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Chemical Composition and Antinociceptive Effect of the Essential Oil of *Dracocephalum moldavica* L.

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ABSTRACT

Background: *Dracocephalum moldavica* L. (Lamiaceae) is a widely used remedy for treatment of pain and gastrointestinal disturbances. The present study aimed to investigate antinociceptive effect of the essential oil of *D. moldavica* (EODM) in various experimental models. ***Methods:*** The antinociceptive effect of EODM was assessed using chemical (formalin and acetic acid) and thermal (hot-plate) nociceptive tests in mice and rats. ***Results:*** The intraperitoneal LD_{50} of the EODM in mice was calculated to be 600 mg/kg. The EODM administration at 5-20 mg/kg produced a significant antinociceptive effect in the formalin test and at 20 mg/kg in the acetic acid-induced writhing test. The essential oil failed to demonstrate any significant influence on hot-plate reaction latency. ***Conclusion:*** The results suggest that the EODM possess analgesic properties that support the folk medicinal use of this plant.

Introduction

The Moldavian dragon's head (*Dracocephalum moldavica* L.) is an annual, herbaceous, essential oil-producing, spicy aromatic medicinal plant of the deadnettle family (*Lamiaceae*), which reaches 25 - 75 cm in height.[1] Its use was reported in West Azerbaijan (Iran) folk medicine as a general tonic, stomachic, digestive, antiemetic, sedative and diaphoretic.[2] Tincture of the dry herb has been used for ages in Uyghur folk medicine to treat heart disease, blood pressure, angina, tracheitis, atherosclerosis, neuralgia, migraine, and headache and toothache.[3]Since the 1970s, 246 compounds, including terpenoids,steroids, flavonoids, alkaloids, lignans, phenols, coumarins, and cyanogenicglucosides, have been identified from the genus *Dracocephalum*, and terpenoids are the dominant constituents within the genus.[4]

D. moldavica contains 0.06–0.92% essential oil, with the maximal level during flowering. Its lemon-like scented essential oil consists mainly of oxygenated acyclic monoterpenes, e.g. geraniol, geranyl acetate, geranial, neral and nerylacetate.[5]

It has been reported that the plant possesses antibacterial, antioxidant and cardioprotective effects.[6,7] A recent study showed that *D. moldavica* also has sedative effect.[8] Although, the *D. moldavica* was popularly used in traditional medicine for pain relief, there have been no published reports regarding its antinociceptive effect. Therefore the present study aims to examine antinociceptive effect and mechanism of analgesic activity of the essential oil of *D. moldavica* (EODM) in chemical (formalin and writhing) and thermal (hot-plate) nociceptive tests. In addition, we describe the oil constituents by GC/MS analyses.

Materials and Methods

Plant Material

The aerial parts of *D. moldavica* was collected from Maraghe of East Azerbaijan province (situated in Iran) and identified by direct comparison with a herbarium sample. A voucher specimen (713 Tbz-Fph) has been deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tabriz University of Medical Sciences, East Azerbaijan, Iran.

Extraction of Essential Oil

The volatile fraction of *D. moldavica* aerial parts was obtained by steam distillation for 2 h by a Clevenger apparatus and the pale yellow essential oil produced. The oil was dried over anhydrous sodium sulfate and stored in the refrigerator (4 °C).

Gas Chromatography /Mass Spectrometry (GC/MS) Analysis

Identification of the oil constituents was performed by a Shimadzu GCMS- QP 5050A gas chromatograph (Shimadzu Corporation, Kyoto, Japan). The column used for the analysis was a 60 m × 0.25 mm DB-1 capillary column coated with a film of

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dimethylpolysiloxane (J&W Scientific, Folsom, CA, USA). 100 µl of the EODM was diluted with 400 µl of dichloromethane and was injected into the GC-MS system in the split mode (split ratio 1: 33). Helium was used as the carrier gas with a flow rate of 0.7 ml/min. The column temperature was maintained at 50 ℃ for 2 min. Then, it was programmed to 140 ℃ at a rate of 3℃/min, then it was increased up to 160 ℃ at a rate of 0.5 ℃/min and the final temperature, 260 ℃ at a rate of 3℃/min,was held for 2 min. Injector and detector temperatures were optimized at 230 ℃ and 260 ℃, respectively. The MS operating parameters were as follows: ionization energy, 70 eV; ion source temperature, 200 ℃; quadrupole, 100 ℃; solvent delay, 8.0 min; scan speed 2000/us and scan range 30–600 u, EV voltage 3000 volts. Determination of the components was based on direct comparison of the retention times and MS data with those for standard compounds, and matching with the combined Wiley 229, Nist 107 and Nist 21 libraries (Version 1998).

