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ANTI-PROLIFERATIVE AND ANTI-ESTROGENIC EFFECTS OF *BOERHAAVIA DIFFUSA* L. IN HUMAN BREAST CANCER CELL LINES

PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

KUMARAGURU COLLEGE OF TECHNOLOGY

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PROJECT WORK -PHASE II

MAY 2010

This is to certify that the project entitled **ANTI-PROLIFERATIVE AND ANTI-ESTROGENIC EFFECTS OF *BOERHAAVIA DIFFUSA* L. IN HUMAN BREAST CANCER CELL LINES** is the bonafide record of project work done by **M.SHEENA EVANGALINE Register No: 0820203013** of M.Tech during the year 2009-2010.

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CERTIFICATE

This is to certify that **SHEENA EVANGALINE M**, Kumaraguru College of Technology, Coimbatore, has successfully completed her final year project work as a dissertation in partial fulfillment of the award of degree of Master of Technology in Biotechnology on **"ANTI-PROLIFERATIVE AND ANTI-ESTROGENIC EFFECTS OF *BOERHAAVIA DIFFUSA L.* EXTRACTS IN HUMAN BREAST CANCER CELL LINES"** under my guidance as per the **MOU** signed by her. If any publication or patent comes out of her work, **RGCB** will have the right to decide on its claim.

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I affirm that the project work titled **Anti-proliferative and anti-estrogenic effects of *boerhaavia diffusa* L. in human breast cancer cell lines** being submitted in partial fulfilment for the award of **M.Tech (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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First and foremost I thank God Almighty for his blessings for the successful completion of my project.

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M.SHEENA EVANGALINE

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Boerhaavia diffusa L. is a plant of tropical region used in Indian traditional medicine for the treatment of human ailments including abdominal tumor, jaundice, menstrual disorders etc. This plant also has anti-lymphoproliferative, anti-metastatic and immuno-modulatory effects. This study aims to assess the anti-proliferative and anti-estrogenic effects of various solvent extracts of *B.diffusa* in breast cancer cell lines. The effective concentration range of the extract in cell viability will be analyzed by MTT Assay. The ability of the extracts to alter the cell cycle phases and distribution will be studied using FACS analysis. The effect of the extracts on the selected estrogen responsive genes will be analyzed by RT-PCR. Treatment with varying concentrations of methanol extract (20-320 μg/ml) resulted in moderate to very strong growth inhibition in MCF-7 cell lines. Methanol extract treatment resulted in a remarkable increase in the number of MCF-7 cells in the G0-G1 fraction from 69.1 % to 75.8 %, with a reciprocal decrease of cells in all other phases indicating cell cycle arrest at G0-G1 phase. RT-PCR analysis revealed that the methanol extract reduced the mRNA expression of pS2 indicating its antiestrogenic action. The results demonstrate that B. diffusa possess antiproliferative and antiestrogenic properties and that it resides in the most polar extractant used ie., methanol and suggest that it may have therapeutic potential in estrogen dependent breast cancers

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AF1	Ligand-independent Activation Function1
ACS	American Cancer Society
ATCC	American Type Culture Collection
BCAS3	Breast Carcinoma Amplified Sequence 3
BDM	*Boerhaavia diffusa* Methanol
BMD	Bone Mineral Density
BMC	Bone Mineral Content
BRAC1	Breast Cancer Gene 1
BRAC2	Breast Cancer Gene 2
CBE	Clinical Breast Examination
CRC	Colorectal Carcinoma
CTS	Charcoal Treated Serum
cDNA	Complimentary DNA
dNTP	deoxynucleotide Triphosphate
DNA	deoxy-ribonucleic acid
DCIS	Ductal Carcinoma in Situ
DMBA	Dimethylbenzanthracene
DES	Diethylstillbesrol
DMSO	Dimethyl Sulphoxide
DMEM	Dulbecco's Modified Eagles Medium
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol

E2	Estradiol
E3	Estriol
EGF	Epidermal Growth Factor
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
ESR1	Estrogen receptor1
ESR2	Estrogen Receptor2
ERE	Estrogen Response Element
EtBr	Ethidium Bromide
ELISA	Enzyme Linked Immuno Sorbant Assay
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
FACS	Fluorescent Activated cell Sorting
HDL	High Density Lipoprotein
HRT	Hormone Replacement Therapy
HER	Human Epidermal Growth Factor
HeLa	Henrietta Lacks
IBC	Inflammatory Breast Carcinoma
LH	Leuteinizing hormone
LDL	Low Density Lipoprotein
LCIS	Lobular Carcinoma in Situ
MCF-7	Michigan Cancer Foundation

MTT	3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide
NR3A1	Nuclear Receptor Subfamily3, group A, member1
NR3A2	Nuclear Receptor Subfamily3, group A, member2
NTX	Urinary n-telopeptide
OCs	Oral Contraceptive Use
PTEN	Phosphatase and Tensin Homolog
PBMC	Peripheral Blood Mononuclear Cell
PBS-EDTA	Phosphate buffered saline- Ethylene Diamine Tetra Acetic acid
RNase	Ribonuclease
RIPA	Radio Immuno Percipitate Assay
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribo Nucleic Acid
SRC1	Steroid Receptor Co-Activator
SERMs	Selective Estrogen Receptor Modulators
TBE	Tris Borate EDTA
TGF-α	Transforming Growth Factor- Alpha
TP53	Tumor Protein 53
WHO	World Health Organization
XRE	Xenobiotic Response Element

INTRODUCTION

Breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death. Breast cancer is about 100 times as frequent among women as among men, but survival rates are equal in both sexes. It is now believed that genetic and/or hormonal factors are the primary risk factors for breast cancer. Breast cancer affects one in eight women during their lives. Breast cancer is mainly caused due to increase in estrogen in body.

Estrogens are a group of steroid compounds. Like all steroid hormones, estrogens readily diffuse across the cell membrane. Estrogens mainly 17β-estradiol exerts numerous pharmacological effects in a large number of targets including the breast, uterus, bone, brain and cardiovascular tissues. Most of their actions are mediated by estrogen receptor (ER) which exists in two different forms Er-α and Er-β which reside predominantly in the nucleus of target cells (Cheng *et al.,* 2004). Recently ERs have also been detected in the cellular membrane (Sreeja *et al.,* 2003; Zivadinovic *et al.,* 2005). About 80% of breast cancers rely on supplies of the hormone estrogen to grow, they are known as hormone-sensitive or hormone-receptor-positive cancers. Suppression of production in the body of estrogen is a treatment for these cancers. Antiestrogens work by binding to estrogen receptors, blocking estrogen from binding to these receptors. This also blocks estrogen from activating genes for specific growth-promoting proteins. So the genes that produce proteins involved in breast cancer are suppressed at translation level.

Phytoestrogens are widely known plant compounds, which bind to ER and affect the physiology of various systems. Phytoestrogens are secondary metabolites produced in a wide variety of plants that induce biologic responses in vertebrates and can mimic or modulate the actions of endogenous estrogens, usually by binding to estrogen receptors (Setchell *et al.*, 1998). The broad classes of phytoestrogenic compounds include isoflavonoids, coumestans,

quercetin, kaempferol), flavanones (e.g., naringenin), isoflavones (e.g., genistein, daidzein, equol), and anthocyanidins. Coumestrol is a coumestan and resveratrol, a stilbene (Kurzer and Xu 1997, Ross, Yang *et al.,* 2001). All are biphenolic compounds with structures resembling natural and synthetic estrogens. Soybeans and clover, alfalfa sprouts, and oilseeds (e.g., flaxseed) are the most significant dietary sources of isoflavones, coumestans, and lignans, respectively, but phytoestrogens are found in a wide number of foodstuffs such as soy beans, black beans, onions, and citrus fruits.

Epidemiologic evidence supports a protective effect of high phytoestrogen diets to reduce the incidence of certain hormone-responsive cancers, such as breast and prostate cancer (Adlercreutz, 2002). Relative to Asian countries, the incidence and mortality from breast cancer is much higher in the Western world. The contributing factors to this differential effect are various; however, several studies have focused on the environmental, and specifically dietary, differences among these two parts of the world. Immigration studies have shown that rates of breast cancer in first-generation immigrants are low, but they increase in the second and subsequent generations, presumably due to intake of an increasingly Western diet (Ziegler *et al.,* 1993, Shimizu *et al.,* 1991). Much of the epidemiologic studies have focused on isoflavones and soy, but interest is increasing in the protective effects of lignans in breast cancer (Adlercreutz, 2002). Average daily dietary intake of soy and isoflavones in Asian countries is estimated to be 50 g daily and 30 mg daily, respectively, while in the West the intake is closer to 1 g daily and 1 mg daily of each (Cos, 2003). The lower breast cancer incidence in Asian countries has also been correlated to early exposure to phytoestrogens through breast milk (Lamartiniere, 2002).

ERs can reduce the effect of potent endogenous estrogens such as estradiol when they are present in sufficient quantities, with the net effect of antagonizing the estrogen-responsive system (Katzenellenbogen *et al.,* 2003). The abundance of isoflavones relative to endogenous estrogens could explain their bioactivity despite their weaker binding. Setchell and Cassidy (Setchell *et al.,*1999) showed that consumption of 50 mg/day of isoflavones in an adult can give rise to plasma concentrations ranging from 200-3200 nM, whereas endogenous plasma estradiol levels range from 0.14–0.28 pmol/ml (Rowland *et al.,*2003). In addition, phytoestrogens have a weaker affinity for serum proteins such as albumin and sex hormone-binding globulin, which renders a relatively greater proportion of unbound phytoestrogens (Rowland *et al.,* 2003). A number of the pleiotropic molecular effects of phytoestrogens, including modulation of cell-signaling pathways, regulation of the cell cycle, stimulation of apoptosis, and antioxidant activities, may occur independently of ER binding (Sarkar *et al.,*2003, Magee *et al.,*2004).

Mounting evidence points out that phytoestrogens exert estrogenic effects on skeleton, affect favorably the cardiovascular function and menopausal symptoms while demonstrate antiestrogenic action on breast and prostate tissue (Finkel, 1998; Lissin *et al.,* 2000). This has led to the hypothesis that phytoestrogens may act as selective estrogen receptor modulators (SERMs) and can be considered therapeutically for the prevention of breast and prostate cancer, osteoporosis and cardiovascular diseases (Ewies, 2002; Middleton *et al.,* 2000).

However, the health consequences of phytoestrogens exposure is perhaps not universally beneficial and, in certain situations, could increase disease risk, particularly with use of dietary supplements that could provide exceedingly high levels of phytoestrogens relative to those provided in whole

to ameliorate menopausal symptoms, potential effects of natural antiestrogens on reproduction, and developmental effects on children prenatally (in the diet of the mother) and postnatally (e.g., in soy-based infant formulas; Whitten *et al.*,1995). The known divergent chemical and biological properties already identified of the phytoestrogens make it important to evaluate each individually and in the context of delivery as food or dietary supplement.

