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Anti-nociceptive and anti-inflammatory effects of *Ferulago angulata*

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Abstract

Introduction: *Ferulago angulata* from the Apiaceae family, has high flavonoid content and is detected to have anti-nociceptive and anti-inflammatory effects.
Objectives: In this study, we sought to determine the components of essential oil and to estimate total phenol and flavonoid contents of its various extracts. We also aimed to find out the anti-nociceptive and anti-inflammatory effects of essential oil, hydro-alcoholic and phenolic extracts of *F. angulata* aerial parts.
Materials and Methods: The plant's essential oil and extracts were prepared according to standard methods. Acetic acid, hot plate and formalin tests were used to investigate anti-nociceptive effects. Additionally, carrageenan and croton oil tests were used to evaluate anti-inflammatory effects.
Results: *Ferulago angulata* aerial parts yielded 0.2% (v/w) yellowish essential oil. The gas chromatography/mass spectrometry (GC-MS) of essential oil identified 82 compounds, which represented 98.9% of the essential oil. Thymol (7.9%), spathulenol (6.5%), trans-anethol (6.4%), myristicin (5.1%) and alpha-pinene (4.5%) were the main components. In acetic acid and formalin tests, the essential oil, hydro-alcoholic and phenolic extracts showed significant anti-nociceptive effects ($P<0.001$). In hot plate test, morphine which was used as standard drug, revealed significant anti-nociceptive effect while the plant extracts and essential oil were ineffective. High dose of the extracts and essential oil in croton oil test ($P<0.001$) and high dose of hydro-alcoholic and phenolic extracts in carrageenan test ($P<0.05$) reduced the inflammation.
Conclusion: *Ferulago angulata* extracts and essential oil have anti-nociceptive and anti-inflammatory effects. However, further studies are needed to clarify their mechanisms of actions.

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Introduction

Tissue damage, irritation and infection can lead to inflammation and pain. This process is followed by release of inflammatory mediators. Nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen and opioids are usually administered to control pain. The NSAIDs such as ibuprofen, naproxen, aspirin and celecoxib have multiple adverse effects including gastric ulcer, gastrointestinal bleeding, acute renal failure, cardiac infarction and stroke. Opioids that are used as anti-nociceptive agents also have side effects such as constipation, drug dependence and respiratory depression (1,2). The complication and adverse effects of these drugs caused more attention to use plant remedies to reduce pain and inflammation.

Ferulago angulata which is traditionally used to reduce pain and inflammation, belongs to Apiaceae family (3). *Ferulago* species have traditionally been used as flavoring agent, sedative, tonic and anti-nociceptive remedies (4,5). Previous studies have shown that various parts of this plant including stems, leaves, flowers and seeds have essential oil (5,6). Recent findings showed that 93.4% of the total essential oil of this plant is related to the monoterpenes (73.85%) and sesquiterpenes (6.29%). The main compounds of the essential oil include

Key point

The essential oil components of *Ferulago angulata* aerial parts and the total phenol and flavonoid contents of its various extracts were determined. The anti-nociceptive and anti-inflammatory effects of essential oil, hydro-alcoholic and phenolic extracts of *F. angulata* aerial parts were also determined. *F. angulata* aerial parts yielded 0.2% (v/w) yellowish essential oil, containing 82 compounds, which represented 98.9% of the essential oil. Thymol (7.9%), spathulenol (6.5%), trans-anethol (6.4%), myristicin (5.1%) and alpha-pinene (4.5%) were the main components. *F. angulata* extracts and essential oil showed anti-nociceptive and anti-inflammatory effects.

a-pinene (24.1%), b-pinene (22.7%), b-phellandrene (20.5%) and a- phellandrene (12.1%) (7).

Studies have been conducted to determine the amount of phenolic and flavonoid components of *F. angulata* leaves and stems. The total flavonoids content measured by the aluminum chloride test in the aqua extract of leaf was approximately equal to 108.5 mg and in the methanol extract was approximately 3.5 mg of quercetin per gram of dried leaf powder. Amount of flavonoids in the aqua extract was equal to 539 mg and in methanol extract was equal to 0.5 mg of quercetin per gram of dried stem powder (8).

