International Journal of Medical Toxicology & Legal Medicine Volume 27, No. 2, 2024
e-ISSN: 0974-4614
p-ISSN: 0972-0448

Effect of Pomegranate extract punicalagin as a chemopreventive modality on experimentally induced hamster buccal pouch carcinogenesis

Mohamed Alaa Al-Dosoki[1],Emad Soliman Al-qalshy[2], Ahmed Abd-Alshakor Abd-Alhafez[3],Ashraf Yehia Shamia[4],Amr Mahmoud Mohamed[5], Ashraf Mohamad Emran[6], Ahmed Mostafa Abbas[7], Ehab Hamed Mostafa Elwardany[8], Ahmed Hussein Gaber[9], Galal Abou Elyazied Nasr[10].

[1]Lecturer; Department of Oral and Dental Pathology Department, Faculty of Dental Medicine, (Boys- Cairo), Al-Azhar University, Egypt.
[2]Assistant Professor; Department of Oral and Dental Pathology Department, Faculty of Dental Medicine, (Boys-Cairo), Al-Azhar University, Egypt.
[3]Assistant Professor; Department of Oral and Dental Pathology Department, Faculty of Dental Medicine, (Boys-Cairo), Al-Azhar University, Egypt.
[4]lecturer, Department of Oral and Dental Pathology, Faculty of Dental Medicine, Al-Azhar University, Palestine.
[5]Lecturer; Department of Oral and Dental Pathology Department, Faculty of Dental Medicine, (Boys- Cairo), Al-Azhar University, Egypt.
[6]Lecturer of Oral and Dental Biology Department, Faculty of Dental Medicine, (Boys- Cairo), Al-Azhar University, Egypt. Email: Ashrafemran88@gmail.com
[7]Lecturer of Oral and Dental Biology Department, Faculty of Dental Medicine, (Boys- Cairo), Al-Azhar University, Egypt. Email: dentist.ahmedmostafa@gmail.com
[8]Lecturer of Oral and Dental Biology Department, Faculty of Dental Medicine, (Boys- Assiut), Al-Azhar University, Egypt. Email: drehab.elwardany@gmail.com
[9]Assistant Professor; Department of Oral and Dental Pathology Department, Faculty of Dental Medicine, Assiut, Al-Azhar University, Egypt. Email: ahmed_gaber@azhar.edu.eg
[10]Assistant Professorof Oral and Dental Biology Department, Faculty of Dental Medicine, (Boys- Cairo), Al-Azhar University, Egypt. Email: galalyonis61@gmail.com

Received: 15.03.2024 Revised: 10.04.2024 Accepted: 25.04.2024

ABSTRACT

Objectives: The current study sought to assess the impact ofpunicalagin (PU)as a chemo-preventive method in artificially generated hamster buccal pouch (HBP) carcinogenesis.

Material and methods: 40 five-week-old Syrian male hamsters weighing between 80 and 120 grams apiece were split up into four groups of 10 hamsters in every group. Group I involved applying liquid paraffin topically three times per week during 14 weeks, whereas Group II involved applying 7, 12-dimethylbenz[a]anthracene (DMBA) topically 3 times a week for 14 weeks at a concentration of 0.5% in liquid paraffin, Group III: Local placement of DMBA (0.5% in liquid paraffin, three times per week during 14 weeks) + Oral administration of punicalagin (18.5 mg/kg bw by oral ingestion, three times a week for 14 weeks), Group IV: Oral administration of PU alone (18.5 mg/kg bw by oral ingestion, three times per week during 14 weeks on alternate days of DMBA application).

Results: The results of gross observation and histological analysis showed that: a) the standard stratified squamous epithelium was present in Group I; b) the SCC was well and moderately differentiated; c) the tumor occurrence was significantly reversed in Group III; and d) the tumor occurrence was normal and comparable to Group I.According to immunohistochemical data, oral PU treatment to hamsters administered with DMBA recovered the normal expression of Bcl-2, Bax, and PCNA, suggesting that PU might possess a chemo-preventive process through pro-apoptotic actions and/or anti-proliferative activity.

Conclusion: PU could work across a variety of ways, including selectively causing apoptosis in cancer cells, anti-inflammatory and antioxidant actions, and inhibition of cell migration and proliferation.

Keywords: HBP carcinoma, punicalagin, 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, 177,757 people die from OSCC each year, and approximately 377,713 instances fresh are diagnosed[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 experimental model for examining bio-chemical, histological, immune-histochemical (IHC), and molecular alterations, oral carcinogenesis caused by 7,12-Dimethylbenz(a)anthracene (DMBA) has been discovered[4]. DMBA is frequently used as a main carcinogen to cause tumors in the buccal pouch of golden Syrian hamsters (HBP) [5]. A well-developed OSCC was the outcome of repeated topical DMBA administrations to the buccal pouches, a pocket-like structure. By producing extensive oxidative damage to DNA and causing serious inflammation and dysplasia in the buccal pouches, DMBA results in neoplasia [6]. Accumulated evidencerevealed DMBA-induced oral cancers and human oral malignancies had histological, morphological, biochemical, and molecular characteristics[7].

Cancer chemoprevention is the utilization of chemical intermediaries, whether synthetic, organic, or biological, to stop, slow, or reverse the growth of cancer [8]. These substances include pleiotropic polyphenols that, in line with the multifactorial nature of cancer, target distinct inflammatory, redox-sensitive, and energy-sensing metabolic pathways by modifying the activity of various transcription influences[9, 10].

As an old, mystical, and quite different fruit, a deciduous tree in the Lythraceae group yields the ancient fruit known as the pomegranate (Punica granatum L.). The health benefits of pomegranates as fruit have long been recognized. The peel, the leaf, the root, and the pulp of pomegranates have all been utilized in medication. Its antiatherosclerosis and antioxidant properties might be the cause of this [11].

Numerous phytochemicals, found in distinct anatomical parts like peel (pericarp or husk), juice, and seeds, are linked to a variety of pomegranate medicinal properties. These include hydrolyzed tannins as well as associated substances (ellagitannin, punicalagin, pedunculagin, punicalin, gallagic acid, ellagic acid, and gallic acid), flavonoids (anthocyanins and catechins), ketones (quercetin and kaempferol), flavones that (apigenin and luteolin), and attached fatty acids (punicic acid) [12]. The anticarcinogenic and cancer chemo-preventive properties of pomegranate juice, gathers, and phytoconstituents have been thoroughly investigated preclinically in relation to prostate, skin, lung, and colon cancer [12, 13]. Punicalagin (PU) is a particularly abundant extract found in pomegranates. In vitro conditions, the PU inhibited human lung, breast, colon, and cervical cancer cell proliferation. It caused apoptosis in cancer cells in vitro conditions, lowered the volume and diversity of estrogen-induced mammary tumors, and diminished the incidence of chemically created lung and mammary cancers. These findings demonstrated that pomegranate phytochemicals offer defense versus a variety of cancer-related mechanisms [14].

