J Oral Med Oral Surg 2023;29:6
https://doi.org/10.1051/mbcb/2023005

https://www.jomos.org

JOMOS
Journal of Oral Medicine and Oral Surgery

Original Research Article

Evaluation of differential white blood cell count and cheek pouch epithelium in 7,12-dimethylbenza[a]anthracene hamster carcinogenesis model, managed with three phytochemicals

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(Received: 8 May 2022, accepted: 5 January 2023)

Keywords: Epigallocatechin-3-gallate / HCPE-DMBA / Nigella sativa / thymoquinone / peripheral WBCs / phytochemicals

Abstract – Objectives: Nigella sativa (NS), thymoquinone (TQ), and epigallocatechin-3-gallate (EGCG) are phytochemicals that might have antioxidant protective potentials on the hamster cheek pouch epithelium (HCPE). We aimed at evaluating and comparing the potential therapeutic outcomes of these 3 phytochemicals by analysis of peripheral white blood cells (WBCs) counts. **Materials and Methods:** NS whole oil, TQ and EGCG were administered before, with or after 7,12-dimethylbenza[a]anthracene (DMBA) painting the hamster left cheek pouch. Before sacrificing each animal, 2 ml of blood was withdrawn into a fine heparin-containing tube to estimate the total WBCs, lymphocytes, MID cells, and granulocytes counts by an automatic count system. All cheek pouches were surgically excised and examined with light microscope. **Results:** Severe epithelial dysplasia was evident after 6 weeks of DMBA administration, and when NS was given for 2 weeks followed by DMBA for 6 weeks. When NS or EGCG were given for 2 weeks then continued with DMBA for 6 weeks, mild dysplasia was seen. When DMBA was given for 6 weeks followed by NS or TQ for 6 weeks, mild dysplasia was noted. Administration of DMBA for 6 weeks resulted in significant reduction in total WBCs and lymphocytes counts compared to healthy controls. Administration of NS or TQ for 2 weeks resulted in significant elevation in lymphocytes count compared to healthy controls. Significant elevation in total WBCS and lymphocytes counts was noted when EGCG was given for 2 weeks and continued with DMBA for other 6 weeks. Similar results were noted when DMBA was given for 6 weeks followed by TQ for 6 weeks when compared to NS, DMBA or healthy controls. **Discussion:** The three phytochemicals showed different levels of protection against DMBA carcinogenic activity, more specifically, TQ and NS had higher therapeutic potential and might be used for treatment and/or preventive management of oral cancer in the future. **Conclusion:** However, further investigations are required to address the mechanism of action and feasibility of clinical application of each phytochemical.

Introduction

Oral cancer ranks the 6th most common type of cancer spreading worldwide and it forms around 25% of cancer types [1]. The first model of oral squamous cell carcinoma (SCC) was produced in the hamster cheek pouch (HCP) using 7,12-dimethylbenza[a]anthracene (DMBA), in 1954. This model was extensively used to study the biologic pathways, prevention, and treatments of oral SCC [2,3]. Nagini *et al.* (2009) had studied important biologic markers of oral SCC in hamster cheek pouch and human where they found comparable key signaling pathways. They reported similar oxidative DNA damage, cell survival and proliferation markers in both human and hamster. In addition, apoptosis markers were downregulated while invasiveness and angiogenesis markers were upregulated in both models [3]. This confirms the importance of HCP carcinogenesis model in studying human oral SCC.

Phytochemical agents are widely studied to test their chemo-preventive or therapeutic effects on different diseases. Nigella Sativa L whole oil (NS) [4] and one of its active ingredients; Thymoquinone (TQ) [2] are among the most used phytochemicals. An active ingredient of green tea, (−)-epi-gallocatechin-3-gallate (EGCG) [5] is another valuable phyto-chemical. Researchers consider these agents as antioxidant cytotoxic materials that may prevent, delay or co-treat malignancies, including ovarian and breast cancer cells [6].

Burits and Bucar (2000) found that NS essential oil and its four constituents (thymoquinone, carvacrol, t-anethol, and

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4-terpineol) had antioxidant effects in different chemical assays [7]. Medenica *et al.* (1997) confirmed that NS extract inhibited endothelial cell progression, decreased production of the angiogenic protein-fibroblastic growth factor made by tumor cells, and inhibited the vascular endothelial growth factor [8]. In a rat model, Nazrul Islam *et al.* (2004) tested the volatile oil of NS seeds and reported significant decrease in the antibody titer, splenocytes and neutrophils counts, but a significant increase in peripheral lymphocytes and monocytes [9]. In addition, NS oil was found to be cytotoxic to fibroblasts and five human cancer cell lines. Thymoquinone (TQ) effects were documented in different health and disease conditions [9]. Mansour *et al.* (2002) investigated the effect of oral TQ and its metabolite (dihydro-TQ) on hepatic, renal and cardiac antioxidant enzymes activities in healthy mice [10]. They provided evidence that TQ and its metabolite acted as superoxide anion and general free radical scavengers which suggests that they might have endogenous antioxidant effects [10].

Green tea (GT) consumption has been associated with decreased risk of certain human cancers. GT-catechins are reported to have antibiotic, anti-inflammatory, antioxidative and anticancer effects [11]. Shimizu *et al.* (2010) had suggested that GT-catechins intake prevented the experimental tumor metastasis in aged mice via inhibition of reduction in immune surveillance potential with aging [11]. Sherry-Chow *et al.* (2007) reported that GT polyphenols may enhance the detoxification of carcinogens in individuals with low baseline detoxification capacity [12]. Brown (1999) proposed that GT polyphenolic compounds, specially catechins, may act as antioxidants, free-radical scavengers, and stimulants of the detoxification systems through selective induction or modification of phase I and II metabolic enzymes [13]. In addition, GT polyphenols may inhibit neoplasms growth and development via inhibition of some biochemical components involved in tumor initiation and cell proliferation [13]. Furthermore, Li *et al.* (2002b) reported that green tea decreased neoplasms number by 35.1% and volume by 41.6% when given for 18 weeks following 6 weeks of DMBA treatment in golden hamsters [14]. Luo *et al.* (2010) found that the GT active ingredient EGCG may sensitize breast cancer cells to paclitaxel chemotherapy, both in vitro and in vivo [15].

