WET-LAB VALIDATION OF *IN SILICO* PREDICTED ANTICANCER POTENTIAL OF SELECTED COMPOUNDS OF CURRY LEAF (*Murraya koenigii L.*)

By

MULLAI V R
(2019-11-003)

DEPARTMENT OF PLANT BIOTECHNOLOGY
CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF AGRICULTURE
VELLANIKKARA, THRISSUR – 680 656
KERALA, INDIA
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THESIS

Submitted in partial fulfillment of the requirement for the degree of

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DEPARTMENT OF PLANT BIOTECHNOLOGY

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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KERALA, INDIA

2021

DECLARATION

I hereby declare that the thesis entitled **"Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya koenigii* L.)"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara

Date: 31/01/2022

Mullai V R

(2019-11-003)

CERTIFICATE

Certified that the thesis entitled **"Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya koenigii* L.)"** is a bonafide record of research work done independently by **Ms. Mullai V R (2019-11-003)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellanikkara

Date: 31/01/2022

Dr. Deepu Mathew
(Chairman, Advisory Committee)
Associate Professor
Department of Plant Biotechnology
College of Agriculture
Vellanikkara,Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Mullai V R (2019-11-003)**, a candidate for the degree of **Master of Science in Agriculture,** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya koenigii* L.)"** may be submitted by **Ms. Mullai V R,** in partial fulfillment of the requirement for the degree.

Dr. Deepu Mathew

(Chairman, Advisory Committee)
Associate Professor
Department of Plant Biotechnology
College of Agriculture
Vellanikkara, Thrissur

Dr. Abida P S

(Member, Advisory Committee)
Professor and Head
Department of Plant Biotechnology
College of Agriculture
Vellanikkara, Thrissur

Mrs. Smitha Nair

(Member, Advisory Committee)
Assistant Professor
Department of Plant Biotechnology
College of Agriculture
Vellanikkara, Thrissur

Dr. Babu T D

(Member, Advisory Committee)
Associate Professor
Department of Biochemistry
Amala Cancer Research Centre
Thrissur

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"A, as its first of letters, every speech maintains;
The "Primal Deity" is first through all the world's domains"
Thirukkural: 1, Thiruvalluvar

I feel overwhelmed with gratitude at this moment and hope these words express my feelings.

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ABBREVIATIONS

%	: Per cent
<	: Less than
µg	: Microgram
µl	: Microlitre
ml	: Millilitre
α	: Alpha
β	: Beta
CPBMB	: Centre for Plant Biotechnology and Molecular Biology
cDNA	: Complementary deoxyribonucleic acid
DMBA	: Dimethylbenz[a]anthracene
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DSS	: Doxylamine Succinate Salt
ER	: Estrogen Receptor
FBS	: Fetal Bovine Serum
g	: Gram
IEAC	: Institutional Animal Ethical Committee
LHD	: L-Histidinol Dihydrochloride
mg	: Milligram
ml	: Millilitre
mM	: Millimolar
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	: Phosphate-buffered saline
PMS	: Pheniramine Maleate Salt
PMSLD	: Pheniramine Maleate Salt Low Dose
PMSHD	: Pheniramine Maleate Salt High Dose
qRT-PCR	: Quantitative Real-Time Polymerase Chain Reaction
RNA	: Ribonucleic acid

INTRODUCTION

1. INTRODUCTION

Worldwide, around 100 million people live with one or other type of cancer. Most common human cancers are lung, stomach, liver, breast, prostate and colon cancers. All around the world, research works are being actively carried out on cancer genomics, detection, diagnosis, prevention and treatment. The existing treatment approaches for cancer include surgery, radiation, hormone therapy, chemotherapy, targeted therapy, immunotherapy, photodynamic therapy and stem cell transplantation. Though these treatments are differentially successful to contain the progression of cancer, side effects such as infection, weakened immune system, bleeding, hair loss, nausea, vomiting, diarrhea and constipation, are common, especially from the chemicals used in chemotherapy. For the past few decades, the efficacy of synthesized drugs has become low due to drug resistance and the overall survival rate is also not satisfactory (Yuan *et al,* 2017). Hence, there is a demand for the identification of cancer specific compounds with no side-effects. Plants are excellent sources of compounds possessing anti-cancer properties. Many plant derived compounds are being tested for their efficacy in treating various types of cancers. One of the efficient methods of drug discovery, both in terms of cost and money, is *in silico* drug designing. Several methods such as homology modeling, molecular docking, virtual high-throughput screening, molecular dynamic simulation, microarray analysis are used in *in silico* approach of drug designing (Wadood *et al.,* 2013).

The *Murraya koenigii* L. (family Rutaceae), commonly called curry leaf, is known to have medicinal and nutraceutical properties. The chemical constituents of curry leaves include alkaloids, phenolic compounds, flavonoids, saponins, proteins, sterols and triterpenes (Jain *et al.,* 2017). The active constituents of curry leaves are reported to target disorganized signaling pathways such as JAK (janus kinase)/STAT (signal transducer and activator of transcription) pathway, PI3K (phosphatidylinositol 3 kinase)/Akt (protein kinase B) pathway and mTOR (mammalian target of rapamycin) pathway. These pathways are associated with cell growth, proliferation and apoptosis thereby play a significant role in cancer cell proliferation (Aniqa *et al.,* 2021).

Molecular docking studies carried out by Bhamare (2019), on the antioxidant fractions of the curry leaves identified through LC-MS/MS, have revealed their anticancer properties against few cancers. Seven compounds of curry leaves, doxylamine, histidinol, pheniramine, flucoxetine, alpha-aminodiphenylacetic acid, valylmethionine and prometon, were identified to have promising interactions with different cancer targets. In the current study entitled 'Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya koenigii* L.)', doxylamine, histidinol and pheniramine in their chemical form, doxylamine succinate salt, L-histidinol dihydrochloride and pheniramine maleate salt respectively, were validated for their anticancer potential through *in vitro*, *in vivo* and gene expression studies.

Objective of the study was to evaluate the anticancer potential of selected curry leaf (*Murraya koenigii*) compounds by *in vitro* and *in vivo* studies using different cancer cell lines and mouse tumour models. In *in vitro* studies, the cytotoxicity of the phytocompounds on cancer cell lines was measured. It was carried out through two assays; trypan blue exclusion assay for analyzing the short-term cytotoxicity of the compounds on murine cancer cell lines and MTT assay for examining the anti-proliferative analysis of the compounds on the breast cancer and colorectal cancer cell lines. The promising compound was validated through *in vivo* and molecular expression studies. *In vivo* studies encompassed acute toxicity study and mouse tumour model study. Relative expression of *ER-α1, Bcl-2, c-Myc* and *Pin 1* genes was measured through quantitative real-time PCR (qRT-PCR), in tumour cells artificially induced in the mice and subjected to different drug treatments.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Introduction to cancer

One of the leading causes of human death around the world is cancer. Nearly 10 million deaths happened in 2020 due to cancer. According to Global Cancer Statistics 2020 (Sung *et al.,* 2021), the mortality rate is highest for lung cancer, amounting to 18% of the total cancer death. Colorectal, liver, stomach and breast cancers occupy the subsequent positions with 9.4%, 8.3%, 7.7% and 6.9%, respectively. India reported 1,392,179 cases of cancer in the year 2020 (Mathur *et al.,* 2020) with lung, breast, mouth, tongue and cervix uteri as the major sites of incidence of cancer.

2.1.1 Etiology of cancer

Global Cancer Observatory (GCO) attributes the major causes of cancer to obesity, infections, UV radiation and alcohol. Danaei *et al.* (2005) identified nine behavioural and environmental risk factors that cause cancer, which are overweight and obesity, low fruit and vegetable intake, lack of physical activity, smoking, alcohol consumption, unprotected sex, air pollution, indoor smoke produced from household usage of solid fuels and contaminated injections. The biggest risk of cancer development is consumption of tobacco and its related products, which account for cancers of the lung, pancreas, liver, stomach, pancreas, kidney *etc.* (Vineis *et al.,* 2004). Although most of the cancers are not hereditary, few tend to run in families. Some of the family cancer syndromes include Hereditary Breast and Ovarian Cancer (HBOC) syndrome, Lynch syndrome (Hereditary Non-Polyposis Colorectal Cancer, HNPCC) and Li-Fraumeni syndrome.

2.1.2 Types of Cancers

Cancers can be broadly grouped as carcinoma, sarcoma, lymphoma, melanoma and leukemia, according to the tissues from where they originate. Most common cancers are lung cancer, breast cancer, prostate cancer, stomach cancer, colon cancer and liver cancer. In accordance with ICD-O-3 (International Classification of Diseases for

Oncology, 3rd edition), ICCC-3 (International Classification of Childhood Cancer, 3rd edition) classified tumours into 12 major groups (Steliarova-Foucher *et al.,* 2005).

Lung cancer is extremely lethal, occupying first position in mortality among the cancer patients in both the sexes. The subtypes of lung cancer include adenocarcinoma, squamous cell carcinoma, small cell undifferentiated carcinoma and large cell carcinoma. The single most risk factor of lung cancer is cigarette smoking. Other risk factors include radiation, air pollution, diet and environmental exposures (Alberg and Samet, 2003).

Breast cancer is the second leading cause of cancer death in women and rarely in men, most of which are diagnosed early and can be treated. The subtypes include luminal A, luminal B, HER2 enriched and basal like (Harbeck and Gnant, 2016). Menarche and menstrual cycle, childbearing, breastfeeding, menopause, endogenous hormone, oral contraceptives, environmental oestrogen, family history and diet are some of the risk factors (Key *et al.,* 2001).

Colon cancer is the third most common cancer, most of which are adenocarcinoma. Incidence of colon cancer is associated with inflammatory bowel disease (Crohn's disease), diet having high red meat and low vegetables, family history and genes like *APC* (Labianca *et al.,* 2010).

Stomach cancer is the fourth common cancer, which occurs more in men than women. Infection with the bacterium *Helicobacter pylori,* is commonly associated with stomach cancer along with other factors such as diet and lifestyle (Brenner *et al.,* 2009).

Prostate cancer is common in men, with risk higher beyond 65 years. Prostate cancer is also associated with ethnicity, with higher incidence among African American men. A genetic linkage is found at *HPC1* locus among high-risk family members. High intake of red meat and low intake of vegetables are also associated with development of prostate cancer (Crawford, 2003).

The risk of liver cancer increases with age and is often associated with chronic viral hepatitis caused by Hepatitis B and Hepatitis C viruses. Exposure to aflatoxins, alcohol and tobacco consumption also cause liver cancer (Bosch *et al.,* 1999).

2.1.3 Molecular basis of cancer

Generally speaking, Cancer is a group of diseases which results in the abnormal growth of cells, called tumour and spreads to various parts of the body. Disregulation of cell cycle is one of the major causes of cancer. Eukaryotic cells progress through various phases of cell cycle (G_1, S, G_2 and M) through activation and inactivation of Cyclin-dependent kinases (Cdks) which are complexed with Cyclins (Arellano and Moreno, 1997). Changes in control machinery, which directs the progression of cells from G_0 to G_1 phase and G_1 to S phase, are often associated with tumour growth. These changes are brought about by loss of pRB, a tumour repressor protein, hyperactivation of cdk4/6, overexpression of cyclin D1, D2 and D3 and deactivation of p16INK4a, an inhibitor belongs to INK4^{cdk} inhibitor family (Deshpande *et al.*, 2005).

Major genetic events that take place in cancer pathway are gain of function and loss of function genetic events (Oppenheimer, 2006). While gain of function genetic event is mediated by oncogenes that promote cancer, loss of function genetic event is mediated by tumour suppressor genes, which protect the body against cancer. Oncogenes are developed as a result of mutations occurring in proto-oncogenes which ultimately increase its expression. Some of the examples of proto-ocogenes include *Ras, HER2, Myc*, and *Cyclin D*. An increase in the growth factor receptor encoded by *HER-2/neu* proto-oncogene is found in 25-30% of human breast cancer (Slamon, 1989). Another frequently mutated proto-oncogene found in breast cancer and the first to get discovered is *Ras* gene. It has four protein isoforms *viz.* HRAS, NRAS, KRAS4A and KRAS4B, with highest mutation rate in *KRAS* gene (Galie, 2019). Activation of *Myc* gene in transgenic mouse induced by Activation-Induced Deaminase (AID) caused tumourigenesis in conditional mouse model (Chesi *et al.,* 2008). *PRAD1*, a Cyclin D1 gene was found to be associated with parathyroid adenomas, B cell neoplasm and centrocytic lymphoma (Motokura and Arnold, 1993).

Tumour suppressor genes are required to inhibit cell proliferation in normal cells. Mutation in these genes results in uncontrolled cell growth leading to cancer development. *BRCA1* and *BRCA2* are the two significant tumour suppressor genes, the mutation in which increases the predisposition of a woman to breast cancer and ovarian

cancer. While breast tumours developed by BRCA1 are triple negative [Estrogen Receptor (ER), Progesterone Receptor (PR) and Epidermal Growth Factor 2 (HER2) negative] and p53 positive, tumours developed by *BRCA2* are often ER positive and p53 negative (Paul and Paul, 2014).

One of the frequently mutated genes, the loss of which leads to cancer development, is *p16*. It is a cyclin-dependent kinase inhibitor (CDKI) which goes by the names, Inhibitor of cyclin-dependent kinase 4a (INK4a) or major tumour-suppressor 1 (MTS1) or cyclin-dependent kinase inhibitor 2A (CDKN2A). Silencing of *p16* in human neoplasms happens mainly through homozygous deletion and methylation of the promoter. Point mutations also contribute to the loss of *p16* (Liggett and Sidransky, 1998).

In addition, aneuploidy, which is the addition or deletion of a complete or a segment of a chromosome, is also associated with cancer. The karyotype of the breast cancer cell line MDA 231 showed various marker chromosomes. These marker chromosomes are formed by the rearrangements of the gained chromosome segment with the same or different chromosome (Duesberg *et al.,* 2005).

Apart from genetic mechanisms, epigenetic mechanisms such as DNA methylation, covalent histone modifications, nucleosome remodeling and miRNAs induced gene silencing also play a vital role in cancer development (Sharma *et al.,* 2010). DNA hypomethylation across the genome is found to be associated with various carcinomas. Instability in the genome was found to be associated with the insertion of an intra-cisternal A particle (IAP), an endogenous retroviral particle, in the *Notch1* genomic locus of the mice, which was brought by DNA hypomethylation (Howard, 2008). RNAi induced knockdown of two Histone H3 lysine 9 methyltransferases, SUV39H1 and G9a inhibited the growth of PC3 cancer cell line which was characterized by shortened telomere (Kondo *et al.,* 2008).

2.1.4 Hallmarks of Cancer

National Cancer Institute, Maryland, USA defines cancer as a disease associated with uncontrolled growth of some of the body cells and their spread to other parts of the

body. Hanahan and Weinberg (2000) proposed six hallmark capabilities of cancer such as self-sufficiency in growth signals, tissue invasion and metastasis, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential and evading apoptosis. Further, deregulation of cellular energetics and avoidance of immune destruction have been added as emerging hallmarks along with genomic instability and mutation and tumour-promoting inflammation as enabling characteristics of cancer (Hanahan and Weinberg, 2011).

2.1.5 Treatment

Treatments to cancer include surgery, hormonal therapy, radiation therapy, adjuvant therapy and immunotherapy. Modern targeted treatments use drugs that can inhibit growth signal and angiogenesis and can induce apoptosis (Sudhakar, 2009). More often, these treatments are associated with side effects such as fatigue, pain and vomiting (Henry *et al.,* 2008). Over 65% of the patients undergoing cancer treatment suffer from Alopecia (hair loss) (Dua *et al.,* 2015). New approaches on cancer treatment are being developed to reduce the side effects and to increase the efficiency.

2.1.5.1 Diagnosis and treatment for breast cancer

Early diagnosis increases the chance of complete recovery from breast cancer. This can be done by mammography and clinical examination, which results in 25 to 30 % decrease in the mortality rate of women over 50 years of age (Hortobagyi, 1998). Breast cancer subtypes play an important role in the successful treatment. The surgical approaches for breast cancer include mastectomy (complete removal of breast) or lumpectomy (removal of breast tumour with a margin of normal surrounding tissue). In radiation therapy, whole breast or a portion of the breast (after lumpectomy) will be exposed to high-energy radiation. Several cytotoxic drugs are employed in neoadjuvant and adjuvant chemotherapy. Some of them include tamoxifen, olaparib, trastuzumab, pertuzumab, fulvestrant, lapatinib *etc.* They are often used in combination with each other, depending upon the stage of the breast cancer and also with other approaches like surgery and chemotherapy (Burguin *et al.,* 2021).

2.2 Phytocompounds as cancer drugs

2.2.1 Screening the plant extracts/fractions

Plants and their extracts occupy a significant position in traditional medicine and ethnopharmacology. They are excellent sources for developing the drugs (Fabricant and Farnsworth, 2001). Phytocompounds such as alkaloids, phenolics, flavonoids, terpenoids, phytosterols, tannins, lignans, saponins and quassinoids have potential anticancer activities and have proved their efficiency as potent drugs for cancer treatment over the years (Sofi and Nabi, 2018).

The hexane and ethanol extracts derived from Toothed-leaf limonia, *Naringi crenulata*, showed significant cytotoxicity against SK-BR3, a HER2 positive breast cancer line, in cell viability assay at 24 hours of exposure. The cytotoxicity assay resulted that the Fraction 11 of *Naringi crenulata* extract (NCE) identified through GC-MS has IC_{50} value at 24.5 μg/ml (Vallinayagam *et al.,* 2021).

The metabolic extract of *Orabanche crenulata* exhibited antioxidant property and anticancer activity (Hegazy *et al*., 2020). A dose dependent development inhibition of MCF-7 (breast cancer), PC3 (prostate cancer), HCT-116 (colon cancer) and HepG2 (hepatocellular carcinoma) cell lines was observed in cell viability analyses, with 50% inhibition at the concentration of 89.6, 111, 28.6 and 30.3 μg/ml, respectively. LDH assay conducted on HCT-116 cell line showed that the activity of LDH increased with the concentration of the extract of *Orabanche crenulata*.

Chemopreventive efficiency of Luteolin-7-O-Glucoside (LUT7G) against colon cancer was studied by Baskar *et al.* (2014). LUT7G is a phytocompound isolated from the methanol extract of *Ophiorrhiza mungos* Linn. DPPH scavenging assay and MTT assay conducted on COLO320DM (colon cancer cell line) showed that LUT7G has antiradical activity with IC_{50} at 161 μM and anti-proliferative activity with IC_{50} at 512.4 μM. DMH (1,2-dimethylhydrazine) was used to induce carcinogenesis in male albino Wistar rats. The number of ACF (Aberrant crypt foci - early neoplastic lesion in colon carcinogenesis) was found to be less in LUT7G treated rats than in control rats treated with only DMH.

2.2.2 Screening the phytocompounds through molecular docking

Molecular docking is one of the powerful tools in drug designing. It predicts the binding affinity between the ligand and the target protein which will be used in lead optimization. The approach of molecular docking is being extensively used in developing drugs against cancer. It mainly focuses on finding potential inhibitors of proteins whose genes get overexpressed in the development of cancer. Based on the results of molecular docking and binding energy analysis, Yousuf *et al.,* (2017) identified five chemical compounds *viz.* S-258002927, S-258012947, S-258282355, S-259411474 and S-259417539 which could act as inhibitors against the targets of human breast cancer; human epidermal growth factor receptors EGFR and HER2/neu receptor and a co-chaperone HSP90. The elevated expression of these targets is reported to be in association with the breast cancer.

Ahmed *et al.,* (2014) reported that ten phytocompounds namely taxifolin 3-O-acetate, secundifloran, kushenol K, kushenol N, kurarinol, leachianone G, AC1LCW2L, silybin, resemarinic acid and podophyllotoxone could be potential antagonists for the breast cancer target ER-α. They had less docking score and strong bonding as compared to tamoxifen, a standard drug for breast cancer.

2.3 Curry leaf

Murraya koenigii (L.), commonly called curry leaf, is a tree native to India and belongs to the family Rutaceae. The leaves are used for both medicinal and culinary purposes. It has antioxidant, antimicrobial, anti-inflammatory, anti-diabetic and anti-fungal properties, making their significance in medicine and pharmacology (Parthasarathy *et al.,* 2008). So far, alkaloids, coumarin glucoside, phosphorous, iron, calcium, riboflavin, niacin, thiamine, vitamin C, carotene, oxalic acid and essential oils have been isolated as the major phytoconstituents from the leaves (Reddy *et al.,* 2018).

2.3.1 Anticancer potential of curry leaves

The hydro-methanolic extract of curry leaves (CLE) was found to be effective against the breast carcinoma cell lines MCF-7 and MDA-MB-231. The results of Colony formation assay, MTT assay, Cell cyle analysis and Annexin V binding, conducted on theses cell lines, showed that the CLE altered the growth kinetics and induced cell death

in the breast cancer cell lines in a dose-dependent manner by inhibiting the 26S proteasome activity (Noolu *et al.,* 2013).

