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Original article

Cytotoxic effects of *Annona squamosa* leaves against breast cancer cells via apoptotic signaling proteins

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ABSTRACT

Annona squamosa L. is an important medicinal plant used in traditional medicine for the treatment of various diseases. Different parts of *A. squamosa* L. have various therapeutic effects; however, the anticancer activity of the leaves has not yet been identified. In *vitro*, MTT, nuclear staining, and LDH assays were used to evaluate cell survival and proliferation in cells exposed to the extracts. The effect of the extracts on cell migration was investigated using a monolayer wound repair assay, and the apoptotic effects were evaluated using flow cytometry. A breast cancer model was used to study the effect of the extract on the tumor size, and the expression of different proliferative and apoptotic markers was evaluated by immunohistochemical analysis. At a concentration of 100 µg/mL, *A. squamosa* leaf extracts exerted strong antiproliferative and cytotoxic effects against various cell lines. The extracts reduced wound closure and strongly induced apoptosis. *In vivo* study, rats were sacrificed 24 h after the last injection, and tumor size, as well as the expression of proliferative and apoptotic markers, were observed to be greatly affected by treatment with the extracts. Therefore, *A. squamosa* leaf extract may be developed as a potential novel drug to treat breast cancer in the future.

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1. Introduction

Breast cancer is the second most common cancer and the most common cause of death in women worldwide (Ferlay et al., 2015). Nevertheless, surgery, chemotherapy, and radiotherapy remain the cornerstone of breast cancer treatment (Wang et al., 2014). Chemotherapeutic agents have several disadvantages and significant side effects, such as the inability to differentiate between cancer and normal cells and drug resistance associated with repeated treatment. Hence, there is an urgent need for new drugs. It is necessary to identify natural products that target multiple signaling pathways and cause growth inhibitory effects on cancer cells with fewer harmful effects on healthy cells and the biological environment (Maya et al., 2013). Plant medicines have gained recognition over the last 40 years, and have been widely used as alternative or complementary medicines in many countries and ethnic groups worldwide (Heinrich et al., 2012). Even today, plant-derived compounds play a major role in primary healthcare as natural remedies in most countries, owing to their safety, effectiveness, quality, and availability (Verpoorte, 2009). It is reported that 49% of the 175 small molecules, which were approved for cancer treatment, were natural products or modified natural products. Additionally, it is estimated that almost 80% of people use plant medicines to treat inflammatory diseases (Tundis et al., 2017). Plants with a long history of use in ethnomedicine can be considered a vast resource of bioactive compounds, providing medicinal and health benefits against different diseases (Efferth et al., 2007; Ma et al., 2017). Therefore, in the field of phytoscience, researchers are working to elucidate the side effects, calculate appropriate dosages, and find

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the best method to extract and identify bioactive components. The bioactive compounds are secondary plant metabolites that elicit pharmacological or toxicological effects in humans and animals; however, one of the most critical challenges that researchers have faced is that a single plant may contain many bioactive compounds (Aḥmad et al., 2006). Currently, there is a global interest in medicinal plants as natural products that may yield the next generation of semi-synthetic derivatives, despite the decrease in the popularity of this approach in the 20th century (Ghazi-Moghadam et al., 2012).

Annona squamosa L. is a small group of an edible plant of the genus *Annona* and the family Annonaceae, commonly known as a sugar apple or custard apple (Lim, 2012). *A. squamosa* L. is a small tree that grows to 3–8 m in height and has conical fruits. The leaves are brilliant green on the top and bluish green on the bottom, with petioles of 0.7–1.5 cm long. The shape of leaves is oblong-elliptical; they measure 5 to 17 cm in length and 2 to 7 cm in width, with an obtuse or acuminate apex. The blade has 15 to 17 pairs of veins (Pinto et al., 2005). Different parts of *A. squamosa* L., such as the bark, root, seed, fruits, flowers, and leaves have been used in traditional medicine to treat various diseases (Kalidindi et al., 2015). Several bioactive compounds have been isolated from *A. squamosa* L. leaves, including alkaloids, steroids, annonaceous acetogenins, terpenoids, glycosides, saponins, flavonoids, and phenolics (Gowdhami et al., 2014), and these compounds were found to be responsible for various biological activities (Chen et al., 2012a; Wang et al., 2014).

A. squamosa L. plant parts are known to have various biological activities, although there are fewer studies of the leaves. To provide a clear understanding of the mechanism action of *A. squamosa* L leaf extracts, screening for the anticancer activity should confirm its use as an alternative to chemotherapeutic agents for breast cancer therapy. This study's objective was to investigate the *in vitro* and *in vivo* anti-breast cancer activity of *A. squamosa* leaf extracts.

2. Materials and methods

2.1. Plant material and extraction process

A. squamosa L. leaves were obtained from a local plant nursery in Ta'if City, Saudi Arabia, in December 2016. The identification and authentication were performed by Ibrahim Al-Dakhil, an agronomist, and confirmed by the Department of Botany and Microbiology, College of Science, King Saud University (KSU). A voucher specimen (KSU-No. 12068) was deposited at the Herbarium of the College of Science, KSU. The shade-dried leaves were ground into powder using an electric blender. Later, the extract was prepared by maceration of equal amounts of powdered leaves and methanol, acetone, or water (1:10 w/v) for 48 h and then filtered. The filtrate was collected in methanol or acetone and evaporated at 18–21 °C under a fume hood to obtain the crude extracts. For the aqueous filtrate, FreeZone 4.5 Liter Benchtop Freeze Dry System (Labconco, USA) was used for lyophilization. All extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) for further experiments.

2.2. Cell lines and culture medium

MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 2×10^{-3} v/v penicillin–streptomycin (composed of 31 g/L penicillin and 50 g/L streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C throughout the assays.

2.3. Cell viability assay

The antiproliferative activity of the extracts was determined by MTT assay following the method of Semlali et al. (2011). The extracts were dissolved in DMSO and then diluted in a culture medium. Briefly, the cells were seeded in 6-well plates (4×10^5 cells/well) and incubated at 37 °C for overnight growth. When cells reached 80% confluence, the culture medium was removed and replaced with 1 mL of fresh culture medium, and the cells were treated with various concentrations of the leaf extract (1 μg/mL, 10 μg/mL, and 100 μg/mL) for 24 h. The untreated cells were assayed as a negative control. After that, cells were incubated with 100 μL MTT (5 mg/mL; Sigma) in fresh medium for 3 h. The colored MTT-formazan crystals were dissolved by the addition of 0.04 N HCl in isopropanol (500 μL) to each well. Finally, 100 μL of the reaction mixture was transferred to a 96-well plate, and the maximum absorbance at 550 nm was detected using an ELISA plate reader (X-Mark Microplate Spectrophotometer, Bio-Rad, USA). The experiment was conducted in triplicate.