Animals

Male albino mice with a weight of 20-30 g and male Wistar rats with a weight of 180 ± 20 g were purchased from animal house of Faculty of Veterinary Medicine, Urmia University. The animals were acclimatized for one week before the beginning of the experiment and were kept under controlled conditions (temperature 22±2 ℃, normal lighting and relative humidity 55%). All of the animals were given standard rodent pellets and tap water ad libitum.

Chemicals

Morphine, naloxone hydrochloride and tween 80 were purchased from Sigma-Aldrich. All other chemicals were purchased from Merck Co.

Acute Toxicity Test

The LD_{50} of the EODM was determined by Lorke's method.[9] In the first stage, the EODM was intraperitoneally administered at doses of 1000, 100 and 10 mg/kg to three groups of mice (n = 3). One group as a control received 1% tween 80 in normal saline. The animals were observed for 24 h for signs of toxicity. Later observations were made three times daily for 7 days. In the second stage, doses of 600, 370, 225 and 140 mg/kg were administered to four groups of mice. These mice were observed until get their health or death. The surviving animals were monitored for 7 days.

Analgesic Activity

This was assessed by chemical (acetic acid-induced writhing and formalin) and thermal (hot-plate) nociceptive methods. All experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigation of experimental pain in conscious animals.[10]

Acetic Acid-Induced Writhing Test

The test was accomplished using a modification of the method as formerly described.[11] Acetic acid (0.6%, v/v) was administered intraperitoneally in a volume of 10 ml/kg. Vehicle (1% tween 80 in normal saline), EODM (5, 10 and 20 mg/kg) and morphine (10mg/kg), were injected intraperitoneally 30 min before acetic acid injection. In this test, mice (n= 30) were divided into 5 groups, each contain 6 mice. The total number of writhes (abdominal contraction and stretching) was counted over a period of 20 min following administration of acetic acid.

Formalin Test

The test was performed as previously described.[12] Animals were injected with 50 µl of 2.5% formalin into subplantar space of the right hind paw. Vehicle (1% tween 80 in normal saline), EODM (5, 10 and 20 mg/kg) and morphine (10 mg/kg) were injected intraperitoneally 30 min before formalin injection. In this test, rats (n= 30) were divided into 5 groups, each contain 6 animals. The time each rat has spent licking or biting of the injected paw were recorded. The formalin-induced behavioral responses to nociception were biphasic. The initial acute phase (neurogenic phase, 0–5 min) was pursued by a relatively short quiet period. The second phase was observed 15-30 min after formalin injection and entitled late phase (inflammatory phase).

Hot-Plate Test

The hot-plate test was carried out using a modification of the method previously described.[13] In this test, rats (n= 30) were divided into 5 groups, each contain 6 animals. Rats were placed on the hot-plate maintained at 53℃ and the time of hind paw licking or jumping was recorded as the index of response latency. A cut-off time of 50 s was chosen to avoid tissue damage. Rats were treated with vehicle (1% tween 80 in normal saline), EODM (5, 10 and 20 mg/kg), and morphine (10 mg/kg). The reaction time (s) for each animal was recorded before and after treatment at intervals of 30 min for a total period of 120 min.

Opioid Antagonist Study

The formalin test in mice was selected for this purpose. The sequence of the experiment was similar to formalin test except the opioid antagonist naloxone (2 mg/kg) was administered subcutaneously 10 min before EODM (10 and 20 mg/kg) and morphine (10 mg/kg) injection. 1% tween 80 in normal saline was used as the vehicle control.[14]

Statistical Analysis

All data were expressed as mean±SEM. The statistical analysis was performed using one-way ANOVA (SPSS 17) followed by Dunnett post hoc test. P values of less than 0.05 were considered significant.

