Antiproliferative activity of *Asparagus racemosus* extracts

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In

Department of Pharmaceutical Sciences & Natural Products

By

Ram Sharma

(16mpharm02)

Supervisor

Dr. Vikas Jaitak

Department of Pharmaceutical Sciences and Natural Products

School of Basic and Applied Sciences

Central University of Punjab, Bathinda

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Declaration

I declare that thesis entitled "Antiproliferative activity of *Asparagus racemosus* extracts" has been prepared by me under the guidance of Dr.Vikas Jaitak, Assistant Professor, Department of Pharmaceutical Sciences and Natural Products, School of Basic and applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Ram Sharma

16mpharm02

Department of Pharmaceutical Sciences and Natural Products

School of Basic and Applied Sciences

Central University of Punjab, Bathinda-151001

Date

Certificate

I certify that Ram Sharma has prepared his thesis entitled "Antiproliferative activity of *Asparagus racemosus* extracts" for the award of Master of Pharmacy (Medicinal Chemistry) degree of the Central University of Punjab, under my guidance. He has carried out this work at the Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Vikas Jaitak

Assistant Professor

Department of Pharmaceutical Sciences and Natural Products

School of Basic and Applied Sciences

Central University of Punjab, Bathinda-151001

Date

Abstract

Antiproliferative activity of *Asparagus racemosus* extracts

Name of Student : Ram Sharma

Registration Number : 16Mpharm02

Degree for which submitted : Master of Pharmacy (Medicinal Chemistry)

Name of Supervisor : Dr. Vikas Jaitak

Department : Pharmaceutical Sciences & Natural Products

School of Studies : School of Basic and Applied Science

Keywords : *Asparagus racemosus,* Phytoestrogens, Estrogen receptor, Breast cancer, *In–vitro* activity, *In-silico* activity

Cancer is regarded as uncontrolled progression and spread of cells. Cancer is not a singular, specific disease but a group of variable tissue responses that result in uncontrolled cell growth. Healthy cells have a specific size, structure, function and growth rate that best serves the needs of the tissues they compose. Cancer is one of the leading of morbidity and mortality worldwide, with approximately 14 million new cases in 2017. Breast cancer (BC) is a disease where cells in the tissue of the breast cancer grow and divide without normal control.

Estrogen receptor is a group of proteins (or a twelve helix protein) present inside the cells of the female reproductive tissues or located in the nucleus of cells. ERα, ERβ and ER gamma have different responses and they are located in different tissues. Quinone forms of catechol estrogen binds to DNA and forms adduct. Semi Quinone intermediates are free radicals can bind with oxygen to producing superoxide radicals. Superoxide radicals can attack and alter the structure of DNA and causing Breast cancer. Various synthetic drugs (Tamoxifen & Raloxifene) are used for treatment of breast cancer, but numerous side effects like menopausal symptoms, vaginal dryness, low libido, mood swings and Nausea. The discovery of novel natural drugs is important for reduction of side-effects, high selectivity, low toxicity, and better killing of cancer cells. Phytoestrogens are one the best category of natural products used for treatment of breast cancer. Phytoestrogens have similar structure to the endogenous estrogen. Distance between the hydroxyl groups is 14.5 A^0 is similar to estrogen. *Asparagus racemosus* contain large number of phytoestrogens. In this context, the aim of the present study was to explore the roots of *Asparagus racemosus* in the terms of its medicinal values for Breast cancer. Anticancer activity of different extracts were evaluated by performing *In vitro* study by using Breast cancer cell lines T-47 D. from the Preliminary phytochemical investigation of extracts demonstrated that methanolic extract and Aqueous methanolic extract contain large number of phytoestrogens. Aqueous methanolic extract and methanolic extract showed maximum IC_{50} value as compare to other extract. Isolation of molecules from methanol extract, total four molecules isolated from methanol extract and three molecules from aqueous methanol extract. Moreover, *in silico* study of reported phytoestrogen from *Asparagus racemosus* was also carried out using glide docking to investigate interaction pattern with estrogen receptor α and estrogen receptor β. The top docking score was obtained for Rutin (Estrogen receptor α) and Quercetin (Estrogen receptor β). Tamoxifen and raloxifene used as standard for estrogen receptor α and oestradiol used as standard for estrogen receptor β. From the ADME study demonstrated that maximum flavonoids has highest oral absorption as compare to other. The results showed that phytoestrogens are expected prospective candidate for regulatory tumor progression with a special emphasis in breast cancer progression.

Ram Sharma Dr. Vikas Jaitak

To Mom and Dad

Who always picked me up on time

And encouraged me to go on every adventure

Especially this one

Dedicated to my Family

Guide and Friends

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I humbly present this work to the ALMIGHTY GOD. Indeed my project is a small work done with help of primitive several people. So it is my bounded duty to thank them individually.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVATION

Serial Number	Full Form	Abbreviation
1	Breast Cancer Susceptibility Gene 1	BRCA1
2	Breast Cancer Susceptibility Gene 2	BRCA2
3	World Health Organization	WHO
4	Deoxyribonucleic Acid	DNA
5	Breast Cancer	BC
6	Human Epidermal Growth factor Receptor	HER
7	Progesterone Receptor	PR
8	Estrogen Receptor	ER
9	Dichloro diphenyldichloro ethylene	DDE
10	Poly Chlorinated Biphenyls	PCB
11	Phosphatase and Tensin Homolog	PTEN
12	Estrogen Receptor Alpha	ERα
13	Estrogen Receptor Beta	ERβ
14	Activation Factor 1	AF-1
15	Activation Factor 2	AF-2
16	Ligand Binding Domain	LBD
17	Ribonucleic Acid	RNA
18	Dichloro Diphenyl Trichloroethane	DDT
19	Micro Tubule Associated Protein	MAP
20	Extracellular Signal Regulated Kinase	ERK
21	Estrogen Response Element	ERE
22	Selective Estrogen Receptor Modulator	SERM
23	Selective Estrogen Receptor Down Regulator	SERD
24	Steroid Sulfatase	STS
25	Michigan Cancer Foundation -7	MCF-7
26	Food and Drug Administration	FDA
27	Low Density Lipoproteins	LDL

28	Lipid Hydro Peroxide	LOOH
29	Thio Barbituric Acid Reactive Substance	TBARS
30	Gamma Aminobutyric Acid	GABA
31	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
32	Very Low Density Lipoprotein	VLDL
33	Super Oxide Dismutase	SOD
34	3-Hydroxy-3-methyl-glutaryl-coenzyme	HMG-CoA
35	Reactive Oxygen Species	ROS
36	Follicle Stimulating Hormone	FSH
37	Luteinizing Hormone	LH
38	National Medical Plants Board	NMPB
39	Thin Layer Chromatography	TLC
40	S D fine Chemical Ltd	SDFCL
41	Dimethyl Sulfoxide	DMSO
42	Protein Data Bank	PDB
43	Absorption, Distribution, Metabolism and Excretion	ADME
44	Glutamate	GLU
45	Glycine	GLY
46	Leucine	LEU
47	Aspartate	ASP

CHAPTER 1.0

INTRODUCTION

Chapter 1.0

Introduction

1.1 Cancer

Cancer is not just one disease, but a common term used to contain a group of more than two hundred diseases sharing common characteristics (Gabriel, 2008). Cancers (carcinomas) are described by their unregulated growth and spread of cells to other parts of the body (V. Kumar *et al.*, 2017). Treatment of an individual diagnosed with cancer is not only dependent upon which type of malignancy (cancer) they have, but also on the extent of its spread, together with its sensitivity to treatment (Kohn *et al.*, 1995).

As humans we are encompassed of many millions of cells. Some cells are specific to certain tissues, for example epithelial cells are found throughout the gastrointestinal tract, bladder, lungs, vagina, breast and skin (Gabriel, 2007) This group of cells accounts for approximately 70% of cancers However, any cell has the potential to suffer malignant changes and lead to the development of a carcinoma (Bierie *et al.*, 2017). Cancerous cells are not confined to localized 'overgrowth' and infiltration of surrounding tissue, but can spread to other parts of the body through the lymphatic system and bloodstream, generating secondary deposits known as 'metastases'(Rizzo *et al.*, 2017) This can occur when 'normal' cell control mechanisms become disrupted. Surgical removal of the original tumour is not always a successful treatment in malignant disease, due to microscopic spread (Wagner *et al.*, 1999).

A word for ailments in which irregular cells divide without control and can enter nearby tissues. Malignant cells can also spread to other parts of the body through the blood and lymph systems. There are several main types of malignancy. Carcinoma is malignancy that initiates in the skin or in tissues that line or cover inner organs. Sarcoma is a malignancy that start in bone, muscle, blood vessels. Leukemia is a malignancy that starts in blood forming tissue Lymphoma and multiple myeloma are malignancies that activate in the cells of the immune system.

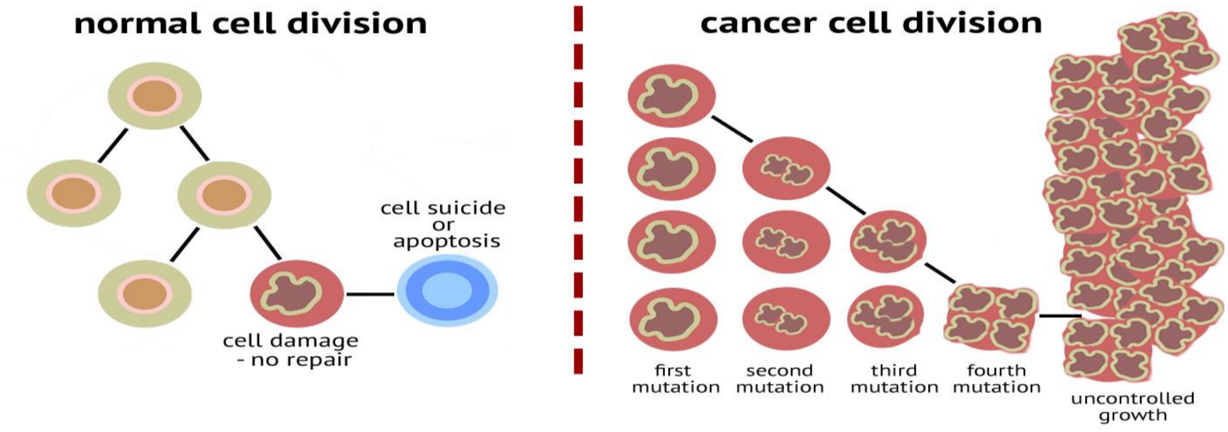


Figure 1.1:- Shown that cell division of cells in both normal and cancerous conditions, adopted from www.philpoteducation.com on 14/04/2018.

Microscopic investigation of the surgical resection margins can expose the presence of malignant cells. If left untouched, these cells will result in localized repetition of the cancer and eventual spread (metastasis) (Saito *et al.*, 2017) The spread of the malignant cells extends outward from the original tumour, and has been described as approaching the appearance of a crab. This is the origin of the term 'cancer', which was derived from the Latin meaning 'crab'. The earlier a cancer is detected, the less likely it is to metastasize, and so the more favorable the prognosis for the individual (Corner *et al.*, 2009)

1.2 Statistic about Cancer

Cancer is one of the top of sickness and death worldwide, with approximately 14 million new cases in 2017. Number of new cases of cancer is expected to rise by about 70% over the next decades. Cancer is a leading cause of death and was responsible for 8.8 million death in 2016-2018 (Siegel *et al.*, 2018). According to world health organization in the world nearly 1 in 6 deaths is due to cancer. Nearly 70% of deaths from cancer in the low and middle income countries like African countries, around one third of deaths from cancer due to the 5 leading behavioral and dietary risks, high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use is the most commonly risk factor for cancer and responsible for approximately 22% of cancer death (Organization, 2016). In 2016 an predictable 1,685,210 new cases of cancer was identified in the United States of America and 595,690 people was died from the cancer (Siegel *et al.*, 2015) According the statistic released by WHO most dangerous types of cancer is breast cancer in developed countries, lung and bronchus cancer, prostate cancer, colon cancer, melanoma of skin and thyroid cancer. Cancer mortality is higher among men than women (207.9 per 100,000 and 145.4 per 100,000). It is highest in African American men (261.5 per 100,000) and lowest in Asian/ Pacific Islander women (91.2 per 100,000) (Siegel *et al.*, 2016). According to National cancer Institute the total number of people living past a cancer diagnosis touched approximately 14.5 million in 2016 and expected to rise to almost 19 million by 2024. Approximately 39.6 of men and women was diagnosed with the cancer at the some point during their lifetimes. More than 60% of the world's new cancer cases occur in only African countries, Asian countries and Central and South American countries and 70% of the world's cancer deaths also occur in these regions (Oncology, 2016).

Various factors which are responsible for the developing of cancer mainly are diet that is low in fibre and high in animal fats contributed to the development of corectal cancer. Lung cancer is one of the most dangerous type of cancer according to world health organization developed by smoking and use of tobacco. Smoking and tobacco leads to damage the genetic and developed the breast cancer. These genes are BRCA1 & BRCA2 which are responsible for inducing breast cancer. According to world health organization there are over 200 types of cancer is bladder cancer, anal cancer, skin cancer, oral cancer, leukemia, lung cancer, stomach cancer and breast cancer (Jung *et al.*, 2017).

1.3 Breast Cancer

The growth of the human female breast initiates during gestation but is not complete at the time of birth. Advance development and differentiation of breast tissue arises over time and especially in response to unstable estrogen and other hormonal signals beginning in puberty, continuing through the reproductive years, during pregnancy and lactation, and at menopause (Rudel *et al.*, 2007).

Monthly ovulatory cycles are supplemented by cyclical changes in the form and behavior of cells and structures in the breast, including progressive differentiation. Pregnancy and lactation activate maximal differentiation of the breast. When a pregnancy and lactation end, as well as at menopause, breast tissue reverts to a less differentiated state (Marmot *et al.*, 2007).

Inside the breast are adipose and connective tissues that surround multiple groups of lobules in which milk is produced during lactation. Milk moves to the nipple through ductal structures. The ducts are lined by luminal epithelial cells and have an outer layer of myoepithelial cells (Witkowska-Zimny *et al.*, 2017) Populations of stem cells that can give rise to either luminal or myoepithelial cells are also found in the ductal tissue (Breyer *et al.*, 2018). The ducts are fixed to a basement membrane, which contributes to both the structure and the function of the ductal tissue. Connective tissue within and between the lobules, known as the stroma, additional contributes to the structure of the breast and plays an important role in regulating both normal and abnormal breast cell growth and function(Świderski *et al.*, 2017). Cell types within the stroma include fibroblasts, adipocytes, macrophages, and lymphocytes. These cells and structures in the breast generate and respond to a various mix of hormones, especially estrogen, and other regulatory factors (Azizi *et al.*, 2018).

Certain interruptions in the complex progressions that govern the structure and function of breast tissue may set the platform for breast cancer. Some carcinogenic events occur impulsively in the passage of normal biological processes and others are activated by external factors. Although the body has well-organized protective responses, like DNA repair and immune system, that can reduce the effect of such events, these protective responses are not always successful (Wieczorek *et al.*, 2017).

Particular mechanisms that may play a role in breast cancer are well-known. The contribution of genetic mutations to cancer is well known. They may be inherited (e.g., germline mutations in the *BRCA1* or *BRCA2* genes, which normally have a role in DNA repair) or grow in some cells during a person's lifetime (somatic mutations) as a result of reactive by-products of normal biological processes, or from the effects of external exposures (Corso *et al.*, 2018). Other mechanisms include epigenetic changes that can change gene expression without changes to DNA, promotion of cell growth by estrogen and other hormones or cell-signaling proteins, and evasion of the immune system (Tung *et al.*, 2015).

Breast cancer (BC) is a disease where cells in the tissue of the breast cancer grow and divide without normal control. It is a malignant growth of cells (tumor) that starts in the cells of the breast. A malignant tumor is a collection of cancer cells that can produce into (invade) surroundings tissues or blowout (metastasize) to distant organs of body (Wculek *et al.*, 2015). This type of disease occurs almost in women, but men can get it, too. Breast cancer is categorized by the uncontrolled growth of irregular cells in the milk generating glands of the breast or in passages (ducts) that deliver milk to the nipples. An expected 27% of female breast cancer is related to life style influences comprising overweight and obesity (9%), alcohol and certain occupational exposures (5%) (Baralt *et al.*, 2010). Breast cancer is also known as carcinoma of the breast. Breast cancer can either be invasive or non-invasive (in situ). The major types of breast cancer are ductal carcinoma in situ, invasive ductal breast cancer, invasive lobular breast cancer and inflammatory breast cancer. Less common categories of breast cancer comprise medullary carcinoma, tubular carcinoma and mucinous carcinoma. In situ, the breast cancer have characteristics of both ductal and lobular carcinomas or have unspecified origins (Baglia *et al.*, 2018). The breast cancer in which cancer cells are inside the milk ducts (in situ), known as ductal carcinoma in situ. While the breast cancer that one developed from lobules, known as lobular carcinoma of breast. There are number of causes of breast cancer due to which it can grow, spread and also affects the other parts of body in the multiple ways (Narod *et al.*, 2015) The important causes of breast cancer are unhealthy lifestyles, environmental factors, genetic factors, gender, family history, radiation exposure, early menarche and late menopause and first pregnancy after the age of 30. Over expression of the leptin in adipose tissue may also play a role in the development of cancer in the breast tissue (Coyle, 2004).

Breast cancer is the most common in women worldwide, with some 5- 10% of all cases due to inherited mutations in BRCA1 and BRCA2 genes. There are some other genes associated with BC and play an active role in causes of breast cancer. A variation of genes such as CDHI, STK11 and p53 increase the risk of breast cancer. The AR, ATM, CHEK2, BARDI, BRIP1, NBN, PLB2, RAD50, RAD51, ERBB2 and DIRAS3 genes are associated with breast cancer (Ford *et al.*, 1998). A woman total lifetime exposure to estrogen is the major risk factor for the development of breast cancer. Breast cancer most commonly spreads to the normal lymph nodes. These lymph nodes are of the three types: axillary, cervical and internal mammary. When cancer arises in breast tissue and metastasizes outside the breast, the cancer cells mainly found in axillary nodes. There is a higher chance getting the cancer cells could into the bloodstream and spread to the other sites of the body e.g. other lymph nodes and other organs such as bones, liver and lungs. In the USA, one in eight women will be diagnosed with breast cancer in her lifetime. The standard treatments that are used for the treatment of breast cancer are surgery, sentinel lymph node biopsy followed by surgery, radiation therapy, chemotherapy, hormone therapy and targeted. Some drugs such as nolvadex (tamoxifen citrate), keoxifene, aromasin, Cytoxan, raloxifene hydrochloride and fluorouracil. There are some natural compounds which show antitumor activity in breast cancer like resveratrol, marine resources like algae, flavonoids (Maughan *et al.*, 2010).

1.4 Types of breast cancer on the basis of receptors given as below

1.4.1 Hormone Receptor Positive Breast Cancer

Approximately 75% of total breast cancers are Estrogen receptor positive. They develop in reaction to the hormone estrogen. About 65% of these are PR active. They raise in reply to the hormone progesterone. The breast cancer tumors that ER/PR positive are 60% respond to endocrine therapy (Burstein *et al.*, 2016).

1.4.2 HER2- Positive Breast Cancer

About 20% to 25% of all breast cancer are HER2 positive breast cancer. The cancer cells made of a protein, known as HER2. These breast cancers tend to be much more aggressive and fast growing (Loibl *et al.*, 2017).

1.4.3 Triple- Negative Breast Cancer

The breast cancer that estimate 10% to 17% are known as triple negative breast cancer. They lack estrogen and progesterone receptors and do not overexpress the HER2 protein. The majority of breast cancers are associated with the gene BRCA1 are triple negative (Dent *et al.*, 2007).

1.5 Causes of Breast Cancer

The breast cancer is caused by mainly four factors that described below:

1.5.1 Lifestyle factors:-

The unhealthy lifestyle also increase the risk of breast cancer. The factors are including poor diet, alcohol consumption, lack of physical activity, irregular sleeping, overweight and no breast feeding (Bodai *et al.*, 2015)The hormone replacement therapy and no childbirth or first pregnancy after the age of > 30 years also increase the risk of breast cancer. Dense breasts have a risk of breast cancer is two times that of women with average breast density. The treatment with DES (diethylstilbestrol), radiation exposure and use of birth control pills also increase the risk in the development of breast cancer. Tobacco smoking may increase the breast cancer (Dal Maso *et al.*, 2008).

1.5.2 Environmental factors

Some environmental factors also contribute to the development of breast cancer. The use of certain cosmetics, personal care products, pesticides (such as DDE) and PCBs (polychlorinated biphenyl) increase the risk of breast cancer (Nickels *et al.*, 2013). It has been found that exposure to DDT before puberty increases the risk of breast cancer later in life. Passive smoking increases breast cancer risk by 70% in younger, primarily premenopausal women (McPherson *et al.*, 2000).

1.5.3 Genetic factors

5% to 10% of breast cancer are thought to be caused by inherited genetic mutations in the genes BRCA1 & BRCA2. An inherited mutation in the genes, p53 and PTEN involves a high risk of breast cancer but are rare. Inherited mutations in CHEK2 gene also include the development of breast cancer (Stephens *et al.*, 2012). Heterozygous female ATM gene mutation that carries in ataxia telangiectasia families, also have an increased risk of developing the breast cancer. Research published in 2014 found that the increase in breast cancer by an abnormal PALB2 gene is 5 to 10 times higher than average, almost as high as an abnormal genes BRCA1 & BRCA2. The other genes like NBN, RAD50, RINTI1, MRE11A and CDH1 also associated with the development of breast cancer (Miki *et al.*, 1994).

1.5.4 Reproductive factors

Most of the female breast cancers are related to the female hormone. The potential risk factor is that increase exposure to these hormones. The reproductive factors are associated with an increase exposure to the endogenous estrogens that produced by the ovaries (Anderson *et al.*, 2014). These factors include earlier menarche, late menopause, low parity and late age at first birth. One of the most established risk factors for breast cancer is the woman’s total life time exposure to estrogen. Estrogen involved in the development of breast cancer via stimulation and proliferation of breast cells (Khalis *et al.*, 2018).

1.6 Statistic of Breast Cancer

Breast cancer is the most common cancer amid women with an probable 1.7 million new cancer cases detected in 2016 and ranks second overall (11.9 % of all cancers), death has increased by 14%. Breast cancer ranks as the fifth cause of death in developed and developing countries (DeSantis *et al.*, 2014). Overall risk of breast cancer doubles each decade until the menopause. Breast cancer is more common after the menopause. Some studies demonstrated that women who migrate from areas of low risk to areas of high risk, will be attain the rate in the host countries one or two generations. This studies shows that environmental factors are important in the progression of the disease. According to world health organization (WHO), International agency for Research of cancer statistics 2016, total breast cancer in India recorded 70,218 deaths (Siegel *et al.*, 2018). According to the American cancer society survival rates vary worldwide, but are improving overall. The rate is 80 to 90 percent for those with a first stage. In the 2016, Belgium had the highest rate of breast cancer in the world with 111.9 cases per 100,000 adult women (Torre *et al.*, 2015). The higher number of cases in developing countries due to their larger portions of the world's population (Siegel *et al.*, 2018).

Table 1:- Shown that incidence and mortality rate of Breast cancer in various countries

Nation	**Incidence**	**Mortality**
China	18.7	5.5
Africa	19.0	14.1
India	19.1	10.4
Japan	32.7	8.3
Brazil	46.0	14.1
Singapore	48.7	15.8
Italy	74.4	18.9
Switzerland	81.7	19.8
Australia	83.2	18.4
Canada	84.3	21.1
Netherlands	86.7	27.5
England	87.2	24.3
Sweden	87.8	17.3
Denmark	88.7	27.8
France	91.9	21.5
United states of America	101.1	19.0

. Source – World health organization Breast cancer statistic 2016

According to the centers for disease control and prevention (CDC) 230,815 women and 2109 men in the United States of America were diagnosed with the breast cancer in 2016. In the same year, some 40,860 women and 464 men in the United States of America died from breast cancer. According to Global cancer facts and figures countries with highest incidence are Netherlands 95.3, France 94.6 and United States of America 90.6. Countries with lowest incidence are Thailand 25.6, Algeria 29.8 and India 30.6 (Parks *et al.*, 2018).

1.7 Estrogen Receptors

Estrogen receptor is a group of proteins (or a twelve helix protein) present inside the cells of the female reproductive tissues or located in the nucleus of cells. In the region it functions as a ligand activated transcriptional regulator. These receptors are activated by the hormone estrogen (17β- estradiol). There are mainly three types of estrogen receptors which play an important role in breast cancer estrogen alpha, estrogen beta and estrogen gamma. The estrogen receptors ERα and ERβ show significant overall sequence homology (Wolff *et al.*, 2013). Estrogen receptors for sex steroids, thyroid hormone and retinoid. ERα, ERβ and ER gamma have different responses and they are located in different tissues. The ERα found in major female organ such as ovary, uterus, vagina, mammary glands and certain areas of central nervous system, especially in hypothalamus. ERβ is located on human chromosome 14. ERβs are located in the prostate, testis, and ovary and in some areas of brain (Rollerova *et al.*, 2000).

1.7.1 Structure of Estrogen Receptor

There two activation domains in the estrogen receptors, an N- terminal activation function (AF-1) and C- terminal activation function (AF-2). These regions act synergistically to recruit co- activators or co- repressors and thus regulate gene transcription. The receptors ERα and ERβ are composed of six domains A-F: (1) a variable NH_2 terminal region (A/B), (2) a conserved DNA binding domain (Dutta *et al.,* 2007), (3) a hinge region,(4) a ligand binding domain region (Dutta *et al.,* 2007) and a COOH terminal region. The AF-1 region of the receptor is involved in protein- protein interactions (e.g. phosphorylation) and can activate transcriptional activity of target genes in the absence of estrogen or other estrogenic ligand. Co activator interact to AF-1 leads to either limited or no activation of ERα, although full activation of this receptor involves requirement of co activators to both AF-1 and AF-2 regions (Enmark *et al.*, 1997). This requires binding of estrogen ligand. The transcriptional activity of AF-1 in ERβ is weak or negligible compared with ERα, while that of AF-2 is similar in both receptors (Yi *et al.*, 2015).

The AF-1 domain in ERα is very active and stimulation of reporter gene expression from the variety of estrogen receptor response elements in different cell lines. But under same conditions the activity of AF-1 domain of ERβ is negligible. The DNA binding domain contains a two zinc finger structure (Lee *et al.*, 2017).The structure plays an important role in receptor dimerization and binding of receptors to specific DNA sequences. The DNA binding domains of ERα and ERβ are highly homologous. The hinge and hyper variable D region allows the receptor protein to bend or alter conformation. The C terminal E domain contains a ligand binding domain (LBD), which determines the ligand binding specificity of ER. The LBD consists of 12α helices and two short β sheets (Chen *et al.*, 2016). The ligand binding domain (LBD) resolve the ligand binding, receptor dimerization, nuclear translocation and transactivation of target gene expression. ER containing a large (42- amino acid) F domain which is not well conserved among different vertebrate ER species. This domain is important in the transcription activation and repression activities of antiestrogens (Yi *et al.*, 2015).

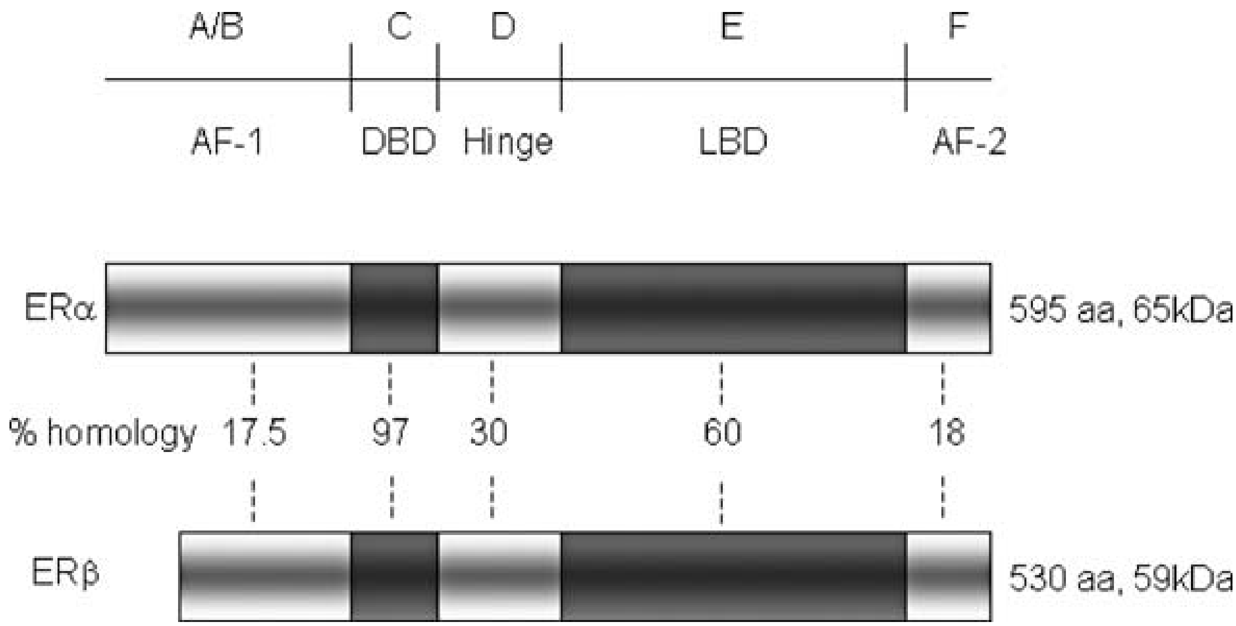


Figure 1.2:- Structure of Estrogen receptor, Adopted from Cancer Treatment and Research by Steven T. Rosen, M.D., Series Editor (2016).

1.7.2 Mechanism of action of Estrogen Receptor

The classical mechanism of activated estrogen receptors regulating gene transcription through binding to the estrogen response element (Shukla *et al.,*2005) on the DNA, they can also regulate transcription by binding to the other promoter elements on the DNA, such as AP1- binding sites. The cAMP response element and SF-1 response element to which other transcription factor bind. The sequence of events leading to the starting of gene transcription by a steroid element as follows: (1) binding of hormone to the receptor (2) formation of a homo dimer from two molecules of receptor (3) transport to the nucleus, if necessary (e.g. in case of estrogen hormone) (4) binding to the response element; (5) recruitment of co-activators; and (6) final activation factors to start transcription. The ultimate consequence is the synthesis of mRNA molecules and corresponding protein which triggers the observed biological response (Khan *et al.*, 2016). For example estrogen enters the cell and attach to at least two receptors and the two loaded receptors have to link together for receptor activation. When the receptors are triggered, they assign to the sponsor sequence of specific genes and turn on the procedure of gene transcription reading the DNA code and using it to make an RNA template known as messenger RNA. The mRNA is read by other kinds of RNA molecules that put together protein. This process of converting RNA code into protein is translation (Mosselman *et al.*, 1996).