2.4. Nuclear staining

The morphology of the treated cells was assessed using a Hoechst 33,342 staining assay (H42), as previously described by Semlali et al. (2011). The cells were seeded in 6-well tissue culture plates (3×10^5 cells/well). After overnight incubation, the medium was removed and replaced with fresh medium. Different concentrations of the extracts were added to the medium, and the cells were incubated for 24 h. The supernatant was then removed, and the cells were washed twice with PBS and fixed in cold methanol for 15 min. After fixing, the cells were washed twice with PBS, and then stained with 2 μg/mL Hoechst 33,342 (Thermo Fisher Scientific, DE) for 15 min. The stained nuclei were washed twice with PBS and observed and photographed using Leica DM2500 & DM2500 LED optical microscopes (LEICA Microsystems, DE).

2.5. Cytotoxicity assay

Breast cancer cell lines were cultured for 24 h in 6-well plates (4×10^5 cells/well). After overnight growth, the culture medium was refreshed, and cells were treated with various concentrations of the leaf extract (1 μg/mL, 10 μg/mL, and 100 μg/mL) for 24 h. Cell injury was assessed by the measurement of the release of lactate dehydrogenase (LDH) (Smith et al., 2011). 10 μL of the culture supernatant was transferred to a 96-well plate, and the enzyme reaction was conducted in accordance with the manufacturer's instructions (LDH cytotoxicity colorimetric assay kit II, BioVision, USA). Briefly, LDH reaction mixture (100 μL) was added to each well and the plate was incubated for 30 min at 18–21 °C (room temperature) in the dark; thereafter, 10 μL of stop solution was added to terminate the reaction. The absorbance was measured at 450 nm using an X-Mark microplate spectrophotometer (Bio-Rad). Negative, positive, and background controls for total LDH activity were included in the experiment.

2.6. Monolayer wound repair assay

Breast cancer cell lines were seeded in 6-well plates and grown to confluence. A 10 μL pipette tip was used to made scratch wounds. The detached cells were removed by washing with PBS; thereafter, the cells were treated with 50 μg/mL of the methanolic, acetonic, or aqueous extracts. The migration of cells into the wounded region was observed using a LEICA DFC450 C digital camera (LEICA Microsystems, DE) at 0 and 24 h Semlali et al. (2010). The percentage of wound closure was calculated following the formula:

$$\text{Wound closure}(\%) = (\text{initial scratch size} - - \text{size of the scratch after an identified culture period}) / (\text{initial scratch size}) \times 100$$

2.7. Annexin V/PI apoptosis assay

To discriminate the type of cell death induced by *A. squamosa* leaf extracts, APC Annexin V Apoptosis Detection Kit with PI (BioLegend, USA) was used. This assay is based on the detection of morphological features associated with apoptosis or necrosis. First, the cells were treated with methanolic, acetonic, or aqueous extracts (50 μg/mL) for 24 h; then, adherent cells were collected, rinsed twice with cold PBS, and resuspended in 100 μL of annexin-binding buffer. This was followed by the addition of 5 μL APC Annexin V and 10 μL PI solution, and incubation at room temperature for 15 min. Subsequently, 400 μL of annexin-binding buffer was added to the reaction mixture, and the cells were immediately analyzed using BD FACSCalibur™ flow cytometer (Becton Dickinson, USA).

2.8. Cell culture conditions for RNA extraction

The breast cancer cell lines were cultured for 24 h in a T25 flask (1×10^6 cells/flask). The culture medium was replaced with medium containing 1% FBS for 24 h, and the cells were then treated with 50 μg/mL of the leaf extracts in 2 mL fresh medium containing 1% FBS, or 0.25% DMSO as the control. The cells were allowed to grow overnight. After washing with PBS, adherent cells were detached by the application of trypsin (Gibco, USA).

2.9. Reverse-Transcription PCR (RT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, DE). The RNA quantity, purity, and quality were determined using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). One microgram of RNA from each sample was reverse-transcribed into cDNA using the Hyperscript Kit (GeneAll, KR) and random hexamers (GeneAll, KR), as described in the manufacturer's protocol.

2.10. Quantitative real-times PCR

The mRNA expression of *Bcl-2* and *Bax* was measured using a QuantStudio 7Flex Detection System (Applied Biosystems, USA). The reactions were performed using iTaq™ Universal SYBER Green Supermix (Bio-Rad). Primers (Table 1) were added to the reaction mix at a final concentration of 10 pM. Five microliters of each cDNA sample were added to a 20 μL PCR mixture containing 12.5 μL of 2 × iTaq™ Universal SYBER Green Supermix, 0.5 μL of primers for *Bcl-2*, *Bax*, *GAPDH* (Macrogen, KR), and 7 μL RNase/DNase-free water (Qiagen, DE). The thermal cycling conditions for *Bcl-2* and *Bax* were established as 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and 30 s at 60 °C, and final 10 s at 95 °C. The specificity of each primer pair was verified by the presence of a single melting temperature peak. The Applied Biosystems software automatically determined the threshold cycle. The expression of *GAPDH*, a housekeeping gene, was used as an endogenous control for this study.