Phytoestrogens have the ability to bind the ER due to their biphenolic structure, which is required for the ligand receptor association (Leclercq *et al.*, 1979). They can act like partial ER agonists or antagonists. Several phytoestrogens show a pattern of differential binding to the two ER subtypes, ERa and ERb, with stronger binding to ERb as evaluated by radioligand-binding assay (Kuiper *et al.*, 1998). The ER is part of the steroid/retinoid receptor gene superfamily, a class of soluble DNA-binding proteins that acts as ligand-activated enhancer factors (Weihua *et al.*, 2003). On ligand binding, the ER initiates transcriptional activation by binding to specific palindromic sequences called estrogen-response elements (EREs) in the promoters of target genes (Klein-Hitpass *et al.*, 1986). However, non-ERE-driven transcriptional activation (e.g., via AP-1) has also been described (Paech *et al.*, 1997). The differential effects of agonists and antagonists on receptor activity in a given cell context have been ascribed to different conformations of the receptor ligand complex, as well as by differences in interaction with transcriptional coactivator and corepressor proteins and other transcription factors (Benassayag *et al.*,2002). Compounds showing varying degrees of agonist-antagonist activity, depending on the cell type and target gene, are referred to as selective ER modulators (Paech K, 1997, Jordan, 2003). Many phytoestrogenic compounds have additional cellular activities not ascribed to activation of the ERs, such as regulation of cell-signaling pathways, and can have activity in inhibiting proliferation and inducing apoptosis in ER-negative

Boerhaavia diffusa L. commonly known as 'Punarnava' is an abundant creeping weed found all over India (Chopra *et al.,* 1958). *Boerhaavia diffusa* L. (Spreading Hogweed in English), belonging to the family of the Nyctaginaceae, is mainly a diffused perennial herbaceous creeping weed of India (known also under its traditional name as *Punarnava*) and of Brazil (known as *Erva tostao*). *Boerhaavia diffusa* L. is up to 1 m long or more, having spreading branches. The stem is prostrate, woody or succulent, cylindrical, often purplish, hairy, and thickened at its nodes. The leaves are simple, thick, fleshy, and hairy, arranged in unequal pairs, green and glabrous above and usually white underneath. The shape of the leaves varies considerably ovate - oblong, round, or subcordate at the base and smooth above. The margins of the leaves are smooth, wavy or undulate. The upper surface of the leaves is green, smooth and glabrous, whereas it is pinkish white and hairy beneath. The flowers are minute, subcapitate, present 4-10 together in small bracteolate umbrellas, mainly red or rose, but the white varieties are also known. The achene fruit is detachable, ovate, oblong, pubescent, five-ribbed and glandular, anthocarpous and viscid on the ribs (Thakur *et al.,* 1989). The seeds germinate before the onset of the monsoon. The plant grows profusely in the rainy season and mature seeds are formed in October-November. Due to its sticky nature, the plant gets stuck on the clothes of humans and on the legs of animals, which helps in its dispersal from one place to another. It has a large root system bearing rootlets. The tap root is tuberous, cylindrical to narrowly fusiform, conical or tapering, light yellow, brown or brownish grey.

The plant has drawn lot of attention due to its uses in Indian traditional medicine. The various parts of the plant are used in the treatment of cancer, jaundice, dyspepsia, inflammation, enlargement of spleen, abdominal pain and

several monocytic, lymphoblastoid, fibroblast and erythroleukemic cell lines of mouse and human origin (Mehrotra *et al.,* 2002). Ethanolic extract of the plant is well demonstrated to have anti-mitotic activity in *in vitro* systems.

In view of the above findings, the present study was designed to explore the anti-proliferative and anti-estrogenic activity of *B. diffusa* extracts using ER-positive and ER-negative breast cancer cell lines. The ability of the extracts to affect the cell viability, cell cycle phases and distributions were studied using cytotoxicity assay and FACS analysis. The effect of the extracts on estrogen dependent gene expression, the *pS2* gene was examined by semi quantitative RT-PCR.

1. To assess the anti-proliferative properties of extracts of *Boerhaavia diffusa* in breast cancer cell lines
2. To analyze the phytoestrogenic property of the extracts of *Boerhaavia diffusa*
3. To evaluate the transcriptional regulatory activity of the active extract in breast carcinoma cell lines
4. Phytochemical analysis of the active phytoestrogenic fraction

REVIEW OF LITERATURE

3.1 Breast Cancer

Cancer is a group of diseases that cause cells in the body to change and grow out of control. Most types of cancer cells eventually form a lump or mass called a tumor, and are named after the part of the body where the tumor originates. Breast cancer begins in breast tissue, which is made up of glands for milk production, called lobules. Breast cancer is the most common cause of death among women around the globe (Parkin *et al.,* 2002). Each year, breast cancer is newly diagnosed in more than 1.1 million women, and these cases represent more than10% of all new cancer cases. With more than 410,000 deaths each year, the disease accounts for more than 1.6% of all female deaths worldwide. Breast cancer is an urgent public health problem in high-resource regions and is becoming an increasingly urgent problem in low resource regions, where incidence rates have been increasing by up to 5% per year (Stewart *et al.,* 2003). Early breast cancer detection and comprehensive cancer treatment appear to play synergistic roles in creating improved outcomes in these countries (Robert *et al.*, 2005).

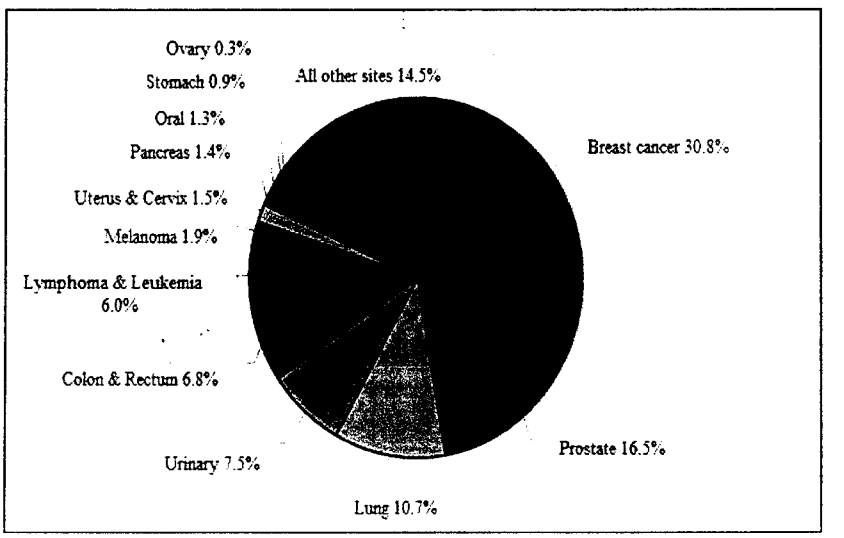

Figure: 3.1 Incidence of Cancer in 2007

in recent decades (Parkin *et al.,* 2002). Early breast cancer detection and comprehensive cancer treatment appears to play synergistic roles in creating improved outcomes in these countries. Because cancer is an inevitable social and health care burden, and because its incidence is increasing, the World Health Organization (WHO) recently passed an important and sweeping cancer prevention and control resolution that creates a mandate for member countries and the WHO director general to address cancer care, including prevention, early detection, diagnosis, treatment, and palliation of symptoms of cancer, around the globe (Parkin *et al.*, 2002).

3.2 Risk Factors

A "risk factor" is anything that increases your risk of developing breast cancer. Many of the most important risk factors for breast cancer are beyond control, such as age, family history, and medical history. However, there are some controllable risk factors, such as weight, physical activity, and alcohol consumption.

3.2.1 Controllable Risk factors

3.2.1.1 Weight

Being overweight is associated with increased risk of breast cancer, especially for women after menopause. Fat tissue is the body's main source of estrogen after menopause, when the ovaries stop producing the hormone. Having more fat tissue means having higher estrogen levels, which can increase breast cancer risk.

3.2.1.2 Diet

Diet is a suspected risk factor for many types of cancer, including breast cancer, but studies have yet to show for sure which types of foods increase

factors, antibiotics, and pesticides. Some researchers believe that eating too much cholesterol and other fats are risk factors for cancer, and studies show that eating a lot of red and/or processed meats is associated with a higher risk of breast cancer. A low-fat diet rich in fruits and vegetables is generally recommended.

3.2.1.3 Exercise

Physical activity reduces a woman's breast cancer risk so regular exercise is advisable (Reeves *et al.*, 2007). The American Cancer Society recommends engaging in 45-60 minutes of physical exercise 5 or more days a week.

3.2.1.4 Alcohol consumption

Studies have shown that breast cancer risk increases with the amount of alcohol a woman drinks. Alcohol can limit liver's ability to control blood levels of the hormone estrogen, which in turn can increase risk. A study of more than 1,280,000 middle-aged British women concluded that for every additional drink regularly consumed per day, the incidence of breast cancer increases by 11 per 1000 (Allen *et al.*, 2009).

3.2.1.5 Smoking

Smoking is associated with a small increase in breast cancer risk (Kristina Fiore, 2009). Exposure to second-hand smoke (passive smoking) has been found to slightly increase breast cancer risk (Johnson, 2005).

3.2.1.6 Exposure to estrogen

One of the most established risk factors for breast cancer is a woman's total lifetime exposure to estrogen (Travis *et al.*, 2003). Because the female

3.2.1.7 Oral contraceptive use

Using oral contraceptives appears to slightly increase a woman's risk for breast cancer, but only for a limited period of time. Women who stopped using oral contraceptives more than 10 years ago do not appear to have any increased breast cancer risk. The risk of endometrial and ovarian cancers is reduced with the use of OCs, while the risk of breast and cervical cancers is increased (Burkman *et al.*, 2004).

3.2.1.8 Stress and anxiety

There is no clear proof that stress and anxiety can increase breast cancer risk. However, anything that reduces stress and which enhances comfort, joy, and satisfaction has a major effect on the quality of life. So-called "mindful measures" (such as meditation, yoga, visualization exercises, and prayer) may be valuable additions to daily or weekly routine.

3.2.2 Uncontrollable Risk factors

3.2.2.1 Gender

Being a woman is the most significant risk factor for developing breast cancer (Giordano *et al.*, 2004). Although men can get breast cancer, too, women's breast cells are constantly changing and growing, mainly due to the activity of the female hormones estrogen and progesterone. This activity puts them at much greater risk for breast cancer.

3.2.2.2 Age

Breast cancer incidence and death rates greatly increase with age. During 1998-2002, women aged 20-24 had a lower risk of breast cancer than women

people in their 60s.

3.2.2.3 Family history of breast cancer

A higher frequency of breast cancer is expected with people who have a first-degree relative (mother, daughter, sister) who has had breast cancer, or have multiple relatives affected by breast or ovarian cancer (especially before they turned age 50).

3.2.2.4 Personal history of breast cancer

If the patient has already been diagnosed with breast cancer, the risk of developing it again, either in the same breast or the other breast, is higher than people who never had the disease.

3.2.2.5 Race

White women are slightly more likely to develop breast cancer than are African or American women. Asian, Hispanic, and Native American women have a lower risk of developing and dying from breast cancer.

3.2.2.6 Radiation therapy to the chest

Having radiation therapy to the chest area as a child or young adult as treatment for another cancer significantly increases breast cancer risk. The increase in risk seems to be highest if the radiation was given while the breasts were still developing (during the teen years).

3.2.2.7 Breast cellular changes

Unusual changes in breast cells found during a breast biopsy (removal of suspicious tissue for examination under a microscope) can be a risk factor for

3.2.2.8 Pregnancy and breastfeeding

Pregnancy and breastfeeding reduce the overall number of menstrual cycles in a woman's lifetime, and this appears to reduce future breast cancer risk. Women who have never had a full-term pregnancy, or had their first full-term pregnancy after age 30, have an increased risk of breast cancer. For women who do have children, breastfeeding may slightly lower their breast cancer risk, especially if they continue breastfeeding for 1 1/2 to 2 years. For many women, however, breastfeeding for this long is neither possible nor practical (Bentley *et al.*, 2001).

3.2.2.9 DES exposure

Women who took a medication called diethylstilbestrol (DES), used to prevent miscarriage from the 1940s through the 1960s, have a slightly increased risk of breast cancer. Women whose mothers took DES during pregnancy may have a higher risk of breast cancer as well (Dutton *et al.*, 1988).

