The Antiangiogenic Activity of Flaxseed Oil Alone and Combination with Mefenamic Acid in Vivo and in Vitro Assay

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Abstract

Background: Antiangiogenic agents are commonly used for the management of many types of cancers. Flaxseed oil is a healthy source of omega-3, omega-6, and lignans, and possesses anticancerous and antioxidant activities. Objective: To investigate the antiangiogenic activity of flaxseed oil alone and in combination with mefenamic acid in a dose-response study. Methods: Oil was extracted from flaxseeds. The ex vivo rat aorta ring assay was used to screen the flaxseed oil alone and in combination with mefenamic acid for possible antiangiogenic activity. Also, the assay was used to determine the dose–response effect. An in vivo chick chorioallantoic membrane (CAM) assay was used to quantify the zone of inhibition of blood vessel by flaxseed oil. Results: The 200, 100, 50, 25, 12.5, and 6.25 µg/ml of the flaxseed oil inhibited blood vessel growth with values of 91±0.42, 84±2.06, 36±2.71, 21±2.15, 23±1.56, and 5±0.93%, respectively, with an IC50 value of 45.695 µg/ml. These concentrations showed a dose-dependent inhibition of angiogenesis. At 200, 100, and 50 µg/ml of flaxseed oil in combination with 50 µg/ml of mefenamic acid inhibited blood vessel growth with inhibition percentages of 81.48±0.82, 79.63±0.75, and 77.78±1.26, respectively, with an IC50 of 2.27 µg/ml. Also, these concentrations indicated a dose-dependent inhibition of angiogenesis. Flaxseed oil produced a significant inhibition zone of blood vessels in the CAM assay. The LD50 for flaxseed oil was 5.656 g/kg. Conclusion: Flaxseed oil alone and in combination with mefenamic acid exhibited antiangiogenic activity both in ex vivo and in vivo assays. At doses of 2.5, 1.25, 0.625, and 0.312 g/kg of flaxseed oil intraperitoneally injected into mice, no symptoms of toxicity or death of mice due to acute toxicity were observed. However, doses of 5 and 10 g/kg of oil resulted in the death of the majority of mice.

Keywords: Flaxseed oil- Mefenamicacid- assay-angiogenic activity

Introduction

Angiogenesis is the physiological process that involves the formation of new blood vessels from pre-existing ones (Birbrairetal., 2015). Endothelial cell replication stimulated by pathological stimulatory diseases. In disease cases such as malignancy, ophthalmic, and inflammatory conditions, the angiogenic stimulatory signals become too much and the equilibrium concerning the inhibitors and inducers of angiogenesis is disturbed, and this subsequently leads to angiogenesis witch (Carmeliet, 2005). Antiangiogenesis are thought to be the most effective in the treatment of cancer when used in conjunction with chemotherapy because they inhibit the growth of blood vessels rather than tumor cells (Yoo and Kwon, 2013).

Plants offer new hope in the treatment of a large number of angiogenesis-related diseases. Linumusitatis Linn., belonging to the family Linaceae, is frequently well-known as linseed or flaxseed oil is extracted from flaxseed, which is a rich source of omega-3, omega-6, and lignans (secoisolariciresinoldiglycoside; SDG) (Bekhit et al., 2018). Flaxseed oil possesses anticancerous and antioxidant activities. It also contains vitamins such as vitamins A, C, F, and E (Rizvi, 2021). The aim of this study was to investigate the antiangiogenic activity of flaxseed oil alone and in combination with mefenamic acid in both in vivo and in vitro assays.

Materials and Methods

Source of flaxseed

Flaxseed oil is a drying oil, meaning it can polymerize into a solid form. Generally, linseed oil is obtained by pressing, sometimes followed by solvent extraction (Bekhit et al., 2018). However, in this study, the flaxseed oil was obtained from the Hemani Group, which has extensive experience in the herbal trade. The product’s trade name is linseed oil.

Source of experimental animals and ethical approval

Albino rats (12–14 weeks of age) were obtained from

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Preparation of flaxseed oil

Flaxseed oil was extracted from the flaxseeds using the method described by mechanical pressing for extracting flaxseed oil. Because of the high levels of -linolenic acid (ALA) in flaxseed oil, high temperatures must be avoided during pressing. Usually, flaxseed oil obtained through cold pressing has high levels of ALA. Numerous types of flaxseed oil presses have been developed, which range from the simple hydraulic press to the more sophisticated continuous screw press (Bekhit et al., 2018). But cold pressing can also bring negative effects on the quality of oil. Due to low pressing temperatures, microorganisms may not be killed completely through the pressing, which can decrease the quality of flaxseed oil. Furthermore, due to low mass transfer under cold pressing, the contents of vitamins, phospholipids, phytosterols, and antioxidants in the oil are minor. These compounds contribute to the stability of flaxseed oil. So, to prolong the shelf life of flaxseed oil, it is highly proposed that flaxseed oil must be kept in a container with dark colour, and incorporated with the antioxidants (Tang et al., 2021).

