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Expression of Vascular Endothelial Growth Factor and Anti-Proliferative Activity of Flaxseed Oil Alone and In Combination with Mefenamic Acid in Cell Lines

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Abstract

The flaxseed oil contains alpha-linolenic acid, lignans and flavonoids**.** It possesses anti-inflammatory, antioxidant, and tumor-suppressive effects. The purpose of this research was to explore the anti-proliferative effect of flaxseed oil alone and flaxseed oil in combination with mefenamic acid on human umbilical vein endothelial cells and Kaposi sarcoma cells and to identify the vascular endothelial growth factor gene expression. The anti-proliferative effects of flaxseed oil alone and in combination with mefenamic acid were investigated *in vitro* by the 3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. The effects of flaxseed oil alone and its combination with mefenamic acid were tested on the expression of vascular endothelial growth factor genes using real-time polymerase chain reaction. The results showed that the IC_{50} of flaxseed oil for human umbilical vein endothelial cells was 29.72 µg/ml and for Kaposi sarcoma cells was 16.48 µg/ml; moreover, the IC_{50} for flaxseed oil in combination with mefenamic acid for human umbilical vein endothelial cells was 252 µg/ml and for Kaposi sarcoma cells was 147.6 µg/ml. These outcomes revealed that flaxseed oil alone and its combination with mefenamic acid possess anti-angiogenic activity in a concentration-dependent manner which supported by significant decline in vascular endothelial growth factor expression.

Keywords: Flaxseed oil, Flaxseed oil in combination with mefenamic acid, Vascular endothelial growth factor.

التعبيرالجيني لعامل نمو بطانة الأوعية الدموية والنشاط المضاد لتكاثرخطوط الخلايا لزيت بذور الكتان بمفرده وبالاشتراك مع حامض الميفينياميك

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الخلاصة

يحتوي زيت بذور الكتان على حامض ألفا لينولينيك والقشور والفلافونويد، و يمتلك تأثيرات مضادة للالتهابات و الأكسدة والأورام. ان الهدف من هذه الدراسة هو لاستكشاف هل لزيت بذور الكتان لوحده و مع حامض الميفينياميك القدرة على منع تكاثرالخلايا البطانية للوريد السري البشري وخلايا كابوسي ساركوما مع قياس التعبير الجيني لعامل نمو بطانة الأوعية الدموية. تمت دراسة التأثيرات المضادة للتكاثر لزيت بذور الكتان لوحده و بالاشتراك مع حامض الميفينياميك في المختبر باستعمال طريقة (٣- (٤ ، ٥- ثنائي ميثيل ثيازول-٢- يل) -٢ ، ٥- ثنائي فينيل رباعي بروميد بروميد). كما تم اختبار تأثيرات زيت بذور الكتان لوحده وبالأشتراك مع حامض الميفينياميك على التعبير الجيني لعامل نمو بطانة الأوعية الدموية باستخدام تفاعل البلمرة المتسلسل. اظهرت النتائج ان التركيز اللازم لتثبيط نصف الاستجابة لزيت بذور الكتان تساوي ٢٩.٧٢ ميكروغرام / مل للخلايا البطانية للوريد السري البشري و لخلايا كابوسي ساركوما كان ١٦.٤٨ميكروغرام / مل, اما التركيز اللازم لتثبيط نصف الاستجابة لزيت بذور الكتان بالأشتراك مع حامض الميفينياميك على الخلايا البطانية للوريد السري البشري تساوي ٢٥٢ ميكروغرام / مل اما لخلايا كابوسي ساركوما فكان ١٤٧.٦ميكروغرام / مل, كما أظهرت النتائج ان كل من زيت بذور الكتان لوحده وبالاشتراك مع حامض الميفينياميك يثبط تكون الأوعية الدموية بطريقة تعتمد على التركيز ويدعم ذلك انخفاض كبيرفي التعبير الجيني لعامل نمو بطانة الأوعية الدموية.

الكلمات المفتاحية : زيت بذور الكتان ، زيت بذور الكتان بالاشتراك مع حمض الميفينياميك ، التعبير الجيني لعامل نمو بطانة الأوعية الدموية.

