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Effect Of Thymoquinone-Loaded Chitosan Nanoparticles On Chemically- Induced Epithelial Dysplasia In Hamster Pouch

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Abstract:
Background*: Oral epithelial dysplasia has a high risk for proceeding into squamous cell carcinoma (SCC) which accounts for 90% of all oral cancers. Thymoquinone (TQ) is a bioactive phytochemical with antineoplastic properties. This study aims to evaluate the effect of loading TQ on chitosan nanoparticles (CS NPs) in treating oral epithelial dysplastic lesions induced in hamster buccal pouch.*
Materials and Methods*: This study was carried out on fifty-five male Syrian golden hamsters divided into 7 groups. Group A (negative control) 5 untreated hamsters euthanized at the end of the 6th week of the experiment. The remaining 50 animals were painted with 7,12-dimethylbenz-(a)-anthracene (DMBA) for 6 weeks then divided into 6 groups. Group BI: 5hamsters euthanized at end of the 6th week, group BII: 5 hamsters left without further treatment for 6 weeks and euthanized at end of the 12th weeks. The remaining hamsters were assigned into 4 groups (C,D,E and F) treated with TQ, CS NPs, TQ-CS-NPs (0.1 mg/kg) and TQ-CS-NPs (0.01 mg/kg) respectively for 6 weeks and euthanized at the end of the 12th week.The left buccal pouches of all animals were surgically removed and processed for H&E and Bcl-2 immunohistochemical (IHC) staining.*
Results*: Loading thymoquinone (TQ) on chitosan nanoparticles (CS-NPs) had improved antitumor activity of TQ with best results obtained from TQ-CS-NPs with lower concentration (0.01 mg/kg) that ends with only focal mild epithelial dysplastic changes with a significant decrease in Bcl-2 expression.*
Conclusion:*TQ-CS-NPs is a promising remedy for oral epithelial dysplasia. It successfully prevented the progression of induced epithelial dysplasia toOSCC anddecreased Bcl-2 expression.*
Keyword*: Bcl-2, chitosan nanoparticles, epithelial dysplasia,Oralsquamous cell carcinoma,thymoquinone.*

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I. Introduction

Oral squamous cell carcinoma is usually preceded by mucosal precancerous changes that might be visible as white (leukoplakia) or red (erythroplakia) lesions, but are mostly not macroscopically visible, which explains that most OSCC seem to develop de novo. However, the preceding precancerous changes can present under the microscope as abnormal mucosal epithelium, indicated as dysplasia [1]. Severe epithelial dysplasia has an overall malignant transformation rate of about 7–50%, moderate dysplasias have a malignant transformation potential of 3–15%, whereas mild epithelial dysplasia shows a very low risk (<5%) [2]. OSCC is the most common oral carcinoma with varied clinical presentations, it accounts for more than 90% of all malignant lesions in the oral cavity [3]. Despite the improvement in diagnostic techniques, as well as new therapies and protocols, the 5-year survival rate remains around 50–60% [4]. Oral cancer is the sixth most common malignancy worldwide. Globally, over 400,000 estimated new cases of oral cancer are diagnosed each year [5].

DMBA-induced hamster buccal pouch (HBP) carcinogenesis model is the most accepted model to study the histopathological and biochemical alterations in oral carcinogenesis. Cancer in HBP shows a highly reproducible sequence from dysplastic changes to SCC [6,7].

Thymoquinone (TQ) is a bioactive phytochemical, obtained from black seed (Nigella sativa), that has beenshown to have multifunction properties**,** including antimicrobial, anti-inflammatory, anti-allergic, anti-diabetic, antioxidant, and anti-neoplastic properties [8,9].The main drawbacks of TQ that decline its effect are poor bioavailability and hydrophobicity. Hence, various nano-drug delivery carriers such ascompatible lipids and polymers' NPs have been used with TQ to enhance its targeting effect [10].

Chitosan (CS)is an abundant natural polysaccharide extracted from chitin. It has several favorable biological characteristics such as biodegradability, non-toxicity, and biocompatibility. Most importantly, chitosan nanoparticles (CS NPs) can increase the cellular uptake of loaded drugs due to their positive surface

charge which can enhance the interaction with a negatively charged mucus membrane surface. Another advantage of CS NPs is their enhancement of the mucosal permeation of hydrophobic drugs and promotion of the drugs' retention in the mucosa [11,12].

B-cell lymphoma -2 (Bcl-2) family includes both anti- and pro-apoptotic members. The pro-apoptotic proteins are Bak and Bax, while the anti-apoptotic proteins comprise Bcl-2 and Bcl-XL [13]. Bcl-2 is normally expressed in stem cell zones like the basal layers, where it works to prevent death of cells in the regenerative compartment [14]. Increased expression of the Bcl-2 gene's protein product appears in the early carcinogenesis, causing apoptosis impairment and, as a result, the propagation of neoplastic changes [15].

Considering the above-mentionedinformation, thisstudy is a novel study intended to evaluate the effect of loading thymoquinone on chitosan nanoparticles and its application topically in treatment of oral epithelial dysplastic lesions induced in the hamster buccal pouch by application of DMBA through histological and Bcl-2 immunohistochemical expression.

II. Material And Methods

Approval of the Ethics Committee of Scientific Research - Faculty of Dentistry - Suez Canal University - has been obtained before starting the search (377/2021).
This study was performed in the Animal House, Faculty of Dentistry, Suez Canal University.