Carbazole alkaloids *viz.* Koenimbine, Murrayazoline, O-methy murrayamine A, Koenidine and Mahanimbine extracted from the curry leaves were tested on eight different cancer cell lines such as MCF-7, MDA-MB-231, A549, DLD-1, U937, PC3 and HepG2. They all showed significant anti-cancer activity towards the cell lines with highest activity on DLD-1 (colon cancer cell line) through the activation of caspase-dependent apoptosis by inducing caspase-3 protein and upregulating Bax/Bcl-2 protein expression ratio (Arun *et al.,* 2017).

Curry leaf ethanol extract (CLEE) increased *caspase-3* expression on Sparague-Dawley rats, which expressed high level of Human epidermal growth factor receptor 2 (HER2) as a result of the injection of the carcinogen, DMBA (7,12-dimethylbenz(a)-anthracene) (Aisyah *et al.,* 2021).

Molecular docking studies on the antioxidant fractions of the curry leaves identified through LC-MS/MS identified seven compounds of curry leaves such as Alpha-aminodiphenylacetic acid, Doxylamine, Histidinol, Flucoxetine, Pheniramine, Valylmethionine and Prometon which interacted with different cancer targets like 17-beta HSD (breast cancer), Polo-like kinase 1 (breast cancer), Human estrogen receptor ligand-binding domain (breast cancer), Dihydropholate reductase (colon cancer), Exchange protein directly activated by CAMP (pancreatic cancer), Human androgen receptor (prostate cancer), Phosphoinositide-3 kinase (all types of cancers), and Nat-2 receptor (all types of cancers), with maximum inhibition in 17-beta HSD and Polo-like kinase 1 proteins (Bhamare, 2019).

2.4 *In vitro* assays

Various *in vitro* assays have been developed to test the effect of drugs in cancer cells. These assays are done *ex vivo* under controlled conditions. They can be broadly classified into cell viability/cytotoxicity and anti-proliferative assays, cell migration and invasion assays, assays for the detection of apoptosis, genome alterations, cellular senescence and energy metabolism in cancer cells and techniques for monitoring

angiogenesis, oxidative stress, and expression of protein and reporter genes (Ediriweera, 2018).

2.4.1 Cytotoxicity and cell viability assays

Cell viability assays employ certain markers which give indication of metabolically active cells. Generally cell viability and cytotoxicity assays are classified into dye exclusion, colorimetric, luminometric and fluorometric assays (Aslanturk, 2018).

Tryphan blue exclusion assay is one of the widely used dye exclusion assays in which the dye gets excluded by the intact cell membranes, which is characteristic of the live cells. Bellamakondi *et al.* (2014) studied the short-term cytotoxicity of the methanolic extract of *Caralluma* species *viz. C. umbellata, C. lasiantha, C. attenuate* and *C. diffusa,* on the murine cancer cells, EAC (Ehrlich Ascites Carcinoma). The methanolic extracts of all the four species exhibited significant cytotoxicity with CTC_{50} value ranged between 191.3 ± 0.92 and 291.8 ± 3.17 µg/ml.

Similarly, another murine cancer cell line, Dalton Lymphoma Ascites (DLA) was treated with the extracts of the *Ayuvedic* herbs, *Ocimum sanctum, Curcuma longa, Zizyphus mauritiana* and *Tinospora cordifolia* and tested for the cytotoxicity action of these herbs using Tryphan blue exclusion assay. Both *Tinospora cordifolia* and *Curcuma longa* showed cytotoxicity on par with 5-FU, which is a standard chemotherapeutic agent (Adhvaryu, 2008).

Another important *in vitro* assay is MTT assay, which measures the viability of the cells. It is a colorimetric assay that relies on the mitochondrial enzyme, NADPH dependent cellular oxidoreductase, which reduces the yellow colored dye MTT to a purple colored insoluble formazan. This formazan dissolves in dimethyl sulfoxide (DMSO) and has its absorption at 570 nm. The intensity of the color corresponds to the number of viable cells (Kumar *et al.,* 2018). Bahuguna *et al.* (2017) evaluated the cytotoxic potential of a drug against A549 lung adenocarcinoma cell line using MTT assay. The cells were seeded at the density of $1x10^4$ cells/well in a 96 well microtiter plate and treated with drug at different concentrations. The plate was incubated with MTT for 24 hours followed by DMSO for 30 minutes at 37 °C in a CO_2 incubator.

Absorbance values at 540 nm showed that there was a decrease in the value with increase in the concentration of the drug, suggesting the cytotoxicity potential of the drug.

2.5 *In vivo* studies

Mouse models are crucial in the development of drugs having chemo-preventive potential. The molecular mechanisms underlying the development of cancer and its metastasis can be best studied in the animal system. Cespedes *et al.* (2006) defined the characteristics of an ideal model of cancer as the one i) displaying histopathologic characters similar to that of human cancer ii) undergoing similar stages in cancer progression and its expression iii) employing same pathways and genes iv) reflecting the same response to the cancer therapy and v) predicting the efficiency of the chemo-preventive treatments in human clinical assays.

Broadly the animal models are divided into two types *viz.* models in which the mice are transplanted with the tumours that are already developed *in vitro* and models in which the mice develop the tumours *in situ* which is either spontaneous or induced (Blatt *et al.,* 2013). Currently, genetically engineered mouse models (GEMM) are widely used in which *de novo* tumours are developed in an immune-proficient microenvironment naturally (Kersten *et al.,* 2016).

2.5.1 Breast cancer mouse models

Zhang *et al.* (2011) broadly classified the mouse models for cancer as transgenic mouse model, gene targeting mouse model, conditional and inducible mouse model, RNA interference mouse model, chromosomal engineering mouse model and replication-competent avian sarcoma-leukosis virus long terminal repeat with a splice acceptor/avian subtype A receptor mouse model.

One of the most frequently used mammary tumour models is MNU (methylnitrosourea) induced mammary carcinogenesis model. It develops ER^+ (Estrogen receptor positive) breast cancer in rats. Another potent carcinogen which develops ER^+ mammary adenocarcinoma in rats is DMBA (7, 12-Dimethylbenz(a)anthracene). Some of the genetically induced mouse models include *Neu+*, *BRCA1* and p53 knock out mouse

models which are associated with ER⁻ cancer i.e. nonresponsive breast cancer (Steele and Lubet, 2010).

In a study conducted by Banerjee *et al.* (2002) for testing the chemo-preventive effect of Resveratol, DMBA was used to induce the mammary cancer in female Sprague Dawley rats. These rats were divided into three groups *viz.* negative control group receiving normal diet, positive control group receiving DMBA and normal diet and test group receiving DMBA and resveratol. 10 mg of DMBA was dissolved in sesame oil and administered to rats. After 120 days of administering DMBA, the study was completed. The rats were sacrificed and tumours were isolated for further characterization like tumour grading, tumour incidence and multiplicity and measurement of tumour volume.

In another study, Minari and Okeke (2014), tested the ethanolic extract of *Annona murica* against breast cancer cells induced in female albino mice using DMBA. Both the DMBA and the extract were given intra-gastrically for 6 weeks and gel electrophoresis carried out using the DNA obtained from the control group and treated group (100 mg/ml) suggested that the extract was found to be effective against proliferation of breast cells. Histological assays carried out in the mice tissues exhibited changes in the globular alveolar hyperplasia and fibro-adenomatoid hyperplasia among different groups, which again confirmed the preventive effect of the extract on the tumour cells.

2.6 Expression studies on candidate genes

Gene expression studies are critical in better understanding the molecular mechanisms underlying cancer development. As already discussed, certain genes are upregulated during cancer progression while some genes are shut off. Some of the genes which are closely linked with breast cancer include *AIB1, KRT5, KRT15, RAF1, WIF1, MSH7, CKMT1B, DDX21PRKDC, PTPN1, YWHAE etc.* (Simino *et al.,* 2008). Moreover, carcinogen also upregulates some genes when administered into mouse. For example, DMBA increases the expression of *c-Myc, cyclin D* (Karimi *et al.,* 2020), *Pdk1, Akt1, Pik3r1, Map3k1, PIk3ca, Erbb2* (Nassan *et al.,* 2018), *Bcl2* (Shain *et al.,* 2010) and *NF-κB* (Karnam *et al.,* 2017). Hence, chemo-preventive potential of anticancer agents will be tested by analyzing the expression of these genes and looking for their downregulation.

2.6.1 qRT-PCR analysis

The expression of genes corresponds to the translation of functional mRNA transcript. RNA detection and quantification is thus important in analyzing gene expression. Some of the most widely used techniques which measure mRNA include Northern blotting, Ribonuclease-protein assay and qRT-PCR (Dvorak *et al.,* 2003).

Quantitative real time reverse transcription PCR (qRT-PCR) technique has proven itself highly useful in clinical diagnosis owing to its potential of detecting various viral pathogens from the mRNA present in them. qRT-PCR is the favorable option for analyzing gene duplications or deletions that occur in cancer markers. Small mutations up to single base pair change can be determined through melt curve analysis which is usually done after PCR amplification (Bernard and Wittwer, 2002).

Analysis of qRT-PCR is done as a relative measurement between two genes. One of the genes is the reporter gene, whose expression is altered due to the treatment and the other one is the reference gene whose expression is constant. The expression ratio of these genes is compared in two or more samples. This method is referred to as $\Delta\Delta Ct$ method (Stahlberg *et al.,* 2005). In the current study, the expression ratio of the reporter and reference genes of the treatment groups are compared with that of normal group.

From the reviewed literature, it is observed that the extract of many plants has been tested for their anticancer potential. The individual active components of the extract have to be identified and tested for the same, using *in silico* analysis and further validation can be done through *in vitro, in vivo* and gene expression studies.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study "Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of curry leaf (*Murraya koenigii* L.)" was carried out in three stages *viz. in vitro, in vivo* and molecular expression studies.

In vitro study was conducted at Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, using cancer cell lines to find out the cytotoxic and anti-proliferative effects of the selected phytocompounds of curry leaf on the cell lines. The most promising compound was carried forward for *in vivo* study, which was performed at Amala Cancer Research Institute, Thrissur. Gene expression was studied through qRT-PCR, at Kerala Veterinary and Animal Sciences University, Mannuthy.

3.1 Materials

3.1.1 *In vitro* study

3.1.1.1 Laboratory chemicals, instruments and glassware

The AR grade phytocompounds, chemicals and glassware used in this study were procured from HiMedia, Sigma Aldrich, ThermoFisher scientific, Tarsons and Sisco Research Laboratories, India (Annexure I). The instruments used for the *in vitro* study were hemocytometer, membrane filtration assembly, Olympus CK40 inverted phase contrast microscope, Genesys 20 spectrophotometer, incubator and laminar air flow chamber.

3.1.1.2 Cancer cell lines

MCF-7 (breast cancer cell line) and HCT-116 (colorectal cancer cell line) were procured form Amala Cancer Research Centre, Thrissur and used in this study. For short-term cytotoxicity analysis, Daltons Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC), maintained in the peritoneal cavity of the mouse were used. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), maintained at 37 ºC in the incubator and used for MTT assay.

3.1.2 *In vivo* studies

Thirty female Swiss albino mice were procured from Small Animal Breeding Station, Kerala Veterinary and Animal Science University, Mannuthy, Thrissur and housed at Amala Cancer Research Centre, Thrissur, under standard conditions of 60-70 % humidity, 24-28 °C and 12 h dark/light cycle. They were fed with standard mice feed (Sai Durga Feeds, Bangalore, India) and water *ad libitum.* The carcinogen used to induce breast cancer in the mice was 7,12-Dimethylbenz[a]anthracene [DMBA (D3254-100MG)]. It was purchased from Sigma Aldrich. All the animal experiments were carried out with the approval of the Institutional Animal Ethical Committee (IAEC) (Approval No. ACRC/IAEC/21(2)-P15) in accordance with the rules and regulations of Committee For The Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

3.1.3 Gene expression studies

ThermoFisher's RevertAid first strand cDNA synthesis kit was used for first strand cDNA preparation from the RNA isolated from mammary pad of the mouse. qRT-PCR was done in StepOnePlus™ Real-Time PCR machine using Biorad's SsoADV Univer SYBR GRN SMX 500 kit. The software used was StepOne V2.2.2.

3.2 Methods

3.2.1 Preparation of drugs

The stock solutions of the drugs, Doxylamine Succinate Salt (DSS), L-Histidinol Dihydrochloride (LHD) and Pheniramine Maleate Salt (PMS) were prepared at the concentration of 1 mg/ml for LHD and 10 mg/ml for DSS and PMS by mixing the drug in autoclaved distilled water in laminar air flow chamber.

3.2.2 Cytotoxicity analysis of phytocompounds on cancer cell lines *in vitro*

3.2.2.1 Short-term cytotoxicity analysis by Trypan blue exclusion assay

Trypan blue exclusion assay was done to assess the cytotoxic effect of the drugs on the murine cancer cells EAC and DLA (Gupta *et al.*, 2002).

Protocol for Trypan blue exclusion assay

❖ The EAC (Ehrlich Ascites Carcinoma) cell line was extracted from the peritoneal cavity of the mice

❖ It was dissolved in PBS (Phosphate buffered saline) buffer (pH of 7.4)

❖ The cell suspension was centrifuged at 2,000 rpm for 5 minutes, supernatant discarded using micropipette. 3 ml of PBS was added and used as stock

❖ From the stock, 100 µl cells were taken, to which 800 µl of PBS and 100 µl of tryphan blue were added. The drugs DSS, LHD and PMS were added at different concentrations of 100, 200, 300 and 400 µg/ml

❖ The solution was kept for 2 minutes and placed in the counting chamber of hemocytometer and viewed under the microscope

❖ Those which had 100-121 cells were taken as sample and placed in an incubator for 3 hours in 37 ºC

❖ After incubation, 100 µl trypan blue was added and the cells were counted after 3 minutes waiting period

❖ Dead cells appeared in dark blue colour whereas viable cells appeared in transparent colour

❖ Percentage of cytotoxicity was calculated using the formula, $\frac{\text{No.of dead cells}}{\text{Total no.of cells}}$ X 100

❖ The same procedure was followed for DLA (Daltons Lymphoma Ascites) also

3.2.2.2 MTT Assay

MTT Assay was carried out to assess the anti-proliferative effect of the drugs on cancer cell lines.

3.2.2.2.1 Preparation of Dulbecco's Modified Eagle medium

The components required for preparing DMEM are listed in Annexure II (Dulbecco and Freeman, 1959).

Protocol for the preparation of DMEM

❖ DMEM powder was dissolved in 600 ml of autoclaved distilled water

❖ The required quantity of FBS, HEPES, sodium pyruvate, non-essential amino acids and glucose were added and stirred well

❖ 300 ml autoclaved distilled water was added to this solution

❖ pH of the solution was adjusted to 7.0 using sodium bicarbonate (1 g/l)

❖ The solution was mixed well and filter sterilized in filtration apparatus having 0.22 μm filter

❖ The media were checked for contamination and finally the antibiotic Streptomycin was added to the media

3.2.2.2.2 Culture of cell lines

❖ The cell lines MCF-7 and HCT-116 were cultured in the T25 cell culture flask, placed in the incubator at 37 °C, until it reached 100 % confluence, which was measured by looking at the cells in the culture flask through the microscope

❖ Once the cells became 100% confluent i.e. 100 % of the surface of the culture flask was covered with cells, they were passaged after trypsinization

3.2.2.2.3 Trypsinization

Trypsinization was done to detach the cell from the culture flasks before passaging. The procedure for trypsinization was as following

❖ The medium was removed and the cells were washed with PBS buffer

❖ 600 μl Trypsin was added to the flask and spread

❖ It was incubated for 2-3 minutes at 35 °C in the incubator and observed under microscope for cell detachment

❖ 1.5 ml DMEM was added in the flask and mixed well to make single cell suspension

❖ From this, 100 μl cells were taken in 1.5 ml Eppendorf tube and 100 μl trypan blue solution was added

❖ The solution was loaded into hemocytometer and the cells were counted.

❖ Based on the count, required amount of cells were mixed with media such that when added into 24 well plate it would count 1×10^5 cells per well

3.2.2.2.4 Protocol for MTT assay

❖ In each well of the 24-well plate, 500 µl of medium was added and incubated for 24 hours at 37 °C

❖ After 24 hours incubation, the drugs were added to the wells at different concentrations of 40, 80, 120, 160, 200, 240 and 280 µg/ml and incubated at 37 °C for 24, 48 and 72 hours

❖ After incubation, fresh medium was added to the plates and 50 µl of MTT was added to each well and incubated for 4 hours

❖ After 4 hours incubation, 1 ml DMSO was added to each well and again incubated for 15 to 20 minutes

❖ The absorbance value of the medium was read at 570 nm in a spectrophotometer

❖ Percentage of inhibition was calculated using the equation $\frac{\text{OD of control} - \text{OD of sample}}{\text{OD of sample}}$ x 100

❖ The experiment was repeated thrice

3.2.3 *In vivo* analyses

Pheniramine maleate salt showed better results in *in vitro* assays and it was further *in vivo* tested.

3.2.3.1 Acute toxicity study

Acute toxicity study was done to find the toxic effects of the drug on the animals. Pheniramine maleate salt was one time orally administered to the mice weighing 25-30 g, at 100 mg/ kg b. wt., in accordance with the guidelines formulated by Organization of Economic Co-operation and Development (OECD) (OECD, 2001). Mice were observed for 14 days for the changes in the body weight, food and water intake, behavior, hair loss *etc*.

3.2.3.2 Anticancer efficacy analysis for Pheniramine maleate salt using *in vivo* mammary tumour model

Mammary tumour model was selected to analyze the anticancer efficiency of Pheniramine maleate. Mammary tumour was induced by orally administrating

dimethylbenz(a)anthracene (DMBA) (20 mg/kg b. wt.) in female Swiss albino mice (Minari *et. al.*, 2015). As DMBA was insoluble in water, it was dissolved in sesame oil (vehicle) and given to all groups of animals (Table 1), except normal, once in a week for six weeks. For rest of the weeks, mice were orally administered with the drug.

Table 1. List of animal groups used for *in vivo* anticancer efficiency analysis

Groups	Treatment	No. of animals
1	Normal	6
2	Control	6
3	Vehicle Control (Sesame Oil)	6
4	Standard (Tamoxifen)	6
5	Drug (low dose -10 mg/kg b. wt.)	6
6	Drug (high dose - 20 mg/kg b. wt.)	6
	Total no. of animals	36

The treated and control animals were carefully examined daily. Mice were sacrificed, the blood and serum collected and analyzed for complete blood count, Liver function test and Renal function test. Histopathology of the breast, liver and kidney tissues was performed.

3.2.4 Gene expression studies

Total RNA was isolated from the breast tissue of all the animal groups using standard TRIZOL method. From the isolated RNA, cDNA was prepared using RevertAid cDNA first strand synthesis kit (ThermoFisher, India).

3.2.4.1 RNA isolation

❖ The tissue samples were homogenized in 1.0 ml TRIZOL reagent using pestle and mortar and transferred to 1.5 ml Eppendorf tubes. The homogenized samples were incubated for 5 min at room temperature.

❖ To the homogenized sample, 0.2 ml of chloroform per ml of TRIZOL reagent was added, mixed thoroughly for 15 seconds and incubated for 2-3 minutes at room temperature

❖ The tubes were centrifuged at 12000 x g for 15 minutes at 2-8 ℃

❖ The upper aqueous phase was carefully transferred into fresh tube and the solution was precipitated by mixing 0.5 ml of isopropyl alcohol per ml of TRIZOL reagent used

❖ The samples were incubated for 10 minutes at room temperature and centrifuged at 12000 x g for 10 min at 2 to 4 ℃

❖ The supernatant was removed completely and the pellet was homogenized by washing once with 1 ml of 75 % ethanol per ml of TRIZOL initially used

❖ The sample was mixed and centrifuged at 7500 x g for 5 min at 2-4 ℃

❖ All the left over ethanol was removed and the RNA pellet was air dried for 5 to 10 minutes

❖ The RNA was dissolved in 30 µl DEPC treated water and stored under -20 ℃

❖ Integrity of the isolated RNA was checked by electrophoresis in 1.0 % gel

❖ The quantity of the RNA was checked using NanoDrop ND-1000 spectrophotometer

3.2.4.2 First strand cDNA synthesis

The cDNA synthesis was carried out from the RNA isolated from the mammary tissues. The procedure followed was

❖ Four µl of template DNA, 1 µl of OligodT primers and 7 µl of nuclease free water were pipetted into a PCR tube and mixed by a brief spin

❖ Tubes were incubated at 65 ℃ for 5 minutes

❖ After incubation, the solution was chilled on ice and 4 µl of 5X reaction buffer, 1 µl of RiboLock RNase inhibitor, 2 µl of 10mM dNTP mix and 1 µl of Revert Aid M-MulV RT were added

❖ The solution was briefly spun and incubated for 60 minutes at 42 ℃ and 5 minutes at 70 ℃

❖ After the incubation the cDNA was stored at -80 ℃

3.2.4.3 Quantitative Real-Time PCR (qRT-PCR)

The gene expression profile of four genes *viz. ER-α1, Bcl-2, c-Myc* and *Pin 1* were studied using qRT-PCR. $\beta - actin$ was used as house-keeping gene in the analysis, for minimizing the errors in quantification of sample, thus increasing the reliability of qRT-PCR. The primers used for qRTPCR are given in Table 2.

