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ORIGINAL ARTICLE

Antitumoral properties of the pomegranate peel and blueberry extracts against tongue carcinoma (*in vitro* study)

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KEYWORDS

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Cancer treatment;
Cell proliferation;
Metastasis;
Apoptosis

Abstract *Background:* Oral squamous cell carcinoma (OSCC) is one of the most common cancers globally. Considering the drawbacks of the traditional treatment phytochemicals have been introduced to the research field with consideration of their minimal, or no side effects and good efficacy against cancer cells. Pomegranate peel and blueberries are very well-known phytochemicals in this context.

Material and methods: Methanolic extracts are prepared from both pomegranate peel (PPE) and blueberry (BE) dried powders. The antioxidant content was determined by ORAC assay for both extracts. The cytotoxic levels of the extracts and IC50 on the HNO-97 cell line were defined using the colorimetric SRB assay. Then flow cytometric apoptosis analysis of the IC50 of both extracts was performed. Finally, an investigation of the metastasis through the wound healing assay was performed for both extracts.

Results: A significant difference in the antioxidant content was found between PPE and BE ethanolic extracts. The Ic50 for the PPE-treated cell line was 73.35 μg/ml while the BE showed it at 525.38 μg/ml with a significant difference between them. Both PPE and BE showed significant induction of cancer cell apoptosis with much better results with PPE treatment. The wound healing assay showed significant inhibition of cell migration when treated with PPE while there was not any significant effect on cell migration when treated with BE.

Conclusion: With the consideration that, the Phytochemicals used are well-known fruits with no harm on normal tissues. Low doses of PPE exert incredibly significant alteration in the HNO-97 tongue cancer cell proliferation, inducing apoptosis and inhibition of cancer cell migration. Meanwhile, treatment with BE needs much higher doses for showing anti-proliferative properties of can-

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cer cells and induce cancer cell apoptosis. This gives promising results for further investigations about using them as a treatment or adjunctive treatment for oral cancer cases.

1. Introduction

Cancer is an intimidating disease that has spread widely in recent days (Balwan et al., 2021). Oral squamous cell carcinoma (OSCC) is the 11th most common cancer forming 90% of detected oral cancers (Tomita, 2022). The dilemma with oral cancer (OC) is that most cases are detected at late stages, costing the country (Amarasinghe et al., 2019). The treatment of OSCC includes surgical intervention, chemotherapy, and radiotherapy (Petrosyan et al., 2019). Complications include dysgeusia and xerostomia associated with salivary gland dysfunction, infectious diseases, and oral mucositis. Regarding these adverse effects, the need for therapeutic modalities without side effects has emerged using novel approaches.

After *in vitro* studies have been introduced to the cancer research field, they gave many advantages in studying the responses to specific drugs (Maeser et al., 2021). These studies present fast screening for the behavior of certain cancers in particular situations such as cell death, metastasis, and even the genes and pathways responsible for these behaviors (Pijuan et al., 2019).

Precision medicine was introduced to OC treatment as a treatment modality. Phytochemicals are the naturally occurring plant-based compounds that can be used as a whole or derivative in cancer therapy (Choudhari et al., 2020). The minimal or no adverse effects of these drugs on normal tissues beside their good efficacy have been a strength point in research work. Some phytochemicals were studied as chemotherapeutic agents as pomegranate fruit extracts (Khwairakpam et al., 2018), blueberry extracts (Davidson et al., 2018) Graviola extract (Ioannis et al., 2015), capsaicin (Mao et al., 2018), resveratrol (Huminiecki & Horbańczuk, 2018).

Pomegranate is one species belonging to the Punica Genus which is in the family of Lythraceae (Puniceae) (Das & Barman, 2012). It is a rich plant in phytochemicals and nutrients. The peel form about 50% of the fruit's total mass, and this part is usually omitted as agricultural wasting (Usha et al., 2020). Phytochemicals present in the peel include tannins (punicalin, pedunculagin, gallic acid, punicalagin, and casuarinin), flavonoids, alkaloids, and polyphenolic acids. This content gives the peel the superior anti-oxidative property (Rahmani et al., 2017). These fruit aspects to the antioxidant, anti-bacterial, anti-viral, antibacterial, and anti-neoplastic effects of the pomegranate peel (PPE) (Khwairakpam et al., 2018). Many studies have been conducted to study the outcome of PPE on different carcinomatous cell lines. Breast cancer is the most commonly investigated cancer using different types of cell lines MDA-MB-231 cells, MCF-7, HepG-2 and PC-3 (Badawi et al., 2018; Bagheri et al., 2018; Ahmadiankia et al., 2018). coming to the colorectal cancer, in-vitro studies using HT-29 CRC cell line and in vivo design on dark agouti rats and Apc-mutated Pirc rats' experiments have been done (Chen et al., 2018; Keta et al., 2020). In addition, ovarian carcinoma, hepatocellular carcinoma, bladder carcinoma, cervical carcinoma, osteosarcoma, thyroid carcinoma, and skin cancer (Li et al., 2014; El-Ashmawy et al., 2016; Song et al., 2016; Keta et al., 2020; Sharifi-Rad et al., 2020; Gonzalez-Castillo et al., 2021).

