Antiproliferative potential of bioactive molecule from *Murraya koenigii* L. Spreng against breast cancer: *In vitro* and *in vivo* studies and gene expression analyses

Mullai Valli Ramamoorthy¹, Bhamare Deepak Prashant¹, Deepu Mathew¹,²*†, Babu Thekkekara Devassy³, Shylaja Muthangaparambil Raman¹, Pareeth Chennattu Muhammed³ & Smita Nair¹

¹Centre for Plant Biotechnology and Molecular Biology; ²Bioinformatics Centre, Kerala Agricultural University, Thrissur - 680 656, Kerala, India
³Department of Biochemistry, Amala Cancer Research Centre, Thrissur - 680 555, Kerala, India

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The curry leaf extract is known to have anticancer property against breast cancer. Identification of the specific compound therein the curry leaf and its validation is essential for successful discovery of drugs. In that content, here, we extracted oleoresin from the mature curry leaves was subjected to antioxidant fractionation using column chromatography. Fraction obtained using 60:40 hexane and ethyl acetate solvent system, showing the maximum inhibition of DPPH, was sub-fractionated and those with the highest antioxidant property was analyzed in LC-MS/MS. Spectrum of molecules identified, along with FDA approved drugs, were docked with target proteins for breast cancer. *In vitro* screening of candidate phytocompounds doxylamine, histidinol and pheniramine, in their commercially available form, through Trypan blue exclusion assay against murine cancer cell lines EAC and DLA had shown that they have no cytotoxicity. Pheniramine maleate salt (PMS), doxylamine succinate salt (DSS) and L-histidinol dihydrochloride (LHD) have shown dose-dependent inhibition of proliferation of MCF-7 cells, with 280 μg/mL PMS at 72 h of incubation giving the maximum of 98.46%. Acute toxicity studies in Swiss albino mice (100 mg PMS/kg body wt.) have confirmed that the drug has no toxicity. Mouse mammary pad tumour model has shown that PMS significantly reduces the WBC count in the tumour induced mice. Liver function tests, histopathological analyses of liver, mammary pad and kidney tissues and expression analysis of oncogenes ER-α₁, Bcl-2, c-Myc and Pin1 have confirmed the drug candidature of PMS.

**Keywords:** Antioxidant fraction, Drug design, Mammary tumour model, Molecular docking, Oncogene expression

Breast cancer is the cancer with the highest incidence globally, with 300,590 estimated new cases and 43,700 estimated deaths in 2023 in the United States. It accounts for 15.81% of total estimated new cases and 7.95% of total estimated cancer deaths, respectively¹,². In 2022, India recorded the estimated breast cancer incidences year of 2,21,757 in both the sexes³. The existing treatment strategies are 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. In the past few decades, there have been reports of resistance to synthesized drugs, resulting reduced efficacy and survival rates⁴⁻⁶. Hence, there is a demand for the identification of cancer drugs with no side effects.

Plants have been excellent sources for therapeutic molecules and many phytocompounds are being tested for their efficacy in treating various cancers. An efficient method of drug discovery, both in terms of cost and money, is *in silico* designing through homology modeling, molecular docking, virtual high-throughput screening, molecular dynamic simulation or microarray analysis⁷.
Curry leaf (Murraya koenigii L. Spreng., Rutaceae), is a medicinal plant rich in alkaloids, phenolics, flavonoids, saponins, proteins, sterols and triterpenes and used in the Ayurvedic preparations. Extensive use of curry leaf based preparations in these traditional medicines to cure different types of cancers has invited the scientific efforts to explore the basis of the anti-cancer potential of this plant. The active constituents of curry leaves target disorganized signaling pathways with profound roles in cancer proliferation 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, regulating cell growth, proliferation and apoptosis. Many similar studies followed have proven the treatment potential of curry leaf extracts against breast cancer, colon cancer, prostate cancer, cervical cancer and ovarian cancer.

Even with all these information, efforts on a systematic attempt to identify the cancer drug molecules from this potential plant is still on. In the present study, we have made an attempt to isolate the antioxidant oleoresin fraction and sub-fractions from curry leaves, identify potential breast cancer drug candidate through chromatographic, spectrometric techniques and in silico analyses, to assess the toxicity and antiproliferative potential of drug candidates using in vitro cell line studies and to confirm the candidature through induced mouse tumour models and candidate gene expression analyses.

**Materials and Methods**

**Oleoresin extraction**

The medium mature and mature leaves from the in vitro raised curry leaf plantlets (cv. Suvasini) were used in the study. Oleoresin was extracted from the shade dried and powdered leaves, using Soxhlet apparatus. Ten gram of curry leaf powder was weighed, packed in a coarse filter paper and placed in the extraction chamber of the apparatus. Extraction was carried out using 100% acetone for 9 h till the solvent becomes colourless. After extraction, the extract was transferred to a pre-weighed beaker, kept open for evaporation to remove the traces of acetone, and recorded the final weight of the beaker. Oleoresin recovery (%) was calculated using the following formula:

\[
\text{Oleoresin recovery} \, (\%) = \frac{\text{Weight of oleoresin}}{\text{Weight of curry leaf powder}} \times 100
\]

**Antioxidant assay**

Potential of the oleoresin in scavenging the reactive oxygen species (ROS) was assessed by DPPH assay, using UV spectrophotometer. Radical scavenging activity was calculated using the following formula:

\[
\text{Radical scavenging activity} \, (\%) = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

**Separation of the antioxidant fraction**

Oleoresin with maximum radical scavenging activity was subjected to fraction separation using silica gel column chromatography. The sample was prepared by mixing two grams of the oleoresin extracted from the mature-leaf powder with 20 mL of hexane. Solvent system was prepared using hexane (nonpolar) and ethyl acetate (polar) in the proportions 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100 (hexane: ethyl acetate). The samples were loaded over the top of the packed column and subsequently allowed to move into. Afterwards, the column was filled with 75 mL of 80:20, 60:40, 40:60, 20:80 and 0:100 (hexane: ethyl acetate) and each fraction was collected separately. DPPH assay was performed in each fraction to find out the one with maximum antioxidant activity.

