Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.03.024

Anti-Proliferative Activity of Prunus Dulcis Seed Oil Alone and in Combination with Aspirin on Human umbilical vein endothelial (HUVECs) and Kaposi sarcoma (KS) cells

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Submitted: 14 November 2022; Accepted: 16 December 2022; Published: 20 January 2023

ABSTRACT

This study's objective was to investigate the potential anticancer, and antiproliferative influence of Iraqi Sweet almond oil (SAO) and aspirin, alone and in combination, on human umbilical vein endothelial cells (HUVECs) and Kaposi sarcoma (KS) cells. The researchers tested different concentrations of SAO and the combination of SAO and aspirin on the cells for 24 hours and measured their viability, cytotoxicity, and cell growth using MTT assays. They also determined the IC50 values for the anticancer and antiproliferative actions of SAO and measured the gene descried of vascular endothelial growth factor 2 (VEGF2) in KS cells using real-time polymerase chain reaction (RT-PCR). The results showed that the SAO had significant antiproliferative and anticancer activity against KS cells and demonstrated selective toxicity against HUVEC cancer cells. The combination of SAO and aspirin had low inhibitory effects. Gas chromatography analysis identified various compounds in SAO, including fatty acids and vitamin E. The researchers concluded that SAO from Iraq may have anticancer, antiangiogenic, and antiproliferative properties on KS cells through its molecular signaling pathways, as demonstrated by the suppression of VEGF2 expression in KS cells.

Keywords: *HUVEC, KS, MTT assay, RT- PCR, SAO*

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INTRODUCTION

Cancer was cause of death globally [1]. As the number of cancer-related deaths rises daily, research concerning anticancer medications has become critical for cancer patients. Currently, the standard treatments for cancer patients include chemotherapy and radiation. However, these techniques elicit undesirable side effects and toxicity, as they harm both healthy and cancerous cells. Furthermore, some individuals develop drug resistance throughout treatment, which results in a recurrence [2]. There is an urgent need for extensive studies on the development of new anticancer medications derived from herbal therapies as medicinal plants have emerged as an intriguing source of anticancer chemicals that are safe, have few side effects, and are readily available [2,3][4].

The almond, Prunus dulcis (Mill.) D.A. Webb, is native to the Mediterranean and other hot climate areas. It belongs to the Rosaceae family's subfamily Prunoideae [5,6] A fixed oil containing phenolic compounds, vitamins, minerals, and micronutrients with a variety of pharmacological and biological activities is present in almond seeds[7] ,[8] ,[9]. In vivo investigations of the hepatoprotective and anticancer properties of SAO has been performed[10] .The fatty acids found in nuts, like the sweet almond and its oil, may act as a cancer preventative. Animal studies have demonstrated that SAO is correlated to a lower risk of colon cancer[11]. There is a possibility that the additive effect of mixtures of bioactive components and derivatives in almonds increases the effect of plant products compared to the individual ingredients. VEGF is the major mediator of angiogenesis. Hypoxic conditions trigger the expression of VEGF in a multistage process [12].

Aspirin, widely utilized nonsteroidal anti-inflammatory drug (NSAID), has been demonstrated to possess potential anticancer properties through various mechanisms. Previous investigations have suggested that the suppression of cyclooxygenase-2 (COX-2), an enzyme upregulated in cancer cells, may be responsible for these effects [13] [14]. Aspirin inhibits cell growth, induces cell cycle arrest, and induces apoptosis in many cancer cell lines independent of COX-2 expression levels, according to in vitro studies. [15].While there exists little published data regarding protective effects of essential oils as adjuvant therapy for Kaposi sarcoma (KS) cell prevention, no published study exists exploring the in vitro antiproliferative activity of Iraqi SAO and its combination with aspirin (COMB) on KS cells, and human umbilical vein endothelial cells (HUVECs). Also in this work, the investigation of the gene expression of VEGF2 in KS cells by RT-PCR has the potential to impart novel knowledge for cancer prevention purposes

MATERIALS AND METHODS

Sweet almond seeds and SAO

Almond trees in northern Iraq (Sulaymaniyah) provided the sweet almond seeds used in this study. The SAO was extracted by hydraulic presses, a cold extraction method that preserves the pleasant physical features of almonds [16].

