Anticancer effects of punicalagin and 5-fluorouracil on laryngeal squamous cell carcinoma: an *in vitro* study

Amany M. Taha,[1] Eman Abd-Elshafy,[2] Asmaa M. Khalifa,[2] Najla Dar-Odeh,[3] Shadia Elsayed,[1,4] Daniah S. Alharkan,[4] Aishah Alhodhodi,[4] Ahmed Mohammed Sapri,[5] Yassmeen SalahEldin Ragheb,[6] Gihan A Balbola[1]

[1]Department of Oral and Dental Pathology, Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt; [2]Department of Botany and Microbiology, Faculty of Science (Girls), Al Azhar University, Cairo, Egypt; [3]College of Dentistry, Taibah University, Al-Madinah Al-Munawwarah, Saudi Arabia; [4]Department of Oral and Maxillofacial Diagnostic Sciences, College of Dentistry, Taibah University, Al-Madinah Al-Munawwarah, Saudi Arabia; [5]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Mansoura University, Egypt; [6]Department of Clinical Pharmacy, Al-Munirah General Hospital, Ministry of Health, Cairo, Egypt

Correspondence: Najla Dar-Odeh, School of Dentistry, The University of Jordan, Amman, Jordan, and College of Dentistry, Taibah University, Almadinah Almunawwarah, Madinah, Saudi Arabia.
E-mail: najla@ju.edu.jo

Key words: laryngeal squamous cell carcinoma; Hep-2 cell line; punicalagin; caspase-3; vascular endothelial growth factor.

Acknowledgments: the authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the scientific research department at Taibah University.

Contributions: AMT, EAE, conceptualization, AMT, GAB, methodology; EAE, AMK, AMS, investigation; NDO, SE, writing, review and editing; DSA, AA, YSER, writing original draft; AMS, resources; GAB, supervision. All the authors approved the final version to be published.

Funding: none.

Conflict of interest: the authors declare no potential conflict of interest.

Ethical approval and consent to participate: this study has received ethical approval from Human Research Ethics Committee of Al Azhar University for Girls, Faculty of Dental Medicine, code # P-PD-23-07.

Availability of data and material: data and materials are available by the authors.

Informed consent: the manuscript does not contain any individual person's data in any form.

Received: 1 February 2024.
Accepted: 1 February 2024.

Publisher's note: all claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.

Licensee PAGEPress, Italy
Italian Journal of Medicine 2024; 18:1699
doi:10.4081/itjm.2024.1699

ABSTRACT

The purpose of this study was to assess the apoptotic effects of punicalagin alone and in combination with 5-fluorouracil (5-FU) on laryngeal squamous cell carcinoma (Hep-2) cell line. Hep-2 cells were cultured and divided into four groups: Group 1 received no therapy and served as control, Group 2 received 5-FU only, Group 3 received punicalagin only, and Group 4 received a combination of 5-FU and punicalagin. After 48 hours of incubation, cellular changes were examined under an inverted microscope. The methyl thiazolyl tetrazolium assay, caspase-3 gene level, and vascular endothelial growth factor (VEGF) level were assessed. The control group showed the highest mean value of cancer cell proliferation rate (1.595±0.58), followed by the punicalagin group (1.263±0.447), then the 5-FU group (0.827±0.256), while the combination group showed the lowest proliferation rate (0.253±0.111). The combination group showed the highest mean value of caspase-3 concentration (3.177±0.736), followed by the 5-FU group (1.830±0.646), and punicalagin group (0.741±0.302), while the control group showed the lowest mean value (0.359±0.117). Regarding VEGF levels, the control group had a statistically significant higher mean value, followed by the punicalagin and 5-FU groups, and finally, the combination group which showed the lowest value. Punicalagin exerts an anticancer effect through anti-proliferative action and induction of apoptosis on Hep-2 cell line. Combining punicalagin with 5-FU potentiates its anti-proliferative, apoptotic, and anti-angiogenic actions. It, further, helps in mitigating the putative side effects of 5-FU by reducing the dose required for its therapeutic effects.