Rat aorta ring anti-angiogenesis assay

The angiogenesis assay, which was developed by Brown and co-workers in 1993, was used in the present study. Freshly excised thoracic tissues were washed with Hanks Balanced Salt Solution containing 2.5µg/ml amphotericin B (Sigma, St. Louis, MO). The fibro adipose apparent materials, as well as any residual clots, were removed from the tissue sample. Then, they were cut into 1mm thick aortic ring pieces under a dissecting microscope (Motic, Taiwan). Forty-eight-well tissue culture was used in this assay (Coster Corning, USA). An aliquot of 500µl of 3mg/ml fibrinogen (Calbiochem, USA) in serum-free M199 growth medium (Gibco, UK) was placed into each well with 5mg/ml of aprotinin (Sigma Aldrich, Germany). Each tissue segment was placed into each well with 5mg/ml of amphotinin (Sigma Aldrich, Germany). Each tissue segment was placed in the center of the well and 15µl of thrombin (50NIH U/ml) (Sigma-Aldrich, Germany) in 0.15M NaCl was added. Following the embedding of the vessel fragment in the fibrin gels, each well was filled with 0.5 ml of medium M199 containing 20% HIFCS (Gibco, UK), 0.1% aminoacaproic acid (Sigma-Aldrich, Germany), 1% amphotericin (Sigma-Aldrich, Germany), 1% L-glutamine (Sigma-Aldrich, Germany), and 0.6% gentamcicin (Sigma-Aldrich, Germany). Six replicates were used to test flaxseed oil alone and in combination with mefenamic acid.

Control cultures were grown in media that did not contain the test oil. To make the final DMSO concentration, the flaxseed oil was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) and diluted in an M199 growth medium. For five days, 1% of vessels were cultivated at 37°C with 5% CO2 in a humidified incubator (Binder, Germany). On the fourth day, a fresh medium was added to each well. The positive control used in the current study was Suramin at 100 μg/ml, which is well known as an antiangiogenic agent (Sigma-Aldrich, Germany). An inverted microscope (Olympus, Japan) was used to quantify the limit at which blood vessels grow under a magnification of 40x on the fifth day of the experiment with the aid of a camera (Leica CCD, Japan) and Leica QWin software packages. The technique established by Nicosia and co-workers in 1997 was employed in the current investigation to calculate the magnitude of vessel growth inhibition. The length of the small blood vessel outgrowths from the primary explants was determined. Using six replicates per sample, the procedure was repeated three times.

The percentage of blood vessel inhibition was determined according to the formula:

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\text{Blood vessel inhibition} = 1 - \left( \frac{A_0}{A} \right) \times 100
\]

where A0 denotes the distance of blood vessel growth in meters, and A denotes the distance of blood vessel growth in the control in meters (Brown et al., 1993).

Dose-response study of flaxseed oil alone and in combination with mefenamic acid with rat aorta assay

A serial dilution of the flaxseed oil alone and in combination with mefenamic acid was prepared. The IC50 was calculated by using the linear regression equation for flaxseed oil (Stiffey-Wilusz et al., 2001).
Lethal dose 50 test on experimental animals treated with flaxseed oil

The toxicity study was conducted using 35 male albino mice weighing a range of 20 to 25 g each. The mice were divided into seven groups in an equal and random manner, with one group serving as a control and the remaining six receiving flaxseed oil. Each group had five mice. All the mice in the toxicity study were given water and food, and they were given seven days to adjust to the laboratory conditions before the experiment. The control group received only 0.3 ml of 2% Tween 80 solution orally, whereas each mouse in the treated group received flaxseed oil through the intraperitoneal route, which was made by dissolving 8.0 g in a 10 ml volume of 2% Tween 80 in the following doses: 0.625, 1.25, 2.5, 5, and 10g/kg. To keep track of any deaths or changes in general behaviour or other physiological processes, the mice were observed continuously for the first four hours and then every 24 hours for the next 48 hours after receiving the flaxseed oil (Alsina-Sanchis et al., 2021).