Serial dilution of SAO and COMB

The SAO was obtained in concentrations of 1.6, 3.1, 6.25, 12.5, 25, and 50 µg/ml. The aspirin concentrations used in the COMB solutions were 400, 200, 100, 50, 25, and 12.5 µg/ml .

Cell line and culture

The American Type Culture Collection supplied the HUVECs and KS cells (ATCC, Manassas, VA, USA). Al-Nahrain University, Iraq, Department of Pharmacology/College of Medicine conducted the experiments. The cells were cultured in M199 medium containing 10% fetal bovine serum (FBS), 1% penicillin 100 international units per milliliter (IU/mL), 1% streptomycin 100 micrograms per milliliter (g/mL), and 1% glutamine. At 37°C and in a humid atmosphere containing 5% CO2, cells were cultivated. When the cells reached 80% confluence, they were typically subcultured with a 0.25 percent trypsin-EDTA (Ethylenediaminetetraacetic acid) solution [17].

Evaluation of cellular viability

To test the viability and proliferation of cells using the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was reduced to a purple formazan product. SOA was diluted in a culture media containing HUVECs double diluted with the same concentration. In 96-well culture plates, cell suspensions were prepared in triplicate and seeded at densities of 1 × 104 cells/mL per well for the SAO and aspirin exposures. Plating was conducted to compare the cytotoxicity of the SAO and aspirin against KS cancer cells and HUVECs. The medium without SAO was regarded as the positive control, which contained 100 µL of M199, and an individual medium not containing any cells or SAO was used as the negative control. Different concentrations of SAO and aspirin were incubated with HUVECs and KS cells for 24 hours. Cells incubated at 37°C in humidifiied 5%, CO2 were analyzed by MTT assay performed within the first two hours after the culture period. The potencies of the SAO, aspirin, and COMB against the growth of HUVECs and KS cells were determined through IC50 values (50% concentration inhibited 50% of the cells), which were calculated using the statistical program Graph Pad Prism[18,19].

Gene expression by real time quantitative polymerase chain reaction (VEGF)

Q-PCR was applied in IQ4 real time PCR (Bio-Rad, USA). The composition of reaction mixture was 1X Go-Taq qPCR "Master Mix 12.5µl", "Promega, USA", 2.5 µl primers, and 1.0 µl of complementary DNA in a complete volume to 25 µl. VEGF Quanti-Tect- Sybr green primers were obtained from "Qiagen, Germany". GAPDH "Glyceraldehyde-3-Phosphate Dehydrogenase" act as "housekeeping gene,". GAPDH primers "Invitrogen-USA" sequences were used in this work [20] , [21] ,[22] .The PCR environment for GAPDH and VEGF included of first growth at" 95°C for 15 min", "40 cycles of denaturation at 95°C for 15 sec", (annealing at 55°C for 30 sec, delay at 72°C for 30 sec). Fluorescence was verified at the finished of extension. "A negative control without c-DNA template was used with every analyze. To create a standard curve, template c-DNA from untreated-control Kaposi sarcoma cells was used in this assay. Amount of gene expression was measured by "the standard curve, and cycle threshold of each concentration of sweet almond oil. The gene expression values were controlled to the reference gene expression, and the fold exchange was calculated in comparison to the untreated cell control. This experiment was repeated twice, with each gene having a duplicated reading. Following QPCR, a melt curve analysis was performed to ensure the specificity of the PCR result [23][24,25].

Analysis of the data

Differences in concentrations were analyzed statistically by one-way ANOVA. The IC50 were determined using the statistical program GraphPad Prism. Data is reported as the mean ± SD. A p-value of $p < .05$ was measured significant.

RESULTS

The viability and cytotoxicity of cells

The HUVECs and KS cells were exposed to SAO and COMB for 24 hours at changed concentrations. Cell viability was measured using an MTT assay, as previously described. The SAO blocked the proliferation of KS cells in a concentration-dependent manner. The results display a drop in viability of the KS cells at concentrations of 50 and 25 µg to 43.79% and 48.92%, respectively, when dosed with SAO. The COMB exhibited selective toxicity and was more effective at inhibiting growth of the KS cells than of the HUVECs when compared with other concentrations.

Furthermore, effect of SAO and COMB on growth and inhibition activity against HUVECs and KS cells was investigated. They demonstrated dose- and time-dependent properties,

The results revealed that 50 µg of SAO and 400 µg of the COMB were more effective in inhibiting KS cell growth than other concentrations. The effects of the SAO on HUVECs and KS cells were shown in in vitro studies. In this study, the SAO at 50 and 25 µg was more effective than the other concentrations against KS cells.