When estradiol binds to the estrogen receptor then there is a conformational change in estrogen receptor occurs due to this estrogen receptors dimerization takes place (Revankar *et al.*, 2005). Simultaneously both the domains like AF-1 and AF-2 are activating. After that, this dimer binds to the estrogen receptor element (Shukla *et al.,*2005) which is already present on DNA then co activators are recruited and fully transcription process will start. Subsequently, transcription followed by translation process occurs (Lubahn *et al.*, 1993).

1.7.3 Estrogen receptor signaling pathway

Estrogen receptor mainly follows two types of pathway genomic and non-genomic. In the genomic pathway the estrogen directly attacks on the genes or at the genomic level. Then regulates the co regulators like co activators and co repressors. Genomic ER action occurs in nucleus when the receptors binds to GNA directly (classical pathway) and indirectly (non-classical pathway). In estrogen classical pathway, 17β- estradiol (E2) enters the cytoplasm at which estrogen receptors are present (Hall *et al.*, 2001). Where it causes the heat shock protein (HSP) like HSP27, HSP90 and HSP70. After it the conformation of ligand bounded activated ER changes and dimerization take place. Then it binds to specific DNA sequence called estrogen response element in order to control transcription (Bjornstrom *et al.*, 2005).

In non-classical pathway the ER does not interact directly with DNA but interact with other DNA bound transcription factors such as C-jun or C fos and with other co activator proteins (Matthews *et al.*, 2006). The identical ligand have different effects on AP-1(jun/fos) dependent transcription depending on the receptor. For example E2 is an agonist for ERα and not for ERβ at AP-1 elements. As a result of these differences, the overall response of a cell expressing ERα and ERβ to a particular ligand cannot be basically predicted where it stimulate the cell proliferation, differentiation and inhibition of cell growth (Filardo, 2002). The AP-1 and SP-1 are alternative regulator DNA sequence used by estrogen receptors ERα and ERβ to alter gene expression. Both classical and non- classical pathway possesses recruitment of co activators and component of RNA polymerase transcription initiation complex that enhances target gene transcription. DNA acts as allosteric factor whose binding changes ER conformation such that different EREs would be hypothesized to regulate ER interaction with other proteins like co activators and co repressors (Márquez-Garbán *et al.*, 2007). In non-genomic pathway there are some mechanism proposed for membrane initiated ER signaling. This pathway acts through a potential membranes ER regulations MAP kinase, ERK/Akt and no synthase response (Acconcia *et al.*, 2006).

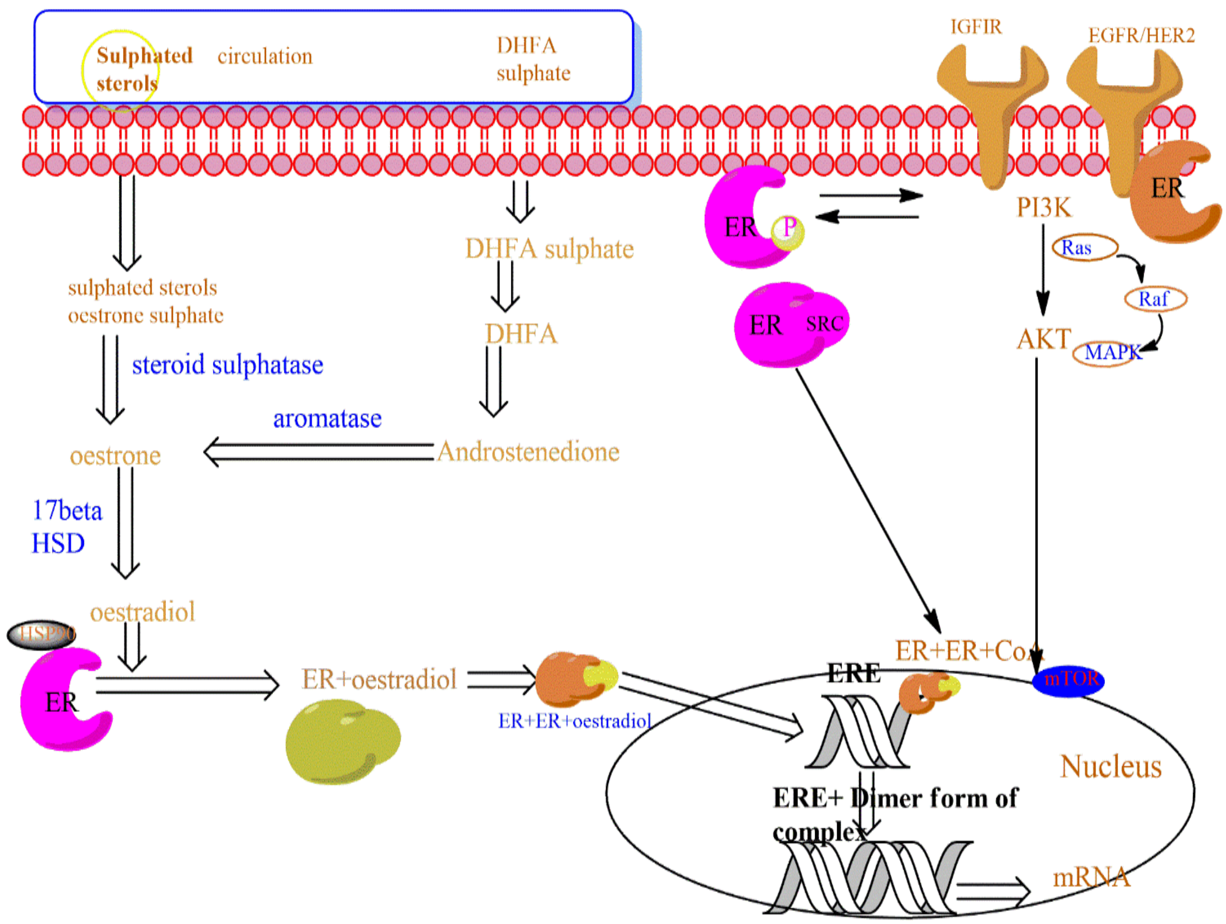


Figure 1.3:- Shown that Estrogen signaling pathways

Source: - Sketched on the Chem Draw software on dated 25/04/2018

1.7.4 Role of Estrogen receptor in Breast cancer

Increase the level of estrogen can cause the risk of breast cancer as such mechanism is not clear. A physiological functional level of estrogen is not initiator of breast carcinogenesis. However certain metabolites of estrogen can bind to DNA and induce mutations (Bailey *et al.*, 1998). Estrogen can be hydroxylated by the cytochrome p450 system to hydroxy catechol estrogen further oxidized to the quinone and semi quinone forms. The quinone forms of catechol estrogen interact with DNA and produced DNA adducts. Semi quinone intermediates are free radicals can bind with oxygen to producing superoxide radicals. Superoxide radicals can attack and alter the structure of DNA (Zhang *et al.*, 2007).

Another mechanism by which estrogen can initiate breast cancer by stimulating breast epithelial cell proliferation. Cell division is important for the propagation of genetic error and elevated the mitogenesis increases the risk of genetic alteration. Estrogen increase the growth of specific types of breast cancer cell (Russo *et al.*, 2004). Estrogen α is infrequently found in normal breast cells enduring proliferation. On the other side cell surrounding normal estrogen alpha positive cells undergo proliferation, suggesting that paracrine factors mediate the mitotic effects of estrogen. In the distinction to the alienation between cell proliferation and the presence of estrogen alpha in normal epithelial cells, proliferating breast cancer cells précised estrogen alpha suggesting that estrogen can directly stimulate the growth of breast cancer cells (Clavel, 2007). Literature suggest that estrogen alpha and estrogen beta have different roles in the progression of breast cancer. Paruthiyil suggested that estrogen stimulated cell proliferation and augmented tumor formation when only estrogen alpha was present(Warasiha, 2008). In the presence of estrogen beta, estrogen inhibited cell proliferation and prevented tumor formation. The estrogen mediated suppression of proliferation was associated only with decreased expression of c-myc, cyclin D1, and cyclin A genes and increased expression of p21 and p27 (Ko, 2014). Studies concluded that estrogen alpha was allied with breast cancer cell proliferation while estrogen beta was associated to decreased proliferation (Duffy, 2006).

1.8 Breast cancer targeted therapies

1.8.1 Endocrine Therapy

Endocrine therapy is the first “targeted” medical treatment in oncology with antitumor activity restricted to patients whose breast tumors express estrogen receptors and/or progesterone receptors (PRs). It is an extremely powerful treatment modality prescribed to two-thirds of the breast cancer population, both in advanced and early disease stages It is also recognized as an effective prevention approach of the disease but with a low uptake by women at risk in view of its side effects (Johnston *et al.*, 2009). One distinguishes three main classes of endocrine agents, based on their mechanism of action:

- The selective estrogen receptor modulators (SERMs), which bind the ER and interfere with its transcriptional activity
- The selective estrogen receptor down regulator Fulvestrant, which binds the ER and accelerates its destruction.
- The aromatase inhibitors, which inhibit the enzyme aromatase and, as a result, profoundly reduce estrogen level in postmenopausal women.

1.8.1.1 Aromatase

The aromatase enzyme is the key enzyme in the biosynthesis of estrogen. Therefore, it is also called estrogen synthase. The enzyme catalyzes the rate limiting step in the production of 17β estradiol (E2). If the aromatase complex made up of cytochrome P450 super family of enzymes proteins known as cytochrome P450 aromatase produced by CYP19 gene (Noble *et al.*, 1997) Aromatase is involved in the aromatization of androgens to estrogens. This enzyme can be found in many tissues like gonads, brain, adipose tissue, placenta, blood vessels, skin and bone (Carreau *et al.*, 2003). Aromatase can also found in the tissue of endometriosis, uterine fibroids, breast cancer and endometrial cancer. The CYP450 enzymes share a common tertiary fold consisting of 12-15 α-helices and four β- sheets (James *et al.*, 1987).

1.8.1.2 Role of Aromatase in Breast Cancer

Over 60% of breast carcinomas express this enzymes with higher levels of mRNA expression and activity compared with the non-malignant tissue. It has been found that aromatase activity is highest in intratumoral stromal cells rather than the epithelial component in breast cancer (Catalano *et al.*, 2003). Some studies have shown that aromatase was immunolocalized either in both cell types or predominantly in carcinoma cell. The activity of aromatase also regulate the concentration of estrogens with endocrine, paracrine, and autocrine effects on target tissues including bone (Smith *et al.*, 2003). It catalyzes the last steps of estrogen biosynthesis androgens to estrogens specially transformation of testosterone to estradiol and androstenedione as follows:

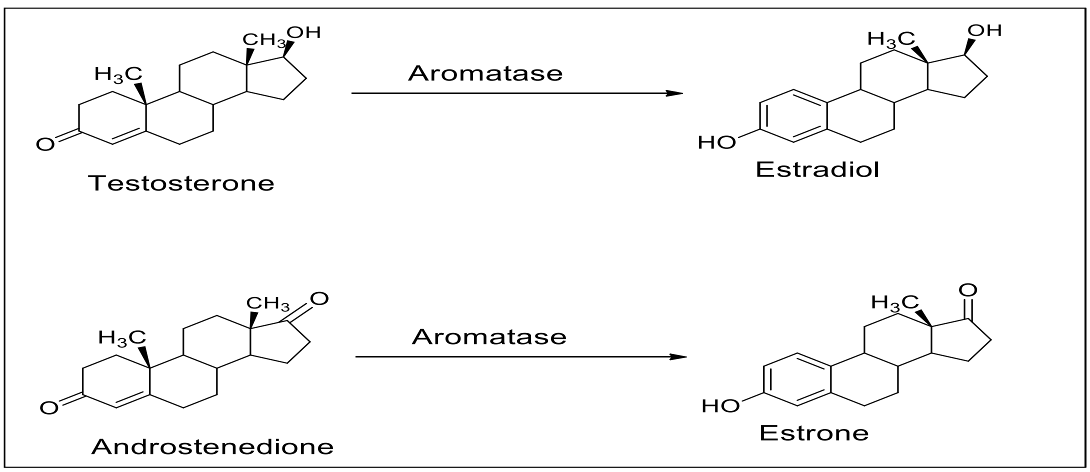


Figure 1.4:- Demonstrated that role of Aromatase in various reaction of biosynthesis of estrogen.

These steps involve the three successive hydroxylation of 19 methyl group of androgens by simultaneous elimination of the methyl group as formate and aromatization of the ring A (Simpson *et al.*, 1997).

1.8.1.3 Aromatase Inhibitors

Aromatase inhibitors are the class of drugs which are used for the treatment of breast cancer and ovarian cancer by reducing the excess of estradiol synthesis during ovarian stimulation (Mitwally *et al.*, 2001)

On the basis of chemical structure aromatase inhibitors are classified into two categories such as steroidal inhibitors. All inhibitors inhibit the synthesis of estrogen by inhibit the aromatase activity. They are different from each other either class independent differences in term of potency or class specific differences in terms of the mechanisms of binding to the aromatase (Geisler *et al.*, 1998). The most used inhibitors are anastrozole, vorozole and letrozole are non-steroidal inhibitors, whereas exemestane and formestane are steroidal inhibitors. The steroidal aromatase inhibitors bind non-covalently and reversibly (Winer *et al.*, 2005). These aromatase inhibitors are used for the treatment of both early and late stage ER positive breast cancer in post-menopausal women (Winer *et al.*, 2005).

1.8.2. Steroid Sulfatase

1.8.2.1 Sulfatase enzyme

Steroid sulfatase (STS) is an enzyme that hydrolyzes several steroids, including E_1S and DHEA-S. its catalyzes the biological inactive estrone 3 sulfate to estrone in the breast tissue (Utsumi *et al.*, 1999). The Steroid sulfatase activity is identified in the mostly in breast cancers and this activity has been correlated with the stages of Steroid sulfatase mRNA and protein expression in breast cells (Nakata *et al.*, 2003). The activity of Steroid sulfatase is advanced in carcinoma tissue associated with normal breast tissue. The significance of E_1S and STS in postmenopausal breast cancer is due to the resulting facts.

- E_1S is the most abundant circulating estrogen in postmenopausal women.
- It has a longer half-life in plasma as compared to the estrogen.
- The levels of E_1S in the breast tumors are considerably higher than in the plasma.
- The activity of STS is 50-200 times that of aromatase.

- The STS expression is detected in 90% of the breast tumors.

1.8.2.2 Biosynthesis of estrogen mediated by STS enzyme

The estrogen sulfotransferase (Shrestha *et al.*) is an enzyme that converts estrogens to inactive estrogen sulphate. It is present in both normal and cancerous breast tissue. The 3β- HSD activity and immuno reactivity have been detected in breast carcinoma cells in a minority of breast cancer tissues (Stanway *et al.*, 2007). Thus this enzyme may be important in increasing the local concentration of androstenedione, a substrate for estrogen synthesis. Aromatase as discussed above it catalyzes the last rate limiting steps in the biosynthesis of estrogen that is it converts the androgens to estrogens (Mungenast *et al.*, 2017).

1.8.2.3 Steroid sulfatase inhibitors

The steroid sulfatase inhibitors play an important role in the future treatment of hormone dependent breast cancer. There are three classes of STS inhibitors: - alternative substrates (including competitive reversible inhibitors), reversible inhibitors and irreversible inhibitors (Piccinato *et al.*, 2016) The alternative substrates examined in the late 1980s, were a 2-(hydroxyphenyl) indole sulfates one of which exhibited an IC_{50} of 80μM. Naturally occurring steroids have been identified having STS inhibitory activity and most potent at 2 μm. the reversible inhibitors whose breakdown products were unlikely to be estrogenic in nature. Initially the replacement of the sulphate group (OSO_{3-}) of E1S with a range of mimics such as phosphate, phosphonates, sulfonates, sodium methyl sulfonates, sulphonyl halides, sulfonamide and methylene sulphonyl group The majority of these compounds were competitive inhibitors that compete with E_1S for STS enzyme active site. The COUMATE (4- methylcoumarin-7-O-sulfamate) is the synthesized non-steroidal sulfamate STS inhibitor without estrogenic properties. It inhibits STS activity in MCF-7 breast cancer cells by >90% at 10μm. further on a series of tricyclic coumarin sulfates identified 667 COUMATE STS inhibitor that was more potent than EMATE. A steroid based selective STS inhibitor, 17-diisopropylcarbamoyl-1, 3, 5(10), 16- estra-tetra-en-3-yl sulfamate also known as

KW2581. This compound has the ability to inhibit the hormone receptor positive human breast cancer cell lines ZR-75-1 and BT-474 (Nussbaumer *et al.*, 2004).

1.8.2.4 Estrogen sulfotransferase

It converts the estrogen to inactive estrogen sulfates. It is present in both normal and cancerous breast tissue. From the immuno reactivity it was detected in the carcinoma cells of 44% patients with breast cancer (Piccinato *et al.*, 2016). The recent studies demonstrate that the presence of very strong estrogen sulfotransferase activity has been shown in breast cancer cell line the MDA-MB 468. Both STS and EST plays an important role in regulating the *in situ* production of estrogens in human breast carcinoma tissues (Suzuki *et al.*, 2005)

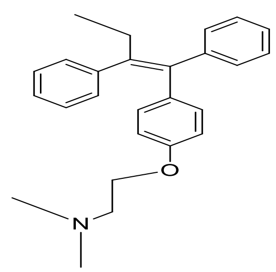
1.8.3 Selective estrogen receptor modulator (SERM)

The selective estrogen receptor modulators deficiency the steroid structure and retain a tertiary structure that permits binding to the estrogen receptor. Depending on the target tissue, these compounds have both selective agonist and antagonist effects. Thus SERMs block the effects of estrogen in breast tissue by binding to the estrogen receptor. If estrogen does not attached to the breast cell, the cells does not receive estrogen's signal to grow and multiply. In this way SERMs reducing the risk of breast cancer. By binding with estrogen receptor and activates it, in turn the conformation of ER changes and then it dimerises. The AF-1 site of the receptor activated while AF-2 site remains inactivated due to the binding of SERM as a ligand. The DNA bound estrogen receptor then regulates target gene transcription, either positively or negatively. The pharmacology of SERMs can be explained by three interactive mechanisms (1) differential estrogen receptor expression in a given target tissue (2) differential estrogen receptor conformation on ligand binding (3) differential expression and binding to the estrogen receptor of co regulated proteins (Diez-Perez, 2006).

Tamoxifen is a ground-breaking selective estrogen receptor modulator which has been a long therapeutics application for all steps of ER positive breast cancer. There are three categories of SERMs (1) triphenylethylene derivatives like tamoxifen (2) non-steroidal compounds like raloxifene (3) steroidal compounds that have more anti-estrogenic activity.

1.8.3.1 Triphenylethylene derivatives (Tamoxifen)

Tamoxifen is the currently most commonly prescribed SERM which is synthesized in 1960. It is approved by the U.S food and drug administration (FDA) in 1977 for the treatment of both early and advanced stages of breast cancer. It has large efficacy of premenopausal and postmenopausal patients with metastatic breast cancer. It is most effective for the ER+ and PR+. It decreased the incident of mammary tumors in rodents, but increase in liver tumors (Dutertre *et al.*, 2000).



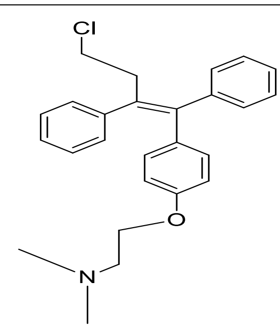
Tamoxifen

There is no increase in liver cancer in humans. Tamoxifen decrease the overall risk of developing invasive breast cancer by 50% with a median follow up of 54.4 months. The occurrence of estrogen receptor positive tumors decreased by 69%. It is a non-steroidal agent with potent antiestrogenic properties that causes cells to remain in the G_0 to G_1 phase of the cell cycle. Because it prevents the proliferation of cancerous cells but does not cause cell death, it is a cytostatic.

Tamoxifen has little affinity towards its target protein, the estrogen receptor because it is prodrug. Its active metabolites such as 4- hydroxy tamoxifen and N-desmethyl-4- hydroxy tamoxifen (endoxifen) have 30-100 times additional affinity for binding to the estrogen receptors than tamoxifen itself. These active metabolites compete with estrogen for binding to estrogen receptor. 4- Hydroxy tamoxifen in breast tissue acts as an estrogen receptor antagonist so that transcription of estrogen responsive genes is inhibited (Lewis *et al.*, 2005).

1.8.3.2 Toremifene

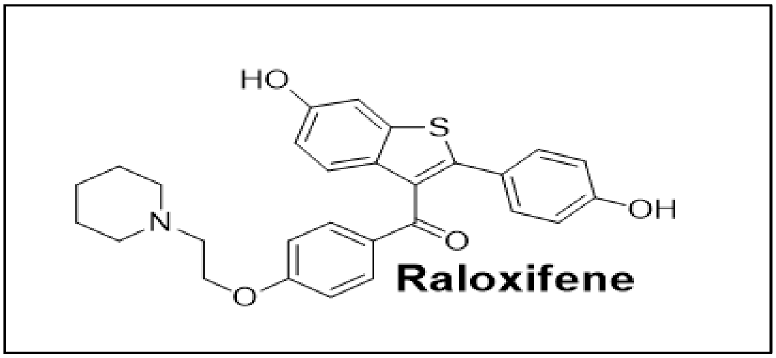
It is a chlorinated tamoxifen used for the treatment of hormone dependent breast cancer. It competes with estradiol for estrogen receptor and has growth inhibitory effects on the MCF-7 breast cells. At high concentrations Toremifene is cytotoxic. It is primarily an antiestrogen but it has some estrogen agonistic properties in postmenopausal women. Its clinical and preclinical activities are very similar to tamoxifen. It is somewhat more expensive to use than tamoxifen but it is an effective and well tolerated in comparison to tamoxifen for the treatment of postmenopausal women with hormone dependent breast cancer. Toremifene is potentially safer than tamoxifen in relation to the carcinogenic effects (Wärri *et al.*, 1993).



Toremifene

1.8.3.3 Raloxifene

The EVISTA (raloxifene hydrochloride) is an estrogen agonist or antagonist depending upon the selectively of selective estrogen receptor modulator that belongs to the benzothiophene class of compounds(Cummings *et al.*, 1999). Raloxifene is an efficacious as tamoxifen in reducing the risk of invasive and non-invasive breast cancer. Some other examples of selective estrogen receptor modulator are raloxifene (3-hydroxytamoxyfene) and 4- iodotamoxifene. These are derivatives of tamoxifen which shows no DNA adduct forming ability and are also less stable compared with that of tamoxifen.



1.8.3.4 Mechanism of action

When tamoxifen binds to the estrogen receptor, then the conformation of estrogen receptor changes and dimerization takes places. Simultaneously, AF-1 domain is in active state and AF-2 domain remains inactive. After that, this dimer binds to the ERE which is present on DNA and the co activators are recruited only at some extent. Then partially transcription takes place.

1.8.4. Selective estrogen receptor down regulator

Selective estrogen receptor down regulator can act as a pure estrogen agonist. Fulvestrant is the mostly commonly used Selective estrogen receptor down regulator. It is used for the treatment of hormone receptor positive metastatic breast cancer in post-menopausal women. Fulvestrant is a 7- alkyl analog of estradiol, it acts quite differently binding to ER and causes dissociation of heat shock proteins. The bulky side chain of Fulvestrant prevents dimerization and results in degradation of ER. It is not only inhibit the ER activity but also down the ER expression levels. Due to the down regulatory activity, Fulvestrant has been termed a SERD. Fulvestrant includes affinity for ER 100 times more than tamoxifen. The steroidal antiestrogen bind to the ER but due to their bulky side chains at the 7 α and 11β positions the receptor dimerization seems to be sterically hindered. There is evidence that ER molecules in the cell both *in vivo* and *in vitro. In vivo* studies demonstrate that tamoxifen resistant breast cell lines remain sensitive to growth inhibition by Fulvestrant and *in vivo* tamoxifen resistant tumors remain sensitive to fulvestrane (Howell *et al.*, 2004).

The SERD fulvestrane has a chance its exclusive endocrine activity because of its capability to down regulate the hypersensitive and activate ER present in LTED tumor cells. There is recognized a novel orally active non-steroidal SERD, the thiochroman derived which is structurally unrelated to fulvestrane and tamoxifen. This may also help overcome drug resistance from endocrine treatment sequence for breast cancer patients.

1.8.4.1 Mechanism of action of SERD

When Fulvestrant binds to the estrogen receptor, there is no conformational changes of ER occur then, no dimerization takes place. Both the activation domains like AF-1 and AF-2 remains inactive. Thus, there is no recruitment of co-activators take place. Due to this transcriptional process does not occur and BC will be totally suppressed.

1.9 Side effects of endocrine therapy

1.9.1 Gynecologic Side Effects

SERMs display estrogen agonist effects in some organs such as the uterus. Endometrial abnormalities include benign hyperplasia, benign uterine polyps, or endometrial carcinoma. The risk of endometrial cancer with long-term tamoxifen use is low and extends several years beyond treatment completion. Fewer gynecologic symptoms have been reported with fulvestrant than with tamoxifen (3.9 % vs. 6.3 %). Aromatase inhibitors are used an alternative for postmenopausal women, but they induce vaginal dryness, contributing to the loss of libido. Non hormonal lubricants may be used to release symptoms. Due to the risk of systemic absorption, estrogen containing vaginal preparations should be avoided (Moore *et al.*, 2003).

1.9.2 Thromboembolic Disease

Several adjuvant and prevention trials have demonstrated an increased risk for venous thromboembolic events during tamoxifen treatment (Hindi *et al.*, 2013).

1.9.3 Hot Flashes

Vasomotor symptoms are frequent complications consecutive to estrogen depletion in women treated for breast cancer, producing impairment of quality of life and leading to noncompliance. Non estrogenic pharmacological interventions, such as the selective serotonin-norepinephrine reuptake inhibitor venlafaxine, at 75 mg/day, and the antihypertensive centrally acting adrenergic agonist clonidine, at 0.1 mg/day, show some efficacy in reducing hot flashes in a recent trial (Mom *et al.*, 2006).

1.9.4 Eye Problems

The rate of cataract was significantly increased by tamoxifen compared to placebo in the large NSABP P-1 preventive study. This complication occurred in 2.77 % of women treated with tamoxifen, while the incidence of cataract surgery was 1 %. Women should be asked to report any visual abnormality, and ophthalmological investigations should be ordered in symptomatic patients. Four cases of retinopathy were reported in 63 patients prospectively followed for ocular toxicity. Retinal opacities were not reversible with tamoxifen withdrawal (Paganini-Hill *et al.*, 2000).

1.9.5 Cardiovascular disease

Hormone therapy is responsible lowers the LDL cholesterol and increases the HDL cholesterol. Hormone therapy raise risk of heart attacks in women who already have heart disease as well as in women who do not have known heart disease (Hooning *et al.*, 2007).

1.9.6 Stroke:

Hormone therapy slightly increased the risk of stroke in women. Study expected that there were 8 extra strokes per 10,000 women taking hormone therapy for one year, compared to women taking a placebo (sugar pill). Because of the opportunity of amplified breast cancer, stroke, and heart disease risks, women who have no chief menopause symptoms may indicate to avoid hormone therapy (HT). The effects of other types of hormone therapy on breast cancer risk are still unclear (Bushnell *et al.*, 2004).

1.10 Natural Products used as Anticancer Agents

Nature has provided us with a rich source of compounds for various disease treatments. Such naturally-derived molecules have been utilized in formal drug discovery stages of the pharmaceutical industry. More than 60% of new chemical entries at the National Cancer Institute from 1981-2002 were either natural products or were derived from natural products. Such naturally-occurring sources can be defined by their origin and include biotic (i.e.: forests, plants, animals, birds and marine organisms) and abiotic (i.e.: land, water, air and minerals such as gold, iron, copper, and silver) components. Within these categories, plants have proven to be a rich source of lead compounds (i.e.: alkaloids, morphine, cocaine) or the basis for synthetic drugs (i.e.: anesthetics from cocaine) (Da Rocha *et al.*, 2001). The discovery of novel natural drugs is important for reduction of side-effects, high selectivity, low toxicity, and better Killing of cancer cells. Previous studies on the effects of curcumin, sauchinone, lycopene, denbinobin, genipin, capsaicin, and ursolic acid against breast cancer. The structure of these natural products are shown in below. These natural compounds possess anti-inflammatory, anti-metastatic, anti-proliferative, anti-angiogenic, and anti-cancer properties in breast cancer. Palmatine an alkaloid isolated from *Guatteria friesiana* exhibited a cytostatic effect against on MCF-7 (breast cancer line). Isobractatin isolated from *Garinia bracteata* has been shown to have anti-proliferative effect on human breast cancer cell lines, the antiproliferative effect is exerted via cell cycle arrest induction apoptosis. Furanodiene, isolated from *Curcuma wenyujin* showed anticancer effects in breast cancer *in vitro* as well as *in vivo* (Harvey *et al.*, 2015).