Most of the plants from Apiaceae family such as *Pimpinella anisum* (9), *Anethum graveolens* (10) have been shown to present anti-inflammatory and anti-nociceptive effects. Additionally, several studies have shown that plants with flavonoids such as citrus sp (11), *Lavandula angustifolia* (12) and *Ziziphus lotus* (13) have proper anti-nociceptive and anti-inflammatory effects. *Ferulago angulata* has high flavonoid content (14) and seems to have anti-nociceptive and anti-inflammatory effects, too.

Objectives

Phenolic compounds that have antioxidant properties and inhibit expression of inflammatory cytokines, have anti-inflammatory activities (15). Therefore, this study was conducted to evaluate the anti-nociceptive and anti-inflammatory effects of *F. angulata* in animal models.

Materials and Methods

Preparation of plant and hydro-alcoholic extract

Ferulago angulata was purchased from Yasouj, Iran in June 2017. It was authenticated by an expert in faculty of pharmacy and with a voucher sample of 1138 was deposited in the herbarium of pharmacognosy department of Isfahan University of Medical Sciences, Isfahan, Iran.

First, the plant was dried in laboratory and then powdered. To prepare the hydro-alcoholic extract, 600 g powder of *F. angulata* was added to 3600 mL of EtOH: H_2O (8:2) for 72 hours. The process of extracting was continued by three hours shaking. Then, the extract was filtered by Buchner funnel and condensed in a rotary evaporator under reduced pressure (16).

Preparation of phenolic extract

To prepare phenolic extract, 400 g of the dried powder of aerial parts was extracted three times with EtOH: H_2O (8:2) while each time the mixture was left for 48 hours and then shaken for three hours. The mixture of extract with residual of plant was filtered by Buchner funnel and then the extract condensed to 1/3 of the initial volume by a rotary evaporator. It was washed five times with chloroform to separate nonpolar materials. Finally the extract was concentrated by rotary for phenolic fraction (16).

Preparation of essential oil

Hydrodistillation method was used to isolate the essential oil of the aerial parts of the *F. angulata* (17). The prepared essential oil was kept in the refrigerator (4°C) until use.

Analysis of essential oil

The prepared essential oil was phytochemically analyzed by gas chromatography/mass spectrometry (GC-MS). The instrument was the Agilent 5975C with a mass selective detector which was coupled with Agilent 6890 GC and a capillary column (HP-5MS, film thickness 0.25 µm, 0.25 mm*30 m). Oven temperature of the GC started from 60°C and gradually increased to 280°C at 4°C/min. The gas carrier of the GC was helium (2 mL/min). The injector and detector temperature of the instrument was set up to 280°C. The operating parameters of MS were 200°C for the ion source temperature and 70 eV for the ionization voltage.

To identify the essential oil components (based on the retention time relative to n-alkanes (C_8-C_{24}),) matching of the computer with NIST (national institute of standards and technology) 08 and Wiley 275 libraries is necessary. Then, the mass spectra fragmentation patterns to literature reports should be compared (18).

Standardization of plant extracts

Total phenolic measurements

The phenolic contents of the extracts were determined, according to the Folin Ciocalteu procedure (19) with some modifications. In brief, 10 mg of dried extract was dissolved in 10 mL of methanol (60%) and then 500 µL of Folin Ciocalteu reagent (10%) was added to 100 µL extract solution and kept at laboratory temperature for 4 to 8 minutes. Then, 400 µL of aqueous sodium bicarbonate solution (7.5%) was added and after 30 minutes, the absorbance was measured at 765 nm using a spectrophotometer (Unico UV2100, USA). The results were presented as mg of gallic acid equivalent/g of the dried extract (GAE/g).

