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Chemotherapeutic effect of Ficus carica latex on experimentally induced hamster buccal pouch carcinogenesis

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ABSTRACT
The presentresearch aimed to assess the chemotherapeutic effect ofFicus carica latex (fig latex)on experimentally induced hamster buccal pouch (HBP) carcinogenesis.
Material and methods: Thirty five-week-old Syrian male hamsters weighing between 80 and 120 grams apiece were split up into three groups (n=10):Group I: left untreated, Group II: 7, 12-dimethyl benz[a]anthracene (DMBA) applied topically on three occasions a week for fourteen weeks at a concentration of 0.5% in liquid paraffin, Group III: DMBA (0.5% in liquid paraffin) was applied topically on three occasions a week for fourteen weeks, and 0.5 cc of fig latex had been administered intramuscularly every day for 4 weeks.
Results: The present research findings demonstrated that fig latex had a positive regression impact on tumor growth. The occurrence of oral squamous cell cancer (OSCC) was considerably decreased by the fig latex, going from 100% to 50%. The immunohistochemical analysis displayed that the fig latex decreased the expression of the antiapoptic marker Bcl-2 with non-significant variationin comparison to GII (p value > 0.05).
Conclusion: These results suggest that the latex of Ficus carica may offer a new chemotherapeutic medication since it inhibits proliferation and induces apoptosis in human oral cancer cells.

Keywords: HBP carcinoma,fig latex, apoptosis.

1. INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a major public health concern because of its high prevalence worldwide. According to GLOBOCAN 2020 estimates, approximately every year, 377,713 fresh instances of OSCC are detected,as well as 177,757 fatalities each year [(1)]. In Egypt, Abd El-Aziz et al. (2020)[(2)]study among 1664 smoker participants revealedthat 1.5% had OSCC and 0.12% had verrucous carcinoma.
Oral carcinogenesis is a multifactorial disease arising from the interaction of lifestyle, environmental, genetic, and epigenetic factors[(3)]. In golden Syrian hamsters, a renowned and widely used laboratory model for examining biochemical, histological, immunohistochemical (IHC), and molecular alterations, oral carcinogenesis caused by 7,12-Dimethylbenz(a)anthracene (DMBA) was recently discovered [(4)]. DMBA is commonly utilized like a significant carcinogen to develop tumors in the golden Syrian hamster buccal pouch (HBP) [(5)]. A well-developed OSCC was produced by the buccal pouches, a pocket-like structure subjected to repeated local DMBA administrations. By producing extensive oxidative damage to DNA and causing extensive inflammations and dysplasia within the buccal pouches, DMBA results in neoplasia [(6)]. Evidence gathered showed that DMBA-induced oral cancers and human oral malignancies had histological, morphological, biochemical, and molecular characteristics[(7)].

Approximately 12,000 plant varieties' laticifer cells release plant latex, a secondary metabolite which includes different concentrations of proteinases, alkaloids, tannins, polyphenols, and hydrolytic enzymes that stop insect eggs from growing and hatching. Additionally, the antifungal, antiviral, and antibacterial properties of certain latex were confirmed [8].

In Persian conventional medication, the latex released by young fig (ficus carica) leaves is highly valuable as a treatment for a variety of ailments. This latex includes rennin, sugar, malic acid, 6-O-palmitoyl-β-D-glucosyl-β-sitosterol, caoutchouc, cerin, albumin, resin, and proteolytic enzymes like diastase, esterase, lipase, catalase, and peroxidase [9]. Avicenna's book, the Canon, suggests consuming fig latex for the treatment of a variety of illnesses, such as papillomatosis, hypoglycemia, and warts, as well as to get rid of parasitic worms [10].

It has been demonstrated that fig latex has anticancer properties towards esophageal cancer cell lines [11],Cervical cancer[12],lung cancer[13], Burkitt B- cell lymphoma[9], human glioblastoma, hepatocellular carcinoma[14], gastric cancer cell lines[15] and hypopharynx squamous carcinoma cells[16].