3.3 Types of breast cancer

Breast cancer is not just one disease, but rather is a general term used to describe a number of different types of cancers that occur in the breast. The majority of breast cancers can be classified into infiltrating ductal carcinoma, infiltrating lobular carcinoma, ductal carcinoma in situ, lobular carcinoma in situ, inflammatory carcinoma, Paget's disease, Cystosarcoma phyllodes. There are other tumors of the breast, such as angiosarcoma, squamous cell cancer and lymphoma, but they are quite rare. These categories are based on the microscopic appearance of the breast tissue obtained with a biopsy sample.

breast. It is the most common form of breast cancer, comprising about 65-85% of all cases. On a mammogram, invasive ductal carcinoma is usually found as an irregular mass, or as a group of small white irregular dots called micro calcifications, or a combination of both. It may also appear as a lump in the breast. On physical examination, this lump usually feels much harder or firmer than other benign causes of lumps in the breast.

3.3.2 Infiltrating Lobular Carcinoma

Infiltrating Lobular Carcinoma comprises 5 to 10 percent of breast cancers. This type of breast cancer can appear similar to infiltrating ductal carcinoma on mammography, but on examination of the breast there is usually not a hard mass, but rather a vague thickening of the breast tissue. Lobular carcinoma can occur in more than one site in the breast (multicentric) or in both breasts simultaneously (bilateral).

3.3.3 Ductal Carcinoma in Situ (DCIS)

Ductal Carcinoma in Situ (DCIS) is a pre-invasive form of breast cancer. It is commonly seen in association with an invasive breast cancer. If it occurs without an invasive cancer there is usually no lump associated with it. On mammography, there may be fine micro calcifications which can signal its presence. DCIS is frequently multifocal, meaning it is located in more than one area of the breast. Approximately one-third of DCIS cases are multifocal. If DCIS is treated with biopsy alone, about 40% of women will ultimately develop an invasive cancer of that breast in the future.

3.3.4 Lobular Carcinoma in situ (LCIS)

Lobular Carcinoma in situ (LCIS) is usually encountered as an incidental finding in a breast biopsy. It has no symptoms, and has no characteristic

opposite breast. The risk of developing an invasive cancer of the breast with LCIS is approximately 1% per year. The invasive cancer that develops has about an equal chance of being in either breast regardless as to which breast the LCIS was initially found. A large percentage (38%) of women with LCIS may not develop an invasive cancer until more than 20 years after the initial diagnosis.

3.3.5 Inflammatory Carcinoma

Inflammatory carcinoma of the breast is a subtype of infiltrating ductal carcinoma, but is named for its typical clinical presentation. The breast becomes red, swollen, and warm, and the skin becomes quite thickened. The breast appears as if it were infected. This appearance is due to the rapid growth of the cancer which blocks the lymphatic's in the breast, causing it to swell and appear infected. The cancer has already spread to the lymph nodes in 90% of the cases at the time of diagnosis. The prognosis for this cancer is very poor, and is fortunately relatively uncommon.

3.3.6 Paget's Disease

Paget's disease of the breast accounts for about one to four percent of all breast cancers. It occurs typically as a crusting and scaling of the nipple. It can be mistaken for a benign skin condition unless there is a high index of suspicion.

3.3.7 Cystosarcoma Phyllodes

Cystosarcoma Phyllodes is a firm tumor that resembles a benign fibro adenoma. This cancer is very different than other cancers of the breast. It seldom spreads to the lymph nodes, but can metastasize to other parts of the body by way of the blood.

Cancer occurs as a result of mutations, or abnormal changes, in the genes responsible for regulating the growth of cells and keeping them healthy. Some of the genetic elements that have been shown to be important in the development of breast cancer are BRCA1 and BRCA2 Genes, HER-2/neu Gene, Estrogen Receptor (ER), PTEN Gene and Cowden Syndrome, TP53 Gene and Li-Fraumeni Syndrome, ATM Gene and Ataxia-Telangiectasia.

3.5 Symptoms

Sometimes there are no symptoms of breast cancer when it is in the early stages. The first symptom, or subjective sign, of breast cancer is typically a lump that feels different from the rest of the breast tissue. It is unique because it often does not present with a lump and therefore often is not detected by mammography or ultrasound. Reported breast cancer symptoms include discharge from the nipple(clear or bloody), persistent tenderness of the breast, pain in the nipple , swelling or mass in the armpit (lymph nodes) , inverted or scaly nipples, a lump that can be felt, no matter how small, swelling of the breast, change in the color or feel of the skin of the breast, areola, or nipple.

3.5.1 Symptoms of Early Stage Breast Cancer Advanced Disease and Recurrence

A small clump of cancer cells are too tiny to be felt, so the earliest stages of breast cancer usually have no symptoms. Some benign breast conditions can seem like cancer. The classic symptom for breast cancer is a lump found in the breast or armpit. An aggressive type of this disease, inflammatory breast cancer (IBC), grows in sheets of tumor cells that invade the skin and resembles a rash. Early detection is the best way to protect your health and improve your odds of survival. Clinical breast exam (CBE) can be done at regular intervals.

3.5.2 Symptoms of Metastatic Breast Cancer

Metastasis is the most advanced stage of this disease. Metastatic breast cancer is defined as having spread beyond the breast and underarm lymph nodes into other parts of the body (Andrea Markowitz, 2009). The symptoms include bone pain, shortness of breath, and drop in appetite, unintentional weight loss, headaches, neurological pain or weakness.

3.5.3 Symptoms of Breast Cancer Recurrence

Recurrence of breast cancer is classified as local, regional, and distant. A distant recurrence is the same as advanced (metastatic) breast cancer. A local recurrence is breast cancer that has returned after treatment, in or close to the original tumor location (Yongqiang, 2008). It can often be effectively treated. Regional recurrence may be in the chest wall muscles, or in lymph nodes located beneath your sternum, just above your collarbone, and around your neck. Local Recurrence Symptoms include a small lump or rash in the excision scar, on or under the skin. Regional Recurrence Symptoms include a swollen lymph node in the same armpit where cancer was previously removed and also above the collarbones or on the sides of neck.

3.6 Treatment

The following are the treatment modalities available for breast cancer

3.6.1 Surgery

Surgery is the first line of attack against breast cancer. Types of surgeries include

tissue.

3.6.1.2 Mastectomy

This is the removal of all of the breast tissue. Mastectomy is more refined and less intrusive than it used to be because in most cases, the muscles under the breast are no longer removed.

3.6.1.3 Lymph node removal

This can take place during lumpectomy and mastectomy if the biopsy shows that breast cancer has spread outside the milk duct.

3.6.1.4 Breast reconstruction

This is the rebuilding of the breast after mastectomy and sometimes lumpectomy. Reconstruction can take place at the same time as cancer-removing surgery, or months to years later.

3.6.1.5 Prophylactic mastectomy

This is preventive removal of the breast to lower the risk of breast cancer in high-risk people

3.6.1.6 Prophylactic ovary removal

This is a preventive surgery that lowers the amount of estrogen in the body, making it harder for estrogen to stimulate the development of breast cancer.

3.6.2 Chemotherapy

Chemotherapy treatment uses medicine to weaken and destroy cancer cells in the body, including cells at the original cancer site and any cancer cells that may have spread to another part of the body. Chemotherapy is used to treat

Chemotherapy medications for breast cancer include abraxane, adriamcin, carboplatin, cytoxan, taxol.

3.6.3 Radiation therapy

Radiation therapy is a highly targeted, highly effective way to destroy cancer cells in the breast that may stick around after surgery. Radiation can reduce the risk of breast cancer recurrence by about 70%. Radiation therapy is relatively easy to tolerate and its side effects are limited to the treated area. There are two main types of radiation

3.6.3.1 External Radiation

External radiation is the most common type of radiation, typically given after lumpectomy and sometimes mastectomy. In this technique, a linear accelerator aims a beam of high-energy radiation at the area affected by the cancer.

3.6.3.2 Internal Radiation

Internal radiation is a less common method of giving radiation. It is being studied for use after lumpectomy. Internal radiation methods typically use small pieces of radioactive material, called seeds, which are placed in the area around where the cancer was. The seeds emit radiation into the surrounding tissue. It is used to compare the current standard of external radiation to the whole breast. Internal radiation is most commonly delivered using multiple small tubes or catheters, or using a balloon-catheter device called Mammo Site.

3.6.4 Hormone therapy

Hormonal (anti-estrogen) therapy works against hormone-receptor-positive breast cancer (Christina, 2009). Hormonal therapy medicines treat hormone-

estrogen on breast cancer cells. Estrogen makes hormone-receptor-positive breast cancers grow. So reducing the amount of estrogen or blocking its action can reduce the risk of early-stage hormone-receptor-positive breast cancers coming back (recurring) after surgery.

3.7 Estrogen

Estrogens are a group of steroid compounds, named for their importance in the estrous cycle, and functioning as the primary female sex hormone. Estrogen has various physiological roles such as growth and differentiation. Thought the ovary is the major source of systemic estrogen in the premonopausal non-pregnant females, estrogen is produced also in the extraovarian tissues including skin, testis, brain, adipose tissue, T cells of spleen. The extraovarian tissues donot produce sufficient estrogen to affect the circulating level. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate the production of estrogen in the ovaries. Estrogen maintains the secondary sex characters and organs, such as mammary glands, uterus, vagina, and fallopian tubes, the corpus luteum, and the placenta of mammalian females. They are produced and secreted directly into the bloodstream by the ovary, testis, adrenal, and placenta. The major estrogen secreted by the ovary is 17β-estradiol; this is converted to estrone in the blood. Estriol is the principal estrogen formed by the placenta during pregnancy. Though estrogen is a sex hormone, they act on organ systems such as cardiovascular, skeletal, immune, gastrointestinal and neural sites through nuclear estrogen receptors (Deroo *et al.,* 2006). Estrogen composed of four interconnected rings of atoms that readily pass through the cell membrane and bind to the intracellular receptors, or recognition site of hormone molecules. Estrogen plays an important role in normal development.

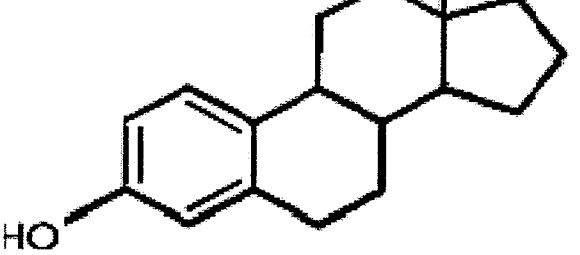

Figure: 3.2 Estrogen

3.8 Estrogen synthesis

Estrogens are produced primarily by developing follicles in the ovaries, the corpus luteum, and the placenta. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulates the production of estrogen in the ovaries. Some estrogens are also produced in smaller amounts by other tissues such as the liver, adrenal glands, and the breasts. In females, synthesis of estrogens starts in theca internal cells in the ovary, by the synthesis of androstenedione from cholesterol. Androstenedione is a substance of moderate androgenic activity. This compound crosses the basal membrane into the surrounding granulosa cells, where it is converted to estrone or estradiol, either immediately or through testosterone. The conversion of testosterone to estradiol, and of androstenedione to estrone, is catalyzed by the enzyme aromatase.

