



## Original Article

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Methanolic extract of *Ephedra alata* inhibits breast cancer cells *in vitro* and *in vivo*Fairouz Sioud<sup>1,2✉</sup>, Aida Lahmer<sup>1,2</sup>, Mouna Selmi<sup>1</sup>, Fadwa Chaabane<sup>1,2</sup>, Leila Chekir–Ghedira<sup>1,2</sup><sup>1</sup>Research Unit Bioactive Natural Products and Biotechnology UR17ES49, Faculty of Dental Medicine of Monastir, University of Monastir, Avicenne Street, 5000 Monastir, Tunisia<sup>2</sup>Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Monastir, Avicenne Street, 5000 Monastir, Tunisia

## ABSTRACT

**Objective:** To determine the anticancer potential of the methanolic extract from *Ephedra alata* against breast cancer both *in vitro* and *in vivo*.

**Methods:** The effects of the methanolic extract of *Ephedra alata* on the viability, migration as well as apoptosis of breast cancer 4T1 cells were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Transwell assay, and annexin V-FITC staining assay, respectively. Histological examination was also carried out. Moreover, a murine breast cancer model was established to evaluate the inhibitory effect of the extract. Biochemical parameters including hepatic and non-hepatic enzymes, malondialdehyde, and glutathione were investigated.

**Results:** The methanolic extract of *Ephedra alata* showed a strong anti-proliferative and anti-migratory activity against 4T1 cells in a dose-dependent manner. It also induced apoptosis in 4T1 cells. In an *in vivo* mouse model, the extract markedly inhibited tumor growth, reduced malondialdehyde, and hepatic and non-hepatic enzymes as well as increased glutathione level.

**Conclusions:** The methanolic extract of *Ephedra alata* inhibits breast cancer *in vitro* and *in vivo*, which may be a promising anticancer agent.

**KEYWORDS:** *Ephedra*; Breast cancer; Apoptosis; Antioxidant; Migration

## 1. Introduction

Malignancies and cancer have recently become a primary focus for health-active communities all around the world. Following lung tumor, breast cancer has been recorded as the second prevalent cause

of death among women[1]. In 2020, 2.3 million cases and 685 000 deaths were recorded[2], and 4.4 million cases are anticipated to be reported in 2070[3].

Cancer is the uncontrolled growth of aberrant cells that can be distinguished from benign and malignant tumors. Breast and colon cancer are two of the most prevalent malignancies discovered. Furthermore, malignant cells frequently exhibit irregular metabolism. Increased formation of reactive oxygen species (ROS) has been detected when cancer progresses, contributing to excessive cell proliferation and invasion[4].

ROS plays an essential role in tumor promotion through direct chemical reactions or perturbation of cellular metabolic processes. Superoxide dismutase, catalase, and glutathione (GSH) are

## Significance

*Ephedra alata* is a natural source of many bioactive phytonutrients with high antioxidant, cytotoxic, and growth-inhibitory effects against cancer cells and tumors. The current investigation shows that the methanolic extract of *Ephedra alata* suppressed the proliferation and migration of breast cancer cells as well as induced cell apoptosis. It also inhibited tumor growth in mice, reduced oxidative stress, and increased glutathione levels. Therefore, it may be a potential therapeutic agent for the treatment of breast cancer, which needs further investigation.

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ROS scavengers that serve as inhibitors at various phases of carcinogenesis[5].

Finding a drug that can modify the redox system could be helpful and useful for tumor eradication. The metabolic imbalance produced by free radicals can potentially be reduced by bioactive substances derived from plants. Recently, researchers have become interested in discovering powerful anti-cancer drugs inside natural items. Several plant extracts and products have been found to protect against free radicals by inducing the expression of antioxidant genes[6,7].

Recently, we have shown the abundance of flavonoids, phenolic acids, and proanthocyanidins in the methanolic extract of *Ephedra alata* (*E. alata*). The LC-MS/MS analysis showed a predominant presence of quercetin 3-*O*-[6''-(3-hydroxy-3-methylglutaryl)]-*D*-galactoside[8]. We have also demonstrated that this extract plays a significant role in preventing and neutralizing free radicals, actively preventing cisplatin-induced oxidative damage *in vivo*[9]. Thus, the objective of this work was to assess the antitumor and antioxidant effects of the methanolic extract of *E. alata in vivo*.