2.11. Animal model

To confirm the *in vitro* results, twelve female Wistar albino rats (*Rattus norvegicus*), aged 5–8 weeks of age and with a bodyweight of 110 ± 20 g were used. The rats were divided into three groups, with four animals in each group. The rats were obtained from the breeding facility at the College of Pharmacy at King Saud University, Riyadh, and the written informed consent to use the animals in our study was obtained from this facility. The animals were housed in plastic cages at room temperature (25 ± 2 °C) with a constant relative humidity (45% ± 5%) under a 12 h light/dark cycle. Mammary tumors were induced in two groups of rats (groups I and II) by a single subcutaneous injection in the pectoral area with the potent carcinogen, dimethylbenzanthracene (DMBA), as described previously (Bersch et al., 2009). After tumor palpation (8 weeks post-injection), animals in group I received corn oil as vehicle control, whereas animals in group II were injected with *A. squamosa* aqueous extract dissolved in corn oil at a dose of 300 mg/kg directly into the tumor. A single dose was injected for 4 weeks every 48 h, following a previously described protocol (Ait M'Barek et al., 2007; Zyad et al., 1995). After treatment, the rats were euthanized by administering a ketamine (125 mg/kg), and xylazine (10 mg/kg) overdose and mammary gland tissues were collected from all sacrificed rats. All animal procedure was reviewed and approved in accordance with ICH GCP guidelines by the Institutional Review Board at King Fahd Medical City, Riyadh, KSA (IRB log number: 18–586, registration number with KACST, KSU: H-01–12-012 and registration number with OHRP/NIH, USA: IRB00010471).

2.12. Immunohistochemistry (IHC) analysis

Polyclonal antibodies were used to assess the expression of different tumor markers, including the genes involved in the proliferation signaling pathway, such as *Ki67* and *p53*. Briefly, IHC was performed on 3-μm slices of deparaffinized and rehydrated breast tissue by a Ventana Benchmark XT automated staining (Ventana Medical Systems Inc., Tucson, AZ) for the assessment of *Ki67* and *p53* (Santa Cruz Biotechnology, CA, USA), as previously described by Semlali et al. (Semlali et al., 2016). The immuno-stained sections were analyzed by an Olympus BX51 light microscope and DP72 Olympus digital camera with powers 400X (Olympus America Inc, Center Valley, PA, USA).

2.13. Statistical analysis

Data are presented as an arithmetical mean ± standard deviation (±SD) using Statistical Package for the Social Sciences (SPSS v.21, Chicago, IL, USA). All statistical differences were evaluated with one-way analysis of variance (ANOVA) followed by student's

Table 1
Description of primers used for qPCR.

Gene		Primer sequence (5′ to 3′)	Product size (bp)
BCL-2	Sense	5′- ACTGAGTACCTGAACCGGCATC-3′	108
	Antisense	5′- GGAGAAATCAAACAGAGGTCGC-3′	
Bax	Sense	5′- AGTGTCTCAGGCGAATTGGC-3′	102
	Antisense	5′- CACGGAAGAAGACCTCTCGG-3′	
GAPDH	Sense	5′- GGTATCGTGGAAGGACTCATGAC-3′	188
	Antisense	5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′	

t-test. $p \leq 0.05$ was considered as statistically significant, while value ≤ 0.005 was taken as statistically highly significant compared to relevant control.

3. Results

3.1. The effect of A. squamosa extract on cell survival

Treatment with *A. squamosa* leaf extracts did not induce clear changes in the morphology of either breast cancer cell line; however, the treatment affected the number of viable cells (Fig. 1). Therefore, the antiproliferative activity of various concentrations of *A. squamosa* extracts was determined against MCF-7 and MDA-MB-231 breast cancer cell lines using MTT assay. The methanolic extract resulted in a highly significant decrease in MCF-7 cell viability at 1 μg/mL, 10 μg/mL, and 100 μg/mL (Fig. 2-a). The 10 μg/mL of the methanolic and acetonic extracts resulted in a highly significant decrease in the viability of MDA-MB-321 cells (Fig. 2-b). However, it was found that all three extracts of *A. squamosa* leaf at 100 μg/mL resulted in a highly significant decrease in MCF-7 and MDA-MB-321 cell viability, with cell proliferation inhibition of approximately 60%. Furthermore, to determine whether the antiproliferative activities of *A. squamosa* extracts were related to the induction of apoptosis, the morphological changes of cells were investigated using a nuclear staining assay. In both cell lines, the number of cells decreased with an increase in the extract concentration applied. Besides, nuclear damage occurred in a dose-dependent manner (Fig. 2-c). These results clearly showed the antiproliferative activity of *A. squamosa* extracts in ER^+ breast cancer cells, MCF-7, and ER^- cells, MDA-MB-231.

3.2. The cytotoxic effects of A. squamosa extracts

The cytotoxicity of *A. squamosa* extracts was determined through the measurement of LDH leakage from degraded cells, which resulted from the cytotoxic effects of the extract. As shown in Fig. 3, the extracts exerted cytotoxicity against MCF-7 and MDA-MB-231 in a dose-dependent manner. Overall, the three different extracts showed highly significant cytotoxic effects in ER^+ cells, MCF-7, compared with the ER^- cells, MDA-MB-231. At the highest concentration tested (100 μg/mL), the methanolic and acetonic extracts induced 100% cell death in MCF-7 cells, and the aqueous extract induced approximately 60% cell death. *A. squamosa* extracts resulted in approximately 70%–80% cell death of MDA-MB-231 cell lines at the highest concentration tested (100 μg/mL).

3.3. The effect of A. squamosa extracts on cell migration

To clarify the potential of *A. squamosa* extracts as anticancer agents, the effect of the extracts on the migration of cancer cells was tested using a monolayer wound healing assay (Fig. 4). After 6 h, the cells treated with extracts had not migrated as far as the untreated cells. Furthermore, MCF-7 cells treated with the three different extracts showed 30%–33% less closure after 6 h than the DMSO-treated control cells ($p < 0.005$). It appears that the healing of ER- cells occurred much more rapidly than ER + cells; therefore the migration of MDA-MB-231 treated cells was not greatly affected. In other words, the decrease in cell migration resulting from *A. squamosa* extract treatment was more considerable in ER + cells.

3.4. The apoptotic effect of A. squamosa extracts

The effect methanolic, acetonic, and aqueous extracts of *A. squamosa* leaves on apoptosis induction in the breast cancer cell lines were measured using Annexin V/PI staining after 24 h of treatment at 50 μg/mL (Fig. 5). For MCF-7 cells, the percentage of normal cells decreased by 65.8%, 92%, and 92.6% after treatment with the methanolic, acetonic, and aqueous extracts. The methanolic extracts induced apoptosis in 27.5% of the cells, and necrosis in 38.3% of the cells. Apoptosis was induced in 52.8% of cells and necrosis was induced in 39.1% of cells treated with the acetonic extract. However, the aqueous extract was found to have the most potent effect on MCF-7, inducing necrosis in 60.2% of cells and apoptosis in 32.3% of cells. In contrast, the methanolic extract induced apoptosis in almost all MDA-MB-231 cells (99.5%), the acetonic extract induced apoptosis in 91.3% of cells, and the aqueous extract induced apoptosis in 89% of cells.