Table 1 shows the in vitro screening of the SAO on HUVECs and KS cells, which were in passage four. The concentrations used were 1.6, 3.1, 6.25, 12.5, 25, and 50 µg. The results exposed a dose-dependent effect on the cell growth after 24 hours. The data are reported as the mean, standard deviation ± SD.The percentages of HUVEC proliferation were 72.69 and 86.81% of SAO at concentrations of 50 and 25 µg, individually. The IC50 of SAO was 28.25 µg/mL, and it inhibited the growth of the HUVECs.

TABLE 1. Serial concentrations and their respective viability percentage of sweet almond oil (SAO) on human umbilical vein endothelial cell (HUVEC) line and Kaposi sarcoma (KS) cells.

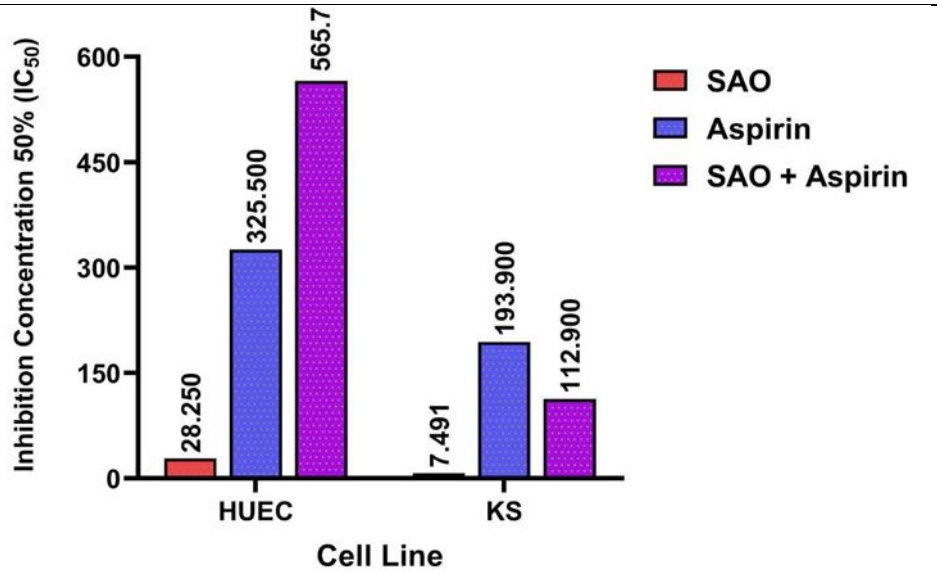
Concentration (µg/ml) of SAO	**HUVEC % Of viability ± SD**	**KS % Of viability ± SD**
50	72.69 ± 1.71	43.79 ± 2.45
25	86.81 ± 3.75	48.92 ± 1.20
12.5	94.29 ± 1.83	63.12 ± 2.95
6.25	95.18 ± 0.96	74.46 ± 3.99
3.1	95.06 ± 1.24	86.81 ± 5.22
1.6	93.56 ± 0.64	96.33 ± 0.41

Table 2 shows that there was a dose-dependent activity on the cell proliferation after 24 hours of 400, 200, 100, 50, 25, and 12.5 µg/mL of aspirin exposure and 1.6, 3.1, 6.25, 12.5, 25, and 50 µg of SAO exposure, sequentially.

TABLE 2. Serial concentrations and their respective viability percentage for sweet almond oil (SAO) and combination with aspirin on human umbilical vein endothelial cell (HUVEC) line and Kaposi sarcoma (KS) cells.

