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EVALUATION OF ANALGESIC ACTIVITY OF METHANOLIC EXTRACT OF *CROTALARIA BURHIA* BUCH.-HAM. ROOTS IN RATS AND MICE

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Key words:

Analgesic activity, *Crotalaria burhia*, MECB, Morphine

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ABSTRACT: Aim: Present study was aimed to evaluate the analgesic activity of methanolic extract of *Crotalaria Burhia* roots (Henceforth, MECB) using various experimental animal model of pain. **Materials & Methods:** Analgesic activity was tested using acetic-acid induced writhing test, formalin test, hot-plate test and tail-flick test for pain in mice and rats. In this study we investigated analgesic activity of MECB at test doses of 100, 150 and 300 mg/kg, p.o. The effects following pretreatment with aspirin, morphine and naloxone were also studied. Standard methodologies were used for the screening of preliminary phytochemical constituents of extract. **Results:** Result showed significant analgesic activity of MECB in all paradigms. The MECB at the dose 150 mg/kg was shown stronger analgesic activity compared to MECB at the dose of 100 and 300 mg/kg. Moreover, analgesic activity of MECB was note in similar manner to aspirin (100 mg/kg, p.o.) and morphine (5 mg/kg, s.c.). Naloxone (2 mg/kg, i.p.) abolished the analgesic activity of both morphine (5 mg/kg, s.c.) and MECB (150 mg/kg, p.o.) in a similar manner. **Conclusion:** Results revealed that the MECB had significant analgesic activity in experimental animals (rats & mice). The mechanism of action of MECB appears to both peripheral and centrally mediated action (may be through opioid receptor). Our studies support the traditional use of *Crotalaria burhia* and the roots of *Crotalaria buria* can be a good source as analgesic.

INTRODUCTION: Medicinal plants are essential sources for development newer pharmacological substances. Medicinal plants derived drug exhibit greater safety and efficacy with stronger therapeutic action. Most people living in developing countries are almost completely dependent on herbal or traditional medical practices to meet their primary health care needs. Medicinal plants are prime source for therapy in herbal or traditional medicine system [1].

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The genus *Crotalaria* (Fabaceae) has 30 species world-wide with only about 18 species reported in India. The genus produces mainly pyrrolizidine alkaloids along with flavonoids and steroids [2]. *C. burhia* or *Khip* is an under shrub, fibrous plant, common in the arid parts of West Pakistan, India and Afghanistan [3, 4].

In antique Indian medical system of Ayurveda, *khip* has been mentioned as a medicinal plant and various parts of the plant are used. The leaves and branches are useful as a cooling medicine (antipyretic), while fresh plant juice is applied on eczema, plant is also very useful in gout, hydrophobia, pain and swelling (inflammation) [4, 5]. Roots extract with sugar is used to cure chronic kidney pain and root decoction is used in typhoid [6].

The pyrrolizidine alkaloids are main active components in *Crotalaria burhia* [7]. In addition quercetin and β-sitosterol have been identified from this plant [3, 8]. Various parts of *C. burhia* have shown a wide array of activities such as anticancer, anti-inflammatory and antimicrobial activities [5, 8, 9]. The whole plant is reported with antibacterial and antifungal activities [5, 10].

Reason for selection of plant:
In the present study *Crotalaria burhia* roots was selected because of traditionally this whole plant is used as remedies to treat gout, pain, tumor, swelling, kidney pain and typhoid [11]. The detailed investigation on the methanolic extract of *C. burhia* roots (MECB) was lacking to supports analgesic potential of *C. burhia* roots. Therefore, present study was designed to establish scientific basis for the traditional uses of *Crotalaria buriha* roots against pain.

MATERIALS AND METHODS:

Plant:
Crotalaria burhia Buch.-Ham. roots were collected from Rajasthan University Campus, Jaipur, Rajasthan, during month of Oct-Nov 2010. The plant was identified by Mr. P.J. Parmar, Joint Director in Botanical Survey of India (BSI), Jodhpur (Rajasthan, India). Authenticated voucher specimen (JNU/JPR/PC/SK-1) was deposited in the BSI, Jodhpur, India.

Animal:
Healthy, W*istar albino* rats and Swiss albino mice were procured from the departmental animal house for experimentation. The animals were grouped and housed in poly acrylic cages under standard laboratory conditions (temperature 25±2 ºC, dark-light cycle 14-10 hrs). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The animals were maintained in accordance with CPCSEA guidelines. All the procedures described were reviewed and approved by Institutional Animal Ethical Committee of Gyanvihar University, Jaipur, India.