The blueberries belong to the Ericaceous Vaccinium genus and the Cyanococcus subgenus. The blueberries contain many bioactive compounds, polyphenolic compounds, and flavones. The anthocyanins are forming a major part of phenolic compounds and a very important flavonoid subclass, which are very rich in blueberries (Koh et al., 2020; Yang et al., 2022). A high level of antioxidants was found upon studying the blueberries (Tian et al., 2021). Berries are one of the most common anthocyanin-rich fruits with anti-tumor effects. This effect is determined by their bioavailability, metabolization, and structure (Shi et al., 2021). Blueberries or extracts from it were studied on different cell lines including melanoma cell lines, skin cancer, cervical cancer, hepatocellular carcinoma, colon carcinoma, colorectal carcinoma, breast cancer, prostate cancer, renal cancer, and lung cancer (Wang et al., 2017; Pan et al., 2019; Lamdan et al., 2020; Lin et al., 2020; Alsadi et al., 2021).

There is a lack of studies of phytochemicals on the OC cell lines. This is the first time studying the effect of PPE and Blueberries extract on the HNO-97 tongue cancer cell line regarding the Antioxidant content of extracts and their cytotoxic and metastatic effects on the cell line.

2. Material and methods

2.1. Material

2.1.1. Pomegranate peel extract

The PPE powder of natural origin was provided by AYURVEDA DISTRIBUTORS LIMITED 160 city road, London, UK- ECLV2NX.

2.1.2. Blueberry Extract

Blueberry powder (BE) of natural origin was provided from Asia and analyzed in the EU provided by NATURE GOLD LTD LONDON, UK.

2.1.3. The cancer cell line

Human tongue squamous cell carcinoma cell line (HNO-97, CVCL_D227) was obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt).

2.2. Methods

The PPE and BE are prepared in methanolic suspension with different concentrations. Trolox standard for ORAC assay Trolox stock solution of 1 mM in methanol was prepared, and 5 serial dilutions were prepared in the concentrations of 400, 300, 200, 100, and 50 μM.

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Table 1 The Antioxidant Content by ORAC Assay/ The cell Viability through the SRB Assay with different concentrations of PPE &BE/ The Flow Cytometry (Cell Apoptosis) for the PPE &BE.

Antioxidant content mean ± SD	BE					PPE				Test of significance	
	218.37 ± 7.04					609.42 ± 25.52				t = 33.03 p < 0.001*	
		PPE	Test of significance between PPE& control	BE	Test of significance between BE & control	comparison between PPE&BE	Gate		Flow cytometry PPE	Flow cytometry BE	Control	Significance within groups
Viability % mean ± SD	control	100 ± 0.0		100 ± 0.0			P*	count	3.78 ± 0.06	7.43 ± 0.59	8.38 ± 0.17	
	0.1	96.69 ± 0.81	t = 9.14 p < 0.001*	98.42 ± 0.1	t = 35.33 p < 0.001*	t = 1.73 p = 0.02*		%	100 ± 0.0	100 ± 0.0	100 ± 0.0	
	1	82.37 ± 2.99	t = 10.25 p < 0.001*	97.99 ± 0.55	t = 8.17 p < 0.001*	t = 8.89 p = 0.009*	Q2	count	418.67 ± 10.97	93.67 ± 8.5	60.0 ± 9.54	P1 < 0.001* P2 = 0.06
	10	81.82 ± 1.61	t = 25.45 p < 0.001*	93.68 ± 0.18	t = 78.51 p < 0.001*	t = 11.86 p = 0.002*		%	11.06 ± 0.474	1.26 ± 0.06	0.717 ± 0.117	P3 < 0.001*
	100	50.28 ± 1.52	t = 73.14 p < 0.001*	93.05 ± 1.53	t = 10.16 p < 0.001*	t = 34.34 p = 0.001*	Q4	count	83.33 ± 3.06	130 ± 3.61	78 ± 8.72	P1 < 0.001* P2 < 0.001*
	1000	3.61 ± 0.47	t = 458.58 p < 0.001*	27.18 ± 2.43	t = 67.0 p < 0.001*	t = 16.49 p < 0.001*		%	2.20 ± 0.09	1.75 ± 0.11	0.930 ± 0.098	P3 = 0.002*

t: Student *t* test, *statistically significant

One-way ANOVA test, *: statistically significant.
P*: population of cells. P1: Difference of significance between the PPE and control group, P2: Difference of significance between the BE and control group, P3: Difference of significance between the PPE and BE group.

