

Involvement of Opioidergic System in the Anti-Nociceptive Potentials of Flavonoids Isolated from *Artemisia macrocephala* Jacquem.

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Abstract: Chromatographic separation of chloroform soluble fraction of the methanolic extract of *Artemisia macrocephala* yielded a flavonoids 5-hydroxy-7-methoxy-2-(4-methoxy phenyl)-4*H*-chromen-4-one (1). It was isolated and then characterized *via* spectroscopic data including ¹H-NMR, 2D NMR (HSQC, HMBC, COSY and NOESY) and mass spectrometry techniques. The compound was tested for *in-vivo* ant-inociceptive inhibitory activity in animal model which exhibited significant antinociceptive activity.

Key words: *Artemisia macrocephala* • Writing • Isolation • Flavonoids • Characterization • Anti-nociceptive

INTRODUCTION

Flavonoids are mostly the products of secondary metabolism and are naturally occurring polyphenolic compounds that are widely distributed in the plant kingdom. They represent one of the most abundant classes of phytochemicals that are generally present in vegetables, fruits, medicinal plants and their products. As per reported literature, thousands of flavonoids have been characterized [1].

Flavonoids are gaining importance for their medicinal value and are extensively investigated for their pharmacological importance. They have shown an impressive array of biological activities, including anti-inflammatory [2], antiviral [3], antioxidant [4], hepatoprotective [5], antileishmanial [6,7], antibacterial [8], antihypertensive [9], antihyperglycemic [10], anticancer or antimutagenic effects [11-15] and analgesic [16-18].

Based upon the significance of flavonoids in the wellbeing of humanity, in this study, we have attempted to isolate flavonides from *Artemisia macrocephala* Jacquem and study for possible analgesic activity.

MATERIALS AND METHODS

Aerial parts of *Artemisia macrocephala* Jacquem were collected from the hills of Badwan Chowk, Dir Lower, Khyber Pakhtunkhwa, Pakistan in August, 2014. It was identified by Professor Jehandar Shah. A voucher specimen “Am-01-2014” was submitted to the herbarium of Department of Botany, University of Malakand.

Extraction: The plant material was dried in shade at room temperature followed by mechanical pulverization and then maceration in commercial grade methanol with intermittent shaking for 22 days at room temperature. Filtered off the material. This process was repeated thrice. The filtrates were combined and evaporated under reduced pressure, via rotary evaporator, till a dark greenish color crude Am.Cme. was obtained.

Fractionation and Isolation: The crude Am.Cme. was suspended in distilled water (400 ml) and successively fractionated with *n*-hexane (400 ml). Repeated the process till complete fractionation. Similar procedure was followed for fractionation with chloroform and ethyl acetate till it

successively gave *n*-hexane fraction (Am.*n*-hex), chloroform fraction (Am.Chf), ethyl acetate (Am.EtOAc) and of residual aqueous fraction (Am.Aq). Stored the fractions in refrigerator.

The chloroform fraction was subjected to gravity column chromatography with elution solvent system ethyl acetate: *n*-hexane, starting from pure *n*-hexane with gradual increase of ethyl acetate yielding 12 fractions. Fraction no. 3 was again subjected to chromatography eluted with 20% ethyl acetate: *n*-hexane. TLC plate was used for examining the purity of the compound and visualized under UV lamp.

Animals: Balb C mice obtained from animal house of Department of Pharmacy, University of Malakand, Chakdara, Dir Lower, KPK, Pakistan, were used. Standard laboratory conditions were provided to them with free access of standard food and water *ad libitum*. All the protocols of the experiment were approved by ethical committee of Department of Pharmacy, University of Malakand.

Acute Toxicity: The isolated flavonoids derivative (Ism-6) was tested for acute toxicity as per standard protocol [19]. The mice, in first phase were given 25, 50, 100 mg/kg and in second phase 125, 250 and 500 mg/kg of the isolated compound. Control group was given normal saline. Observed them continuously for 6 hours for changes in the behavioral or autonomic responses followed by another examination after 24 hours. Noted any mortality for the next 14 days.

Analgesic Activity

Acetic Acid Induced Writhing Test: The peripheral antinociceptive activity was carried out via acetic acid induced writhing test. Two groups of mice ($n = 6$) were prepared. They were given 30 mg/kg of the isolated flavonoids 1 h before the injection of 10 ml/kg of 1% acetic acid intraperitoneally. Negative control group was given 10 ml/kg of 1% solution of Tween 80 while positive control group was given 10 mg/kg of diclofenac sodium intraperitoneally to overnight fasting mice. The number of writhing and stretching was noted and percent protection was determined from the data [20].

Formalin Test: This test was carried out in Balb C mice using formalin. A dose of 30 mg/kg of isolated compound in each group was given to the pre labeled groups.

After a time of half an hour of test samples treatment, 2.5% formalin (20 μ l) was injected (s.c) in hind paw of the mice. Recorded the time spent in licking the injected paw in early phase (0–5 min) and late phase (15–30 min). Opioid antagonist, naloxone, (2 mg/kg, s.c) and standard drugs morphine (5 mg/kg) and indomethacin (10 mg/kg) was also used [21].

