Anticancer Potential of Ethyl Acetate Extract Fractions of *Ipomoea horsfalliae* Hook on DMBA-Induced Breast Cancer Model

Muhammed ashraf V. K. 1,*, Kalaichelvan V. K. 1, Venkatachalam V. V. 1

1 Department of Pharmacy, Annamalai University, Annamalai Nagar-608002, Tamil Nadu, India
* Correspondence: ashrafvkclt@gmail.com (M.A.V.K.);

Abstract: The genus *Ipomoea* is distributed globally and honored as the largest genus of the family Convolvulaceae. Several varieties of this family have been shown to be effective in treating various diseases, including cancer. This research aimed to explore the anticancer activity of ethyl acetate fractions of *Ipomoea horsfalliae* Hook (EAIH) in female Sprague-Dawley rats. 7, 12-dimethylbenz(a)anthracene (DMBA) was used to produce breast cancer. The Fractions were selected based on the cytotoxicity analysis in vitro, which was reported in our earlier studies. The study employed two dosages of EAIH (25 and 50 mg/kg). Biochemical, hematological, and antioxidant characteristics were investigated. A decrease in mean tumor volume and tumor weight was detected in EAIH treated groups. The blood parameters were seen as normal. In both DMBA and doxorubicin groups, malondialdehyde was increased, and the level was significantly reduced in EAIH-treated groups. The effect of catalase was shown to be diminished in the groups given DMBA and doxorubicin but normal in the EAIH groups. Nitrate and nitrite levels increased in the DMBA control groups but were normal in the others. There was less necrosis and infiltration in breast tissues treated with doxorubicin as well as in EAIH. In animals treated with EAIH, the therapeutic effect was found to be dose-dependent. The therapies helped to repair some of the altered breast patterns. The study concludes that *I. horsfalliae* may be a potential anticancer candidate and need to explore further.

Keywords: *Ipomoea horsfalliae* Hook; breast cancer; EAIH; anticancer potential; DMBA induced model.

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1. Introduction

Extreme cell proliferation, wrong regulation of cellular differentiation, and insufficient apoptosis are all features of breast cancer [1]. Breast cancer is one of the most common cancers in women, accounting for 25% of all new cases (1.7 million) and 15% of cancer deaths [2]. On the other hand, inflammation and angiogenesis have been proven to be critical for primary cancer development, invasion, and metastases in a number of investigations over the last decade [3,4,5]. Chemically incited rat models of mammary malignancy have been widely utilized to imitate human breast carcinogenesis for many years. The mammary glands of a few rodent strains, primarily Sprague-Dawley and Wistar rats, are more vulnerable to chemical carcinogens, with DMBA and N-methyl nitrosourea being the two most commonly used breast cancer inducers [6,7]. The tumor-induced by this model resembles human estrogen-dependent breast cancer in terms of morphology and histology [8]. Several chemoprevention indicators
have been developed. They depend on how chemopreventive drugs can use one or more combination mechanisms to prevent carcinogenic cascade, such as promoting enzymes to detoxify carcinogenic substances, scavenge reactive oxygen species, enhance apoptosis, and suppress apoptosis the proliferation of cells [9,10].

Free radicals play a critical role in the development of tumors by direct chemical reactions or changes in cell metabolism, and their scavengers can suppress cancer cells in various stages of carcinosis. Antioxidants can also regulate reactive oxygen (ROS) toxicity as an impediment to ROS formation [11]. The original strategy to chemotherapy involves individual alkylating and antimetabolite therapy which is now replaced by combination therapy since two or more medicines were demonstrated in conjunction with improved outcomes[12]. Antioxidants can be used to limit the toxicity of reactive oxygen (ROS) by the impediment in ROS generation. Modern breast cancer management or treatment includes surgery, chemotherapy, radiation treatment, hormonal treatment, and anti-Her-2 medication [13,14]. However, the enormous treatment options and breakthroughs in breast malignancy therapy continue to generate several reactions that badly affect the quality of life [15]. Thus, the search for treatments that can reduce side effects, either alone or in conjunction with medications already used to treat breast cancer, has a lot of potential in the drug discovery process.

