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In-Vivo Inhibitory Potential of Argemone Mexicana Leaf Extract on Chemical Induced Mammary Tumors in Wister Rats.

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ABSTRACT:

Background: Breast cancer, major threat worldwide, even with a variety of treatment approaches, the death rate is still high. *Argemone mexicana,* traditionally used in several conditions, experimentally, plant extract showed anti-microbial, antioxidant, cytotoxic, and anti-cancer properties. There was lack of reports regarding its anticancer activity in-vivo. **Aim:** This study is aimed at investigate anticancer properties of *Argemone mexicana* leaves (AML) extract against MNU-induced mammary tumors by comparing the tumor volume, external features and histopathological results of the experimental groups. **Methodology:** Experimental rats divided into 4-groups: Group I-normal control, Group II-tumor control (MNU alone, 50 mg/kg/b.w.), Group III-treated with 500 mg/kg/b.w. of AML, and Group IV- treated with Tamoxifen (TAM) 10 mg/kg/b.w. Treatment were started immediately nest to tumour induction. After the experimental period, collected mammary tumor mass, calculated weight and volume of the tumor, and compared external features and histopathological studies. All data were analyzed by Students t-test and results were statistically significance ($P<0.05$). **Results:** Tumor weight and volume were reduced in AML and TAM-treated groups compared to tumor control. External features were observed in tumour control rats, tumor mass under growing beneath the skin, deep wound with ulcerated skin compare to treated rats. Microscopic studies revealed that there was cellular improvement and fewer pleomorphic changes in extract-treated groups, like TAM-treated rats, compared to MNU-treated rats. There was severe infiltration of hyperchromatic and pleomorphic nuclei in tumour induced rats. In rats treated with MNU-induced AML, there was a large rise in the expression of caspase-3 and Bax proteins, but a significant decrease in PCNA and, Bcl-2 protein expression. The aforementioned proteins' expression is restored after AML treatment. **Conclusion:** From the results, *Argemone mexicana* leaf extract might have antitumor/anticancer activity by reducing tumor volume, and showing improvement in external features and under microscope. But isolation and identifying individual compounds for anticancer properties can be continued as extended activities.

Keywords: *Argemone mexicana* leaves (AML), Anticancer activities, MNU, Tumour volume, external features and histopathology, Tumour protein expression.

1. Introduction:

Herbal extracts were broadly accepted for availability, lower cost, safe, rare side effects. Plants, contains phytochemicals, which occurs naturally in all medicinal plants, and potential to protect from several conditions. Alkaloids, flavonoids, steroids, volatile oils, and other phenolic compounds are examples of phytochemicals (1). The traditional medical system and the modern medical system are two different areas of health care that make substantial use of medicinal plants worldwide (2). Approximately half of the pharmaceuticals currently in clinical use come from natural compounds and their derivatives, with about 25% coming from higher plants (3). According to WHO estimates, 855 traditional medications contain crude plant extracts, and an average of 80% of people in underdeveloped nations rely on herbal medicinal plants for traditional remedies for everyday needs. The world's population, estimated at 3.5 to 4 billion, depends on plant resources for medication (4). A few notable examples of pharmaceuticals originating from plants are aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine, and vinblastine (5).

Approximately 460 different plant species can be used as herbs for medicinal purposes, including various types of anti-cancer plant are Wood Apple (*Aegle marmelos*), American Aloe *(Agave Americana),* Garlic (*Allium sativum*), Graviola (*Annona muricata*), Naked ladies (*Antumn crocus*), Birch (*Betula alba*), Green tea (*Camellia sinensis*), Cinnamon bark (*Cinnamomum cassia*), Coriander (*Coriandrum sativum*), Curcumin (*curcuma longa*), Bamboo Grass (*Loathatreum Gràcies*), Sunflower (*Helianthus annus)* and others (6). Several species, including *A. mexicana*, *A. pleiacantha,* and *A. ochroleuca,* have demonstrated a range of therapeutic benefits, including antimicrobial, analgesic, antibiotic, antimalarial, anti-inflammatory, and anti-tumor consequences (7). Almost every traditional medical system, including ayurveda, unani, and sidha, has used *A. mexicana.* Indigenous, rural, and tribal people in our nation have long used this herb to treat a variety of ailments (8).