Tail Immersion Test: For determination of central antinociceptive activity of the sample, the animals were given 30 mg/kg of the sample intraperitoneally, 2% vehicle and 50 mg/kg of diclofenac sodium, 30 minutes before the immersion of the tail (3 cm) in hot water ($55 \pm 0.5^\circ\text{C}$). To investigate the possible involvement of opioid receptor, morphine (agonist, 5 mg) and naloxone (antagonist, 2 mg) were used. Noted the time of reaction taken at 15, 30, 45, 60, 75 and 90 minutes after administration of sample [22].

Statistical Analysis: All the values are expressed as mean \pm SEM (standard error of mean, $n=6$). Applied One-way ANOVA test for comparing the treatment group with the control groups.

RESULTS

5-hydroxy-7-methoxy-2-(4-methoxy phenyl)-4H-chromen-4-one (1)

Compound (1), Amorphous powder, EI MS m/z (%) 284 (M^+), 255, $^1\text{H-NMR}$ (500 MHz, acetone- d_6) δ 12.98 (s, 1H, H-5/OH), 7.96 (d, 2H, $J_{2,3'/6,5'} = 8.0$ Hz, H-2/H-6), 7.03 (d, 2H, $J_{3,2'/5,6'} = 8.0$ Hz, H-3/H-5), 6.68 (s, 1H, H-3), 6.66 (d, 1H, $J_{8,6} = 2.0$ Hz, H-8), 6.32 (d, 1H, $J_{6,8} = 2.0$ Hz, H-6), 3.92 (s, 3H, H-4'/OCH $_3$).

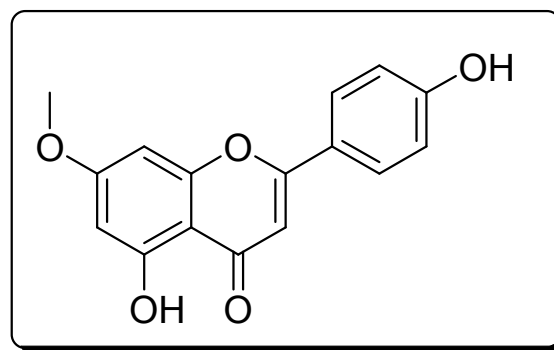


Fig. 1: 5-hydroxy-7-methoxy-2-(4-methoxy phenyl)-4H-chromen-4-one (1).

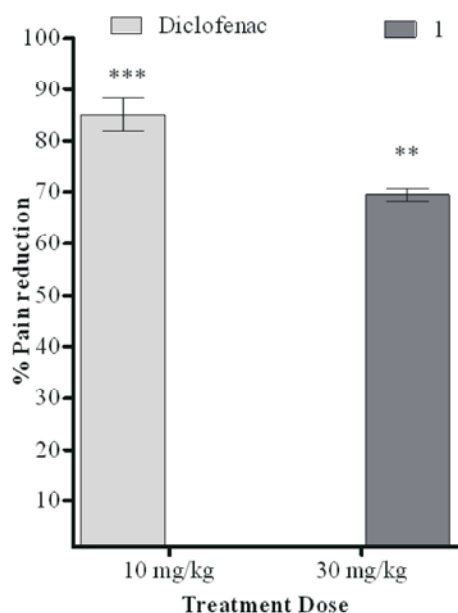


Fig. 2: Acetic acid induced analgesic activity

The isolated pure compound showed no adverse effects at 500 mg/kg on the behavioral responses in the tested mice for 14 days observation. No weight change/ mortality observed. Therefore, a highest dose of 30 mg/kg was considered to be safe in this study.

While performing the acetic acid induced writhing essay the sample showed a significant effect. The acetic acid induced writhing was significantly inhibited by the sample to 69.45 % (P<0.01, n=6) as shown in Fig. 2. The results were compared to the standard (diclofenac sodium, 10 mg/kg) with 84.96 % response.

Formalin treated animals when tested with the samples significantly inhibited both the phases with 43.23 % (**P<0.001, n=6) in the first phase and 64.65 % (**P<0.001, n=6) in the second phase.

Morphine (5 mg) treated animals inhibited significantly both the phases with 87.16 % (**P<0.001, n=6) and 96.34 % (**P<0.001, n=6) for first phase and second phase respectively as shown in Table 1.

Table 1: Formalin-induced paw-licking response.

Treatment/Dose	Licking time (Sec)		Inhibition (%)	
	1 st Phase	2 nd Phase	1 st Phase	2 nd Phase
Control (2% Tween 80)	50.65 ± 1.25	73.90 ± 1.45	----	----
1 30mg	28.75±1.231***	26.12±1.145***	43.23	64.65
Indomethacin (10mg)	39.05±1.26**	19.00±1.342***	22.90	74.28
Morphine (5mg)	6.50±1.153***	2.70±1.340***	87.16	96.34
N + 1 (2mg + 30 mg)	47.82±1.33	66.85±1.40	5.58	9.53
N + Indomethacin (2mg + 10mg)	44.80±1.76**	24.95 ±1.249***	11.54	66.23
N + Morphine (2mg + 5mg)	49.29±1.52	72.83±1.152	2.68	1.44

All the values were expressed as mean ±SEM. **P<0.01 and ***P<0.001 when compared to control group (one way ANOVA followed by Dunnetts: compare all vs control test).

