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Tumor suppressive effect of *Artemisia annua* extract in 7, 12-dimethylbenz[a] anthracene-induced breast cancer in Wistar rats

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ABSTRACT

Objective: This study aimed to detect the genetic and immunohistochemical effects of the extract of *Artemisia annua*, a popular medicinal herb in a chemical model of breast cancer. **Materials and Methods:** Breast cancer was induced in female rats by 7, 12-dimethylbenz[a] anthracene (DMBA). The rats were then treated with 100 and 200 mg/kg *A. annua* extract after 4 months of DMBA administration, and the genetic and immunohistochemical effects of *A. annua* extract were examined on DMBA-induced breast cancer. **Results:** We observed the upregulation of *Nkap*, proliferating cell nuclear antigen, and phosphoinositide-dependent protein kinase 1 genes in DMBA administered rats, which were significantly normalized in the treated groups, particularly with 100 and 200 mg/kg of *A. annua* extract. A significant decrease in p53 expression was detected in DMBA administered rats, which was normalized in the treated groups ($P < 0.05$). Further, the expression of calponin-h2 was enhanced, and that of p63 was declined in the breast tissue of DMBA administered rats, and both levels were normalized in the treated groups. **Conclusion:** We recommend the use of *A. annua* extract as a promising complementary drug in breast cancer treatment.

Keywords: *Artemisia annua*, breast cancer, calponin, DMBA, p63

INTRODUCTION

Breast cancer is spreading in developing countries rapidly; it is the second most common form of malignancy in the world and the most common form of carcinoma in developed countries. The incidence of breast cancer is increasing in young females. Therefore, the world needs a treatment that is more effective and less toxic.[1] Oxidative stress plays an essential role by affecting apoptosis, initiation, promotion, and progression of breast carcinoma by inducing DNA damage and interfering with the intracellular signal transduction pathways.[2]

The beneficial effects of natural herbs with health-promoting purposes were evaluated in several studies through the use of derived products from these traditional plants. In the past few years, *Artemisia* spp. has been increasingly used for reducing and combating the risk of different cancers.[3] The action of artemisinin and its derivatives found in *Artemisia annua* extract have been studied in the induction of apoptosis, inhibition of angiogenesis, inhibition of cell cycle, and in the production of reactive oxygen species.[4] Safety studies have confirmed the absence of clear toxicity and little adverse effects of *A. annua* extract. It also inhibits the growth of tumor masses and has been suggested as a promising therapy.[4] Artesunate and dihydroartemisinin (DHA) have *in vivo* chemosensitizing effects in breast, pancreas, glioma, and lung cancer cells. *A. annua* extract has been recently suggested to be used in combination with anticancer therapy. Therefore, this study was planned and designed to chemically simulate a model of breast cancer using 7, 12-dimethylbenz[a]anthracene (DMBA) and monitor the genetic and immunohistochemical effects of *A. annua* extract on this model. *Nkap* is a 47 kD, nuclear protein involved in NF

kappa (NF-κB) activation in many cells. *Nkap* has a vital role in transcriptional suppression, T-cell development, maturation, maintenance and survival of hematopoietic stem cells, and RNA splicing.[5] P53 works as a tumor suppressor with a significant role in DNA repair mechanisms, working to initiate arrest, repair, and apoptotic pathways.[6] Proliferating cell nuclear antigen (*PCNA*) is an essential proliferation biomarker in many types of cancer, and immunohistochemical staining of *PCNA* has been extensively used in diagnosis and prognosis of breast cancer. *PCNA* has a central role in regulation of the cell cycle in breast cancer proliferation.[7] Phosphoinositide-dependent protein kinase 1 (*PDK1*) plays a key role in the regulation of cell metabolism, proliferation, and survival of cancer cells. In addition, *PDK1* is extremely expressed in many invasive human breast cancers.[8] Calponin-h2 is a significant indicator of breast cancer more than the benign breast disease and healthy control groups. The combination of calponin-h2, carbohydrate antigen 15–3, and carcinoembryonic antigen improved the breast cancer diagnosis.[9] It is an actin cytoskeleton binding protein which is found in smooth muscle and non-muscle cells. It is upregulated in many cancers as breast cancer due to its effect on cell proliferation and metastasis.[10] P63 is a member of the p53 gene family. P63 has an essential role in the development of the stratified squamous epithelium cells in the breast. Breast cancer may be due to the changes in the expression of p63 as it plays a role either as a tumor suppressor.[11] P63 is used as a breast cancer marker without regard to the presence or activity of other p63 isoforms. In mammary adenocarcinoma, there was no expression of p63 or the levels of the expression were small. This was confirmed that p63 is a tumor suppressor.[12]

To the best of our knowledge, this is the first study to use *A. annua* extract in *in vivo* model of breast cancer. This work evaluated the effect of *A. annua* extract on the expression of *Nkap*, p53, *PCNA*, and *PDK1* genes together with the histopathological and immunohistochemical examination of calponin-h2, p63, and Bcl2 expression in the breast tissue of the control, A. annua extract, and DMBA-treated groups.

