Chapter VIII

Evaluation of

Anticancer activity
8. ANTICANCER ACTIVITY

8.1 Introduction

Cancer, a cellular malignancy that results in the loss of normal cell-cycle control, such as unregulated growth and the lack of differentiation, can develop in any tissue of any organ and at any time. Globally, cancer is one of the top ten leading causes of death. It is estimated that 7.9 million people died of cancer (or around 13% of all deaths worldwide) in the year of 2007 and if current trends continue, 83.2 million more will have to die by 2015.

8.1.1 Carcinogenesis

Cancer is caused by DNA damage in genes that regulate cell growth and division. These changes are due to the interaction between genetic factors and also due to three categories of external agents which are physical carcinogens (ultraviolet and ionizing radiation), chemical carcinogens (asbestos, components of tobacco smoke etc.,) and biological carcinogens (infection from certain viruses, bacteria or parasites).

Mutations can also occur as a result of mistakes that are made during normal cells duplication of its DNA molecules prior to cell division. When cells acquire mutations in genes such as proto-oncogenes or tumor suppressor genes, these changes are copied to new generation of cells. More mutations in these altered cells lead to uncontrolled cell proliferation and finally lead to cancer (Zimney, 2008).

Breast cancer is a leading cause of death in women. Worldwide it accounts for 548,000 deaths of cancer mortality each year. There were 3738 female breast cancer cases reported during the year 2003. This accounted for 31% of newly diagnosed female cases. It is most prominent among the Chinese, followed by Indians and Malays. This statistics was sourced from the National Cancer Registry Reports 2002 and 2003. The incidence of breast cancer is steadily increasing (Gerard et al., 2008; Brinton et al., 2002). Breast cancer is common from the age of 15 years in all ethnic groups. Statistics in 2003 showed a peak incidence rate at the 50-59 age group and the rates then declined in the older age groups (Gerard et al., 2004; Brinton et al., 2002).
8.1.2 Factors

1. **Age:**
   
   Increasing age is one of the biggest risks of breast cancer development. The cumulative risk of developing breast cancer from birth to age 39 is less than 0.5% while from age 40-59 the risk is 1 in 25 i.e., 4%. From the age 60-79 the risk is 1 in 15 i.e., 7% and up to an age of 90 is 1 in 8, with overall lifetime risk of 12.5% (Ries et al., 2002).

2. **Gender:**
   
   The risk of developing the breast cancer is more in woman. However this malignancy can also be found in men.

3. **Abnormal genes:**
   
   The history of breast cancer revealed that the family history of breast cancer is an inherited genetic risk factor to the next generations for developing breast cancer. It accounts for 5-10% breast cancer cases (Easton et al., 1994)

4. **Menstrual and Reproductive factors:**
   
   The early age of menarche, nulliparity, non-lactation and late age of menopause increase the risk. The late age at first pregnancy i.e., after 30 years increase the breast cancer risk. Higher serum estrogen and testosterone levels are associated with risk of breast cancer.

5. **Hormone therapy:**
   
   The risk of breast cancer is more in cases under combination therapy than with oestrogen only therapy. Undergoing this hormone replacement therapy for over 5 years increases the risk by 5-30% (Chen et al., 2002).

6. **Exposure to radiation:**
   
   The median time to breast cancer development following radiation is approximately 15 years. The peak sensitivity period for developing breast cancer on exposure to radiation was found to be puberty-adolescence years (10-19 years), during the years of peak breast growth.

7. **Other factors:**
   
   Other factors associated with breast cancer include smoking, alcohol intake, obesity and sedentary lifestyle. High consumption of fats and high energy diets increases the IGF-1 levels, which are associated with increased breast cancer (Hankinson et al., 1998).
8.1.3 Risk profiling

Gail and Claus developed two different models for risk profiling of breast cancer in individual woman. The Gail model categorizes women into low, moderate and high risk groups and he developed a model to estimate the 5 year or lifetime probability of disease developing in individual woman (Gail et al., 1989). The Clauss model helps in predicting the individual’s life time risk for the development of breast cancer. These two models are the basis for breast cancer risk assessment at National Cancer Institute, U.S.A. (Domcheck et al., 2003 and Rubenstein et al., 2002).

8.1.4 Mammary gland development in human beings

The mammary gland is unique organ because most of its development occurs after the birth. In humans the mammary gland starts to develop during the fourth week of gestation. Although the mammary epithelium is derived from the epidermis its initiation is dependent on the presence of specialized mesenchyme called mammary fat pad. The epidermis direct epidermal cells to mammary differentiation pathway and induce their migration into the mammary pat fad. The anatomical structure of mammary gland was shown in Fig. 8.1.

**Fig. 8.1. Anatomy of Breast** (www.breastcancer.org).

**Breast profile**

A-ducts, B-lobules, C-dilated section of duct to hold milk, D-nipple, E-fat,

F-pectoralis major muscle, G-chest wall/rib cage.

**Enlargement:** A normal duct cells, B basement membrane, C lumen (center of duct). During embryogenesis only few, poorly branched mammary ducts or formed. After
birth the mammary gland remains in this rudimentary form until puberty when 
hormones, particularly oestrogen and progesterone induce further elongation, 
branching and extension of the already existing ducts. This leads to the generation of 
lobules that contain a terminal ducts splitting in to alveoli. However, most of these 
lobules in nulliparous woman or relatively simple but not highly branched structure.

![Mammary duct showing putative mammary stem cells and lobules](www.breastcancer.org)

The breast of post pubertal nulliparous women is composed of lobular 
structure reflecting different stages of development. The lobule type I, also called 
terminal ductal lobular unit (TDLU). It is the most and differentiated structure and it 
is composed cluster of 6 to 11 ductules per lobule. It evolves to lobule type II, which 
has a more complex morphology, being composed of higher number of ductules per 
lobule. Both lobules I & II types are present in the breast of mature nulliparous 
women. Extensive branching, alveologenesis and terminal differentiation of 
mammary epithelial cells only occur during full- term pregnancy.

During pregnancy, lobule I & II rapidly progress to lobule type III, which are 
characterized by having an average of 80 ductules are alveoli per lobule and to lobule 
type IV which become present only during the last trimester of pregnancy and the 
lactation period. After weaning, the paranchymal structure of the mammary gland 
regress to lobule III & lobule II, acquiring the appearance of lobule I after menopause.
8.1.6 Aetiology of Breast Cancer

Breast cancer results from a combination of many factors including inherited mutation or polymorphism of cancer susceptibility genes, environmental agents that influence the acquisition of somatic genetic changes and several other systemic and local factors.

![Aetiology of Breast Cancer Diagram](image)

**Fig. 8.3. Aetiology of Breast Cancer** (Kornelia Polyak, 2001)

8.1.7 Inherited mutation

A certain number of breast cancer cases (~10%) are attributable to inherited mutations in highly penetrant breast cancer susceptibility genes, two of which (BRCA 1 & BRCA 2) have been identified based on genetic linkage studies of affected families (Marcus *et al.*, 1996; Miki *et al.*, 1994).

In addition germ line mutations of PTEN, LKBI, ATM, P53, MS112/MLH1, Chk 2 & BACH-1 are associated with breast cancer but to a much remitted extent than the BRCA genes.
8.1.8 Acquired mutations

The majority of the tumors occur in women with no family history and the molecular basis of these sporadic breast cancers is still poorly defined. Genes responsible for inherited cancer predisposition (with the exception of P53) are not frequently genetically inactivated in sporadic tumors. Epigenetic inactivation of BRCA1 gene due to promoter hypermethylation has been described in a fraction of breast and ovarian carcinomas. Since the phenotype of these BRCA1 methylated sporadic carcinomas highly resemble that of tumors of patients with hereditary inactivation of the BRCA1 gene. It is hypothesized that epigenetic silencing is a mechanism if inactivation of BRCA1 in sporadic tumors.

