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ORIGINAL ARTICLE

Anti-nociceptive Effect of 7-methoxy Coumarin from *Eupatorium Triplinerve vahl* (Asteraceae)

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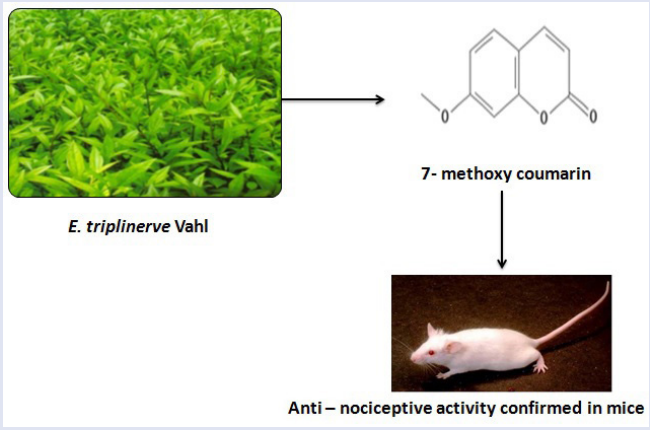
ABSTRACT

Aim: To evaluate the anti-nociceptive activity of 7-methoxy coumarin isolated from ethyl acetate fraction of the alcoholic extract of *Eupatorium triplinerve Vahl.* **Materials and Methods:** The shade dried leaves of *E. triplinerve* were extracted with ethyl alcohol and the extract was condensed. This extract was fractionated with n-hexane, ethyl acetate, and n-butanol. The ethyl acetate fraction was subjected to column chromatography which yielded a crystalline compound-A, which was investigated for spectral characteristics. **Pharmacological studies:** The isolated compound-A was subjected to behavioral studies and anti-nociceptive evaluation in mice by acetic acid induced writhing and formalin induced nociception. **Results:** The spectral studies indicated that the structure of compound-A complies with 7- methoxy coumarin. Pre-treatment with 7-methoxy coumarin reduced the number of abdominal constrictions in mice and decreased the time spent in paw licking and biting response in formalin assay. There were no significant behavioral changes. **Conclusion:** A dose dependent anti-nociceptive action of 7- methoxy coumarin was revealed by the present experiments which support the traditional use of *E. triplinerve* in pain and inflammatory disorders.

Key words: anti-nociception, ayapanin, eupatorium triplinerve (E. triplinerve), herniarin, 7-methoxy coumarin

SUMMARY

- Bio-guided fractionation of alcoholic extract of *E. triplinerve* yielded 7-methoxy coumarin.
- 7-methoxy coumarin was evaluated for its anti-nociceptive potential by acetic acid induced writhing and formalin induced nociception assays.
- 7-methoxy coumarin exhibited significant inhibition of acetic acid induced writhing response and the second phase of formalin nociception.
- The anti-nociceptive action of 7-methoxy coumarin revealed by the present experiments supports the traditional use of *E. triplinerve* in pain and inflammatory disorders.



Abbreviation used: TLC-Thin layer chromatography, Kg-kilogram, g-gram, TXB_2-Thromboxane B2, UV-Ultraviolet, IgE-Immunoglobulin E, s.c-subcutaneous, p.o-oral route

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INTRODUCTION

Ayurvedic practitioners in India use the leaves and whole plant of *E. triplinerve* for the treatment of pain and inflammatory disorders.[1] The extract of *E. triplinerve* has shown hepatoprotective, antioxidant,[2] analgesic,[3] and anti-inflammatory[4] activity in experimental animals. Previous phytochemical studies revealed that *E. triplinerve* is rich in coumarins and phytosterols.[5-7] Authors have reported the anti-inflammatory[4] and anti-nociceptive[3] properties of the alcoholic extract of *E. triplinerve*, its fractions, and identified the ethyl acetate fraction as more bioactive. Since 7- methoxy coumarin has been isolated as the main constituent of ethyl acetate fraction of *E. triplinerve*, it was felt interesting to investigate this compound for its anti-nociceptive action.

