INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 16, No. 3, p. 566-575, 2020

RESEARCH PAPER OPEN ACCESS

In-vivo biological investigations of methanolic extract of *Himalrandia tetrasperma* in animal model

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Key words: *Himalrandia tetrasperma*, Anti-inflammatory, Antipyretic, Antinociceptive, Acute toxicity

http://dx.doi.org/10.12692/ijb/16.3.566-575 Article published on March 30, 2020

Abstract

The study in reference was planned to evaluate the anti-inflammatory, antipyretic and antinociceptive activities of methanolic extract of *Himalrandia tetrasperma* (MEHT), as well as acute toxicity. Anti-inflammatory, antipyretic, antinociceptive and acute toxicity activities were evaluated using mice animal model. Anti-inflammatory activity was investigated using carrageenan-induced edema model of inflammation. Brewer, s yeast induced pyrexia method was employed to evaluate the antipyretic activity. To assess the antinociceptive property of extract two models were employed, acetic acid-induced writhing model and hot plate model. Acute toxicity was conducted with the help of well-established method. The extract showed significant ($p < 0.01$) anti-inflammatory effect. The extract exhibited prominent ($p < 0.01$) antipyretic activity. It also evoked substantial ($p < 0.01$) anti-nociceptive activity (both peripheral and central analgesia). Acute toxicity data revealed that the extract was non-toxic up to a dose of 1500 mg/kg. From these findings it has been found that MEHT could be used to develop a natural drug to cure these ailments.

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Introduction

Since the inception of human life, plant derived products have been used for the remedy of myriad ailments. Medicinal plants are gaining tremendous attention in the primary health care of individuals and communities throughout the world. Plants and their derivatives have been a valuable and benign source of pharmacologically active agents for curing different ailments since prehistoric times. A majority of the world's population in developing countries relies on herbal medicines to meet its health needs. Herbal medicines are often used to provide first-line and basic health service, both to the people living in remote areas where it is the only available health service, and to the people living in poor areas where it offers the only affordable remedy.

Ancient Unani manuscripts, Egyptian papyrus and Chinese writings described the use of herbs. Evidences exist that Unani hakims, Indian Vedas, European and Mediterranean cultures were using herbs for over 4000 years as medicines. Different cultures such as Rome, Egypt, Iran, and Africa used herbs in their healing rituals, while other developed traditional medical systems such as Unani, Ayurveda and Chinese Medicines in which herbal therapies were used systematically. Traditional systems of medicines continue to be widely practiced on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as source of medicines for a wide variety of human ailments. (Guimarães *et al.*, 2011, Velavan and Hazeena Begum, 2011)

Recently, WHO estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary health care needs. According to WHO, around 21,000 plant species have the potentials for being used as medicinal plants. Treatment with medicinal plants is considered very safe as there is no or minimal side-effects, eco-friendly and locally available. These remedies are in sync with nature which is the biggest advantage. The golden fact is that use of herbal treatments is independent of any age groups or sexes (Ayyanar and Ignacimuthu, 2009, Ahmed *et al.*, 2011, Khan *et al.*, 2012).

Without scientific knowledge, the conventional therapists employ these plant derived products on the basis of their vast personal practice. According to latest research, the phytochemical investigation of about 15% of medicinally valuable plants and biological evaluation of around six percent have been conducted (Dhami, 2013). The remaining have been left untouched. For exploration of novel and pharmacologically active entities, the study of phytochemical composition of medicinal plants is indispensable (Saeed *et al.*, 2016).

There are two main groups of phytochemicals. The primary group contains proteins, sugars and chlorophyll etc. and secondary group contains saponins, flavonoids, tannins, terpenoids, alkaloids, essential oils and phenolic compounds etc. (Krishnaiah *et al.*, 2007) Pharmacological activities of plants such as anti-inflammatory, antipyretic, antinociceptive activities are due to the presence of their saponins , flavonoids, tannins and steroids contents (Das *et al.*, 1989, Bruneton, 1995, Bhaskar and Balakrishnan, 2015).