According to sample preparation Procedure, the assay was carried out according to the method of Liang et al (2014).

The SRB cytotoxicity assay was performed following the methods of Skehan et al. and Allam et al. (Skehan et al., 1990; Allam et al., 2018). The aliquots are prepared and treated with the PPE and BE with different concentrations of 0.1, 1.0, 10, 100, and 1000 µg/ml.

In the flowcytometric assay (Alaufi et al., 2017; Mohamed et al., 2017; Bashmail et al., 2018; Fekry et al., 2019), the apoptosis including early and late apoptosis in addition to necrosis is determined using "Annexin V-FITC apoptosis detection kit" (Abcam Inc., Cambridge Science Park, Cambridge, UK) linked with two channeled fluorescent flow cytometry. The cell lines were treated with the PPE and BE at concentrations of the IC50 for 24-48 and 72 hours (about 3 days). Each experiment is repeated three times and the average and standard deviation is calculated.

The Biological Significance of each phase: (Q4): early phase apoptotic cells. (Q2): late-phase apoptotic cells. (Q1): cell necrosis. (Q3): Normal intact cells.

According to the wound healing assay (Jonkman et al., 2014; Main et al., 2019; Dudley, 2020), Scratches were established into the merging monolayer. The control wells were filled with the fresh medium, while the extracts' wells were treated with PPE and BE at the concentration of Ic50.

The Wound Area: Wound area can be calculated by tracing the cell-free area in images using (Fiji-ImageJ software). Under normal conditions, the wound area will decrease over time.

The Wound Closure percentile: Wound closure%: (At = 0hr − At = Δh/At = 0 h) × 100, where At = 0hr is the average area of the wound measured immediately after scratching (time zero), and At = Δh is the average area of the wound measured h hours after the scratch is performed. All the experiments are repeated in triplets.

All the tests were done at Nawah Scientific Inc. labs: street 9, Al-Mokatam, Cairo, Egypt.

2.3. Statistical analysis and data interpretation

Data analysis was performed by SPSS software, version 18 (SPSS Inc., PASW statistics for Windows version 18. Chicago: SPSS Inc.).

3. Results

3.1. The ORAC Assay

Regarding the oxygen radical absorbance assay (ORAC), the PPE showed 218.37 ± 7.04 µM eq/mg, the BE showed 609.4 2 ± 25.52 µM eq/mg and there was a high level of significant difference between the two extracts (Table 1).

3.2. The SRB assay

There was a concentration and time-dependent decrease in the cell viability percentile for both extracts with a high level of significant difference between the control groups at all concentrations and both extracts. The PPE showed a higher significant decrease in cell viability at all concentrations. The PPE IC50 (half-cell population death concentration) was 73.35 µg/ml, while the BE was 525.38 µg/ml. (Table 1, Chart1, Fig. 1).

3.3. The flow cytometry (cell apoptosis) assay

The flow cytometric analysis of the HNO-97 treated with PPE showed an induced increase of 2% in the early apoptosis and 11% in late apoptosis,while the treated BE group showed an induced increase of 1.75% in early apoptosis and 1.26% in late apoptosis. Concerning the percentile of cell apoptosis between groups, there was a significant difference in increasing cancer cell line apoptosis between the PPE-treated group from the control group in early and late stages. While with the BE-treated group, there was a slightly significant difference cumulatilvly in the early and late apoptosis together. There was also a slightly significant difference in cell apoptosis between PPE and BE-treated cell line groups. (Table1, charts2).

3.4. The wound healing assay (cell migration)

After the treatment of the HNO-97 cell line with the PPE, the cell line showed highly significant results regarding both wound areas and wound closure percentile in time dependent manner. After 72 h of treatment, the wound area was increased

