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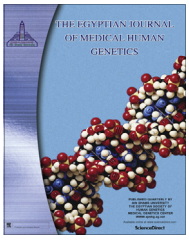

ORIGINAL ARTICLE

Chemopreventive effect of *Annona muricata* on DMBA-induced cell proliferation in the breast tissues of female albino mice

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KEYWORDS

Annona muricata;
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Abstract *Background:* Breast cancer is the most common type of cancer and leading cause of cancer death in women. Breast cancer and cancer related diseases have been treated using surgery, chemotherapy, and radiation therapy, or a combination of these. Despite these therapeutic options, cancer remains associated with high mortality. Traditional medicine which involves the use of herbs has been used to treat various types of cancer and this has been found to be effective with minimal or no side effects.

Aim: This research was aimed at evaluating the potential chemopreventive effect of an ethanolic extract of *Annona muricata* leaves on 7,12-dimethylbenzeneanthracene (DMBA)-induced cell proliferation in the breast tissues of female albino mice.

Materials and methods: *A. muricata* leaves, thirty (30) female albino mice, and 7,12-dimethylbenzeneanthracene (DMBA) were used for this study. Crude extraction protocol was employed in the preparation of an ethanolic extract of *A. muricata* leaves. Qualitative and quantitative phytochemical screening of an ethanolic extract of *A. muricata* leaves was carried out using standard protocol. Agarose gel electrophoresis was used to analyze deoxyribonucleic acid (DNA) extracted from the breast tissues of experimental mice while hematoxylin and eosin staining was used for histological assay.

Results: Phytochemical screening revealed the presence of terpenoid, steriod, flavonoids, cardiac glycoside, tannin, phenol, alkaloid, and reducing sugar. Phenol was quantitatively determined to be present in the highest amount. DNA smears obtained from agarose gel electrophoresis suggested possible DMBA-induced damage which was significantly prevented owing to the effect of the leaf extract of *A. muricata* leaves. Histological assay revealed the presence of DMBA induced lobular alveolar hyperplasia, adenomatoid hyperplasia, fibro adipose stroma, and proliferating sebaceous gland in the histological sections of the breast tissues of treated mice, however, these changes were found to vary in occurrence among the different groups of treated animals.

Abbreviations: DMBA, 7,12-dimethylbenzene[a]anthracene; HE, hematoxylin and eosin; *A. muricata*, *Annona muricata*; DNA, deoxyribonucleic acid

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Conclusion: This study has shown that the leaf extract of *A. muricata* could be used as a prophylactic measure against DMBA-induced cell proliferation in the breast tissues of female albino mice.

1. Introduction

Cancer is a multifactorial disease arising from the accumulative effects of gene products of mutant proto-oncogenes, tumor suppressor genes and DNA repair genes, leading to the uncontrolled growth and spread of cancer cells [1,2]. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected, and these include cervical cancer, skin cancer, leukemia, lung cancer, prostate cancer, and so on [3].

Breast cancer represents the most common neoplastic disease in females, accounting for up to one third of new diagnoses of women's cancer in certain regions of the world [4]. In developing countries traditionally known for low incidence of breast cancer, increase in both incidence and mortality has been recently detected [5]. Azubuike and Okwuokei [6] observed that in Nigeria, the peak age of breast cancer is about ten years earlier than the experience of many western women and were attributed to increasing adoption of western life style and diet. Contrary to previous reports indicating breast cancer as the second leading cause of cancer deaths, breast cancer is now the leading cause of cancer deaths in Nigerian women [7–9].

Breast cancer and cancer related diseases have been treated using surgery, chemotherapy, and radiation therapy, or a combination of these. But despite these therapeutic options, cancer remains associated with high mortality [10]. This is basically due to difficulties in early diagnosis, exorbitant cost of treatment, with the often late presentation of breast cancer that generally characterizes cancer diagnosis among Nigerian and other African women [10–13]. Owing to these several shortcomings, there is a need for better therapeutic options which will increase the chances of survival of breast cancer patients with minimal or no side effects of treatment. Early diagnoses of breast cancer and its prevention have been suggested as a better means of managing breast cancer because about 5% of human breast cancers have been attributed to inheritance of breast cancer susceptibility genes [2,14]. Therefore, people with familial history of breast cancer have better options of diagnosing and preventing its occurrence within their life time.

