EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 70, 251:260, JANUARY, 2024
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 04-10-2023 • Accept Date : 31-10-2023 • Available online: 01-01-2024 • DOI : 10.21608/EDJ.2023.240685.2735

TOPICAL CHEMOTHERAPEUTIC EFFECT OF THYMOQUINONE, EPIGALLOCATECHIN-3-GALLATE, AND CURCUMIN ON CHEMICALLY INDUCED ORAL EPITHELIAL DYSPLASIA IN HAMSTERS

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ABSTRACT

This study aimed to compare the effects of Thymoquinone (TQ), Epigallocatechin gallate (EGCG) and Curcumin (CUR) on the inhibition of progression of oral premalignant lesions to invasive carcinoma through detection of PCNA and Caspase 3 expression. Thirty Syrian golden hamsters were divided into 5 groups (n=6). G.A: no treatment was given. The remaining hamsters were painted three times per week for six weeks with 0.5% DMBA. Then, they were subdivided into: G.B 1: did not receive any treatment till 12th week. G.B 2, 3, 4: were painted by TQ, EGCG, and CUR 3 times/week for another 6 weeks respectively. In G.B1; 100% of hamsters revealed OSCC. In G.B2 (TQ); 16.6% hamsters showed superficial invasion, 33.3% revealed carcinoma in situ and 50% showed severe dysplasia. In G.B3 (EGCG), 66.6% of hamsters showed severe dysplasia and 33.3% exhibited moderate dysplasia. In G.B4 (CUR), 66.6% hamsters showed well-differentiated OSCC, and 33.3% revealed carcinoma in situ. Significant decrease of PCNA immunoreactivity in GA, GB3, and GB2 (8.9 ± 2.2, 16.1 ± 2.4, and 32.5 ± 1.3 respectively) in comparison with G.B1 (53.7 ± 1.4). While GB4, showed statistically significant decrease (40.7 ± 0.8) in comparison to G.B1. Significant increase of Caspase 3 expression in GB3, GB2, GB4, and GA (120.3 ± 2.1, 100 ± 3.1, 70.5 ± 0.9, and 59.9 ± 11.7 respectively) in comparison with G.B1. Topical EGCG had a superior therapeutic effect than TQ, and CUR against the progression of oral premalignant lesions.

KEYWORDS: Oral squamous cell carcinoma, phytotherapy, thymoquinone, epigallocatechin gallate, curcumin.

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INTRODUCTION

Head and neck squamous cell carcinomas are the world's sixth most common cancer type. It is expected to increase by 30% by 2030.[1] The term "epithelial precursor lesion" is considered an altered epithelium with an increased likelihood of progression to squamous cell carcinoma. Its incidence correlates with exposure to tobacco or excessive alcohol intake. [2] An ideal phytochemical must be non-toxic, low cost, highly multi-site effective, as well as with known mechanism of action. [3] The bioactive component of the volatile oil of *Nigella Sativa L. seeds* is thymoquinone (TQ), also known as 2-isopropyl-5-methyl-1, 4-benzoquinone. It has outstanding activity against different carcinomas because of its negligible toxicity to normal cells. [4] Epigallocatechin-3-gallate (EGCG) is one of the four major phenols found in green tea. It allows the inhibition of cancer cell growth, survival, and metastasis. [5] It is considered a dose-dependent drug that allows the inhibition of invasion and migration of oral cancer due to marked decrease in the production of matrix metalloproteinase-2/9.[6] The natural source of curcumin (CUR) is Curcuma longa or turmeric. CUR exhibits a significant inhibitory growth effect on oral cancer cell lines. It has anti-inflammatory, antioxidant, anticancer, anti-ischemic, antifungal, and antibacterial effects. [7,8] One of the hallmarks of tumor progression is altered rates of cell proliferation through detection of proliferating cell nuclear antigen (PCNA). Also, dysregulation of apoptosis was found to be involved in the pathogenesis of cancer.[9] The goal of this research was to inhibit the progression of premalignant lesions to invasive carcinoma by using phytotherapeutic agents.