The species *I. horsfalliae* belongs to the family Convolvulaceae, which is well known as “Morning glory” which has simple and dark green alternate leaves. Its inflorescences have the color of deep fuchsia and grow on the end of the branches [16]. This species comes from the West Indian islands and is grown worldwide as a blooming climber in the tropics. They have attractive red-purple flowers and bright dark green leaf and are therefore used mainly for ornamental purposes [17]. The *I. horsfalliae* species are not edible; their flowers are reported for photoprotective activity [18]. In addition to medicinal importance, certain plants also belong to the *Ipomoea* genus used for crops and industrial applications. For example, *I. Aquatic* for removing Auramine O dye as a green adsorbent [19]. In the paper industry, *I. carnea* is used [20]. The role of *I. Cairica* and *I. tricolor* for allelopathic crop weed effect have been reported [21,22], *I. Carnea* is used as a fuel source [23]. The literature does not contain scientific evidence or reports about the benefits of *I. horsfalliae* in breast cancer. We hypothesized that *I. horsfalliae* aerial parts may include numerous phytochemicals with anticancer potential and can heal breast cancer. Therefore, this work aims to assess the anticancer activity of *I. horsfalliae* using the DMBA induced model for breast cancer.

2. Materials and Methods

2.1. Chemicals.

The DMBA was supplied from sigma chemical company St. Louis, MO, USA. All other chemical materials used in this study are of analytical quality, obtained from Himedia private laboratories ltd., Mumbai, India

2.2. Collection, extraction, and fractionation of plant material.

Aerial parts of *Ipomoea horsfalliae* Hook were obtained locally from Calicut district (Kerala, India). Extraction and fractionation methods used have already been reported in our previously published paper [24]. The fractions with better cytotoxicity on Human breast cancer cells (MCF-7) in *in vitro* studies were used for the present studies. This study used two doses of ethyl acetate fractions of *I. horsfalliae* (EAIH) (25 and 50 mg/kg).
2.3. Selection of experimental animals.

Female Sprague–Dawley rats (125-175 g body weight) were employed. The studies were prepared and carried out in accordance with ethical guidelines approved by Institutional Animal Ethical Committee (IAEC).

2.4. Tumor induction and drug treatment.

DMBA is a potent carcinogenic agent used in animals to induce tumors at a single dose (30 mg/kg). The DMBA was administered orally with an intragastric tube to the respective groups. They developed tumors after 14 weeks. The EAIH 25 mg/kg and EAIH 50 mg/kg (test samples) were administered orally for four weeks. Rats selected for the study were fasted overnight and sacrificed. The blood was collected for further biochemical studies, and the serum was isolated.

2.5. Experimental design.

For animal experiments and evaluation of different parameters, previously reported methods were carried out [25]. Animals are grouped, and treatment was given as follows:

- Group I: 0.25% of carboxymethyl cellulose (CMC) was received orally and is considered a normal control;
- Group II: Mammary tumor-induced rats by DMBA;
- Group III: DMBA administered mammary tumor groups. Animals are treated with doxorubicin once weekly for 4 weeks (2 mg/ kg, i.p);
- Groups IV and V: DMBA administered rats treated with EAIH 25 mg /kg and EAIH 50 mg /kg respectively for 4 weeks (suspended at 0.25% CMC) p.o. Drug administration began when tumors became stable and palpable.

2.6. General observations.

Throughout the experiment, the animal’s total body weight gain was recorded every week. At the end of the study, animals were sacrificed, and different organs were dissected and weighed out, including the liver, kidneys, spleen, and heart.

2.7. Tumor weight and tumor volume.

To assess tumors, Vernier calipers were used [26], and the volume of tumors was measured as follows,

\[ \text{Volume of tumor} = \frac{(L \times W^2)}{2} \]

where L and W deliberated as length and width in centimeter, respectively.