Statistical analysis

The obtained data were expressed as mean±standard deviation of the mean (SD).

The results were reported as mean standard deviation (SDV). One way analysis of variance (ANOVA) followed by a Tukey test comparison t-test (2-tailed) was used to compare between treatments groups. Statistical analysis was carried out using native functions in the Microsoft software. The differences between the means are considered significant at the 5% confidence level. The concentration that inhibits 50% of the blood vessels growth and cell proliferation (IC50) was analyzed by a logarithmic equation. The statistical analysis was carried out by using SSPS 16.0. The level of significance was set at P<0.05.

Results

Effect of flaxseed oil alone and in combination with mefenamic acid on rat aorta rings, on day five of the experiment, flaxseed oil and its combination with mefenamic acid (COM) inhibited blood vessel growth. When compared to the other concentrations, flaxseed oil at 200 g/ml demonstrated a high percentage of antiangiogenic action as shown in Table 1 and Figure 1. Flaxseed oil in concentrations of 200, 100, 50, 25, 12.5, and 6.25 g/ml decreased blood vessel growth by 91±0.42, 84±2.06, 36±2.71, 21±2.15, 23±1.56, and 5±0.93%, respectively. When combined, 200, 100, and 50 g/ml of flaxseed oil and 50 g/ml of mefenamic acid were utilized in different concentrations of flaxseed oil. For the above concentrations, percentages of inhibition for COM were 81.48±0.82, 79.63±0.75, and 77.78±1.26%, respectively, Table 2.

Dose-response curve of flaxseed oil alone and in combination with mefenamic acid on rat aorta rings

In Figure 2, the dose-response curve of the serially diluted flaxseed oil on the rat aorta ring assay is presented. There were six concentrations used: 200, 100, 50, 25, 12.5 and 6.25 g/ml. These concentrations showed a dose-dependent inhibitory activity, with percentages of inhibition as follows: 91±0.42, 84±2.06, 36±2.71, 21±2.15, 23±1.56, and 5±0.93%, respectively. Blood vessels were quantified after 5 days of flaxseed oil addition, where the IC50 value was calculated from the linear regression equation Y=25.886ln(x)-48.962 equating to 45.695 µg/ml. Where Y denotes percentage of inhibition and X denotes concentration. Serial concentrations (200, 100, and 50µg/ml) of flaxseed oil in combination with 50µg/ml of mefenamic acid were added to the rat aorta rings. The percentages of inhibition for the aforementioned concentrations were 81.48±0.82, 79.63±0.75, and 77.78±1.26%, respectively, indicating a dose-dependent inhibitory action. After 5 days of combination addition, the IC50 value of blood vessel quantification was 2.27g/ml.

In vivo CAM assay with flaxseed oil

On the seventh day of the in vivo assay, the zone of inhibition for flaxseed oil was enlarged. Because of the flaxseed oil’s impact, blood vessel regression in the CAM started; disorganization in the vessel occurred with a yellowish pale appearance. The zone of inhibition was detected by and a vascular zone around the disc that had previously been soaked in flaxseed oil, and the size of this zone was amplified according to the prior scoring.

Table 1. Serial Concentrations and Their Respective Inhibition Percentage for Flaxseed Oil on Rat Aorta Ring

| Concentration μg/ml | Percentage of inhibition ± SD |
|---------------------|-------------------------------|
| 200                 | 91±0.42                       |
| 100                 | 84±2.06                       |
| 50                  | 36±2.71                       |
| 25                  | 21±2.15                       |
| 12.5                | 23±1.56                       |
| 6.25                | 5±0.93                        |

Table 2. The Concentrations and Their Respective Inhibition Percentage for Flaxseed Oil in Combination With Mefenamic Acid on Rat Aorta Ring

| Concentration (µg/ml) Mefenamic acid | Concentration (µg/ml) Flaxseed oil | Percentage of inhibition ± SD |
|--------------------------------------|-----------------------------------|------------------------------|
| 50                                   | 200                               | 81.48±0.82                   |
|                                      | 100                               | 79.63±0.75                   |
|                                      | 50                                | 77.78±1.26                   |

Table 3. The Scoring for the Inhibition Zone of Blood Vessels Growth in in vivo (CAM) Assay for Flaxseed Oil

| No. | Scoring for the inhibition zone |
|-----|---------------------------------|
| 1   | 8                               |
| 2   | 10                              |
| 3   | 12                              |
| 4   | 15                              |
| Mean| 11.25                           |
| + SD| 2.764                           |

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System. At a score of (+++), flaxseed oil produces a strong inhibitory zone of blood vessels in the CAM (Table 3).