Total flavonoids measurement

Aluminum chloride colorimetric procedure by Bouterfas et al with some modifications was employed to measure the total flavonoid content of the extract (20). In brief, 100 mg dried extract was dissolved in 10 mL of methanol (60%), after that, 1 mL of the final solution was added to one ml aluminum chloride (2%) and 6 mL potassium acetate (5%) and incubated for 40 minutes. Then, the absorbance of the mixture was determined at 415 nm using a spectrophotometer. In the blank, the same amount of distilled water was used instead of aluminum chloride. In this experiment, total flavonoids were presented as mg/g rutin equivalent (RE).

Animals

For evaluating anti-nociceptive and anti-inflammatory

effects, male Swiss mice (20-30 g) and Wistar rats (180-200 g) were prepared from School of Pharmacy, Isfahan University of Medical Science, Isfahan, Iran. The animals were kept in standard cages (6 animals per cage) in air temperature (22 ±2°C), with a 12 hours light/dark cycle. They had access to water and food freely. The animals were transferred to laboratory two days before starting tests for adaptation. All tests and experiments were performed based on the guidelines of caring laboratory animals instructed in Ethics Committee of Isfahan University of Medical Science.

Anti-nociceptive and anti-inflammatory evaluations

To evaluate the anti-nociceptive activity of the plant's essential oil and extracts, acetic acid test, formalin test and hotplate test were carried out. Croton oil and carrageenan tests were applied to assess anti-inflammatory activity.

Acetic acid test

For each extract and essential oil, 30 mice were designated into five equal groups. The first group, as a control group, received normal saline 0.9% (10 mL/kg); the second, third and fourth groups respectively received 100, 200 and 400 mg/kg extract (doses of essential oil were 50, 100 and 200 µL/kg) and the fifth group as a standard group received indomethacin (10 mg/kg), intraperitoneally (i.p). After half an hour, acetic acid 1% was injected i.p. and ten minutes after injection of acetic acid, the number of writhes and stretching was counted for 10 minutes (21).

Formalin test

Like previous test, five groups of six mice were selected and intraperitoneally injected normal saline (negative control group), morphine (10 mg/kg; positive control group), extracts (100, 200 and 400 mg/kg) or essential oil (50, 100 and 200 µg/kg). Half an hour later, 20 µL of formalin 2.5% was injected into the right foot of each animal. The time spent for paw licking was measured and compared at 0-5 and 20-40 minutes after injection (22).

Hot plate test

First, the time that animal showed a pain response was recorded as the base time. Then, 400 mg/kg of each extract and 200 µL/kg of the essential oil and standard drug (morphine, 10 mg/kg) were administered intraperitoneally. The animals in the control group received 10 mL/kg, normal saline, intraperitoneally (i.p.). The reaction time of animal was measured during two hours of injection at 30 minutes intervals. The percentage of response was calculated with MPE% formula (percent of maximal possible effect) and compared in various groups (23).

Croton oil test

First a 2.5% solution of croton oil in acetone was prepared. Animals received three doses of each extract or essential oil (as mentioned before) and indomethacin (10 mg/kg), intraperitoneally. The control group received normal saline. Half an hour later, 20 µL croton oil solution was spread with the sampler on inside part of the animal's right ear. Six hours later the animals were sacrificed. Then, from their right and left ears six mm disks were removed and weighed with an analytical scale. The difference between weights of these two disks was considered as an inflammation index (24).

Carrageenan test

For this part of study 180-200 g Wistar rats were used. Study groups received 400 mg/kg of each extract or 200 µL/kg essential oil and the positive control group received indomethacin (10 mg/kg), intraperitoneally. The animals in the control group received normal saline. Half an hour later, carrageenan (1% w/v) was injected (100 µL) into the right foot. The foot volume was measured by water plethysmometer (Borj Sanat, Iran), just prior and four hours after injection of carrageenan and compared. The increase in volume was considered as a measure of edema (swelling) (25).

Ethical issues

All experimental protocols and steps of this study were conducted in accordance with the regulations of the research ethics committee of Isfahan University of Medical Sciences and Iranian ethical guidelines for the use of animals in research. Accordingly all animal experiments were in accordance with protocols approved by the United States National Institutes of Health (NIH, 1978). This study has the permission of ethical committee of Isfahan University of Medical Sciences and was conducted as the pharmacy thesis of Samira Rafieian Kopaei in School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences (Thesis # 396817).