Chemicals and reagents:

Chemicals used in this study included;Thymoquinone (Sigma Aldrich Chemical Company, Saint Louis, USA, Cat. No, 490-91-5). Propylene glycol (Loba Chemie, India, Cat. No, 57-55-6). Chitosan (CS) (Degree of deacetylation: 92%, Molecular weight: 161.61 KDa) (Oxford Lab Fine Chem Llp, Vasai, Maharashtra, India). Acetic acid (Chemajet Chemical Company, Alexandria, Egypt, Cat. No. 8012-89-3). Tripolyphosphate (TPP) (Sigma Aldrich Chemical Company, Saint Louis, USA, Cat No, 7758-29-4). 6-7,12-Dimethylbenz-(a)-anthracene (DMBA) (Sigma Chemical Company, Saint Louis, Mo, USA, Cat. No. D3254). Mineral oil (Loba Chemie, India, Cat. No, 8012-95-1). Bcl-2 antibody (Gene Tex International Corporation, Cat. No, GTX100064). Mouse/Rabbit PolyDetector DAB HRP Brown Detection System (Bio SB, USA, Cat. No, BSB 0201S).

Drug Synthesis:

Preparation of chitosan nanoparticles:

Half gram of chitosan was dissolved in 35 ml distilled water containing 2% ml glacial acetic acid and stirred on a magnetic stirrer for 30 minutes then, 15 ml sodium tripolyphosphate (STPP) solution was added. The chitosan solution was magnetically stirred for 45 minutes. Chitosan nanoparticles were centrifuged using a cooling centrifuge at 4˚C for 20 mins at 4500 rpm to collect nanoparticles` pellets.

Preparation of TQ solution:

Thymoquinone was dissolved in propylene glycol to get a concentration of 0.1 mg/kg body weight, by a magnetic stirrer.

Preparation of TQ-loaded CS NPs:

Nanoparticles were prepared using the ionic gelation (IG) method according to *Alam et al.,* [16].

Characterization of nanoparticles:

UV Spectrophotometer analysis of free thymoquinone:

The weight of free TQ in the supernatant of TQ-CS NPs was quantified by using a T90+UV-VIS Spectrometer. To calculate encapsulation efficiency and drug loadin capacity.

Fourier transform infrared spectroscopy (FTIR):

Bruker Alpha II FTIR spectrometer (Bruker Corp., Massachusetts, USA).was used to confirm the synthesis of CS NPs.

Dynamic light scattering (DLS):

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential measurements were performed at room temperature by Malvern Zetasize Nano-zs90.

Transmission electron microscopy (TEM):

The prepared TQ-CS NPs were examined by TEM to characterize their microstructure. The images were recorded using JEOL JEM-2100 Electron Microscope.

The Experimental Study:

Carcinogen:

The chemical carcinogen (DMBA) was dissolved in heavy mineral oil using a magnetic stirrer to get a solution of 0.5% concentration. The carcinogen was topically painted to the hamster buccal pouch (HBP) of Syrian golden hamsters (*(Mesocricetus auratus)* by using a number (4) camel hairbrush.

Experimental design and animal grouping:

The study was carried out on fifty-five male Syrian golden hamsters weighing 90-100 grams (with 10% more hamsters to compensate for the possible mortalities), The hamsters were purchased from the Holding Company for Biological Products and Vaccines (VACSERA), Helwan, Cairo, Egypt.The animals were housed in groups of five in a well-ventilated room with controlled temperature, 50-70% humidity, and a 12-hour day/night cycle. All animals were given water and recommended diet, *ad libitum*.

The hamsters were divided into six (6) groups as follows: **Group A** (5 animals) did not receive any treatment and served as the negative control group, which were euthanized at the end of the 6^{th} week. **Group B** (10 animals) served as the positive control group. The left buccal pouches were painted with DMBA 3 times/week/6 weeks then, the hamsters were subdivided into two groups as follows: Group BI (5 animals) were euthanized at the end of the 6^{th} week. Group BII (5 animals) were left without further treatment after DMBA application and were euthanized at the end of the 12^{th} week.

All the following groups were treated as group B, then treated with different drugsas follows: **Group C** (10 animals) were painted with TQ 0.1 mg/kg 3 times/week/6 weeks. **Group D** (10 animals) were painted with CS NPs 0.235 mg/kg 3 times/week/6 weeks.**Group E** (10 animals) were painted with TQ-CS-NPs 0.1 mg/kg 3 times/week/6 weeks.**Group F** (10 animals) were painted with TQ-CS-NPs 0.01 mg/kg 3 times/week/6 weeks.

All animals were euthanized the at end of the 12^{th} week (except group A and group BI). The left buccal pouches were surgically removed for H&E and Bcl-2 immunohistochemical evaluation.

Histopathological evaluation: Leftpouches of all animals were surgically excised, fixed in 10% neutral formalin solution, embedded in soft paraffin wax, sectioned serially into 5µm, and routinely stained with H&E. Grading of epithelial dysplasia (OED) was done according to Dakhakhny& Aziz [17].

Bcl-2 immunohistochemical staining procedures: From each paraffin block, 5 µm thick sections were cut and mounted on positively charged slides for Bcl-2 immuno-histochemical staining as following manufacturers' instructions. Regarding the analysis of Bcl-2 expression, Bcl-2 is expressed as a granular cytoplasmic brownish stain. The immunohistochemical stain intensity was quantitatively evaluated using image analysis software (Image J, 1.41a, NIH, USA) at a magnification of X40.