Peripheral blood contains a large pool of circulating leukocytes that perform a multitude of functions and are used for diagnosis and treatment of many diseases, including cancer. Circulating cells include lymphocytes, neutrophils, eosinophils, basophils, monocytes and other less abundant cells. This study aimed to compare the immune-boosting potential of Nigella Sativa, thymoquinone and epigallocatechin-3-gallate against the carcinogenic effect of 7,12-dimethylbenza[a]anthracene on hamster cheek pouch epithelium, through evaluation of different peripheral white blood cells.

Materials and methods

This study received an approval for the animal use from the university animal care committee in accordance with the guidelines of the university. One hundred and fifty male Syrian hamsters, aged 6–8 weeks and weighed 100–120 g, were purchased from the Central Animal House, Theodor Bilharz Research Institute, Cairo, Egypt. Animals were housed as 5 hamsters per cage and were provided water and standard food ad libitum, at the animal house of the university. Nigella Sativa whole oil and Thymoquinone materials were kindly supplied by Dr Mohamed El-Dakhakhny, Faculty of Medicine, Alexandria University, and EGCG was kindly supplied by MEPACO, Arab Company for Pharmaceutical and Medicine Plants, Enshas El-Raml, Sharkeiya, Egypt.

Animals grouping

Animals were placed in transpicuous glass jars to receive gas anesthesia at a volume of 1 mL diethyl ether for 20 mL jar volume until achieving a stable anesthetic level. All experiments were performed using diethyl ether as a general anesthesia and control animals were exposed to the same dose to exclude the effect of anesthesia. Table I summarizes the grouping of animals used in this study with the main histopathologic results.

Animals were divided into 5 groups as follows:

- Negative control group: marked as group GI and included 10 healthy untreated animals.
- Positive control or carcinogen-treated group: marked as group GII and included 10 animals. In this group, animals were treated with 0.5% DMBA dissolved in mineral oil; number 4 camel hairbrush was used to paint the left cheek pouch of each hamster, 3 times per week [16] for 6 weeks.
- Nigella Sativa L. (NS) whole oil group: marked as group A and included 50 animals which were further divided into 4 subgroups to be treated with NS oil (5 mg/kg body weight) via an intragastric tube. Figure 1A shows group A subgroups: subgroup Ai contained 20 animals treated with NS oil alone (daily for 2 weeks). Ten animals were sacrificed with no further treatment (Aia) and the other 10 were treated with the DMBA carcinogen (3 times a week) for other 6 weeks before being sacrificed (Aib). Subgroup Aii contained 10 animals treated with NS oil (daily) plus DMBA (3 times a week) for 6 weeks. Subgroup Aiii contained 10 animals treated with NS oil only (daily for 2 weeks), then treated with both NS oil (daily) and DMBA (3 times a week) for 6 weeks. Subgroup Aiv contained 10 animals treated with DMBA (3 times a week) for 6 weeks then NS (daily) for other 6 weeks.
- Thymoquinone (TQ) group: marked as group B and included 40 animals. TQ was dissolved in propylene glycol and diluted in sterile saline solution and injected daily via the intraperitoneal route, in a dose of 0.1 mg/kg body weight. The 40 animals in group B were further divided into 4 subgroups as shown in Figure 1B. Subgroup Bia contained 10 animals treated with TQ alone, (daily for 2 weeks). Subgroup Bib contained 10 animals treated with TQ alone (daily for 2 weeks), then DMBA (3 times a week) for 6 weeks. Subgroup Bii contained 10 animals treated with TQ (daily) plus DMBA (3 times a week) for 6 weeks. Subgroup Biv contained 10 animals treated with DMBA (3 times a week) for 6 weeks, then TQ (daily) for other 6 weeks.

Table I. shows summary of the study groups, number of animals in each group, treatments provided to each group and the main histopathologic observations. Nigella Sativa (NS); Thymoquinone (TQ); (–)-epigallocatechin-3-gallate (EGCG); 7,12-dimethylbenza [a] anthracene (DMBA).

Group	Treatment and Animals' Numbers (N)	Histopathologic observations
G I	Untreated healthy control. *N* = 10	Normal thin keratinized stratified squamous epithelium, no rete ridges, and no appendages.
G II	DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia, hyperkeratinization, signs of severe epithelial dysplasia and fibrotic connective tissue with moderate inflammatory cell infiltrate.
G Aia	NS for 2 weeks. *N* = 10	Similar to control with slight hyperkeratinization.
G Aib	NS for 2 weeks then DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia and severe dysplasia.
G Aii	NS plus DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia and moderate dysplasia.
G Aiii	NS for 2 weeks then, DMBA plus NS for 6 weeks. *N* = 10	Epithelial hyperplasia and mild dysplasia.
G Aiv	DMBA for 6 weeks then, NS for 6 weeks. *N* = 10	Epithelial hyperplasia and mild dysplasia.
G Bia	TQ for 2 weeks. *N* = 10	Similar to control with slight hyperkeratinization.
G Bib	TQ alone for 2 weeks then, DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia and mild dysplasia.
G Bii	TQ plus DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia and moderate dysplasia.
G Biv	DMBA for 6 weeks then, TQ for 6 weeks. *N* = 10	Epithelial hyperplasia and mild dysplasia.
G Cia	EGCG for 2 weeks. *N* = 10	Similar to control with slight hyperkeratinization.
G Cib	EGCG for 2 weeks then, DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia and moderate dysplasia.
G Cii	EGCG plus DMBA for 6 weeks. *N* = 10	Epithelial hyperplasia and moderate dysplasia.
G Ciii	EGCG for 2 weeks, then DMBA plus EGCG for 6 weeks. *N* = 10	Epithelial hyperplasia, hyperkeratinization and mild dysplasia.

– Green tea active ingredient (–)-epigallocatechin-3-gallate (EGCG) group: marked as group C and included 40 animals which were treated with EGCG (1.5 mg/animal) via an intragastric tube. Animals in group C were further divided into 4 subgroups as shown in Figure 1C. Subgroup Cia contained 10 animals treated (daily) with EGCG for 2 weeks. Subgroup Cib contained 10 animals treated (daily) with EGCG alone for 2 weeks, then DMBA (3 times a week) for 6 weeks. Subgroup Cii contained 10 animals treated with EGCG (daily) plus DMBA (3 times a week) for 6 weeks. Subgroup Ciii contained 10 animals treated with EGCG only (daily for 2 weeks), then EGCG (daily) and DMBA (3 times a week) for 6 weeks.