Interestingly, investigation of the effects of pomegranate on oral cancer is limited primarily to in vitro studies.In terms of oral cancer, ellagic acid supplementation inhibited the growth of KB and CAL27 oral cancer cell lines by 45–88%, PU by 0–42%, and tannins by 0–27%. When ellagic acid, PU, and tannins were present, CAL27 growth was decreased by 26–69%, 10–96%, and 17–97%, respectively [15].According to related research, the pomegranate extract demonstrated cytotoxic effect in CAL27 cells at 50 μg/mL, in SCC1483 and HSC-2 cells at 75 μg/mL, and in HSC-3 and Ca9-22 cells at 0–50 μg/mL.In contrast, the pomegranate extract proved cytotoxic at 125 μg/mL in normal HF-1 cells [16, 17]. Additionally, it was discovered that apoptosis was triggered and the cells' viability was decreased [18, 19]. Furthermore, Peng et al.[19] shown that polyphenolic pomegranate extract stimulates mitochondrial damage pathways to produce an apoptotic impact. The extract's IC50 values versus Ca-22, HSC-2, and OC-2 cancer cell lines were 80.53, 100.34, and 108.12 μg/mL, respectively, according to Peng's study[19], which used a 24-hour ATP test to validate its antiproliferative solid action.

Among various influences, it was discovered that apoptosis, or programmed cell death, was essential in regulating OSCC [20]. Pro-apoptotic proteins like Bcl-2 Associated X Protein (Bax) are anticipated to work as tumor-suppressors, while B cell lymphoma-2 (Bcl-2) is an anti-apoptotic protein that is predicted to behave as an oncogene in cancer cells. These assumptions suggest that tumors overexpressing Bax ought to be linked to better clinical results, whereas malignancies overexpressing Bcl-2 should have a poorer prognosis. Nevertheless, research examining the prognostic relevance of apoptotic proteins in OSCC has shown inconsistent findings [21].

As far as we are aware and believe, no published study has been made about the in vivo chemo-preventive activity of PU against OSCC. Therefore, using male golden Syrian hamsters and DMBA, the present research set out to (a) assess the chemo-preventive potential of PU and (b) outline the putative method or processes of action in a well-established, in vivo preclinical oral cancer model.

2. MATERIAL AND METHODS

The contemporaryresearch used golden Syrian hamsters as experimental animals. Theseanimals were employed as a model for inducing carcinomas using the chemical carcinogen DMBA. Then,PU was used as a chemo-

preventive agent through oral gavage. Following that, numerous investigations were conducted, including staining with hematoxylin and eosin (H&E) and assessment of proliferation using proliferating cell nuclear antigen (PCNA) and apoptosis using specific markers (Bax and Bcl-2).

Animals: 40 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 animalswere housed in typical enclosures with bedding made of sawdust under circumstances of regulated light (12 hours of light and 12 hours of darkness), temperature (20–2°C), and humidity (30–40%). All test hamsters received standard diet and unlimited water admission.

Sample size: Based on the Abd Al-Wahabet al. (2020) study[22], a sample size of 10 per group in the present research provides an 80% power for identifying a variance amongst means of 0.53 at 95% confidence intervals and a significance level (alpha) of 0.05 (two-tailed). The p-value was less than 0.05 (two-tailed) in 80% (the power) of those tests, deemed that the results were "statistically significant." The mean difference in the remaining 20% of the tests was considered "statistically nonsignificant." GraphPad StatMate 2.00 produced the report.

Chemicals: We bought DMBA (0.5%) dissolved in paraffin oil from Sigma Aldrich. PU was purchased from Xi'an Rongsheng Biotechnology Co., Ltd. (82 Keji Road, Xi'an Hi-tech Zone, China) and acquired from a purity of 80% HPLC.

Experimental design: After adaption for one week, the animals will be split randomly into 4groups, every have10 hamsters. **Group I** (control group) the animals will serve as negative controls and only receive food and water. **Group II** (DMBA), in which positive controls will be painted three times a week utilizing a camel's hair brush and 0.5% DMBA in liquid paraffin for 14 weeks.

Group III oral delivery of PU (18.5 mg/kg bw by oral gavage) combined with topical treatment of DMBA (0.5% in liquid paraffin, three times per week for 14 weeks) [23], three times per week on different days of the DMBA usage for 14 weeks [22].

Group IV: Oral administration of PU alone (18.5 mg /kg b.w by oral gavage, three times per week for 14 weeks).

Investigations: Following the end of the study, the animals were put to sleep, their cheek pouches removed and preserved in 10% neutral buffered formalin, treated routinely, and inserted into paraffin blocks in order to be ready for histological and immunohistochemical analysis.

For histological assessment: To create paraffin blocks, the preserved specimens were immersed in paraffin wax and dehydrated in an increasing ethanol sequence. Regarding light microscopic analysis, tissue slices of 4 µm in thickness were sliced on a rotary microtome, placed on slides, treated, and stained with H&E.

For immunohistochemical assessment: The expression of Bcl-2, Bax, and PCNA antibodies was shown by cutting more tissue slices and using 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). The main antibodies (Bax, Bcl-2, and PCNA) were then diluted 1:100 in Tris buffer solution and applied to the tissue slices in single or double drops. They spent the evening at 4°C in a humid environment at ambient temperature. A biotinylated second antibody was incorporated following PBS cleaning, and it was then allowed to sit at ambient temperature for half an hour.Tissue slices underwent diaminobenzidine (Sigma, USA) for two-four minutes to produce color following being rinsed using PBS. The slides were cleaned, counter-stained via hematoxylin, and covered using mounting media once the color brightness was deemed appropriate.

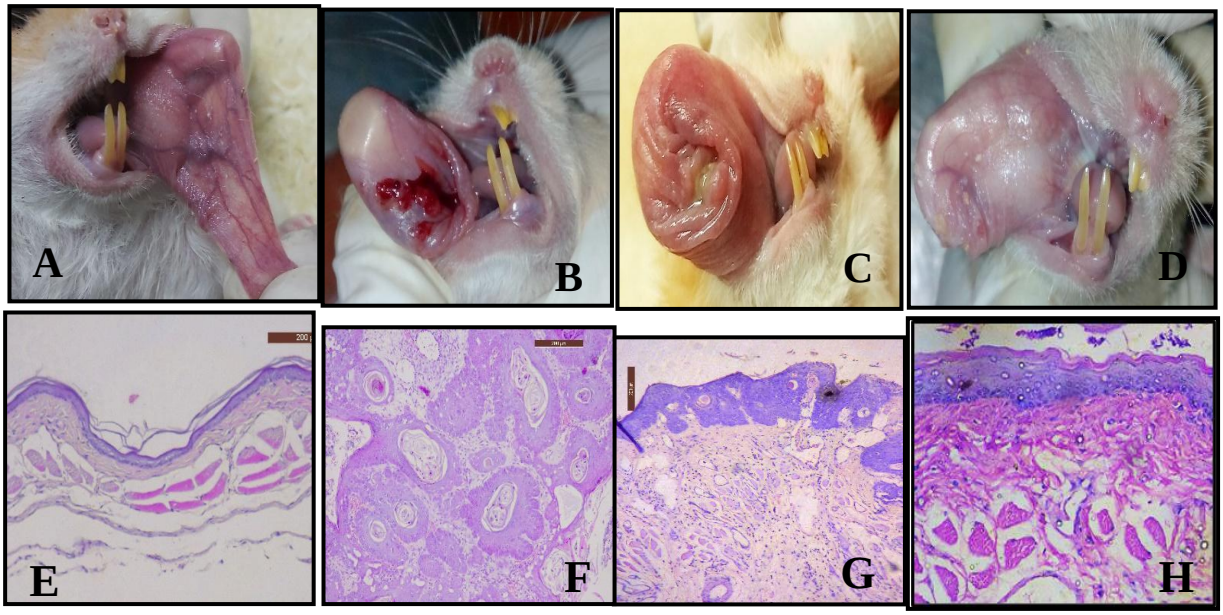
To decide the position of immunostaining inside the tissues and the frequency of positive instances, the immune-stained slices were viewed beneath a light microscope. Additionally, the area percentage of immunostaining-positive cells was calculated using an image processing program. This was carried out at Al-Azhar University's Oral and Dental Pathology Department, Faculty of Dental Medicine, Boys, Cairo.