Statistical analysis: Data was subjected to outliers' detections and normality statistical tests to detect whether the data were parametric or nonparametric. Data was analyzed for descriptive statistics both graphical and numerical descriptions. Inferential statistics for evaluating and comparing different treatments were performed by one-way analysis of variance (ANOVA) at significance levels of 0.05. ANOVA was followed by Duncan multiple range tests (DMRTs) as a post hoc test to further compare treatment groups then, a comparison between groups E & F was done with an independent T-test. Data analyses were carried out using the computer software Statistical Package for Social Science SPSS (IBM-SPSS ver. 26.0 for Mac (OS)[18].

III.Results

A. Chemical and physical characterization of the prepared NPs:

Encapsulation efficiency and loading capacity:

The weight of free TQ in the supernatant was 1±0.01mg/ml obtained by UV spectrophotometer analysis. The total free TQ in the supernatant was 42.5±0.42 mg. While added TQ during preparation was 200 mg and the total weight of the lyophilized NPs was 527.5 mg. Hence, the encapsulation efficiency (EE) and drug loading capacity (DLC) of TQ were calculated according to the following equation [16].

Encapsulation efficiency (EE%) = [(A− B)/ A] x 100

= [(200− 42.5)/ 200] x 100 =78.75±0.21.

Drug loading capacity (DLC%) = [(A− B)/ C] x 100 =29.85±0.08.

= [(200− 42.5)/ 527] x 100 =29.85±0.08

"A" is the weight of TQ used for preparation, "B" is the free TQ calculated in the supernatant, and "C" is the total weight of the *lyph olized* NPs.

After calculating DLC the dose of CS NPs was calculated.

Fourier transform infrared spectroscopy (FTIR):

FTIR was performed to confirm formation of chitosan nanoparticles.**(Fig. 1A)** The peak at 3020.05 cm−1 is the O-H stretch, which overlapped with the N-H_2 stretch from the primary amine groups of CS NPs. The peak at 1650.34 cm−1 was C=O, while the peak at 1548.04cm−1 was C=C. The peak at 1080.05 cm−1was P=O which proved the reaction of inorganic phosphate groups from sodium tripolyphosphate (STPP) with CS. The presence of OH, NH2, and P=O absorption bands confirmed that CS NPs were successfully formed. The appearance of absorption bands at 1548.04 cm−1 and 1650.34cm−1 that are assigned to the C=C and amide II functional groups, respectively, proved the formation of TQ - CS NPs.

Dynamic light scattering (DLS):

The mean zeta potential for TQ-CS NPs was found to be 13.5±4.36mV. (**Fig. 1B**)The mean hydrodynamic particle size for the TQ-CS NPs was found to be603.9±106.1 nm, while the polydispersity index (PdI) was found to be 0.338. (**Fig. 1C**)

Transmission electron microscope (TEM):

TEM images of the prepared TQ-CS NPs showed spherical particles with an average diameter of 15.61±6.2 nm. (**Fig. 1D**)

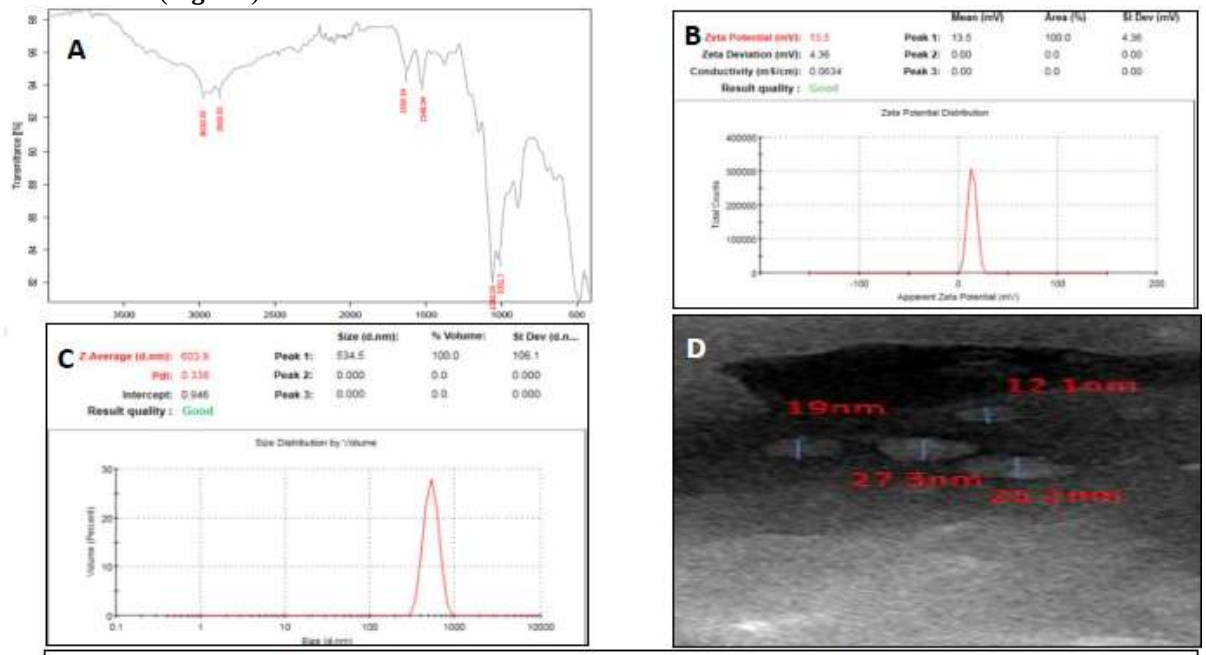

Figure 1: Results of drug characterization (A) FTIR spectra of TQ-CS NPs. **(B)** Zeta potential of TQ-CS-NPs. **(C)** Particle size distribution curve of TQ-CS NPs. **(D)** TEM image of TQ-CS NPs.

