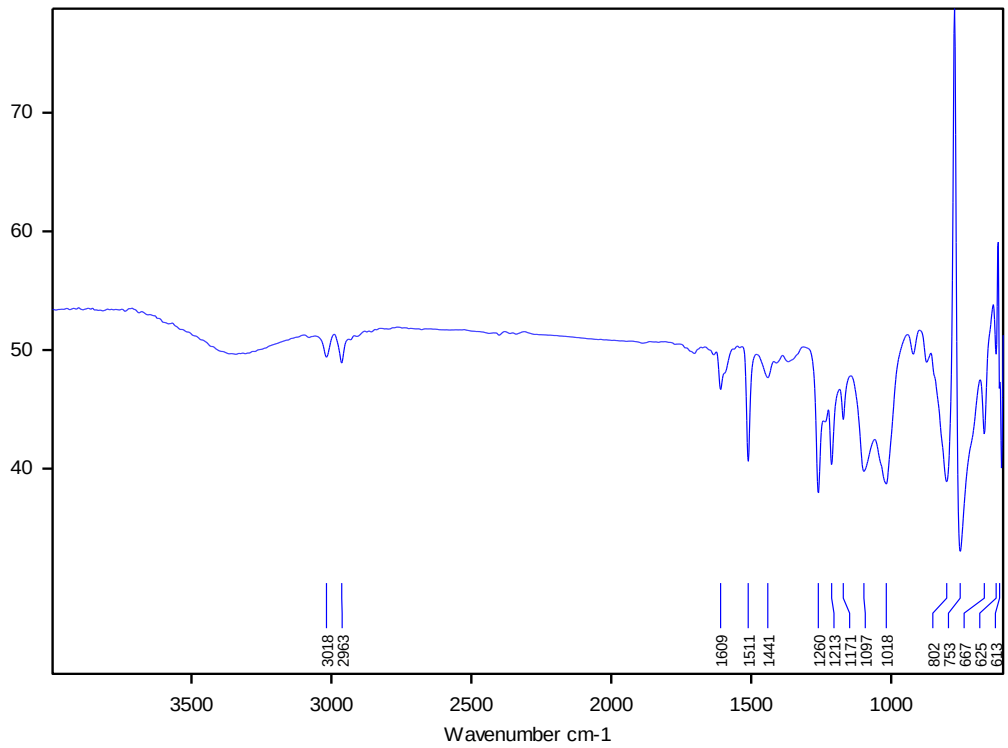

Figure 13: IR spectrum of compound RVA-1

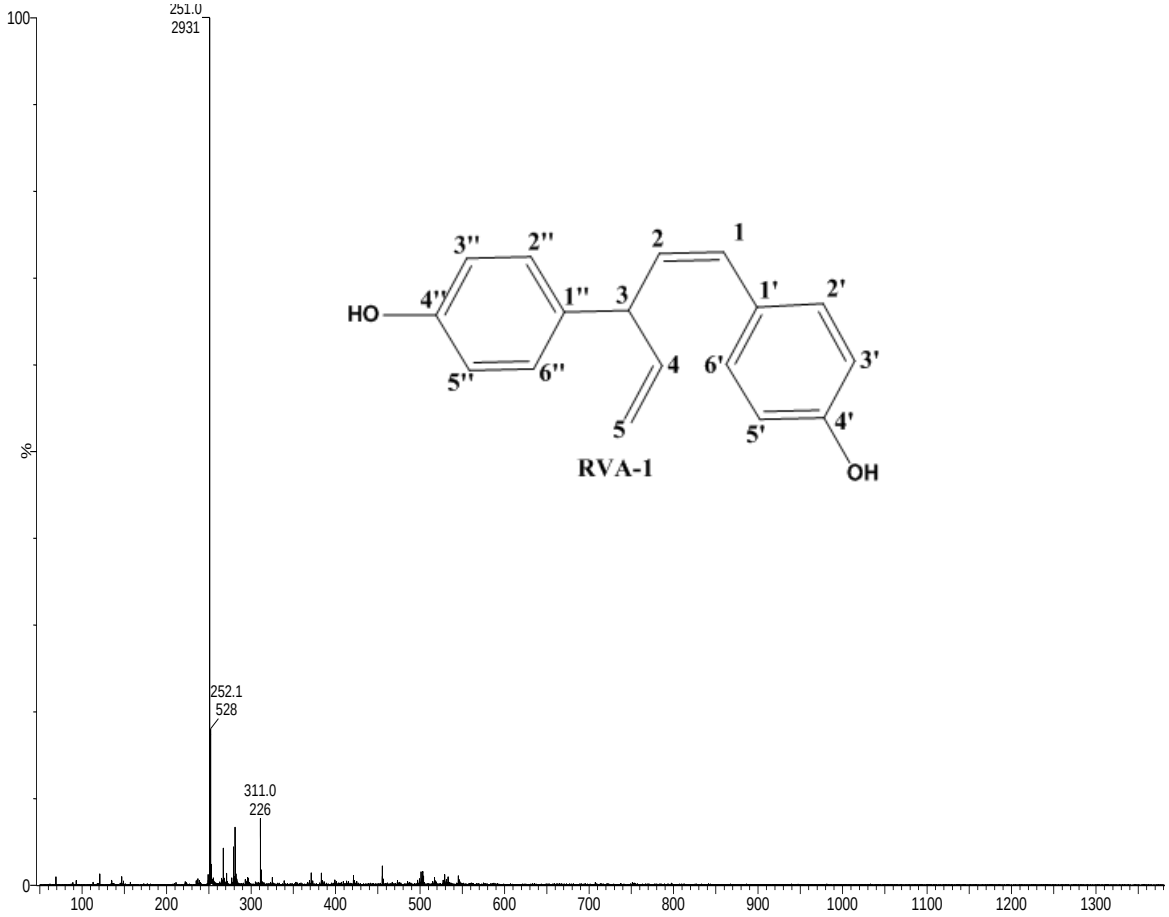