Introduction

The *Linum usitatissimum Linn*., or flax, is a member of the *Linaceae* family [(1)]. It contains alpha-linolenic acid (a type of omega-3 fatty acid), the lignin, secoisolariciresinol diglucoside (SDG), and flavonoids [(2)]. Like other edible oils , fatty acids make up the majority of FO; furthermore, phenolic substances, tocopherol, phytosterols, and triacyl glycerides (TAG) are also present in FO [(3)].The effects of phytosterol are anti-inflammatory, immunomodulatory, and anticancer [(4)]. Despite the fact that phytosterols have antioxidant properties [(5)] and prevent apoptosis from being induced, as well as cell growth, proliferation, and metastasis [(6)], such sterols are also inhibiting the production of carcinogens, and angiogenesis [(7)]. The primary elements that represented the antioxidant capabilities of FO were thought to be the phenolic compounds and tocopherols [(8)];

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where, the majority of the natural tocopherols found in FO were γ-tocopherol [9].

The anti-cancer actions of γ-tocopherol are due to its antiangiogenic properties [10]. Moreover, SDG in flaxseed has anti-cancer [11] and antioxidant effects by scavenging the free radicals, which can cause cell damage and a variety of diseases, including cancer [12].

Furthermore, the FO is represented as a rich source of the omega-3 poly-unsaturated fatty acid alpha-linolenic acid [13]. Moreover, numerous studies have demonstrated the anti-inflammatory properties of omega 3 and their beneficial effects on a range of human diseases, including autoimmune disorders, tumors and stroke [14].

The key angiogenesis mediators are vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1 and VEGFR-2) [15].

The human umbilical ventricular endothelial cell lines (HUVECs) are taken from the endothelial layer of the veins of the umbilical cord [16]; and these cells can proliferate easily in a laboratory [17]; and are considered suiTable for *in vitro* studies and research on the vasculature and angiogenesis [18].

The objectives of this study are to explore the anti-proliferative effect and cytotoxic activity of FO alone and in combination with mefenamic acid (COMB) on HUVECs and Kaposi sarcoma (KS) and to identify the VEGF gene expression.

Materials and Methods

The FO was purchased from Iraqi market from trade name HEMANI Linseed oil manufactured by Hemani International KEP7, Karachi, Pakistan. This study had been done in the Department of Pharmacology in the College of Medicine / Al-Nahrain University in Baghdad /Iraq.

Assessment of proliferation of cancer and normal cell line [19]

The 3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is frequently employed to assess the proliferation of a variety of cell lines. The cell passages must range from 4 to 7; and these cells were treated with serial concentrations of FO: 50, 25, 12.5, 6.25, 3.1, and 1.6 µg/ml and the COMB: 400, 200, 100, 50, 25, and 12.5 µg/ml of mefenamic acid with 50, 25, 12.5, 6.25, 3.1, and 1.6 µg/ml of FO for 24 hours. Moreover, the untreated cells received only dimethyl sulfoxide (DMSO) as a negative control.

Moreover, the phosphate buffered saline (PBS) at 5mg/ml is going to be added to the MTT to be ready for use later in the cell line; and each well of a 96-well plate received 20µl of the prepared MTT, and the plates were incubated at 37°C with 5% CO_2 for 4 hours. Later, these plates were removed from the incubator, and the supernatant was aspirated; and after that, 200µl of DMSO was added to each well; and then, all the plates were vigorously-shaken for approximately one minute. The absorbance of cells was read at 570nm by the microplate reader, with a value of 650nm as the reference reading. The absorbance of cells cultivated in control media is 100% viability. Furthermore, the experiment was carried out twice, with quadruplicate tests of each concentration; and each well contained $1x10^4$ cells, and the mean minus the standard deviation was used to calculate the percentage of cell viability:

Cell viability % =Mean optical density/control optical density × 100%

Inhibition %= 1- cell viability%

The cell growth extent was determined by counting with a hemocytometer. Following 24 hours of treatments with FO alone and COMB, the IC_{50} (the concentration required to prevent 50% of cell growth) was determined by counting viable cells of HUVEC and KS. The calculation of IC_{50} as µg/ml was accomplished with a sigmoidal plotting function by the Graph Pad Prism software program.

