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Preventive Effect of *Commiphora molmol* on Rat Mammary Carcinogenesis Induced by 7, 12-Dimethylbenz(A)Anthracene (DMBA) in Comparison with Melatonin

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Abstract: *Commiphora molmol* is one of the most common herbs consumed in the Arabian countries. In this study the efficacy of *C. molmol* in preventing mammary tumorigenesis was assessed by comparing its pretreatment effects in the DMBA-induced rat mammary carcinogenesis model to pretreatment with melatonin. Virgin female Sprague-Dawley rats (5-6 weeks-old) were randomly divided into one of five groups: (1) melatonin (10 mg/ kg.b.wt; n=20); (2) Oleo-gum-resin of *C. molmol* (500mg/ Kg.bwt; n=20); (3) 0.5% ethanolic phosphate-buffered saline (PBS; n=20); (4) distilled water (n=20); (5) no treatment (n=10). All treatments started 15 days prior to DMBA and ended at the same day of DMBA administration. 1st- 4th groups received a single oral dose of 75 mg DMBA in 1ml of corn oil and 5th group received a single dose of 1ml corn oil. After five months, DMBA disrupts hematopoiesis in the bone marrow as indicated by the significant ($P < 0.05$) decrease in leukocytes count and by the appearance of atypical lymphocytes and ring cells. It also produced ductal carcinoma *in situ* (DCIS), as well as intraductal proliferations (IDPs) in 100% of mammary glands of all treated rats. Daily administration of either melatonin or *C. molmol* appeared to protect bone marrow from damaging effect of DMBA as revealed by increasing the number of leukocytes and decreasing the number of atypical lymphocytes. Also, treatment with melatonin and *C. molmol* alone resulted in 66.7% and 25% inhibition of DCIS incidence respectively. The frequency of occurrence of mammary IDPs and DCIS was decreased as well in melatonin and *C.molmol* treated rats. Overall, melatonin appears to be stronger than *Commiphora molmol* in inhibiting the carcinogenic effects of DMBA. This is the first report of chemopreventive potential of *C. molmol* in DMBA-induced mammary carcinogenesis and the results suggest that *C. molmol* may be a good candidate for possible use in the prevention of breast cancer. Further studies are needed to clarify its mode of action and safety for medicinal use in cancer prevention and treatment.

Key words: *Commiphora molmol* · Melatonin · DMBA · Mammary · Carcinogenesis · Hematological Parameters

INTRODUCTION

Breast cancer is the most common cancer in women worldwide and is a leading cause of mortality in women both in developed and the developing world [1, 2]. The incidence of breast cancer is increasing in the developing countries due to increase urbanization and adoption of western life styles. In Egypt, the number of people suffering from breast cancer is alarming. According to official statistics of the National Cancer Institute, breast cancer is the most common cancer among women, representing 35.1% of total cancer cases [3]. As for most of the cancer types, conventional therapeutic and surgical approaches have not been able to control properly breast cancer. Therefore, there is an urgent need to develop mechanism-based approaches for the management of breast cancer. Chemoprevention by means of non-toxic agents could be one such approach, in which the incidence of this disease can be prevented by administration of one or several natural or synthetic compounds [4].

Many naturally occurring agents have shown cancer chemopreventive potential in a variety of bioassays and animal models that having relevance to human disease [5].

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They may inhibit, delay and/or reverse cancer evoked by either environmental insults and/or life style [6]. Several of these chempreventive agents act at the initiation, promotion and/or progression stages conceptually associated with the ontogeny of multistage carcinogenesis [7]. From these substances melatonin (the principal hormone of the pineal gland) is known for its antitumor activities [8].

Melatonin, referred to as "the darkness hormone", is synthesized from tryptophan and released mainly at night reaching its nadir during the daytime [9], thus the peak duration of nightly melatonin faithfully codes the day length [10]. The pineal melatonin rhythm persists under continuous darkness (DD). On the other hand, constant light (LL) abolishes the melatonin rhythm. Melatonin is lipophilic in nature; therefore, it reaches all parts of the body and can affect all aspects of physiological and pathophysiological processes, including circadian rhythm regulation, seasonal reproduction [9] immune function [11] and tumorigenesis [12]. Many studies have shown that melatonin inhibits the growth of breast cancer cells and it also inhibits the development and growth of DMBA-induced mammary cancer in female rats [8, 13].

The search for new natural chemopreventive substances that can attenuate the risk of mammary tumors has recently received an increased interest. These substances have been tested in experimental animals against mammary gland carcinomas induced by chemical carcinogens in order to use them for possible application in treatment strategy in humans. The oleo–gum–resin of *Commiphora molmol* Engler (Burceraceae) is locally known as ‘‘mur’’ (*i.e.*, “bitter”) or ‘‘myrrh’’. Traditionally, the myrrh resin was in use by the ancient Egyptian, Greek and Romans. Also, early Muslim writers recorded many medicinal uses for the oleo-gum resin of *C. molmol*. Specifically, it has been used to treat wounds, intestinal disorders, diarrhea and cough. In the Arabian countries today, it is one of the most common herbs and it is employed as antipyretic, antiseptic, mouth wash and for the treatment of some inflammatory conditions [14]. Also, many reports are available on the folklore importance of *Commiphora* species in the treatment of malignant tumors and cancer of the breast, spleen, liver, stomach, head, nose and eye [14, 15]. Accumulating evidence derived from laboratory studies indicated that *C. molmol* have cytotoxic and anti-tumor activity on Ehrlich ascites carcinoma cell-bearing mice [16, 17]. To our knowledge, there is no available data on the chemopreventive potential of *C. molmol* in mammary carcinogenesis.

Rat mammary gland carcinogenesis has been considered as a model that mimics the human mammary tumors and induction of mammary tumors in rats by DMBA is one of the most models that utilized for analyzing the various aspects of mammary carcinogenesis [18]. Consequently, the aim of the present study is to address the role of preventive treatment of *Chommiphora molmol* in modulating the carcinogenic effects of DMBA on mammary gland, compared with melatonin in virgin female albino rats. Also the current work addresses the effects of each of *C. molmol* and melatonin on certain hematological parameters that may emerge during DMBA-induced mammary carcinogenesis.

MATERIAL AND METHODS

Animals and Housing Condition: Virgin female Sprague-Dawely rats were obtained from the breeding colony of the Ministry of Health (Helwan-Egypt) at 28-35 days old. Upon arrival, the animals were weighed, group-housed (5 animals per cage) and kept in a well-ventilated animal facility under natural photoperiod conditions. They were acclimatized for one week prior to experimentation. Food and water were available *ad libitum* throughout the study. All procedures were in accordance with institutional guidelines and follow the Guide for Care and Use of Laboratory Animals.