A. mexicana is recognized as a significant medicinal plant in India; the yellow liquid that the plant releases when it is wounded has long been used as a traditional remedy for dermatological diseases, jaundice, ophthalmia, dropsy, and scabies (9). It has also been stated that leaves and seeds can help the human body maintain regular blood circulation and cholesterol levels (10). The entire *A. mexicana* plant's aqueous extract has strong hepatotoxicity-prevention properties (11). The alkaloid that was separated from the aerial portion was cytotoxic to human gastric cancer and nasopharyngeal carcinoma cell lines (12). Varun and Sudha, (2014) also reported Based on results of the MTT test, the methanolic extract of *A. mexicana* leaves has anticancer action against the cancer cell lines HeLa and MCF-7. Investigations into the effects of *A. mexicana* extract on cell viability and apoptosis induction revealed that treated MCF-7 cells showed a considerable increase in apoptosis in comparison to the untreated group, and the results indicated that *A. mexicana* treatment greatly increased caspase activity (13). It has been observed that *A. mexicana's* ethanolic extract has inhibitory effect against human cancer cell lines. This cytotoxic activity is apoptotic rather than necrosis-related, and it could be caused by the flavonoid components found in leaves. (14).

With breast cancer making up 23% of all female cancer cases worldwide, it is the most frequent cancer among women, occurs among women 35 - 54 years of age in which 2% breast cancer occurs in women between 20 - 34 years and 10% breast cancer occur in women over 80 of age (15). Because breast cancer is variable in terms of both genetic makeup and histology, it is still unknown what mechanism(s) underlies the development of breast cancer (16). Targeted therapy for many malignancies remain inadequate despite advances in medication delivery and cancer research. Contemporary anticancer treatments have enhanced clinical effectiveness and selectivity for cancer cells; yet, their efficiency is restricted by the emergence

of resistance and a multitude of adverse effects, making their utilization in chemotherapy regimens more challenging (17). The potential of manufactured and naturally occurring compounds found in food or medicinal plants to protect against cancer remains a prominent subject of scientific research (18). The present study investigated the *Argemone mexicana* leaf (AML) extract as reducing agent and studied the anti-tumour activity in rat mammary tumours.

2. Materials & Methods:

Plant collection and preparation of extract

A.mexicana plants that were fresh and in good condition were gathered from the Tamil Nadu district of Coimbatore. Botanical Survey of India, Coimbatore, recognized it (survey no: BSI/SRC/5/23/2021/Tech/260. After the leaves were cleaned under running water, they were allowed to air dry in the shadow for seven days. After that, they were ground into a fine powder and sealed in bottles. Using a clean and sterile soxhlet apparatus, 50g of fine power was extracted using 250 mL of distilled water. The extracts were collected for additional analysis following extraction.

In-Vivo Experiment:

40-day-old virgin female Wistar albino rats weighing between 100 and 120 grams were obtained from JKKN college of pharmacy and research institute, Kumarapalayam, Namakkal. Tamilnadu, India. Throughout the trial, the six animals in each group were given a normal pellet diet (Hindustan Lever Limited, Mumbai, India) and had access to free, daily-refilled water. The animals were kept in spacious, well-ventilated polypropylene cages with controlled lighting (12 hours of light and 12 hours of darkness), humidity (50–55%), and temperature (25–2°C). In accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) rules, the animal experiments were conducted. The experimental strategy used in this investigation, Wister albino rats as an animal model for anticancer activity, was authorized by the Institutional Animal Ethics Committee (JKKN/IAEC/Ph.D./04/2021). In terms of mortality and reported as LD50, the toxicological effects were noted at a maximal dose of 2000 mg/kg. It was recorded how many animals died during that time and LD50 was determined.

Experimental groups and induction of mammary tumor

Before the trial began, the rats were given fifteen days to get used to the lab environment. To attain balance with regard to pretest body weights, animals were allocated at random to treatment groups according to their most recent pretest body weight. The animals were split up into 4 groups, each with 6 animals.

Group I: Controls (Rats treated with 2ml/kg Saline)

Group II: Tumour induced rats (Rats given a single intraperitoneal (IP) injection of MNU at a dose of 50 mg/kg as a carcinogen (19).

Group III: Treated rats (Rats treated with MNU and then AML administered at 500 mg/kg/day for 24 weeks)

Group IV: Drug controls (Rats given tamoxifen for 24 weeks at a dose of 25 mg/kg per day according to Faustino, 2017) (20).