Among the major problems of breast cancer therapy is the fact that a majority of patients suffering from the disease cannot afford the high cost of therapy [15]. It has also been discovered that more than 70% of all cancer deaths occurred in low and middle-income earners [16]. Nigeria as one of the countries found in the tropics is rich with plants that have been found to possess anticancer therapeutic activity [10]. *Annona muricata* commonly called soursop or "sharp sharp" is a small erect evergreen tropical fruit tree plant belonging to the family Annonaceae, growing 5–6 m in height [17]. It is one of the easily found plants used traditionally in treating cancer. The leaf decoction is usually taken to lessen the symptoms of cancer [18].

In order to develop a better and cheaper means of preventing breast cancer in people liable to suffering the disease in their life time, this work was therefore aimed at evaluating the chemopreventive effect of an ethanolic extract of *A. muricata* leaves on 7,12-dimethylbenzeneanthracene (DMBA)-induced cell proliferation in the breast tissues of female albino mice.

2. Materials and methods

2.1. Experimental animals

The 30 adult mice used for this study were obtained from the Nigeria Institute of Medical Research, Lagos, Nigeria. The animals were housed in standard clean mice cages at 25 °C, fed with standard pellet and tap water *ad libitum*. They were maintained under uniform conditions of natural photo period (12 h light/dark cycle) and humidity (61–95%).

2.2. Plant collection and identification

Leaves of *A. muricata* were collected in the month of May 2013, from the botanical garden of the University of Lagos, Lagos, Nigeria. Leaves were identified and confirmed taxonomically at the Department of Botany, University of Lagos, Lagos, Nigeria. A voucher specimen number LUH 6070 of the plant was deposited in the Herbarium of the same department.

2.3. Plant preparation and extraction

A. muricata leaves were washed in a running tap and air-dried in the Cell Biology and Genetics laboratory, University of Lagos. The dried leaves were later blended using an electric blender which had been sterilized with 70% ethanol. The leaf extract was obtained using Crude Extraction Protocol. About 350 g of *A. muricata* powder was soaked in 70% ethanol for 72 h. This was later filtered using a sieve. The filtrate was concentrated using a water bath set at 40 °C. The final mass of concentrated extract obtained was 40 g. The concentrate was used to prepare the different concentrations used for the experiment and phytochemical screening.

3. Methodology

At the commencement of this work, thirty (30) adult female albino mice weighing between 21 and 28 g, were divided into 5 groups, each group had 6 mice. The experimental groups received different concentrations of an ethanolic extract of *A. muricata* prepared with respect to the LD_{50} result as documented by Arthur et al. [17]. Mice were treated with increasing doses of extract.

Group MA: Mice treated with 20 mg/ml/week of DMBA + 200 mg/ml/day of extract.
Group MB: Mice treated with 20 mg/ml/week of DMBA + 100 mg/ml/day of extract.
Group MC: Mice treated with 20 mg/ml/week of DMBA + 50 mg/ml/day of extract.
Group NC (Negative Control): Mice treated with 20 mg/ml/week of DMBA only.
Group PC (Positive Control): Mice treated with distilled water only.

DMBA and extract were given intragastrically by gavage using a cannula fitted to a feeding needle. Treatment of animals lasted for six (6) weeks. The experimental and control animals were carefully checked daily and their weight taken weekly. Each mouse had 6 pairs of mammary glands that were checked by inspection, touching and palpitation [4]. Mice were sacrificed at the end of the sixth week by cervical dislocation. The breast tissues from each animal were sliced off and divided into two portions using a surgical blade. One portion was fixed in formalin saline for histology using hematoxylin and eosin staining while the other portion was fixed in ethanol for agarose gel electrophoresis. The work was carried out in the animal house of the University of Lagos, Lagos, Nigeria in accordance with the Code of Ethics of The World Medical Association (Declaration of Helsinki) for animal experiment with consent from the University of Lagos Ethics Committee guidelines for experiments with whole animals [19].

3.1. Phytochemical screening

This was carried out using standard procedures in accordance with Vimala et al. [20].

3.2. DNA extraction

The DNA extraction protocol used for this study was a modified procedure of Nishiguchi et al. [21].

Group MA: Mice treated with 20 mg/ml/week of DMBA + 200 mg/ml/day of extract.
Group MB: Mice treated with 20 mg/ml/week of DMBA + 100 mg/ml/day of extract.
Group MC: Mice treated with 20 mg/ml/week of DMBA + 50 mg/ml/day of extract.
Group NC (Negative Control): Mice treated with 20 mg/ml/week of DMBA only.
Group PC (Positive Control): Mice treated with distilled water only.