Statistical analysis

Data were presented as mean and standard error of mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Scheffe post hoc test using SPSS (version 22). The $P < 0.05$ was considered as significant.

Results

The extract yield

The yields of hydro-alcoholic and phenolic extracts were respectively 10.6% and 9%.

Analysis of the Ferulago angulata essential oil

Ferulago angulata aerial parts yielded 0.2% (v/w) yellowish essential oil with penetrating smell. The GC-MS analysis of the yielded essential oil identified 82 compounds that represented 98.9% of it. Thirty of the most important compounds of the essential oil are seen in Table 1. Thymol (7.9%), spathulenol (6.5%), trans-anethol (6.4), myristicin (5.1%) and alpha-pinene (4.5%) were the main components.

Table 1. Percentage composition of essential oil of *Ferulago angulata*

RT	Compound	%*	RI
3.86	α-Pinene	4.5	940
4.12	Camphene	1.1	954
4.29	Verbenene	0.9	964
4.93	Myrcene	0.9	993
5.73	p-Cymene	2.1	1027
5.83	Limonene	1.4	1032
6.56	γ-Terpinene	1.0	1062
7.78	Linalool	1.6	1101
8.34	p-Menth-2-en-1-ol	1.8	1125
9.00	Camphor	3.6	1147
9.28	p-Menth-3-en-8-ol	5.2	1153
9.89	p-Mentha-1,5-dien-8-ol	3.8	1174
10.10	4-Terpineol	1.5	1181
10.66	Methyl chavicol	1.4	1199
11.01	*trans*-piperitol	0.9	1211
11.38	*trans*-carveol	0.9	1221
11.69	Citronellol	1.1	1229
11.94	Cuminal	0.9	1243
12.09	Carvone	1.2	1247
13.51	*trans*-anethol	6.4	1289
13.96	Thymol	7.9	1294
14.18	Carvacrol	2.6	1304
17.17	Methyl eugenol	1.8	1407
19.96	Methyl *cis*-isougenol	1.4	1500
20.73	Myristicin	5.1	1523
22.36	Spathulenol	6.5	1581
24.19	β-Eudesmol	1.4	1649
25.22	Apiole	3.1	1682
32.66	Hexadecanoic acid	1.5	1985
37.95	Suberosin	1.7	2222

* The calculated percentages from total chromatogram data.
RI= Retention time based on HP-5MS capillary column.

Estimation of total phenolic and flavonoid compounds

Total phenolic and flavonoid components of hydro-alcoholic extract of *F. angulata* were 28.7 ± 2.1 mg GAE/g and 19.8 ± 1.7 mg RTN/g of dry extract respectively and also total phenolic and flavonoid components of polyphenolic extract were 53.2 ± 3.9 mg GAE/g and 39.4 ± 1.4 mg RTN/g of dry extract in order.

Pharmacologic results

In the acid-acetic induced writhing test, hydro-alcoholic extract (200, 400 mg/kg), phenolic extract (100, 200, 400) and essential oil (50, 100, 200 µL/kg) of *F. angulata* obviously inhibited abdominal twitches ($P<0.001$). In this test indomethacin at a dose of 10 mg/kg as a reference drug inhibited twitches by 93.38% (Figures 1 to 3).

