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EVALUATION OF ANTINOCICEPTIVE ACTIVITY OF METHANOLIC EXTRACT ISOLATED FROM THE LEAVES OF *MALUS PUMILA* IN MICE

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ABSTRACT:

Background: ***Malus pumila*** is commonly known as Apple. Family, Myrtaceae, primarily cultivated in Jammu & Kashmir; Himachal Pradesh; hills of Uttar Pradesh and Uttaranchal and also cultivated to a small extent in Arunachal Pradesh; Nagaland; Punjab and Sikkim. Traditional uses of apple includes treatment for cancer, diabetes, dysentery, constipation, fever, heart ailments, scurvy, and warts.
Aim: To evaluate antinociceptive activity of methanolic extract of *Malus pumila* (MEMP)isolated from the leaves in mice.
Materials and Methods: Dried pulverized leaves of *Malus pumila* were first defatted with petroleum ether and then extracted with methanol. Antinociceptive activity of the methanolic extract was evaluated in swiss albino mice after an acute oral toxicity study as per OECD-423 guideline. Two animal model; Formalin induced paw licking and Tail immersion test were performed and MEPS was orally given at a dose of 200mg/kg and 400mg/kg of body weight in 4 groups of animals, each consists of 6 animals. Morphine 10mg/kg body weight was used as standard drug.

Results: The results obtained demonstrated that methanolic extract of *Malus pumila(MEPS)* produced significant(P<0.001) antinociceptive response in dose dependent manner by reducing the number of leaking in Formalin induced paw licking and delayed in response in nociceptive stimuli in tail immersion test.

Conclusion: MEPS having antinociceptive activity involves activation of the peripheral and central mechanisms.

Key Words: Antinociceptive activity, Pain, MEPS

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INTRODUCTION:

Pain is defined by International association for the study of pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. In humans the pain experience consists of three dimensions: sensory- discriminative, motivational-affective and cognitive-evaluative.**(1)**In a general manner, there are four types of pain: (a) nociceptive pain, due to excessive stimulation of nociceptors localized in the skin, viscera, and other organs. (b) neurogenic pain, pain reflecting damage to neuronal tissue in the periphery or CNS. (c) neuropathic pain, due to a dysfunction of, or damage to, a nerve or group of nerves. (d) psychogenic pain, not due to an identifiable, somatic origin and which may reflect psychological factors. Pain is usually elicited by the activation of specific nociceptors (nociceptive pain). However, it may also result from injury to sensory fibres or from damage to the CNS itself (neuropathic pain)**[2]**

Cutaneous injury due to heat and mechanical stimuli elicits hyperalgesia that occurs at the site of injury and is referred to primary hyperalgesia while the hyperalgesia felt in the area surrounding the injury site is referred to as secondary hyperalgesia(3,4,5).

Primary hyperalgesia due to heat stimuli is believed to be as a result of peripheral sensitization of A-delta and C nociceptors(6,7,8)**.**

Secondary hyperalgesia is due to central sensitization of neurons in the spinal cord caused by discharges of nocicieptors(9). When nociceptors are stimulated they release excitatory amino acids (EAAs) and peptides like substance P (SP), neurokinin-A, vasoactive intestinal peptide (VIP) and calcitoningene – related peptide (CGRP) in the central nervous system**.** These agents have a sensitizing effect on nociceptors and can cause hyperalgesia.

Hyperalgesia can also occur in the viscera and can exist in three forms. Visceral hyperalgesia can be a form of primary hyperalgesia which involves the site of injury; it can be caused by inflammation or excess stimulation of the visceral structures or it can be referred hyperalgesia where by the pain from the viscera is referred to somatic tissues. Viscero visceral hyperalgesia is hyperalgesia of one visceral organ that manifest clinically on another visceral organ whose segmental afferent innervations partially overlaps (10)**.**

While pain is defined as a subjective experience of noxious stimuli, the physiological and pharmacological activities that lead to a painful sensation are denominated nociception. Pain also has a physiological component which is called nociception, i.e., the process by which intense thermal, mechanical, or chemical stimuli are detected by a subpopulation of peripheral nerve fibers called nociceptors(11)**.**

The nociceptive pathway can be described as a three- neuron chain that transmits nociceptive information from the periphery to the cerebral cortex**.** The *first order neurons* have hair cell bodies in the dorsal root ganglion from where two axon sproject, one to the peripheral tissues and the other one to the dorsal horn of the spinal cord. The *second order neurons* originate from the spinal cord and ascend to the thalamus or the regions of the brainstem. From the thalamus the *third order neurons* project to the cerebrocortex**.(12)**

According to WHO, nearly 75-80% of world population still depends on herbal medicines. Active constituents from plant sources directly used as therapeutic agent and phytoconstituents are also served as lead molecule for the synthesis of various drugs.