Clinical findings: Animals in **group A** were healthy and active. (**Fig. 2A**) Animals in **group BI** in the 2nd week showed extensive necrotic material and hair loss in the left perioral area. (**Fig. 2B**) Animals showed skin ulcers and abscesses formation at the 4th week, while in animals in **group BII** at the 7th and 8th weeks, the hair loss was extended to the abdomen and the right side of the body in some hamsters with skin eruptions, **(Fig. 2c)** animals were severely debilitated, inactive, and had severe weight loss.Animals in **group C**, after treatment with TQ, animals were moderately debilitated, hair loss was limited to the perioral area. (**Fig.2D**) Animals in **group D** were as severely debilitated as those in group BII, while animals in **group E** were in good health with moderate hair recovery, (**Fig. 2E**) no skin ulcers or eruptions, and finally animals in **group F**, after treatment with TQ CSNPs, animals were in a good health with marked hair recovery, **(Fig. 2F)** no skin ulcers or eruptions.

Histopathological (H&E) and Bcl-2 immunohistochemical (IHC) results:

Group AH&E sections showed normal buccal mucosa,(**Fig.3A**) with mild positive Bcl-2 expression confined to the basal cell layer. (**Fig.3B**)

Group BI (DMBA 6 weeks) H&E sections showed severe dysplastic epithelial changes with focal areas of carcinoma in situ (CIS) and superficial invasion of the underlying connective tissue.(**Fig 4A**)The epithelium showed hyperplasia, hyper-keratinization, drop-shaped rete ridges, loss of polarity and hyperplasia of the basal cell layer, individual cell keratinization, cellular & nuclear pleomorphism, prominent nucleoli, and increased normal and abnormal mitosis. The underlying connective tissue showed moderate chronic inflammatory cellular infiltration and dilated blood vessels, with positive cytoplasmic Bcl-2 expression extended all over the epithelial thickness. (**Fig 5A**)

Group BII (DMBA 6 weeks then, 6 weeks with no treatment) H&E sections showed areas of papillomatous overgrowths represented well/moderately differentiated squamous cell carcinoma with islands of dysplastic cells invading the underlying connective tissue. (**Fig 4B**) The rest of the pouches showed severe dysplastic epithelium. The underlying connective tissue showed severe chronic inflammatory cellular infiltration and dilated blood vessels, with positive cytoplasmic Bcl-2 expression all over the epithelial thickness and the islands invading the connective tissue. (**Fig 5B**)

Group C (TQ 0.1 mg/kg) H&E sections showed areas of epithelial hyperplasia and hyper-keratinization, severe dysplastic changes with focal areas of carcinoma in situ. (**Fig 4C**) The underlying connective tissue showed moderate chronic inflammatory cellular infiltrate and dilated blood vessels, with positive Bcl-2 expression all over the epithelial thickness. (**Fig 5C**)

Group D (CS-NPs) H&E section revealed focal papillomatous overgrowths represented well/moderately differentiated squamous cell carcinoma with invasive islands in the connective tissue, formed of dysplastic squamous cells. (**Fig 4D**) The rest of the pouches' lining showed severe dysplasia with epithelial hyperplasia and hyper-keratinization. The underlying connective tissue showed moderate chronic inflammatory cellular infiltration and dilated blood vessels, with positive Bcl-2 expression all over the epithelial thickness and the epithelial islands invading the connective tissue. (**Fig 5D**)

Group E (TQ-CS-NPs 0.1 mg\kg) H&E sections revealed hyper-keratinization, and moderate to severe dysplastic changes with loss of polarity and hyperplasia of the basal cell layer, hyperchromatism, and prominent nucleoli. The underlying connective tissue showed mild to moderate chronic inflammatory cellular infiltrate,**(Fig 4E)**with moderate positive Bcl-2 expression all over the epithelial thickness. (**Fig 5E**)

Group F (TQ-CS-NPs 0.01 mg\kg) revealed H&E sections showed mild to moderate dysplastic changes in the form of loss of polarity and hyperplasia of the basal cell layer, nuclear pleomorphism, nuclear hyperchromatism and prominent nucleoli. The underlying connective tissue showed mild chronic inflammatory cellular infiltration, (**Fig 4F**)with mild positive Bcl-2 expression all over the thickness of the epithelium. (**Fig 5F**)

Thymoquinone -loaded chitosan nanoparticles were fabricated to investigate their topical effect on chemically induced epithelial dysplasia through histological and Bcl-2 immunohistochemical expression analysis. Best results were found in group F (TQ CS-NPs 0.01 mg/kg) which showed mild to moderate epithelial dysplasia and mild positive Bcl-2 expression.

