Assessment of Dolichandrone falcata Seem. Leaves for Anti-cancer Potential in Experimental Animal Models

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ABSTRACT
Background: Mammary cancer is the most common type of cancer and the leading cause of cancer-related death in women. Despite of numerous therapeutic options, cancer remains associated with high mortality. Traditional herb medicine has been found effective with minimal or no side effects. Materials and Methods: The leaves of Dolichandrone falcata Seem. (Bignoniaceae family) contains chrysin-7-rutinoside, flavanoids which possess Anti-cancer potential. The extract obtained from Soxhlet extraction process is then assessed against the chemical carcinogen (7,12-Dimethyl benzene anthracene) induced mammary carcinoma in rats. Results and Conclusion: The dose dependent study and statistical comparison with the help of graph pad prism, version 8.03 by one way ANOVA followed by Dunnett’s multiple comparison test has revealed that the significant reduction (p<0.05) is observed in the mammary tumor volume of the group treated with the extract and hence the Anti-cancer Potential of Dolichandrone falcata Seem. is justified.

Keywords: Dolichandrone falcata, Anti-cancer, Flavonoids, Chemical carcinogen, Rats.

INTRODUCTION
Cancer is the uncontrolled growth of cells having potential to infiltrate the normal tissues. Mammary cancer is a type of carcinoma that arises in the inner lining of milk ducts or the lobules that supply milk. Lobular carcinoma and ductal carcinoma are the types of mammmary cancer where lobular carcinoma begins in the lobules and progress to the ducts, whereas ductal carcinoma begins in the ducts.¹ Currently, the available treatment choices for mammary cancer include surgery, chemotherapy radiation therapy or a combination of these. Despite these therapeutic options, the death rate for mammary cancer remains relatively high, which sets the need for improved therapeutic choices that, would rate the likelihood of mammary cancer patients surviving with minimum or no therapy adverse effects.²

National Cancer Institute has evaluated about 35,000 plant species for Anti-cancer properties. Around 3,000 plant species have been found to have Anti-cancer action that can be replicated.³ Dolichandrone falcata Seem. (Family-Bignoniaceae) is a medicinal herb mentioned in Ayurveda and it contains a variety of phytochemicals constituents that have been linked to a variety of pharmacological effects like anxiolytic, analgesic, anti-diabetic, anti-estrogenic, anti-inflammatory and immunomodulatory. The leaves of the plant contain Quecertin, Lutein, Apigenin and Ericocitrin which are known for their therapeutic benefits. The flavonoids named Chrysin-7-rutinoside present in the leaves of Dolichandrone falcata possess potential Anti-cancer effect.⁴,⁵

MATERIALS AND METHODS
Plant material
Dolichandrone falcata Seem. plant belonging to family Bignoniaceae was collected from the premises of Savitribai Phule Pune University Ganeshkhind, Pune. Herbarium was prepared and authenticated through Botanical Survey of India, Western circle, Koregaon Park, Pune city, Maharashtra.

Experimental Animals, approvals and housing
Normal, healthy adult Sprague Dawley female rats weighing 150-200 gm were bought from National Institute of Biosciences, Dhangawadi, Tal-Bhor, Dist-Pune (412205) and study protocol was sanctioned by the Institutional Animal Ethics Committee and regulation was approved by CPCSEA (Protocol No- RDCOP/PCOL-02/IAEC/2018-2019/03).
All the animals were kept in animal houses under standard laboratory conditions in clean polypropylene cages maintained at 25±03ºC temperature with 46±06% relative Humidity with clean paddy husk budding (12 hr light-dark cycle). All animals were fed with a standard pellet diet and had unlimited access to water throughout the study period. Before the tests, the animals were acclimatized to laboratory conditions for 2 weeks. All animals in the research were cared and handled humanely in accordance with laboratory animal care guidelines.

**Drying and pulverizing of plant material**

The leaves of *Dolichandrone falcata* Seem. plant were dried in shade for 2 weeks and triturated to a fine powder. The powder was further passed through a 2 mm sieve to obtain finer particles.