DMBA: DMBA (7,12-dimethylbenz(a)anthracene) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in corn oil, to get a final concentration of 75mg/ 1ml of corn oil.

Melatonin: Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Due to its instability in non-sterile solutions it was prepared in ethanol and diluted to a final concentration of 10 mg melatonin/ 1ml of 0.5% ethanolic phosphate-buffered saline (PBS). The bottles of melatonin solution were covered with aluminum foil and kept in refrigerator; fresh solutions were prepared every two days.

Commiphora Molmol: The oleo–gum–resin of *Commiphora molmol* was obtained, in the purest form available commercially, from a local market in El-Minia city, Egypt. Five grams of the resin was crushed into a fine powder using a sterile pestle and mortar. The plant powder was then suspended in distilled water to have a final concentration of 500 mg/ ml.

Experimental Design: After one week of acclimatization, virgin female rats (5-6 weeks-old) were randomly divided into five groups. The first group of animals [melatonin (Mel)/DMBA Group; n=20] received a daily dose of melatonin (10 mg/ kg. b.wt) by oral intubation for 15 consecutive days in the late afternoon (4.00-6.00 pm). This regimen of melatonin treatment was based on the study of Lenoir *et al.* [19] which demonstrated that preventive treatment of melatonin before the induction of DMBA, in a similar protocol, has caused long term inhibition of mammary adenocarcinoma promotion.

The second group of rats [*Commiphora molmol* (*C. mol*)/DMBA Group; n=20] received a dose of 500mg/ Kg.bwt of the oleo-gum-resin of *Commiphora molmol* by oral intubation for 15 consecutive days. The selection of this dose level was based on previous studies which showed that a dose of 500mg/kg.bwt of *Commiphora molmol* has a high cytotoxic and antitumor activity in rats without inducing toxic effects [16, 17]. According to published information, two weeks time period ensure sufficient time for the substances to achieve their proper concentration in the different tissues [20]. Contrary to melatonin and to the best of our knowledge, there was no data available on the cancer preventive pretreatment effects of *Commiphora molmol* in DMBA treated rats. Accordingly, a pretreatment of 15-day time period of 500 mg *Commiphora molmol* was thought to be adequate to provide a preventive proliferative activity.

Two of the remaining three groups (n=20 each) were used as controls for each of the experimental groups. They received the same vehicle corresponding to the dose, route of administration and treatment time period. The last group of rats (fifth Group, n=10) did not receive any substances or undergo any experimental manipulations.

On the 16th day, rats from the 1st - 4th groups (now at 51-58 days of age) were subjected to the carcinogenic effect of DMBA on the same day (16th day) by receiving a single oral dose of 75mg /kg. b.wt. of DMBA in 1ml of corn oil [19]. Hence, rats of the 3th – 4th groups served as positive control for the incidence and histopathology of neoplasm because they were pretreated with vehicles and were not subjected to any experimental manipulation prior to the administration of DMBA. Animals of the fifth group were treated with a single oral dose of corn oil alone and served as negative control. Based on previous studies, the susceptibility of the mammary gland to DMBA carcinogenesis has been shown to be strongly age-dependent and is maximal when DMBA is administered to animals between the ages of approximately 45-60 days [18]. Consequently, the ages of the rats in the current study were thought to be suitable for induction of carcinogenesis.

All animals were weighed weekly and checked daily for any signs of toxicity such as lethargy, weight loss or mortality. Palpation of the mammary glands was performed every week starting two weeks post DMBA administration for the detection of mammary tumors.

At the end of the study (5 months) animals were sacrificed by decapitation under light ether anesthesia. Upon sacrifice, blood samples were collected in heparinized tubes for hematological analysis and mammary glands were harvested for histopathological studies. Rats that died before the end of the experiment were excluded from the study.

Hematological Studies: Total red blood cells (RBCs) count, hemoglobin concentration (gm/dl), total leukocyte (WBCs) count and differential leukocyte count were determined by standard techniques [21].

Histopathological Analysis

Tissue Preparation: Both right and left cervical (#1), thoracic (#2 and #3), abdominal (#4) and inguinal (#5 and #6) mammary glands attached to the skin were removed from each rat according to the procedure of Russo *et al.* [22] and processed for routine histopathological analysis.

Classifying Mammary Lesions: Based on previous studies, histopathological pictures of various benign, premalignant and malignant mammary lesions produced by carcinogen treatment to rats are quite similar to that seen in breast biopsy specimens from human [23- 25]. Accordingly it was recommended to use the same terminology and criteria applied for human breast diseases in classifying mammary lesions of the rat [24]. Therefore, the histopathological changes of the mammary glands were diagnosed and evaluated in a blinded protocol and classified according to established histological and cytological criteria [24- 26] into three groups: usual ductal hyperplasia, atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS).

Incidence and Frequency of Mammary Lesions: Based on a preliminary experiment (data not shown), no palpable tumors were detected until the end of the study and only ductal carcinoma *in situ* (DCIS) were identified ; therefore incidence was calculated as the number of animals with lesions that meet the criteria for ductal carcinoma *in situ* (DCIS) compared to the total number of animals in the group.

The frequency of occurrence of various mammary lesions was assessed in the mammary glands according to the method of Murray *et al.* [27] with some modifications. Briefly, three 5μm sections of mammary tissue separated by 50μm were used and a box of 4mm^2 was drawn on each slide. Ducts located within this specific area were counted and classified according to established criteria. The frequency of mammary lesions was expressed as the number of lesions in comparison to the total number of ductal structures found in the specified 4mm^2 area. Lesions that meet the criteria for usual ductal hyperplasia or atypical ductal hyperplasia were counted as intraductal proliferations (IDPs) without categorization [28]. The frequency of IDP and DCIS was calculated per gland (cervical, thoracic, abdominal and inguinal) per animal. Then, the values obtained for each of the glands were pooled and expressed as an average value for each animal. Finally, the mean and standard error were calculated for each group.

Statistical Analysis: Statistical analysis was performed using ANOVA (SPSS program, version 10.0; SPSS Inc., Chicago, IL, USA). Post-Hoc comparisons of pair-wise means were made using Least Significant Difference (LSD) test. The results were presented as mean ± standard error. Differences between group means were considered statistically significant if *P*-value < 0.05.

RESULTS

Hematological Studies

Total Erythrocyte Count (RBCs) and Hemoglobin Concentration (Hb): As shown in Figure 1 treatment of rats with DMBA induced a non significant decrease ($P > 0.05$) in RBCs count and hemoglobin concentration determined at five months post-treatment. Daily administration of either melatonin (Mel/DMBA group) or *Commiphora molmol* (*C. mol*/DMBA group) for 15 consecutive days prior to DMBA-treatment did not affect RBCs count or hemoglobin concentration; although there was a tendency toward increasing these values, this increase was not significant ($P> 0.05$) and they did not reach the control values.

