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Original article

Fatty acid composition of *Opuntia ficus-indica* seed oil control angiogenic activity in colon carcinoma cell lines

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Abstract. *Opuntia ficus-indica* belongs to the family Cactaceae that particularly rich in unsaturated fatty acids. The aim of this study was to investigate the composition and *in vitro* anti-angiogenic activity of spiny and thornless *Opuntia ficus-indica* seed (CPS) oils. Spiny and thornless CPS oils were obtained by supercritical CO_2 extraction and analyzed by GC-MS. Cell growth and cytotoxicity were measured with MTT assay with different concentrations of spiny and thornless CPS oils in Colo-320 and Colo-741 cell lines. Anti-angiogenic activity of CPS oils were investigated by immunocytochemistry using antibodies directed against to Flt-1, Flk-1, eNOS, iNOS, IL-6, PGE_2, TNF-α and VEGF. Oleic acid (17.6%; 15.6%), linoleic acid (55.9%; 60.1%), palmitic acid (12.4%; 12.3%), elaidic acid (4%; 4.1%) were determined as the major compounds of spiny and thornless CPS oils, respectively. In the MTT assay, 1:16 dilution spiny CPS oil was found to be active against Colo-320 and Colo-741 cells for 48h incubation. Also, 1:8 and 1:16 dilutions of thornless CPS oil were more effective at inhibiting Colo-320 and Colo-741 for 48 h, respectively.We conclude that spiny CPS oil decreased signaling molecules which play in PGE_2 mediated and VEGF-dependent angiogenesis. Interestingly, thornless CPS oil increased angiogenesis thought signaling molecules in colon cancer cells.

Key words: *Opuntia ficus-indica*, colon, cancer, angiogenesis, signaling pathway

Introduction

Opuntia ficus-indica is a cactus which belongs to Cactaceae family in botany and commonly known as prickly pear. It is highly consumed in South Africa, Australia, India, Cyprus, Mediterranean areas, Mexico, Latin America and Middle Eastern countries (1). Different part of *Opuntia ficus-indica* (flowers, fruit, roots and leaves) are used in the food and cosmetic industry (2-4). Also, its fruits and stems (cladodes) have been used in folk medicine for medical benefits such as hypocholesterolemic, anti-inflammatory and hypoglycemic agent (5-7). Spiny (wild) and thornless (cultivated) form of *Opuntia ficus-indica* grow in Cyprus.

Opuntia ficus-indica fruit (cactus pear) compose of pulp, peel and seeds. Chemical composition and many pharmacological effect of pulp and peel of cactus pear have been studied widely. However, cactus pear seed oil (CPS oil) has been less investigated part. The previous studies had shown CPS oil chemical composition from different countries (8-10). Especially, animal studies have provided antidiabetic and antioxidant effect of CPS oil. To date, with respect to the specific effects of CPS oil in colon carcinoma cells, one unique study demonstrated that thornless CPS oil induced apoptosis in primer colon adenocarcinoma cell lines (11).

Colorectal cancer is the third most common cancer type worldwide. Tumor growth, proliferation,

differentiation and metastasization depend on different pathways in colorectal cancer (12). The vascular endothelial growth factor (VEGF) signaling is the one of the major pathway that plays key role in colorectal cancer development and progression. This signaling cascade is activated by VEGF that bind with different cell membrane receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), and VEGFR-3 (Flt4). VEGF/VEGFR signaling mediate growth, migration and angiogenesis of tumor cells (13). VEGFR-2 activates PI3K/AMPK/Akt/eNOS pathway and nitric oxide (NO) production and tumor angiogenesis in cancer (14). Furthermore, inflammatory mediators and cytokines such as prostaglandin E2 (PGE2), interleukin 6 (IL-6) and tumor necrosis factor (TNF)-α have been shown to have a significant effect on the angiogenesis of tumor throught VEGFR-1 (15). According to this, therapeutic agents targeting VEGF/VEGFR signaling have been intensively investigated and improve patients' survival in colorectal cancer (16).

To the best of our knowledge, there are no reports addressing the specifc effects of spiny and thornless CPS oils with respect to signalling molecules which play a role during tumour viability, angiogenesis, metastasis and cell proliferation in colon carcinoma cells through VEGF/VEGFR signaling. This study determines the fatty acid composition of spiny and thornless CPS oil from Cyprus. In addition, we aimed to examine spiny and thornless CPS oils antiproliferative effects which decrease of Ki-67 expression and their protective effects toward relevant molecular mechanisms, including VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), eNOS, iNOS, IL-6, PGE2, TNF-α and VEGF that play an important role during migration, growth and angiogenesis in primary (Colo-320) and metastatic (Colo-741) colon adenocarcinoma cell lines.