The use of medicinal plants as analgesic and anti-inflammatory drugs in folk medicine is a practice common in many countries, although, in most cases, the active principles of the plants are unknown. However, evaluation of the pharmacological effects of the herbal crude extracts can still be used as a logical research strategy for searching of new drugs (13).

An apple is a sweet, edible fruit produced by apple tree. Apple tree are cultivated worldwide. The tree originated in Asia , Europe .In India the apple growing areas do not fall in temperate zone but the prevailing temperate climate of the region is due to the Himalayan ranges and high altitudes.

Plants of medicinal value in ethnopharmacology are an important source of natural products with potential therapeutic effects (14,15). Study of plant species that are used in traditional herbal medicine as pain killers therefore form a logical search strategy for new analgesic drugs (16). Compounds derived from medicinal extracts are appealing for several reasons; they are often stereo chemically complex, multi- or macro cyclic molecules with limited likelihood of prior chemical synthesis ,and they tend to have interesting biological properties. But perhaps most importantly, parent extracts have been "clinically" tested in their traditional milieu, in some cases over millennia(17). Various anti-nociceptive drugs are available in market that are effective but they exert side effects such as heart burn and gastric ulceretc(18).Traditional uses of apples include treatment for cancer, diabetes, dysentery, constipation, fever, heart ailments, scurvy, and warts. However, there are no clinical trials to support the use of apple for these conditions. There is increasing evidence suggesting apple consumption may be protective against cancer, particularly colorectal, lung, and possibly other types; may prevent cardiovascular disease by virtue of its beneficial effects on cardiovascular risk factors (eg, atherosclerosis, hypercholesterolemia, obesity, diabetes); and may have beneficial effects on pulmonary function, including preventing asthma. In addition, there is preliminary evidence that the antioxidant and anti-inflammatory effects of apples may provide benefits in a range of other conditions.(19). An infusion is used in the treatment of intermittent, remittent and bilious fevers. The fruit is said to dispel gas, dissolve mucous, cure flux and be a tonic for anaemia, bilious disorders and colic(20). The leaves contain up to 2.4% of an antibacterial substance called 'phloretin. This inhibits the growth of a number of gram-positive and gram-negative bacteria in as low a concentration as 30 ppm (21). However there are no established scientific reports of nociceptive activity on the plantpart chosen for the study, hence scientific data may be established for the plant for its anti-nociceptive activity.

MATERIAL AND METHODE

Collection of plant materials and authentication of plant part: Leaves of *Malus pumila* was collected from Himalaya region, (Uttarakhand) India, Herbarium of plant was prepared graciously and submitted to Department of Botany, Safia College of Science, Bhopal India, for authentication. Plants were authenticated by Dr. Zia-Ul-Hasan, Head, Department of Botany, Safia College of Science,

Bhopal, India. Plant authentication number obtained was **112/Bot/Saf/18.**Dried pulverised leaves of *Malus pumila*were placed in thimble of Soxhlet apparatus. Soxhlation was performed at 60°C using Petroleum ether (40 - 60ºC) as non polar solvent at first. Exhausted plant material (marc) was dried and then extracted with methanol.