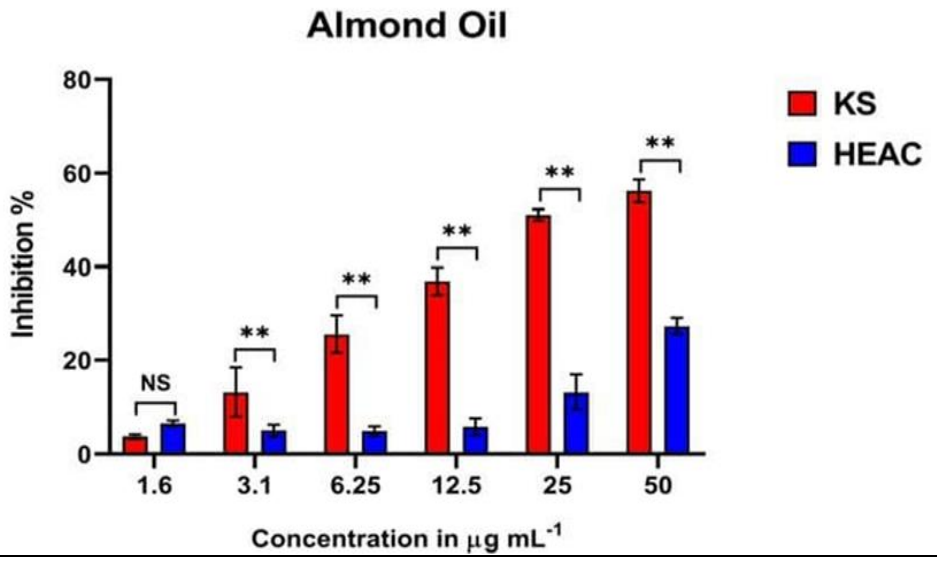
Concentration (µg/mL) Aspirin	**Concentration (µg) SAO**	**HUVEC % Of viability ± SD**	**KS %Of viability ± SD**
400	50	71.18 ± 0.69	39.35 ± 4.78
200	25	83.64 ± 1.46	48.03 ± 2.55
100	12.5	89.35 ± 2.06	71.49 ± 3.40
50	6.25	95.33 ± 1.18	90.70 ± 3.18
25	3.1	95.22 ± 0.82	95.72 ± 0.81
12.5	1.6	95.95 ± 1.03	95.18 ± 1.28

The comparison of IC50 values between SAO, aspirin, and COMB on the HUVECs is shown in Fig. 1a. In Fig. 1b, dose prevention of 50% of proliferation KS and HUVEC by SAO, aspirin, and COMB reveals that SAO was more potent than the COMB, which was safe against cells. Contrastingly, the KS cells exposed to SAO appear more cytotoxic compared to aspirin and COMB exposures.



a. The activity of sweet almond oil (SAO), aspirin, and their combination on human umbilical vein endothelial cell (HUVEC) line and Kaposi sarcoma (KS) cells.

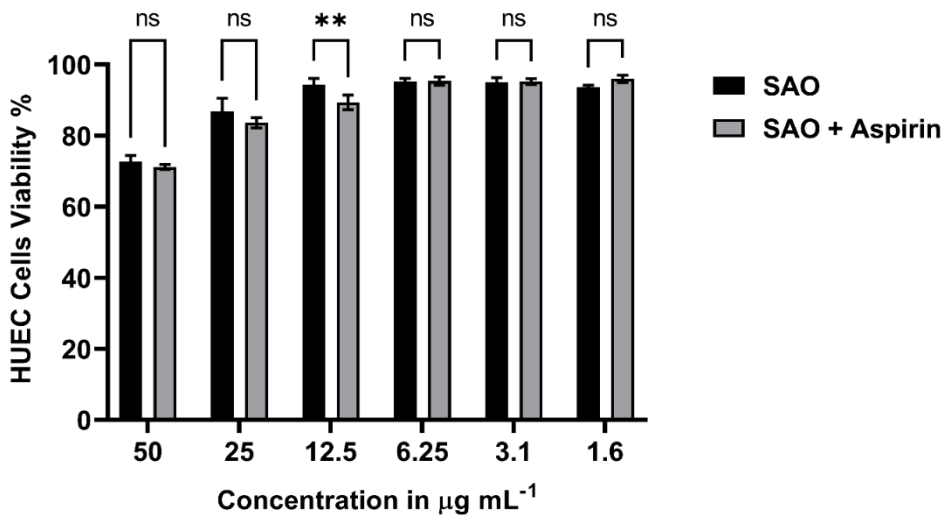
b.