As far as we are aware and believe, no publicly available research has been made about the in vivo chemotherapeutic impact of fig latex against OSCC. Therefore, using male golden Syrian hamsters and DMBA, the presentresearch set out to (a) assess the chemotherapeutic effectiveness of fig latex and (b) outline the putative process or processes of action in a well-established, in vivo preclinical oral cancer model.

2. MATERIAL AND METHODS

Preparation of fig latex: Following slicing young fig tree leaves, which bring from private farm in New Damietta City, Damietta, Egypt, Ficus carica latex was gathered drop by drop. Throughout the therapeutic phase, the new latex had been mixed 1:1 with distilled water.

Animals: Thirty-five-week-old Syrian male hamsters weighing between 80 and 120 grams apiece, were acquired from Cairo University's animal department (Cairo, Egypt). The test hamsters were housed in typical cages with bedding made of sawdust under circumstances of monitored humidity (30-40%), temperature (20-2°C), and light (12-h light/12-h dark). Every experimental hamster was received fed regular food and had unlimited usage of water.

Sample size:Based on the Abd Al-Wahabet al. (2020) study[17, 18], in the present research, a sample size of 10 per group provides an 80% power for identifying a variation among means of 0.53 at 95% confidence intervals and a significance level (alpha) of 0.05 (two-tailed). The two-tailed p-value in 80% (the power) of those tests was less than 0.05, therefore the results were deemed "statistically significant."The mean difference in the remaining 20% of the tests was considered "not statistically significant." GraphPad StatMate 2.00 produced the analysis.

Chemicals: DMBA (0.5%) was purchased dissolved in paraffin oil from the Sigma Aldrich company.

Experimental design: After adaption for one week, the animals split randomly into threegroups, each with ten hamsters. **Group I** (negative control group) hamsters were just fed and watered. **Group II** (DMBA), in which positive controls painted three times a week utilizing a camel's hair brush and 0.5% DMBA in liquid paraffin for 14 weeks. **Group III**(fig latex) The OSCC had beengenerated in such animals similar to how the DMBA group for 14 weeks. Following that, for four weeks, 0.5 cc of fig latex had been administered intratumorally every day [19].

General health examinations: Modifications in the hamster's overall health were documented throughout the trial. When dealing with disease or injury, animals showed one or more of the following symptoms: Wetness around the tail, diarrhea, hair loss, sneezing, coughing, huddling in a corner, loss of appetite, and/or discharge from the eyes or nose. Animal body weights were recorded weekly during the experimental period.

Investigations: Following the end of the study, the hamsters were put to sleep, the right cheek pouch was inverted, the dimension for every tumor was determined using a Vernier caliper, and gross observations were noted, including mucosal thickness, exudation, ulcers, and tumors. The equation Vmm3 = (4/3) π [(D1/2) (D2/2) (D3/2)] was used to determine the tumor dimension, with D1, D2, and D3 representing the tumor's three diameters (mm) [20].The right cheek pouch was then removed, preserved in a 10% neutral buffered formalin solution, treated as usual, and inserted into paraffin blocks for histological and immunohistochemical analysis.

Histopathological examinations: The preserved samples were incorporated in paraffin wax to create paraffin blocks after being dehydrated in an increasing ethanol series. To the light microscopic assessment, tissue slices that were 4 µm thick were cut using a rotary microtome, placed on glass slides, handled, and dyed using hematoxylin and eosin (H&E).

Immunohistochemical examination: To show the expression of Bcl-2 antibodies, additional tissue slices were cut and subjected to the conventional labeled streptavidin-biotin technique. Graded ethanol to distilled water was used to dewax and rehydrate the tissue slices fixed in paraffin. After 10 minutes of treatment with 3% H2O2 in methanol, endogenous peroxidase was inhibited. After applying citrate buffer solution (pH 6.0) and heating it to 95°C for three separate 5-minute bursts, the antigen was recovered and washed using phosphate-buffered saline (PBS). After that, single or double drops of the main antibody (Bcl-2) diluted 1:100 in Tris buffer solution were applied to the tissue slices. They spent the evening at 4°C in a humid environment at ambient temperature. A biotinylated secondary antibody had been incorporated following PBS rinsing, and it was then incubated for 30