Figure 14: MS of compound RVA-1

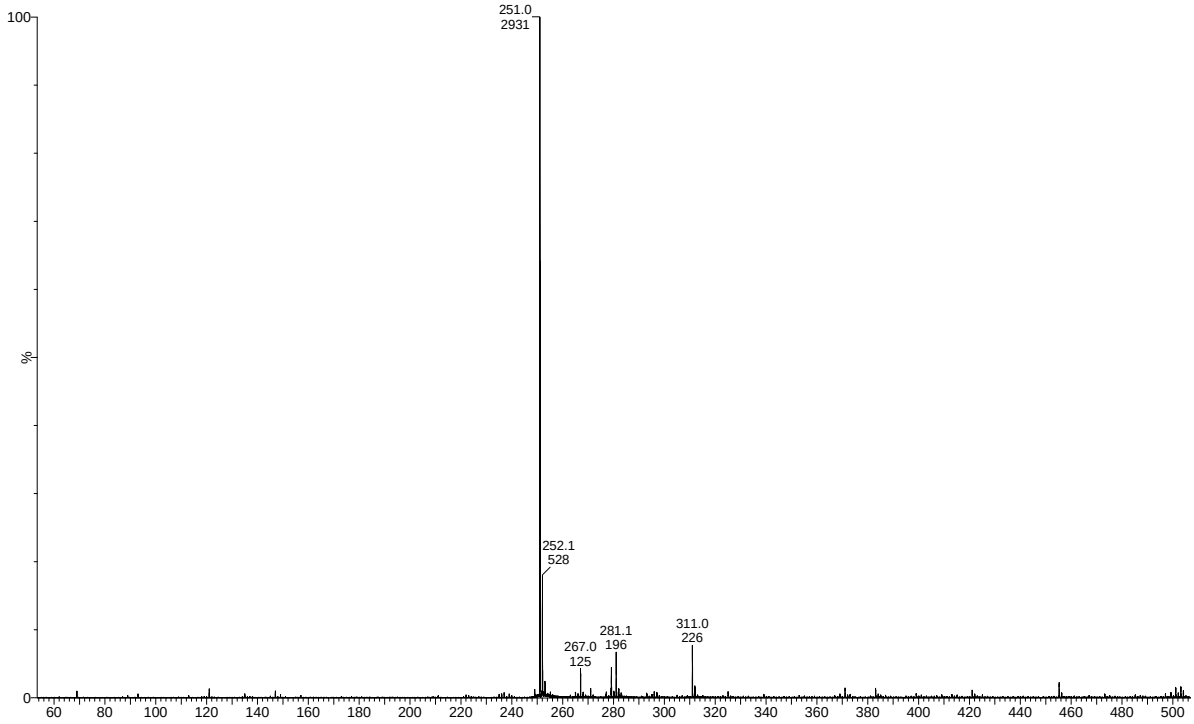

Figure 15: MS of compound RVA-1 (Expanded)

