International Journal for Pharmaceutical Research Scholars (IJPRS)

V-3, I-1, 2014 ISSN No: 2277 - 7873

RESEARCH ARTICLE

Comparative Evaluation of Analgesic Activities of Aniseed Essential and Fixed Oils

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Manuscript No: IJPRS/V3/I1/00025, Received On: 23/01/2014, Accepted On: 01/02/2014

ABSTRACT

Different dosage forms of Aniseed oil have been used in Persian traditional medicine for their pain killer and analgesic properties but their effects have not been compared scientifically. Aniseed essential oil was extracted using hydro-steam distillation method. Fixed oil was extracted with petroleum ether and oils composition was analyzed by GC-MS. Phenolic content of fixed oil was determines spectrophotometrically. Different concentrations of essential and fixed oil were injected i.p. to the rats and animals were evaluated for analgesic effect at three times (30, 60 and 120 min) after injection by tail-flick and formalin tests. Major constituents of essential and fixed oil were anethole (87.58%) and 9-octadecenoic acid (74.55%). All doses of fixed and essential oil after 60 min had the highest significant analgesic effect compared to the control group ($P < 0.01$). The analgesic effect of the essential oil (500 mg/kg) was higher than paracetamol (100 mg/kg) in chronic phase of pain in formalin test ($P < 0.05$). Also, in both tests analgesic effects of essential oil was higher than fixed oil in all doses but it was significant just at 500 mg/kg ($P < 0.05$). Analgesic effects of essential and fixed oils of *P. anisum* are comparable with paracetamol but essential oil in higher doses was significantly more effective than fixed oil.

KEYWORDS

Aniseed, Analgesia, Essential oil, Fixed oil, Pain, *Pimpinella anisum*, Rat

INTRODUCTION

Pain is an unpleasant feeling often caused by intense or damaging stimuli. The management of pain is considered as a major clinical problem.[1] Acute pain indicates injury and has essential biological value as an alert of imminent or ongoing tissue lesion, leading the individual to fight, flee, or attempt to remove the causal agent.[2] Chronic pain is considered a disease that results from extensive inflammatory reactions that usually results in tissue damage and a loss of function.[3]

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Medicinal plants and their active components have been increasingly explored to find promising alternatives to treat pain and inflammation processes.

Pimpinella anisum L. (Anise), member of Apiaceae family, is an annual herb with white flowers and small green-yellow seeds, which grow in warm regions of the world.[4] Whole aniseed, its extracts or volatile oils are used as a food spice in many countries.[5] The aniseed also has a long list of traditional medicinal uses such as carminative, diuretic, diaphoretic, expectorant antiseptic and antispasmodic.[6] Both the extracts and essential oil of *Pimpinella* are known to be rich in pseudoisoeugenol type phenylpropanoids which is unique to the genus. Aniseed oil has been used in Persian traditional

medicine for its pain killer and analgesic properties but these effects have not had been proved scientifically. The aim of this study was to evaluate and compare phytochemical composition and analgesic effects of essential and fixed oils of aniseed in different *in vivo* models of pain.

MATERIALS AND METHOD

Plant Material and Extraction of Fixed Oil

Aniseed was purchased from a local market in Shiraz, Iran. The seeds were authenticated by Miss Sedigheh Khademyan (taxonomist at Shiraz School of Pharmacy) and its voucher specimen was hold in department of Pharmacognosy, Shiraz University of Medical Sciences with the code 184 PM. Seeds (200 g) were milled and extracted with petroleum ether applying Soxhlet apparatus for 6 h. The extract was concentrated by a rotary evaporator at 40ºC and finally dried by speed vacuum for 48 h at 40°C. The solvent free fixed oil was placed in well closed tubes and stored at -20°C.

Extraction of Essential Oil

Clevenger apparatus with a round bottle flask was applied for 5 h to extract volatile constituents of the seeds (100 g). One milliliter of n-hexane was added to trap the volatile oil. The essential oil was collected, dried under anhydrous sodium sulfate. The n-hexane was evaporated under a stream of N_2 and then solvent free essential oil was stored at well closed tubes at -20°C.

Estimation of Total Phenolic Compounds

For determination of total polyphenols, 50 ml of n-hexane was added to 7 grams of dried petroleum ether extract (fixed oil), and then 60% methanol was added (100 ml×3). Metabolic phase was dried, dissolved in water, and then extracted with petroleum ether. Separated aqueous phase was saturated with NaCl and extracted four times by ethyl acetate (each time with double volume of aqueous phase). Ethyl acetate Fractions was collected dried with anhydrous sodium sulfate fallowed by rotary operator. Total phenol content was determined by the Folic Ciocalteu method.[7] UV absorbance was read at 740 nm and serial dilutions of standard gallic acid were used to draw calibration curve. All experiments were carried out in triplicate.