**Sub-fractionation and identification of compounds through LC-MS/MS**

Fraction with the highest antioxidant activity was sub-fractionated using column chromatography. The fraction was loaded into silica column and subjected to column chromatography. Fractions were collected at five min. interval and a total of 47 fractions were obtained. DPPH assay was done for all the 47 sub-fractions and five sub-fractions with the highest antioxidant activity were identified. Those sub-fractions along with the main fraction were analyzed using LC-MS/MS. The samples were analyzed with Agilent G6550A with triple quadrupole mass spectrophotometer. The samples were mixed with water:acetonitrile in the ratio 95:5 and 3 µL of sample was injected to the machine. Electrospray ionization with positive polarity (ES+) was given at 3500 V capacity voltage, 1000 V nozzle voltage, and gas at 13 L/min. with source temperature 250°C.

**Molecular docking**

Phytochemicals identified through LC-MS/MS were considered as ligands and docked against breast cancer targets, 17-beta HSD, polo-like kinase 1, exchange protein directly activated by CAMP, NAT-2 receptor, phosphoinitrate-3 kinase, human androgen receptor, dihydrofolate reductase and human estrogen.
receptor ligand-binding domain. Interactions were also compared with that of commercial drugs available for cancer viz. fulvestrant, ribociclib, diphenylamine, NSC-54767, tamoxifen, fenritinide and trimetrexate. Structures of the phytocompounds and commercial drugs were retrieved from PubChem and ChemSpider databases. Structures of the cancer targets were retrieved from Brookhaven National Laboratory’s database on protein structures.

The 3D structures of the ligands and targets were processed using the protein preparation wizard of Discovery Studio 4.0. Energy minimization of the proteins was done using CHARMM force field. The ligands were filtered as per Lipinski-Veber’s protocol. Receptor cavity and Prediction tool has been used to predict the active site of the target protein based on the active amino acid present in the binding site and the docking was done using CDOCKER protocol. The best pose of the phytocompound and the interacting target protein was identified from the minimum difference between C-DOCKER and C-DOCKER interaction energies. The binding affinity of each compound against their targets has been identified (Ligand binding energy = $E_{\text{complex}} - E_{\text{ligand}} - E_{\text{protein}}$) and the scoring function was based on these binding energies. ADMET analysis was done using ADME/T Descriptor algorithm, by which the pharmacokinetic properties such as aqueous solubility, human intestinal absorption, blood brain barrier (BBB) penetration, cytochrome P450 inhibition (CYP2D6) and hepatotoxicity levels were estimated for the successful ligands.

**In vitro cytotoxicity and anti-proliferative analyses**

The promising molecules identified through molecular docking were tested for their cytotoxicity and anti-proliferative properties. They were purchased in their chemical form (Sigma Aldrich) and used in the analyses. Trypan blue exclusion assay in murine cancer cell lines and MTT assay in human breast cancer cell line were used in cytotoxicity and anti-proliferative studies, respectively. murine cancer cell lines, ehrlich ascites carcinoma (EAC) and daltons lymphoma ascites (DLA) and Human breast cancer cell line, MCF-7 were procured from Amala Cancer Research Centre, Thrissur, India. EAC and DLA were maintained in the peritoneal cavity of the mouse whereas human cell lines were cultured in Dulbecco’s modified eagle medium (DMEM), maintained at 37°C in the incubator.

Approximately, 1×10^6 tumour cells of DLA and EAC were incubated in 1 mL PBS (phosphate buffered saline) containing different concentrations of test materials at 37°C for 3 h. The percentage of dead cells was determined by trypan blue exclusion method.

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 formazan. For MTT assay, the MCF-7 cells were seeded at a density of 5×10^3 cells/ well in 500 µL of the medium in a 24-well culture plate and cultured for 24 h. Then, the cells were treated with 40, 80, 120, 160, 200, 240 and 280 µg/mL of test materials for 24, 48 and 72 h. After treatment, the cells were incubated with 0.5 mg/ mL of MTT at 37°C for 4 h and with DMSO at room temperature for 15 min. The absorbance of the samples was measured at 570 nm on a scanning multi-well spectrophotometer. Percentage of inhibition was calculated as the ratio of the difference in the OD values of the control sample and test sample and the OD value of the test sample. Experiments were performed at least three times, mean±SE was calculated and statistically analyzed using Graph Pad software followed by Dunnett’s test, with $P < 0.05$* and $P < 0.01$** considered significant.

**Anticancer efficacy analysis using mouse mammary tumour model**

Based on the in vitro results, the most promising phytocompound was carried forward to the in vivo analyses in mouse mammary tumour model. All the animal experiments were conducted with the approval of the Institutional Animal Ethical Committee, Amala Cancer Research Centre, Thrissur, India, according to 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. Thirty-six female Swiss Albino mice (procured from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, India) were housed under standard conditions of 24-28°C, 60-70% humidity and 12 h dark/light cycle, and fed with mouse feed and water ad libitum.