Acquired mutation are caused by chemical carcinogens present in the environment or by physical carcinogens like UV radiation or ionizing radiation which includes X- Rays, α, β, r – rays or indiscriminate use of hormones and growth factors.

8.1.9 Polymorphism

Polymorphism in several metabolic and detoxifying enzymes (GSTML, CYP1A1, CYP17, NAT2, SULTLAL, COMT, SOD), components of hormonal signaling pathways (estrogen & androgen receptor), proto-oncogenes (H – cis VNTR), DNA repair genes (XRCC1, XRCC3) and HLA alleles have been shown to influence breast cancer susceptibility (Weber et al., 2000; Seth et al., 2000; Zheng et al., 2001).

8.2 Pathophysiology of Breast Cancer

8.2.1 Initiation and progression of Breast cancer

The natural history of breast cancer involves a sequential progression through defined clinical and pathological stages starting with atypical hyper-proliferation, progressing into in situ then into invasive carcinomas and culminating in metabolic disease (Fig.8.4).
8.2.2 Clinical and pathological stages of mammary cancer

Tumor progression is driven by the sequential acquisition of various genetic changes in a single cell followed by expansion and evolution into a well-defined carcinoma. This mammary tumor progression model is strongly supported by human epidemiological studies as well as by studies in animal models of breast cancer. Ductal carcinoma in situ (DCIS) is believed to be the true precursor of invasive ductal carcinoma (IDC) based on its frequent coexistence with invasive lesions and on its high rate recurrence as an invasive tumor at its original site. The histology of micro invasive carcinoma, invasive cancer cells measuring less than 1mm protruding from ducts of DCIS, is also consistent with DCIS being a precursor of invasive cancer. Recent comparative molecular analysis of DCIS and invasive tumors further supports this hypothesis and confirms that most invasive tumors arise through evolution from pre-existing in-situ disease (James et al., 1997)

In addition of genetic changes epigenetic alteration are also involved in the initiation and progression of breast carcinomas. Several genes have been demonstrated to be hypermethylated in breast carcinomas, including E-cartherin, BRCA1, estrogen receptor, GSTP1, MDGI(Mammary derived growth inhibitor), HoxA5, 14-3-3 sigma, and cyclin D2 (Graff et al., 1995; Esteller et al., 2000; Ferguson et al., 2000). Among these only 14-3-3σ is methylated in more than 50% of primary invasive breast carcinomas and only cyclin D2 has been implicated in pre-invasive lesions.
8.2.3 The Role of non-epithelial cells in breast cancer initiation and progression

Mammary drugs and alveoli are comprised of luminal epithelial cells and myoepithelial cells that surround and separate luminal cells from the basement membrane and stromal cells. The close cellular contact of luminal and myoepithelial cells enables autocrine and paracrine interactions mediated by secreted and are cell-cell contact dependent factors. Several observations indicate the roll of myoepithelial cells in breast tumorigenesis and indicate that they may have a tumor suppressive
function (Sternlicht et al., 1997). Consistent with this, myoepithelial cells have been shown to have anti-proliferative, anti-invasive and anti-angiogenic effects both in vitro and in vivo mediated by the expression of unidentified factors, protease inhibitors (Serpins, TIMP-1) and angiogenesis inhibitors (Thrombospondin 1, soluble hFGF receptors), respectively (Alpaugh et al., 2000). In addition, myoepithelial cells are necessary for the maintenance of organized breast ducts and may regulate the morphogenesis of luminal epithelial cells. It is possible that the elimination of myoepithelial cells and the paracrine factors produced by them; in breast tumors may promote cell proliferation, invasive behavior and angiogenesis.

Another well characterized stromal cell type with an important role in mammary development and tumor genesis is the mammary fibroblast. Embryonic mesenchyme induces the differentiation of normal mammary epithelial cells and even transplanted mammary carcinomas (De Cosse et al., 1973 and 1975). Several hormones including progesterone and growth and differentiation factors like inhibins and activins regulate mammary epithelial cells indirectly through mesenchymal cells. In addition, fibroblasts contribute to the formation of proper extracellular matrix, which plays an essential role in mammary cell differentiation and morphogenesis. There are several indications that fibroblasts derived from normal mammary gland behave differently from those derived from peri-tumoral stroma. Based on in vitro co-culture experiments normal fibroblasts inhibit, while tumor derived fibroblasts stimulate the growth of breast carcinomas (Dong-Le Bourthis et al., 1997). Molecular characterization of peri-tumoral stromal cells has led to the identification of chromosomal abnormalities.

8.3 Classification of Tumors

Tumors are categorized into benign or malignant based on certain morphological features and biological properties of the tumor. The characteristics of tumors may be described as follows:

1. Macroscopic features
2. Microscopic features
3. Growth rate
4. Local invasion
5. Metastasis
8.4 Macroscopic features

The gross appearance of a tumor although not diagnostic may help in differentiating benign from malignant tumors. Benign tumors are generally spherical / ovoid, well circumscribed/ encapsulated and freely mobile with homogenous cut surface. Malignant tumors on the other hand are irregularly shaped, unencapsulated and poorly circumscribed with infiltrating margins. Secondary changes like ulceration, hemorrhage and infraction are more common with malignant tumors. Table 8.1 shows some characteristics of typical benign and malignant tumors.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate</td>
<td>Slow</td>
<td>Rapid</td>
</tr>
<tr>
<td>Mitosis</td>
<td>Few</td>
<td>Many</td>
</tr>
<tr>
<td>Nuclear chromatin</td>
<td>Normal</td>
<td>Increased</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Local growth</td>
<td>Expansile</td>
<td>Invasive</td>
</tr>
<tr>
<td>Encapsulation</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Destruction of tissue</td>
<td>Little</td>
<td>Considerable</td>
</tr>
<tr>
<td>Vessel invasion</td>
<td>None</td>
<td>Frequent</td>
</tr>
<tr>
<td>Metastases</td>
<td>None</td>
<td>Frequent</td>
</tr>
<tr>
<td>Effect of host</td>
<td>Often insignificant</td>
<td>Significant</td>
</tr>
</tbody>
</table>

8.5 Experimental Models of Breast Cancer

In order to establish experimental models for carcinogenesis of mammary glands or any other organ, the following goals must be met.

1. The tumors produced must have histological and biological similarities to human lesions.
2. The resulting tumors have to be induced with a single dose (or at most only a few carcinogenic doses)
3. The tumor induction has to be specific for the target organ and should not be compromised by induction of tumors at several sites.
The experimental approach to evaluate chemo preventive agent of breast cancer in laboratory includes

1. *In vitro* models
2. *In vivo* models

### 8.5.1 *In Vitro* Models

There are two types of *in vitro* models

1. Cell culture Models
2. Organ culture models

#### 8.5.1.1 Cell Culture Models

Cell culture models would be useful in studying the effects of chemopreventive agents since they represent a homogenous cell population and the effects of a given chemopreventive agent can be studied in a controlled environment.

Several human breast cancer cell lines which can be grown in different culture media in laboratory have been developed to study the anti-proliferative and anti-carcinogenic activity of the various test drugs.

Some of the important breast cancer cells lines are:

MDA-MB 468, MDA-MB 231, MDA-MB 435, HBL-100, MCF-7, A2l2, BT-474 etc.