4. Results

Table 1 shows the result obtained from the qualitative phytochemical screening of the ethanolic extract of *A. muricata* leaf. Phytochemical screening revealed that terpenoid, steroid, flavonoids, cardiac glycoside, tannin, phenol, alkaloid, and reducing sugar were present while phlobatannin and saponin were absent.

The quantitative phytochemical screening of selected phytochemicals present in the ethanolic extract of *Annona muuricata* is shown in Table 2. Phenol was discovered to be present in the highest amount while cardiac glycoside was found to be in the lowest amount.

The effects of an ethanolic extract of *A. muricata* leaves on the survival rate of mice administered with DMBA are shown in Fig. 1. No death was recorded in any group during the first 2 weeks. The highest number of deaths was recorded in NC in the fifth week. No death was recorded in MB throughout the 6 weeks of this experiment.

Plate 2 is the genomic DNA smear obtained from the electrophoresis of deoxyribonucleotides (DNAs) obtained from the breast tissues of mice from different experimental groups. Genomic DNA smear from PC had normal smear typical of agarose gel electrophoresis. But smears displayed by other groups deviated from normal; with the deviation being highest in NC. Genomic DNA smear obtained from PC and MB are similar and have better smears compared to NC and MC. DNA obtained from MA showed no smear.

The histological section of the breast tissues of mouse in the positive control is shown in Plate 3i. This revealed the presence of normal epidermal tissue, sweat glands, sebaceous gland, alveolar duct and terminal bronchiole.

Plate 3ii shows the histological section of the breast tissues of DMBA-induced cell proliferation in mouse from the negative control group. Normal keratinocytes, sebaceous glands, ecrine glands and subdermal layer with prominent DMBA-induced lobular alveolar hyperplasia were seen to be present.

Plate 4i reveals the histological section of the breast tissue of DMBA-induced cell proliferation of mice that were treated with 200 mg/ml of extract. The presence of DMBA-induced

Table 1 Qualitative analysis of an ethanolic extract of *Annona muricata* leaves.

S. No.	Phytochemical components	Ethanolic extract
1	Saponin	–
2	Terpenoid	+
3	Steroid	+
4	Flavonoids	+
5	Cardiac glycoside	+
6	Tannin	+
7	Phenol	+
8	Phlobatannin	–
9	Alkaloid	+
10	Reducing sugar	+

Key: (+), Presence of phytochemical; (–), Absence of phytochemical.

Table 2 Quantitative analyses of selected phytochemicals present in an ethanolic extract of *Annona muricata* leaves.

S. No.	Phytochemical	*Annona muricata* (mg/100 g)
1	Phenol	162.99
2	Cardiac glycosides	12.92
3	Tannin	121.98
4	Alkaloid	13.74
5	Flavonoids	16.26

lobular alveolar hyperplasia, fibrocystic change and DMBA-induced fibroadenomatoid hyperplasia were identified in Plate 4i while lobular alveolar hyperplasia and fibro adipose stroma were identified in Plate 4ii.

The histological section of the breast tissue of DMBA-induced cell proliferation in mouse that was treated with 100 mg/ml of extract is shown in Plate 5. The section revealed the presence of DMBA-induced lobular alveolar hyperplasia.

Plate 6 shows the histological section of the breast tissue of DMBA-induced cell proliferation in mouse that was treated with 50 mg/ml of ethanolic extract of *A. muricata*. This reveals the presence of proliferating sebaceous glands and lobular alveolar hyperplasia.

5. Discussion

Breast cancer is the most common type of cancer and the leading cause of cancer death in Nigerian women [7,8]. Owing to this fact, in addition to a long latency period of many years prior to the development of metastatic disease, this form of cancer could be identified as an ideal target for chemoprevention and/or early intervention. A general concept put forward by several researchers is that the anticancer activity of compounds which are typically present in plants at sub-pharmaceutical doses could synergize to delay or disrupt the development of aggressive disease [22,23]. The chemopreventive effect of an ethanolic extract of *A. muricata* leaf extract