Material and Methods

Spiny and thornless cactus pear seed oils

Spiny and thornless cactus pear fruits were picked in August from İskele province of Northern Cyprus. Cactus pear fruit pulps were removed from seeds. The seeds were washed and dries in atmospheric conditions. Spiny and thornless cactus pear seed oils were obtained by using supercritical CO_2 extraction (Super critical fluid extraction 100-2- FMC system) (Thar Instruments Inc.) [17].

Determination of fatty acids

Fatty acid composition of spiny and thornless cactus pear seed oils were investigated by GC-MS analysis. CPS oils were methylated according to the method described previously [18]. The fatty acid methyl esters were analyzed using an Agilent 5975C VL MSD with a triple-axis detector system and an Agilent 7890A GC system. The column was HP-Innowax FSC (60 m x 0.25 mm, 0.25 lm film thickness). Carrier gas was helium (1.4 mL/min). GC oven temperature was programed as 10 min at 60 ˚C/4 ˚C/min to 220 ˚C/10 min at 220 ˚C/1 ˚C/min to 240 ˚C/20 min at 240 ˚C (total 100 min). One microliter sample (10% in hexane v/V) was injected into the system. Injection mode: split mode (40:1). Injector temperature:250 ˚C. Transfer line temperature: 280 ˚C. Mass spectrum: 70 eV. Mass range: m/z 35 to 450 GC Detector: FID at 300 ˚C. In order to obtain the same elution order with GC-MS, the column outlet was split into two, one for FID and the other for MS detector.

Identification and quantification of fatty acids

The fatty acid constituents were identificated by parallel comparison of their retention indices and mass spectra with data stored in the Wiley/NIST GC/MS Library (W908N.L, New York, NY). n-Alkanes (C8-C40) were used as reference points in the calculation of retention indices (RI). Quantification of volatiles components was performed on the basis of their GC/FID peak areas using integration data.

Cell line and cell culture

Colo-320 (human colon adenocarcinoma, ATCC catalog: CCL 220) and Colo-741 cell lines (ECACC 93052621) were cultured in RPMI-1640 medium (Biochrom, FG1215), 10% FBS (Capricorn Scientific, FBS-11B), 1% penicillin-streptomycin (Biochrom,

A2213) and 1% glutamine (EMD Millipore, K0282). They were cultured in a humidified atmosphere at 37 °C in 5% CO_2. When the cells were 80% confluent, they were routinely subcultured using 0.25% trypsin-EDTA solution (Biochrom, L2143).

Colo-320 cells are semi-adhesive, rounded and refractile cells in standard culture conditions (Figure 1B). Colo-741 cells are fibroblast-like cells that grow with typical fibroblast colony morphology after 24 h in culture (Figure 1A).

Cell viability and growth assays

Cell viability and growth of Colo-320 and Colo-741 cells under various treatment conditions was evaluated by using the colorimetric MTT assay. Pure spiny and thornless CPS oils diluted in culture medium with four dilutions (1:1, 1:2, 1:4, 1:8, 1:16) at different time points (24 and 48 h). Cell suspensions were first prepared at densities of $5x10^4$/mL cells per each well of 96-well culture dishes and plated in triplicate for each oil concentration. Medium (100 µL) without CPS oil was used as a positive control, and only medium which did not contain any cells and CPS oil was used as a negative control. Cells were incubated for 4 h at 5% CO_2 (in air) and 37 °C with MTT solution. The medium was then decanted and insoluble formazan crystals were dissolved by 200 µL dimethylsulfoxide (DMSO, Sigma-Aldrich) to each well. The absorbance of each well was immediately read at 570 nm in an UV–visible spectrophotometer multiplate reader (Versa Max, Molecular Device, Sunnyvale, CA).

Cultivation of cells with spiny and thornless cactus pear seed oils

Colo-320 and Colo-741 cells were divided into three subgroups; the first group was the control group which was cultured with standard culture medium. The second group was treated with spiny CPS oil and third group was treated with thornless CPS oil.

Immunocytochemistry

Cultured Colo-320 and Colo-741 cells were assessed immunocytochemically for binding of antibodies against VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), eNOS, iNOS, IL-6, PGE_2, TNF-α and VEGF. Cells were fixed with 4% paraformaldehyde in PBS at 4 °C for 30 min. Tween 20 (Sigma-Aldich) was added for permeabilization for 15 min. The cells were washed with PBS and endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 5 min at room temperature. After washing cells with PBS three times for 5 min, primary antibodies VEGFR-1 (Flt-1) (RB-1527, Thermo Fisher Scientific, USA), VEGFR-2 (Flk-1) (sc-6251, Santa Cruz Biotechnology, USA), eNOS (*RB-1711*-P, Neomarkers, Inc.