MATERIAL AND METHODS

Ethical statement: The present study was carried out after the approval of Research Ethics Committee (REC) number 551/2022, Faculty of Dentistry, Suez Canal University, Ismailia, Egypt. The methodology for this study was described in compliance with the ARRIVE (Animal research: Reporting in vivo experiments) criteria for animal research reporting. [10]

AIM OF THE STUDY

In this work, the chemopreventive effects of thymoquinone, epigallocatechin-3-gallate, and curcumin on chemically induced oral epithelial dysplasia in hamsters were compared histologically and immunohistochemically.

Study setting: This study was carried out at the animal house of Faculty of Dentistry, Suez Canal University (Ismailia, Egypt). The histological and immunohistochemical section of the study was conducted in the Oral Pathology Department of the Faculty of Dentistry, Suez Canal University.

Study design: It is a comparative animal experiment; 30 Syrian golden male hamsters were divided into five groups where each group had 6 hamsters. The groups were negative control, positive control and three treated groups; according to materials used in treatment of oral epithelial dysplasia and these materials were tested after one time interval (six weeks).

Sample size: Sample size calculation was done utilizing G*Power version 3.1.9.2.[11] The effect size was 1.25 using alpha (α) level of 0.05 and Beta (β) level of 0.05, i.e., power = 95%; the estimated minimum sample size (n) was a add up to 30 samples, 6 samples for each group (table 1).

TABLE (1) Sample size calculation

Analysis:	A priori: Compute required sample size	
Input:	Tail(s)	One
	Effect size d	1.25
	α err prob	0.05
	Power (1-β err prob)	0.05
Output:	Non-centrality parameter δ	3.58
	Critical t	1.73
	Sample size	6.0
	Total sample size	30.0
	Actual power	95%

Inclusion and exclusion criteria: Inclusion criteria consisted of healthy Syrian golden male hamsters with an approximate weight of 80–125 gram and with normal buccal pouches without wounds, inflammation, or any lesions. Any hamsters showing systemic illness, diarrhea, traumatic injury, infections, was excluded. All animals were purchased from VACSERA, Helwan, Cairo, Egypt

Housing and husbandry: Standard cages were used to keep hamsters with sawdust bedding (three animals per cage) at a controlled room temperature (20±2°C), humidity (30-40%), and light (12 h light/12 h dark cycles). Hamsters were given feed and water *ad libitum*.

Randomization and Allocation Concealment: The randomization plan was created by the pathologist (R.E) who was not involved in the treatment procedures. Each hamster was provided with a number from 1 to 30. The treatment assignments were held in opaque sealed envelopes. After the animals were painted with carcinogenic material for six weeks, the envelopes were opened, and the treatment assignment was disclosed to the pathologist (M.E).

Experimental procedures: In the present study, 30 hamsters were divided into 5 groups, each group had 6 hamsters. G.A: (control group): no treatment was given to animals. G.B: 24 animals were painted with 0.5% DMBA (3 times per week for six weeks) in their left buccal pouches using a number 4 camel hairbrush. After that, they were subdivided into 4 subgroups: G.B 1: did not receive any type of treatment or painting till the end of the experiment. G.B 2: was painted by TQ solution/ 3 times per week for six weeks. G.B 3: was painted by EGCG solution/ 3 times per week for six weeks. G.B 4: was painted by CUR solution/ 3 times per week for six weeks.

Clinical evaluation: Clinical features were observed and recorded during the experiment as white and red lesions, exophytic masses and pouch necrosis.

Euthanasia: The hamsters were anesthetized and then euthanized with carbon dioxide (CO2)

Biopsy collection: To remove the buccal pouches, a cut was made behind the left ear. From the mouth, a blunt forceps was inserted into the pouch's depth. The fascia and muscles surrounding the pouch were cut using precise scissors to release it. Towards the oral cavity, a piece of gauze was rolled, dipped in neutral formalin, and inserted into the pouch from the skin side. To eliminate food that had been stored inside the excised pouches, saline was applied to the pouches. Samples were fixed in 10% neutral buffered formalin for 24 hours prepared into paraffin blocks for routine haematoxylin and eosin (H&E) staining, PCNA immunohistochemistry, and caspase 3 immunohistochemistry.