Statistical analysis: The level of statistical significance was set at 5%. R and R Studio were used for statistical analysis. To organize, manipulate, and summarize the data, the "tidyverse" R package was used. The mean and standard deviation were used to summarize continuous data. The normality of data distribution was explored using the Shapiro-Wilk test function from the "rstatix" R package. Box plots were constructed and graphed using the "ggpubr" R package. The homogeneity of variance assumption was checked using Levene`s test function from the "rstatix" R package. Inter-group comparison for each biomarker expression was made using the one-way ANOVA test followed by the proper posthoc tests in case of a significant ANOVA test, using the "rstatix" R package. Correlation between the biomarkers expression levels in each group was assessed by the Pearson correlation test using the "correlation" R package, and the correlation matrix was plotted by the "ggally" R package.

3.RESULTS

Gross observation Results: **Group I** (control group), Animals showed no gross alterations, exhibiting vigorous and healthy activity; the length of both buccal pouches was around 5 cm. The right HBP mucosa seemspink,having a flawless surface and no discernible imperfections (Fig.1A). **Group II** (DMBA-treated group),Animals emitted white particles from their mouths and a foul odor. Some hamsters having substantial body weight showed considerable perioral loss of hair up to the belly, which could have been brought on by inadequate food consumption as a result of DMBA-induced oral cavity inflammation. Up to 2 cm, the pouch depth started to drop and stayed constant till the end of the study. The hamsters were debilitated and skinny. The right HBP mucosa showed a pouch mucosa has a granular surface and a white membrane, erythema of various degrees, and numerous elevated nodules also present **(Fig.1B)**. In**Group III**(punicalagintreated group),variations in the HBP mucosa were varied,including erythematous mucosal surface to standardshadehaving smooth surfaceand the hamsters seemed to be in good health **(Fig.1C)**. In **Group IV**, the HBP mucosa seemed normal, much like Group I **(Fig.1D)**.

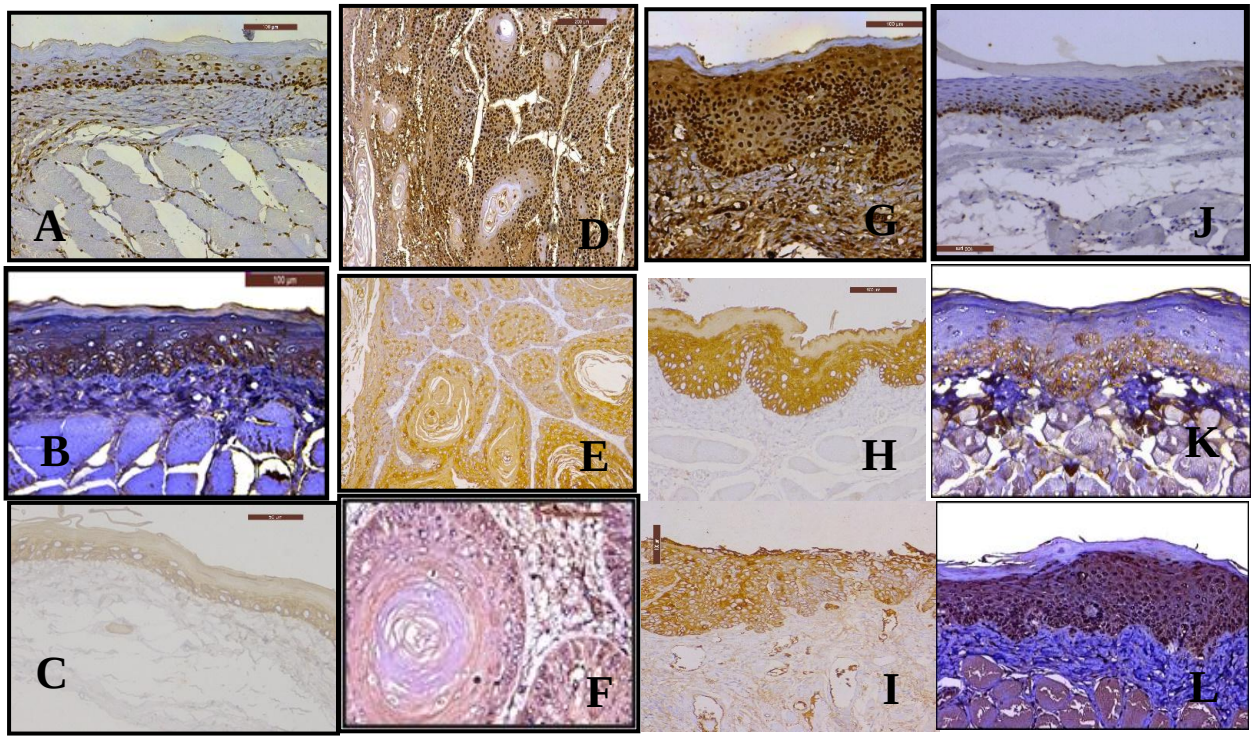
Histopathologic findings: In **Group I,**the HBP mucosa's lining epithelium is very thin, having keratinized stratified squamous epithelium that is 3–5 cells thick, according to H&E staining. Flattened granular cells having smallkeratohyaline granules, one or two layers of polyhedral spinous cells, and only one layer of cuboidal basal cells were visible in the epithelial layers. The epithelium was sparsely coated with keratin. Without rete processes, the epithelium's connective tissue interaction was comparatively flat. The submucosa was composed of delicate and loose connective tissue and a layer of striated muscle fibers**(Fig. 1E)**. In **Group II,**the H&E stain indicated that eighthamsters displayedwell-differentiated SCC and 2 cases exhibited moderate SCC. Histologically, well-differentiated SCC showsmalignant epithelial cells that deeply penetrate the connective tissue. The invasive epithelial cells appeared as nests of cells or as dispersed, unattached cells exhibiting keratin development.Tumor cells with pleomorphic, hyperchromatic nuclei demonstrated an abnormal nuclear/cytoplasmic ratio. **(Fig.1F)**. In **Group III**, the H&E stain indicated that eight out of ten mice showed hyperkeratosis to normal epithelium, and two out of ten showed minor epithelial dysplasia **(Fig.1G)**. In **Group IV**, the HBP mucosa seemed normal, much like Group I **(Fig.1H)**.