**Extraction**

100 gm powdered sample was macerated in 1000 mL petroleum ether and well shaken to remove lipids, oil and fatty acid. The insoluble residue was dried and further Soxhlet extracted for 72 hr using 1000 mL ethanol. After 72 hr of post-incubation, the *Dolichandrone falcata* Leaves Extract (DFLE) was concentrated in the rota evaporator under vacumm condition and kept in refrigerator at 2ºC to 4ºC and used for further study.

**Physical characterization and Phytochemical study**

Besides the authentication, the crude drug is tested for the quality and purity parameters which include total ash value, acid insoluble ash value, water soluble ash, sulphated ash, loss on drying, alcohol soluble extractive value, water soluble extractive value, petroleum ether extractive value, foaming index. The extract so collected is evaluated for Carbohydrates, Proteins Fats and Oils, Glycosides, Flavonoids, Alkaloids, Terpenoids, Steroids, Saponins, Tannins and Phenolic Compounds.

**Thin layer chromatography**

Thin layer chromatography was performed by the reported methods where various solvent systems were tried and tested. Combination of Chloroform: Ethyl acetate: Methanol (6:2:2) has identified as the suitable solvent system and the spots were detected using UV light at 254 nm.

**Pharmacological study**

**Determination of IC<sub>50</sub> by using MTT assay (in vitro assessment)**

**Cell line:** MCF-7 (Human Breast Cancer cell line).

**Media:** Dulbecco’s Modified Eagle Medium (DMEM) with high glucose, Fetal Bovine Serum (FBS), Antibiotic-Antimycotic 100 x solution.

**Experimental procedure**

MCF-7 (Human Breast Cancer cell line) was incubated at a concentration of 1×10<sup>4</sup> cells/mL in culture medium for 24 hr at 37°C and 5% CO<sub>2</sub>. Cells were seeded at a concentration (70 µL) of 10<sup>3</sup> cells/well in 100 µL culture medium and cells were incubated at a concentration of 1×10<sup>6</sup> cells/mL in culture medium for 24 hr at 37°C and 5% CO<sub>2</sub>. Cells were seeded at a concentration (70 µL) 10<sup>4</sup> cells/well in 100 µL culture medium and 100 µL sample of extracts A to D, in (10, 30, 100 µg/mL) into micro plates respectively (tissue culture grade, and 96 wells). Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate. Controls were maintained to determine the control cell survival and the percentage of live cells after culture. Cell cultures were incubated for 24 hr at 37°C and 5% CO<sub>2</sub> in CO<sub>2</sub> incubator (Thermo scientific BB150). After incubation, the medium was completely removed and 20 µL of MTT reagent (5 mg/min PBS) was added into it. After addition of MTT, cells were incubated for 4 hr at 37°C in CO<sub>2</sub> incubator and the wells for formazan crystal formation were observed under microscope. The yellowish MTT was reduced to dark coloured formazan by viable cells only. Remove the medium and add 200 µL of DMSO (kept for 10 min) and incubate at 37°C (wrapped with aluminum foil). Triplicate samples were analyzed by measuring the absorbance of each sample by a microplate reader (Benesphera E21) at a wavelength of 550 nm.

**In vivo assessment of Anti-cancer potential**

**Acute oral toxicity study in animal**

Acute oral toxicity study was under taken in *Sprague Dawley* female rats as per standard protocol given in OECD guideline-25. Animals were housed in standard environmental conditions with temperature (22±30ºC), humidity (60±5%) and a 12 hr light/dark cycle. All the animals were fasted for 24 hr prior dosing. All the animals were weighed and dose was calculated accordingly. The higher dose of 2000 mg/kg was given to animal by oral gavage feeding needle. The animals were seen for toxic effect for the first 4 hr after the dosing. Further, animals were investigated for 14 days for any toxic effect and mortality. Behavioral changes and other parameters such as body weight, urination, food intake, water intake, respiration, constipation, changes in eye and skin colors, etc. were observed.