## 2. Materials and methods

### 2.1. Chemicals and reagents

DMEM medium, trypsin, streptomycin, penicillin, and fetal bovine serum were purchased from Sigma Cell Culture (Courtaboeuf, France). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Euromedex (Mundolsheim, France). Triton X-100, ribonuclease A (RNase), phosphate-buffered saline (PBS), sodium hydroxide and propidium iodide (PI) were purchased from Sigma-Aldrich (Steinheim, Germany).

### 2.2. Preparation of methanolic extracts of *E. alata*

*E. alata* was sourced from the Sahara region of Tataouine, located in the southeast of Tunisia, in February 2017. The plant material was shade dried, powdered and stored at room temperature. The plant was taxonomically identified by Pr. Fethia Skhiri (Higher Institute of Biotechnology of Monastir, Tunisia) according to the Tunisian Guideon Flora. A voucher specimen (EA001) was deposited in our laboratory.

To prepare the methanolic extract, 100 g sample of dried aerial parts of the plant was extracted by maceration in 1 L of methanol for 7 d at room temperature. Following this, the liquid was filtered, and the solvent was removed using a rotary evaporator under reduced pressure at 40 °C, resulting in a methanol extract[8].

### 2.3. MTT assay

Using the MTT assays, cell proliferation was evaluated. In a 96-well plate, we seeded the 4T1 cells [a murine mammary carcinoma

cell line that was obtained from American Tissue Culture Collection (ATCC, Molsheim, France)] at a density of  $1 \times 10^4$  cells per well. These cells were subsequently cultured in 200  $\mu$ L of DMEM media including various doses of the methanolic extract of *E. alata* (0, 20, 40, 60, 80, and 100  $\mu$ g/mL). The MTT solution was applied to each well and incubated for 24 and 48 h[10]. A Thermo Scientific (Vantaa, Finland) plate reader was used to measure the solution's absorbance at a wavelength of 492 nm.

### 2.4. Annexin V-FITC staining assay for apoptosis analysis

PI and annexin V-FITC staining were used to measure the apoptosis of 4T1 cells. First, different doses of the methanolic extract of *E. alata* were applied to 4T1 cells over 48 h. After incubation, the cultivated cells were completely cleaned with PBS three times, stained for 5 min in the dark with annexin V-FITC and PI, and then analyzed on a flow cytometer (FACS system, Switzerland)[11].

### 2.5. Transwell chamber assay

To assess the migrative capacity of 4T1 cells, we employed a Transwell chamber test. Cells were treated with various concentrations (0, 30, and 60  $\mu$ g/mL) of the methanolic extract of *E. alata* for 48 h. The cells were then seeded to the upper chamber with serum-free DMEM medium and serum-containing DMEM medium was added to the lower chamber as a chemoattractant for 24 h. After incubation, the cells under the membrane were stained with 0.1% crystal violet and photographed[12].

### 2.6. Experimental animals

A total of 24 female BALB/c mice (8 weeks old and weighing  $\approx 25$  g) were received from the Pasteur Institute in Tunisia. Mice were housed in an approved pathogen-free facility with conventional settings of 24 °C, 50% humidity, and 12 hours of light/dark. All animals had free access to normal rodent food and filtered water.

### 2.7. Experimental design and tumorigenesis assessment

To generate a breast tumor,  $1 \times 10^6$  4T1 cells were subcutaneously injected into the mammary fat pad area. All mice were checked by palpation every week to monitor tumorigenesis. Treatment started on the seventh day. All mice were treated for two weeks (three times per week) by intraperitoneal injection. Animals were randomly divided into four groups: the untreated group without a breast tumor (as a negative control), the untreated group with a breast tumor (as a positive control), the tumor group treated group with 50 or 150 mg/kg of the methanolic extract of *E. alata*. For all groups, dimethyl sulfoxide (DMSO) was used at the final concentration of 0.1% and the two control groups received a saline solution with 0.1% of DMSO.