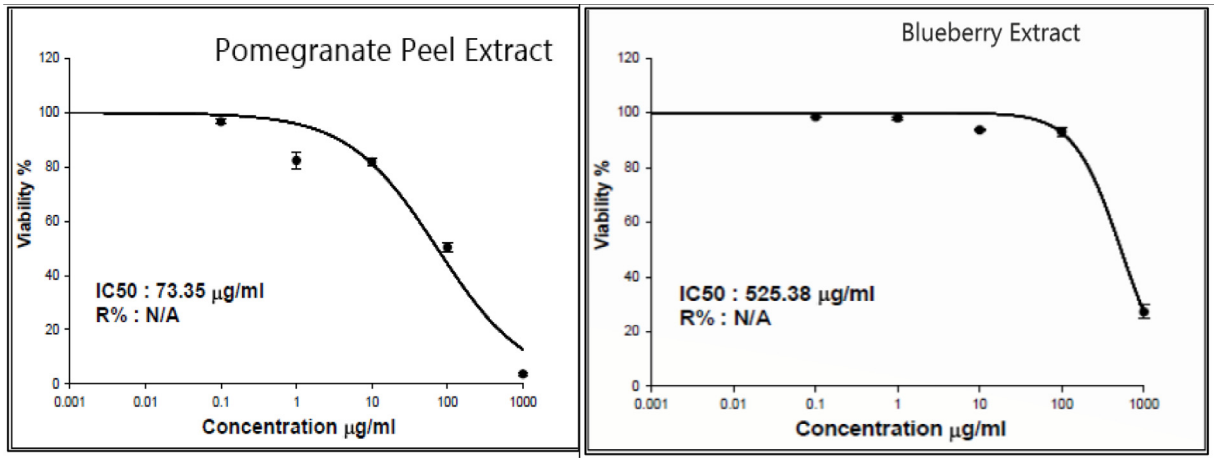

Chart 1 The cell viability effect on the HNO-97 cell line treated with PPE and BE and IC50 for both extracts. The HNO-97 showed declination in the cell viability with both PPE and BE at different rates.

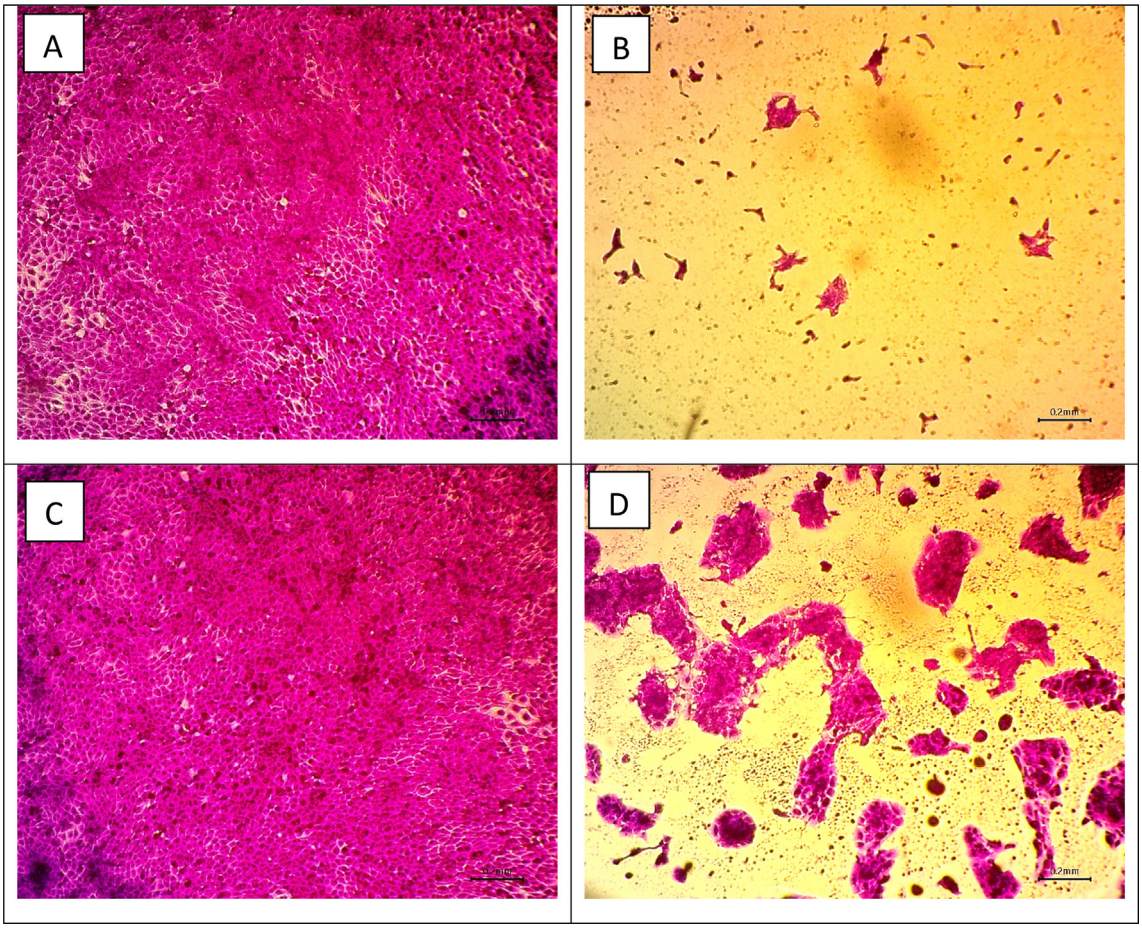

Fig. 1 The SRB Microscopic Photomicrograph showing the HNO-97 Cell line stained with H&E after treatment with extracts at different concentrations after 72 h of exposure to extract. A: PPE at concentration 0.1 ug/ml, B: PPE at concentration 1000 ug/ml. C: BE at concentration 100 ug/ml, D: BE at concentration 1000 ug/ml. The cell line showed a reduction of the cell population with both extracts at different rates.

by almost seven folds which means a decrease in cell migration. While with BE the wound area and wound closure were only significant at 24 h of treatment and then considered statistically in significant (Table 2, Fig. 2).