Methyl Ester Preparation of Fixed Oil Fatty Acids

For analysis of fatty acids by GC-MS methyl ester derivatives were prepared. Dried aniseed extract (0.2 g) was dissolved in 1 ml of toluene, and then 2 ml of 1% sulfuric acid in methanol was added to sample. Palmitic acid (0.1 ml of 2 g/l solution) was added as internal standard (experiment was carried out in triplicate). Test tubes were incubated at 50 °C, overnight. Sodium chloride solution (5 ml of 5%) was added to each tube and esters were extracted with n-hexane fallowed by washing with 4 ml of 2% sodium bicarbonate solution. Methyl ester derivatives of fatty acids were analyzed by the gas chromatography-mass spectroscopy (GC/MS).[8]

GC-MS Analysis

GC/MS analysis was carried out using Agilent technologies 7890 gas chromatograph with a mass detector Agilent technologies model 5975 C), The gas chromatograph was equipped with a HP-5MS capillary column (phenyl methyl siloxan, 30 m×0.25 mm i.d., Agilent technologies 19091S – 433 (60 to 325/350 °C).

The mass spectrometer (Agilent technologies 5975 C) was operating in EI mode at 70 eV. The interface temperature was 280 °C; mass range was 30–600 m/z. Analysis of essential oils was performed with oven temperature program A and the Kovats index (KI) of components was calculated for all compounds using a homologous series of n-alkanes under the same operational conditions of analysis. Analysis of essential components was based on a comparison of their retention time, KI, mass spectra and comparison of them with Wiley, Adams libraries spectra and Pherobase Kovats Index Database.[9]

Program B was applied for determination of fatty acids profile of the fixed oil and analysis was based on comparison of peaks retention

time and mass spectra with standard fatty acids and Wiley (257).

A: The oven temperature was programmed from 60 °C (4 min) to 250 °C at a rate of 3 °C/min and increased at a rate of 5 °C/min to 280 °C and held for 10 min. The carrier gas was helium with a flow rate of 1.2 ml/min.

B: The oven temperature was held at 160 °C for 2 min and then increased from 160 to 230 °C at a rate of 8 °C/min. and was held at this temperature for 20 min., the injector temperature was 250 °C. Helium was the carrier gas with the flow rate of 1 ml/min. and split ratio was 1:10.

Experimental Animals

Sprague-Dawley female rats (180-220 g) were sampled from the laboratory animal center of Shiraz University of Medical Sciences, Shiraz, Iran. The animals were acclimated for one week under 12 h light and 12 h dark cycle at room temperature of 25-30 °C. Chow diet and water were provided ad libitum. Animal care and treatment procedures conformed to the Institutional Guidelines and Animal Ordinance (Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran).

Analgesic Tests

In this study, two types of painful stimuli were used to induce pain in the rats by direct stimulation of pain receptors and they included: thermally (tail-flick test) and chemically (formalin) induced pain.

Tail Flick Test

In order to assess the acute analgesic models, Tail flick (Borje Sanat, Iran) was used. 70% of light intensity and sensitivity were used. A 15 second cut-off was imposed to avoid tail damage. Duration of time in tail-flick latency was measured as a response to pain. In this test, rats were randomly divided into different groups of six animals. In negative control group, the animals received dimethyl sulfoxide as vehicle (DMSO 250 µl/kg, i.p.) and in positive control group rats received the standard drug paracetamol 100 mg/kg, i.p. In test groups; analgesic activity of essential oil at doses of 125, 250 and 500 mg/kg, i.p. and fixed oil at doses 125, 250 and 500 mg/kg, i.p. was assessed by observing the reaction time. Following the administration of vehicle, paracetamol and different doses of oils, the reaction time was noted at 30, 60 and 120 min.[10]

Formalin Test

In this model, in negative control group, the animals received vehicle (DMSO 250 mg/kg, i.p.) and in positive control group rats received the standard drug paracetamol 100 mg/kg, i.p. Analgesic activity of essential and fixed oils at doses of 250 and 500 mg/kg, i.p. was assessed by observing the reaction time in the test groups. 60 minutes after vehicle, paracetamol and oils treatment, fifty micro liters of 2.5% formalin (V/V in distilled water) was subcutaneously injected into the plantar surface of the animals' right hind paw. Nociception was rated using the original formalin test protocol.[11]

Briefly, the pain scoring measurement was follows: 0, no response behavior of the injected paw; 1, limping during locomotion or resting the paw lightly on the floor; 2, elevation of the injected paw; 3, licking or biting of the injected paw, or grooming. Behavioral responses were observed and recorded for 1 h after the formalin injection. The first 5 min was considered as the early phase and the period between 20 and 60 min as the second phase.