Cell lines

The HUVEC and KS were cell lines bought from the American Type Culture Collection (ATCC). The cells were cultured in Roswell Park Memorial Institute (RBMI) medium containing 10% heat-inactivated fetal calf serum (HIFCS) (Gibco, UK), 1% Penicillin/streptomycin (Sigma-Aldrich, Germany) and 1% glutamine. The HUVEC was used to assess cell viability against the FO and COMB which were kept alive in ECM-2 (Science Cell, USA).

Ten milligrams (10mg) of tested agents (FO, COMB) dissolved in 1ml DMSO then diluted with RBMI to make serial concentrations; then the cells treated with the serial concentrations of FO: 50, 25, 12.5, 6.25, 3.1, and 1.6 µg/ml and the COMB: 400, 200, 100, 50, 25, and 12.5 µg/ml of mefenamic acid plus 50, 25, 12.5, 6.25, 3.1, and 1.6 µg/ml of FO. The negative control wells got 200µl of the medium with 1% DMSO, the samples were introduced to the well in quadruplicate and incubated in the incubator for 24 hours at 37 °C with 5% CO_2; then the MTT was applied to these cells and incubated for 4 hours before measuring absorbance at 570nm [19].

Gene Expression

The KS cells were seeded at 1×10^4 cells on the day before treatment and then exposed to serial concentrations of both FO (50, 25, 12.5, 6.25, 3.1, and 1.6 µg/ml) and COMB (400, 200, 100, 50, 25, and 12.5 µg/ml of mefenamic acid and 50,25,12.5,6.25,3.1and 1.6 µg/ml of FO) for 24 hours.

The RNA was extracted from 1×10^4 treated cells using high-purity RNA isolation kit (Roche); and according to the manufacturer's instructions, the mRNA was subjected to DNase I treatment (Fermentas, Lithuania) to eliminate genomic contamination. Moreover, the RNA concentrations can be measured by using of UV spectrophotometer (Germany), and cDNA synthesis was achieved by

using a Revert Aid TM first strand cDNA synthesis kit (Fermentas, Lithuania).

The appropriate primers for the VEGFR gene (used as an internal control) were designed by Allele ID software for the analysis of PCR quantities.

Then 10µl of SYBR Green master mix (Japan) was added to 2µl of cDNA samples, 0.5 µl forward, 10 pmol reverse primers, and 7µl nuclease-free water (Germany) to perform PCR in 20µl of reaction mixture using real-time PCR apparatus (USA).

The PCR reaction involved the following steps:

- Denaturation at 95 degrees Celsius for 1 minute
- Followed by the initiation of about 40 cycles at 95 degrees Celsius for 10 seconds.
- Then annealing at 56 degrees Celsius for 15 seconds.
- Elongation at 72 degrees Celsius for 20 seconds.
- A single final step at 58 degrees Celsius for 90 seconds.

At the end of the program, the melting curve (measure the change of fluorescence that occurred when double strand DNA with merged dye dissociates into single strand DNA) was checked and the data were analyzed by CT calculation [20].

Statistical Analysis

Data were displayed as the Mean±SD, and were statistically-analyzed by one-way analysis of variance (ANOVA). The IC_{50} value was calculated by the utilization of the statistical program Graph Pad Prism; and the statistical analysis was carried out by using SSPS 16.0. A p-value less than 0.05 ($p<0.05$) was considered significant. Moreover, the Applied Biosystems Sequence Detection Software (SDS v1.3.1) was used to analyze the fluorescence signals, and the cycle threshold (CT), ΔCT, ΔΔCT, and RQ (relative expression) fold readings were automatically-determined using the $2^{-\Delta\Delta CT}$ method.

Results and Discussion

Antiproliferative activity of the FO alone and the COMB on HUVECs

The percentages of viability of FO on the HUVECs were 71.10±1.82%, 83.68±0.76%, 92.21±2.78%, 94.17±0.77%, 94.25±1.71%, and 94.29±2.98%, for the concentrations of FO, respectively that were shown in Table 1.

Table 1. Serial concentrations and their respective viability percentage of flaxseed oil (FO) on human umbilical vein endothelial cell (HUVEC) line and Kaposi sarcoma (KS) cells.

