Gene expression and histopathology alterations during rat mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and the protective role of Neem (*Azadirachta indica*) leaf extract

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Abstract: The present study was investigated to evaluate the protective role of ethanolic neem leaf extract (ENLE) against 7,12-dimethylbenz[a]anthracene (DMBA)-induced expression alterations of the Bcl-2, CK8, CK19, p53, p21, p27 and PCNA genes and histopathological lesions in the mammary tissues of female rats. Eighty Swiss albino female rats were divided into eight groups. Group 1 supplemented with corn oil as control. Group 2 females received DMBA. Groups 3, 4 and 5 females received 100, 200 and 400 mg/kg of ENLE alternate to the DMBA application from the beginning of the experimental period, respectively. Groups 6, 7, and 8 females treated similar to groups 3, 4 and 5 plus DMBA. All the animals were sacrificed after an experimental period of 12 weeks. The expression of Bcl-2, CK8, CK19, p53, p21, p27 and PCNA genes was investigated using reverse transcription polymerase chain reaction (RT-PCR). In addition, histopathology analysis of the mammary tissues was confirmed. The results revealed that DMBA treatment induced expression alterations of genes related to cancer. Also histopathological lesions were found in mammary tissues of female rats. These alterations of the gene expression as well as the histopathological lesions were markedly suppressed when female rats were treated with ENLE combined with DMBA. Conclusion: These findings suggest that ENLE exerts its anticancer properties by inhibiting alterations in the gene expression and the histopathological lesions in the mammary tissues of female rats exposed to DMBA. [Journal of American Science 2010; 6(9):843-859]. (ISSN: 1545-1003).

Keywords: Bcl-2, CK8, CK19, p53, p21, p27, PCNA genes; RT-PCR; Rat mammary carcinogenesis; Neem leaf, Histopathology.

Introduction

Human breast cancer is a heterogeneous disease with respect to pathology, biochemistry and etiology. While environmental carcinogen exposure is often regarded as contributing to human cancer risk, identifying specific etiological factors in breast cancer remains a challenge in cancer research [1-4]. The rat has served as a valuable model for understanding the development of human breast cancer because of similarities in pathology, cell of origin and hormone dependency [5,6]. Furthermore, the rat model provides a means to study specific etiological factors in breast carcinogenesis in a way not feasible in humans. Breast cancer is the most frequent malignancy among women worldwide [7]. It is a highly heterogeneous disease represented by tumors that have a diverse natural history, complex histology and a variable response to therapy.

Although the molecular events that trigger breast cancer progression, including its initiation, promotion and progression to a fully malignant state, are not fully understood, many genetic alterations have been described [8-10]. These changes primarily encompass mutations, chromosomal amplifications and deletions involving oncogenes and tumor suppressor genes. Altered expression of several key genes in breast cancer also form the basis for clinically useful subdivisions. These genes include the estrogen receptor and erbB2 (HER2/neu). A clearer understanding of the molecular events underlying this complex disease has been accomplished using gene expression profiling. This global approach provides evidence of the biologic diversity of the disease and has led to the classification of breast tumors into informative subtypes [11-13].

The induction of rodent mammary tumors following the administration of N-methyl-N-nitrosourea (NMU) or 7,12-dimethylbenz[a]anthracene (DMBA) is a widely used experimental animal model for investigating breast cancer in women [14—20]. These carcinogen-induced tumors arise from terminal end buds [21-22], an analogous structure to the terminal ductal lobular unit in humans, which is the proposed site of origin of ductal carcinoma in situ (DCIS) [15,23]. Substantial evidence suggests that this animal model mimics human breast cancer. The rat tumor's histopathology, origination from mammary ductal epithelial cells, and

dependency on ovarian hormones for tumor development all correlate with human breast cancer [22,24,27]. Comparative studies have also shown that chemically-induced mammary carcinomas, like their human counterparts, have altered expression of TGFB, erbB2 and cyclin D1 [27]. Furthermore, some human and most rat mammary tumors express estrogen and progesterone receptors [28,29].

Unlike human breast cancer, the majority of carcinogen induced rat tumors are thought to have carcinogen-specific mutations in the H-ras oncogene [30]. In particular an H-ras mutation at codon 12 has been identified in NMU-induced tumors, while DMBA-induced tumors harbor a mutation at codon 61. In the rat model these mutations have been implicated in tumor initiation based upon observations of ras oncogene activation preceding the onset of neoplasia [30-32]. Nonetheless, this model has been used extensively to evaluate preventative and therapeutic agents for human breast cancer despite the near absence of ras mutations in human breast cancer. On a more global scale, little is known about the similarities in gene expression between human breast cancer and carcinogen-induced rat tumors.

Many plant-based chemopreventive agents are recognized to exert their anticarcinogenic effects by inhibiting cell proliferation and inducing cell differentiation and apoptosis. However, the chemopreventive efficacies of these plants need to be tested in well established experimental animal tumour models [33].

Of late, medicinal plants rich in antioxidant phytochemicals are being explored for antiproliferative and apoptosis-inducing properties. *Azadirachta indica* A. Juss, commonly known as neem, is one of the most versatile medicinal plants that has gained worldwide prominence owing to its medicinal properties. Although all parts of the neem tree-leaves, flowers, seeds, fruits, roots and bark are known to possess a wide range of pharmacological properties, the medicinal utilities have been described especially for neem leaf. Extracts of neem leaf have been reported to be non-toxic, non-mutagenic and found to possess immunomodulatory, anti-inflammatory and anticarcinogenic properties [34]. Previously, we documented the chemopreventive potential of aqueous neem leaf extract against experimental animal tumour models for oral and stomach carcinogenesis [35,36].

Several experimental studies have demonstrated that the alcoholic extracts of neem leaf are more effective than aqueous extracts and exhibit a wide range of pharmacological properties [34,37].

Development of malignant tumors is associated with excessive cell proliferation, deregulation of cellular differentiation, insufficient apoptosis and genomic instability [38]. Proteins that play a pivotal role in each of these processes are useful as surrogate endpoint biomarkers (SEBs) of carcinogenesis as well as chemoprevention. Proliferating cell nuclear antigen (PCNA), a nuclear protein present in proliferating cells is essential for cell replication and acts as a marker for cell proliferation [39]. Cytokeratins (CK8 and CK19) are sensitive and specific indicators of epithelial differentiation [40]. p53, a protein encoded by the TSG p53 functions as a guardian of the genome facilitating cell cycle arrest, differentiation and apoptosis thereby decreasing the accumulation of mutant cell populations [41]. p21 and p27, cyclin dependent kinase inhibitors are important regulators of cell cycle progression. The Bcl-2 family of proteins plays a central role in regulating apoptosis [42]. We demonstrated the protective effects of ethanolic neem leaf extract (ENLE) against 7,12-dimethylbenz[a]anthracene-induced oxidative stress and mammary carcinogenesis. The present study was designed to evaluate the histopathological lesions and gene expression analysis of the Bcl-2, CK8, CK19, p53, p21, p27 and PCNA genes to biomonitor chemoprevention.