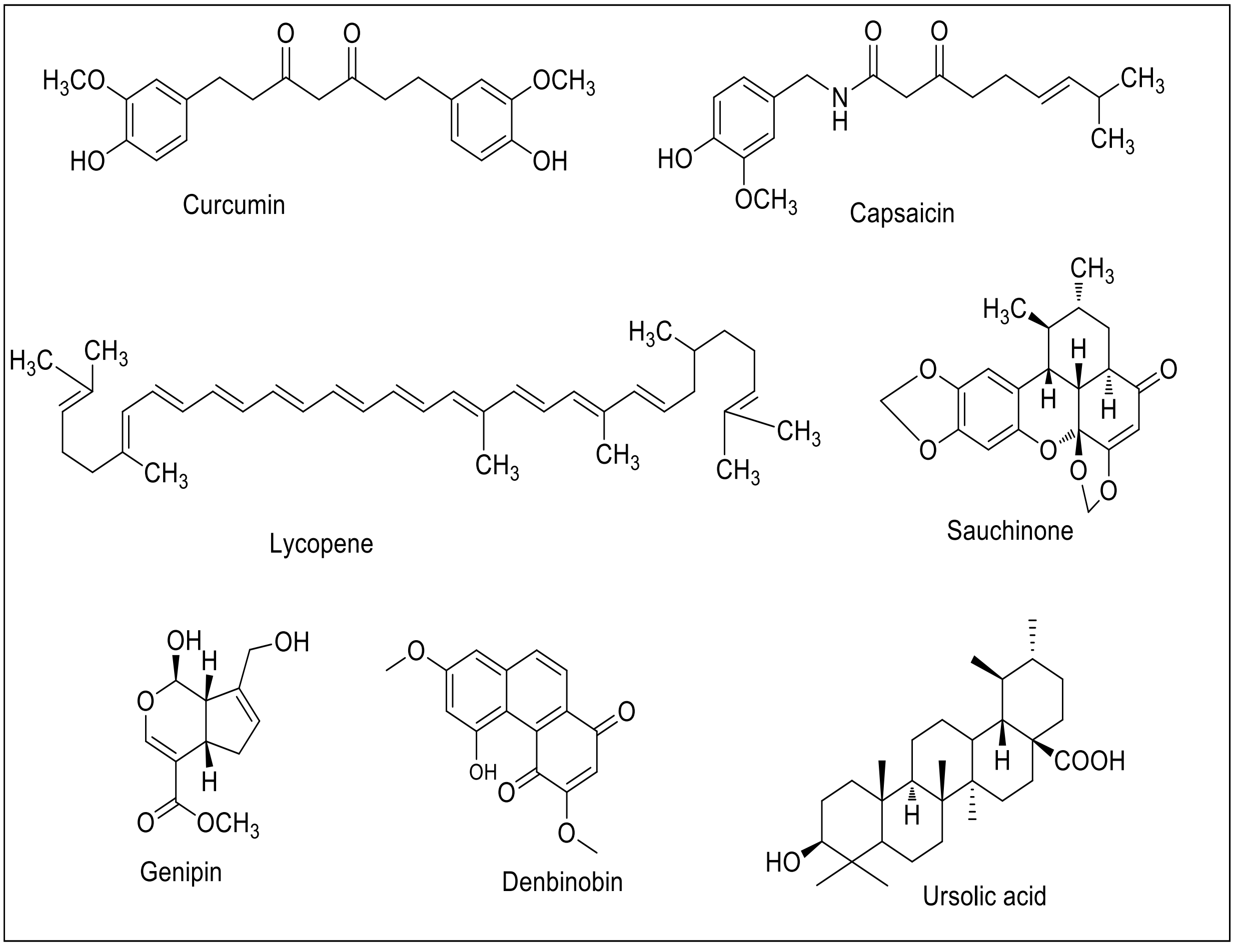


Figure 1.5:- Show that various natural products have anticancer activity.

1.10.1 Phytoestrogen

Natural products consists of important class of category that are called phytoestrogens. Phytoestrogens are also called xenoestrogens, which are derived from the plants. These are sometimes referred as dietary estrogens (Blanton, 2018). Phytoestrogens include flavonoids (isoflavones, coumestans, prenyl flavonoids) and non-flavonoids (lignans, stilbenes, phytosterols, saponins, resorcyclic acid), which are all polyphenolic compounds. Phytoestrogens denoted one of many significant bioactive non-nutrients found in many plants regularly consumed in human diet (Duke *et al.*, 2016). Phytoestrogens are naturally arising chemicals of plant source that retain weak estrogenic properties due to their structural likenesses to the human hormone estradiol. Bearing a structural resemblance to 17α-estradiol, they can interfere *in vivo* with mechanisms controlled by the hormone through competition for its receptors. They bind the estrogen receptor and exert hormonal and anti-hormonal effects. In addition, phytoestrogens may be important antioxidants (Sirotkin *et al.*, 2014). They disturb the action of DNA topoisomerase II and ribosomal S6 kinase, which could explain their observed effects on cell cycle, differentiation, proliferation, and apoptosis. Moreover, genistein is a potent tyrosine kinase inhibitor. It has been proposed that through such mechanisms phytoestrogens protect against a wide range of ailments, including breast, prostate, bowel, and other cancers, cardiovascular diseases, osteoporosis, and menopausal symptoms (Setchell, 1998) Of the flavonoids group of phytoestrogens, coumestan and isoflavone classes possess the greatest estrogenic breast, prostate, bowel, and other cancers, cardiovascular diseases, osteoporosis, and menopausal symptoms. Of the flavonoids group of phytoestrogens, coumestan and isoflavone classes possess the greatest estrogenic activity. Since coumestans are originate principally in clover and alfalfa plants and so are fewer constituents of the human diet, genistein, which has been reported to have the most potent anti-carcinogenic and estrogenic effect among isoflavones, attracts a great deal of interest in current studies (Magee *et al.*, 2004).

Almost all the phytoestrogens are non-steroidal heterocyclic phenols with the structure resembles to β estradiol figures show similarity of most common phytoestrogens (Genistein and Daidzein) to estrogen (Rodríguez-Landa *et al.*, 2017). Isoflavones are most common form of phytoestrogens. Isoflavones have diphenolic structure that resemble the structure of synthetic estrogen like diethylstilbestrol and hexestrol. The phenolic ring is the most important element of structure of most compound that bind to estrogen receptor. Lignans are compounds having a 2, 3-dibenzylbutane structure and occur as minor constituents of many plants. The chemical structure of plant lignans differs slightly from that of mammalian lignans in that they have phenolic hydroxyl groups in the Meta position only in their aromatic rings (Lima *et al.*, 2017).

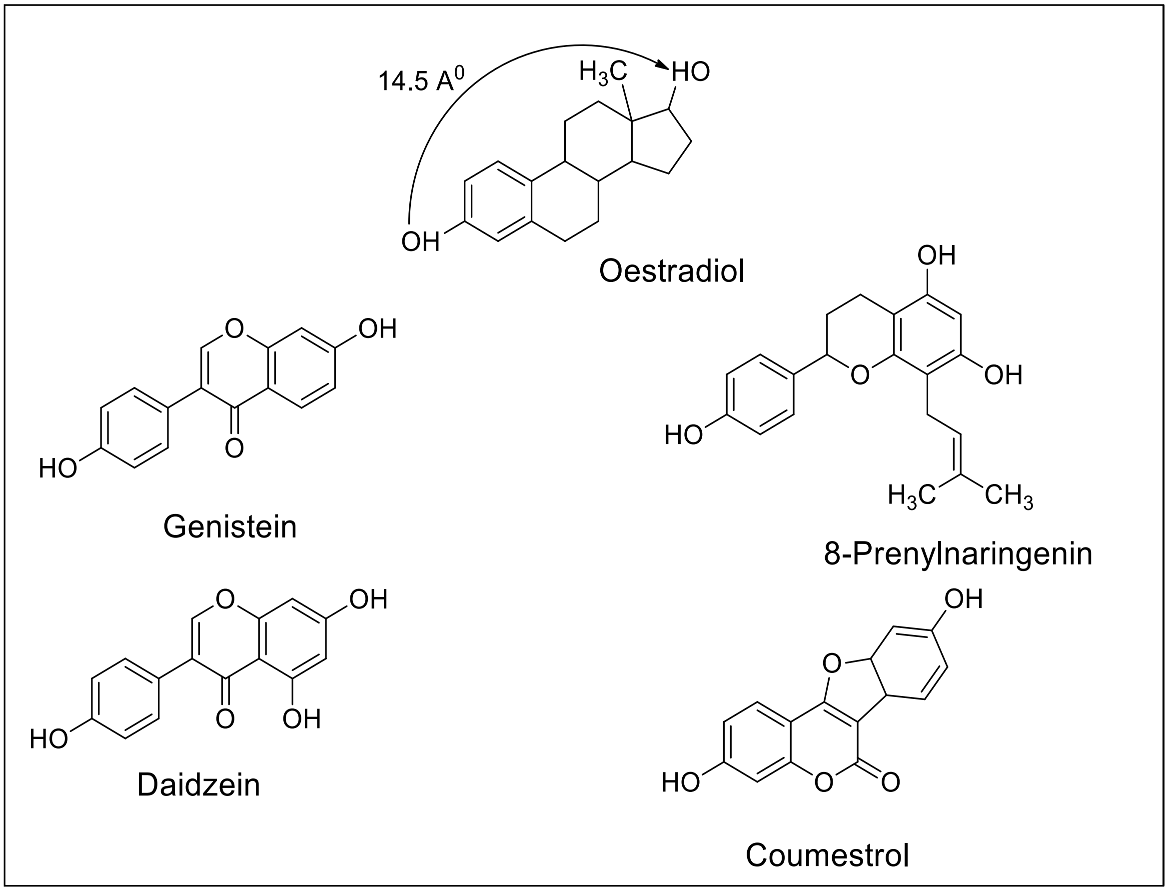


Figure 1.6:- Show that the structural similarities of Phytoestrogens to estradiol.

Some other key structural elements crucial for Oestradiol like activity

- ❖ Low molecular weights, similar to that of Oestradiol (C18H2402) (MW=272)
- ❖ Distance between two aromatic hydroxyl groups in the nucleus of the isoflavones equal to the distance between two hydroxyl groups of oestradiol
- ❖ Ideal pattern of hydroxylation, i.e., hydroxyl substituents at 4, 5 and 7 positions (Genistein).

1.10.1.1 Classification of Phytoestrogens

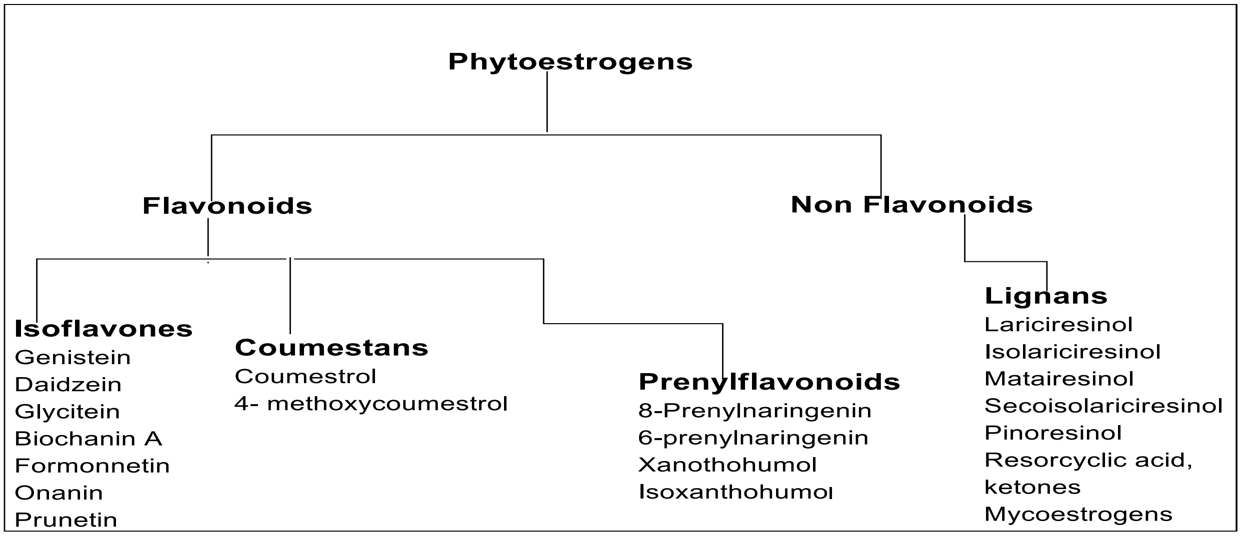


Figure 1.7:- Show that classification of Phytoestrogens.

Phytoestrogen exhibit anticancer activity via mechanisms including estrogen receptor modulation, aromatase inhibition, and anti- angiogenesis (Sirotkin & Harrath, 2014).

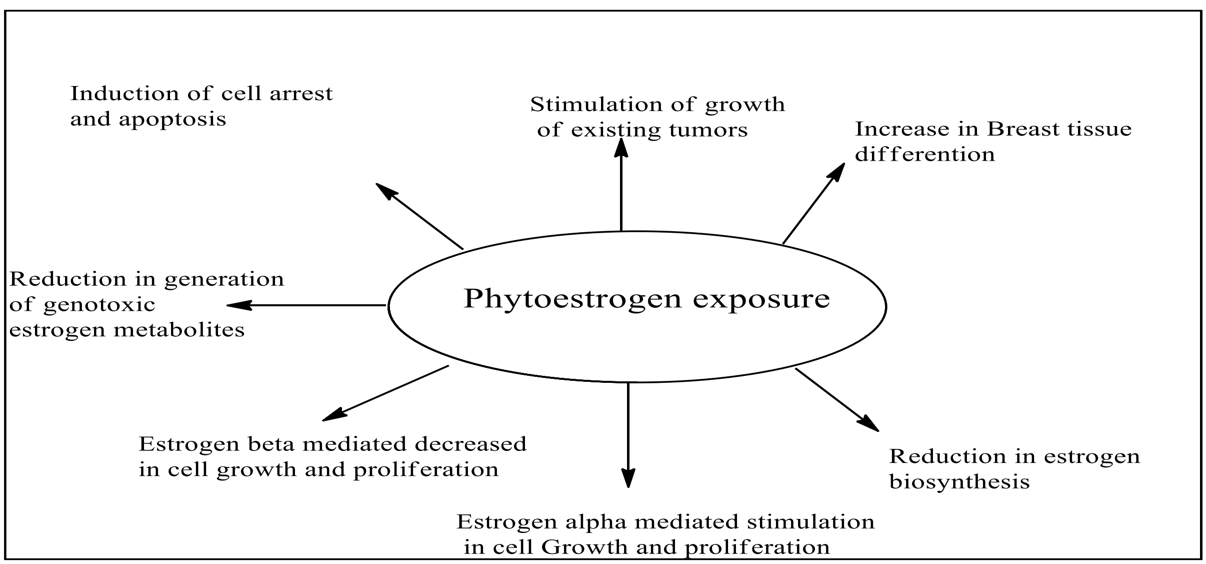


Figure 1.8:- Show that importance of Phytoestrogens, sketched on Chem-Draw software on 27/04/2018.

Studies carried out indicated that the dietary intake of phytoestrogens reduced the incidence of breast cancer. Genistein, an isoflavone which regarded as phytoestrogens at higher doses reduced the proliferation of MCF-7 (Breast cancer cell line). Phytoestrogen binds preferentially to estrogen receptor beta therapy activating the down regulated receptor. Geldanamycin, a phytoestrogen act as an inhibitor of HSP90, was observed to decrease the hormone binding to the estrogen receptor (Pons *et al.*, 2016).

Concentration of Phytoestrogens in some foods (µg/100g)

Table 1.2:- Show that Concentration of Phytoestrogens in some foods, data adopted from gynecomastia.org.uk on 27/04/2018

Serial number	Sources of food	Isoflavones		Lignans	Coumestrol's
		Genistein	Daidzein		
1	Soy flower	93900	67400	130	----
2	Soy drink	2100	700	----	----
3	Soymilk	300	30	----	----
4	Linseeds	---------	----	369900	----
5	Red clover	323	178	13	----
6	Alfalfa sprouts	117.6	151.7	TR	105.3
7	*Asparagus racemosus*	157.6	257.8	68.4	25
8	Carrots	ND	ND	38.3	ND
9	Cauliflower	ND	TR	30.2	ND
10	Garlic	TR	ND	ND	ND
11	Mung bean sprouts	424.1	387.2	26.1	2000.4
12	Sweet potatoes	TR	ND	ND	ND
13	Bran oat	6.9	3.5	----	----
14	Meal oat	----	0.2	----	----
15	Rye Bran	----	9.7	132	----
16	Pumpkin seeds	153	0.56	21370	----
17	Chick peas	76.3	11.4	8.4	5

ND= Not detected TR= Trace as 25µ/100g.

From the various source of food, *Asparagus racemosus* contain large concentration of phytoestrogens. Large fraction of genistein (157.6 µg/100g), Daidzein (257.8µg/100g) are the form of isoflavones present in *Asparagus racemosus*. *Therefore Asparagus racemosus* was selected for investigation of anticancer activity.

RATIONALE FOR SELECTION OF THE PLANT

Now a day's lots of herbal plants are being upgraded by validating the traditional claims and establishing its medicinal value. Various plants are yet to be scientifically proven for their therapeutic efficacy. *Asparagus racemosus* is one such plant belonging to the family Liliaceae. This plant is commonly known as Satavari. The plant has a long history of numerous traditional and ethno botanical applications in diverse cultures. *Asparagus racemosus* (Liliaceae) is well known for their medicinal value. *Asparagus racemosus* contain flavonoids, steroidal glycosides, and other phytochemical constituent act as phytoestrogen. Phytoestrogens are used as alternative therapy for treatment of breast cancer. Because phytoestrogens have structure similarity to estradiol and the same distance (14.5Å) between two hydroxy groups.

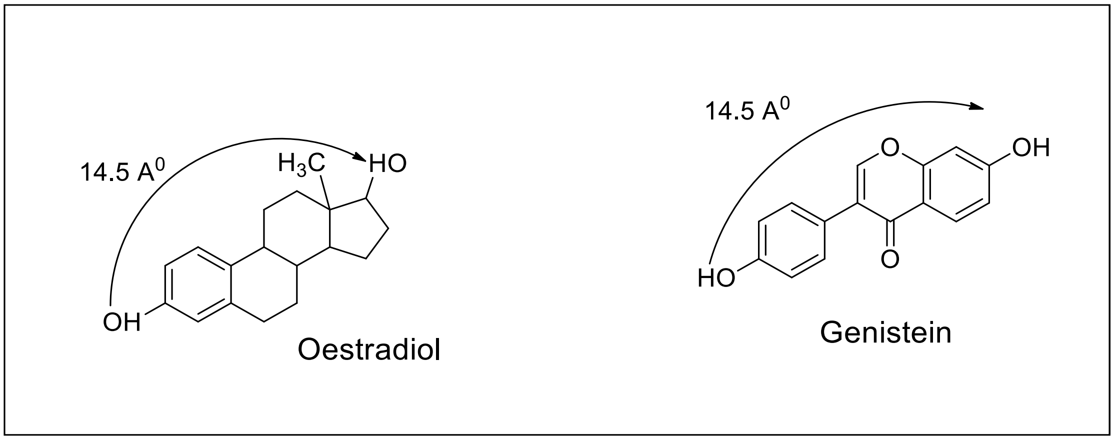


Figure: - Structural similarity of Oestradiol and Genistein.

Phytoestrogens have good binding affinities for estrogen receptors. *Asparagus racemosus* has less side effects than synthetic drugs like tamoxifen. The powder of roots are shown maximum content of phytoestrogens. Traditionally the roots part has been used for the treatment of cancer. So, the present study was undertaken to investigate the effects of *Asparagus racemosus* roots on anticancer activity.

Aim & Objectives

- Preliminary Phytochemical investigation of *Asparagus racemosus* extracts by various chemical test
- *In- vitro* study of different extracts of *Asparagus racemosus.*
- *In-silico* Study of the interaction pattern of reported Phytochemicals from the *Asparagus racemosus* with Estrogen receptors.

CHAPTER 2.0

REVIEW OF LITERATURE

CHAPTER 2.0

Review of Literature

2.1 *Asparagus racemosus*

Asparagus racemosus is a perennial climbing plant with straight and hooked spines, extending to a height of 1- 2 meters. This is a sweet and bitter herb. Its young stems are faint and brittle. The flowers having fragrance, white in color with small thorns. The roots are white tuberous, radish shaped, tapered at end and are found in clusters. Older plants have longer and thicker roots. Its leaves are thin and pine needles. Fruits are in the form of small berries that appear purple to black in color. The herb have antioxidant, diuretic, antidepressant, antiepileptic, antitussive, antileshmanial, anti HIV, antibacterial, antiulcer activity. The plant also used as an immunostimulant, heptoprotective, during pregnancy, cardio protective and in neurodegenerative disorders. Various types of secondary metabolite such as steroids, alkaloids, flavonoids, furan derivatives, dihydrophenanthrene derivatives and numerous volatile oils are present in *Asparagus racemosus.* The main secondary metabolite are present in *Asparagus racemosus* is steroidal saponins, racemosides and flavonoids which are considered to be responsible for the pharmacological activities (Edwards *et al.*, 2015).

Approximately 300 species of *Asparagus racemosus* are found in the world, out of 300 species 22 species of *Asparagus racemosus* are reported in India. *Asparagus racemosus* related to family Liliaceae is one such essential medicinal plant which is called as a rasayana. It promoting wellness and increase cellular strength. Consumption of *Asparagus racemosus* was revealed in the prehistoric literature of Ayurveda. It is generally called satavari, satawar or satmuli in Hindi language, 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, Chatavari in Malayam, Majjigegadde or Aheruballi in Kannada, Kairuwa in Kumaon, Narbodh or Satmooli in Madhya Pradesh and Norkanto or Satawar in Rajasthan (Singla *et al.*, 2014).

Use of *Asparagus racemosus* is reported in the ancient literature of Ayurveda. *Asparagus racemosus* is very supportive in epilepsy, brain tonic. It regulated the cardiac function and related disorder. *Asparagus racemosus* is broadly useful in sexual related disorder like male genital dysfunctional, oligospermia, spermatogenic irregulaties and painful micturition. Numerous herbal formulation has been prepared for digestive discomfort, amoebiasis and piles. Researchers of current times have demonstrated through investigational evidences that shatavari has been used for treatment of diarrhoea and spasmodic infection. It is used as antidysenteric demulcent, diuretic, galactagogue, nutritive, mucilaginous, refrigerant, stomachic properties and acts as a tonic for human beings. It improves immunity and protects the heart brain and other vital organs of the body. *Asparagus racemosus* is mentioned in Ayurveda for general weakness due to prolong illness (Goyal *et al.*, 2003).

The name shatavari interprets she who possesses 100 husbands starting herb with rejuvenative effects upon the female reproductive organs not only for young woman, even for women in their middle and elder years, to help them graceful transition through the natural phases of life, including menopause. Shatavari efficiently helps in balancing Pitta Dosh. Ayurveda has entitled shatavari as the queen of herbs. It is the primary herb recommended for female health (Singh *et al.*, 2018).

Classification

Kingdom : Plantae

Order : Asparagales

Family : Asparagaceae

Sub family : Asparagoideae

Genus : *Asparagus*

Species : *Asparagus racemosus*

2.2 Ethnobotany

2.2.1 Habitat

This climber growing in low jungles is found all over India; especially in Northern India. The plant is a climber growing to 1-2m in length found all over India (Bopana *et al.*, 2007).

2.2.2 Distribution

Throughout India, Tropical and subtropical parts including Andaman's and ascending in the Himalayas up to an altitude of 1500m (Bopana & Saxena, 2007).

2.2.3 Propagation and Cultivation

Average black and slack soil is an essential for cultivation and farming *of Asparagus racemosus.* Farming need tropical warm climatic conditions. In the month of May – June raised beds of *Asparagus racemosus* about 3 m in length are harvested. Plant produce small flower in month of July. Flowers are unisexual in nature. It begins to bear fruits which are spherical or obscurely 3 lobed, fruits are soft berries and converted into purplish black on the time ripening. Seeds of this plant generally hard and brittle. Crops of *Asparagus racemosus* are not affected with pest and viruses. After 1.5 – 2 years of transplanting, plants are harvested (Singla & Jaitak, 2014). Shatavari can also be propagated by shoot tip culture on MS medium supplemented with BAP (0.5mg/l).Shoot tip proliferates into a number of offshoots supplemented with NAA (1.0 mg/l) + KN (0.5 mg/l).

2.3 Morphology

2.3.1 Macroscopy

Asparagus racemosus (Shatavari) is a plant comprising of tuberous roots. According to Indian pharmacopoeia, dried weight of *Asparagus racemosus* contain not less than 0.1 per cent of Shatavarin IV. The air dried roots are brown in color, tuberous, elongated, and tapering at both the ends, up to 30-100 cm long. The fresh roots are fleshy and white in color; while on drying it become shrinked, longitudinal ridges appeared and the color turned light brown. Outer surface of the fresh root is soft and contains epidermal hairs. Taste is mucilaginous, fracture brittle. The powder drug swells on moistening with water. Roots are cylinderly-shaped, corpulent tuberous straight or slightly bent, tapering towards the base & swollen in the middle; white buff colour, 5- 15cm in length 1-2 cm diameter (Singla & Jaitak, 2014).

Figure 2.1:- Photographs of *Asparagus racemosus* showing (A) Roots (B) Shoot, leaves, flowers and fruits adopted from http://www.plantsrescue.com.

2.3.2 Microscopy

Transverse section of the root is circular or elliptical, periderm is composed of 5-6 layers of compact cells, tangentially elongated thin walled phellem. About 2-3 peripheral layers of cork cells followed by a single layer of phelloderm. The phelloderm is followed by 6-7 layers of cortical cells. Vascular bundles are arranged in the center forming a circular ring. Protoxylems are arranged toward the center; while the metaxylem toward the outer side. There is a wide zone of secondary phloem composed of sieve tubes, companion cells and phloem parenchyma. A wide zone of secondary xylem, which is composed of vessels, tracheids and xylem parenchyma, follows secondary phloem. The epidermal layers contain numerous epidermal hairs (Chawla *et al.*, 2011).

2.4 Phytochemicals

Asparagus racemosus is known to possess a wide range of phytochemical constituents. The major bioactive constituents are a group of steroidal saponins.

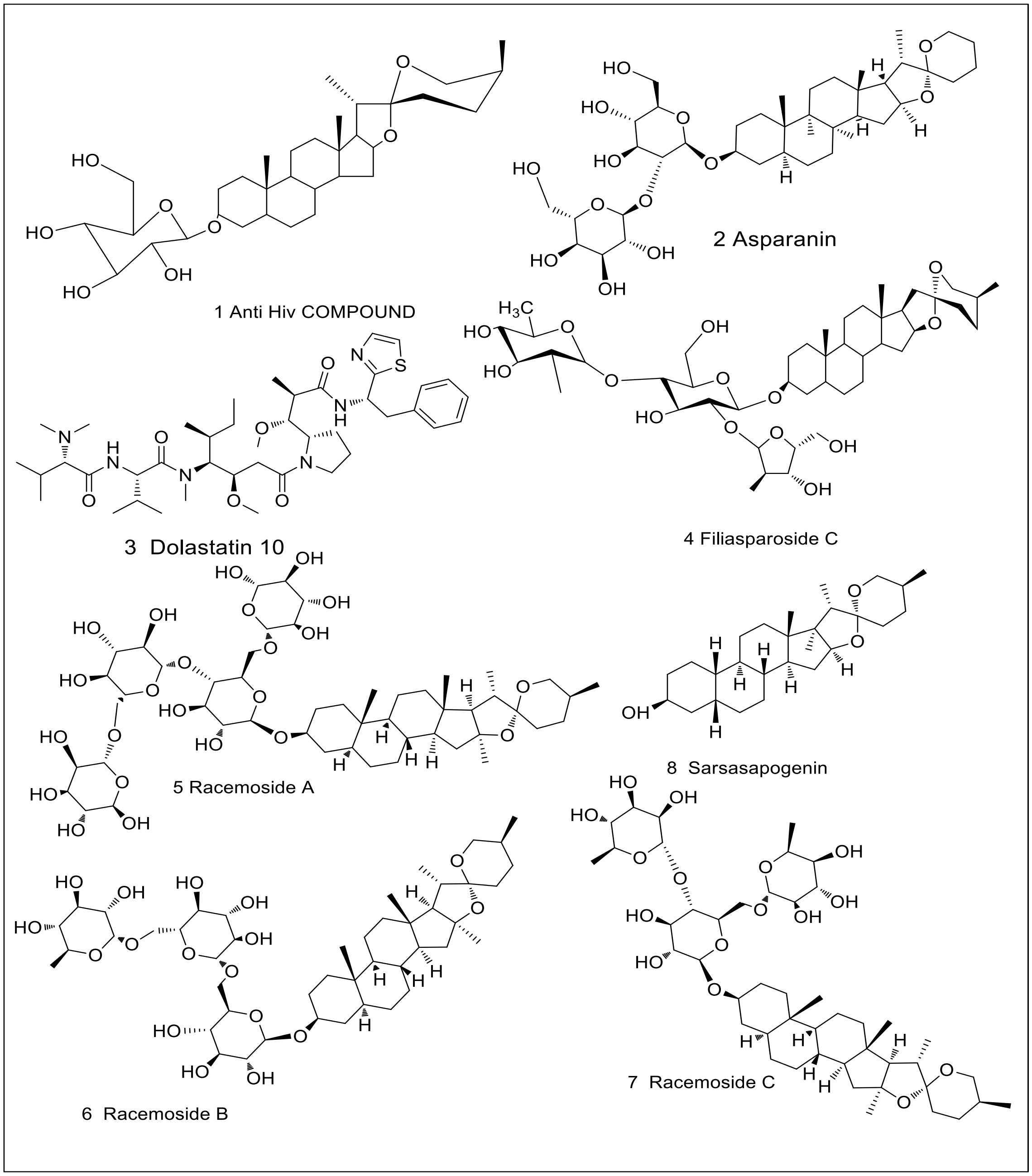
Other secondary metabolites are essential oils, asparagine, arginine, tyrosine, alkaloids, furan derivatives flavonoids (Kaempferol, Quercetin, and Rutin), resin, and tannin. The powdered root contains 2.95% protein, 5.44% saponins, 52.89% carbohydrate, 17.93% crude fiber, 4.18% inorganic matter, and 5% volatile oil (Sachan *et al.*, 2012).

2.4.1 Steroidal Saponins

Previous research shown twenty nine steroidal saponins has been reported in *Asparagus racemosus.* An Oligospirostanoside named 3-O-[α- L-rhamnopyranosyl-(1-2)- α-L- rhamnopyranosyl-(1-4)-O-β-D-glucopyranosyl]-25(S)- spirosta-3 β-oil from, in immune compromised animals oral administration of *Asparagus racemosus* formulations induce antibody development and increased cell mediated immune reaction. Racemosides A, B, C were obtained from the n-BuOH soluble fraction of the methanol extract of the defatted fruits of *A. racemosus*, according to decreasing order of polarity. All the three compounds gave positive Liebermann–Burchard test for steroids and Molisch test for sugar (Joshi, 2016).