Concentration of FO (µg/ml)	HUVEC % of viability ± SD	KS % of viability ± SD
50	71.10±1.82%	54.05±1.53%
25	83.68±0.76%	62.23±2.58%
12.5	92.21±2.78%	79.75±6.52%
6.25	94.17±0.77%	86.07±3.76%
3.1	94.25±1.71%	95.99±0.98%
1.6	94.29±2.98%	95.99±0.94%,

The IC_{50} value for the FO for the HUVECs was 29.72 µg/ml; and the percentages of viability of COMB on the HUVECs were 80.13±4.98%, 87.92±3.58%, 92.21±2.78%, 96.49±1.29%, 96.95±1.14%, and 94.29±2.98% for the concentrations of COMB, respectively as shown in Table 2. The IC_{50} value for the COMB was 252µg/ml**;** and the number of viable cells decreased in a concentration-dependent manner for FO and COMB after 24 hours, as shown in Figures 1 and 2.

Carcinogenesis and metastasis greatly-depend on the angiogenesis which is a favorable target for anti-angiogenic drugs [21].

The MTT results showed that FO is not cytotoxic towards the endothelial cells, and the 50% inhibition equals 29.72µg/ml. The National Cancer Institute (NCI) states that herbs with IC_{50} values greater than 20 µg/ml are not regarded as cytotoxic [22], which may indicate that the tested oil had an IC_{50} value higher than 20µg/ml and had no detecTable cytotoxic impact on HUVECs; therefore, the anti-angiogenic effect of FO was not due to the cytotoxic nature of the compound but may be due to blocking angiogenesis mediators or receptors.

Moreover, Shen and coworkers observed an increase in the inflammatory response without causing cytotoxicity in HUVECs treated with 100ng/ml of a lipopolysaccharide of FO; and this observation was consistent with the results of the ongoing investigation [23].

Furthermore, the *in vitro* screening was conducted on the effects of COMB on the HUVECs; where, the IC50 value for the COMB on HUVECs was 252µg/ml, and this was higher than the IC50 for the FO alone; therefore, the antiproliferative of the COMB declined and may have an antagonistic effect, and this may be brought on by the presence of other chemicals or other active compositions with lower concentrations that could antagonize the active constituents [24]. As a result, the COMB had a

lower anti-proliferative effect on HUVECs than the FO alone; but, there was no significant difference between the addition of FO alone and COMB to HUVEC as shown in Figures 1 and 2.

Antiproliferative activity of the FO alone and the COMB on KS cells

The percentages of viability of FO on the KS cells were 54.05±1.53%, 62.23±2.58%, 79.75±6.52%, 86.07±3.76%, 95.99±0.98%, and 95.99±0.94% for the concentrations of FO, respectively as shown in Table 1.

Moreover, the IC_{50} value for the FO for the KS cells was 16.48µg/ml; and the percentages of viability of COMB on the KS cells were 64.20±2.66%, 73.53±1.27%, 83.29±2.80%, 92.13±1.59%, 96.80±0.94%, and 95.02±3.04% for the concentrations of COMB, respectively that are shown in Table 2; and the IC_{50} value for the COMB was 147.6µg/ml.

The number of viable cells decreased in a concentration-dependent manner for FO and COMB after 24 hours, as shown in Figure 1 and 2.

The *in vivo* and *in vitro* studies revealed that the FO have strong anti-angiogenic activity [25]. Furthermore, the anti-angiogenic compounds may have anti-tumor effect; and most anti-tumor compounds have a significant cytotoxic impact in cell culture systems [26].

The IC50 of a substance must be less than 20µg/ml in order to be considered cytotoxic to cell lines [22]. The IC50 value for FO on KS cells was 16.48µg/ml, (Figure 1); therefore, the FO can be considered a cytotoxic agent against KS cells. and it may possess anti-tumor activity.

In the current research, when the concentrations of FO and COMB increased, the KS cell viability decreased. This finding showed that FO and COMB had dose-dependent efficacy against KS cells.

The anti-tumor activity of FO may be due to the presence of many constituents such as lignin [11], omega-3 fatty acids [14], vitamin E [12], and phytosterols [4]; and the results of the current study are agreed with those performed by others; where, the flaxseed orbitides (a new formulation of FO that consist of cyclic octa-, nona-, and decapeptides) showed considerable cytotoxicity against the triple-negative subtype of human breast cancer [27].