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MATERIALS AND METHODS

Plant collection and identification

The leaves of *E. triplinerve Vahl* (Asteraceae) were collected from Kollam district, Kerala in the month of October, and the sample was authenticated by herbarium department of Tropical botanical garden and research institute (TBGRI), Thiruvanathapuram, India (collection no 31691, 31692 account no 20391, 20392). The samples were deposited at Herbarium Department of TBGRI and Department of Pharmacology, Meenakshi Medical College and Research Institute Kanchipuram, for future reference.

Drugs and chemicals

Acetic acid (Merck, USA), formalin (S.D fine chemicals, India), and morphine sulphate (Pharma-chemico lab, India) were used in the experiments. All other chemicals employed in this work were of analytical grade.

Animals

The male Swiss albino mice (25-30 g) were used for the study. The animals were maintained at 25 ± 2ºC in natural light-dark cycle with water and food *ad libitum*. The studies were carried out in accordance with the institutional animal ethics committee guidelines. Each mouse was used only once for experiment.

Preparation of extract and its fractions

The powdered leaves of *E.triplinerve* (1.0 kg) were exhaustively extracted by using 99% alcohol and concentrated under reduced pressure. The concentrated alcoholic extract (106 g) was then subjected to successive fractionation with different solvents in sequence and the yield were; n-hexane-46 g, ethylacetate-24 g, and n-butanol-33 g.

Isolation and identification of compound-A from ethyl acetate fraction

The ethyl acetate fraction (20 g) was chromatographed over silica gel by column chromatography and eluted sequentially with n-hexane/ ethyl acetate/ isopropylalcohol gradients. The fractions were monitored by thin layer chromatography (TLC). The TLC plates were sprayed with dilute sulfuric acid followed by heating at 100ºC and viewed under UV light. Similar fractions were grouped according to their TLC profile. The fractions that were eluted with n-hexane: ethyl acetate (90:10) yielded compound-A with melting point in the range of 117-119ºC. The spectral data of compound-A (mass,^{1}HNMR and[13] C NMR) were compared with values available in literature and the compound-A was identified as 7-methoxy coumarin.

Drug administration in animals

7-methoxy coumarin (compound-A) was prepared as a suspension in1% Tween-80 and administered orally to mice. The doses of 7-methoxy coumarin (3.5 and 7 mg/kg) were calculated based on its ratio in the ethyl acetate fraction of *E. triplinerve* which was earlier studied for anti-nociceptive action.[3] Morphine sulphate (1 or 5 mg/kg s.c.) was used as a standard analgesic drug.

Evaluation of loco motor activity by open-field test[8]

The ambulatory behavior was assessed in an open-field apparatus which consists of a wooden box (90x90x30cm) with the floor divided into 16 equal squares. Mice were treated with 7- methoxy coumarin in doses of 3 .5 and 7 mg/ kg, p.o, or vehicle (1% Tween-80). The number of squares crossed with all paws were counted for a period of 5 min initially and 60 min after the administration of test compounds and compared with vehicle treated group.

Assessment of motor co-ordination by rotarod test[9]

The effect of 7-methoxy coumarin on motor co-ordination in mice was assessed using a rotarod test (15 rpm). Mice were treated with 7-methoxy coumarin in doses of 3 .5 and 7 mg/ kg, p.o or vehicle. Each animal was tested on the rota rod and the time for balancing on the rod was recorded initially and 60 min after administration of the test drug. The balancing time of the animals on the rotarod was measured and compared with vehicle treated animals. A cut off time of 5 min was maintained throughout the experiment.