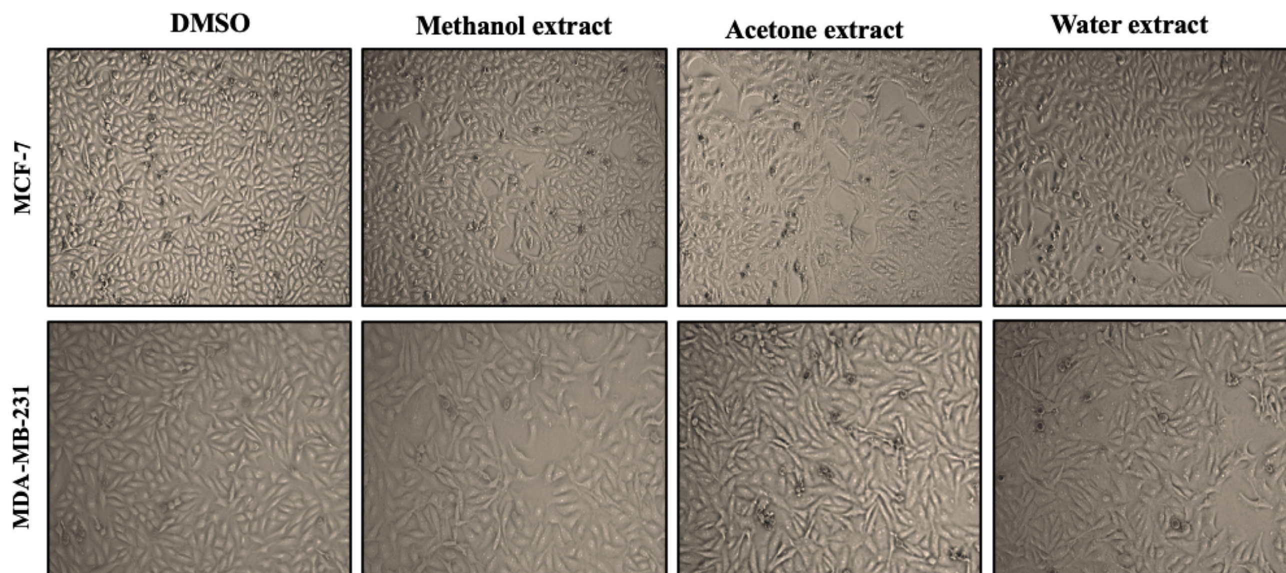

Fig. 1. The effect of *A. squamosa* extracts on the morphology of breast cancer cell lines. MCF-7 and MDA-MB-231 cells were seeded at 4×10^5 cells/well and treated with 50 μg/mL methanol, acetone, or aqueous extract in DMEM medium with 10% FBS. Photomicrographs were taken at 24 h.

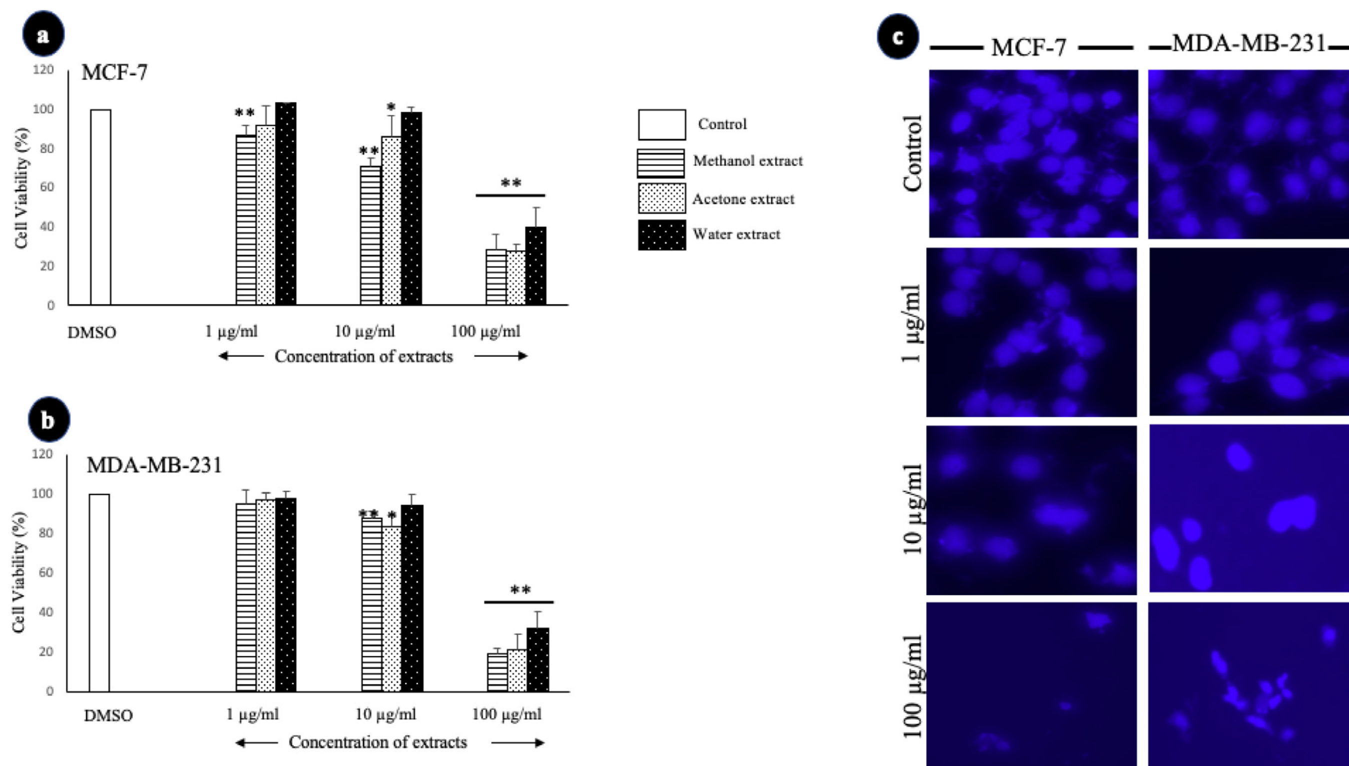

Fig. 2. The effect of *A. squamosa* extracts on the proliferation of breast cancer cell lines as determined by MTT and nuclear staining assays. The cell viability of MCF-7 cells (a) and MDA-MB-231 cells (b) in the MTT assay (n = 5). (c) Photomicrographs of breast cell lines treated with three different concentrations of methanolic extract of *A. squamosa* leaves. The control cells were treated with DMSO (*$p < 0.05$, **$p < 0.005$).