(Fig.1A): Picture of Group I (normal). Presenting a rosy, smooth buccal pouch mucosa (arrow). **(Fig.1B):**Picture of Group II (DMBA) several polypoid papillary tumor masses encircled by hemorrhage areas (arrow). **(Fig.1C):**Picture of Group III (PU chemopreventive group)mucosa showedvarying alterations, including restoring the natural color of the erythematous mucosal surface and smooth surface (arrow). **(Fig.1D):** Photomicrograph of Group IV, the HBP mucosa seemed normal.**(Fig.1E):** Photomicrograph of Group I (normal) showing: The epithelium is composed of two to four layers: 5 superficial keratinized squamous cells with flattened rete ridges, the C.T layer, the muscular layer, and the deep layer of loose areolar connective tissue. (arrow). (H&E stain X400) **(Fig.1F):** Photomicrograph of Group II (DMBA) displaying tumor islands that are profoundly invasive through the connective tissue and well-differentiated SCC (arrow). (H&E stain X100). **(Fig.1G):** Photomicrograph of Group III (PU chemopreventive group)displaying mild to moderate dysplasia. (arrow). (H&E stain X200). **(Fig.1H):** Photomicrograph of Group IV displaying normal epithelium without any dysplastic changes. (arrow). (H&E stain X200).

Immunohistochemical (IHC) results

The IHC staining in **group I** showed that although some PCNA-positive cells have been identified and dispersed across the basal cell layer, PCNA antigen was only isolated in the nuclei of epithelial cells. There was no cytoplasmic response.PCNA exhibited positive nuclear expression (10.04%) throughout the basal cell layer (**Fig.2A**).Bcl-2 antibody IHC labeling revealed positive expression (mean = 6.08), but only within the basal and supra-basal layers **(Fig.2B)**.On the other hand, the Bax expression, which is found in all epithelial layers, demonstrated positive expression (mean = 45.68) **(Fig.2C).**In **Group II**,Utilizing PCNA for IHC labeling, the epithelial layers showed positive nuclear expression (71.33%) **(Fig.2D)**.Meanwhile, the basal and suprabasal epithelial layers showed positive cytoplasmic expression (mean = 66.41) when Bcl-2 was used for IHC labeling**(Fig.2E).**Meanwhile,across all tumor cells, Bax showed positive cytoplasmic expression (mean=10.23) **(Fig.2F)**. In **Group III**,utilizing PCNA for IHC labeling, the epithelial layers showed positive nuclear expression (33.41%) **(Fig.2G)**. At the same time,Bcl-2 IHC labeling demonstrated positive cytoplasmic expression across all epithelial layers (mean 28.67) **(Fig.2H)**.Meanwhile,across all epithelial layers, the Bax expression demonstrated positive cytoplasmic expression (mean = 26.15) **(Fig.2I)**. In **Group IV**,utilizing PCNA for IHC labeling, the basal cell layer showed positive nuclear expression (11.49%) **(Fig.2J)**.Bcl-2 IHC labeling demonstrated strong cytoplasmic expression across all epithelial layers (mean = 8.05) **(Fig.2K),**whereas the epithelial layers' Bax expression demonstrated strong cytoplasmic expression (mean = 41.2) **(Fig.2L).**



(Fig.2A):IHC expression of PCNA in group I displaying positive nuclear expression in basal epithelial (Streptavidin biotin peroxidase, x200). **(Fig.2B):**The basal and supra-basal epithelial layers in group I exhibit positive cytoplasmic expression of Bcl-2 according to IHC analysis (Streptavidin biotin peroxidase, x200). **(Fig.2C):**Bax in group I has positive cytoplasmic expression across all epithelial layers, according to IHC analysis (Streptavidin biotin peroxidase, x200). **(Fig.2D):**PCNA's IHC expression in group II demonstrates positive nuclear expression across tumor cells and epithelial layers. (Biotin peroxidase streptavidin, x200). **(Fig.2E):**Using streptavidin biotin peroxidase (x200), Bcl-2 IHC expression in group II demonstrates positive cytoplasmic expression across the tumor cells. **(Fig.2F):**The tumor cells in group II exhibit positive cytoplasmic expression of Bax, according to IHC analysis (Streptavidin biotin peroxidase, x200). **(Fig.2G):**Positive nuclear expression across all epithelial layers is demonstrated by PCNA IHC expression in group III (Streptavidin biotin peroxidase, x200). (**Fig.2H):**Group III's Bcl-2 IHC reveals cytoplasmic expression that is positive across all epithelial layers (Streptavidin biotin peroxidase, x200). **(Fig.2I):**Using Streptavidin biotin peroxidase (x200), the IHC expression of Bax in group III demonstrates positive cytoplasmic expression across all epithelial layers. **(Fig.2J):**PCNA IHC expression in group IV demonstrates basal epithelial nuclear expression that is positive (Streptavidin biotin peroxidase, x200). (**Fig.2K):**The basal and supra-basal epithelial layers exhibit positive cytoplasmic expression of Bcl-2 in group IV, according to IHC analysis (Streptavidin biotin peroxidase, x200). **(Fig.2L):**Bax in group IV has positive cytoplasmic expression across all epithelial layers, according to IHC analysis (Streptavidin biotin peroxidase, x200).

Statistical analysis outcomes of PCNA, Bcl-2 and Bax expression

Data was normally distributed, as evidenced by a non-statistically significant Shapiro-Wilk test ($p > 0.05$). However, the homogeneity of variance assumption was not met, as evidenced by a significant Leven`s test ($p < 0.05$) leading to the Welch one-way ANOVA test for intergroup comparisons followed by the Games-Howell post-hoc test.

According to our findings, GI had the smallest average area ratio (6.08%) and GII had the greatest average area percentage (66.41%) for Bcl-2 expression. GII had the smallest average area ratio (10.23%) in terms of Bax expression, whereas GI had the greatest average area ratio (45.68%), furthermore, regarding PCNA expression, GIIshowed the greatest average area ratio (71.33%), whereasGIshowed the smallest average area ratio (10.04%). **Table (1),Figure (3).**

Table 1: 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	Group IV	P-value
BCL-2	6.08 (1.47)	66.41 (18.90)	28.67 (7.84)	8.05 (1.83)	0.00025*
BAX	45.68 (15.44)	10.23 (3.23)	26.15 (6.17)	41.20 (11.26)	0.00017*
PCNA	10.04 (2.16)	71.33 (12.54)	33.41 (9.98)	11.49 (1.80)	0.00063*