4. Discussion

In this study, the antioxidant level in PPE was higher than the results of Elfalleh et al. concerning that, in their study the highest level of antioxidants was found in the peel (Elfalleh et al., 2011). Zhai et al. and Peršurić et al. concluded that very potent scavenging activity was found against free radicals (Zhai et al., 2018; Peršurić et al., 2020). Almost all studies are in line with the high antioxidant content of the PPE with minimal differences that may owe to different factors (Vaneková et al., 2020). For the BE extract, these results of the antioxidant level agree with Nemzer et al. who ranged the results from 243.33 to 392.25 µmol Trolox eq/mg,and with Richards et al. whose results were 222 µmol Trolox eq/mg (Nemzer et al., 2018; Rickards et al., 2022).

The cytotoxicity and IC50 results of this study on the HNO-97 with PPE treatment are in accordance with the results of colon carcinoma cell line HCT116 where the IC50 was about 74.84 µg/mg (Keta et al., 2020) with the results of Elbakry et al. in which liver cancer cell line HepG2 was 83.9 µg/mg (Elbakry et al., 2023) and also with the POMx powder (capsules loaded pomegranate Juice powder),which was 80.53 µg/mg with Ca9-22 oral carcinoma cell line (Peng et al., 2021). These results disagree with the results of melanoma (HTB140), large cell lung carcinoma (HTB177), and breast cancer (MCF7) (Keta et al., 2020). The PPE is considered extraordinarily rich in antioxidant content with great benefits in the treatment of many cancer types. It showed very promising anticancer effects in the treatment of cervical cancer (Teniente et al., 2023). Recent advances have been introduced to this field carrying PPE on chitosan nanoparticles increasing their efficacy against breast cancer (Monika et al., 2022).

The results of this study with treating the HNO-97 with the BE regarding the cytotoxicity and IC50 showed disagreement with Lamdan et al. whose research results showed lower levels of the IC50 with variability according to cancer type, for example with mouse colon carcinoma CT26 was 19.1 µg/ml, 53.2 µg/ml with Human breast carcinoma MDA-MB-231, 49.2 µg/ml with prostate cancer PC3 and 26 µg/ml with human lung adenocarcinoma HCL-H125 (H. Lamdan et al., 2020). The difference