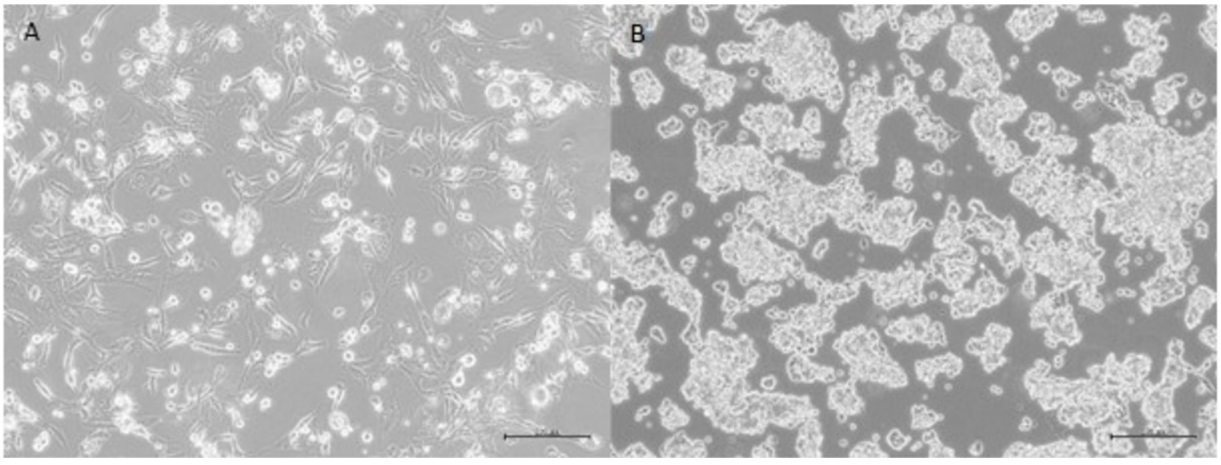

Figure 1. Colo-320 and Colo-741 cells imaged under an inverted microscope: (A) Colo-741 cells and (B) Colo-320 cells. Scale bars=200 µm.

Fremont, CA, USA), iNOS (RB-1605, Thermo Fisher Scientific, USA), IL-6 (sc-1265, Santa Cruz Biotechnology, USA), PGE_2 (ab2318, Abcam), TNF-α (sc-52746, Santa Cruz Biotechnology, USA) and VEGF (sc-7269, Santa Cruz Biotechnology, USA) were added and incubated overnight at 4 °C. Biotinylated secondary antibody (Histostain-Plus, IHC Kit, HRP, 859043, Thermo Fischer) was added and incubated for 30 min followed by PBS wash (×3) for 5 min. Streptavidin-peroxidase complex (100 µl) was added to cultured cells. Cells then washed by PBS and diaminobenzidine (DAB) (ScyTec, Logan, Utah,. USA, REF: ACK125, LOT: 15827) was added and incubated for 5 min for enhancement of immuno-labelling. Diaminobenzidine was washed with distilled water. Cells were counterstained with Mayer's hematoxylin for 5 min and mounted with mounting medium (Merck Millipore, 107961, Germany). All specimens were examined using a light microscope (Olympus BX40, Tokyo, Japan). Staining of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), eNOS, iNOS, IL-6, PGE_2, TNF-α and VEGF was also graded semi quantitatively using the H-SCORE that was calculated with the following equation: HSCORE=Σл (i+1), where i is the intensity of staining with a value of 1, 2 or 3 (mild, moderate, or strong, respectively) and л is the percentage of cells stained with each intensity, varying between 0 and 100%.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). The results were analyzed using GraphPad Prism 7 software. Differences among groups were analyzed statistically with Kruskal-Wallis where appropriate. A p value of<0.05 was considered as statistically significant.

Results

Analysis of spiny and thornless cactus pear seed oils

Spiny and thornless cactus pear seeds yielded fixed oil. Major compounds of spiny cactus pear seed oil were oleic acid (17.6%), linoleic acid (55,9%), palmitic acid (12.4%) and elaidic acid (4%). Palmitic acid and elaidic acid contents of thornless CPS oil are almost similar to spiny CPS oil as shown on Table 1. Total lipid extracted from thornless CPS oil show that oleic acid, linoleic acid, palmitic acid, elaidic acid contribute 15.6%, 60.1%, 12.3% and 4.1% of the total fatty acid content, respectively.

Cell viability and cytotoxicity

Colo-320 and Colo-741 cells were treated with different dilutions of spiny and thornless CPS oils for 24 and 48 h. Cell viability was determined by the MTT assay. Spiny CPS oil inhibited the growth of Colo-320 and Colo-741 cells in a dose-and time-dependent manner. In our study, 1:16 dilution spiny CPS oil was more effective in inhibiting Colo-320 and Colo 741 cell growth when compared with other dilutions for 48 h incubation (Figure 2). Additionally, our results showed that 1:8 and 1:16 dilutions of thornless CPS oil were more effective in inhibiting Colo-320 and Colo 741 cell growth when compared with other dilutions for 48 h, respectively (Figure 3).