The demand of plant derived medication is increasing in Pakistan just like other developing countries because of the ease in accessibility and affordability of these products (Saeed *et al.*, 2010). Pakistan has above 6,000 species, of higher plants (Ali and Qaiser, 1986, Shinwari and Qaiser, 2011). In Pakistan approximately 80% of population rely mostly on plants to get medicinal therapy (Qureshi *et al.*, 2007).

Himalrandia tetrasperma is a very important medicinal plant also known as *Randia tetrasperma* (Salman *et al.*, 2015). It belongs to family Rubiaceae, commonly known as coffee family (Ajaib *et al.*, 2016). *Himalrandia tetrasperma* is found in temperate and sub-tropical Himalayas, Salt region, Pakistan, Nepal, Sikkim, Bhutan, and India. In Pakistan, it is found in Hazara and Malakand regions, Khyber Pakhtunkhwa.

It has been reported that *Himalrandia tetrasperma* possesses antibacterial, antifungal (Salman *et al.*, 2015) and anti-diabetic activities (Ajaib *et al.*, 2016). *Himalrandia tetrasperma* has many ethno-medicinal uses. Pulp of fruits is used in dysentery, as antihelmintic and abortificient (Salman *et al.*, 2015). It is also used as fuel or making fire and fencing/ hedge plant (Haq *et al.*, 2010).

In the current research work, we have aimed to study various pharmacological activities of the methanolic extract of *Himalrandia tetrasperma* on different animal models in order to ascertain any scientific basis for the use of this plant in cure of inflammation, pyrexia and analgesia.

Materials and methods

Plant material

Fresh Aerial parts of *Himalrandia tetrasperma* were collected from Butti, Tehsil Oghi, District Mansehra, Khyber Pakhtunkhwa, Pakistan, in May 2016. These were authenticated by a taxonomist in the Department of Botany, University of Peshawar.

Chemicals

The chemicals used in this study and their sources are Paracetamol (Tianjin Bofa Pharmaceutical Co., Ltd., China), Aspirin (Merck); Acetic acid, Naloxone^{R} hydrochloride and Sodium chloride (Sigma Chemicals Company, St Louis, USA); Carrageenan (Sigma Lambda, USA) and Tranmadol (Searle Pakistan Ltd). Sterile normal saline was used as control in all studies.

Animals

Albino mice (*Balb-C*), weighing 17-26g of either sex, were used. They were fed laboratory diet ad libitum and allowed free access to drinking water under standard environmental condition of temperature (25°C) in a 12 h-dark/12 h-light cycle.

Preparation of plant extract

The plant was dried in a shade, chopped into small pieces and powdered. The powdered plant material was extracted by maceration with methanol at ambient temperature for 2 weeks.

The methanol-soluble material was filtered through filter paper and the residue obtained was again macerated with methanol. This process was repeated three times and the combined filtrate was concentrated under vacuum at low temperature (40°C) using a rotary evaporator to get the concentrated methanolic extract.

Acute toxicity studies

The acute toxicity test was conducted for MEHT to ascertain its any possible toxicity. Albino mice (n=6) of either sex were used. Animals were treated with various intra-peritoneal (i.p) doses of the MEHT. The dose levels administered were ranging from 50 to 1500mg/kg. The control group is treated only with normal saline (10ml/kg). For a period of 24 hours, all the groups were observed for any gross effect or mortality (Saeed *et al.*, 2016).

Anti-inflammatory activity

Carrageenan-induced edema model

Carrageenan-induced hind paw edema test was conducted to investigate the anti-inflammatory potential of MEHT (Muhammad *et al.*, 2012, Saeed *et al.*, 2016). The animals were randomly distributed in five groups, each containing six animals. Group I was treated with normal saline (10ml/kg), as negative control group, group II with Aspirin (100 mg/kg), as a positive control group, while the other three groups were treated with MEHT at dose levels of 100, 200, and 400mg/kg (i.p) respectively.