Following subcutaneous intraplantar injection of formalin, the animals were immediately placed in a chamber with a mirror placed under it, with a 45° angle underneath the floor to allow an unobstructed view of the formalin injected paw. All animals were brought to the test chamber 1 h prior to the experiment.

Statistical Analysis

Results were expressed as mean values ± SD and statistical comparison of data was performed one way analysis of analysis of variance (ANOVA) followed by Dunnets test. All levels of significance were set at $P < 0.05$.

RESULT AND DISCUSSION

The Content and Composition of Aniseed Essential Oil

The yield of essential oil from aniseed was 1.50% (v/w). GC-MS analysis revealed the presence of 15 compounds which represented 98.92% of the oil (Table 1). Trans-anethole (87.58%) was the major constituent of aniseed oil, followed by carvotanacetone (2.51%), γ-himachalene (1.77%), estragole (1.56%), limonene (1.36%) and dillapiole (1.11%).

Table 1: Constituents of aniseed essential oil

Compounds	KI	Percentage
Limonene	1029.86	1.36
Estragole	1200.45	1.56
Dihydrocarvone	1207.97	0.31
Carvotanacetone	1246.92	2.51
Anis aldehyde	1256.21	0.50
Trans –Anethole	1292.71	87.58
1H-Benzocycloheptene	1455.44	0.15
Gama-himachalene	1484.00	1.77
Germacrene	1486.40	0.70
Methyleugenol	1493.37	0.09
Alpha –zingiberene	1498.33	0.83
Beta-Hemachalene	1506.40	0.12
Beta-bisabolene	1511.95	0.22
Beta-sesquiphellandrene	1527.61	0.12
Unknown	1596.29	0.30
Dillapiole	1628.9	1.11
Unknown	1845.687	0.69
Unknown	1900.31	0.09

The Fatty Acid and Polyphenol Content of Aniseed Fixed Oil

In order to extract fixed oil, aniseed was extracted with petroleum ether which its yield was 7% (W/W, in dry matter). Total phenolic content of fixed oil was calculated 0.01±0.001 (W/W %). The GC-MS analysis revealed the presence of 9 components which represent 85.15% of fixed oil (Table 2). As it can be seen, 74.55±3 (W/W %) of the fixed oil was 9-octadecenoic acid (oleic acid) as the major component. Anethole was also detected in fixed oil (0.32±0.04, W/W %).

Table 2: Fatty acids and anethole content of aniseed fixed oil

Compound	Formula	Percentage
7-Hexadecenoic acid	C_{16}:1	0.89
Hexadecanoic acid	C_{16}:0	6.76
15-Methyl-11-hexadecnoic acid	$C_{17}H_{32}O_2$	0.14
9-Octadecenoic acid	C_{18}:1	74.55
Octadecanoic acid	C_{18}:0	1.30
11,13-Eicosenoic acid	C_{20}:2	0.58
Cis-Anethole	$C_{10}H_{12}O$	0.32
Eicosanoic acid	C_{20}:0	0.30
Docosanoic acid	C_{22}:0	0.31

Tail-Flick Test

As shown in Fig. 1, injection of different doses of essential oil (125, 250, 250 mg/kg; i.p.) to rats significantly enhanced the tail flick reaction time compared to control group (DMSO) in 30, 60, 120 min after injection, as compared with paracetamol (100mg/kg), the antinociceptive effect of essential oil at dose 500 mg at times 60 and 120 min after injection was significantly higher than paracetamol.

As shown in Fig.2. Injection of different doses (125, 250 and 500 mg/kg; i.p.), of fixed oil to rats caused a significant increase in time of latency compared to control group in 30, 60, 120 min after injection, this antinociceptive effect was not significantly different when compared with paracetamol.

Also, analgesic effect (30, 60, 120 min after injection) of essential oil was higher than fixed oil in all doses but it was significant just at 500 mg/kg, $P < 0.05$ (Fig. 3).