Introduction

Head and neck cancers are a heterogeneous group of cancers. Most of them derive from the epithelium of the mucosal lining of the mouth, pharynx, and larynx.[1] Ranking the sixth among the most frequently diagnosed carcinomas globally,[2] a high annual incidence rate of 30% of head and neck squamous cell carcinoma (HNSCC) is reported, with approxi-

mately 890,000 new cases, and 450,000 deaths identified yearly.[3] Laryngeal squamous cell carcinoma (LSCC) is the second most common type of head and neck cancer,[4] and it constitutes 95% of laryngeal cancer cases.[2] Disease outcomes are influenced by the disease stage, wherein patients with stage IV have a 5-year survival rate of 40%, however, a cure rate of 80-90% can be achieved in stage I/II patients.[5] Therefore, early detection and timely initiation of treatment contribute greatly to improving disease outcomes. A multimodal approach is adopted in management including surgery, radiation therapy, and chemotherapy; however, concurrent chemoradiotherapy is considered the primary mode of treatment for LSCC, and it is the standard treatment option for locally advanced laryngeal cancers (stage III/IV).[1] Aims behind chemotherapy in the context of laryngeal cancer are systemic disease cytoreduction; and/or locoregional radiosensitization.[6] Nevertheless, success rates of chemotherapy may be jeopardized by the lack of specificity leading to recurrence and metastasis, worsening the prognosis.[7] Therefore, targeted chemotherapy could reduce complications of adverse effects on normal tissues and insufficient therapeutic doses in tumor areas.[8] Moreover, combination therapies have shown promising efficacy in cancer therapy.[8]

5-fluorouracil (5-FU) is a commonly used chemotherapeutic drug for the treatment of laryngeal cancer, and it has been widely used alone or in combination with other anti-cancer drugs to treat cancer for many decades.[9] However, the therapeutic efficacy of 5-FU is limited by dose-dependent toxicities and chemoresistance, which is associated with reduced curative potential, constituting a major obstacle to conservative cancer chemotherapy. To overcome these problems, improving the utilization ratio of 5-FU and combining it with other anti-cancer drugs with different mechanisms of action could be promising strategies to enhance its anti-cancer efficiency. Employing naturally derived agents in cancer chemotherapy was extensively investigated,[10] and recommended due to the associated therapeutic efficacy and minimal side effects.[11]

The chemo-preventive and antitumor effects of pomegranate were documented in previous studies,[12] for the treatment of various types of cancers.[13] Punicalagin (2,3-hexahydroxydiphenoylgallagyl-D-glucose) is the abundant bioactive tannin compound isolated from pomegranate. It is highly bioavailable,[14] and it shows antioxidant, antiproliferative, antiviral, anti-inflammatory, and anticancer properties.[15-18] It was also shown to induce apoptosis in leukemic cells, colon cancer lines and glioma cells.[15] The majority of research on the therapeutic efficacy of dietary antioxidants for cancer is derived from epidemiological studies. Only a few studies have investigated the anti-cancer efficacy of punicalagin on HNSCC. Therefore, this study was conducted to evaluate the antitumor effects of punicalagin alone or in combination with 5-FU on LSCC represented by the Hep-2 cell line. Effects were assessed by determining the morphological antiproliferative effects, VEGF levels, and caspase-3 concentrations.

Materials and Methods

This study has received ethical approval from the Human Research Ethics Committee of Al Azhar University for Girls, Faculty of Dental Medicine, code # P-PD-23-07.

Cell line and culture setting

Biological products & vaccines Holding Company in Egypt (VACSERA) provided the Hep-2 cell line. Cells were imported from the American type culture collection in the form of a frozen vial. All procedures were carried out in a laminar flow hood with perfect aseptic conditions, at 37°C and 7.4 pH values, utilizing T25 flasks with 10% heat-inactivated fetal bovine serum, 2% sodium bicarbonate, and 2% streptomycin. Penicillin was used to cultivate the cells in Dulbecco's Modified Eagle Medium, which was then incubated at 37°C and 5% CO2.