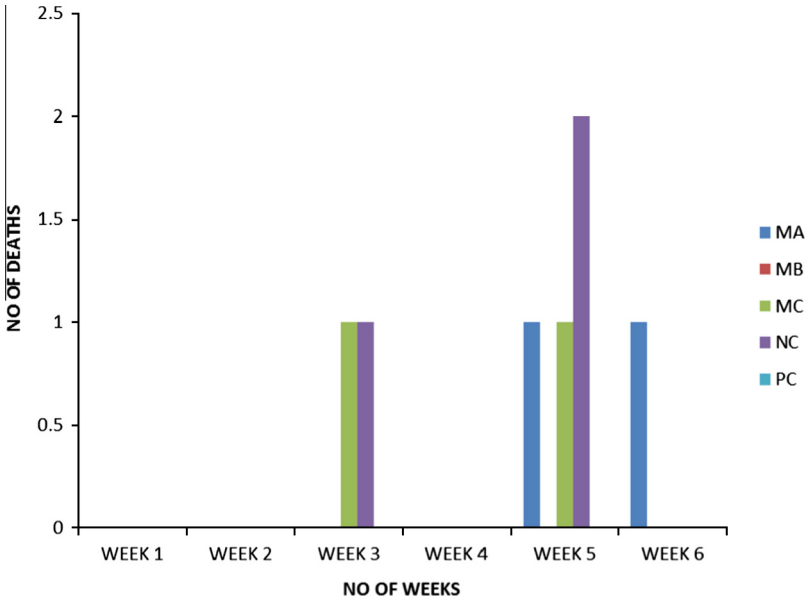


Figure 1 Effects of an ethanolic extract of *Annona muricata* leaves on the survival rate of DMBA-induced cell proliferation in mice.

Plate 1 DMBA induced-papilloma on the hind limbs of mouse treated with DMBA only. Arrows indicate the location of DMBA induced papilloma.

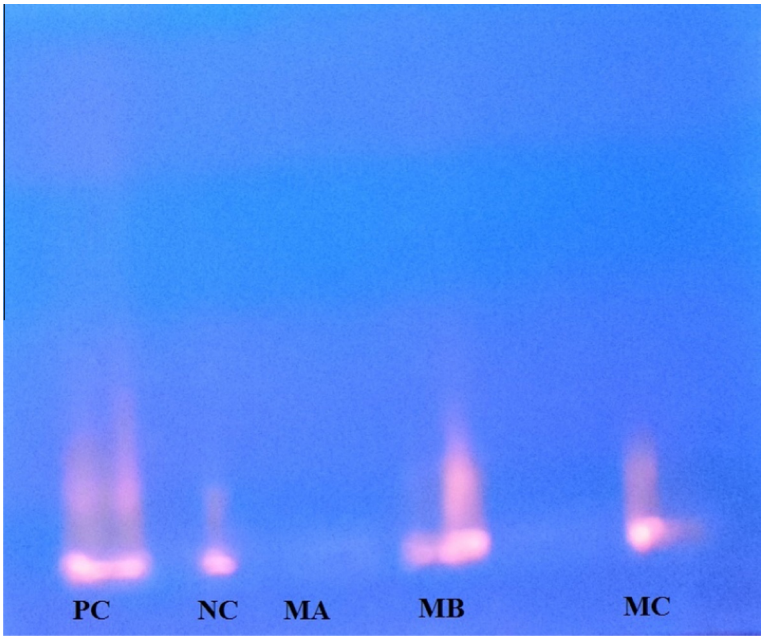
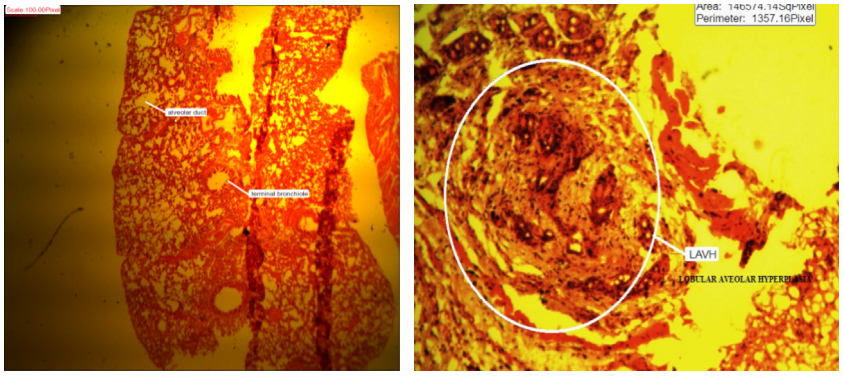


Plate 2 Genomic DNA smear obtained from the electrophoresis DNAs obtained from the breast tissues of DMBA-induced cell proliferation in mice.