Acute toxicity study was done to find out the toxic effects of the selected drug. The drug was orally administered at 100 mg/ kg body wt. for one day and monitored for 14 days.

Mammary tumour was induced by the oral administration of dimethylbenz[a]anthracene (DMBA,
DMBA was dissolved in sesame oil and given to all the groups of animals once in a week for six weeks. There were six animal groups, each with six mice. Group I: Normal, Group II: control-DMBA treated, Group III: vehicle control-sesame oil, Group IV: tamoxifen (10 mg/kg body wt.), and Group V: drug-low dose and group VI: drug-high dose. Drug selected was also given orally, each day for six weeks. The experimental and control animals were carefully examined daily and sacrificed after the sixth week. Blood and serum were collected for blood tests, liver function test and renal function test. Histopathology of the mammary pad, kidney and liver tissues were performed.

Gene expression analyses
From the mammary pad of the mice, RNA was isolated using TRIzol method and cDNA was prepared (RevertAid First Strand cDNA synthesis kit, ThermoFisher Scientific, India). The qRT-PCR analyses were performed (StepOnePlus™ Real-Time PCR system, ThermoFisher Scientific) using SsoAdvanced Universal SYBR Green Supermix (BioRad) to study the expression profile of the genes, ER-1, Bcl-2, c-Myc and Pin 1 with reference to the house keeping gene β-actin. The primer details are presented in Supplementary Table 1. All supplementary data are available only online along with the respective paper at the journal website (http://ijeb.res.in) as well as NOPR repository at http://nopr.res.in.

Results

Oleoresin extraction
Medium mature and mature leaves have yielded 7.87 and 9.16% oleoresin, respectively. DPPH assay was conducted on the extracted oleoresin along with the control, butylated hydroxyanisole (BHA). Percent inhibition of ROS was highest for BHA (91.14%) followed by oleoresin extracted from mature leaves (85.19%) and oleoresin extracted from medium mature leaves (83.30 %) (Suppl. Table S2). As oleoresin extracted from mature leaves showed better results, it was used for further studies.

Separation of antioxidant fraction by column chromatography
Oleoresin was separated by column chromatography using a solvent system composed of different concentrations of hexane and ethyl acetate. Yield of the fraction extracted in each solvent system, after complete evaporation of the solvent, is shown in Suppl. Table S3. The highest yield was recorded for the fraction extracted using 60:40 (hexane: ethyl acetate) solvent system (706.4 mg) and the lowest was found in the fraction extracted using 100 percent ethyl acetate (50.6 mg). DPPH assay conducted on each fraction had shown that the standard antioxidant butylated hydroxyanisole possess the highest free radical inhibition (91.147%) (Suppl. Table S4). Among the fractions, 60:40 (hexane: ethyl acetate) fraction recorded the highest inhibition of DPPH (88.680 %) followed by 40:60 fraction (85.140%).

The whole fraction of 60:40 (hexane: ethyl acetate) was subjected to sub-fractionation at five min. interval using column chromatography. A total of 47 sub-fractions were collected, evaporated and subjected to DPPH assay. Sub-fractions 26, 28, 34, 38 and 40, collected at 130, 140, 170, 190 and 200 min, respectively have given the highest percent inhibition for DPPH (91.080, 91.510, 91.080, 89.535 and 89.535, respectively) (Suppl. Table S5) and were selected for LC-MS/MS analysis. LC-MS/MS analysis was carried out for the 60:40 hexane:ethyl acetate whole fraction and five sub-fractions. The number and details of compounds identified from these six samples are presented in Suppl. Tables S6 A and B. From each fraction, 100 compounds have been analyzed and a total of 98 compounds were identified.

Molecular docking
Sixty-nine ligands identified through LC-MS/MS, seven FDA approved drugs and eight target proteins for cancer were used in molecular docking studies. Among the ligands, 43 passed the filtering using Lipinski rule and among the cancer drugs, Fulvestrant failed. The docking scores of the selected ligands with the cancer targets are given in Table 1.

Ligand valylmethionine has shown good interaction with the cancer target 17-beta HSD, with minimum deviation between CDOCKER energy and CDOCKER interaction energy (0.0388 kcal/mol) and good binding energy (-66.7903 kcal/mol). Hence, valylmethionine can be used as a drug candidate to target 17-beta HSD. With the target polo like kinase 1 (PLK-1), both valylmethionine and pheniramine had good interaction. Valylmethionine recorded the least deviation between CDOCKER energy and CDOCKER interaction energy and interacted at the critical amino acid (Lys82) of active site of PLK-1. Alpha-aminodiphenylacetic acid and Valylmethionine interacted with EPAC2 at Glu404 residue while commercial drug diphenylamine failed to from any
Table 1 — Docking scores for different cancer targets with selected ligands