Results

Essential Oil Constituents

The GC–MS chromatogram yielded for the extracted volatile compounds from the EODM is demonstrated in figure 1. Thirty-nine compounds, representing 99.84% of the essential oil, have been identified (Table 1). The major components in *Dracocephalum Moldavica* essential oil were identified as citral (31.14%), 3,7-dimethyl -2,6 octadienal (21.43%), cis-geraniol (17.08%), neral (9.63%) and neryl acetate (4.03%).

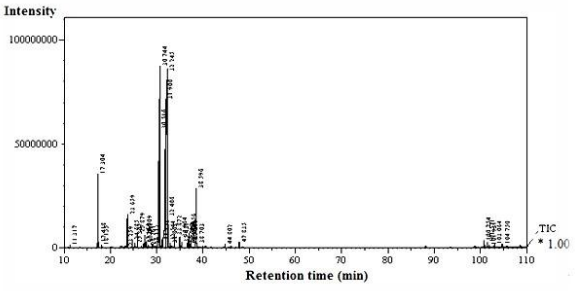

Figure 1. GC–MS chromatogram of essential oil of *D.moldavica* L.

Table 1. Volatile organic compounds identified in the essential oil of *Dracocephalum Moldavica*

No.	Compound	Retention time (min)	Relative area (%)	Chemical formula	Molecular weight
1	3-Hexen-1-ol	11.31	0.09	$C_6H_{12}O$	100
2	**6-Methyl-5-hepten**	17.30	**3.30**	**$C_8H_{14}O$**	126
3	3-Octanone	17.41	0.17	$C_8H_{16}O$	128
4	Ethylamylcarbinol	18.15	0.09	$C_8H_{18}O$	130
5	1-Cyclohexene-1-acetaldehyde	23.25	0.09	$C_{10}H_{16}O$	152
6	**Linalool**	23.65	**1.54**	**$C_{10}H_{18}O$**	154
7	Cyclohexene	24.68	0.38	C_9H_{14}	122
8	Cyclodecene	25.36	0.18	$C_{12}H_{22}$	166
9	1-Cyclohexene-1-acetaldehyde	25.87	0.62	$C_{10}H_{16}O$	152
10	Bicyclo heptane	27.16	0.17	$C_{10}H_{16}$	136
11	Cis-Myrtanol	27.50	0.72	$C_{10}H_{18}O$	154
12	Cis-Carveol	27.72	0.28	$C_{10}H_{16}O$	152
13	α-Terpineol	28.26	0.20	$C_{10}H_{18}O$	154
14	2-Cyclohexene-1-methanol	29.11	0.44	$C_{10}H_{18}O$	154
15	**Neral**	30.51	**9.62**	**$C_{10}H_{16}O$**	152
16	**2,6-Octadienal, 3,7-dimethyl-,(Z)-**	30.74	**21.43**	**$C_{10}H_{16}O$**	152
17	2-Cyclohexen-1-one	31.17	0.35	$C_{10}H_{16}O$	152
18	**Cis-Geraniol**	31.90	**17.08**	**$C_{10}H_{18}O$**	154
19	**Citral**	32.24	**31.14**	**$C_{10}H_{16}O$**	152
20	6-Methyl-5-hepten-2-one	32.40	1.26	$C_8H_{14}O$	126
21	Epoxy-linalooloxide	32.54	0.30	$C_{10}H_{18}O_3$	186
22	Neryl acetate	32.86	0.26	$C_{12}H_{20}O_2$	196
23	Geraniolformate	33.87	0.53	$C_{11}H_{18}O_2$	182
24	2-Pentadecyn-1-ol	34.96	0.63	$C_{15}H_{28}O$	224
25	Oxiranemethanol	35.43	0.25	$C_{10}H_{18}O_2$	170
26	2-Cyclohexen-1-one	36.61	0.31	$C_{10}H_{16}O_2$	168
27	Terpendiol	36.82	0.35	$C_{10}H_{18}O_2$	170
28	Ethanone	36.95	0.96	$C_{11}H_{20}O$	168
29	Geranic acid	37.20	0.43	$C_{10}H_{16}O_2$	168
30	Cis-Geranyl acetate	37.37	0.48	$C_{12}H_{20}O_2$	196
31	**Neryl acetate**	38.59	**4.03**	**$C_{12}H_{20}O_2$**	196
32	2(5H)-Furanone	38.70	0.24	$C_{10}H_{14}O_2$	166
33	Furan	44.80	0.26	$C_7H_{12}O$	112
34	2-Nitrohept-2-en-1-ol	47.83	0.47	$C_7H_{13}NO_3$	159
35	Farnesol	100.75	0.45	$C_{15}H_{26}O$	222
36	2-Buten-1-one	101.52	0.28	$C_{13}H_{20}O_2$	208
37	2-Buten-1-one	101.93	0.13	$C_{13}H_{20}O_2$	208
38	Beta-Myrcene	103.06	0.14	$C_{10}H_{16}$	136
39	Nerolidol Isomer	104.75	0.19	$C_{15}H_{26}O$	222