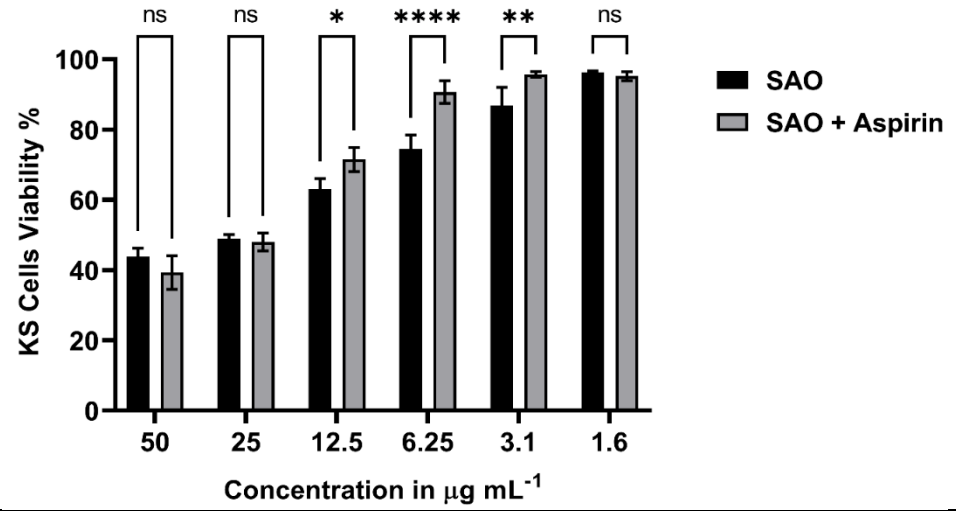
b. The inhibitory effects of 1.6, 3.1, 6.25, 12.5, 25, and 50 µg of sweet almond oil (SAO) on human umbilical vein endothelial cell (HUVEC) line and Kaposi sarcoma (KS) cells.

FIG. 1. The cytotoxic effect of sweet almond oil and with combination with aspirin against human umbilical vein endothelial cell line and Kaposi sarcoma cells.

Fig. 2a displays the effects of the SAO and COMB on HUVECs, demonstrating that SAO was safer against normal cells. Alternatively, Fig. 2b shows the activity of SAO and COMB on KS cells, and SAO was more potent in inhibiting the cancer KS cells than was COMB, showing an antagonism effect. In Fig. 2a, the inhibitory effect values are reported as mean ± SD from a lowest of three independent experiments (*$p < 0.05$ and **$p < 0.01$ changed to the control).



a. Effect of sweet almond oil (SAO) and there combination with aspirin against human umbilical vein endothelial cell (HUVEC) line.



b. Effect of sweet almond oil (SAO) and there combination with aspirin against Kaposi sarcoma (KS) cells.

FIG. 2. The effect of sweet almond oil and its combination with aspirin on cells treated with a serial dose for 24 hours. Viability was measured via an MTT assay.

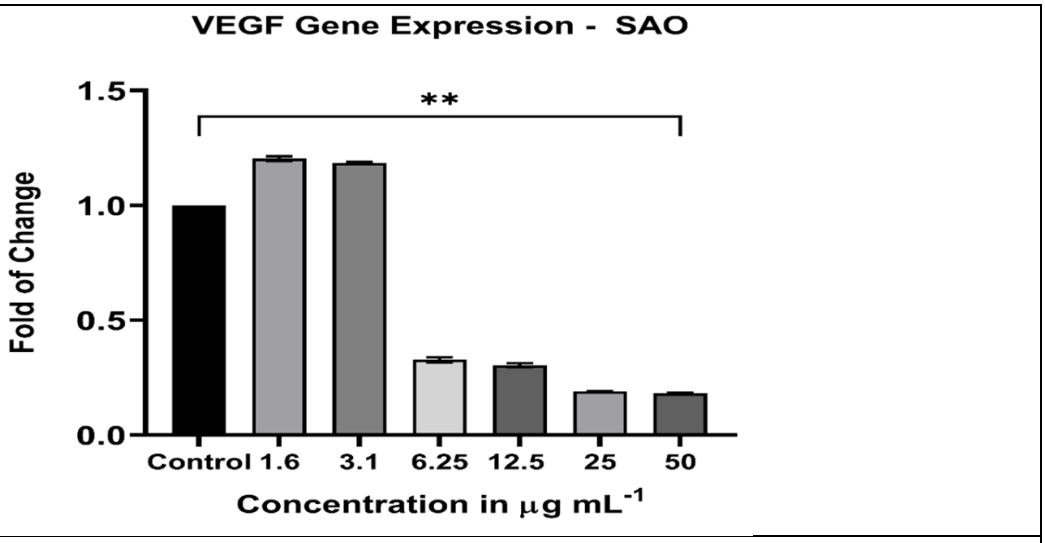
Gene expression of vascular endothelial growth factors (VEGF) for Kaposi's sarcoma (KS) using RT-PCR, and the activity of SAO and COMB on VEGF gene expression