Experimental groups

Punicalagin and 5-FU were purchased and supplied to the national center of research in Egypt from Sigma Aldrich in Munich. In a concentration of 5 mg/ml, punicalagin was dispersed in methanol, and 5g of 5-FU powder was supplied in a vial with a molecular weight of 130.1 g/ mole and a molecular formula of C4H3FN2O2.

Four experimental groups of Hep-2 cell line were divided as follows: Group 1 received no treatment and served as control; Group 2 received 5-FU (5mg/ml) alone; Group 3 received punicalagin (20 µg/ml) alone, and Group 4 received punicalagin (20 µg/ml) and 5-FU (5mg/ml).

Proliferation assay

The methyl thiazolyl tetrazolium (MTT) assay determines cell viability by detecting the optical absorbance caused by a change in formazan concentration as a result of altered mitochondrial activity. Reagents of proliferation assay kit were purchased from Sigma Aldrich which included: MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) 15 mg/ml serum vial and MTT solubilization solution 10% Triton X-100 plus 0.1 NHCl in anhydrous isopropanol, 125 ml was used for cytotoxicity testing.

The test is based on metabolically active cells reducing yellow tetrazolium salt (MTT) to purple formazan crystals by the action of NADH-dependent oxidoreductase enzymes present in living cells. An enzyme-linked immunosorbent assay (ELISA) plate reader is used to measure absorbance when the insoluble crystals are dissolved using a solubilization solution; the more metabolically active cells present, the darker the color of the resulting solution. For the assay cells were placed in the form of $5x10^3$ cells per well and grown to >80% confluence before adding treatment as indicated in the experimental groups. Viable cells were determined 48 hours later by the MTT assay.

For the MTT assay, one ml of Hep-2 cells ($5x10^4$ to $1x10^5$ cells) was plated into each well of a 96-well culture plate for 24 hours. Each test should include a blank containing a complete medium without cells. Cells were incubated for 24h in CO_2 incubator. Media were carefully discarded and plates were washed in phosphate-buffered saline. Punicalagin and 5-FU were separately added to Hep-2 cell line with predetermined concentrations in experimental groups and plates were incubated for 48h. Media were carefully aspirated from all plates. Plates were filled with 50 µL of serum-free media and 50 µL of MTT solution. The plates were incubated for three hours at 37°C. 150 µL of MTT solvent were added to each well following incubation. For 15 minutes, the plates were

shaken on a shaker with foil covering them to completely dissolve the MTT formazan. Within an hour, the absorbance at optical density (OD) =590 nm was measured. By dividing the total OD of the column wells by the number of wells, the mean OD of each column on the 96-well plate was determined. To calculate the percentage of viability and cytotoxicity for each concentration treatment, the mean OD of each column with a particular concentration treatment was divided by the mean of the untreated control cells.

Quantitative real-time polymerase chain reaction

For evaluating the caspase-3 gene and the amounts of nucleic acids (DNA, cDNA, and RNA) quantitative real-time polymerase chain reaction (qRT-PCR) was used. This is achieved by quantification and detection of fluorescence emitted from a reporter molecule in real-time. With every cycle of amplification, the PCR product accumulates and is detected. An applied biosystem with software version 3.1 (Step OneTM, USA) was used for the qRT-PCR amplification and analysis. This assay was performed as explained by Overbergh *et al.* using the same reagents and kits.[19]

Enzyme-linked immunosorbent assay technique for assessment of vascular endothelial growth factor

The human VEGF ELISA kit was used following the manufacturer's instructions. The human VEGF level can be measured quantitatively using *in vitro* quantitative sandwich enzyme-linked immunosorbent assay. The protocol for the human VEGF ELISA kit was done in the following steps: i) cells were grown as mentioned in experimental groups for 48 hours; ii) supernatants were collected from each group and placed in 96 well plates for ELISA assays. Supernatants were added to appropriate wells; iii) after washing the plates, a prepared biotin antibody was added to every well; iv) after washing the plates, the ready streptavidin horseradish peroxidase enzyme (HRP) conjugate solution was added; v) each well received an addition of tetra-methyl benzidine one-step development solution; vi) each well received a dose of stop solution. Plates were immediately read at 450 nm.