While the ovaries are the principal source of systemic estrogen in the premenopausal non-pregnant woman, other sites of estrogen biosynthesis are present throughout the body and these become the major sources of estrogen beyond menopause. These sites include the mesenchymal cells of the adipose tissue and skin, osteoblasts and perhaps osteoclasts in bone, possibly vascular endothelial and aortic smooth muscle cells as well as a number of sites in the brain including the medial preoptic/anterior hypothalamus, the medial basal hypothalamus and the amygdala. Principally, these sites are dependent on

unlike the ovaries they lack the ability to synthesize C19 precursors. Hence, estrogen production in adipose, bone and brain is totally dependent on the availability of circulating C19 precursors (Simpson *et al.*, 1999).

3.9 Estrogen Types

3.9.1 Steroidal

The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol (E2) is the predominant form in nonpregnant females, estrone is produced during menopause, and estriol is the primary estrogen of pregnancy. In the body these are all produced from androgens through actions of enzymes.

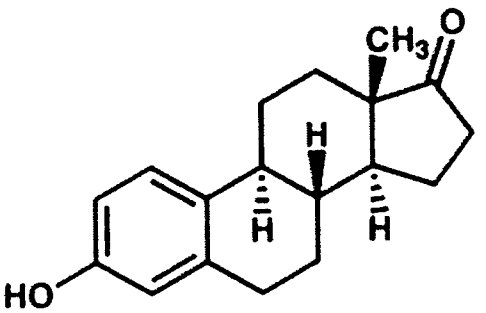

Figure: 3.3 Structure of Estrone

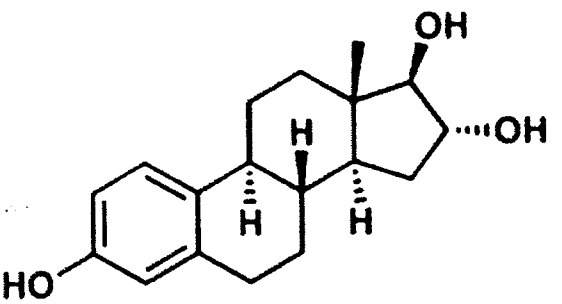

Figure: 3.4 Structure of Estriol

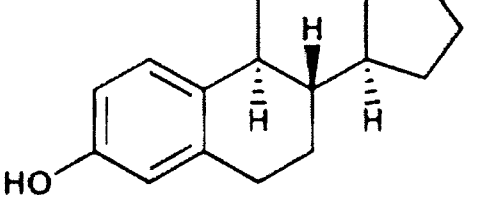

Figure: 3.5 Structure of Estradiol

3.9.2 Nonsteroidal

A range of synthetic and natural substances have been identified that also possess estrogenic activity (Fang *et al.,* 2009). They are xenoestrogens, phytoestrogens, mycoestrogens.

3.10 Estrogen receptor

The types of Estrogen Receptors are ER-α and ER-ß.

3.10.1 Estrogen receptor-α

Estrogen receptor alpha (ER-α), also known as NR3A1 (nuclear receptor subfamily 3, group A, member 1), is a nuclear receptor which is activated by the sex hormone estrogen. ER- α is encoded by the human gene ESR1. The natural ligand for ER is the estrogenic compound 17 ß-estradiol. The translated receptors show less variability (Greene *et al.,* 1986). ER- α is expressed in a wide variety of tissues such as bones, uterus, bladder, ovary, prostate, testis, epididymis, kidney, breast, heart, vessel wall, pituitary and hypothalamus. The estrogen receptor has a variety of central physiological roles, including those involved in maintenance of the reproductive, cardiovascular, musculoskeletal and central nervous systems.

Estrogen receptor beta is also known as NR3A2 (nuclear receptor subfamily 3, group A, member 2), is a nuclear receptor which is activated by the sex hormone estrogen. ER-β is encoded by the human gene ESR2 (Estrogen Receptor 2).. The gene product contains an N-terminal DNA binding domain and C-terminal ligand binding domain and is localized to the nucleus, cytoplasm, and mitochondria.

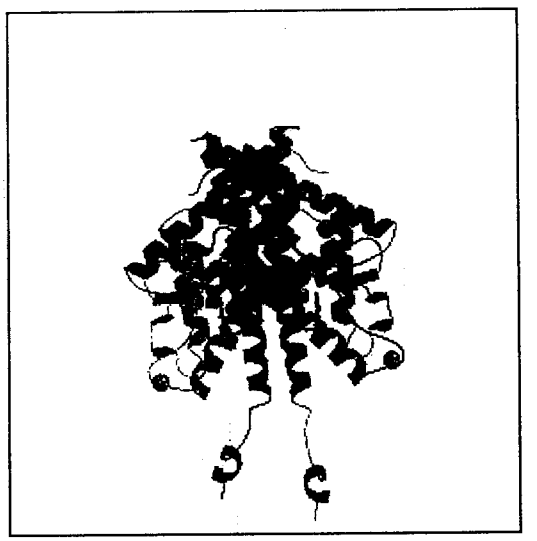

Figure: 3.6 Structure of ER- α

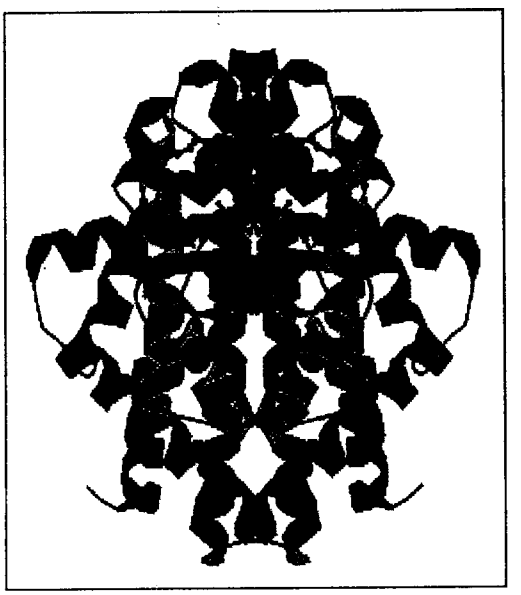

Figure: 3.7 Structure of ER-α and ER-β

two separate gene products and share a relationship to one another that is similar to those between, for instance, the glucocorticoid receptor and the mineralocorticoid receptor, or the glucocorticoid receptor and the progesterone receptor, which shows a homology between their ligand-binding domains that corresponds to those of ER-α and ER-β. ER-β has biological roles greater than ER-α. In vascular tissue, where both ER-α and ER-β appear to be expressed, denudation involving removal of the endothelial cell layer leading to smooth muscle cell proliferation is accompanied by a huge increase (up to 80-fold) of ER-β expression in smooth muscle cells and endothelial cells, whereas the expression of ER-α in these cells is unaffected.

3.11 Mechanism of action

ER-α and ER-β belong to the steroid hormone superfamily of nuclear receptors, members of which share a common structural architecture (Evans, 1988). They are composed of three independent but interacting functional domains the NH2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain. Binding of a ligand to ER triggers conformational changes in the receptor and this leads, via a number of events, to changes in the rate of transcription of estrogen-regulated genes. These events, and the order in which they occur in the overall process, include receptor dimerization, receptor-DNA interaction, recruitment of and interaction with coactivators and other transcription factors, and formation of a preinitiation complex (Beato, 1989).

Figure 3.8: Diagrammatic representation of the domain structure of nuclear receptors. The A/B domain at the NH2 terminus contains the AF-1 site where other transcription factors interact. The C/D domain contains the two-zinc finger structure that binds to DNA, and the C/F domain contains the ligand binding pocket as well as the AF-2 domain that directly contacts coactivator peptides.

The N-terminal domain of nuclear receptors encodes a ligand-independent activation function (AF1) (Kraus *et al., 1995*), a region of the receptor involved in protein-protein interactions (Webb *et al.*, 1998) and transcriptional activation of target-gene expression. Comparison of the AF1 domains of the two estrogen receptors has revealed that, in ER-α, this domain is very active in stimulation of reporter-gene expression from a variety of estrogen response element (ERE)-reporter constructs, in different cell lines (Cowley *et al.*, 1999), but the activity of the AF1 domain of ER-β under the same conditions is negligible. On an ERE-based reporter gene, these ligands are partial E2 agonists with ER alpha but are pure E2 antagonists with ER-β (Barkhem *et al.*, 1998). Dissimilarity in the NH2-terminal regions of ER-α and ER- β is one possible explanation for the difference between the two receptors in their response to various ligands. In ER-α, two distinct parts of AF1 are required for the agonism of E2 and the partial agonism of tamoxifen, respectively (McDonnell *et al.*, 1995).

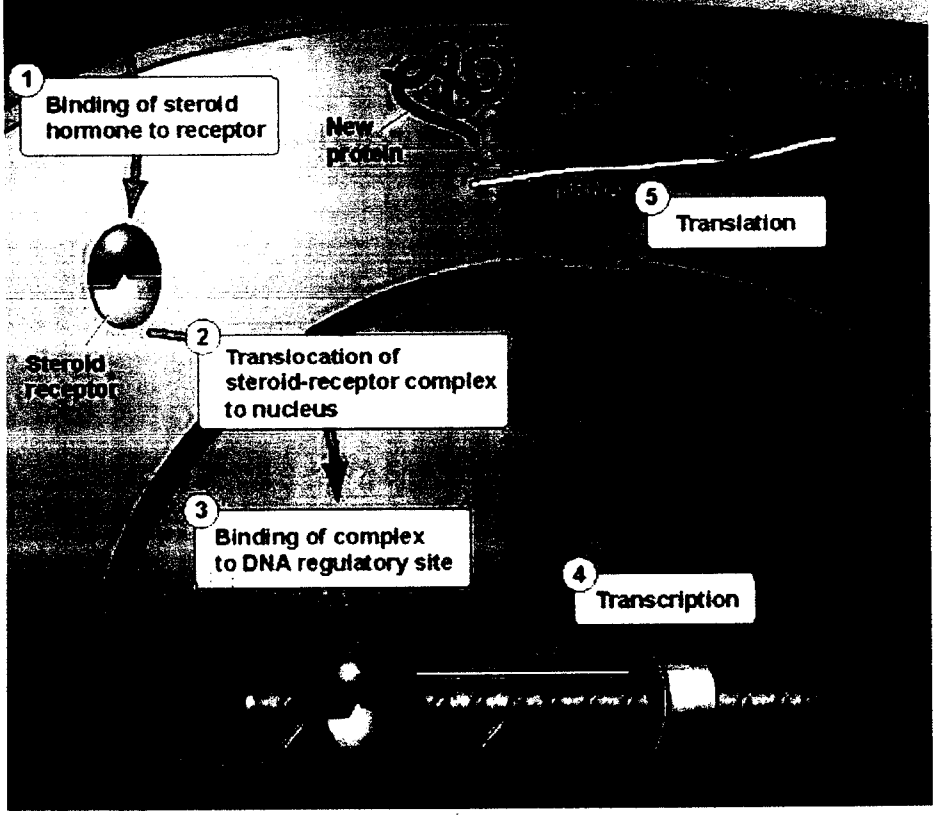

Figure: 3.9 Mechanism of Estrogen action

3.12 Physiological role

Estrogen promotes the development of female secondary sexual characteristics, such as breasts, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. In males, estrogen regulates certain functions of the reproductive system important to the maturation of sperm (Hess *et al.*, 1997). Estrogens promote formation of female secondary sex characteristics, decelerate height growth, reduce muscle mass, stimulate endometrial growth, increase uterine growth, increase vaginal lubrication, thicken the vaginal wall, increase circulating level of factors 2, 7, 9, 10, plasminogen, decrease antithrombin III, increase HDL, triglyceride, decrease LDL, fat deposition, support hormone-sensitive breast cancers , fetal development , mental health in women.