minutes at an ambient temperature. Tissue slices were rinsed with PBS and then exposed to diaminobenzidine (Sigma, USA) for two to four minutes in order to produce color. The slides were cleaned, counter-stained with hematoxylin, and coated using mounting media once the color intensity was deemed appropriate.
To determine the location of immunostaining inside the tissues and the frequency of positive instances, the immune-stained slices were viewed under a light microscope. Additionally, the region's percentage of immunostaining-positive cells was calculated using an image analysis computer system. This was carried out at Al-Azhar University's Oral and Dental Pathology Department, Faculty of Dental Medicine, Boys, Cairo.
Statistical analysis: After statistical analysis, the data was collected as the mean ± standard deviation (SD). The SPSS version 17.0 for Windows was used to conduct a one-way analysis of variance (ANOVA). ANOVA and post hoc analysis utilizing the LSD test were used to compare farther than two independent groups using quantitative data and parametric distribution. The subsequent criteria were used to determine if a p value was significant: $p > 0.05$ represented non-significant, $p < 0.05$ represented significant, and $p < 0.001$ represented extremely significant.

3. RESULTS

Gross observations and tumor volume: Group I (control group) The animals' behavior was healthy and lively, and they had no gross abnormalities. The length of both buccal pouches was around 5 cm. The right HBP mucosa showed pink with a smooth surface and no observable abnormalities **(Fig.2A)**. **Group II** (DMBA-treated group) Animals emitted white particles from their mouths and a foul odor. Some hamsters with substantial body weight showed considerable perioral loss of hair up to the belly, which could have been brought on by a lack of nutrition as a result of DMBA-induced oral cavity inflammation. Up to 2 cm, the pouch depth started to drop and stayed constant until the study was finished. The animals were debilitated and skinny. The right HBP mucosa showed a whitish membrane and granular surface on the pouch mucosa, erythema of various degrees, and numerous elevated nodules also present. Using vernier caliper, the average tumor dimension of tumor-bearing hamsters was 769.5 mm^3**(Fig.2B)**. In**Group III**(fig latextreated group), HBP mucosa displayedfluctuating alterations,including erythematous mucosal surface to standardcolor with smooth surfaceand the hamsters seemed to be in good health. Using vernier caliper, the hamsters with tumors had an average tumor volume of 340.8 mm^3**(Fig.2C)**. The general health examinations and common clinical outcomes in the tested groups were summarized in **table 1.**

Table 1. Common clinical outcomes detected in the tested groups.

Features	Groups		
	GI	GII	GIII
loss of appetite	-	+++	++
Inactivity	-	+++	++
corner huddling	-	+++	++
sneezing, wheezing, and/or discharge from the nose or eyes,	-	+++	++
wetness around the tail, diarrhea	-	+++	++
hair loss	-	+++	++
Gross observations			
Papillomatous lesion	-	+++	++
Pouch length	5 cm	1.5-2cm	2.5cm
Ulcers	-	+++	++
Exudation	-	+++	++
Tumors	-	100 %	50 %

− = no change; + = mild; ++ = moderate; +++ = severe

The mean body weight was highly significant increase in the fig latex group and normal group (group 1) in comparison to the DMBA group ($p< 0.001$). There were no significant variations across controlgroups and the remaining groups **(Table 2, Fig.1).** It was displayed a highly significant decrease ($p < 0.001$) in the tumor size in fig latex-treated hamsters compared to the sizes of tumors in hamsters of cancerous group**(Table 2, Fig.1).**

Table 2: Comparison between studied groups as regard tumor volume and Body weight.