Key: N = Naloxone 2mg

Table 2: Analgesic activity (tail flick method) data

Treatment/Dose	Time in Sec (Tail Flick)/ Response (%)					
	15 min	30 min	45 min	60 min	75 min	90 min
Control (2% Tween 80)	0.81±0.015	0.88±0.035	0.98±0.026	0.95±0.034	0.88±0.025	0.91±0.043
1 30mg	1.05±0.78* (29.62%)	1.27±0.123* (44.31%)	1.47±0.134** (50.00%)	1.57±0.119** (65.26%)	1.54±0.163*** (75.00%)	1.49±0.138*** (63.73%)
(Morphine 5mg)	1.19 ±0.025 (46.91%)	2.05 ±0.064 (57.07%)	3.94±0.034 (75.12%)	6.34±0.054 (85.01%)	4.43±0.050 (80.13%)	4.30±0.074 (78.83%)
N + 1 (2mg + 30 mg)	0.93±0.039	0.95±0.026	1.19±0.030	0.97±0.040	0.91±0.034	0.95±0.060
N + Morphine (2mg + 5mg)	0.79±0.046	0.87±0.037	0.93±0.045	0.90±0.033	0.89±0.034	0.90±0.045

All the values were expressed as mean ±SEM. *P<0.05, **P<0.01 and ***P<0.001 when compared to control group (one way ANOVA followed by Dunnetts: compare all vs control test).

Key: N = Naloxone 2mg.

A reversal of inhibitory effect was observed in animals pre-treated with naloxone. Naloxone caused a prominent reversal effect in the analgesic activity of morphine in both the phases. Indomethacin (10 mg/kg) slightly inhibited the first phase with 22.90 % (**P<0.01, n=6) and significantly inhibited the second phase with 74.28 % (**P<0.001, n=6).

The sample when tested for its effectiveness in tail flick model, significantly increased the latency time to 65.26 % (**P<0.01, n=6) at 60 min at which morphine (opioid analgesic, centrally acting), showed 85.01 % (**P<0.001, n=6) activity. Naloxone treated animals significantly reduced the analgesic potentials of morphine and the isolated compound (Table 2).

DISCUSSION

Various natural products are used in traditional medicines in different countries. Alternative system of medicine is attracting more attention. A lot of plants produce a significant effect like synthetic drugs. Therefore, natural products with little side effects are compulsory to take place of chemical therapeutics [23]. Several species of the genera *Artemisia* have analgesic activity is attributed mainly to flavonoids, alkaloids, sesquiterpene lactones and essential oils [24].

We focused on isolation of constituents having antinociceptive potentials from *A. macrocephala*. Therefore, isolation was carried out through column chromatography and antinociceptive activity was carried out through three different methods. The results obtained from this study showed the presence of constituent with antinociceptive potentials in all animal models used. Acetic acid induced abdominal constriction is a quite sensitive practice that enables the detection of peripheral antinociceptive activity [25]. This model is based on several nociceptive mechanisms like sympathetic system (biogenic amines release), cyclooxygenases and their metabolites [26] and opioid mechanisms [27]. Therefore, the formalin and tail immersion tests were used to find out if the plant possesses constituents any central analgesic potential.

The formalin test is considered a valid model for clinical pain [28] and in this model; the isolated constituents effectively inhibited the licking response in both early and late phases, in a manner more similar to that of morphine. An opioid antagonist, naloxone, greatly reversed the analgesic effect of the isolated constituents indicating that opioid receptors are involved in the

activity. Anyhow a complete antagonism was not observed, therefore, it may be concluded that flavonoids possess such effect [29].

In tail flick test isolated constituents again showed morphine like response indicating that the presence of spinal effect. NSAIDs (Non-steroidal anti-inflammatory drugs) act by decreasing the sensitivity of pain receptors which is caused by prostaglandins [30]. Though the exact mechanism is not known, the observed activity may be due to morphine like effect of the samples. A lot of substances with spinal analgesic activity are present in literature among which the most familiar one is morphine, obtained from opium [31]. In a similar way, we tried to find out on scientific basis the antinociceptive potentials of isolated constituents from *A. macrocephala*, which showed morphine like antinociceptive activity which increases the list of pharmacological activities already mentioned for *A. macrocephala*. This discovery encourages our previous work of relaxant activity of the crude extract and essential oil of *A. macrocephala* [32].

CONCLUSION

A known flavonoids was isolated for the first time from the dried aerial parts of *A. macrocephala*. It possess potent antinociceptive activity; this proves the use of the plant in folk medicine as analgesic agent.

Competing interests It is declared that the authors have no competing interests.

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