Histopathological evaluation (H&E stain): By microtome, 5μm serial sections were obtained and stained with H&E. Examination was done by a light microscope and images were taken by E-330 Digital Photography camera using Olympus BX50 Microscope.

Immunohistochemical evaluation (IHC): Tissue sections of 5µm thickness were cut to demonstrate PCNA (AB-clonal, Cat No. A0264) and caspase 3 (AB-clonal, Cat No. A11953) antibodies expression using the usual labelled streptavidin-biotin technique. Dewaxed paraffin-embedded tissue sections were rehydrated in distilled water after being dewaxed in graded ethanol.

Sections were treated with primary anti-PCNA antibody (PC-10, diluted 1:80, USA) for 18 hours, incubation in secondary serum, incubation in streptoavidin-biotin complex for 30 minutes at room temperature, and development of the reaction with diaminobenzidine for the PCNA protein after being washed in phosphate-buffered saline. The material was submerged in a pH 7.4 trisphosphate buffer solution (trisphosphate buffer solution) between stages. Sections were cleaned and counterstained with Mayers hematoxylin after the reaction had developed.

Sections were washed in phosphate-buffered saline for 5 min in order to remove the caspase 3 protein. Antigen retrieval was carried out in a pressure cooker at 125°C for 10 minutes while immersed in Tris-EDTA (10 mM, pH 9.0). With 3% hydrogen peroxide in methanol, endogenous peroxidase activity was stopped for 30 minutes. The anti-caspase-3 antibody for the cleaved form (dilution 1:100) and the anti-caspase-3 antibody in Tris-buffered saline solution with 5% bovine serum albumin were incubated overnight on the slides at 4°C in a humid environment. The sections were counterstained with hematoxylin after the color was developed for 2 minutes at room temperature with a solution of 0.03% diaminobenzidine.All data were analyzed through using Image analyzer computer system (image J / Fiji 1.46) by digitizing the slides under 400X objective magnification. The analysis was performed through using the fraction of the positive cells by counting the number of immunopositive cells in relation to the number of the unstained cells.

Blinding: The first investigator (M.E.) applied materials while being blinded to the allocation of hamsters and materials. The first investigator (M.E.) did not take part in either the immunohistochemical analysis or the histological examination. The histological and immunohistochemical investigation of the tissue was carried out by the other pathologists (R.E. and M.S.) according to a blinded methodology.

Statistical analysis: The statistical analysis was performed for comparison between different Treatments. The data were collected, checked, revised, and organized using Microsoft Excel 356. Data were subjected to outliers' detections and normality statistical tests to detect whether the data were parametric or nonparametric. Utilizing one way analysis of variance (ANOVA), comparisons between more than two independent groups with quantitative data were made. at significance levels of 0.05 followed by Duncan multiple range tests (DMRTs) as a post hoc test to further compare between treatment groups. Data analyses were carried out using computer software Statistical Package for Social Science SPSS (IBM-SPSS ver. 26.0 for Mac OS).

Materials:

The carcinogen, DMBA (7,12-Dimethylbenz-(a)-anthracene) was purchased from Sigma-Aldrich Company, Saint Louiss, USA. It was dissolved in heavy mineral oil (U.S.P) to get 0.5% DMBA solution. Thymoquinone (Sigma-Aldrich Company, Saint Louiss, USA) was dissolved in propylene glycol (0.01 mg/kg) and the mixture was stirred by a magnetic stirrer until a uniform consistency was achieved.[12] EGCG was prepared by dissolving EGCG with polyethene glycol hydrogel base with 90 ppm concentration that was purchased from Nawah Scientific laboratory, Al-mokattam St., Cairo, Egypt.[13] Curcumin powder was dissolved in propylene glycol (15 mg/kg) with tween 80 (Sigma-Aldrich Company, Saint Louiss, USA).[14]