**Determination of Anticancer potential by 7, 12-Dimethyl benzene anthracene induced carcinogen**

7, 12-Dimethyl Benzene Anthracene (DMBA) was weighed and dissolved in olive oil to have 25mg/kg/mL concentration and injected subcutaneously in the abdomen and flank region to 4 groups of animals. All the animals were observed regularly for tumor formation by touching. Physical inspection, palpitation and body weight were measured weekly to monitor difference. The test drug DFLE 200 and 400 mg/kg were administered orally with predetermined doses as mentioned in following Table 1. The doses were freshly prepared just before oral feeding throughout...
the 21 days of treatment. The standard metal oral gavage needle was used for oral feeding. Simultaneously the standard drug 5-Flurouracil 25 mg/kg was administered by intraperitoneal route to rats. All the animals were weighed weekly at the time of treatment to adjust the gavage volume to monitor their general health. Tumor volume was measured weekly to determine the tumor volume difference in 4 groups. Animals were sacrificed at the end of the experiment by cervical dislocation and subjected to biochemical, and hematological study.13

**Induction of mammary carcinogenesis**

Mammary carcinogenesis was induced in Sprague Dawley female rats by subcutaneous route with a dose of 25 mg of DMBA dissolved in 1 mL emulsion of olive oil (0.5 mL) and physiological saline solution (0.5 mL) beneath the mammary gland on abdomen and flank region on either side of rat. Tumor yield and size were stabilized with the initiation of DMBA, and these were served in 5 groups.14

**Morphological examination**

Morphological examination comprises of weekly examination of body weight, body organ weight, total number of tumors that appeared till the end of experiment, tumor incidence, average number of tumor per animal, latency period of tumor, antitumor capacity (% CAT), tumor volume calculated by $4/3r^2$.15

**Hematological examination**

After 21 days of treatment with DFLE and 5-Flurouracil, the animal were fasted for 12 hr and blood was collected from retro-orbital plexus with the help of capillary tube and hematological parameters like mean corpuscular volume, hemoglobin, hematocrit, mean corpuscular hemoglobin, monocytes, lymphocytes, basophiles, eosinophiles, total erythrocyte count, total leukocyte count, mean corpuscular hemoglobin concentration, neutrophils were studied.

**Biochemical examination**

The biochemical tests including liver function tests (SGPT, SGOT, and ALP) and kidney function tests were performed using commercially available kits as per the manufacturer’s instructions.

**Statistical analysis**

All the data was expressed as Mean±SEM. Statistical comparison was performed on graph pad prism, version 8.03 by one way ANOVA followed by Dunnett's multiple comparison test. Result $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ were considered as statistically significant.

**RESULTS AND DISCUSSION**

**Physical characterization and Phytochemical study**

The phytochemical analysis of the extract has confirmed the presence of flavonoids in the extract and the flavonoids are accounted for the majority of the anti-mammary activity.

**Thin layer chromatography**

The TLC has identified the flavonoids named Rutin and Chrysin. The Rf value of DFLE was found to be 0.76 which matches with that of the standard and hence presence of Rutin and Chrysin is confirmed.

**Effect of DFLE on MCF-7 (Human breast cancer) cell line**

The percent cell viability counts of DFLE on MCF-7 cell line was calculated by using the formula (100 X Control-Sample/Control). A significant difference is observed in cytotoxicity on cancer cell lines when the DFLE is compared with control and standard (5-Flourouracil). Similarly, more pronounced effect was observed in estrogen receptor-positive cells. At the concentration of 1000 µg/mL, DFLE has significantly reduced the growth of cancerous cell line and found effective against MCF-7 Human breast cancer cell line. So, it has been concluded that the DFLE acts as potential Anti-cancer agent against MCF-7 (Human breast cancer) cell line (Figure 1).

Where, a-5-Flurouracil 100 µg/mL, b-DFLE 200 µg/mL, c-DFLE 400 µg/mL, d-DFLE 600 µg/mL, e-DFLE 800 µg/mL, f-DFLE 1000 µg/mL.