Blood analysis

Blood analysis of each animal was performed by withdrawal of 2 mL blood sample, from the orbital sinus through the mesial angel of the eye, into a heparin-containing tube. Samples were immediately analyzed by an automatic count system (Cell-DYN1700) Hematology Lab, in the university. The following blood cells counts were evaluated: total WBCs, lymphocytes, MID (monocytes, basophils, and eosinophils), and granulocytes (polymorphonuclear leukocytes).

Histopathologic evaluation

All animals were sacrificed by an overdose of diethyl ether anesthesia, after 24 h of fasting, at the end of the experiment. Both buccal pouches of each animal were surgically excised, fixed in 10% buffered neutral formalin for 24 h, processed routinely and embedded in paraffin wax. Sections were cut at 5 μm thickness and stained with Hematoxylin and Eosin for examination under light microscope at different magnifications.

Statistical analysis

Data was collected and statistically analyzed using the SPSS program for Windows (Standard version 21); One-way ANOVA test was used to compare results within each group followed by Least Square Difference test for multiple comparisons among groups. Data is presented as mean ± standard deviation and P values < 0.05 were considered statistically significant.

Results

Clinical observations

The overall health performance of all animals which were given NS, TQ or EGCG was better than those which were given

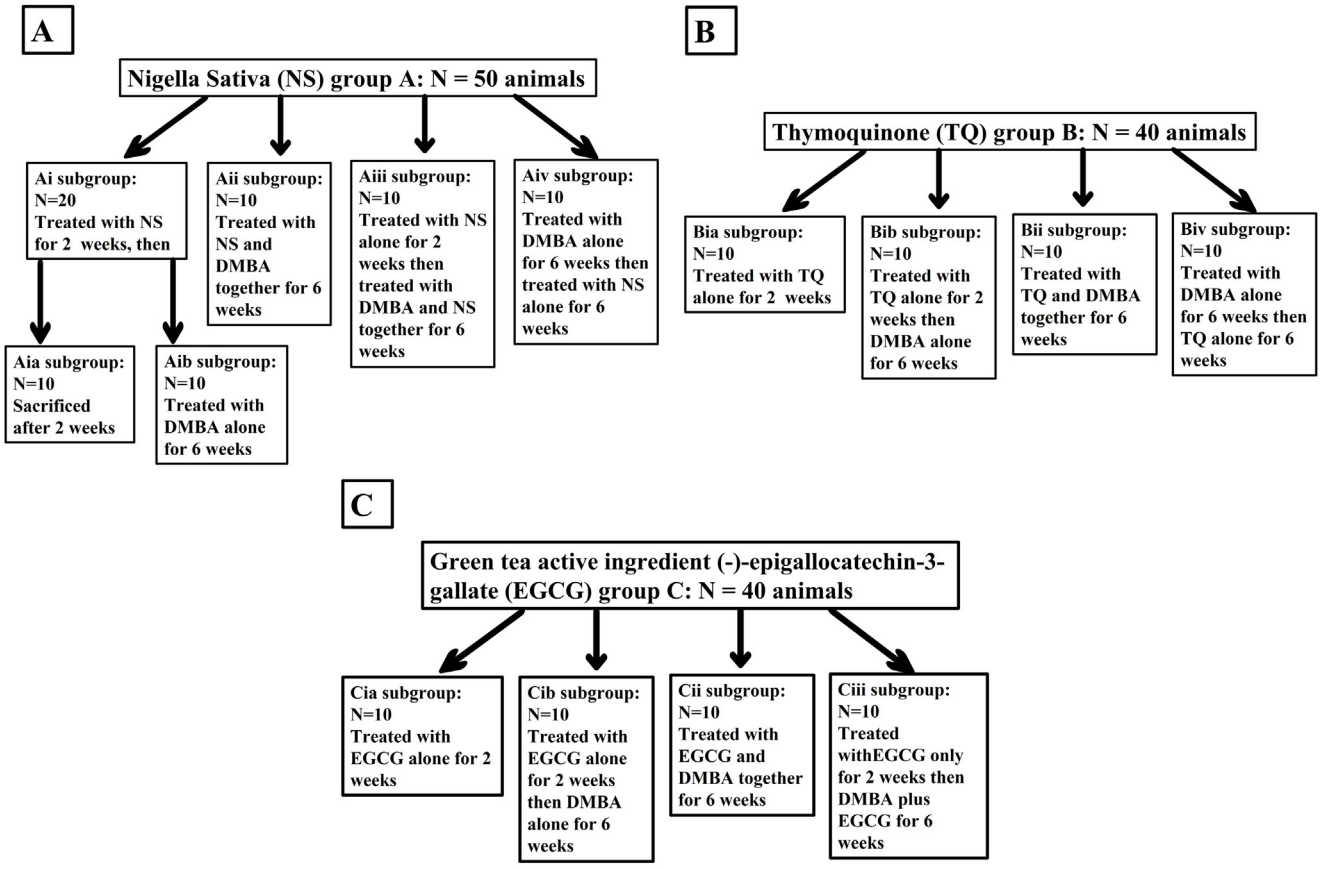

Fig. 1. Temporal scales A, B, and C explain the subdivisions, animals' numbers and phytochemical treatments which were given to subgroups of groups A, B, and C. Group A: 50 animals were treated with Nigella sativa (NS), Group B: 40 animals were treated with Thymoquinone (TQ), and Group C: 40 animals were treated with green tea active ingredient (–)-EpiGalloCatechin-3-Gallate (EGCG).

DMBA. DMBA-treated animals showed one death while the other 9 animals were very weak, lost weight and hair around the oral cavity, and all animals showed skin eruptions at the hair loss areas that turned to be squamous cell carcinoma. After a week of treatment with DMBA, a necrotic white material with fetid odor was seen at the painted left cheek pouch, it was peeled off when it was touched with a brush. This necrotic area disappeared after another week, leaving a marked shrinkage of the cheek pouch mucosa.

Milder clinical observations were encountered in animals treated with the three phytochemical ingredients with or after the carcinogen, while such observations were delayed in groups given the carcinogen after stopping the three phytochemicals. In addition, neither deaths nor skin carcinomas were seen in the negative control or the phytochemical groups.

Histopathologic analysis

Table I summarizes the histopathologic observations of all groups. The cheek pouches of the control animals (GI) showed normal thin keratinized stratified squamous epithelium with 2–4 cell thickness and no rete ridges or appendages, Figure 2. Cheek pouches of animals treated with phytochemical ingredients alone (NS Aia, TQ Bia, EGCG Cia) showed normal histologic appearance with slight hyperkeratinization. The connective tissue was loose and free of inflammatory cells, and normal striated muscle layer was observed in the submucosa.