Acetic acid induced writhing method[10]

The acetic acid induced abdominal writhing is considered as a sensitive method with minimal noxious stimulus for anti-nociception study. The acid (0.6%v/v) in a dose of 10 ml/kg was injected i.p to mice, and the number of abdominal constrictions (writhings) during the following 15 min period was counted. Mice were treated with 7-methoxy coumarin (3 .5 and 7 mg/ kg p.o), or vehicle (1% Tween-80) 60 min prior to acetic acid challenge. The standard drug morphine (1 mg/kg s.c) was administered 30 min before acetic acid administration. Any significant reduction in the number of abdominal constrictions in any treatment group compared with vehicle treatment was considered as an anti-nociceptive response.

Results were expressed as mean ± S.E.M. of six animals *per* group.

The percent inhibition of abdominal constrictions produced by any treatment groups was calculated using the formula: (C-T / C) X 100.

C = Number of abdominal constriction in vehicle treated group.

T = Number of abdominal constriction in test group.

Formalin test[11]

20 µl of 1% formalin in saline was injected s.c into the plantar surface of the left hind paw of the mouse. The animal reacts with a licking or biting response of injected paw. The time spent in licking or biting of the injected paw was recorded every 5 min for a period of 30 min. The summation of responses of first 10 min was taken as acute phase and 10 to 30 min was considered as chronic phase. The mice were treated orally with 7-methoxy coumarin (3 .5 and 7 mg/ kg), or vehicle (1% Tween-80) 60 min prior to formalin injection. The standard drug morphine (5mg/ kg s.c) was administered 30 min before formalin sub planatar injection. Any significant reduction in the licking/biting response time in any treatment group compared with vehicle treatment was considered as an anti-nociceptive response.

The results were expressed as mean ± S.E.M. of six animals *per* group. The percent inhibition of licking/biting response produced by any treatment was calculated using the formula: (C-T / C) X 100

C = Paw licking/ biting response time in vehicle treated group.

T = paw licking/biting response time in test group.

Statistical analysis

All values were expressed as mean ± SEM. The data from animal experiments were statistically analyzed (spss12) using one way ANOVA followed by Bonferonni test and $p<0.05$ was considered to be significant.

RESULTS

Phytochemical studies: The *E. triplinerve* leaves were extracted with ethyl alcohol at room temperature and the alcoholic extract obtained was partitioned into n- hexane, ethylacetate, and n-butanol fractions. The repeated fractionation over silica gel columns of the ethyl acetate-fraction

yielded compound-A. The structure of compound-A was elucidated by investigating spectroscopic details like ^1H,[13] CNMR, and mass. The mass spectrum showed the fragmentation pattern of compound with base peak of 176, m/z 148, 133, and 105. The mass spectrum revealed the empirical formula $C_{10}H_8O_3$ with the molecular ion peak M^+at 176 and fragment ions at M/Z148 and 133 mass units corresponding to $[M\text{-}CO]^+$ and $[M\text{-}(CH3\text{-}OH)]^+$, respectively.[5,6] The ^1H- NMR displayed a singlet at 3.90 assignable to the methoxy group at 7th position. The doublet peaks at 6.25, 7.82, 7.54, 6.82 are assignable HNMR at 3rd, 4th ,5th, and 8th position protons. The peak at 6.52 confirmed the presence of protons at H-6 one doublet of doublet (ortho and meta coupled protons) and a weal doublet (meta coupled proton).[13] C-NMR Spectrum showed the presence of 10 carbon atoms. The peak at 55.01 is assigned to methoxyl group. The peak at 161.1, 112.5, 143.3, 128.7, 113, 162.8, 100.8, 155.8, 112.5 are assigned to C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, and C-10 respectively. The proton and carbon NMR spectral study of compound-A satisfies the splitting of aromatic protons, which has three doublets: a methoxy proton and 10 carbon signals.[5,6] These data were compared with literature values and based on this the structure was proposed as 7-methoxy coumarin. which is in complete unison with previously published data [Figure 1]. 7-methoxy coumarin has been previously isolated from this plant and designated as herniarin or ayapanin.[5,6]