2.8. Hematological parameters.

The blood from the animals’ retro-orbital plexus was collected and measured with the help of a veterinary blood cell counter.

2.9. Antioxidant and biochemical evaluation in breast tissue.

The breast tissues were removed, and super cold PBS was used to wipe, blot, and gauge the blood. The tissue homogenate was prepared in a potassium chloride solution. The homogenate obtained was centrifuged for 10 min at 8000 rpm (4 °C) to get a clear supernatant, which was used to estimate antioxidant and biochemical parameters.

2.10. Assessment of nitric oxide (NO).
The nitrogen oxide levels of the breast tissue were evaluated by measuring the cumulative nitrate and nitrite concentrations. To estimate the nitrite content, the Griess reagent method was used. The Griess reagent (100 μL) and the homogeneous tissue (100 μL) were incubated at 37 °C for 20 min, the absorbance was read at 540 nm, and the nitrite content was derived from the standard sodium nitrite curve [27]. 100 μL of homogenate from breast tissue was incubated at 45 °C for 60 min with Griess reagent and vanadium (III) chloride. To measure absorbance at 540 nm, a microplate reader was employed.

2.11. Lipid peroxidation analysis.

Briefly, 8.1 percent of sodium dodecyl sulfate (200 μL), 20 percent acetic acid (1.5 ml) solution, and 0.8 percent aqueous thiobarbituric acid solution (1.5 mL) were treated with breast tissue homogenate (200 μL). The total volume is then composed of up to 4 ml of distilled water and then heated at 95 °C for 1 h. The cooled mixture was added to the distilled water (1 ml), n-butanol (5ml), and pyridine mixture. The mixture was heavily shaken and then centrifuged for 5 minutes at a rate of 5000 rpm. The top layer was discarded, and the absorbance measurement was at 532 nm using the UV spectrophotometer [28].

2.12. Assessment of catalase level.

The breast tissue homogenate was then added to 3 ml of hydrogen peroxide solution for catalase level evaluation. At 240 nm, the absorbance was recorded [29].

2.13. Assessment of serum biomarkers.

For the assessment of Creatinine, Alanine transaminase (ALT), Urea and Aspartate aminotransferase (AST), appropriate autoanalyzer kits (Roche Diagnostics, Indianapolis, USA) were used.

2.14. Histopathological evaluation of breast tissues.

In order to support the histopathological assessment of the breast, tissues were fixed in neutral buffered formalin (10 percent), washed with alcohol, and cleared with xylene. (RM22545, Leica Microsystems GmbH, Wetzlar) Paraffin wax impregnated tissue, and a 5 μm section were then fixed on the slides and dewaxed with the help of xylene and rehydrated by using alcohol using a rotating microtome. The tissues were then stained with hematoxylin and eosin. In the tubular alveolar pattern, the slides were monitored and interpreted under a microscope by an expert (pathologist) to identify the type of carcinoma, immune cell infiltration, necrosis, and hemorrhage.

2.15. Statistical analysis.

The one-way variance analysis (ANOVA), followed by "Tukey's post hoc test" with GraphPad Prism 6.05 version, was used to evaluate statistical comparisons and significance. Two-way ANOVA was used to examine the effect on tumor growth, followed by "Tukey's post hoc test." All outcomes were presented as Mean ± SEM, considering the significance value of p >0.05.
3. Results and Discussion

3.1. Treatment impact on organ weight and body mass.

In our study, the liver, heart, and kidneys’ weight between treatment and control groups were not significantly changed. However, the spleen’s weight was significantly decreased in the doxorubicin administered groups compared with DMBA and normal control groups. There was no remarkable variation in body weight seen among all groups (Table 1).