Acute Toxicity Testing

EODM was toxic (LD_{50}= 600 mg/kg) according to Lorke's method when it was administered intraperitoneally.

Effect of EODM on Acetic Acid-Induced Writhing Test

This test revealed that the mean number of writhings induced by a 0.6% acetic acid solution in mice treated with vehicle, 5, 10 and 20 mg/kg of EODM were 43,

37, 23 and 21 respectively (Figure 2). This result was significant only at 20 mg/kg of the EODM ($p<0.05$). Morphine significantly reduced the mean number of writhings when compared to control group ($p<0.001$).

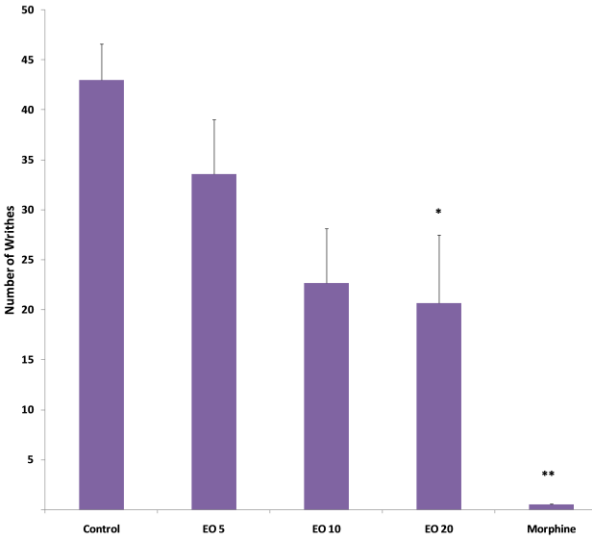

Figure 2. Effect of the essential oil of *D. moldavica* (EODM) on acetic acid-induced writhing in mice. The vehicle (Control, 10 ml/kg), the EODM (5, 10 and 20 mg/kg), morphine (M, 10 mg/kg) were administered 30 min before the intraperitoneal administration of acetic acid and the number of writhes were counted over a period of 20 min. Values are mean± S.E.M., n=6,*P<0.05, **P<0.001 . (ANOVA followed by Dunnett's test).

Effect of EODM on Formalin Test

As shown in figure 3 the mean paw licking time in vehicle treated control group was 65 s in the early phase while this time at dose of 5 mg/kg of EODM decreased to 39 s, at 10 mg/kg reduced to 31 s and at 20 mg/kg declined to 26 s ($p<0.01$). Morphine treatment resulted in a considerable reduction of paw licking time to 14 s in the early phase ($p<0.01$). In the second phase of the formalin test, no significant difference was found in licking time at dose of 5 mg/kg of EODM while this time was significantly reduced from 50 s in vehicle treated control group to 31 s at dose of 10 mg/kg and 13 s at dose of 20 mg/kg ($p<0.01$). In the morphine treated animals the mean paw licking time reduced to 24 s in the second phase of formalin test ($p<0.01$).