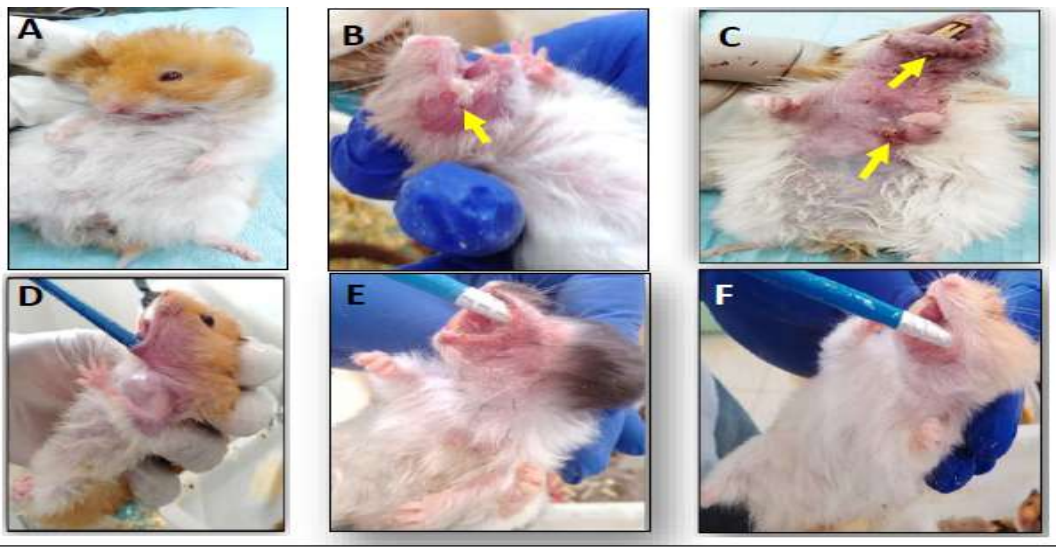

Figure 2: Clinical findings in different groups (A) Normal hamster. (B) Necrotic material (arrow) and hair loss in group BI. (C) Extensive hair loss & skin eruptions (arrows) in group BII. (D) Hair loss in group C. (E) Moderate hair recovery in group E. (F) Marked hair recovery in group F.

Statistical analysis of Bcl-2 immunohistochemical results:

The Bcl-2 immunohistochemical expression was evaluated and presented in **(Table 1A)** in terms of mean, standard deviations (SD), minimum and maximum values.

The overall difference between the seven groups was highly significant (F=267.99; p-value<0.001) as revealed by one-way ANOVA. There was a significant difference between the scores of groups BI, BII, C, D, E, and F compared to the negative control group as revealed by Duncan's Multiple Range tests (DMRTs). There was a significant increase in Bcl-2 expression in groups BI, BII, C, D, E, and F by 504%, 784%, 486.1%, 770.1%, and 212.7%,93.6%; respectively compared to group A (negative control group).There was a significant difference between the scores of groups C, E, and F compared to the positive control group (BII), as revealed by Duncan's Multiple Range test (DMRTs). However, there was a non-significant difference between group D compared to the positive control group (BII).There was a significant decrease in Groups C, E and F by -33.7, -64.6 and -78.1; respectively, compared to group BII (positive control group).

Comparison between E and F groups for Bcl-2 immunohistochemical expression. Statistical analysis using independent T-test at P<0.05 showed significant difference between the two groups. Also, the F group gave the lower value (40.8 ±2.98) than the E group (66.0±4.25).**(Table 1B)**

Table 1A: Statistical analysis of Bcl-2 immunohistochemical expression

Groups	Mean	SD	Letters
A (negative control)	21.1	1.32	e
B1 (DMBA; positive control 6 weeks)	127.6	20.09	b
BII (DMBA; positive control 12 weeks)	186.5	13.76	a
C (DMBA+ TQ 0.1 mg/Kg)	123.7	9.25	b
D (DMBA+ CS NPs)	183.6	14.48	a
E (DMBA+ TQ-CS NPs 0.1 mg/kg)	66.0	4.25	c
F (DMBA+ TQ-CS-NPs 0.01mg/Kg)	40.8	2.98	d
ANOVA	F= 267.99; p<0.001***		
Note: Different letters are significantly different according to DMRTs at 0.05 level.			

Table 1B: Comparison between E and F groups for Bcl-2 immunohistochemical expression

Groups	Mean	Std. Deviation	mean difference	indep-T test	P value
E	66.0	4.25	25.20000	11.95	<0.001**
F	40.8	2.98			
Test used: T-test at P value<0.05. ** means significant					

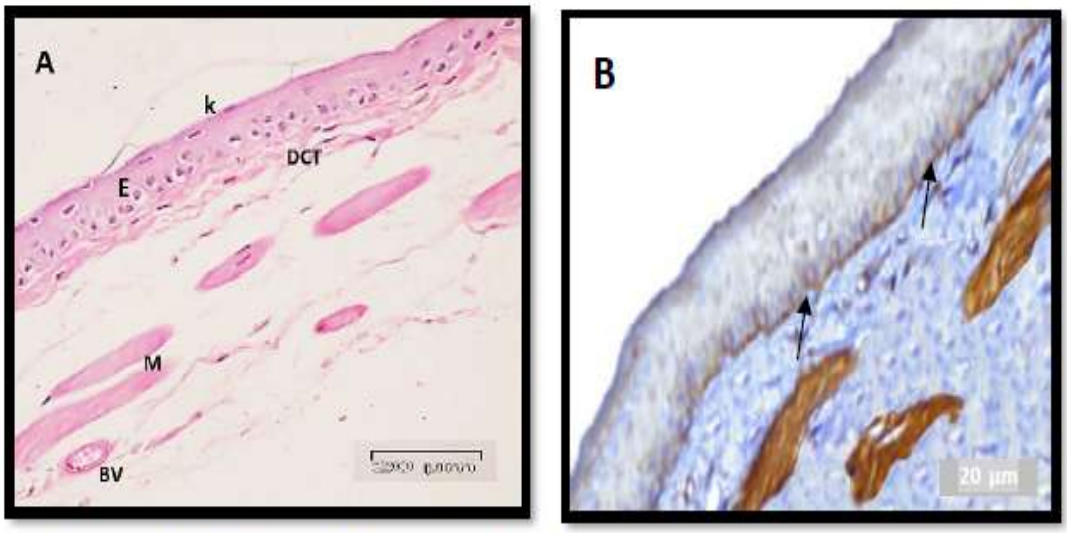

Figure 3: H&E and Bcl-2 immunohistochemical sections of negative control group (A) H&E section of group A shows thin layer of stratified squamous epithelium (E) with thin parakeratin layer (K) covering dense collagenous fibrous tissue (lamina propria) (DCT), loose striated muscle fibers(M), and blood vessels (BV) deeper in the connective tissue (H&E X40). (B) Immunohistochemical expression of Bcl-2 in group A in the epithelium confined to the basal layer (arrows) (IHC X40).