RESULTS

Clinical findings (table 2)

The control group (G.A) showed normal pink color with no inflammatory or pathological changes. All hamsters in the positive control group (G.B1) revealed scattered large exophytic masses with lobulated smooth surfaces (100%), the rest of the pouches showed white and red lesions (i.e., leukoplakia and erythroplakia). G.B2 (TQ) exhibited exophytic masses in 5 hamsters (83.3%) while one hamster (16.6%) showed only red patches. G.B3 group (EGCG) revealed small exophytic masses in 4 hamsters (66.6%), while 2 hamsters (33.3%) showed white and red patches without masses. G.B 4 group (CUR) showed scattered exophytic masses with different sizes in all hamsters (100%). (Fig. 1)

Histopathological findings (table 2)

All hamsters in G.A as shown in figure 2 revealed normal mucosa composed of four distinct layers of keratinized stratified squamous epithelium without rete ridges. The subepithelial layer consisted of loose

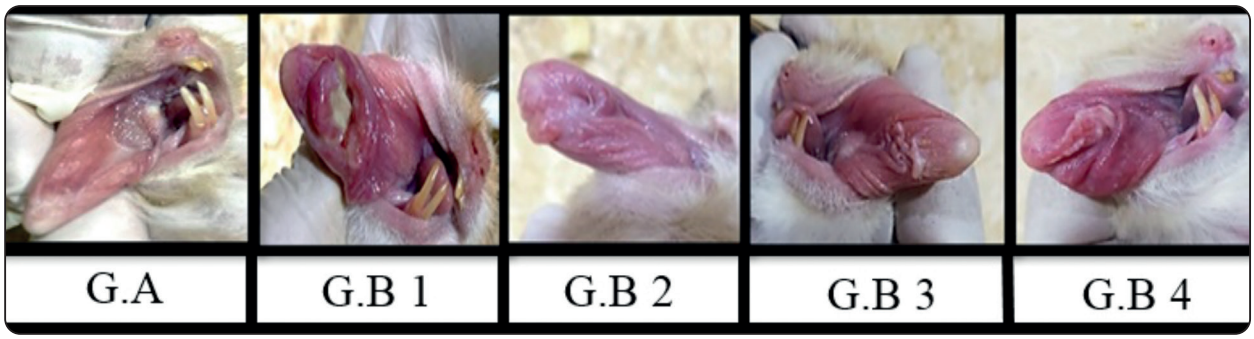

Fig. (1) Gross pictures of hamster pouches in different groups. G.A (control group) showed no pathological lesions. G.B 1 (DMBA group) revealed large exophytic masses with lobulated smooth surfaces. G.B 2 (TQ group) exhibited exophytic masses. G.B 3 (EGCG group) revealed small exophytic masses. G.B 4 (CUR group) showed scattered exophytic masses with different sizes in all hamsters.

fibrous connective tissue and muscular layer (100%). In G.B1, all hamsters revealed well to moderate differentiated OSCC in the form of numerous papillomatous overgrowths with dysplastic criteria besides invasion of the malignant epithelial nests. The rest of the pouches' lining showed moderate to severe dysplasia (100%). In G.B2 (TQ), hamsters showed small papillomatous overgrowths, one of them showed superficial invasion limited the nodule (16.6%), two of them revealed carcinoma in situ with no invasion into the underlying connective tissue (33.3%) and the rest showed severe dysplasia (50%). In G.B3 (EGCG), four hamsters showed small papillomatous overgrowths with severe dysplasia (66.6%) and two hamsters exhibited only moderate dysplasia with no invasion into the underlying connective tissue (33.3%). In G.B4 (CUR), four hamsters showed well-differentiated OSCC with different papillomatous overgrowths (66.6%) and two hamsters revealed carcinoma in situ (33.3%) as shown in (Table 2).