<table>
<thead>
<tr>
<th>Type of compound</th>
<th>Ligand</th>
<th>(-) CDOCKER energy (kcal/mol)</th>
<th>(-) CDOCKER interaction energy (kcal/mol)</th>
<th>No. of H bonds</th>
<th>Amino acid bound to H bond</th>
<th>Distance (Å)</th>
<th>Binding energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target: 17-beta HSD</td>
<td>Curry leaf phyto-compound</td>
<td>Valylmethionine</td>
<td>34.9551</td>
<td>34.9939</td>
<td>4</td>
<td>Ser&lt;sub&gt;15&lt;/sub&gt;*</td>
<td>2.02656</td>
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<td></td>
<td></td>
<td>Fluoxetine</td>
<td>23.9072</td>
<td>33.6213</td>
<td>3</td>
<td>Ser&lt;sub&gt;15&lt;/sub&gt;*</td>
<td>2.07231</td>
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<td></td>
<td></td>
<td>Prometon</td>
<td>25.9838</td>
<td>28.5795</td>
<td>1</td>
<td>Ser&lt;sub&gt;15&lt;/sub&gt;*</td>
<td>2.38996</td>
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<tr>
<td></td>
<td></td>
<td>Alpha-amino diphenylacetic acid</td>
<td>23.1847</td>
<td>30.4697</td>
<td>5</td>
<td>Lys&lt;sub&gt;159&lt;/sub&gt;*</td>
<td>2.37818</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standard antioxidant</td>
<td>BHA</td>
<td>16.4736</td>
<td>24.9752</td>
<td>3</td>
<td>Gly&lt;sub&gt;15&lt;/sub&gt;*</td>
</tr>
<tr>
<td></td>
<td>Commercial drug</td>
<td>Fluvestrant</td>
<td>Failed in Lipinski Veber rule</td>
<td></td>
<td></td>
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<tr>
<td>Target: Polo-like kinase 1</td>
<td>Curry leaf phyto-compounds</td>
<td>Valylmethionine</td>
<td>38.9120</td>
<td>40.8400</td>
<td>1</td>
<td>Lys&lt;sub&gt;82&lt;/sub&gt;*</td>
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<td></td>
<td></td>
<td>Pheniramine</td>
<td>28.9006</td>
<td>37.2325</td>
<td>1</td>
<td>Ser&lt;sub&gt;287&lt;/sub&gt;*</td>
<td>1.92422</td>
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<td></td>
<td></td>
<td>Alpha-amino diphenylacetic acid</td>
<td>21.8506</td>
<td>27.2960</td>
<td>1</td>
<td>Arg&lt;sub&gt;136&lt;/sub&gt;</td>
<td>2.07521</td>
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<td></td>
<td></td>
<td>Standard antioxidant</td>
<td>BHA</td>
<td>17.5853</td>
<td>28.2903</td>
<td>1</td>
<td>Cys&lt;sub&gt;287&lt;/sub&gt;*</td>
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<tr>
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<td>Commercial drug</td>
<td>Ribociclib</td>
<td>-1.32815</td>
<td>55.2089</td>
<td>1</td>
<td>Asp&lt;sub&gt;104&lt;/sub&gt;</td>
<td>2.10157</td>
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<td>Target: Exchange protein directly activated by CAMP</td>
<td>Curry leaf phyto-compounds</td>
<td>Alpha-amino diphenylacetic acid</td>
<td>33.6452</td>
<td>41.6221</td>
<td>2</td>
<td>Gly&lt;sub&gt;404&lt;/sub&gt;*</td>
<td>1.81371</td>
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<tr>
<td></td>
<td></td>
<td>Histidinol</td>
<td>22.7564</td>
<td>29.5362</td>
<td>3</td>
<td>Val&lt;sub&gt;406&lt;/sub&gt;*</td>
<td>2.07356</td>
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<tr>
<td></td>
<td>Standard antioxidant</td>
<td>BHA</td>
<td>12.3313</td>
<td>21.8548</td>
<td>4</td>
<td>Val&lt;sub&gt;406&lt;/sub&gt;*</td>
<td>1.81371</td>
</tr>
<tr>
<td></td>
<td>Commercial drug</td>
<td>Diphenylamine</td>
<td>19.0380</td>
<td>26.2256</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Target: NAT-2 receptor</td>
<td>Curry leaf phyto-compounds</td>
<td>Pheniramine</td>
<td>28.9006</td>
<td>37.2325</td>
<td>1</td>
<td>Ser&lt;sub&gt;287&lt;/sub&gt;*</td>
<td>1.92422</td>
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<tr>
<td></td>
<td></td>
<td>Alpha-amino diphenylacetic acid</td>
<td>26.9884</td>
<td>31.9594</td>
<td>4</td>
<td>Ser&lt;sub&gt;216&lt;/sub&gt;(3)*</td>
<td>2.3497</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histidinol</td>
<td>20.5092</td>
<td>27.1549</td>
<td>2</td>
<td>Thr&lt;sub&gt;214&lt;/sub&gt;</td>
<td>2.08209</td>
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<tr>
<td></td>
<td>Standard antioxidant</td>
<td>BHA</td>
<td>22.1629</td>
<td>30.2810</td>
<td>1</td>
<td>Cys&lt;sub&gt;287&lt;/sub&gt;*</td>
<td>1.99432</td>
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<td></td>
<td>Commercial drug</td>
<td>NSC-54767</td>
<td>19.0380</td>
<td>26.2256</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Target: Phosphoinositide-3 kinase</td>
<td>Curry leaf phyto-compounds</td>
<td>Histidinol</td>
<td>23.7478</td>
<td>30.5784</td>
<td>3</td>
<td>Gly&lt;sub&gt;380&lt;/sub&gt;(2)*</td>
<td>2.29569</td>
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<td></td>
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<td>Pheniramine</td>
<td>22.7752</td>
<td>30.9258</td>
<td>1</td>
<td>Met&lt;sub&gt;353&lt;/sub&gt;</td>
<td>2.28098</td>
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<td>Doxylamine</td>
<td>22.4484</td>
<td>31.5954</td>
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<td>Ser&lt;sub&gt;306&lt;/sub&gt;</td>
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<td>Standard antioxidant</td>
<td>BHA</td>
<td>17.6706</td>
<td>25.5117</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Target: Human androgen receptor</td>
<td>Curry leaf phyto-compounds</td>
<td>Histidinol</td>
<td>28.3878</td>
<td>33.2952</td>
<td>3</td>
<td>Thr&lt;sub&gt;377&lt;/sub&gt;*</td>
<td>1.89568</td>
</tr>
</tbody>
</table>