Table 1. Impact of the treatments in Sprague Dawley rats on body weight and organ weight.

| Groups            | Heart (g) | Spleen (g) | Kidney (g) | Liver (g) | Growth in body weight (%) |
|-------------------|-----------|------------|------------|-----------|---------------------------|
| Normal Control    | 0.85 ± 0.77 | 0.74 ± 0.64 | 0.77 ± 0.32 | 5.23 ± 0.32 | 116.67 ± 5.76             |
| DMBA control      | 0.81 ± 0.28 | 0.77 ± 0.33 | 0.73 ± 0.23 | 5.45 ± 0.23 | 126.32 ± 5.22             |
| Doxorubicin       | 0.78 ± 0.67 | 0.34 ± 0.45b | 0.78 ± 0.34 | 5.34 ± 0.24 | 117.78 ± 5.82             |
| EAIH 25mg/kg      | 0.83 ± 0.24 | 0.73 ± 0.47 | 0.74 ± 0.75 | 5.55 ± 0.67 | 120.47 ± 4.65             |
| EAIH 50mg/kg      | 0.88 ± 0.35 | 0.75 ± 0.63 | 0.76 ± 0.48 | 5.78 ± 0.37 | 121.17 ± 5.75             |

All the values are measured in mean ± SEM; a,p < 0.05 compared to normal control groups; b,p < 0.05 compared to DMBA Control; n= 6.

3.2. Treatment impact on tumor growth and the weight of tumors.

The impression of the treatment on tumor progression was measured by a reliable (each week) evaluation of tumor volume. The rats were sacrificed, and parameters such as mean tumor weight and mean tumor volume was observed. After one week of treatment, doxorubicin decreased the tumor volume significantly. Compared to control rats treated with DMBA alone, all animals treated with EAIH significantly reduced the mean tumor volume and mean tumor weight at both doses (Figures 1 and 2). In rats treated with EAIH 25 mg/kg and EAIH 50 mg/kg, tumor volume decreased significantly after three weeks of EAIH treatment when connected with DMBA control groups. In the fourth week, the most significant decrease in tumor volume was seen.

Figure 1. Influence of EAIH and Doxorubicin on tumor development. The drug treatments were continued for four weeks period. All treatments began after the tumor growth was palpable, and the start of drug treatment was measured as 0th week. All values are expressed as mean ± SEM; *p < 0.05 compared to DMBA Control; n=6.
Figure 1. Influence of the treatments on mean tumor weight; All the values are given as mean ± SEM; *p < 0.05 compared to DMBA Control; n =6.

3.3. Impact of treatment on blood parameters.

The Red blood cell counts (RBC) and hemoglobin levels were significantly reduced in groups administered with doxorubicin when compared with normal control. It may be a clear consequence of the bone marrow toxicity of doxorubicin. No significant changes in any other blood parameters were produced at both doses of EAIH (Table 2).

| Groups          | RBC (×10⁶/μl) | WBC (×10³/μl) | Platelets (×10³/μl) | Hb (g/dl)   |
|-----------------|---------------|---------------|---------------------|-------------|
| Normal Control  | 8.35 ± 0.32   | 12.45 ± 0.24  | 417 ± 19.37         | 12.23 ± 0.76|
| DMBA control    | 7.76 ± 0.54   | 11.53 ± 0.38  | 465 ± 23.76         | 11.53 ±0.56 |
| Doxorubicin     | 5.44 ± 0.74   | 11.64 ± 0.54  | 417 ± 14.56         | 8.54 ± 0.34 |
| EAIH 25mg/kg    | 7.77 ± 0.85   | 12.56 ± 0.34  | 426 ± 22.64         | 12.67 ± 0.21|
| EAIH 50mg/kg    | 8.54 ±0.76    | 11.63 ± 0.84  | 433 ± 16.76         | 12.48 ± 0.56|

All the values given as mean ± SEM; *p < 0.05 compared to normal control; n =6.