Total Leukocyte Count: The data for total WBCs count are detailed in Table 1. By five months, total WBCs count of DMBA-treated rats was significantly lower than that of the control animals ($P < 0.05$). In contrast, a marked increase ($P < 0.05$) in the total WBCs count was recorded in Mel/DMBA and *C. mol*/DMBA groups when compared with that of the corresponding DMBA-alone-treated rats. Accordingly, WBCs count of Mel/DMBA and *C. mol*/DMBA groups approached the control value.

Differential Leukocyte Count: As shown in Fig. 2 A the lymphocytes were the prevalent type of leucocytes in blood smears of control rats representing a mean percentage of 67.9% ± 0.34. This lymphocyte percentage was significantly decreased ($P < 0.05$) in DMBA-treated rats as compared with respective control group (Fig 2A). However, large lymphocytes with irregular nuclei were observed in all blood smears of DMBA-treated rats. The cytoplasm of most of these cells tends to be indented by surrounding RBC's. These cells were identified as atypical lymphocytes (Figs. 2B and 3A). Daily administration of melatonin for 15 days before DMBA-treatment caused a significant increase in the percentage of lymphocytes accompanied with a significant decrease in the percentage of atypical lymphocytes ($P< 0.05$) when compared with DMBA-alone-treated animals (Fig. 2). This inhibitory effect of melatonin on the presence of atypical lymphocytes in the peripheral blood was more pronounced in 66.7% of rats in which no atypical lymphocytes were detected in any of their peripheral blood smears (Fig. 3B). As a result, these cells were only determined in the blood smears of 33.3% of Mel/DMBA treated rats.

Rats treated with *C. molmol* for 15 consecutive days prior to DMBA-administration exhibited a marked increase ($P< 0.05$) in lymphocytes percentage and a significant decrease in the percentage of atypical lymphocytes ($P< 0.05$) compared with DMBA-treated animals (Fig. 2). It is noteworthy to mention that atypical lymphocytes appeared in the blood smears of only 75% of animals pre-treated with *C. molmol* before DMBA administration (Fig. 3C).

Table 1: Changes in total leukocyte count (x 10^3), determined after 5 months of DMBA administration.

	Groups			
Parameter	Control	DMBA	Mel / DMBA	*C.mol* / DMBA
WBCs (x10^3)	4.5 ± 0.28	2.93^a ± 0.58	4.95^b ± 0.2	5.05^b ± 1.03

Data represent means ± SEM. Control = vehicle (oil-treated) control; DMBA= 7,12-dimethylbenz(a)anthracene; Mel= melatonin; *C.mol= Commiphora molmol*. ^{a}= $P < 0.05$ *vs.* control animals, ^{b}= $P < 0.05$ *vs.* DMBA-alone-treated rats.

Table 2: Changes in differential leukocyte count (mean percentage ± SEM), determined after 5 months of DMBA administration.

	Groups			
Parameters	Control	DMBA	Mel / DMBA	C.*mol* / DMBA
Neutrophils	25.75 ± 0.43	6.48[a] ± 0.52	9.38[b] ± 0.25	7 ± 0.16
Monocytes	3.52 ± 0.19	1.85[a] ± 0.35	3.1[b]± 0.06	3[b] ± 0.16
Eosinophils	1.41± 0.25	0.93 ± 0.25	0[b] ± 0	1 ± 0.04
Basiophils	1.41 ± 0.25	0[a] ± 0	0 ± 0	1[b] ± 0.04

Data represent means ± SEM. Control = vehicle (oil-treated) control; DMBA= 7,12-dimethylbenz(a)anthracene; Mel= melatonin; *C.mol*= *Commiphora molmol*. [a]= $P < 0.05$ *vs.* control animals, [b]= $P < 0.05$ *vs.* DMBA-alone-treated rats.

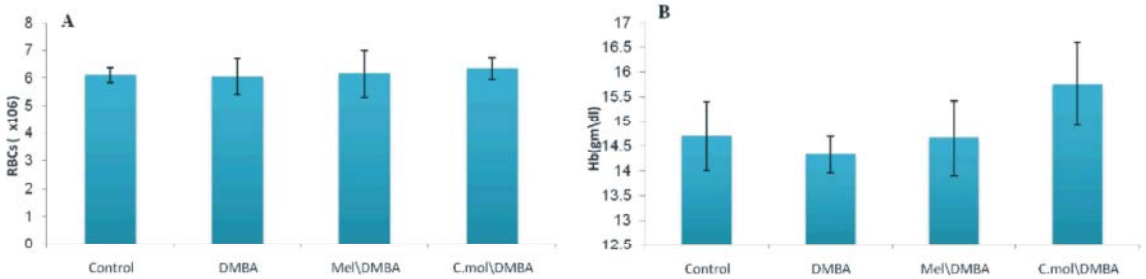

Fig. 1: (A) Changes in total erythrocyte count (x 10^6); (B) Changes in hemoglobin concentrations (gm/dl) determined 5 months post-DMBA administration. Data represent means ± SEM. Control = vehicle (corn oil); DMBA= 7,12-dimethylbenz(a)anthracene; Mel= melatonin; *C.mol*= *Commiphora molmol*. [a] = $P < 0.05$ *vs.* control animals, [b]= $P < 0.05$ *vs.* DMBA-alone-treated rats.

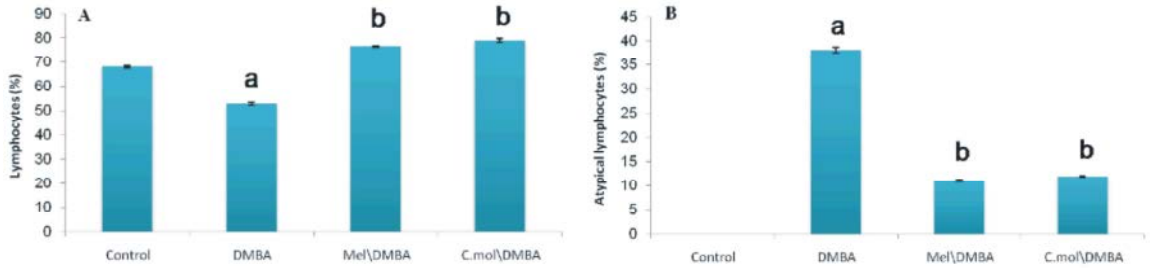

Fig. 2: (A) Lymphocyte percentages of control and experimental animals; (B) Atypical Lymphocyte percentages, determined 5 months post-DMBA treatment. The data are expressed as means± SEM. Control = vehicle (corn oil); DMBA= 7,12-dimethylbenz(a)anthracene; Mel= melatonin; *C.mol*= *Commiphora molmol*. [a]= $P < 0.05$ *vs.* control animals, [b]= $P < 0.05$ *vs.* DMBA-alone-treated rats (see text for details).