Materials and Methods

Chemicals

Reagents and solvents used in the current study were of the highest possible grade available. The DMBA was purchased from Sigma-Aldrich (USA). Reagents for RT-PCR method were purchased from Invitrogen (UK) and Fermentas (Germany).

Experimental Animals

Eighty Swiss albino female rats weighing 80-100 g were obtained from the Animal House Colony, University of king Abdulaziz, Jeddah, Saudi Arabian. The animals were kept individually in wire bottomed cages at room temperature (25 ± 2 ºC) under 12 h dark-light cycle. They were maintained on standard laboratory diet and water *ad libitum*. The animals were allowed to acclimatize their new conditions for one week before commencing experiment, then they were distributed into eight groups (10 rats/ group). All animals received human care in compliance with the guidelines of the Ethical Committee of Medical Research, University of king Abdulaziz, Jeddah, Saudi Arabian.

Preparation of neem leaf extract

Ethanolic neem leaf extract (ENLE) was prepared according to the procedure described by Chattopadhyay [43]. Air-dried powder (1 kg) of *A. Indica* leaves was mixed with 3 L of 70% ethyl alcohol and kept at room temperature for 36 h. The

slurry was stirred intermittently for 2 h and left overnight. The mixture was then filtered and the filtrate was concentrated under reduced pressure (bath temperature 50°C) and finally dried in a vacuum dessicator. The residue collected (yield 48 g/kg of neem leaf powder) was a thick paste, green in colour and gummaceous in nature. The extract was suspended in normal saline to obtain a final concentration of 20 mg/mL and used for the experiment. The dose administered in the present study (100 - 400 mg/kg bw) is based on the literatures [43-45]. This dose is also far less than the oral LD50 for ENLE, which was found to be 4.57 g/kg bw in acute toxicity studies [43].

Experimental design

After an acclimation period of one week, female albino rats 60-day-old (n=10 per group) were treated for 12 weeks and divided into the following groups: (1) corn oil (0.5 ml/ animal, i.g.) treated as control group, (2) females received 100 mg/kg DMBA (in corn oil)/kg body weight (i.g.), (3, 4, 5) females received 100, 200 and 400 mg/kg bw, i.g., of ENLE extract three times a week on days alternate to the DMBA application from the beginning of the experimental period, respectively, (6, 7, 8) females treated similar to groups 3, 4 and 5 plus100 mg/kg DMBA (in corn oil)/kg body weight (i.g.). In this study, the dose of DMBA was selected following the previous study of Huggins et al. [46]. Rats were palpated every day for mammary tumors until they were 150 days old. In each group, the cumulative number of tumor masses was calculated by each day's palpation of rats.

During treatment, animals were observed twice daily for signs of moribundity and mortality. Body weights were recorded initially, once weekly, and at termination. At the end of the experimental period, the animals were rapidly sacrificed and the samples of the mammary tissues of each animal were dissected, thoroughly washed with isotonic saline and placed in a Petri dish containing PBS buffer (137 mM NaCl, 3 mM KCl, 4 mM Na2HPO4 and 1.5 mM KH2PO4, pH 7.4) to prevent desiccation. The tissues were gently blotted to remove excess PBS. Tissues were snap-frozen in liquid nitrogen and were kept at -80°C until analysis.

Histopathological examination

Samples of the mammary tissues were obtained from rats under deep anesthesia with diethyl ether. Representative sections of each tumor were fixed in 10% phosphate-buffered formalin and routinely processed for hematoxylin–eosin staining. Pathological diagnoses of the mammary lesions were classified as described previously [47]. In addition, other representative sections were frozen without fixation and stored at −80 ◦C.

Semi-quantitative Reverse Transcription-PCR
RNA extraction

Stored mammary tissue samples (at −80 °C prior to extraction), were used to extract the total RNA. Total RNA was isolated from 100 mg of tissues by the standard TRIzol extraction method (Invitrogen, UK) and recovered in 100 µl molecular biology grade water. In order to remove any possible genomic DNA contamination, the total RNA samples were pre-treated using DNA-free™ DNase treatment and removal reagents kit (Ambion, Austin, TX, USA) following the manufacturer's protocol. The RNA concentration was determined by spectrophotometric absorption at 260 nm.

Synthesize of First-strand cDNA

To synthesize the first-strand cDNA, 5 µg of the complete Poly(A)$^+$ RNA isolated from rat samples was reverse transcribed into cDNA in a total volume of 20 µl using 1 µl oligo (poly(deoxythymidine)$_{18}$) primer [48] . The composition of the reaction mixture consisted of 50 mM MgCl2, 10x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 200 U/ µl reverse transcriptase (RNase H free), 10 mM of each dNTP, and 50 µM of oligo(dT) primer. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through polymerase chain reaction (PCR) [49].

RT-PCR assay

The first strand cDNA from different mammary tissue samples was used as templates for the semi-quantitative RT-PCR with a pair of specific primers in a 25-µl reaction volume. The sequences of specific primer and product sizes are listed in Table 1. β-Actin was used as a housekeeping gene for normalizing mRNA levels of the target genes. The reaction mixture for RT-PCR was consisted of 10 mM dNTP's, 50 mM MgCl2, 10x PCR buffer (50 mM KCl; 20 mM Tris-HCl; pH 8.3), 1U/ µl taq polymerase, and autoclaved water. The PCR cycling parameters of the studied genes (Bcl-2, CK8, CK19, p53, p21, p27 and PCNA) were performed as the PCR condition summarized in Table 1. The PCR products were then loaded onto 2.0% agarose gel, with PCR products derived from β-actin of the different rat samples**.** Each reaction of the RT-PCR was repeated

with ten rats, generating new cDNA products at least ten times per each group.

Statistical Analysis:

All data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System [50] followed by Scheffé-test to assess significant differences between groups. The values are expressed as mean ± SEM. All statements of significant were based on probability of $P < 0.05$.

Results

Rat survival and body weight

The results revealed that no significant differences in survival were observed between the corn oil-treated control, ENLE and ENLE+DMBA groups, with approximately 97% of the animals surviving to study termination (range = 89–99%). However, the survival rate between DMBA animals was decreased compared with control which reached 76%.

The mean body weights of rats receiving 200 and 400 mg/kg of ENLE did not significantly differ from controls over time. However, the mean body weight of rats exposed to DMBA and 100 mg/kg of ENLE plus DMBA groups was only 69% and 87% that of controls by the end of the study (376 g vs. 458 g and 398 g vs. 458 g, respectively).

Semi-quantitative RT-PCR

Reverse transcription polymerase chain reaction was conducted to verify the expression of the Bcl-2, CK8, CK19, P53, P21, P27 and PCNA genes related to cancer progression in female rats using gene expression analysis (Table 1).in the mammary tissues female rats exposed to DMBA with or without ENLE for 12 weeks.

The results of the present study revealed that expression level of Bcl2 gene was significantly higher in mammary tissue of DMBA group than control and other ENLE groups (Fig. 1). Moreover, the expression level of Bcl2 gene in 100 and 200 mg/kg of ENLE plus DMBA groups was also significantly higher than control group. However, this expression was significantly lower in 400 mg/kg of ENLE plus DMBA group than control group. On the other hand, the Bcl2 expression in 100, 200 and 400 mg/kg of ENLE alone was similar to control group (Fig. 1).