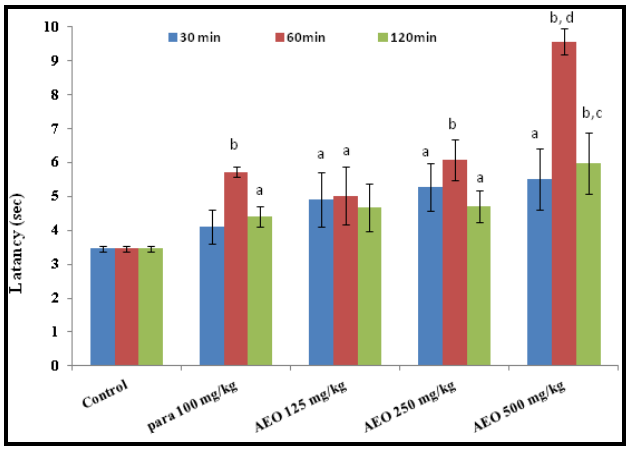

Figure 1: Analgesic effect of aniseed essential oil in tail flick test on rats (30, 60 and 120 min after injection), data are expressed as mean ± SEM (n = 5), Control: DMSO 250 µl/kg, Para: Paracetamol, AEO: Aniseed essential oil, [a]Significantly different with the control group ($P < 0.05$), [b]Significantly different with the control group ($P < 0.01$), [c]Significantly different with the paracetamol group ($P < 0.05$), [d]Significantly different with the paracetamol group ($P < 0.01$)

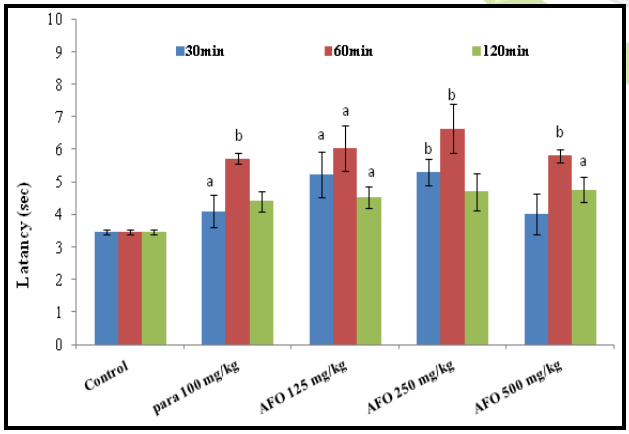

Figure 2: Analgesic effect of aniseed fixed oil in tail flick test on rats (30, 60 and 120 min after injection), data are expressed as mean ± SEM (n= 5). Control: DMSO 250 µl/kg, Para: Paracetamol, AFO: Aniseed fixed oil, a significantly different with the control group (P< 0.05), bSignificantly different with the control group (P < 0.01)

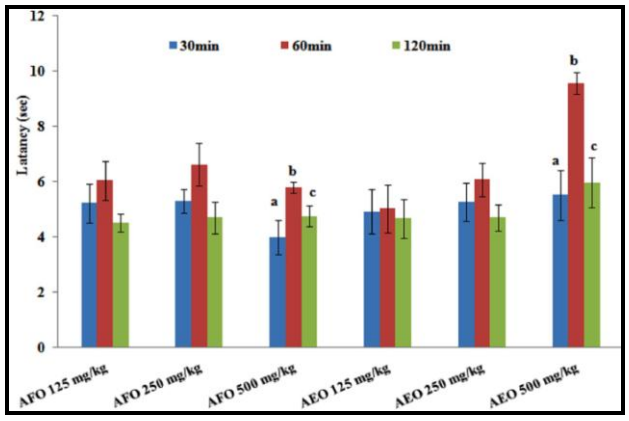

Figure 3: Comparative evaluation of analgesic activities of aniseed essential and fixed oils in tail flick test on rats (30, 60 and 120 min after injection), data are expressed as mean ± SEM (n= 5). AFO: Aniseed fixed oil, [a]significantly different with each other ($P < 0.01$), [b]significantly different each other ($P < 0.0001$), [c]significantly different with each other ($P<0.05$)

Formalin Test

The effect of essential oil in early (0–5 min) and late (20–60 min) phases of formalin test are shown in Table 3.