Data of Table 2 showed that neutrophils represent the second predominant leukocyte type in peripheral blood smears of control rats showing a mean percentage of 25.75% ± 0.43. Similar to lymphocytes, it was noticed that neutrophil percentage exhibited a significant decrease ($P < 0.05$) in DMBA-treated rats when compared with those of the corresponding controls. Administration of melatonin for 15 consecutive days prior DMBA-treatment induced a significant increase ($P < 0.05$) in neutrophil percentage but it did not approach that of the control animals. On the contrary, when rats were subjected to *C. molmol* treatment for 15 consecutive days before DMBA-administration, no changes ($P > 0.05$) were recorded in neutrophil percentages compared with DMBA-treated animals.

The percentage of monocyte in peripheral blood smears of DMBA-treated rats showed a significant decrease ($P < 0.05$) in comparison with that of the corresponding control group (Table 2). However, a significant increase ($P < 0.05$) in the percentage of monocytes was detected when melatonin or *C. molmol* was administered prior to DMBA-administration (Table 2). Thus, the percentages of monocytes in these later two groups were close to the control values.

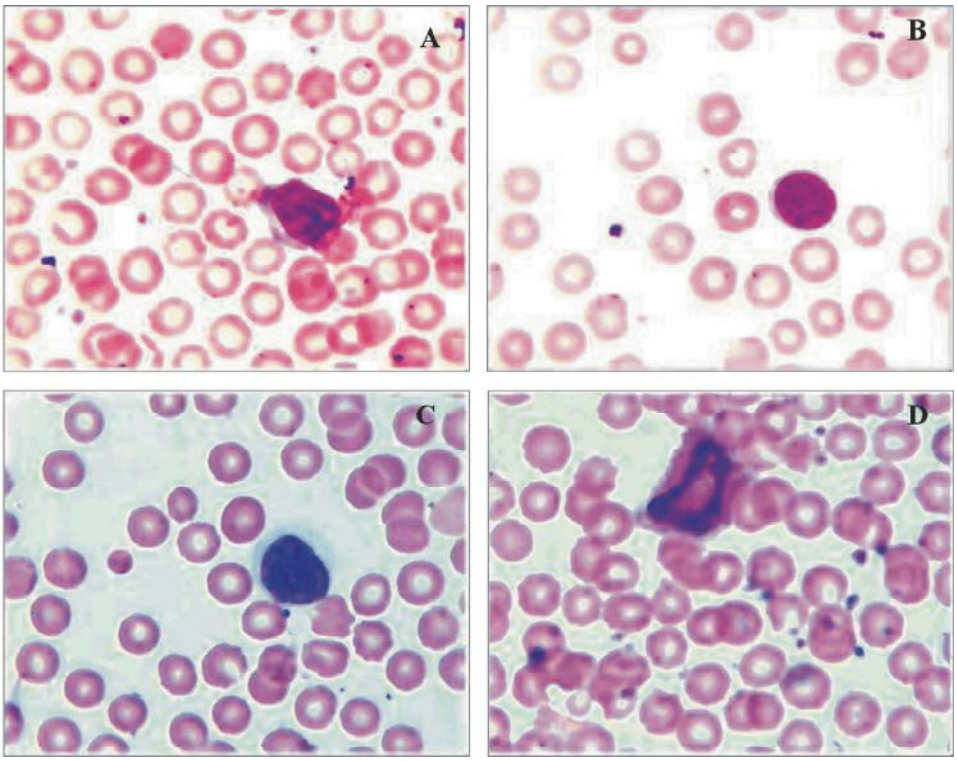

Fig. 3: Peripheral blood smears from experimental animals, 5 months post-DMBA administration. (A) Atypical lymphocyte with large folded nuclear membrane from a DMBA-treated rat; (B) normal lymphocyte from a rat treated with melatonin followed by DMBA; (C) atypical lymphocyte from a rat treated with *C. molmol* prior to DMBA administration; (D) a leukocyte with ring-shaped nucleus from a DMBA-treated rat. (Giemsa stain, x 1000).

The data for eosionphil and basophil percentages in peripheral blood were also given in Table 2. DMBA treatment caused a decrease in the percentage of eosinophils, but this decrease was not statistically significant ($P > 0.05$) when compared with the control value. Moreover, the percentage of eosinophils in *C. molmol*/ DMBA group was not statically different ($P > 0.05$) from that of DMBA-treated rats. In contrast, esinophils were not recorded ($P < 0.05$) in the blood smears of Mel/DMBA animals. Basophils were only recorded in the blood smears of *C. molmol*/DMBA animals, but this still in the range of normal physiological values.

Regarding the morphology of leukocytes, beside atypical lymphocytes (described earlier) leukocytes with ring-shaped nuclei (ring cells) were detected in the blood smears of DMBA- treated rats (Fig 3D).

Histopathological Studies: The incidence of ductal carcinoma *in situ* (DCIS) as well as the frequency of occurrence of mammary intraductal proliferations (IDPs) and DCIS in virgin female rats treated with DMBA are shown in Fig. 4. Specifically a single oral dose (75mg/ k.b.wt) of DMBA alone induced DCIS in 100% of animals (Fig. 4A). In fact, DCIS were present in all (cervical, thoracic, abdominal and inguinal) mammary glands of the rats. The frequency of DCIS occurrence per rat was 77.29±1.11 whereas the frequency of occurrence of IDPs per rat was 18.27 ± 1.053 (Fig. 4B). On the contrary, no palpable mass was detected in any animal examined.

Histopathologically, the mammary glands of the control virgin female rats administered vehicle in the present study were similar to those of the normal rats consisting of excretory ducts and acini distributed in fibro-fatty stroma (Fig. 5 A and B). DCIS lesion in DMBA-treated rats consisted of a malignant population of cells extending within the mammary ducts without invading through the basement membrane (Fig 5 C-F). The histopathologic picture of DCIS was variable, but the major histologic variant was solid type in which the ducts were expanded and filled with solid plugs of neoplastic cells that appeared monomorphic with nuclear hyperchromasia and varying nuclear grades from low to high. Frequent mitotic activity, central necrosis and minimal central degeneration were evident (Fig 5 D-F).