Statistical analysis

The statistical package for social science V23.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The ranges and mean ± standard deviation were calculated for the quantitative data. Frequencies and percentages were also used to represent qualitative variables. A one-way analysis of variance (ANOVA) and the Post Hoc test were used to compare group means. Multiple comparisons between various variables were conducted using Tukey's test.

Results

Morphological assessment

The cells were examined under the inverted microscope phase to assess their viability and possible morphological changes that occurred after exposure to punicalagin and 5-FU in the four groups. Expected morphological changes during apoptotic cell death include nuclear shrinkage and fragmentation, chromatin condensation, and irregular nuclear outline. In group I (control group), Hep-2 cells showed a monolayer of densely packed cohesive slightly elongated fusiform and angular malignant cells. In group 2, there was a reduced number of viable malignant cells with obvious necrotic areas and a significant number of cells showing morphological changes and appearing rounded with decreased size (shrunken) which indicated apoptotic changes. No obvious changes were detected in group 3 when compared to the control group with small areas of necrosis and a slight increase in apoptotic cell numbers. The number of apoptotic cells increased in group 4 with large areas of necrosis (Figures 1-4).

Cell proliferation evaluation by methyl thiazolyl tetrazolium assay

After 48h of incubation, the ANOVA test revealed a statistically significant difference between groups ($P<0.001$) (Table 1). The control group showed the highest mean value of Hep-2 cell proliferation, followed by punicalagin, then 5-FU, with the least mean value recorded in punicalagin-5-FU combination.

Caspase-3 concentration

The concentration of caspase-3 was measured in different groups to assess the apoptotic effect. There was a highly statistically significant difference ($P<0.001$) between groups, with the combination group showing the highest mean value, followed by punicalagin alone, and the least mean value identified in the control group. The efficacy of the punicalagin-5-FU group was 173.6% more than 5-FU alone (Table 1).

Table 1. Values of cell viability, vascular endothelial growth factor and caspase-3, and statistical significance of difference among experimental groups.

Groups		Cell viability (µg/ml) Mean±SD	VEGF Mean±SD	Caspase-3 (µg/ml) Mean±SD
Control group		1.595±0.582 A	52.110±16.423 A	0.359±0.117 D
5-FU		0.827±0.256 C	28.459±4.091 C	1.830±0.646 B
Punicalagin		1.263±0.447 B	34.348±7.060 B	0.741±0.302 C
Punicalagin + 5-FU		0.253±0.111 D	13.940±2.486 D	3.177±0.736 A
ANOVA	F-test	21.851	29.139	60.110
	P-value	<0.001**	<0.001**	<0.001**

SD, standard deviation; VEGF, vascular endothelial growth factor; 5-FU, 5-fluorouracil.

IV-vascular endothelial growth factor level

A highly statistically significant difference is noted between groups for the VEGF levels with the highest VEGF mean value in the control group, followed by punicalagin group, 5-FU group, then punicalagin+ 5-FU, which showed the lowest value (P<0.001) (Table 1).

Discussion

This study explored the potential antitumor efficacy of punicalagin and 5-FU when used on the Hep-2 cell line. Previous research highlighted the antitumor roles of punicalagin in a few types of cancer. Within the context of laryngeal cancer, one study concluded that cytotoxicity of punicalagin is only achieved at higher concentrations;[20] however, there were no conclusive results. The laryngeal squamous cell carcinoma (Hep-2) cell line was used as a model similar to other studies because it is widely used to represent head and neck malignancies as squamous cell carcinoma of the larynx that arises from the intra-oral site and has similar genetic phenotypes.