Mammary gland organ culture models have been developed on the basis that when mammary glands from young virgin female mice were exposed to chemical carcinogen 7, 12-dimethylbenz (a) anthracene (DMBA) for 24 h in culture, they develop hyperplasic alveolar nodule-like mammary lesions under appropriate hormonal conditions (Lin *et al.*, 1976). These cell transformation models can be used to evaluate the effects of potential chemopreventive agents.
8.5.2 In Vivo models

Mammary carcinogenesis in animal models is of the following types:

8.5.2.1 Physically induced mammary carcinoma models

Mammary carcinomas can be developed in mice, rats etc by irradiation of the animals with UV-B rays and γ-rays. The animals are irradiated locally in the area of the mammary gland, which leads to the development of mammary tumors in those areas.

8.5.2.2 Hormonally induced mammary carcinoma models

Medroxyprogesterone acetate is administered to the BALB/c mice, which leads to the development of mammary carcinoma.

8.5.2.3 Biologically induced mammary carcinoma models

Mammary carcinoma can be developed by the inoculation of malignant cells or virus in animals. The development of immuno deficient mouse models engrafted with human tumors can be used to evaluate anticarcinogenic activity of drugs e.g A2L2 cells, MDA-MB-435 cells, ascites in mice lead the development of mammary carcinoma. HBL-100 human breast epithelial cells transformed with SV 40 virus are inoculated into animals, which result in the development of mammary carcinoma.

8.5.2.4 Transgenic or genetically modified animal models

Transgenic and knockout animal models with a genetically engineered predisposition to develop cancer are being used increasingly in the breast cancer research.

e.g. The p53 knockout mouse is predisposed to develop neoplasms relatively early in life.

8.5.2.5 Chemically induced mammary carcinoma models

The most widely used mammary carcinogenesis models include polycyclic hydrocarbons, such as Dimethylbenz (a) anthracene (DMBA) or benzo (a) pyrene-induced or directly-acting carcinogen N-methyl N-nitrosourea (MNU)-induced mammary cancers in female Sprague Dawley rats. Other carcinogens such as
parathion, methion, serine and other rat species such as Fisher or Wistar Furth rats have been studied as possible animal models to induce mammary cancers. However, due to poor incidence and reduced specificity, these models have not been frequently employed.

DMBA and MNU induced mammary carcinogenesis models are similar in many respects. Both carcinogens induce mammary adenocarcinoma with a single dose. The tumor induction is mammary gland specific. There is well-established dose-dependence and tumors are developed without any systemic toxicity. Both models are extremely reproducible. The multiplicity can also be adjusted with carcinogen dose.

In majority of MNU-induced rat mammary carcinomas, the H-ras onchogene is activated by a G to A transition at the second nucleotide of codon 12 encoding glutamic acid in place of glycine. These result in the single point mutations, which are responsible for the MNU-induced mammary cancer.

8.6 Literature Review

Although there are many cytotoxic drugs available, many cancer patients develop resistance to the drugs. These drugs also have a wide range of side effects. Many anticancer drug studies are done in order to find new anticancer drugs with broader the therapeutic spectrum and with minimum side effects (Abdille et al., 2005).

Natural products are well known to exhibit many biological properties and one of them is antitumor property. Natural products with antitumor properties are known to have the ability to trigger cell death pathways such as apoptosis of the cancer cells. Betulinic acid is one of the natural products found to exhibit potent antitumor activities by triggering the mitochondrial path to apoptosis (Abdille et al., 2005; Kommera et al., 2010; Drag et al., 2009; Csuk et al., 2010; Fulda et al., 2008; Sami et al., 2006).

Despite the therapeutic advances made in understanding the processes involved in carcinogenesis, cancer has become one of the most serious medical problems today. The worldwide mortality rate was increasing annually and seven million deaths were occurring every year. For this reason, cancer chemotherapy has become a major focus area of research. Different life styles, risk factors (such as age,
gender, race, genetic disposition) and the exposure to different environmental carcinogens, lead to the varying patterns of cancer incidence (Chang and Kinghorn, 2001).

The discovery and development of plant-derived compounds led to the first cures of human cancer, specifically upon administration of these compounds in combination with synthetic agents, of the 121 medications being prescribed for use in cancer treatment, 90% are sourced from plants and 74% of these discoveries were as a result of an investigation into the claims made by folkloric tradition (Shishodia and Aggarwal, 2004). Examples of some compounds used as cytotoxic drugs are given in Table 8.2.

A number of mechanisms exist by which phytochemicals aid in the prevention of cancer. This preventative action most probably results from the additive or synergistic effects of a number of phytochemicals, since cancer is a multi-step process.

Proposed mechanisms by which phytochemicals may prevent cancer include:

(i) Anti-oxidant and free radical scavenging activity.
(ii) Antiproliferative activity.
(iii) Cell-cycle arresting activity.
(iv) Induction of apoptosis.
(v) Activity as enzyme cofactors.
(vi) Enzyme inhibition.
(vii) Gene regulation.
(viii) Activity as hepatic phase I enzyme inducers.
(ix) Activity as hepatic phase II enzyme inducers.

Oxidative damage to DNA, proteins and lipids, resulting from an increase in oxidative stress, is considered to be one of the most important mechanisms contributing to the development of cancer. As the oxidative damage is linked to the multistep process of carcinogenesis, this may be prevented or at least limited, by the consumption of anti-oxidants (mechanism (i), as described above) (Liu, 2004).
<table>
<thead>
<tr>
<th>Therapeutic agent</th>
<th>Chemical compound</th>
<th>Mechanism of action</th>
<th>Treatment of cancer type</th>
<th>Plant source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vinblastine,</strong> vincristine</td>
<td>Alkaloids</td>
<td>Inhibition of tubulin polymerization</td>
<td>Hodgkin’s disease</td>
<td>Catharanthus roseus</td>
<td>Mans et al. 2000</td>
</tr>
<tr>
<td><strong>Etoposide,</strong> teniposide</td>
<td>Epipodophyllotoxin</td>
<td>Inhibition of topoisomerase II</td>
<td>Testicular cancer, and small cell lung carcinoma, lymphomas</td>
<td>Podophyllum peltatum</td>
<td>Lee 1999, Mans et al. 2000</td>
</tr>
<tr>
<td><strong>Paclitaxel,</strong> docetaxel</td>
<td>Taxanes</td>
<td>Promotion of tubular stabilization</td>
<td>Ovarian and breast cancer</td>
<td>Taxus brevifolia</td>
<td>Mans et al. 2000</td>
</tr>
<tr>
<td><strong>Irinotecan,</strong> topotecan, 9-aminocamptothecin, 9-nitrocamptothecin</td>
<td>Alkaloids</td>
<td>Inhibition of topoisomerase I</td>
<td>Colorectal, lung, cervix and ovarian cancer</td>
<td>Camptotheca acuminate</td>
<td>Srivastava et al. 2005</td>
</tr>
<tr>
<td><strong>Homoharringtonine</strong></td>
<td>Alkaloid</td>
<td>Inhibition of DNA polymerase</td>
<td>Various leukemias</td>
<td>Harringtonia cephalotaxus</td>
<td>Mans et al. 2000</td>
</tr>
<tr>
<td><strong>4-Ipomeanol</strong></td>
<td>Pneumotoxic furan derivative</td>
<td>Cytochrome P-450-mediated conversion into DNA-binding metabolites</td>
<td>Lung cancer</td>
<td>Ipomoea batatas</td>
<td>Mans et al. 2000</td>
</tr>
<tr>
<td><strong>Elliptinium</strong></td>
<td>Semi-synthetic derivative of ellipticine</td>
<td>Inhibition of topoisomerase II</td>
<td>Advanced breast cancer</td>
<td>Bleekeria vitensis</td>
<td>Mans et al. 2000</td>
</tr>
<tr>
<td><strong>Flavopiridol</strong></td>
<td>Synthetic flavone derived from plant alkaloid rohitukine</td>
<td>Inhibition of cyclin dependent kinases</td>
<td>Colorectal, prostate, lung, renal carcinoma</td>
<td>Amoora rohituka, Dysoxylum binectariferum</td>
<td>Mans et al., 2000</td>
</tr>
</tbody>
</table>

Table 8.2: Cytotoxic drugs developed from plant sources.
8.6.1 Role of lignans in the treatment of cancer

Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals present in a wide variety of plants and display a large number of biochemical and pharmacological properties, including protective effects against cancer. Phytoestrogens are non-steroidal plant molecules with estrogen-like activity and they are capable of interacting with estrogen receptors, showing both agonist and antagonist methods of action. Hence, these are very useful in the treatment of breast cancer thus regulating the levels of gonadal harmones. A number of mechanisms by which lignans are able to prevent carcinogenesis have been reported. These mechanisms include their free radical scavenging ability (Krithika et al., 2009), the modification of enzymes to activate or detoxify carcinogens (Hari Kumar and Kuttan, 2006) and the lignans are capable of induction of apoptosis in conjunction with their antimetastastic action (Lee et al., 2011).