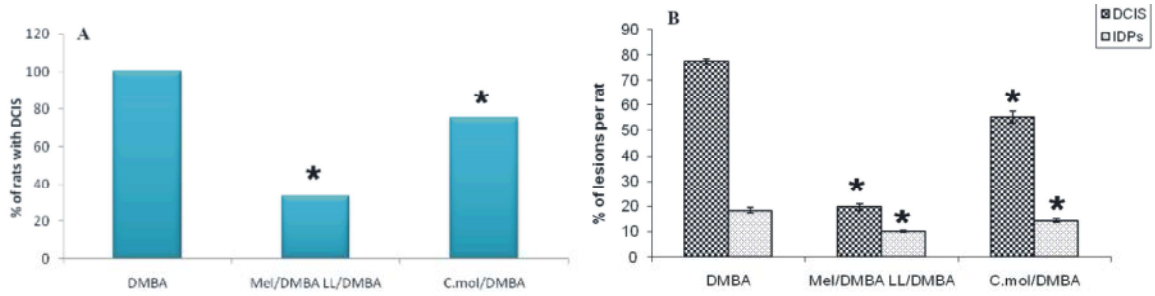

Fig. 4: (A) Incidence of ductal carcinoma *in situ* (DCIS); plotted is the percent of rats with (DCIS) for each treatment group. (B) Frequency of occurrence of lesions following DMBA exposure; Plotted is the percent ± SEM of intraductal proliferations (IDPs) and ductal carcinoma *in situ* (DCIS) per rat for each treatment group. Data represent means ± SEM determined after 5 months of DMBA administration. DMBA=7,12-dimethylbenz(a)anthracene; Mel= melatonin; *C.mol= Commiphora molmol*. *=$P < 0.05$ *vs.* positive control group (DMBA-treated rats). No DCIS or intraductual proliferations were detected in the mammary gland of control animals.

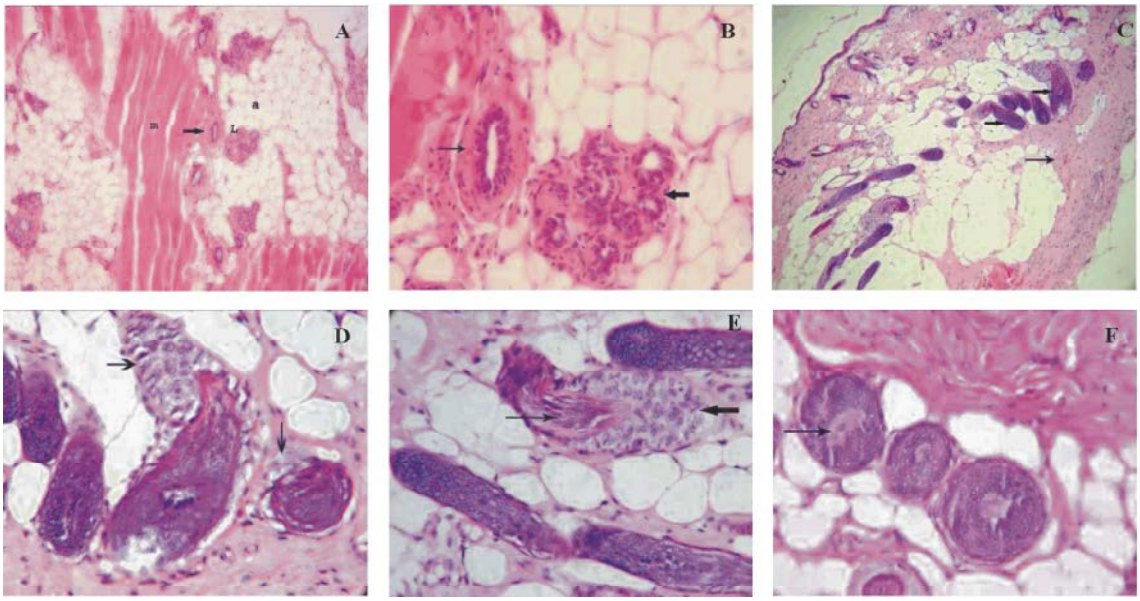

Fig. 5: (A) Histological section of a mammary gland from virgin corn oil-treated rat; note muscle tissue (m) lobules (L), excretory ducts (arrow) and adipose tissue (a). (x 100); (B) is a higher magnification of panel A (x 400), the thin arrow indicates excretory ducts and the thick arrow indicates acinar structures. Panels C-F are Sections of mammary glands from DMBA-treated rats (5 months post-DMBA exposure); the thick arrows in panel C indicate DCIS and the thin arrow shows stromal fibrosis (x 100); (D) is a higher magnification of panel C showing DCIS of solid pattern with central necrosis; arrows indicate myoepithelial hyperplasia (x 400); (E) DCIS of solid type; thin arrows shows hyperplasia of usual type and thick arrow indicates myoepithelial hyperplasia (x 400); (F) DCIS with minimal central degeneration (arrow; x 400). All sections stained with H & E.

In addition, myoepithelial hyperplasia was detected in some mammary ducts (Fig. 5 D and E). Frequently, the hyperplastic ducts as well as atypical hyperplastic ducts and DCIS were surrounded by periductal fibrosis; consequently, the amount of mammary fat in relation to the stroma appeared markedly decreased (Fig 5 C and D). The periductal stroma consisted of dense collagenous fibers (Fig. 5 D and E)., with fibroblasts, lymphocytes and mast cells

Daily administration of melatonin for 15 days prior to DMBA- treatment resulted in a significant decrease ($P < 0.05$) in the incidence of ductal carcinoma *in situ*