SAO

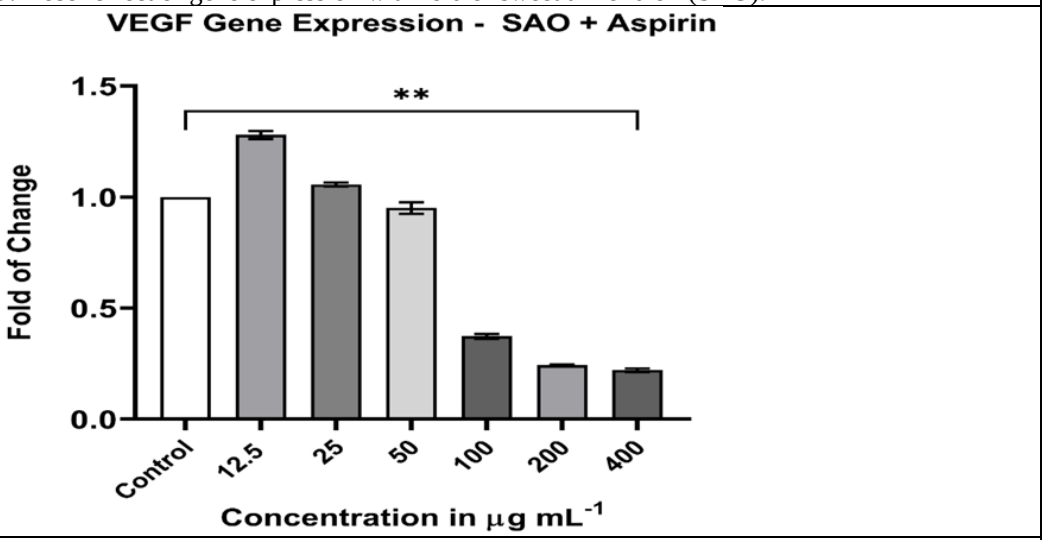
Analysis by RT-PCR displayed a down-regulation or a reduction in the transcription levels of VEGFR-2 in KS cell cultures, showing that the SAO and COMB had a dose -dependent efficacy. As described in Fig. 3a, the dose response effect of SAO against VEGF gene expression was by fold, meaning the value was decreased compared to control. It is worth noting that there was more inhibition against KS cells at lower concentrations of 1.6 µg/mL compared to the other concentrations. On the other hand, in

Fig. 3b, the activity of COMB against KS cells is shown, which was less inhibitory than SAO. The COMB had a synergistic effect, but the SAO was more effective in reducing the expression of VEGFR-2 due to the existence of polyphenols, phytosterols, and flavonoids in the oil, also have antiproliferative and anticancer activities [26];[27].

Furthermore, as shown in Table 4, SAO concentrations of 50, 25, and 6.25 µg/mL reduced VEGFR-2 transcription by 18.4% ($p < .05$), 19% ($p < .005$), and 13.9% ($p < .005$), respectively, when compared to the control, which was non-significant on KS cells.



a. Dose- effect of gene expression with fold of sweet almond oil (SAO).



b. Dose-effect of gene expression with fold of sweet almond oil (SAO) in combination with aspirin.

FIG. 3. Different concentration of sweet almond oil and its combination with aspirin on VEGF gene expression in Kaposi sarcoma cells.

Also, the effects of different doses of SAO and COMB on VEGF expression as explained by percentage of expression vs. control and fold (COMB at aspirin concentrations at 400, 200, and 100 µg/mL plus SAO at 12.5, 25, and 50 µg) reduced the Messenger RNA expression of VEGFR-2 to 22.2% ($p < .05$), 24.3% ($p < .005$), and 37% ($p < .001$), respectively, compared with the control on KS cells (Table 4).

This study indicates that the SAO's fold ratio had decreased from 1.0 to 0.18 and 0.19. This translates to approximately 10-fold reductions for the first two concentration treatments.