MATERIALS AND METHODS

Materials

DMBA, agarose, ethidium bromide, and TriZol reagents for the RNA extraction were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kits for CEA and CA15–3 detection were purchased from LifeSpan BioSciences (Seattle, WA, USA). Oligo dT, ethanol, and chloroform were purchased from Wako Pure Chemicals (Osaka, Japan), and primers were obtained from the Macrogen Company (Seoul, South Korea). The reverse transcriptase enzyme was from SibEnzyme, Ltd. (Ak, Novosibirsk, Russia). The master mix for polymerase chain reaction (PCR) was purchased from Promega Corporation (Madison, WI, USA).

Preparation of *A. annua* Aqueous Extract

The collected samples were identified by a botanist (Prof, Yassin Asoudani, Taif University) and a sample was added to the herbarium of Taif University under voucher number 42–215. The plant stems and leaves of *A. annua* were dried and ground to a powder. The dried material was processed for extraction by soaking (1:10 weight/volume) in hot distilled water (90°C). The soaked material was left for 24 h at normal room temperature and then filtered using the Whatman number 1 filter paper. The solvent was evaporated under reduced pressure in a rotary evaporator to get the final extract, which was lyophilized to achieve constant weight. The dried final extract was stored in a dark bottle in a desiccator.[13]

Experimental Design

The Scientific Deanship of Zagazig University, Egypt, along with its Ethical Committee, approved all procedures used in this study #1–440–1567. The present work was carried out on 60 healthy adult female Wistar rats (110–170 g) which were obtained from Laboratory Animals Unit, Fac. Vet. Med., Zagazig University, Egypt. Rats were incubated at 25–30°C and exposed to 12 h/12 h day-night cycle with free access to food and water, then were divided into six groups of 10 rats each as Group 1: Negative control group received no treatment; Group 2: Received 100 mg/kg BW *A. annua* extract by oral gavage,[14] for 1 month (at the same time of extract administration in Groups 5 and 6), Group 3: Received 200 mg/kg BW *A. annua* extract by oral gavage;[14] Group 4: Received 50 mg/kg BW DMBA as a single dose in sesame oil orally on the 55th day of age;[15] Group 5: Received 50 mg/kg BW DMBA in sesame oil orally at the 55th day of age, left for 4 months, and then treated with 100 mg/kg *A. annua* extract by gavage daily for 1 month; and Group 6: Received 50 mg/kg BW DMBA in sesame oil orally at the 55th day of age then left for 4 months then treated with 200 mg/kg *A. annua* extract for 1 month. Groups 1–3 received a single dose of sesame oil orally on the 55th day of age. The animals were weighed daily. The palpation of the animals was performed twice weekly to ensure tumor incidence. Animals were sacrificed by CO_2 asphyxiation 5 months after DMBA treatment. Then, neoplastic and treated tissues were harvested and stored at −80°C for gene analysis and in buffered neutral formalin 10% for immunohistochemical examination.

Gene Analysis and Reverse Transcription PCR (RT-PCR)

Extraction of RNA

About 100 mg of breast tissue samples were collected from each rat, frozen in liquid nitrogen, and stored in 1 mL Qiazol at –70°C followed by homogenization. Chloroform was added, mixed, and centrifuged for 20 min at 12,500 rpm/4°C. The resulting supernatant was mixed with the same volume of isopropanol, adequately shaken, and then centrifuged. Remaining RNA pellets were washed, dried, and dissolved in diethyl pyrocarbonate (DEPC) water.[16] The concentration of RNA was determined spectrophotometrically (260 nm).

cDNA synthesis

For denaturation, 2 μg total RNA was added to 0.5 ng oligo dT primer in a final volume of 11 μL sterilized DEPC water then incubated in PCR machine at 65°C/10 min. Of 5X RT Buffer, 4 μL was added to 2 μL of 10 mM dNTP mix and 100 U Reverse Transcriptase in a final volume of 20 μL by DEPC water. This mixture was incubated again in the PCR machine at 37°C for 1 h, then at 90°C for 10 min to inactivate the enzyme.