## 2.8. Determination of tumor inhibition rate

Throughout the investigation, the tumor volumes of mice were calculated after 18 days using the following formula:

$$Tv\text{ (mm}^3\text{)} = d^2 \times D/2$$

where d and D are the shortest and the longest diameters, respectively. 24 h after the last administration, mice were sacrificed by cervical dislocation under anesthesia with 2% isoflurane inhalation, and tumors were peeled off and weighed.

The tumor inhibition rate was calculated according to the following formula:

Tumor inhibition rate = (average tumor weight of the control group – average tumor weight of the experimental group)/average tumor weight of the control group × 100

## 2.9. Histological study

Collected tumors were fixed with 10% formaldehyde. They were then placed in various baths with alcohol to eliminate the water. This will then allow the impregnation of the formalin-containing paraffin in the tissue to dehydrate and dissolve the fats in the tissue. The samples are finely cut into 5 µm thick sections with a microtome (KD-2268 Manual Microtome). The sections are then stained with hematoxylin and eosin (H&E) for observation using an optical microscope (Leica Microsystems, Germany)[13].

## 2.10. Biochemical analysis

Aspartate transaminase (AST), alanine transaminase (ALT), creatinine, and alkaline phosphatase (ALP) were analyzed by the Biochemistry Department Laboratory of the University Hospital Center using an automated analysis with a commercial Cobas Integra kit (Roche, Boulogne-Billancourt, France).

## 2.11. Non-enzymatic antioxidant assay

The Moron technique was used to determine the glutathione (GSH) content present in liver and kidney homogenates[9]. Briefly, to precipitate the tissue proteins, 100 µL of homogenate was mixed with 10% sulfosalicylic acid and vortexed. The mixture was centrifuged at 5000 rpm for 20 min. Subsequently, supernatant, sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>), and 5,5'-dithiobis-(2-nitrobenzoic acid) were added successively. The absorbance was measured within 10 min at 412 nm. Commercially available GSH was used as a standard. The level of GSH was expressed as µmol GSH/mg of protein.

## 2.12. Evaluation of lipid peroxidation status

Kidney and liver homogenates were mixed with trichloroacetic acid and thiobarbituric acid and then incubated at 95 °C for 2 h. The reaction was stopped by immediate cooling in ice, and then

the samples were centrifuged at 2500 rpm for 10 min at 4 °C. The reaction between thiobarbituric acid and the lipids produced by oxidative degradation generates red complexes measured using a microplate reader (Vantaa, Finland) at 530 nm[9]. The concentration of malondialdehyde (MDA) (µmol per mg proteins) was obtained by extrapolating absorbance values to concentrations of the standard curve of MDA.

## 2.13. Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical comparisons between groups were conducted using one-way and two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A *P*-value < 0.05 was considered statistically significant.

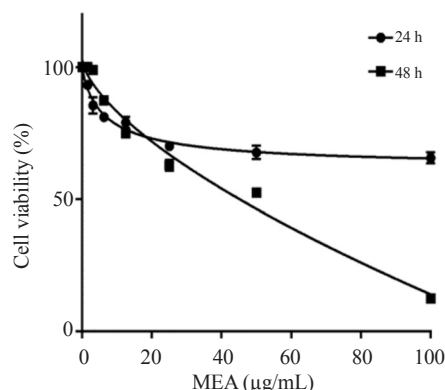
## 2.14. Ethical statement

Directives regulating the welfare of experimental animals, and experiments were approved by the Life Sciences and Health Research Ethics Committee of the Institute of Biotechnology (University of Monastir, Tunisia; Reference ethical approval: CER-SVS/ISBM 009/2020) in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no.85-23, revised 1996).

# 3. Results

## 3.1. Methanolic extract of *E. alata* inhibits the proliferation of 4T1 cells

The methanolic extract of *E. alata* inhibited the proliferation of 4T1 cells in a dose-dependent manner. The IC<sub>50</sub> value was 65 µg/mL after 48 h of incubation (Figure 1).



**Figure 1.** Antiproliferative effect of the methanolic extract of *Ephedra alata* on 4T1 cells. Cells were treated with different concentrations of *Ephedra alata* for 24 and 48 h and cell proliferation was measured by MTT assay. MEA: Methanolic extract of *Ephedra alata*.