Rats received single i.p. injections of NMU, dissolved in 0.9% of saline, and heated to 50–60°C at the age of 50 days. Following the carcinogen injection, AML treatment was

initiated right away and continued until the studies were completed. The animals were palpated once a week for six weeks following their NMU injection in order to track the development of mammary tumors. For histological results, treatment was continued for up to 24 weeks, and for molecular findings, up to 35 weeks. Biweekly application of phorbol myristate acetate (PMA, 1.83ml/ml of acetone) was continued upto induced epidermal tumors in precancerous skin with emergence of palpable tumors free of necrosis (21). Animals were sacrificed by cervical dislocation at the conclusion of the studies, and their bodies were autopsied. After tumors were removed, half of them were preserved at -70 degrees by freezing in liquid nitrogen, and the other half was preserved for histological analysis in 10% neutral buffered formalin.

Morphometric study

The food and water intake of the experimental rats, as well as any obvious toxicity, weight loss, or mortality, were routinely observed. Six weeks following NMU treatment, palpation and the timing of the emergence of the breast tumor were started. Every week, the rats were given a mild ether anesthesia, and the tumors were measured using a Vernier calibre to obtain two-dimensional measurements (Calculation of volume: Tumor width (W) and tumor length (L) are represented by the formula $V = (W2 \times L)/2$, denoted in cm^3) (22). Incidence of tumor and multiplicity were recorded. Observed tumours external features in mammary gland, excised tumours were compared with morphology and its histology.

Histological study

Formalin fixed (10%) tissues were embedded in low-melting-point paraffin after being dehydrated with graded alcohol. Cut sections, each 5 μm thick, were arranged in a serial fashion on glass slides. After sections were deparaffinized in xylene and rehydrated with 100%, 90%, and 70% alcohol, each mammary tissue sample was divided into three adjacent sections, which were then stained with hematoxylin and eosin (23). A coverslip containing DPX was placed under a light microscope (Olympus CH 20i Tr) for histological examination.

Western blotting method:

Tris–HCl (pH 7.5) 20mM, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulphonyl fluoride, and 1 mg/mL aprotinin were added to the homogenized mammary tissue. Centrifuging the homogenate for ten minutes at 4 oC at 14,000 rpm was done. Sohlenius-Sternbeck (2006) approach was utilized to determine the protein concentration in the homogenate (24). The materials underwent a 5-minute boil in sample solubilizing buffer before being separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis by the method of Kielkopf et al., (2021) (25). After the gel was placed onto a nitrocellulose membrane and exposed to 30 V for three hours, it was thrice cleaned with phosphate-buffered saline and blocked using Tris-buffered saline containing 5% nonfat dried milk and Tween-20. Following that, the membrane was gently shaken at room temperature for three hours while being treated with primary antibodies (rabbit monoclonal antibody, Bax, Bcl-2, PCNA, and caspase-3). Following the primary antibody incubation, the blots were buffer-washed and then incubated at room temperature for 75 minutes with a secondary goat rabbit anti-goat horseradish peroxidase conjugated antibody. Chemical luminescence was used to identify the bands (26).

Statistical analysis

Fischer's exact probability test was used to compare the incidence of mammary tumors statistically among the various experimental groups. Student's t-test was used to statistically

assess all other data. The threshold for significance was set at $p < 0.05$. Three replicated quantitative data were used to calculate the average, standard deviation, and standard error.

3. Results and Discussion

Morphometric findings

General observations

Over the course of the investigation, there was no discernible change in the animals' daily consumption of food or water due to therapy. Since there was no discernible difference between the final body weights and the usual range, the animals' growth was unaffected throughout the entire investigation of any of the treatments. The outcomes align with previous literature (27,28,29).

Tumor incidence & multiplicity

Four of the six rats in the tumor control group (group II) acquired a tumor at least once, for a total of nine tumors in that group. On the other hand, there were five tumors in the group of rats treated with AML, and two of them were totally tumor-free. The incidence of NMU-induced mammary tumors and the total number of tumors per rat were both dramatically decreased by treatment with AML (Table 1).

Table 1. Incidence and multiplicity of tumors in rats treated with AML and controls

Rat group	0 Tumors	1 Tumor	2 Tumors	3 Tumors	Total
Normal control	**0**	**0**	**0**	**0**	**0**
MNU Controls (n = 6)	**1**	**4**	**1**	**1**	**9**
AML treated (n = 6)	**2**	**3**	**1**	**0**	**5**
TAM treated (n = 6)	3	2	0	0	**2**

Tumor weight

Tumor weight decreased, which was also indicative of AML's inhibitory action (Table 2). Tumor weight/ group and per rat, the tumour control group's mean tumor weight per rat (5.54 g) was noticeably higher than the AML group's (1.25 g) tumor weight. There was a significant difference in the mean individual tumor weight between the groups.