Semi-quantitative PCR analysis

Specific primers for genes expressed in breast tissues were designed using the Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, Gasan-dong and Geumcheon-gu, Korea) [Table 1]. PCR was performed as reported in the study of Ibrahim *et al*.[16]

Histopathological Examination

Breast tissue samples were fixed in 10% buffered neutral formalin solution for at least 24 h and then routinely processed. Paraffin sections of 5-micron thickness were prepared, stained with hematoxylin and eosin, and examined microscopically.

Immunohistochemical Examination of p63, Calponin-h2, and Bcl2

The breast sections were deparaffinized and treated with 3% H_2O_2 for 10 min to inactivate the peroxidases. Subsequently, the tissue samples were heated in 10 mM citrate buffer at 121°C for 30 min for antigen retrieval, blocked in 5% normal serum for 20 min, and incubated overnight with a rabbit polyclonal antip63 or calponin or Bcl2 primary antibody in phosphate-buffered saline (PBS) at 4°C. After three extensive washes with PBS, the sections were incubated with a goat anti-rabbit IgG biotin-conjugated secondary antibody (1:2000; sc–2040; Santa Cruz Biotechnology, Inc.) for 20 min at 32°C. After further incubation with horseradish peroxidase-labeled streptavidin, visualization of antibody binding was done using diaminobenzidine, and the sections were counterstained with hematoxylin. Five fields/section and four sections from four rats per group were examined under a light microscope. Investigations were conducted in a blinded manner, and sample scores were estimated according to routine diagnostic guidelines as follows: Score 1= weak; <10 positive stained cells of the three high-power fields (HPF) at ×40; score 2= moderate; 11–20 positive stained cells/HPF; and score 3= Strong; >20 positive stained cells/HPF.[17]

Statistical Analysis

The results are presented as means ± standard error of means. Data analysis was performed through SPSS software version 11.5 for Windows (SPSS, IBM, Chicago, IL, U.S.A.) using analysis of variance and *post hoc* descriptive tests with $P < 0.05$ considered as statistically significant. Regression analysis was calculated using the same software.

Table 1: The primers used for amplification

Primer	Forward	Reverse
G3PDH	AGATCCACAACGGATA CATT	TCCCTCAAGATTGTCA GCAA
Nkap	GGTTCAGCTCA CACCACCAT	CGCGAGTAGCCGCCATA ATA
p53	AGGCAACTATGGCTT CCACC	GACCTCAGGTGGCTCAT ACG
PCNA	ACCTCGCTCCCCTTAC AGT	GTCCCGGCATATACGTG }CAA
PDK1	AAATGCGAAATCACCA GGAC	ATATGGGCAATCCGTAA CCA

RESULTS

All animals survived till the end of study with symptoms of fatigue and diminished activity appeared in DMBA received group with no significant change of weight between different groups, tumors were palpated in all groups received DMBA.

Histopathological Examination

Tumor weights decreased significantly in the treated groups when compared to the DMBA group, particularly in DMBA administered adult female rats treated with 200 mg/kg *A. annua* extract [Table 2]. Control group showed normal tissue architecture with normal secretory acini and ductules lined by low columnar and cuboidal mammary epithelial cells and surrounded by myoepithelial cells with loose connective tissue in between [Figure 1a]. Groups of 100 and 200 mg/kg *A. annua* showed normal lobules and acini surrounded by adipose tissue [Figure 1b and c]. DMBA administered rats showed cancer cells invading intralobular stroma [Figure 1d]. DMBA administered rats followed by 100 and 200 mg/kg *A. annua* groups showed regression in tumor mass and the regression increased by increasing *A. annua* concentration [Figure 1e and f], respectively.

Immunohistochemical Examination of Calponin-h2

Figure 2a-c shows faint calponin-h2 immunostaining of myoepithelium of mammary glands of control and 100 and 200 mg/kg *A. annua* extract received groups, respectively. Figure 2d shows dense calponin-h2 immunostaining of myoepithelium of tumor mass of DMBA administered group rats. Figure 2e and 2f shows regression of tumor mass with mild calponin-h2 immunostaining of DMBA administered rats followed by 100 and 200 mg/kg *A. annua,* respectively.