The expression level of CK8 and CK19 genes in the mammary tissues of female rats exposed to DMBA and 100 mg/kg of ENLE plus DMBA groups was significantly higher than control and other ENLE groups (Fig. 2 and 3). However, these genes showed expression level significantly lower in 400 mg/kg of ENLE plus DMBA group than control group. In contrast, the CK8 and CK19 expression in 100, 200 and 400 mg/kg of ENLE alone was similar to control group (Fig. 2 and 3).

From the determination of the expression level of gene p53 the results revealed that the expression level was significantly higher in mammary tissue of DMBA group than control and other ENLE groups (Fig. 4). However, the expression level of p53 gene in 100 mg/kg of ENLE plus DMBA group was significantly higher than control group. However, this expression was significantly lower in 200 and 400 mg/kg of ENLE plus DMBA groups than control group. Also, the p53 expression in 100, 200 and 400 mg/kg of ENLE alone was similar to control group (Fig. 4).

The expression level of p21 gene in the mammary tissues of female rats exposed to DMBA and 100 mg/kg of ENLE plus DMBA groups was significantly higher than control and other ENLE groups (Fig. 5). However, the expression of p21 gene showed level of expression did not significantly change in 200 and 400 mg/kg of ENLE plus DMBA groups compared with control group. In the same trend, the p21 expression in 100, 200 and 400 mg/kg of ENLE alone was similar to control group (Fig.5).

The expression profile of p27 gene was significantly higher in mammary tissue of DMBA group than control and other ENLE groups (Fig. 6). However, the expression level of p27 gene in 100, 200 and 400 mg/kg of ENLE plus DMBA groups was not significantly different compared with control group. Also, this expression in 100, 200 and 400 mg/kg of ENLE alone was similar to control group (Fig. 6).

The expression findings of PCNA gene found that the expression level was significantly higher in mammary tissue of DMBA group than control and other ENLE groups (Fig. 7). However, the expression level of PCNA gene in 100 mg/kg of ENLE plus DMBA group was significantly higher than control group. However, this expression was lower in 200 and 400 mg/kg of ENLE plus DMBA groups than control group. Moreover, the PCNA expression in 100, 200 and 400 mg/kg of ENLE alone was similar to control group (Fig. 4).

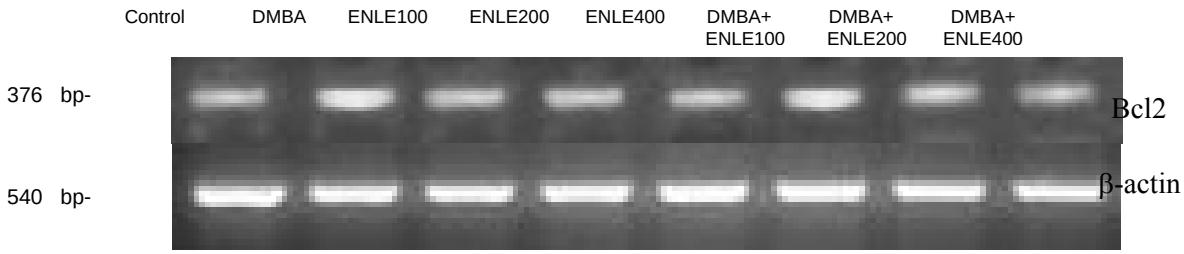
Histopathological findings

The present study revealed that the development of the mammary gland of control and 10, 200 and 400 mg/kg of ENLE rats at the ages of 160 days found significant advanced lobular maturation in the mammary gland with numerous type 2 lobules and even a few type 3 lobules, reminiscent of a pseudo-pregnant state (Fig. 8a, b and c). All of the animals at 160 days of age had fluid filled lobules and fat globules within the epithelium, but none had

undergone the proliferation necessary to form fully lactational type 4 lobules (Fig. 8a, b and c).

In contrast, the mammary gland at the ages of 160 days after exposure to DMBA alone and 100 mg/kg of ENLE plus DMBA for 90 days found enlargement of the lobular maturation in the mammary gland (Fig. 8d, e and f). Histological examination of these groups found enlarged lymph node of female rats. Lymphatic nodules are enlarged with lymphoid cell accumulation in the periphery of germinal centres. Proliferation of medullary cords and the stromal tissue has resulted in compression of medullary sinuses (Fig. 8d, e and f). Mammary adenocarcinoma indicated small epithelial cells are arranged to form small round cavities or elongated tubules. Cords of cells form a loose glandular formation adjacent to nests of cells. The dark area is a cystic cavity filled with blood (Fig. 8d, e and f). These lesions did not occur in the mammary tissues of female rats exposed to 200 and 400 mg/kg of ENLE plus DMBA.

a



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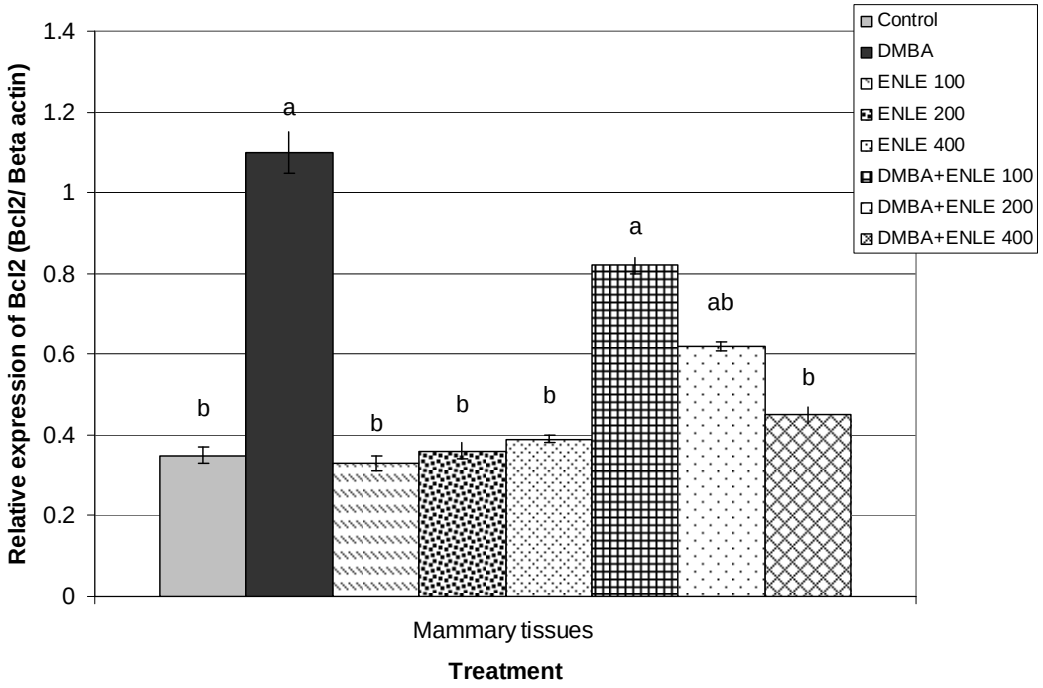


Figure 1: Semi-quantitative RT-PCR confirmation of Bcl2 gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