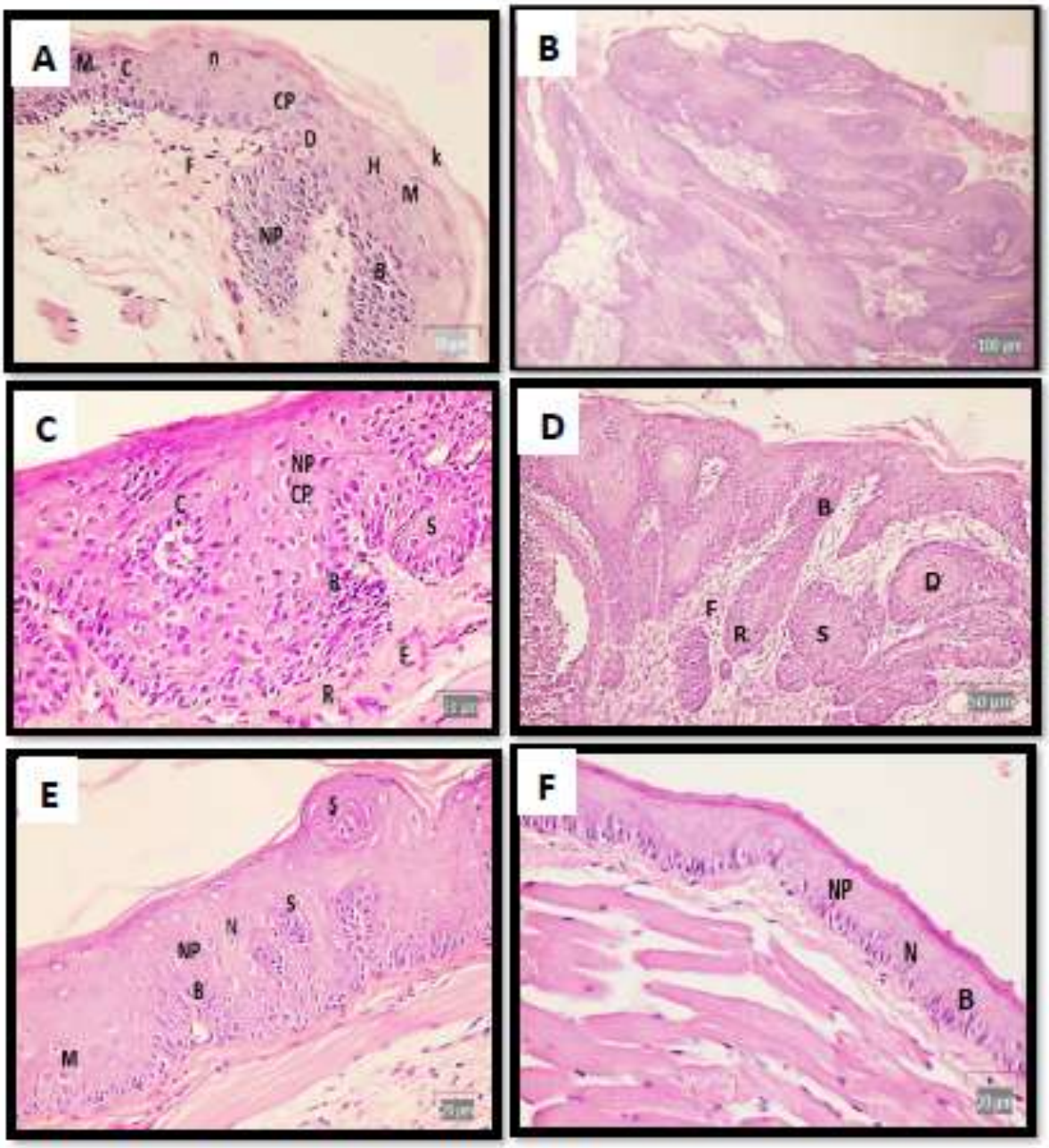

Figure 4: H&E sections of different groups (A) H&E section of group BI shows severe dysplasia (H&E×40). **(B)** H&E section of group BII shows papillomatous overgrowth of squamous cell carcinoma (H&E ×10). **(C)** H&E section of group C Shows severe epithelial dysplasia (H&E X40). **(D)** H&E section of group D shows well/ moderate differentiated squamous cell carcinoma (H&E X20). **(E)** H&E section of group E shows moderate epithelial dysplasia (H&E X40). **(F)** H&E section of group F shows mild epithelial dysplasia (H&E X40). **Note**: Epithelial hyperplasia (H), hyper-keratinization (k), drop shaped rete ridges (R), basal layer hyperplasia and loss of polarity (B), increased normal and abnormal mitosis (M), prominent nucleoli (n), nuclear pleomorphism (NP), cellular pleomorphism (CP), individual cell keratinization (D), nuclear hyperchromatism (C), swirling of prickle cells (S), invading islands (S) and chronic inflammatory infiltrate (F).