Effect of EODM on Hot-Plate Test

Figure 4 shows the effect of EODM and morphine on hot-plate test. In this test, different doses of EODM (5, 10 and 20 mg/kg) and control group in i.p. administration did not exhibit any antinociceptive effect while morphine (10 mg/kg) significantly increased ($p<0.001$) the latency time to the nociceptive response at 30 and 60 min after injection.

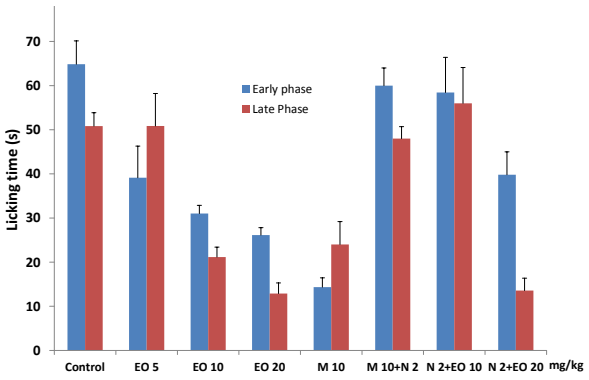

Figure 3. Effect of the essential oil of *D. moldavica* (EODM) on formalin-induced nociception in rats. The total time spent in licking the injected hind-paw was measured in the early phase (0-5 min) and the late phase (15-30 min). The vehicle (Control, 10 ml/kg), the EODM (5, 10 and 20 mg/kg) or morphine (M, 10 mg/kg) were administered intraperitoneally. The effects of naloxone on EODM and morphine antinociception are shown in the right side of the panel. Naloxone (N, 2 mg/kg s.c.) was administered 10 min before EODM or morphine. Asterisks indicate significant difference from control. Each column represented the mean±S.E.M., n=6, *P< 0.001. (ANOVA followed by Dunnett's test).

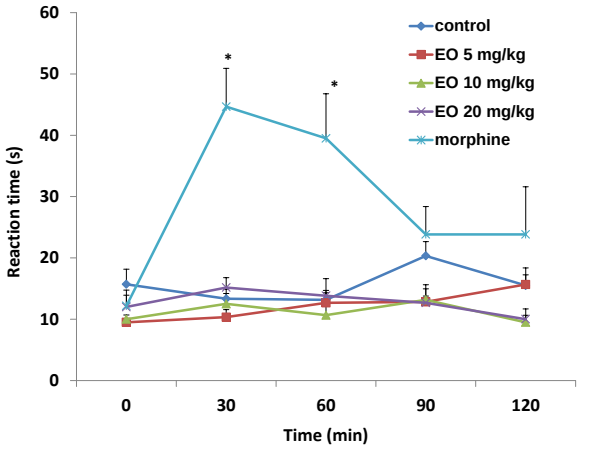

Figure 4. Effect of the essential oil of *D. moldavica* (EODM) in the hot-plate test in rats. The reaction time was measured in seconds (s) before (0 min) and 30, 60, 90 and 120 min after drug treatment. Horizontal axis shows time intervals (min) and the lines represent reaction time (s) in each animal group treated with the vehicle (Control, 10 ml/kg), the EOAD (5, 10 and 20 mg/kg) or morphine 10 mg/kg. The EODM indicated no significant difference from control. Asterisks indicate significant difference from control. Values are mean± S.E.M., n = 6, *P<0.01. (ANOVA followed by Dunnett's test).

Result of Opioid Antagonist Study

In order to evaluate the antinociceptive activity of EODM mediated by opioid receptors, the formalin test was performed using the opioid antagonist naloxone hydrochloride. In both early and late phase of the formalin test, naloxone administration was completely removed analgesic effect of the morphine. Likewise, it reversed the analgesic effect of EODM at dose of 10 mg/kg in both early and late phase of the formalin test. Pretreatment with naloxone did not entirely reverse the

analgesic effect of EODM when the animals were treated i.p. with 20 mg/kg dose (Figure 3). There was a significant difference in licking time between dose of 20 mg/kg of EODM and control group ($p<0.01$).