(Contd.)
hydrogen bond with the target. Alpha-amino diphenylacetic acid, pheniramine and histidinol interacted with NAT-2 protein. Alpha-amino diphenylacetic acid recorded the least difference between CDOCKER and CDOCKER interaction energy and formed three hydrogen bonds with Ser 216, amino acid at the active site, which may cause inhibition of the target NAT-2 protein.

With the target phosphoinositide-3 phosphate (PI3K), histidinol and doxylamine have interacted through Glu880 and Met 953 suggesting that the binding of these compounds inhibits PI3K. Only histidinol has interacted with human androgen receptor (AR). It showed high binding energy and interacted with critical amino acids (Thr877, Asn705 and Leu 704) in the binding site of AR. Surprisingly, the commercial drug fenretinide had shown a positive CDOCKER energy. Histidinol interacted with the target DHFR at amino acid Ile 5 and Asp 27 while commercial drug Trimextrexate interacted at Ile 5, Asp 27 and Ile 94 with more binding energy than histidinol. With estrogen receptor ligand binding domain, only histidinol has interacted with high docking energy, forming a hydrogen bond with Leu346 and Glu353.

**ADME/T analysis of ligands**

The pharmacokinetic properties of the ligands and approved drugs were studied through ADME/T analysis (Table 2). Based on ADME/T values, ligands and commercial drugs were grouped into Acceptable (A, compounds not in acceptable range for ≤2 parameters), Highly acceptable (HA, compounds satisfy the acceptable range for all parameters) and Non-acceptable (NA, compounds not in acceptable range for ≥3 parameters.).

Among the seven curry leaf ligands which interacted with cancer targets, only two compounds, alpha-amino diphenylacetic acid and valylmethionine, were highly acceptable. They were easily absorbed in body, easily soluble in body fluids, non-inhibitor in action on CYP2D6 drug metabolizing enzyme with less penetration in CNS and exhibited non-toxic effect in liver. Remaining phytocompounds were found acceptable, either too soluble as in histidinol or interfere with CYP2D6 enzyme as in doxylamine. Pheniramine and doxylamine had high penetration in the central nervous system while prometon showed hepatotoxicity. Among the commercial drugs, only trimetrexate was highly acceptable and others were acceptable. Of the eight targets, histidinol interacted with seven targets and was found to be a promising phytocompound with anticancer activity, even though it was too soluble in ADME/T analysis.

**Short-term cytotoxicity analysis by Trypan blue exclusion assay**

Of the seven phytocompounds that interacted better with the cancer targets, doxylamine, histidinol and pheniramine were tested in vitro against breast cancer cell line MCF-7 and murine cancer cell lines DLA and EAC. They were purchased in their chemical form as doxylamine succinate salt, L-histidinol dihydrochloride and pheniramine maleate salt, respectively. Trypan blue exclusion assay, the earliest and widely used method for measuring the viability of the cells, was used to screen the drug candidates. In cases of daltons lymphoma ascites (DLA) and ehrlich...
ascites carcinoma (EAC) cells, the number of dead cells was very low when treated with the drug candidates even at the highest concentration of 400 μg/mL. Only pheniramine maleate salt showed some cytotoxicity with 15 and 10% inhibition on DLA and (EAC) cells, respectively. Since the inhibition observed was less than 50% in all the treatments, it was concluded that these drug candidates have no significant cytotoxicity against murine cancer cells.

Anti-proliferative analysis by MTT assay
The percent inhibition of proliferation (mean±SD) of MCF-7 cells by the drug candidates pheniramine maleate salt (PMS), doxylamine succinate salt (DSS) and L-histidinol dihydrochloride (LHD) is presented in Table 3. Inhibition responses were dose-dependent and based on this, IC50 values were calculated.

When MCF-7 was incubated with PMS for 24 h, the maximum inhibition obtained at the concentration of 280 μg/mL was only 38.0±2.2%. At 48 h, maximum inhibition at 280 μg/mL was 71.2±2.5%, with IC50 at 108 μg/mL. More than 90% inhibition was seen in all the concentrations of PMS at 72 h with the maximum of 98.46±3.6% at 280 μg/mL, and the corresponding IC50 value was 14 μg/mL.

At 24 h of incubation of MCF-7 cells with 280 μg/mL DSS, maximum inhibition obtained was 38.22±1.5%, nearly the same as that obtained with PMS. At 48 and 72 h, maximum inhibition were 58.23±2.1 and 80.49±3.4%, with IC50 values of 252 and 61 μg/mL, respectively.

LHD did not inhibit the proliferation of the breast cancer cells. The maximum percentage of inhibition even at 72 h of incubation at 280 μg/mL was 28.82±1.5%.