In Table 3, SAO and COMB effects on the gene expression of VEGF were found to be significant statistically. It is also important to highlight that SAO showed more inhibition than COMB on VEGF gene expression. It is worth noting that SOA concentrations of 50 and 25 µg/mL had more effect than COMB, with significant effects at concentrations of 400 and 200 µg/mL.

TABLE 3. Gene expression for VEGF by Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glycolysis pathways of sweet almond oil (SAO) and its combination with aspirin (COMB).

SAO					**COMB**				
Construction of sweet almond oil µg	**D CT**	**DD CT**	**% Of gene expression**	**FOLD**	**Construction of SAO and aspirin µg**	**D CT**	**DD CT**	**% Of gene expression**	**FOLD**
Control	-	-	100	1	Control	-	-	100	1
50	5.1	2.44	18.4	0.184	400	4.83	2.17	22	0.222
25	5.05	2.39	19	0.190	200	4.7	2.04	24.3	0.243
12.5	4.33	1.67	31.4	0.314	100	4.07	1.41	37.6	0.376
6.25	5.5	2.84	13.9	0.139	50	2.29	-0.37	129	1.292
3.1	2.41	-0.25	118	1.189	25	2.7	0.04	97	0.972
1.6	2.38	-0.28	121	1.214	12.5	2.57	-0.09	106	1.064

TABLE 4. Effect of sweet almond oil (SAO) and its combination with aspirin (COMB) against Gene expression of growth factor VEGF on Kaposi sarcoma cancer cells

Statistical parameter	**SAO on gene expression of VEGF**							**COMB on gene expression on VEGF**					
	Control	1.6 b	3.1 b	6.25 c	12.5 d	25 e	50 e	12.5 b	25 c	50 d	100 e	200 f	400 f
Mean	1.000	1.204	1.185	0.328	0.304	0.190	0.182	1.280	1.057	0.951	0.373	0.243	0.2208
Std. Deviation	0.000	0.010	0.005	0.010	0.008	0.001	0.003	0.018	0.009	0.026	0.010	0.004	0.008
Std. Error of Mean	0.000	0.006	0.003	0.006	0.005	0.000	0.001	0.010	0.005	0.015	0.006	0.002	0.004

SAO and aspirin comb

The results observed that the fold ratio of the COMB was decrease from 1.0 to 0.22, 0.24, and 0.37. This indicates approximately an 8- to 7-fold change (Table 4, Fig 3).

DISCUSSION

Extraction of SAO

SAO has been extracted by hydraulic presses with cold extraction methods that keep the pleasant sensory features of almonds[16] [28,29]. SAO is rich in vitamin E and tocopherols. All tocopherols are potent antioxidants and reduce oxidative stress through induced DNA damage[30] ,[31]. Antioxidants, including vitamin E, polyphenols, and fiber, protect against cancer and heart disease.

Anti-proliferative activity against KS

SAO was more effective against KS cell cytotoxic activity, with an IC50 of 7.491 µg/mL. The IC50 value was less than 20 µg/mL, revealing that this tested substance had a significant cytotoxic effect. This compound is well-recognized as being cytotoxic, according to the National Cancer Institute [32]. The IC50 of COMB was 112.9 µg/mL. This IC50 value is higher than 20 µg/mL, and therefore, COMB had no significant cytotoxic effect or antagonistic effect. This might have been due to the existence of other active mixtures in fewer concentrations or other compounds that might have antagonized the active constituents [33] . Aspirin had an IC50 = 193.9 µg/mL and had no cytotoxicity activity against KS cells, which may be a potent antagonized effect of SAO.