APPENDIX B

Docking Score of Phytoestrogens

Table 1: Docking score of phytoestrogens with Estrogen receptor β

S.No	Ligand	DockScore (kcal/mol)	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)
1	Rutin	-11.01	-1.52	-4.59
2	Shatavarin V	-9.41	-1.8	-4.76
3	Shatavarin IX	-8.04	-2.74	-3.35
4	Immunoside	-7.92	-2.15	-6.39
5	Shathavaroside A	-7.05	0.03	-3.85
6	Shathavaroside B	-6.71	-1.58	-3.17
7	Racemoside A	-6.69	-2.41	-2.24
8	Oligospantanoside	-6.5	-2.88	-4.32
9	Shatavarin VI	-6.49	-2.16	-2.36
10	Imunostimulant 1	-6.26	-2.05	-1.92
11	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-6.06	-1.09	-3.34
12	Querecetin	-6.06	-2.09	-1.44
13	Querecetin-3-glucouronide	-5.99	-3.49	-3.38
14	Sarsapogenin	-5.91	-3.2	0
15	Shatavarin I	-5.82	-0.29	-3.48
16	Shatavarin X	-5.81	-1.51	-2.12
17	Cyanidine-3-galactoside	-5.5	-1.23	-2.14
18	Filiasparoside C	-5.45	-0.92	-2.21
19	Racemoside B	-5.43	-1.38	-5.81
20	Kaempferol	-5.16	-1.48	-0.96
21	Shatavarin VIII	-5.01	0.19	-3.3
22	Shatavarin VII	-4.91	-1.59	-4.32
23	Shatavarin IV	-4.6	-1.51	-3.84
24	Diosgenin	-4.44	-2.33	0
25	Sitosterol	-4.2	-1.62	-0.5
26	Hyperside	-3.92	-1	-4.03
27	Racemoside C	-3.87	-1.8	-3.09
28	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-3.51	-1.38	-3.08
29	Imunostimulant 2	-2.69	-2.96	-0.48
30	oestradiol	-0.14	-0.68	0

Table 2: Docking score of phytoestrogens with HSP90

S.No	Ligand	DockScore (kcal/mol)	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)
1	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-12.01	-2.7	-4.21
2	Shatavarin VIII	-11.89	-2.38	-7.58
3	Racemoside A	-10.74	-2.33	-7.71
4	Immunoside	-10.57	-1.72	-7.01
5	Shatavarin IV	-9.43	-1.89	-5.86
6	Racemoside B	-9.04	-2.03	-6.01
7	Shathavaroside B	-9.04	-1.35	-6.62
8	Querecetin-3-glucouronide	-8.78	-2.48	-4.41
9	Racemoside C	-7.85	-1.57	-5.1
10	Oligospantanoside	-7.77	-1.79	-4.67
11	Shatavarin V	-7.57	-1.72	-4.21
12	Hyperside	-7.55	-2.53	-3.52
13	Shatavarin VI	-7.46	-0.25	-5.34
14	Imunostimulant 1	-7.38	-2.63	-2.83
15	Shatavarin IX	-7.35	-1.18	-5.34
16	Cyanidine-3-galactoside	-7.16	-1.98	-3.39
17	Shatavarin VII	-6.99	-1.93	-3.84
18	Rutin	-6.82	-1.45	-3.64
19	Kaempferol	-6.54	-2.53	-2.01
20	Shathavaroside A	-6.26	-0.9	-3.77
21	Shatavarin I	-6.2	-1.35	-5.3
22	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-6.09	-1.69	-2.93
23	Filiasparoside C	-5.67	-1.53	-4.35
24	Querecetin	-5.38	-1.15	-1.98
25	Geldanamycin	-3.74	-1.19	-1.05
26	Imunostimulant 2	-3.68	-1.83	-1.03
27	Sitosterol	-3.3	-1.31	-0.35
28	Sarsapogenin	-3.29	-1.38	-1.33
29	Diosgenin	-2.18	-2.2	-0.35
30	Imunostimulant 3	-1.65	-2.04	-0.8
31	Shatavarin X	-0.49	-0.93	-2.98