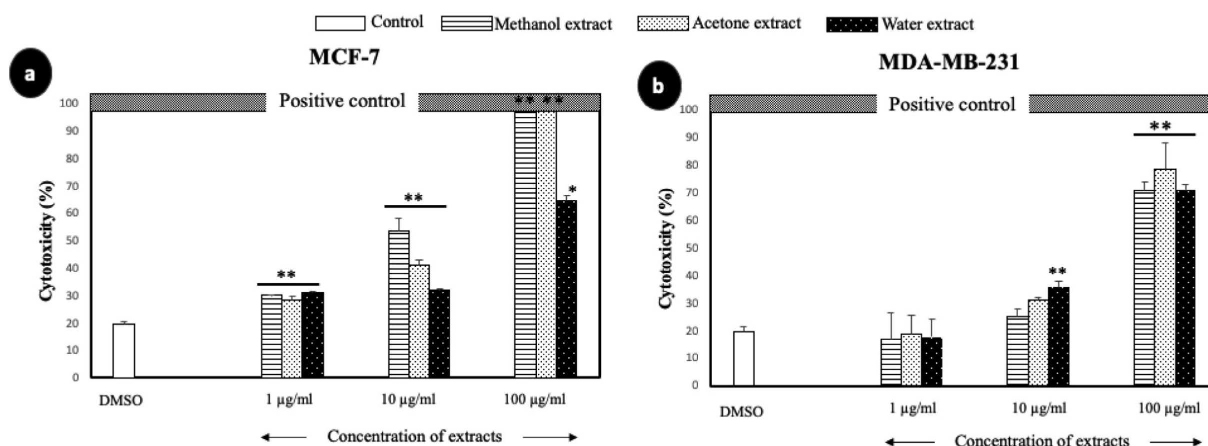

Fig. 3. The cytotoxic effect of *A. squamosa* extracts against breast cancer cell lines as determined by LDH assay. MCF-7 (a) and MDA-MB-231 (b) breast cancer cell lines. DMSO and Triton X-100 were used as negative and positive controls, respectively (*$p < 0.05$, **$p < 0.005$).

3.5. The effect of A. squamosa extracts on anti-apoptotic/pro-apoptotic gene expression

To detect the apoptotic effects of the methanolic, acetonic, and aqueous extracts, the expression of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) genes in breast cancer cells were measured using qPCR. The exposure of MCF-7 cells to the extracts caused downregulation of the Bcl-2 mRNA; this change was significant after treatment with the methanolic and acetonic extracts. All three extracts upregulated the expression of Bax; however, the change was only significant after treatment with the aqueous extract (Fig. 6-a). In MDA-MB-231 cells, the expression of Bcl-2 was downregulated, and that of Bax was upregulated (Fig. 6-b). Therefore, these findings showed that *A. squamosa* extracts induced apoptosis through the suppression of Bcl-2 and the activation of Bax. Consequently, the Bcl-2/Bax ratios showed a highly significant decrease ($P < 0.005$).

3.6. In vivo effect of A. squamosa extract on tumor growth

The therapeutic effects of *A. squamosa* extract in a rat model of breast cancer were assessed after treatment, via local injection, with the aqueous extract for one month. Aqueous extract of *A. squamosa* was used as a starting point, to eliminate any side effects of using the organic solvents in the existing system. As shown in Fig. 7, the tumor size in the treatment group was significantly