A number of natural products are used as chemo protective agents against commonly occurring cancers. The phytochemicals, which act against cancer are curcumin, genistein, resveratrol, diallyl sulfide, (S)-allyl cystein, allicin, lycopene, ellagic acid, ursolic acid, catechins, eugenol, isoeugenol, isoflavones, protease inhibitors, saponins, phytosterols, vitamin C, lutein, folic acid, carotene, vitamin E and flavonoids (Reddy et al., 2003).

8.6.2 Brief review of anticancer activity of Phyllanthus species

The ethanolic extracts of three Phyllanthus species, P. urinaria, P. amarus and P. debilis at the concentration of 10µg/mL significantly inhibited the proliferation of the HepG2 cells by inducing TNF-α production from the hepatocellular carcinoma cells while inhibiting production of potent anti-apoptotic genes IL-8 and COX-2 (Sureban et al., 2006). The extracts induced apoptosis by inducing caspase-3. Phyllanthus amarus inhibited the N-methyl-N-nitrosoguanidine (MNNG) induced stomach cancer in male Wistar rats. It also reduced the incidence of gastric neoplasms in rats (44%) as well as their numbers (Raphael et al., 2006).

The methanolic extract of P. amarus hairy roots revealed potent antiproliferative activity in the MCF-7 cells through induction of apoptosis mediated by increased intracellular reactive oxygen species (ROS) in conjunction with decreased mitochondrial membrane potential (Abhyankar et al., 2010).
In vitro, apoptotic effect of 75% methanolic extract of aerial parts (stem and leaves) of *P. amarus* against Dalton’s Lymphoma Ascites (DLA) cells in culture produced significant reduction in cell viability as determined by the MTT assay (Harikumar *et al.*, 2009). Oral administration of 75% methanolic extract of aerial parts (stem and leaves) of *P. amarus* was found to enhance the life span of leukemia harboring animals and decrease the incidence of anemia (Harikumar and Kuttan, 2009).

A mixture of phyllanthin and hypophyllanthin (1:1) isolated from *P. amarus* exhibited antitumor activities against EAC in Swiss albino mice. The decrement of tumor volume and packed cell volume and viable cell count were observed in lignan treated mice when compared only to EAC tumor bearing mice. Treatment with test compounds increased the survival time and normal peritoneal cell count. Hematological parameters, PCV which were altered by tumor volume inoculation, were restored considerably (Islam *et al.*, 2008a).

The objective of this study was to determine the in vitro anti-cancer activities of *P. amarus* aerial part extracts (PAHE, PAEA & PAME) and isolated compounds (phyllanthin & hypophyllanthin) on human estrogen-receptor positive breast cancer (MCF-7) cell line and human estrogen-receptor negative breast cancer (MDA-MB-231) cell line. This study also includes the in vivo anticancer evaluation of potent extract which showed better results in in vitro studies and isolated compounds phyllanthin (PAPH) and hypophyllanthin (PAHP) in MNU induced breast cancer model in Sprague Dawley rats.

### 8.6.3 Phyllanthin and Hypophyllanthin

Phyllanthin and hypophyllanthin are the two major bioactive lignans available in almost all parts of the plants belonging to *Phyllanthus* species. Phyllanthin is a bitter constituent and hypophyllanthin is a non-bitter constituent. Phyllanthin and hypophyllanthin were isolated by column chromatography as given in 4.3.
8.7 IN VITRO ANTICANCER ACTIVITY

8.7.1 Materials and methods

Human estrogen-receptor positive breast cancer (MCF-7) cell lines, Human estrogen-receptor negative breast cancer (MDA-MB-231) cell lines were collected from the American Type Culture Collection (ATCC), USA. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and Dulbecco’s Modified Eagle’s Medium (DMEM) were obtained from Sigma Chemical Co., St.Louis, MO. Fetal bovine serum (FBS) from Arrow labs, penicillin-G, sodium streptomycin sulphate, phosphate buffered saline (PBS), trypsin-EDTA from Highveld Biological, L-glutamine from Cambrex Bioproducts. Trypan blue from Sigma-Aldrich Co., St.Louis, Mo. 96 well flat bottom tissue culture plates- Tarson, Centrifuge Sigma 2-16KC, Refrigerated -Sartorius AG, Germany. UV-visible spectrophotometer- Shimadzu Corporation, Japan. Neubauer haemocytometer - Superior Marienfeld, Germany.

8.7.2 Preparation of plant samples

Stock solutions of P.amarus extracts (PAHE, PAEA & PAME), phyllanthin (PAPH) and hypophyllanthin (PAHP) in methanol at a concentration of 100 mg/mL were prepared. The final well concentrations of 10μg/mL, 20μg/mL, 40μg/mL and 80μg/mL were prepared using experimental medium.

8.7.3 Principle of the MTT assay

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay is an antiproliferative assay used to assess the growth inhibition of cells and used to measure the activity of enzymes in mitochondria to reduce the yellow tetrazolium salt MTT to purple formazan crystal. Mitochondrial dehydrogenase enzymes from viable cells cleaved tetrazolium rings then form crystals which were largely impermeable to a cell membrane. Impermeability causes accumulation of healthy cells which were then solubilized by Dimethyl Sulfoxide (DMSO). The number of surviving cells is directly proportional to the level of the formazan produced (Mosmann, 1983).
This colorimetric assay indirectly estimates the viable cell number by staining total cellular proteins (Mosmann, 1983; Swarnalatha et al., 2010). The ability of *P. amarus* hexane, ethyl acetate and methanolic extracts, phyllanthin and hypophyllanthin to inhibit the *in vitro* growth of two human cancer cell lines (MCF-7 & MDA-MB-231) was evaluated.

### 8.7.4 Experimental procedure (Swarnalatha et al., 2010)

#### 8.7.4.1 Cell lines and cell culture

The MCF-7 and MDA-MB-231 cell lines were collected from the American Type Culture Collection (ATCC), USA. All cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% FBS, 1mL per 500mL media of 10mg/mL penicillin-G, 10mg/mL sodium streptomycin sulphate and 5mL per 500mL media of 2 mM L-glutamine. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2. Once the cells were at 75% confluency, they were subcultured by aspirating the media and replacing it with 0.1 mM (500μL) phosphate buffered saline (PBS) (pH 7.4) and 1mL 0.05% (2 mg/mL) trypsin-EDTA. The flasks were incubated at 37°C for 10 - 15 min, until the majority of cells had lifted. The trypsin was then inactivated by the addition of experimental media (antibiotic-free, serum supplemented DMEM medium).

A single cell suspension was formed by the gentle pipetting action. The cell suspension was centrifuged at 1000 rpm for 3 minutes and the supernatant discarded. The pellet was resuspended in its respective medium, an aliquot of which was stained with 0.2% (w/v) trypan blue and the cells were counted using a haemocytometer. This single cell suspension, with a cell viability of greater than 95%, was then diluted in culture medium to obtain a standard cell suspension of 1,50,000 cells/mL.