Table 2. Primers used in qRT-PCR analysis

Sl. No.	Gene	Primer Sequences (5'-3')	Reference
1	*ER-α1*	F: GGCACATGAGTAACAAAGGCA	Zeweil *et al.* (2019)
		R: GGCATGAAGACGATGAGCAT	
2	*Bcl-2*	F: GTATGATAACCGGGAGATCG	Zeweil *et al.* (2019)
		R: AGCCAGGAGAAATCAAACAG	
3	*c-Myc*	F: CTCCACTCACCAGCACAACT	Karimi *et al.* (2020)
		R: CGTTCCTCCTCTGACGTTCC	
4	*Pin 1*	F: TGATCAACGCTACATCCAG	Wang *et al.* (2016)
		R: CAAACGAGGCGTCTTCAAAT	
5	*β − actin*	F: TCTTCCAGCCTTCCTTCCTG	Zeweil *et al.* (2019)
		R: CACACAGAGTACTTGCGCTC	

PCR amplification was carried out in 20 µl reaction mixture containing

a) SsoAdvanced^{TM} Universal SYBR$^®$ Green Supermix – 10 µl

b) Forward primer – 1 µl

c) Reverse primer – 1 µl

d) DNA Template – 5 µl

e) Nuclease free water – 3 µl

Total volume – 20 µl

The following programme was used for amplification:

Stage	Temperature (ºC)	Time (Sec.)
1. Polymerase Activation and DNA Denaturation	95	30
2. Denaturation	95	15
3. Annealing/Extension	60	30
4. Melt Curve Analysis	95	15
	60	60
	95	15

Steps 2 and 3 were carried out for 45 cycles

3.2.4.3.1 Calculation of fold change (Schmittgen and Livak, 2008)

Ct values obtained from the results were further used for calculating ΔCt and ΔΔCt of the target genes compared to the house-keeping gene using the formula,
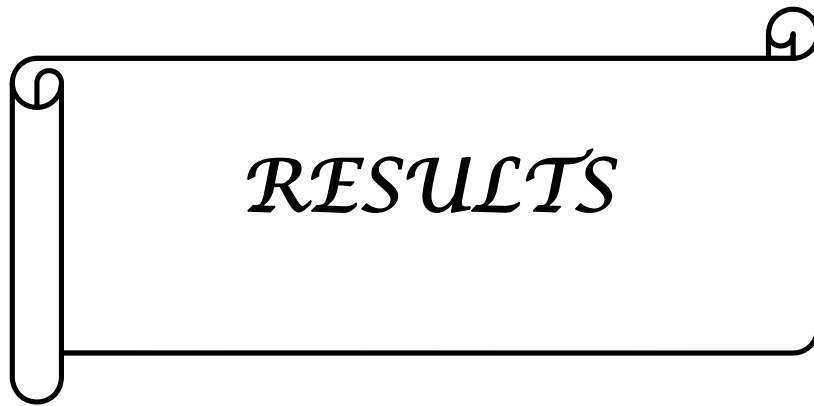
$$\Delta Ct = Ct \text{ gene of interest} - Ct \text{ internal control}$$

where, internal control refers to the reference gene

$$\Delta\Delta Ct = \Delta Ct \text{ (Sample A)} - \Delta Ct \text{ (Sample B)} = [(Ct \text{ gene of interest} - Ct \text{ internal control}) \text{ Sample A} - (Ct \text{ gene of interest} - Ct \text{ internal control}) \text{ Sample B}]$$

where, Sample A corresponds to the treated sample and sample B corresponds to the untreated sample i.e., normal group

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

RESULTS

4. RESULTS

The observations and results obtained from the experiments in the study "Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya keonigii* L.)" are presented in this chapter.

4.1 Cytotoxicity analysis of phytocompounds on cancer cell lines *in vitro*

4.1.1 Short-term cytotoxicity analysis by Trypan blue exclusion assay

No significant cytotoxicity was observed even at the concentration of 400 μg/ ml of the drugs. Only Pheniramine maleate salt showed some cytotoxicity with 15 % and 10 % inhibition on Daltons Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells, respectively.

4.1.2 Anti-proliferative analysis by MTT assay

The anti-proliferative property of each drug was measured in terms of % of inhibition of the cancer cells. The mean values ± Standard Deviation of percentage of inhibition of Pheniramine maleate salt (PMS), Doxylamine succinate salt (DSS) and L-Histidinol dihydrochloride (LHD) on MCF-7 and HCT-116 cells are presented in Tables 3-8.

Table 3. Percentage of inhibition by PMS on MCF-7 cells

Concentration (μg/ ml)	% of inhibition		
	24 hours	48 hours	72 hours
40	4.25 ± 2.2	27.92 ± 2.4***	93.63 ± 3.2***
80	5.91 ± 2.5	35.52 ± 3.3***	95.08 ± 2.3***
120	30.00 ± 3.4	55.63 ± 2.5***	95.18 ± 2.6***
160	32.06 ± 2.7	62.34 ± 2.8***	96.55 ± 3.6***
200	37.37 ± 2.1	67.10 ± 3.1***	97.24 ± 3.1***
240	37.58 ± 2.5	69.05 ± 3.4***	98.41 ± 4.0***
280	38.00 ± 2.2	71.21 ± 2.5***	98.46 ± 3.6***

Mean±SD (n=3) in comparison with 24 hours group. ***significant at $p < 0.001$

Table 4. Percentage of inhibition by PMS on HCT-116 cells

Concentration (µg/ ml)	% of inhibition		
	24 hours	**48 hours**	**72 hours**
40	5.88 ± 2.3	9.45 ± 2.2	21.19 ± 2.5***
80	16.03 ± 2.7	23.52 ± 2**	26.58 ± 3.1***
120	27.21 ± 3.4	32.45 ± 2.8	37.07 ± 2.9
160	32.35 ± 2.3	41.79 ± 2.5***	48.87 ± 3.4***
200	38.5 ± 3.6	48.12 ± 3.2***	61.58 ± 3.3***
240	42.38 ± 2.8	56.51 ± 2.9***	64.97 ± 2.8***
280	46.62 ± 3	67.16 ± 2.7***	70.34 ± 2.4***

Mean±SD (n=3) in comparison with 24 hours group. **significant at $p<0.01$ and ***significant at $p<0.001$

Table 5. Percentage of inhibition by DSS on MCF-7 cells

Concentration (µg/ ml)	% of inhibition		
	24 hours	**48 hours**	**72 hours**
40	2.34 ± 0.9	11.26 ± 1.9**	44.96 ± 3.3***
80	5.31 ± 0.7	24.07 ± 1.4***	55.56 ± 3.6***
120	8.92 ± 0.5	31.82 ± 2.2***	62.89 ± 3.2***
160	17.83 ± 1.2	40.69 ± 2.6***	68.99 ± 3.7***
200	26.90 ± 1.8	46.32 ± 2.0***	73.38 ± 4.1***
240	33.97 ± 1.9	46.75 ± 1.8***	76.92 ± 3.6***
280	38.22 ± 1.5	58.23 ± 2.1***	80.49 ± 3.4***

Mean±SD (n=3) in comparison with 24 hours group. **significant at $p<0.01$ and ***significant at $p<0.001$

Table 6. Percentage of inhibition by DSS on HCT-116 cells

Concentration (µg/ ml)	% of inhibition		
	24 hours	**48 hours**	**72 hours**
40	4.5 ± 2.4	9.03 ± 2.6	16.95 ± 2.5***

80	9.12 ± 2.7	16.58 ± 3**	31.64 ± 2.8***
120	13.71 ± 3.1	34.48 ± 2.6***	38.98 ± 2.9***
160	18.12± 3.4	42.05 ± 3.1***	49.83 ± 2.6***
200	25.29 ± 2.9	51.98 ± 2.6***	55.48 ± 2.5***
240	28.97 ± 2.6	55.1 ± 2.9***	58.21 ± 3.1***
280	31.03 ± 2.1	60.59 ± 3.2***	63.28 ± 3.5***

Mean±SD (n=3) in comparison with 24 hours group. **significant at p<0.01 and ***significant at p<0.001

Table 7. Percentage of inhibition by LHD on MCF-7 cells

Concentration	% of inhibition		
(µg/ ml)	24 hours	48 hours	72 hours
40	1.06 ± 0.3	1.30 ± 0.2	6.32 ± 0.3
80	1.49 ± 0.5	2.43 ± 0.6	8.28 ± 0.5
120	2.55 ± 0.1	4.11 ± 0.7	14.06 ± 0.9
160	3.18 ± 0.5	6.93 ± 0.5	19.81 ± 1.2
200	4.25 ± 0.6	7.14 ± 0.2	20.11 ± 1.3
240	5.31 ± 1.2	12.97 ± 1.3	24.16 ± 1.9
280	5.94 ± 1.6	14.72 ± 1.5	28.82 ± 1.5

Table 8. Percentage of inhibition by LHD on HCT-116 cells

Concentration	% of inhibition		
(µg/ ml)	24 hours	48 hours	72 hours
40	1.24 ± 0.2	4.65 ± 0.5	6.50 ± 0.7
80	1.94 ± 0.3	5.71 ± 0.9	9.93 ± 1.1
120	1.97 ± 0.1	7.11 ± 0.3	12.13 ± 0.7
160	2.29 ± 0.5	7.55 ± 0.6	15.37 ± 1.4
200	2.88 ± 0.3	9.10 ± 0.8	15.63 ± 1.3
240	3.85 ± 0.4	10.43 ± 1.4	16.67 ± 1.5
280	4.29 ± 0.6	12.32 ± 1.2	17.34 ± 1.7

An increase in the percentage of inhibition of cells was observed when the concentration was increased. Based on the values, graphs were drawn and IC_{50} values were calculated. IC_{50} refers to the concentration of the drug at which 50 % inhibition of the cells is observed. Figures 1-6 show the graphs indicating % of inhibition induced by PMS, DSS and LHD on the MCF-7 and HCT-116 cancer cell lines at various concentrations and time interval. The IC_{50} of PMS on MCF-7 was found to be 108 μg/ml for 48 hours and 14 μg/ml for 72 hours while for HCT-116, IC_{50} value was 211 μg/ml for 48 hours and 164 μg/ml for 72 hours. DSS had IC_{50} at 252 μg/ml for 48 hours and 61 μg/ml for 72 hours on MCF-7 cell line and at 195 μg/ml for 48 hours and 162 μg/ml for 72 hours on HCT-116 cell line. LHD could not cause 50 % inhibition in both the cell lines even at 280 μg/ml concentration.

For the treatments which could cause 50 % inhibition of the cells i.e. PMS on MCF-7 and HCT-116 and DSS on MCF-7 and HCT-116 the values were compared with control. The statistical analysis was done by two way analysis of variance (ANOVA) using Graph Pad Prism 8.02 software followed by Dunnetts test. P values of $p < 0.05*$ and $p < 0.01**$ were considered significant and $p > 0.05$ non-significant. Plates 1-6 show the images of cell lines treated with the drugs at different concentrations, captured by Phase contrast microscope.

4.2 Acute toxicity study

After 14 days of monitoring, no significant changes in the behavior of the animals were observed and they were all alive. The general appearance and behavioral observations of the animals are listed in the Table 9. There were no changes in the appearance, body weight, food and water intake and skin and there were no symptoms of dizziness or diarrhea. Hence $1/10^{th}$ (10 mg/ kg b. wt.) of the concentration of drug initially given for acute toxicity study (100 mg/ kg b. wt.) was used as low dose and $1/5^{th}$, high dose (20 mg/ kg b. wt.).

Table 9. General appearance and behavior of the animals after 14 days of treatment
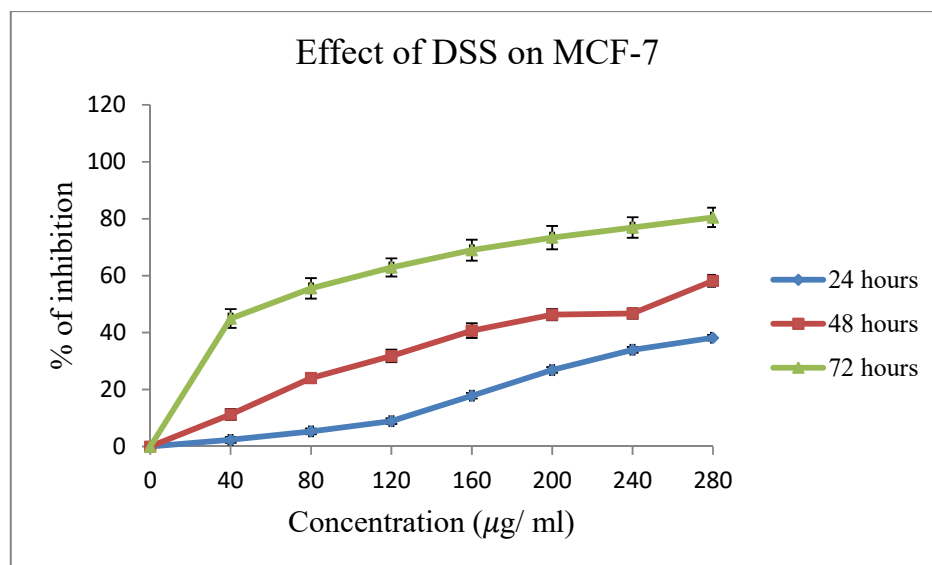
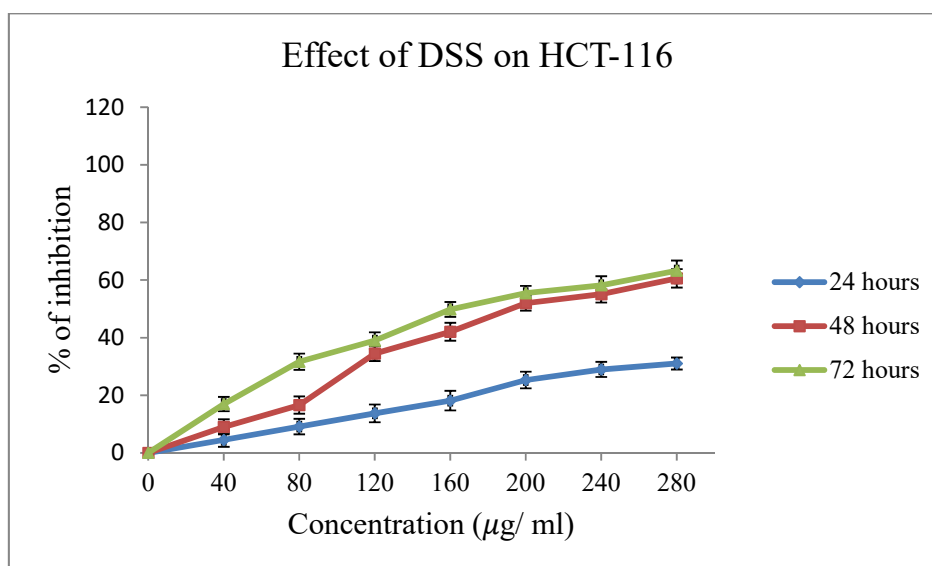| Observations	Normal group	Treated group
Body weight	Normal	Normal
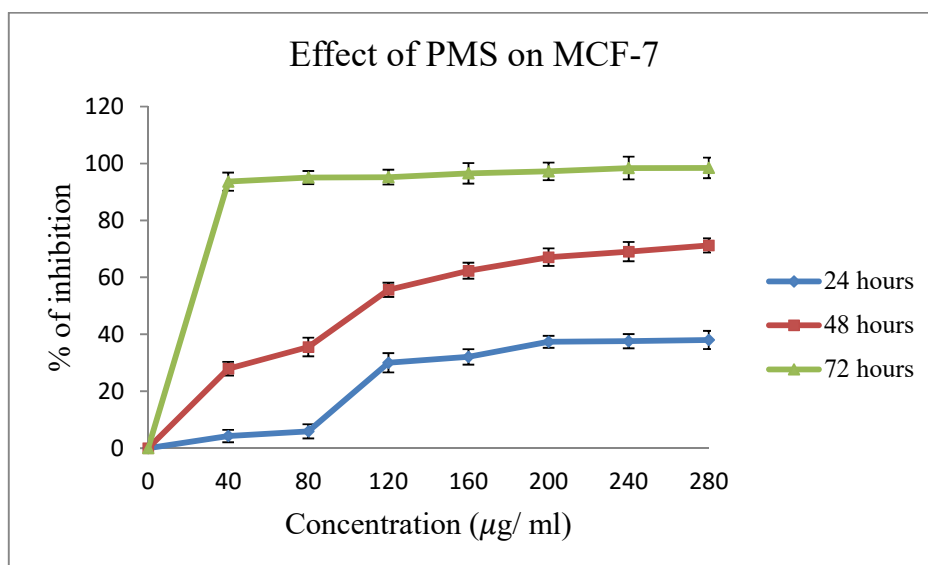
Figure 1. Cytotoxicity on MCF-7 cell line treated with Pheniramine maleate salt
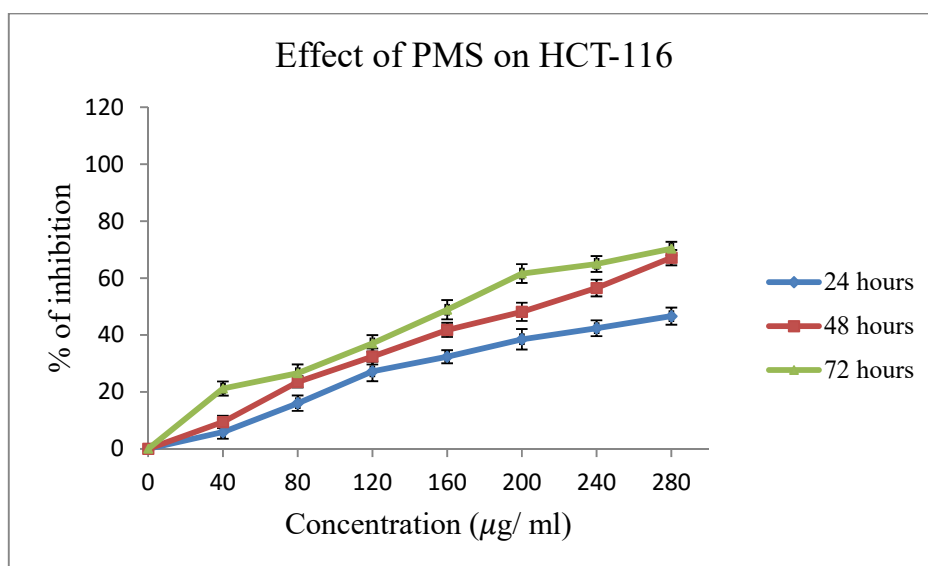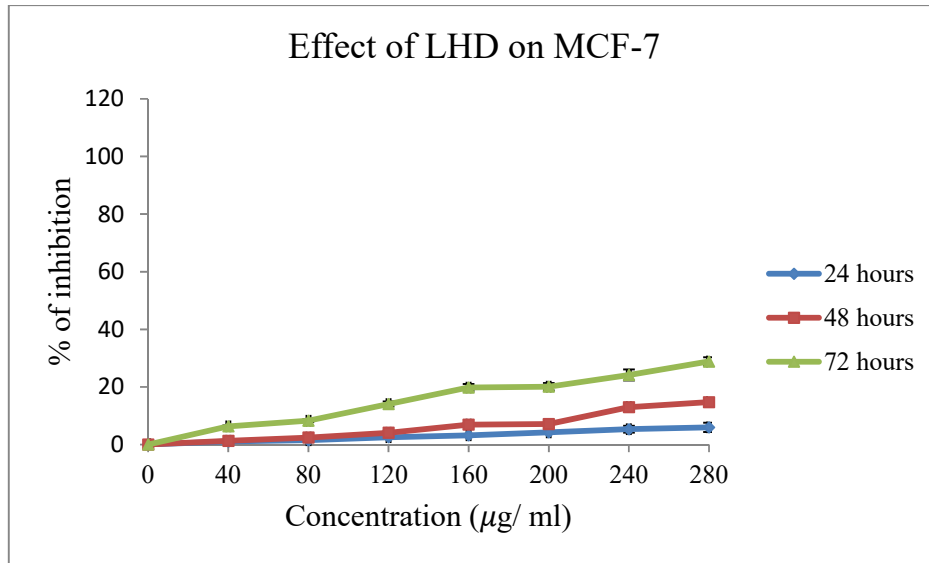