Immunohistochemical Examination of p63

Figure 3a-c shows positive p63 immunostaining of myoepithelium of mammary glands acini in control and 100 and 200 mg/kg *A. annua* extract received groups, respectively. In contrast, the mammary glands of DMBA administered adult female rats immunostained with anti-p63 antibody showed mostly negative staining of tumor mass [Figure 3d]. The mammary gland of DMBA administered adult female rats treated with 100 and 200 mg/kg *A. annua* extract immunostained with anti-p63 antibody showed tumor mass regression with positive immunostaining of myoepithelium [Figure 3e and f], respectively.

Table 2: Effect of administrating *A. annua* extract on tumor weight (g) in the six groups

Group	Control	*A. annua* 100 mg/kg	*A. annua* 200 mg/kg	DMBA	DMBA+ *A. annua* 100 mg/kg	DMBA+ *A. annua* 200 mg/kg
Tumor weight	0	0	0	19.1±1.3*	9.6±1.4*#	7.2±1.1*#

Values are means±standard error of 10 rats. *P<0.05 versus control group; #P<0.05 versus DMBA group. *A. annua: Artemisia annua*

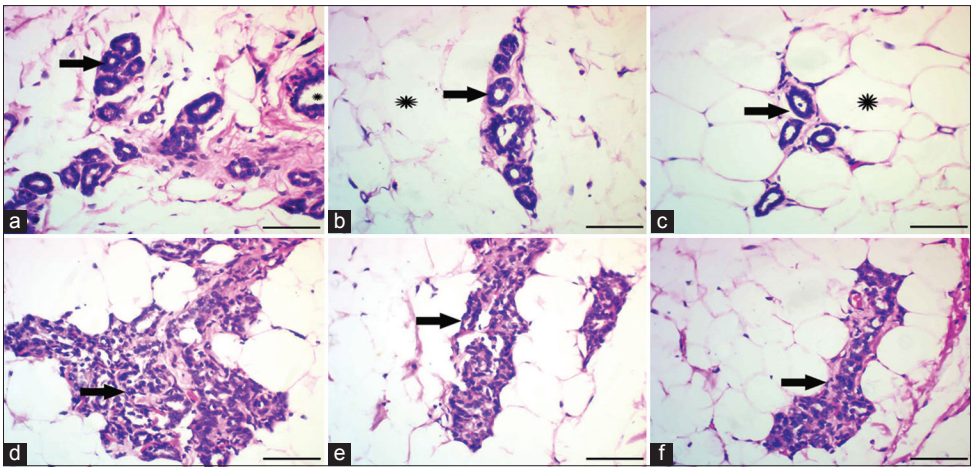

Figure 1: Histopathological examination of mammary gland in the six groups. (a) Control mammary gland with normal acini (arrow) and ductules (*). (b and c) *Artemisia annua* extract group (100 and 200 mg/kg, respectively) showing normal architecture. (d) 7, 12-dimethylbenz[a] anthracene (DMBA) group showing tumor mass (arrow). (e and f) DMBA group treated with (100 and 200 mg/kg, respectively) *A. annua* extract showing decreased tumor mass (arrow) (Hematoxylin and eosin). Scale bar =50 μm

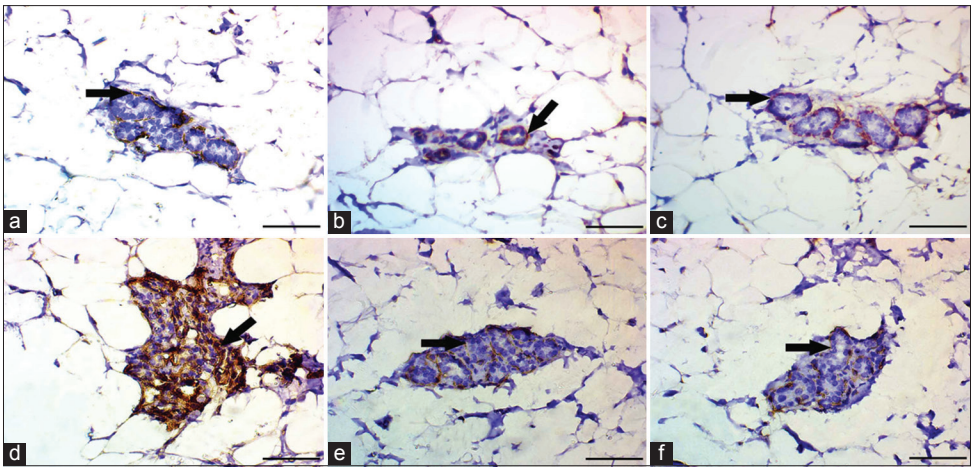

Figure 2: Immunohistochemical examination of calponin-h2 in mammary gland of the six groups. (a) Control group showing the mild reaction of myoepithelium (arrow). (b and c) *Artemisia annua* extract group (100 and 200 mg/kg, respectively) showing the same effect (arrow). (d) 7, 12-dimethylbenz[a]anthracene (DMBA) group showing increased immunostaining of tumor mass (arrow). (e and f) DMBA administered rat treated with 100 and 200 mg/kg *A. annua* extract, respectively, showing decreased tumor mass with mild immunostaining (arrow). Scale bar =50 μm