#### 8.7.4.2 The MTT assay

Aliquots of 100μL of 150000 cells were seeded into a 96-well microtiter plate. The plates were incubated at 37°C for 24 hours to facilitate the attachment of the cells to the bottom of the wells. Plant extract or compound to be tested was added at different concentrations of 10, 20, 40 and 80μg/mL with serial two-fold dilutions of
cell culture medium (100μL) to the wells already containing 100μL of cell suspension, in triplicate.

The plates were incubated for a further 48 hours at 37°C. On completion of the 48 hour incubation period, 10μL of MTT reagent was added to each well. The plates were then incubated at 4°C for 1 hour, after which the supernatant was washed from the wells (washed out 5 times) with PBS to remove any excess amounts of MTT reagent, experimental media or any other low molecular weight metabolites. The plates were inverted and left overnight to air dry. Once dried, 100μL of DMSO was added to all wells to stain the fixed cells and aid in assessing the cell growth and the plate was shaken at 960 rpm for 3 min on a Tecan microtiter plate reader, dissolving bound dye present in the wells. The absorbance was then read at 570nm. The colour intensity of each well corresponds to the number of viable cells, an indication of the inhibitory effect of the extracts or test compounds added. The percentage growth inhibitions \( \left[ \frac{(\text{absorbance of control cells} - \text{absorbance of treated cells})}{\text{absorbance of control cells}} \right] \times 100 \) were calculated and plotted against the concentrations. The IC\(_{50}\) values were calculated by using Graph pad prism-5 software.

### 8.7.5 Results and Discussion

The inhibition of cancer cell proliferation and cellular viability by *P. amarus* hexane, ethyl acetate, methanolic extracts, phyllanthin and hypophyllanthin against two human cancer cell lines was evaluated and is detailed in Table 8.3.

The evaluation of the cytotoxic potential of *P. amarus* hexane extract, ethyl acetate, methanolic extracts, phyllanthin and hypophyllanthin was investigated using the MTT assay. The results are expressed as percentage cell growth inhibition at 80μg/mL, while those indicating a percentage inhibition of greater than 80% at 80μg/mL were expressed as IC\(_{50}\) values, the latter defined as the concentration causing 50% cell growth inhibition. After a continuous 48 hour exposure of the cells to the test extracts and compounds, in accordance with the “NCI-cell line screen”, the activity observed was most certainly reflective of a cancer type-specific sensitivity.

The percentage cell growth inhibition at 80μg/mL of *P. amarus* hexane, ethyl acetate, methanolic extracts, phyllanthin and hypophyllanthin against the tested cell lines were found to be 82.42±3.14, 81.65±2.69, 86.74±2.25, 92.27±2.84 and
90.17±1.62 (MCF-7) and 80.25±2.94, 79.54±2.15, 87.92±1.84, 94.14±3.14 and 89.56±2.85 (MDA-MB-231). The *P.amarus* aerial part extracts and isolated compounds exhibited an inhibitory effect greater than 80% at concentration of 80μg/mL on the tested cancer cell lines. The inhibitory activity of the extracts showed concentration-dependent activity (Fig. 6.05 and 6.06), which increased with an increase in extract or compound concentration. The IC$_{50}$ values of *P.amarus* hexane, ethyl acetate, methanolic extracts, phyllanthin and hypophyllanthin against the tested cell lines were found to be 54.4±1.12, 54.68±1.09, 50.27±1.87, 32.51±0.95 and 35.18±1.48 (MCF-7) and 56.85±2.26, 56.64±1.98, 49.17±2.34, 36.22±1.17 and 38.74±1.24 (MDA-MB-231). Among the tested extracts and two isolated compounds the most promising activity was observed for phyllanthin against the MCF-7 and MDA-MB-231 cells. The order of activity is found to be phyllanthin > hypophyllanthin > methanolic extract > ethylacetate extract > hexane extract.

The hexane, ethylacetate and methanolic extracts of *P.amarus* aerial parts showed inhibition of cell growth when treated against breast cancer cell lines i.e., MCF-7 and MDA-MB-231. Upon phytochemical screening, these extracts showed the presence of lignans, sterols, terpenoids, flavonoids, alkaloids and glycosides (Chapter 3; Table 3.2). The anti-proliferative effects of *P.amarus* could be due to the presence of these different classes of bioactive compounds.

It has been previously reported that the four plant species of *Phyllanthus* (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) showed anti-proliferative effects on different cancer cells; without cytotoxic effects on their respective normal cells (Lee *et al.*, 2011 and Sureban *et al.*, 2006). Several studies reported that the effectiveness of organic-soluble compounds in inhibiting or being lethal to cancerous cells is because most bioactive compounds are more likely to dissolve in organic solvents such as hexane, ethylacetate and methanol and only partially dissolve in polar solvents such as water (Wu *et al.*, 2009; Saetung *et al.*, 2005; Ojala *et al.*, 2000). Their anti-proliferative effects are always associated with their natural antioxidant activity. The role of these bioactive compounds on cancer was well documented as they can reduce the chance of cancer development by preventing mutation to occur in normal cells, which was caused by free radicals. Due to their protective role on normal cells, the anti-proliferative effects on these cells were diminished.
Lignan rich fractions and the isolated lignans of *P. amarus* like phyllanthin, hypophyllanthin, niranthin and nirtetralin were known to have good antioxidant (Madhukiran *et al.*, 2013 and krithika *et al.*, 2009) and anticancer activities (Leite *et al.*, 2006). Various other phytochemical groups of *P. amarus* like flavonoids, glycosides, phenolic compounds and alkaloids (Moon *et al.*, 2006; Rusak *et al.*, 2005; Dixit *et al.*, 2011 and Lee *et al.*, 2011) are reported to have antioxidant and anticancer activities, all these together may contribute to the observed anticancer activity.

Existing research has indicated that a tumour is a dynamic system, consisting of cancer cells, often of multiple classes, supporting stroma normal cells and frequently lymphocytes. Tumors are highly individualistic and researchers have concluded that each tumour has its own individual drug response spectrum (Hoffman, 1991). This phenomenon was shown to be true by the current comparison of inhibitory activity of the two different cell lines, and their sensitivity to the tested extracts and isolated compounds of *P. amarus*. The methanolic extract (PAME) showed better activity when compared to the hexane (PAHE) and ethylacetate (PAEA) extracts with percentage cell growth inhibition of greater than 80% in tested cell lines at 80µg/mL concentration. The phyllanthin showed potent inhibitory activity than hypophyllanthin against the growth of tested two cancer cell lines. The IC<sub>50</sub> values (Table 8.3) indicates that the concentration dependent inhibitory effect of the tested extracts and compounds of *P. amarus* aerial parts.

