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Chemical composition and analgesic activity of the essential oil of *Mentha mozaffarianii* jamzad leaves

H. Sam-Daliri[1], Z. Mousavi[1], N. Naderi[2], J. Asgarpanah[3*]

[1] *Department of Pharmacology & Toxicology, Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran - Iran (IAUPS).*

[2] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, ShahidBeheshti University of Medical Sciences, Tehran - Iran.*

[3] *Department of Pharmacognosy, Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran - Iran (IAUPS).*

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The analgesic property of *Mentha mozaffariani* essential oil was investigated on mice and rats. The analgesic activity of the oil was assessed by acetic acid-induced writhing and formalin-induced paw licking methods. *M. mozaffariani* oil significantly decreased the number of acetic acid-induced writhes in mice compared to animals that received vehicle only. In formalin test, a dose of 100 mg/kg significantly reduced the pain score of first phase in comparison to control ($p<0.05$). The inhibitory activity of *M. mozaffariani* essential oil was found to be very close to that of the standard drug, aspirin (100mg/kg). The studied oil was analyzed by GC and GC–MS and twenty two constituents, representing 99.4 % of the oil, were identified. The major component of the oil was characterized as piperitone (51.0%) which might be responsible for the observed activity. The results suggest that *M. mozaffariani* essential oil possesses biologically active constituent(s) that have significant analgesic effects which support the ethnomedicinal claims of the plant application in the management of pain.

Keywords: Analgesic activity, *Mentha mozaffariani*, Essential oil, Piperitone

INTRODUCTION

The Iranian endemic plant *Mentha mozaffarianii*Jamzad. belongs to the *Lamiaceae* family and is known locally as "Pooneh-Koohi" [1]. Six species and several subspecies of the genus *Mentha* are found in Iran, among which just *M. mozaffarianii* is endemic. It has a limited geographical range in the south of Iran and is found in Siyahoo, Qotb-Abad, Damtang and Sikhoran in Hormozgan Province [2]. The leaves have been commonly used in Iranian traditional medicine as antiseptic, analgesic means,for treating painful menstruation, dyspepsia, arthralgia, fever, headache, common cold and for healing wounds [1-3]. This plant is also among the medicinal plants which have served as natural remedies for Huntington's disease in medieval Persian medicine [4].

Due to the widespread use of *M. mozaffarianii* in Iranian traditional medicine for the relief and treatment of pain, we were prompted to evaluate the analgesic activity of this plant. As the plant's leaves contain high amount of essential oil (2-4% v/w), the analgesic effect of the essential oil extracted from *M. mozaffarianii* leaves was investigated to confirm the pharmacological basis for its folkloric use as a natural pain killer agent. This study explores the analgesic property of *M. mozaffarianii* oil by two standard experimental test models. The oil was also analyzed by GC and GC–MS in order to identify the potentially responsible compounds for the observed property. This is the first attempt addressing such ethnopharmacological properties of *M. mozaffarianii* essential oil in a comprehensive manner.

EXPERIMENTAL

Plant Materials

Leaves of *M. mozaffariani* were collected in March 2014 from Genow protected area, Bandar Abbas, Hormozgan Province, south of Iran. The leaves were identified by R. Asadpour. A voucher specimen has been deposited at the herbarium of the Department of Pharmacognosy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran, under code number 1011-AUPF.

Fresh leaves were separately submitted to hydrodistillation in a Clevenger-type apparatus for 3 hours. At the end of distillation the oils were collected, dried with anhydrous Na_2SO_4, measured,

* To whom all correspondence should be sent:
E-mail: taxolfa@yahoo.com

transferred to clean glass vials and kept at a temperature of −18°C for further analyses.

Experimental animals

Male NMRI mice (20–25 g) and male Wistar rats (200-250 g) were used throughout this study. Animals were housed separately in groups of 5-6 and were allowed free access to food and water except for the short time that animals were removed from their cages for testing. All experiments were conducted during the period between 10.00 a.m. and 13 p.m. with normal room light (12 h regular light/dark cycle) and temperature (22 ± 1 °C). All procedures were carried out in accordance with the institutional guidelines for animal care and use (ethical approval number: 3183). Each mouse was used only once.

Analgesic activity

Acetic acid-induced writhing in mice

The writhing acetic acid test was performed as originally described by Siegmund*et al.* [5]. This method was employed to preferentially evaluate possible peripheral effects of *M. mozaffarianii* essential oil as an analgesic substance. Four groups of 10 mice were fasted overnight prior to the start of the experiment, while given free access to water. The vehicle, tween 80 (2%, 10 ml/kg), aspirin (100 mg/kg) and the essential oil (50 and 100 mg/kg) were orally administered to the first, second, third and fourth group of mice, respectively, 45 min prior to the injection of acetic acid (1%, 10 ml/kg). Aspirin is a well-known peripheral analgesic drug and it was used as a positive control in the present investigation. The mice were then placed in an observation box, and the number of writhes was counted for 30 min after acetic acid injection [6].