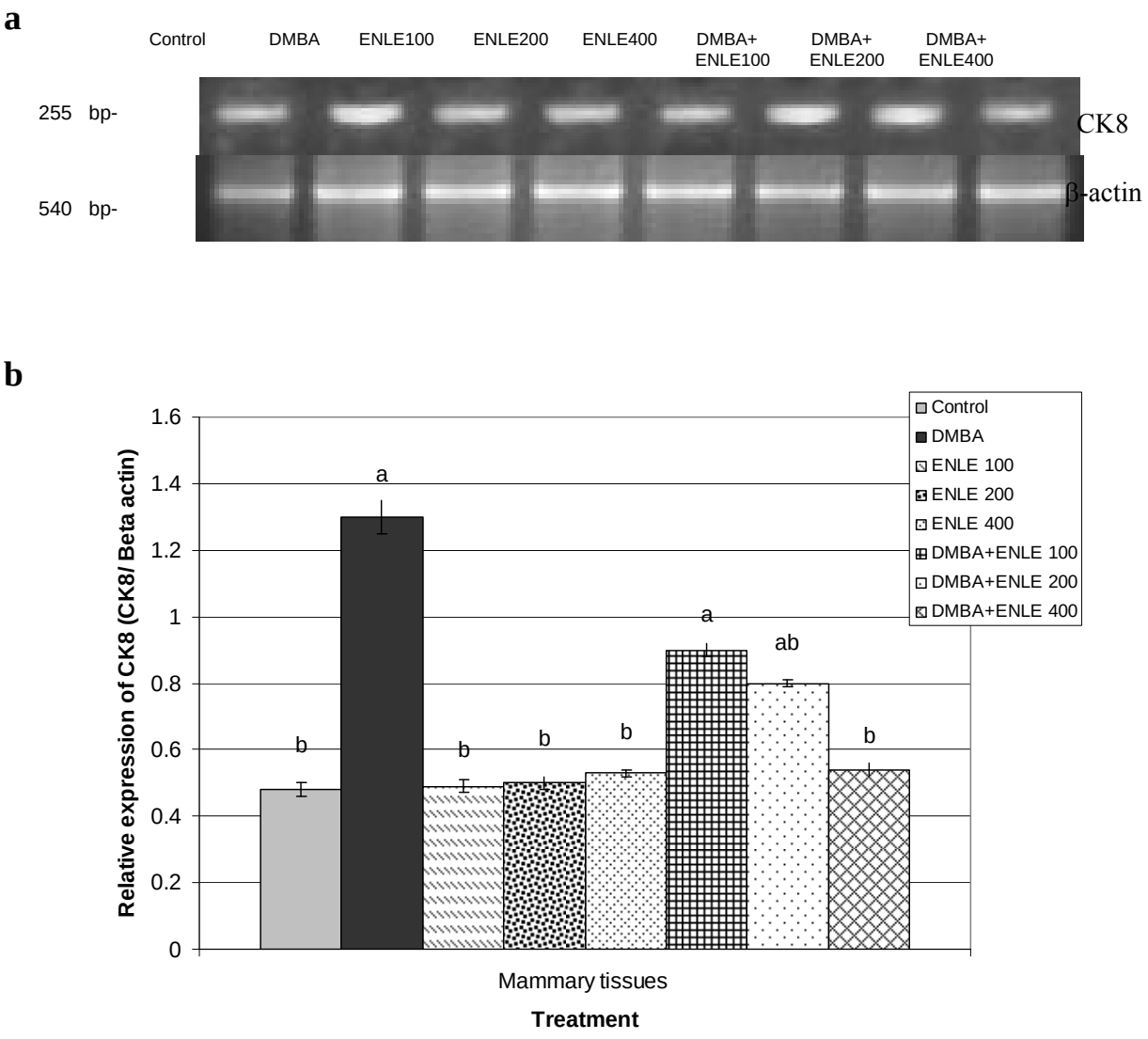


Figure 2: Semi-quantitative RT-PCR confirmation of CK8 gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

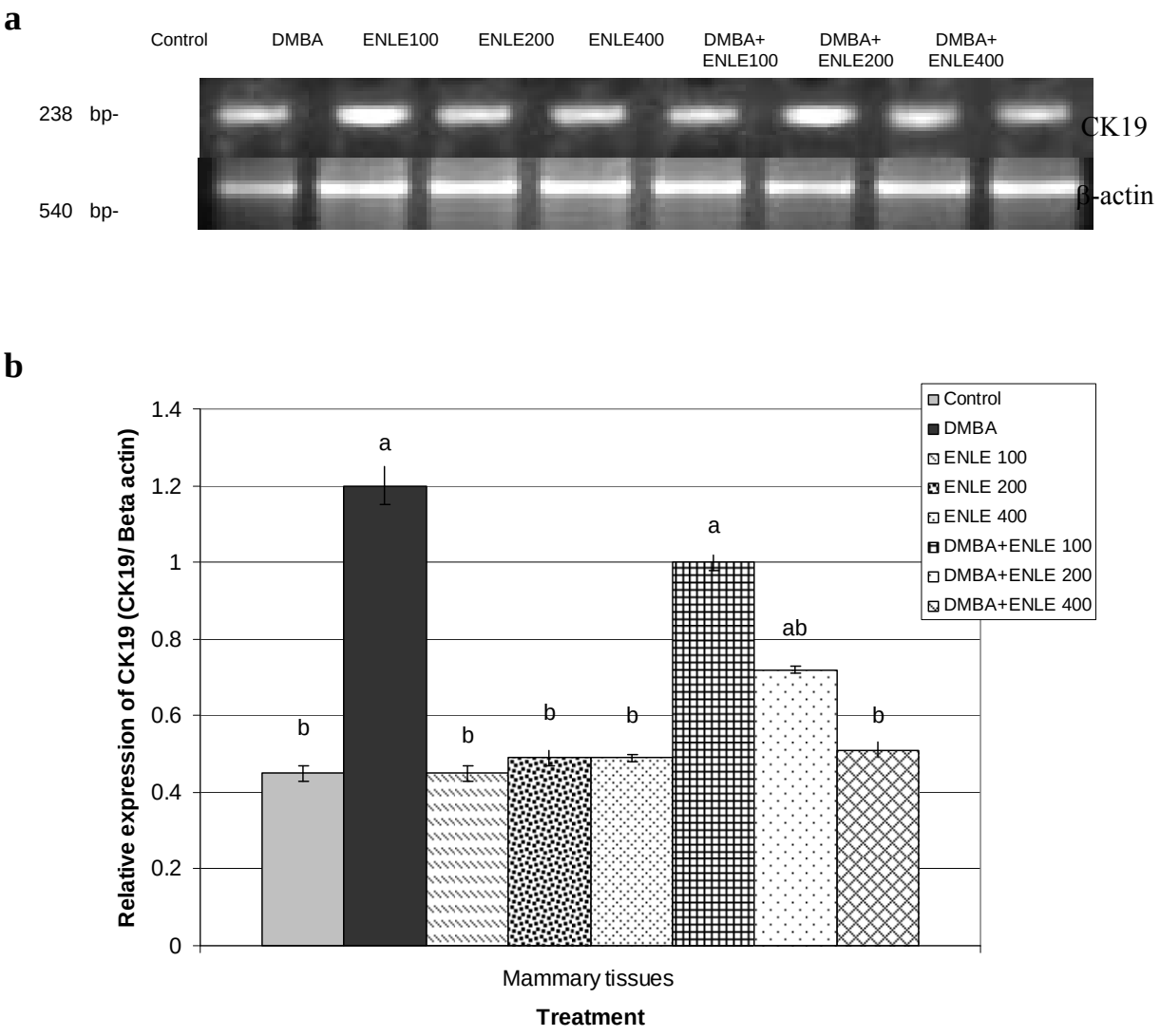


Figure 3: Semi-quantitative RT-PCR confirmation of CK19 gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