The results of acute and chronic phases of formalin test are revealed in Table 2. In acute phase, the paw licking time reduced by hydro-alcoholic extract at dose of 400 mg/kg, essential oil at doses of 100, 200 mg/kg ($P<0.001$) and phenolic extract at dose of 400 mg/kg ($P<0.05$). In the chronic phase, all doses of the extracts and essential oil had anti-nociceptive effects and significantly reduced paw licking time ($P<0.001$). However, there was no significant

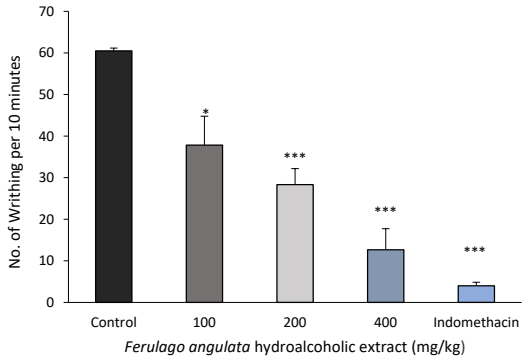

Figure 1. Effect of intrperitoneal injection of hydro-alcoholic extract of *F. angulata* on acetic acid-induced writhing test in mice. Extract (100, 200 and 400 mg/kg), indomethacin (10 mg/kg) and the vehicle were injected (i.p.) 30 min prior to acetic acid (1%) injection and the number of abdominal contractions was counted in a 10 min period starting 10 min after acetic acid injection. The values represent mean ± SEM. *P<0.05; ***P<0.001 compared with control group.

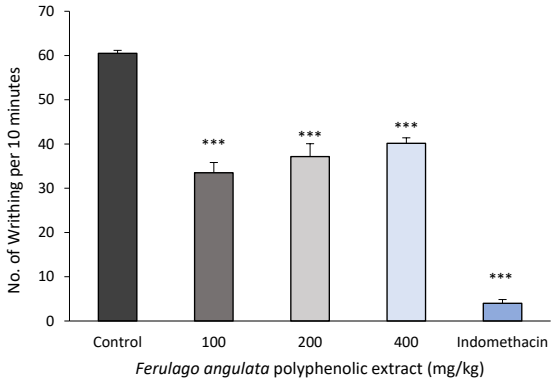

Figure 2. Effect of intrperitoneal injection of polyphenolic extract of *F. angulata* on acetic acid-induced writhing test in mice. Extract (100, 200 and 400 mg/kg), indomethacin (10 mg/kg) and the vehicle were injected (i.p.) 30 min prior to acetic acid (1%) injection and the number of abdominal contractions was counted in a 10 min period starting 10 min after acetic acid injection. The values represent mean ± SEM. ***P<0.001 compared with control group.

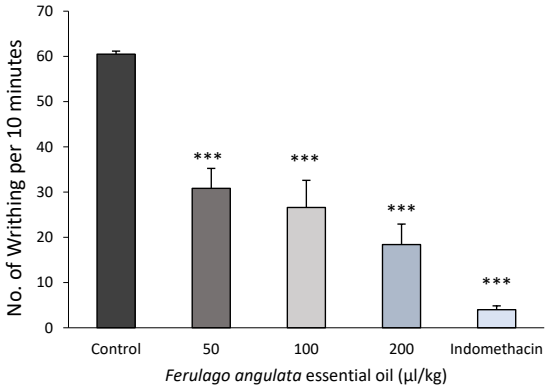

Figure 3. Effect of intrperitoneal injection of essential oil of *F. angulata* on acetic acid-induced writhing test in mice. Essential oil (50, 100 and 200 µL/kg), indomethacin (10 mg/kg) and the vehicle were injected (i.p.) 30 min prior to acetic acid (1%) injection and the number of abdominal contractions was counted in a 10 min period starting 10 min after acetic acid injection. The values represent mean ± SEM. ***P<0.001 compared with control group.

Table 2. Anti-nociceptive properties of *Ferulago angulata* extracts and essential oil in formalin test