*Table 8.3 Percentage cell growth inhibition (% CGI) and the IC<sub>50</sub> values of human cancer cell lines of *P. amarus* aerial part extracts and isolated lignans.*

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th><em>P. amarus</em> Extracts/compounds</th>
<th>MCF-7</th>
<th></th>
<th>MDA-MB-231</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% CGI</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>% CGI</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µg)</td>
</tr>
<tr>
<td>1</td>
<td>Hexane extract (PAHE)</td>
<td>82.42±3.14</td>
<td>54.4±1.12</td>
<td>80.25±2.94</td>
<td>56.85±2.26</td>
</tr>
<tr>
<td>2</td>
<td>Ethylacetate extract (PAEA)</td>
<td>81.65±2.69</td>
<td>54.68±1.09</td>
<td>79.54±2.15</td>
<td>56.64±1.98</td>
</tr>
<tr>
<td>3</td>
<td>Methanolic extract (PAME)</td>
<td>86.74±2.25</td>
<td>50.27±1.87</td>
<td>87.92±1.84</td>
<td>49.17±2.34</td>
</tr>
<tr>
<td>4</td>
<td>Phyllanthin (PAPH)</td>
<td>92.27±2.84</td>
<td>32.51±0.95</td>
<td>94.14±3.14</td>
<td>36.22±1.17</td>
</tr>
<tr>
<td>5</td>
<td>Hypophyllanthin (PAHP)</td>
<td>90.17±1.62</td>
<td>35.18±1.48</td>
<td>89.56±2.85</td>
<td>38.74±1.24</td>
</tr>
</tbody>
</table>

MCF-7: Human estrogen receptor positive breast cancer cell lines; MDA-MB-231: Human estrogen receptor negative breast cancer cell lines; Results are given as Mean ± SEM, n=3.
Fig. 8.5 Percentage cell growth inhibition of the MCF-7 cells by hexane (PAHE), ethylacetate (PAEA), methanol extracts (PAME), phyllanthin (PAPH), hypophyllanthin (PAHP) of *P. amarus* aerial parts.

Fig. 8.6 Percentage cell growth inhibition of MDA-MB-231 cells by hexane (PAHE), ethylacetate (PAEA), methanol extracts (PAME), phyllanthin (PAPH), hypophyllanthin (PAHP) of *P. amarus* aerial parts.
8.8 IN VIVO ANTICANCER ACTIVITY

The methanolic extract of *P. amarus* aerial parts (PAME), phyllanthin (PAPH) and hypophyllanthin (PAHP) showed prominent anticancer activity in *in vitro* studies on breast cancer cell lines MDA-MB-231 and MCF-7 (Table 8.3). Since the tested extracts and lignan molecules showed *in vitro* anticancer activity, it was also aimed to evaluate their anticancer activity *in vivo*, particularly in mammary carcinoma model. Hence, the present study was designed to evaluate the anticancer activity of methanolic extract of *P. amarus* aerial parts (PAME), phyllanthin (PAPH) and hypophyllanthin (PAHP) in *N*-methyl *N*-nitrosourea (MNU) induced mammary cancer in female Sprague-Dawley rats.

8.8.1 Materials and methods

Animals and Diet

Virgin female Sprague-Dawley rats of 35 days old were obtained from Mahaveer enterprises, Hyderabad. The animals were housed in plastic cages with husk-bedding in a temperature (22±2°C) and humidity (60±10%) controlled animal room under a 12h light/12h dark cycles. The animals were fed a commercial diet (Nutrimix Std-1020, Nutravet Pvt. Ltd, Pune) *ad libitum* and water freely throughout the study. The animals were experimented with prior approval of the institutional ethics committee (Regd No. 516/PO/c/01/ CPCSEA).

8.8.2 CHEMICALS

Carcinogen

*N*-methyl *N*-nitrosourea (MNU) was obtained from Sigma Chemical Co. St. Louis, MO, USA. Up on arrival MNU was stored at -20°C in the dark.

8.8.3 *P. amarus* aerial part extracts, Phyllanthin and Hypophyllanthin

The aerial parts of the *P. amarus* was collected, shade dried and successively extracted with hexane, ethylacetate and methanol using Soxhlet apparatus. The extraction of *P. amarus* aerial parts was given in 3.3.1. The concentrated methanolic extract (PAME) was used in this study. The bioactive lignans phyllanthin (PAPH) and
hypophyllanthin (PAHP) were isolated from *P. amarus* hexane extract by column chromatography as given in 4.3.

**8.8.4 Induction of Mammary Carcinogenesis**

MNU was dissolved immediately prior to its use in 0.9% NaCl (Normal Saline) containing 0.05% acetic acid (pH 5) for systemic exposure. The intraperitoneal injection of 50mg/kg body weight, a dose known to produce a high incidence of mammary cancer (Mc Cormick *et al.*, 1981; Takahiko Gotoh *et al.*, 1998) was made along the ventral midline of the 50-days old rats.

As MNU is listed as the Hazardous Substance, MNU was handled as a carcinogen and a teratogen with extreme caution.

The following precautions were taken during the induction of MNU

1. In the laboratory utmost care was taken to avoid any skin contact with MNU. Protective gloves, facemask, shoe covers, and protective clothing were put on before the induction process.
2. The induction was done in an enclosed area with barrier protection so as to avoid any chance in inhalation.
3. The clothing, gloves face mask containers, syringes etc used in the process of induction were disposed by incarnation at a remote area.

**8.8.4.1 Preparation of sodium CMC suspension**

Stock suspension of sodium CMC was prepared by triturating the powder sodium CMC (1g) finely in 2.5 mL of water, 1:10 dilution of this stock solution made in distilled water was used for suspending the test and standard drugs. Different doses of PAME, PAPH and PAHP (Table 8.4) were weighed accurately and suspended in 2mL of aqueous solution of sodium.CMC by vortexing (Vortex, Genei, India) for 5 minutes. The volume for each oral dose of test extract or compound was made to 2mL/kg body weight.

**8.8.5 Dosage Frequency** (Ronald *et al.*, 2005)

After the appearance of first tumor after induction of mammary carcinogenesis, the animals were orally administered daily for 4 weeks with single dose (500mg/kg) of PAME and two doses (5mg/kg and 10mg/kg) of phyllanthin
(PAPH) and hypophyllanthin (PAHP) suspended in aqueous solution of Sodium CMC. The dose was administered through oral gavage.

8.8.6 Experimental Protocol

Female Sprague-Dawley rats were randomized into following groups (n=8).

Table 8.4 Grouping and Treatment of rats with MNU induced mammary carcinogenesis

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>GROUPS</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>Control (Tween &amp; DV )</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>MNU</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>MNU + Tamoxifen-2mg/kg b.w</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>MNU + PAME-500mg/kg b.w</td>
</tr>
<tr>
<td>5</td>
<td>Group V</td>
<td>MNU + PAPH-5 mg/kg b.w</td>
</tr>
<tr>
<td>6</td>
<td>Group VI</td>
<td>MNU + PAPH-10 mg/kg b.w</td>
</tr>
<tr>
<td>7</td>
<td>Group VII</td>
<td>MNU + PAHP-5 mg/kg b.w</td>
</tr>
<tr>
<td>8</td>
<td>Group VIII</td>
<td>MNU + PAHP-10 mg/kg b.w</td>
</tr>
</tbody>
</table>

PAME- *P. amarus* methanolic extract; PAPH-phyllanthin; PAHP-hypophyllanthin; MNU- N-methyl N-nitrosourea and DV-drug vehicle.

8.8.7 Parameters estimated

1. *Tumor incidence*
   Tumor incidence is the number of animals with tumors at the end of the experiment. The incidence rates in different groups were calculated and tabulated.

2. *Tumor multiplicity*
   It is the number of tumors per rat observed in different treated groups.

3. *Tumor weight*
   The tumor weight per each group was determined in different treated groups.
   The tumor incidence, tumor multiplicity and tumor weight of different groups as shown in Table 8.5 were measured according to Takahiko Gotoh *et al.*, 1998; Roomi *et al.*, 2004; Jagatheesh *et al.*, 2010.

4. *Haemotological Parameters*
   In these 4 weeks of study, different groups of animals were given different treatments via oral route as shown in Table.8.4. After the completion of treatment, blood was collected from retro orbital puncture and the hematological parameters
like red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), and platelets (PLTs) were estimated by fully auto hematology analyzer (Erba Scientific, India) (Perse et al., 2009; Jagatheesh et al., 2010).