Immunohistochemical Examination of Bcl2

Mammary tissue of control and *A. annua* 100 and 200 mg/kg received groups did not show significant difference in Bcl2 expression. The same picture was detected in DMBA received rats. On the other hand, DMBA received rats treated with 100 and 200 mg/kg *A. annua* extract showed significant decline in Bcl2 expression, particularly in the group treated with 200 mg/kg *A. annua* extract [Figure 4]. Immunohistochemical scores of calponin-h2 and p53 are recorded in Table 3.

Effect of *A. annua* Extract on mRNA Expression of *Nkap* and p53 in Breast Cancer-induced Rats

Expression of NF-KBNF-KB activating protein (*Nkap*) exhibited no significant difference in the control and 100 and 200 mg/kg *A. annua* extract-administered groups. Adult female rats administered with DMBA showed marked mRNA overexpression of *Nkap* in breast tissues. Treatment of DMBA administered female rats with 100 and 200 mg/kg *A. annua* extract led to significant normalization of *Nkap* mRNA expression in breast tissues, especially in the 200 mg/kg group ($P < 0.05$) as is evident from Figure 5. Meanwhile, the p53 mRNA expression in the breast tissues of the control and 100 and 200 mg/kg *A. annua* extract-administered groups revealed no significant differences. DMBA administered female rats showed significant regression of p53 mRNA expression in breast tissue ($P < 0.05$) in comparison to the control group. The treatment of DMBA administered female rats with 100 and 200 mg/kg *A. annua* extract resulted in the restoration of p53 mRNA expression in breast tissue in comparison to the DMBA group ($P < 0.05$), and the expression was more enhanced in the 200 mg/kg group [Figure 5].

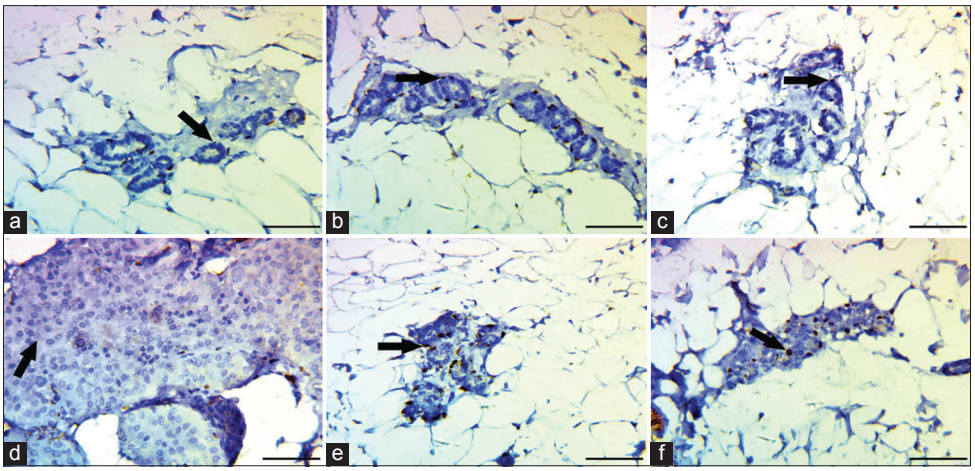

Figure 3: Immunohistochemical examination of p63 in mammary tissue of the six groups. (a) Control group showing positive reactivity (arrow). (b and c) *Artemisia annua* extract group (100 and 200 mg/kg, respectively) showing the same effect (arrow). (d) 7, 12-dimethylbenz[a]anthracene (DMBA) group showing the absence of reactivity (arrow). (e and f) DMBA group treated with *A. annua* extract (100 and 200 mg/kg, respectively) showing the regression of tumor mass with positive immunostaining (arrow). Scale bar =50 μm