The selective toxicity of FO on the malignant cell proliferation was due to its cytotoxic effect on KS cells with no adverse toxic effect on endothelial cells, and therefore this finding agreed with the results of the other investigation, which stated that FO can selectively inhibit cancerous cells [28].

According to another study, FO inhibited the development of many different malignant cell lines in a dose-dependent manner [29].

Furthermore, the *in vitro* screening was conducted on the effects of COMB on KS cells; since, on KS cells, the IC50 value for the COMB was 147.6g/ml; and this was higher than the IC50 for the FO alone in KS (Figures 1 and 2). Therefore, the antiproliferative activity of the COMB declined and was considered non-cytotoxic and may have an antagonistic effect, which may be brought on by the presence of other chemicals or low concentration of other compositions that could antagonize the active constituents [24].

In the present study, there was a significant difference ($p<0.05$) between the addition of FO alone in KS and HUVEC at concentration 50, 25, 12.5, 6.25µg/ml as shown in Table 1.

Also, there was a significant difference ($p<0.05$) between the addition of COMB in KS and HUVEC at concentration 50, 25, 12.5µg/ml of FO and 400, 200, 100µg/ml of mefenamic acid as shown in Table 2.

Table 2. Serial concentrations and their respective viability percentage of flaxseed oil in combination with mefenamic acid (COMB) on human umbilical vein endothelial cell (HUVEC) line and Kaposi sarcoma (KS) cells.

Concentration of FO (µg/ml)	Concentration of mefenamic acid (µg/mL)	HUVEC % of viability ± SD	KS %of viability ± SD
50	400	80.13±4.98%	64.20±2.66%
25	200	87.92±3.58%	73.53±1.27%
12.5	100	92.21±2.78%	83.29±2.80%
6.25	50	96.49±1.29%	92.13±1.59%
3.1	25	96.95±1.14%	96.80±0.94%
1.6	12.5	94.29±2.98	95.02±3.04%

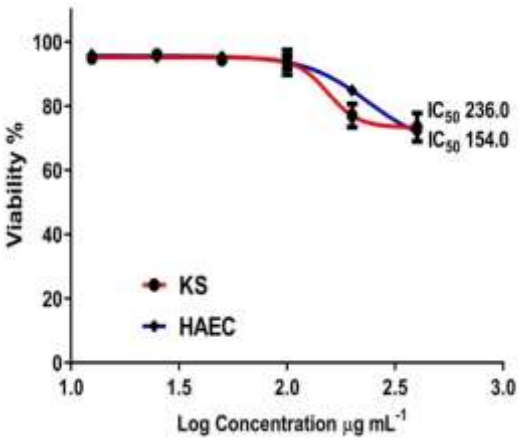

Figure 1. The percentage of viability of FO on HUVEC and KS

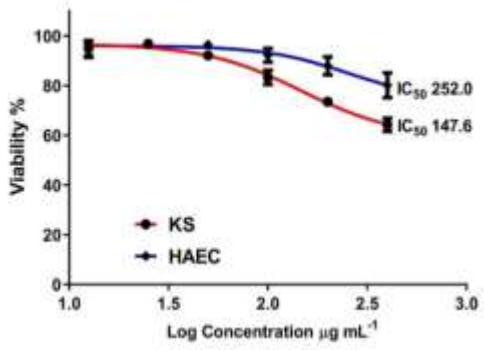

Figure 2. The percentage of viability of COMB on HUVEC and KS

Gene expression of VEGF for KS cells by FO alone and COMB using RT- PCR

The concentrations of FO 50, 25, 12.5 and 6.25 µg/ml significantly diminished the transcription of the VEGFs to 16.2%, 17.9%, 48.9% and 88.2%, respectively ($p < 0.05$). At 3.1 and 1.6 µg/ml of FO no significant effect on gene expression as shown in Table 3 and Figure 3.

Moreover, Figure 4 summarized the RT-PCR cycle numbers in the serially diluted FO samples.