8.8.9 Histopathological Evaluation

After four weeks of the treatment, the animals were sacrificed by the method of cervical dislocation. The animals were dissected along the ventral midline and were grossly examined for the presence of tumors. All the summary tumors and suspected areas were fixed in 10% buffered formalin. The tissue fragments, which were fixed in formalin, were cut into 5-micrometer sections using microtome on paraffin blocks and stained with haematoxylin and eosin (H & E) for histological examination. Breast cancer disease pathology and histological types were evaluated.

8.8.10 Statistical Analysis

The difference in percentage tumor incidence, tumor multiplicity per rat and tumor weight per group were statistically evaluated by one-way analysis of variance (ANOVA), followed Dunett’s test. Values of P<0.05 were considered to be significant.

8.8.11 Results

Tumor Incidence

The incidence rate in the MNU control group was found to be 85.7%. Out of eight rats in the MNU control group, one rat died after four days of MNU injection. The mammary tumors first appeared after 12 weeks of the MNU injection and after 14 weeks of induction, six rats developed at least one tumor out of the remaining seven rats in the MNU control group. The tumor incidence in the Tamoxifen (2mg/kg) treated group was 25% where as the tumor incidence rates in the methanolic extract (PAME), phyllanthin (PAPH) and hypophyllanthin (PAHP) treated groups were found to be 75% (PAME), 50% (PAPH-5mg/kg), 31.5% (PAPH-10mg/kg), 62.5% (PAHP-5mg/kg) and 50% (PAHP-10mg/kg) (Table 8.5).
From the tested extract and isolated lignans, the phyllanthin at a dose of 10mg/kg showed highest reduction of mammary tumor incidence of 31.5% next to Tamoxifen treated group which showed a tumor incidence of 25%. There was nearly 54.2% reduction in the incidence rates in the PAPH (10mg/kg) treatment group when compared to the MNU control group which shows the anticancer potential of phyllanthin.

**Tumor Multiplicity**

The tumor multiplicity was determined per rat in different treated groups and the mean tumor multiplicity per rat was reported in Table 8.5. The mean tumor multiplicity of MNU control group was 2.14±0.58. The Tamoxifen treated group showed significant (P<0.05) decrease in the tumor multiplicity and is found to be 0.5±0.26. The mean tumor multiplicity in methanolic extract (PAME) treated group was 1.5±0.33. The phyllanthin treated groups showed comparably lower tumor multiplicities [1.12±0.47 (PAPH-5mg/kg) and 0.87±0.27 (PAPH-10mg/kg)] than hypophyllanthin treated groups which showed tumor multiplicities of 1.38±0.32 (PAHP-5mg/kg) and 1.25±0.41(PAHP-10mg/kg).

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment/groups</th>
<th>Num. of rats with Tumor</th>
<th>Incidence (%)</th>
<th>Tumor Multiplicity/rat</th>
<th>Tumor weight(g)/group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNU</td>
<td>6/7</td>
<td>85.7</td>
<td>2.14±0.58</td>
<td>35.85</td>
</tr>
<tr>
<td>2</td>
<td>MNU + Tamoxifen-2mg/kg b.w</td>
<td>2/8</td>
<td>25</td>
<td>0.5±0.26c</td>
<td>4.67b</td>
</tr>
<tr>
<td>3</td>
<td>MNU + PAME-500mg/kg b.w</td>
<td>6/8</td>
<td>75</td>
<td>1.5±0.33</td>
<td>21.08</td>
</tr>
<tr>
<td>4</td>
<td>MNU + PAPH-5 mg/kg b.w</td>
<td>4/8</td>
<td>50</td>
<td>1.12±0.47a</td>
<td>11.95a</td>
</tr>
<tr>
<td>5</td>
<td>MNU + PAPH-10 mg/kg b.w</td>
<td>3/8</td>
<td>31.5</td>
<td>0.87±0.27</td>
<td>8.87a</td>
</tr>
<tr>
<td>6</td>
<td>MNU + PAHP-5 mg/kg b.w</td>
<td>5/8</td>
<td>62.5</td>
<td>1.38±0.32a</td>
<td>12.82a</td>
</tr>
<tr>
<td>7</td>
<td>MNU + PAHP-10 mg/kg b.w</td>
<td>4/8</td>
<td>50</td>
<td>1.25±0.41</td>
<td>12.06a</td>
</tr>
</tbody>
</table>

a***P<0.001; b**P<0.01; c*P<0.05. dResults are given as mean±SEM.
PAME- *P. amarus* methanolic extract; PAPH-phyllanthin; PAHP-hypophyllanthin; MNU- N-methyl N-nitrosourea and DV-drug vehicle.
Tumor weight

The weight of grossly detectable mammary tumors in MNU control group was 35.85 g ranging from 0.06 g to 4.9 g per tumour. The mean tumor weight per rat in MNU control group was 5.12±0.26. The weight of total mammary tumors per group (total tumour mass) ranged from 4.67 g to 35.85 g. The majority of rats (65%) had a total tumour mass lower than 4.5 g per rat. Tamoxifen treated group showed significant (P<0.01) decrease of total tumor weight to 4.67 g when compared to MNU control group (total tumor weight of MNU control group is 35.85 g). The phyllanthin and hypophyllanthin treated groups showed significant (P<0.001) decrease in tumor weight when compared to MNU control group. The total tumor weight of phyllanthin treated group was decreased from 11.95 g to 8.87 g with increase in dose of phyllanthin from 5 mg/kg to 10 mg/kg body weight. Whereas, no remarkable change was observed in the total tumor weight of hypophyllanthin treated groups though the dose of hypophyllanthin was increased from 5 mg/kg to 10 mg/kg.

Hematological Parameters

The changes in the various blood parameters like white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb) and platelets (PLTs) for different treatment groups were given in the Table 8.6.

Table 8.6 Haematological analysis of tumor bearing rats treated for 4 weeks with *P. amarus* extract (PAME) and its isolated lignans (PAPH & PAHP)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment/groups</th>
<th>WBCs (10^3/µL)</th>
<th>RBCs (10^6/µL)</th>
<th>Hb (g/L)</th>
<th>PLTs (10^3/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (DV oral)</td>
<td>6.52±1.27</td>
<td>7.17±1.22</td>
<td>13.81±2.08</td>
<td>660.07±31.61</td>
</tr>
<tr>
<td>2</td>
<td>MNU</td>
<td>12.62±2.65</td>
<td>6.18±2.21</td>
<td>11.22±2.35</td>
<td>448.24±28.11</td>
</tr>
<tr>
<td>3</td>
<td>MNU + Tamoxifen-2mg/kg b.w</td>
<td>7.51±1.31^a</td>
<td>6.97±1.17^c</td>
<td>13.25±3.12</td>
<td>571.7±21.25^b</td>
</tr>
<tr>
<td>4</td>
<td>MNU + PAME-500mg/kg b.w</td>
<td>9.87±1.35^a</td>
<td>6.63±1.57</td>
<td>11.65±2.18</td>
<td>509.25±19.89</td>
</tr>
<tr>
<td>5</td>
<td>MNU + PAPH-5 mg/kg b.w</td>
<td>8.95±1.87^a</td>
<td>6.75±1.64</td>
<td>12.25±2.46</td>
<td>517.58±17.50</td>
</tr>
<tr>
<td>6</td>
<td>MNU + PAPH-10 mg/kg b.w</td>
<td>8.63±1.24^a</td>
<td>6.82±1.71</td>
<td>12.72±2.77</td>
<td>547.58±25.84^c</td>
</tr>
<tr>
<td>7</td>
<td>MNU + PAHP-5 mg/kg b.w</td>
<td>9.25±0.98^a</td>
<td>6.73±1.74</td>
<td>11.95±2.29</td>
<td>522.41±32.24</td>
</tr>
<tr>
<td>8</td>
<td>MNU + PAHP-10 mg/kg b.w</td>
<td>8.77±1.35^a</td>
<td>6.79±1.67</td>
<td>12.48±2.51</td>
<td>536.08±31.97</td>
</tr>
</tbody>
</table>