3.13.1 Oral contraceptives

The ability of estrogens to suppress secretion of follicle-stimulating hormone (FSH) by the pituitary gland and thereby inhibit ovulation makes estrogen and estrogen like compounds major components in oral contraceptives.

3.13.2 Hormone replacement therapy

Hormone replacement therapy, estrogen and other hormones are given to postmenopausal women in order to prevent osteoporosis as well as treat the symptoms of menopause such as hot flashes, vaginal dryness, urinary stress incontinence, chilly sensations, dizziness, fatigue, irritability, and sweating.

3.13.3 Prostate cancer

Under certain circumstances, estrogen may also be used in males for treatment of prostate cancer. A decrease in the ratio of androgens to estrogens with aging could be responsible for prostate carcinogenesis. Estrogen produced by peripheral aromatization of testosterone exert their efforts on prostatic tissue by interaction with their receptors, ER-α and β (Geraldine *et al.,* 2009).

3.13.4 Osteoporosis

Osteoporosis is a disease of bone that leads to an increased risk of fracture. In osteoporosis the bone mineral density (BMD) is reduced, bone micro architecture is disrupted, and the amount and variety of proteins in bone is altered.In humans two types of osteoporosis are recognized. Type I occurs in post menopausal women, Type II is age related and occurs both in men and women at the age of 70. Type I osteoporosis is associated with declining estrogen postmenopausally, is characterized by an accelerated resorption of bone for about 10year followed by a slower steady loss. The resulting pathophysiology is caused by increasing bone sensitivity to parathyroid hormone and decreased calcium absorption from the gut. Estrogen replacement therapy remains a good treatment for prevention of osteoporosis. SERMs produce some estrogen like effect on bones.

Phytoestrogens, sometimes called dietary [illegible]

group of naturally occurring non steroidal plant compounds that, because of their structural similarity with estradiol (17-β-estradiol), have the ability to cause estrogenic or/and antiestrogenic effects . The similarities, at molecular level, of estrogens and phytoestrogens allow them to mildly mimic and sometimes act as antagonists of estrogen. Phytoestrogens were first observed in 1926 but it was unknown if they could have any effect in human or animal metabolism. Phytoestrogens mainly belong to a large group of substituted phenolic compounds known as flavonoids the coumestans, prenylated flavonoids and isoflavones are three of the most active in estrogenic effects in this class. The best-researched are isoflavones, which are commonly found in soy and red clover. Lignans have also been identified as phytoestrogens, although they are not flavonoids.

Phytoestrogen content varies in different foods, and may vary significantly within the same group of foods (e.g. soy beverages, tofu) depending on processing mechanisms and type of soy bean used. Legumes (in particular soybeans), whole grain cereals, and some seeds are high in phytoestrogen. A more comprehensive list of foods known to contain phytoestrogens includes soy beans, tofu, soy beverages, linseed, sesame seeds, wheat, berries, oats, barley, dried beans, lentils, yams, rice, alfalfa, mung beans, apples, carrots, pomegranates (Van Elswijk *et al.*,2004),wheat germ, ricebran, soy linseed bread, ginseng, hops(Chadwick *et al.*,2004) bourbon and beer(Rosenblum *et al.*,1993) fennel and anise(Albert-Puleo,1980). The activity of phytoestrogens depends on its structure and mechanism, its concentration relative to that of endogenous estrogen and the biological function being assessed.

Phytoestrogens show a protective effect against breast cancer (Ingram *et al.*, 2008). Phytoestrogenic plants have been used for centuries in the treatment of menstrual and menopausal problems as well as for fertility problems.

3.14.1 Types of phytoestrogens

The following are the classes of Phytoestrogens

- Isoflavones
- Flavanols
- Flavones
- Flavanones
- Lignans

3.14.2 Phytoestrogens in breast cancer

Phytoestrogens could have a protective effect on the initiation or progression of breast cancer by inhibiting the local production of oestrogens from circulating precursors in breast tissue. Indeed, in vitro experiments have shown that phytoestrogens inhibit the activity of key steroidogenic enzymes involved in the synthesis of estradiol from circulating androgens and oestrogen sulfate. Amongst the phytoestrogens, the flavones and flavonones are the most potent inhibitors of aromatase.

The chemical structure of phytoestrogens and their structural relationship with estrogens allows them to bind to estrogen receptors (ER). Phytoestrogens can be considered as weak estrogens, presenting an activity 100 to 1000 times lower than that of 17β-estradiol (E2), depending on the system studied (Martin *et al.,* 1978, Markiewicz *et al.,* 1993, Zava & Duwe 1997). However, in individuals with a moderate soy intake, plasma concentrations of phytoestrogens are 1000 times higher than endogenous estrogen concentrations in women of reproductive age. Plasma genistein concentrations are of the order of 0.1 to 3 μM/l (Zava & Duwe, 1997). The affinities of genistein and daidzein for ER are also lower than that of estradiol. The order of magnitude varies according to the system studied; for example, the affinity of genistein for ER is 20 to 100 times lower than that of E2 (Martin *et al.,* 1978, Kuiper *et al.,* 1997). Finally, a second type of ER has recently

appear to have a higher affinity than for ERα (Kuiper *et al.*, 1997).

Dietary changes present one of the few socially acceptable modifiable risk factors for breast cancer, the second leading cause of cancer deaths in women. Hence, even a modest protective role of phytoestrogens could have important implications for public health. In addition to its potential role in preventing breast cancer, there has been much interest in using phytoestrogens for menopausal symptoms among breast cancer survivors. Women diagnosed with breast cancer report more menopausal symptoms than women who undergo menopause naturally, yet they are generally advised not to use hormone therapy because of concerns that hormone treatment may increase risk of recurrence. Women often seek out complementary and alternative therapies in place of hormone therapy for menopausal symptoms, particularly phytoestrogens, in the belief they are more "natural". There have been concerns that phytoestrogens, through their estrogenic properties, may increase the risk of recurrence or stimulate the growth of existing tumors.

3.15 *Boerhaavia diffusa*

Boerhaavia diffusa L. commonly known as 'Punarnava' is an abundant creeping weed found all over India. *Boerhaavia diffusa* is a species of flowering plant in the four o'clock family which is commonly known as Tar vine or Red spiderling. It is found all over the world. It is commonly called as hog weed, pig weed, thazhuthama etc. *Boerhavia diffusa* .L is mainly used for the treatment of liver disorders, gallbladder disorders, kidney and urinary tract disorders, and menstrual disorders and to tone, balance, and strengthen the adrenals.

Figure:3.8 *Boerhaavia diffusa* L.

3.15.1 Collection

Boerhavia diffusa L. was collected from RGCB Campus, Trivandrum.

3.15.2 Habitat

Boerhavia diffusa prefers light (sandy) and medium (loamy) soils and requires well-drained soil. The plant prefers acidic, neutral and basic (alkaline) soils. It cannot grow in the shade. It requires dry or moist soil and can tolerate drought.

3.15.3 Description of *Boerhaavia diffusa* L.

Boerhaavia diffusa is a perennial creeping weed, prostate or ascending herb, up to 1 m long or more, having spreading branches (Fig. 2.7). The roots are stout and fusiform with a woody root stock. The stem is prostrate, woody or succulent, cylindrical, often purplish, hairy, and thickened at the nodes. Leaves are simple, thick, fleshy, and hairy, arranged in unequal pairs, green and glabrous above and usually white underneath. The shape of the leaves varies considerably - ovate-oblong, round, or subcordate at the base and smooth above. The flowers are hermaphrodite (have both male and female

bitter in taste.

3.15.4 Edible uses

Tender young leaves and shoots are cooked and used as a vegetable (Kunkel, 1984). Root is baked, it is rich in carbohydrate and protein, though the flavour is bland and the texture sometimes woody. Seed is cooked. It can be ground into a powder and added to cereals when making bread, cakes etc.

3.15.5 Medicinal Uses

According to Ayurveda Boerhavia is bitter, anaemia, cooling, heart diseases, astringent to bowels, useful in biliousness, blood impurities, leucorrhoea, asthma, alternatives etc. The leaves are useful in dyspepsia, tumours, abdominal pains, and spleen enlargement. According to Unani system of medicine, the leaves are appetizers, alexiteric, useful in opthalmia and in joint pains. Seeds are tonic expectorant, carminative, useful in lumbago, scabies. The seeds are considered as promising blood purifier. Roots are used to treat gonorrhea, all internal inflammation and edema. Roots stimulate the emptying of the gallbladder, as a diuretic, for all types of liver disorders (including jaundice and hepatitis), gallbladder pain and stones, urinary tract disorders, renal disorders, kidney stones, cystitis, and nephritis.

The plant has drawn lot of attention due to its uses in Indian traditional medicine. The various parts of the plant are used in the treatment of cancer, jaundice, dyspepsia, inflammation, enlargement of spleen, abdominal pain and as an anti-stress agent (Chakraborti *et al.,* 1989; Kirtikar and Basu, 1956; Leslie Taylor, 2005). Root extracts of *B. diffusa* inhibited the growth of several monocytic, lymphoblastoid, fibroblast and erythroleukemic cell lines of mouse and human origin. *B. diffusa* extracts prevented DMBA-induced skin carcinogenesis in mice (Bharali *et al.,* 2003). Ethanolic extract of the plant is

found to be effective in reducing the metastases formation by B167-10 melanoma cells and Punarnavine, an alkaloid from *B. diffusa* enhanced the immune response against metastatic progression of B16F-10 melanoma cells in mice (Leyon *et al.,* 2005; Manu and Kuttan, 2007).

3.15.6 Chemical composition of *Boerhaavia diffusa* L.

The *Boerhaavia diffusa* plant contains a large number of such compounds as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins. Punarnavine $C_{17}H_{22}N_2O$, mp is 236-237°C (Agarwal and Dutt, 1936; Basu *et al.*, 1947),boeravinone A.F (Kadota *et al.*, 1989; Lami *et al.*, 1990; 1992), hypoxanthine 9-L-arabinofuranoside(Ahmad and Hossain, 1968), ursolic acid (Mishra and Tiwari, 1971), punarnavoside (Jain and Khanna, 1989), liirodendrin (Aftab *et al.*, 1996), and a glycoprotein having a molecular weight of 16-20 kDa (Verma *et al.*, 1979).

3.15.7 Antiviral activity of *Boerhaavia diffusa* L.

In view of the medicinal potential of this plant, the root, leaf, stem, flower, and seed samples were screened (collected at different stages of plant growth and from different locations, both fresh and dried) for their antiviral activity against a number of isometric as well as anisometric phytopathogenic viruses, in various host/virus combinations both in vitro and in vivo (Verma and Awasthi, 1979). Maximum antiviral activity, in each case, was recorded with the aqueous extract of dried root powder applied before virus inoculation. The active principle was purified and isolated (Verma *et al.*, 1979). The roots of *B. diffusa* are a rich source of a basic protein, which is used for inducing systemic resistance in many susceptible crops against commonly occurring viruses (Verma and Awasthi, 1979). This protein or antiviral agent was active

NP31); sunnhemp rosette virus in *Cyamopsis tetragonoloba*, *Vigna unguiculata,* and *Crotalaria juncea*; and gomphrena mosaic virus in *Chenopodium amaranticolor, Vigna unguiculata,* and *Gomphrena globosa* when applied a few hours (2-24 h) before inoculation by the respective inocula of viruses (Verma and Awasthi, 1979). The antiviral agent was a basic glycoprotein (70-80% protein and 8-13% carbohydrates) with a molecular weight of 16-20 kDa as determined by gel filtration chromatography (Verma *et al.*, 1979). The resistance-inducing protein was found to be extremely thermostable (Verma and Awasthi, 1979). Following treatment with the systemic resistance inducing protein, the susceptible healthy host produced a virus inhibitory agent (VIA). The VIA showed the characteristics of the protein, and upon incubation with the virus, reduced infectivity of the viruses both in vitro and in vivo. Upon gel filtration on Sephadex G-75®, two active fractions, exhibiting protein characteristics, were recovered. The VIA was present both in treated as well as untreated leaves. The biophysical characteristics of induced VIA were also studied and it was found to be a basic protein. The glycoprotein occurring in *B. diffusa* roots functions as a signal molecule, and is of great interest as it has a role in stimulating the defence systems of plants against viruses.