*= significant difference

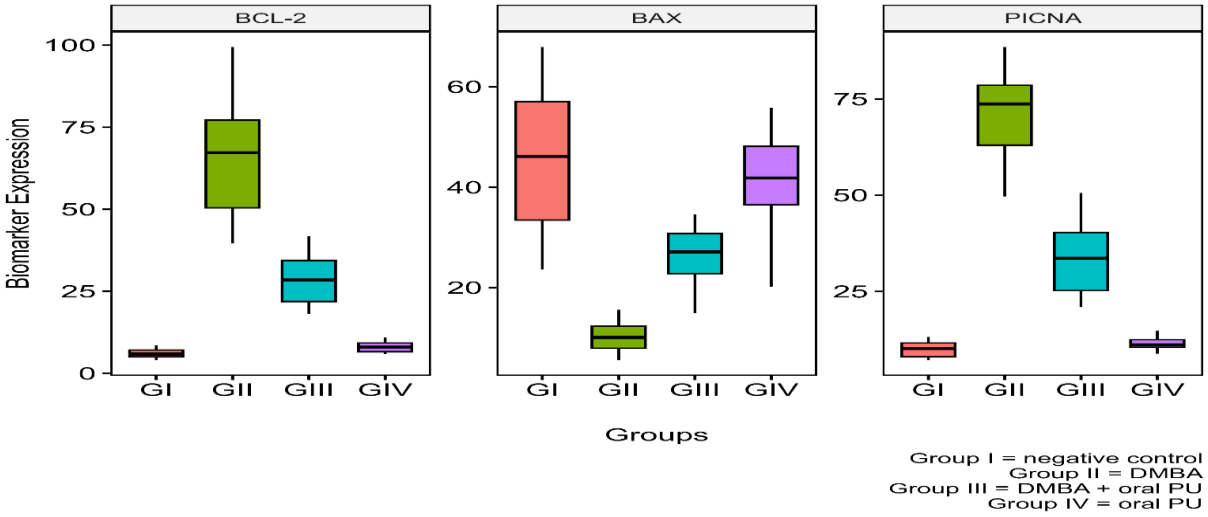


Figure 3. boxplot for each biomarker expression value

The results showed an extremely significant variation in PCNA, Bcl-2, and Bax between the DMBA treated group (GII) and GI (normal) and GIV; the P value was < 0.001. In comparison the GIII versus GI (normal) and GIV, the results showed a significant variation in Bax across the same groups (P value = 0.014), however there was an extremely significant variation in Bcl-2 and PCNA (P value < 0.001).There was an extremely significant variation in PCNA, Bcl-2, and Bax between the GIII (PU chemo-preventive group) and GII (DMBA treated group); the P value was < 0.001. **Table (2)**

Table 2. Games-Howell posthoc test for significant welch-ANOVA test

Biomarker	Comparison	MD, 95% CI	P value
BCL-2	GI vs. GII	60.33 [41.66 to 78.99]	0.00001*
	GI vs GIII	22.59 [14.82 to 30.36]	0.00003*
	GI vs GIV	1.97 [-0.13 to 4.08]	0.07
	GII vs GIII	-37.74 [-56.94 to -18.53]	0.0004*
	GII vs GIV	-58.36 [-77.03 to -39.68]	0.00002*
	GIII vs GIV	-20.62 [-28.41 to -12.83]	0.00005*
BAX	GI vs. GII	-35.45 [-50.77 to -20.14]	0.00018*
	GI vs GIII	-19.53 [-35.17 to -3.88]	0.014*
	GI vs GIV	-4.48 [-21.71 to 12.75]	0.879
	GII vs GIII	15.92 [9.49 to 22.35]	0.00003*

	GII vs GIV	30.97 [19.73 to 42.21]	0.00003*
	GIII vs GIV	15.05 [3.25 to 26.86]	0.011*
PCNA	GI vs. GII	61.28 [48.86 to 73.71]	0.00003*
	GI vs GIII	23.37 [13.46 to 33.28]	0.00015*
	GI vs GIV	1.45 [-1.07 to 3.97]	0.38
	GII vs GIII	-37.92 [-52.31 to -23.52]	0.00026*
	GII vs GIV	-59.83 [-72.24 to -47.43]	0.00031*
	GIII vs GIV	-21.92 [-31.80 to -12.03]	0.00027*

MD: mean difference, 95% CI: 95% confidence interval, *: significant difference

Correlation analysis:

The results demonstrated a statistically high significant negative (converse) association across area % of Bax and area % of Bcl-2 expression as well as between area % of Bax and area % of PCNA(p value > 0.001)**(Table 3, Figure.4).**This indicates that a rise in one of the factors is linked to a fall in another, and vice versa.

The results demonstrated a statistically high significant positive (direct) association either across area % of Bcl-2 and area % ofPCNA (p value > 0.001) **(Table 3, Figure.4).**This indicates that a rise in a single factor is linked to a rise in another, and vice versa.

Table 3. correlation coefficient and 95% confidence interval values

Correlation parameter	Pearson Correlation coefficient (r), 95% CI	P value
BCL_2 & BAX	-0.75 [-0.86 to -0.57]	0.0002*
BCL_2 & PCNA	0.96 [0.92 to 0.98]	0.0009*
BAX & PCNA	-0.77 [-0.87 to -0.61]	0.0008*

95% CI: 95% confidence interval, *: significant difference

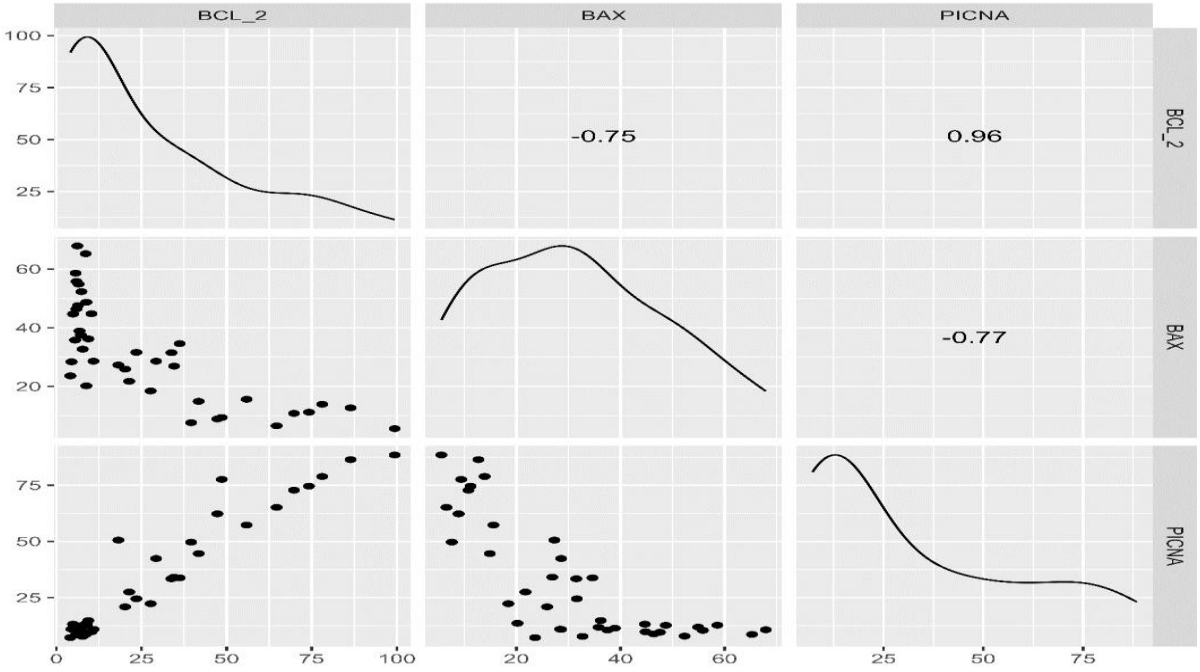


Figure 4. correlation matrix for different biomarker expression values

4. DISCUSSION

Many studies have been conducted on oral carcinogenesis having the goal of creating biomarkers for early detection or efficient therapy. The HBP model works well because it closely resembles the histological and molecular processes of oral carcinogenesis in humans. Furthermore, since DMBA has an identical etiologicimpact on hamster SCC like the alcohol and tobacco do in human OSCC, it was selected as the chemical carcinogen. This work was the initial attempt to assess the impact of PU as a novel chemo-preventive strategy on DMBA-induced HBP carcinogenesis in the open literature of English. Varying findings were found by the outcomes of gross observations, H&E staining, and immunohistochemical staining using Bcl-2, Bax, and PCNA antibodies.