Group	Dose	Paw licking time (s)			
		First phase (0-5 min)		Second phase (20-30 min)	
		Mean ± SEM	Inhibition (%)	Mean ± SEM	Inhibition (%)
Control		77.66 ± 6.17		67.33 ± 8.8	
HE	100 mg/kg	57.80 ± 7.66	25.6	10.60 ± 4.16***	84.3
	200 mg/kg	55.66 ± 6.55	28.3	10.75 ± 6.48***	84.0
	400 mg/kg	33.20 ± 6.25***	57.2	4.60 ± 1.88***	93.2
PE	100 mg/kg	68.40 ± 4.76	11.9	7.80 ± 1.01***	88.4
	200 mg/kg	59.33 ± 6.32	23.6	6.60 ± 1.43***	90.2
	400 mg/kg	51.50 ± 3.51*	33.7	5.16 ± 2.32***	92.3
EO	50 µL/kg	59.60 ± 6.39	23.3	11 ± 5.20***	83.7
	100 µL/kg	47 ± 3.51***	39.5	9.60 ± 5.20***	85.7
	200 µL/kg	40 ± 4.26***	48.5	9.10 ± 4.68***	86.5
Morphine	10 mg/kg	3.50 ± 1.12***	95.5	2.66 ± 1.02***	96.0

*P <0.05; ***P <0.001 in comparison to control group. PE, Polyphenolic extract; HE, Hydro-alcoholic extract; EO, Essential oil.

difference response between various doses. Morphine, used as a standard drug, was able to inhibit pain in both phases ($P<0.001$).

In hot plate test, none of the extracts and essential oil of *F. angulata* produced significant anti-nociceptive activity (Figure 4).

Figure 5 shows the results of intraperitoneal administration of *F. angulata* extracts and essential oil on croton oil test. Hydro-alcoholic extract at dose of 400 mg/kg and essential oil at doses of 200 and 100 µL/kg significantly reduced ear edema ($P<0.001$), and produced 74%, 66% and 66% inhibition of ear edema, respectively. Phenolic extract at doses of 200 and 400 mg/kg and hydro-alcoholic extract at dose of 200 mg/kg also inhibited edema ($P<0.05$). Indomethacin inhibited 82% of ear edema in croton oil test.

The hydro-alcoholic and phenolic extracts (at high doses) inhibited paw edema 48% and 47%, respectively in carrageenan induced paw edema test). It should be noted that indomethacin used as a reference drug was able to inhibit 87% of paw edema (Table 3).

Discussion

In this study the essential oil and extracts of *F. angulate*, showed anti-nociceptive effect in formalin and acetic acid tests. Acetic acid test is not a specific anti-nociceptive test while it has also applied for other substances such as some drugs that have not anti-nociceptive activity, like neuroleptics, muscle relaxants, adrenergic blockers and antihistamines. Nevertheless, investigators believe that acetic acid-induced abdominal contractions have similarities with visceral pain in human; On the other

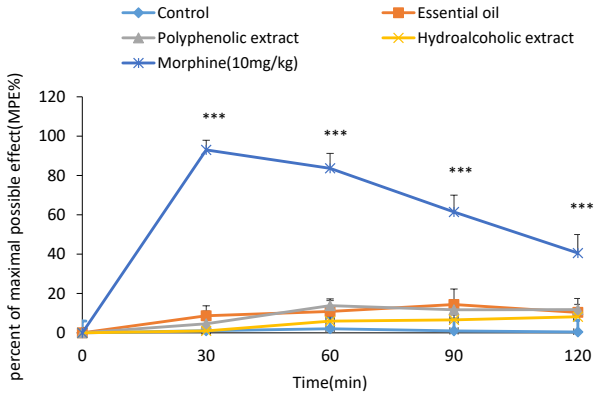

Figure 4. The antinociceptive activity of extracts and essential oil of *F. angulata* in hot plate test. Doses of 400 mg/kg of the Hydro-alcoholic and polyphenolic extracts, 200 µL/kg of the essential oil and 10 mg/kg of standard drug (morphine) were administered 30 min prior to placement of the animal on hot plate and reaction time of mice was measured at 30 min intervals until 2 h and percent of maximal possible antinociceptive effect (MPE%) was calculated for each time and compared. Data are mean ± SEM. ***P<0.001 compared with control group.