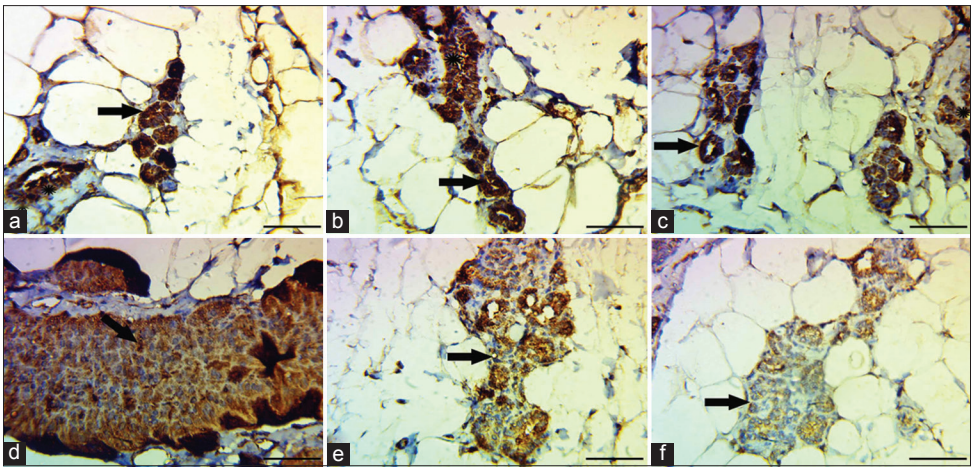

Figure 4: Immunohistochemical examination of Bcl2 in mammary tissue. (a-c) Breast tissue of control and *Artemisia annua* 100 and 200 mg/kg received groups, respectively, with increased expression of Bcl2 in acini (arrows) and ducts (*). (d) 7, 12-dimethylbenz[a]anthracene (DMBA) received rats showed increased Bcl2 expression in solid tumor mass (arrow). (e and f) DMBA group treated with *A. annua* 100 and 200 mg/kg, respectively, showed decreased Bcl2 expression (arrows). Scale bar =50 μm

Table 3: Immunochemical scores of calponin-h2 and p53 in the mammary gland of the six groups (mean±standard error)

Immunohistochemical staining	Control	*A. annua* 100 mg/kg	*A. annua* 200 mg/kg	DMBA	DMBA+ *A. annua* 100 mg/kg	DMBA+ *A. annua* 200 mg/kg
Calponin-h2	1±0.011	1±0.013	1± 0.026	3±0.012*	1±0.01#	1±0.015#
p63	2±0.013	2±0.012	2±0.015	1±0.014*	2±0.009#	2±0.011#
Bcl2	3±0.012	3±0.017	3±0.009	3±0.010	2±0.012#	1±0.014#

Values are means±standard error of 10 rats. *P<0.05 versus control group; #P<0.05 versus DMBA group

Effect of *A. annua* Extract on mRNA Expression of *PCNA* and *PDK1* in Breast Cancer-induced Rats

Evaluation of the genetic effect of DMBA on *PCNA* mRNA expression in the breast tissue of adult female rats resulted in a significant increase in its expression in comparison to the control group (P < 0.05). No significant variations were detected between the control and 100 and 200 mg *A. annua* extract-administered groups. Treatment of DMBA group with 100 and 200 mg/kg *A. annua* extract led to a significant decline in the elevated *PCNA* mRNA expression (P < 0.05), as is evident from Figure 6. In addition, no significant difference in *PDK1* expression was detected between the control and 100 and 200 mg *A. annua* extract-administered groups. DMBA administration led to a significant increase in *PDK1* mRNA expression in the breast tissue of adult female rats. Treatment of DMBA group with 100 and 200 mg/kg *A. annua* extract led to a significant decline of the elevated mRNA expression in comparison to the DMBA group (P < 0.05), as shown in Figure 6.