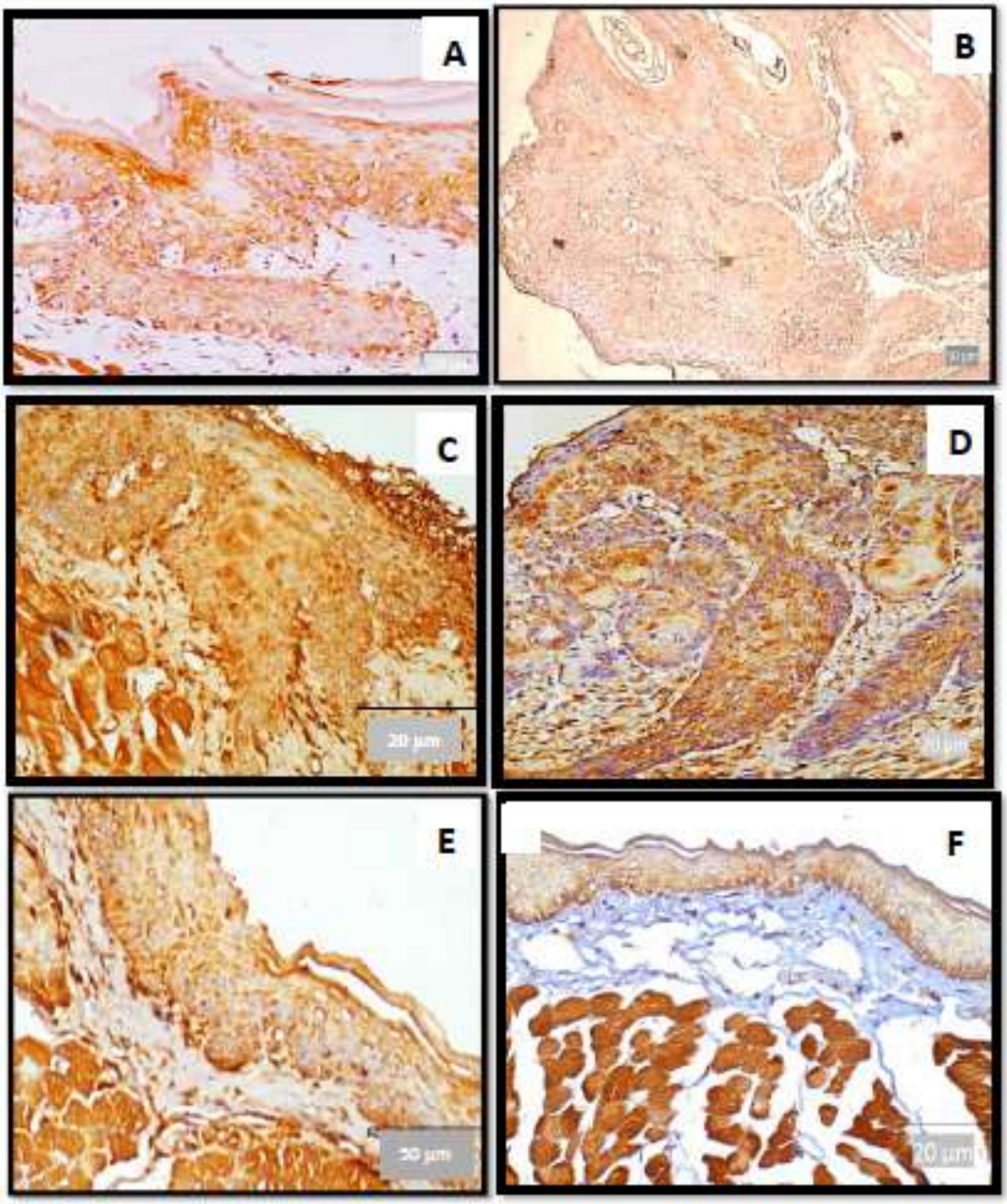

Figure 5: H&E and Bcl-2 immunohistochemical sections of treatment groups (A) Bcl-2 expression in group BI in the epithelium extended all over the epithelium thickness with cytoplasmic pattern (IHC× 40). **(B)** Positive Bcl-2 expression in group BII extended all over the epithelium and the invading islands (IHC ×20). **(C)** Positive Bcl-2 expression in group C extending all over the epithelial thickness in granular cytoplasmic pattern (IHC X40). **(D)** Group D showing expression of Bcl-2 all over the thickness of epithelium and the invading islands in a granular cytoplasmic pattern (IHC X40). **(E)** Group E shows Bcl-2 moderate expression in the epithelial cells with cytoplasmic pattern (IHC X20). **(F)** Group F shows Bcl-2 mild expression in the epithelial cells with granular cytoplasmic pattern (IHC X40).

IV. Discussion

In this study, the tumor induction protocol of ***Shklar*** was followed as it closely correlates with common events involved in the development of premalignant, and malignant human oral cancers [19].

Chitosan nanoparticles were chosen as a carrier for TQ because of the excellent mucoadhesive and muco-permeable properties of chitosan which allow the binding of a drug to the mucus layer and releasing it over time, In addition to its biocompatible and biodegradable properties [20].

Clinical findings of the present study showed that the worst results were obtained in animals treated with DMBA, and CS NPs. All animals were severely debilitated, with marked hair loss. The DMBA-treated animals results agreed with other similar studies [6,7,21]. Poor results with CS-NPs may be a result of its low concentration 0.235 mg/kg (the same concentration used in TQ-CS NPs). Chitosan has a dose-dependent anticancer effect as reported in previous research. [22,23] This concentration was used to ensure the sharing effect of CS as used in groups E & F.