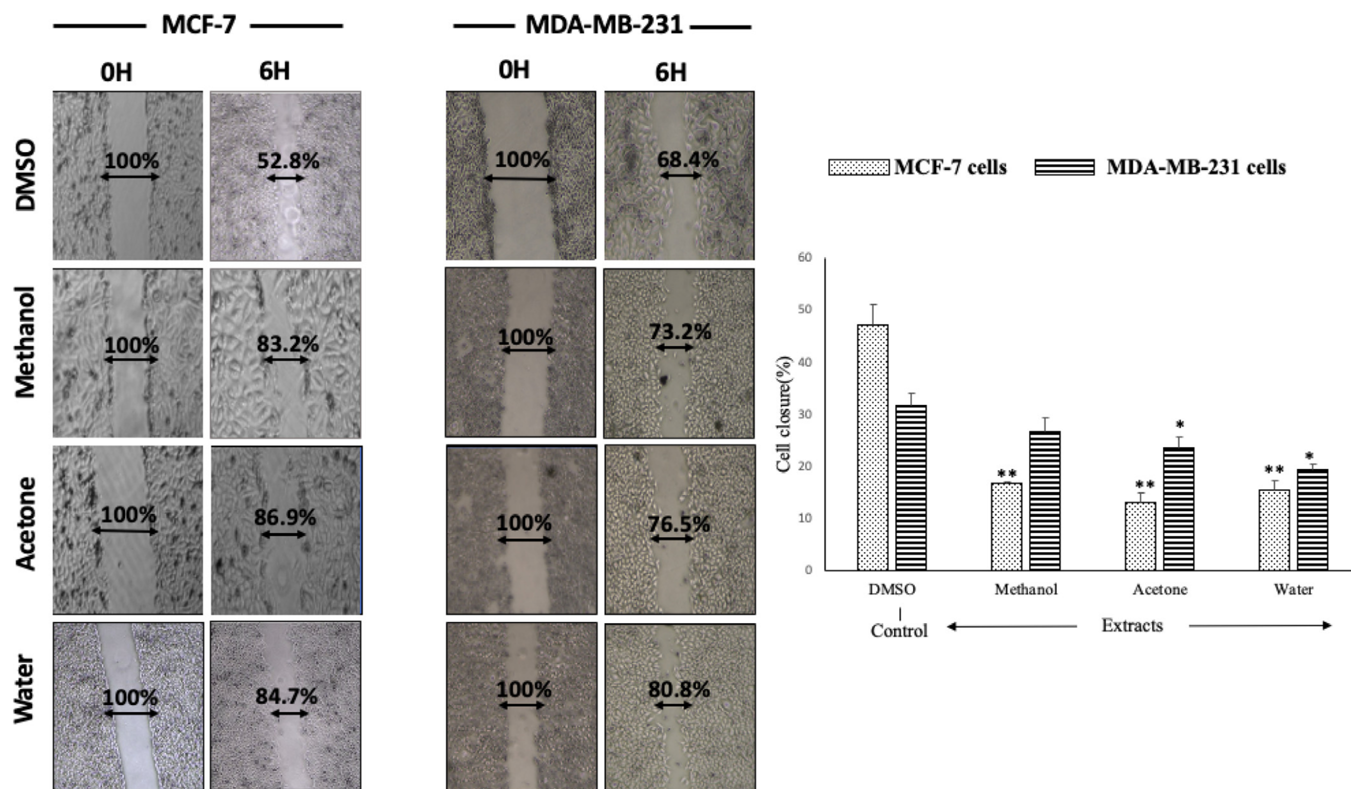

Fig. 4. The migration of breast cancer cell lines (MCF-7 and MDA-MB-231) after treatment with different *A. squamosa* extracts. A scratch was made on each monolayer, the culture medium was refreshed, and 50 μg/mL of extract was added. The cultures were maintained under the appropriate conditions, observed, and photographed at 0 and 24 h (n = 3, $^{*}p < 0.05$, and $^{**}p < 0.005$).

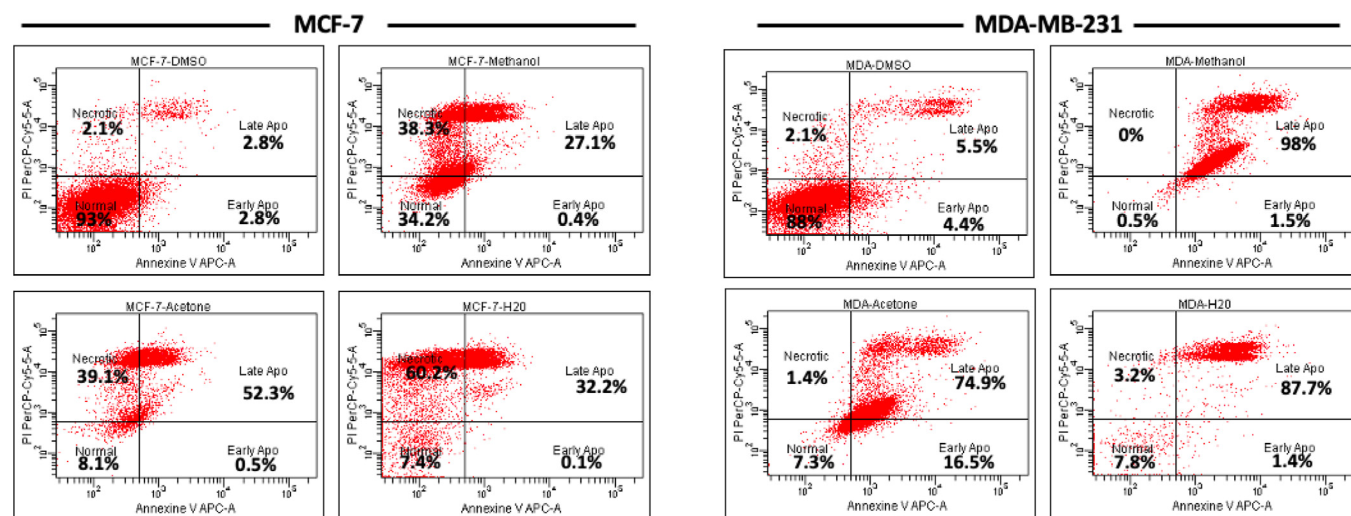

Fig. 5. Survival of breast cancer cells after treatment with *A. squamosa* extracts for 24 h. MCF-7 and MDA-MB-231 cells were seeded at 4 × 10^5 cells/well and treated with 50 μg/mL methanol, acetone, or aqueous extract in DMEM medium with 10% FBS for 24 h. The apoptotic effect was measured using Annexin V-PI staining kit and flow cytometry.

reduced compared with that of the control (corn oil-treated) group, 1083 mm^3, and 6583 mm^3, respectively. These data suggested that the local treatment of *A. squamosa* extract significantly reduced the development and progress of tumor formation.

These results were confirmed by histological analysis of the tumor tissues, which revealed a more aggressive metaplastic carcinoma in the untreated rats than the treated rats (Fig. 8). In the immunohistochemical analysis, less intense positive immunostaining of Ki67, a well-established marker for tumor cell proliferation, in treated tissue compared to untreated tumor tissues. Moreover, the expression of p53, a tumor suppressor gene, was more intense in the treated tumor tissues than in the untreated tumor tissues. Overall results were indicating a pronounced decrease in proliferation and increase in apoptosis, in the treated tumor tissues.

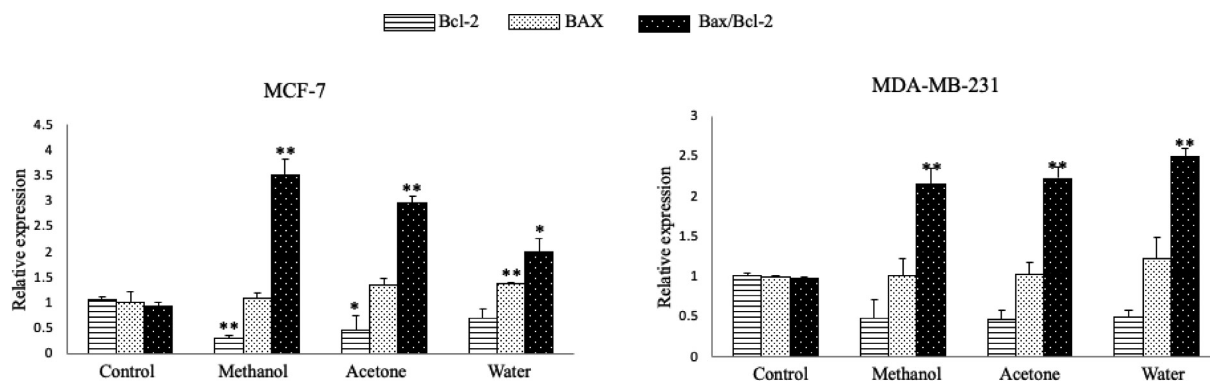

Fig. 6. Relative mRNA expression of *Bax* and *Bcl-2* in breast cancer cells treated and untreated with *A. squamosa* extracts for 24 h. (a) MCF-7 cell lines. (b) MDA-MB-231 cell lines. The data are expressed relative to the values for untreated control cells and represent the mean ± standard deviation (n = 3, $^{*}p < 0.05$, and $^{**}p < 0.005$).