Immunohistochemical findings (IHC)

The immunoreactivity of PCNA nuclear proliferative marker in G.B1 treated with DMBA only showed intense nuclear immunoreactivity. Quantitative analysis revealed highly statistically significant decrease of PCNA immunoreactivity in GA, GB3, and GB2 respectively (8.9 ±2.2, 16.1±2.4, and 32.5±1.3 respectively) in comparison with G.B1 (53.7±1.4). While GB4, only showed statistically significant decrease (40.7±0.8) in comparison to G.B1. (Figure 3 and 5)

Caspase-3 positive immunoreactivity could be observed as brown cytoplasmic expression of epithelial cells. Weak expression was exhibited in G.B1 throughout the epithelial layers and invading tumor cells (31.6±1.8). Quantitative analysis showed a highly statistically significant increase of Caspase 3 expression in GB3, GB2, GB4, and GA respectively (120.3 ± 2.1, 100 ± 3.1, 70.5 ± 0.9, and 59.9±11.7 respectively) in comparison with G.B1. (Figure 4 and 5).

TABLE (2) Gross observations and histological results in various groups:

Groups	Exophytic masses	Red / white lesions	No lesions	SCC	CIS	Severe dysplasia	Moderate dysplasia
G. A	-	-	100%	-	-	-	-
G. B1	100%	-	-	100%	-	-	-
G. B2	83.3%	16.6%	-	16.6%	33.3%	50%	-
G. B3	66.6%	33.3%	-	-	-	66.6%	33.3%
G. B4	100%	-	-	66.6%	33.3%	-	-