Table 2. Mean tumor weight for control and AML treated rats

Groups	Total tumor weight/ group	Mean tumor weight/ rat
Normal control	0	0
MNU Controls (n = 6)	36.72 g	5.54 ± 1.2 g*
AML treated (n = 6)	12.60 g	1.25 ± 0.9 g
TAM treated (n = 6)	10.89g	1.09 ± 0.14g

Standard deviation ± mean is used to express values. Significant differences were observed within the groups ($P < 0.05$).

Tumor size

Changes in tumor volume in four groups of rat were assessed. Data indicated that the tumor growth was significantly suppressed in rat treated with methanol extract of *Argemone mexicana* leaf (AML) in comparison with other groups. Enlargement in tumor size there is an increase in tissue vascularization. Treatment of animals with extract of *Argemone mexicana* leaf extract markedly inhibited the tumor growth and/or induced cancer cell death. The

majority of the tumors were smaller than 10 mm in size, and it was common to find numerous tumors of varying sizes in one animal. The number of 5 and 3 mm tumors in NMU-induced rats was significantly reduced by AML therapy, suggesting a decreased rate of tumor growth (Table 3).

Table 3. Tumor size per rat

Groups	**Mean tumor size/ rat**
Normal control (*n* = 6)	0
MNU Controls(*n* = 6)	**28.2**4 ± **3**.2 **mm***
AML treated (*n* = 6)	19.**17** ± **1**.8 **mm**
TAM treated (*n* = 6)	14.78 ± **2**.8 **mm**

Standard deviation ± mean is used to express values. Significant differences were observed within the groups ($P < 0.05$).

When compared to the carcinogen control group, there was a substantial ($p<0.05$) decrease in tumor volume between 30 and 32 weeks, as well as a highly significant ($p<0.05$) decrease in the percentage of tumors between 27 and 30 weeks.The normal controls showed no tumors at any point in time.

Rats treated with AML exhibited a noteworthy decrease in tumor volume in contrast to rats that were stimulated with MNU to develop mammary cancer. This could be because of the flavonoids found in Argemone mexicana leaves, which have been shown to exhibit a wide range of cellular processes and can regulate the entire carcinogenesis process through a number of methods. This may be in charge of tumor regression in AML-treated rats and includes altering survival/proliferation pathways, activating caspases, downregulating the expression of Bcl-2 and Bcl-xL and enhancing the expression of Bax and Bak, as well as modifying nuclear factor kB. (30-34)

Morphology and Histopathological evaluation of the effect of AML on MNU treated rat mammary tissues.

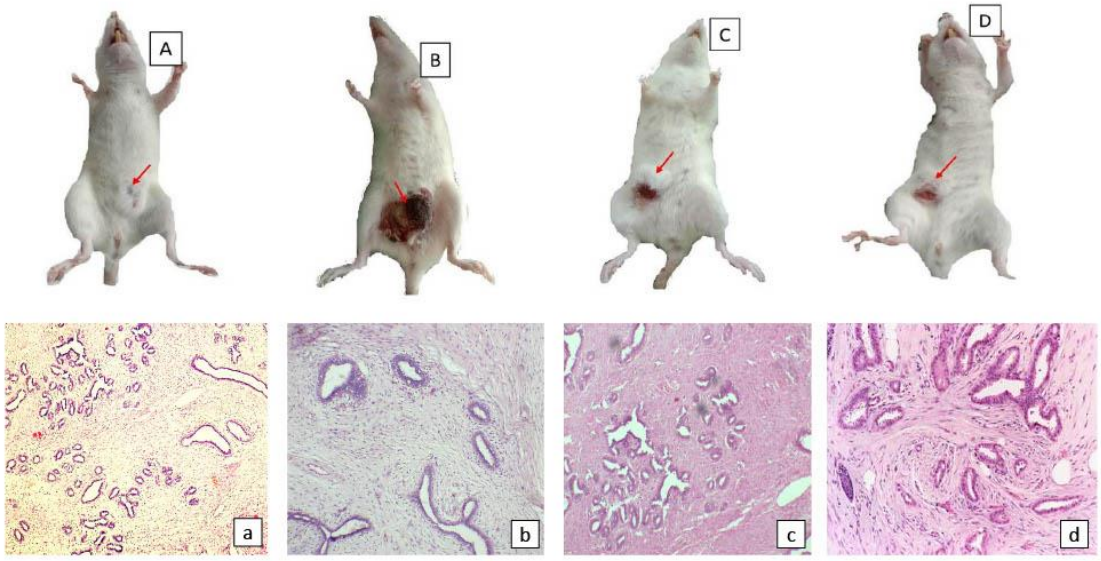

Figure 1. Shows external features of experimental animals mammary tumours and its histological examination: A,a; tumour induced at the beginning stage of nodule developed