Figure 2. Cytotoxicity on HCT-116 cell line treated with Pheniramine maleate salt
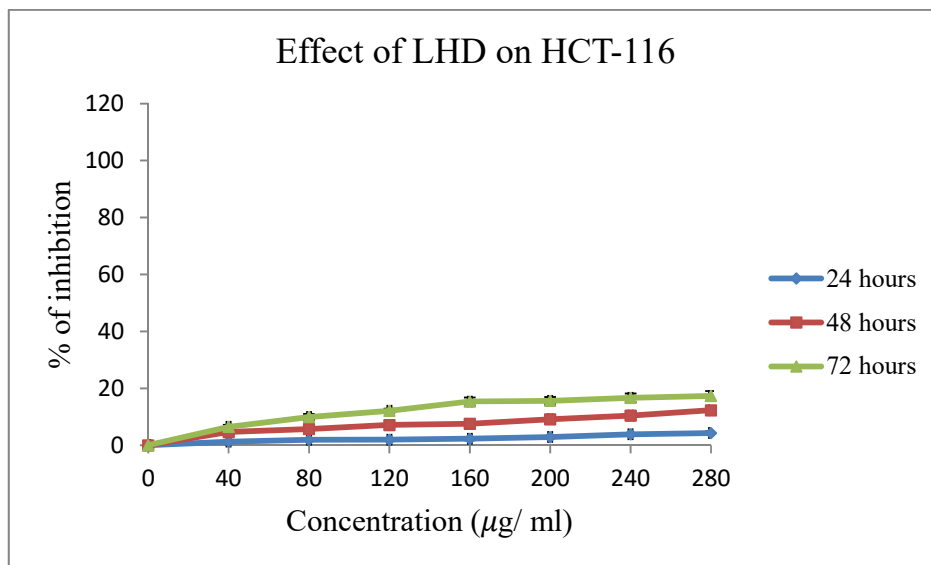
Figure 3. Cytotoxicity on MCF-7 cell line treated with Doxylamine succinate salt
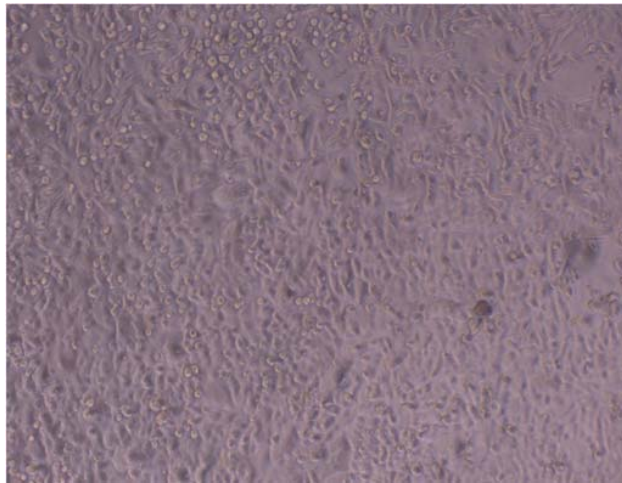

Figure 4. Cytotoxicity on HCT-116 cell line treated with Doxylamine succinate salt
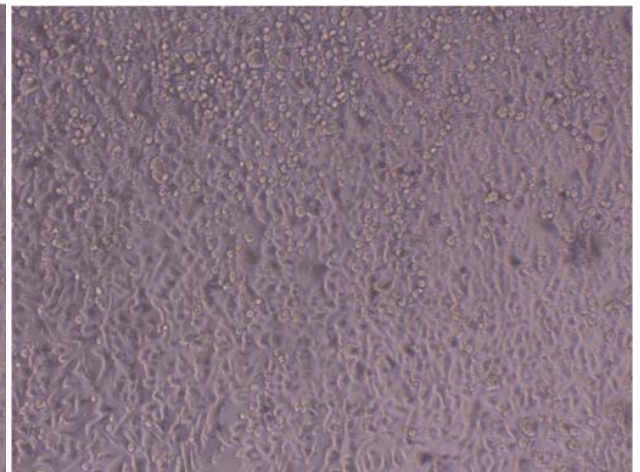
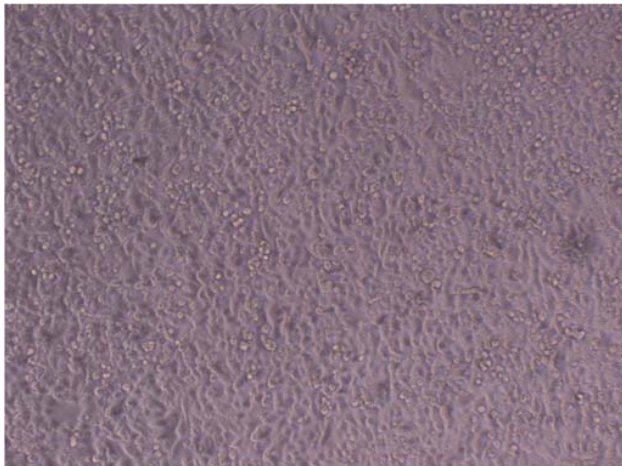**Figure 5.** Cytotoxicity on MCF-7 cell line treated with L-histidinol dihydrochloride

Figure 6. Cytotoxicity on HCT-116 cell line treated with L-histidinol dihydrochloride
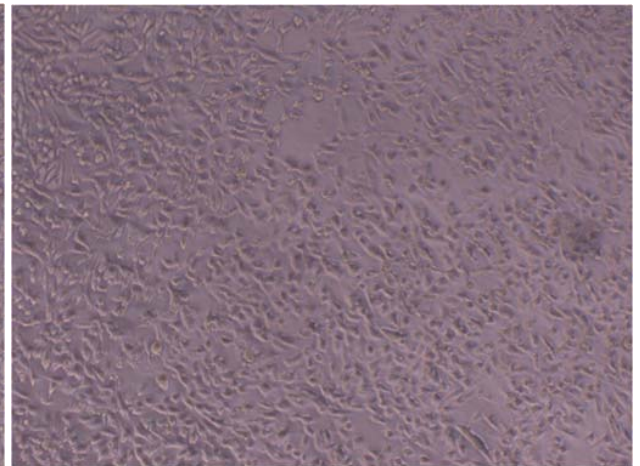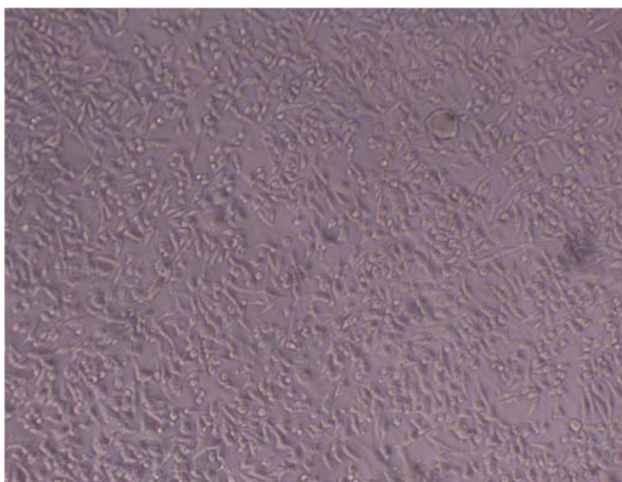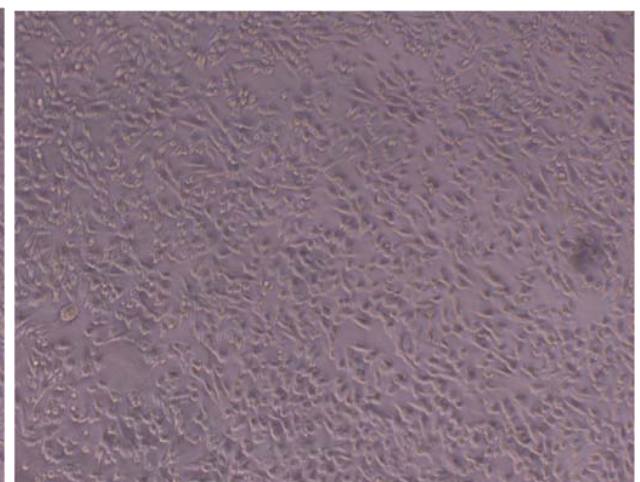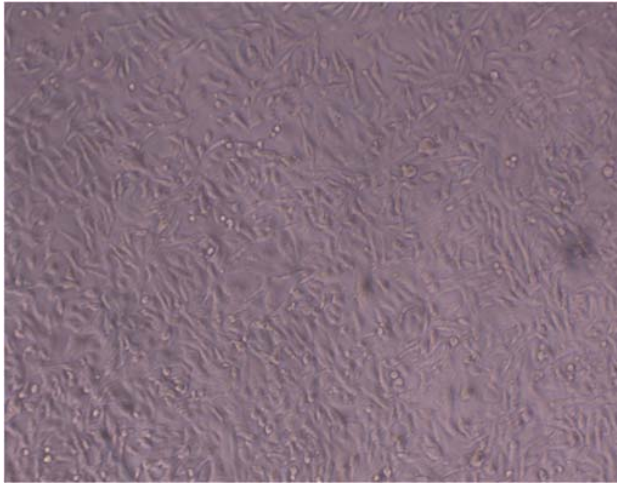
Control
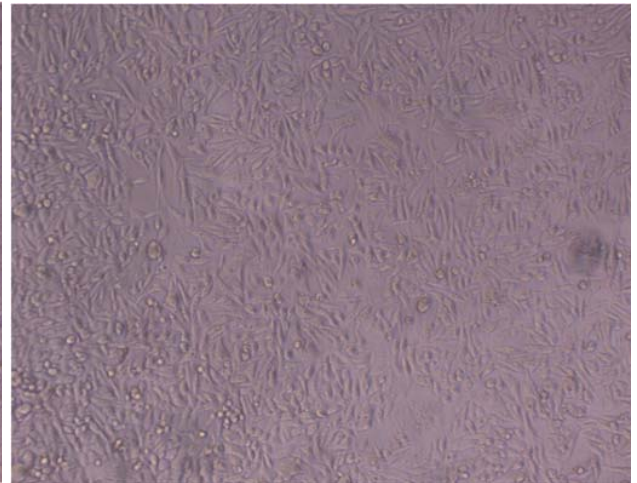
40 μg/ml

80 μg/ml

160 μg/ml

240 μg/ml

280 μg/ml

Plate 1. MCF-7 cells treated with Doxylamine succinate salt at different concentrations

40 µg/ml

80 µg/ml
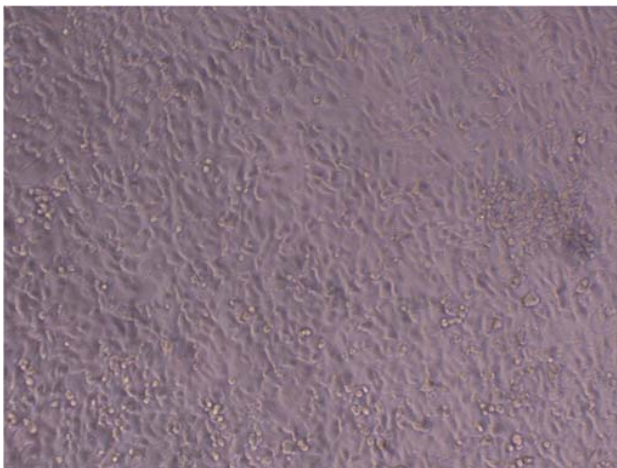
120 µg/ml

200 µg/ml

240 µg/ml

280 µg/ml

Plate 2. MCF-7 cells treated with L-histidinol dihydrochloride at different concentrations

40 µg/ml

80 µg/ml

120 µg/ml

160 µg/ml
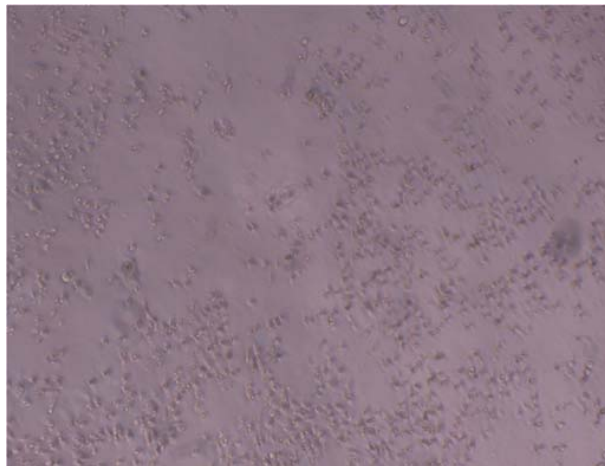
200 µg/ml

280 µg/ml

Plate 3. MCF-7 cells treated with Pheniramine maleate salt at different concentrations

Control
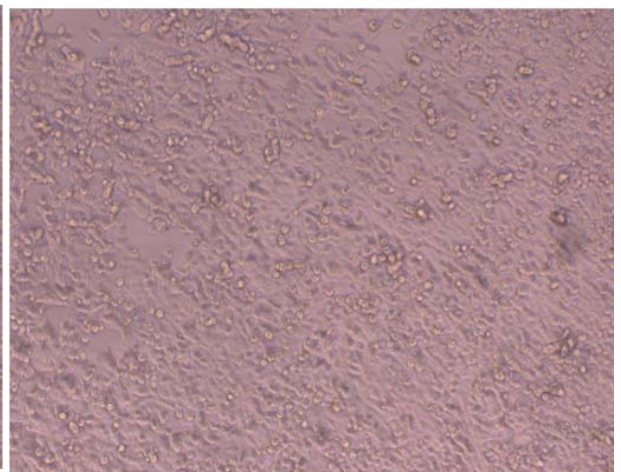
40 µg/ml
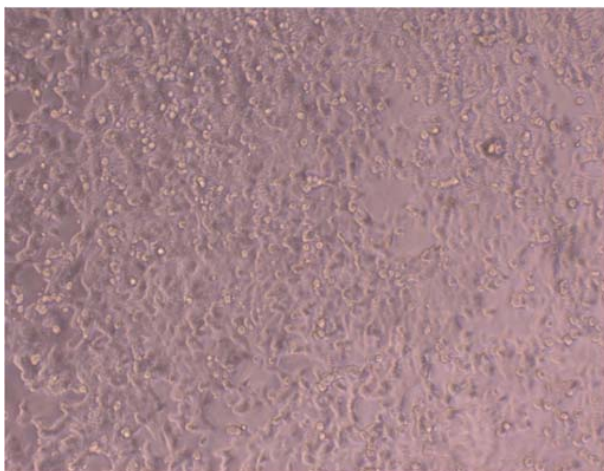
80 µg/ml
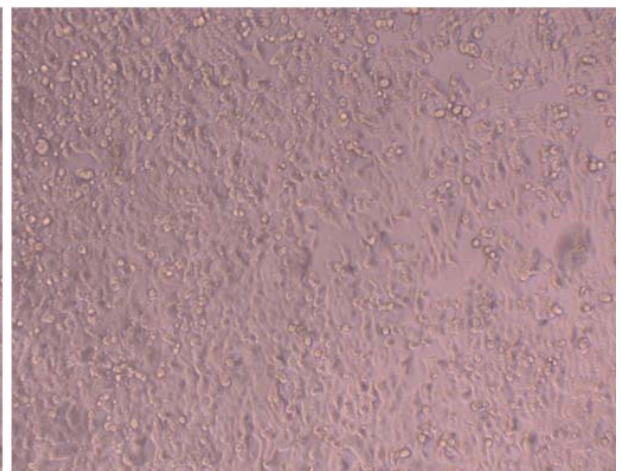
120 µg/ml
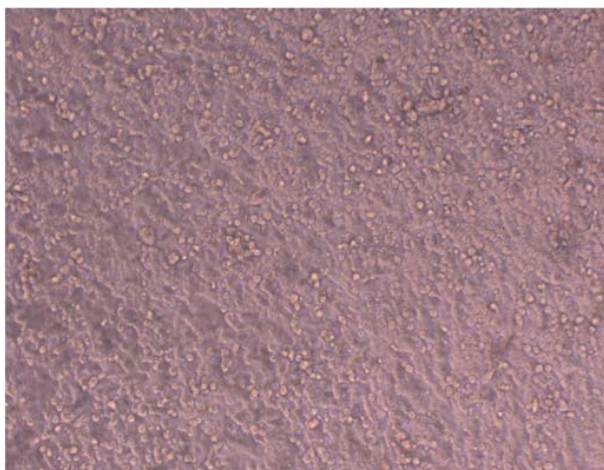
200 µg/ml
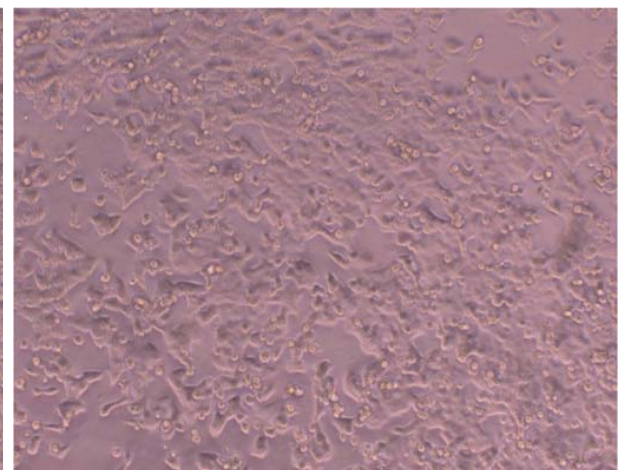
280 µg/ml

Plate 4. HCT-116 cells treated with L-histidinol dihydrochloride at different concentrations
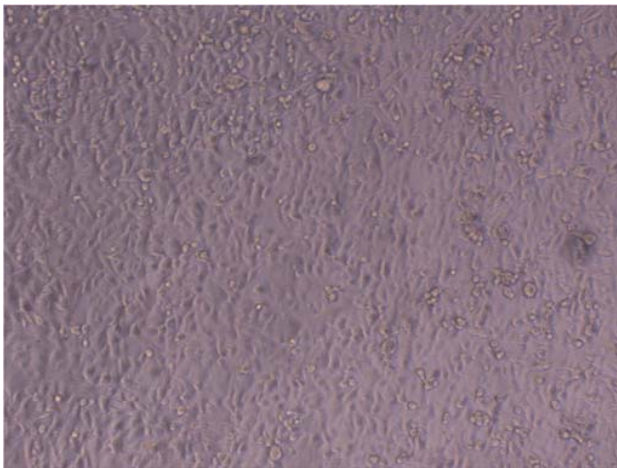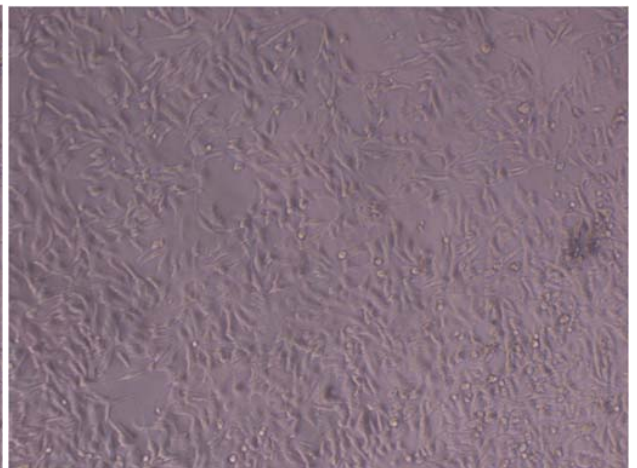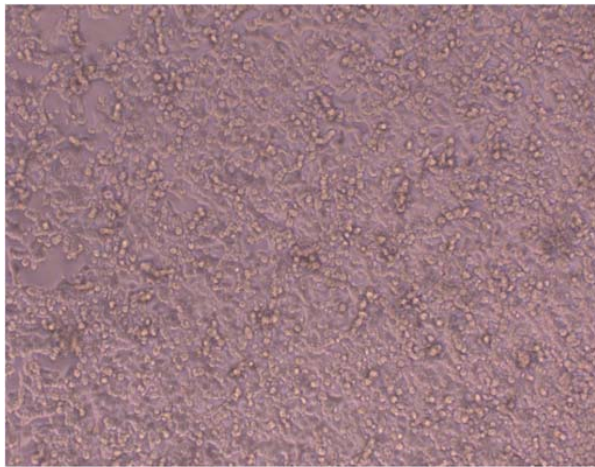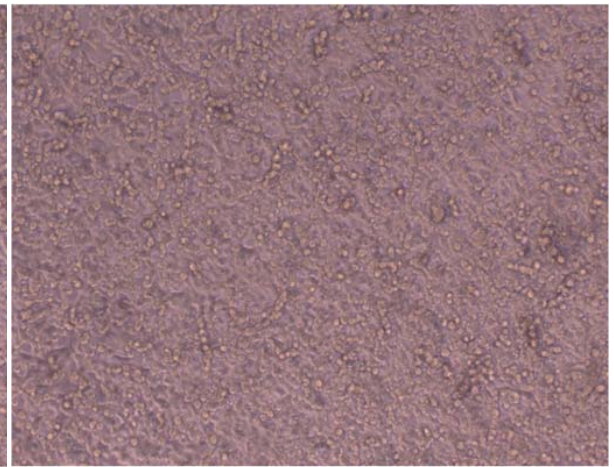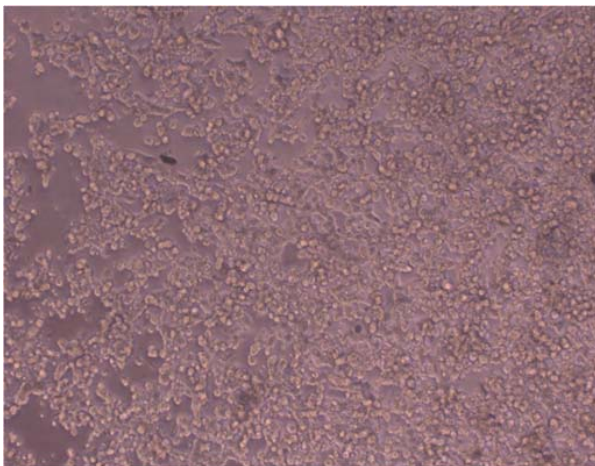
40 µg/ml
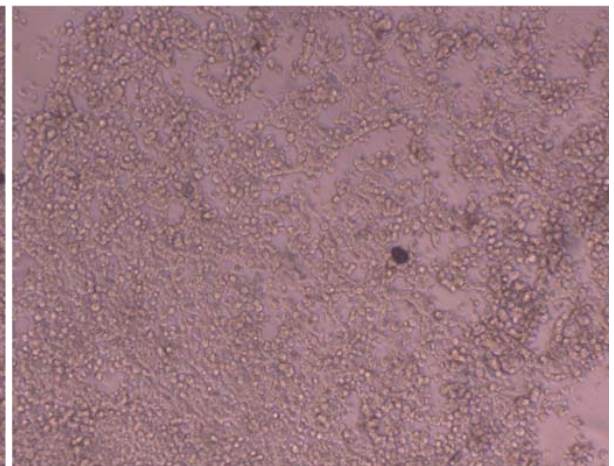
80 µg/ml