3.4. Impact on catalase levels and lipid peroxidation in breast tissue.

In the animal groups treated with DMBA and doxorubicin, catalase activity in breast tissue was significantly reduced compared with normal controls. However, in comparison with normal control, the catalase activity of the EAIH treated animal groups was not significant. In both doxorubicin and DMBA control-treated rats, the level of malondialdehyde (MDA) was significantly increased. Compared to the normal control groups, the difference in MDA levels in the EAIH treated groups at both doses did not produce any significant changes (Table 3).

| Groups          | Catalase (U/mg of protein) | MDA (nmol MDA/mg of protein) |
|-----------------|-----------------------------|-----------------------------|
| Normal Control  | 43.36 ± 1.42                | 1.08 ± 0.21                 |
| DMBA control    | 24.47 ±1.75                 | 2.52 ± 0.86                 |
| Doxorubicin     | 24.03 ± 1.32                | 2.79 ± 0.48                 |
| EAIH 25mg/kg    | 43.63 ± 1.86                | 0.93 ± 0.31                 |
| EAIH 50mg/kg    | 46.53 ± 1.67                | 0.90 ± 0.28                 |

All the values are given as mean ± SEM; *p < 0.05 compared to normal control; b*p < 0.05 compared to DMBA control; n =6.
3.5. Treatment impacts biochemical parameters.

Aspartate transaminase (AST) was increased in the DMBA control group and the doxorubicin-treated group compared to the normal group, but the level was normal in the EAIH-treated group. This can be attributed to the toxic effect on the liver from DMBA and doxorubicin. In both the DMBA and doxorubicin treatment groups, the level of urea was significantly increased compared to the normal control groups. However, the level of urea was almost normal in groups with EAIH. There were no significant alterations in the levels of alanine aminotransferase (ALT) and creatinine in any group (Table 4).

| Groups             | Urea (mg/dL)   | Creatinine (μmol/L) | AST (U/L)     | ALT (U/L)  |
|--------------------|----------------|---------------------|---------------|------------|
| Normal Control     | 27.83 ± 0.64   | 31.24 ± 0.32        | 127.83 ± 2.22 | 63.34 ± 0.34 |
| DMBA control       | 35.13 ± 0.39   | 30.34 ± 0.42        | 153.36 ± 1.63 | 62.76 ± 0.32 |
| Doxorubicin        | 33.25 ± 0.25   | 30.15 ± 0.25        | 159.63 ± 2.25 | 60.34 ± 1.73 |
| EAIH 25mg/kg       | 30.76 ± 0.54   | 29.23 ± 0.27        | 128.53 ± 2.53 | 62.64 ± 0.35 |
| EAIH 50mg/kg       | 29.65 ± 0.75   | 31.86 ± 0.33        | 131.74 ± 1.48 | 63.97 ± 0.85 |

All the values are given as mean ± SEM; *p < 0.05 compared to Normal control; n=6.

3.6. Treatment impact on levels of nitrate and nitrite in breast tissue.

The levels of nitrate and nitrites were elevated in the DMBA-treated control group, indicating oxidative stress. However, the levels for all other treatment groups were near normal (Figure 3).

3.7. Histopathology studies on breast tissue.

Tissue infiltrated into lobules, abundant fibrous stroma, and proliferated ductules were shown in breast tissue sections from the DMBA control tumor groups. In large areas, necrosis has been recognized as well. These typical characteristics have been designated as invasive ductal carcinoma. The breast tissues collected from doxorubicin-treated rats showed less necrosis and infiltration; similarly, the EAIH 25 mg/kg and EAIH 50 mg/kg tested samples also showed less necrosis and infiltration. Some of the damaged breast patterns were refurbished to normal by the treatments (Figure 4).
Currently, breast cancer is a common neoplasm among females and has a great level of occurrence of mortality [30]. At present, breast cancer managements consist of surgery, chemotherapies, radiation therapy, hormone, and immunotherapy, though these remedies have many toxic effects [31]. Herbal products are used and examined to identify and enhance therapeutic anticancer agents. The use of animal models in experiments is mostly valuable for learning about human mammary cancer. To induce a tumor, we employed a single dosage (30 mg/kg) of 7, 12-dimethylbenz (a) anthracenes (DMBA) produced in olive oil administered using an intragastric tube. The ductal region of the human breast is where malignant growth begins, and the DMBA-induced mammary tumor model follows the same [32]. DMBA-instigated carcinogenesis had been recorded to be related to ductal carcinoma, fibroadenoma, and papilloma [33]. Multiplication in human breast carcinomas occurs predominantly in epithelial cells [34,35,36]. Therefore, the rat model is a precious animal model for human breast cancer studies. The mammalian rat glans then have a good chance of developing neoplasms that closely imitate human breast cancer [37]. The toxicity of DMBA is due to its oxidative metabolism, which produces free radicals that bind to nucleophilic sites via covalently connecting with cellular macromolecules, producing carcinogenic processes. Our research used two dosages of 25 mg/kg and 50 mg/kg EAIH body weight. Doxorubicin was chosen as the reference medicine (standard), and it was given at a dose of 2 mg/kg i. p. Animals treated
with EAIH at any dose had no significant increases in body weight or organs such as the kidney, liver, spleen, or heart, according to the findings. All of the EAIH animals treated showed a significant reduction in tumor volume and weight compared to the DMBA control rats.