**Group wise Percent cytotoxicity on MCF-7 cell line**

**Acute oral toxicity study**

The body weight of experimental animal remains unchanged which signs non-toxicity on the animal during 14 days and zero

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Groups</th>
<th>Treatment received</th>
<th>Dose of drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal control</td>
<td>Distilled water</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Negative control</td>
<td>DMBA</td>
<td>25 mg/kg in 1 mL emulsion of saline and olive oil (sc).</td>
</tr>
<tr>
<td>3.</td>
<td>Standard</td>
<td>DMBA+5-Flurouracil</td>
<td>25 mg/kg (i.p).</td>
</tr>
<tr>
<td>4.</td>
<td>Test-1</td>
<td>DMBA+DFLE</td>
<td>200 mg/kg (oral).</td>
</tr>
<tr>
<td>5.</td>
<td>Test-2</td>
<td>DMBA+DFLE</td>
<td>400 mg/kg (oral).</td>
</tr>
</tbody>
</table>

**Table 1: Animal grouping for treatment.**
mortality was observed. Based on this the lethal dose LD<sub>50</sub> was calculated for the higher proportion i.e. 2000 mg/kg. The effective dose ED<sub>50</sub> was calculated as 1/10<sup>th</sup> of lethal dose and thus the final dose regime was kept in the range of s 200 mg/kg and 400 mg/kg.

**Effect of DFLE on 7, 12-Dimethylbenzeneanthracene induced carcinogen in rats**

**Tumor incidence**

The 7, 12-Dimethylbenzeneanthracene (DMBA) dissolved in Olive oil was given through subcutaneous route (25 mg/kg/mL body weight) in abdomen and flank region to develop the mammary tumor. Total 30 female Sprague Dawley rats were used in experiment. A first sign of tumor was observed after 6<sup>th</sup> week in total 90 days of study period. All animals were checked by touching, palpitation, inspection and tumor were measured by Vernier caliper. Tumor with 1.0 mm or more diameters were considered as positive.

At the end of experiment tumor development in each group were analyzed and recorded (Table 2).

**Anti-tumor activity**

The oral administration of DFLE in DMBA induced tumor bearing rats and intraperitoneal administration of 5-Flurouracil, showed that there was a significant decrease (<i>p</i>&lt;0.05) in the tumor incidence to 68% and 55% after DFLE 400 and 200 mg/kg treatment and 75% after 5-Flurouracil treatment and decrease tumor volume when compared with DMBA treated group rats.

### Number of tumors developed

In the DMBA treated group 6 out of 6 animals developed a tumor, in DMBA+5-Flurouracil treated group 3 out of 6 animals developed a tumor and in DMBA+DFLE 200 and 400 mg/kg treated group 5 and 3 out of 6 animals developed a tumor. The study recorded 100% tumor incidence in DMBA treated group.

### Latency period of tumor

Latency period of tumor formation in DMBA treated groups was observed after 6<sup>th</sup> week of tumor induction in 90 days study period.

### Tumor yield

The average numbers of tumor were found to be 2 to 4 tumors individually per animal in mammary gland at both sides.

### Group wise morphological examination of tumor

#### Body weight

The average body weight of all animals in different groups was recorded weekly. The development was observed in body weights from 0<sup>th</sup> day till the end of experiment. The body weights were found to be significantly reduced in DMBA treated tumor bearing animals. Whereas, administration of 5-Flurouracil and DFLE 200 mg/kg and 400 mg/kg showed significant increases in the body weight (<i>p</i>&lt;0.05). No significant body weight changes were observed in control and treatment groups of rats. The value did not differ significantly (<i>p</i>&lt;0.05) among the groups (Figure 2).