This study explored the antitumor effects by morphological assessment, and evaluating caspase 3 and VEGF levels. We used punicalagin with constant concentration (20 µg/ml ≈ 18.5 µM). Tang *et al*. used punicalagin with increasing concentrations of 0, 12.5, 25, 50, 100, and 200 µM, for 24, 36, and 48h in the treatment of HeLa cells to evaluate cell viability.[15] They found that after 48h inhibition of the viability of HeLa cells *in vitro* became statistically significant at 12.5 µM. Similarly, Seeram *et al.* found that punicalagin, ellagic acid, a total pomegranate tannin extracts reduced cell proliferation in human oral, prostate, and colon cell lines in a dose-dependent manner ranging from 12.5 to 100 g/ml.[21]

In order to clarify the punicalagin anti-carcinogenic effect in comparison to 5-FU, morphological changes of Hep-2 cells were assessed under the inverted phase microscope. According to Sangour *et al.*, cell viability was assessed at 48 hours to enable adequate time for the substance to inhibit or kill the cells.

Regarding morphological changes of cancerous cells, signs of apoptosis in treated groups were identified such as cell body shrinkage, reduction in cell volume, and the detection of a circular shape, as well as nuclear shrinkage, frag-

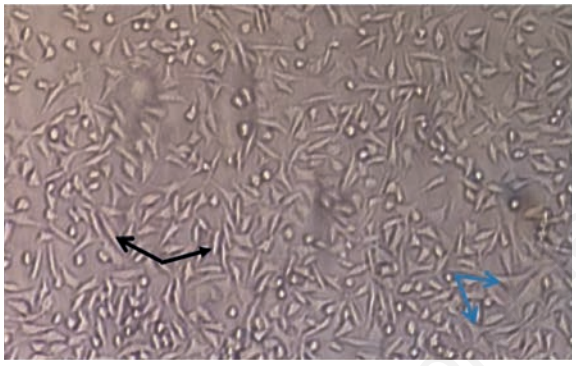

Figure 1. Photomicrograph of Hep-2 cells (control group) taken with an inverted phase microscope revealing a large proportion of viable elongated fusiform cells (black arrows) and angular cells (blue arrows) (original magnification X20).

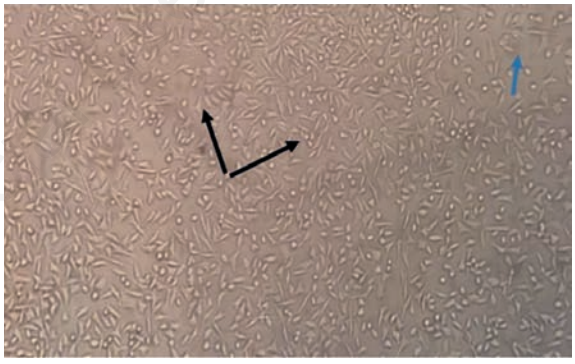

Figure 3. Inverted phase microscope photomicrograph of Hep-2 cells after addition of punicalagin (III) showing many fusiform and stellate angular cells (black arrows) and small area of necrosis (blue arrow) (original magnification X10).

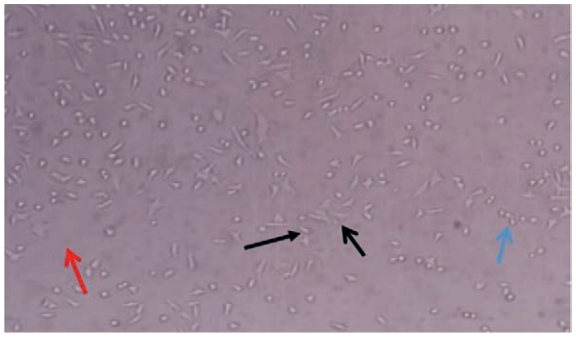

Figure 2. Photomicrograph of Hep-2 cells after addition of 5-fluorouracil (group II) revealing a decrease in viable cells (black arrows) and a change in cell morphology (rounded cells) (blue arrow) as well as areas of necrosis (red arrow) (original magnification X10).