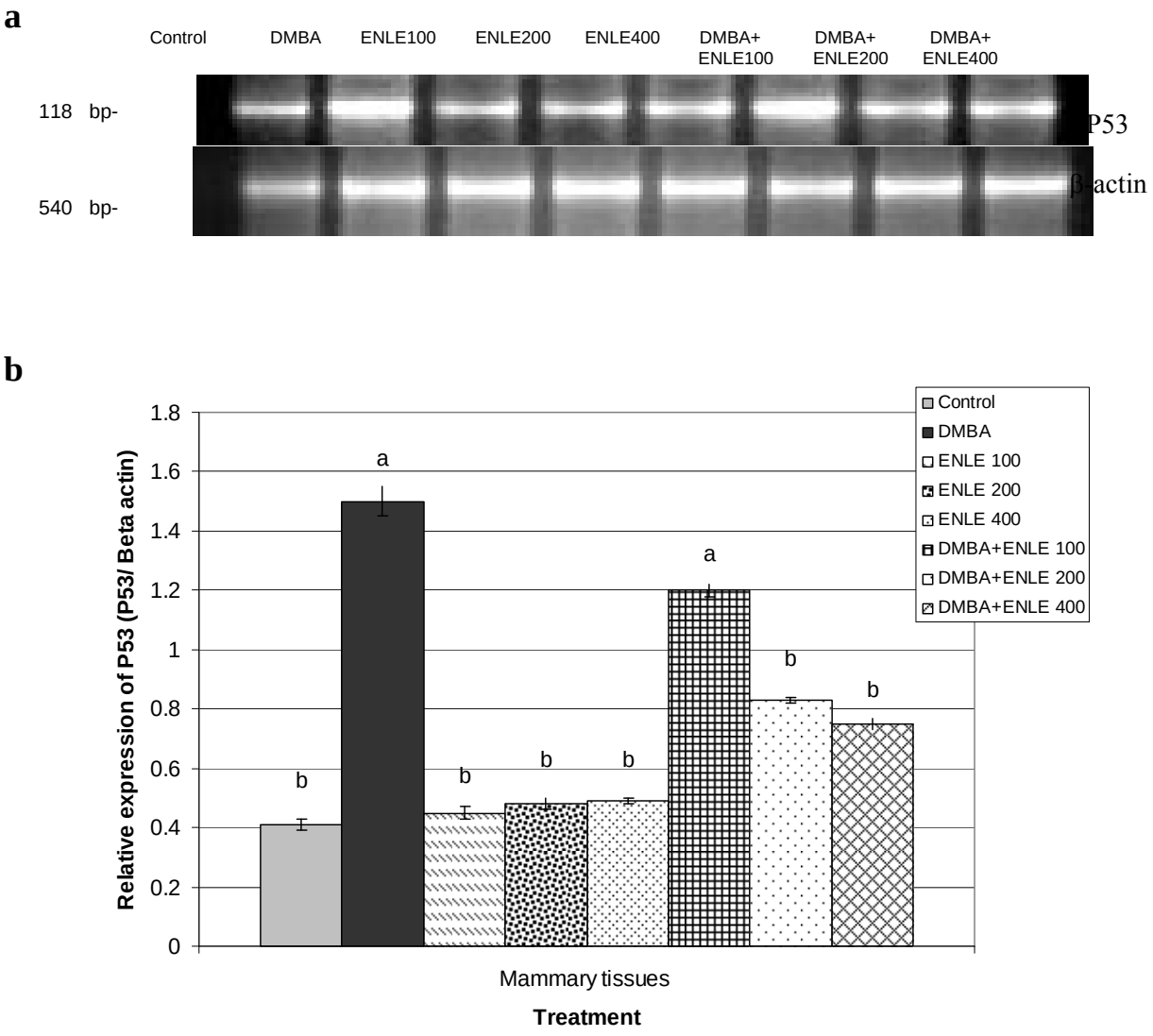
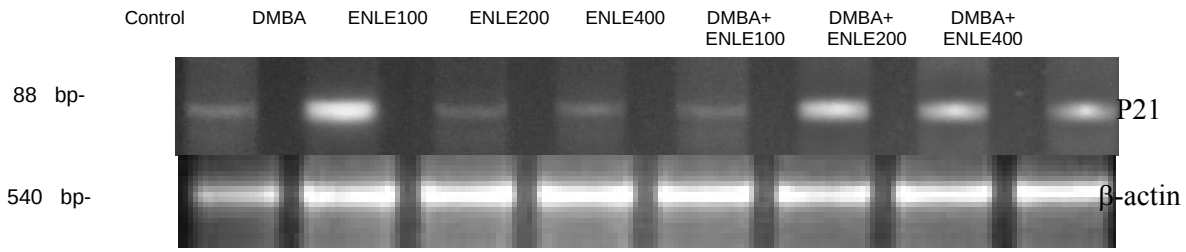


Figure 4: Semi-quantitative RT-PCR confirmation of P53 gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

a



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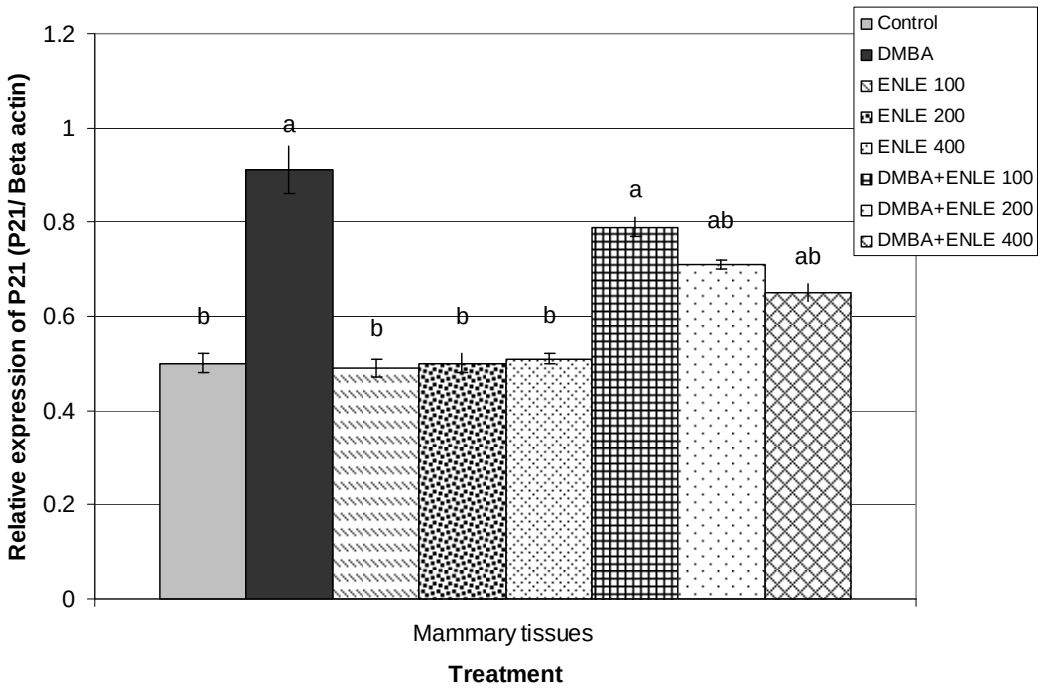