DMBA-painted pouches in group GII showed focal epithelial hyperplasia and hyper-keratinization. In addition, signs of severe epithelial dysplastic changes were evident, such as drop-shaped rete ridges, basal cell hyperplasia, loss of polarity, hyper-chromatism, cellular and nuclear pleomorphism, prominent nucleoli, loss of cellular adhesion, and abnormal mitotic figures in the form of multinucleated epithelial cells. The connective tissue was fibrous with moderate inflammatory cell infiltrate (Fig. 3). Similar findings were evident in animals treated with NS for 2 weeks then DMBA for 6 weeks; subgroup Aib.

Mild epithelial dysplasia was seen in pouches of animals treated with NS or TQ for 6 weeks after the carcinogen was administered for 6 weeks; subgroups Aiv and Biv (Fig. 4). The same findings were evident when either NS and DMBA or EGCG and DMBA were given for 6 weeks after NS or EGCG alone, respectively, for 2 weeks; subgroups Aiii (Fig. 5), and Ciii, and when TQ was given alone for 2 weeks followed by DMBA for 6 weeks (subgroup Bib).

Moderate epithelial dysplasia with hyperplasia were seen in cheek pouches of animals given EGCG for 2 weeks followed by DMBA for 6 weeks; subgroup Cib (Fig. 6). The same observation

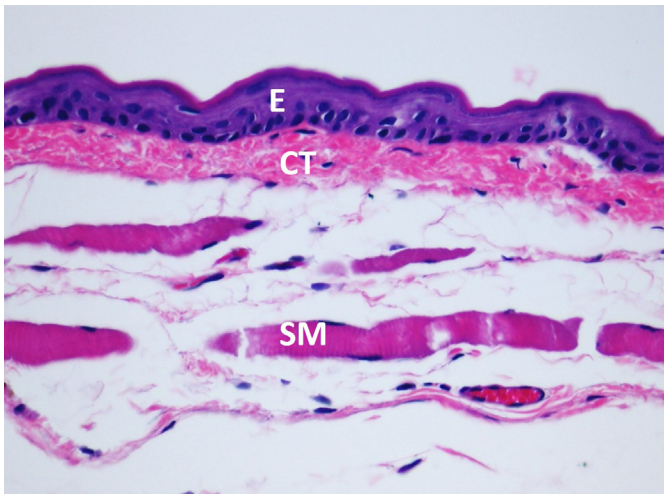

Fig. 2. Shows Hematoxylin and Eosin-stained cheek pouch section from a healthy untreated control animal. Normal thin keratinized stratified squamous epithelium (E) on top of an uninflamed connective tissue (CT) and a layer of normal striated muscles (SM). Magnification: 20×.

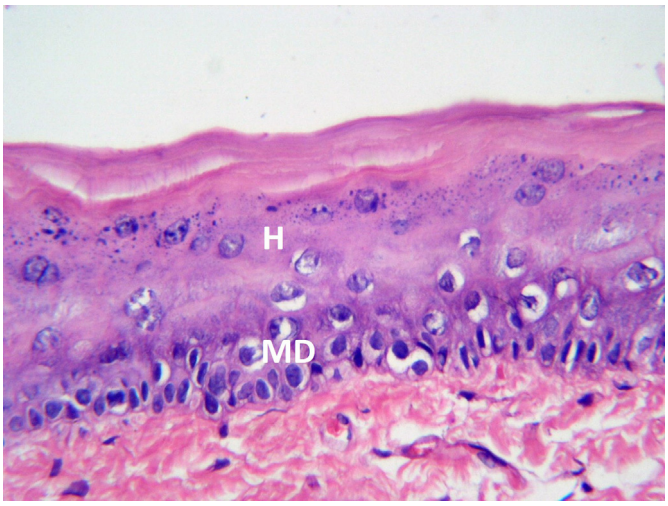

Fig. 4. Shows hematoxylin and eosin-stained cheek pouch section from an animal treated for 6 weeks with 7,12-dimethylbenza[a] anthracene (DMBA) followed by thymoquinone for 6 weeks. Epithelial hyperplasia (H) and mild dysplasia (MD) are evident. Magnification: 40×.

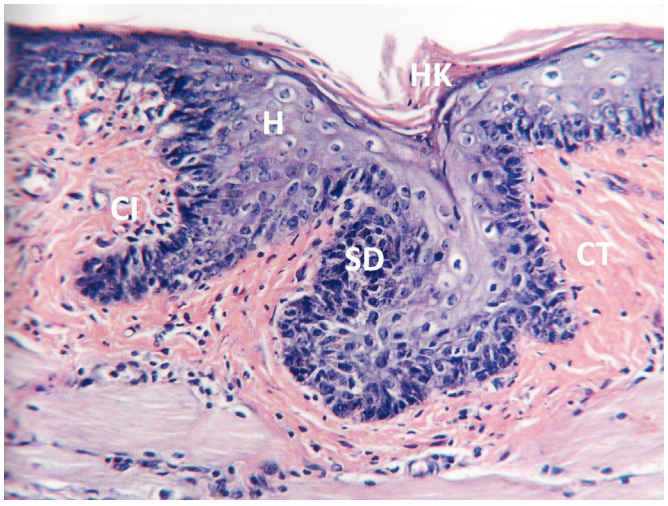

Fig. 3. Shows hematoxylin and eosin-stained cheek pouch section from an animal treated for 6 weeks with 7,12-dimethylbenza[a] anthracene (DMBA). Focal epithelial hyperplasia (H), hyperkeratinization (HK) and severe epithelial dysplastic changes (SD) are evident. The connective tissue (CT) looks more fibrous with moderate inflammatory cell infiltrate (CI). Magnification: 20×.

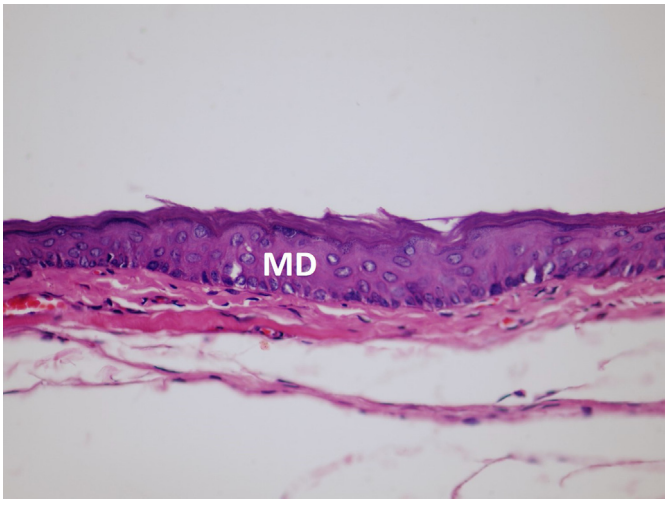

Fig. 5. Shows hematoxylin and eosin-stained cheek pouch section from an animal treated for 2 weeks with Nigella sativa (NS) followed by NS and 7,12-dimethylbenza[a]anthracene (DMBA) for 6 weeks. Mild dysplasia (MD) is noted. Magnification: 20×.

was evident in animals treated with DMBA plus either NS or TQ or EGCG for 6 weeks; subgroups Aii, Bii and Cii.