Formalin test in rats

The formalin test differs from most other nociceptive tests as it enables evaluation of analgesic activity towards moderate continuous pain generated by injured tissue [7]. This method was employed to preferentially evaluate possible central effects of *M. mozaffarianii* essential oil as an analgesic substance. Five groups of 6 rats were fasted overnight prior to the start of the experiment, while given free access to water. 30 min after oral administration of the vehicle, tween 80 (2%, 10 ml/kg), aspirin (100 mg/kg) and the essential oil (50, 100 and 200 mg/kg) to the first, second, third, fourth and fifth group of rats, respectively, the right hind paw was subcutaneously injected with 0.05 ml of 5% formalin in saline. Aspirin is a well-known central analgesic drug and it was used as a positive control in the present investigation. The animals were placed in a transparent polypropylene cage next to a mirror so that they could be observed from all angles. The animals were continuously observed from the time of formalin injection to 60 min. The behaviors were quantified as described by Dubuisson and Dennis [8] as 0 = normal weight bearing on the injected paw, 1=limping during locomotion or resting the paw lightly on the floor, 2=elevation of the injected paw so that at most the nails touch the floor, and 3=licking, biting or shaking the injected paw. Subjects' behaviors were continuously scored in 5 min intervals by a trained observer. The pain score was then calculated for a 5-min interval using the following equation:

$\Sigma n/20$ = average score in 5 min

Subcutaneous formalin injection resulted in a biphasic response of nociceptive behavior in rats. The early phase starts immediately after formalin application, followed by an intermediate phase in which the pain-related behavior was relatively decreased and finally by a more prolonged but delayed phase of increasing pain-related behavior. The area-under-the-curve (AUC) of the pain score of the first 10 min was considered as phase 1 and the AUC of pain scores during 10–60 min after formalin injection was considered as phase 2.

Statistical analysis

The results were presented as mean+SEM. Statistical analysis was performed using Prism 5 (Graphpad Software Inc.). One-way analysis of variance (ANOVA) followed by Dunnett'stests was used and a p-value less than 0.05 was considered as statistically significant.

Analysis of the essential oil

Oil sample analysis was performed on a HP-6890 gas chromatograph (GC) equipped with a FID and a DB-5 capillary column, 30 m × 0.25 mm, 0.25 μm film thickness, temperature programmed as follows: 60°−240°C at 4°C/min. The carrier gas was N_2 at a flow rate of 2.0 ml/min; injector port and detector temperature were 250°C and 300°C, respectively. Sample was injected by splitting and the split ratio was 1:10.

GC/MS analysis was performed on a Hewlett-Packard 6890 /5972 system with a DB-5 capillary column (30 m × 0.25 mm; 0.25 μm film thickness). The operating conditions were as described above but the carrier gas was He. Mass spectra were taken at 70 eV. Scan mass range was 40-400 m/z at a sampling rate of 1.0 scan/s. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the oils were

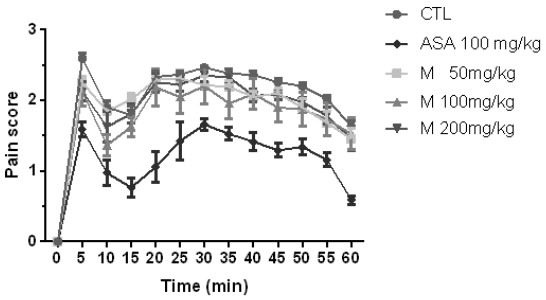
identified by their retention times, retention indices, relative to C_9-C_{28}*n*-alkanes, computer matching with the WILEY275.L library and by comparison of their mass spectra with data already available in the literature [9-10]. The percentage composition of the identified compounds was computed from the GC peak areas without any correction factors and was calculated relatively. The result of the oil analysis is the average of three replicates.

RESULTS AND DISCUSSION

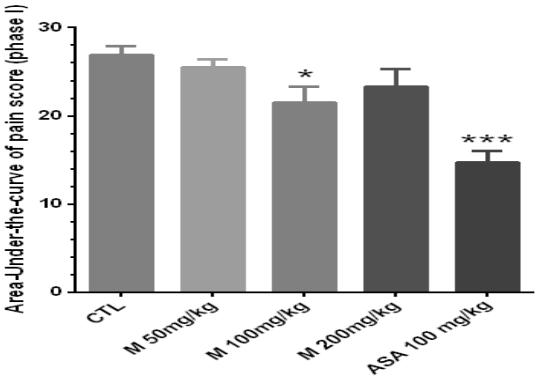
In this study, we evaluated the efficacy of the essential oil of *M. mozaffarianii* aerial parts since the volatile constituents comprise the main components of this plant. Oral administration of *M. mozaffarianii* oil (50 and100 mg/kg) decreased the number of acetic acid-induced writhes in mice compared to the animals that received vehicle only (Table 1). The writhe inhibitory effects of the oil ranged from 68 to 78%. The best results were observed by 100 mg/kg dose of the oil. By comparison, 100 mg/kg aspirin produced a little less (i.e. 77.68% effectiveness) analgesia in this nociception model. The analgesic effect induced by *M. mozaffarianii* oil was dose-related.