Figure 5: Semi-quantitative RT-PCR confirmation of P21 gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

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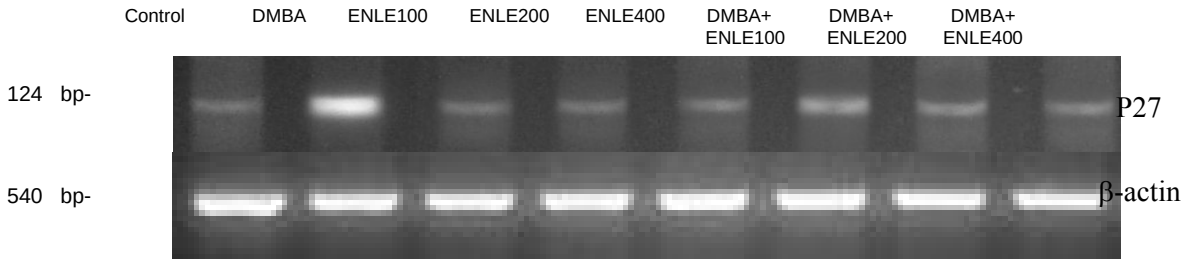

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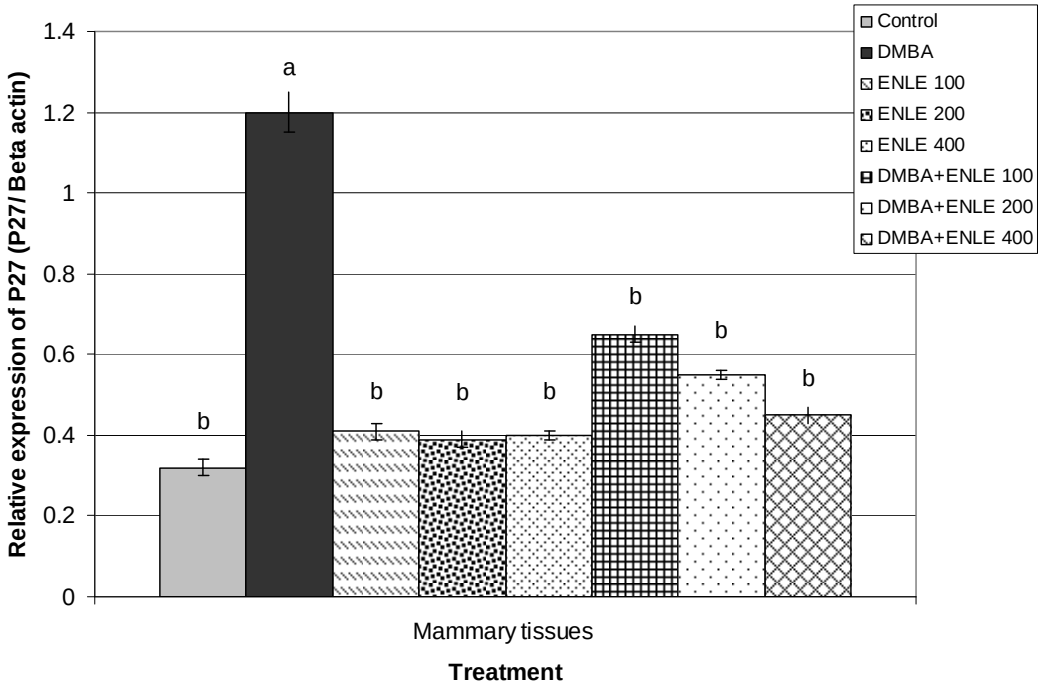

Figure 6: Semi-quantitative RT-PCR confirmation of P27 gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

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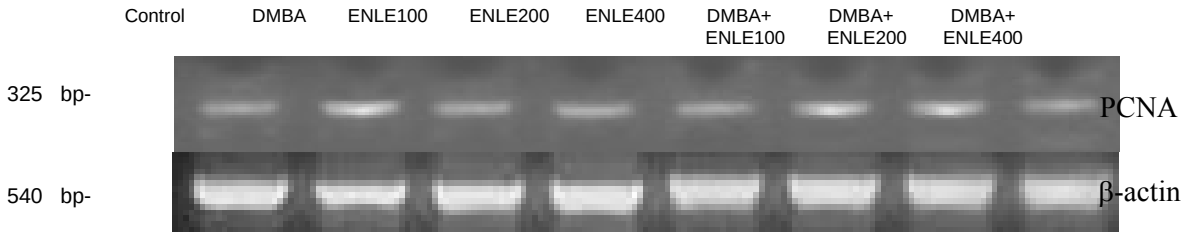

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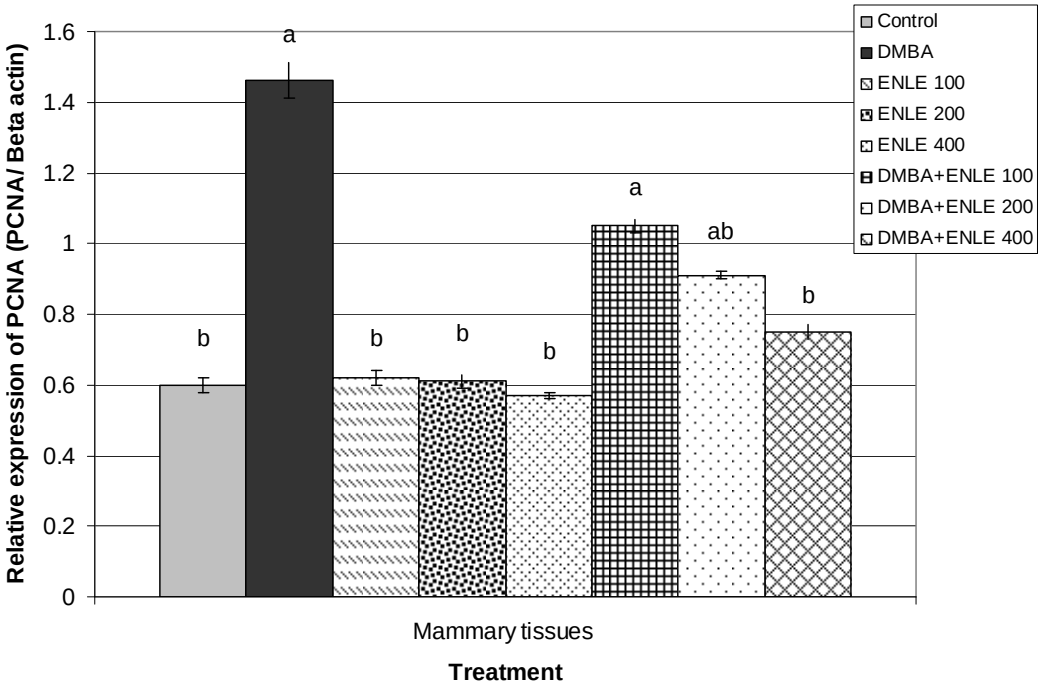

Figure 7: Semi-quantitative RT-PCR confirmation of PCNA gene in mammary tissues of female rats treated with DMBA with or without ENLE (100, 200 and 400 mg/kg) for 12 weeks (a&b). Within each column means superscripts with different letters are significantly different ($P \leq 0.05$).