Immunocytochemical evaluation

The immunoreactivity of Flk-1 was significantly decreased in Colo-741 cells treated with spiny CPS oil for 48 h compared to control group (Figure 4 A1, A2); the intensity of Flk-1 was significant in Colo-741 cells

Table 1. Fatty acid composition of spiny and thornless CPS oils (%).

Fatty acid	Spiny CPS oil value	Thornless CPS oil value
Oleic acid (Omega-9)	17.6	15.6
Linoleic acid (Omega-6)	55.9	60.1
Palmitic acid	12.4	12.3
Elaidic acid (Omega-9)	4	4.1

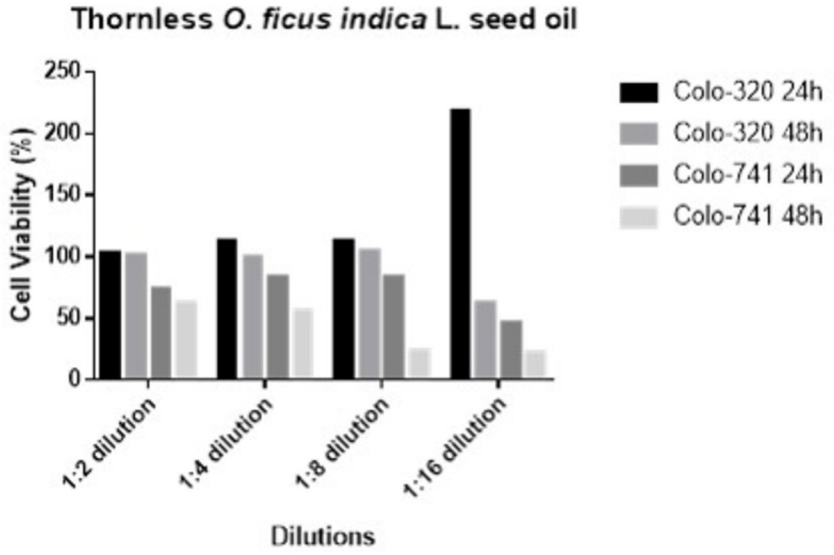

Figure 2. Effect of thornless CPS oil on cell viability of Colo-741 and Colo-320 cells. Colo-741 and Colo-320 cells were treated with different concentrations of thornless CPS oil for 24 or 48 h. Viability was quantitated by the MTT assay.

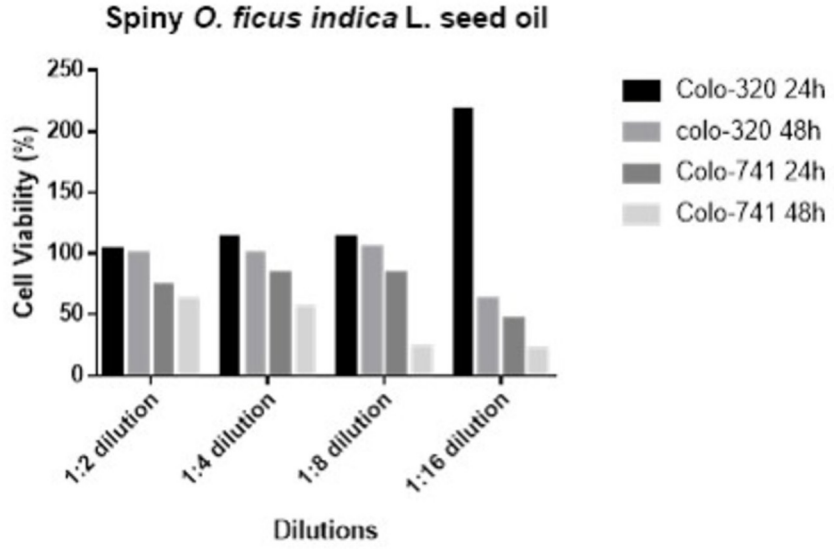

Figure 3. Effect of spiny CPS oil on cell viability of Colo-741 and Colo-320 cells. Colo-741 and Colo-320 cells were treated with different concentrations of spines CPS oil for 24 or 48 h. Viability was quantitated by the MTT assay.

(p=0.0011, Table 2). The H-SCORE of Flk-1 immunoreactivity was significantly decreased in Colo-320 cells which were treated with spiny and thornless CPS oils for 48 h compared to control group, respectively (Figure 5 J1-J3) (p=0.018, p=0.023, Table 2).