In the sub-plantar region of right hind paw of the mice of each group, 0.05 ml of 1% suspension of carrageenan was injected, 30 minutes after administration of the normal saline, aspirin and test samples. Paw volume was measured using digital plethysmometer (LE 7500 Panlab S.L) before and after 1, 2, 3, 4 and 5 hours after the carrageenan injection. Percent inhibition was measured according to formula as given below:

$$\% \text{ inhibition} = \Delta C - \Delta T/\Delta C \times 100$$

Where, ΔC represents increase in paw volume of control and ΔT increase in paw volume of tested group after induced inflammation.

Antipyretic activity

Brewer's yeast induced pyrexia method

MEHT was subjected to antipyretic activity using albino mice of either sex. The animals were of sound health (Muhammad *et al.*, 2012, Hassan *et al.*, 2019). Prior to the start of experiment animals were acclimatized to laboratory condition. The animals were distributed into five groups, each group consisting of six mice. With the help of digital thermometer, the normal body temperature of each mouse was noted and then by administering subcutaneously (s.c.) of 20% aqueous suspension of Brewer's yeast at the dose level of 10ml/kg produced pyrexia in all mice. Animals of all groups were fasted overnight but allowed free access to portable water and rectal temperature of each mouse was noted after 24h. The induction of pyrexia was confirmed by increase in temperature more than 0.5°C, while animals showed increase in temperature less than 0.5°C were exempted from experiment. Group I was treated with normal saline (10ml/kg), as a negative control, Group II was treated with paracetamol (150mg/kg) as a standard drug (positive control) while the other three groups III, IV and V were treated with 100, 200, 400mg/kg intraperitoneally (i.p.) MEHT respectively. After drug treatment, rectal temperature was again noted regularly at 1, 2, 3, 4, and 5 h of the drug treatment. The percent reduction in pyrexia was calculated by the following formula.

Percent reduction = $Y - T_n / Y - X \times 100$

Where, Y represents temperature after pyrexia; T_n temperature after 1, 2, 3, 4, and 5 h and X normal body temperature.

Antinociceptive activity

Acetic acid-induced writhing test

The antinociceptive property of the extract was evaluated by acetic acid-induced abdominal constriction test in mice (Saeed *et al.*, 2016, Hassan *et al.*, 2019). Albino mice of either sex were used. All animals were removed from food for 2 h prior to the start of experiment and were randomly distributed in five groups (six per group). Group I was treated with normal saline (10ml/kg), as a negative control, Group II was treated with the standard drug aspirin (100mg.kg), as a positive control, while the other three groups III, IV and V were treated with 100, 200 and 400mg/kg i.p. of MEHT, respectively. After lapse of 30 minutes of saline, aspirin and MEHT treatment, the animals were injected i.p. with 1% acetic acid (10 ml/kg). After 5 min of acetic acid injection the number of writhes (abdominal constrictions) was counted for a period of 20 min. Percent protection against nociception was calculated by the following formula.

Percent protection = $C - T/C \times 100$

Where, C represents number of writhing in control and T number of writhing in tested groups.

Hot plate test

Albino mice of either sex (n=6) were acclimatized to laboratory conditions two hours prior to the start of experiment with food and water available *ad libitum* (Saeed *et al.*, 2016). Animals were then subjected to pre-testing on hot plate (Harvard apparatus) kept at 55±0.1°C Animals have latency time greater than 15 seconds on the hot plate during pre-testing were rejected (latency time). All the animals were distributed in eight groups, each containing six mice. Group I was treated with saline (10ml/kg), group II was treated with Tramadol (30mg/kg). group III, IV and V were treated with 100, 200 and 400mg/kg MEHT, i.p. respectively. After 30min of treatment animals were placed on hot plate and the latency time (time for which mouse remains on the hot plate (55±0.1°C) without licking or flicking of hind limb or jumping) was measured in seconds. In order to avoid tissue damage a cut-off time of 30 seconds was applied for all animals.