120 µg/ml

240 µg/ml
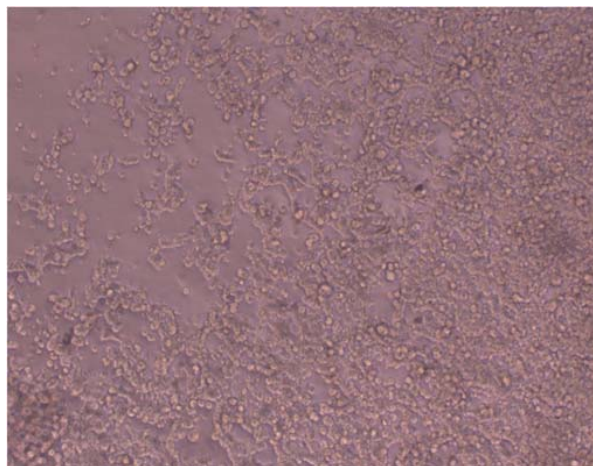
280 µg/mLl

Plate 5. HCT-116 cells treated with Doxylamine succinate salt at different concentrations
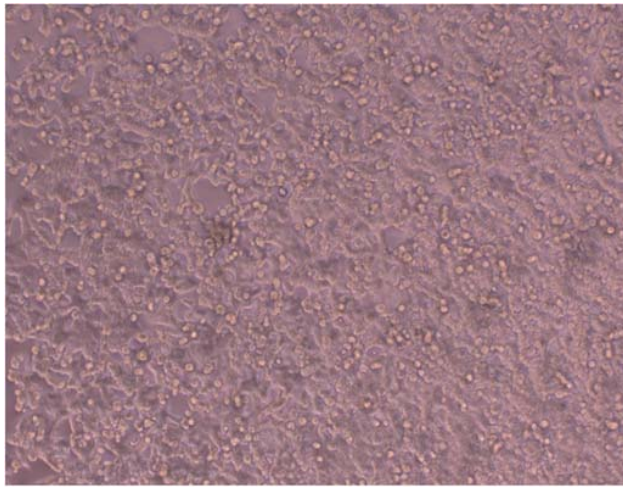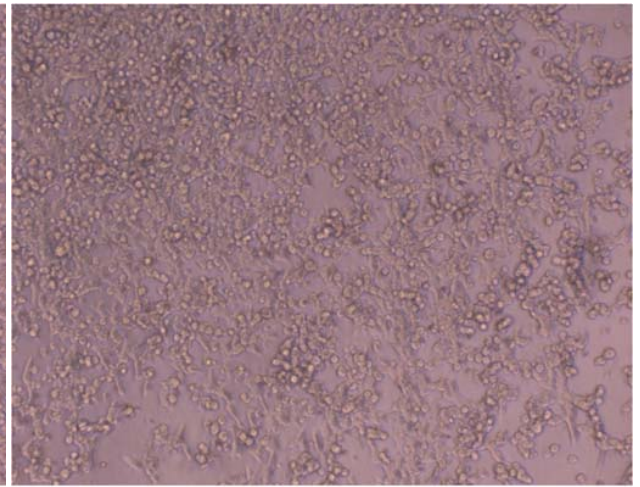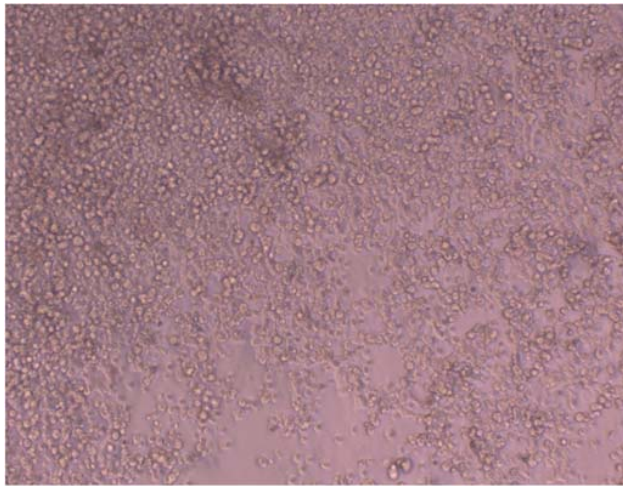
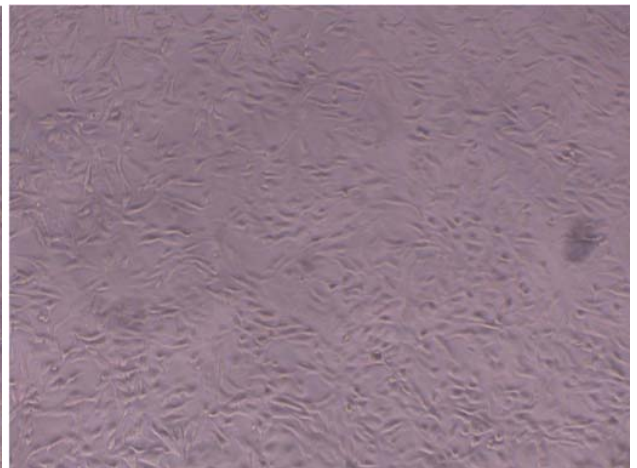40 µg/ml

80 µg/ml

120 µg/ml
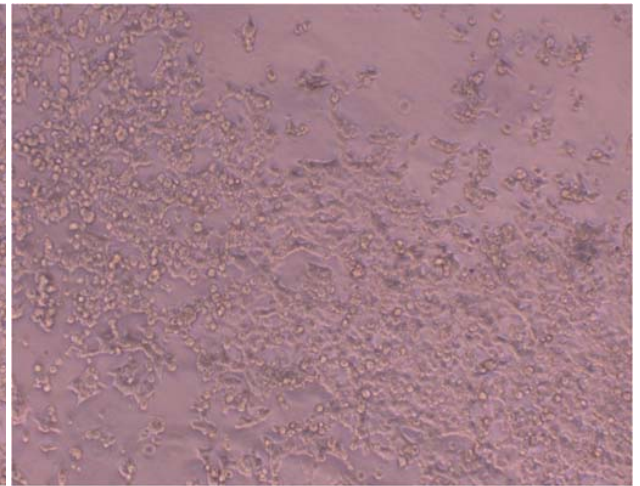
160 µg/ml
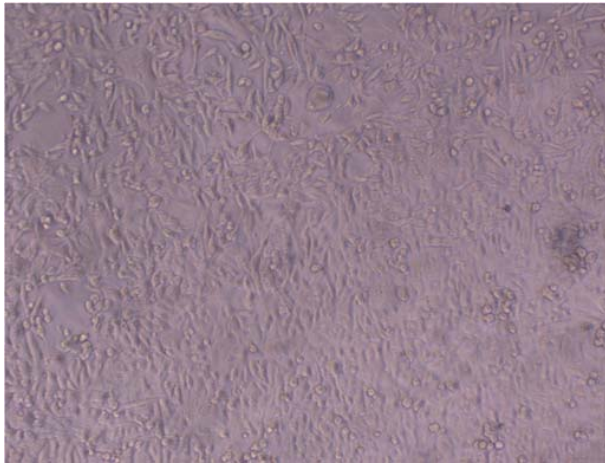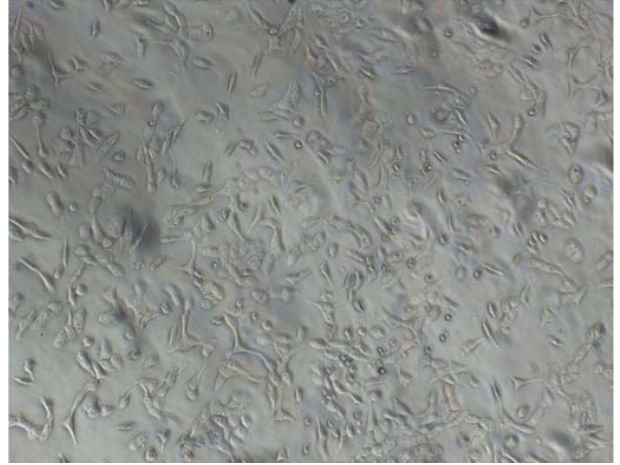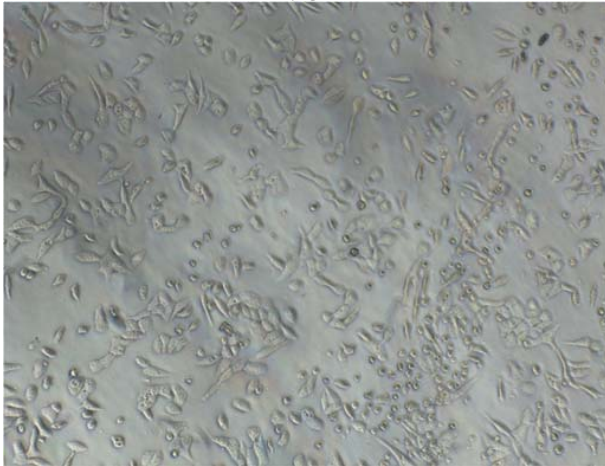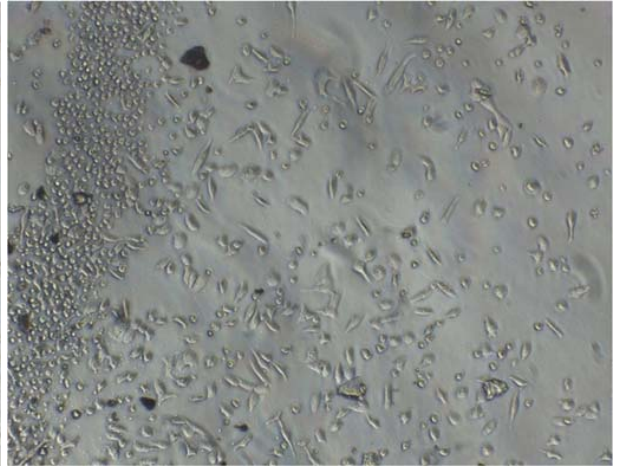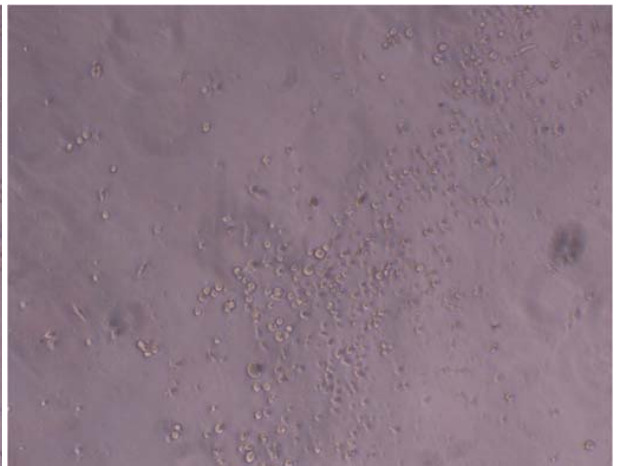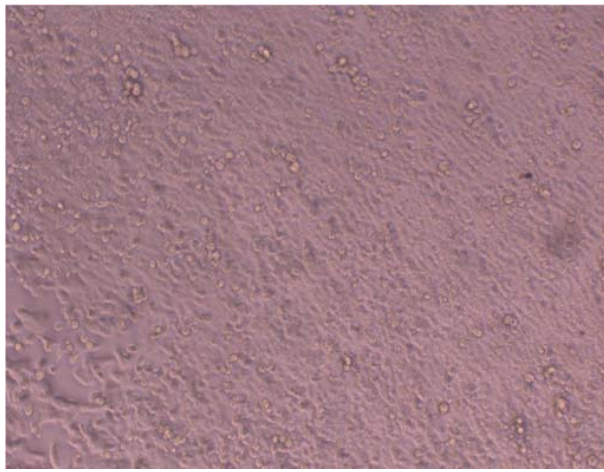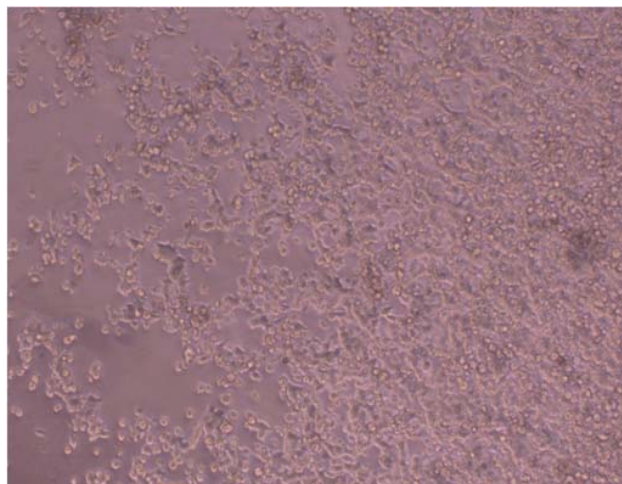
280 µg/ml

Plate 6. HCT-116 cells treated with Pheniramine maleate salt at different concentrations

Death	Alive	Alive
Dizziness	Absent	Absent
Diarrhea	Absent	Absent
General appearance	Normal	Normal
Intake of food	Normal	Normal
Intake of water	Normal	Normal
Sedation	No effect	No effect
Skin changes	No change	No change

4.3 Anticancer efficacy analysis of phyto-compounds on *in vivo* mouse tumour model

After the tumour development, mice were sacrificed in CO_2 chamber. There were six groups *viz.* normal, control, vehicle control, standard, drug (low dose) and drug (high dose). Each group had six animals. The development of tumour in the mammary pad of the mouse fed only with DMBA is shown in Plate 7 along with that of normal mouse. Three animals were used for the collection of the blood and three were used for serum collection. Blood was used for Haematogram and serum was used for Liver function test and renal function test. The haematogram, liver function and renal function profile of the DMBA induced mammary tumour mice and normal mice are listed in the Tables 10, 11 and 12.

Table 10. Haematological parameters of DMBA induced breast tumour mice and normal mice

Haematological parameters	Normal	Control	Vehicle Control	Standard	PMSLD	PMSHD
Haemoglobin (g/dl)	14.3±0.2	13.2±0.2	12.9±0.2	11.3±0.1	14.6±0.2	13.9±0.2
Total RBC count (millions/cu mm)	8.2±0.1	8.1±0.2	7.8±0.2	7±0.2	8.5±0.2	8.1±0.1
Platelet count (lakhs/cu mm)	10.9±0.2	10.2±0.2	12.9±0.2	11.1±0.2	9.5±0.2	9±0.1
Total WBC count	5600±205	8600±270	8100±190	6800±230	8300±510	6300±550

(cells/cu mm)				***	**	**
Neutrophils (%)	8±3	18±2.8	25±2.5	12±8	16±1.5	20±2.1
Lymphocytes (%)	87±2.5	80±2.2	67±1.7	85±3.2	81±3.1	77±4.2
Eosinophils (%)	5±0.1	2±0.1	8±0.2	3±0.2	3±0.1	3±0.1

Mean±SD (n=3) in comparison with control group. **significant at $p<0.01$ and ***significant at $p<0.001$

Table 11. Liver function test of DMBA induced breast tumour mice and normal mice

Biochemical parameters	Normal	Control	Vehicle Control	Standard	PMSLD	PMSHD
SGOT (U/L)	312±5.3	380±4.8	316±3	272±3.5**	303±8.9**	297±4.3
SGPT (U/L)	60±3.1	101±1.5	68±2.2	72±1.5***	65±4.2***	76±2.5***
Alkaline phosphatase (U/L)	234±2.5	128±2.1	79±4.4	146±7.8***	73±2.5***	156±2.4***
Total protein (g/dl)	7±0.4	8±0.9	8.3±0.8	7.7±2.5	7.1±1.5	7.7±0.5
Albumin (g/dl)	3.4±0.2	3.3±0.2	2.9±0.2	3.1±0.1	3.2±0.1	3.4±0.2
Globulin (g/dl)	3.6±0.2	4.7±0.1	5.4±0.2	4.6±0.2	3.9±0.1	4.3±0.3
Total Bilirubin (mg/dl)	0.3±0.1	0.3±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.1

Mean±SD (n=3) in comparison with control group. **significant at $p<0.01$ and ***significant at $p<0.001$

Table 12. Renal function test

Biochemical parameters	Normal	Control	Vehicle Control	Standard	PMSLD	PMSHD
Urea (mg/dL)	53 ± 0.6	43±0.9	37±0.5	40±0.7	35±0.4	31±0.3
Creatinine (mg/dL)	0.46±0.02	0.48±0.03	0.45±0.01	0.45±0.02	0.44±0.01	0.47±0.03
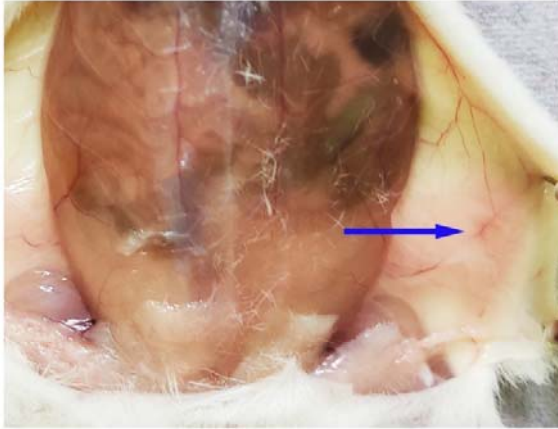
Plate 7. Comparison of tumour developed in the mammary pad of the DMBA treated mouse with normal mouse

Blue arrow – Mammary pad

The values were expressed as Mean±SD and compared with control. The statistical analysis was done by two way analysis of variance (ANOVA) followed by Dunnetts test using Graph Pad Prism 8.02 software. P values of $p < 0.05*$ and $p < 0.01**$ were considered significant and $p > 0.05$ non-significant.

Only in total WBC count (cells/cu mm), a significant decrease was found in standard (6800 ± 230 ***), PMSLD (8300 ± 510 **) and PMSHD (6300 ± 550 **) as compared with that of control (8600 ± 270). For rest of the haematological parameters *viz.* haemoglobin (g/dl), total RBC count (millions/cu mm), platelet count (lakhs/cu mm), neutrophils (%), lymphocytes (%) and eosinophils (%) no significant difference was found in any of the groups with reference to control group.

In case of biochemical parameters of liver function test, there was a significant decrease in SGOT (Serum glutamic-oxaloacetic transaminase) (U/L) level of the standard (272 ± 3.5**) and PMS low dose (303 ± 8.9**), SGPT (Serum glutamic pyruvic transaminase) (U/L) level of standard (72 ± 1.5***), PMS low dose (65 ± 4.2 ***) and PMS high dose (76 ± 2.5***) and ALP (Alkaline phosphatase) (U/L)level of standard (146 ± 7.8***), PMS low dose (73 ± 2.5***) and PMS high dose (156 ± 2.4***). The respective values for control group were 380 ± 4.8 U/L, 101 ± 1.5 U/L and 128 ± 2.1 U/L. Other parameters like total protein (g/dl), albumin (g/dl), globulin (g/dl), total bilirubin (mg/dl) were found to be non-significant.