Chemical:
All chemicals used in the experiment were of analytical grade.

Preparation of extract and reference drug:
The air and shadow dried powdered roots of *Crotalaria burhia* (5 Kg) was exhaustively extracted with methanol by continuous extraction using Soxhlet-apparatus. The final extract (henceforth MECB) was concentrated under reduced pressure, on a rotary evaporator at 40-45^{o}C, to get a yield of 2.1 % w/w.

The MECB doses of 100, 150 and 300mg/kg were prepared by suspending the residue in the cosolvent (propylene glycol:Tween 80:distilled water; 4:1:4). The reference drugs used in this study were aspirin (100 mg/kg), morphine sulphate (5 mg/kg) and naloxone (2 mg/kg). All of reference drugs were prepared for administration by dissolving in 0.9% normal saline.

Acute toxicity study:
The up-and-down method for acute toxicity testing was carried out according to method described by Bruce (1985) [12]. In this study, the first dose of MECB was administered orally at 300 mg/kg body weight and adjusted by a constant multiplicative factor of 1.5 up-to 5 g/kg body weight. After dosing animals were observed for behavior parameters such as convulsion, hyperactivity, sedation, grooming, loss of righting reflex and increased or decreased respiration during a period of 7 days. No mortality was observed up to 7 days of monitoring. Food and water were provided *ad libitum*.

Acetic acid-induced writhing in mice:
Mice were grouped (n=6) and writhing test was performed according to method described by Koster *et al* (1959) [13]. The MECB at doses of 100, 150 or 300 mg/kg were administered orally to each animal before 30 min of induction of writing by intra-peritoneal injection of 0.6% acetic acid in 0.9% normal saline (10 ml/kg body weight). Co-solvent (10 ml/kg, p.o.) and aspirin (100 mg/kg, p.o.) were administered to control and reference groups, respectively. The mice were observed and the number of abdominal constrictions and stretching were counted for a period of 0–20 min. The inhibition of writhing was calculated and reported in the results as a percentage.

Formalin test in mice:
Mice were grouped (n=6) and formalin test was conducted according to method described by Hunskaar *et al* (1985) [14]. The control group was treated with cosolvent 002810 ml/kg, p.o.) and reference groups were treated with aspirin (100 mg/kg, p.o.) and morphine sulphate (5 mg/kg, s.c.). Test groups were treated with MECB at dose of 100, 150 and 300mg/kg (p.o). After 30 min of treatment (after 15 min for morphine), 20 µl of 2.5% formalin in saline was injected subcutaneously into a hind paw of each animal. Formalin injected paw was observed for time spend for licking and expressed as the total licking time in early phase (0-5 min) and late phase (15-30 min) after injection of formalin.

Hot plate test in mice:
The hot plate test was carried out according method described by Woolfe and MacDonald (1944) [15]. Animals were grouped (n=6), control group was received cosolvent (10ml/kg, p.o.) while the reference group was treated with morphine sulphate (5 mg/kg, s.c.) and naloxone (2 mg/kg, i.p.). The animals in test groups were treated with different doses of MECB i.e 100, 150 and 300mg/kg, p.o.. In separate groups, the animals received naloxone (2 mg/kg, i.p.) 10 min before morphine (5 mg/kg, s.c.) or the extract (200 mg/kg, p.o.). After 30 min of treatment with all test drugs and before 15 min for morphine and 10 min for naloxone, mice were placed on a hot plate (maintained at 55±1 ◦C).

The latency of nociceptive response (reaction time) of each animal was recorded by identifying the time for licking and flicking of a hind limb or jumping. The reaction time was measured for the period of 90-min with intervals of 15 min. The cut-off time of observation was 45 s. Only the mice shown nociceptive response within 15 s was used in the experiment.

Tail-flick test in rats:
The tail-flick test conducted in rats by the method described D'Amour and Smith [16]. The experiment was carried out in the same manner as the hot plate test. After 30min of treatment with all test drugs and after 15min of treatment with morphine, and after 10 min of treatment with naloxone, the tail-flick response was measured by gently placing rat tail at a central position of a light beam. The time taken by the animals to withdraw (flick) the tail from heat was recorded as the reaction time. The cut-off time was 20 s to prevent injury to tail.

Data analysis
The data were expressed as a mean ± S.E.M. and statistically significant differences between groups were calculated by the application of analysis of variance (ANOVA) by following Bonferroni's test (using SPSS software program version 11.5). The unpaired t-test was used for comparison between two groups. The $P<0.05$ was considered as the significant.