To determine the opiodergic mechanism in the analgesic activity of MEHT, Group VI and VII were treated with MEHT (200 and 400mg/kg i.p.), while group VIII was treated with Tramadol (30mg/kg i.p.) after 10min of naloxone injection. The latency time for all groups was noted at 0, 30, 60, 90 and 120 min. Percent analgesia was calculated using the following formula:

% Analgesia = (Test latency – Control latency)/(Cut off time – Control latency) × 100

Statistical analysis

All values obtained are expressed as mean ± SEM (n=6). One-way analysis of variance (ANOVA), followed by post hoc Dennett's test for multiple comparison was used for evaluation of activities. The value of $p < 0.05$ was considered to be statistically significant in all cases. Graph Pad Prism 6.0 version was used for statistical analysis of the data.

Results and discussion

Acute toxicity

Administration of MEHT upto 1500 mg/kg did not produce any mortality and gross behavioural changes (Table 1). The animals were found secure and normal during 24 hr assessment. The result obtained from acute toxicity reflects that the extract is secure within the therapeutic dose levels used in the current study.

Table 1. Acute Toxicity of MEHT.

Group	Samples	Dose/kg	Mortality after 24 hrs
I	Saline	10 ml	0
II	Meht	50mg	0
III		100mg	0
IV		150mg	0
V		200mg	0
VI		300mg	0
VII		400mg	0
VII		500mg	0
VII		1000mg	0
IX		1500mg	0

Anti-inflammatory activity

Carrageenan-induced paw edema

The anti-inflammatory activity of the extract is shown in Table 2. The percent inhibition of inflammation is shown in Fig. 1. Administration of carrageenan in the mice paws produced inflammatory edema which increased gradually. At a dose of 400 mg/kg the Meht exhibited prominent anti-inflammatory effect ($p < 0.01$) which was highest at 3 hr after carrageenan injection (63.85% inhibition). The anti-inflammatory effect of Aspirin (100 mg/kg) was greater than that of the Meht.

The MEHT exhibited significant inhibition of carrageenan-induced inflammation in mice. Carrageenan-induced inflammatory process is thought to be biphasic. The primary phase observed in the 1st hr is due to the discharge of histamine and serotonin. It is overwhelmingly a non-phagocytic phase (Maity *et al.*, 1998). The second enhancing phase of inflammation (peripheral inflammation) is due to the discharge of prostaglandins (PGs), bradykinin and lysozyme (Pérez-Guerrero *et al.*, 2001). It has been reported that both medically valuable steroidal and non-steroidal anti-inflammatory agents (NSAIDs) are effective in the second phase of edema (Wan *et al.*, 2013, Saeed *et al.*, 2016).

Table 2 Carrageenan-induced mice paw edema model of anti-inflammatory activity.

Groups	Treatment	Dose/kg	Paw volume (ml)					
			NPV	After administration				
				1h	2h	3h	4h	5h
I	Saline (Control)	10 ml	0.1085±0.031	0.2286±0.033	0.2485±0.036	0.2670±0.035	0.2800±0.044	0.2917±0.048
II	Aspirin	100 mg	0.1118±0.033	0.1461**±0.044	0.1471**±0.050	0.1500**±0.051	0.1545**±0.059	0.1576**±0.062
III	MEHT	100 mg	0.1145±0.032	0.2256±0.052	0.2365±0.045	0.2427±0.043	0.2549±0.049	0.2662±0.040
IV	MEHT	200 mg	0.1019±0.030	0.1996±0.042	0.2058*±0.43	0.1936**±0.033	0.2062**±0.046	0.2193**±0.043
V	MEHT	400 mg	0.1056±0.022	0.1828**±0.026	0.1732**±0.036	0.1629**±0.039	0.1788**±0.043	0.1879**±0.048

NPV= Normal paw volume. *P<0.05 and **P<0.01 indicate statistically significant values from control (negative).

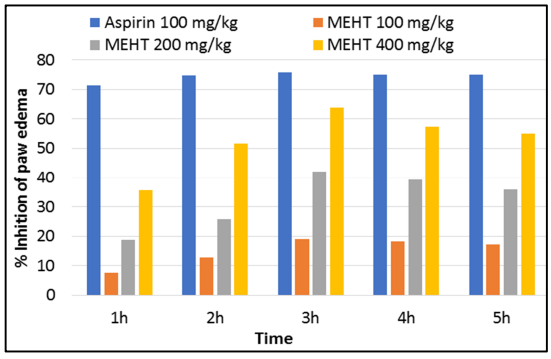

Fig. 1. Anti-inflammtory activity of MEHT.