(fibroadenoma). B,b; MNU control after 34 weeks developed ductal carcinoma. C,c; treated with AML (500mg/kg), D,d; treated with TAM drug (25mg/kg). (Hematoxylin and Eosin stain, 40 X magnification).

Control group shows no observable distinct changes. A specimen stained with H&E reveals many lobules inside the breast's dense connective tissue. The lobule's branching duct system constitutes the epithelial component. Adipose cells are seen in the clear sections, and some inflammatory cells are also visible (Fig1.A,a). The external features of the tumour control (NMU treated) revealed an undergrown, massive tumour accompanied by thick, ulcerated, edematous skin. The cellular architecture was discovered to have changed, and the alveolus was detected to grow. Cells exhibiting nuclear pleomorphism, which is characterized by nuclear enlargement, chromatid clumping, and a characteristic epithelial hyperplasia, were also observed. In animals treated with MNU carcinogen, epithelial cells displayed fluctuation in nuclear size with uneven chromatin and conspicuous nucleoli (Fig1.B,b). Histological analysis revealed that the AML treatment group has localized epithelial hyperplasia and moderate duct proliferation. Anisonucleosis and mitosis were not present, and epithelial cells had homogeneous sizes.In rats treated with AML, tumors were developed beneath the skin in external characteristics, but there was no undergrowth (Fig1.C,c). In the neighboring duct, there was little epithelial hyperplasia. Rats given TAM had ulcerated skin and moderate tumor-related inflammatory alterations on their skin's surface. Histological observations revealed benign-appearing glandular elements surrounded by cuboidal to low columnar epithelium with round to oval nuclei sitting on the myoepithelial layer, as well as stromal expansion with moderately cellular stroma hypercellularity. The stroma was thick and displayed gaps that resembled clefts and were made up of cells with oval to spindle shapes (Fig1.D,d).

Animals treated with NMU alone showed typical hyperplasia and invasive ductal carcinoma while the AML treatment was found to inhibit the necrosis of epithelial cells in the present study. According to the histological classification to the total number of lesions classified were in-vasive (35 - 44 %). Carcinomas penetrated the surrounding tissues while maintaining the gland's natural architecture. The invasion of tissue was mainly localized. Certain invasive tumors were identified by solid sheets of epithelial cells or projections that resembled fingers penetrating into the surrounding stroma (35). Often seen massive stromal reaction characterized by fibrosis and mononuclear infiltration. Even within the same tumor, individual neoplastic cells were found to exhibit varying degrees of neoplasia. The nucleus/cytoplasm ratio and the nucleoli of epithelial cells were both larger. The tumors in sections H and E were categorized and classified using the WHO's criteria for mammary neoplasms. Though they are a small percentage of tumors brought on by the frequently employed regimens, the most highly malignant tumors in rats share certain characteristics with intraductal and infiltrating ductal carcinomas in humans (36).

Effect of AML extract on tumour initiator and tumour depressor protein levels in MNU treated rats' mammary tissues.