Acute oral toxicity study (OECD 423):

The acute oral toxicity study was performed as per the guidelines of Organization for Economic Cooperation and Development (OECD; guideline 423). Nulliparous healthy female mice were used for this study.3 animals per step was selected. Dose selected 5,50,300,2000 mg/kg body weight. Immediately after administration of extract, all of the animals were observed for a total of 14 days based on established criteria, observations of behaviour patternchanges in skinand eye, respriration, tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.With special attention given during the first 4 hours, clinical signs or mortality were noted. On day 15, all of the animals were euthanized by cervical dislocation**.(22)**

***In vivo* evaluation of anti-nociceptive activity:**

Selection of animals and housing condition

Animals (Swiss Albino Mice, either sex Body weight - 25±5 gm) were selected randomly from animal house of PBRI, Bhopal, India and further divided into various treatment groups randomly and kept in propylene cage with sterile husk as bedding. Relative humidity of 30.7 % at 22 ± 2 °C and 12:12 light and dark cycle was maintained in the animal house and fed with standard pellets (Golden feeds, New Delhi, India) and water was available ad libitum. Institutional Animal Ethics Committee (IAEC) of PBRI, Bhopal has approved all animal experiments with CPCSEA **(Reg. No. 1824/PO/ERe/S/15/CPCSEA).**Protocol approval reference number is **PBRI/IAEC/PN-18013.**

Experimental Design:

A randomized design was used. The animals and the treatment were randomly assigned to an experimental unit. Each experimental unit comprised of a treated group and a control group. The experimenter was blind to the extract or negative control to be administered. In all the experiments each animal was used once.

Formalin induced paw licking (23):

Mice fasted overnight with the provision of water were used. Then the overnight fasted mice were randomly selected and assigned into group four, each group with six mice.

Group 1: receiving Distilled Water (10 ml/kg) was assigned as the control.

Group 2: mice receiving Morphine at a dose of 10 mg/kg served as positive control.

Group 3: were given the test extract *Malus pumila* at a dose of 200mg/kg.

Group4: were given the test extract *Malus pumila* at a dose of 400mg/kg.

Before starting the experiment, mice in each group were allowed 20-min acclimatization in a transparent observation cage. After acclimatization, formalin solution (0.02 mL of 5%) was administered by intraplantar injection into the mice dorsal surface of the right hind paw. Two phases of nociception, namely the early and late phase, were observed during the course of the experiment. The first phase was recorded by taking the time of the animals spent licking their paw for 0–5 min after the injection of formalin. The second phase was recorded by taking

the time the animal spent licking its paw for 15–30 min after formalin injection. The percentage inhibition of nociception for the two phases was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Control mean} - \text{Test mean}) \times 100}{\text{Control mean}}$$

Tail immersion test (24)

Mice used in this experiment were screened by dipping the lower 5 cm portion of the tail into hot water bath maintained at 55 °C ± 0.5 to induce pain and animal that failed to withdraw the tail in 10 seconds were discarded. Eligible mice were divided into four groups of six animals each. Treatment was then carried out accordingly:

Group 1: receiving Distilled Water (10 ml/kg) was assigned as the control.

Group 2: mice receiving Morphine at a dose of 10 mg/kg served as positive control.

Group 3: were given the test extract *Malus pumila* at a dose of 200mg/kg.

Group4: were given the test extract *Malus pumila* at a dose of 400mg/kg.

The time required for the animal to withdraw the tail clearly out of the water was taken as the reaction time. Reaction time was taken after oral administration of the extract at 30, 60, 90, 120, and 150 minutes. A post treatment cut-off time of 30 s was used.

Results:

Acute Oral Toxicity:

The acute oral toxicity study was carried out according to OECD 423 guidelines. Four ranges of dose were used for toxicity studies, i.e 5mg/Kg, 50 mg/Kg, 300 mg/Kg, 2000 mg/Kg. and no mortality were observed.

Table 1: Mortality Rate

SL.No	Group	Observations/Mortality
1	5mg/Kg body weight	0/3
2	50mg/Kg body weight	0/3
3	300mg/Kg body weight	0/3
4	2000mg/Kg body weight	0/3

In Vivo Anti Nociceptive Activity

Formalin induced lick test

Formalin-induced behaviour is characterised by two phases relevant to acute and tonic pain. Morphine was administered systemically before or after the early phase, and its ability to affect the late phase was investigated. Inhibitory effects of morphine injected immediately after the early phase were significantly stronger compared to the preemptive administration. It appears that some neural and/or behavioural changes during the early phase limit effects of morphine on the late phase. In both phases of the formalin test, MPME caused a dose-dependent inhibition of the licking number induced by formalin. The effect is significant ($p < 0.001$) with all of the experimental doses, where 35.66 ± 2.94 of licking in the first phase and 45.33 ± 3.54 in second phase were observed with the dose of 400 mg/kg of MPME.Similarly, the effect is significant with all of the experimental doses, where 82.5 ± 4.958 of licking in the first phase and in 132