The anti-inflammatory activity exerted by MEHT suggests that it could affect prostaglandins, and bradykinin and lysozyme synthesis as caused by NSAIDs. Percent inhibition of the extract at the dose of 400mg/kg on carrageenan-induced inflammation over period of 5 h is greater as compared to inhibition produced at a dose of 100 mg/kg or at 200mg/kg.

This shows a dose-related inhibitory effect of the extract on inflammation.

Antipyretic activity

Hyperthermia induced by yeast was prominently ($p < 0.01$) attenuated by MEHT. The inhibition was dose reliant and remained significant upto 3h of administration as reflected in Table 3. The maximum antipyretic activity was observed at 400 mg/kg i.e. 70.13% while the antipyretic activity of paracetamol was 88.71%. The percent pyrexia inhibition is presented in Fig. 2.

Table 3. Yeast induced pyrexia model of antipyretic activity.

Groups	Treatment	Dose/kg	Rectal temperature (°C)						
			Normal (X)	After administration of drug					
				After24hr (Y)	1h (T_1)	2h (T_2)	3h (T_3)	4h (T_4)	5h (T_5)
I	Saline (Control)	10 ml	37.35±0.19	39.91±0.24	39.83± 0.18	39.80± 0.17	39.72± 0.15	39.77± 0.23	39.69± 0.21
II	Paracetamol	150 mg	36.90±0.11	38.76±0.09	37.86**±0.37	387.54**±0.42	37.11**±0.44	37.17**±0.48	37.18**±0.47
III	MEHT	100 mg	36.78±0.06	38.69±0.25	38.59±0.50	38.37*±0.42	37.88**±0.34	37.94**±0.39	37.97**±0.37
IV	MEHT	200 mg	37.14±0.20	39.53±0.35	39.30±0.30	39.10*±0.32	38.44**±0.27	38.54**±0.33	38.56**±0.30
V	MEHT	400 mg	37.11±0.14	39.62±0.40	39.22*±0.28	38.80**±0.27	37.86**±0.33	37.98**±0.40	38.00**±0.39

*P<0.05 and **P<0.01 indicate statistically significant values from control (negative).

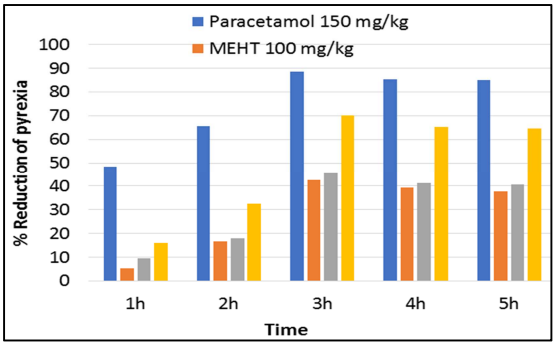

Fig. 2. Antipyretic activity of MEHT.

Brewer's yeast model for pyrexia induction is widely used for evaluation of the antipyretic activity of different substances obtained from plants or synthetic drugs, (Devi *et al.*, 2003). The injection of brewer's yeast (s.c. route) causes increase production of prostaglandins, which induces hyperthermia (Singh *et al.*, 1996). Paracetamol, the available antipyretic drugs in the market, suppress the production of PGs through suppression of cyclooxygenases (COXs) pathway. Hyperthermia is caused by a large number of substances, which act as a mediator. Antipyretic effect will produce by blocking of these mediators by different agents/drugs (Moltz, 1993). An ample decrease in rectal temperature of mice is caused by the MEHT, which shows the presence of some chemical agents, which may be capable for suppression of PGs.