So far as renal function test was concerned, values of urea and creatinine for all the groups were non-significant when compared with that of control (53 ± 0.6 mg/dL urea and 0.46 ± 0.02 mg/dL creatinine)

4.3.1 Histopathology of liver, mammary pad and kidney

The histopathological architecture of mammary pad, liver and kidney of the animals from all the six groups, photographed under light microscope at 200 X magnification are shown in the Plates 8, 9 and 10. The histology of mammary pads of untreated groups revealed the presence of normal adipose tissue, mammary duct and stromal tissue. In control and low dose group, the stroma shows carcinomatous growth. In

high dose treatment group the tumour size was relatively smaller and tumour initiation and progression were much reduced compared to control groups.

Histopathological examination of liver tissues from mouse liver section of normal group shows typical hepatic architecture. The control groups and low treatment group showed cytoplasmic degeneration and aggregation of inflammatory cells, whereas high treatment group showed less cytoplasmic degeneration. The standard group shows hepatocellular injury.

The kidney histopathology of normal group showed normal glomerulus, distal and proximal tubules. The control and treated groups didn't show much change in the renal architecture.

4.4 Molecular pathway analysis

Total RNA from the mice of all the groups was isolated using TRIZOL method. From the RNA cDNA was prepared and qRT-PCR was done to measure the extent of expression of the genes, *ER-α1, Bcl-2, c-Myc* and *Pin 1* in all the groups of mice in order to study the difference in the gene expression among treated and normal animal groups. *β-actin* was used as the reference gene. The qRT-PCR data was analysed using comparative Ct method (Schmittgen and Livak, 2008). The Ct, ΔΔCt and fold change values obtained for different set of gene primers for the animal groups are listed in the Tables 13-16.

Table 13. Ct and Fold change values obtained for *ER-α1* of animal groups through qRT-PCR

Animal Groups	Sample A (Treated groups)		Sample B (Normal group)		ΔCt (Sample A)	ΔCt (Sample B)	ΔΔCt	Fold change = 2^-ΔΔCt
	Ct gene	Ct control	Ct gene	Ct control				
Normal	40.26	17.57	40.26	17.57	22.70	22.69	0.00	1.00
Control	40.37	25.79	40.26	17.57	14.58	22.69	-8.11	276.75
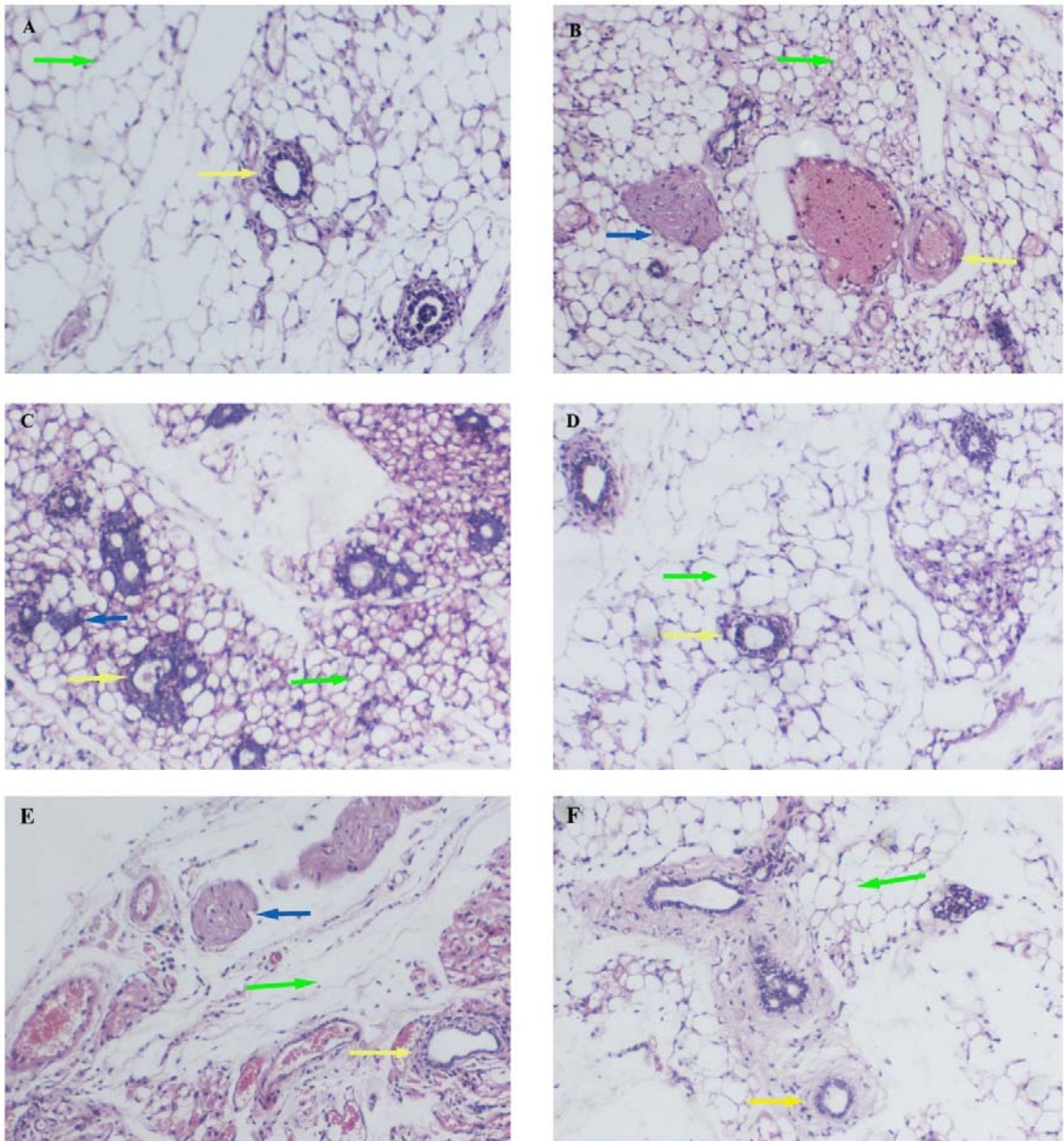
Plate 8. Histological section of mammary gland of normal mice and DMBA induced breast cancer mice - A: normal, B: control, C: vehicle control, D: standard, E: PMSLD, F: PMSHD

Yellow arrow- Duct, Green arrow- Adipose tissue, Blue arrow- Hyperplastic areas
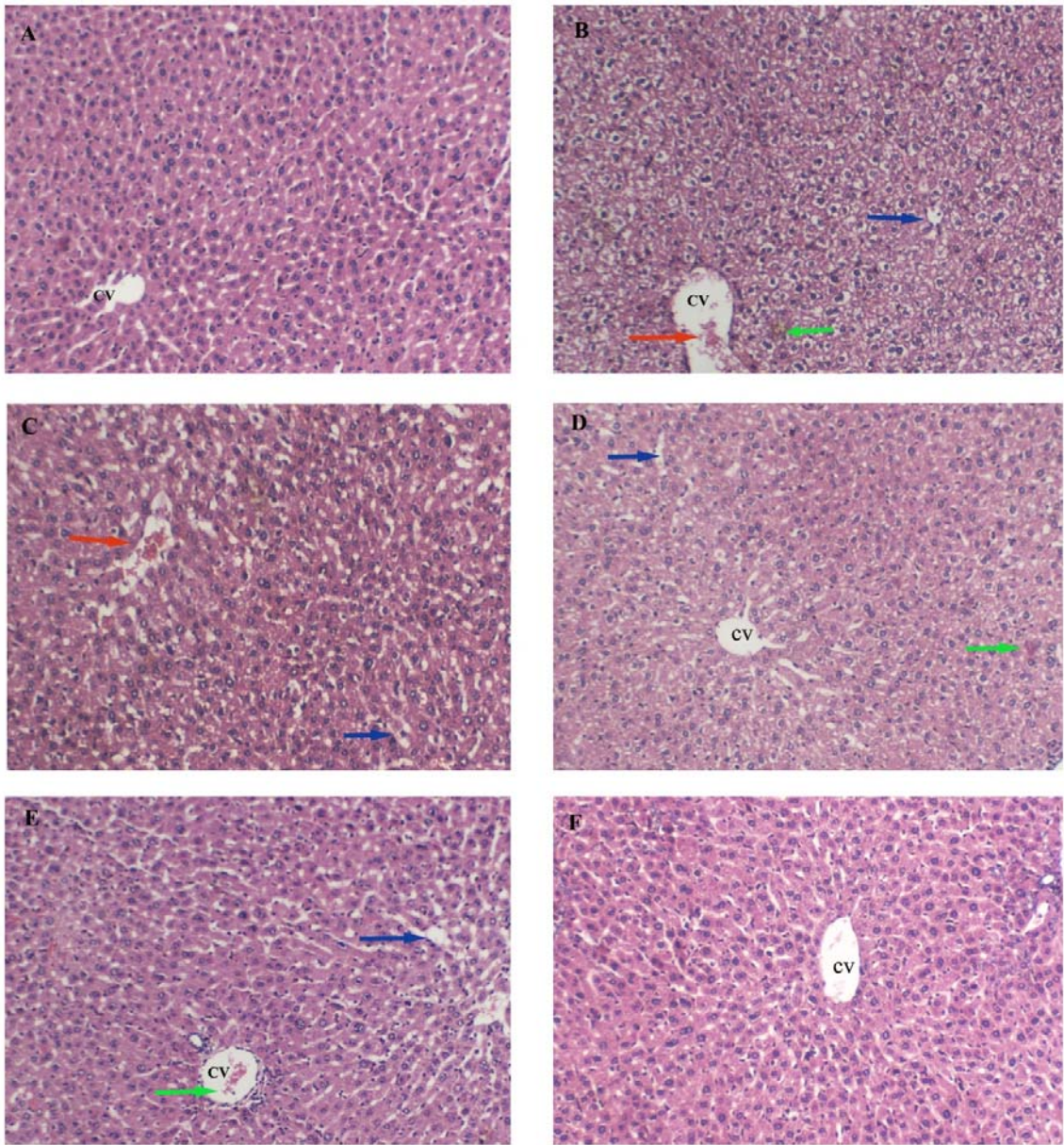
Plate 9. Histological section of liver of normal mice and DMBA induced breast cancer mice - A: normal, B: control, C: vehicle control, D: standard, E: PMSLD, F: PMSHD

Red arrow- infiltration of inflammatory cells, Blue arrow- cytoplasmic degeneration, Green arrow- necrosis, CV- central vein
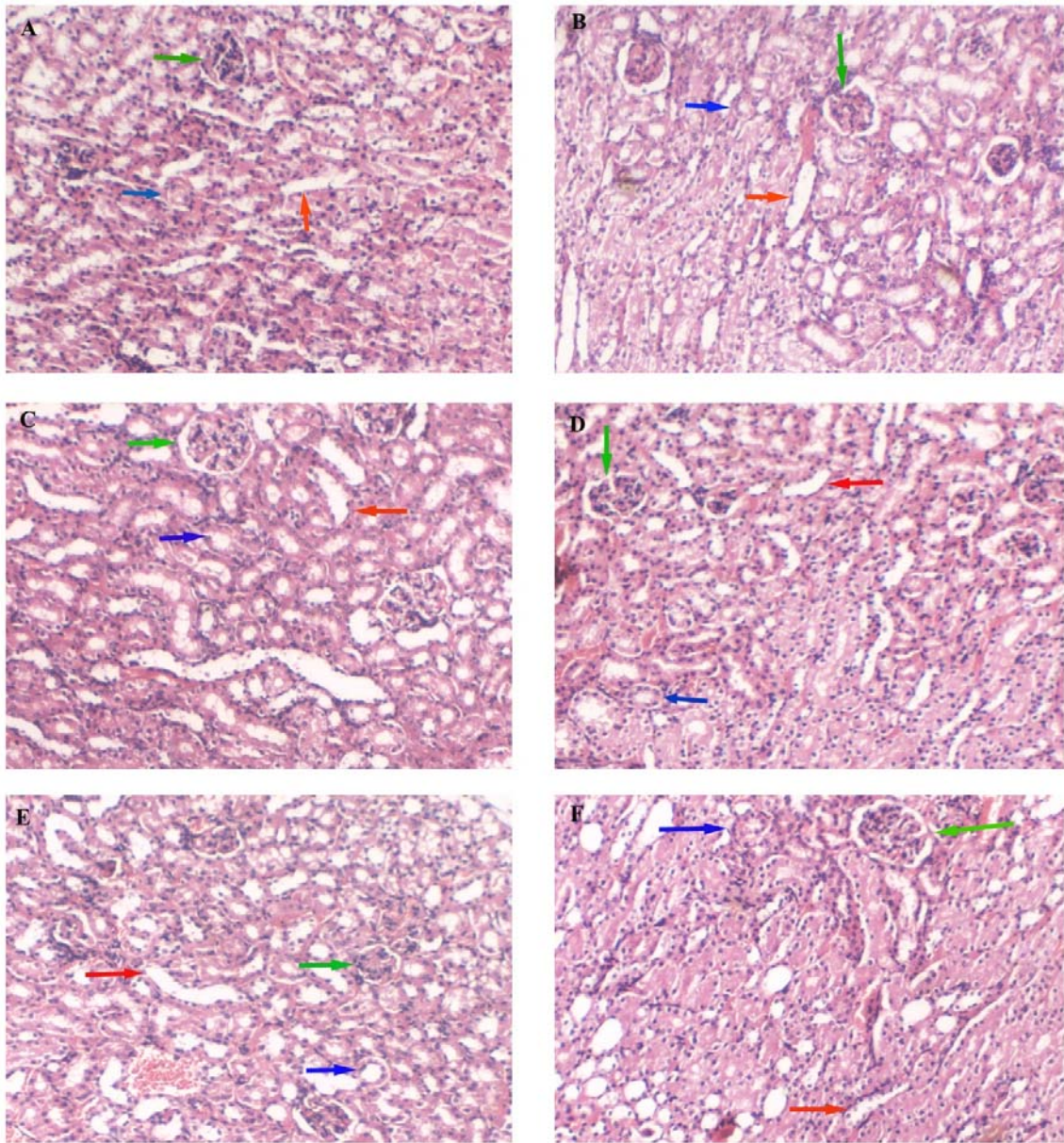
Plate 10. Histological section of kidney of normal mice and DMBA induced mice- A: normal, B: control, C: vehicle control, D: standard, E: PMSLD, F: PMSHD

Green arrow- glomerulus, Red arrow- distal tubules and Blue arrow- proximal tubules

	Sample A (Treated groups)		Sample B (Normal group)		ΔCt (Sample A)	ΔCt (Sample B)	ΔΔCt	Fold change = 2^-ΔΔCt
	Ct gene	Ct control	Ct gene	Ct control				
V. control	38.22	23.29	40.26	17.57	14.93	22.69	-7.76	216.28
Standard	39.53	22.54	40.26	17.57	16.99	22.69	-5.70	51.98
PMSLD	36.53	18.57	40.26	17.57	17.96	22.69	-4.73	26.61
PMSHD	38.02	19.77	40.26	17.57	18.25	22.69	-4.44	21.69

Table 14. Ct and Fold change values obtained for *Bcl-2* of animal groups through qRT-PCR

Animal Groups	Sample A (Treated groups)		Sample B (Normal group)		ΔCt (Sample A)	ΔCt (Sample B)	ΔΔCt	Fold change = 2^-ΔΔCt
	Ct gene	Ct control	Ct gene	Ct control				
Normal	43.66	17.57	43.66	17.57	26.09	26.09	0.00	1.00
Control	30.12	25.79	43.66	17.57	4.34	26.09	-21.75	3535599.11
V. control	31.33	23.29	43.66	17.57	8.04	26.09	-18.05	270476.36
Standard	32.18	22.54	43.66	17.57	9.64	26.09	-16.45	89524.81
PMSLD	28.08	18.57	43.66	17.57	9.51	26.09	-16.58	97762.77
PMSHD	37.67	19.77	43.66	17.57	17.9	26.09	-8.19	292.42

Table 15. Ct and Fold change values obtained for *Pin1* of animal groups through qRT-PCR

Animal Groups	Sample A (Treated groups)		Sample B (Normal group)		ΔCt (Sample A)	ΔCt (Sample B)	ΔΔCt	Fold change = 2^-ΔΔCt
	Ct gene	Ct control	Ct gene	Ct control				
Normal	44.66	17.57	44.66	17.57	27.09	27.09	0.00	1.00

| Control	34.26	25.79	44.66	17.57	8.47	27.09	-18.7	402749.5
V. control	33.19	23.29	44.66	17.57	9.9	27.09	-17.2	149798.86
Standard	43.05	22.54	44.66	17.57	20.51	27.09	-6.58	95.51
PMSLD	35.63	18.57	44.66	17.57	17.06	27.09	-10.03	1048.79
PMSHD	40.27	19.77	44.66	17.57	20.5	27.09	-6.6	96.65

Table 16. Ct and Fold change values obtained for *c-Myc* of animal groups through qRT-PCR

Animal Groups	Sample A (Treated groups)		Sample B (Normal group)		ΔCt (Sample A)	ΔCt (Sample B)	ΔΔCt	Fold change = $2^{-\Delta\Delta Ct}$
	Ct gene	Ct control	Ct gene	Ct control				
Normal	28.32	17.57	28.32	17.57	10.75	10.75	0.00	1.00
Control	27.89	25.79	28.32	17.57	2.10	10.75	-8.65	401.06
V. control	25.32	23.29	28.32	17.57	2.03	10.75	-8.72	421.34
Standard	34.85	22.54	28.32	17.57	12.31	10.75	1.56	0.34
PMSLD	29.34	18.57	28.32	17.57	10.77	10.75	0.02	0.99
PMSHD	38.54	19.77	28.32	17.57	8.77	10.75	-1.98	3.95