Acute toxicity study
Acute toxicity studies were done in Swiss albino mice using a high dose of 100 mg PMS/kg body wt. Monitoring for 14 days after the drug administration
had shown no significant differences in the behavioural and general appearance between the normal and treated groups, indicating that the drug has no toxicity. Hence, \( \frac{1}{6} \) th and \( \frac{1}{5} \) th of the dose (10 and 20 mg/kg body wt.) were used as low and high doses, respectively.

**Anticancer efficacy analysis using mouse tumour model**

After six weeks of carcinogen administration, the tumour developed (Fig. 1) and the mice were sacrificed in CO2 chamber. The average values of uptake of feed, water and changes in the weight of the animal groups over the six weeks are presented in Suppl. Tables S7-S9. Three mice each from each category were used to collect the blood to analyze the haematological parameters and serum for liver function and renal function tests. The mean values for the haematological and biochemical parameters from the liver and renal function tests were recorded and compared with the control.

PMS administration significantly reduced the WBC count in the tumour induced mice, with the standard \( (P < 0.001) \), PMS low dose \( (P < 0.01) \) and high dose \( (P < 0.01) \) groups, showing 6800, 8300 and 6300 cells/ cu mm, respectively (Table 4). Tamoxifen treated animals exhibited a significant decrease \( (P < 0.05) \) in the WBC count compared to the normal and DMBA-treated animals. Variations observed among the different treatments for haemoglobin, platelet count, total RBC count, neutrophils, lymphocytes and eosinophils, were not significant.

In liver function tests, SGOT (Serum glutamic-oxaloacetic transaminase) levels were found to have reduced significantly in the standard and PMS low dose treatments (272 and 303 U/L, respectively). Similarly, for SGPT (Serum glutamic pyruvic transaminase), levels of standard, PMS low dose and PMS high dose were significantly lower (72, 65 and 76 U/L, respectively) than that of the control group (101 U/L).

For ALP (Alkaline phosphatase), levels of standard, PMS low dose and PMS high dose were 146, 73 and 156 U/L, respectively compared to the control groups with 234, 128 and 79 U/L (Table 5). Variations among the treatments for total protein, albumin, globulin, total bilirubin were insignificant.

---

**Table 4 — Haematological parameters of breast tumour induced and normal mice**

<table>
<thead>
<tr>
<th>Haematological parameters</th>
<th>Normal</th>
<th>Control</th>
<th>Vehicle control</th>
<th>Standard</th>
<th>PMS LD</th>
<th>PMS HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>14.3±0.2</td>
<td>13.2±0.2</td>
<td>12.9±0.2</td>
<td>11.3±0.1</td>
<td>14.6±0.2</td>
<td>13.9±0.2</td>
</tr>
<tr>
<td>Total RBC count (millions/cu mm)</td>
<td>8.2±0.1</td>
<td>8.1±0.2</td>
<td>7.8±0.2</td>
<td>7±0.2</td>
<td>8.5±0.2</td>
<td>8.1±0.1</td>
</tr>
<tr>
<td>Platelet count (lakhs/cu mm)</td>
<td>10.9±0.2</td>
<td>10.2±0.2</td>
<td>12.9±0.2</td>
<td>11.1±0.2</td>
<td>9.5±0.2</td>
<td>9±0.1</td>
</tr>
<tr>
<td>Total WBC count (cells/cu mm)</td>
<td>5600±205</td>
<td>8600±270</td>
<td>8100±190</td>
<td>6800±230***</td>
<td>8300±510**</td>
<td>6300±550***</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>8±3</td>
<td>18±2.8</td>
<td>25±2.5</td>
<td>12±8</td>
<td>9±0.8</td>
<td>9±1.5</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>87±2.5</td>
<td>80±2.2</td>
<td>67±1.7</td>
<td>85±3.2</td>
<td>81±3.1</td>
<td>77±4.2</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>5±0.1</td>
<td>2±0.1</td>
<td>8±0.2</td>
<td>3±0.2</td>
<td>3±0.1</td>
<td>3±0.1</td>
</tr>
</tbody>
</table>

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

**Table 5 — Liver function and renal function tests of breast tumour induced and normal mice**

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Normal</th>
<th>Control</th>
<th>Vehicle Control</th>
<th>Standard</th>
<th>PMS LD</th>
<th>PMS HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGOT (U/L)</td>
<td>312±5.3</td>
<td>380±4.8</td>
<td>316±3</td>
<td>272±3.5**</td>
<td>303±8.9**</td>
<td>297±4.3</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>60±3.1</td>
<td>101±1.5</td>
<td>68±2.2</td>
<td>72±1.5***</td>
<td>65±4.2***</td>
<td>76±2.5***</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>234±2.5</td>
<td>128±2.1</td>
<td>79±4.4</td>
<td>146±7.8***</td>
<td>73±2.5***</td>
<td>156±2.4***</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>7±0.4</td>
<td>8±0.9</td>
<td>8±0.8</td>
<td>7.7±2.5</td>
<td>7.1±1.5</td>
<td>7±0.5</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.4±0.2</td>
<td>3.3±0.2</td>
<td>2.9±0.2</td>
<td>3.1±0.1</td>
<td>3.2±0.1</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
<td>3.6±0.2</td>
<td>4.7±0.1</td>
<td>5.4±0.2</td>
<td>4.6±0.2</td>
<td>3.9±0.1</td>
<td>4.3±0.3</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>53±0.6</td>
<td>43±0.9</td>
<td>37±0.5</td>
<td>40±0.7</td>
<td>35±0.4</td>
<td>31±0.3</td>
</tr>
<tr>
<td>Creatinine (mg/DL)</td>
<td>0.46±0.02</td>
<td>0.48±0.03</td>
<td>0.45±0.01</td>
<td>0.45±0.02</td>
<td>0.44±0.01</td>
<td>0.47±0.03</td>
</tr>
</tbody>
</table>

Mean±SD (n=3) in comparison with control group. **significant at P <0.01 and ***significant at P <0.001.
In the renal function test, variations in urea and creatinine levels in all the groups were also insignificant, compared to that of control (53 mg/dL urea and 0.46 mg/dL creatinine).