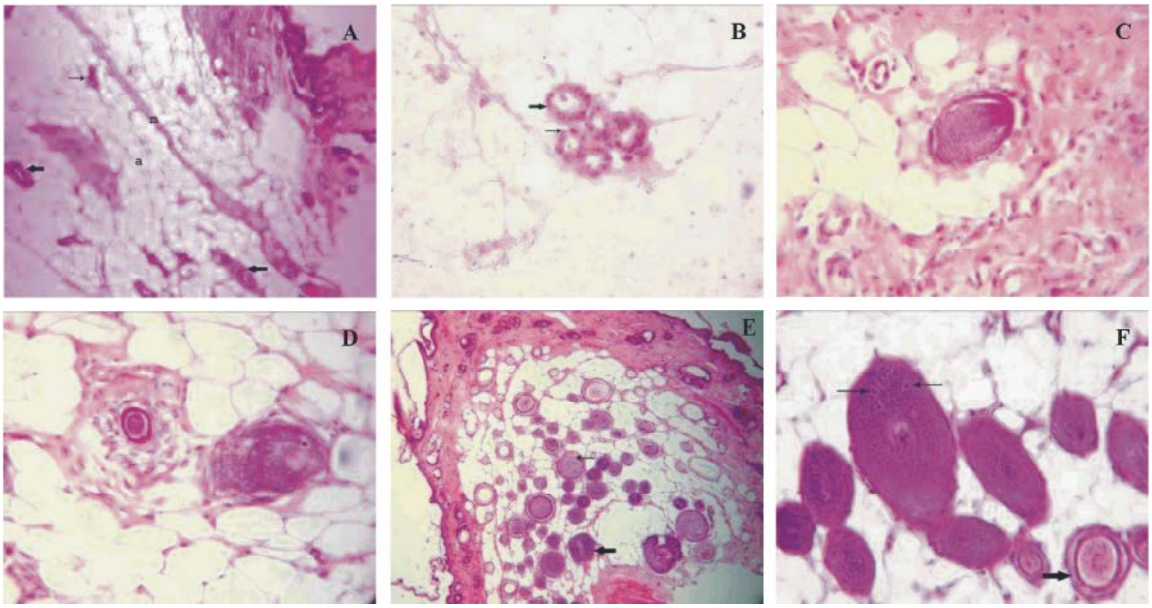

Fig. 6: (A-F) Histological sections of mammary glands from female rats treated with melatonin for 15 consecutive days prior to DMBA administration (5 months post-DMBA exposure). (A) Normal structure showing excretory ducts (thick arrows) acini (thin arrow), muscle tissue (m) and adipose tissue (a; x 100); (B) Acini lined by a layer of cuboidal epithelial cells (thick arrow) and a discontinuous layer of myoepithelial cells (thin arrow; x400). (C & D) Atypical ductal hyperplasia (ADH; x 400). (E) DCIS (thick arrow) combined with dilated ducts (thin arrow x 200); (F) DCIS of solid pattern with frequent mitotic figures (thin arrows) and dilated ducts with calcified secretion (thick arrow; x 400). All sections stained with H & E

(DCIS; Fig. 4A). Thus, compared with 100% of rats with DCIS in the positive control (DMBA-treated group), only 33.3% of animals in the melatonin-treated group had DCIS, accounting for 66.7% inhibition of DCIS incidence.

Similarly, as shown in figure 4B, when the data were evaluated for the frequency of occurrence of mammary intraductal proliferations (IDPs) and DCIS, melatonin induced a significant decrease in the percentages of mammary IDPs ($P < 0.05$) and DCIS ($P < 0.05$) compared with positive control rats.

As a consequence, histopathological analysis of the mammary glands revealed that among the animals pretreated with melatonin, two groups were identified. The first group constitutes 66.7% of melatonin-treated rats in which melatonin was able to suppress neoplastic proliferation; accordingly no atypical cells, malignant cells or other pathological changes were detected (Figs. 6 A and B) in the mammary glands.

On the other hand, the second group (33.3%) of melatonin-administered animals showed significant pathological changes in the mammary tissue. Specifically, atypical lobular and ductal hyperplasia were frequently seen (Figs. 6 C and D). Focal fibrosis (Fig. 6C) and stromal reaction were also noticed (Figs. 6 C and D). In some glands, cystic dilatations with minimal focal secretory changes and calcifications were observed (Fig. 6E) along with DCIS (Figs. 6 E and F).

Again, data shown in figure 4A indicated that pre-treatment of *C.molmol* for 15 days before the administration of DMBA caused a significant decrease ($P < 0.05$) in the incidence of ductal carcinoma *in situ* when compared with DMBA alone-treated group (positive control). That is seventy five percent of the *C. molmol*-treated rats were found to have DCIS, accounting for 25% inhibition ($P < 0.05$) of DCIS incidence.

Similarly, the frequency of occurrence of mammary intraductal proliferations and DCIS were found to be significantly lower ($P< 0.05$; Fig. 4B) compared with IDPs and DCIS in the positive control rats.

Histopathological examination of the mammary glands revealed that 25% of rats were free of DCIS, atypical hyperplasia or any other significant pathological changes. The mammary glands had a similar histologic architecture to that of normal rats. Briefly, the ductal and acinar structures appeared normal and were distributed in fibro-fatty stroma (Figs. 7 A and B). However, in some glands

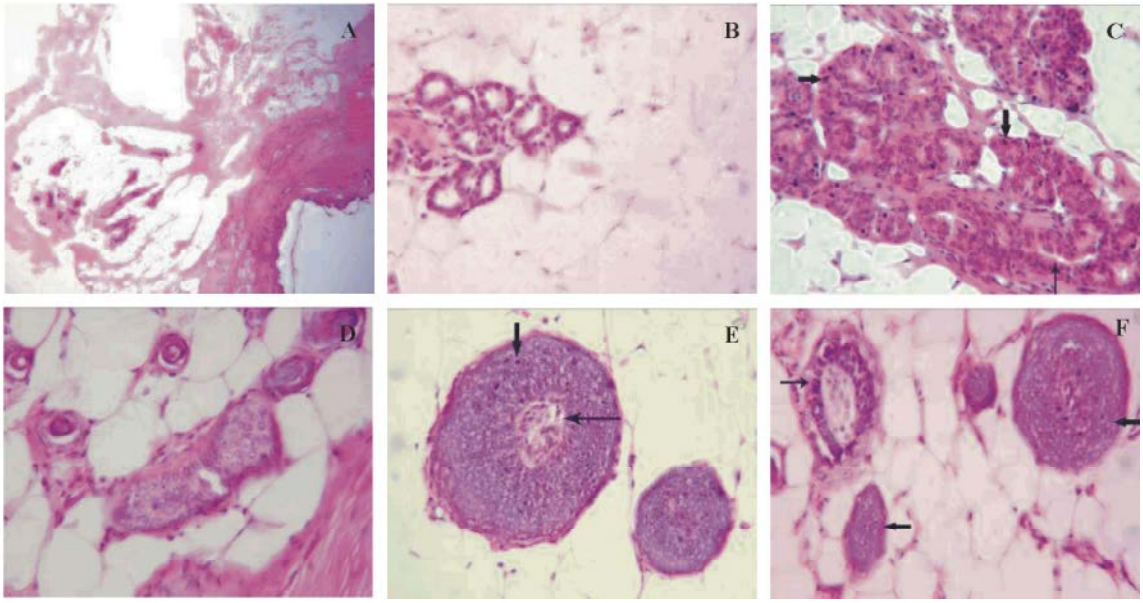

Fig. 7: (A-F) Histological sections of mammary glands from female rats treated with *C. molmol* for 15 consecutive days prior to DMBA administration (5 months post-DMBA exposure). (A) Normal structure of mammary gland (x 100); (B) Acini lined by a layer of cuboidal epithelial cells and a discontinuous layer of myoepithelial cell (x 400); (C) Hyperplasia of acinar epithelium; thin arrow indicates slit-like lumina and thick arrows show pyknosis (x 400); (D) Atypical ductal hyperplasia (ADH; x 400); (E) DCIS with mitotic figures (thick arrow) and central area of cellular degeneration (thin arrow; x 400). (F) DCIS of solid type (thick arrows) and DCIS of comedo type with central necrosis (thin arrow; x 400). All sections stained with H & E.

ductal and acinar hyperplasia were marked and occasionally the lumina appeared irregular in both size and shape (Fig. 7C). Pyknotic nuclei were also detected (Fig. 7C).