		GI (n = 10)	GII (n = 10)	GIII (n = 10)	F	P-value
Tumor volume	**Mean**	-----	769.5	340.8	**144.9**	**< 0.001 HS**
	±SD	-----	109.3	79.1		
Body weight	**Mean**	495	330	409	**11.02**	**< 0.001 HS**
	±SD	18	13.3	16.1		

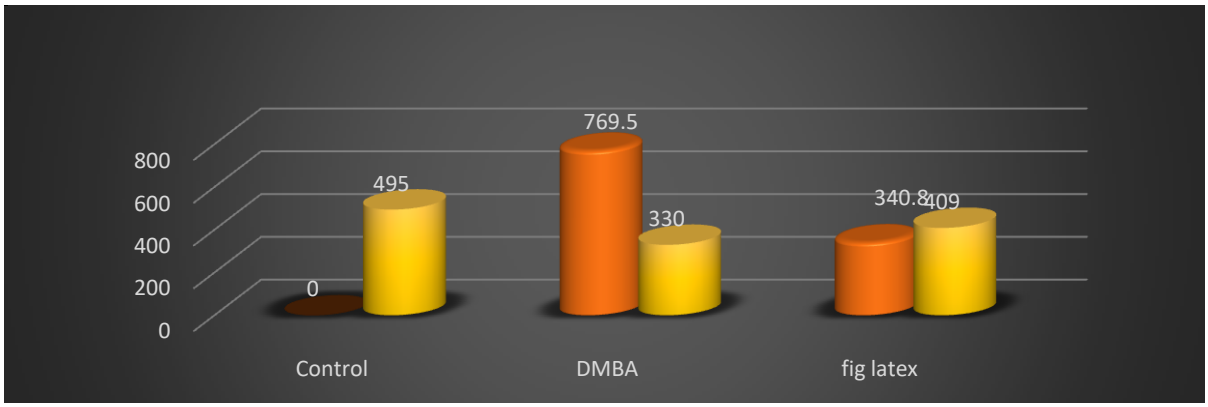
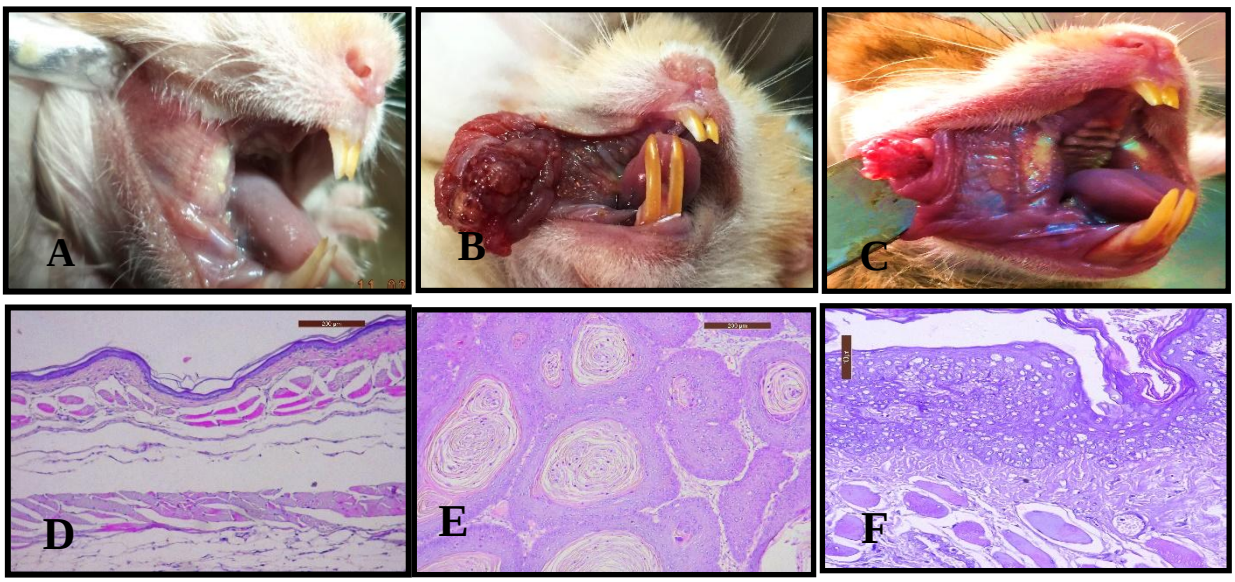


Fig.1: Bar chart representing tumor volume and body weight in the studied groups.