Analysis of the lethal dose 50 test on animals treated with flaxseed oil

Table 4 shows the numbers of dead and alive male mice after 14 days of experimentation. For each group of mice, serial concentrations of flaxseed oil were administered. The lethal dose of flaxseed oil required to kill 50% of male mice is called the LD50. The LD50 was determined as 5.656g/kg using the equation $y = 9.8291x - 5.5989$ and $y=50$.

Acute toxicity study of flaxseed oil on mice

During the 14-day study period, no obvious signs of toxicity or mortality of mice were identified after the mice were intraperitoneally injected with the doses of flaxseed oil at 0.625, 1.25, and 2.5g/kg, except for a little variance in weight growth in comparison to the control (Figure 4). Meanwhile, the majority of the mice that were intraperitoneally injected with doses of 10 and 5g/kg died and only gained a minor amount of weight. The weight growth of the mice is shown in Figure 5.

| Group | NO. Mice | Dose of flaxseed oil g/kg | NO. of Dead Mice | Cumulative percentage of Dead Mice |
|-------|----------|---------------------------|------------------|----------------------------------|
| 1     | 5        | 10                        | 4                | 80                               |
| 2     | 5        | 5                         | 4                | 80                               |
| 3     | 5        | 2.5                       | 0                | 0                                |
| 4     | 5        | 1.25                      | 0                | 0                                |
| 5     | 5        | 0.625                     | 0                | 0                                |
| 6     | 5        | 0.312                     | 0                | 0                                |
| 7     | 5        | Control                   | 0                | 0                                |

Figure 1. Effects of Different Concentrations of Flaxseed Oil on Blood Vessel Growth

Figure 2. Dose-response Curve of Flaxseed Oil on Vascular Growth
of male mice was evaluated at day zero and day 14, and there was no significant difference in weight between the oily and control groups (P>0.05) as presented in Table 5.

### Discussion

Angiogenesis has a central role in tumour growth. In addition, it is the main player in tumour invasion and metastasis. For that reason, agents with the antiangiogenic property are one of the important lines for cancer disease treatment (Ren et al., 2019). In recent years, this antiangiogenic assay has been broadly used to evaluate angiogenesis in part or entire organ culture as an ex vivo assay (Carlyn et al., 2009). Mefenamic acid is one of the drugs belonging to the non-steroidal anti-inflammatory drug (NSAID) class, which are known to hinder angiogenesis (Wong, 2019; Frassanito et al., 2019) and proliferation (Khan et al., 2022). In the present study, the main objective was to investigate whether flaxseed oil and its combination with mefenamic acid have antiangiogenic activity or not. The results reveal that all of the flaxseed oil test concentrations in this investigation retained antiangiogenic action, with 200 of g/ml flaxseed oil having the highest percentage of antiangiogenic activity when compared to the other concentrations employed. The dosage response study was carried out to identify the concentration of flaxseed oil that would prevent vessel development by 50%. The flaxseed oil has an IC$_{50}$ value of 45.695 g/ml and decreases blood vessel growth.
in a dose-dependent manner. The presence of several phytochemicals in flaxseed oil can be attributed to its antiangiogenic effect. Flax oil is a wonderful source of omega-6 and omega-3 fatty acids (Bekhit et al., 2018). Also, it contains lignans (SDG) (Al-Mathkury et al., 2016), terpene, alcohols, phenolic compounds, caffeic acid, p-coumaric acid, ferulic acid, Ɣ-tocopherol, phytosterols (campesterol, stigmasterol, and sitosterol), and vitamins A, C (Rizvi, 2021).

Omega-3 is one of the flaxseed oil constituents that possesses potent antiangiogenic effects by inhibiting the production of different angiogenic mediators like; vascular endothelial growth factor (VEGF), cyclo-oxygenase-2 (COX-2), prostaglandin-E2 (PGE2), and others (Spencer et al., 2009). Vitamin C has antiangiogenic activity (Shanmugam et al., 2014). Vitamin A possesses antiangiogenic and antiproliferative potentials through VEGFR-2 kinase inhibition (Metibemu et al., 2021). There are several pathways for antiangiogenic effect of phytosterols in flaxseed oil, including limiting carcinogenic generation, malignant cell development, cell angiogenesis, cancer cell invasion, and cancer cell metastasis. Lignans in oil may also reduce cancer risk by limiting angiogenesis and metastasis and blocking pre-cancerous alterations in cells. Flax oil contains vitamin E, which inhibits inflammation and angiogenesis. In the ex vivo administration of flaxseed oil and mefenamic acid to the rat aortic ring, the IC₅₀ value was 2.27µg/ml, which is lower than the IC₅₀ for flaxseed oil alone. Therefore, the combination of flaxseed oil with mefenamic acid may have a synergistic effect, and the combination of flaxseed oil with mefenamic acid exhibited potent antiangiogenic activity. The synergistic effect of such a combination may be due to mefenamic acid, having a COX-2 inhibitory activity that hinders inflammation and angiogenesis in vivo and in vitro. Many elements of flaxseed oil have antiangiogenic activity, including vitamin E, lignans, omega-3 (Imamura et al., 2018), and others, resulting in stronger antiangiogenic activity than using a drug or oil alone.