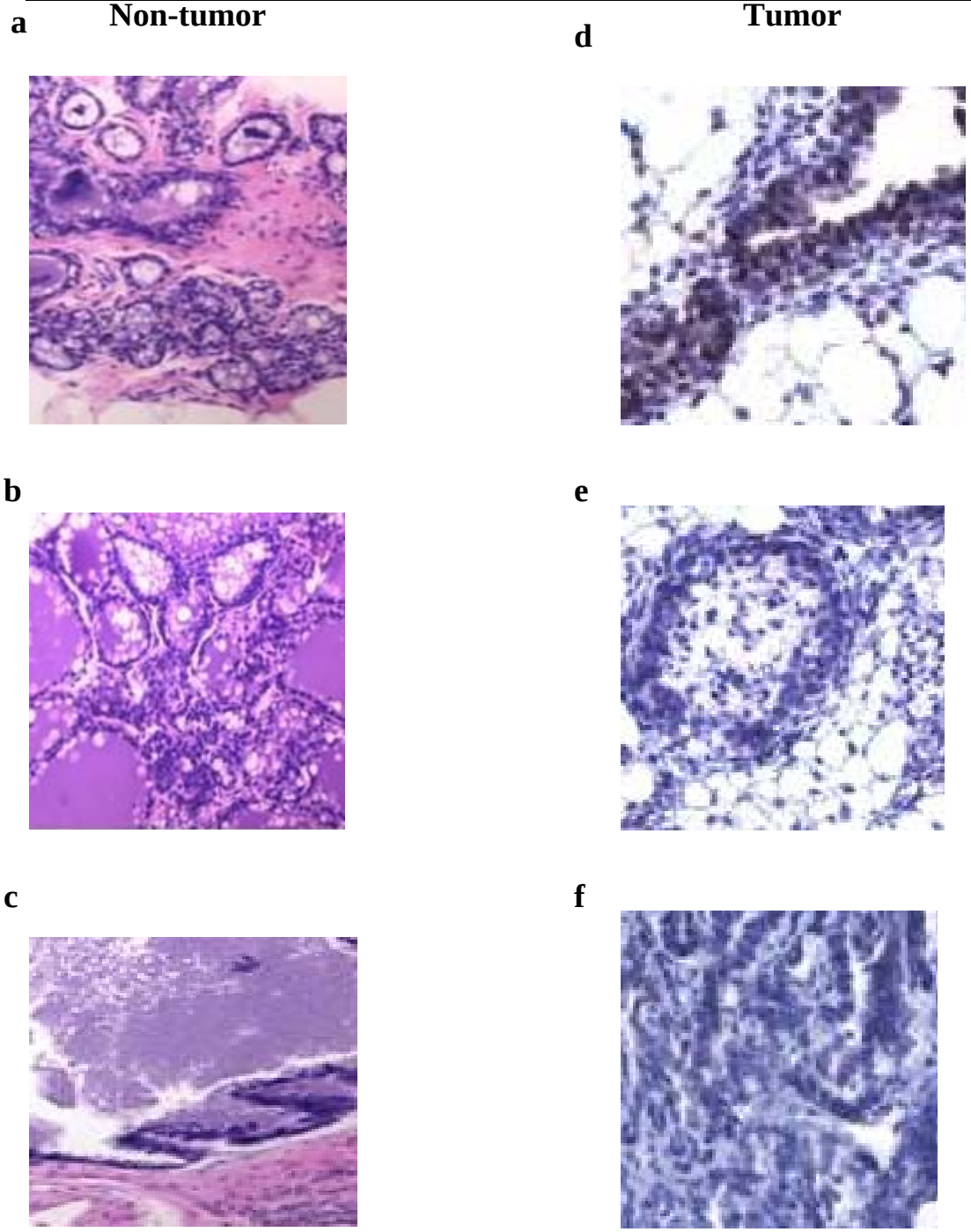

Figure 8: Panels (a) (b) and (c) are representative samples of histological sections of mammary glands from control corn oil treated rat at time of sacrifice (approx. 160 days old), x 20 magnification. The panel (a) indicate milk-filled cystic spaces; panel (b) indicates specific lobules which are massively expanded by milk; panel (c) shows milk filled ducts. The panels (a, b, c) are expressed in the nuclei of epithelial cells lining the ducts and lobules of nontumor tissue in the surrounding area but expression is lost in regions showing atypical hyperplasia and in the tumors (d,e,f).

Table I. Primers and PCR thermocycling parameters

Primer	Sequence (5′-3′)	PCR conditions	RT-PCR (bp)
Bcl-2	GGT GCC ACC TGT GGT CCA CCT G CTT CAC TTG TGG CCC AGA TAG G	42°C for 1 h, 95°C for 15 min, 32 cycles of (i) 94°C for 30 s, (ii) 62°C for 30 min, (iii) 72°C for 1 min and 72°C for 10 min	376
Cytokeratin 8 CK8	TTCCTGGAGCAGCAGAACAA GAGG ACAAATTCGTTCTCCAT	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	255
Cytokeratin 19 CK19	A TTCTTGG TGCCACCATTGA TCCTCATGGTTCTTC TTCAGG	30 cycles: 94°C, 30 s; 65°C, 30 s; 72°C, 1 min Final extension: 72°C, 2 min	238
p53	CGCAAAAGAAGAAGCCACTA TCCACTCTGGGCATCCTT	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	118
p21	ACCTCTCAGGGCCGAAAAC TAGGGCTTCCTCTTGGAGAA	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	88
p27	CAGAGGACACACACTTGGTAGA TCTTTTGTTTTGAGGAGAGGAA	35 cycles: 93°C, 30 s; 56°C, 45 s; 74°C, 45 s Final extension: 74°C, 10 min	124
PCNA	GGTGCTTGGCGGGAGC ATCGCTTGAGCCCAGAAGT	30 cycles: 93°C, 30 s; 55°C, 45 s; 74°C, 45 s Final extension: 74°C, 10 min	325
β-Actin	GTGGGCCGCTCTAGGCACCAA CTCTTTGATGTCACGCACGATTTC	25 cycles: 94°C, 30 s; 65°C, 30 s; 68°C, 1 min Final extension: 68°C, 2 min	540

Discussion

The current work was carried out to investigate the effect of DMBA on the expression alterations of the genes (Bcl-2, CK8, CK19, P53, P21, P27 and PCNA) related to cancer progression in female rats using RT-PCR assay. Also the protective role of ENLE was determined to inhibit the alterations in the gene expression as carcinogenic effect of DMBA.