On the other hand, the majority of rats (75%) treated with *C. molmol* for 15 consecutive days before DMBA-administration developed DCIS and atypical ductal hyperplasia (ADH); however, the number of these lesions were markedly decreased compared to those in DMBA-alone-treated rats. Atypical hyperplasia appeared as intraductal irregular proliferations that displayed irregular lumen formations (Fig. 7D). The most prevailing patterns of DCIS noticed in this group were the solid (Figs. 7 E and F) and comedo (Fig. 7F) types. The later is characterized by high-grade malignant cells filled the expanded ducts with central necrosis. Increased number of mitotic figures and cellular degeneration were also present (Figs. 7 E and F).

DISCUSSION

In recent years, finding natural effective chemopreventive substances that can suppress malignant transformation of cells has been the goal of many cancer researches. The present study investigates the chemopreventive action of the oleo-gum -resin of *Commiphora molmol* compared with melatonin in a model of chemical carcinogenesis that closely mimics the human disease. To the best of our knowledge, this is the first report showing chempopreventive effects of *C. molmol* on DMBA-induced mammary carcinogenesis. In the current study oral administration of a single dose of DMBA (75mg / Kg. b.wt) disrupted hematopoiesis in the bone marrow as indicated by changes in blood cell indices. Also DMBA produced ductal carcinoma *in situ*, as well as ductal hyperplasia and atypical ductal hyperplasia in 100% of mammary glands of all treated rats. This model mimics the human breast cancer which evolves in a linear progression through sequential stages of hyperplastic lesions with and without cellular atypia, carcinoma in situ and finally invasive carcinoma. Interestingly, the data here indicated that melatonin and *C. molmol* given separately for 15 consecutive days prior to DMBA-treatment protected bone marrow from damaging effects of DMBA and stimulates the suppressed bone marrow. They also inhibited the incidence of DCIS as well as the frequency of occurrence of intraductal proliferations (IDPs) and DCIS. However, melatonin effects were more pronounced than those of *C. molmol*.

Hematological Studies: DMBA administration in this study did not affect red blood cells count or hemoglobin concentration of female rats, although it induced a marked decrease in the total leukocyte count, as well as in lymphocyte, monocyte and granulocyte percentages. Similar findings have been reported by N'jai *et al.* [29] who demonstrated that neither intraperitoneal (IP) nor oral DMBA (50mg/kg) treatment has caused any significant changes in red blood cell numbers, hematocrit value, or platelet numbers, but it produced a reduction by 50% in total numbers of WBCs, as well as numbers of neutrophils and lymphocytes. They suggested that DMBA selectively targets white blood cell rather than red cell or thrombocyte precursors in the bone marrow.

Previous studies revealed that IP administration of DMBA to mice has resulted in a substantial hypocellularity of the bone marrow at 48 h after exposure. This response was dependent on local metabolism of the DMBA by cytochrome P450 1B1 (CYP1B1) enzyme that is expressed in the bone marrow, spleen, thymus and peripheral blood leukocytes [30]. DMBA metabolites have been shown to inhibit proliferation or differentiation of hematopoietic stem cells (HSCs), resulting in impaired maturation into committed lymphoid progenitor and committed myeloid progenitor cells [31]. This may explain the presence of leucocytes with ring-shaped nuclei, which indicate myeloproliferative disorders [32], in the peripheral blood smears from DMBA-treated rats in this study.

Furthermore, in the present work a high percentage of atypical lymphocytes was detected in peripheral blood smears from DMBA-treated rats. Atypical lymphocytes are reactive lymphocytes that play an essential role in the immune response [33]. According to Inman and Cooper [34] atypical lymphocytes are small nonmalignant lymphocytes that become larger in size and capable of dividing. They are seen in peripheral blood as a nonspecific response to stress from a variety of stimuli and may be associated with malignant diseases [35].

Melatonin regulates dynamics of virtually all hemopoietic and immune cell lineages, including, T and B lymphocytes, granulocytes and monocytes in bone marrow as well as in tissues. In particular, melatonin has been shown to possess a strong antiapoptotic property, thereby promoting the survival of normal granulocytes and B lymphocytes [36]. Evening injection of melatonin significantly induced lymphoproliferation of splenocytes and thymocytes and consequently increased the circulating level of lymphocytes in peripheral blood and also in bone marrow, thereby increasing its immune status [37]. Those observations may explain the significant increase in the total number of leucocytes as well as in lymphocyte, monocyte, neutrophil and eosionophil percentages reported in the present study when melatonin was administered for 15 consecutive days prior to DMBA administration. Actually, these results were expected in terms of the well known immunoenhansing effects of melatonin.

Previous studies showed that administration of melatonin in therapeutic doses resulted in an increase of the number of lymphocytes and a decrease of the number of morphologically altered cells [38]. This is consistent with the results of the current work in which a significant decrease in the percentage of atypical lymphocytes was detected in blood smears of female rats treated with melatonin for 15 consecutive days before the administration of DMBA as compared with DMBA-treated rats.

Moreover, in this study, administration of *C. molmol* for 15 consecutive days prior DMBA-treatment (*C. mol* / DMBA group) produced a significant increase in the total number of leucocytes and in the percentage of lymphocytes, monocytes and basiophils.. These results are in agreement with those of Haffor (2010) [39] who found that *C. molmol* supplementation in drinking water for four weeks induced an increase in the proliferation of all types of leukocytes, with high relative rate in lymphocytes, neutrophils and monocytes, respectively. Therefore, it has been suggested that *C. molmol* stimulates the early steps of both lymphoid and meolyed maturation pathways for leukocytes. The observation that *C. molmol* also caused a decrease in the percentage of DMBA-induced atypical lymphocytes may indicate cytotoxic effects for *C. molmol*. This strengthens the finding of Al-Harbi *et al.* [40] that the anti-tumor potential of *C. molmol* was similar to the standard cytotoxic drug cyclophosphamide.