**Histopathology of liver, mammary pad and kidney**

The histopathological architecture of mammary pad, liver and kidney of the animals from the six groups were photographed under light microscope (200X). The histology of mammary pads of untreated groups revealed the presence of normal adipose tissue, mammary duct and stromal tissue (Fig. 2). In control and low dose groups, the stroma had shown 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 the normal group had shown typical hepatic architecture (Fig. 3). The control and low dose treatment groups had cytoplasmic degeneration and aggregation of inflammatory cells, whereas standard group showed hepatocellular injury. High dose treatment group showed less cytoplasmic degeneration.

The histopathology of kidney tissues from the normal group showed normal glomerulus and distal and proximal tubules (Fig. 4). The control and treated
groups had no visible differences in their renal architecture.

Expression analysis of candidate oncogenes

From the mice belonging to each treatment, total RNA was isolated, cDNA was prepared and qRT-PCR was done to measure the relative expression of the candidate oncogenes, ER-α1, Bcl-2, c-Myc and Pin1, using β-actin as the reference gene.

The relative expression of the genes among different treated groups and normal group is shown in the Figs. 5A-D. Fold change values for normal group was 1 with which the fold change in all other groups were calculated. Expression of all the genes were increased in the control and vehicle control groups 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 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.

In general, higher level of expression was observed for these genes in the control and vehicle control groups. Relatively lower expression of the oncogenes in PMSLD and PMSHD groups indicates that the drug PMS is efficient to reduce the progress of carcinogenesis.

Discussion

Natural compounds derived from the plants are excellent sources of drugs against cancer. Various phytochemicals have been characterized for their anti-cancerous properties. Few plant derived cancer drugs, such as paclitaxel from Taxus brevifolia Nutt., are already available in the market 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, Nimboide 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 cancer20. The anticancer activity of curry leaf compounds are identified and validated in this study.

Oleoresin extraction

The oleoresin content in black pepper leaves is reported to increase with maturity, with 1.29% increase in mature leaves compared to the emerging ones21. This was found to be true with curry leaf also. The anti-oxidant property of the oleoresin also gets enhanced since the total phenol content increases significantly. The oleoresin extract from mature curry leaves possess a high quantity of total phenols22.

Separation of antioxidant fraction by column chromatography

Column chromatography is a standard methodology followed to fractionate the secondary metabolites. Researchers have been extensively using the various chromatographic strategies to isolate the carbazole alkaloids from curry leaves23.

DPPH assay is a widely preferred method for evaluating the antioxidant potential of the plant extracts25 and butylated hydroxianisole is the standard antioxidant used for the comparison24. DPPH inhibition by the sub-fractions was higher than that by the main fraction (60: 40 hexane: ethyl acetate), which might be due to the presence of large number of compounds in the fraction. Individual compound isolated from the curry leaf oleoresin will have higher DPPH inhibition than the total oleoresin23.

Molecular docking

Of the 69 ligands identified, 43 have passed the Lipinski rule. Selection of active inhibitors was based on low binding energy25. The difference of more than...
10 between CDOCKER energy and CDOCKER interaction energy for ligand and target protein interaction was unstable, hence neglected. All the ADMET parameters were calculated using standard mathematical formulae. Among the phyto-coumpounds interacting with cancer targets, alpha-amino diphenylacetic acid and valylmethionine, were highly acceptable and among the commercial drugs, only trimetrexate was highly acceptable. Histidinol interacted with seven of the eight targets studied and hence found promising in cancer treatment, even with its too soluble nature. Solubility of the drugs can be modified by physical and chemical methods and hence efforts can be made to improve its solubility while drug formulation.

**Short-term cytotoxicity and Anti-proliferative analyses**

The compounds pheniramine, doxylamine and histidinol were selected based on their dock scores against cancer targets polo-like kinase, NAT-2 receptor, phosphoinositide-3 kinase and Human estrogen receptor ligand-binding domain, which are the common targets of breast cancer.

Three phyto-compounds in its chemical form, 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. Pheniramine maleate salt showed cytotoxicity of 15% in DLA cells and 10% in EAC cells.

In a short-term cytotoxicity study conducted by Bellamakondi et al.27, the methanolic extracts of *Caralluma* species were tested against EAC cells (Ehrlich ascites carcinoma) and the CTC₅₀ value ranged between 191.3±0.92 and 291.8±3.17 μM. 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 these three molecules do not have considerable cytotoxicity against murine cancer cells.