Discussion

The present study was designed to determine the effect of the EODM in chemical (formalin and writhing) and thermal (hot-plate) nociceptive tests. In the current work, we have shown that the main components of the EODM are citral (31.14%), 3,7- dimethyl -2,6 octadienal (21.43%), cis-geraniol (17.08%), neral (9.63%) and neryl acetate (4.03%). This is in good agreement with Sonboli, et al.[15] who reported the composition of the EODM grown in Iran. Initially, antinociceptive activity of the EODM was assessed by using the writhing test. Although, i.p. administration of the EODM, in all doses, 30 min before the acetic acid injection, reduced abdominal constrictions in mice in dose-dependent manner, this result was significant only at 20 mg/kg of the EODM. Morphine (10 mg/kg) also produced significant inhibition of acetic acid-induced writhing response. The writhing behavior, in mice, by the intraperitoneally injection of acetic acid in the chemical nociception, is used to evaluate, essentially, central and peripheral analgesic activity.[16] Acetic acid induced abdominal writhing involves the production and release of arachidonic acid metabolites via cycloxygenase (COX) and prostaglandin biosynthesis.[17] It is already well documented that 30 min after i.p. administration of acetic acid into peritoneal cavity high levels of PGE_2 and $PGF_{2\alpha}$ were produced.[18] Therefore, the effect of the EODM might be modulated by the inhibition of prostaglandin biosynthesis. The major constituent of the EODM is citral which is a naturally occurring aliphatic aldehyde of the terpene series and is an isomeric mixture of geranial and neral.[19] It has been demonstrated that citral possesses peripheral antinociceptive property as well as anti-inflammatory activity and has beneficial effect in the management of inflammatory pain conditions.[20] Therefore, the antinociceptive activity of the EODM in writhing test might be related to citral. Although, the writhing test has a good sensitivity, it presents poor specificity.[21] To prevent confusion of the results, the formalin test was selected to evaluate analgesic activity of the EODM. The formalin-induced nociceptive response possesses two phases that can involve different mechanisms. The first phase (neurogenic nociception) is elicited by the direct stimulation of nociceptive afferent fibers, mainly C fibers which reflects centrally mediated pain, while the second phase (inflammatory phase) is mediated by the release of inflammatory mediators.[12,22] It is well documented that centrally-acting drugs such as narcotics inhibited nociception in both phases equally, while peripherally acting drugs such as NSAIDs, which blocked prostaglandin synthesis, only inhibited the second phase.[20] This is in accordance with our results showing that morphine could prevent pain in both phases equally. Taking together the ability of EODM to produce antinociceptive effects in the acetic acid-induced abdominal writhing test and in both phases of the formalin-induced paw licking test showed that EODM acting both centrally and peripherally. It also implied that it possessed not only antinociceptive but also anti-inflammatory activities. However, naloxone, a non-selective antagonist of opioid receptors, reversed the antinociceptive effect of morphine in both phases and the EODM at dose of 10 mg/kg, it revealed no influence on the analgesic activity of the EODM at dose of 20 mg/kg. The findings presented herein suggested that the opioid system is partially involved in the analgesic mechanism of the EODM. Nevertheless, in the hot plate test, a central model that has selectivity for opioid-derived analgesic compounds,[23] the EODM had no effect; however, the latency time was significantly increased by morphine. These findings reveal that this compound does not possess similar action to morphine or derivatives when evaluated in this assay suggesting a different mechanism of action which merits further investigation. According to the data of GC/MS analysis about EODM, the main components are the Terpenoids in which citral is the highest one. Several studies have demonstrated that citral possesses central[19] and peripheral effects which mechanism of action is mediated through inhibition of NO production[24] or may be related to the arachidonic acid cascade.[20] In another study synergistic effect of the interaction between naproxen and citral on inflammation in rat was reported.[25] Therefore, the antinociceptive and anti inflammatory properties of the EODM may be related to citral.

Conclusion

The results of the present study indicate, for the first time, that the EODM antinociceptive activity in chemical models of nociception in two species of rodents, suggesting that the essential oil of EODM might represent potential therapeutic options for the treatment of pain related diseases. This study reinforces the folk medicinal use of this plant in pain and inflammatory disorders. Further studies will be undertaken to establish the mechanisms of action for EODM and its active constituents.

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