Blood analysis

Figure 7 shows the results of WBCs count of subgroups treated with the three phytochemicals alone for two weeks compared to healthy controls (GI) and DMBA-treated animals (GII). There was a significant reduction in total WBCs and lymphocytes counts after 6 weeks of DMBA treatment (GII) compared to healthy control animals (GI). However, significant elevation of WBCs and lymphocytes counts was found when animals were treated with any of the phytochemicals (for 2 weeks) when compared to GII. There was no significant difference between WBCs count of any subgroup when compared to GI. Significant elevation was found in lymphocytes count of Aia and Bia subgroups when compared to GI. No significant difference in MID or granulocytes counts was found when any subgroup in Figure 7 was compared to GI or GII.

Figure 8 presents the results of WBCs count of subgroups treated with DMBA for 6 weeks then, NS (Aiv) or TQ (Biv) for 6 weeks as compared to each other and to healthy untreated animals (GI). Significant elevation in WBCs count was noted when DMBA was given for 6 weeks followed by TQ (Biv) as

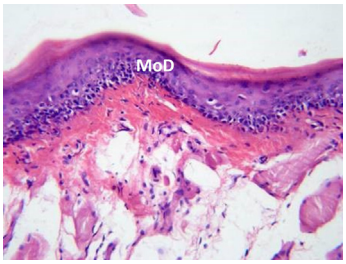

Fig. 6. Shows hematoxylin and eosin-stained cheek pouch section from an animal treated for 2 weeks with epigallocatechin-3-gallate followed by 7,12-dimethylbenza[a]anthracene (DMBA) for 6 weeks. Moderate epithelial dysplasia (MoD) is noted. Magnification: 10×.

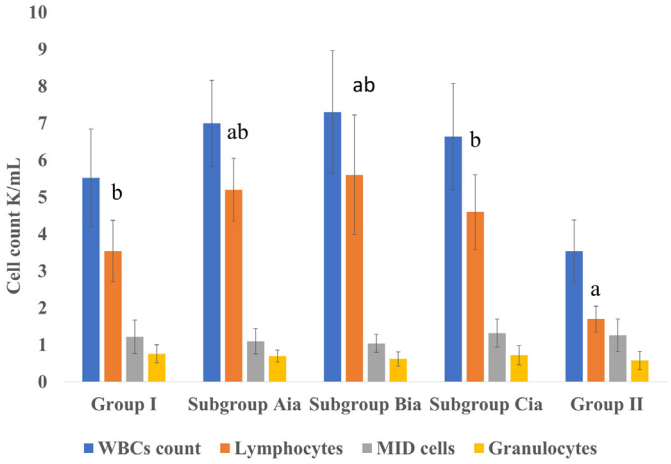

Fig. 7. A chart shows WBCs count results (mean ± standard deviation) of different experimental subgroups compared to group I and II. Group I included healthy untreated animals, Group II included animals treated with 7,12-dimethylbenza[a]anthracene (DMBA) for 6 weeks, Subgroup Aia included animals treated with Nigella Sativa for 2 weeks, subgroup Bia included animals treated with Thymoquinone for 2 weeks and subgroup C included animals treated with (−)-Epigallocatechin-3-gallate for 2 weeks. MID: Less frequently occurring cells such as monocytes, eosinophils, basophils and other precursor white cells. [a] Indicates that a significant difference (P value < 0.05) exists with group I. [b] Indicates that a significant difference (P value < 0.05) exists with group II.

compared to NS subgroup (Aiv). In addition, significant elevation in lymphocytes count in TQ subgroup (Biv) was noted when compared to NS (Aiv) and GI. No significant difference was noted in MID or granulocytes counts among all tested subgroups presented in this figure.

Figure 9 shows the results of WBCs count of subgroups treated with DMBA for 6 weeks then, NS (Aiv) or TQ (Biv) for 6 weeks as compared to each other and to DMBA-treated group (GII). Significant elevation in WBCs count was noted when TQ (Biv) was given for 6 weeks following 6 weeks of DMBA-treatment, when compared to GII and to NS (Aiv). In addition, significant elevation in lymphocytes count was noted when NS (Aiv) or TQ (Biv) were given for 6 weeks following 6 weeks of

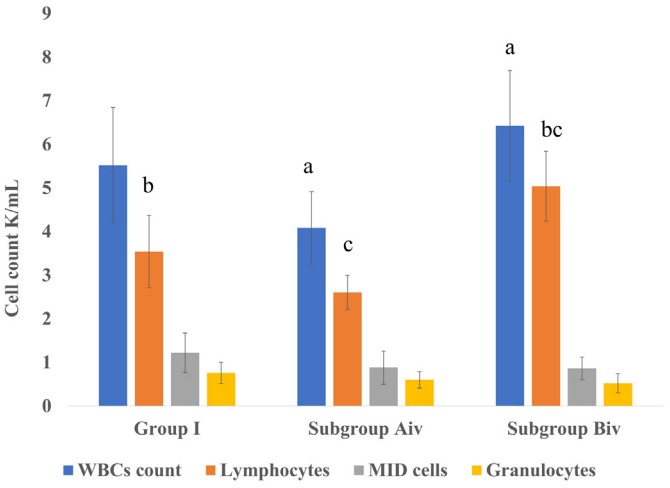

Fig. 8. A chart shows WBCs count results (mean ± standard deviation) of subgroups treated with 7,12-dimethylbenza[a]anthracene (DMBA) for 6 weeks then, Nigella Sativa (Aiv) or Thymoquinone (Biv) for 6 weeks in comparison to each other and to healthy untreated group (GI). MID: Less frequently occurring cells such monocytes, eosinophils, basophils and other precursor white cells. [abc] Indicate that a significant difference (P value < 0.05) exists between compared sub/groups that are marked with the same letter.