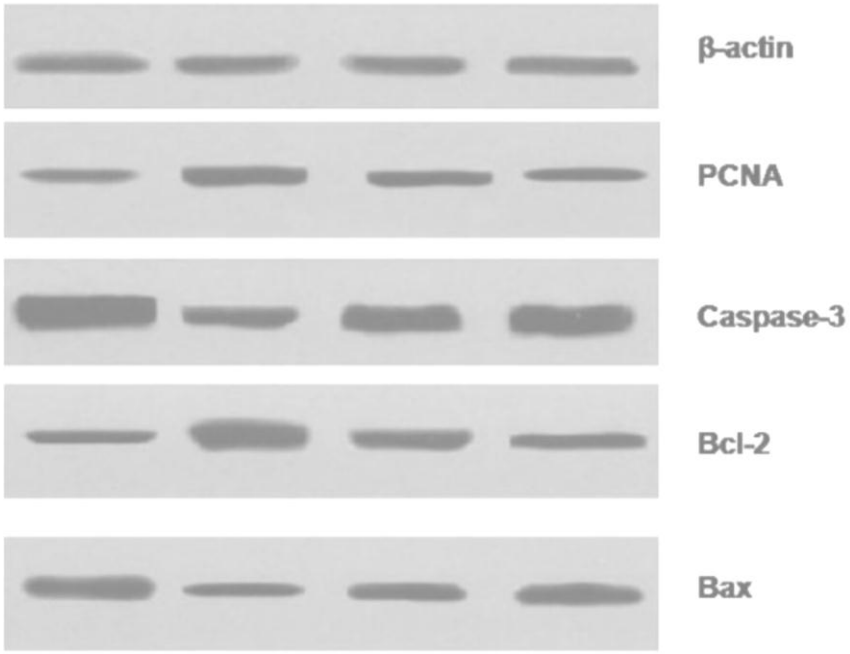

Figure 2. AML effect on expression of PCNA, Caspase 3, Bcl2, Bax in mammary tissues of experimental rats by Western blot technique. Lanes 1: Control, 2: MNU control, 3: AML treated, 4: TAM treated.

AML's impact on the presence of PCNA, caspase 3, Bax, and Bcl-2 proteins in the mammary glands of experimental and control rats. In rats with MNU-induced AML-treated mammary tumors, there was a large rise in Bax and caspase-3 proteins and a significant decrease in PCNA and Bcl-2 proteins. AML treatment reestablishes the expression of the above-mentioned proteins. In the mammary tumor tissues of MNU-induced rats, overexpression of PCNA and Bcl-2 was seen in comparison to AML-treated animals. This suggests aberrant cell proliferation and resistance of apoptosis. Proliferating cell nuclear antigen (PCNA), protein that functions as a cofactor of DNA polymerase (37). proliferating cell nuclear antigen (PCNA), a protein that aids DNA polymerase in its activity plays a crucial part in the cell cycle and is a proliferative marker that is useful for assessing a number of malignancies, including breast cancer.

When cells are actively multiplying during the S-phase of the cell cycle, it is highly expressed.The tremendous multiplication of tumor cells is shown by the elevated expression of PCNA in the mammary tissues of MNU-induced rats, which is consistent with other researchers' similar findings (38). The antiproliferative efficaciousness of AML was shown in the reduced expression of this proliferative marker in rats treated with the mammary tumours. Compared to animals given MNU, AML therapy resulted in higher expression levels of caspase-3 and Bax. To highlight the pro-apoptotic effects of AML in our investigation, we assessed the protein expression of caspase-3, pro-apoptotic Bax, and anti-apoptotic Bcl-2 in specimens of mammary tissues from each experimental group. In rats given MNU, the upregulation of Bcl-2 expression in conjunction with downregulation of Bax (rise in the Bcl-2/Bax ratio) indicates that the rats are evading apoptosis (39). It has been proposed that one factor in the pathophysiology of breast cancer is the dysregulation of apoptosis brought on by

an increase in the Bcl-2/Bax ratio (40). Our investigation confirmed a pro-apoptotic shift in the Bax/Bcl-2 protein expression ratio upon AML therapy (500 mg/kg body weight). The combination of caspase-3 activation and a decrease in Bcl-2 expression, which is a good measure of a cell's general propensity to undergo apoptosis, highlights the ability of AML to induce apoptosis.

4. Conclusion

The potential of dietary polyphenols as anticarcinogenic agents has been demonstrated by several research conducted in various cell lines, animal models, and human epidemiological trials. The growth of AML may be influenced by bioactive components such as flavonoids, polyphenolic compounds, ascorbic acid, b-carotene, and others because of their anti-inflammatory, antioxidant, and immunopotentiating qualities. By inducing apoptosis linked to caspase-3 activation and dysregulating Bcl-2 and Bax in rat mammary tumors generated by MNU, AML extract demonstrates an anti-cancer activity. These findings support the potential of AML as a chemotherapeutic and cytostatic agent in breast cancer cells, since apoptosis has emerged as a novel therapeutic target in cancer research. However, more studies are needed to elucidate the mechanism AML with the aim of developing new strategies for the treatment of cancer with lower cost and effectiveness. Still, the mechanism and pathways by which way AML induce cytotoxic activity on rat mammary tumour need further investigation.

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Authors' contributions: All author equally contributed for concept, data collection, statistical analysis, writing manuscript, review and publication.
Data Availability: All datasets of this study included in the manuscript.

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