Our outcomes demonstrated no noticeable alterations in the gross observation of Group I (control group) in the current investigation.The surface of the HBP mucosa seemed smooth and normal. Following scarification, the length of each hamster's buccal pouch was around 5 cm. H&E staining, which revealed a thin keratinized stratified squamous epithelium, supported this conclusion. Subepithelial C.T. is developed by several sporadic fibrocytes and blood vessels that lie between the epithelium and the muscle layer. Brunaet al.,2017[24]revealed the similar discovery within the present investigation,Group I (control group),according to the Bcl-2 immunohistochemistry data, only the basal and supra-basal layers exhibited positive expression (mean = 6.08). In contrast, across all epithelial layers, the Bax expression was positive (mean = 45.68). Our findings agreedwithKarthikeyan,2014[25]whom discovered that,within typical conditions, the Bcl-2/Bax ratio decides whether a cell will survive or die by controlling theCyt C's discharge from the mitochondria. This findingmight be due to Bcl-2 prevents keratinocyte stem cells from dying, thereby regulating the final differentiation of keratinocytes [25].

Within present research, Group I (normal group), IHC staining of average hamster mucosal tissue displayed positive PCNA expression (mean = 10.04) that was restricted to the basal, parabasal layer and negative in the layers of epithelial cells that remain. Liu et al.[26]stated that the expression of the proliferative marker, only the germinative layers included the normal epithelium. Cells in the superficial layer did not express proliferation markers[26].

Gross examination of Group II (the DMBA-treated group) in this research showed many exophytic masses of varying sizes encircled by ulceration, hemorrhage, stiffs, necrosis, and the buccal pouch constricted. This outcomewas supported by H&E staining, which demonstrated characteristics SCC that is both well and moderate differentiated, including increased cellular and nuclear pleomorphism in the epithelium, loss of stratification, frequent mitotic figures, hyperchromatia, disruption of the keratin layer alongside drop-shaped rete pigs, discontinuous basement membrane, and a high number of inflammatory cells. Thesefindings areconsistent with those shown by otherstudies[24, 27].As a consequence of recurrent exposure to tumor promoters, a chronic inflammatory state with a prolonged production of ROS, which leads to chronic oxidative stress, suchoutcomes could be described as a greater amount of intracellular ROS throughout DMBA administration. Adjacent epithelial cells may sustain damage from free radicals and non-radical ROS, such as H2O2 generated by phagocytic cells, including lipid peroxidation, protein alterations, sister chromatid exchanges, DNA strand breakage, and mutations.Furthermore, transcriptional regulation, apoptosis, metabolism, cell signaling, and DNA repair ability might all be impacted by protein alterations brought via free radicals or ROS [28].

In the current study, Group II (DMBA treated group), the Bcl-2 IHCfindingsdisplayed positive cytoplasmic expression (mean=66.41) that was observed in the invasive tumor cells and epithelial layers, that displayedextremely significant difference in comparison to GI (p value < 0.001). Such findingsagree with previousinvestigations[29, 30]. Anti-apoptotic Bcl-2 overexpression might be a factor in that. By blocking the mitochondria's ability to release Cyt C, Bcl-2 inhibits apoptosis and prolongs cell survival. It may also promote the growth of tumors. Group II (the DMBA-treated group). The Bax IHC findings in Group II (the group treated with DMBA) in this investigation indicated a extremely significant difference from GI (p value < 0.001), with positive cytoplasmic expression (mean=10.2) across the tumor cells. Suchoutcomesagreewith other investigations[31][32]. Increased development and a greater reduction in apoptotic cell death might be the cause of the reduced expression of Bax in malignant tissues.

The development of oral epithelial dysplasia to OSCC could be demonstrated by the higher expression of Bcl-2 and lower expression of Bax in OSCC relative to oral epithelial dysplasia, because the study's findings point to alterations in the Bcl-2 family proteins' expression, which is favorable the development of cancer. The present study's findings displayed that suppression of apoptosis is common in oral carcinogenesis. The final differentiation of keratinocytes seems to be controlled by the Bcl-2 protein family.Final differentiating was followed by a decrease in Bcl-2 expression and an increase in Bax.By preventing the tumor cells from going through apoptosis, overexpression of the antiapoptotic protein Bcl-2 and downexpression of the proapoptotic protein Bax let the tumor cells survive [32].

Within the present research, Group II (DMBA treated group), the PCNA IHC results revealed positive nuclear expression (mean=71.33) that was observed across the invasive tumor cells and epithelial layers, demonstrating a extremely significant difference from GI (p value < 0.001). This agrees with other investigations[33, 34]. In Group II, it has been demonstrated that precancerous and cancerous oral cavity lesions have aberrant PCNA expression,this may be attributed to the dysregulation of genes related to the proliferation and differentiation of cells [38]. Lawall M et al. discovered that when normal epithelium progressed from hyperplasia to dysplasia to oral squamous cell cancer, PCNA expression gradually increased[39]. Therefore, in DMBA-induced malignancies, overexpression of PCNA indicates enhanced cell proliferation,which agrees with other investigations[40].

In this study, animalsreceived DMBA, oral PU treatment at a dosage of 18.5 mg/kg bw not only completely avoided the development of oral tumors but also markedly reduced the incidence of preneoplastic abnormalities like hyperplasia, keratosis, and dysplasia. The current outcomes imply that PU potently prevents oral carcinogenesis (chemopreventive potential). Suchoutcomes disagreed with H&E staining, showing that eight mice had hyperkeratosis to normal and just a couple mices had mild epithelial dysplasia. Such outcomes align with Gómez-

García et al., 2020[41]. Who observed an apparentreduction in the PU group's macroscopic skin lesions. Inmicroscopy analysis, the incidence of SCC was 100% in the control group, whereasPU-treated mice only developed one cancer. The PU treatment group and the control group showed statistically significant variations. The animals who received PU showed a surprising degree of skin affection, with their skin staying normal [41].

The effectiveness of pomegranate extracts to prevent skin cancer was also assessed in the chemical carcinogenesis model that was induced by DMBA and accelerated by 12-O-tetradecanoylphorbol-3-acetate (TPA). When pomegranate extract was applied topically to mouse skin, the development of skin tumors was delayed, and the frequency and burden of tumors in mice were greatly decreased [42]. Pomegranate phytochemicals shown exceptional anti-proliferative and pro-apoptotic properties in DMBA-induced mammary cancer in rats, resulting in a chemo-preventive impact[43]. Additionally, after treating DMBA-induced tumor lesions in the mouse mammary gland using polyphenols derived from pomegranate fermented juice, in vivo experiments demonstrated a 47% reduction in the growth of malignant lesions [44, 45].