Table 3. Concentration of FO, their folds and % of VEGF expression

Concentration of FO µg/ml	**Fold**	**% of VEGF expression**
Control gene (house keeping gene)	1	100
50	0.162668	16.2
25	0.179244	17.9
12.5	0.48971	48.9
6.25	0.882703	88.2
3.1	0.997287	99.7
1.6	0.986233	98.6

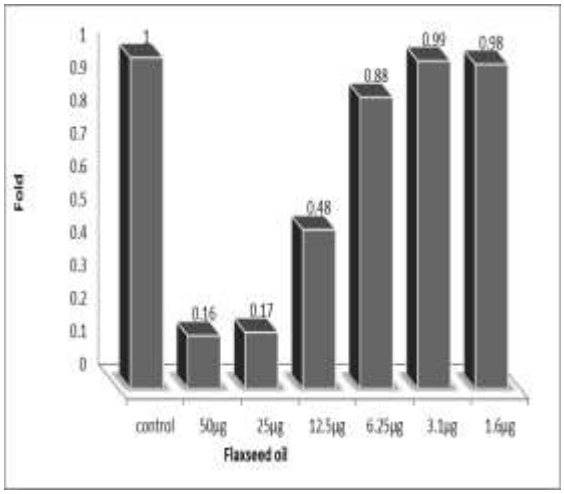

Figure 3. Dose response effect of gene expression with fold for FO

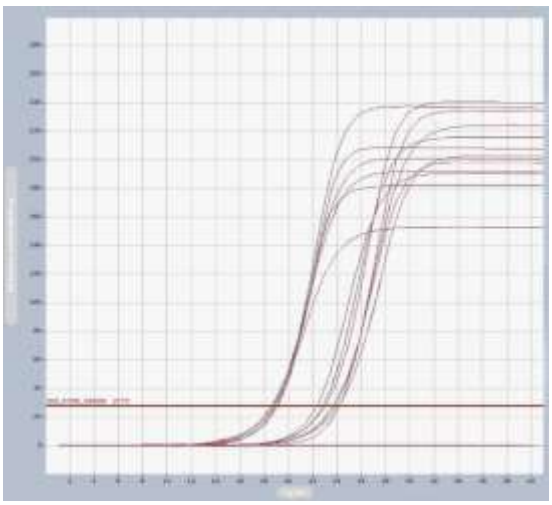

Figure 4. The RT-PCR cycle numbers in the serially diluted FO samples.

The concentrations of COMB at 400, 200, 100, 50 and 12.5µg/ml of mefenamic acid plus 50, 25, 12.5, 6.25 and 1.6µg/ml of FO significantly-diminished the transcription of VEGF to 10.3 %, 14.9%, 16.8%, 65.5% and 80.6%, respectively ($p< 0.05$). At 25µg of mefenamic acid plus 3.1µg of FO as COMB showed a non-significant effect on the gene expression as shown in Table 4 and Figure 5.

The transcription of VEGF is not significantly different between the FO alone and the COMB.

Figure 6 summarized The RT-PCR cycle numbers in the serially diluted COMB samples.

Table 4. The concentration of COMB, their folds and % of VEGF expression

Concentration of COMB µg/ml		**Fold**	**% of VEGF expression**
Flaxseed oil	**Mefenamic acid**		
Control gene (house keeping gene)		1	100
50	400	0.103665	10.3
25	200	0.149685	14.9
12.5	100	0.168404	16.8
6.25	50	0.655197	65.5
3.1	25	0.972655	97.2
1.6	12.5	0.806642	80.6