The chemical structures of shatavarins I and IV reported by others earlier, was revised by Hayes and coworker later as: 3-*O*-{[β-d-glucopyranosyl(1→2)][α-l-rhamnopyranosyl(1→4)]-β-dglucopyranosyl}-26-*O*-(β-d glucopyranosyl)-(25*S*)-5β-furostan-3β,22α,26-triol and, 3-*O*-{[β-dglucopyranosyl(1→2)][α-l-rhamnopyranosyl(1→4)]-β-d-glucopyranosyl}-(25*S*)-5β-spirostan-3β-ol respectively. In the same year Hayes and coworker isolated a new steroidal saponin, Shatavarin V: (3-*O*-{[α-l-rhamnopyranosyl (1→2)][β-d-glucopyranosyl (1→4)]-β-d-glucopyranosyl}-(25*S*)-5β-spirostan-3β-ol), from the roots of *Asparagus racemosus* by reverse phase-high performance liquid chromatography (RP-HPLC). The structure of Shatavarin V was determined by nuclear magnetic resonance (NMR) studies. These NMR studies also permit clarification of the structures reported earlier for several known saponins: asparagines A and B, asparosides A and B, curillin H, curillosides G and H, and shatavarins I and IV. Shatavarins VI to X, together with five known saponins: Shatavarin I (or asparoside B), Shatavarin IV, Shatavarin V, Immunoside and asparanin A are isolated from the roots of

Asparagus racemosus (Hayes *et al.*, 2008). The dried roots yielded sitosterol which was reported to possess antiprotozoal and spasmolytic property. Anti HIV compounds was isolated from the ethyl acetate, butanol and aqueous root extracts. The methanolic root extract of *Asparagus racemosus* yielded filiasparoside C. Shatavaroside A and Shatavaroside B from the alcoholic root extract of *Asparagus racemosus* which possess an Immunomodulatory activity (D. Mandal *et al.*, 2006).



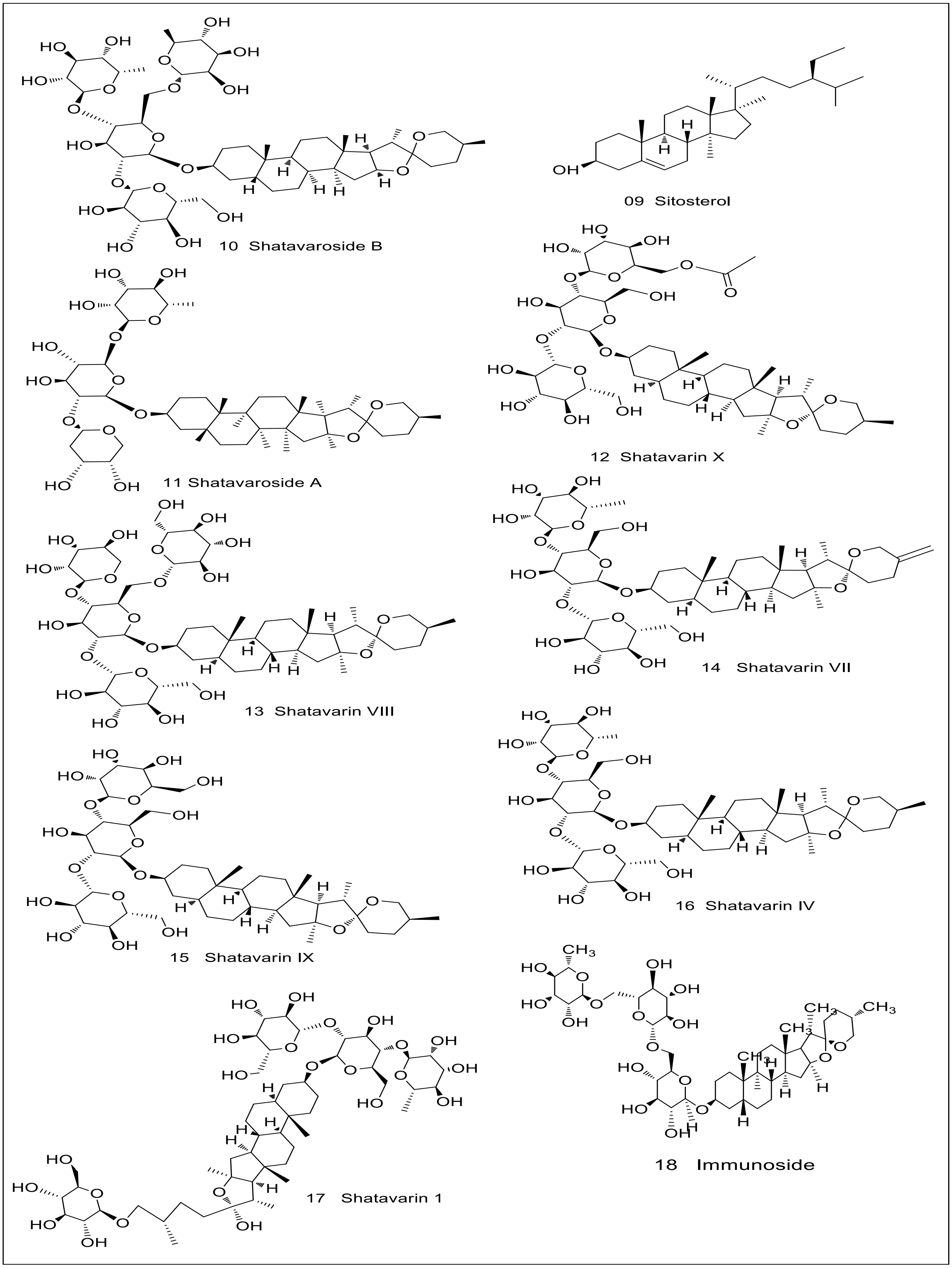
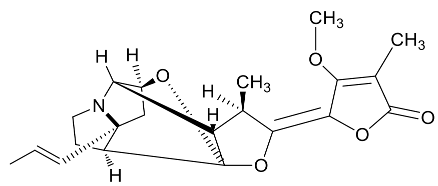


Figure 2.2:- Structures of Steroidal saponins from *Asparagus racemosus*.

2.4.2 Alkaloids

Skeine and coworker reported the isolation and characterization of a polycyclic alkaloid called ‘Asparagamine’ from the roots of *Asparagus racemosus*. The alkaloid exhibited a unique cage-type structure with outstanding anti-oxytocic activity. Later, a new 9, 10- dihydrophenanthrene derivative called racemosol, was also isolated from the roots of *Asparagus racemosus*. Its structure was elucidated by spectroscopic analysis as: 9, 10-dihydro-1, 5-dimethoxy-8-methyl-2, 7-phenanthrenediol (Baldwin, 2004).

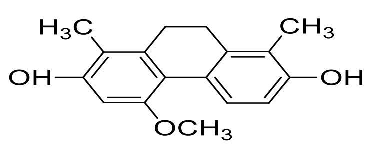


19

Figure 2.3:- Structure of Alkaloids from *Asparagus racemosus*

2.4.3 Dihydrophenanthrene Derivative

A dihydrophenanthrene derivative, racemosol was isolated from methanolic root extract of *Asparagus racemosus* (Singla & Jaitak, 2014).



20 Racemosol

Figure 2.4:- Structure of Dihydrophenanthrene from *Asparagus racemosus*.

2.4.4 Furan Derivative

A benzofuran identified as Racemofuran which was reported to possess antioxidant activity, isolated from the chloroform root extract(Singla & Jaitak, 2014).

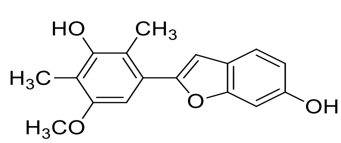


Figure 2.5:- Structure of Furan derivative from *Asparagus racemosus*.

2.4.5 Flavonoids

Quantitative analysis of *A. racemosus* root extract discovered the presence of flavonoids (36.7±3.9 mg/100ml), polyphenols (88.2±9.3 mg/100ml) and vitamin-C (42.4±5.1 mg/100ml), while Visavadiya and Narasimhacharya reported presence of phytosterols (0.79 %), saponin (8.83 %), polyphenols, 1.69 %, flavonoids, 0.47 % and ascorbic acid, 0.76%). An isoflavone 8 methoxy-5, 5, 4- trihydroxyisoflavone was isolated from the roots of the plant. Cyanidine-3-galatoside and kaempferol was also being isolated from the woody portions of tuberous roots. Quercetin, Rutin and hyperoside were found in the flowers and fruits along with Quercetin -3-glucuronide which was obtained from the leaves (Sarabjot Kaur *et al.*, 2014).

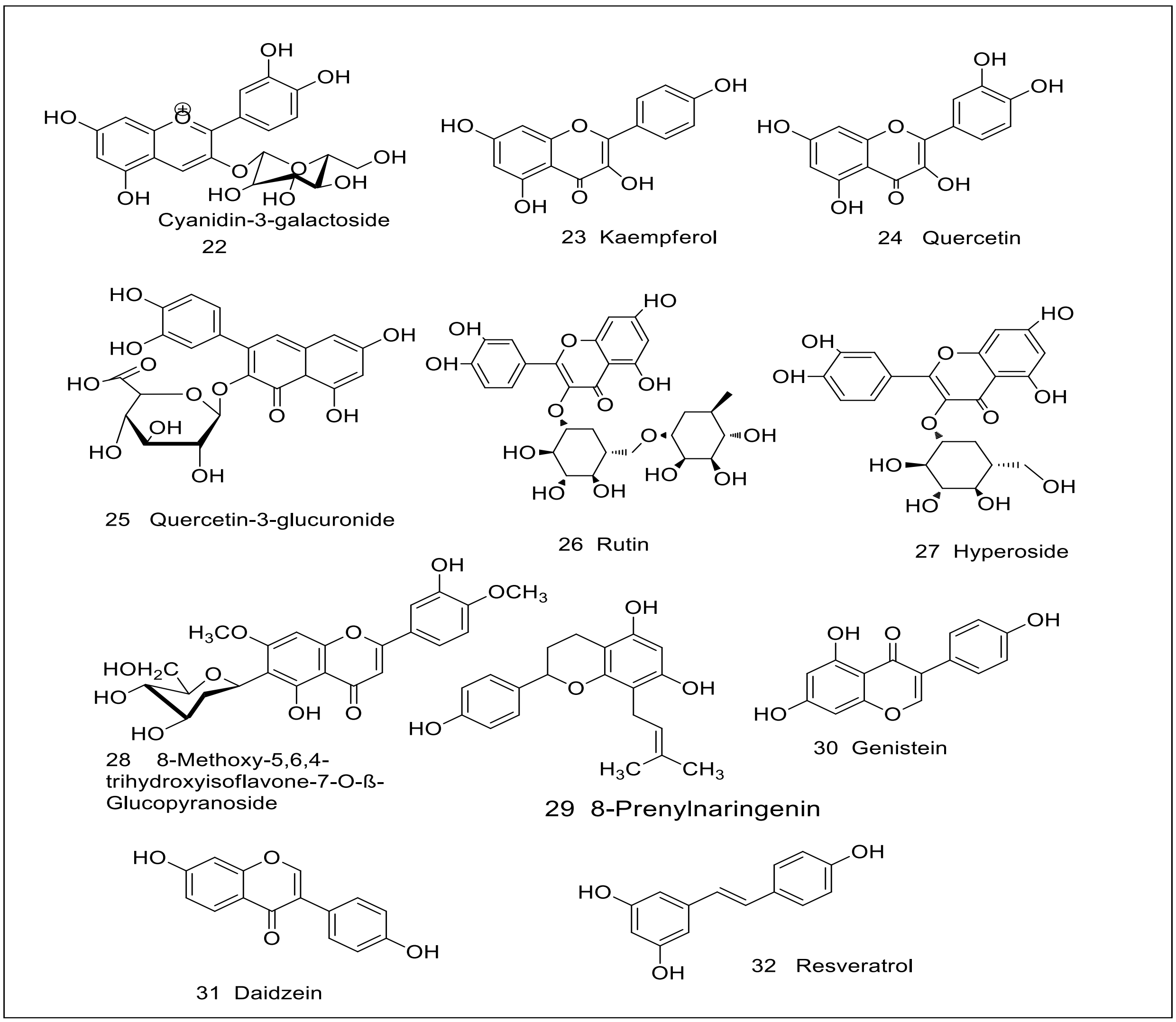
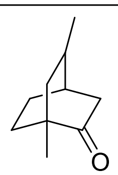


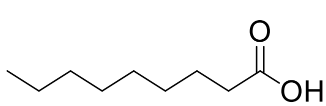
Figure 2.6:- Structure of Flavonoids from *Asparagus racemosus*.

2.4.6 Essential oil constituents

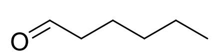
Volatile constituents removed were having a place with a different scope of chemicals classes, for example, acids, alcohol, aldehyde, ester, hydrocarbon, ketone, N-containing compounds. Alcoholic compounds, accounts 49.82% of the aggregate oil content; in which the major ones were borneol (26.40%), myrtanol (13.72%), and pinocarveol (2.37%) and 2-ethylhexanol (1.76%). Aldehydes were the second most abundant chemical described, containing 16.70% of aggregate volatile oil content in which the significant one was perillaldehyde (8.97%), 4-[1-hydroxyethyl] benzaldehyde (1.55%), hexanal (1.34%) and furfural (1.17%). Essential oil contained 8.97% of corrosive and 8.97% of ketone content. The class of acids, comprised of decanoic corrosive (4.19%). Ketonic essential oil included camphor (3.33%) and 6, 10, 14-trimethyl pentadecanone (1.71%) were major ones. The hydrocarbons secluded with the exception of [E]-4-hexadecane-6-yne were mono terpenoidal in nature. Remaining compound were identified at levels lower than 3%. Just three compounds; borneol, myrtanol and paraldehyde could possess 45.09% of the entire content. Roots were additionally reported to contain benzaldehyde and undecanyl cetanoate. The fourteen essential oils were acquired from the biodeteriorated *Asparagus racemosus*. Thymol, eugenol, menthol, gerayl acetic acid derivation, linalool, β-asarone, 1, 8-cineol, E-citral, β-caryophyllene, α-pinene, carvone, P-cymene, carvacrol, ocimene (Sarabjot Kaur & Mondal, 2014).



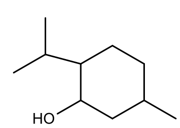
33 Camphor



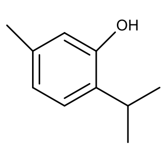
34 Decanoic acid



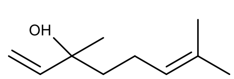
35 Hexanal



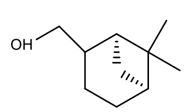
36 Menthol



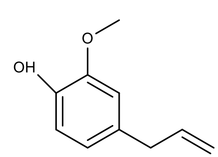
37 Thymol



38 Linalool



39 Myrtenol



40 Eugenol

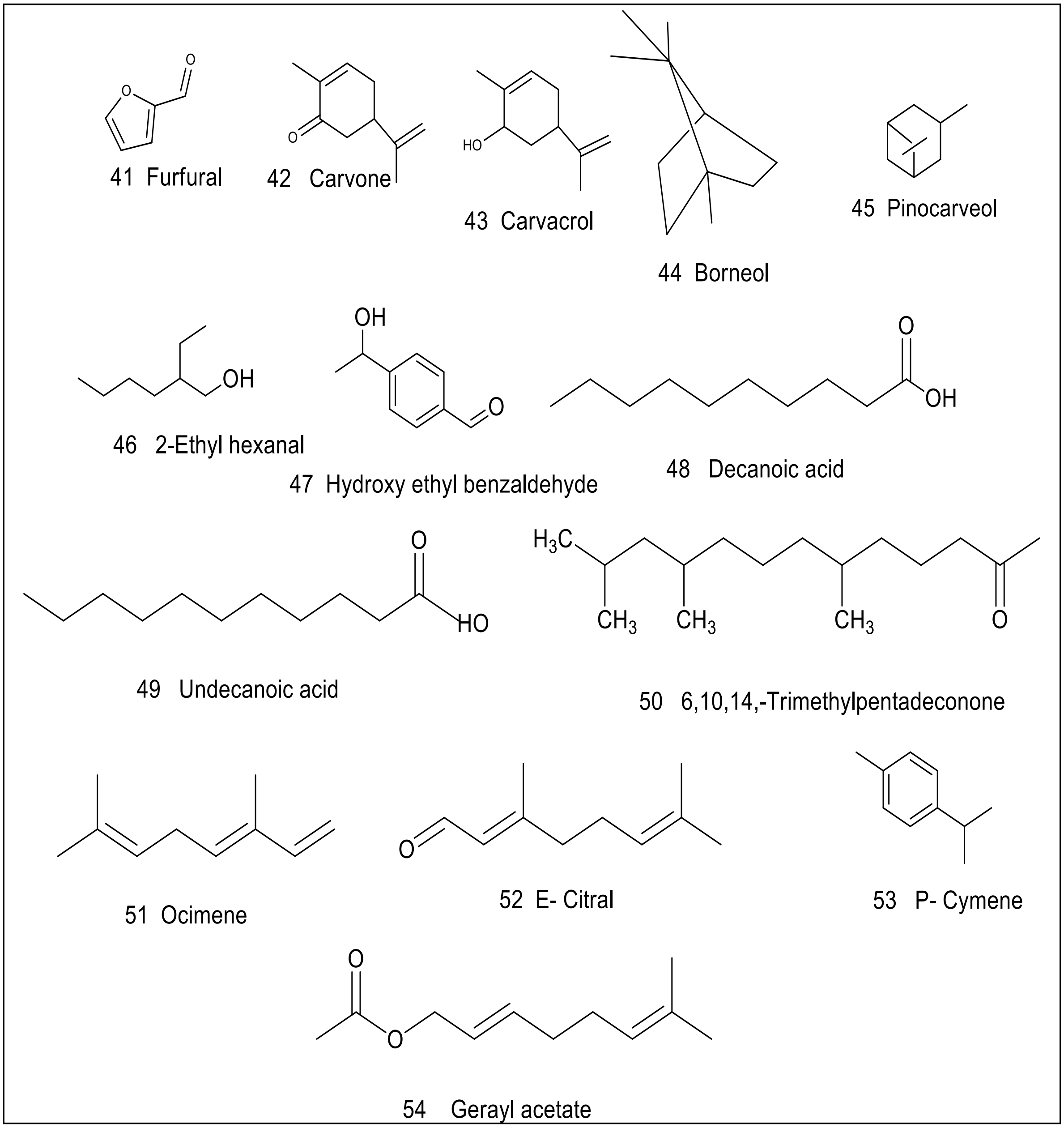


Figure 2.7:- Structure of essential oils from *Asparagus racemosus.*

2.4.7 Trace minerals

Analysis of elemental constituents at trace levels of *Asparagus racemosus* roots ensued in the detection of Fe (746±5), zinc (120±15), manganese (17±1), copper (28±1), Nickel (22±1), Cobalt (7±1.8), micrograms per grams respectively) along with boron, calcium, rubidium and strontium. Further, accumulation and variation of trace elements in roots and leaves of *Asparagus racemosus* collected from cultivates at four different altitudes in Uttarakhand, India, shown the presence of metals such as Zn, Cu, Mn, Fe, Co, Na, K, Ca and Li. The concentration level of Fe in leaves was found to be highest at an altitude of 2,250 m whereas, the level of Cu in the roots was lowest at this altitude (Singla & Jaitak, 2014).

Table 2.1:- Summary of the trace elements from the *Asparagus racemosus*.

Serial No.	Metal	Roots mg/kg	Leave mg/kg
1	Zinc	120±15	53±0.2
2	Manganese	17±1	15.0±0.6
3	Copper	28±1	14±0.4
4	Nickel	22±1	505.0±0.2
5	Cobalt	7±1.8	85±0.3
6	Iron	746±5	28.0±0.6

2.5 Pharmacological Activities

Shatavari is a traditional Ayurvedic herb. The root is used medicinally. In the Indian medicine system, shatavari is assumed to "give her capacity to have a hundred husbands. The famous physician Galen described *Asparagus racemosus* as cleansing and healing (Chawla *et al.*, 2011). Further studies shown that *Asparagus racemosus* is a source of low calorie, folate and potassium. *Asparagus racemosus* got most admirable care because of medicinal value. *Asparagus racemosus* is used in the numerous Ayurvedic medicinal formulations such as *shatavari Kalpa, Phalaghrita, Vishu taila. Asparagus racemosus* has numerous pharmacological activities described below

2.5.1 Antioxidant Property

The harmful effects of ionizing radiation, particularly those having low linear energy transfer in biological systems are mainly facilitated through the generation of oxygen-derived intermediates in the form of free radicals and excited states. Membranes are highly prone for oxidative damage induced by reactive oxygen species (Wagner *et al.*), generated during radiation due to presence of polyunsaturated fatty acids. *Asparagus racemosus* has been shown antioxidant effects of the methanolic extract (Wiboonpun *et al.*, 2004).

Extracts of *Asparagus racemosus* shown inhibitory effect against Gamma-radiation induced oxidative damage using rat liver mitochondria as model systems. Kamat and co-worker demonstrated that radiation induced lipid peroxidation in mitochondria by estimating two different product of peroxidation LOOH unstable product and TBARS is stable product, studies show that *Asparagus racemosus* inhibit the both product LOOH and TBARS. Various enzyme like superoxide dismutase induced oxidation stress, free radical, *Asparagus racemosus* prevent from oxidation stress and inhibit the generation of free radical at low concentration 10µg/ml. it is clear that crude extract and alcoholic extract are effective in protection against lipid peroxidation and protein oxidation. The mechanism responsible for antioxidant properties of *Asparagus racemosus* are still not clear. Various studies demonstrate that polysaccharides derived from plants exhibit protent antioxidant as well as radio protective properties. The polysaccharides kreskin also has been shown to have inhibitor effects on the oxidation of LDL (Kamat *et al.*, 2000).

2.5.2 Antiurolithiatic activity

Kumar and co-worker worked on Antiurolithiatic effect of *Asparagus racemosus*. Calcium oxalate urolithiatic induced by either ethylene glycol alone or in combination with ammonium chloride are most commonly used to study the pathogenesis of urolithiatic. Kumar and co-worker analyzed the body weight, serum concentration of calcium, phosphorus, urea, creatinine and histopathology of kidneys of rats. The possible mechanism of *Asparagus racemosus* in urolithiatic is excessive secretion or decreased in the urinary concentration of urinary salts that prevent super saturation of the crystallizing salts (M. Kumar *et al.*, 2010). The study done on lemon juice in rat urolithiatis model has shown that it has protective activity against urolithiasis due to its high antioxidant property due to the presence of vitamin E and vitamin C. Several studies have reported the antioxidant activity of *A. racemosus*. Thus, it is possible that ethanolic extract of *A. racemosus* also prevents stone formation via its antioxidant effects. Moreover, the antibacterial activity of the *Asparagus racemosus* probably contributes for its antiurolithiasis activity as bacterial infection also promotes urolithiasis *racemosus* probably contributes for its antiurolithiasis activity as bacterial infection also promotes urolithiasis (Jagannath *et al.*, 2012).

2.5.3 Diuretic activity

Asparagus racemosus is used as diuretic. Research demonstrated that consuming three dose vials of aqueous extract of *Asparagus racemosus* 800mg/kg, 1600mg/kg and 3200mg/kg showing mild diuretic activity by comparison with standard drug furosemide. Aqueous extract show excellent diuretic activity at 3200mg/kg without any acute toxicity (M. Kumar *et al.*, 2010).

2.5.4 Antitussive effect

Methanolic extract of *Asparagus racemosus* prepared and phytochemical screening gave positive tests for steroids and teiterpenoids. Methanolic extract of *Asparagus racemosus* possess antitussive activity. Antitussive activity was proved against sulphur dioxide induced cough in mice. The mice were divided into five groups of 10 each. Group 1 animals (Dal Maso *et al.*), received the vehicle (saline; 10 ml/kg), groups 2 and 3 were treated with the test extract 200 and 400 mg/kg, respectively and groups 4 and 5 with codeine phosphate 10 and 20 mg/kg, respectively., as a standard antitussive agent. The animals were exposed to SO_2 for 1 min and then the frequency of the cough was observed for 5 min. 40% to 58.5% inhibition of SO_2 induced cough at a dose 200 to 400mg/kg respectively by using oral administration of methanolic extract(S. C. Mandal *et al.*, 2000).

2.5.5 Anti-epileptic effect

The anticonvulsant activity was evaluated using the methanol and aqueous extracts of the *Asparagus racemosus* on seizures. In the rat models seizures were induced by highest electroshock and pentylenetetrazole. In the test carried out methanolic extract revealed the presence of alkaloids, steroids, flavonoids, tannis and saponins as the major phytochemical. Flavonoids enhance (GABA) neurotransmission and it is the main inhibitory neurotransmitter, which suppressed in epilepsy. Another study demonstrated diazepam synergized anticonvulsant activity of methanolic rich fraction. Thus indicating *Asparagus racemosus* as conventional antiepileptic drug therapy (Gohil *et al.*, 2010).

2.5.6 Ameliorative effect against Depression and memory defict

Rajesh Kumar Goel and Priyanka Pahwa, 2016 conducted a study on mice using tail suspension test, passive shock avoidance paradigm and estimate the neurochemicals to determine the antidepressants activity. The hydroethanolic extract of *Asparagus racemosus* decreased immobility periods significantly in tail suspension test, which indicated the antidepressant activity (Pahwa *et al.*, 2016). Norepinephrine and valproate were used as standard reference drugs in the study. Animals treated with hydroethanolic extract of *Asparagus racemosus* (200,400,800 mg/kg) shown the significant reduction in the immobility times. Treatment with valproate to another groups of animal shown decrease in immobility times. Study also proved that memory improvement with hydroethanolic extract of *Asparagus racemosus*. Treatment with hydroethanolic extract of *Asparagus racemosus* and valproate significant increased the noradrenaline, dopamine and serotonin level in cortex and hippocampus. Hydroethanolic extract reduced the total nitrile level, acetylcholinestrase in the cortex and hippocampus (Sithisarn *et al.*, 2013).

2.5.7 Hepatotoxicity

Administration of *Asparagus racemosus* to hepatotoxicity induced rats decrease the serum Alanine amino transferase and Aspartate amino transferase activities when compared with paracetamol treated group. In liver damage by paracetamol, the determination of enzyme activities such as Alanine amino transferase and Aspartate amino transferase is largely used. In the present study, the increase in serum activities of Alanine amino transferase, Aspartate amino transferase and Alkaline phosphatase activities in paracetamol treated rats had been contributed to the damaged structural integrity of the liver, because these are normally located in the cytoplasm, mitochondria or microsome and released into the circulation after cellular damage or due to alterations in the permeability of cell membrane and increased synthesis or decreased catabolism of aminotransferases (Palanisamy *et al.*, 2012). On the other hand, reported that, the reduction of Alanine amino transferase and Aspartate amino transferase activities by the *Asparagus racemosus* extracts is an indication of repair of hepatic tissue damage induced by paracetamol. The aqueous extract induced suppression of increased Alanine amino transferase and Aspartate amino transferase activities. Thus, administration of *Asparagus racemosus* exposed heptoprotective activity against the toxic effect of paracetamol. These results showed improvement in activities of Alanine amino transferase, Aspartate amino transferase and alkaline phosphatase enzymes was recorded in *Asparagus racemosus* group that due to its antioxidant effect. Study showed that paracetamol at dose 1g/kg wt. significantly decreased hepatic catalase, and superoxide dismutase when compared with control group. Similarly the activities of Superoxide dismutase (Agrawal *et al.*, 2008).

Catalase in liver tissue were decreased in the paracetamol group. Also, reported that, paracetamol induced hepatotoxicity in rats resulted in decreased level of hepatic (Parsons *et al.*) activity as compared to control rats. However found that during cell membrane damage, various enzymes leak down to the circulatory fluid and their assessment in serum serves as markers in clinical studies. Superoxide dismutase is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide to hydrogen peroxide, while Catalase is a Peroxisomal haem protein that catalyzes the removal of hydrogen peroxide formed during the reaction catalyzed by Superoxide dismutase. Thus, Superoxide dismutase Catalase act as antioxidative enzymes, which provide protective defense against reactive oxygen species. From the obtain Results indicate the heptoprotective properties of *Asparagus racemosus* against paracetamol-induced hepatotoxicity in rats (Acharya *et al.*, 2012).

2.5.8 Antidiarrhoeal activity

Several studies have shown that prior administration with some plants extracts (*Andrographis paniculata*, *Cassia auriculta, Butea monosperma)* was evaluated for its antidiarrhoeal potential in contradiction of castor oil prompted diarrhea, gastrointestinal motility. These extracts had a comparable activity as loperamide. When tested at 200 and 250mg/kg and statistically substantial decrease in the occurrence of defection and the wetness of the faecal droppings. It is widely known castor oil or its dynamic component ricinoleic acid encourages permeability changes in mucosal fluid and electrolyte transport that outcomes in the hypersecretory response and diarrhoea. Ricinoleic acid improved the PGE_2 content in the gut lumen and also produced a rise of net excretion of water and electrolytes into the small intestine. The release of ricinoleic acid from castor oil effects in irascibility mucosa, prominent to discharge of prostaglandins, which motivate motility and excretion. Inhibitors of prostaglandin biosynthesis delayed castor oil induced diarrhoea. Study suggest that the anti- diarrhoeal result of ethanol and aqueous extracts may be due to the reticence of prostaglandin biosynthesis. Earlier information have demonstrated the antidiarrhoeal activity of tannin, flavonoids, and alkaloids, saponins, reducing sugars and sterols and terpenes containing plant extract (Alok *et al.*, 2013). The phytochemical investigation of the extract exposed the occurrence of alkaloids, saponins, flavonoids, sterols, terpenes and sugars. These ingredients may answerable for the antidiarrhoeal activity of *Asparagus racemosus.* The antidiarrhoeal activity of flavonoids has been predictable to their ability to inhibit intestinal motility and hydro-electrolytic secretion, which are acknowledged to be rehabilitated in this intestinal condition. *In vitro* and *in vivo* experiments have presented that flavonoids are capable to prevent the intestinal secretary reaction, tempted by prostaglandins E_2. In addition, flavonoids existing antioxidant properties which are imaginary to be responsible for the inhibitory effects utilized upon several enzymes including those involved in the arachidonic acid metabolism. The crude extract and a purified faction of *Asparagus racemosus* exhibited antioxidant activity against impairment convinced by gamma radiation. In particular cases, it has been established that anti-diarrhoeal action is allied with antimicrobial. Previous study show that the crude methanol extract of *Asparagus racemosus* root demonstrated substantial antimicrobial activity against *Escherichia coli, Salmonella typhimurium* and *vibrio cholerae*. The three pathogens reason of variety of diseases comprising diarrhoea and gastroenteritis in human (Venkatesan *et al.*, 2005).