Assessment of per cent inhibition of proliferation of MCF-7 cancer cells by PMS, DSS and LHD by *in vitro* MTT assay had shown that PMS and DSS inhibit the cancer cell proliferation up to 98.46%. When Kumar et al.28 have tested the anticancer potential of ethanolic extract and essential oil of *Syzygium aromaticum* L. in MCF-7 breast cancer cell lines, IC₅₀ values of ethanolic extract were 61.29 and 16.71 μg/mL for 24 and 48 h incubation, respectively. The essential oil achieved IC₅₀ at 36.43 and 17.6 μg/mL in 24 and 48 h, respectively. Compared to this, the concentration required for both PMS and DSS to achieve 50% inhibition at 48 hours of inhibition was higher (108 and 252 μg/mL, respectively) but with 72 h incubation, IC₅₀ values were only 14 μg/mL for PMS and 21 μg/mL for DSS. Similarly, Urdiales et al.29 have tested the effects of chlorpheniramine on the activity of ornithine decarboxylase (ODC) enzyme in cultured Ehrlich carcinoma cells and found that the ODC enzyme activity decreased with increase in drug concentration. Among the PMS and DSS lines, PMS performed better as it had lower IC₅₀ compared to that of DSS. Hence, PMS was selected for the *in vivo* analysis.

**Acute toxicity study**

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 studies were done to find whether the selected drug impose any sort of toxicity in the animals. In order to study the anti-histaminic effect of *Bauhinia racemosa* leaves, Nirmal et al.30 had used 10 mg/kg b.wt. of pheniramine maleate as a standard in male albino mice. Similar quantity of pheniramine maleate salt was administered in various other studies.31, Hence, a high-dose of 100 mg/ kg body wt. of the drug PMS was administered once to the animals and monitored for 14 days for the changes in the behavioural and general appearance.

**Anticancer efficacy analysis using mouse tumour model**

PMS administration has significantly reduced the WBC count in the tumour induced mice. Zinge et al.32 has shown that DMBA treatment increases the WBC count significantly compared to that in normal mice.

Liver function test is used to detect damage and inflammation of the liver by analysing the enzymes such as serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP). A significant reduction in SGPT, SGOT and ALP levels was observed in the treated mice than that of the normal mice. Reduced level of these serum enzymes indicate the hepato-protective activity of the drug candidate. Similar kind of hepato-protective activity of *Cyathea gigantea* was observed by Kiran et al.33 in paracetamol-induced Wistar albino rats.
Histopathology of liver, mammary pad and kidney

Histopathology, a routine method in cancer diagnosis, provides an insight into the architecture of the tissues in which the disease is manifested. Comparing 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. 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 has produced significant changes in the tissue morphology of the DMBA treated animals. The identifiable changes suggest the definite roles of the drug candidate in cell proliferation and organization. Under chronic conditions, lower doses of the drug is reported to produce promising effects compared to the higher doses.

Counts on haemoglobin, total RBC, platelet, total WBC, neutrophils, lymphocytes and eosinophils were made under the haematological analyses. A reduction in the RBC count implicated that there is reduction in the transport of oxygen and carbon dioxide. WBCs 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.

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 bilirubin, 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., 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 increases 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 SGPT (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.

Expression analysis of candidate oncogenes

All the genes analyzed were oncogenes with profound roles in breast cancer. The ER-(Estrogen receptor-α) signaling plays a major role in the breast cancer proliferation. It promotes the expression of other oncogenic proteins such as cyclin-D and c-Myc and inhibits the expression of cell cycle inhibitors such as P21. The standard drug tamoxifen is a modulator of ER-α. In DMBA induced Wistar albino rats, the ER-α activity is reported to have down regulated by Graviola.

The Bel-2 is an anti-apoptotic gene and prognostic marker in breast cancer. The higher expression of this protein is shown to help the cancer cells evade apoptosis. Extract of Taraxacum officinale on DMBA induced Wistar rats resulted in a reduced expression of Bel-2 gene. The c-Myc codes for a pleiotropic transcription factor, upregulated by ER-α. Simvastatin treatment is shown to reduce the c-Myc expression compared to the control mice receiving only DMBA. The level of Pin1 protein has role in deciding the transcription of cyclin D1, another
oncogene of breast cancer. Overexpression of Pin1 activates the promoter of cyclin D1. Also, Pin1 is shown to modulate the chemo-resistance by upregulating FoxM1 and involved in Wnt/β-catenin signaling pathway. Kim et al. showed that amuresin G from Vitis amurensis could inhibit angiogenesis of tamoxifen-resistant cancer by inhibiting the expression of Pin1.

Through comparative C_T method, the fold change in the expression of these genes in the tumour cells were assessed in each animal group. Relatively lower expression of the oncogenes in PMSLD and PMSHD groups indicated that the PMS is a promising drug to reduce the carcinogenesis.

Conclusion

Though curry leaf is known to have activity against breast cancer, potential of individual molecules has not been validated until now. This work details the identification of pheniramine maleate salt, the chemical derivative of pheniramine, as a potential breast cancer drug candidate from curry leaf. Protocols and results in extraction and fractionation of the antioxidant principles from curry leaves, their characterization using LC-MS/MS, identification of molecules with anticancer properties through molecular docking, in vitro antiproliferative analysis, in vivo studies using mouse breast cancer tumour model and expression analysis of candidate oncogenes, leading to the identification of the drug candidate, are detailed. PMS suppresses the expression of the oncogenes, thus reducing the progression of cancer. In gene expression analyses, action of PMS was comparable with the standard drug, thereby emphasizing its potential.

Acknowledgement

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Ethical approval

This study was conducted with the approval of the Institutional Animal Ethical Committee, Amala Cancer Research Centre, India (No. ACRC/IAEC/21(2)-P15 dtd.19.11.2021), according to the rules and regulations of the Committee For The Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Conflict of Interest

Authors declare no competing interests.

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