Immunoreactivity of Flt-1 was decreased in Colo-741 cells compared to the control group after treatment with thornless CPS oil for 48 h (Figure 4 B1, B3) (p=0.0086, Table 2). Also, both Flt-1 expression decreased in Colo-320 cells after incubation

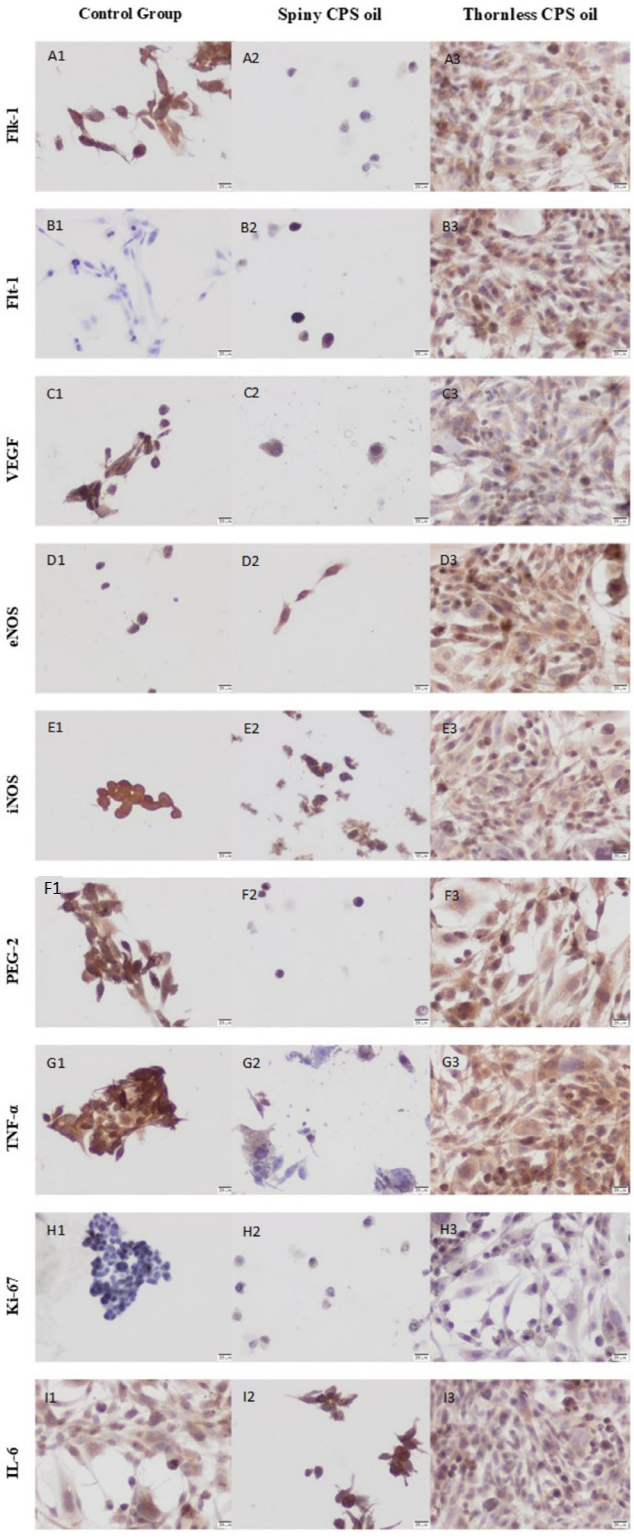

Figure 4. Immunoreactivity of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), eNOS, iNOS, IL-6, PGE-2, TNF-α and VEGF in Colo-741 for 48 h culture with with standard culture conditions (A1-I1) or spiny CPS oil (A2-I2) or thornless CPS oil (A3-I3). (Scale Bars=20μm)