2.5.9 Antileshmanial activity

Leishmaniasis can arise in various medical forms such as cutaneous, mucosal, visceral leishmaniasis. Visceral leishmaniasis most dangerous and become major health problem in tropical and subtropical areas. Almost 350 million people are suffering from visceral leishmaniasis (Sachdeva *et al.*, 2014). By using MTT assay capability of promastigotes after treatment with Racemoside was estimated. Racemoside A decline the viability of cells by indicating the formazan formation in the promastigotes (Sukhbir Kaur *et al.*, 2010). *L.donovani* is treated with Racemoside A have exposed signs of programmed cell death, i.e. the flagellated promastigotes shortened and became a flogged, oval or round with an proliferation in vacuole (Sachdeva *et al.*, 2014). There was also translocation of phosphatidyl serine from the interior side of the outer coating of the plasma membrane which is a surveillance of cell death (Dutta *et al.*, 2007).

2.5.10 Antibacterial activity

Plant based antimicrobial compounds have vast therapeutically potential as they can serve the purpose without any side effects. *Asparagus racemosus* roots were subjected to a preliminary screening for antimicrobial activity against the gram positive and gram negative bacteria. The reason behind this could be the presence of different active molecules in different parts of the plant. The preliminary tests for the methanol extract showed maximum antimicrobial activity. The antibacterial activity of diverse extract like Petroleum ether, Methanol, Chloroform, Acetone, Ethyl acetate and Water of root extract of *Asparagus racemosus* plant with different concentration 100µg/ml, 50µg/ml, 25µg/ml was very well comparable with standard reference drug streptomycin 5µg/ml for antibacterial activity.

The extracts were also tested for antifungal activity and are active against *candida utilis* is comparable to that of standard drug fluconazole 5µg/ml. In the present study, we evaluated the antibacterial and antifungal investigations were carried out of the crude extracts obtained from the root of *Asparagus racemosus* Willd. using different solvents like Petroleum ether, Methanol, Chloroform, Acetone, Ethyl acetate and Water were subjected to antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus,, Escherichia coli, Pseudomonas aeruginosa* and *candida utilis*. (Patel *et al.*, 2013). Different extract of *Asparagus racemosus* roots have potent antimicrobial activity against Gram positive and Gram negative bacteria were indicating the presence of broad spectrum antibacterial substance in the plant. The presence of antifungal and antimicrobial substances in the higher plants is well established as they have provided a source of stimulation for novel drug compound as plant derived medicines have made significant contribution towards human the treatment of diseases as is done in cases of Unani and Ayurvedic system of medicines (Shrestha *et al.*, 2018).

2.5.11 Cardio protective Activity

The augmented concentration of serum lipids, expressly cholesterol and the development of reactive oxygen species are main causes for the development of coronary artery disease and atherosclerosis. Herbo-mineral formulation 'Abana' containing 10 milligram of *Asparagus racemosus* extract in each tablet showed a significant hypocholesterolaemic effect in rats, and thus has the potential of use in the cardio preventive treatment. 37 to 45% decrease of total cholesterol, phospholipids and triglycerides has been described compared to the control samples (Dzubak *et al.*, 2006). Due to the multi-components of 'Abana' further research is needed to indicate the participation of *Asparagus racemosus* in this hypolipidaemic action. The effect of *Asparagus racemosus* on the lowering of cholesterol in hypercholesterolemia in rats. Supplementation with powder from the roots of *Asparagus racemosus* caused in a reduction of lipid peroxidation. Supplementation with *Asparagus racemosus* root powder diminished lipid peroxidation and initiated a lessening in lipid profiles depending on the dose. The level of complete lipids, full cholesterol and triglycerides in plasma and liver, as well as plasma LDL and VLDL-cholesterol lessened by more than 30%. There is a hypothesis that hypercholesterolemia in this case is tempered by decreased exogenous cholesterol absorption and an increased conversion of endogenous cholesterol into bile acids (Khanna *et al.*, 1991).

2.5.12 Anti- plasmodial activity

Various extract of the roots of *Asparagus racemosus* used as anti- plasmodial activity. Ethyl acetate extract shown excellent activity with yield value of 7.9% per 100mg dose dependent inhibition of chloroquine resistant strain of *Plasmodium falciparum* with an IC_{50} value of 29 µg/ml (Kaushik *et al.*, 2013).

2.5.13 Anticandidal activity

Methanol extracts had high anticandidal activity against different *Candida albicans, Candida tropicalis, Candida krusei, Candida guillermondii, Candida parapsilosis* and *Candida stellatoida.* The disc diffusion method was elected for antifungal susceptibility assessments by taking fluconazole as a standard drug (antifungal). *Candida* strains were isolated from vaginal thrush patients, and the species were recognized using their predictable tests. Zone of inhibition observed ranged from 13 to 16mm. Minimum inhibitory concentration values were between 2.5 to 0.312 mg/mL, while Minimum fungicidal concentration values were between 5 to 0.625mg/mL. (Uma *et al.*, 2009).

2.5.14 Hypocholesteremic and Hyperlipidemic Activity

Hyperlipidemia/hypercholesteremia is major causes for atherosclerosis and cardiovascular diseases. The major phytochemical present in *Asparagus racemosus* root i.e. phytosterols, saponins, polyphenols, flavonoids and ascorbic acid, could be responsible for increased bile acid production, abolition of excess cholesterol and elevation of hepatic antioxidant status in hypolipidaemic and hypercholesteremia conditions. Normal and hypercholesteremia rats were administered with root powder of A. racemosus (5 and 10g % dose levels) along with normal and hypercholesteremic diets, individually, for duration of 4 weeks. Plasma and hepatic lipid profiles, fecal sterol, bile acid excretion and hepatic antioxidant activity were evaluated (Visavadiya *et al.*, 2005). Additionally *Asparagus racemosus* root powder in diet, resulted in a dose-dependent lessening in plasma and hepatic lipid profiles, amplified fecal excretion of cholesterol, neutral sterol and bile acid along with increases in hepatic 3-hydroxy-3- methylglutaryl coenzyme A (HMG-CoA) reductase activity and bile acid content in hypercholesteremic rats. Further, *A. racemosus* root also improved the hepatic antioxidant status (catalase, SOD and ascorbic acid levels). A. racemosus root appeared to be useful as a dietary *A. racemosus* root appeared to be useful as a dietary supplement that offers a protection against hyperlipidemia/hypercholesteremia in hypercholesteremic animals (Zhu *et al.*, 2011).

2.5.15 Antidiabetic activity

Diabetes mellitus is a major cause of disability and hospitalization that presents a significant burden on worldwide. Herbal medicines for the treatment of diabetes is very important. *Asparagus racemosus* roots have been reported to reduce blood glucose levels in rats and rabbits. The ethanol extract stimulated insulin secretion in isolated perfused rat pancreas, isolated rat islet cells, and clonal β cells (Perez G *et al.*, 1998). Ethanol extract increased intracellular Ca^{2+} consistent with the observed abolition of insulin secretory effects under free Ca^{2+} conditions.

2.5.16 Teratogenic effects

Methanolic extracts of *Asparagus racemosus* showing increased mortality and delayed development against teratogenic effects. Symptoms of teratogenic effects such as swelling of legs, slow growth of fetal body and placental part an increase in resorption of fetus (Goel *et al.*, 2006).

2.5.17 Anti- inflammatory effects

Aqueous extracts of *Asparagus racemosus* administered 200mg/kg in mice reduction in skin thickness tissues, weight and inflammatory, cytokine production, neutrophil mediated myeloperoxidase activity. Aqueous extract of *Asparagus racemosus* maintain angiotensin converting enzyme, these enzyme helpful reducing inflammatory damage (Plangsombat *et al.*, 2016).

2.5.18 Aphrodisiac activity

Asparagus racemosus is considered as vajikarak (aphrodisiac) in Ayurvedic and used for treatment of sexual dysfunctions (Thakur *et al.*, 2007)Aqueous extract of *Asparagus racemosus* improving the sexual performance (Thakur *et al.*, 2009).The experimental anabolic activity demonstrated by increase in body and organ weight combined with presence of steroidal saponins in the extract (Shukla *et al.*, 2005) Aqueous extract help in the secretion of testosterone and make it better available to gonads. Testosterone production could be a result of gonadotropic activity as well as an increased availability of precursors in the form of steroidal components. The mechanism of action of testosterone spans from an increased rate of protein formation in targets cells (Thakur *et al.*, 2009).

2.5.19 Anti HIV activity

Asparagus racemosus has been shown Immunomodulatory activity. The ethyl acetate, butanolic and aqueous extracts of roots were found to be active against HIV. Steroidal saponin glycosides have been reported from these extracts. The glycoside with one sugar was isolated from the ethanolic extract, while glycosides with two, three and four sugar units were isolated from butanolic extract. Glycoside with one sugar displayed the highest anti HIV activity (53.3% inhibition at 6μg/mL). Glycoside with two sugars molecule revealed feeble anti HIV activity at 6μg/ml and saponins with three sugar units exhibited pathetic to no activity structurally comparable compounds have been testified to have anti HIV protease activity (Sabde *et al.*, 2011).

2.5.20 Immunomodulatory activity

Th1 and Th2 immunity is most important parameter to assess therapeutic effectiveness of Immunomodulator. Aqueous extract of *Asparagus racemosus* roots is known to exhibit immunological activities under different biological responses, however its efficacy towards Th1 and Th2 immunity has not been investigated. Regional research laboratory Jammu has done study demonstrate that aqueous extract of *Asparagus racemosus* showing mixed response towards Th1 and Th2 adjuvant properties. T cells (CD3 positive cells) is helpful for cytokine secretion, B cells help through CD4 (T helper) and CD8 positive (cytotoxic T cells) cells. Several studies has been shown significant positive correlation between peripheral cellular or hormonal responses in immune compromised conditions cancer, AIDS and tuberculosis. Aqueous extract of *Asparagus racemosus* up regulated CD3 and CD4/ CD8 positive percentages in peripheral blood suggesting its immunological potential (Gautam *et al.*, 2009). Previous study shown the adjuvant role of *Asparagus racemosus* extract in boosting protective immunity against pertussis. Based upon antigenic/adjuvant stimulus CD4 T cells differentiate into functionally dissimilar subsets known as Th1 or Th2 that can be identified by observing their signature cytokines. *Asparagus racemosus* produced higher antibody titers, cytoprotection and increased host resistance to tumors. *Asparagus racemosus* extract showed important proliferative effects on T and B lymphocytes in presence of antigens stimulus which supports its adjuvant roles to activated lymphocytes. Maximum tolerable dose of (2000mg/kg) with no toxicological consequences of *Asparagus racemosus* extract is safe to use. Ginenosides is very close related steroidal saponins, mediated Immunomodulatory effects through different targets. Standardized extracts such as aqueous extract may provide newer adjuvant moieties for safer modulation of host Immunity (Gautam *et al.*, 2004).

2.5.21 Neurodegenerative disorder activity

Excitotoxicity and oxidative stress are major mechanism of neuronal cells death that play an important role in Alzheimer and Parkinson diseases, therefore protection from neurodegenerative disorder there is a need for compound that can hinder or converse the neuronal damage. Innumerable formulations of *Asparagus racemosus* have effective neurodegenerative likely such as Mentat, EuMil. Mentat is mainly active in treatment of alcohol asceticism convinced withdrawal symptoms such as tremor, convulsions, hallucinations and anxiety. EuMil regularized noradrenaline, dopamine and 5-hydroxytryptamine concentration in brain. Parihar and Hemnani demonstrated that prospective of methanolic extract of *Asparagus racemosus* in contradiction of kainic acid prompted hippocampal and striatal neuronal destruction in animals. Intrahippocampal and intrastriated injections of Kainic acid increased development of excitotoxic lesions in the brain. Impairment of hippocampus and striatal sections of brain was detected. Amplified lipid peroxidation, better protein carbonyl content shrinked glutathione peroxidase activity and decreased glutathione hormone content. Glutathione hormone is a significant antioxidant which acts as nucleophilic scavenger of poisonous compounds and as a substrate in the glutathione peroxidase intermediated devastation of hyper oxides. *Asparagus racemosus* extract exposed amplified the glutathione activity and glutathione hormone content and reduction in membranal lipid peroxidation and protein carbonyl. Methanolic extract of *Asparagus racemosus* implicate the adrenergic system and boosts the serotonergic facilitated behavior representative the involvement of serotonergic pathway in the antidepressant activity (Jalsrai *et al.*, 2016).

2.5.22 Galactogogue activity

Alcoholic extract of *Asparagus racemosus* have a significant effect on lactating mother to increase milk production and increased growth of mammary glands, alveolar tissues and acini. The growth of lobuloaveolar tissue and milk secretion in the estrogen primed rats was thought to be due to the action of released corticoids or prolactin (S. Kumar *et al.*, 2008).

2.5.23 Anticancer activity

Qualitative analysis shows the presence of alkaloids, tannin, flavonoids, saponin, glycosides, and phenolic compounds in methanol and aqueous extract of *Asparagus racemosus*. They may help to prevent diseases like cancer and heart diseases also their role to inhibit the microorganisms causing many diseases in human beings. Some of the secondary metabolites act as scavengers of free radicals and reduce the damage due to oxidants by neutralizing the free radicals. Free radicals and other reactive oxygen species (Wagner *et al.*) are formed constantly in human body during normal metabolic processes. They help to destroy micro-organisms and fight against infections. However gathering of ROS is toxic. Increased ROS results in oxidative stress, which may lead to widespread cellular damage through covalent binding and lipid peroxidation. The ability of the plant extract in fighting the free radicals mainly due to the secondary metabolites of *Asparagus racemosus.* Methanol extract of *Asparagus racemosus* exhibited anticancer by MTT assay (Park *et al.*, 2011). The cytotoxicity of total methanol extract of *Asparagus racemosus* was observed on malignant cell lines MCF- 7 in a different concentration was evaluated by MTT assay. The IC_{50} value of the *Asparagus racemosus* was found to be 300ug/ml. The inhibitory effect of *Asparagus racemosus* was less than that of the standard drug taxol. MTT assay has been most widely used in different cancers, and is sensitive, accurate, and efficient in the *in vitro* evaluation of anticancer or immunological agents prior to the preclinical and clinical testing. Different concentration of the plant extracts was tested for its cytotoxic effect on MCF-7 by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay using taxol as standard (IC_{50} 300 μg/ml). The result shown that the methanolic extract of *Asparagus racemosus* showed marked anticancer activity against MCF-7cell line. This study shown that the antioxidant and anticancer properties of *Asparagus racemosus* might be due to the phytochemicals present in it. Another study was carried out to evaluate the anticancer activity of major shatavarins (Shatavarin IV) from methanolic extract of *Asparagus racemosus* roots. *In vitro* cytotoxicity study using MCF7, HT29, and A498 cell lines showed potent activity with methanolic extract of *Asparagus racemosus* roots (5.05% Shatavarin IV) (Mitra *et al.*, 2012).

2.5.24 Reproductive activity

Asparagus racemosus is also called “Hundred husband” in Sanskrit for its beneficial effects in women and reproductive function. *Asparagus racemosus* is also well known for treatment of reproductive problems such as sexual debility, amenorrhea, dysmenorrhea, dysfunctional uterine bleeding, menopause pelvic inflammatory disease like endometriosis and gonorrhea (Sharma, 2011). It also supports deeper tissue and builds blood and so it helps to remove infertility prepare the womb for conception, prevents miscarriage and acts as a postpartum tonic where it helps to increase lactation and normalize the uterus (Bansode *et al.*, 2015).

Asparagus racemosus also used to stimulant of endometrium and ovarian tissues regulating menstruation and ovulation, balance of various hormonal level like TSH, Estrogen, FSH, LH. The extract of *Asparagus racemosus* amplified the weight of mammary glands in postpartum and estrogen well-informed group and the uterine weight in estrogen primed group (Sharma, 2011).

2.5.25 Phytoestrogenic properties of *Asparagus racemosus*

Numerous studies provide evidence that synthetic hormones increase the risk of endometrial cancer, breast cancer, venous thromboembolic events and gall bladder disease in women. Due to undesirable effects of synthetic hormones has increased an interest in phytoestrogen i.e. plant derived estrogens. The most frequently used raw materials are plants that contain the most of active hormonal compounds. They are soybeans *Actaea racemosa* (Menofem- dry extract of roots of black cohosh) and red clover (Singh *et al.*, 2018).

Asparagus racemosus is also a plant known for its phytoestrogenic properties and for use as hormone modulator. In studies on rats showed a diet containing 2% of root extract of this plant obtained by extraction with chloroform and methanol (1:1) led to a significant decrease in the incidence of this cancer as well as the decrease in the average number of tumors.

Joglekar detected an increase in milk secretion after administration of *A. racemosus* in the form of Ricalax tablets (Aphali Pharmaceutical's, 40 mg of root extract per tablet). (Sharma's *et al)*. Controlled clinical trials performed on randomly selected women giving due birth, without complications, with an insufficient amount of milk secreted did not give a similar result. Four-week treatment with *A. racemosus* extract had no effect on the increase in lactation (Pandey *et al.*, 2005)
EveCare capsules confirmed their effectiveness in the treatment of dysfunctional uterine bleeding. Dysfunctional uterine bleeding are regular or acyclic bleedings from the uterus with no organic cause such as neoplasia, inflammation of the

endometrium, submucous fibroids. Dysfunctional uterine bleedings may occur throughout the reproductive period. 20% of them are observed in teenagers, 40% occur at the age of 18-45, the other 40% - in premenopausal stage. The Menosan formulation that is suggested in the treatment of postmenopausal symptoms was also confirmed. In the group of 27 women who used this preparation, a significant relief from post-menopausal symptoms such as depression (90% relief), insomnia (83.33% relief), irritability (50%), weight gain (50%), sweating (37.88%), and hot flashes (37.03%) were observed. (Tuszyńska, 2010).

2.6 Herbal formulation made from *Asparagus racemosus*

Asparagus racemosus due to its several applications is an important constituent of many Ayurvedic formulations. The demand of the plant is on significant rise and thus the annual growth rate is 15.1% (NMPB, 2002). It is being overharvested for its roots containing its essential properties and is thus considered as vulnerable in its natural habitat. The growing demand of plant has caused a serious reduction in native populations. The general use of *Asparagus racemosus* has brought it to the list of 32 prioritized medicinal plants for conservation and development by National Medicinal Plant Board (NMPB, 2002). Conservation of this species thus assumes paramount urgency. The Himalayan herbal health care company and various currently manufactures formulations containing significant amount of extract of the plant. *Asparagus racemosus* is not only a research oriented plant; the marketing status displays its economic importance worldwide.

Table 2.2:- *Asparagus racemosus* containing herbal formulations

Serial no.	**Herbal formulation**	**Content of *Asparagus racemosus***	**Medicinal property**	**Reference**
1	Abana formulation	10 mg shatavari root powder extract per tablet	Hyperlipidemic condition hypertension	venkataramaiah,2002 Verma & Bordia,1992
2	Banyan	Powder of *Asparagus racemosus* roots	Antioxidant	www.banyanbotanicals.com
3	Cystone tablets	25 mg shatavari roots extract per tablet	Dissolve kidney stone, possess diuretic	www.himalayastore.com
4	Diabecon	20 mg shatavari roots extract per tablet	Insulin dependent diabetes mellitus hyperlipidemia	Ganguly *et al*, 1995 Kohli *et al*, 2004 Mitra *et al*, 1996
5	Eve care	32 mg shatavari roots extract per 5 ml syrup	Dysmenorrhea	Swarup & Umadevi,1998 Sarda *et al*, 2007
6	EuMil (capsules)	30 mg of shatavari roots extract per 5ml syrup	Used for cough, common cold Hay fever	www.himalayastore.com
7	Geriforte	20mg shatavari roots extract per tablet	Antioxidant Enhances immunity	Venugopal,1998 Ghosh & Mitra,1985 Vaidya,1979
8	Gasex	45 mg shatavari roots extract per tablet	Hepatoprotective Antimicrobial Anti-inflammatory	www.himalayastore.com
9	Himplasia	80 mg shatavari roots extract per tablet	Benign prostatic hyperplasia	Sahu & Kulkarni,2003
10	Lukol	40 mg shatavari root extract per tablet	Leucorrhea inflammatory disease	Bhatnagar & Bhatnagar,1984
11	Menosan	110 mg shatavari roots	Natural	Sarkar, 2004

		extract per tablet	menopause Surgical menopause	Singh & Kulkarni, 2002
12	Mentat	55 mg of shatavari roots extract per 20 ml syrup	Used as antiparkinsonian Used in Alzheimer Improve memory	www.himalayastore.com
13	Renalka	50 mg shatavari root extract per tablet	Burning micturition Cystitis, urinary tract infection, dysuria	Sahu & Kulkarni,2003
14	Rumalaya	60 mg shatavari root extract per tablet	Treating joint gout, arthritis, analgesic properties	www.himalayastore.com
15	Shatavari women wellness Tablets (Himalaya)	75 mg shatavari root extract per tablet	Improving lactation, promotes physical and mental health Acts as phytoestrogen	www.himalayastore.com
16	Shatavari churna (Patanjali)	*Asparagus racemosus* powder	Enhancing breast milk Useful in debility, weakness and loss immunity	www.patanjaliayurved.net
17	Shatavari gulam	*Asparagus racemosus* content 65%	Used in the gynecological problem	www.himalayastore.com

CHAPTER 3.0

MATERIAL AND METHODS

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Material and Methods

3.1 Chemical and Instruments

Solvent methanol and ethyl acetate (laboratory grade) was procured from Finar limited India, petroleum ether (laboratory grade) was procured from SDFCL (s d fine-chem limited) Mumbai India. Solvent Chloroform laboratory grade was procured from Thomas baker chemicals Pvt. Ltd India. RPMI 1640 and DMEM, antibiotic solution, Phosphate buffer and bovine serum media were used to culture cancer cell lines. Sulphuric acid (91%) was purchased from Loba Chemie Pvt Ltd. Silica gel 60\120 for column chromatography was procured from SDFCL, glassware of fine quality were used and procured from Borosil JSGW, readymade TLC plates F_{254} from Merck were used, Rota vapor instrument LABINDIA was used. Laboratory grade reverse osmosis water R.O system was used. T-47 (Breast Cancer) cancer cell lines were selected for biological activity. These cell lines procured from National Cell Repository located at NCCS Pune. For visualizing the TLC plates procured UV chamber from Mac Company, characterization of structured of isolated compound NMR (H_1 and C_{13}) 400 MHz were used. Isolation of compounds flash chromatography is used procured from Biotage. Other instruments were used for thesis work such as Incubator for incubation, automatic cell counter, UV-Vis spectrophotometer, laminar airflow. For docking studies of reported phytoestrogens and isolated compounds Maestro software 2015 procured from Schrodinger Company.

3.2 Computer and software

Apple Mac book work station with configuration of Intel (R) xenon (R) X5660@2.80 GHz 2.79 GHz (2 processor) was used in In-silico study of the compounds. The operating system used included centox-6.5. Software used in the study include latest release of Schrödinger LLC (New York, United states of America) and ChemBio Draw Ultra-12.

3.3 Procurement of Plant Material

The powder of *Asparagus racemosus* were collected from Herbal Health Research Consortium Pvt. Ltd. Amritsar in month of August 2017. Herbal Health Research consortium institutes run under the Ministry of Ayush and Ministry of Health and Family Welfare, Government of India.

3.4 Extraction of plant material

4.0 kg of *Asparagus racemosus* powdered material was used for extraction purpose. Extraction was done using solvents in increasing order of polarity using petroleum ether, ethyl acetate, methanol and aqueous methanol. Percolation process was used for extraction where plant material was percolated three times with each solvents. Extracted fractions were dried and weighed. Weight of various extracts obtained included petroleum ether (9 g), Ethyl acetate (8g), Methanol (46g) and Aqueous Methanol (34.4g).

Figure 3.1:- Powdered root of *Asparagus racemosus* packed in Aspirator

3.5 Preliminary Phytochemical Investigation of Extracts

Preliminary screening of phytochemicals is a valuable step, in the detection of the bioactive principles present in medicinal plants and subsequently may lead to drug discovery and development. Various tests were performed for detection of phytochemicals present in *Asparagus racemosus*. For detection for bioactive phytochemicals four extracts of *Asparagus racemosus* were selected for preliminary screening. Fresh, clean and dry test tubes were used for preliminary screening. Various tests were performed for detection of phytochemicals are written below.

3.5.1 General test for Glycosides: - The general test for glycosides is as follows

Test A; - Dissolve the 200 mg of *Asparagus racemosus* powder with sulphuric acid. Then add 5% NaOH solution for neutralization. Add Fehling solution A and B to the above mixture Red colour is produced.

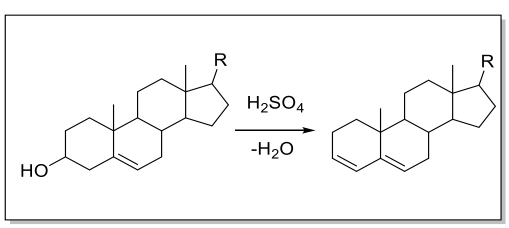
Test B; - Dissolve the 200 mg of *Asparagus racemosus* powder with sufficient amount of the water. Add further water to dilute the solution. This solution is tested with Fehling solution A and B. Red colour is produced from the reducing sugar present in the *Asparagus racemosus* powder.

Compare the red colour from the two tests of the plant material, if the colour of test A is more intense than Test B glycoside presence confirmed.

3.5.2 Chemical test for Steroids and Terpenoids

3.5.2.1 Salwoski Test; - *Asparagus racemosus* powder is mixed with sulphuric acid. Upper layer is steroids which are red in colour and lower coloured layer represent trirepenes and steroids.

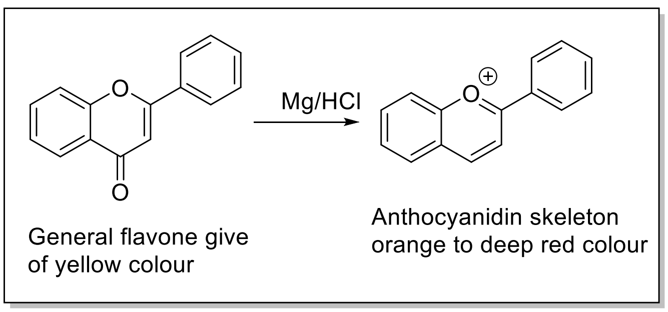
Sterols are alcohols, the Salwoski test involves treating sterols like cholesterol with strong acid which leads to removal of water from the cholesterol structure. Colour change occur to the formation of charge transfer complexes in the resulting conjugated dienes.



3.5.3 Chemical test for Flavonoids

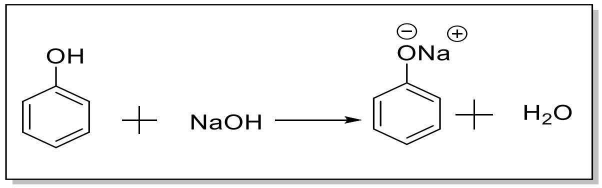
3.5.3.1 Shinoda Test; - Magnesium were added to *Asparagus racemosus* powder followed by a few drops of concentrated hydrochloric acid. A reddish colour indicate the presence of flavonoids Red.

The reaction includes the conversion of flavonoids to the corresponding anthocyanidin. The structure of that resulting anthocyanidin contains an extended systems of conjugated π-π electrons which constituents a chromophore. As it happens, the electrons of molecule covering conjugated π systems have energy level transitions corresponding to absorbance in the visible region of the electromagnetic spectrum. This accounts for their perceived colour.



The Shinoda test is similar to the clemmensen reduction in that a carbonyl group is reduced. However in this case, reductive elimination occur. Due to stability of the anthocyanidin products. Instead of the reduction going all the way to the corresponding methylene compound. Thee hydroxyl like intermediate undergoes elimination to the create the stable anthocyanidin. The conjugation in flavonoid compounds produces a yellow colour, while the extended conjugation in the resultant anthocyanidin shifts the colour further out to red region of the visible spectrum. The change in colour makes this a simple visual test for the presence of flavones.

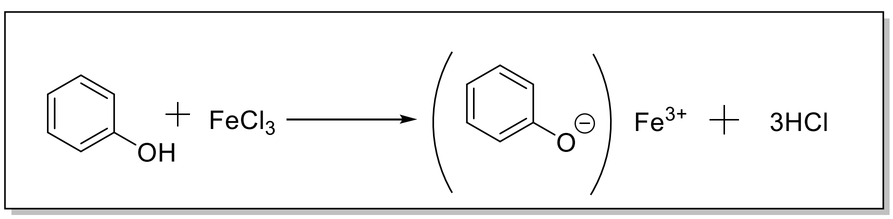
3.5.3.2 Alkaline Reagent Test; - As the name suggests, an alkaline reagent is used for this test sodium hydroxide is added to the *Asparagus racemosus* powder. Yellow colour is produced, if on addition of dilute acid this colour disappears then it confirms the presence of flavonoids.



Polyphenolic flavonoids which are weakly acidic in nature. Sodium hydroxide is completely ionic, containing sodium cations and hydroxide anions. It is sufficient strong base which deprotonate phenol entirely. The purpose of sodium hydroxide in this test was to deprotonate the polyphenolic molecules contained in flavonoids. Sodium hydroxide turns phenols into phenoxides which are much more soluble in water than phenols. The alkaline condition may have been necessary to disrupt bonding between the flavonoids. Other reason is treatment of a flavonoid with a strong base will result in ionization of its hydroxyl groups with resulting large bathochromic shifts of both absorption maxima.

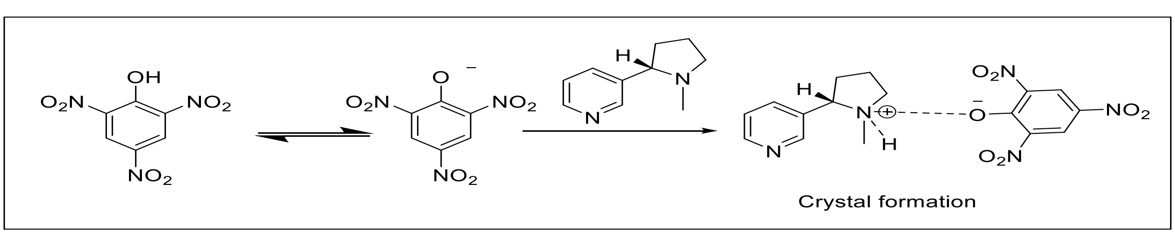
3.5.4 Chemical Tests for Tannins:-

3.5.4.1 Ferric chloride test:- Ferric Chloride Test for the presence of a phenol. yellow colour is produced with $FeCl_3$ in the case of hydrolysable tannins, whereas condensed tannins give green colors.