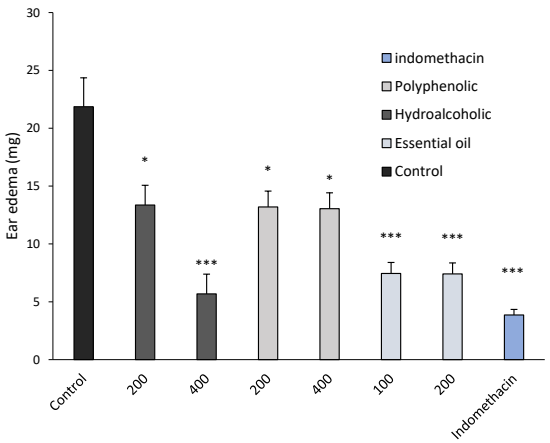

Figure 5. The anti-inflammatory effect of extracts and essential oil of *F. angulata* on croton oil-induced ear edema. Extracts (200 and 400 mg/kg), essential oil (100 and 200 µL/kg) and indomethacin (10 mg/kg) were injected i.p. 30 minutes prior to topical application of 20 µL croton oil solution (2.5% v/v in acetone) on right ears of animals. Six hours later animals were sacrificed and 6 mm disks were removed from both ears and weighed. The difference in weight of the disks was considered as an inflammation index. Data are mean ± SEM. *P<0.05; ***P<0.001 compared with control group.

Table 3. Effect of *Ferulago angulata* extracts on carrageenan-induced rat paw edema

	Dose (mg/kg)	Paw volume (ml)	Inhibition %
Control	-	0.83 ± 0.04	
HE	400 mg/kg	0.48 ± 0.11*	42.16 %
PE	400 mg/kg	0.47 ± 0.11*	43.37 %
Indomethacin	10 mg/kg	0.10 ± 0.01	87 %

*P <0.05 in comparison to control group. PE, Polyphenolic extract; HE, hydro-alcoholic extract.

hand, drugs that show effectiveness in this test, have promising potential for controlling visceral pain including abdominal or renal colic (26,27).

In formalin test, after subcutaneous formalin administration, the results show a typical biphasic behavior (22). This behavioral test has two phases (28). The first phase (first 5 minutes) is caused by direct stimulation of pain receptors which activate c-fibers, while some studies showed that second phase (20 minutes after formalin injection) resulted from CNS processes that are triggered via neural activation during first phase (29). However, some researchers have suggested that second phase results from combination of peripheral inflammatory reaction and central functional changes (30). In this experiment, high doses of the extracts and essential oil reduced the nociceptive behavior of the first phase and all doses inhibited the second phase of formalin test. Opioids have shown anti-nociceptive effect in both phases; however the second phase was more sensitive to them. NSAIDs seem to inhibit only the second phase (22).

In hot plate test, licking paw and jumping are considered as pain responses. This model is just affected by opioids and is not sensitive to anti-nociceptive effects of NSAIDs. Extracts and essential oil of *F. angulata* in high doses could not inhibit pain in this model (27).

There are several tests to detect the anti-inflammatory effect of drugs. Croton oil test and carrageenan test are two of the most common ones. High dose of hydro-alcoholic extract and essential oil in croton oil test and high dose of phenolic and hydro-alcoholic extract in carrageenan test inhibited inflammatory response. Recent studies have revealed that flavonoids and polyphenolic compounds have some pharmacological activities like antioxidant effect (31), reduction of histamine release from mast cells (32) and prevention of metabolism of arachidonic acid (33). Furthermore, croton oil activates phospholipase A_2 and increases the release of arachidonic acid from cell membrane that lead to production of prostaglandins which are inflammatory mediators (34). Fachini-Queiroz et al showed that thymol can increase edema volume induced by croton oil (35), since thymol is a major component of *F. angulata* essential oil.

Conclusion

This paper showed that *F. angulata* extracts and essential oil had a proper anti-nociceptive effect compared to NSAIDs and a moderate anti-inflammatory effect. However, more experiments are needed to clarify the mechanisms involved.

Authors' contribution

VH and SES supervised and designed the project and SRK promoted laboratory works, analyzed the data and prepared the first draft of the article, VH edited and completed the manuscript. All authors read and confirmed the final version of the manuscript and confirmed its publication.

Conflicts of interest

The authors declare no conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the author.

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