In the current study, Group III (PU chemopreventive group), the IHCoutcomes of Bcl-2 displayed positive expression (mean = 28.67), throughout the epithelial layers, that displayed extremely significant difference in comparison to GII (p value < 0.001). In contrast, across all epithelial layers, the Bax expression was positive (mean = 26.15), indicating an extremely significant variation from GII (p value < 0.001). The present outcomes showed that by drastically downregulating Bcl-2 and upregulating Bax expression, PU administration to DMBA-treated rats halted the course of the pathological lesions by inducing the apoptotic process.This finding agree with Ghani et al.[46], who discovered that pomegranates might activate caspase-3 and release cytochrome C, which would cause apoptosis with the intrinsic mechanism. Hussein et al.[23] suggested that because PU reduces GST activity in the livers of DENA-treated rats, it may make the hepatocytes additional vulnerable to TNF-α-induced (extrinsic) apoptosis, which is indirectly linked to the reduction of hepatocyte GSH conjugates. Tang et al.[47] found that by up-regulating Bax and down-regulating Bcl-2 expression, punicalagin was shown to suppress cell growth and cause a cell cycle stoppage at the G1/S phase, which in turn promotes death.

Positive nuclear expression (mean=33.41) was seen in all epithelial layers in Group III (PU chemo-preventive group) in the current research. This was a extremely significant variation from GII (p value < 0.001). PCNA expression was downregulated in the PU group, significantly different from Group II. These results concur with other studies[41, 48].Additionally, oral PU reduced UVB-induced stimulation of important inflammatory and cell-proliferative pathways including MAPK and NFκB. The anti-inflammatory and anti-proliferative possessions of PU were further substantiated by a decrease in UVB-induced protein production of COX-2, iNOS, PCNA, cyclin D1, and MMPs in skin of mice [49].In the B(a)P-induced lung cancer model, punicalagin and ellagic acid exhibit strong anti-mutagenic and anti-proliferative properties [50].

CONCLUSIONS

PU may be an attractive chemo-prevention drug since it avoided and reduced the likelihood of HBP carcinogenesis to varying degrees when administered orally. Higher expression levels of Bax and a decrease of Bcl-2 and PCNA expressions throughout PU chemoprevention demonstrate the beneficial effects of PU on apoptosis and proliferation rate. Additionally, there is aextremely significant variation in both apoptosis and proliferation rate among the carvacrol chemoprevention group and the DMBA painted group.Pomegranate extract may thus be thought of as an abundant source of possible options to be incorporated into the diet, with potential benefits at pre-determined levels while preventing toxicity, given the factors that were already stated.

References

1. Sung H, Ferlay J, Siegel R, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin.2021;71(3):209-49.
2. El-Aziz A, AbouShousha A, Ali S, Zahran F. Prevalence of Potentially Malignant Lesions and Oral Cancer Among Smokers in an Egyptian cohort: A Hospital-based Cross-Sectional Study. Adv Dent J. 2020;2(3):93-100.
3. Mello F, Melo G, Pasetto J, Silva C, Warnakulasuriya S, Rivero E. The synergistic effect of tobacco and alcohol consumption on oral squamous cell carcinoma: a systematic review and meta-analysis. Clin Oral Investig. 2019;23(7):2849-59.
4. Manoharan S, VasanthaSelvan M, Silvan S, Baskaran N, Singh A, Kumar V. Carnosic acid: a potent chemopreventive agent against oral carcinogenesis. Chem Biol Interact. 2010;188(3):616-22.
5. Karthikeyan S, Srinivasan R, Wani S, Manoharan S. Chemopreventive potential of chrysin in 7, 12-dimethylbenz (a) anthracene-induced hamster buccal pouch carcinogenesis. Int. J. Nutr. Pharmacol. Neurol. Dis.2013;3(1):46-53.
6. Manoharan S, Karthikeyan S, Essa MM, Manimaran A, Selvasundram R. An overview of oral carcinogenesis. Int. J. Nutr. Pharmacol. Neurol. Dis.2016;6(2):51-62.
7. Tanaka T, Ishigamori R. Understanding carcinogenesis for fighting oral cancer. J Oncol. 2011;2011.

8. Sporn M, Dunlop N, Newton D, Smith J. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed proc. 1976 ;35(6):1332-38.
9. Barrajón-Catalán E, Herranz-López M, Joven J, Segura-Carretero A, Alonso-Villaverde C, Menéndez JA, et al. Molecular promiscuity of plant polyphenols in the management of age-related diseases: far beyond their antioxidant properties. Adv Exp Med Biol. 2014:824:141-59.
10. Menendez J, Joven J, Aragonès G, Barrajón-Catalán E, Beltrán-Debón R, Borrás-Linares I, et al. Xenohormetic and anti-aging activity of secoiridoid polyphenols present in extra virgin olive oil: a new family of gerosuppressant agents. Cell Cycle. 2013;12(4):555-78.
11. Sharma P, McClees S, Afaq F. Pomegranate for prevention and treatment of cancer: an update. Molecules. 2017 ;22(1):177.
12. Viladomiu M, Hontecillas R, Lu P, Bassaganya-Riera J. Preventive and prophylactic mechanisms of action of pomegranate bioactive constituents. Evid Based Complement Alternat Med. 2013:2013:789764.
13. Adhami V, Khan N, Mukhtar H. Cancer chemoprevention by pomegranate: laboratory and clinical evidence. Nutr Cancer. 2009;61(6):811-15.
14. Sreekumar S, Sithul H, Muraleedharan P, Azeez J, Sreeharshan S. Pomegranate fruit as a rich source of biologically active compounds. Biomed Res Int. 2014:2014:686921.
15. Jurenka J. Therapeutic applications of pomegranate (Punica granatum L.): a review. Altern Med Rev. 2008 Jun;13(2):128-44.
16. Weisburg J, Schuck A, Silverman M, Ovits-Levy C, Solodokin L, Zuckerbraun H, et al. Pomegranate extract, a prooxidant with antiproliferative and proapoptotic activities preferentially towards carcinoma cells. Anticancer Agents Med Chem. 2010;10(8):634-44.
17. Peng S, Hsiao C, Lan T, Yen C, Farooqi A, Cheng C, et al. Pomegranate extract inhibits migration and invasion of oral cancer cells by downregulating matrix metalloproteinase-2/9 and epithelial-mesenchymal transition. Environ Toxicol. 2020;35(6):673-82.
18. Wang F, Chen J, Xiang D, Lian X, Wu C, Quan J. Ellagic acid inhibits cell proliferation, migration, and invasion in melanoma via EGFR pathway. Am J Transl Res. 2020;12(5):2295-304.
19. Peng S, Lin L, Chen S, Farooqi A, Cheng Y, Tang J, et al. Pomegranate extract (POMx) induces mitochondrial dysfunction and apoptosis of oral cancer cells. Antioxidants. 2021;10(7):1117.
20. Lo Muzio L, Sartini D, Santarelli A, Rocchetti R, Morganti S, Pozzi V, et al. Expression and prognostic significance of apoptotic genes in oral squamous cell carcinoma. Mol carcinog. 2014;53(4):264-71.
21. Bose P, Klimowicz A, Kornaga E, Petrillo S, Matthews T, Chandarana S, et al. Bax expression measured by AQUAnalysis is an independent prognostic marker in oral squamous cell carcinoma. BMC cancer. 2012;12(1):332.
22. Al-Wahab A, Gobran H, Dameer H. Effect of rosmarinic acid as a chemopreventive modality on experimentally induced hamster buccal pouch carcinogenesis. Egypt Dent J. 2020;66(4):2411-21.
23. Hussein A, El-Beih N, Swellam M, El-Hussieny E. Pomegranate juice and punicalagin-mediated chemoprevention of hepatocellular carcinogenesis via regulating miR-21 and NF-κB-p65 in a rat model. Cancer Cell Int. 2022;22(1):333.
24. Bruna F, Arango-Rodríguez M, Plaza A, Espinoza I, Conget P. The administration of multipotent stromal cells at precancerous stage precludes tumor growth and epithelial dedifferentiation of oral squamous cell carcinoma. Stem Cell Res. 2017;18:5-13.
25. Karthikeyan S, Manoharan S. Cromolyn inhibits 7, 12-dimethylbenz (a) anthracene induced oral cancer through apoptotic induction and suppression of cell proliferation. Int J Pharm Bio Sci. 2016; 7(1):35-42.
26. Liu S, Klein-Szanto A. Markers of proliferation in normal and leukoplakic oral epithelia. Oral Oncol. 2000 ;36(2):145-51.
27. Sophia J. Nimbolide, a neem limonoid inhibits Phosphatidyl Inositol-3 Kinase to activate Glycogen Synthase Kinase-3β in a hamster model of oral oncogenesis. Sci Rep. 2016;6(1):1-13.
28. Rundhaug J, Fischer S. Molecular mechanisms of mouse skin tumor promotion. Cancers. 2010;2(2):436-82.
29. Kathiresan S, Mariadoss A, Muthusamy R, Kathiresan S. Protective Effects of [6]-Shogaol on Histological and Immunohistochemical Gene Expression in DMBA Induced Hamster Buccal Pouch Carcinogenesis. Asian Pac. J. Cancer Prev., 14 (2013), pp. 3123-29.
30. Manoharan S, Sindhu G, Nirmal M, Vetrichelvi V, Balakrishnan S. Protective effect of berberine on expression pattern of apoptotic, cell proliferative, inflammatory and angiogenic markers during 7, 12-dimethylbenz (a) anthracene induced hamster buccal pouch carcinogenesis. Pak J Biol Sci. 2011;14(20):918-32.
31. Manoharan S, Rajasekaran D, Prabhakar MM, Karthikeyan S, Manimaran A. Modulating effect of Enicostemma littorale on the expression pattern of apoptotic, cell proliferative, inflammatory and angiogenic markers During 7, 12-Dimethylbenz (a) anthracene induced hamster buccal pouch carcinogenesis. Toxicol Int. 2015;22(1):130-40.