MATERIALS AND METHODS

4.1 Soxhlet Extraction

A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by *Franz von Soxhlet.* Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. It has mainly three parts. They are,

- Solvent Flask
- Extractor
- Condenser

Figure : 4.1 Soxhlet Extraction

powdered using a blender. 25g of powdered sample was weighed and placed inside a thimble made of thick cotton cloth. The thimble was then assembled inside the extractor. 250ml of Methanol was placed in the solvent flask. The Soxhlet extractor was placed onto a flask containing the extraction solvent. The Soxhlet was then equipped with a condenser. The solvent was heated to reflux and the solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser condensed the solvent vapour and drips back down into the thimble. The desired compound dissolved in the warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle was repeated for 20 hours. After extraction, the solvent was removed from the extractor. The extract was the centrifuged at 2500 rpm for 5 minutes. The supernatant was then collected and transferred in to a 250ml flask and was covered with foil paper. The flask was then allowed for evaporation by making holes on the foil paper. After evaporation, the extract was stored at a concentration of 160 mg/ml in DMSO and was stored at -20°C for further use.

4.2 Animal Cell Culture

Cell culture is the process by which cells are grown under controlled conditions. Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2 for mammalian cells) in a cell incubator.

PBS (1L)

Reagents	Volume(g/l)
Sodium chloride	8 g
Potassium chloride	0.2 g
Di sodium hydrogen phosphate	1.44 g
Potassium di hydrogen phosphate	0.2 g

Table 4.2.2

PBS-EDTA(1X-1L)

Reagents	Volume(g/l)
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.2g
EDTA	0.2g

4.2.1 Revival of cells

The freezed cells in cryo vials were placed in ice. The cells were resuspended in 1ml of 20% DMEM. Cell suspension was transferred into a centrifuge tube and the volume was made upto 2ml with DMEM. The mixture was centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and

cells were then incubated at 37°C for 24 hours in CO_2 incubator. After 24 hours, medium was decanted and the cells were washed using PBS-EDTA. Then, the cells were provided with 10% DMEM and incubated at 37°C for 24 hours in CO_2 incubator.

4.2.2 Sub-culturing

The medium was decanted. The cells were washed with PBS-EDTA and were trypsinized with 1ml of 0.2% trypsin. The cells are centrifuged at 2500rpm for 5minute. The supernatant was discarded and the pellet was resuspended in 1ml of 10% DMEM. The cells were then transferred to a fresh T-flask and the volume was made upto 4ml with 10% DMEM. The cells were then incubated at 37°C for 24 hours in CO_2 incubator.

4.2.3 Cryopreservation

The medium was decanted. The cells were washed with PBS-EDTA and were trypsinized with 1ml of 0.2% trypsin. The cells were centrifuged at 2500rpm for 5minute.The supernatant was discarded and the pellet was resuspended in 2ml of freezing mixture. The cells were transferred into cryo vials and were kept at -80°C.

4.3 Cytotoxic Assay

4.3.1 MTT Assay

To evaluate the effect of the extracts on cell proliferation, MTT assay was performed (Haridas *et al.,* 1998). The MTT assay is standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan, giving a purple colour. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials, since those agents would result in cell toxicity and therefore metabolic dysfunction and therefore

yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in living cells. The dye is cleaved to a coloured product by the activity of dehydrogenase enzymes and this indicates high level of mitochondrial cell's activity.

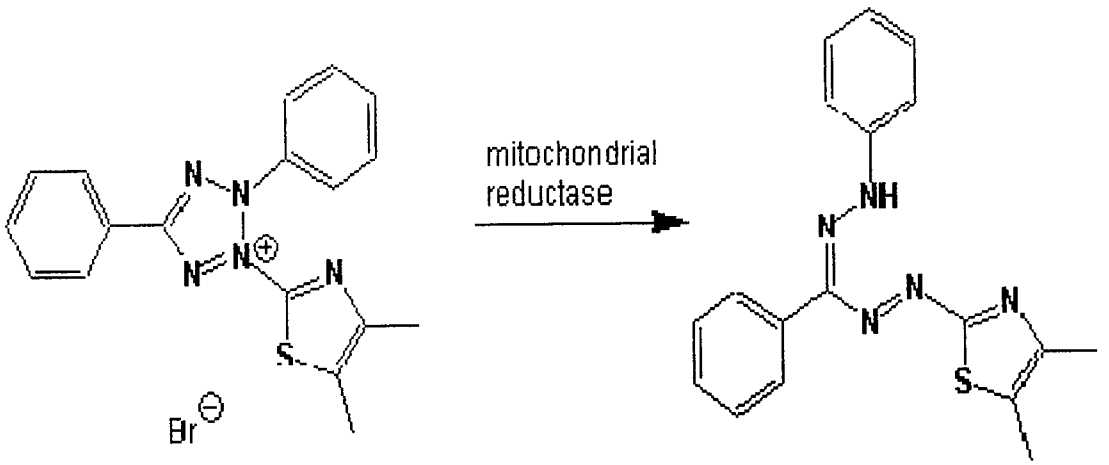

Figure 4.2 Mechanism of action of MTT

Table: 4.3.3

MTT Drug(14ml)

Stock concentration for DMSO = 160mg/ml

Dilutions	Preparation	Concentration
1: 8000	1 µl drug in 8000 µl 0 % DMEM	20 µg/ml
1:4000	1 µl drug in 4000 µl 0 % DMEM	40 µg/ml
1:2000	1 µl drug in 2000 µl 0 % DMEM	80 µg/ml
1:1000	1 µl drug in 1000 µl 0 % DMEM	160 µg/ml
1 :500	1 µl drug in 500 µl 0 % DMEM	320 µg/ml

Reagents	Volume(l)
Sodium Dodecyl Sulphate	20%
Dimethyl Formamide	50%
Distilled water	500ml

4.3.1.1 Procedure

The medium was decanted. The cells were washed with PBS-EDTA. The cells were trypsinized with 1ml of 0.2% trypsin. About 800µl of trypsin was discarded and 2ml of DMEM was added. The cells were centrifuged at 2500rpm for 5minutes. The supernatant was discarded. The pellet was resuspended in 2ml of DMEM. The cells were counted using a haemocytometer. Cells were seeded in 96-well plates at a density of 5 x 10^3 cells per well and treated with 0, 20, 40, 80, 160 and 320 □g/mL of the different solvent extracts.460 µl of cell suspension was taken and made up to 7ml with 10% DMEM. 100 µl of cell suspension was added to each well of a 96 well plate. The plate was incubated for 24hours in a CO_2 incubator. Remove the media using a micro pipette. 100 µl of drug was added at varying concentrations. The plate was then incubated for 48hours in a CO_2 incubator. The drug solution was removed and 100 µl of MTT (2mg/mL) was added. The plate was incubated for 2hours in CO_2 incubator. 100 µl of MTT lysis buffer was added and incubated for 4hours at 37°C. The plates were read using an ELISA reader 570 nm. Cell survival was expressed as percentage over the untreated control.

4.4 Fluorescent Activated Cell Sorting

The medium was decanted. The cells were washed with PBS - EDTA. Trypsinization was done. The cells were centrifuged at 2500rpm for 10 minutes. The pellet was resuspended in 2ml 10% DMEM and the volume was made up to 7ml using 10%DMEM. 1ml from the resuspended medium was transfered into 5 petriplates and then 6ml of 10% DMEM was added to all the plates. Then it was kept for 24 hours incubation in CO_2 incubator. The medium was decanted. 7ml of 2.5% CTS was taken in 5tubes. The drugs were added to 4tubes with 80µg/ml concentration, one plate was used as control. The drugs added were extracts with Acetone, Methanol, Hexane, and Chloroform. The CTS containing drug was added to each plates with one plate as control. The plates were then incubated in CO_2 incubator for 48 hours. The 48 hours treated medium was collected in a 15ml centrifuge tube. The plate was washed with 2ml PBS- EDTA, and kept for 1minute and transferred in to the centrifuge tube. 1ml of trypsin was added to the plate and kept for 1minute. To that 1ml of 1x PBS was added and the cells were resuspended and transferred in to the centrifuge tube. 1ml of 1x PBS was added and transferred in to the tube. Centrifuged at 3500rpm for 7minutes at 4°C. The pellet was resuspended in 1ml 1x PBS. Centrifuged at 3500rpm for 7minutes at 4°C. 450µl 1x PBS and 1050µl 70% ethanol was added drop wise. Kept for half hour incubation at 4°C. This was spinned at 3500rpm, 4°C, 7 minutes. The supernatant was discarded and the pellet was resuspended in 1ml 1x PBS. This was centrifuged at 3500rpm, 4°C, 7 minutes. The pellet was resuspended in 250µl PBS and 5µl of 10mg/ml RNase A was added. Incubate at 37°C for 30minutes. 10µl of 1mg/ml Propidium Iodide (20 µg/mL) was added and was kept for 5minutes. Then it was filtered it in to the FACS tube and analysis was carried out.

The medium was decanted. The cells were washed with PBS-EDTA and were trypsinized with 1ml of 0.2% trypsin. Trypsin was discarded and added 2ml of DMEM was added. The cells were centrifuged at 2500 rpm for 5minutes. The supernatant was discarded. The pellet was resuspended in 1ml of 10% DMEM. The cell suspension was made up to 6ml using 10% DMEM. 1ml of cell suspension was added to 6 Petri plates (100ml) and the volume was made up to 7ml using 10% DMEM. One plate was kept as control (7ml DMEM). The plates were incubated for 24 hours in a CO_2 incubator. After incubation the medium was decanted and CTS treatment for 24hours. The drug of 80µg/ml was added to 5 plates and one was kept as control. The petriplates were kept for incubation for 24hours. Medium was decanted and cells were stored in 1ml of trizol. The cells in trizol maintained at -80°C was thawed. The cells were centrifuged at 10,000rpm, 4°C for 10 minutes. To the aqueous layer, 200µl of chloroform was added, mixed vigorously. This was incubated at room temperature for 10 minutes. The cells were centrifuged at 10,000rpm, 4°C for 10 minutes. To the aqueous layer 400 µl of chloroform isoamylalcohol was added and mixed vigorously. The cells were centrifuged at 10,000rpm, 4°C for 10 minutes. To the aqueous layer, 400 µl of isopropanol was added, inverse mixing was done. Then it was kept in ice for 10minutes. The cells were centrifuged at 12,000rpm, 4°C for 12 minutes. The pellet was washed in 1ml of 70% ethanol. Centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded and the pellet was dried. 20 µl of nuclease free water was added and kept for 5 minutes. Pellet was resuspended in nuclease free water and stored at -80°C.

0.2g of agarose was added in to 25ml of 0.5x TBE in DEPC water. Agarose was dissolved by boiling. Agarose was allowed to cool to 55°C and 2µl of Ethidium bromide was added. Agarose was then transferred to gel boat and allowed to polymerise. 2µl of RNA samples was taken in 6 tubes. 2µl of gel loading dye was added and mixed well. The samples were then loaded to each well. The gel was then allowed to run at 70V for one hour. The gel was then documented.