Table 3: Docking score of phytoestrogens with human placental Estrone sulphatase

S.No	Ligand	DockScore (kcal/mol)	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)
1	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-11.06	-3.3	-4.81
2	Rutin	-10.74	-2.54	-5.76
3	Shathavaroside A	-10.46	-3.37	-4.47
4	Racemoside B	-10.41	-2.1	-7.27
5	Hyperside	-9.91	-2.14	-5.51
6	Querecetin-3-glucouronide	-9.46	-2.52	-4.81
7	Immunoside	-9.22	-1.72	-6.09
8	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-9	-2.96	-3.75
9	Shatavarin I	-8.81	-1.9	-5.83
10	Shatavarin VI	-8.41	-2.34	-5.94
11	Filiasparoside C	-7.56	-2.08	-3.84
12	Shatavarin IV	-7.55	-2.58	-4.23
13	Imunostimulant 1	-7.5	-3.35	-2.27
14	Oligospantanoside	-7.02	-2.49	-3.84
15	Shatavarin IX	-6.48	-1.2	-3.77
16	Cyanidine-3-galactoside	-6.45	-1.79	-3.04
17	Shatavarin V	-6.26	-1.41	-5.19
18	Shatavarin VII	-6.19	-2.88	-3.61
19	Querecetin	-5.82	-1.49	-1.92
20	Kaempferol	-5.61	-1.57	-1.57
21	Imunostimulant 2	-5.33	-2.67	-1.63
22	Imunostimulant 3	-4.99	-2.74	-1.19
23	Sarsapogenin	-4.91	-3.31	-0.7
24	Racemoside C	-4.77	-1.71	-3.52
25	Diosgenin	-4.68	-2.49	-0.7
26	Estrone sulphate	-4.4	-2.68	-0.5
27	Shatavarin X	-4.19	-1.45	-3.68
28	KW-2581	-3.45	-1.84	-0.7
29	Racemoside A	-1.98	-5.07	-6.31
30	Shathavaroside B	7.78	-0.46	-6.25
31	Shatavarin VIII	8	-2.24	-5.42

Table 4: Docking score of phytoestrogens with 17β-hydroxydehydrogenase

S.No	Ligand	DockScore (kcal/mol)	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)
1	Shatavarin X	-14.15	-5.34	-6.7
2	Shatavarin VI	-13.85	-4.9	-7.24
3	Shatavarin VIII	-13.04	-5.74	-5.53
4	Shathavaroside B	-13	-4.54	-5.81
5	Racemoside B	-12.28	-5.22	-4.85
6	Racemoside A	-12.21	-4.95	-5.28
7	Rutin	-12.04	-2.48	-7.36
8	Shathavaroside A	-11.74	-4.36	-5.32
9	Shatavarin V	-11.74	-5.12	-4.48
10	Oligospantanoside	-11.69	-5.15	-5.01
11	Filiasparoside C	-11.56	-5.29	-4.75
12	Shatavarin X	-11.52	-5.52	-5.4
13	Shatavarin I	-11.22	-3.6	-7.47
14	Shatavarin IV	-10.75	-3.88	-5.24
15	Immunoside	-10.44	-5.09	-3.23
16	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-9.11	-3.08	-4.03
17	Querecetin-3-glucouronide	-8.84	-2.86	-4.17
18	Racemoside C	-8.27	-3.6	-3.45
19	Hyperside	-8.13	-1.82	-4.42
20	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-7.87	-3.05	-4.57
21	Imunostimulant 1	-7.49	-4.25	-2.41
22	Shatavarin VII	-7.13	-2.42	-2.94
23	Imunostimulant 2	-6.79	-4.03	-1.36
24	Inhibitor	-6.74	-3.73	-1.87
25	Querecetin	-6.68	-1.87	-2.56
26	Cyanidine-3-galactoside	-6.1	-1.03	-3.66
27	Imunostimulant 3	-5.91	-3.61	-1.39
28	Sarsapogenin	-5.7	-4.53	-0.7
29	Kaempferol	-5.64	-1.58	-1.66
30	Sitosterol	-5.34	-4.54	-0.7
31	Diosgenin	-5.17	-4.14	-0.58