32. Al-Dosoki M, Mansour A, Ahmed M. Effect of oxygenated water as a new treatment modality on experimentally induced hamster buccal pouch carcinogenesis. Al-Azhar J Dent Sci.2018;21(3):261-73.
33. Botti E, Spallone G, Moretti F, Marinari B, Pinetti V, Galanti S, et al. Developmental factor IRF6 exhibits tumor suppressor activity in squamous cell carcinomas. Developmental factor IRF6 exhibits tumor suppressor activity in squamous cell carcinomas. Proc Natl Acad Sci U S A. 2011;108(33):13710-15.
34. Tsuji T, Mimura Y, Wen S, Li X, Kanekawa A, Sasaki K, et al. The significance of PCNA and p53 protein in some oral tumors. Int J Oral Maxillofac Surg. 1995;24(3):221-25.
35. Kaur M, Saxena S, Bansal P. Expression of proliferating cell nuclear antigen in normal oral mucosa, oral submucous fibrosis and leukoplakia with or without dysplasia. Int J Oral Med Sci. 2011;10(3):149-55.
36. Baldasquin-Caceres B, Gomez-Garcia F, López-Jornet P, Castillo-Sanchez J, Vicente-Ortega V. Chemopreventive potential of phenolic compounds in oral carcinogenesis. Arch Oral Biol. 2014;59(10):1101-07.
37. Silvan S, Manoharan SJAoob. Apigenin prevents deregulation in the expression pattern of cell-proliferative, apoptotic, inflammatory and angiogenic markers during 7, 12-dimethylbenz [a] anthracene-induced hamster buccal pouch carcinogenesis. Arch Oral Biol.2013;58(1):94-101.
38. Mallick S, Agarwal J, Kannan S, Pawar S, Kane S, Teni T. PCNA and anti-apoptotic Mcl-1 proteins predict disease-free survival in oral cancer patients treated with definitive radiotherapy. Oral Oncol.2010;46(9):688-93.
39. Lawall M, Crivelini M. PCNA and p53 expression in oral leukoplakia with different degrees of keratinization. J Appl Oral Sci. 2006;14:276-80.
40. Kaplan I, Hochstadt T, Dayan D. PCNA in palate and tongue mucosal dysplastic lesions induced by topically applied 4NQO in desalivated rat. Med Oral.2002;7(5):336-43.
41. Gómez-García F, López López A, Guerrero-Sánchez Y, Sánchez Siles M, Martínez Díaz F, Camacho Alonso F. Chemopreventive effect of pomegranate and cocoa extracts on ultraviolet radiation-induced photocarcinogenesis in SKH-1 mice. PLoS One. 2020;15(4):e0232009.
42. Afaq F, Saleem M, Krueger C, Reed J, Mukhtar H. Anthocyanin-and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-κB pathways and inhibits skin tumorigenesis in CD-1 mice. Int J Cancer.2005;113(3):423-33.
43. Mandal A, Bishayee A. Mechanism of breast cancer preventive action of pomegranate: disruption of estrogen receptor and Wnt/β-catenin signaling pathways. Molecules.2015;20(12):22315-28.
44. Kim N, Mehta R, Yu W, Neeman I, Livney T, Amichay A, et al. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast Cancer Res Treat.2002;71:203-17.
45. Mehta R, Lansky E. Breast cancer chemopreventive properties of pomegranate (Punica granatum) fruit extracts in a mouse mammary organ culture. Eur J Cancer Prev.2004;13(4):345-8.
46. Ghani R, Malek N, Ashari N, Abdullah N. Pomegranate juice induced cell cycle arrest and apoptosis via mitochondrial pathway in human lung adenocarcinoma A549 cells. Inte J Eng Technol,2018;7:287-92.
47. Tang J, Min J, Li B, Hong S, Liu C, Hu M, et al. Therapeutic effects of punicalagin against ovarian carcinoma cells in association with β-catenin signaling inhibition. Int J Gynecol Cancer. 2016;26(9):1557-63.
48. Afaq F, Khan N, Syed D, Mukhtar H. Oral feeding of pomegranate fruit extract inhibits early biomarkers of UVB radiation-induced carcinogenesis in SKH-1 hairless mouse epidermis. Photochem Photobiol.2010;86(6):1318-26.
49. Khan N, Syed D, Pal H, Mukhtar H, Afaq F. Pomegranate fruit extract inhibits UVB-induced inflammation and proliferation by modulating NF-κB and MAPK signaling pathways in mouse skin. Photochem Photobiol.2012;88(5):1126-34.
50. Zahin M, Ahmad I, Gupta R, Aqil F. Punicalagin and ellagic acid demonstrate antimutagenic activity and inhibition of benzo [a] pyrene induced DNA adducts. Biomed Res Int. 2014:2014:467465.