### 3.2. Methanolic extract of *E. alata* induces apoptosis in 4T1 cells

The flow cytometry analysis showed that the methanolic extract of *E. alata* markedly increased the early and late stages of apoptosis in 4T1 cells (Figure 2). Specifically, apoptotic cells significantly increased at 60  $\mu\text{g/mL}$  and 120  $\mu\text{g/mL}$ . More than 70% apoptotic cells were observed in cells treated with 120  $\mu\text{g/mL}$  of the methanolic extract of *E. alata*.

### 3.3. Methanolic extract of *E. alata* inhibits the migration of 4T1 cells

The methanolic extract of *E. alata* efficiently inhibited 4T1 migration in a dose-dependent manner (Figure 3A) and the inhibition rates were 76% and 85% after treatment with 30 and 60  $\mu\text{g/mL}$  of the methanolic extract, respectively (Figure 3B).

### 3.4. Methanolic extract of *E. alata* inhibits tumor growth in mice

After 6 days of treatment, the growth of tumors was significantly decreased compared to the control group (Figure 4). After 12 days, this difference was sharply accentuated. The inhibition rates were

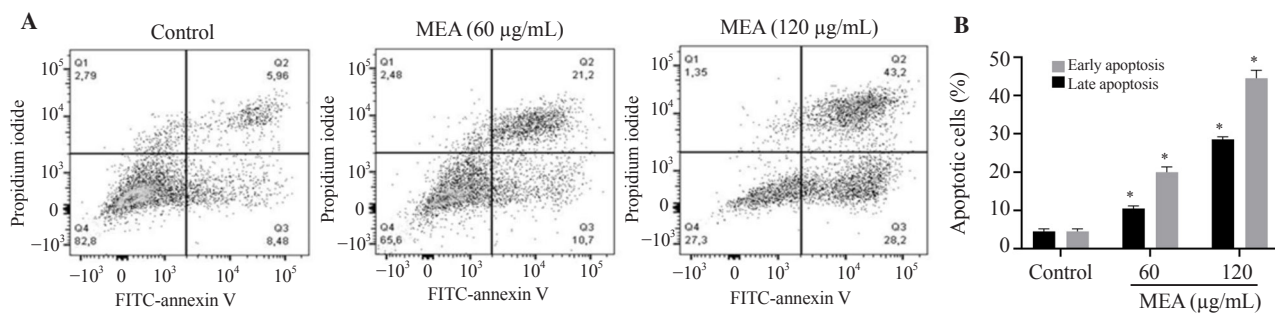
46.16% and 68.8%, for the group treated with 50 and 150 mg/kg of the methanolic extract of *E. alata*, respectively.

### 3.5. Histopathological results

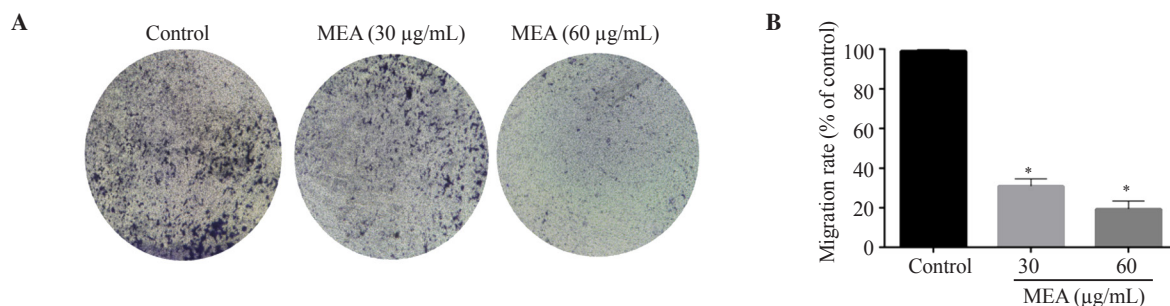
The sections of the positive control group showed infiltration of tumor cells. In neoplastic cells, large hyperchromatic nuclei and a relatively small amount of cytoplasm were noted. These neoplastic cells showed special characteristics, including pleomorphic cells and nuclei, and giant cells. The results indicate cells under mitotic division in some areas (Figure 5A). In most areas of the group treated with the extract, a significantly increased degree of cell necrosis could be observed. The tumor section of mice treated with 150 mg/kg of the extract showed larger areas of necrosis which exceeded half of the total area of the tumor (Figure 5B).