### Table 2: Morphological examination of tumor.

<table>
<thead>
<tr>
<th>Morphological examination</th>
<th>Normal control</th>
<th>Negative control</th>
<th>Positive control</th>
<th>DMBA+DFLE 200</th>
<th>DMBA+DFLE 400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence</td>
<td>-</td>
<td>100%</td>
<td>50%</td>
<td>90%</td>
<td>50%</td>
</tr>
<tr>
<td>No. of tumors</td>
<td>-</td>
<td>6/6</td>
<td>3/6</td>
<td>5/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Antitumor activity</td>
<td>-</td>
<td>0%</td>
<td>75%</td>
<td>55%</td>
<td>68%</td>
</tr>
<tr>
<td>Latency period of tumor</td>
<td>-</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; week</td>
<td>6.2 week</td>
<td>6.4 week</td>
<td>6.2 week</td>
</tr>
<tr>
<td>Tumor yield</td>
<td>-</td>
<td>2/1</td>
<td>2/1</td>
<td>2/1</td>
<td>2/1</td>
</tr>
</tbody>
</table>

![Figure 1: Percentage cytotoxicity on MCF-7 cell line.](image1)

![Figure 2: Body weight of animals (in gms).](image2)
Hematological examination

The mean values of various hematological parameters at end of experiment are as shown in Figure 3.

Where, a-hemoglobin (g/dL), b-mean corpuscular volume (fl), c-hematocrit (%), d-mean corpuscular hemoglobin(g/dL), e-Neutrophils (%), f-Lymphocytes (%), g-Total Leukocyte Count (/L), h-Transient erythroblastopenia of childhood (mcL).

The mean values of hemoglobin (Hb) were significantly \( p<0.05 \) lowered in group 2 as compared to group 1, 3, 4, 5. Treatment groups 4, 5 had comparable values with that of group 1. In group 2, Hb value is significantly decreased. This might be due to oxidative stress caused by DMBA. Compared to group 1 and 3 herbal group showed significantly comparable mean values this might indicate that herbal extract have helped in maintaining the Hb values.

The Mean Corpuscular Volume (MCV) values did not show any significant difference among various groups when compared to control group. The result suggests that there was no significant effect of administration of herbal extract.

The mean Hematocrit values were significantly decreased \( p<0.05 \) in group 2 as compared to group 1 and group 3, 4, 5. Among the group 1 and group 3, 4, 5 there was no significant difference in their mean hematocrit values.

The mean Total Erythrocyte Count (TEC) was significantly \( p<0.05 \) lowered in group 2 as compared to group 1 and group
The mean TEC of groups 4, 5 were non-significant from each other.

All the groups other than group 1 showed significant increase \( p < 0.05 \) in Mean Corpuscular Hematocrit (MCHC) values.

Group 2 showed significantly decrease \( p < 0.05 \) in the leukocyte count compared to all other groups. All groups' values were significantly comparable to each other.

The mean lymphocyte, neutrophils, Monocyte and Eosinophil values did not show any significant difference among the various groups when compared to group 1.

The mean monocyte values did not show any significant differences among the various groups when compared to group 1.

**Biochemical examination**

The mean values of various biochemical parameters at the end of experiment are presented in Figure 4.

Where, a-SGPT (U/mL), b-SGOT (U/mL), c-ALP (U/L), d-Albumin (g/dL), e-Creatinine (mg/dL), f-BUN (mg/dL).

The liver function biomarker parameters showed significantly \( p < 0.05 \) higher SGPT, SGOT, ALP levels and non-significant decrease levels in the serum albumin in DMBA treated group as compared to control group. However, after administration of DFLE there was significant reduction in SGPT, SGOT and ALP levels in comparison to the DMBA treated group.

The kidney function biomarker parameters showed significant \( p < 0.05 \) higher serum creatinine, blood, urea, nitrogen levels in the DMBA group as compared to control group. However, after administration of DFLE significantly reduction of creatinine, blood, urea, nitrogen levels as compared to DMBA treated group.

**Tumor volume and body organ examination**

**Tumor volume**

The tumour volume was calculated using Vernier caliper. Tumor volume has increased in DMBA treated group as compared to control group. However, after the completion of the treatment, a significant reduction \( p < 0.05 \) in the mammary tumor volume of the DFLE treated group was observed as compared to standard DFLE 200 mg/kg and 400 mg/kg dose has shown significant reduction in tumor volume as similar to standard.