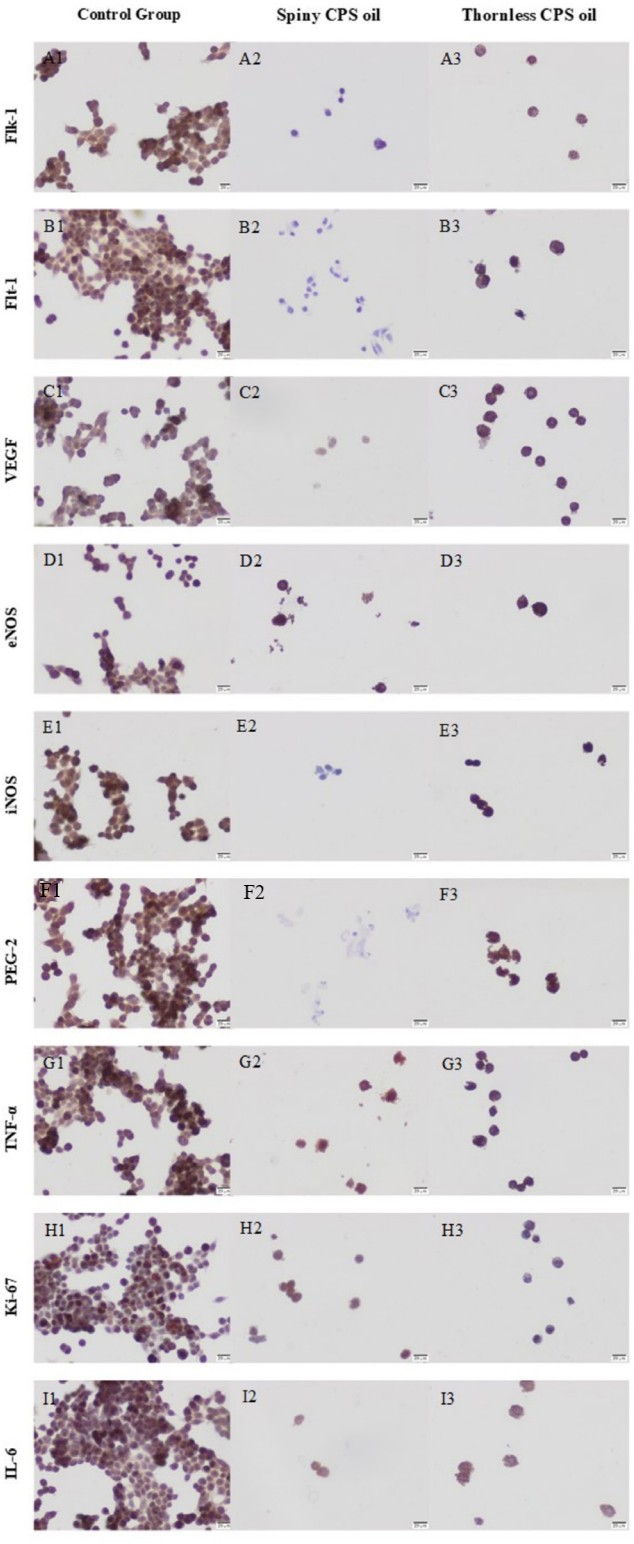

Figure 5. Immunoreactivity of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), eNOS, iNOS, IL-6, PGE-2, TNF-α and VEGF in Colo-320 for 48 h culture with with standard culture conditions (A1-I1) or spiny CPS oil (A2-I2) or thornless CPS oil (A3-I3). (Scale Bars=20μm)

Table 2. The intensity of Flt-1, Flk-1, eNOS, iNOS, IL-6, PGE-2, TNF-α and VEGF immunolabelling in Colo-320 and Colo-741 cells treated with spiny and thornless CPS oils for 48h

	Control Group		Spiny CPS oil		Thornless CPS oil	
	Colo-741	**Colo-320**	**Colo-741**	**Colo-320**	**Colo-741**	**Colo-320**
Flk-1	325±46,81	239,6±48,59	110±22,36a	121±12b	215,4±4,79	252,4±92,56c
Flt-1	104,2±9,41	236,9±24,05	135,2±57,89	110±23	254,8±31,59d	307,7±96,68e
VEGF	243,1±60,63	225,2±16,77	260±54,77	174±69,86f	238,1±12,06	375,4±25,99
eNOS	275±43,3	206,9±22,8	290±57,11	260±151,7	229,2±13,3	320±134,2
iNOS	390±22,36	251,6±57,5	235±41,83	260±151,7	178,5±12,55g	312,4±92,86
PEG-2	317,3±50,02	235,7±25,25	201,7±54,76h	120,5±27,78ı	276,8±24,6	292,5±115,8
TNF-α	255,5±42,52	226,9±9,95	199±113,3	387,4±19,03j	248,8±22,84	380±44,72k
Ki-67	120±21,37	239,3±60,48	168,3±19l	220±28,28	138,8±31,54	164,7±43,31
IL-6	183±36,87	223,8±12,95	178±42,33	280±164,3	205,7±44,89	297,9±60,74

Data are expressed as means ± SD and were compared by Kruskal-Wallis.

[a] The data was significant when compared with control group (p=0.0011)
[b] The data was significant when compared with control group (p= 0.018)
[c] The data was significant when compared with control group (p=0.023)
[d] The data was significant when compared with control group (p=0.0086)
[e] The data was significant when compared with spiny CPS oil threated cell group (p=0.0073)
[f] The data was significant when compared with thornless CPS oil threated cell group (p=0.0029)
[g] The data was significant when compared with control group (p=0.0011)
[h] The data was significant when compared with control group (p=0.047)
[ı] The data was significant when compared with thornless CPS oil threated cell group (p=0.026)
[j] The data was significant when compared with control group (p=0.02)
[k] The data was significant when compared with control group (p=0.011)
[l] The data was significant when compared with control group (p=0.047)

with spiny CPS oil compared to control group but HSCORE median did not differ. The HSCORE of Flt-1 significantly decreased in Colo-320 cells which were incubated with spiny CPS oil for 48 h, compared with thornless CPS oil treated Colo-320 cells (Figure 5 B1, B2) (p=0.0073, Table 2).