Histopathological Studies: The present study demonstrated that all mammary glands from rats received DMBA alone developed intraductal carcinoma (ductal carcinoma *in situ*; DCIS) of solid type which did not invade the surrounding stroma. In addition, treatment of rats with DMBA alone induced ductal hyperplasias, atypical hyperplasias and myoepithelial hypeplaias in the mammary glands. These results are similar to those of previous studies [27, 41]. The detection of proliferation in the epithelial and myoepithelial cells of the mammary gland strongly suggests that DMBA acts on different cells in the breast tissue [42].

There is accumulating evidence that DMBA-induced rat mammary carcinomas are hormone-dependent adenocarcinomas that are histologically similar to human

breast tumors [43]. The susceptibility of the rat mammary gland to carcinogenesis is strongly age-dependent and is maximal when the carcinogens are administered to animals between the ages of approximately 45 and 60 days in which the mammary gland exhibits a high rate of proliferation of the glandular epithelium. Therefore, administration of DMBA to virgin rats at these ages has been reported to induce intraductal carcinomas that progress to invasive carcinomas, developing various patterns [22, 43]. Unexpectedly, no palpable masses or invasive adenocarcinomas were detected in the current study until the end of the experiment (5 month post-DMBA administration) although DMBA was administered to rats at ages of 51-58 days.

These unexpected findings are puzzling because they are somewhat different from what have been reported in a similarly designed study of Lenoir *et al.* [19], where DMBA administration induced palpable mammary tumors generally classified as adenocarcinomas (ADKs). In that study, the first palpable mammary ADK (latency period) appeared 2 months after DMBA administration. By 6 months after the administration of DMBA, 75% of control rats exhibited at least one palpable mammary ADK. Given that the protocols were identical between the two studies, it is not precisely known why the results of the present study were not similar to those reported in that study. The difference could be due to the direct effect of photoperiod; the present study started in December with short winter days in comparison with Lenoir (2005) experiment which performed under conditions of controlled light; 12 hours light / 12 hours darkness. This suggestion may be supported by the finding of Löscher *et al.* [44] who observed a seasonal variability in the incidence of DMBA-induced mammary carcinogenesis in female Sprague-Dawley rats, with a maximum rate in April to July and a minimum rate in September to December. Moreover, Kubatka *et al.* [45] indicated that season may play a substantial role in experimental mammary carcinogenesis in female rats. Circannual oscillations in the pineal melatonin production might be a possible explanation for this phenomenon.

In This study, administration of melatonin for 15 consecutive days prior to DMBA-treatment markedly reduced the proliferative activity in the mammary gland. Specifically, it inhibited the incidence of DCIS as well as the frequency of occurrence of IDPs and DCIS. These findings are in agreement with reported data of previous investigations. In a previous study, the maximal inhibitory effect of administration of melatonin for 15 days prior to DMBA-treatment (preventive effect) on the percentage of female rats with mammary tumors was a reduction to 62% relative to DMBA group [19]. In another study, animals with enhanced pineal function or those treated with melatonin have an increase in tumor latency or time elapsing between the administration of the carcinogen and the appearance of palpable mammary tumors; they also have a lower tumor incidence and a lower rate of tumor growth, in contrast to pinealectomized animals or to those animals with decreased melatonin levels [46].

The antineoplastic action of melatonin arises through infinite possible routes, including antioxidant, antimitotic and/or antiangiogenic activity, as well as its ability to modulate the immune system and alter fat metabolism [8]. Melatonin may inhibit DMBA-induced adduct formation with DNA because of its free radical scavenging/antioxidant action [47]. In that view, it may be expected that the strong artificial amplification of the intensity of the circadian rhythm of melatonin, provoked by an exogenous supply, before the administration of DMBA could play a major preventive role against the induction of the carcinogenic process [48]. In addition it has been proposed that melatonin reduces the development of breast cancer throughout its interactions with the estrogen-signaling pathways [46].

In the present work, oral administration *C.molmol* for 15 days before DMBA treatment caused a 25% inhibition of DCIS incidence. It also induced a marked decrease in the frequency of occurrence of mammary IDPs and DCIS. The antitumor effects noticed for *C.molmol* in the current study are supported by previous studies [16, 17, 40] in which *C. molmol* showed significant antitumor, antimutagenic, antioxidative and cytoprotective properties. Nevertheless, in this study the preventive treatment with *C. molmol* persisted for only 15 days prior to DMBA administration, a longer period of preventive treatment and/or long term treatment after DMBA administration might have led to a more pronounced inhibitory effect on DMBA-induced mammary carcinogenesis.

The mechanism by which *C. molmol* inhibits mammary carcinogenesis is not clear. Certain chemical constituents of *C. molmol* such as Aldehydes and eugenols have been described to possess good oxygen radical scavenging and antimutagenic potential [40]. It has been suggested that DMBA mammary carcinogenesis is induced throughout the formation of a cascade of metabolites which ends in 3,4-dihydro-diol-1,2-epoxide, before adduct formation with DNA occurs [49]. Consequently, *C. molmol* may inhibit DNA adduct formation by its free radical scavenging activity.

The oleo–gum–resin of *C. molmol* contains volatile oils (up to 17%), resins (up to 40%) and gum (up to 60%). In the volatile oil fraction different terpenes, sesquiterpenes, esters, cinnamaldehyde, cuminaldehyde, cumic alcohol, eugenol, heerabolene, limonine, dipentene, pinene, m-cresol and cadinene were identified. The resins were found to contain á-, â- and ã-commiphoric acids, commiphorinic acid, á, â-herrabomyrrhols, heeraboresene, commiferin, ketosteroids, compesterol, â-sitosterol, cholesterol, á-amyrone and 3-epi-á-amyrin. The gum on hydrolysis yielded arabinose, galactose, xylose and 4-O-methylglucuronic acid [50]. Therefore, the carcinogenic-inhibitory effect exhibited by *C. molmol* in the present study may be related to certain active molecules in its phytoconstituents capable of inhibiting cancer cell proliferation.

Conclusively, the data presented herein suggest that melatonin and *C. molmol* given separately for 15 consecutive days prior to DMBA-treatment protect bone marrow from damaging effect of DMBA and stimulates the suppressed bone marrow, as indicated by the increase of the number of leukocytes and the decrease of the number of morphologically altered cells. Additionally, without a doubt both of *Commiphora molmol* and melatonin inhibited the carcinogenic effects of DMBA on mammary glands. However, melatonin was more efficacious than *C. molmol*.

Interestingly, it was found that, similar to melatonin, the administration of *C. molmol* was not accompanied by side effects and this finding together with its ability to decrease the incidence and frequency of occurrence of DCIS and mammary intraductal proliferations (IDPs) in rats make *C. molmol* a good candidate for possible use in the prevention of breast cancer. Further studies are needed to clarify its mode of action and safety for medicinal use in cancer prevention and treatment.

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