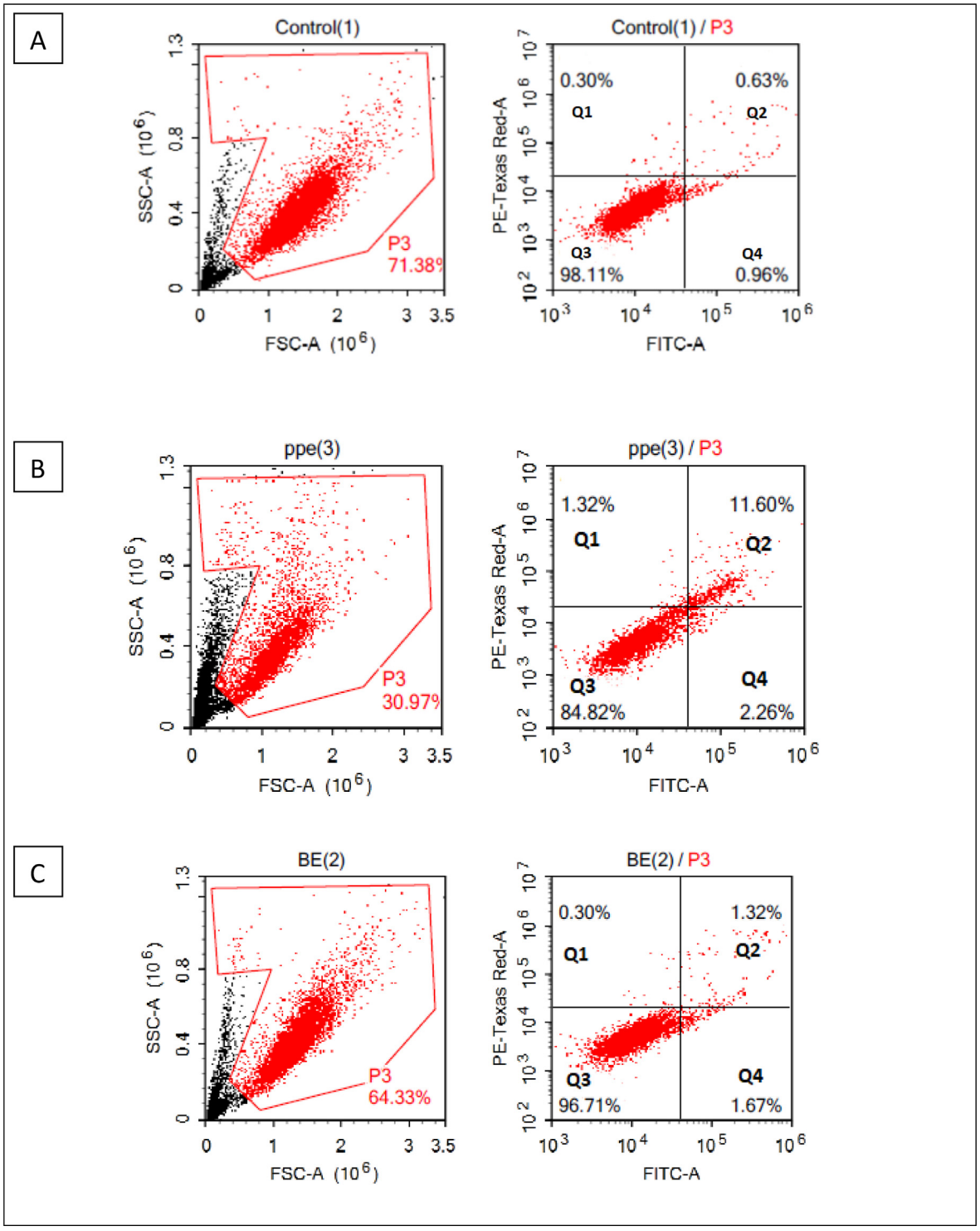

Chart 2 The flow cytometric analysis (apoptosis assay). A: Control group HNO-97 cell line without any phytochemicals, B: The HNO-97 treated with PPE at concentration IC50. C: The HNO-97 treated with be at concentration IC50. The highest levels of apoptosis noticed with PPE.

in results can be owed to the difference in preparation methods (Reyes-Díaz et al., 2016). The IC50 results of Diaconeasa et al. were found to be 281, 224, and 224 μg/mg with HeLa (cervical cancer), B16F16 (melanoma), and A2780 (ovarian carcinoma) respectively (Diaconeasa et al., 2015).

The flowcytometric analysis apoptosis assays significant results performed on HNO-97 with the PPE can be conducted to results of hepatocellular carcinoma HEPG2 cell line, Prostate cancer, and cervical cancer (Deng et al., 2017; Fazio et al., 2018; Chaves et al., 2020; Elbakry et al., 2023). These

Table 2 The Wound Area (Cell Migration) Assay: Wound Area and Wound closure percentile with PPE and BE:

Wound area	Control	PPE	Test of significance PPE	BE	Test of significance BE	Wound closure	Control	PPE	Test of significance	BE	Test of significance
0H	42.89	47.38	z = 4.69961 p = 0.000003*	46.94	z = 1.96 p = 0.05*						
24H	22.33	66.13	z = 6.56367 p ≈ 0.0*	9.95	z = -1.9684 p ≈ 0.049*	24H	45.027 ± 12.758%	−34.3 ± 16.209%	Z = -6.2178 P ≈ 0.0*	73.802 ± 16.209%	Z = 2.25545 P ≈ 0.0241*
48H	9.97	61.07	z = 9.48404 p ≈ 0.0*	0.692	z = -0.7243 p ≈ 0.4688*	48H	76.871 ± 13.646%	−39.284 ± 20.6%	Z = -8.512 P ≈ 0.000009*	87.372 ± 20.608%	Z = 0.7695 P ≈ 0.4416
72H	9.27	75.84	z = 9.30163 p ≈ 0.0*	0.0	z = -0.65216 p ≈ 0.5143	72H	76.65 ± 15.49%	−52.957 ± 21.902%	Z = -7.786 P ≈ 0.0*	87.355 ± 21.902%	Z = 0.78448 P ≈ 0.4328
For the PPE: Two-Tailed U Test Mann-Whitney: Test statistics U_1 = 16 The critical region:{0}U [14,16] So, since U_1 lies in the critical region, the findings are found statistically significant. (At significance level 0.05)			For BE: Mann-Whitney two-tailed *U* test: Test statistics U_1 = 6 The critical region:{0}U [14,16] So, since U_1 lies outside the critical region, the findings are found statistically insignificant. (At significance level 0.05)					For the PPE: Two-tailed *U* test (Mann-Whitney): Test statistics U_1 = 99 Critical region:[0,23]U[74,99] Since U_1 lies outside the critical region, the findings are considered statistically significant (at a significance level of 0.05)		For BE: Two-tailed *U* test (Mann-Whitney): Test statistics U_1 = 21 Critical region:[0,17]U[62,81] Since U_1 lies outside the critical region, the findings are considered statistically insignificant (at a significance level of 0.05)	
The wound area is described as mean ± SD, Z: Mann Whitney *U* test, decisions made at significance level α = 0.05						wound closure % is described as mean ± SD, Z: Mann Whitney *U* test, decisions made at significance level α = 0.05					