Anti-proliferative activity against HUVEC

SAO, with an IC50 = 28.25 µg/mL, and aspirin, with an IC50 = 325.5 µg/mL, had no significant cytotoxic effects, as they were higher than 20 µg/mL. However, COMB had an IC50 =565.7 µg/mL and a synergistic effect to HUVECs. In the exist study, the dilutions of SAO to 50 and 25 µg/mL were more effective than other concentrations in KS cell lines according to viability (43.79%, 48.92%, respectively); these concentrations were less effective in HUVECs, with viabilities of 72.69% and 86.81%, respectively. The COMB viability on the KS cells and HUVECs at two higher doses (400 µg/mL aspirin and 50 µg/mL SAO) and (200 µg/mL aspirin and 25 µg/mL SAO) were 39.35% and 48.03%, respectively, which expressed higher toxicity on KS cells than in HUVECs with viability of 71.18% and 83.64%, respectively, demonstrating selective toxicity for KS cells. Therefore, the IC50 of SAO in KS cells was 7.491 µg/mL, which was more effective than in combination with aspirin that had an antagonism effect. However, in HUVECs exposed to SAO, the IC50 value was 28.25 µg/mL. In HUVECs, SAO and COMB exposures had no significant differences ($p > .05$). There was low antiproliferative activity. SAO may be capable of preventing colon cancer, regarded as chemoprevention. The results of a recent study found that almonds were an effective chemo preventative agent against colon carcinogenesis [31,34] A reduced IC50 value indicates higher antiproliferative activity and more inhibition of cell growth. Alternatively, substantial changes were displaced in the composition of phenolics and flavonoids in SAO and their antioxidant and antiproliferative activities. These variations were mostly attributed to a developing environment and genotype, geoclimatic factors, harvesting time period, and plant physiology during the extraction as well as analytical techniques used for this study [35,36],[37]. These findings show that SAO had significant dose-dependent effects on the growth of KS cells. Together, these agents have cytotoxic activity at a practical dose. In combination with aspirin, the cytotoxic activity decreased (antagonism effect). SAO exposure has a higher percentage of antiproliferative activity than COMB or aspirin exposures against KS cells.

The Expression of Vascular endothelial growth factor by RT-PCR

One of the major agonists of angiogenesis is VEGF. It is a secreted protein that increases endothelial cell growth and vascular penetrability. In both conditions physiological and pathological, VEGFR-2 is a key modulator of angiogenesis. By attaching to receptors, VEGF triggers a series of signaling process that led to proliferation, migration, survival, and increased vascular permeability. VEGF and its recepitors were the molecules most frequently targeted by antiangiogenic medicines in many different types of tumor growth progressions[38].

SAO, at the first two concentrations, exemplified effectiveness in inhibiting gene expression. It inhibited proliferation, migration, and tube formation process of KS cells by preventing the VEGFR-2 from attached to the surface of receptors in KS cells, and decreasing the VEGFR-2 receptors expression.

COMB reduced the expression of the VEGFR-2 receptor in different types of tumor cells, RT-PCR used to analyze the effect of SAO and COMB against VEGFR-2 gene expression levels. Results showed that SAO and COMB significantly decreased the VEGFR-2 transcript gene expression. These results were due to presence of fatty acids, flavonoids, phenolic compounds, lipid-soluble vitamins, and phytosterols; their antiradical, antiproliferative, and anticancer properties have a significant effect on health[39] . In this exposure, a gradient was observed in the treatment toward gene expression, which indicated the efficiency of the experiment.

CONCLUSION

Sweet almond (Prunus dulcis) seeds were collected from the north of Iraq. They have higher cytotoxic and antiproliferative activity against KS cells alone than in combination with aspirin (antagonism effect). The concentrations of 50 and 25 µg/mL of SAO were particularly effective against of KS and HUVEC cells line viability, therefore used for in-vivo studies. In in -vitro, and in-vivo assays, SAO had developed activity than COMB at inhibiting angiogenesis.

The findings of the angiogenesis experiments were determined using RT-PCR and mRNA gene expression, as evidenced by a decrease in VEGFR-2 transcription levels in KS cells. As demonstrated by these findings, SAO may be a capable antiangiogenic agent in complementary chemo-therapy. SAO has unsaturated fatty acids, mostly oleic acids, that have anticancer effects. It has antiproliferative activity, which reduces VEGFR-2 expression that plays an active role in transcription factors, metastasis, and the viability of tumors. Specifically, SAO showed more inhibition in KS cells than in HUVECs.

ACKNOWLEDGEMENTS

Non.

CONFLICTS OF INTEREST

The authors state that they have no known competing financial interests or personal ties that could be perceived as having influenced the work described in this study.

Author Contributions

Conceptualization, Zainab K. Ali and Hayder B Sahib; methodology, Zainab K. Ali; validation, Hayder B Sahib.; formal analysis, Zainab K. Ali; investigation, S.M.A.; resources, Zainab K. Ali data curation, Zainab K. Ali; writing—original draft preparation, Zainab K. Ali; supervision, Hayder B Sahib. All authors have read and approved the final manuscript version.

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J Popul Ther Clin Pharmacol Vol 30(3):e220–e231; 20 January 2023.