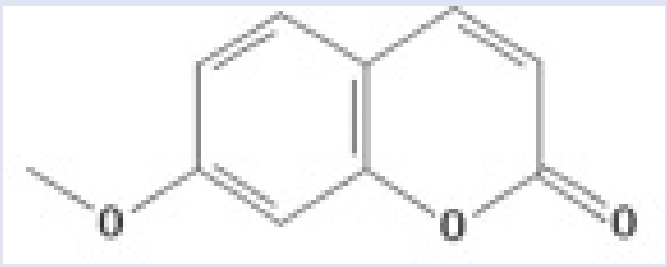

Figure 1: Chemical structure of 7- methoxy coumarin

Table 1: Effect of 7-methoxy coumarin on acetic acid induced abdominal constrictions in mice:

Treatment (*n*=6)	Number of abdominal constrictions
vehicle	39.0 ± 0.37
Morphine 1mg/kg, s.c	3.80 ± 0.90* (90.25%)
7-methoxy coumarin 3.5 mg/kg, p.o	15.24 ± 0.52* (60.92%)
7-methoxy coumarin 7 mg/kg, p.o	12.02 ± 0.49* (69.17%)

Each value represents the mean ± SEM of six observations. *p<0.05 compared to vehicle treated control group. Values in the parenthesis indicates the percentage inhibition

Effect of 7- methoxy coumarin on locomotor activity and motor coordination in mice: The treatment with 7-methoxy coumarin in doses of 3.5 and 7 mg/kg did not significantly modify the ambulatory score recorded in open field apparatus or balancing time of mice on rotarod, compared to vehicle treatment (data not shown).

Effect of 7-methoxy coumarin on acetic acid induced abdominal constriction in mice: In vehicle treated animals the number of abdominal constrictions after acetic acid challenge was 39.0 ± 0.37 [Table 1]. Treatment with morphine (1mg/kg s.c) significantly (p< 0.05) reduced the number of abdominal constrictions to 3.80 ± 0.90 and the inhibition of writhing response by morphine was 90.25%. 7- methoxy coumarin in both the doses (3.5 and 7 mg/kg) significantly reduced the number of abdominal constrictions to 15.24 ± 0.52 and 12.02 ± 0.49 and the inhibition of writhing response in the above doses were found to be 60.92% and 69.17% respectively.

Effect of 7- methoxy coumarin on formalin nociception in mice: In vehicle treated animals, the paw licking response time in early phase was 53.3 ± 1.33 sec and 85.0 ± 0.77 sec in the late phase [Table 2]. In Morphine treated animals, the paw licking response time was significantly (p< 0.05) reduced both in early phase (10.0 ± 0.73 sec) and in late phase (3.33 ± 0.49 sec) when compared with vehicle treated group. There was 81.25% inhibition of formalin nociception in the acute phase and 96.08% inhibition in the late phase after morphine treatment. Oral administration of 7-methoxy coumarin in doses of 3.5 mg/kg and 7 mg/kg did not significantly alter the early phase of formalin induced nociception. However, a significant and dose dependent reduction in paw licking / biting response time was observed in the late phase (34.14 ± 0.84 sec and 26.82 ± 1.05 sec). The inhibition of response was to an extent of 60.0% and 68.47% in the above doses of 7-methoxy coumarin.

DISCUSSION

Medicinal herbs have been used as a form of therapy for the relief of pain throughout history.[12] Considering that, the most important analgesic prototypes salicylic acid and morphine, which were originally derived from plant source, investigation of plant species traditionally used as pain relievers is considered a useful strategy in the search for new analgesic and anti-inflammatory drugs.[13]

In India, a leaf infusion of *E. triplinerve* is considered as cardio tonic, diaphoretic, emetic, haemostatic, laxative, anti-inflammatory, analgesic, anti dote for snake bite poisoning, and for the treatment for piles and