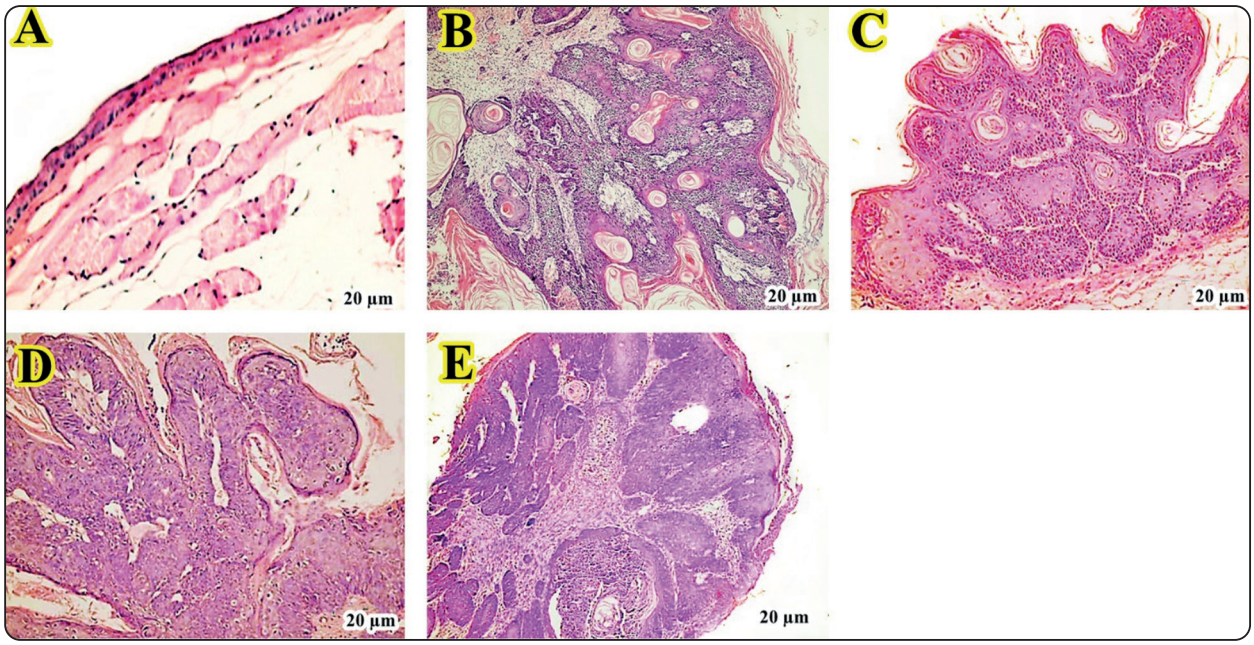

Fig (2) Error! Main Document Only.: Photomicrographs showing histological sections in various groups. (A) Negative control showing normal hamster pouch lining of stratified squamous epithelium. (B) positive control showing well differentiated OSCC. (C) TQ-treated group, showing small papillomatous overgrowths with severe dysplasia and carcinoma in situ. (D) EGCG-treated group, showing moderate to severe dysplasia within papillomatous overgrowths. (E) CUR-treated group showing multiple lesions of well differentiated OSCC.

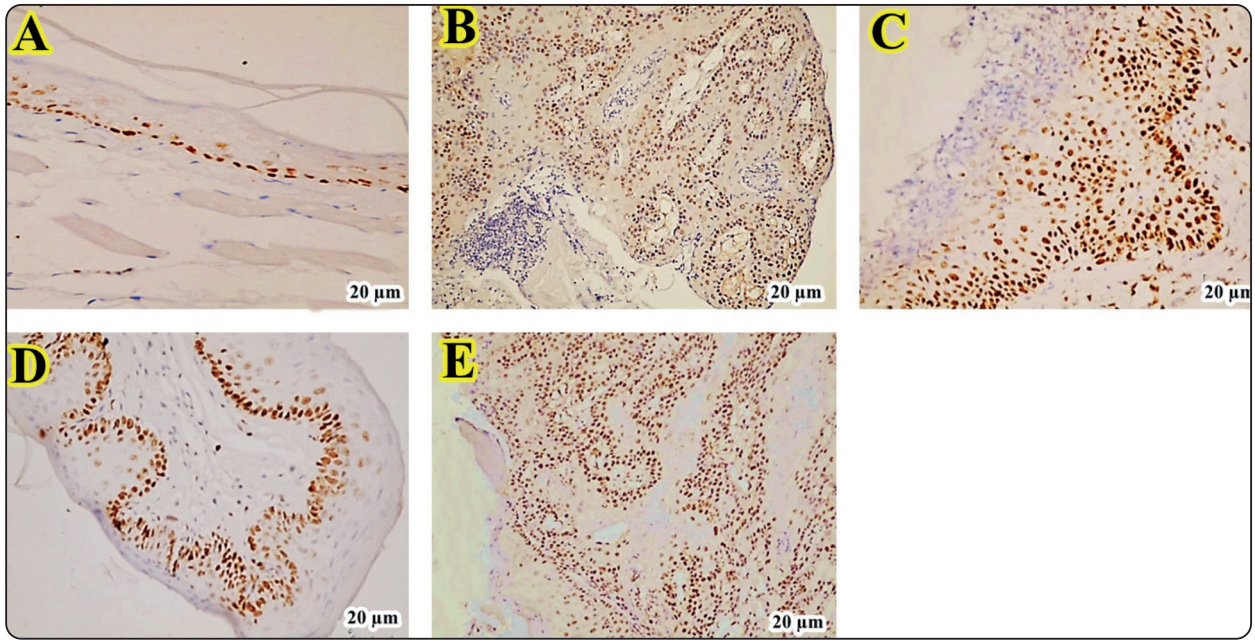

Fig. (3): Photomicrographs showing immunohistochemical expression in various groups. (A) Negative control showing significant decrease in PCNA expression. (B) positive control with strong expression to PCNA in all epithelial layers. (C) TQ-treated group showed expression of PCNA limited to basal and suprabasal cell layer. (D) EGCG-treated group, showing weak nuclear expression of PCNA limited to basal layer. (D) CUR-treated group showing positive expression of PCNA in all epithelial layers.