This study was associated with over-expression of the all tested genes in the DMBA treated rats. These results were in great agreement of the results reported by Subapriya et al [33], who found that topical application of DMBA to the hamster cheek pouch for 14 weeks induced squamous cell carcinomas with a very high tumour burden. This was associated with over-expression of Bcl-2, mutant p53 as well as PCNA genes in hamster tissues. Our results showed also over-expression of Bcl-2, p53, p21, p27, CK8, CK19 as well as PCNA genes in the mammary tissues collected from DMBA treated rats.

Bcl-2, a prominent anti-apoptotic member of the Bcl-2 family inhibits the release of pro-apoptotic molecules and cytochrome C from the mitochondria thereby inhibiting apoptosis and permitting persistence of tumour cells. In addition, overexpression of both p53 and Bcl-2 has been reported to inhibit transcriptional activation of Bax gene [51,52]. Thus, overexpression of Bcl-2, p53, p21, p27, CK8, CK19 as well as PCNA genes in the mammary carcinomas may confer a selective growth advantage on tumour cells. Administration of ENLE reduced the incidence of DMBA induced rat mammary tissues (RMT) carcinomas. Where, the ENLE was able significantly to down-regulate Bcl-2, p53, p21, p27, CK8, CK19 as well as PCNA expression in the RMT carcinomas at the dose of 400 mg/kg of body weight. These results agreed the results of Subapriya et al [33], who found that ENLE significantly down-regulated PCNA, p53 and Bcl-2 expression in the buccal pouch in the hamster. The results of the present study substantiate the anticarcinogenic properties of neem preparations reported in literature. An extract of neem leaf was found to inhibit benzo[a]pyrene-induced fore-stomach tumors and DMBA-induced skin papillomagenesis in a murine model [53]. Dietary administration of 10% neem flowers significantly inhibited DMBA-induced mammary tumors by inducing GGT activity [54]. Treatment with aqueous neem leaf extract was shown to reduce the growth of Ehrlich carcinoma and B16 melanoma in Swiss mice [55].

To understand the regulation of the p53, p21 and p27 in the present study that it is known to induce apoptosis through inhibition of Bcl-2, amplification of death signals and activation of caspases [56]. Mutations in p53 have been reported to occur in 40% of all human tumors. While wild type p53 functions as a TSG, mutant p53 functions as an oncogene. Mutant

p53 protein is reported to have lost the ability to act as a growth suppressor and gained the ability to promote cell proliferation. Over-expression of p53, p21 and p27 may enhance genetic instability by facilitating cell proliferation and inhibiting DNA repair and apoptosis. Furthermore, p53 activates telomerase and factors involved in angiogenesis as well as metastasis. In particular, p53 mutations have been reported to be associated with over-expression of Bcl-2 [52,56].

PCNA is a 36 kDa nonhistone nuclear acidic protein expressed in the nuclei of proliferating cells during S-phase. PCNA functions as a sliding clamp increasing the processivity of DNA polymerase δ [39]. Besides proliferation, PCNA also exhibits anti-apoptotic functions. PCNA interacts with Gadd 45, a growth arrest and DNA damage protein, as well as MyD118, a myeloid differentiation primary response protein. These interactions inhibit apoptosis and differentiation and promote tumor cell growth [57]. Furthermore, PCNA binds to MeCTr, an enzyme responsible for inheritance of the methylated CpG sequences thereby altering gene expression and facilitating cell proliferation [39]. PCNA expression has been considered to reflect the proliferation rate of tumor cells. Shin et al. [58] found a gradual increase in PCNA expression during progression of normal epithelium from hyperplasia through dysplasia to oral squamous cell carcinoma. Over-expression of PCNA has been reported in a wide range of human tumors as well as in DMBA-induced HBP carcinomas [58]. Thus, over-expression of PCNA observed in the present study reflects increased cell proliferation in RMT tumors of female rats.

The present study clearly demonstrates that inhibition of cell proliferation and induction of differentiation and apoptosis may be major mechanisms through which ENLE exerts its anticarcinogenic properties. An extensive survey of literature revealed that ours is the first report on the effect of ENLE on cell proliferation, differentiation and apoptosis in the mammary tissues of female rats treated with DMBA. Although there is paucity of information with respect to neem preparations, this study substantiates the anti-proliferative and apoptosis-inducing effects of a wide range of natural products and phytochemicals reported by us and other workers [59].

The chemopreventive effects of ENLE observed in the present study may be related to its constituent phytochemicals. ENLE contains a number of potent antioxidants and anticarcinogens including β-carotene, flavonoids, terpenoids, carotenes and various limonoids [31]. Azadirachtin, a limonoid constituent of neem leaf has inhibitory effects on cell proliferation [60]. Akudugu et al. [61] have reported the cytotoxicity of azadirachtin A in human glioblastoma cell lines. Azadirone 1 has been found to possess cytotoxic activity against breast, melanoma and prostate cancer cell lines [62]. Nimbolide and 28-deoxonimbolide have been identified as cytotoxic constituents of neem leaves [63]. In particular, 70% ENLE used in this study is reported to contain six major compounds: (i) quercetin-3-O-β-D-glucoside; (ii) myricetin-3-O-rutinoside; (iii) quercetin-3-O-rutinoside; (iv) kaempferol-3-O-rutinoside; (v) kaempferol-3-O-β-D-glucoside; (vi) quercetin-3-O-L-rhamnoside [34]. Quercetin and kaempferol, the flavonoids present in neem leaf have been documented to retard carcinogenesis [64]. The antiproliferative effects of quercetin have been documented in experimental animal models and humans. Quercetin has been reported to inhibit the growth of tumor cells in malignant cell lines and down-regulate the expression of Bcl-2 and mutant p53 protein [65]. Kaempferol was found to significantly inhibit Bcl-2 expression in the human lung cancer cell line A549 [66].

The current results clearly demonstrate that ENLE acts as a suppressing agent by inhibiting cell proliferation and inducing differentiation and apoptosis as revealed by the down-regulation of Bcl-2, p53, p21, p27, CK8, CK19 as well as PCNA genes, associated with inhibition of the histopathological lesions in the mammary tissues. Agents that inhibit cell proliferation and induce apoptosis are known to have immense potential in chemoprevention and chemotherapy. While cancer cells evade apoptosis, induction of apoptosis is an attractive strategy to arrest proliferation of cancer cells. Although downregulation of Bcl-2 by ENLE is an indicator of apoptosis induction, additional studies on Bcl-2 family proteins, caspases, Fas and NF-κB signalling pathways and cell cycle-associated proteins are required to unravel the differential response of neem leaf extract in normal versus cancer cells to validate its chemopreventive potential.

Conclusion: Expression alterations of genes related to cancer and histopathological lesions were found in mammary tissues of female rats exposed to DMBA. These alterations of the gene expression as well as the histopathological lesions were markedly suppressed when female rats were treated with ENLE combined with DMBA.

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