4.5.2 RNA quantification

The nucleic acid, after purification can be assumed for purity and concentration by UV spectroscopy. They have a characteristic absorption spectrum and even a small amount of protein contamination can alter this spectrum. The ratio of absorbance at 260 nm and 280nm, the A_{260}/A_{280} ratio is the commonly used estimate for the purity of the nucleic acid precipitation. The ratio if falls between 1.8 and 2.0 is an indicative of a relatively pure precipitation of nucleic acid. The concentration of the nucleic acid can be determined from A_{260} using the conversion factor of 40µg/ml of RNA for every 1 OD.

4.5.3 cDNA synthesis

cDNA is being synthesized from 4□g of isolated RNA using AccuScript™ 1st strand cDNA synthesis kit (Stratagene,USA) according to the manufacturer's instructions.

Reagents	C1	C2	C3	C4	C5	C6
RNase free water(µl)	10.35	10.64	10.08	10.77	10.55	11.1
RT Buffer(µl)	2.0	2.0	2.0	2.0	2.0	2.0
Oligo dT(µl)	1.0	1.0	1.0	1.0	1.0	1.0
dNTP(µl)	0.8	0.8	0.8	0.8	0.8	0.8
RNA(µl)	2.35	2.06	2.62	1.93	2.15	1.60

4.6 Polymerase chain reaction

The *pS2* cDNA fragments were amplified using the primer pairs, (Fotovati *et al.,* 2006) forward-5'TTTGGAGCAGAGAGGAGGCAATGG3', reverse-5'TGGTATTAGGATAGAA GCACCAGGG3' (product size 240 bp). The reaction mixture containing 10 mM Tris HCl (pH 8.5), 50 mM KCl, 2 mM MgCl, 200 µM dNTPs, primers (2.5 pM each) and 2.5 units of *Taq* polymerase were subjected to amplification cycles of 94°C for 4 min, followed by 30 cycles of 94 °C for 1 min, 59 °C for 30 s, 72 °C for 1min in a (Eppendorf) thermal cycler. Aliquots (5 µL) of each PCR mixture were analyzed by electrophoresis in a 1.2% agarose gel and fragments were visualized by ethidium bromide staining. The intensity of bands was quantified in a Flour-S multi imager (BioRad, Hercules, USA) by using Quantity one densitometry software. The transcripts were normalized with *gapdh* expression level. The gene expression was shown as ratio of densitometric value of target mRNA to that of *gapdh*

H_20	12.5µl
Buffer	5µl
$MgCl_2$	2.5 µl
dNTP	1 µl
Forward Primer	1 µl
Reverse Primer	1 µl
cDNA Template	1.5 µl
Taq Polymerase	0.5 µl

4.7 Thin Layer Chromatography

Thin layer chromatography (TLC) is a chromatographic technique that is useful for separating organic compounds. Thin layer chromatography consists of stationary phase immobilized on a glass or plastic plate, and an organic solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The constituents of a sample can be identified by simultaneously running standards with the unknown. The different components in the mixture move the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are visualized with ultra violet light or by placing the plate in iodine vapor, the movement of analyte is expressed by its retardation factor such that

Distance moved by solvent front from the origin

Table: 4.7.7

Thin Layer Chromatography

Extract	Solvent system
Methanol	Toluene Ethylacetate Formic acid (6 3.8 2)

4.7.1 Procedure

A strip of alumina sheet of size 7cm×2cm was taken. Suitable dilution of the sample (plant extract-1 - 25) was prepared. 2µl of the sample was loaded on the spot marked 2cm away from the bottom. 2 ml of the suitable solvent system was added to the TLC chamber. After air-drying the sample, the TLC plate was placed in the TLC chamber containing the solvent system and TLC chamber was then closed. When the solvent front reached near the other edge of the stationary phase, the plate was removed from the solvent reservoir. Identified the bands on the plate under UV transilluminator. The R_f value of the sample was calculated and compared it with that of standard (estradiol).

RESULTS AND DISCUSSIONS

5.1 Effect of the extracts on cell proliferation

MCF-7 and MDA MB-231 cells were treated with varying concentration of extracts (hexane, chloroform, acetone, and methanol), (20 – 320 µg/ ml) for 48 h to study its anti-proliferative effects. The effect of the extracts on growth inhibition was assessed as percentage cell viability where DMSO treated cells was taken as 100 % viable. As shown in Fig. 5.1 there was a decrease in viability of cells treated with the extracts especially by the acetone and methanol extracts in comparison to the DMSO treated control. In ER negative cell line MDA MB-231 the extracts failed to elicit an inhibition of cell viability (Fig 5.2).

Estrogen level is a critical factor in the human physiology in that it influences the development, sexual differentiation, and fertility, control of the female reproductive tract, organ responsiveness and female diseases due to hormone imbalances. In the classical ER mediated pathway, the ligand binds to the ligand-binding domain of the ER followed by the formation of a liganded receptor homodimeric complex. The complex binds to the distal ERE's in the 5'-promoter regions of the E2-responsive genes, further interacts with the components of the general transcription factor complex and recruit coactivators or adaptors and finally induces transcription (Korach *et al.*, 1997). Estrogen level is a critical factor in the human physiology in that it influences the development, sexual differentiation, fertility, control of the female reproductive tract, organ responsiveness and female diseases due to hormone imbalances. In the classical ER mediated pathway, the ligand binds to the ligand-binding domain of the ER followed by the formation of a liganded receptor homodimeric complex. The complex binds to the distal ERE's in the 5'-promoter regions of the E2-responsive genes, further interacts with the

Estrogens are known stimulants of breast cancer growth whereas antiestrogens arrest the growth of cells (Pratt and Pollak, 1993). To assess the anti-proliferative effects of the extracts we applied MTT assay which depends on the reduction of tetrazolium salt by the mitochondria of living cells to form a blue formazan product (Denizot and Lang, 1986). The results depend on both number of cells present and on the mitochondrial activity per cell and showed a dose dependent decrease in viability of MCF-7 cells and not in MDA MB-231 cells treated with the methanol and acetone extract of *B. diffusa* in comparison to the control. These findings emphasize further the involvement of ER regulated genes in controlling cell proliferation.

5.2 Cell cycle analysis by flow cytometry

The effect of the extracts on cell cycle progression on MCF-7 cells was determined by flow cytometry. MCF-7 cells were treated with the different solvent extracts (hexane, chloroform, acetone, methanol), (100 µg/ ml) or varying concentration of methanol extract (20 – 320 µg/ ml) for 48 hours. As shown in Fig. 4, the methanol extract increased the population of cells in the G0- G1 phase of the cell cycle from 63. 7 % to 71.1 % compared to untreated control. When treated with varying concentration of methanol extracts the population of cells in the G0-G1 phase increased from 69.1 % to 75.8 % as compared with that of the untreated control(Fig 5.3). E2 via ER influence the genes controlling cell proliferation, cell cycle progression or apoptosis resulting in uncontrolled proliferation leading to cancers. Many antiestrogenic anticancer agents from plants inhibit cancer cell growth through cell cycle regulation including G0/G1 accumulation (Cariou *et al.*, 2000). MCF-7 cells treated with the methanol extract caused G0-G1 arrest by increasing the population of G0-G1 phase from 69.1 % to 75.8 % as compared with that of

mediated pathways.

5.3 Methanol extract downregulates the ER mediated transcription

The effect of the extracts on *pS2* gene expression was studied as a model for endogenous estrogen responsive gene expressed by MCF-7 cells. E2 and ICI 182-780 were used as agonistic control antagonistic control respectively. MCF-7 cells were incubated with the extracts (100 µg/mL) or estradiol (10 nM) or ICI 182-780 (10 nM) for 24 h and RT PCR was performed to amplify the *pS2* mRNA using *gapdh* as endogenous control. As shown in Fig. 5.5 and 5.6, in comparison to other extracts the methanol extract decreased the expression of *pS2* gene to levels approximately as great as those produced by ICI 182-780. E2 up-regulated the expression of the gene. Estrogen induces the expression of various genes such as *c-fos, c-erb-b2, ER, PR, pS2* etc. *pS2* gene transcription is a primary response to E2 in human breast cancer cell line MCF-7 (Brown *et al.,* 1984). *pS2* gene is an estrogen related gene whose expression can be increased by estrogen and inhibited by antiestrogen (Masiakowski *et al.,* 1982). Since methanol extract down regulated *pS2* gene transcription, it was confirmed that methanol extract has some potential compounds that could down regulate the ER mediated transcription and cause antiestrogenic effects in breast tissue.

FIGURE 5.3

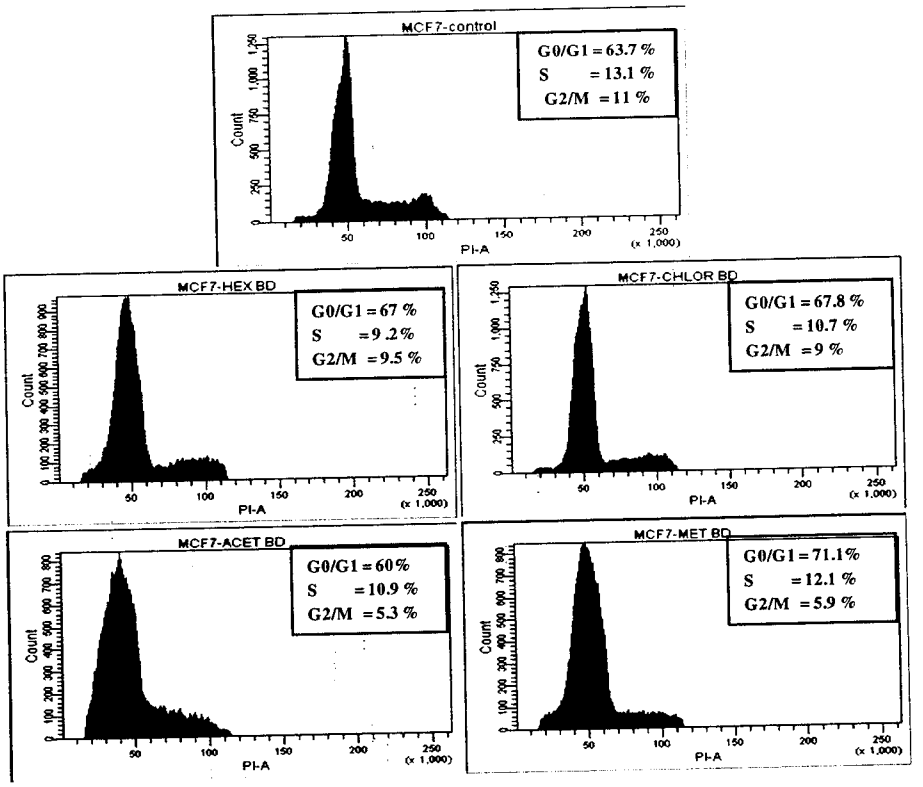

Fig.5.3. Inhibition of cell cycle progression in breast cancer cell lines as analyzed by flow cytometry. Flow cytometric analysis (propidium iodide staining) of the cell cycle distribution of MCF-7 cells without the extracts (control) and after treatment with 80 μg/ml hexane, cloroform, acetone and methanol extract of *B. diffusa* for 48 h.