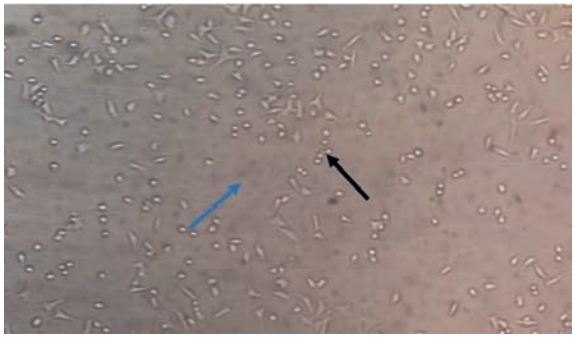

Figure 4. Photomicrograph of Hep-2 cells following addition of punicalagin with 5-fluorouracil (group 4) showing numerous cells experiencing apoptosis with change in cell shape (rounded cell) (black arrow) and increasing areas of necrosis (blue arrow) (original magnification X20).

mented irregular outline, and chromatin condensation and margination. These signs were markedly noticed in the punicalagin+ 5-FU treated group followed by 5-FU and punicalagin monotherapy groups, respectively. These changes were not detected in the control group. Similarly, Li *et al.* found the same morphological changes after treating thyroid cancer cell lines with pomegranate (punica granatum) extract.[22] In the current study, the highest mean value of cell proliferation assay was identified in the control group, followed by punicalagin, and finally, 5-FU while the least mean value was recorded in the combination group. This indicates that punicalagin amplifies the anti-proliferative effect of 5-FU. Punicalagin with a high 5-FU concentration induced the highest cytotoxic effect with the lowest percentage detected for viable and proliferating cells. This could be attributed to the antioxidant effect of punicalagin, which eventually undergoes reactive oxygen species modulation in cancer cells.[23] Furthermore, Mirzaei *et al*. reported that Punicalagin was capable of reducing oxidative phosphorylation and glycolysis leading to autophagy of cancer cells.[24] 5-FU is a widely used chemotherapeutic medication that has remained an important treatment for a variety of solid tumors, including LSCC. However, the systemic use of 5-FU as chemotherapy is associated with considerable toxic side effects and the development of drug resistance. The main mechanisms of action of 5-FU include the inhibition of thymidylate synthase activity, DNA synthesis, and DNA repair via the incorporation of its metabolites into the DNA/RNA of cancer cells.[25] All of these activities could ultimately lead to apoptosis.[9] Punicalagin was previously used as an adjuvant chemo-preventive agent in combination with 5-FU, to reduce the 5-FU dose, thereby, decreasing its toxic side effects and improving treatment outcomes.[26]