Histopathologic findings:Group I The HBP mucosa's lining epithelium is very thin, having keratinized stratified squamous epithelium ranging from 3–5 cells thick, according to H&E staining. One layer of cuboidal basal cells, 1-2 layers of polyhedral spinous cells, and flattened -granular cells havingsmall keratohyaline granules were visible in the epithelial layers. The epithelium was sparsely coated with keratin. The epithelium connective tissue interaction was quite flat without rete operations. The submucosa was composed of delicate and loose connective tissue and a layer of striated muscle fibers**(Fig. 2D)**. In **Group II,**the H&E stain indicated that 8 animalsdisplayedwell-differentiated SCC and 2 animals displayed moderate SCC. Histologically, well-differentiated SCC shows deeply invading malignant epithelial cells into the connective tissue. The invasive epithelial cells appeared signs of keratin production and manifested as cell nests or as detached, dispersed cells.The nuclear/cytoplasmic ratio was demonstrated in tumor cells having pleomorphic, hyperchromatic nuclei **(Fig.2E)**. In **Group III**, Five animals had moderate to serious epithelial dysplasia with top-to-bottom alterations or carcinoma in situ (CIS), according to the H&E stain, whereas the remaining five animals had well-differentiated SCC, which was a superficial invasion of malignant cells that only spread to the nodules and not deeper**(Fig.2F)**.

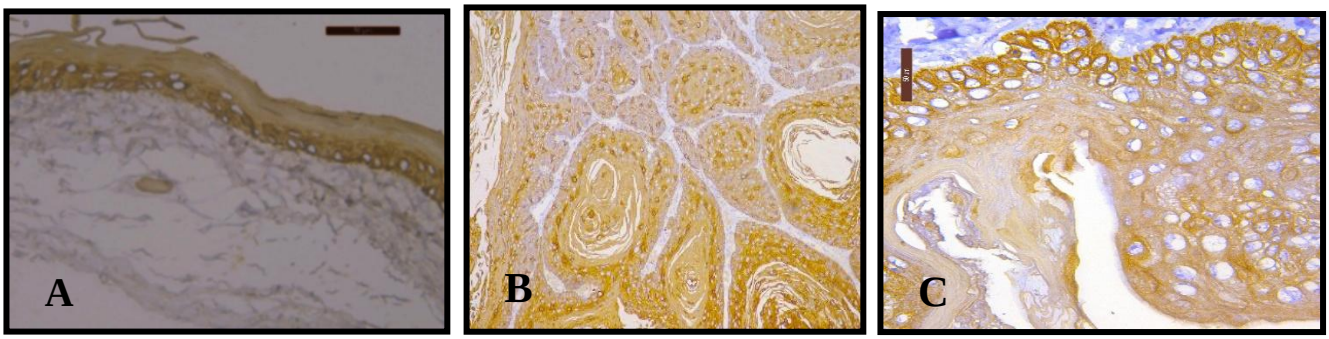


(Fig.2A): Photograph of Group I (normal). Displaying a rosy, smooth buccal pouch mucosa. **(Fig.2B):** Photograph of Group II (DMBA) showed several polypoid papillary tumor masses encircled by hemorrhage regions. **(Fig.2C):**Picture of Group III (fig latex group)mucosa showedvarious alterations, including erythematous mucosal surface to normal color with smoothtexture. **(Fig.2D):**Photomicrograph of Group I (normal) displaying: The epithelium is composed of 2-4 layers and superficial keratinized squamous cells with flattened rete ridges, the C.T layer, the muscular layer, and the deep layer of loose areolar connective tissue. (H&E stain X400) **(Fig.2E):** Photomicrograph of Group II (DMBA) showing well-differentiated SCC with deeply invasive tumor islands into the connective tissue. (H&E stain X100). **(Fig.2F):** Photomicrograph of Group III (fig latex group) showing moderate to severe dysplasia. (H&E stain X200).

Immunohistochemical (IHC) results: The IHC staining using Bcl-2 in **Group I**revealed that basal and suprabasal layers were the only ones with positive expression (mean = 6.07) **(Fig.3A)**.