The effects of the systemic administration of different doses of the oil on the behavioral responses in the first and second phases of formalin test were evaluated (Figure 1. A.). In both phases of the pain, behaviors were separately analyzed as the area under the curve pain score *versus* time. As shown in Figure 1. B, in the first phase of the formalin test 100mg/kg dose of the essential oil reduced the pain score ($p<0.05$) and aspirin as a standard analgesic drug significantly ($p<0.001$) reduced pain behavior. Doses of 50, 100 and 200 mg/kg of the essential oil did not affect the area-under-the-curve pain score in the second phase of the formalin test when compared to aspirin as the standard analgesic drug which significantly ($p<0.001$) reduced pain behavior (Figure 1. C.).

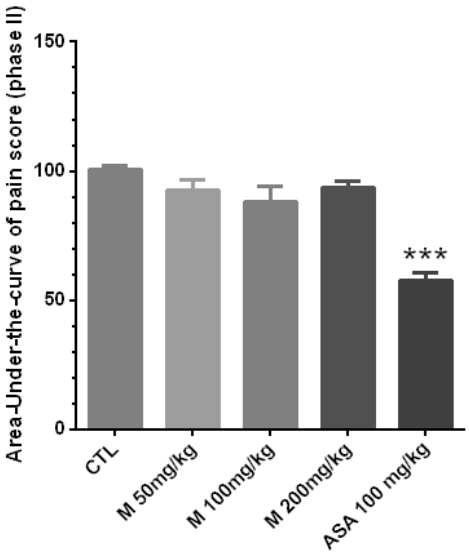
M. mozaffarianii essential oil was analyzed by GC and GC/MS to determine the possible compounds responsible for the observed antinociceptive activity. The hydrodistillation of *M. mozaffarianii* aerial parts gave yellowish oil with a pleasant odor and a yield of 4.3% (v/w). As shown in Table 2, twenty two components were identified in this oil, which represented about 99.4% of the total chromatographic material. The major constituent of the oil was piperitone (51.0%). Different preparations made from *M. mozaffarianii* leaves are commonly used in ethnobotanical practices for the treatment of pain in Iran.



A: Changes in pain scores during 60 min of the observation.



B: Overall changes in pain scores as the area-under-the-curve (AUC) of the first phase of the formalin test.



C:Overall changes in pain scores as the area-under-the-curve (AUC) of the second phase of the formalin test.

Fig. 1. Effect of oral administration of *M. mozaffarianii* essential oil (50, 100 and 200 mg/kg) on pain related behavior in formalin test. The oil or its vehicle (control group) was administered 30 min before the formalin test. Data are shown as mean ± SEM (n = 6). *P<0.05, **P<0.01, ***P<0.001 significant difference compared to control group.

Table 1. Effect of *M. mozaffarianii* essential oil on acetic acid-induced writhing in mice.

Group	Dose (mg/kg)	Number of writhings	Inhibition (%)
Control	10	45.71±6.94	-
Essential oil	50	14.50±8.75*	68.27
	100	10.12±11.51**	77.89
Aspirin	100	10.23±4.57***	77.68

Each value represents the mean±S.E.M. of 5 mice. *P< 0.05, **P<0.01 and ***P<0.001 compared to control group using one way ANOVA followed by Dunnett's multiple comparison test.

Table 2. GC-MS analysis of the essential oil of *M. mozaffariani* aerial parts.

Compound[a]	KI[b]	KI[c]	Percentage
α-Pinene	938	939	0.6
Camphene	952	954	0.2
Sabinene	971	975	0.5
β-Pinene	977	979	1.0
Myrcene	990	991	0.3
Ocymene	998	999	0.6
Limonene	1028	1029	0.4
1,8-Cineol	1033	1031	11.7
Linalool	1097	1097	11.1
Menthone	1149	1153	1.9
δ-Terpineol	1162	1166	0.3
Borneol	1165	1169	1.0
4-Terpineol	1178	1177	0.2
α-Terpineol	1190	1189	3.4
Pulegone	1237	1237	0.3
Piperitone	1251	1253	51.0
Thymol	1290	1290	1.0
Piperitenone	1339	1343	8.6
Piperitenone oxide	1371	1369	2.3
trans-Jasmone	1390	1391	1.9
β-Caryophyllene	1419	1419	0.8
Bicyclogermacrene	1500	1500	0.3
Total			99.4

[a]Compounds listed in order of elution.