RESULTS:
Acute toxicity study:
MECB was found to be safe up-to the dose of 2000 mg/kg in mice. There were no signs of convulsions, hyperactivity, sedation, grooming, loss of righting reflex and increased or decreased respiration. In further no mortality was observed during monitoring period.

Acetic acid induced writhing:
Oral administration of MECB at different doses showed significant inhibition against acetic acid induced writhing when compared to control group ($P<0.05$). The MECB at dose of 150 mg/kg (↓47.38%) was found with superior analgesic activity than MECB at 100 mg/kg (↓25.5%) and 300 mg/kg (↓38.95%) when compared to control group. The MECB was shown significant inhibitory effect against acetic acid induced writhing in mice and inhibitory response was noted in similar manner to aspirin (↓56.02%) (**Table 1**).

TABLE 1: EFFECT OF MECB ON ACETIC ACID INDUCED WRITHING IN MICE

Treatment	Dose (mg/kg)	Number of writhing	% Inhibition
Control	-	49.8 ± 3.12	-
MECB	100	37.1 ± 1.81	25.50%
MECB	150	26.2 ± 1.31**	47.38%
MECB	300	30.4 ± 2.4**	38.95%
Aspirin	100	21.9 ± 1.67*	56.02%

Value are presented as the mean±SEM (n=6), *$P<0.05$, **$P<0.01$ when compared to control

Formalin test:

Oral administration of MECB at different doses was shown significant analgesic activity in this paradigm when compared to control group (P<0.05). Interestingly in this test also MECB at dose of 150 mg/kg was shown superior analgesic activity than MECB at 100 mg/kg and 300 mg/kg when compared to control group. Enchantingly, the MECB at dose of 150 mg/kg was shown superior analgesic activity than aspirin at the dose of 100 mg/kg in both early and late phases of pain stimulus. Analgesic activity of MECB was in similar mode to reference drug when compared to control **(Table 2**).

TABLE 2: EFFECT OF MECB IN FORMALIN TEST

Treatment group (n=6)	Doses (mg/kg)	Licking time			
		Early phase (0-5 min)	% Inhibition	Late phase (15-30 min	% Inhibition
Control	-	75.89 ± 2.65	-	95.4 ± 2.98	-
MECB	100	58.76 ± 3.21*	22.57	67.3 ± 1.87*	29.45
MECB	150	36.8 ± 2.73**	51.5	48.3 ± 2.86**	49.37
MECB	300	45.3 ± 1.7**	40.3	56.1 ± 3.41**	41.19
Morphine	5	24.39 ± 2.3*	67.86	19.3 ± 3.56**	79.76
Aspirin	100	40.3 ± 1.56**	46.89	71.4 ± 2.34*	25.15

Value are presented as the mean±SEM, *P<0.05, **P<0.01 when compared to control.

Hot-plate test:

The analgesic activity of MECB was evaluated by hot-plate method. The results showed that the MECB caused significantly prolongation in reaction time against heat stimulus was noted. The reaction time against heat stimulus was significantly increased after 30 min of administration of MECB at 100, 150, 300 mg/kg and it was sustained up to 90 min when compared to control. In this paradigm also the MECB at the dose of 150 mg/kg was noted superior compared to other test doses. Morphine markedly increased pain latency (P<0.01) at each time point after dosing and maximum effect was noted after 60 min of dosing. Interestingly, the MECB with naloxone was shown equivalent analgesic activity to morphin and naloxone (**Table 3**).

TABLE 3: EFFECT OF MECB ON THE REACTION TIME IN HOT PLATE TEST

Treatment group (n=6)	Doses (mg/kg)	Reaction time (s)				
		30 min	45 min	60 min	75 min	90 min
Cosolvent		7.93 ± 1.3	8.89 ± 0.7	10.43 ± 0.4	11.3 ± 0.9	9.57 ± 0.54
Morphine	5	11.3 ± 0.47**	13.92 ± 1.56*	16.71 ± 0.83**	17.3 ± 0.52*	15.3 ± 0.41**
Nalaxone	2	7.9 ± 0.3	8.64 ± 0.75	8.98 ± 0.9	9.32 ± 0.23*	8.87 ± 0.42*
MECB	100	8.15 ± 0.5**	9.1 ± 0.52*	10.95 ± 0.34**	12.62 ± 0.32**	12.31 ± 0.12**
MECB	150	10.42 ± 0.5**	11.63 ± 0.65**	14.53 ± 0.2**	15.3 ± 0.32**	14.3 ± 0.54*
MECB	300	8.98 ± 1.3*	9.1 ± 0.4**	11.67 ± 0.32*	13.4 ± 0.71*	13.72 ± 0.28**
Nalaxone + Morphine	2 + 5	10.95 ± 0.2**[a]	13.1 ± 0.59*	14.73 ± 0.1*[b]	15.9 ± 0.84*[a]	15.23 ± 0.41*
Nalaxone +MECB	2 + 150	10.71 ± 0.62*	12.61 ± 0.73*[†]	13.54 ± 0.52*[†]	14.81 ± 0.3**[†]	14.16 ± 0.51**