In **Group II**,the basal and suprabasal epithelial layers showed positive cytoplasmic expression (mean = 65.45) in the Bcl-2 IHC staining **(Fig.3B).**

In **Group III**,the Bcl-2 IHC labeling revealed positive cytoplasmic expression across overall epithelial layers (mean 55.67) **(Fig. 3C)**.



(Fig.3A): Group I's Bcl-2 IHC expression demonstrates positive cytoplasmic expression in the basal and suprabasal epithelial layers. **(Fig.3B):**Bcl-2 IHC expression demonstrates positive cytoplasmic expression across all tumor cells. (**Fig.3C):**Group III's Bcl-2 IHC expression demonstrates positive cytoplasmic expression across all epithelial layers.

Statistical analysis results of Bcl-2 expression:

According to the findings of the statistical examination, GI had the smallest average region percentage (6.07%) and GII had the greatest average region percentage (65.45%) with respect to Bcl-2 expression. **Table (3),Fig(4).**

Table 3: mean and standard deviation for biomarker expression and statistical significance using the Welch one-way ANOVA test.

Biomarker/Group	Group I	Group II	Group III	P-value
BCL-2	6.07 (1.47)	65.45 (18.90)	55.67 (7.84)	0.00025*

*= significant difference

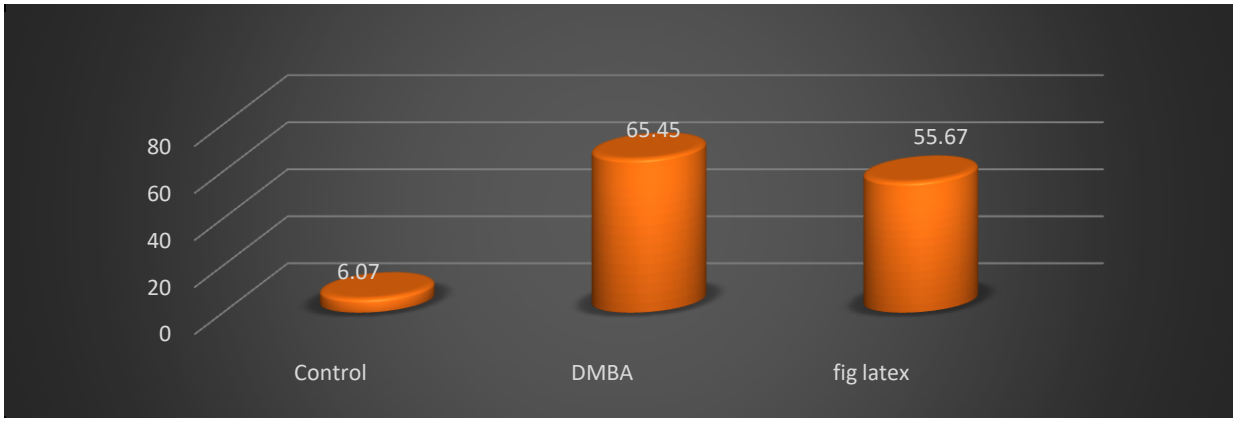


Fig.4: Bar chart representing Bcl-2 experssion in the studied groups.

There was a highly significant variation between the DMBA-treated group (GII) and GI (normal); the p-value was less than 0.001. The p-value was less than 0.001, indicating a very significant variation between the GIII and GI (normal). The p-value observed was more than 0.05, indicating that there was no significant variation between the GII (DMBA treated group) and GIII (fig latex group).**Table (4)**

Table 4: Games-Howell posthoc test for significant welch-ANOVA test

Biomarker	Comparison	P value
BCL-2	GI vs. GII	0.00001*
	GI vs GIII	0.00001*
	GII vs GIII	0.167*

4. DISCUSSION

Animal models are thought to be crucial for examining the course of illnesses and the evolution of diagnostic or treatment procedures.There has been increasing interest in the utilization of natural items including green tea, fruit and vegetable extraction from grapes, apples, and tomatoes for the treatment and chemoprevention of oral cancer [21-23]. The anticancer efficacy of the natural medicinal herb Ficus carica extract on oral cancer has not yet been documented. In this work, the chemotherapeutic properties of the Ficus carica extract were assessed in an animal model that was previously developed for oral cancer utilizing DMBA.