In the current investigation, the antiangiogenic efficacy was assessed by evaluating VEGF levels which is used to evaluate the angiogenic potential of cancer cells. The greatest mean value for VEGF was recorded in the control group, followed by punicalagin, then 5-FU, with the lowest mean value recorded in the Punicalagin+ 5-FU group, indicating that punicalagin enhances the anti-angiogenic activity of 5-FU. Punicalagin has also shown promise as an adjuvant treatment with 5-fluorouracil (5-FU) to decrease its negative effects. In rats and human beings, Souchon *et al*. (1975) observed that intravenous hyperalimentation, a method of delivering nutrients straight into the bloodstream, reduced the gastrointestinal toxicity of 5-FU.[27] This suggested that punicalagin, which protects against diabetes and brain impairment could also help to minimize the adverse effects of 5-FU.[28] Several investigations have demonstrated that the combination of punicalagin with 5-fluorouracil has promising antitumor effects. Anitha concluded that 5-FU and curcumin, a chemical related to punicalagin, have improved anticancer effects in colon cancer.[29] Ghiringhelli also emphasized 5-FU's ability to improve immune responses and remove immunosuppressive cells, indicating a possible synergy with punicalagin.[30] Both Huang *et al*. and Suruli *et al.* showed that punicalagin can decrease cancer cell growth and invasion, in addition to lowering inflammatory cytokines which support its potential as a supplemental treatment to 5-FU.[31,32] In both Liu *et al*. and Tang *et al*. studies, punicalagin caused apoptosis and limited proliferation in human cancer cells, including HepG2 hepatoma cells and HeLa cervical cancer cells.[15,22] These effects were linked to mitochondrial targeting and suppression of the catenin pathway, respectively.[15] Yan *et al.* and Zhang *et al*. demonstrated punicalagin's ability to protect against lipotoxicity, a condition linked with nonalcoholic fatty liver disease, by activating the Keap1-Nrf2 antioxidant defense system and the SIRT1/autophagy pathway.[28,33]

Conclusions

These findings emphasize that punicalagin has a broad therapeutic potential in cancer management. Punicalagin exerts an anticancer effect through anti-proliferative action and induction of apoptosis on the Hep-2 cell line. The addition of punicalagin to 5-FU potentiates its anti-proliferative, apoptotic, and anti-angiogenic actions. Punicalagin could be used as adjunctive treatment with 5-FU to diminish its side effects.

References

1. Johnson DE, Burtness B, Leemans CR, et al. Head and neck squamous cell carcinoma. Nat Rev Dis Primer 2020;6.
2. Gao P, Gong L, Wang X. Induction chemotherapy in patients with resectable laryngeal cancer: A meta-analysis. Mol Clin Oncol 2018;9:155-62.
3. Li Z, Li T, Zhang P, Wang X. A practical online prediction platform to predict the survival status of laryngeal squamous cell carcinoma after 5 years. Am J Otolaryngol 2024;45:104209.
4. Gamez ME, Blakaj A, Zoller W, et al. Emerging Concepts and Novel Strategies in Radiation Therapy for Laryngeal Cancer Management. Cancers 2020;12:1651.
5. Megwalu UC, Sikora AG. Survival outcomes in advanced laryngeal cancer. JAMA Otolaryngol Head Neck Surg 2014;140:855-60.
6. Haigentz MJr, Silver CE, Hartl DM, et al. Chemotherapy regimens and treatment protocols for laryngeal cancer. Expert Opin Pharmacother 2010;11:1305-16.
7. Iizuka Y, Yoshimnura M, Inokuchi H, et al. Recurrence patterns after postoperative radiotherapy for squamous cell carcinoma of the pharynx and larynx. Acta Otolaryngol (Stockh.) 2015;135:96-102.
8. Wang D, Yu D, Liu X, et al. Targeting laryngeal cancer cells with 5-fluorouracil and curcumin using mesoporous silica nanoparticles. Technol Cancer Res Treat 2020;19: 1533033820962114.
9. Echanique KA, Evans LK, Han AY, et al. Cancer of the Larynx and Hypopharynx. Head Neck Cancer 2021; 35:933-47.
10. Demain AL, Vaishnav P. Natural products for cancer chemotherapy. Microb Biotechnol 2011;4:687-99.
11. Iqbal J, et al. Plant-derived anticancer agents: A green anticancer approach. Asian Pac J Trop Biomed 2017;7: 1129-50.
12. Sharma P, McClees SF, Afaq F. Pomegranate for Prevention and Treatment of Cancer: An Update. Molecules 2017;22:177.
13. Syed DN, Chamcheu JC, Adhami VM, Mukhtar H. Pomegranate extracts and cancer prevention: Molecular and cellular activities. Anticancer Agents Med Chem 2013;13:1149-61.
14. Lin LT, Chen TY, Lin SC, et al. Broad-spectrum antiviral