The free radicals and their biochemical reactions are part of cancer growth at each metabolic stage [38]. Herbal polyphenols have been shown to have anticancer properties in previous studies, particularly in human cancer cells [39,40,41]. Carcinogenesis has been linked to oxidative stress, specifically lipid peroxidation [42,43]. Catalase is found throughout the body and causes hydrogen peroxide to break down in tumor cells. SOD and catalase levels have been found to drop in a variety of carcinogenic situations [44]. The decisive result of lipid peroxidation, malondialdehyde (MDA), was shown to be higher in carcinomatous tissue than in normal tissue [45]. A raised degree of lipid peroxide level in the control group reflects the overproduction of free radicals and the failure of the antioxidant protection system to execute sufficiently during the occasion of tumor growth [46].

RBC and hemoglobin levels in doxorubicin-treated rats were considerably lower than in normal control groups in our study. This could have been an immediate result of doxorubicin's bone marrow poisoning. Lipid peroxidation in tissues can reveal the degree of oxidative danger [47]. The levels of MDA in the breast tissue were measured, and the DMBA control and doxorubicin-given groups had significantly higher amounts than the normal control animals. When compared to normal control, the change in MDA levels in EAIH treated groups was not significant. In comparison to the normal animal group, the amount of breast tissue catalase in the DMBA control and doxorubicin controlled groups was significantly reduced. A high sensitivity-free radical present within the body is nitric oxide (NO) which causes malignant growth and genotoxicity [48]. NO's metabolites, such as nitrate and nitrite, can be measured in physiological fluids and tissues. The DMBA control groups' nitrate and nitrite levels showed signs of oxidative stress, and the levels were found to be higher in those groups in our investigation. The levels of nitrate and nitrite were found to be close to normal in EAIH-treated groups. The scavenging of NO by antioxidant phytoconstituents present may have been the work for this plant. According to the literature review, several plant extracts and isolated compounds such as M. capitatum [49], M. trifoliate [50], P. santalinus [51], A. annua [52], S. vulgare [53], C. Cupreum [54], C. formicarum [55], C. indicum [56], C. glabrum [57], C. viscosum [58], F. grahamiana [59], W. Somnifera [60], C. Annuum [61], A. elata [62], etc., were reported for anticancer activity.

In our investigation, the breast tissues from the rats treated with standard drug doxorubicin and the tested sample EAIH 25 mg/kg and EAIH 50 mg/kg showed less necrosis and infiltration in a dose-dependent way. The EAIH therapies were able to restore some of the typical breast patterns. Herbal remedies have long been a rich source of chemotherapeutic drugs, and I. horsfalliae could be an anticancer candidate.

4. Conclusions

The current study suggests that EAIH may have inhibitory effects on DMBA-induced breast cancers, which could be attributed to the phytochemicals found in this plant. The antioxidant components in EAIH may be responsible for its ability to reduce DMBA-induced oxidative damage. More research is being done to extract and demonstrate the promising elements that contribute to its anticancer properties.
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Conflicts of Interest

The authors declare no conflict of interest.

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