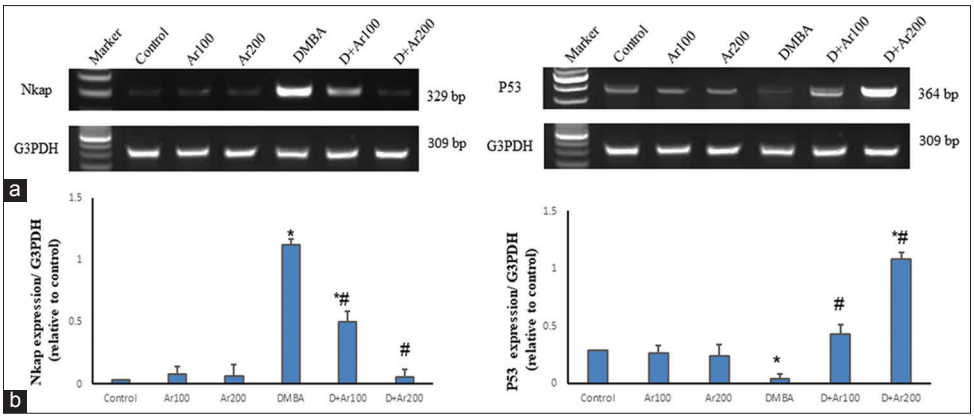

Figure 5: (a and b) Effect of *Artemisia annua* extract on changes in *Nkap* and p53 gene expression induced by DMBA in the breast of the six groups. Values are means ± standard error of 10 rats. *P <0.05 versus control group; #P <0.05 versus 7, 12-dimethylbenz[a]anthracene group. Upper panels show mRNA expression of the examined gene. Lower columns show the densitometric analysis of gene expression

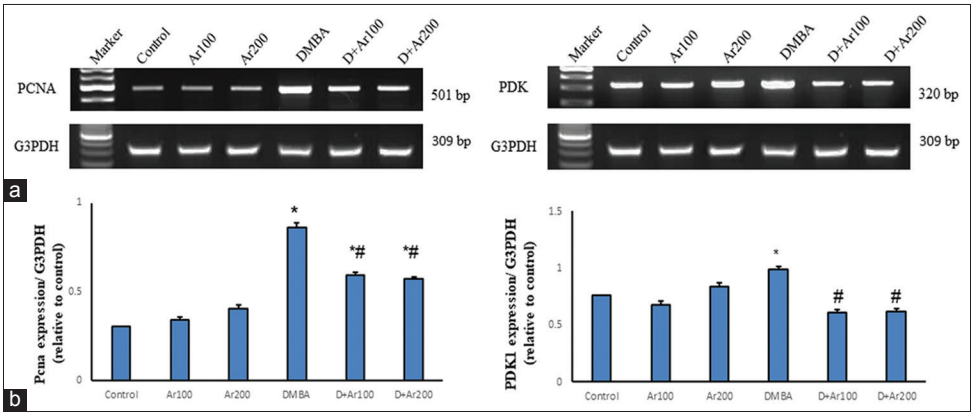

Figure 6: (a and b) Effect of *Artemisia annua* extract on changes in *PCNA* and PDK gene expression induced by 7, 12-dimethylbenz[a]anthracene (DMBA) in the breast of the six groups. Values are means ± standard error of 10 rats. *P <0.05 versus control group; #P <0.05 versus DMBA group. Upper panels show mRNA expression of the examined gene. Lower columns show the densitometric analysis of gene expression

DISCUSSION

Breast cancer is one of the most prevalent cancers occurring worldwide. Mortalities due to this disease are increasing annually with a high level of suffering to a patient. We showed extensive anaplastic changes in the mammary gland when using DMBA to induce breast cancer. DMBA is a well-known toxic agent that chemically induces breast cancer in the rat model. It induces hyperplasia and atypical hyperplasticity of the ductal epithelial cells, with carcinogenesis of the terminal ducts.[15] Several studies have used DMBA for the induction of breast cancer in a rat model.[18-20] The tumor induced by this model is morphologically and histologically similar to that observed in human estrogen-dependent breast cancer.[21] Treatment with *A. annua* extract led to a decrease in tumor mass due to the presence of several active ingredients that combat cancer progressions such as artemisinin and its derivatives, coumarins, flavonoids, and other terpenoid constituents which have been reported to exert several bioactive functions including antitumor and anti-inflammatory activities.[22] *A. annua* L. is known to contain artemisinin and its derivatives. The herb is also used as a source of essential oils, including mainly camphor (44%), germacrene D (16%), trans-pinocarveol (11%), β-selinene (9%), β-caryophyllene (9%), and artemisia ketone (3%).[22] Suppression of immune mediators of angiogenesis by flavonoids and sesquiterpene lactones might be one of the mechanisms of *A. annua* L. anticancer activity.[23] Artemisinin compounds changed the immune response by regulating cytokine release and cell proliferation.[24] DHA is a primary compound of artemisinin isolated from *A. annua* and exhibits anticancer activity in specific types of cancers such as ovarian cancer, breast cancer, prostate cancer, and lung cancer. Among artemisinin and its derivatives, DHA shows the strongest anticancer effects. DHA was found to induce apoptosis and inhibit cell migration, proliferation, and invasion.[25] *A. annua* L. contains many compounds such as flavonoids and terpenoids, which are characteristic secondary metabolites. Many essential oils and other ingredients were detected as α-pinene, 1,8-cineole, camphor, fenchol, camphene hydrate, artemisia alcohol, artemisia ketone, and santolina triene which give the plant a sweet scent.[26]