### 3.6. Effect of the methanolic extract of *E. alata* on liver function markers

An increase in ALT, AST, and ALP activities was observed in the positive control group, confirming liver damage, whereas treatment with 150 mg/kg of the extract decreased ALT and ALP activities, and 50 mg/kg of the extract only reduced ALP activity (Figure 6A-C).

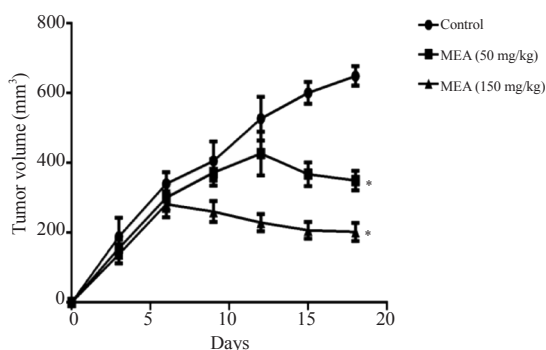


**Figure 2.** Flow cytometric analysis of early and late apoptosis in 4T1 cells treated with the methanolic extract of *Ephedra alata*. After 48 hours of treatment, 4T1 cells were stained with FITC-annexin V/propidium iodide. Apoptosis was assessed using flow cytometry. (A) Apoptosis profile plots. (B) The percentages of early and late apoptosis in 4T1 cells treated with the methanolic extract of *Ephedra alata*. The data are presented as mean  $\pm$  standard deviation (SD) of three independent experiments and analyzed by one way ANOVA followed by Tukey's multiple comparison test. \* $P < 0.01$  compared to the control group.



**Figure 3.** Anti-migratory effect of the methanolic extract of *Ephedra alata* in 4T1 cells. A Transwell chamber was used for the migration assay and images were captured. (A) Representative photomicrographs of stained cells (Magnification,  $\times 40$ ). (B) The percentage of migrative cells. The results are expressed as mean  $\pm$  SD of three independent experiments and analyzed by one way ANOVA followed by Tukey's multiple comparison test. \* $P < 0.01$  compared to the control group.





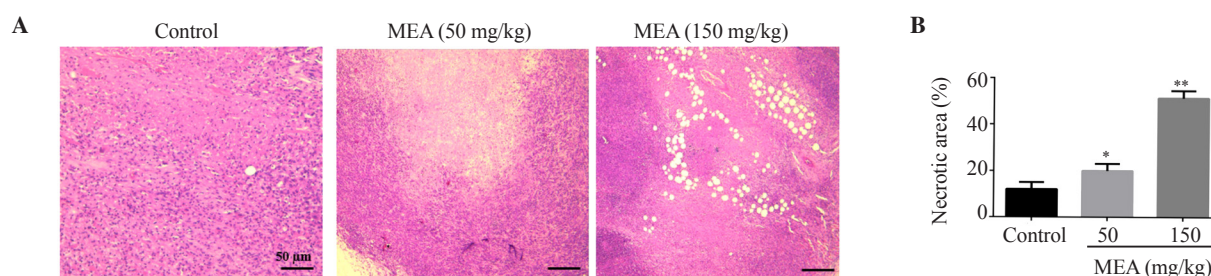
**Figure 4.** Tumor volume after treatment with the methanolic extract of *Ephedra alata*. The results are expressed as mean tumor volume  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. \* $P < 0.01$  compared to the control group.