±3.696 second phase were observed with the dose of 200 mg/kg of MPME. Therefore, Oral administration of both extracts at 200 and 400 mg/kg and standard drug caused a significant decrease in the number of licking induced by formalin in a dose-dependent manner compared to the control group

In vivo Anti Nociceptive Activity

Formalin Induced Lick test

Table 2: Formalin Induced Lick test

Treatment Group	Mean lick time(Sec)±SD	
	Early Phase	Late Phase
Control	102±4.358	240.5±26.113
Standard	28.5±3.593	37.16±2.339
MPME200	82.5±4.958	132±3.696
MPME400	35.66±2.94	45.33±3.543

Tail Immersion Test: Pre-treatment observations of tail immersion test.

Table 3: Pre-treatment observations of tail immersion test

SL NO	Treatment	Observation
1	Control	1.33±0.745
2	Standard	3.16±0.687
3	MPME200	0.816±0.816
4	MPME400	1.83±0.372

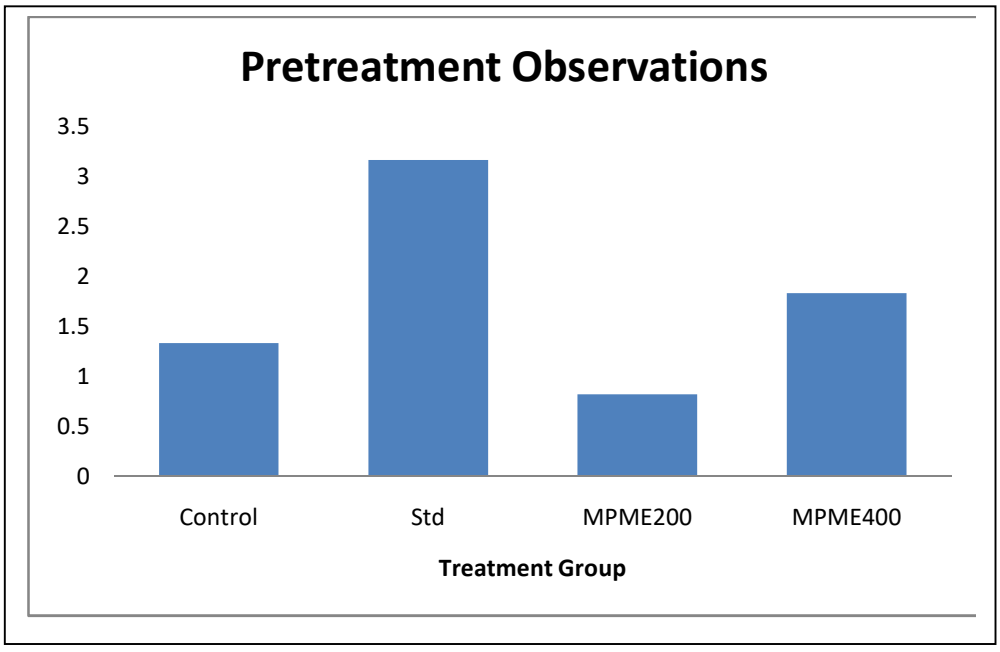

Fig 1: Pretreatment Observations

Table 4: Post treatment with leaves extract of *Malus pumila* at reaction time 60,90,120 and 150 min.

Treatment	60 min	90min	120min	150 min
Control	1.5±0.5	1.66±0.74	1.25±0.901	1.8±0.786
Standard	36.16±4.41	40.16±4.59	41.33±3.636	37.83±3.236
MPME200	8.5±0.763	15.16±2.266	20±2.081	15±1.414
MPME400	16.33±1.247	20±1.29	35.83±2.409	22.83±1.343

In the tail immersion test, MPME showed marked antinociceptive activity in a dose-dependent manner. More specifically, at 60 min after oral administration of MPME at both 200 mg/kg and 400 mg/kg doses significantly delayed the reaction time in response to a nociceptive stimulus. Morphine, the reference drug, exhibited strong antinociceptive activity as well as MPME extract in dose dependent way.