Mild improvement was revealed in animals treated with TQ 0.1 mg/kg compared to animals treated with DMBA only. This finding wasin line with **Shata etal.,** [24] who reported that free TQ has little topical chemo-preventive effect. This may be becauseTQ suffers from some properties including poor bioavailability, hydrophobicity, light, and pH sensitivity [25].

Animals in group E (TQ-CS-NPs 0.1mg/kg) & group F (TQ-CS-NPs 0.01mg/kg) showed marked improvement in the general health of animals. Marked hair recovery.These findings were also reported by researchers who used different carriers to improve the physical properties of TQ. These carriers provided sustained release, protection of TQ from degradation, and enhanced the permeability and retention effect of TQ [6,26].

The histological results of the present work showed wide differences.The untreated control pouches revealed the four distinct layers as first described by ***Salley, (1954)*** [27].

Like clinical results, the poorest findings were reported in DMBAand CS-NPs treated animals.Following DMBA application for 6 weeks, the left pouches showed severe epithelial dysplasia with focal areas of carcinoma in situ, and when animals were left without further treatment for another 6 weeks, the left pouches developed focal areas of well to moderately differentiated squamous cell carcinoma. These findings agreed with previous studies that used the same induction protocol [6,7,21].

Left pouches of animals treated with free TQ 0.1 mg/kg showed severe dysplastic changes with focal areas of carcinoma in situ.In contrast, left pouches in animals treated with TQ-CS NPs 0.1mg/kg showed epithelial hyperplasia, hyper-keratinization, and moderate to severe dysplastic changes of the epithelial cells.

Lowering the dose of TQ-CS NPs 10-fold gave the best results. Histopathological specimens showed only epithelial hyperplasia, hyper-keratinization and mild to moderate dysplastic changes.This could be due to fewer TQ molecules loaded on CS NPs resulting in proper distribution and internalization of TQ-CS NPs molecules. A comparable dose reduction was reported by **Shata el al.,** [24] who found that topical application of thymoquinone loaded on gold nanoparticles (GNP-TQ) 0.001 had a better chemo-preventive effect than higher doses GNP-TQ 0.01 and GNP-TQ 0.05. GNP-TQ 0.001 successfully retarded HBP/DMBA carcinogenesis, improved the general animal health, and inhibited NF kappa B activation.

Lowering TQ concentration was in line with **Siddiqui et al.,** [28]who reported that nano-EGCG (polylactic acid-polyethyleneglycol PLA-PEG NPs encapsulated EGCG), was used against tumor xenograft mouse model, had an anticancer, pro-apoptotic, and anti-angiogenic effects at 10-fold lower dose compared to non-nano-EGCG.

However, **El-Sherbiny*et al*.,** [6]reported that nano-thymoquinone (NTQ) injected intraperitoneally has a chemo-preventive effect in a dose-dependent manner. They found that NTQ with 0.1 mg/kg concentration successfully retarded HBP/DMBA carcinogenesis, resulting in focal mild epithelial dysplastic changes. Lower NTQ concentrations (0.05, 0.025, and 0.01 mg/kg of body weight) did not give the same results this because they used liposomes as nanocarrier for TQ which is a different carrier than ours.

Further insight into the effect of TQ-CS NPs on the DMBA /HPB carcinogenesis model, studying the immunohistochemical expression of Bcl-2 antibody.There were highly statistically significant differences between the negative control group and the positive control group.

There wassignificant increase in Bcl-2 expression for group BI which showed severe epithelial dysplasia, and in group BII which showed well/moderately differentiated squamous cell carcinoma. In these groups, Bcl-2 was expressed in all layers of the epithelium and in the invading nests and sheets. Several studies of oral squamous cell carcinoma and premalignant lesions also revealed similar findings, as well [29,30,31]. Overexpression of Bcl-2 in cancer cells helps evasion of apoptosis and aids oncogenic transformation at multiple stages by facilitating sustained tumor growth and survival during the metastatic process as well as resistance to therapy [32].

The best immunohistochemical result was observed in animals treated with0.01 mg/kg TQ-CS NPs. There was a high statistically significant difference compared to group BII. TQ-CS NPs 0.01 mg/kg decreased

the expression of Bcl-2 by -64.6 compared to group BII and resulted in very week expression of Bcl-2 in all epithelial layers.

Comparable findings were reported by other researchers who used different chemopreventive agents **as, Saad *et al.,***[31] they used the same induction protocol and reported moderate to severe dysplasia with Bcl- 2 moderate positive expression in all epithelial layers. On the other hand, HBPs that developed well-differentiated squamous cell carcinoma showed strong positive expression of Bcl-2 all over the epithelium as well as in the dysplastic epithelial nests. After oral administration of carvacrol for 6 weeks, HBPs showed mild to moderate dysplasia with a significant reduction in Bcl-2 expression all over the epithelial thickness.

Abd Al-Wahab et al.,[33] supported our Bcl-2 expression results in their study inducing SCC after 14 weeks of DMBA application to HBP. The induced well/ moderately differentiated SCC revealed strong positive cytoplasmic Bcl-2 expression. This positive Bcl-2 staining was more significant as compared to normal mucosa. Oral administration of rosmarinic acid showed mild epithelial dysplasia and down-regulation of Bcl-2 expression into weak positive expression, not confined to the basal layer as in the control group.

V. Conclusion

Topical application of 0.01 mg/kg TQ loaded on CS NPs, proved to be effective in preventing the progression of induced HBP epithelial dysplasia into squamous cell carcinoma and reverting the dysplastic changes to be minimal in a period of 6 weeks.

References

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