Values are presented as the mean±S.E (n=6); *P<0.05 & **P<0.01, when compared to control; [a]P<0.05, [b]P<0.01 significantly decreased when compared with morphine alone; † P<0.05, significant reduction when compared with MECB (150 mg/kg).

Tail-flick test in rats:

MECB showed significant analgesic activity (P<0.01) in tail-flick test when compared to control. The response of tail-flick latency was significantly increased at 45 min after administration of MECB at different doses (**Table 4**). The MECB at different doses was found highly effective after 60 min of dosing. The MECB at dose of 150 mg/kg was found more effective in this paradigm when compared other test doses of MECB (**Table 4**). Morphine was significantly increase tail-flick latency at 45 min after dosing (P<0.01). However, the pretreatment of naloxone in combination with morphine or MECB (150 mg/kg) caused significant reduction in tail-flick latency at 45 min after dosing and constant reduction was noted up to 90 min (**Table 4**).

DISCUSSION: The study was principally aimed to establish the scientific basis to support traditional use of *Crotalaria burhia* Buch-Ham. roots against pain. The numerous animal models are used to evaluate analgesic activity of medicinal compounds. In present investigation the MECB was shown to have significant analgesic activity against pain induced by various experimental methods (**Tables 1-4**). In present study the MECB at the dose of 150 mg/kg was found more effective compared to other doses of MECB. Preliminary phytochemical screening of MECB suggests that the plant is rich in alkaloids, flavonoids, phenolic compounds and steroids.

The acetic-acid induced writhing test was used to explore analgesic activity of MECB against peripheral pain. In this model, pain is generated indirectly via endogenous mediators such as bradykinin, serotonin, histamine, prostaglandin and interleukine, all acting by stimulating peripheral nociceptive neurons [17]. This nociceptive effect can be easily prevented by peripherally and centrally acting analgesics. Results of present study imply that the MECB has significant analgesic activity against pain induced by acetic acid. Analgesic activity of MECB in writhing test may be due to inhibition of inflammatory mediators.

The formalin test for assessment of analgesic activity is to discriminate between central and peripheral pain [18]. The early and late phase of formalin test is useful in assessing analgesic substances and also for elucidating mechanism of analgesia [19]. The early phase, associated with non-inflammatory pain, which is caused by direct simulation of nociceptors and reflects centrally mediated pain. The late phase associated with inflammatory pain, is caused by local inflammation with releases of inflammatory and hyperalgesic mediators [19]. In the present investigation MECB showed significant analgesic activity during both phases of formalin test and which was significantly comparable with reference drug. Considering results it appears that the pain inhibitory property of MECB seen in both phases may be due to inhibition of non-inflammatory and inflammatory pain. The hot plate and tail-flick test are most commonly used to evaluate central analgesic activity of medicinal agents [20] at the supraspinal and spinal levels [21], respectively, possibly acting on descending inhibitory pathway. The tail-flick response is believed to be a spinally mediated reflex and paw-licking response in the hot plate is a more complex supraspinally organized behavior [22]. The μ receptor has generally been associated with pain relief and has been reported to be potent in regulating thermal pain [23].

Activation of μ_2 opioid subtype receptor produces spinal analgesia and commonly leads to the adverse effect of producing constipation [24]. The effectiveness of analgesic agents in the tail-flick pain model is highly correlated with relief of human pain. In this study, results indicated that the MECB has analgesic effect against both hot plate and tail-flick tests, therefore the antinociceptive action is expected to mediated centrally (spinally and supraspinally). According to results of present study we believe that the analgesic activity of MECB is most likely to be mediated peripherally and centrally.

CONCLUSION: In conclusion, the results obtained in present study demonstrate that the MECB has significant analgesic activity. While the exact mechanism and component underlying for analgesic activity is remains to be confirmed. Analgesic activity of MECB and naloxane was noted in same manner of analgesic activity of morphine in both hot plate and tail-flick test, indicating partly through opioid mediated mechanism. According to results it may conclude that the analgesic activity may be due to inhibition of inflammatory mediators and binding of MECB to the opioid receptor and that is responsible for centrally acting analgesic activity.

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