3.5.5 Chemical Tests for Alkaloids:-

3.5.5.1 Hager's Test: - This reagent is constituents of picric acid. Alkaloids give yellow precipitate with the Hager's reagent.



3.6 *In vitro* anticancer activity

Extracts of *Asparagus racemosus* were used for evaluated anticancer activity. MTT assay was performed by using T-47 D breast cancer cell lines. The cells of cell lines was treat with extracts in triplicate of concentration and experiment was also repeated three times. The result was demonstrated that there is substantial amount of anticancer activity.

For the evaluation the antiproliferative activity of extract of *Asparagus racemosus* MTT assay was performed. All extracts of *Asparagus racemosus* was dissolved in DMSO (25mg/ml) to form a stock solution and subjected to *in- vitro* antiproliferative activity. MTT assays demonstrated results in varied range of concentration. Cancer cell lines T-47 D was used to establish the antiproliferative potential of the extracts using *in vitro* MTT assay. The cell lines were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) 50U/ml penicillin G and 50µg/ml streptomycin sulphate. The cells were cultured at 37^0C with 5% CO_2 and 95% humidity conditions. Approximately, 7200 cells per well of 96 well plate were seeded and the plate was then cultured properly for 24h. After incubation period of 24 hour the cells were treated with extracts in triplicate with concentration of 2µg/ml, 5µg/ml, 10µg/ml, 25µg/ml and 50µg/ml and cells were incubated for 48h. After treatment media was discarded, cells were washed with 1xPBS and mixed with 100µl per well of MTT and incubated at room temperature in dark for 4h to let production of formation crystals. Finally added 100µl of DMSO in each well to dissolve the formazon crystals followed by analysis using Microplate reader at 570nm. Results were then plotted in graphs to calculate cytotoxic potential and IC_{50} values(Jose *et al.*, 2014).

3.7 Isolation of Phytoestrogens

From the Preliminary phytochemical Investigations of Extracts and *In-vitro* study of extracts demonstrated that methanolic extract was selected for further isolation of compound. 46g methanolic extract was subjected to successive extraction using gradient elution 10%, 25%, 50%, 75%, 100% methanol: chloroform.

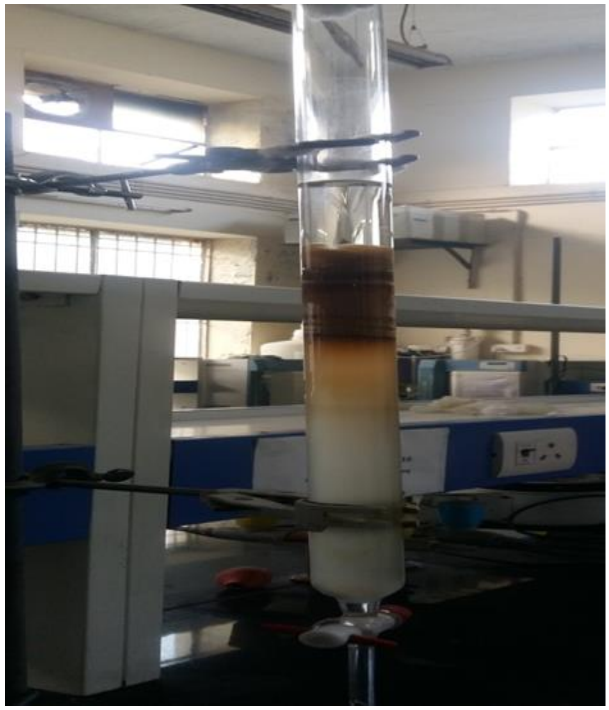


Figure: 3.2-Isolation of compounds from methanolic extract by using column chromatography

On the basis of thin layer chromatography similar compound visualize in 10% and 25%were merged, similarly 50% and 75% were merged. Further, 100% methanolic extract was subjected to column chromatography using gradient elution of 5% to 100% petroleum ether: ethyl acetate. Fractions 30% and 35% were combined and dried on the rotavapour to yield oily mixture, which was further chromatographed over silica gel (60-120) and eluted 100% petroleum ether and 1% ethyl acetate. Sub fractions 5-10 were combined together on the basis of a single spot on precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on the rotavapour yielding (RM01) 127mg as pale yellow oily compound.

The methanolic extract was then chromatographed using gradient elution of 5% to 100% methanol in chloroform. In 5% methanol in chloroform fractions 8 and 9 combined together on the basis of a single spot on precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on the rotavapour yielding (RM02) 35mg as pale yellow solid compound and melting point was 89^0C.

25% methanol in chloroform fractions from 15 to 155 combined together on the basis of a single spot on precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on the rotavapour yielding (RM03) 245mg as yellow solid compound. Melting point of RM03 is 99^0C.

35% methanol in chloroform to 70% methanol in chloroform fractions combined together on the basis of single spot on precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on the rotavapour yielding (RM04) 254 mg as brown solid compound. Melting point of RM04 was 126^oC.

Another extract (Methanol- water) was chosen for further isolation of compounds by using gradient elution of 50% chloroform in Pet. Ether. 25 fractions of 60% chloroform in Pet. Ether were combined on the basis of thin layer chromatography, different type two spots were visualized. For further isolation of two compounds small pencil column was set up and obtained a single compound (RM05) weighed 32.6 mg, using gradient elution 5% ethyl acetate in Pet. Ether and melting point was 109^0C. The methanol- water extract was then chromatographed using gradient elution of 5% and 8% methanol in chloroform, two compounds were isolated (RM06) and (RM07) weighed 40.1mg, 23.8mg and melting point was 118^0C and 85^0C respectively.

3.8 *In-silico* activity

The computational study of molecular recognition is an important part of structure-based drug design. The molecular docking problem is commonly cast as a problem of finding the low-energy binding modes of a small molecule, or ligand, within the active site of a macromolecule, or receptor, whose structure is known (Pagadala *et al.*, 2017). With the help of molecular docking environment, using Maestro software 11. The mechanism behind the antiproliferative activity of phytoestrogens from *Asparagus racemosus* was determined. The targets selected for the study were estrogen receptor alpha and estrogen receptor β. Total 53 molecules selected for molecular docking study.

3.8.1 Ligand preparation

With the aid of ChemBio Draw ultra 12.0 structured of phytoestrogens were sketched and saved in SDF format. The molecules were converted to 3D structure from the 2D using Lig Prep version 2.5 (Maestro version 11.0, 2018). These ligands were imported from workspace option then were subjected to ligand preparation using ‘LigPrep’ wizard application in Maestro 9.6 The Lig Prep developed a single, low energy, 3D structure with correct chiralities for the each input structure. During the performance of this step, chiralities were determined from 3D structure and original states of ionization were retained. Lig Prep application of the Maestro operates OPLS 2005 force field (Lam *et al.*, 2018).

3.8.2 Protein Preparation:

The PDB for the crystal structure of estrogen alpha PDB Id 3ERT were retrieved from RSCB protein data bank. The protein preparation wizard option was used for preparation of protein structure with polar hydrogen. This procedure contain two steps that is preparation and refinement (Singla *et al.*, 2017). In this step, bond orders were consigned, all hydrogen’s were added 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 5Å specific distance. Any missing disulphide bonds were added. The hydrogen bond 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. . Also the reorientation of hydroxyl groups, water molecule and amino acids to the optimization of hydrogen bond network. The refinement process the last step for protein preparation. This steps involves firstly optimization then add hydrogen only and minimization. Thus in this process the restrained impact of minimization of protein was taken and stearic clashes would be exposed. For the determination of druggable pockets of five different receptors as mentioned in which the co-crystalized ligands are present in crystal structures (Yuriev *et al.*, 2015).

3.8.3 Receptor Grid generation

The grid generation at a particular site in the protein is an important parameter to perform docking study. The grid was generated at the site of co-crystalized ligand already has to bound to protein. The ligand molecule has to be selected for grid generation. The grid was generated in which van der Waals scaling was reduced to

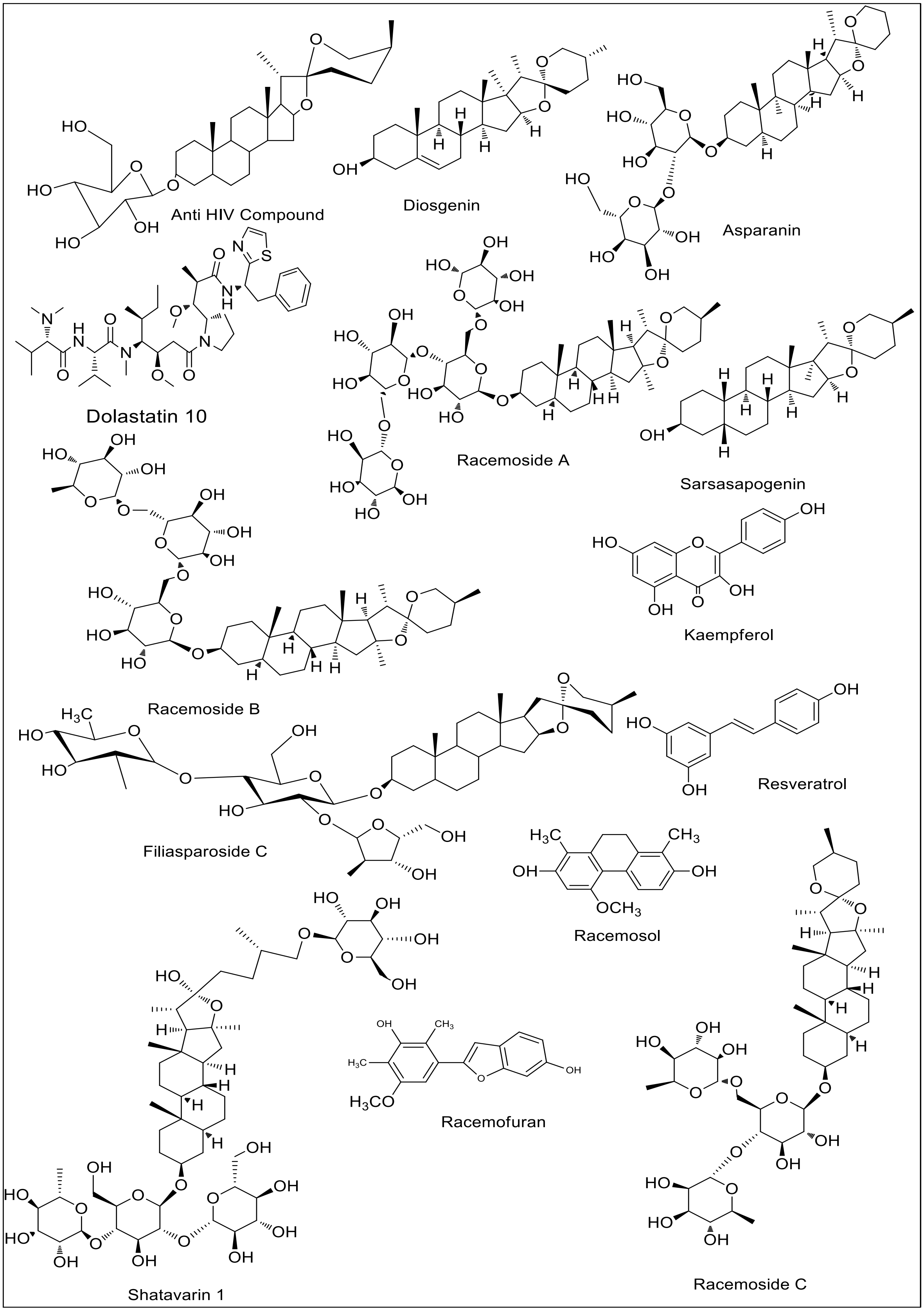
0.20 for estrogen α. The length of ligands which are selected for docking were made to 36Å (Singla *et al.*, 2015).

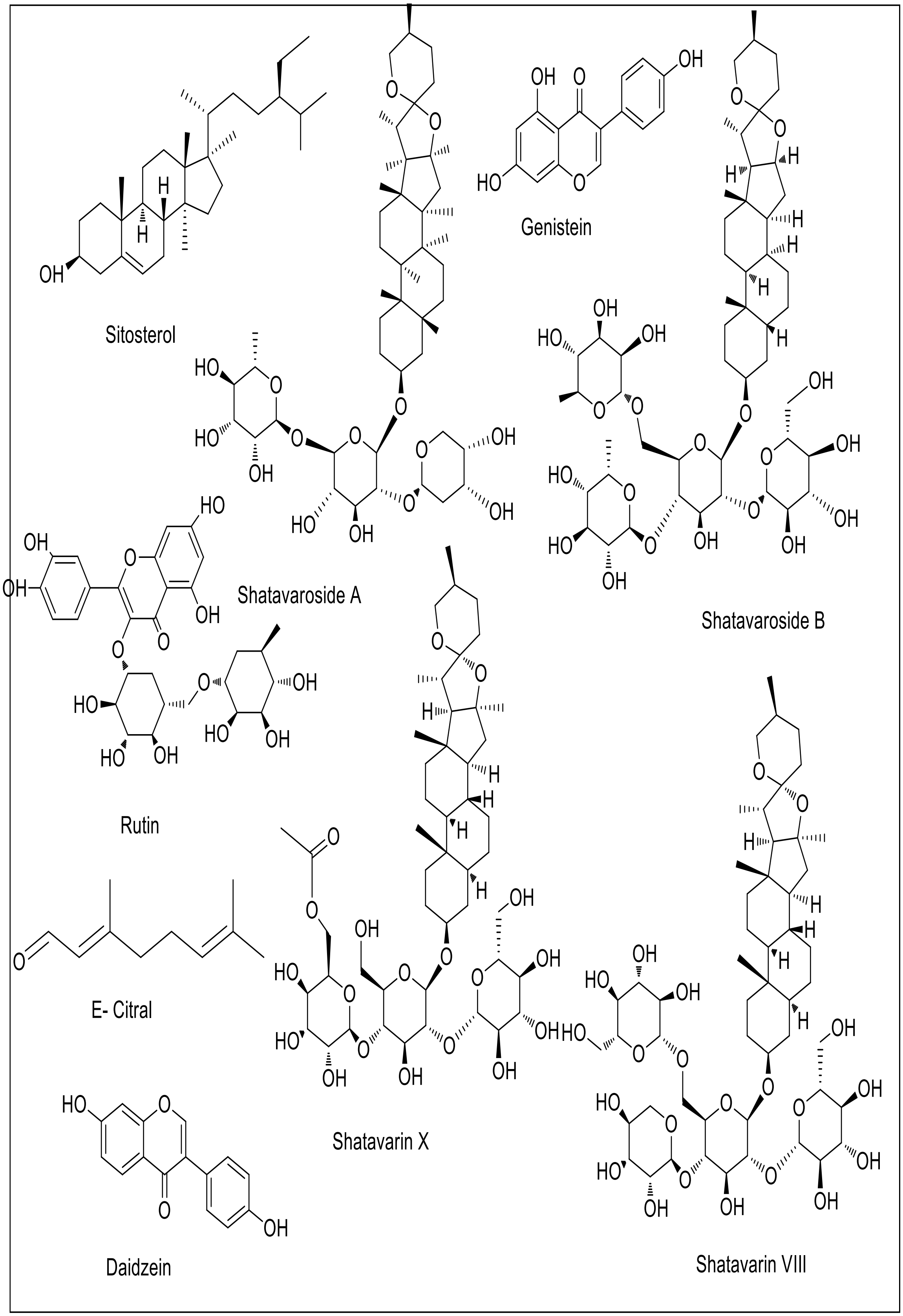
3.8.4 Glide Docking

GLIDE (Grid Based Ligand Docking with Energetics) software was used for docking procedure developed by Schrödinger. Three docking precision is available in GLIDE docking module such as HTVS (High throughout visual screening), standard precision (SP) and Extra precision (XP). HTVS is proposed for quick screening of very large numbers of ligands. It has controlled conformational sampling. SP is set as a default parameter and is used to screen thousands of compounds. Extra precision docking and scoring is more dominated and sharp procedure. Extra precision is developed to locate active compounds that binds to a particular conformation of receptors. Docking of reported phytoestrogens and the inhibitor was done with extra precision. Extra precision descriptions were written, ligand was taken as flexible. Epik penalties were added to the docking score. Docking score was taken into consideration for comparing the results, more negative the docking score more potent the compound and indicates the good binding potential. Various components are present in GLIDE score like hydrogen bonds, hydrophobic contacts, Van der Waals interaction, and Columbic interaction, Polar interaction in the binding site, Metal binding and Freezing rotable bonds (Singla & Jaitak, 2015).

3.8.5 ADME Study

For estimation of absorption, distribution, metabolism and excretion properties of ligands Quikprop module in Schrödinger suit is used. It evaluates the pharmacokinetics properties of ligands by retrieving the drug like properties. The properties include QPlogKhsa, QPlogBB, QPlogPo/w (partition coefficient) log IC_{50} value for the blockage of K^+ channels QPlogHER, molecular weight, gut blood barrier (QPPCaco) and percentage human oral absorption etc. (Singla & Jaitak, 2017).


Anti HIV Compound
Diosgenin
Asparanin
Dolastatin 10
Racemoside A
Sarsasapogenin
Kaempferol
Racemoside B
Resveratrol
Filiasparoside C
Racemosol
Racemofuran
Shatavarin 1
Racemoside C


Sitosterol
Genistein
Shatavaroside A
Shatavaroside B
Rutin
E- Citral
Shatavarin X
Daidzein
Shatavarin VIII

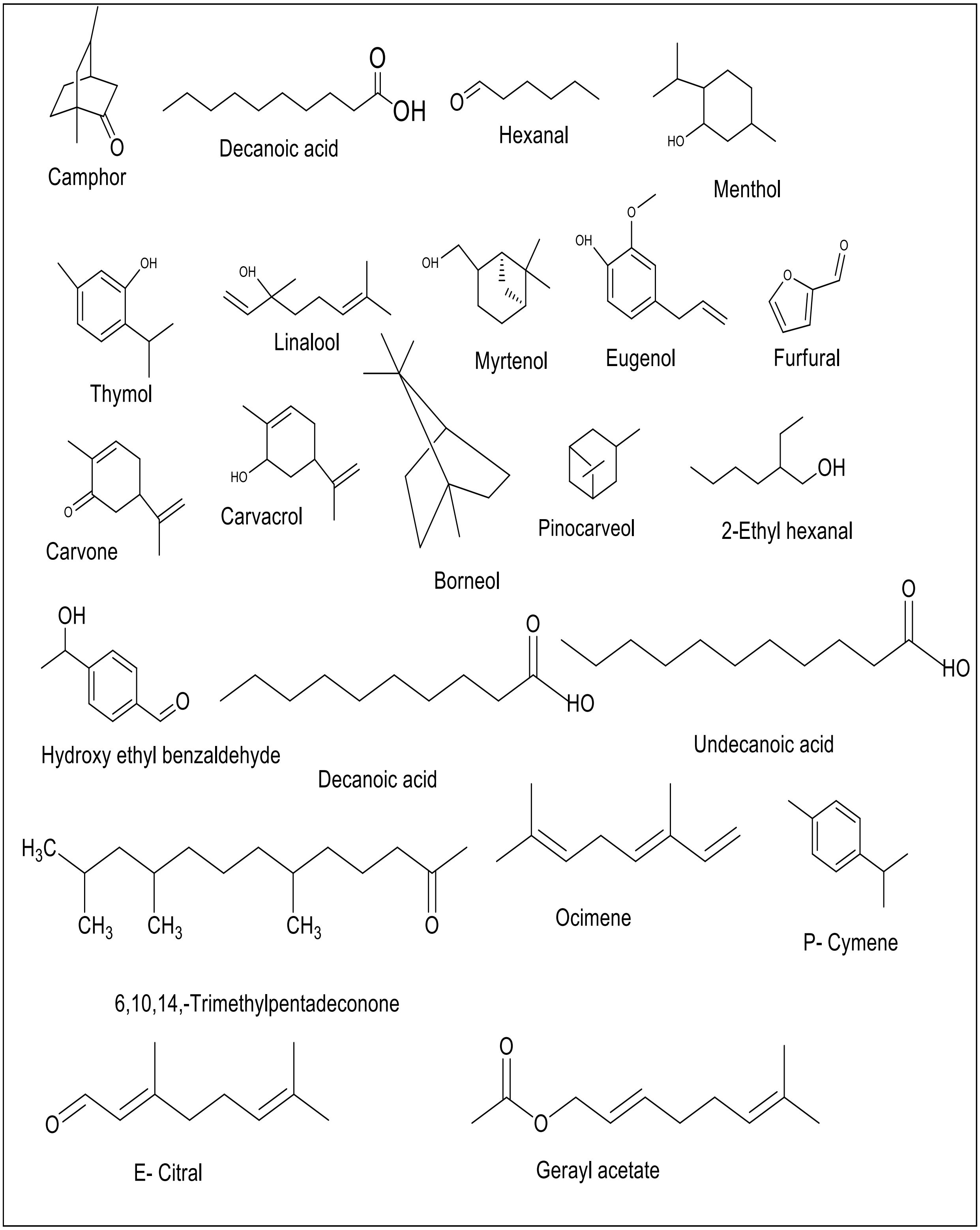


Figure 3.3:- Reported Phytoestrogens in literature used for evaluation of *in silico* activity.

CHAPTER 4.0

RESULTS AND DISCUSSION

CHAPTER 4.0

Results and Discussion

4.1 *Asparagus racemosus* extract preparation

After the percolation process different extracts were obtained. Extracts were dried under reduced pressure by using Rota vapor apparatus and recovered solvent.

Table 4.1:- Weight of different extracts.

Serial number	**Extracts**	**Weight of extract**
1	Petroleum Ether Extract	9g
2	Ethyl acetate Extract	8g
3	Methanolic Extract	46g
4	Methanolic water Extract	34.4g

4.2 Preliminary Phytochemical Investigations of Extracts

Phytochemical test of petroleum ether, ethyl acetate, methanol, methanol water extracts of *Asparagus racemosus* were subjected to tests identify the presence of various phytoconstituents. General test performed for glycoside, Salwoski test for steroids, Shinoda test for flavonoids, ferric chloride test for tannin, Hager test for alkaloids.

Table 4.2:- Preliminary Phytochemical Investigations of Extracts of *Asparagus racemosus*.

Chemical Test	**Pet ether extract**	**Ethyl acetate extract**	**Methanol extract**	**Methanol water extract**
Test for Alkaloid **Hager's Test**	Negative	Negative	Positive	Negative
Test for carbohydrate **(Molisch test)**	Negative	Negative	Negative	Negative
Test for steroid **Salwoski Test**	Positive	Negative	Positive	Positive
Test for Tannin **Ferric chloride test**	Positive	Negative	Positive	Negative
Test for Flavonoid **Shinoda Test**	Negative	Negative	Positive	Positive
Test for glycoside	Negative	Negative	Positive	Positive

From the phytochemical investigations of extracts, the methanol extract and methanol- water showed that the most chemical constituents are present. So methanol extract and methanol extract was selected for further study and isolation of compound.

4.3 *In vitro* activities

For the evaluation the antiproliferative activity of extracts of *Asparagus racemosus* MTT assay was performed. Four different extracts of *Asparagus racemosus* including Petroleum Ether, Ethyl acetate, Methanol and Aqueous Methanol have been assessed for inhibition for proliferative activity. It was found that the most polar aqueous methanol extract was most active with IC_{50} 8.89 µg/ml followed by methanol extract with IC_{50} 27.7µg/ml. therefore it was evident from the study that *Asparagus racemosus* extracts had potent antiproliferative activity. The methanol water extract which has shown impressive activity is known to be source of Rutin, Quercetin, kaempferol, genistein and Daidzein. Petroleum ether extract and ethyl acetate extract were inactive against breast cancer cell lines (T-47 D). Further IC_{50} values of methanol extract and methanol water extract were calculated and tabulated in the table.

Table 4.3:- *In- vitro* activity results of different extracts.

Serial number	Cell lines	Extracts	IC_{50} (µg/ml)
1	T-47D	Methanolic –water extract (MR04)	8.89 µg/ml
		Methanolic extract (MR03)	27.7 µg/ml
		Ethyl acetate extract (MR02)	ND
		Petroleum Ether Extract (MR01)	ND
		Bazedoxifene (Standard)	18.9 µg/ml

4.4 Isolation of compounds and characterization

On the basis of Preliminary Phytochemical Investigations of Extracts and In-vitro antiproliferative activity, methanolic extract was selected to subject to chromatography. Four compounds were isolated from methanolic extract. Three compounds were isolated from Methanol- water extract.

Table 4.4: - List of compounds isolated from different extracts.

Serial number	Compound	Weight of compound	Melting point of compound
1	RM01	127 mg	------
2	RM02	35 mg	89^0 C
3	RM03	245 mg	99^0 C
4	RM04	254 mg	126^0 C
5	RM05	32.6 mg	109^0 C
6	RM06	40.1 mg	118^0 C
7	RM07	23.8 mg	85^0 C

Characterization and spectroscopic analysis of isolated compounds is still in process.

4.5 *In silico* activity

The literature of *Asparagus racemosus* suggest that the extracts of *Asparagus racemosus* possesses anticancer activity specially showing the activity against the breast cancer, due to presence of phytoestrogens. Estrogen receptors like estrogen alpha and estrogen beta play a crucial role in regulation of estrogen hormone in human beings. Irregulaties in these receptors causes to induce the breast cancer and prostate cancer. Docking study experiment demonstrated that phytoestrogens regulate the estrogen receptors. Phytoestrogens prevents the binding of oestradiol with its receptor (Estrogen Beta) therefore down regulating the signaling pathway and these phytoestrogens also acts as inhibitor of enzymes involved in the biosynthesis of endogenous oestradiol. The targets selected for the study are estrogen receptor alpha and estrogen receptor beta. Both targets having a vital role in the breast cancer proliferation.

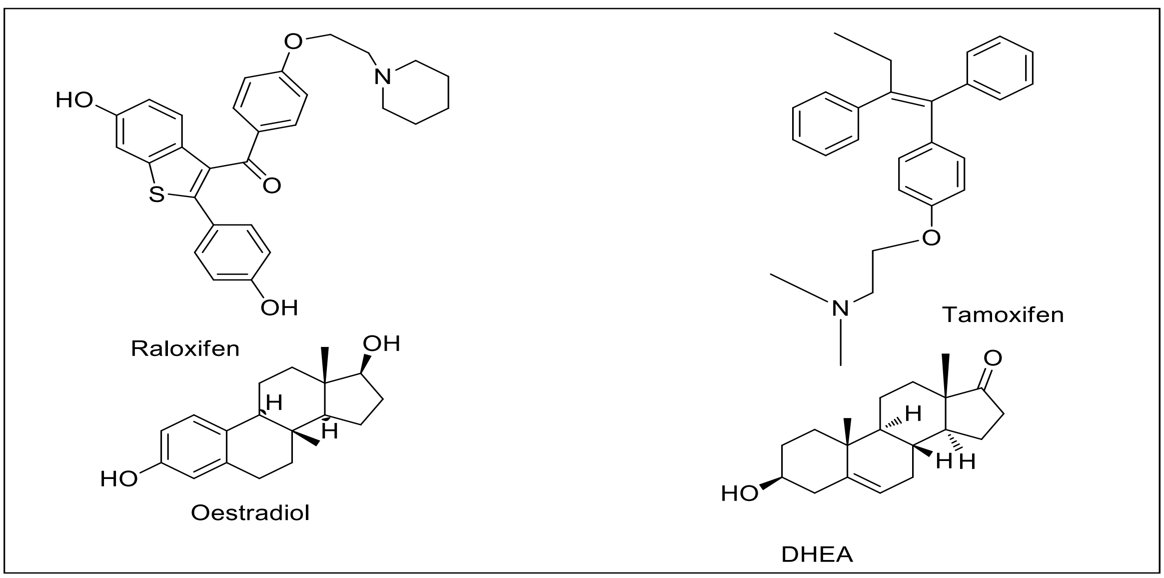


Figure 4.1:- Different standard inhibitors used for *In-silico study*.

Phytoestrogens were docked over estrogen receptor beta and estrogen receptor alpha. The scoring phytoestrogens and their lipophilic EvdW, hydrogen bond value and the residues involved in hydrogen bonding and π-π stacking obtained upon docking over estrogen receptor beta and estrogen receptor alpha.

Table 4.5:- Docking score of molecules of *Asparagus racemosus*.

Serial number	Receptor	Molecule	Dockscore Kcal/mol	Lipophilic EvdW	Hydrogen bond	Residues in hydrogen
1	Estrogen β	Quercetin	-11.55	-4.54	-2.84	GLU_{305},GLY_{472}
		oestradiol	-10.73	-6.27	-0.99	LEU_{339}
2	Estrogen α	Rutin	-13.71	-5.72	-5.66	ASP_{351},GLU_{353}
		Raloxifene	-12.15	-7.49	-1.19	GLY_{420}, GLU_{353},APG_{394}

This docking study of the reported phytoestrogens shown that phytoestrogens from *Asparagus racemosus* have a multiple approach for the protection against breast cancer. Estrogen receptor contained of two subtypes, estrogen receptor α and estrogen receptor β. Estrogen receptor β, antagonize the growth promoting effect of estrogen receptor therefore its activation is favorable in estrogen sensitive tumors cells. It has been revealed that estrogen receptor β is significant down regulation in the breast cancer epithelium as compared to the epithelium of the normal breast therefore activating specifically this receptor will be the beneficiary in tumor suppression. Phytoestrogens have 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.

The high Dockscore of the phytoestrogen in comparison to the oestradiol demonstrated strong binding affinity of the phytoestrogens for estrogen receptor β. The Dockscore of Quercetin was -11.55 kcal/mol and Dockscore of the oestradiol was -10.73 kcal/mol. The interaction diagram are displayed.