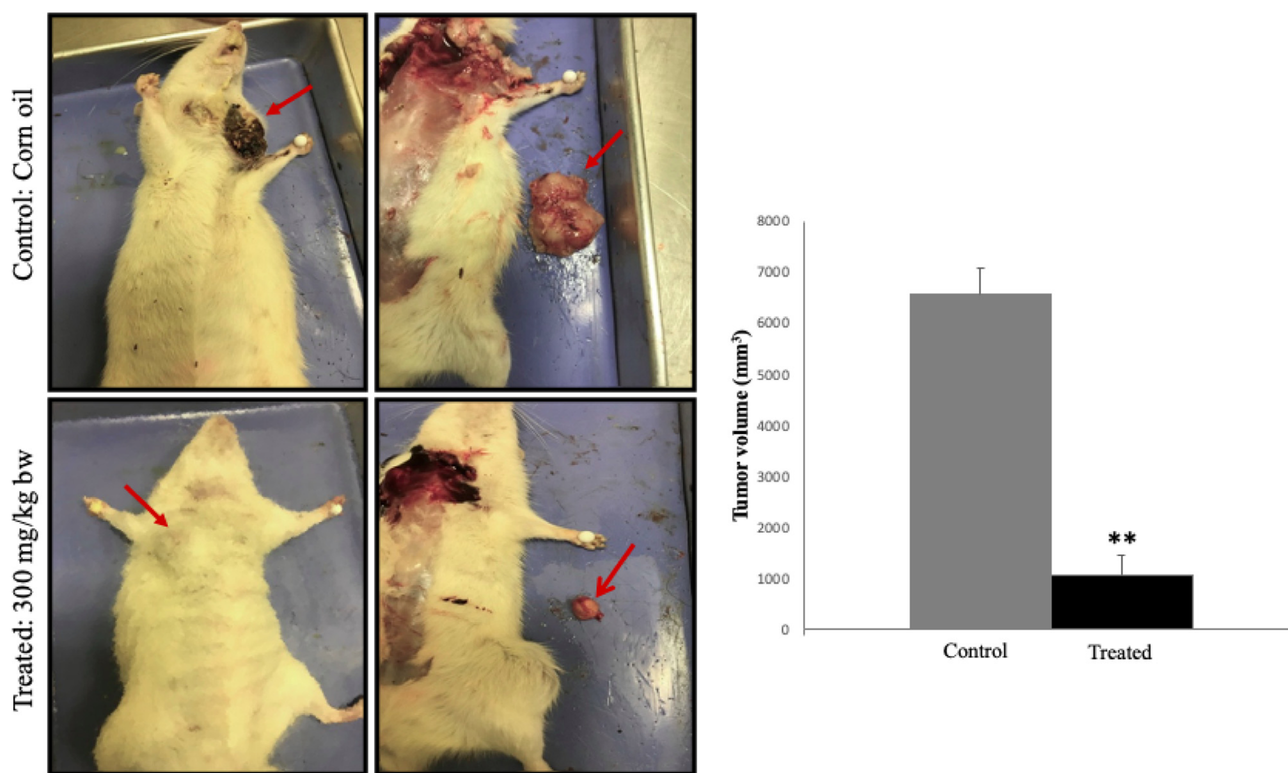

Fig. 7. Test of *A. squamosa* extract treatment on size of breast tumor induced by DMBA. (a) Injecting *A. squamosa* aqueous extract (300 mg/kg body weight) into the breast cancer model inhibited the tumor growth when compared with the control group. (b) Tumor size was measured with a caliper and calculated using the formula: (Length $\times$ Width^{2})/2, (n = 3, $^{*}p < 0.05$, and $^{**}p < 0.005$).

4. Discussion

From ancient times, plants have been used for the treatment of diseases by humans. They are used as complementary medicine or to synthesize chemical compounds. According to the World Health Organization, more than 80% of the world's population depends on nature-derived traditional alternative medicine for their primary healthcare needs (Bailon-Moscoso et al., 2016, Semlali, et al., 2021, Contant, et al., 2021). One of these plants with extensive traditional use is *A. squamosa*; however, biologically, it is less well characterized. Few studies have illustrated the anticancer activity of *A. squamosa* leaf extracts. Therefore, this study aimed to investigate the anticancer effect of *A. squamosa* leaf extract on breast cancer cells.

In the current study, the anticancer activity of *A. squamosa* leaf extracts was investigated in vitro against two breast cancer cell lines; MCF-7 and MDA-MB-231. Our results revealed that the different *A. squamosa* leaf extracts did not induce any changes in the morphology of the cell lines (Fig. 1). However, different extracts were found to induce cytotoxicity and inhibit the proliferation of MCF-7 and MDA-MB-231 cells in a dose-dependent manner (Figs. 2 and 3). In this study, the antiproliferative and cytotoxicity activities of the extracts were confirmed by nuclear staining, which revealed damaged nuclei (Fig. 2-c). These appear to be consistent with the results of a recent study on the isolation of different acetogenin compounds from *A. squamosa* seeds; the compounds were able to suppress the proliferation of multi-drug resistant MCF-7 cells via cell cycle arrest in the G1 phase (Ma