### 3.7. Effect of the methanolic extract of *E. alata* on creatinine level

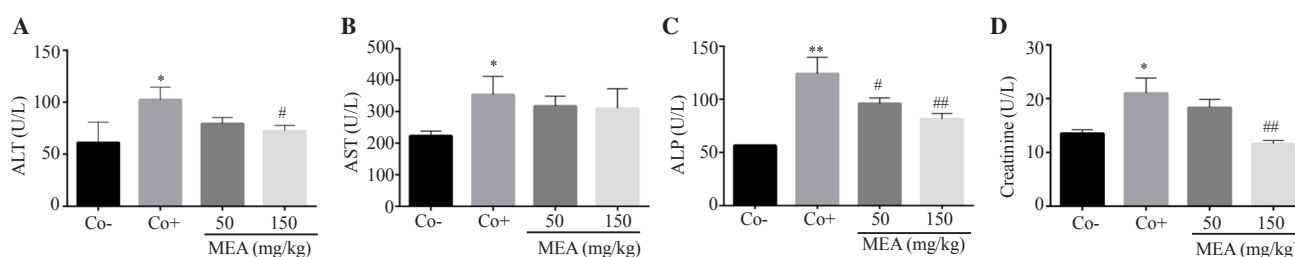
Creatinine level was significantly increased in the positive control group. However, treatment with the methanolic extract of *E. alata* reduced the creatinine level in a dose-dependent manner (Figure 6D).

### 3.8. Effect of the methanolic extract of *Ephedra alata* on lipid peroxidation

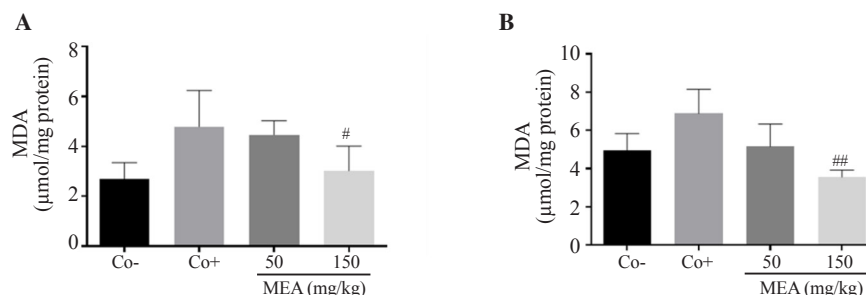
An increase in hepatic and renal MDA levels was observed in the positive control group. Administration of 150 mg/kg of the methanolic extract of *E. alata* attenuated lipid peroxidation in the kidney and liver,



**Figure 5.** Effect of the methanolic extract of *Ephedra alata* on histology in mice with breast tumor. (A) Representative sections of tumors from untreated mice and mice treated with different doses of the methanolic extract of *Ephedra alata* (Magnification: 200 $\times$ ). (B) The percentage of necrotic cells. The results are expressed as mean  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. \* $P < 0.05$ , \*\* $P < 0.01$  compared to the control group.



**Figure 6.** Effect of the methanolic extract of *Ephedra alata* on (A) alanine transaminase (ALT), (B) aspartate transaminase (AST), (C) alkaline phosphatase (ALP), and (D) creatinine. The results are expressed as mean  $\pm$  SD of three independent experiments and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. \* $P < 0.05$ , \*\* $P < 0.01$  compared with the negative control group; # $P < 0.05$ , ## $P < 0.01$  compared with the positive control group. Co-: the negative control group; Co+: the positive control group.



**Figure 7.** Lipid peroxidation level in the (A) kidney and (B) liver of treated mice with 4T1 breast cancer cell-induced tumor. The data are expressed as mean  $\pm$  SD of three independent experiments and analyzed by one way ANOVA followed by Tukey's multiple comparison test. # $P < 0.05$ , ## $P < 0.01$  compared with the positive control group. MDA: malondialdehyde.

thus preventing further damage caused by tumor progression (Figure 7).

### 3.9. Effect of the methanolic extract of *E. alata* on GSH

Mice in the positive control group showed a reduction in the level of GSH. Treatment with 150 mg/kg of the methanolic extract of *E. alata* significantly increased the level of GSH (Figure 8).

## 4. Discussion

Inhibiting the growth of tumor cells or eradicating them without harming healthy cells is the primary goal of the use of anti-cancer drugs. One of the most efficient cancer treatment strategies is the use of natural products, which are less harmful than chemotherapy and radiation treatment[14].

The current study was conducted to assess the anticancer and antioxidant effects of the methanolic extract of *E. alata* in mammary carcinoma-bearing mice. The 4T1 mammary carcinoma is a transplantable tumor cell line that is extremely tumorigenic and invasive. Unlike most tumor models, it can spontaneously metastasize from the initial tumor in the mammary gland to various distant locations including lymph nodes, blood, liver, lung, brain, and bone[15].