The CAM assay is a classical technique for the in vivo study of angiogenesis (Ribatti, 2010). The current investigation discovered that CAM treated with 20 µl of oil had a significant antiangiogenic effect. A huge number of flaxseed oil-treated blood vessels stopped radiating from underneath the disc that carried the 20µl of flaxseed oil. Furthermore, the flaxseed oil-treated blood vessels became sparse, disordered, and pale yellow in appearance. According to the findings of this investigation, flaxseed oil displayed a substantial antiangiogenic effect in vivo. The inclusion of a range of phytochemicals in oil, such as omega-6, omega-3, lignans (SDG), caffeic acid, p-coumaric acid, ferulic acid, Ɣ-tocopherol, phytosterols, and vitamins A, C, and E, may explain the substantial antiangiogenic impact of oil in CAM (Rizvi, 2021). Polyphenols (such as flavonoids and terpenes) have long been recognized to inhibit angiogenic processes and cell metastasis by coordinating many signalling pathways. Flavonoids, in particular, have substantial antiangiogenic effects through regulating the expression of VEGF, matrix metalloproteinases (MMPs), and EGFR, as well as inhibiting the NFB, PI3-K/Akt, and ERK1/2 signaling pathways (Rizvi, 2021).

In a study published in 2020, Keykhasalar and coworkers found that flaxseed oil nanoemulsion reduced the length and number of vessels in a CAM assay, proving that flaxseed oil nanoemulsion has antiangiogenic activity. This study supports the antiangiogenic effect produced by flaxseed oil in the in vivo CAM assay in the current study (Keykhasalar et al., 2020). Lethal dosage (LD50) is the dose that kills 50% of test animals and is used to assess the acute toxicity of drugs and plants. After the mice were intraperitoneally injected with a single dose of flaxseed oil at 2.5, 1.25, 0.625, and 0.312 g/kg, there was no signs of toxicity or death of mice were observed during the 14-day acute toxicity experimental period. On the other hand, in the animals that were intraperitoneally injected with doses of 5 and 10 g/kg of oil, the majority of the mice in these groups died. The LD50 was computed using a linear equation and came out to be 5.656g/kg. The results showed a very tiny gain in weight in comparison to mice in the control group, which could be attributed to the oil’s toxicity, but the difference in weight between groups is not significant (P >0.05) when compared to their control group (Pillai et al., 2021).

In their study, Kaithwas et al. (2011) found no change in body weight in rats given flaxseed oil intraperitoneally, and this observation is in agreement with the findings of the current investigation into acute toxicity.

In conclusion, the findings of this study indicate that in rat aorta angiogenesis sex vivo and CAM in vivo assays, flaxseed oil alone and in combination with mefenamic acid had an antiangiogenic impact. There was no evidence of toxicity or death observed during the 14-days acute toxicity test period, when flaxseed oil was intraperitoneally injected into mice at doses of 2.5, 1.25, 0.625, and 0.312 g/kg. However, doses of 5 and 10 g/kg of oil resulted in the death of the majority of mice.

**Author Contribution Statement**

Noor A. Al-Zubaidy: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft.

Hayder B Sahib (HS): Supervision, Validation, Writing – review & editing.

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**Approval**

This paper is part of the dissertation of the corresponding author at the College of Medicine at Al-Nahrain University in Partial Fulfilment of the Requirements for the Degree of PhD in Science in Pharmacology.

**Ethic statement**

Handling of the animals and the experimental design...
were carried out based on the guidelines reported in “Guide for the care and use of laboratory animal” and according to the protocol approved by the Al-Nahrain University/college of medicine, Baghdad, Iraq with the ethical clearance number 20200970 date 14/03/2022.

Data Availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest
The authors declare that they have no competing interests.

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