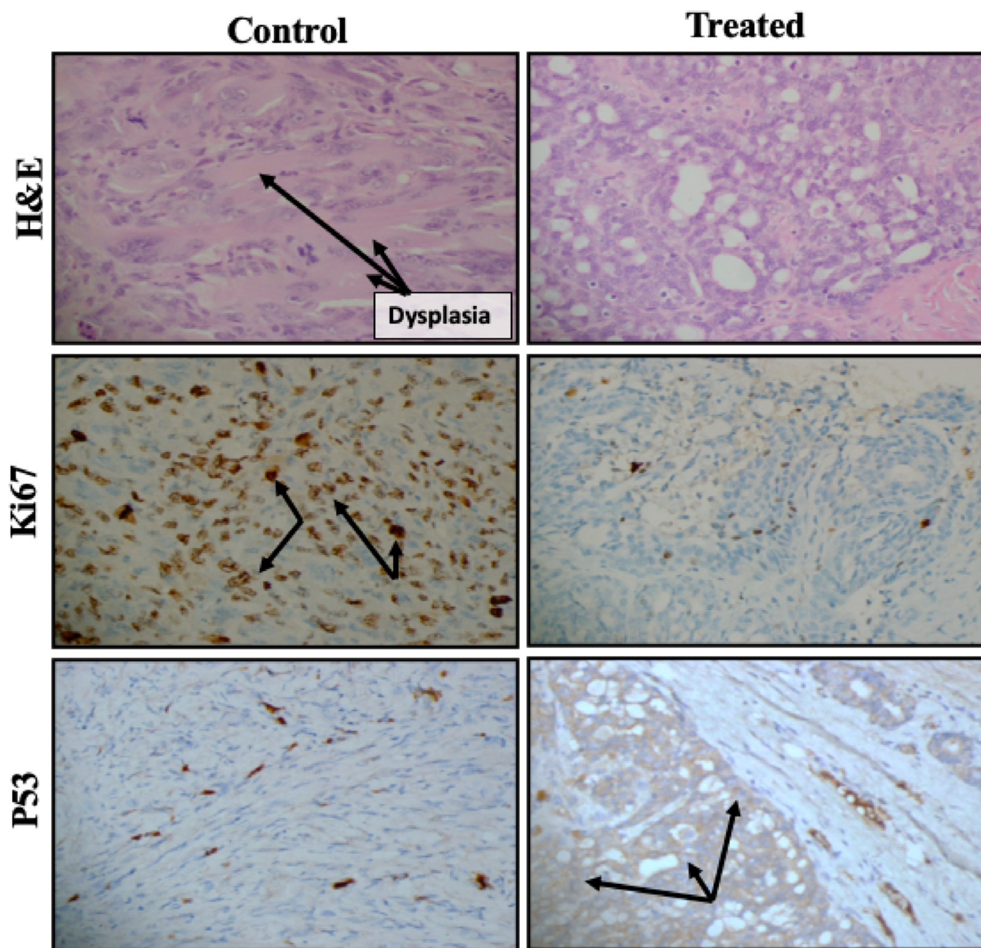

Fig. 8. Histopathological and immunohistochemical evaluation of cell proliferation and apoptotic markers following treatment with aqueous extract (300 mg/kg body weight) in the rat model of breast cancer. Representative treated and untreated rats were used for the evaluation. Hematoxylin and eosin staining was used for the histopathological examination, whereas antibodies for *Ki67* and *p53* were used for immunohistochemical staining. Magnification 400×.

et al., 2017). Indeed, it is well known that the leading cause of death among patients with cancer is the ability of cancer cells to metastasize and invade (Seyfried and Huysentruyt, 2013), and our results present the first demonstration that different *A. squamosa* leaf extracts partially inhibited breast cancer migration (Fig. 4). Furthermore, the current study showed that different extracts induced late apoptosis and necrosis in both MCF-7 and MDA-MB-231 cells (Fig. 5). These results were confirmed by the study of the gene expression of pro-apoptotic and anti-apoptotic markers, and different extracts were found to induce the expression of *Bax* and suppress the expression of *Bcl-2* (Fig. 6). These results agreed with those of Pardhasaradhi et al. (2005), which showed that treatment of MCF-7 with *A. squamosa* seed extracts resulted in nuclear condensation, DNA fragmentation, ROS generation, and apoptosis induction via downregulation of the ratio of *Bcl-2*/Bax. The remarkable anticancer activity of the extracts may due to the high level of germacrene-D on the leaves which exerted anticancer activity against different cell lines, or to the presence of other bioactive compounds which have also been known to have an anticancer activity such as humulene, phytol and/or a combination of these bioactive compounds (Essien et al., 2016; Kim et al., 2015; Pejin et al., 2014). Collectively, methanolic extracts have the highest total phenolic content and germacrene-D, while acetonic extract have the highest total flavonoid content, these differences on the ratio of the bioactive compounds explain the potential differences between the three extracts (Alnemari et al., 2020).

An *in vivo* study can offer conclusive insights about the nature of the biological effect of *A. squamosa* leaf extracts; therefore, a breast cancer model developed in our laboratory was used. It was interesting to note that aqueous extracts significantly decrease tumor size. To better understand the mode of action of the extracts, immunohistochemistry was used to evaluate the expression of proliferative and tumor suppressor genes, *Ki67*, and *p53*. Stronger immunostaining intense for *p53* was found in the treated tissue than the control one, and less intense staining *Ki67* was observed in the treated tissue. This finding was consistent with those of Chen et al. (2012b), which showed that *A. squamosa* seeds extracts inhibited the growth of H22 hepatoma cells by 69.55% in mice without any side effects. According to these results, we can infer that *p53* directly or indirectly targeted either the apoptotic or non-apoptotic cell death pathway. For example, Caspase-3 activation drives the expression of different microRNA species that are known to target the *Bcl-2* or the expression of different genes that are involved in cell cycle arrest (Aubrey et al., 2017).

5. Conclusions

In summary, *A. squamosa* leaf extracts demonstrated potential anticancer activity against breast cancer in both in vitro and *in vivo* experiments; therefore, further investigation may contribute to the development of a potential new anticancer therapy

for both ER + and ER- breast cancer. Further research is needed to fully understand the mechanism of action of *A. squamosa* leaf extracts and its role in cell autophagy, in order to identify the pathway targeted by these extracts.

Author contributions

RA, ABB, and AS conceived and supervised the research. RA conducted the experiments, analyzed, and interpreted the data. RA, ABB and MHA drafted the materials and methods section. NA, MA, and HA helped with animal experiments. RA and AS completed the manuscript. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board, King Fahad Medical City, Riyadh, Saudi Arabia (IRB log number: 18–586, Registration number with KACST, KSU: H-01–12-012 and Registration number with OHRP/NIH, USA: IRB00010471). The rats were obtained from the breeding facility at the College of Pharmacy at King Saud University, Riyadh and the written informed consent to use the animals in our study was obtained from this facility.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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