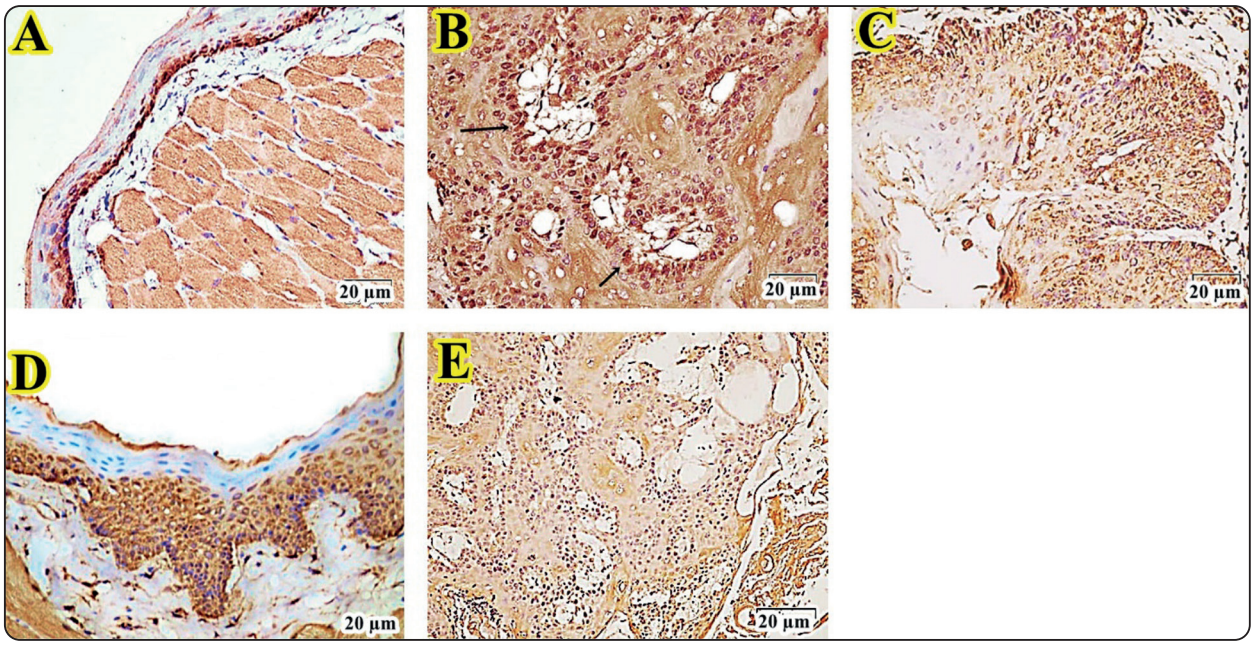

Fig. (4): Photomicrographs showing immunohistochemical expression in various groups. (A) Negative control showing normal hamster pouch lining with significant increase in caspase 3 expression. (B) positive control showing with weak expression of caspase 3 in all epithelial layers. (C) TQ-treated group, showing positive caspase 3 expression in all layers. (D) EGCG-treated group, showing strong positive expression of caspase 3. (E) CUR-treated group showing moderate expression of caspase 3.