DMBA-treatment as compared to each other or to GII. However, no significant difference was noted in MID or granulocytes counts among all subgroups presented in Figure 9.

Figure 10 presents the results of WBCs count of the subgroups treated for 2 weeks with either NS (Aib) or TQ (Bib) or EGCG (Cib) then with DMBA for 6 weeks as compared to healthy untreated group (GI). Significant reduction was noted in the total WBCs and lymphocytes counts in Aib, Bib, Cib subgroups as compared to GI, however, lymphocytes count in these subgroups was still higher than DMBA-treated group (GII). In addition, significant reduction was noted in granulocytes count in Bib and Cib subgroups compared to GI, however, no significant difference in MID counts was noted.

Figure 11 shows the results of WBCs count of subgroups treated for 2 weeks with either NS or EGCG then, with DMBA combined with either NS (Aiii) or EGCG (Ciii) for 6 weeks as compared to each other and to healthy untreated group (GI). Significant differences in WBCs and lymphocytes counts were notes between Aiii and Ciii subgroups, however, no significant difference was found between Aiii or Ciii subgroups when compared to GI. No significant difference was seen in MID or granulocytes counts among all compared subgroups shown in this figure.

Figure 12 presents the results of WBCs count of subgroups treated for 2 weeks with either NS or EGCG then, with DMBA combined with either NS (Aiii) or EGCG (Ciii) for 6 weeks as compared to each other and to DMBA-treated group (GII). Significant elevations in WBCs and lymphocytes counts of Ciii subgroup was noted when compared to GII, however, there was no difference between Aiii subgroup and GII. No significant difference in MID or granulocytes counts was noted among all compared subgroups shown in Figure 12.

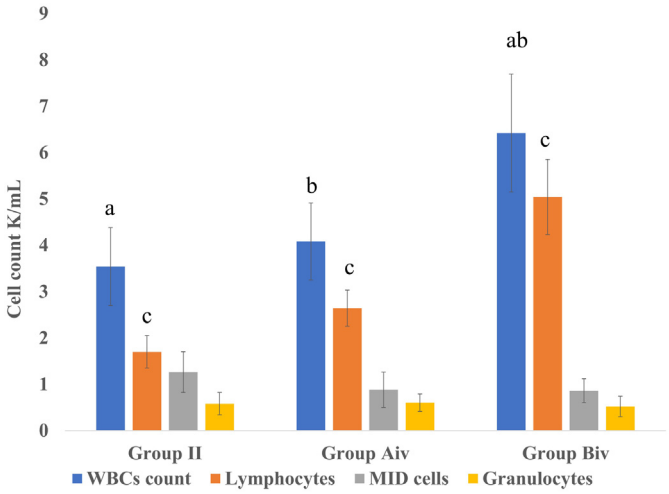

Fig. 9. A chart shows WBCs count results (mean ± standard deviation) of subgroups treated with 7,12-dimethylbenza[a]anthracene (DMBA) for 6 weeks then, Nigella Sativa (Aiv) or Thymoquinone (Biv) for 6 weeks as compared to each other and to DMBA-treated group (GII). MID: Less frequently occurring cells such as monocytes, eosinophils, basophils and other precursor white cells. [abc] Indicate that a significant difference (*P* value < 0.05) exists between compared sub/groups that are marked with the same letter.

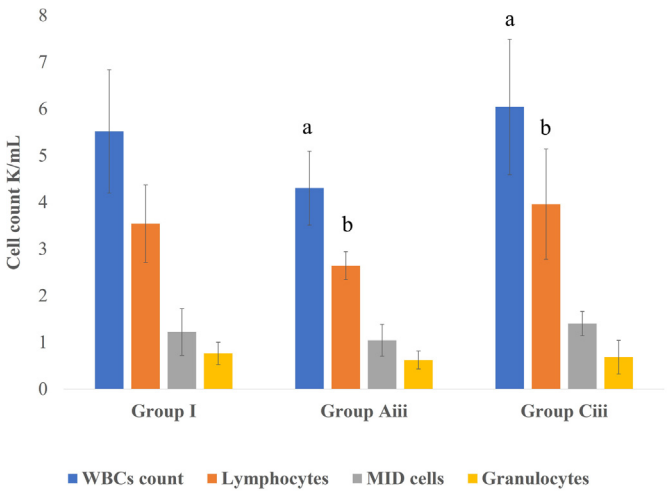

Fig. 11. A chart shows WBCs count results (mean ± standard deviation) of subgroups treated for 2 weeks with either Nigella Sativa (NS) or (−)-epigallocatechin-3-gallate (EGCG) then, with 7,12-dimethylbenza[a]anthracene (DMBA) combined with either NS (Aiii) or EGCG (Ciii) for 6 weeks as compared to each other and to healthy untreated group (G I). MID: Less frequently occurring cells such as monocytes, eosinophils, basophils and other precursor white cells. [ab] Indicate that a significant difference (*P* value < 0.05) exists between compared sub/groups that are marked with the same letter.

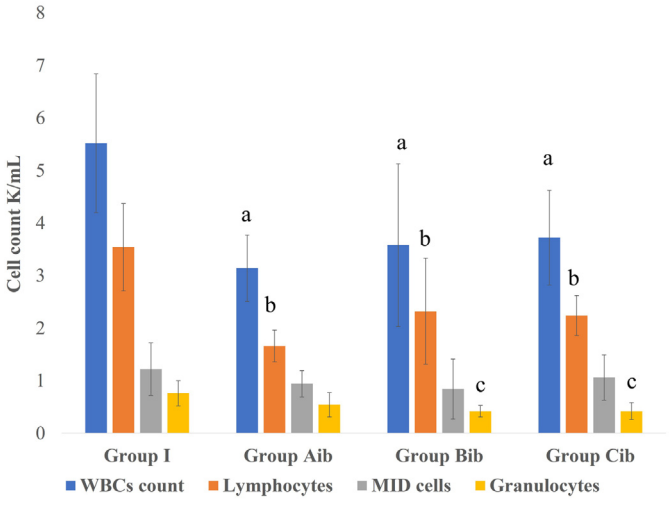

Fig. 10. A chart shows WBCs count results (mean ± standard deviation) of subgroups treated for 2 weeks with either Nigella Sativa (Aib) or Thymoquinone (Bib) or (−)-Epigallocatechin-3-gallate (Cib), then with 7,12-dimethylbenza[a]anthracene (DMBA) for 6 weeks as compared to healthy untreated group (GI). MID: Less frequently occurring cells such as monocytes, eosinophils, basophils and other precursor white cells. [abc] Indicate that a significant difference (*P* value < 0.05) exists between compared subgroups and group I.