Calponin-h2 is encoded by the *CNN2* gene. Calponin-h2 is a significant indicator of breast cancer, with its expression more than that in benign breast disease and healthy control groups.[9] The expression of calponin increased in DMBA administered rats, indicating cancer progression as a result

of the continuous proliferation of cancer cells and complete separation of calponin-h2 from the actin skeleton of the mitotic cells.[27] Treatment with *A. annua* extract normalized the calponin-h2 expression with a decrease in the tumor mass. Rats treated with 100 and 200 mg/kg *A. annua* extract showed regression of tumor mass with positive p63 immunostaining of myoepithelium. P63 is a specific and sensitive marker for the myoepithelial cells.[28] It is a member of the p53 gene family that has an essential role in the development and differentiation of the stratified squamous epithelial cells in the breast and is used as a breast cancer marker.[11,29] P63 is absent or weakly expressed in mammary cancer confirming that p63 is a tumor suppressor.[12] P63 is a vital protein that stimulates cell apoptosis and the absence of p63 as observed in our results in DMBA administered rats with accelerated tumor genesis and cancer spread.[30] Meanwhile, increased expression of p63 in the treated groups suppressed tumor progression.

Our results showed upregulation of *Nkap*, *PCNA*, and *PDK1* genes in DMBA administered rats, which were significantly normalized in the groups, particularly when treated with 100 and 200 mg/kg *A. annua* extract. *Nkap* has been shown to play a role in promoting the progression of breast cancer.[5] *Nkap* (NF-κB activating protein) derived its name from the previous findings that this protein could activate the NF-κB pathway.[31] NF-κB participates in the initiation and progression of the tumor in tissues where cancer-related inflammation occurs easily.[32] It is not only a key coordinator of inflammation and innate immunity but also regarded as an important endogenous tumor promoter. In tumor cells, NF-κB induces the anti-apoptotic gene expression to accelerate cell growth and activates associated inflammatory cytokines, adhesion molecules, and angiogenic factors to facilitate the proliferation of tumor cells.[33] In addition, the expression of NF-κB in treated groups was repressed, indicating tumor suppression. *PCNA* is essential for DNA replication and DNA repair in normal conditions. It is overexpressed in proliferating and cancer cells and is a good proliferation marker. Earlier studies have linked *PCNA* to the degree, stage, and prognosis of cancer.[34] Enhanced expression of *PCNA* indicates abnormal proliferation of breast tissue.[35] Our results were in accordance with those of Qiu *et al*. who detected increased expression (42.2%) of *PCNA* in cancer tissues[34] which is essentially associated with the incidence and progression of breast cancer.[36] Meanwhile, the DMBA groups treated with *A. annua* extract showed repressed *PCNA* expression, which was associated with tumor suppression. *PDK1* is upregulated in certain cancers and helps in activating cancer cell growth and survival. *PDK1* has multiple roles as an Akt-activating agent, a vital oncogenic regulator, and a potential therapeutic target in cancer. *PDK1* regulates tumor formation in breast cancer and confers resistance against many anticancer drugs.[37] Here, its expression in treated groups was reduced, indicating tumor suppression. A significant decline in the p53 expression was detected in DMBA administered rats, which became normal in the treated groups ($P < 0.05$). P53 is a tumor suppressor that plays several roles such as gene transcription, DNA synthesis and repair, cell cycle regulation, aging, and cell death. It can induce cell cycle arrest and apoptotic cell death.[38,39] Therefore, the downregulation of p53 in the DMBA group indicates the suppression of apoptosis and progression of cancer growth, while, its expression was upregulated in treated groups indicating enhanced apoptosis and decreased tumor progression. Thus, we suggest that *A. annua* extract works by downregulating the *Nkap*, *PCNA*, and *PDK1* genes, and calponin-h2 and enhancement of p53 and p63 expression with promising *in vivo* anticancer effect; thus, we suggest its use as a complementary therapy in breast cancer.

CONCLUSION

A. annua extract exerts remarkable effects on DMBA administered adult female rats with significant normalization of mRNA expressions of p53, Knap, *PDK1,* and *PCNA*, and immunohistochemically normalized expression of calponin-h2 and p63, suggesting its potential use as a complementary anticancer agent.

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