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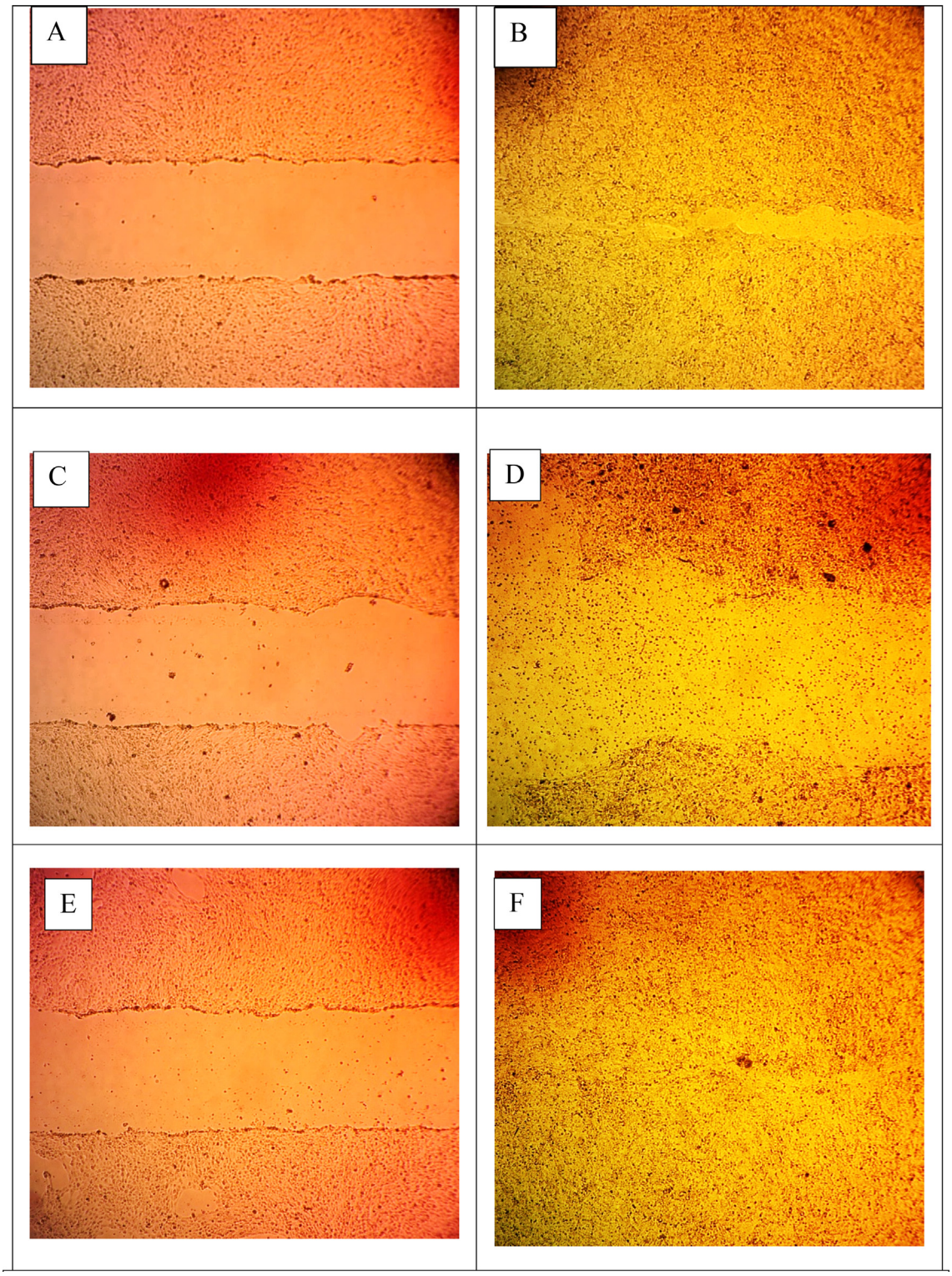

Fig. 2 Photomicrograph showing wound area in wound healing assay of the HNO-97 cell line for the control group "without any extracts", (A: 0 h, B: 72 h)/ for the PPE group (C: 0 h, D: 72 h) at the PPE IC50/ for the BE group (E: 0 h, F: 72 h) at the BE IC50. All microscopic pictures were captured at a magnification of ×100.

results can also be correlated to Peng et al. who studies the effect of POMx, which showed an increasing apoptotic effect of OC (Ca9-22,HSC-3,and OC-2) (Peng et al., 2021). The anti-cancer effect of the PPE punicalagin was explained by interfering with the cell cycle, proliferation and signaling, and catabolic pathways such as autophagy, and apoptosis

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(Berdowska et al., 2021). Later research suggested that the apoptosis among the punicalagin-treated cells is due to the upregulation of caspase-3, 7, 9 and downregulation of e6 and e7 with an effect on the STAT3 proteins as their target (Xie et al., 2022).