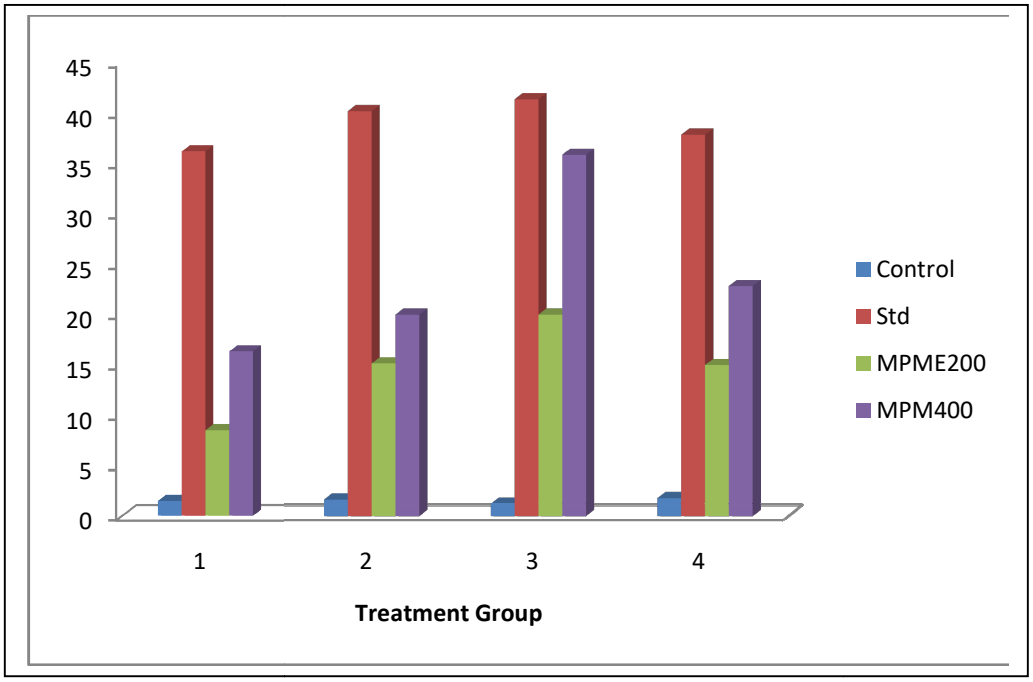

Fig 2: Activity Chart

CONCLUSION:

In the present study antinociceptive activity of methanolic extract of leaves of *Malus pumila(MEPS)* was evaluated in different experimental models of pain. The models for investigating antinociception were selected based on their capacity to investigate both centrally and peripherally mediated effects.The tail immersion method investigates the central activity, while the formalin test investigates both. In this test, our results indicate that antinociceptive activity of extracts may be due to blockade of liberation or receptors of those inflammatory

mediators. Another possible mechanism could be the blockade in the eicosanoid system by blocking cyclooxygenases (COX-1and/or COX-2).The tail flick (spinal analgesia) model in which opioid agents exert their analgesic effects via spinal and supra spinal receptors, respectively. These models are used to test the central antinociceptive activity of *Malus pumila* methanolic Extract.Experimental evidence obtained in this study suggest that the *Malus pumila* Methanolic Extract shows antinociceptive property by acting on both supra spinal and spinal receptors. The formalin test is a model constituted of two distinct phases. The first transient phase, corresponds to acute neurogenic pain, is caused by the direct effect of formalin on sensory C fibers, and the second prolonged phase is associated with the development of an inflammatory response and the release of nociceptive mediator. Substances that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the second phase.

In this test, our results show that mehanolic leaves extract of *Malus pumila* (MEMP) has antinociceptive activity. Although the extracts MEMP contains several chemical constituents, further studies must be carried out to investigate the exact chemical substance present in the MPME that exerts antinociceptive activity.In Conclusion, this study has shown that MPME has antinociceptive activities and these results support the traditional use of MPME in some painful conditions. The mechanism through which MPME exerts antinociceptive activity seems to be mediated, at least in part, by acting on opioid receptors and not by acting on cholinergic receptors.

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