[b]KI (Kovats index) measured relative to *n*-alkanes (C_9-C_{28}) on the non-polar DB-5 column under conditions listed in the experimental section.

[c]KI, (Kovats index) from literature.

Acetic acid-induced and formalin-induced pain models were applied in the present study to evaluate the antinociceptive effect of *M. mozaffarianii* essential oil in experimental mice and rats. The abdominal constriction response induced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics. In general, acetic acid causes pain by liberating endogenous substances such as serotonin histamine, prostaglandins (PGs), bradykinins and substance P endings. Local peritoneal receptors are postulated to be involved in the abdominal constrictions response. The method has also been associated with prostanoids in general, that is, increased levels of PGE2 and PGF2α in peritoneal fluids, as well as lipoxygenase products [11]. The formalin test is an important animal model in the study of acute long-lasting pains comprising two distinct phases. The first phase (neurogenic pain) is caused by direct chemical stimulation of nociceptive afferent fibers, predominantly C fibers, which can be suppressed by opiates like morphine. The second phase (inflammatory pain) results from the action of inflammatory mediators such as prostaglandins, serotonin and bradikinin in the peripheral tissues and from functional changes in the spinal dorsal horn. The level of pain in this model is sensitive to both centrally (phase 1 and phase 2) and peripherally (mostly phase 2) acting analgesics [12].

Results obtained in the present study showed it to be the first report describing the antinociceptive activity of *M. mozaffarianii* oil. The results demonstrated that the essential oil decreased the abdominal constriction, indicating the inhibition of the expression of prostaglandin synthesis by cyclooxygenase pathway [13]. The profile of anti-nociceptive activity of the oil identified in these experiments is different from that of aspirin, decrease of pain behavior in the first phase of formalin test but not in the second phase. Perhaps the simplest explanation is that the oil did not show anti-inflammatory effects. According to the phytochemical results, piperitoneconstituted more than half of the oil composition and it could be concluded that the analgesic effects of *M. mozaffarianii* essential oil may be due to the high content of piperitone. As there are no data on the analgesic activity of piperitone, further work to establish the analgesic activity of pure piperitone is currently going on in our laboratory. 1,8-Cineol as the other main compound of *M. mozaffarianii* essential oil comprising 11.7% of the oil could be considered as one of the active components. Several studies have proven the potent analgesic property of this monoterpenoidalcohol [14-16]. Analgesic activity of linalool which comprises 11.1% of the studied oil has been previously examined in two different pain models including the acetic acid-induced writhing response and the hot plate test in mice and since results have shown marked analgesic activity, it could be concluded that the observed analgesic activity of the studied oil could be also related to the linalool content [17]. These results justified the use of *M. mozaffarianii* in traditional medicine and the plant essential oil could be a potential candidate as an analgesic agent.

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ХИМИЧЕН СЪСТАВ И АНАЛГЕТИЧНО ДЕЙСТВИЕ НА ЕСЕНЦИАЛНО МАСЛО ОТ ЛИСТА НА *Mentha mozaffarianii*

Х. Сам-Далири[1], З. Мусави[1], Н. Надери[2], Дж. Асгарпанах[3*]

[1]*Департамент по фармакология и токсикология, Факултет по фармация, Фармацевтичен научен клон, Ислямски университет „Азад“, Техеран, Иран*
[2]*Департамент по фармакология и токсикология, Факултет по фармация, Медицински университет “ШахидБехещи”, Техеран, Иран*
[3]*Департамент по фармакогнозия, Факултет по фармация, Фармацевтичен научен клон, Ислямски университет „Азад“, Техеран, Иран*

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Аналгетичните свойства на есенциалното масло от *Mentha mozaffariani* са изследвани върху мишки и плъхове.Аналгетичната активност е оценена чрез гърчове, предизвикани от оцетна киселина и близане на лапа, индуцирано от формалин. Маслото от *M. mozaffariani* значително намалява гърчовете от оцетна киселина, в сравнение с контролни животни. Във формалиновия тест дозата от 100 mg/kg значително намалява болките в сравнение с контролата ($p<0.05$). Инхибиторната активност на маслото от *M. mozaffariani* essential е много близка до стандартно лекарство - аспирин (100 mg/kg). Изследваното масло е изследван аналитично с GC и GC–MS, като са идентифицирани 22 съставки, представляващи 99.4 % от маслото.Главна компонента на маслото е пиперитонът (51.0%), който може би е отговорен за наблюдаваната активност. Резултатите показват, че есенциалното масло от *M. mozaffariani* притежава биологично активни компоненти със значителен аналгетичен ефект, което обяснява медицинските му приложения, установени от народната медицинска практика.