Our findings showed that the methanolic extract of *E. alata* had anti-cancer properties *in vitro*. The methanolic extract of *E. alata* greatly inhibited the development of 4T1 cells by preventing cell migration and proliferation as well as inducing apoptosis. The  $IC_{50}$  value of the methanolic extract of *E. alata* was found to be 65  $\mu$ g/mL, substantially lower than those of other plant extracts[16,17]. These findings suggest the methanolic extract of *E. alata* is a potent anticancer agent.

In mice with breast tumors, treatment with 50 and 150 mg/kg of the methanolic extract of *E. alata* significantly reduced the tumor volume compared to the control group. An obvious necrosis was

observed in tumor mice that received the extract treatment.

Serum enzymes have been investigated for a long time as potential early markers of neoplasia[18] as well as a method for early detection of liver metastatic lesion in patient with breast cancer[19].

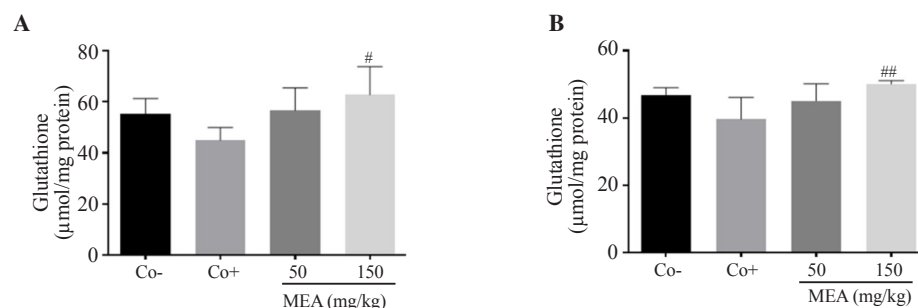
In the present study, the analysis of biochemical markers revealed that the untreated group with tumors showed elevated levels of liver enzymes such as ALT, AST, and ALP due to hepatocellular injury. Treatment with the extract reduced the increased markers, showing protection against tumor cell-induced hepatotoxicity.

Oxidative stress can cause macromolecule damage *in vivo*, such as lipid peroxidation[20]. In the present study, the positive control group showed significantly higher levels of MDA in the liver and kidney, which were lowered to near-normal levels in the extract-treated groups. This reflects the decrease in free radical formation and subsequent reduction in oxidative stress, which are the primary risk factors for the disease.

GSH, an effective inhibitor of neoplastic development, functions as an endogenous antioxidant system. It was detected in significant amounts in the liver and is thought to play an important role in the protective process[15]. Its production increases under oxidative stress and inflammation. The present findings show that GSH levels were decreased in control mice, most likely due to a high level of free radicals. These results are consistent with previous studies that have shown that glutathione levels are significantly reduced in the blood of breast cancer patients compared to those of healthy control subjects[21]. Treatment with the methanolic extract of *E. alata* increased GSH concentration in the liver and kidney.

The findings of the *in vitro* and *in vivo* experiments demonstrated that the methanolic extract of *E. alata* possesses an anti-cancer effect. However, there are some limitations in this study. The anticancer mechanism of the methanolic extract of *E. alata* is not elucidated and the active compounds responsible for the anticancer activity of this plant extract are not determined. Therefore, further studies are needed to unravel these aspects.

In conclusion, the methanolic extract of *E. alata* inhibits the



**Figure 8.** Glutathione level in the (A) kidney and (B) liver of treated mice with 4T1 breast cancer cell-induced tumor. The data are expressed as mean  $\pm$  SD of three independent experiments and analyzed by one way ANOVA followed by Tukey's multiple comparison test. # $P < 0.05$ , ## $P < 0.01$  compared with the positive control group.

migration and proliferation of breast cancer cells and tumor volume in mice, which suggests that it may be a promising anticancer agent for the treatment of breast cancer.

## Conflict of interest statement

The authors have no conflicts of interest to disclose.

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## Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

## Authors' contributions

FS and LCG supervised and designed the study. FS and AL performed *in vivo* study. MS and FC performed data collection and analytic calculation. FS contributed to the final version of the manuscript.

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