The relative expression of the genes among different treated groups and normal group is shown in the Figures 7-10. Fold change value for normal group was 1 with which the fold change in the gene expression of all other groups was calculated. The fold change was increased in the expression of all the four genes for control and vehicle control group compared with that of normal. The fold change values for PMSLD and PMSHD were very less compared to that of the control group and almost similar to that
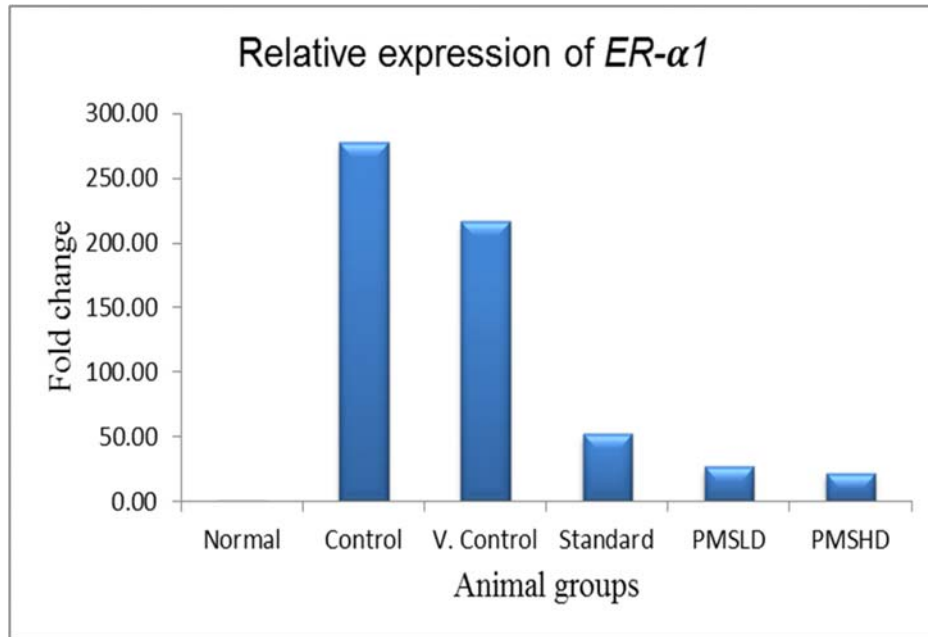
Figure 7. Relative expression of *ER- α1*
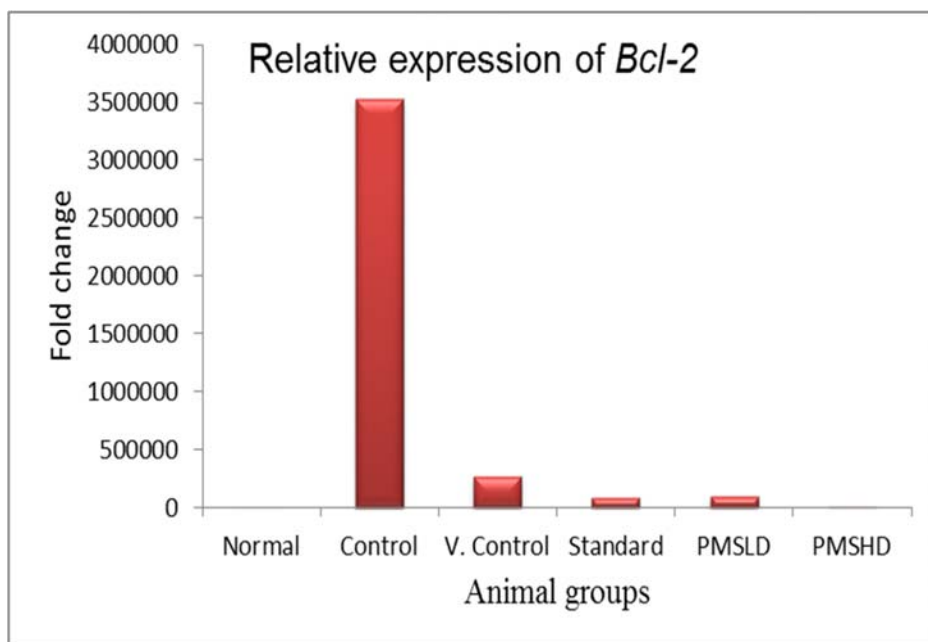
Figure 8. Relative expression of *Bcl-2*
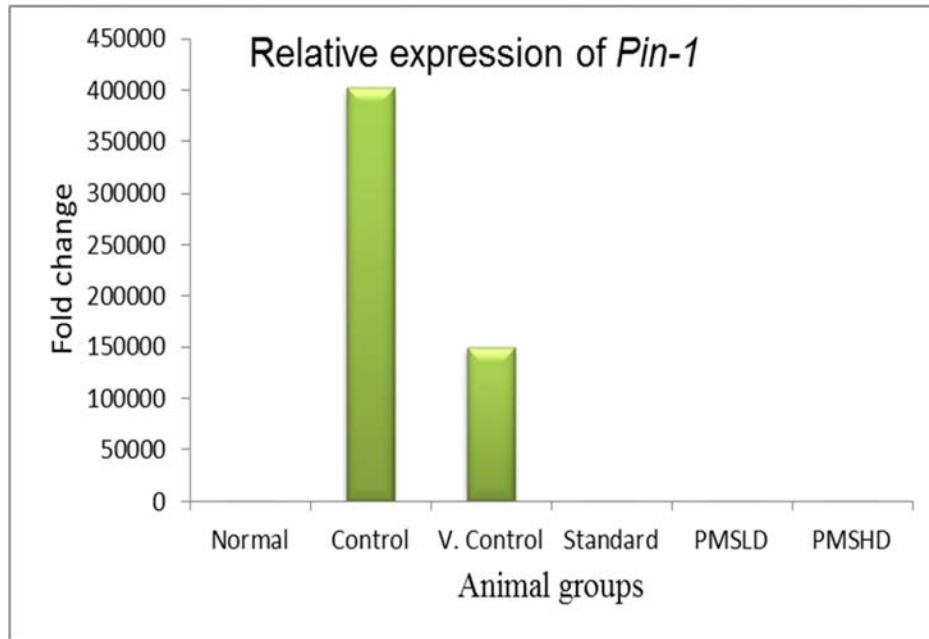
Figure 9. Relative expression of *Pin-1*
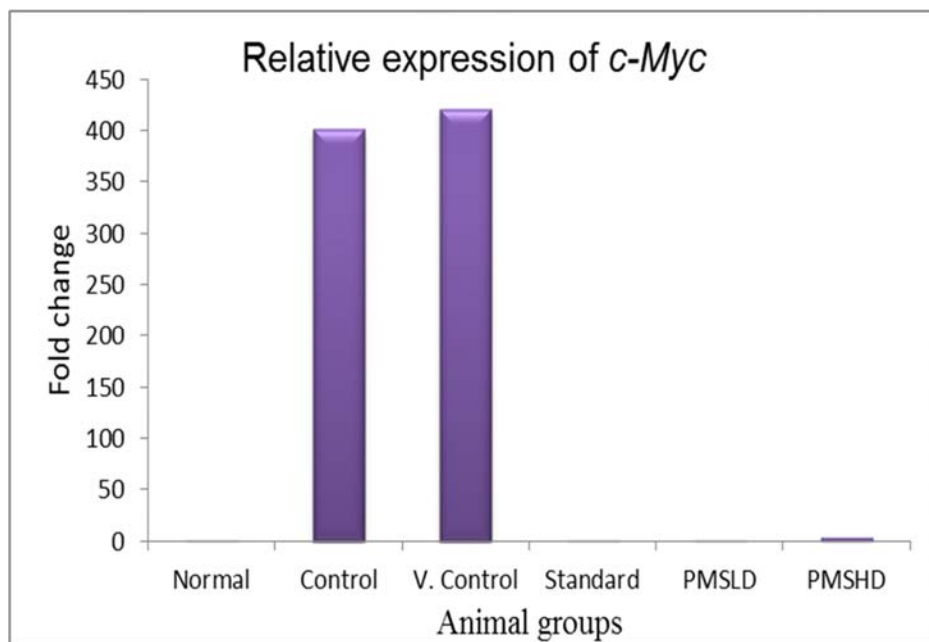
Figure 10. Relative expression of *c-Myc*

of standard. For control group, the fold change in the expression of *ER-α1* was 276.75 while, it was 26.61 for PMSLD and 21.69 for PMSHD. The fold change values for *Bcl-2* gene expression were very high, 3535599.11 for control, 97762.77 for PMSLD and 292.42 for PMSHD. High values of fold change were found in the expression of *Pin1* gene also, 402749.5 for control, 1048.79 for PMSLD and 96.65 for PMSHD. The fold change values for the expression of *c-Myc* were 401.06 for control, 0.99 for PMSLD and 3.95 for PMSHD.

From the results obtained from anti-proliferative analysis through MTT assay pheniramine maleate salt was selected and used for further validation using breast cancer tumour model. Significant difference was there in some of the parameters of haematogram, liver and renal function tests in standard, PMSLD and PMSHD groups in comparison with that of control group. Histopathology analysis of mammary pads and liver showed that there was reduction in tumour size and cytoplasmic degeneration in PMSHD groups. Gene expression analysis also showed significant difference in the expression level of the genes *ER-α1, Bcl-2, Pin 1* and *c-Myc* among the treatment groups.

DISCUSSION

5. DISCUSSION

Cancer is one of the deadliest diseases prevailing in the world. Each year, about 10 million people die of cancer. Cancer mainly arises due to the mutations that occur in the regulatory genes called oncogenes and tumour suppressor genes, which control the cell proliferation, differentiation and cell survival. Activation of proto-oncogenes to oncogenes and inactivation of tumour suppressor genes lead to cancer cell proliferation. At least one third of the cancers that occur commonly among the people can be curable with proper diagnosis and monitoring. Various strategies for cancer treatment include chemotherapy, surgical removal of tumour, radiation therapy, immunotherapy and bone marrow transplantation. Due to various side effects posed by these treatments, scientists are turning their attention towards phytocompounds that have therapeutic potential against cancer. Few plant-derived cancer drugs are already available in market (eg. Paclitaxel from *Taxus brevifolia* Nutt.) whereas various phytochemicals such as Allicin from *Allium sativum,* Andrographolide from *Andrographis paniculata,* Baicalin from *Scutellaria baicalensis*, Curcumin from *Curcuma longa*, Genistein from *Glycine max,* Nimbolide from *Azadirachta indica,* Resveratrol from *Polygonum cuspidatum,* Thymol from *Thymus vulgaris,* Withaferin-A from *Withania somnifera*, are reported to possess anti-cancer properties and are in pre-clinical trials for the treatment of cancer (Choudhari *et al.,* 2020). Many more phytocompounds are to be explored for their potential as anti-cancer drugs.

One of the efficient strategies for initial screening of anticancer potential of phytocompounds is molecular docking of these compounds with the proteins that are specific for the development of cancer (Mathew *et al.,* 2017). Through molecular docking, Bhamare (2019) has identified seven phytocompounds *viz.* α-aminodiphenylacetic acid, Doxylamine, Histidinol, Flucoxetine, Pheniramine, Valylmethionine and Prometon, which interacted with various cancer targets. In this study, the phytocompounds Doxylamine, Histidinol and Pheniramine were tested for their anticancer potential through *in vitro*, *in vivo* and gene expression. The one which performed better in the *in vitro* studies was carried forward for further validation in mouse tumour model and gene expression studies using qRT-PCR.

5.1 Cytotoxicity analysis of phytocompounds on cancer cell lines *in vitro*

5.1.1 Short-term cytotoxicity analysis by Trypan blue exclusion assay

Trypan blue exclusion assay is one of the earliest and widely used methods for measuring the viability of the cells. Living cells possess intact cell membrane while dead cells will not. The intact cell membrane excludes the trypan blue dye, which cannot be done by the dead cells. Hence, they appear blue in colour and the live cells appear white. After treating the cells with the drugs, percentage of dead cells can be calculated by counting the number the live and dead cells, using a hemocytometer under the microscope, and thus the cytotoxic effect of the drug on the cells can be measured.

Three phytocompounds in its chemical form, *viz.* Doxylamine succinate salt, L-Histidinol dihydrochloride and Pheniramine maleate salt were tested for their cytotoxicity against the murine cancer cells; Daltons Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) using Trypan blue exclusion assay. In cases of DLA and EAC, the number of dead cells was very low when treated with the drugs even at the highest concentration of 400 μg/ml. Only pheniramine maleate salt showed some cytotoxicity of 15 % in DLA cells and 10 % in EAC cells.

In a short-term cytotoxicity study conducted by Bellamakondi *et al.* (2014), the methanolic extracts of *Caralluma* species were tested against EAC (Ehrlich Ascites Carcinoma) and the CTC_{50} value ranged between 191.3 ± 0.92 and 291.8 ± 3.17 μg/ml. Inhibition of 50 % of the total cells is required to confirm the cytotoxicity action of the drug. Since 50 % inhibition was not there in any cases, it was concluded that all the three drugs did not show significant cytotoxicity against murine cancer cells.

5.1.2 Anti-proliferative analysis by MTT assay

MTT assay is a colorimetric assay which is based on the conversion of yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a purple formazon. While MTT is water soluble, formazon is insoluble and hence dimethyl sulfoxide (DMSO) is used to solubilize the formazon, the concentration of which can be determined spectrophotometrically by measuring the absorbance at 570 nm. The conversion of MTT to formazon is mainly due to the action of the mitochondrial enzyme

which is characteristic of live cells. Hence the absorbance value of the untreated cells would be high compared to that of the treated cells. For MTT assay, MCF-7 (breast cancer) and HCT-116 (colon cancer) cell lines were used. All the three drugs, PMS, DSS and LHD were tested on these cell lines at the concentrations of 40, 80, 120, 160, 200, 240 and 280 μg/ml for 24, 48 and 72 hours. The percentage of inhibition of the cancer cells induced by the drugs was calculated. A dose-dependent inhibition was observed with both the drugs on the cell lines. When the concentration was increased, the percentage of inhibition was also increased.

5.1.2.1 Inhibition of MCF-7 cells by the drugs

When MCF-7 has been incubated with PMS for 24 hours, the maximum inhibition was 38 ± 2.2 %, only at the concentration of 280 μg/ml . At 48 hours of incubation, the maximum inhibition was 71.21 ± 2.5 % at 280 μg/ml concentration with IC_{50} at 108 μg/ml. More than 90 % inhibition was seen in all the concentrations of PMS at 72 hours with maximum 98.46 ± 3.6 % inhibition at 280 μg/ml concentration. Because of this high inhibition percentage, the IC_{50} value was 14 μg/ml.

At 24 hours of incubation of MCF-7 cells with DSS, maximum inhibition was 38.22 ± 1.5 % which was almost the same with the inhibition caused by PMS at same time of incubation. At 48 hours of incubation, maximum inhibition obtained was 58.23 ± 2.1 % and the IC_{50} value was 252 μg/ml. For 72 hours incubation, the maximum inhibition was 80.49 ± 3.4 % with IC_{50} 61 μg/ml.

LHD couldn't impose any effect in the proliferation of the breast cancer cells. The maximum percentage of inhibition even at 72 hours of incubation at 280 μg/ml was 28.82 ± 1.5.

Two way ANOVA followed by Dunnetts test was done to compare the percentage of inhibition by various concentrations of the drugs which could cause 50 % inhibition in the proliferation of the MCF-7 cells, after 24 hours incubation, with that of 48 and 72 hours of inhibition. For PMS, a significant increase ($p < 0.001$) was found in all the concentrations in both 48 and 72 hours of incubation. DSS also had significant increase

(p<0.001) in all the values except for 40 µg/ml concentration (48 hours), which had significant increase at p<0.01.

Kumar *et al.* (2014) tested the anticancer potential of *Syzygium aromaticum* L. in MCF-7 breast cancer cell lines. They tested both the ethanolic extract and essential oil of cloves. The IC_{50} value of ethanolic extract were 61.29 µg/ml for 24 hours incubation and 16.71 µg/mL for 48 hours incubation. The essential oil achieved IC_{50} at 36.43 µg/ml in 24 hours and 17.6 µg/ml in 48 hours. Lower IC_{50} value indicated higher effect. Hence, essential oil has high potential as anti-cancer agent.

Compared to the above work, the concentration required for both PMS and DSS to achieve 50 % inhibition at 48 hours of inhibition was high (108 and 252 µg/ml, respectively) but with a higher incubation period of 72 hours, IC_{50} values were only 14 µg/ml for PMS and 21 µg/ml for DSS. Among the PMS and DSS lines, PMS performed better as it had lower IC_{50} compared to that of DSS. Hence, for mammary tumour model, PMS was selected.

5.1.2.2 Inhibition of HCT-116 cells by the drugs

The maximum inhibition achieved by PMS on the proliferation of HCT-116 cell line was 46.62 ± 3 % at 24 hours, 67.16 ± 2.7 % at 48 hours and 70.34 ± 2.4 % at 72 hours of incubation with IC_{50} values of 211 µg/ml and 164 µg/ml for 48 and 72 hours incubation, respectively.

DSS inhibited the maximum of 31.03 ± 2.1, 60.59 ± 3.2 and 63.28 ± 3.5 % of colorectal cancer cells when incubated for 24, 48 and 72 hours, respectively and IC_{50} values were achieved at 195 µg/ml for 48 hours and 162 µg/ml for 72 hours. LHD didn't show significant effect in the HCT-116 cells proliferation, the maximum inhibition being 17.34 ± 1.7 % at 280 µg/ml after 72 hours incubation.

Two way ANOVA test was done to compare the per cent inhibition after 24, 48 and 72 hours of incubation for PMS and DSS. For PMS, significant increase at 80 µg/mL for 48 hours (p<0.01) and at 160, 200, 240 and 280 µg/ml for 48 hours and at 40, 80, 160, 200, 240 and 280 µg/ml for 72 hours (p<0.001), were observed. DSS had significant increase

(p<0.01) in all the concentrations except at 80 μg/ml for 48 hours, but 40 μg/ml for 48 hours was not significant.

Grbovic *et al.* (2013) studied the cytotoxic activity of methanolic extract of *Origanum vulgare* L. on HCT-116 cell lines through MTT assay. They had treated the colorectal cancer cell line with the extract for 24 and 72 hours and recorded the effect on cell proliferation. They found a dose-dependent inhibition of the proliferation, with IC_{50} values 140.77 ± 2.13 μg/ml for 24 hours and 109.51 ± 1.28 μg/ml for 72 hours.

5.2 Acute toxicity studies

Swiss albino mice were selected for the animal model study as the development of tumour and its metastasis was similar to that of human. Acute toxicity study was done to find whether the selected drug had any toxicity for the animals. In order to study the anti-histiminic effect of *Bauhinia racemosa* leaves, Nirmal *et al.* (2011) had used 10 mg/ kg b. wt. of pheniramine maleate as a standard in male albino mice. Hence, a maximum quantity of 100 mg/ kg b. wt. of the drug, PMS was given once to the animals and monitored for 14 days for the changes in the behavioural and general appearance. No significant difference was found between the normal and treated groups, indicating that the drug did not have any toxicity effect on the animals at 100 mg/ kg b. wt. concentration. Hence $1/10^{th}$ of the value i.e. 10 mg/ kg b. wt. was used for low dose and $1/5^{th}$ of the value, i.e. 20 mg/ kg b. wt. was used for high dose.

5.3 Anticancer efficacy analysis of phytocompounds on *in vivo* mouse tumour model

Animal models are used to assess the efficiency of the potential agents having chemotherapeutic property. Based on the observations obtained from the models, they will be qualified for clinical trials. One of the reasons for using mouse models is the similarity of mouse genome with that of humans. Other factors such as low cost of maintenance, rapid growth and high fecundity and short gestation period make the mouse models preferable. The animals are grouped, generally with six animals per group and fed with the drug candidates as per the requirements of the study.

In this study, there were six groups of Swiss albino mice, each having six animals. The carcinogen used for inducing breast tumour in the mice was dimethylbenz[a]anthracene.

The Normal group received standard food without drugs or carcinogen. Control group was fed with the carcinogen DMBA only. Vehicle control group was given sesame oil and DMBA. Standard group received DMBA and the drug, tamoxifen. Low dose group received DMBA along with PMS (10 mg/ kg b. wt.), while high dose group was fed with DMBA and PMS (20 mg/ kg b. wt.). At the end of the experiment, all the animals were sacrificed. Three animals from each group were used for the collection of blood to analyse the haematological parameters and three animals for collecting serum for liver function and renal function tests.

Haematological components included haemoglobin, total RBC count, platelet count, total WBC count, neutrophils, lymphocytes and eosinophils. A reduction in the RBC count implicated that there is reduction in the transport of oxygen and carbon dioxide. White blood cells are involved in defending the body from the foreign agents. Reduction in WBC count implied the destruction of immune system and the animal becomes highly prone to infection. An increase in WBC count suggested enhanced production of antibodies and better resistance to infections. Platelets are involved in blood clotting and a decrease in their concentration suggests a prolonged process of blood clotting and excess blood loss (Etim *et al.,* 2014).

Liver function test is used for the detection of damage and inflammation of the liver by analysing the liver enzymes such as serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase. High levels of these enzymes suggested liver damage. Other biochemical parameters included total protein, albumin, globulin and bilirubin. Higher values of bilibrubin, albumin and globulin indicated liver or bile duct problems.

Renal function test included urea and creatinine, levels of which reflected the glomerular filtration rate (GFR). Renal failure is often associated with elevated urea and creatinine.

Two way ANOVA was performed for the test results, with reference to the control group. A significant reduction in the WBC count was found in the standard ($p < 0.001$), PMS low dose ($p < 0.01$) and PMS high dose ($p < 0.01$) groups with values of 6800±230 cells/cu mm, 8300±510 cells/cu mm and 6300±550 cells/ cu mm, respectively. In a study conducted by Zingue *et al.* (2018), there was a significant increase ($p < 0.001$) in white blood count of

the rats treated with DMBA than that of normal ones. Tamoxifen treated animals exhibited a significant decrease ($p < 0.05$) in the WBC count compared with that of normal and DMBA-treated animals. It implicated that WBC count gets increased when there is a tumour growth or inflammation. Hence, the reduction in the WBC count in the PMS treated groups is promising. Other parameters *viz.* haemoglobin, platelet count, total RBC count, neutrophils, lymphocytes and eosinophils were insignificant. In liver function test, there was a significant reduction in SGOT (Serum glutamic-oxaloacetic transaminase) level ($p < 0.01$ for standard and PMS low dose), SGPT (Serum glutamic pyruvic transaminase) level ($p < 0.001$ for standard, PMS low dose and PMS high dose) and ALP (Alkaline phosphatase) level ($p < 0.001$ for standard, PMS low dose and PMS high dose). Higher levels of SGPT, SGOT and ALP indicated liver cell injury. Since these parameters were lower in the animal groups treated with the drug candidates, on par with the standard, the drug candidates are shown to have effect on the tumour induced animals.

5.3.1 Histopathology of liver and mammary pad

Histopathology, a routine method in cancer diagnosis, provides an insight into the architecture of the tissues in which the disease is manifested. Comparison of the tissue slides of mice among different groups helps in inferring the effects of the drug. In this study, histopathological examinations of mammary pad, liver and kidney were carried out.

Mammary duct, stromal tissue and adipose tissues were seen in the histomorphology of the mammary tissues of the mice. A carcinomatous growth was observed in the stroma region of control and PMS low dose groups while the size of tumour in the PMS high dose group was comparatively lower. PMS high dose group also exhibited reduced initiation and development of tumour, compared to the control group.

Histo-architecture of the hepatic tissues of the normal group was normal. There were aggregations of inflammatory cells and cytoplasmic degeneration in the control group and PMS low dose group. But the cytoplasmic degeneration visualized was lower in PMS high dose group. Hepatocellular injury was seen in standard group.

Typical architecture of kidney with glomerulus, and proximal and distal tubules was seen in the histological sections of the kidney of normal group. No significant change in the renal architecture was observed in control and treated groups.