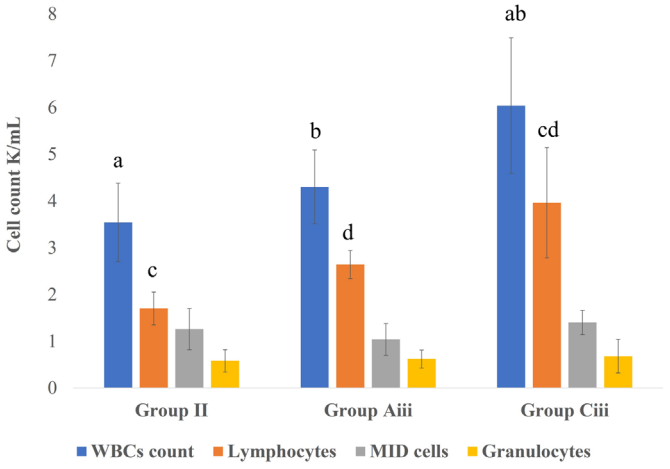

Fig. 12. A chart shows WBCs results (mean ± standard deviation) of subgroups treated for 2 weeks with either Nigella Sativa (NS) or (−)-epigallocatechin-3-gallate (EGCG) then, with 7,12-dimethylbenza[a]anthracene (DMBA) combined with either NS (Aiii) or EGCG (Ciii) for 6 weeks as compared to each other and to DMBA-treated group (G II). MID: Less frequently occurring cells such as monocytes, eosinophils, basophils and other precursor white cells. [abcd] Indicate that a significant difference (*P* value < 0.05) exists between compared sub/groups that are marked with the same letter.

Discussion

In this study, the overall clinical performance of animals treated with either Nigella Sativa (NS) or Thymoquinone (TQ) or (−)-Epigallocatechin-3-gallate (EGCG), and the absence of death in all treatment subgroups, clearly indicated the beneficial therapeutic potential of each agent. Only one animal was found died in GII, treated with DMBA-treated, however, the remaining 9 animals showed weight and hair loss, and skin eruptions that were diagnosed as squamous cell carcinoma. Similar results were reported by other studies [1,16–18].

The DMBA-treated animals (GII) showed severe dysplasia, as documented in similar studies [1,10,16–20]. This was accompanied by a significant reduction in total WBCs and lymphocytes counts as compared to untreated healthy controls (GI), which indicates the high level of local and systemic toxicity of DMBA. Noticeable reduction was seen in MID and granulocytes counts in all treated subgroups but the differences were not significant when compared to untreated animals (GI). In agreement with the present results, Das *et al.* (1986) studied the relation of lymphocytes count and blastogenesis in 70 patients with oral squamous cell carcinoma. Their results correlated with clinical stage, tumor size, lymph node involvement, tumor differentiation, lymphoreticular responses and outcomes during a year of follow-up. Although absolute lymphocytes count and T-cell population were reduced in the primary stage of the disease, the functional capacity of isolated lymphocytes to undergo blast formation was retained. This blastogenesis activity showed significant impairment only when the tumor was well-established and disseminated beyond its local confines [21]. Our results showed evidence of fibrous connective tissue infiltrated with moderate inflammatory cells in the cheek pouches of animals treated with DMBA for 6 weeks (positive control group, Fig. 3) as reported in similar studies [1,10,16–19].

Shahzad *et al.* (2009) reported that intraperitoneal administration of black seed oil caused significant reduction in all markers of allergic inflammation by inhibiting delayed hypersensitivity and T-cell proliferation in the spleen [22]. This might explain the reduction in MID (basophils, eosinophils and monocyte) count in NS- and TQ-treated subgroups (for 2 weeks) as compared to healthy controls or carcinogen-treated groups or EGCG subgroups. In the present study, subgroups treated for 2 weeks with either NS (Aib) or TQ (Bia) or EGCG (Cia) alone, showed normal cheek pouch epithelium with slight hyperkeratosis, which might correlate with a positive differentiation effect of these agents on the cheek epithelial cells [2,23]. In Aia, Bia, and Cia subgroups, there was insignificant increase in total WBCs compared to healthy control group (GI), and subgroup Bia showed the highest WBCs count. On the other side, lymphocytes count showed significant elevation in Aia and Bia subgroups, similar observation reported that NS volatile oil resulted in a rise of peripheral lymphocytes and monocytes [9]. Although all 3 phytochemicals were given for 2 weeks, in the present work, had led to insignificant increase in total WBCs count, EGCG showed the least increase compared to NS or TQ. On the other side, the increase in lymphocytes count was significant when NS or TQ were given for 2 weeks but insignificant when EGCG was given for 2 weeks. Similar findings were reported by Wu *et al.* (2009) who showed that EGCG inhibited T lymphocytes division and cell cycle progression [24]. Variations in elevated WBCs and lymphocytes counts among the 3 phytochemicals, together with milder epithelial dysplastic changes, might reflect a dual role in this animal model. One is local at the epithelial level, and the other is systemic through modulation of peripheral white blood cells activities, such as cytotoxic lymphocytes, natural killer cells and macrophages, rather than just increasing WBCs number.

In the present study, NS or TQ or GT were given at different sequences with the carcinogen (DMBA) had resulted in mild or moderate or severe epithelial dysplasia, accompanied by increased total WBCs and lymphocytes counts. This strongly suggests that all ingredients have protective and/or curative activities. These activities might include anti-inflammatory, detoxification, anti-mutagenic or immunostimulatory effects, at both epithelial and peripheral blood cells levels. Houghton *et al.* (1995) explained a mechanism in rat peritoneal leukocytes, by which both NS and TQ work as anti-inflammatory agents and they noted that TQ was about 10 times more potent than NS [25]. In addition, EGCG was reported to produce an antigenotoxicity effect on cultured human lymphocyte chromosomes [26].

The toxic effect of DMBA was further confirmed when it was given for 6 weeks following 2 weeks of administration of an individual phytochemical in subgroups Aib, Bib, and Cib. Significant reductions were noted in the total WBCs and lymphocytes counts of the 3 subgroups, as well as in granulocytes count of Bib and Cib subgroups, when compared to healthy controls (GI). Locally, severe epithelial dysplasia was seen when DMBA was painted for 6 weeks after stopping NS in subgroup Aib, however, moderate dysplasia when EGCG preceded DMBA (subgroup Cib), and mild dysplasia when TQ preceded DMBA (subgroup Bib). Similar findings were reported by Al-Jawfi *et al.* 2008b [23] and El-Dakhakhny *et al.* 2009b [27].