^a***P<0.001; ^b**P<0.01; ^c*P<0.05; Data was expressed as Mean ± SEM.
PAME- methanolic extract of *P. amarus* aerial parts; PAPH-phyllanthin; PAHP-hypophyllanthin; MNU- N-methyl N-nitrosourea and DV-drug vehicle.
The number of red blood cells (6.18±2.21x10⁶/µL) and haemoglobin (11.22±2.35g/L) were lower in the MNU treated group than in the control group, indicating a tendency to anemia. On the other hand the number of white blood cells (WBC) was considerably increased in MNU control group indicating the diseased state. In the Tamoxifen treated group there was significant (P<0.05) decrease in WBC’s to 7.51±1.31x10³/µL when compared to MNU control group (12.62±2.65 x10³/µL). The number of platelets (PLT) decreased to 448.24±28.11 x10³/µL in the MNU group (Table 8.6) when compared to normal control group (660.07±31.61 x10³/µL).

**Histological Observations**

The histology of normal mammary gland (Fig.8.8) showed the presence of lobules, numerous acini and clear basement membrane. Photomicrographs (Fig.8.9-8.13) of mammary tissue of MNU control rats clearly showed the marked proliferation of stroma which was resulted due to stromal reaction with the carcinogen. Photomicrograph Fig.8.11 demonstrates a well circumscribed tumor composed of tubules which are closely packed giving rise to back to back fashion. The tubules are lined by inner epithelial cells and basal myoepithelial cells. The stroma is very scanty in between the tubules and some of the tubules were showing intraluminal secretions. Within the stroma there was mixed mononuclear cell infiltrate with prominent mast cells (Fig. 8.11).

The epithelial component of the tumors was organized into acinar structures, but the growth pattern was very variable from one region to another in the same tumour, with areas of typical well-differentiated adenocarcinoma merging into areas with a papillary and cribriform pattern (Fig.8.12 & Fig.8.13). The carcinoma sections showed partially capsulated tumor with irregularly arranged glands showing architectural and cytological atypia. Fig.8.13 clearly demonstrated the formation of well defined capsule by fibrous collagenous tissue in between the adipose and cancerous tissues which is a characteristic feature of breast cancer. Fig.8.10 shows the complete tumor tissue necrosis; this tissue necrosis was due to increased tumor size followed by the congestion of blood vessels and insufficient blood supply to the tumor tissue. The phyllanthin and hypophyllanthin treated groups showed small sized
tumors when compared to MNU control group. The phyllanthin treated rats showed small sized tumors with significant decrease in tumor multiplicity and tumor weight. The histology of phyllanthin treated rat tumors showed very few necrotic cells (Fig.8.14), this may be due to potent inhibition of mammary tumor growth by phyllanthin treatment whereas the hypophyllanthin treated rat mammary tumors showed moderate number of necrotic cells as showed in Fig.8.15.

Fig. 8.7 External view of the mammary tumor in Sprague-Dawley rat
Fig. 8.8 Photomicrograph of vehicle control group demonstrating normal mammary gland with basement membrane (B) and Lobules (L) (H & E, x 40).

Fig. 8.9 Photomicrograph of MNU-induced rat mammary tumor demonstrating stroma (S) and congested blood vessels (BV) (H & E, x 100).
Fig. 8.10 Photomicrograph of MNU-induced rat mammary tumor demonstrating tumor tissue necrosis (NC) (H & E, x 100).

Fig. 8.11 Photomicrograph of MNU-induced rat mammary tumor demonstrating stromal proliferation (S), tubules (T) and mast cells (M) (H & E, x 140).
Fig. 8.12 Photomicrograph of MNU-induced rat mammary tumor demonstrating stromal proliferation (S), Tubules (T) and secretions (SE) (H & E, x 100).

Fig. 8.13 Photomicrograph of MNU-induced rat mammary tumor demonstrating Adipose tissue (A), fibrous capsule (C), cancerous tissue (CT) and secretions (SE) (H & E, x 100).
Fig. 8.14 Photomicrograph of MNU-induced rat mammary tumor treated with phyllanthin (10mg/kg b.w) demonstrating cancer tissue necrosis (NC) adjacent to cancer tissue (CT) (H & E, x 100).

Fig. 8.15 Photomicrograph of MNU-induced rat mammary tumor treated with hypophyllanthin (10mg/kg b.w) demonstrating cancer tissue necrosis (NC) adjacent to cancer tissue (CT) (H & E, x 100).
8.8.13 Discussion

MNU-induced mammary cancer model in Sprague-Dawley rats was chosen for the evaluation of methanolic extract (PAME) of \textit{P. amarus} aerial parts, phyllanthin (PAPH) and hypophyllanthin (PAHP) because the histological structure of mammary gland tumors in MNU induced rats closely resembles that of human mammary tumors (Welsch, 1985; Russo \textit{et al.}, 1990; Thompson \textit{et al.}, 1995). The mouse lesions are primarily alveolar and in contrast to these, the rat mammary tumors are predominantly ductal which are similar to human carcinoma (Thompson \textit{et al.}, 1995) and the most highly malignant rat tumors share some features with human intraductal and infiltrating ductal carcinomas (Russo \textit{et al.}, 1990). It has been reported that the MNU model has several advantages, such as reliability of tumor induction, organ site specificity, tumor of ductal origin and predominantly carcinomatous histopathological characterization, and the ability to examine tumor initiation and promotion processes (Thompson \textit{et al.}, 1995).

The results of the present study demonstrate significant inhibition of mammary tumor incidence and multiplicity in Sprague-Dawley female rats by subjecting them to different treatments as showed in Table-8.5. Furthermore, rats treated with phyllanthin and hypophyllanthin showed the decreased number of mammary tumors and also decrease in tumor weight compared with those of MNU control group.

Phyllanthin and hypophyllanthin are naturally occurring phytoestrogens. Previously, many other phytoestrogens showed anticancer activity against breast cancer via several mechanisms including estrogen receptor agonism and possible antagonism both in \textit{in vitro} and \textit{in vivo} studies. Many mechanisms like interference with estradiol biosynthesis by inhibition of key steroid enzymes (steroid dehydrogenase, aromatase) in \textit{in vitro} studies has been reported. (Islam \textit{et al.}, 2008; Adlercreutz \textit{et al.}, 1982; 1988; 1995b; 1998b). Receptor independent anti-oxidant properties (Krithika \textit{et al.}, 2009) and inhibition of several enzymes involved in cell signaling and proliferation. The methanolic extract (PAME) of \textit{P. amarus} showed the presence of flavonoids, glycosides, alkaloids and tanins which may be responsible for the present anticancer activity.

The results of the present study demonstrate significant inhibition of mammary tumor incidence, multiplicity and tumor weight in Sprague-Dawley female
rats up on oral administration of tamoxifen, phyllanthin (PAPH), hypophyllanthin (PAHP) and methanolic extract of *P. amarus* (PAME). Phyllanthin (PAPH) and hypophyllanthin (PAHP) showed significant (p<0.001) dose dependent inhibition of mammary tumor weight as showed in Table 8.5. The numerous tumors in the MNU control rats were larger in size and had characteristic diagnostic of adenocarcinoma and tissue necrosis, in contrast to this, phyllanthin and hypophyllanthin treated rats showed few small adenomas.

Although the mechanism underlying the reduced tumor size in tumor bearing rats was not identified in this experiment, these findings were consistent with our previous *in vitro* studies that demonstrated significant growth inhibition of human breast cancer cell lines MDA-MB-231 and MCF-7.