There were no such significant difference observed in kidney and liver weight of animals in all groups (Figure 5).

**Histopathology of tumor, liver and kidney**

**Histology of mammary tumors**

Normal histomorphological features of mammary gland were observed. Group 2 showed presence of tumor mass with proliferating neoplastic cells throughout the tissue section. Fibrosarcoma and Moderate basophilia with focal inflammatory cellular infiltration and occasional necrotic changes are noted in the proliferating tumor tissue. Group 3 showed mild to moderate degenerative changes in the tumor mass. Multiple foci of degenerative changes of tumor cells and necrosis with cellular debris formation were noted in the tumor mass.

Mild (+2) reduction in the tumor cells was noted by loss of cellular tissue and presence of necrotic changes. Group 4 and 5 showed mild to moderate degenerative changes in the tumor mass with presence of few areas of proliferating neoplastic cells. Mild (+2) reduction in the tumor cells was noted by loss of cellular tissue and presence of necrotic and degenerative changes leading to cellular debris in the tumor tissue sections. These necrotic and degenerative changes could be attributed to the treatment given to the animals in this group.

**Histology of kidney**

Group 1 showed normal renal parenchyma comprised of renal cortex and medulla. The renal tubules showed normal cellular histomorphology of epithelium with intact cell borders and nucleus. There was absence of any pathological or inflammatory changes in the kidney tissue sections. Group 2 showed normal renal parenchyma. The vascular tissue appeared normal with focal congested blood vessels. Focal vascular congestion and interstitial hemorrhages observed. Overall, minimal pathological changes were observed in the kidney section. Group 3 showed normal renal parenchyma comprised of renal cortex and medulla. There was absence of any pathological or inflammatory changes in the kidney tissue sections.

**Histology of liver**

Group 2 showed less glandular differentiation (80% grade 3 Vs 7% grade 3; \( p < 0.001 \), less frequently had fibrin deposition at the tumor-liver parenchyma interface (21 vs 56%), expressed CA9 only in a minority of cases at the interface and had significantly lower macrophage chakley count. The result not showed any difference in other group.

**CONCLUSION**

Breast cancer is a prominent cause of death among women in both industrialized and developing nations. The potential of Dolichandrone falcata Seem. Leaves extract was tested against the mammary carcinoma induced in experimental animal. The results of in vivo assessments, morphological examination, Hematological examination, Biochemical examination and tumor examination are evident to convince the potential of Ethanolic extract of Dolichandrone falcata Seem. leaves against the cancer and it can act as potential Anti-cancer agent against mammary cancer.
ACKNOWLEDGEMENT

The authors are grateful to all the direct and indirect hands that has helped us during every step of study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Study protocol was sanctioned from the Institutional Animal Ethics Committee and regulation was approved by CPCSEA (Protocol No- RDCOP/PCOL-02/IAEC/2018-2019/03).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMBA: 7, 12-Dimethyl benzene anthracene; DFLE: Dolichandrone falcata leaves extract; MCV: Mean corpuscular volume; HB: Hemoglobin; HCT: Hematocrit; MCHC: Mean corpuscular hemoglobin concentration; MCH: mean corpuscular hemoglobin; EO: Eosinophil; BO: Basophil; TEC: Total erythrocyte count; TLC: Total leukocyte count; SGPT: Serum glutamate pyruvic transaminase; SGOT: Serum glutamate oxaloacetic transaminase; ALP: Alanine phosphate; BUN: Blood urea, nitrogen; CPCSEA: Committee for the Purpose of Control and Supervision on Experimental Animals; IAEC: Institutional Animal Ethical Committee.

SUMMARY

The anticancer potential study of Dolichadrone falcata leaves ethanolic extract performed on Sprague Dawley female rats as per standard protocol given in OECD guideline. The results of critical in vivo assessments have convinced the efficacy of the extract against the mammary carcinoma.

REFERENCES