In the present study, TQ possessed the best protective effect against DMBA insult, when compared to NS or EGCG. The same chemoprotective effect of TQ was observed earlier by Hassan and El-Dakhakhny (1992) who tested the effect of TQ and polythymoquinone on DMBA-HCPE model. They reported a significant reduction in tumor burden in animals treated with TQ for a week and TQ with DMBA for 12 weeks, when compared to DMBA-treated group [28]. TQ effect was further documented in other models where TQ was found to have a powerful chemopreventive effect against benzo(a)pyrene-induced forestomach tumors in mice. TQ reduced the incidence and multiplicity of methylcholanthrene-induced fibrosarcoma by 70% and 67%, respectively, in female Swiss albino mice [29,30]. In addition, TQ was suggested to express an antioxidant anti-inflammatory effect and enhance the detoxification processes [29,30]. Thymoquinone was reported to have different beneficial effects, such as; immunoregulatory [31], antioxidant [32], antidiabetic [33], anti-inflammatory [34], pain relieving [35], blood pressure regulatory [36], gastro protecting [37], renal protecting [38], and anti-malignancy [31,39,40].

In other studies, TQ was reported to be effective against human colon cancer cells, by inhibiting cell proliferation, reducing cell viability, and inducing apoptosis [41,42].

The toxic and mutagenic effect of DMBA became significantly less effective when NS or EGCG were given for

2 weeks before DMBA, then continued for 6 weeks in subgroups Aiii, Ciii. Animals in these subgroups expressed mild epithelial dysplasia. Similar observations were reported by Hassan 1985 [43] and Al-Jawfi *et al.* 2008b [23]. Sustained NS or EGCG intake was required for better protection against the carcinogenic effect of DMBA. It was reported that NS had decreased DNA damage and thereby prevented initiation of carcinogenesis in rat colonic tissue following exposure to toxic azoxymethane [44,45]. El-Dahtory (2010) had shown that NS seeds emulsion has a preventive effect against chromosomal breakage in Down syndrome patients, which may decrease the risk of malignancy [46]. In addition, the significant elevation of WBCs and lymphocytes counts in subgroup Ciii as compared to subgroup Aiii and GII might be explained by the EGCG mechanism of action, where EGCG has the ability to arrest cell proliferation, thus counteracts the increased proliferation rate induced earlier by DMBA [43,47–49]. Li *et al.* (2002) suggested that green tea alone or with curcumin, significantly increased apoptosis index in dysplasia and squamous cell carcinoma and inhibited angiogenesis in papilloma and squamous cell carcinoma in HCPE/DMBA model [14]. Hamer (2007) found that EGCG possesses an anti-inflammatory action through its antioxidant capacity which might explain the mild dysplasia seen in our study [3,43,50]. Induction of detoxification enzymes has been suggested as one of the biochemical mechanisms responsible for the cancer-preventive effects of green tea [12]. Brown (1999) indicated that green tea inhibits tumor initiation and promotion, thus, inhibiting the growth and development of neoplasms [13].

In the current study, when DMBA was painted for 6 weeks followed by 6 weeks of administration of either NS (Aiv) or TQ (Biv), this resulted in mild epithelial dysplasia. Similar observations were described in other studies [2,23,28,43]. This finding indicates a promising chemotherapeutic potential of NS and TQ which might be related to their cytotoxic activity against cancerous cells [51]. Our blood results presented significant elevations of WBCs and lymphocytes counts in subgroup Biv as compared to subgroup Aiv or DMBA group (GII). Salomi *et al.* (1992) reported that TQ was cytotoxic against tumor cell lines with little effect against lymphocytes [52]. Furthermore, Mabrouk *et al.* (2002) found that oral NS had protected rats against methylnitrourea-induced oxidative stress and carcinogenesis by 80% and when NS was combined with honey, they showed 100% rat protection [53]. Shoieb *et al.* (2003) reported that TQ kills cancer cells by inducing apoptosis and arresting the cell cycle while non-cancerous cells are relatively resistant to TQ [6]. Furthermore, El-Najjar *et al.* (2010) showed that TQ inhibited the proliferation of a panel of human colon cancer cells, without exhibiting cytotoxicity to normal human intestinal cell line [54]. It was reported that neutrophils and macrophages, the major components of infiltrating leukocytes, migrate to the tumor site, capture cancer cells via tight physical contact, then, destroy them via cytolysis [55]. This mechanism of fighting malignant cells may explain the reduced granulocytes count in the present study, especially in subgroups with mild dysplasia; instead of showing severe dysplasia or advanced malignancy.

Conclusion

In this study, DMBA resulted in severe epithelial dysplasia and significant reduction of total WBCs and lymphocytes counts when given for 6 weeks either alone or after administration of either phytochemical. At the local level, thymoquinone (TQ) showed the best chemotherapeutic potential for the chemically induced malignant transformation in this HCPE-DMBA model. This was evident when TQ was given either for 2 weeks before DMBA or for 6 weeks after 6 weeks of DMBA administration. Nigella Sativa (NS) and epigallocatechin-3-gallate (EGCG) showed protective effects when given 2 weeks before DMBA, then continued with DMBA for 6 weeks. However, EGCG was superior to NS in significantly elevating total WBCs and lymphocytes counts. At the blood level, NS or TQ alone showed significant elevations of lymphocytes count compared to EGCG, DMBA, or healthy controls. In addition, NS and TQ showed significant elevation of total WBCs count as compared to DMBA group. This DMBA-hamster carcinogenesis model can be used to further study SCC mechanism, pathogenesis and develop new diagnostic markers. In addition, these three phytochemicals may be employed in the invention and advancement of SCC chemotherapy or immunotherapy.

Author contribution statement

All authors have shared the design of this study, the interpretation of data, writing and revising the manuscript.

Funding statement

All authors declare receiving no funding for this research project.

Ethical approval

This study received an approval for the animal use from the university animal care committee in accordance with the guidelines of Mansoura university.

Conflict of interest

Authors declare no conflict of interest.

Informed consent

Not applicable to this animal study.

Acknowledgments. The authors are grateful to Dr. Nashwa Yehia, Mathematical Statistics Department, Faculty of Science, Suez Canal University, for performing the statistical analysis of this study.

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