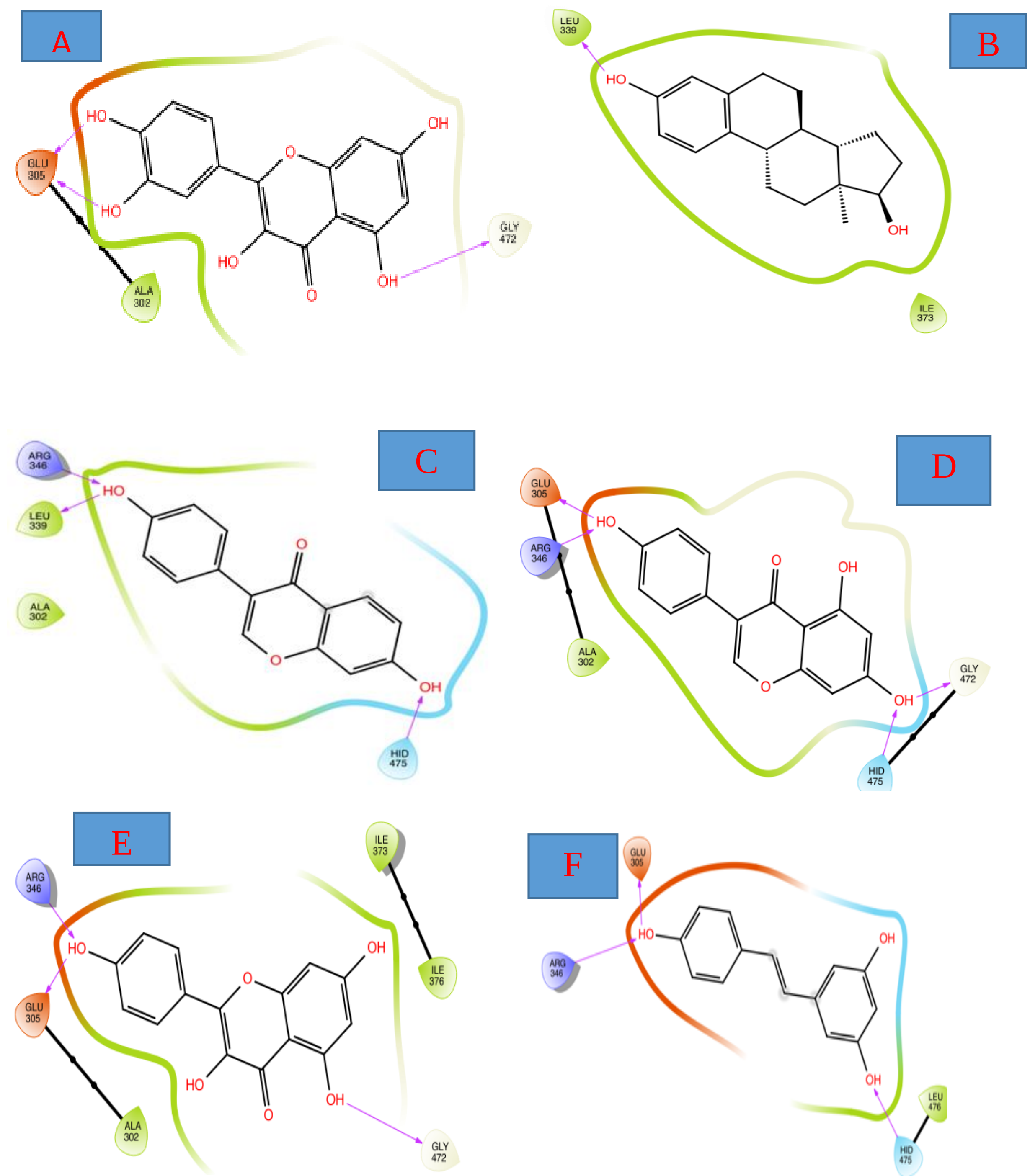


Figure 4.2:- Ligand interaction diagram of (A) Quercetin (B) Oestradiol (C) Genistein (D) Daidzein (E) Kaempferol (F) Resveratrol with Estrogen receptor β.

Table 4.6:- Highest Docking score of molecules of *Asparagus racemosus* with Estrogen receptor β.

Structures	Dock score Kcal/mol	H bond	Lipophilic	Electro
Quercetin	-11.57	-2.84	-4.54	-1.38
Oestradiol	-10.73	-0.99	-6.27	-0.28
Kaempferol	-10.63	-2.04	-4.74	-1.06
Genistein	-10.55	-1.65	-4.79	-1.08
Daidzein	-9.95	-2.16	-4.81	--0.73
Racemoside A	-9.27	-1.28	-4.35	-0.96
Filiasparoside C	-5.45	-0.92	-2.41	-0.256
Shatavarin VIII	-5.01	0.19	-3.3	-0.166
Sitosterol	-4.44	-0.5	-1.62	0.04
Hyperoside	-3.92	-4.93	-1	0.078

Oestradiol was standard inhibitor shows good anticancer activity in the protein 3OLS with dock score, hydrogen bond, lipophilicity and electrophilicity measured -10.73,-0.99,-6.27,-0.28 kcal/mol respectively. In the interaction diagram its shows to hydrogen bonding with GLU_{305}, GLY_{472}. The most active compound is Quercetin give the dock score -11.57 kcal/mol, lipophilicity is -4.54, hydrogen bond -2.84 and electrophilicity -1.38. Kaempferol also showing better anticancer activity but less active than oestradiol showed dock score -10.63 kcal/mol, hydrogen bond -2.04, lipophilicity -4.74 and electrophilicity was -1.06. Shatavarin is saponin glycosides present in *Asparagus racemosus* showing anticancer activity with dock score -5.01kcal/mol, hydrogen bond 0.19 lipophilicity -3.3 and electrophilicity -0.166.

Hyperoside showing least anticancer activity as compared to other phytoestrogens. The dock score of hyperoside -3.92, hydrogen bond -4.93 lipophilicity -1 and electrophilicity 0.078. Molecular docking score of other ligands are shown in appendix

Phytoestrogens have been revealed to have superior affinity toward ER-beta than toward ER-alpha. The binding affinities of genistein, dihydrogenistein, and equol were comparable to 17-estradiol, and equol induced transcription most strongly with ER beta and ER alpha.

Over half of all breast cancers overexpress estrogen alpha and about 70% of these react to anti-estrogen (tamoxifen) therapy. In addition, the presence of raised levels of Estrogen alpha in benign breast epithelium performs to indicate an increased risk of breast cancer, suggesting a role for estrogen alpha in breast cancer initiation, as well as progression. However, a fraction of estrogen alpha-positive tumors does not react to endocrine therapy and the majority of those that do react ultimately become resistant. Most resistant tumors remain estrogen alpha-positive and frequently respond to alternative endocrine treatment, indicative of a continued role for estrogen alpha in breast cancer cell proliferation. The problem of resistance has resulted in the search for and the development of natural products to inhibit ER alpha action, phytoestrogen play an important role for regulating the estrogen alpha. Docking study of the reported phytoestrogens with estrogen receptor alpha (PDB id 3ERT) demonstrated that phytoestrogens present in *Asparagus racemosus* showing inhibitory activity against the breast cancer.

The high Dockscore of the phytoestrogen in comparison to the Raloxifene demonstrated strong binding affinity of the phytoestrogens for estrogen receptor alpha. The Dockscore of Rutin was -13.71kcal/mol and Dockscore of the raloxifene was -12.15. The interaction diagram are displayed are shown below.

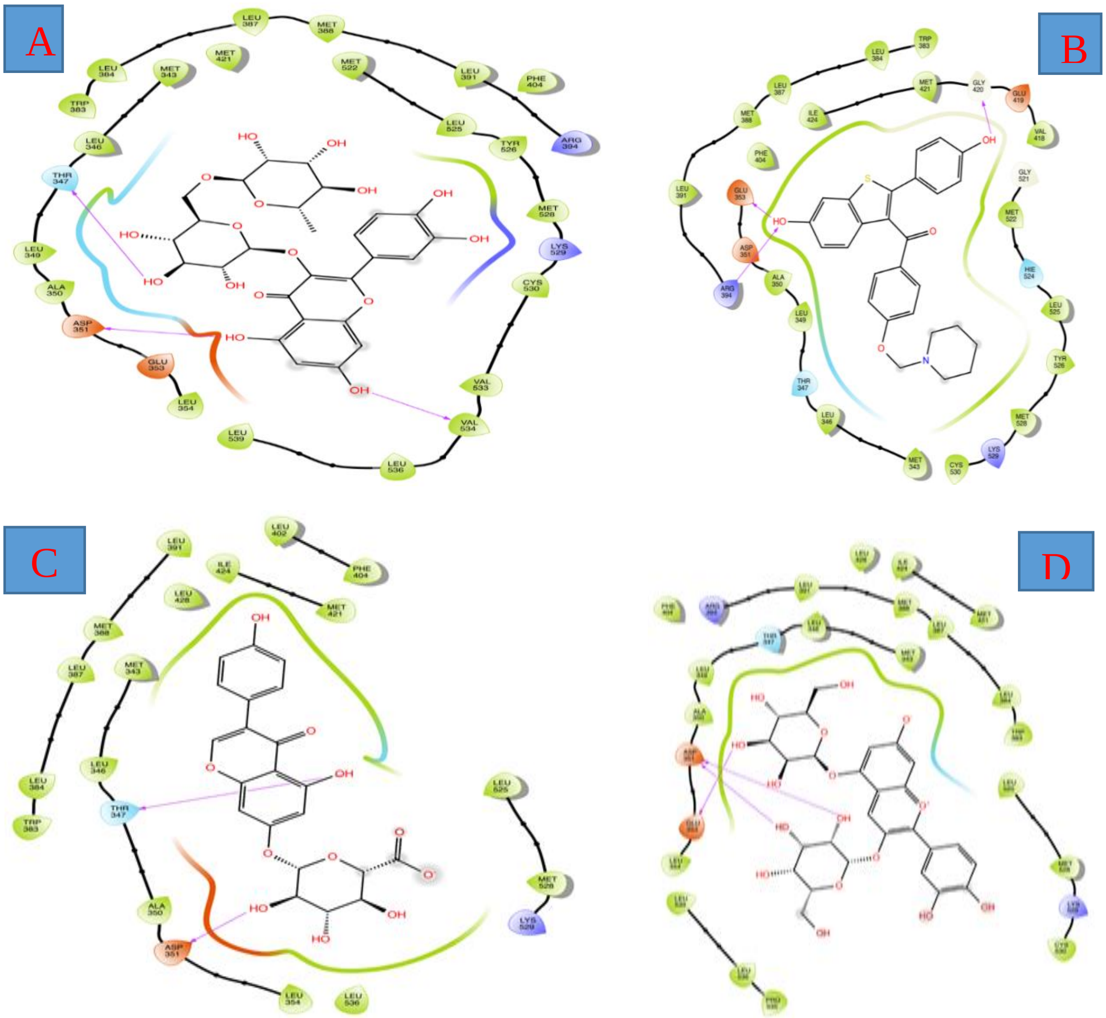


Figure 4.3:- Ligand interaction diagram of (A) Rutin (B) Raloxifene (C) Genistein 7-O-glucuronide (D) Hyperoside with Estrogen receptor α.

Raloxifene was standard inhibitor for estrogen alpha receptor shows good anticancer activity in the protein 3ERT with dock score, hydrogen bond, lipophilicity and electrophilicity measured -12.75,-1.19,-7.49,-1.24kcal/mol respectively. In the interaction diagram its shows to hydrogen bonding with GLU_{353}, GLY_{420} ARG_{394}. The most active compound is Rutin give the dock score -13.7, lipophilicity is -5.72, hydrogen bond -5.66 and electrophilicity -1.5. Kaempferol also showing better anticancer activity but less active than Rutin showed dock score -12.55 kcal/mol, hydrogen bond -5.64, lipophilicity -6.59 and electrophilicity was -0.81. Spiraeoside is showing anticancer activity against the estrogen receptor alpha with dock score -11.18kcal/mol, hydrogen score -3.93, lipophilic -5.47, electrophilic -1.31kcal/mol.

Table 4.7:- Highest Docking score of molecules of *Asparagus racemosus* with Estrogen receptor α.

Structures	Dock score Kcal/mol	Hydrogen bond	Lipophilic	Electrophilic
Rutin	-13.7	-5.66	-5.72	-1.5
Kaempferol	-12.55	-5.64	-6.59	-0.81
Raloxifene	-12.15	-1.19	-7.49	-1.24
Tamoxifen	-11.63	-0.9	-7.64	-0.4
Spiraeoside	-11.18	-3.93	-5.47	-1.31
Genistein	-10.28	-1.69	-5.06	-0.75
Daidzein	-10	-1.09	-2.7	-0.83
Genistein7-O-glucuronide	-9.68	-2.4	-2.13	-0.45
Quercetin-3'-glucuronide	-9.6	-2.88	-1.27	-0.23
Quercetin.	-9.48	-2.83	-0.98	-1.03

Genistein, Quercetin and Daidzein are flavonoids type phytoestrogen also showing anticancer activity. Docking study revealed that flavonoids type phytoestrogens are less active as compared Rutin. Genistein has dock score -10.28 kcal/mol, hydrogen bond -1.69, lipophilicity-5.06 and electrophilicity -0.75. Molecular docking score of other ligands are shown in appendix.

From the docking score genistein revealed that good binding affinities with the estrogen receptor alpha. 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 in comparison to the standard raloxifene and tamoxifen. The molecular docking stimulation results clearly indicated that Rutin, Spiraeoside, Genistein, Cyanidin 3-galactoside-5-

glucoside, Daidzein, Genistein7-O-glucuronide, Quercetin-3'-glucuronide, Quercetin, Resveratrol, Hyperoside, Racemofuran, Beta-Sitosterol, Shatavarin IV, Racemosol, Didehydrostemofoline and Diosgenin having more binding affinity as compared to their respective standards.

4.5.1 *In silico* ADME prediction

ADME properties of all reported phytoestrogens have been predicted using Quikprop application and are represented in Table

Table 4.8:- Predicted molecular descriptor and ADME characteristics of Phytoestrogens present in *Asparagus racemosus*.

Ligand	mol wt.	H-bond donor	H- bond acceptor	QPlogB B Po/w	QPlogH ER HERG	QPP Cacao	QPlogKhs a	%oral absorp tion
Rutin	254.2	2	5.5	-4.213	842.1	-0.571	-3.08	80%
Genistein	270.7	2	5.2	-4.135	468.5	-0.876	-3.55	75%
Genistein7-O-glucuronide	446.3	5	12.2	-4.712	37.43	-2.18	-5.8	17%
Hyperoside	464.3	6	9.7	-6.067	7.201	-3.708	-6.99	9.6%
Kaempferol	286.2	1	4	-4.523	136.1	-1.553	-4.513	67.7%
Quercetin-3'-glucuronide	478.3	5	11.7	-4.991	6.036	-3.154	-7143	2.6%
Quercetin	302.2	1	3.75	-4.796	58.82	-2.074	-5.125	61.01 %
Resveratrol	228.2	3	2.25	-3.004	395.7	-0.807	-3.949	75%
Spiraeoside	464.3	6	12.2	-5.601	23.28	-2.074	-6.1	15%

Pharmacokinetics activities of the most phytoestrogens are found to be excellent, with high oral percentage. Rutin having 80% oral absorption and Quercetin-3'-glucuronide having 2.6% oral absorption. ADME study revealed that most of flavonoids having good oral absorption (Genistein 75% and Resveratrol 75%). All the phytoestrogens compound moderate to lowest cell permeability.

CHAPTER 5.0

Summary

CHAPTER 5.0

Summary

Cancer is one of the leading causes of death worldwide. Breast cancer is the most common type of cancer and second in causes of death in world. Most of cases occur of death in developed countries due to their genetic character and life style. Main cause of Breast cancer is over expression of estrogen receptor. Currently allopathic drugs (selective estrogen receptor modulator and selective estrogen receptor down regulator) for treating Breast cancer cause number of side effects, hence people are now looking towards the herbal medicine. Phytoestrogen is best choice for decrease the side effects of allopathic drugs like tamoxifen and raloxifene. Phytoestrogen is the class of natural products, have structure similarity to estradiol and possess weak estrogenic properties. Genistein an isoflavone which regarded as phytoestrogen at higher doses reduced the proliferation of MCF-7 (Breast cancer cell lines). *Asparagus racemosus* was select for investigation of anticancer activity because presence of large fraction of phytoestrogens like genistein, Daidzein and steroidal saponins.

Various extracts of *Asparagus racemosus* were prepared by using different solvent basis of polarity. The preliminary phytochemical investigation of extracts of *Asparagus racemosus* demonstrated that methanolic extract was contain most of phytochemicals. All extracts were subjected to *in vitro* anticancer activity studies using T-47D cells lines by MTT assay. Methanolic extract and methanolic water extract showed the significant anticancer activity by MTT assay. Methanolic water extract showed maximum activity with IC_{50} 8.89 µg/ml. and methanolic extract and methanolic water were selected for isolation of phytoestrogens. RM01 to RM04 were isolated from methanolic extract and RM05 to RM07 were isolated from methanolic – water extract.

The molecular docking indicated the reported phytoestrogens from *Asparagus racemosus* have shown strong affinity for estrogen receptor α and estrogen receptor β. Phytoestrogens decrease the endogenous availability of the oestradiol by inhibiting its biosynthesis. Rutin showed the maximum docking score (-

13.76kcal/mol) as compare standard drug tamoxifen and raloxifene in estrogen receptor α. In case of estrogen receptor beta Quercetin showed maximum docking score (-11.55kcal/mol) as compare to oestradiol (-10.73kcal/mol). Docking results suggested that phytoestrogens can be a potential candidate for controlling tumor progression with a special emphasis in breast cancer progression. There is additional need to perform *in- vitro* and *in- vivo* bioassays for the establishment of phytoestrogens from *Asparagus racemosus* in the search of lead in the evolution of cancer chemotherapy.

CHAPTER 6.0

REFERENCES

CHAPTER 6.0

References

Acconcia, F., & Kumar, R. (2006). Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Letters,* ***238***(1), 1-14.

Acharya, S., Acharya, N., Bhangale, J., Shah, S., & Pandya, S. (2012). Antioxidant and hepatoprotective action of *Asparagus racemosus* Willd. root extracts. *Indian Journal of Experimental Biology* 795-801.

Agrawal, A., Sharma, M., Rai, S. K., Singh, B., Tiwari, M., & Chandra, R. (2008). The effect of the aqueous extract of the roots of *Asparagus racemosus* on hepatocarcinogenesis initiated by diethylnitrosamine. *Phytotherapy research,* ***22***(9), 1175-1182.

Alok, S., Jain, S. K., Verma, A., Kumar, M., Mahor, A., & Sabharwal, M. (2013). Plant profile, phytochemistry and pharmacology of *Asparagus racemosus* (Shatavari): A review. *Asian Pacific journal of Tropical Disease,* ***3***(3), 242-251.

Anderson, K. N., Schwab, R. B., & Martinez, M. E. (2014). Reproductive risk factors and breast cancer subtypes: a review of the literature. *Breast cancer Research and Treatment,* ***144***(1), 1-10.

Azizi, E., Granger, J., Harouaka, R., Luther, T. K., & Wicha, M. S. (2018). Stem Cells in Breast Development and Cancer *The Breast (Fifth Edition)* (pp. 308-314. e302): Elsevier.

Baglia, M. L., Tang, M.-T. C., Malone, K. E., Porter, P., & Li, C. I. (2018). Family History and Risk of Second Primary Breast Cancer after In Situ Breast Carcinoma. *Cancer Epidemiology and Prevention Biomarkers*, cebp. 0837.2017.

Bailey, L. R., Roodi, N., Verrier, C. S., Yee, C. J., Dupont, W. D., & Parl, F. F. (1998). Breast cancer and CYPIA1, GSTM1, and GSTT1 polymorphisms: evidence of a lack of association in Caucasians and African Americans. *Cancer Research,* ***58***(1), 65-70.

Baldwin, I. T. (2004). A most productive passion for natural history. *Science,* ***303***(5660), 958-960.

Bansode, F. W., Arya, K. R., Singh, R. K., & Narender, T. (2015). Dose-dependent effects of Asparagus adscendens root (AARR) extract on the anabolic, reproductive, and sexual behavioral activity in rats. *Pharmaceutical biology, **53***(2), 192-200.

Baralt, L. B., & McCormick, S. (2010). A review of advocate–scientist collaboration in federally funded environmental breast cancer research centers. *Environmental health perspectives, **118***(12), 1668.

Bierie, B., Pierce, S. E., Kroeger, C., Stover, D. G., Pattabiraman, D. R., Thiru, P., Donaher, J. L., Reinhardt, F., Chaffer, C. L., & Keckesova, Z. (2017). Integrin-β4 identifies cancer stem cell-enriched populations of partially mesenchymal carcinoma cells. *Proceedings of the National Academy of Sciences, **114***(12), E2337-E2346.

Bjornstrom, L., & Sjoberg, M. (2005). Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Molecular endocrinology, **19***(4), 833-842.

Blanton, S. (2018). Do Pharmacological Doses of Phytoestrogens Exert a Negative Effect on Motor Coordination in Aging Brains?

Bodai, B. I., & Tuso, P. (2015). Breast cancer survivorship: a comprehensive review of long-term medical issues and lifestyle recommendations. *The Permanente Journal, **19***(2), 48.

Bopana, N., & Saxena, S. (2007). *Asparagus racemosus*—Ethnopharmacological evaluation and conservation needs. *Journal of Ethnopharmacology, **110***(1), 1-15.

Breyer, J. Z., Wendland, E. M., Kops, N. L., Caleffi, M., & Hammes, L. S. (2018). Assessment of potential risk factors for breast cancer in a population in Southern Brazil. *Breast cancer research and treatment*, 1-7.

Burstein, H. J., Lacchetti, C., & Griggs, J. J. (2016). Adjuvant Endocrine Therapy for Women With Hormone Receptor–Positive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline Update on Ovarian Suppression Summary. *Journal of oncology practice, **12***(4), 390-393.

Bushnell, C. D., & Goldstein, L. B. (2004). Risk of ischemic stroke with tamoxifen treatment for breast cancer A meta-analysis. *Neurology, **63***(7), 1230-1233.

Carreau, S., Lambard, S., Delalande, C., Denis-Galeraud, I., Bilinska, B., & Bourguiba, S. (2003). Aromatase expression and role of estrogens in male gonad: a review. *Reproductive Biology and Endocrinology, **1***(1), 35.

Catalano, S., Marsico, S., Giordano, C., Mauro, L., Rizza, P., Panno, M. L., & Andò, S. (2003). Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *Journal of Biological Chemistry, **278***(31), 28668-28676.

Chawla, A., Chawla, P., Mangalesh, P., & Roy, R. (2011). *Asparagus racemosus* (Willd): biological activities & its active principles. *Indo Global J Pharm Sci, **1***(2), 113-120.

Chen, X., Uzuner, U., Li, M., Shi, W., Yuan, J. S., & Dai, S. Y. (2016). Phytoestrogens and Mycoestrogens Induce Signature Structure Dynamics Changes on Estrogen Receptor α. *International Journal of Environmental Research and Public health, **13***(9), 869.

Clavel, J. (2007). Progress in the epidemiological understanding of gene–environment interactions in major diseases: cancer. *Comptes rendus biologies, **330***(4), 306-317.

Corner, J., & Bailey, C. D. (2009). *Cancer nursing: care in context*: John Wiley & Sons.

Corso, G., Feroce, I., Intra, M., Toesca, A., Magnoni, F., Sargenti, M., Naninato, P., Caldarella, P., Pagani, G., & Vento, A. (2018). BRCA1/2 germline missense mutations: a systematic review. *European Journal of Cancer Prevention, **27***(3), 279-286.

Coyle, Y. M. (2004). The effect of environment on breast cancer risk. *Breast cancer Research and Treatment, **84***(3), 273-288.

Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., Norton, L., Nickelsen, T., Bjarnason, N. H., & Morrow, M. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. *Jama, **281***(23), 2189-2197.

Da Rocha, A. B., Lopes, R. M., & Schwartsmann, G. (2001). Natural products in anticancer therapy. *Current Opinion in Pharmacology, **1***(4), 364-369.

Dal Maso, L., Zucchetto, A., Talamini, R., Serraino, D., Stocco, C. F., Vercelli, M., Falcini, F., Franceschi, S., factors, P. A. o. C. c. s. o. E., & group, h. s. (2008). Effect of obesity and other lifestyle factors on mortality in women with breast cancer. *International Journal of Cancer, **123***(9), 2188-2194.

Dent, R., Trudeau, M., Pritchard, K. I., Hanna, W. M., Kahn, H. K., Sawka, C. A., Lickley, L. A., Rawlinson, E., Sun, P., & Narod, S. A. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clinical Cancer Research, **13***(15), 4429-4434.

DeSantis, C., Ma, J., Bryan, L., & Jemal, A. (2014). Breast cancer statistics, 2013. *CA: a Cancer Journal for Clinicians, **64***(1), 52-62.

Diez-Perez, A. (2006). Selective estrogen receptor modulators (SERMS). *Arquivos Brasileiros de Endocrinologia & Metabologia, **50***(4), 720-734.

Duffy, M. J. (2006). Estrogen receptors: role in breast cancer. *Critical reviews in Clinical Laboratory Sciences, **43***(4), 325-347.

Duke, J. A., Cseke, L. J., Warber, S., Kirakosyan, A., Brielmann, H. L., & Kaufman, P. B. (2016). *Natural Products from Plants*: CRC press.

Dutertre, M., & Smith, C. L. (2000). Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *Journal of Pharmacology and Experimental Therapeutics, **295***(2), 431-437.

Dutta, A., Ghoshal, A., Mandal, D., Mondal, N. B., Banerjee, S., Sahu, N. P., & Mandal, C. (2007). Racemoside A, an anti-leishmanial, water-soluble, natural steroidal saponin, induces programmed cell death in Leishmania donovani. *Journal of Medical Microbiology, **56***(9), 1196-1204.

Dzubak, P., Hajduch, M., Vydra, D., Hustova, A., Kvasnica, M., Biedermann, D., Markova, L., Urban, M., & Sarek, J. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. *Natural product reports, **23***(3), 394-411.

Edwards, S. E., da Costa Rocha, I., Williamson, E. M., & Heinrich, M. (2015). Shatavari *Asparagus racemosus* Willd. *Phytopharmacy: An Evidence-Based Guide to Herbal Medicinal Products*, 354.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjöld, M., & Gustafsson, J.-A. k. (1997). Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. *The Journal of Clinical Endocrinology & Metabolism,* ***82***(12), 4258-4265.

Filardo, E. J. (2002). Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *The Journal of steroid Biochemistry and Molecular Biology,* ***80***(2), 231-238.

Ford, D., Easton, D., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D., Weber, B., Lenoir, G., & Chang-Claude, J. (1998). Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. *The American Journal of Human Genetics,* ***62***(3), 676-689.

Gabriel, J. (2007). Predisposing Factors to Developing Cancer. *The Biology of Cancer, Second Edition*, 11-21.

Gabriel, J. (2008). What is Cancer? *The Biology of Cancer, Second Edition*, 3-9.

Gautam, M., Diwanay, S., Gairola, S., Shinde, Y., Patki, P., & Patwardhan, B. (2004). Immunoadjuvant potential of *Asparagus racemosus* aqueous extract in experimental system. *Journal of Ethnopharmacology,* ***91***(2-3), 251-255.

Gautam, M., Saha, S., Bani, S., Kaul, A., Mishra, S., Patil, D., Satti, N., Suri, K., Gairola, S., & Suresh, K. (2009). Immunomodulatory activity of *Asparagus racemosus* on systemic Th1/Th2 immunity: implications for immunoadjuvant potential. *Journal of Ethnopharmacology,* ***121***(2), 241-247.

Geisler, J., King, N., Anker, G., Ornati, G., Di Salle, E., Lønning, P., & Dowsett, M. (1998). In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. *Clinical Cancer Research,* ***4***(9), 2089-2093.

Goel, R., Prabha, T., Kumar, M. M., Dorababu, M., & Singh, G. (2006). Teratogenicity of *Asparagus racemosus* Willd. root, a herbal medicine.

Gohil, K., & Patel, J. (2010). A review on Bacopa monniera: current research and future prospects. *International Journal of Green Pharmacy,* ***4***(1), 1.

Goyal, R., Singh, J., & Lal, H. (2003). *Asparagus racemosus*-An update. *Indian Journal of Medical Sciences,* ***57***(9), 408-414.

Hall, J. M., Couse, J. F., & Korach, K. S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. *Journal of Biological Chemistry,* ***276***(40), 36869-36872.

Harvey, A. L., Edrada-Ebel, R., & Quinn, R. J. (2015). The re-emergence of natural products for drug discovery in the genomics era. *Nature Reviews Drug Discovery,* ***14***(2), 111.

Hayes, P. Y., Jahidin, A. H., Lehmann, R., Penman, K., Kitching, W., & De Voss, J. J. (2008). Steroidal saponins from the roots of *Asparagus racemosus*. *Phytochemistry,* ***69***(3), 796-804.

Hindi, N., Cordero, N., & Espinosa, E. (2013). Thromboembolic disease in cancer patients. *Supportive Care in Cancer,* ***21***(5), 1481-1486.

Hooning, M. J., Botma, A., Aleman, B. M., Baaijens, M. H., Bartelink, H., Klijn, J. G., Taylor, C. W., & Van Leeuwen, F. E. (2007). Long-term risk of cardiovascular disease in 10-year survivors of breast cancer. *Journal of the National Cancer Institute,* ***99***(5), 365-375.

Howell, S. J., Johnston, S. R., & Howell, A. (2004). The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. *Best practice & Research Clinical Endocrinology & Metabolism,* ***18***(1), 47-66.

Jagannath, N., Chikkannasetty, S. S., Govindadas, D., & Devasankaraiah, G. (2012). Study of antiurolithiatic activity of *Asparagus racemosus* on albino rats. *Indian journal of pharmacology,* ***44***(5), 576.

Jalsrai, A., Numakawa, T., Kunugi, H., Dieterich, D., & Becker, A. (2016). The neuroprotective effects and possible mechanism of action of a methanol extract from *Asparagus cochinchinensis*: *In vitro* and *in vivo* studies. *Neuroscience, **322***, 452-463.

James, V., McNeill, J., Lai, L., Newton, C., Ghilchik, M., & Reed, M. (1987). Aromatase activity in normal breast and breast tumor tissues: *in vivo* and *in vitro studies*. *Steroids, **50***(1-3), 269-279.

Johnston, S., Pippen Jr, J., Pivot, X., Lichinitser, M., Sadeghi, S., Dieras, V., Gomez, H. L., Romieu, G., Manikhas, A., & Kennedy, M. J. (2009). Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor–positive metastatic breast cancer. *Journal of Clinical Oncology, **27***(33), 5538-5546.

Jose, J., Sudhakaran, S., Kumar, S., Jayaraman, S., & Variyar, E. J. (2014). A comparative evaluation of anticancer activities of flavonoids isolated from Mimosa pudica, Aloe vera and Phyllanthus niruri against human breast carcinoma cell line (MCF-7) using MTT assay. *International Journal of Pharmacy and Pharmaceutical Sciences, **6***(2), 319-322.

Joshi, R. K. (2016). *Asparagus racemosus* (Shatawari), phytoconstituents and medicinal importance, future source of economy by cultivation in Uttrakhand: A review. *Inter. J. Herb. Med, **4***(4), 18-21.