Table 2: Effect of 7-methoxy coumarin on formalin nociception in mice

	Paw licking response time (sec)	
Treatment (*n*=6)	Early phase [100-] min	Late phase 10-30 min
vehicle	53.3 ± 1.33	85.0 ± 0.77
Morphine 1mg/kg, s.c	10.0 ± 0.73* (81.25%)	3.33 ± 0.49* (96.08%)
7-methoxy coumarin 3.5 mg/kg, p.o	52.15 ± 1.21 (1%)	34.14 ± 0.84* (60%)
7-methoxy coumarin 7mg/kg, p.o	51.18 ± 1.51 (1%)	26.82 ± 1.05* (68.47%)

Each value represents the mean ± SEM. of six observations. *p<0.05 compared to vehicle treated control group. Values in parenthesis indicates the percentage inhibition

wound healing[1] Phytochemical studies reveal that the plant is rich in coumarins. A total of seven coumarin compounds namely 7-methoxy coumarin, 7-methylene di methoxy coumarin, daphnethin, daphnethin di methyl ether, hydrangetin, daphnethin-7-methyl ether, and umbelliferone have been characterized in this plant.[7] Coumarins are considered to be components of the general defense response to abiotic and biotic stresses, and it has been proved that various substituted coumarins exhibit anti-inflammatory activity and act as inhibitors of numerous enzyme systems.[14]

An earlier study identified the ethyl acetate fraction of *E. triplinerve* to exhibit potent anti-nociceptive[3] action in animals. The present study reports the isolation, structure elucidation and, anti-nociceptive activity of 7-methoxy coumarin obtained from the bioactive ethyl acetate fraction of ethanolic extract of *E. triplinerve*.

The available experimental models for anti-nociception in animals essentially involve the assessment of some kind of motor activity in response to a noxious stimulus. The abdominal constrictions induced by acetic acid and the biting response to the intra plantar injection of formalin require co-ordinated movements. Any substance interfering with motor activity is likely to yield false positive results.[15] Therefore, in the present study the effect of 7-methoxy coumarin on the spontaneous motor activity in mice was tested using an open field apparatus and the effect on muscle co-ordination was tested using a rotarod. The motor activity of mice and the balancing time on a rotarod were not altered by treatment with different doses of 7-methoxy coumarin. Thus, it can be inferred that the test compound does not influence the motor activity in the doses employed.

The nociceptive behavior induced by acetic acid and late phase of formalin nociception are considered due to an inflammatory response, and any agent that suppresses the above responses may be considered to be useful in alleviating inflammatory pain. The inhibition of nociceptive response induced by acetic acid and late phase of formalin nociception clearly indicates that 7-methoxy coumarin will be effective against inflammatory pain. This proposal is supported by previous reports on the effect of 7-methoxy coumarin on certain inflammatory mediators. 7-methoxy coumarin was found to decrease the production of TXB_2 in human platelets[16] by inhibiting cyclo oxygenases. During inflammation and allergic reactions, a variety of mediators are released from mast cells, basophils, and other inflammatory cells. 7-methoxy coumarin has been proved to inhibit IgE mediated β-hexosaminidase (a marker molecule to assess mast cell degranulation) release from RBL-2H3 cells.[17]

The above reports indicate the potential role of 7-methoxy coumarin to suppress inflammation and these data along with the present results support the contention that 7-methoxy coumarin might be useful against inflammatory pain. This observation is further strengthened by a report that coumarin derivatives, columbianadin, columbianetinacetate, bergapten, and umbelliferone isolated from, *Angelica pubescens*, exhibited analgesic activity in mice.[18]

The present findings are from a sequential, bioassay guided fractionation[3] of *E. triplinerve* that has led to the identification of 7-methoxy coumarin as an active ingredient responsible for anti-nociceptive action of this traditionally used herb. Further it may be suggested that coumarin derivatives may serve as a potential source for the development of new anti- inflammatory and analgesic agents.

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Nil

Conflicts of interest

There are no conflicts of interest

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