activity of chebulagic acid and punicalagin against viruses that use glycosaminoglycans for entry. BMC Microbiol 2013;13:187.
15. Tang J, Li B, Hong S, et al. Punicalagin suppresses the proliferation and invasion of cervical cancer cells through inhibition of the β-catenin pathway. Mol Med Rep 2017;161439-44.
16. Lee CJ, Chen LG, Liang WL, Wang CC. Anti-inflammatory effects of Punica granatum Linne in vitro and in vivo. Food Chem 2010;118:315-22.
17. Bialonska D, Ramnani P, Kasimsetty SG, et al. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. Int J of Food Microbiol 2010;140:175-82.
18. Adams LS, Seeram NP, Aggarwal BB, Y, et al. Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. J Agric Food Chem 2006;54:980-5.
19. Overbergh L, Giulietti A, Valckx D, et al. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. J Biomol Tech 2003;1:33-43.
20. Kulkarni AP, Mahal HS, Kapoor S, Aradhya SM. In Vitro Studies on the Binding, Antioxidant, and Cytotoxic Actions of Punicalagin. J Agric Food Chem 2007;55:1491-500.
21. Seeram NP, Adams LS, Henning SM, et al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 2005;16:360-67.
22. Liu W, Ou Y, Yang Y, et al. Inhibitory Effect of Punicalagin on Inflammatory and Angiogenic Activation of Human Umbilical Vein Endothelial Cells. Front Pharmacol 2021;12.
23. Cao D, Zhu G-Y, Lu Y, et al. Luteolin suppresses epithelial-mesenchymal transition and migration of triple-negative breast cancer cells by inhibiting YAP/TAZ activity. Biomed Pharmacother 2020;129:110462.
24. Mirzaei S, Zarrabi A, Hashemi F, et al. Nrf2 Signaling Pathway in Chemoprotection and Doxorubicin Resistance: Potential Application in Drug Discovery. Antioxidants 2021;10.
25. Odell E, et al. European Laryngological Society position paper on laryngeal dysplasia Part I: aetiology and pathological classification. Eur Arch Otorhinolaryngol 2021; 278:1717-22.
26. Berdowska I, Matusiewicz M, Fecka I. Punicalagin in Cancer Prevention—Via Signaling Pathways Targeting. Nutrients 2021;13.
27. Souchon EA, Copeland EM, Watson P, Dudrick SJ. Intravenous hyperalimentation as an adjunct to cancer chemotherapy with 5-fluorouracil. J Surg Res 1975; 18:451-4.
28. Zhang L, Chinnathambi A, Alharbi SA, et al. Punicalagin promotes the apoptosis in human cervical cancer (ME-180) cells through mitochondrial pathway and by inhibiting the NF-kB signaling pathway. Saudi J Biol Sci 2020;27:1100-6.
29. Anitha A, Sreeranganathan M, Chennazhi KP, et al. In vitro combinatorial anticancer effects of 5-fluorouracil and curcumin loaded N,O-carboxymethyl chitosan nanoparticles toward colon cancer and in vivo pharmacokinetic studies. Eur J Pharm Biopharm 2014;88:238-51.
30. Ghiringhelli F, Apetoh L. Enhancing the anticancer effects of 5-fluorouracil: current challenges and future perspectives. Biomed J 2015;38:111-6.
31. Huang T, Zhang X, Wang H. Punicalagin inhibited proliferation, invasion and angiogenesis of osteosarcoma through suppression of NF-κB signaling. Mol Med Rep 2020;22:2386-94.
32. Suruli D, Mirza FBS, Kottaiswamy GJCRA, et al. Naringin and 5-fluorouracil suppress inflammatory Cytokines in human skin cancer cell line. Int J Res Pharm Sci 2021;12:729-33.
33. Yan C, et al. Punicalagin attenuates palmitate-induced lipotoxicity in HepG2 cells by activating the Keap1-Nrf2 antioxidant defense system. Mol Nutr Food Res 2016;60: 1139-49.