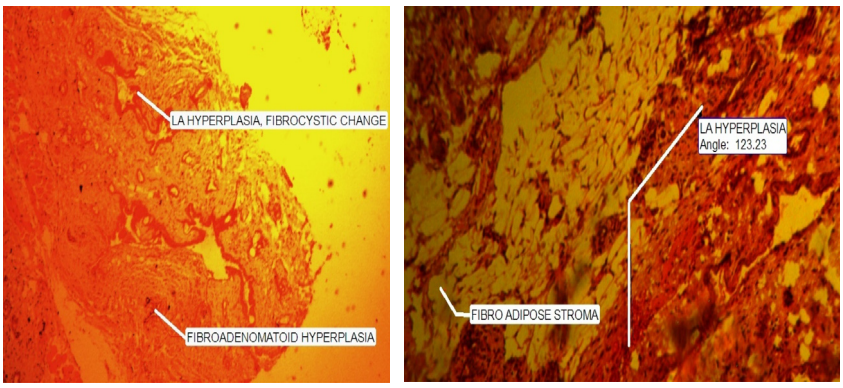
(i) PC (HE-100X) **(ii) NC (HE-100X)**

Plate 3 Histological sections of the breast tissues of mice from the positive (PC) and negative control (NC) showing the alveolar duct and terminal bronchiole in (i) and DMBA-induced lobular alveolar hyperplasia in (ii).

on 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer in mice was evaluated. DMBA is a well-known potent carcinogen which has been used to induce carcinogenesis in the mammary gland or skin of experimental rodents such as rat and mouse [4,24–27].

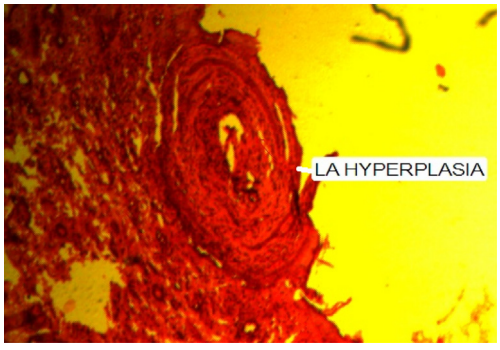
The visible evidence of 7,12-dimethylbenz[a]anthracene administration (DMBA) induced-papilloma was seen in the hind limbs of mouse treated with DMBA only (Plate 1) about 4 weeks after DMBA administration. This occurred earlier than reported by Barros et al. [4]. This could be owing to the fact that the modified method of Barros et al. [4] used for this study was of higher concentration. The high death rate recorded in the fifth week among the group of mice treated with DMBA only might be attributed to the toxicity of the carcinogen. A recent study that focused on the immunotoxicity of DMBA given to experimental animals to induce mammary gland showed that DMBA elicits immunotoxicity in the spleen, thymus and bone marrow [28].

The deviation from normal displayed by the genomic DNA smears obtained after electrophoresis, especially in NC (Plate 2), might have arisen as a result of DMBA-induced deoxyribonucleic acid (DNA) damage or mutation. This is in concordance with the report of Nebert et al. and Rundle et al. [29,30] who described the mechanism of action of DMBA to involve up-regulation of cytochrome P450 enzymes that metabolize DMBA into a mutagenic epoxide intermediate which readily forms DNA-adducts that are associated with DNA mutations and the malignant transformation that leads to carcinogenesis. As cancer cells usually have mutations in genes regulating DNA damage responses or repair pathways,



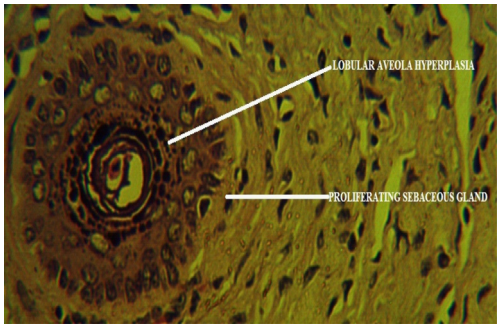
(i) MA (HE-100X) **(ii) MA (HE-100X)**

Plate 4 Histological sections of the breast tissues of DMBA-induced cell proliferation in mouse treated with 200 mg/ml showing fibroadenomatoid hyperplasia in (i) and fibro adipose stroma in (ii).