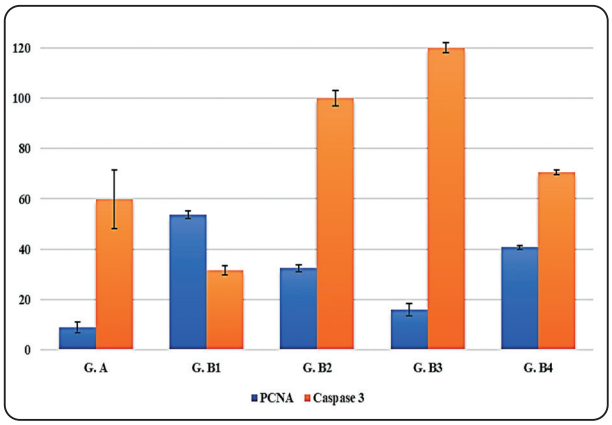

Fig 5: Representing values of PCNA and caspase 3 expression among various groups

DISCUSSION

Oral squamous cell carcinoma is one of the most common malignant tumors that can seriously affect life quality. [15] Conventional surgery and radiotherapy treatments are less successful for patients with advanced cancer. There is still a great need to reduce morbidity and mortality by improving early detection and treatment.[16] In recent years, many studies highlighted the role of phytotherapy against precancerous lesions and malignant tumors.[3] Utilizing natural materials has been deemed promising because to their lower dose and minimal toxicity profiles due to the prolonged period of oral pathogenesis.[17] Several studies were exhibited the antitumor properties of Thymoquinone (TQ),[12] Epigallocatechin-3-gallate (EGCG) [18], and curcumin (CUR) against OSCC. [19]

The present study was aimed to compare the therapeutic effect of TQ, EGCG, and CUR in DMBA-induced oral carcinogenesis. DMBA carcinogen is commonly used to induce OSCC in hamsters due to the similarities between humans and hamsters during cancer progression. [20] Clinical outcomes and histopathological findings of well to moderate OSCC were comparable to our other similar studies. [21,22]

The topical drug delivery system was our choice. It is the preferred method for delivering

medicinal chemicals locally since it is convenient and affordable.[23] In this hopeful work, after induction of oral dysplasia by DMBA, the best results were reported on EGCG, TQ, and CUR groups respectively. All these data were parallel to many previous studies that confirmed the chemo-preventive and chemo-therapeutic effect of these phytochemicals against oral carcinogenesis in experimental models. [20,24] Saleh *et al.,* detected that CUR had a less significant chemo-preventive effect against oral carcinogenesis in comparison with green tea. Moreover, the combination of both phytotherapeutic agents had a better result.[24] In contrast to our study, Sonavane *et al*.[25], reported that CUR treatment resulted in significant and complete inhibition of skin cancer but in dual roots of administrations. Our frustrating results in this group may be due to little CUR concentration in the prepared drug.

In parallel to H&E results, immunohistochemical studies using anti-PCNA antibodies are considered as a prognostic and predictive marker to assess biopsies that have dysplastic criteria. Its expression increases with the severity of oral dysplasia and the grading of oral squamous cell carcinoma. [26] In our study, there was PCNA up-regulation with DMBA group and PCNA down-regulation with EGCG-treated group. Zhang *et al*. [27] reported that EGCG dramatically decreases cell proliferation through reduced PCNA expression in uterine leiomyoma both in vitro and in vivo. In our proven data, TQ showed a moderate effect on PCNA inhibition, whereas TQ has a potent anti-proliferative activity by regulating PCNA expression in other studies. [28,29] on the other hand, CUR in this study has a lower effect on PCNA inhibition, as usual in other studies inverse our data. [30,31]

Online with the present results, oral carcinogenesis, and advanced tumor stages were all linked to decreased caspase 3 expression. [32,33] Roychoudhury *et al*., [34] ensured the effect of EGCG in apoptosis induction through mitochondrial pathway and caspase 3 activation. Islam *et al*., [35] improved the strong inhibitory effects of EGCG on the proliferation and viability of HTB-94 human chondrosarcoma cells and induction of apoptosis through caspase-3 activation. TQ proved to be effective to some extent in inhibition of cellular proliferation based on caspase 3 apoptotic pathway, which was proved in other studies. [36,37] In our study, CUR has the least effect on caspase 3 activation. Kang et al., in contrast to our findings, demonstrated that curcumin causes histone hypoacetylation in brain cancer cells before causing apoptotic cell death via a (PARP)- and caspase 3-mediated mechanism. [38,39]

CONCLUSIONS

Topical administration of EGCG was found to be superior to both TQ, and CUR in induction of apoptosis, and inhibition of tumor cell proliferation in DMBA- induced oral carcinogenesis in hamsters.

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