Table 5: Docking score of phytoestrogens with glucose-6-phosphate dehydrogenase

S.No	ligand	DockScore (kcal/mol)	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)
1	Racemoside A	-11.79	-3.32	-6.6
2	Rutin	-10.3	-2.73	-5.84
3	Shatavarin VIII	-9.88	-2.15	-5.69
4	Racemoside B	-8.71	-2.26	-4.55
5	Querecetin-3-glucouronide	-8.36	-2.8	-3.96
6	Shatavarin X	-8.1	-2.33	-4.28
7	Shathavaroside B	-7.65	-1.29	-6.39
8	Shatavarin V	-7.45	-1.93	-3.88
9	Shatavarin I	-7.45	-2.4	-5.26
10	Hyperside	-7.14	-2.74	-3.84
11	Shatavarin IX	-6.9	-1.3	-5.57
12	Shatavarin VI	-6.86	-2.2	-3.76
13	Immunoside	-6.65	-1.24	-5.91
14	Cyanidine-3-galactoside	-6.35	-2.21	-3.21
15	Shathavaroside A	-6.28	-2.15	-4.8
16	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-6.26	-2.47	-2.89
17	Shatavarin VII	-6.2	-2.26	-3.3
18	Shatavarin IV	-6.17	-1.23	-4.32
19	Racemoside C	-6.08	-1	-5.32
20	Filiasparoside C	-5.84	-1.75	-3.36
21	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-5.79	-2.92	-2.52
22	Oligospantanoside	-5.69	-1.13	-5.03
23	Imunostimulant 2	-5.35	-3.2	-1.09
24	Imunostimulant 1	-5.3	-2.85	-2.07
25	Kaempferol	-5.26	-2.22	-2.07
26	Querecetin	-5.08	-1.83	-2.13
27	Imunostimulant 3	-4.62	-2.9	-0.98
28	Diosgenin	-4.48	-3.35	-0.7
29	Sitosterol	-4.33	-3.36	-0.7
30	DHEA	-3.25	-1.51	-0.68
31	Sarsapogenin	-3.24	-2.71	-0.35

Table 6: Docking score of phytoestrogens with colchicine binding site of tubulin

S.No	ligand	DockScore (kcal/mol)	LipophilicEvdW (kcal/mol)	HBond (kcal/mol)
1	Immunoside	-10.92	-2.06	-6.84
2	Cyanidine-3-galactoside	-10.58	-2.87	-5.91
3	Shathavaroside B	-10.1	0.18	-9.11
4	Querecetin-3-glucouronide	-9.78	-3.41	-4.1
5	Rutin	-9.66	-3.17	-4.91
6	Hyperside	-9.14	-2.15	-5.41
7	Shathavaroside A	-8.9	-2.39	-4.88
8	Shatavarin X	-8.84	-2.67	-4.55
9	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-D-xylopyranoside glucopyranpsyl	-8.84	-4.27	-3.84
10	Oligospantanoside	-8.51	-2.62	-4.32
11	Racemoside B	-8.45	-3.07	-3.56
12	Racemoside A	-8.44	-0.69	-6.13
13	Racemoside C	-8.18	-3.4	-3.39
14	Shatavarin V	-8.08	-3.64	-2.9
15	Filiasparoside C	-8.08	-3.18	-3.52
16	Shatavarin IX	-7.99	-2.49	-3.77
17	Shatavarin I	-7.43	-0.29	-5.51
18	Shatavarin IV	-7.08	-0.27	-5.67
19	Kaempferol	-6.53	-2.27	-1.78
20	Shatavarin VIII	-6.18	-1.63	-3
21	Querecetin	-6.08	-1.68	-2.14
22	8-Methoxy-5,6,4-trihydroxyisoflavone-7-O-β-D-glucopyranoside	-6.07	-2.64	-2.88
23	2-ethoxyestradiol	-5.84	-3.05	-1.15
24	Shatavarin VI	-5.43	-3.31	-1.42
25	Imunostimulant 1	-5.13	-2.45	-1.99
26	Shatavarin VII	-4.95	-0.32	-3.73
27	Diosgenin	-4.58	-3.45	-0.62
28	Imunostimulant 2	-4.13	-2.26	-1.53
29	Sarsapogenin	-3.92	-3.25	-0.55
30	Sitosterol	-3.74	-3.15	-0.7
31	Imunostimulant 3	-3.59	-2.43	-0.58

APPENDIX C

List of Publication

1. Shatavari (*Asparagus racemosus* Wild): A review on its Ethnobotany, Phytochemistry and Pharmacological importance (Accepted International Journal of Pharmaceutical Science and Research).
2. Molecular Docking Simulation Study of Phytoestrogens from *Asparagus racemosus* in Breast Cancer Progression (communicated).
3. Essential oil molecules from *Aconitum heterophyllum* as a target for cancer chemotherapy via *in-silico* approach and *in-vitro* antioxidant activity (communicated).
4. *In-vitro* antimutagenic activity of *Asparagus racemosus*- An Indian medicinal plant (communicated)