Antinociceptive activity

Acetic acid induced writhing test

The results transpired that the mitigation in pain was noticed at all test doses of MEHT (100, 200 and 400mg/kg) as is reflected in Table 4. The inhibition exhibited by our extract was does reliant. At the dose level of 400mg/kg of MEHT the percent inhibition was found to be 69.56%. The percent inhibitory effect of Aspirin (86.09%) was more than that of the maximum dose of our test extract. The percent inhibition of writhing at the entire test doses is reflected in Fig. 3.

Table 4. Acetic acid induced writhing test of antinociceptive activity.

Groups	Treatment	Dose/kg	No. of writhing
I	Saline (Control)	10 ml	59.76±2.94
II	Aspirin	100 mg	8.31±1.20**
III	MEHT	100 mg	41.98±2.73**
IV	MEHT	200 mg	35.07±1.61**
V	MEHT	400 mg	18.19±1.35**

**P<0.01 indicate statistically significant values from control (negative).

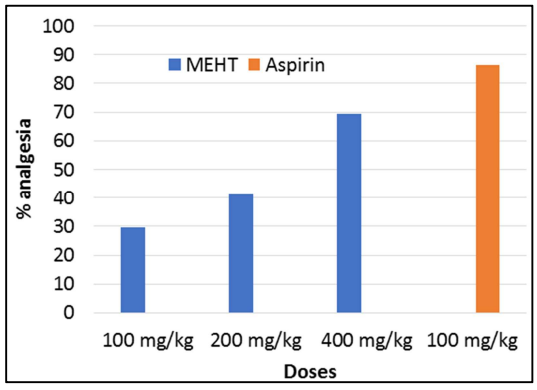

Fig. 3. Antinociceptve activity (writhing method).

For investigation of peripheral antinociceptive activity, acetic acid-induced abdominal constriction model is commonly used (Bentley *et al.*, 1981). In acetic acid-induced writhing model in mice the

MEHT exhibited antinociceptive effect. This indicates that it possesses peripheral mediated antinociceptive activity. It is believed that local peritoneal receptors are involved in abdominal constriction (Mbiantcha *et al.*, 2011). The analgesic effect of extract may be partially due to interference with these receptors. In acetic acid-caused writhing pattern, pain perception is evoked through production of localized inflammatory responses due to discharge of arachidonic acid from phospholipids tissue of abdomen mediated by COX pathway, resulted in synthesis of prostaglandins (PGE2 and PGF2α) , even though the level of lipoxygenase (LOX) derivatives may also increase in peritoneal fluids (Delevalcee and Falhout, 1980). As a result, inflammation and pain is produced by increasing capillary permeability due to these PGs and LOXs derivatives.

Inhibition of writhing results in antinociceptive effect is caused by decreased or inhibition of PGs synthesis, a peripheral mechanism of pain inhibition. The mechanism of antinociceptive activity of the extract may be linked to cyclooxygenases and/or lipoxygenases as acetic acid-induced pain model has been related with increase in the levels of PGs (PGE2 and PGF2α) and lipoxygenases derivatives in peritoneal fluid (Duarte *et al.*, 1988, Ramabadran *et al.*, 1989). The prominent inhibition of writhing reflux produced by the MEHT emphatically shows that MEHT has peripheral antinociceptive activity mediated through inhibition of local peritoneal receptors and COXs. Thus it may be considered that the prominent antinociceptive activity of the extract may be due to the intermeddling of its active agents with discharge of pain mediators.

Hot plate test

The MEHT at the dose levels of 100, 200 and 400mg/kg prominently ($p < 0.05$) increased the latency time by 16.24, 23.90 and 65.81% respectively. After 60 min the highest action was noticed as reflected in Table 5. The effect was dose reliant. The most glaring ($P < 0.01$) increase in latency time observed against 400 mg/kg of the MEHT was 65.81% while, Tramadol (the standard opioid analgesic drug) exhibited the % increase in latency time was 78.21 as reflected in Fig. 4. The antinociceptive effect of Tramadol (30 mg/kg) and extract (200 and 400mg/kg) was reversed deeply in the presence of naloxone (Fig. 5).

Table 5. Hot plate method of antinociceptive activity.