Table 3: Effects of the essential and fixed oils of aniseed in formalin test on rats (n=5, data are expressed as mean ± SEM)

	First phase (0–5 min)	**Second phase (20–60 min)**
Group	Score of pain	Score of pain
Control (DMSO 250 µl/kg)	2.39±0.10	1.74±0.22
Paracetamol, 100 mg/kg	1.66±0.28[a]	1.17±0.26[a]
Essential oil, 250 mg/kg	1.16±0.34[a]	0.81±0.32[a, c]
Essential oil, 500 mg/kg	0.35±0.13[a, d]	0.16±0.08[a, d]
Fixed oil, 250 mg/kg	1.4±0.22[a]	1.24±0.13[a]
Fixed oil, 500 mg/kg	1.4±0.56[b]	1.14±0.20[a]

[a]Significantly different with the control group ($P < 0.01$), [b]Significantly different with the

control group ($P < 0.05$), [c]Significantly different with the paracetamol group ($P < 0.05$), [d]Significantly different with the paracetamol group ($P < 0.01$)

In the both early and late phases of formalin test, doses of 250 and 500 mg/kg of essential oil showed significant effect on the nociception compared to control group. As compared with the paracetamol in early phase, the analgesic effect of essential oil was significant, only at higher dose 500 mg/kg, while in late phase of formalin test both doses of essential oil (250 and 500 mg/kg) was significant. Also, in the both phase of formalin test doses 250 and 500 mg/kg of fixed oil showed significant effect on the nociception compared to control group at 60 min after injection. Analgesic property of essential oil in both phases was significantly higher than fixed oil at 500 mg/kg, $P < 0.05$ (Fig. 4).

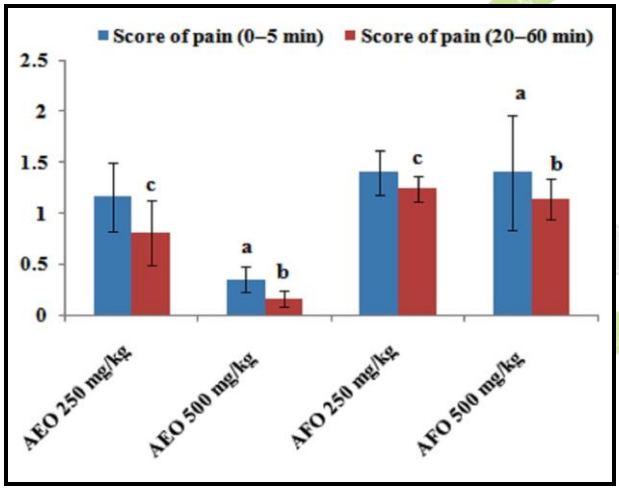

Figure 4: Comparative evaluation of analgesic activities of aniseed essential oil (AEO) and fixed oil (AFO) in formalin test on rats (n=5, data are expressed as mean ± SEM), [a]significantly different with each other ($P<0.01$), [b]significantly different each other ($P < 0.0001$), [c]significantly different with each other ($P<0.05$)

Several medicinal oils are prepared and used as practical therapeutic dosage forms in Persian, Chinese and Indian traditional and folk medicine. But little is done to evaluate their effects scientifically. These oils have been prepared by distillation methods, direct compression, maceration in heated oil or other lipophylic solvents and boiling the aqueous extract in oily vehicles. Thus, the concept of medicinal oil may be different in traditional medicine and current medicine and acceptable scientific investigations may be essential for application of these oils in current medicine.[12]

Aniseed oil has been used in Persian traditional medicine for its pain killer and analgesic properties. Also, this plant is used for variety of conditions such as headaches, melancholy, nightmare, epilepsy, seizure.[13] So far, different studies were performed on the extracts and oil of *P. anisum* to identify the chemical compounds and pharmacological properties of this plant.[14,15] Some recent investigations have reported analgesic or anti-inflammatory properties for the essential or fixed oil of aniseed with different methods. For example, screening of some Iraqi medicinal plants for analgesic activity showed that the extracts of *P. anisum* exhibited significant analgesic activity versus benzoquinone induced writing and in thermal tests.[16] In another study by Tas, essential oil of *P. anisum* exhibited significant analgesic effect comparable to morphine and aspirin.[17] But the aim of this study was to compare analgesic effects of essential and fixed oil of aniseed in different *in vivo* models of pain. Also, since constituent of the essential and fixed oils of the medicinal plants may vary due to genetically biodiversity and ecological factors, and thus affects their biological activities chemical constituent of the oils were primarily analyzed by GC-MS and spectrophotometric methods.[18,19]

Yield of aniseed essential oil and its constituents were similar to previous reports with slight variation,[15] except for the presence of carvotanacetone (5-isopropyl-2-methyl-2-cyclohexen-1-one). This compound was previously reported from essential oil of *Prunus mahaleb*[20], *Psidium salutare*[21] and some *Eucalyptus specie*[22,23] but we could not find any previous report on its presence in aniseed volatile oil.