Staining for VEGF in Colo-320 and Colo-741 cells is summarized in Figure 5 and Figure 4. The H-SCORE results of VEGF did not differ in Colo-741 after incubation with both CPS oils for 48 h (Figure 4, Table 2). The immunoreactivity of VEGF was significantly decreased in Colo-320 cells after incubation with spiny CPS oil compared to thornless CPS oil (Figure 5 C2, C3) (p=0,0029, Table 2).

eNOS immunoreactivity results did not differ in extracted treated Colo-741 and Colo-320 cells and control group after incubation with spiny and thornless CPS oils for 48 h (Figure 4 and 5) (p > 0.05, Table 2). Figure 4 shows that the H-SCORES of iNOS in Colo-741 cells treated with thornless CPS oil for 48 h were significantly decreased versus control groups (p=0.0011, Table 2).

The immunoreactivity of iNOS was significantly decreased in Colo-741 cells incubated with thornless CPS oil compare to control groups (Figure 4 E1, E3). The expression of iNOS was evaluable in Colo-320 cells and the median H-SCORE for 48 h did not differ in Colo-320 after incubation with both spiny and thornless CPS oils for 48 h (Figure 5) (Table 2).

Immunoreactivity of PGE-2 was decreased in Colo-741 cells compared to the control group after treatment with both spiny and thornless CPS oils for 48 h (Figure 4 F1-F3). The HSCORE of PGE-2 significantly decreased in Colo-741 cells which were incubated with spiny CPS oil for 48 h (p=0.047, Table 2). Thornless CPS oil treated Colo-320 cells showed higher expression of PGE-2 at 48 h compared with spiny CPS oil incubated Colo-320 cells (Figure 5 F2, F3)(p=0.026, Table 2).

TNF-α immunoreactivity was decreased in Colo-741 cells incubated with both CPS oils but H-SCORE result was not statistically significant (Figure 4) (p > 0.05, Table 2). Interestingly, TNF-α immunostaining intensity was strong and immunoreactivity was higher in Colo-320 cells treated with spiny and thornless CPS oils than control group as shown in Figure 5 (G1-G3). The H-SCORE results revealed that, there was a significantly increase in H-SCORE in spiny and thornless CPS oils treated Colo-741 cells, respectively (p= 0.02, p=0.011, Table 2).

As shown Figure 6, the immunoreactivity of Ki-67 was significantly elevated in spiny CPS oil treated Colo-741 cells compare to control groups but not for Colo-320 cells (p=0.047, Table 2). Although spiny and thornless CPS oils treated Colo-320 cells showed lower H-SCORE values, the decrease was not statistically significant (p > 0.05, Table 2). The IL-6 immunoreactivity results did not differ in Colo-741 and Colo-320 after incubation with both CPS oils for 48 h (Figure 4, 5) (Table 2).

The H-SCORE of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), eNOS, iNOS, IL-6, PGE-2, TNF-α and VEGF immunolabelling in Colo-741 and Colo-320 cells treated with spiny and thornless CPS oils for 48 h are given in Table 2.

4. Discussion

Opuntia ficus-indica seed oil is a valued bioproduct that has a high degree of unsaturated fatty acids, vitamin E and biophenols (19). The chemical constituents, antidiabetic and hypocholesterolemic activities of the *Opuntia ficus-indica* seed oil have been investigated (20, 21). To date, there is no reported study addressing the effects of spiny and thornless *Opuntia ficus-indica* seed oils with respect to angiogenesis signaling pathway molecules in colon adenocarcinoma cells.

The main component for both form of *Opuntia ficus-indica* seed oils were linoleic acid (spiny 55.9%, thornless 60.1%), oleic acid (spiny 17.6%, thornless 15.6%), palmitic acid (spiny 12.4%, thornless 12.3%), elaidic acid (spiny 4%, thornless 4.1%). These results are partly similar to those of results of Yeddes et al. (2012). They determined higher oleic and palmitic acid contents in both form of CPS oils than our results (8). Also, linoleic acid and oleic acid contents of CPS oil grown in Turkey and Algeria were higher than our both CPS oils contents (9, 22). According to the literature, the observed differnces in the concentration of fatty acids could be due to the degree of the fruit maturation (23).