Blueberry flowcytometric apoptosis results can be correlated to the results of Adams et al. when using the BE on different breast cancer (HCC38, HCC1937, and MDA-MB-231) showing a decrease in the proliferation of these cancer cells (Adams et al., 2010). In vivo study evaluating the effect of the blueberry supplement on the OC induced in hamster animals' buccal pouch. Results showed inhibition in progression targeting oncogenesis signaling pathway and cell apoptosis through dietary blueberry supplements (Baba et al., 2016). The BE compounds were investigated on B16-F10 melanoma cell line. The study found that these extracts have profound effects on the inhibition of cell viability, and cell proliferation, and induced early and late apoptosis (Wang et al., 2017). A later study on BE polymerized Proanthocyanidin showed inhibition of cell proliferation and inducing apoptosis of human adult T-cell leukemia (Ichikawa et al., 2022). Another one with experimented malvidin-3-galactoside, on Huh-7 hepatocellular carcinoma, results showed dose-dependent inhibition on the cell proliferation ability, and inducing the cell cycle arrest and apoptosis (Lin et al., 2020). Recent research by Haytham Dahlawi evaluated the effect of malvidin on leukemia, SUP-B15, and KG-1a. The treatment with malvidin showed significant cell proliferation inhibition in both cell lines inducing cell apoptosis (Dahlawi, 2022).

The wound healing (cell migration) assay of HN0-97 PPE treated cells showed very promising significant results decreasing the OC cell migration giving for further in vivo studies about inhibiting the cancer cell metastasis. This is considered a major step in this process (Majidpoor & Mortezaee, 2021). These results agreed with the results of breast cancer cell line MCF7 and large lung cell carcinoma HTB177 but doesn't agree with the results of melanoma HTB140, and colon cancer HCT116 where there was not a significant effect on cell migration (Keta et al., 2020). Peng et al. results with POMx on OSCC can be conducted to our results explained by downregulation of the matrix metalloproteinase activities (MMP-2 & MMP-9) and epithelial mesenchymal transition signaling (EMT) (Peng et al., 2020; Peng et al., 2021). Results with PPE can be explained through effect of the punicalagin (present in PPE) (Berdowska et al., 2021).

The BE treated HNO-97 cell migration assay results are in line with the results on the murine colon carcinoma (CT26), cervical cancer (HeLa), human renal carcinoma (SN12C), human colorectal carcinoma (HCT-116), and human lung adenocarcinoma (HCI-H125) experimented by Lamdan et al. (H. Lamdan et al., 2020). Results disagree with BE malvidin-3-galactose showed a significant effect on cell migration of hepatocellular carcinoma cell line HepG2 in the *in vitro* and in vivo studies (Wang et al., 2018) and was supported by Lin et al. significantly reducing the invasion of Huh-7 hepatocellular carcinoma cell line (Lin et al., 2020). However, these results can be debated with the difference in concentration of this fraction as concentrated from the whole extract. Generally, the results can be different from one cancer type to another even though they have many common features, though each single cancer type has its own proliferation and invasion pathway. It has been demonstrated that different outcomes of different cell line types even with treatment of the same preparation of extract (Lamdan et al., 2020; Keta et al., 2020; Raina et al., 2022).

Comparing the results of the antioxidant content between the PPE and BE, these results can agree with the results of Kalita et al. who found higher antioxidant content also in PPE than BE using ORAC assay (Kalita & Jayanty, 2014). Another study by Kandyliari et al. can be correlated found higher antioxidant content in pomegranate than the blueberries (Kandyliari et al., 2021).

These results are very promising with OSCC especially with the PPE, and need further investigations for better application in field of cancer treatment.

5. Conclusion

This study found a high antioxidant profile in the PPE extracts and BE with the privilege to the PPE. Treating the tongue cancer cells with PPE extract with minimal concentrations induces cancer cell apoptosis and reduces the cancer cell migration imitating the behavioral effects on cancer cell metastasis. The results were different when using the BE inducing the cancer cell apoptosis with higher concentration without effect on the cancer cell metastasis. Comparing the antioxidant content, apoptotic effect and the cell migration, better results were using the PPE.

Ethical statement

This research was approved by the Faculty of Dentistry, Mansoura University, Dental Research Ethics Committee No. A21100221.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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