Recently, according to various research, fig latex contains a class of proteases, including ficin, caseinolytic, and gelatinolytic enzymes, which cause cancer cells to undergo apoptosis without harming healthy cells [12, 24, 25]. Thus, using an animal model of oral cancer, we assessed how fig latex affected cancer cells. By comparing the mean tumor dimensions and IHC staining utilizing Bcl-2 among the fig latex-treated and cancerous groups, the present research demonstrated the inhibitory impact of fig latex on development of malignant tumors. The presentresearch was the initial research to assess the impact of fig latex as a novel chemotherapeutic approach on DMBA-induced HBP carcinogenesis in open English literature.

According to reports, the OSCC frequency in the HBP was highly repeatable (100%) at 14 weeks after hamsters were administered DMBA (0.5%) for 14 weeks [22]. DMBA may cause various dysplastic and neoplastic lesions involving molecular and morphological changes that might be connected to human carcinogenesis, based on the dosage and length of time the carcinogen is given [26]. After 14 weeks of cancer induction, DMBA caused precancerous and cancerous morphological alterations on the HBP in the present research.

When DMBA is administered to animals to induce cancer, it may additionally cause various clinical findings including loss of appetite, Inactivity, wheeze, sneezing, or visual or nasal discharge, hair loss as well as substantial body weight loss, a rise in metabolic rate, difficulty in eating, and loss of desire due to the occurrence of oral cancer [21, 27]. The administration of DMBA in this investigation resulted in a notable decrease in body weight and great changes in the general health of the cancer-induced animals, in line with other results. Nevertheless, the fig latex treatment was in charge of the weight loss.

Ghandehari et al[19]stated that, it is commonly recognized that cancer cells exhibit failure at any stage of the cell cycling and evasion of apoptosis. Therefore, it is probable that fig latex's activation of apoptosis and its interaction with the cell cycling have lowered tumor growth and cell division.In the presentreserch, fig latex decreased the tumor voulmes of DMBA treated animals with highly significant reduction ($p < 0.001$) in the comparision to the sizes of tumors in hamsters of cancerous group.

Karthikeyan et al[28]discovered that,within normal conditions, the Bcl-2/Bax ratio establishes whether a cell will survive or die by regulating the Cyt C secretion from the mitochondria. This outcomemight be because Bcl-2 controls the terminal differentiation of keratinocytes by preventing the death of their stem cells [28]. A key factor in the development of OSCC is the dysregulation of apoptotic processes, which encourage programmed cell death and favor cell invasion and metastasis. Treatment failure may occur when cancer cells develop resistance to therapies, which allows them to evade apoptotic processes. The poor prognosis of OSCC has been linked to overexpression of Bcl-2, an anti-apoptotic protein essential for maintaining cellular homeostasis [29].

The presentoutcomesdiscovered that fig latex had the ability to downregulate Bcl-2 expression with non-significantvariationin comparison to GII (p value >0.05). Same outcomes were found by Al-koshab et al [30] whom explained that, the non-signficant difference could be attributed to that, the occurrence of oral cancer in their research as the presentresearch waslinkedto the well-differentiated kind of OSCC. Consequently, this might have had a role in the insignificant Bcl-2 expression. Shin et al [16]found that in hypopharynx SCC, fig latex raised anti-survival Bax protein levels and reduced antiapoptotic Bcl-2 protein concentrations and suggested that in hypopharynx SCC cells, the anti-cancer action of fig latex suppresses cell proliferation and induces cell apoptosis via the internal mitochondrial-dependent apoptotic signaling system and the external death receptor.

Ghandehari et al[19]stated that, it is commonly recognized that cancer cells exhibit failure at any stage of the cell cycle and evasion of apoptosis. Therefore, it is probable that fig latex's activation of apoptosis and its interaction with the cell cycle have lowered tumor growth and cell division.

CONCLUSIONS

Based on ouroutcomes, we may conclude that fig (Ficus carica) tree latex may be a viable agent for preventing the growth and development of malignant cells, and that it may have an equivalent impact in clinical trials.

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