Angiogenesis is an crucial role in growth, proliferation and metastasization of colorectal cancer. Vascular endothelial growth factor (VEGF) signaling is an important angiogenesis pathway. VEGFs are family of related growth factors that includes five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (24). Anti-VEGF pathway therapies have been intensively used and investigated in colorectal cancer treatment because of survival benefits and reaponse rate. VEGFs and their receptors are main regulators of tumor associated angiogenesis of colorectal cancer. Moreover, they have been used as marker for colorectal cancer metastasis and progression [24, 25]. VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) are receptors that bind with VEGF and activate growth and migration of human colorectal carcinoma cells (25). Our study found that the immunoreactivity of Flk-1 was significantly decreased in Colo- 741 and Colo-320 cells incubated with spiny CPS oil compared to control group. Additionally, spiny CPS oil exhibited a stronger inhibition effect on VEGF than thornless CPS oil in Colo-320 cells. Interestingly, the immunoreactivity of Flk-1 and Flt-1 increased in Colo-320 cells which were incubated with thornless CPS oil but in Colo-741 cells only Flt-1 increased. These data suggest that spiny CPS oil inhibited angiogenesis in both Colo-320 and Colo-741 cells after 48h incubation with thornless CPS oil activated VEGF signaling

pathway. This difference in the inhibitory effect may due to the variation in chemical content of CPS oils. Spiny is wild but thornless is culturvated form of *Opuntia ficus-indica*.

Prostaglandin E_2 (PGE_2) is a bioactive pro-inflammatory lipid that reveal a wide range of biological effects associated with inflammation and tumor progression. PGE_2 promotes tumor growth in colorectal cancer and is often associated with a poor prognosis (26). In colon tumors, PGE_2 induces angiogenesis with VEGF-independent pathway (27). VEGF dependent and PGE_2 mediated pathway might be effective therapy for blocking tumor angiogenesis in colon cancer treatment. Our results showed that PGE_2 reactivities were decreased in both Colo-741 and Colo-320 cells after incubation with spiny CPS oil. These results suggested that spiny CPS oil shown to inhibite angiogenesis through PGE_2 mediated pathway.

TNF-α and IL-6 are an important cytokine that regulate cell proliferation and inflammation in cancer. These cytokines generate by tumor stromal cells and collect inflamatory cells to the tumor microenviroment. Furthermore, TNF-α and IL-6 enhance the proliferation and survival of cancer cells (28). In the current study, TNF-α immunoreactivity was increased in Colo-320 cells after treatment with both spiny and thornless CPS oils but results did not significantly different in Colo-741 cells. These results indicates that spiny and thornless CPS oils were triggered inflamation in Colo-320 cells. However, the immunoreactivities of IL-6 which is a one of the most consisitent colorectal cancer associated inflammatory marker did not differ in Colo-741 and Colo-320 after incubation with both CPS oils. It can therefore be concluded that both CPS oils initiate inflammatory reactions in Colo-320 cells but did not change tumor microenvironment with cancer promoting prognosis.

Inducible nitric oxide synthase (iNOS) is one of the NOS isoform generating nitric oxide (NO) from the amino acid l-arginine. NO induce apoptosis via iNOS in colorectal cancer cells (29). Considering our results, which showed that iNOS immunoreactivity was decreased in Colo-741 cells after treatment with thornless CPS oil. It can therefore be concluded that low expression level of iNOS may be reduced protective effect against carcinogenesis in metastatic colon carcinoma cancer cells after incubation with thornless CPS oil.

The human Ki-67 is a nuclear and nucleolar protein which immunoreactivity is associated with cell proliferation (30). Our results show that the spiny CPS oil has an proliferative effect on Colo-320 cells.

In conclusion, the fatty acid composition of spiny and thornless *Opuntia ficus-indica* seed oils from Cyprus was determined. Moreover, the anti-angiogenic effects both spiny and thornless *Opuntia ficus-indica* seed oils were investigated for the first time in both primary and metastatic colon carcinoma cells. Interestingly, thornless *Opuntia ficus-indica* seed oil increased angiogenesis thought signaling molecules in colon cancer cells. On the other hand, spiny Opuntia ficus-indica seed oil decreased signalling molecules which play a role in VEGF-dependent and PGE2 mediated angiogenesis. TNF-α and IL-6 are inflammatory cytokines and both spiny and thornless CPS oils increased TNF-α expression in Colo-320 cells but not IL-6. Overall, CPS oils did not change tumour microenvironment. In order to resolve exact different effects of spiny and thornless Opuntia ficus-indica seed oils on colon cancer cells, total active chemical composition must be identified. These *in vitro* results also need to be examined with *in vivo* experimental models for further progress of *Opuntia ficus-indica* seed oils, such as drug development and usage as a complementary agent.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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