Jung, K.-W., Won, Y.-J., Oh, C.-M., Kong, H.-J., Lee, D. H., & Lee, K. H. (2017). Cancer statistics in Korea: incidence, mortality, survival, and prevalence in 2014. *Cancer Research and Treatment: official journal of Korean Cancer Association, **49***(2), 292.

Kamat, J. P., Boloor, K. K., Devasagayam, T. P., & Venkatachalam, S. (2000). Antioxidant properties of *Asparagus racemosus* against damage induced by γ-radiation in rat liver mitochondria. *Journal of ethnopharmacology, **71***(3), 425-435.

Kaur, S., & Mondal, P. (2014). Study of total phenolic and flavonoid content, antioxidant activity and antimicrobial properties of medicinal plants. *J Microbiol Exp, **1***(1), 00005.

Kaur, S., Sachdeva, H., Dhuria, S., Sharma, M., & Kaur, T. (2010). Antileishmanial effect of cisplatin against murine visceral leishmaniasis. *Parasitology international,* ***59***(1), 62-69.

Kaushik, N. K., Bagavan, A., Rahuman, A. A., Mohanakrishnan, D., Kamaraj, C., Elango, G., Zahir, A. A., & Sahal, D. (2013). Antiplasmodial potential of selected medicinal plants from eastern Ghats of South India. *Experimental Parasitology,* ***134***(1), 26-32.

Khalis, M., Charbotel, B., Chajès, V., Rinaldi, S., Moskal, A., Biessy, C., Dossus, L., Huybrechts, I., Fort, E., & Mellas, N. (2018). Menstrual and reproductive factors and risk of breast cancer: A case-control study in the Fez region, Morocco. *PloS one,* ***13***(1), e0191333.

Khan, D., & Ansar Ahmed, S. (2016). The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Frontiers in Immunology,* ***6***, 635.

Khanna, A., Chander, R., & Kapoor, N. (1991). Hypolipidemic activity of Abana in rats. *Fitoterapia,* ***62***(3), 271-275.

Ko, E. (2014). *Effects of Long-acting Insulin Analogues on Breast and Colon Cancer Promotion.*

Kohn, E. C., & Liotta, L. A. (1995). Molecular insights into cancer invasion: strategies for prevention and intervention. *Cancer research,* ***55***(9), 1856-1862.

Kumar, M., Udupa, A., Sammodavardhana, K., Rathnakar, U., Shvetha, U., & Kodancha, G. P. (2010). Acute toxicity and diuretic studies of the roots of *Asparagus racemosus* Willd in rats. *West Indian Medical Journal,* ***59***(1), 03-06.

Kumar, S., Mehla, R., & Dang, A. (2008). Use of shatavari (*Asparagus racemosus*) as a galactopoietic and therapeutic herb–a review. *Agric. Rev,* ***29***(2), 132-138.

Kumar, V., Abbas, A. K., & Aster, J. C. (2017). *Robbins Basic Pathology E-Book*: Elsevier Health Sciences.

Lam, P. C.-H., Abagyan, R., & Totrov, M. (2018). Ligand-biased ensemble receptor docking (LigBEnD): a hybrid ligand/receptor structure-based approach. *Journal of Computer-aided Molecular Design,* ***32***(1), 187-198.

Lee, S., & Barron, M. G. (2017). Structure-based understanding of binding affinity and mode of estrogen receptor α agonists and antagonists. *PloS one,* ***12***(1), e0169607.

Lewis, J. S., & Jordan, V. C. (2005). Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* ***591***(1), 247-263.

Lima, S. M. R. R., Campaner, A. B., & Auge, A. P. F. (2017). Isoflavones derived from Glycine max (L.) Merr. in the treatment of vaginal atrophy: A new frontier. *Revista da Associação Médica Brasileira,* ***63***(9), 727-728.

Loibl, S., & Gianni, L. (2017). HER2-positive breast cancer. *The Lancet,* ***389***(10087), 2415-2429.

Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., & Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences,* ***90***(23), 11162-11166.

Magee, P. J., & Rowland, I. R. (2004). Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *British Journal of Nutrition,* ***91***(4), 513-531.

Mandal, D., Banerjee, S., Mondal, N. B., Chakravarty, A. K., & Sahu, N. P. (2006). Steroidal saponins from the fruits of *Asparagus racemosus*. *Phytochemistry,* ***67***(13), 1316-1321.

Mandal, S. C., CK, A. K., Lakshmi, S. M., Sinha, S., Murugesan, T., Saha, B., & Pal, M. (2000). Antitussive effect of *Asparagus racemosus* root against sulfur dioxide-induced cough in mice. *Fitoterapia,* ***71***(6), 686-689.

Marmot, M., Atinmo, T., Byers, T., Chen, J., Hirohata, T., Jackson, A., James, W., Kolonel, L., Kumanyika, S., & Leitzmann, C. (2007). Food, nutrition, physical activity, and the prevention of cancer: a global perspective.

Márquez-Garbán, D. C., Chen, H.-W., Fishbein, M. C., Goodglick, L., & Pietras, R. J. (2007). Estrogen receptor signaling pathways in human non-small cell lung cancer. *Steroids, **72***(2), 135-143.

Matthews, J., & Gustafsson, J.-Å. (2006). Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nuclear Receptor Signaling, **4***.

Maughan, K. L., Lutterbie, M. A., & Ham, P. S. (2010). Treatment of breast cancer. *Chemotherapy, **51***, 53.

McPherson, K., Steel, C., & Dixon, J. (2000). ABC of breast diseases: breast cancer—epidemiology, risk factors, and genetics. *BMJ: British Medical Journal, **321***(7261), 624.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., & Ding, W. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science, **266***(5182), 66-71.

Mitra, S. K., Prakash, N. S., & Sundaram, R. (2012). Shatavarins (containing Shatavarin IV) with anticancer activity from the roots of *Asparagus racemosus*. *Indian Journal of Pharmacology, **44***(6), 732.

Mitwally, M. F., & Casper, R. F. (2001). Use of an aromatase inhibitor for induction of ovulation in patients with an inadequate response to clomiphene citrate. *Fertility and Sterility, **75***(2), 305-309.

Mom, C. H., Buijs, C., Willemse, P. H., Mourits, M. J., & de Vries, E. G. (2006). Hot flushes in breast cancer patients. *Critical reviews in Oncology/Hematology, **57***(1), 63-77.

Moore, E., Wisniewski, A., & Dobs, A. (2003). Endocrine treatment of transsexual people: a review of treatment regimens, outcomes, and adverse effects. *The Journal of Clinical Endocrinology & Metabolism, **88***(8), 3467-3473.

Mosselman, S., Polman, J., & Dijkema, R. (1996). ERβ: identification and characterization of a novel human estrogen receptor. *FEBS letters, **392***(1), 49-53.

Mungenast, F., Aust, S., Vergote, I., Vanderstichele, A., Sehouli, J., Braicu, E., Mahner, S., Castillo‑Tong, D. C., Zeillinger, R., & Thalhammer, T. (2017). Clinical significance of the estrogen-modifying enzymes steroid sulfatase and estrogen sulfotransferase in epithelial ovarian cancer. *Oncology letters, **13***(6), 4047-4054.

Nakata, T., Takashima, S., Shiotsu, Y., Murakata, C., Ishida, H., Akinaga, S., Li, P.-K., Sasano, H., Suzuki, T., & Saeki, T. (2003). Role of steroid sulfatase in local formation of estrogen in post-menopausal breast cancer patients. *The Journal of Steroid Biochemistry and Molecular Biology, **86***(3-5), 455-460.

Narod, S. A., Iqbal, J., Giannakeas, V., Sopik, V., & Sun, P. (2015). Breast cancer mortality after a diagnosis of ductal carcinoma in situ. *JAMA oncology, **1***(7), 888-896.

Nickels, S., Truong, T., Hein, R., Stevens, K., Buck, K., Behrens, S., Eilber, U., Schmidt, M., Häberle, L., & Vrieling, A. (2013). Evidence of gene–environment interactions between common breast cancer susceptibility loci and established environmental risk factors. *PLoS genetics, **9***(3), e1003284.

Noble, L. S., Takayama, K., Zeitoun, K. M., Putman, J. M., Johns, D. A., Hinshelwood, M. M., Agarwal, V. R., Zhao, Y., Carr, B. R., & Bulun, S. E. (1997). Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *The Journal of Clinical Endocrinology & Metabolism, **82***(2), 600-606.

Nussbaumer, P., & Billich, A. (2004). Steroid sulfatase inhibitors. *Medicinal research reviews, **24***(4), 529-576.

Oncology, A. S. o. C. (2016). The state of cancer care in America, 2016: A report by the American Society of Clinical Oncology. *Journal of oncology practice, **12***(4), 339-383.

Organization, W. H. (2016). *World health statistics 2016: monitoring health for the SDGs sustainable development goals*: World Health Organization.

Pagadala, N. S., Syed, K., & Tuszynski, J. (2017). Software for molecular docking: a review. *Biophysical reviews, **9***(2), 91-102.

Paganini-Hill, A., & Clark, L. J. (2000). Eye problems in breast cancer patients treated with tamoxifen. *Breast Cancer Research and Treatment, **60***(2), 167-172.

Pahwa, P., & Goel, R. K. (2016). Ameliorative effect of *Asparagus racemosus* root extract against pentylenetetrazol-induced kindling and associated depression and memory deficit. *Epilepsy & Behavior, **57***, 196-201.

Palanisamy, N., & Manian, S. (2012). Protective effects of *Asparagus racemosus* on oxidative damage in isoniazid-induced hepatotoxic rats: an in vivo study. *Toxicology and Industrial Health, **28***(3), 238-244.

Pandey, S., Sahay, A., Pandey, R., & Tripathi, Y. (2005). Effect of *Asparagus racemosus* rhizome (Shatavari) on mammary gland and genital organs of pregnant rat. *Phytotherapy Research, **19***(8), 721-724.

Park, M., Cheon, M. S., Kim, S. H., Chun, J. M., Lee, A. Y., Moon, B. C., Yoon, T., Choo, B. K., & Kim, H. K. (2011). Anticancer activity of Asparagus cochinchinensis extract and fractions in HepG2 cells. *Journal of the Korean Society for Applied Biological Chemistry, **54***(2), 188-193.

Parks, R., Derks, M., Bastiaannet, E., & Cheung, K. (2018). Breast Cancer Epidemiology *Breast Cancer Management for Surgeons* (pp. 19-29): Springer.

Parsons, I. C., Lansing, J., Bosques, C. J., & Beccati, D. (2008). Comparative analysis of protein conformations by using 2D NOESY NMR spectra: Google Patents.

Patel, L., & Patel, R. (2013). Antimicrobial Activity of *Asparagus racemosus* Wild From Leaf Extracts–a Medicinal Plant. *International Journal of Scientific and Research Publications, **3***(3), 2250-3153.

Perez G, R., Zavala S, M., Perez G, S., & Perez G, C. (1998). Antidiabetic effect of compounds isolated from plants. *Phytomedicine, **5***(1), 55-75.

Piccinato, C. A., Neme, R. M., Torres, N., Sanches, L. R., Derogis, P. B. M. C., Brudniewski, H. F., e Silva, J. C. R., & Ferriani, R. A. (2016). Effects of steroid hormone on estrogen sulfotransferase and on steroid sulfatase expression in endometriosis tissue and stromal cells. *The Journal of steroid biochemistry and molecular biology, **158***, 117-126.

Plangsombat, N., Rungsardthong, K., Kongkaneramit, L., Waranuch, N., & Sarisuta, N. (2016). Anti-inflammatory activity of liposomes of *Asparagus racemosus* root extracts prepared by various methods. *Experimental and Therapeutic Medicine, **12***(4), 2790-2796.

Pons, D. G., Nadal-Serrano, M., Torrens-Mas, M., Oliver, J., & Roca, P. (2016). The phytoestrogen genistein affects breast cancer cells treatment depending on the ERα/ERβ ratio. *Journal of Cellular Biochemistry, **117***(1), 218-229.

Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., & Prossnitz, E. R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science, **307***(5715), 1625-1630.

Rizzo, F. M., Childs, A., Mandair, D., Khan, M. S., Cives, M., Ensell, L., Lowe, H., Caplin, M. E., Toumpanakis, C., & Meyer, T. (2017). Circulating Tumour Cells (CTCs) are associated with bone metastases in patients with Neuroendocrine Tumours (NET).

Rodríguez-Landa, J. F., Cueto-Escobedo, J., Puga-Olguín, A., Rivadeneyra-Domínguez, E., Bernal-Morales, B., Herrera-Huerta, E. V., & Santos-Torres, A. (2017). The Phytoestrogen Genistein Produces Similar Effects as 17β-Estradiol on Anxiety-Like Behavior in Rats at 12 Weeks after Ovariectomy. *BioMed research international, **2017***.

Rollerova, E., & Urbancikova, M. (2000). Intracellular estrogen receptors, their characterization and function. *Endocr Regul, **34***(4), 203-218.

Rudel, R. A., Attfield, K. R., Schifano, J. N., & Brody, J. G. (2007). Chemicals causing mammary gland tumors in animals signal new directions for epidemiology, chemicals testing, and risk assessment for breast cancer prevention. *Cancer, **109***(S12), 2635-2666.

Russo, J., & Russo, I. H. (2004). *Molecular Basis of Breast Cancer: Prevention and Treatment*: Springer Science & Business Media.

Sabde, S., Bodiwala, H. S., Karmase, A., Deshpande, P. J., Kaur, A., Ahmed, N., Chauthe, S. K., Brahmbhatt, K. G., Phadke, R. U., & Mitra, D. (2011). Anti-HIV activity of Indian medicinal plants. *Journal of Natural Medicines,* ***65***(3-4), 662-669.

Sachan, A. K., Das, D. R., Dohare, S. L., & Shuaib, M. (2012). *Asparagus racemosus* (Shatavari): an overview. *International Journal Of Pharmaceutical And Chemical Sciences,* ***1***(2), 588-592.

Sachdeva, H., Sehgal, R., & Kaur, S. (2014). *Asparagus racemosus* ameliorates cisplatin induced toxicities and augments its antileishmanial activity by immunomodulation in vivo. *Parasitology International,* ***63***(1), 21-30.

Saito, R., Abe, H., Kunita, A., Yamashita, H., Seto, Y., & Fukayama, M. (2017). Overexpression and gene amplification of PD-L1 in cancer cells and PD-L1+ immune cells in Epstein–Barr virus-associated gastric cancer: the prognostic implications. *Modern Pathology,* ***30***(3), 427.

Setchell, K. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *The American Journal of Clinical Nutrition,* ***68***(6), 1333S-1346S.

Sharma, K. (2011). *Asparagus racemosus* (Shatavari): a versatile female tonic. *International Journal of Pharmaceutical & Biological Archive,* ***2***(3).

Shrestha, R., Shakya, A., & Shrestha, K. K. (2018). Phytochemical Screening and Antimicrobial Activity Of *Asparagus racemosus* Willd. and Asparagus Curillus Buch.-Ham. Ex Roxb. *Journal of Natural History Museum,* ***29***, 91-102.

Shukla, N., Jones, R., Persad, R., Angelini, G. D., & Jeremy, J. Y. (2005). Effect of sildenafil citrate and a nitric oxide donating sildenafil derivative, NCX 911, on cavernosal relaxation and superoxide formation in hypercholesterolaemic rabbits. *European Journal of Pharmacology,* ***517***(3), 224-231.

Siegel, R. L., Miller, K. D., & Jemal, A. (2015). Cancer statistics, 2015. *CA: a cancer journal for clinicians,* ***65***(1), 5-29.

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA: a cancer journal for clinicians,* ***66***(1), 7-30.

Siegel, R. L., Miller, K. D., & Jemal, A. (2018). Cancer statistics, 2018. *CA: a cancer journal for clinicians, **68***(1), 7-30.

Simpson, E. R., Michael, M. D., Agarwal, V. R., Hinshelwood, M. M., Bulun, S. E., & Zhao, Y. (1997). Cytochromes P450 11: expression of the CYP19 (aromatase) gene: an unusual case of alternative promoter usage. *The FASEB journal, **11***(1), 29-36.

Singh, A. K., Srivastava, A., Kumar, V., & Singh, K. (2018). Phytochemicals, Medicinal and Food Applications of Shatavari (*Asparagus racemosus*): An Updated Review. *The Natural Products Journal, **8***(1), 32-44.

Singla, R., & Jaitak, V. (2014). Shatavari (*Asparagus racemosus* Wild): a review on its cultivation, morphology, phytochemistry and pharmacological importance. *International Journal of Pharmaceutical Sciences and Research, **5***(3), 742.

Singla, R., & Jaitak, V. (2015). Molecular docking simulation study of phytoestrogens from *Asparagus racemosus* in breast cancer progression. *International Journal of Pharmaceutical Sciences and Research, **6***(1), 172.

Singla, R., & Jaitak, V. (2017). Multitargeted Molecular Docking Study of Natural-Derived Alkaloids on Breast Cancer Pathway Components. *Current Computer-aided Drug Design, **13***(4), 294-302.

Sirotkin, A. V., & Harrath, A. H. (2014). Phytoestrogens and their effects. *European Journal of Pharmacology, **741***, 230-236.

Sithisarn, P., Rojsanga, P., Jarikasem, S., Tanaka, K., & Matsumoto, K. (2013). Ameliorative effects of Acanthopanax trifoliatus on cognitive and emotional deficits in olfactory bulbectomized mice: an animal model of depression and cognitive deficits. *Evidence-Based Complementary and Alternative Medicine, **2013***.

Smith, I. E., & Dowsett, M. (2003). Aromatase inhibitors in breast cancer. *New England Journal of Medicine, **348***(24), 2431-2442.

Stanway, S. J., Delavault, P., Purohit, A., Woo, L. L., Thurieau, C., Potter, B. V., & Reed, M. J. (2007). Steroid sulfatase: a new target for the endocrine therapy of breast cancer. *The oncologist, **12***(4), 370-374.

Stephens, P. J., Tarpey, P. S., Davies, H., Van Loo, P., Greenman, C., Wedge, D. C., Nik-Zainal, S., Martin, S., Varela, I., & Bignell, G. R. (2012). The landscape of cancer genes and mutational processes in breast cancer. *Nature,* ***486***(7403), 400.

Suzuki, T., Miki, Y., Nakamura, Y., Moriya, T., Ito, K., Ohuchi, N., & Sasano, H. (2005). Sex steroid-producing enzymes in human breast cancer. *Endocrine-related Cancer,* ***12***(4), 701-720.

Świderski, B., Kruk, M., Osowski, S., Wieczorek, G., Kurek, J., Chmielewski, L. J., & Orłowski, A. (2017). *Milk duct segmentation in microscopic HE images of Breast cancer Tissues.* Paper presented at the MATEC Web of Conferences.

Thakur, M., Chauhan, N. S., Bhargava, S., & Dixit, V. K. (2009). A comparative study on aphrodisiac activity of some ayurvedic herbs in male albino rats. *Archives of sexual behavior,* ***38***(6), 1009-1015.

Thakur, M., & Dixit, V. (2007). Effect of some vajikaran herbs on pendiculation activities and in vitro sperm count in male. *Sexuality and Disability,* ***25***(4), 203-207.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., & Jemal, A. (2015). Global cancer statistics, 2012. *CA: a cancer journal for clinicians,* ***65***(2), 87-108.

Tung, N., Battelli, C., Allen, B., Kaldate, R., Bhatnagar, S., Bowles, K., Timms, K., Garber, J. E., Herold, C., & Ellisen, L. (2015). Frequency of mutations in individuals with breast cancer referred for BRCA1 and BRCA2 testing using next-generation sequencing with a 25-gene panel. *Cancer,* ***121***(1), 25-33.

Tuszyńska, M. (2010). Pharmacological and therapeutic application of *Asparagus racemosus* Willd. *Herba Polonica,* ***56***(2), 92-104.

Uma, B., Prabhakar, K., & Rajendran, S. (2009). Anticandidal Activity of *Asparagus racemosus*. *Indian Journal of Pharmaceutical Sciences,* ***71***(3), 342-343.

Utsumi, T., Yoshimura, N., Takeuchi, S., Ando, J., Maruta, M., Maeda, K., & Harada, N. (1999). Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer. *Cancer Research,* ***59***(2), 377-381.

Venkatesan, N., Thiyagarajan, V., Narayanan, S., Arul, A., Raja, S., Kumar, S. V., Rajarajan, T., & Perianayagam, J. B. (2005). Anti-diarrhoeal potential of *Asparagus racemosus* wild root extracts in laboratory animals. *J Pharm Pharm Sci,* ***8***(1), 39-46.

Visavadiya, N., & Narasimhacharya, A. R. (2005). Hypolipidemic and antioxidant activities of *Asparagus racemosus* in hypercholesteremic rats. *Indian Journal of Pharmacology,* ***37***(6), 376.

Wagner, J. R., Walther, M. M., Linehan, W. M., White, D. E., Rosenberg, S. A., & Yang, J. C. (1999). Interleukin-2 based immunotherapy for metastatic renal cell carcinoma with the kidney in place. *The Journal of urology,* ***162***(1), 43-45.

Warasiha, B. (2008). Cytochrome P450 mRNA profile in human breast cancer cell lines.

Wärri, A. M., Huovinen, R. L., Laine, A. M., Martikainen, P. M., & Härkönen, P. L. (1993). Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro. *JNCI: Journal of the National Cancer Institute,* ***85***(17), 1412-1418.

Wculek, S. K., & Malanchi, I. (2015). Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature,* ***528***(7582), 413.

Wiboonpun, N., Phuwapraisirisan, P., & Tip-pyang, S. (2004). Identification of antioxidant compound from *Asparagus racemosus*. *Phytotherapy Research,* ***18***(9), 771-773.

Wieczorek, G., Świderski, B., Chmielewski, L. J., Kruk, M., & Orłowski, A. (2017). *Choice of distance function in the segmentation of regions of interest in microscopic images of breast tissues.* Paper presented at the Computational Problems of Electrical Engineering (CPEE), 2017 18th International Conference on.

Winer, E. P., Hudis, C., Burstein, H. J., Wolff, A. C., Pritchard, K. I., Ingle, J. N., Chlebowski, R. T., Gelber, R., Edge, S. B., & Gralow, J. (2005). American Society of Clinical Oncology technology assessment on the use of aromatase inhibitors as adjuvant therapy for postmenopausal women with hormone receptor–positive breast cancer: status report 2004. *Journal of Clinical Oncology, **23***(3), 619-629.

Witkowska-Zimny, M., & Kaminska-El-Hassan, E. (2017). Cells of human breast milk. *Cellular & Molecular Biology Letters, **22***(1), 11.

Wolff, A. C., Hammond, M. E. H., Hicks, D. G., Dowsett, M., McShane, L. M., Allison, K. H., Allred, D. C., Bartlett, J. M., Bilous, M., & Fitzgibbons, P. (2013). Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Archives of Pathology and Laboratory Medicine, **138***(2), 241-256.

Yi, P., Wang, Z., Feng, Q., Pintilie, G. D., Foulds, C. E., Lanz, R. B., Ludtke, S. J., Schmid, M. F., Chiu, W., & O'Malley, B. W. (2015). Structure of a biologically active estrogen receptor-coactivator complex on DNA. *Molecular cell, **57***(6), 1047-1058.

Yuriev, E., Holien, J., & Ramsland, P. A. (2015). Improvements, trends, and new ideas in molecular docking: 2012–2013 in review. *Journal of Molecular Recognition, **28***(10), 581-604.

Zhang, Y., Gaikwad, N. W., Olson, K., Zahid, M., Cavalieri, E. L., & Rogan, E. G. (2007). Cytochrome P450 isoforms catalyze formation of catechol estrogen quinones that react with DNA. *Metabolism-Clinical and Experimental, **56***(7), 887-894.

Zhu, X., Zhang, W., Pang, X., Wang, J., Zhao, J., & Qu, W. (2011). Hypolipidemic Effect of n-Butanol Extract from Asparagus officinalis L. in Mice fed a High-fat Diet. *Phytotherapy research, **25***(8), 1119-1124.

Appendix

Docking score of Phytoestrogens with Estrogen Receptor alpha (ERα)

Serial Number	Structures	Dock score	H bond	Lipophilic	Electro
1	Rutin	-13.7	-5.66	-5.72	-1.5
2	Kaempferol	-12.55	-5.64	-6.59	-0.81
3	Raloxifene	-12.15	-1.19	-7.49	-1.24
4	Tamoxifen	-11.63	-0.9	-7.64	-0.4
5	Spiraeoside	-11.18	-3.93	-5.47	-1.31
6	Genistein	-10.28	-1.69	-5.06	-0.75
7	Cyanidin 3-galactoside-5-glucoside	-10.12	-4.8	-4.53	-0.66
8	Daidzein	-10	-1.09	-2.7	-0.83
9	Genistein7-O-glucuronide	-9.68	-2.4	-2.13	-0.45
10	Quercetin-3'-glucuronide	-9.6	-2.88	-1.27	-0.23
11	Quercetin.	-9.48	-2.83	-0.98	-1.03
12	Shatavarin V	-9.41	-1.8	-4.59	-0.03
13	Shatavarin IX	-8.04	-2.74	-4.76	-1.58
14	Immunoside	-7.92	-2.15	-2.15	-1.09
15	Shatavaroside A	-7.87	-3.05	-4.57	-2.89
16	Shatavarin VIII	-7.49	-4.25	-2.41	-3.3
17	Racemoside B	-7.12	-2.42	-2.94	-4.32
18	Oligospantanoside	-6.79	-4.03	-1.36	-5.32

19	Shatavarin I	-6.74	-3.73	-1.87	-3.36
20	Filiasparoside C	-6.68	-1.87	-2.56	-2.52
21	Geldanamycin	-6.1	-1.03	-3.66	-5.03
22	Imunostimulant	-5.91	-3.61	-1.39	-1.09
23	Sitosterol	-5.7	-4.53	-1.0	-3.36
24	Sarsapogenin	-5.64	-1.58	-0.7	-2.07
25	Diosgenin	-5.34	-4.54	-1.66	-2.13
26	Shatavarin X	-5.17	-4.14	-0.7	-0.98
27	Hyperside	-4.58	-2.92	-0.98	-2.88
28	Racemoside C	-4.08	-3.35	-0.7	-1.15
29	Immunoside	-3.65	-3.36	-0.68	-0.55
30	Shatavarin VII	-2.95	-0.32	-3.73	-0.62

Docking score of Phytoestrogens with Estrogen Receptor Beta (ERβ)

Serial Number	Structures	Dock score	H bond	Lipophilic	Electro
1	Quercetin	-11.57	-2.84	-4.54	-1.38
2	Oestradiol	-10.73	-0.99	-6.27	-0.28
3	Kaempferol	-10.63	-2.04	-4.74	-1.06
4	Genistein	-10.55	-1.65	-4.79	-1.08
5	Shatavaroside A	-10.393	-4.56	-1.9	-2.34
6	Shatavaroside B	-10.341	-2.88	-2.1	-2.08
7	Oligospantanoside	-10.092		-1.72	-2.58
8	Daidzein	-9.95	-2.16	-4.81	--0.73
9	Racemoside A	-9.27	-1.28	-4.35	-0.96
10	Shatavarin VI	-9.884	-2.88	-2.7	-3.35
11	Immunostimulant	-9.517	-4.09	-2.38	-2.49
12	Querecetin-3-glucouronide	-9.472	-4.53	-2.33	-1.2
13	Sarsapogenin	-9.154	-2.53	-1.72	-1.79
14	Shatavarin I	-8.346	-1.92	-1.89	-1.41
15	Shatavarin X	-8.129	-2.41	-2.09	-2.88
16	Cyanidine-3-galactoside	-8.023	-1.85	-1.35	-1.49
18	Racemoside B	-7.618	-1.44	-2.48	-1.57
19	Shatavarin IX	-7.378	-1.62	-1.57	-2.67

20	Shatavarin VII	-7.331	-3.67	-1.79	-2.74
21	Diosgenin	-7.218	-1.48	-1.72	-3.31
22	Racemoside C	-7.061	-2.45	-2.53	-1.71
23	8-Methoxy-5,6,4-trihydroxyisoflavone7-O-β-D glucopyranoside	-6.991	-1.83	-0.25	-2.49
24	Imunostimulant 2	-6.958	-2.59	-2.63	-2.68
26	Geldanamycin	-6.576			-1.45
27	3,6,4'-trimethoxy-7-O-β-D-glucopyranosyl [1→4]-O-α-Dxylopyranoside glucopyranpsyl	-6.382	-0.48	-1.18	-1.84
28	Racemofuran	-6.241	-0.48	-1.98	-5.07
29	Racemosol	-6.241	-1.564	-1.93	-0.46
30 30	Spiraeoside	-6.181	-0.355	-1.45	-2.24
31	Filiasparoside B	-6.045	-0.35	-2.53	-1.9
32	Asparanin A	-5.945	-0.755	-0.9	-2.34
33	Resveratrol	-5.438	-0.642	-1.35	-2.08
34	Filiasparoside C	-5.45	-0.92	-2.41	-0.256
35	Shatavarin VIII	-5.01	0.19	-3.3	-0.166
36	Diosgenin	-4.471	-1.642	-1.69	-5.76

37	Sitosterol	-4.44	-0.77	-1.53	-4.47
38	Undecanoic Acid	-4.288	-0.72	-1.69	-7.27
39	beta-Sitosterol	-4.225	-0.044	-1.15	-5.51
40	Thymol	-4.108	-0.07	-1.19	-4.81
41	Carvone	-4.103	-1.15	-1.83	-6.09
42	Hyperoside	-3.92	-0.7	-1.31	-3.75
43	Eugenol	-3.872	-0.656	-1.38	-5.83
44	Perillaldehyde	-3.793	-0.755	-2.2	-5.94
45	Caryophyllene	-3.781	-0.78	-2.04	-3.84
46	P-Cymene	-3.69	-5.81	-4.54	-4.23
47	Didehydrostemofoline	-3.346	-0.96	-6.27	-2.27
48	Menthol	-3.257	-3.3	-4.74	-3.84
49	Geranyl Acetate	-3.196	-4.32	-4.79	-3.77
50	Linalool	-3.048	-3.84	-4.81	-3.04
51	Pinocarveol	-3.015	0	-4.41	-5.19
52	Geranial	-2.542	-1.59	-5.1	-3.61
53	Myrtenol	-2.506	-1.51	-4.67	-1.92
54	Eucalyptol	-2.224	-2.33	-4.21	-1.57
55	Undecyl Acetate	-2.011	-1.62	-3.52	-1.63