Histopathological analysis of the mammary pad and liver tissues between normal and DMBA treated animal groups indicated that DMBA altered the normal architecture of the tissues. Treatment with PMS at high dose produced significant changes in the tissue morphology of the DMBA treated animals.

5.5 Molecular pathway analysis

Mutations, the major driving factor of cancer, alter the activity of hallmark pathways thereby unlocking the oncogenic properties of the normal cells. The cancer genes are also reported to be involved in many cellular pathways (Reyna *et al.,* 2020). Hence looking into the expression of the genes provides insights into the pathways and the networks interrupted in the cancer and accordingly drugs that target the pathways can be developed.

Expression of the genes, *ER-α1, Bcl-2, c-Myc* and *Pin 1* was analyzed through quantitative real-time polymerase chain reaction (qRT-PCR) with reference to the house keeping gene, *β*-actin. Owing to its high accuracy, sensitivity and ease in use, qRT-PCR has become an important tool in cancer research (Mocellin *et al.,* 2003). All the genes studied in the research are oncogenes and also reported for profound roles in the breast cancer. The *ER-α* (Estrogen receptor-*α*) signaling plays a major role in the breast cancer proliferation. It promotes the expression of other oncogenic proteins like Cyclin-D and c-Myc and inhibits the expression of cell cycle inhibitors like P21 (Xue *et al.,* 2019). The standard drug tamoxifen is a modulator of *ER- α*. The *Bcl-2* is an antiapoptotic gene and prognostic marker in breast cancer. It is found in chromosome 18 and transfer of this gene to other chromosomes is shown to result in the larger expression of this protein thereby the cancer cells evade apoptosis. The *c-Myc* codes for a pleiotropic transcription factor, upregulated by *ER- α*. The *Pin 1* codes for a protein which is in correlation with cyclin D1 levels. Overexpression of *Pin 1* activates the promoter of cyclin D1, another oncogene of breast cancer.

Expression of the genes of each treatment group *viz.* control, vehicle control, standard, PMSLD and PMSHD, was compared with that of normal group. The qRT-PCR data was analysed using comparative Ct method (Schmittgen and Livak, 2008). In all the cases of genes, the expression level was increased in control and vehicle control groups which indicates the higher expression of oncogenes in the tumour cells. PMSLD and PMSHD groups exhibited relatively decreased expression of the oncogenes. These results indicated that the drug PMS could reduce the expression of the oncogenes *ER-α1, Bcl-2, c-Myc* and *Pin 1* in the tumour induced mice. In a study conducted by Karimi *et al.* (2020) a reduced *c-Myc* expression was observed in simvastatin treated group compared with that of control receiving only DMBA. Similar result was found in this case also. The amplification plot and melt curve plots of the genes are shown in Figures 11 and 12.

Thus, the present study highlights the efficacy of the phytocompound, Pheniramine as a therapeutic drug against breast cancer. The anti-proliferative analysis through MTT assay, histopathology of the liver and mammary pad of the animals treated with carcinogen and drugs and gene expression study showed that Pheniramine maleate salt could be used as a therapeutic drug for breast cancer.
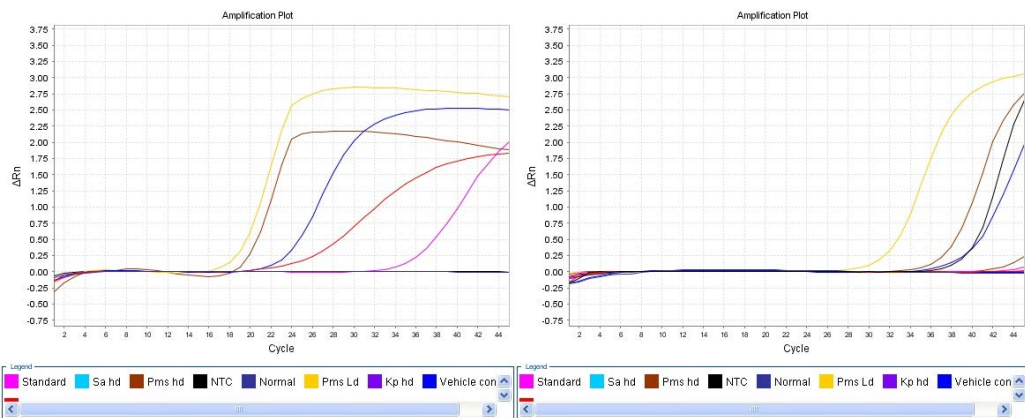
Amplification plot of *β-actin*
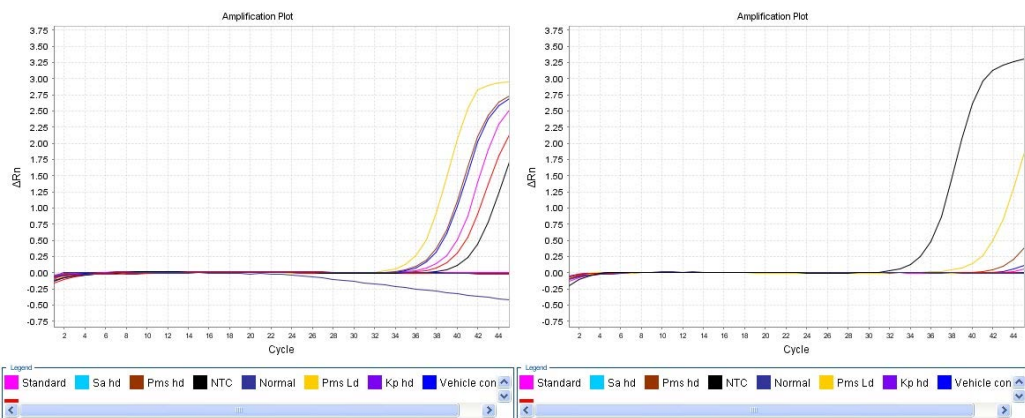
Amplification plot of *Bcl-2*
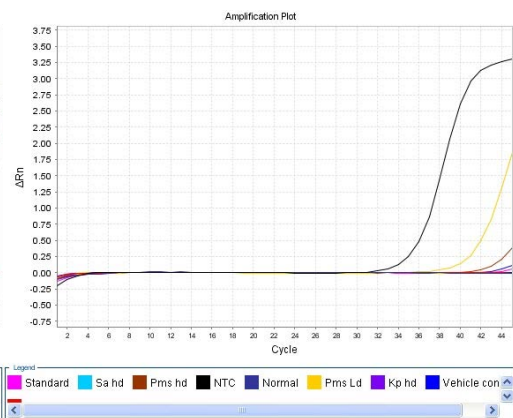
Amplification plot of *ER-α1*
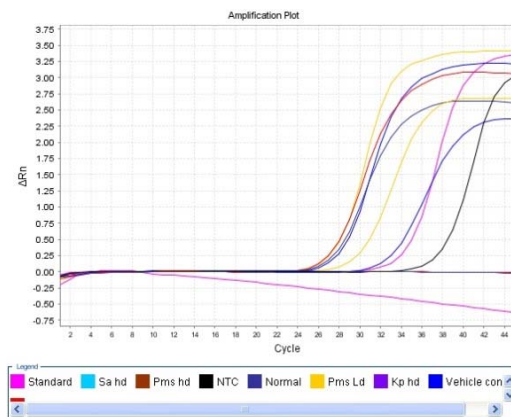
Amplification plot of *Pin1*

Amplification plot of *c-Myc*

Figure 11. Amplification plot of the genes obtained through qRT-PCR
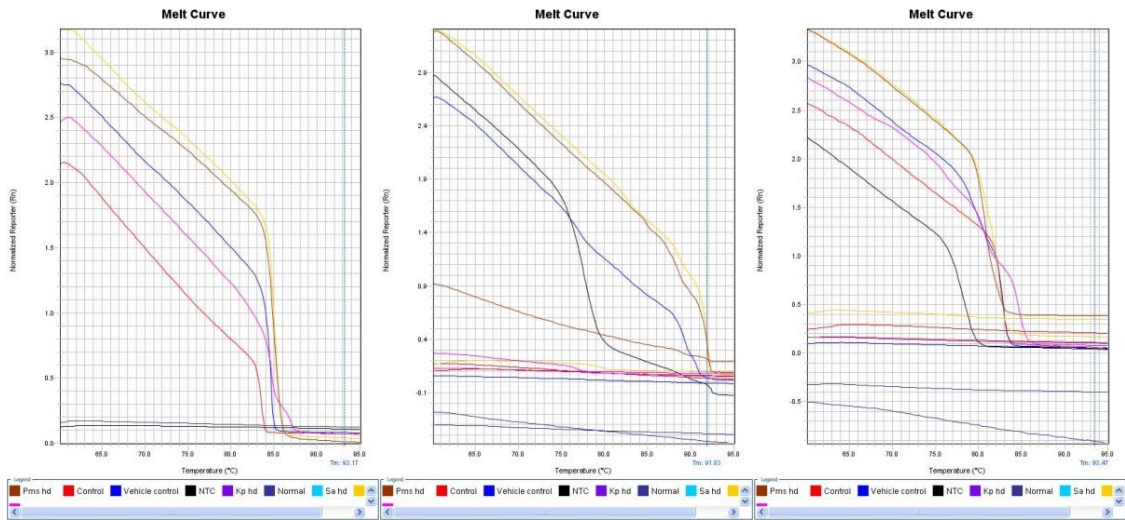
Melt curve plot of *β-actin*   Melt curve plot of *Bcl-2*   Melt curve plot of *ER-α1*
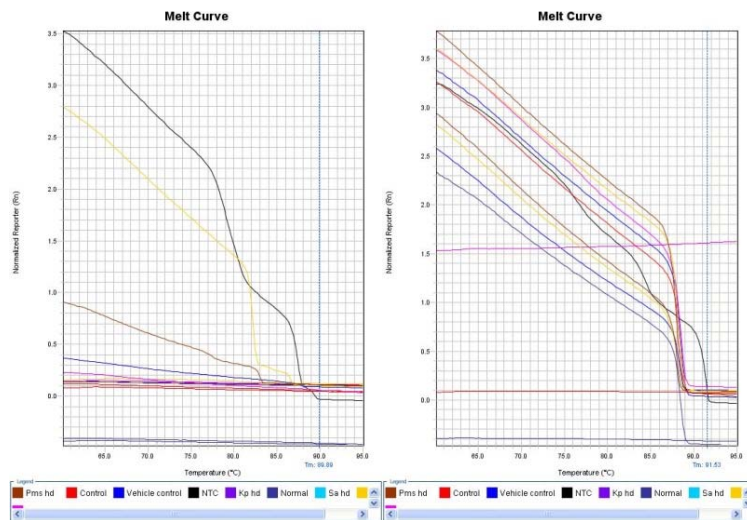
Melt curve plot of *Pin1*   Melt curve plot of *c-Myc*

Figure 12. Melt curve plot of the genes obtained through qRT-PCR
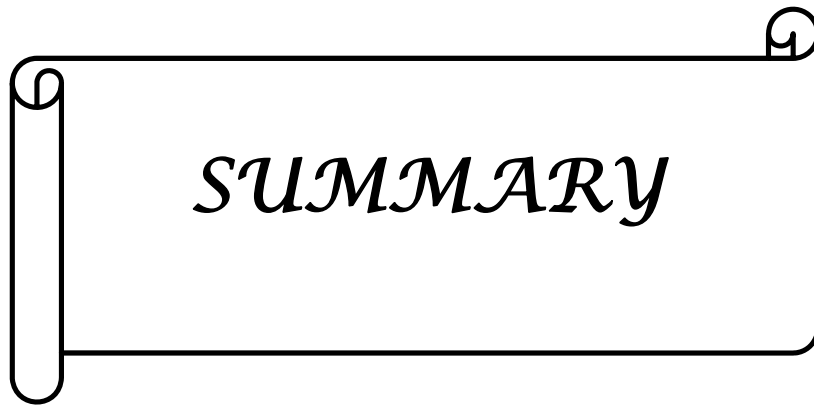
SUMMARY

6. SUMMARY

The study entitled 'Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya koenigii* L.)' was carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agricultural University and Amala Cancer Research Centre, Thrissur, during 2019-2021. The objective of the study was to evaluate the anticancer potential of selected curry leaf (*Murraya koenigii)* compounds by *in vitro* and *in vivo* studies using different cancer cell lines and mouse tumour models.

The salient findings of the study are summarised in this chapter.

Cytotoxicity analysis of phytocompounds on cancer cell lines *in vitro*

❖   In short-term cytotoxicity analysis by trypan blue exclusion assay, cytotoxicity caused on DLA and EAC cells were only 15 and 10 %, respectively by the Pheniramine maleate salt. Other two drugs, Doxylamine succinate salt and L-histidinol dihydrochloride didn't show significant cytotoxicity.

❖   MTT assay conducted for analysing the anti-proliferative efficiency of the phytocompounds showed that PMS had IC_{50} values at 108 μg/ml at 48 hours incubation and 14 μg/ml at 72 hours incubation on MCF-7 breast cancer cell line and at 252 μg/ml on HCT-116 colorectal cancer cell line at 48 hours incubation and 164 μg/ml at 72 hours incubation.

❖   DSS caused 50 % inhibition of MCF-7 cell lines at 252 μg/ml at 48 hours incubation and 61 μg/ml at 72 hours incubation. For HCT-116 cells, it caused 50 % inhibition at 195 μg/ml concentration when incubated for 48 hours and 162 μg/ml for 72 hours

❖   LHD couldn't produce significant effect in the proliferation of the cell lines. The maximum percentage of inhibition caused by LHD was 28.82 ± 1.5 on MCF-7 cells and 17.34 ± 1.7 on HCT-116 cells at the concentration of 280 μg/ml for 72 hours incubation

Acute toxicity study

❖ Based on the cytotoxicity results, PMS was selected for further validation. The acute toxicity study for PMS conducted on female Swiss albino mice suggested that 100 mg/ kg b. wt. of PMS couldn't produce any toxicity on the animals and they remained similar to the normal counterparts. Therefore, for low dose, $1/10^{th}$ of the value (10 mg/ kg b. wt.) and for high dose of $1/5^{th}$ of the value (20 mg/ kg b. wt.) were used.

Anti-cancer efficacy analysis on *in vivo* mouse tumour model

❖ Histopathalogical analyses carried out in the mammary pad, liver and kidney tissues of the mice of different groups *viz.,* normal, control, vehicle control, standard, PMSLD and PMSHD showed that PMSHD could bring significant change in the architecture of mammary pad and liver of the mice induced with mammary tumour using DMBA.

❖ Haematological tests conducted on the blood of the mice groups showed significant reduction in the White Blood Cells count of standard, PMSLD and PMSHD groups, in comparison with that of control group. Other components were found to be non-significant.

❖ Liver function test and renal function test carried out on mice serum showed a significant reduction in the SGOT level of standard and PMSLD groups, SGPT level of standard, PMSLD and PMSHD groups and ALP level of standard, PMSLD and PMSHD groups, compared with that of control group. There were no significant differences in the urea and creatinine levels

Gene expression study

❖ RNA was isolated from the breast tissue of all the mice and cDNA was prepared from it. The qRT-PCR analysis carried out on the cDNA for quantifying the expression of the genes, *ER-α1, Bcl-2, Pin 1* and *c-Myc* with reference to the house keeping gene *β-actin*, indicated a reduction in the expression of all the above mentioned genes with respect to the DMBA treated control group.

- ❖ Results of *in vitro, in vivo* and gene expression studies indicated that Pheniramine maleate salt, at high dose (20 mg/ kg b. wt.), possess anti-cancer properties against breast cancer.

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7. REFERENCES

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ANNEXURES

ANNEXURE I

List of chemicals used for the *in vitro* analyses

Sl. No.	Catalog number	Items	Company
1	RM10432-500ML	Fetal bovine serum Brazil Origin	HiMedia, India
2	TCL021-100ML	HEPES 1M	
3	TCL015-100ML	Sodium pyruvate solution 100mM	
4	ACL005-100ML	MEM Non-essential amino acids solution 50X	
5	TCL048-500ML	Trypsin EDTA solution 1X	
6	D3775-5 G	Doxylamine succinate salt	Sigma Aldrich, India
7	H6647-10 MG	L-histidinol dihydrochloride	
8	P6902-5G	Pheniramine maleate salt	
9	12100046	DMEM Powder - 10X1 L	ThermoFisher Scientific, India
10	15250061	Tryphan blue solution, 0.4 % 100 ml	
11	142475	24 well plates - case of 75	
12	546021	Tarsons tubes (15ml)	Tarsons, India
13	52682 (204844)	Trypsin ex. Bovine pancreas 0.2Anson U/g (2000U/g)	Sisco Research Laboratories Pvt. Ltd., India
14	66120	Dimethyl Sulphoxide (DMSO)	

15	58945	Thiazolyl blue Tetrazolium bromide (MTT) for tissue culture 98%	

ANNEXURE II

Composition of Dulbecco's Modified Eagle medium

Components	Quantity	Concentration
Fetal Bovine Serum	100 ml	10%
HEPES Solution	10 ml	10 mM
Sodium pyruvate	10 ml	1 mM
Glucose powder	3.5 g	25 mM
Non-essential amino acids	10 ml	
Sodium bicarbonate	1 g	

ABSTRACT

WET-LAB VALIDATION OF *IN SILICO* PREDICTED ANTICANCER POTENTIAL OF SELECTED COMPOUNDS OF CURRY LEAF (*Murraya koenigii. L.*)

By

MULLAI V. R.
(2019-11-003)

ABSTRACT OF THE THESIS

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Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR – 680 656

KERALA, INDIA

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ABSTRACT

Cancer is one among the most prevailing human diseases and nearly 0.1 billion people in the world are living with different types of cancers. Four most common cancers are lung, breast, bowel and prostate cancers. Chemotherapy, radiation therapy, surgery, bone marrow transplantation and immunotherapy are the major treatment strategies for cancer. Severe side effects of chemotherapy including fatigue, hair loss, nausea and bleeding, demand the finding of plant based molecules for cancer treatment. Several phytocompounds are identified to have therapeutic potential against multiple human diseases. Molecular docking of these ligand compounds with the target proteins is an efficient strategy for the initial large-scale screening to reveal the drug potential of the phytocompounds. Molecular docking studies on the antioxidant fractions identified through LC-MS/MS on curry leaf extracts have revealed their anticancer properties.

The drug potential of the molecules identified through *in silico* analysis has to be confirmed through subsequent steps such as *in vitro* analyses on cancer cell lines and *in vivo* analyses in mouse tumour models. Hence, the study entitled 'Wet-lab validation of *in silico* predicted anticancer potential of selected compounds of Curry leaf (*Murraya koenigii* L.)' was undertaken at the Department of Plant Biotechnology, CoA, Thrissur, during 2019 to 2022, with the objective of evaluating the anticancer potential of selected curry leaf (*Murraya koenigii)* compounds by *in vitro* and *in vivo* studies using different cancer cell lines and mouse tumour models.

Three compounds *viz.* Doxylamine succinate salt, L-histidinol dihydrochloride and Pheniramine maleate salt were tested *in vitro* on murine cancer cell lines EAC and DLA and human cancer cell lines, MCF-7 (breast cancer) and HCT-116 (colorectal cancer). The compound which performed better was carried forward for *in vivo* study in mouse mammary tumour model. DMBA was used to induce cancer in Swiss albino mice. There were six animal groups such as normal, control, vehicle control, standard, drug-low dose and drug-high dose and each group had six mice. After six weeks, all the animals were sacrificed, blood and serum collected for blood, liver function and renal function tests and tissues of mammary pad, liver and kidney collected for histopathology analysis. From the mammary pad tissue, RNA was isolated and used for cDNA isolation. The

qRT-PCR analysis was done using the cDNA to study the expression of *ER-α*1, *Bcl-2, c-Myc* and *Pin 1* genes, with reference to the house-keeping gene $\beta - actin$.

Trypan blue assay conducted on murine cell lines has exhibited no significant cytotoxicity from the drugs. MTT assay on the cell lines MCF-7 and HCT-116 grown in DMEM showed that PMS efficiently inhibited the proliferation of MCF-7 cells. Hence, PMS was selected for further validation in mouse breast tumour model. The histopathology analysis of the mammary pad, liver and kidney of the mice showed that PMSHD group showed better histo-architecture than that of control group. The results of the blood, liver and renal function tests, analyzed by two way ANOVA, showed a significant reduction in the WBC count (standard, PMSLD and PMSHD groups), SGOT level (standard and PMSLD), SGPT level (standard, PMSLD and PMSHD) and ALT level (standard, PMSLD and PMSHD), in comparison with control group. The qRT-PCR results indicated a reduced expression in all the genes in the drug treated mice compared to that of control.

Overall results obtained from the *in vitro, in vivo* and gene expression studies indicated that Pheniramine maleate salt could be the potential candidate for anti-cancer drug. Other biological properties of Pheniramine maleate salt such as anti-inflammatory and anti-oxidant properties may be further validated in the same way. The molecular mechanisms involved in the downstream signaling pathways may also be explored in the future.