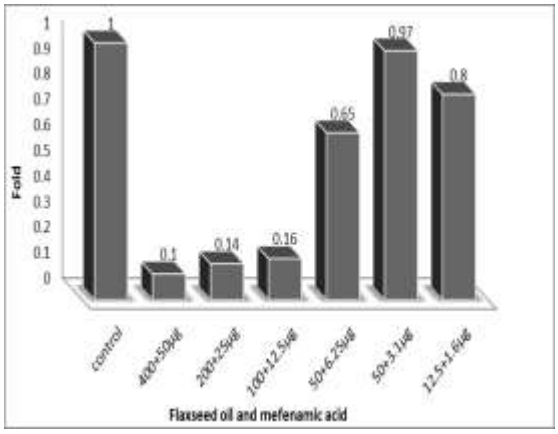

Figure 5. Dose response effect of gene expression with fold of COMB

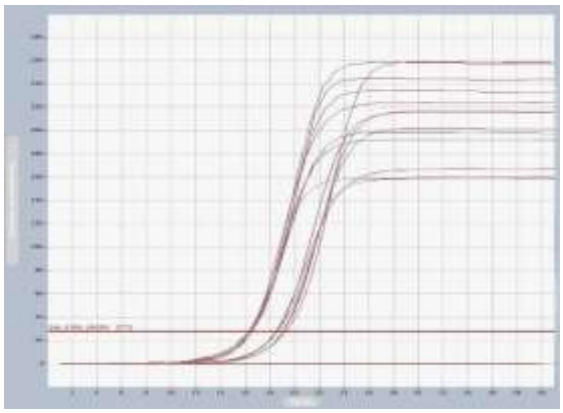

Figure 6.The RT-PCR cycle numbers in the serially diluted COMB samples.

Researchers reported that one of the most crucial pro-angiogenic factors involved in the tumor angiogenesis is the VEGF [30]. Moreover, the VEGF-A, which binds and activates the VEGFR-1 and VEGFR-2 receptors, can regulate vascular permeability, proliferation, migration, and survival of endothelial cells [31]. Furthermore, the antiangiogenic drugs are well recognized to target VEGF and its receptors [32].

The current study demonstrated that, the FO had a significant antiangiogenic impact since it considerablyreduced the expression of VEGF to around 16.2%, 17.9%, 48.9%, and 88.2% for the following concentrations: 50, 25, 12.5, and 6.25 µg/ml, respectively as shown in Table 3.

Moreover, this study also showed that the COMB exhibited a considerable antiangiogenic effect, as it significantly-decreased VEGF expression to roughly 10.3 %, 14.9%, 16.8%, 65.5% and 80.6% for the following concentrations of mefenamic acid: 400, 200, 100, 50 and 12.5µg/ml with FO 50, 25, 12.5, 6.25 and 1.6µg/ml as COMB respectively as shown in Table 4.

The present study, revealed that FO and COMB produced a concentration-dependent decline in VEGF transcription and, in turn a decline in angiogenesis (Tables 3 and 4).

Moreover, in the current study, FO and COMB have been shown to reduce the expression of VEGF on its own receptors by preventing VEGF from interacting with and activating VEGFR on the surface of KS, which in turn inhibits the proliferation, migration, and tube formation of endothelial cells.

The FO have many constituents, which can decline the expression of VEGF; since these compounds are vitamin E [33], lignans [34], phytosterols [35], omega-3 [36] and polyphenol [37], which can verify that the FO has antiangiogenic effect.

Moreover, the consumption of FO can reduce the VEGF expression in the solid Ehrlich ascites carcinoma [38], which supports the results of the ongoing investigation.

Moreover, the COMB displayed as a potent anti-angiogenic activity which may be due to the anti-angiogenic constituents of FO in COMB or may be from the mefenamic acid, which is an anti-inflammatory medication that inhibits angiogenesis by decreasing VEGF expression, inhibiting endothelial cell proliferation, migration, and spreading, as well as inducing endothelial apoptosis [39].

Conclusion

The current study displayed that, the FO and COMB were able to suppress angiogenesis in an *in vitro* assay on HUVECs and KS cells in a concentration-dependent manner which may be attributed to the existence of alpha-linolenic acid, phytosterols, lignans, and flavonoids. FO had selective anti-angiogenic activity on HUVECs.

The antiangiogenic effect of the FO and COMB were confirmed by RT-PCR on mRNA gene expressions, which revealed a significant decrease in transcript levels in KS cultures exposed to FO and COMB. It implies that the FO has the potential to be a promising and successful anti-angiogenic agent in complementary chemotherapy. These findings provide useful information for future research on FO into the therapeutic/preventive modalities for various cancers.

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Conflict of Interest

None

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College of Medicine, Al-Nahrain University.

Ethics Statements

This work approved by animal ethics committee from College of Medicine ,Al-Nahrain University

Author Contribution

Noor A. Al-Zubaidy main researcher and work at lab Hayder B Sahib research designer and aid in statistics

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