Without Naloxone							
Group	Treatment	Dose/kg	Latency time (sec)				
			0min	After treatment			
				30min	60min	90min	120min
I	Saline (Control)	10 ml	10.13± 0.04	10.21± 0.07	10.17± 0.08	10.19± 0.05	10.09± 0.12
II	Tramadol	30 mg	10.13±0.04	25.37**±0.03	25.68***±0.05	25.46***±0.09	25.27***±0.01
III	MEHT	100 mg	10.15±0.28	12.34±0.39	13.39*±0.78	13.22*±0.39	12.98*±0.29
IV	MEHT	200 mg	10.17±0.59	13.00±0.78	14.91**±0.49	14.59**±0.79	14.30**±0.85
V	MEHT	400 mg	10.18±0.72	19.93*±0.20	23.22**±0.09	22.65**±0.10	22.25**±0.63
With Naloxone							
VI	Tramadol	30 mg	10.16±0.03	11.12**±0.04	11.00***±0.08	11.16***±0.05	10.93***±0.01
VII	MEHT	200 mg	10.19±0.62	11.64**±0.73	11.62**±0.79	11.68**±0.58	11.69**±0.80
VIII	MEHT	400 mg	10.14±0.32	11.58**±0.47	11.64**±0.35	11.75**±0.79	11.79**±0.61

*P<0.05, **P<0.01 and ***P<0.001 indicate statistically significant values from control (negative).

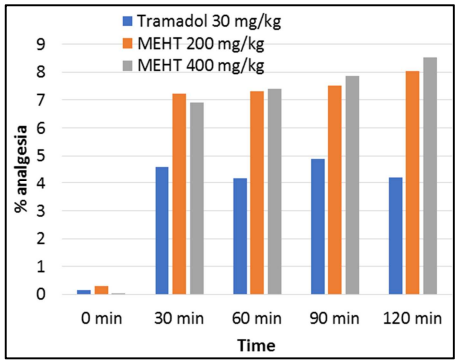

Fig. 4. Hot plate model of Antinociceptive activity (With Naloxone).

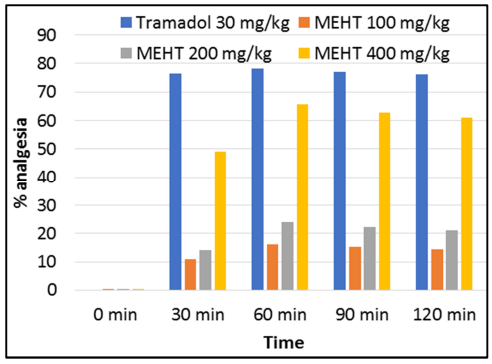

Fig. 5. Hot plate model of Antinociceptive activity (Without Naloxone).

Latency time for thermal stimulation at the test dose levels of 100, 200 and 400mg/kg of MEHT was markedly increased. It exhibited late onset of antinociceptive effects which remained after the 5th hour post-treatment similar to the effect of aspirin. The hot plate test is employed to differentiate between peripheral and central acting antinociceptive agents (Duarte *et al.*, 1988, Ramabadran *et al.*, 1989). Our findings regarding the MEHT extract are indicative of central acting antinociceptive effects. MEHT exhibits a significant inhibition of heat-induced hyperalgesia by producing prominent attenuation when compared with the control. The antinociceptive effect of the standard drug tramadol was higher than MEHT. A non-selective opioid receptor antagonist, Naloxone, reversed the antinociceptive effect of MEHT. This emphatically shows that the activation/ stimulation of opioid receptors produced the antinociceptive effect of MEHT.

Conclusion

The study in reference reveals that MEHT-caused inhibition of inflammation at first and second phase may be due to its ability to inhibit release and/or activity of substances called mediators involved in both phases of inflammation. The extract exhibited prominent antipyretic activity. The extract possesses strong antinociceptive activity involving both peripheral and central mechanisms. The peripheral activity was augmented by opioid-dependent central activity. This study provides support to its ethno-pharmacological uses. However, further studies are required to isolate the active ingredients responsible for these pharmacological activities and to investigate their mechanism of action.

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