FIGURE 5.1

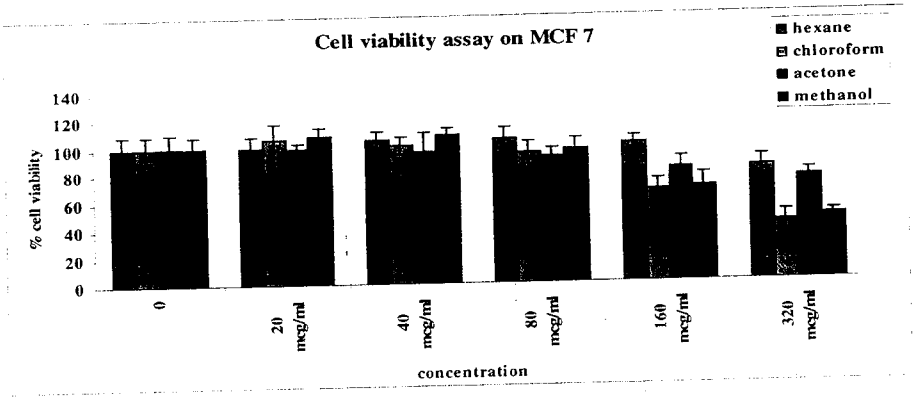

FIGURE 5.2

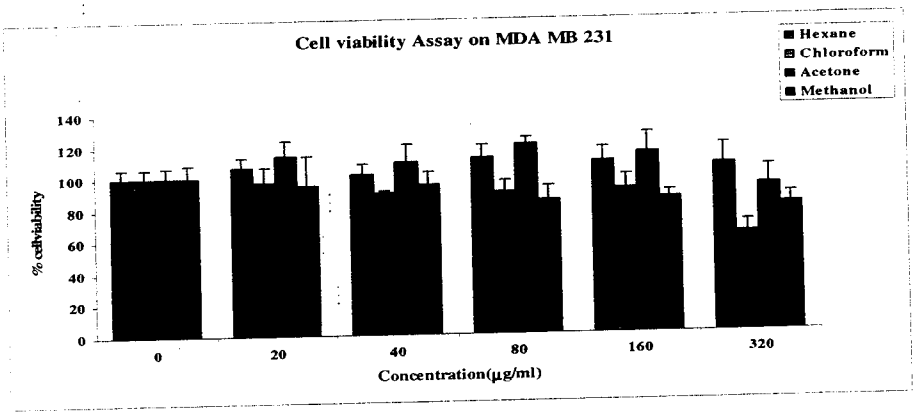

Fig 5.1 & 5.2. Effect of the extracts on cell viability/proliferation. MCF-7 , MDA MB-231 cell lines were treated with 0, 20, 40, 80, 160 and 320 *m*g/ml of the different extracts for 48 h and the cell viability was determined by MTT assay. The cell survival was expressed as % over the untreated control. A dose dependent growth inhibition of MCF-7 cells was observed after treatment with extrcats. Results are mean values ± S.E of five replicates.

FIGURE 5.4

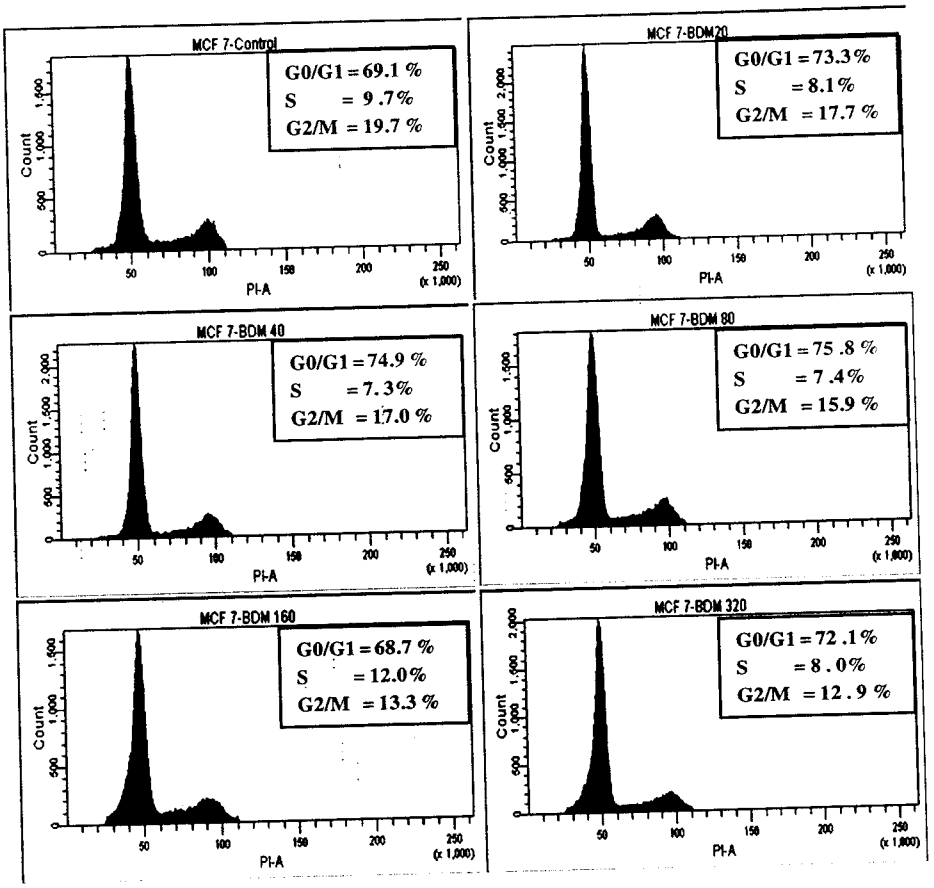

Fig.5.4. Inhibition of cell cycle progression in breast cancer cell lines as analyzed by flow cytometry. Flow cytometric analysis (propidium iodide staining) of the cell cycle distribution of MCF-7 cells without the extracts (control) and after treatment with 20, 40, 80 160 and 320 μg/ml of methanol extract of *B. diffusa* for 48 h.

FIGURE 5.5

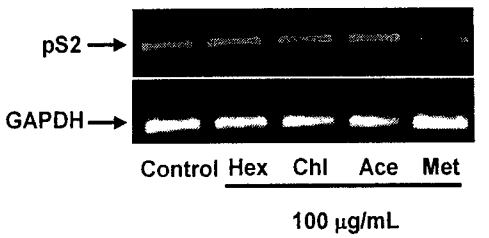

FIGURE 5.6

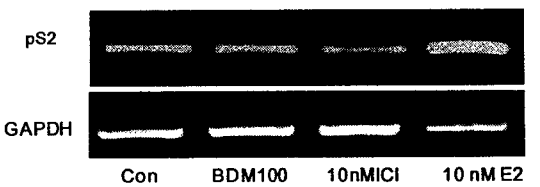

Fig.5.5 & 5.6. Effects of the extracts on *pS2* gene expression in MCF-7 cells. MCF-7 cells were incubated with hexane, cloroform, acetone and methanol extract of *B. diffusa* (100 µg/mL) for 24 h (5.5). MCF-7 cells were incubated with methanol extract of *B. diffusa* (, mg/mL), E2 (10 nM) or ICI 182-780 (10 nM) for 24 h (5.6). The transcripts were normalized with *GAPDH* expression level.

FIGURE 5.7

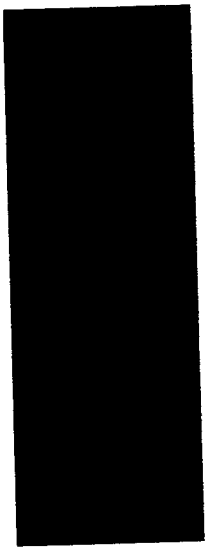

Fig.5.7. Thin layer chromatogram of the methanol extract of *B.diffusa* visualised under long UV.

CONCLUSION

traditional medicine for the treatment of human ailments including abdominal tumor, jaundice, menstrual disorders etc. This study aims to assess the anti-proliferative and anti-estrogenic effects of various solvent extracts of *B.diffusa* in breast cancer cell lines. The effective concentration range of the extract in cell viability will be analyzed by MTT Assay. The ability of the extracts to alter the cell cycle phases and distribution will be studied using FACS analysis. The effect of the extracts on the selected estrogen responsive genes will be analyzed by RT-PCR. Treatment with varying concentrations of methanol extract (20-320 µg/ml) resulted in moderate to very strong growth inhibition in MCF-7 cell lines. Methanol extract treatment resulted in a remarkable increase in the number of MCF-7 cells in the G0-G1 fraction from 69.1 % to 75.8 %, with a reciprocal decrease of cells in all other phases indicating cell cycle arrest at G0-G1 phase. RT-PCR analysis revealed that the methanol extract reduced the mRNA expression of *pS2* indicating its antiestrogenic action.

In conclusion, the potential antiestrogenic activity of *B. diffusa* against human breast cancer cells was investigated in this experimental study. *B. diffusa* extracts exhibited a strong inhibitory effect on the proliferation of human breast cancer cells *in vitro* and the antiestrogenic effects are mediated by ER. The results demonstrate that *B. diffusa* possess antiproliferative and antiestrogenic properties and that it resides in the most polar extractant used ie., methanol and suggest that it may have therapeutic potential in estrogen dependent breast cancers. However it warrants further identification of the active components of the extract and thorough analysis of its antiestrogenic and health promoting effects *in vivo* to demonstrate its suitability in hormone dependent breast cancer chemoprevention.

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APPENDICES

APPENDIX 1

MCF-7

ATCC	HTB-22™
Designation	MCF-7
Shipped	Frozen
Growth Properties	Adherent
Organism	*Homo sapiens*
Morphology	Epithelial
Source	**Organ** Mammary gland;Breast **Disease** Adenocarcinoma **Derived from metastatic site** Plural efflusion **Cell type** Epithelial
Cellular products	Insulin-like Growth Factor Binding Proteins (IGFBP) BP-2; BP-4; BP-5
Application	Transfection Host
Receptors	Estrogen receptor expressed
Comments	The MCF7 line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. The cells express the WNT7B oncogene. Growth of MCF7 cells is inhibited by tumor necrosis factor alpha (TNF alpha).
Preservation	**Freeze medium** Complete growth medium with 5% (v/v) DMSO

MDA MB-231

ATCC Number	HTB [26TM]
Designations	MDA MB- 231
Depositors	R Cailleau
Shipped	Frozen
Growth properties	Adherent
Organism	*Homo sapiens* (Human)
Morphology	Epithelial
Source	**Organ** Mammary gland; Breast **Disease** Adenocarcinoma **Derived from metastatic site** Pleural effusion **Cell Type** Epithelial
Applications	Transfection host
Receptors	Epidermal growth factor (EGF),expressed, transforming growth factor alpha (TGF α), expessed
Propagation	ATCC complete growth medium The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, Catalog No. 30-2008. To make the complete growth medium, add the following components to the base medium fetal bovine serum to a final concentration of 10%. **Atmosphere** air- 100% **Temperature** 37.0°C
Preservation	**Freeze medium** Complete growth medium supplemented with 5% (v/v) DMSO. **Storage temperature** liquid nitrogen vapor phase

Dulbecco's Modified Eagle's Medium (DMEM)

Reagents	Volume(g/l)
DMEM powder	17.3 g
Penicillin	0.1 g
Streptomycin	0.1 g
Sodium bicarbonate	3.7 g
pH	7.0 ± 0.3

APPENDIX 4

Agarose Gel Electrophoresis

Reagents	Volume(g/l)
0.5X DEPC water	1l
Agarose	8
EtBr	2µl

APPENDIX 5

1X TBE Buffer

Reagents	Volume(g/l)
Tris buffer	24.2g
Sodium chloride	146g

10X Electrode Buffer

Reagents	Volume (g/l)
Tris Buffer	30.3
Glycine	144