GC-MS analysis of the oil revealed that the major constituents of the fixed oil are

unsaturated 9-octadecenoic acid (18:1, most probably oleic acid) and palmitic acid which is consistent with previous reports.[24,25]

In this study, analgesic properties of the oils were compared using formalin and tail flick tests and compared statistically. Analgesic property of essential oil in both phases of formalin test was significantly higher than fixed oil at 500 mg/kg ($P < 0.05$).

In the formalin test, interestingly, nociception occurs in two phases. The first phase is probably a direct result of stimulation of nociceptors in the paw, while the second phase may reflect the inflammation process, and at least to some degree, the sensitization of central nociceptive neurons. Drugs such as opioids which act mainly centrally inhibit both phases of formalin induced pain, while drugs, such as non-steroidal anti-inflammatory drugs which are primarily peripherally acting, only inhibit the late phase.[11] The second phase of formalin test is related to a peripheral inflammatory process. In formalin test, administration of 250 and 500 mg/kg (i.p.) of aniseed essential and fixed oil caused decrease in pain responses in the first and the second phases of formalin test. These findings suggest that central mechanisms may be involved in the antinociceptive activity of these oils. Analgesic activities of the essential and fixed oils of aniseed were also, compared by tail flick test. The maximum analgesic activity of the essential and fixed oil was within 60 minutes after injection in this model of analgesia (Fig. 1 and 2).

In tail flick test, analgesic effect of essential oil was higher than fixed oil in all doses but it was significant just at 500 mg/kg ($P < 0.05$). This may be the result of higher concentration of anethol in essential oil than the fixed oil (Table 2). Ritter et al. provided evidence of the anti-inflammatory and analgesic activities of anethole in acute and persistent inflammation models.[1]

Both oils were able to inhibit peripheral inflammation, so it can be deduced that peripheral mechanisms might also, be involved in antinociceptive effects of essential and fixed oil of aniseed. Our findings are consistent with the report by Tas et al. who showed essential oil of *P. anisum* has significant analgesic effect similar to morphine and aspirin.[17] Also, fixed oil of anise was previously investigated for anti-inflammatory properties in mice. The finding showed that the fixed oil of anise has anti-inflammatory effect as strong as indomethacin and it showed analgesic effect comparable to that of 100 mg/kg aspirin and 10 mg/kg morphine at 30th min.[19] The effects of the fixed oil might because of the presence of cis anethole as well as oleic acid and hexadecanoic acid. Some evidence can be found for anti-inflammatory properties of these components for example in a review by Carrillo et al, it was concluded that oleic acid can act as an anti-inflammatory fatty acid by activation of different pathways of immune competent cells.[26] According to analgesic effect of plants and their constituents, hexadecanoic acid and 9-octadecadecenoic was isolated from the seed of *Telfairia occidentalis* that had antinociception activities in mice.[27] Also, previous studies of Yahaya showed that 9,12-octadecadienoic acid, n-hexadecanoic acid and other fatty acids of *Trametes Lactinea* extracts act as the lipoxygenase inhibitor.[28]

The fixed oil also contained 0.01±0.001 (W/W%) polyphenols. We did not determine the structures of the phenolic constituents of fixed oil but previous reports have suggested the presence of quercetin 3-glucuronide, rutin, luteolin 7-glucoside, isoorientin, and isovitexin, apigenin 7-glucoside in aniseed.[29] The presence of these components may also, attribute to analgesic and anti-inflammatory properties of the fixed oil. For example quercetin was reported previously to exhibit dose-related antinociception in several models of chemical pain, through interaction with L-arginine-nitric oxide, serotonin, and GABAergic systems.[30] Rutin and apigenin have been also reported to have analgesic properties.[31,32] In overall, it can be said that different constituents of essential or fixed oil may be responsible for analgesic properties of aniseed and may have synergistic effects with each other.

CONCLUSION

Analgesic effects of essential and fixed oils of *P. anisum* are comparable with paracetamol but essential oil in higher doses was significantly more effective than fixed oil. However, more future studies on these oils are needed to clarifying the exact mechanism(s) responsible for analgesic effects and finding the active constituents of the oils.

ACKNOWLEDGEMENT

This study was part of the Pharm. D. thesis project of Ali Altalqi and was supported by Shiraz University of Medical Sciences (grant # 91-01-70-4357).

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