MB (HE-100X)

Plate 5 Histological section of the breast tissue of DMBA-induced cell proliferation in mouse treated with 100 mg/ml of extract showing the presence of lobular alveolar hyperplasia.



MC (HE-100X)

Plate 6 Histological section of the breast tissue of DMBA induced cell proliferation in mouse treated with 50 mg/ml of extract showing the presence of lobular alveolar hyperplasia and proliferating sebaceous gland.

proto-oncogenes and tumor suppressor genes, they can be more susceptible to cell cycle arrest and death from treatment with carcinogenic agents than normal cells [2,31].

The similarity between the genomic DNA smears displayed by DNAs obtained from PC and MB might be due to the prevention of excessive DMBA-induced damage to DNA by an ethanolic extract of *A. muricata* leaves at a concentration of 100 mg/ml (Plate 2). Many chemotherapeutic agents such as plant-derived compounds have been shown to exert their therapeutic effect by directly interacting with DNA or DNA-binding proteins [31,32]. This interaction triggers DNA damage signaling pathways resulting in the inhibition of cell proliferation or the induction of apoptosis, depending on the extent of the damage [33]. However, the similarity between smears displayed by the DNAs obtained from MC and NC (Plate 2) suggests the plant extract's inability to prevent DMBA-induced DNA damage at a dose of 50 mg/ml. Therefore, the extract could be said to have acted in such a way as to prevent or reduce excessive DMBA-induced DNA damage. The preventive effect of an ethanolic extract of *A. muricata* leaves against DMBA-induced DNA damage could be owing to the presence of the various secondary metabolites (tannins, terpenoids, cardiac glycosides (CGs), and flavonoids) discovered from the phytochemical screening of the ethanolic extract of *A. muricata* (Table 1).

Previous studies have shown that tannins, terpenoids, cardiac glycosides (CGs), and flavonoids possess anticancer activities [34–41]. Epidemiologic evidence suggested that breast cancer patients treated with cardiac glycosides had a significantly lower mortality rate, and their cancer cells had more benign characteristics than those from patients not treated with it [34]. Phenol was determined to be quantitatively present in the highest amount (Table 2), this might have accounted for the preventive effect of the extract on DMBA-induced cell proliferation. This is in agreement with the previous studies showing the anti-proliferative effects of herbal polyphenols, in various human cancer cell lines [42–44].

The presence of DMBA-induced lobular alveolar hyperplasia, and DMBA-induced fibroadenomatoid hyperplasia in the histological sections of the breast tissues of mice treated with DMBA (Plates. 3ii, 4, 5, and 6) suggests a neoplastic transformation which is an indication of DMBA-induced cell proliferation [45]. DMBA-induced ductal hyperplasia has been discovered to be a physiologic precursor to the development of ductal carcinoma in situ [46]. Furthermore, DMBA-induced

carcinogenesis had been documented to be associated with ductal carcinomas, fibroadenomas, adenomas, and papillomas [4]. However, these changes were found to vary in occurrence among the different groups used for the present study. These variations might be due to the preventive effect of the different concentrations of extract administered to mice. Terminal end buds had been shown to be preferential targets of DMBA effects (DMBA–DNA linking) in the neoplastic transformation of the mammary gland [26]. The presence of proliferating cells in the stroma as well as among the epithelial and myoepithelial cells (Plate 4ii) strongly suggests that the carcinogen acts on different cells in the breast tissue and, although the stroma itself does not undergo neoplastic transformation, it plays an important role in the carcinogenic process [47].

6. Conclusion

The present study showed that the ethanolic extract of *A. muricata* leaves can be used as a preventive measure against DMBA-induced breast cell proliferation in the breast tissues of female albino mice. Agarose gel electrophoresis showed that the plant extract prevented DMBA-induced DNA damage to some extent. Histological assay revealed the presence of a neoplastic transformation which suggests the presence of cells undergoing the initial proliferative stage preceding carcinogenesis. The knowledge obtained from this study can be exploited by person(s) suspected to be linked with familial history of breast cancer. Nevertheless, further studies and more research need to be done to optimize the quality of extract, effective dose and its specificity on breast cancer susceptibility genes.

Conflict of interest

The authors declare no conflict of interest.

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