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Antioxidant and Antinociceptive Activity of Methanol Extract of Leaves of *Malus Pumila* Mill in Mice

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ABSTRACT

Conventional medications are expensive and arguably associated with various severe adverse effects, hence the need to develop herbal agents that are effective as alternative. Apple (*Malus pumila* Mill) is the fruit of malus plant. It belongs to the family rosaceae and has been widely cultivated in various parts of the world for centuries. It has a beneficial effect on cardiovascular disease, cancer, pulmonary function and age-related cognitive decline. The leaves of is *M. pumila* rich in resources, but lack of medicinal value research. Chemical constituents of the same family of *M. pumila* have many activities. This study evaluated the antioxidant and antinociceptive effect of the methanolic extract of *M. pumila* leaves in mice. Qualitative phytochemical screening of methanolic extract was carried out to identify the phytoconstituents. The *In vitro* antioxidant activity of methanolic extracts of *M. pumila* leaves was assessed against hydrogen peroxide scavenging assay using standard protocols. The antinociceptive activity of methanolic extracts was investigated in thermal-induced (tail immersion) and chemical-induced (formalin) nociception models in mice at two different doses (200 and 400 mg/kg; p.o.). Morphine sulphate (10mg/kg, i.p.) was used as reference analgesic agents. *M. pumila* extract demonstrated potent and dose-dependent antinociceptive activity in the chemical and heat induced mice models ($p < 0.001$). The findings of this study indicate that the involvement of both peripheral and central antinociceptive mechanisms. Further, the phytochemical screening results showed that the extract had flavonoids, steroids, saponins, phenolics and terpenoids which have been associated with anti-nociceptive activities. Therefore, the study has established that the methanolic extracts of *M. pumila* are effective in the management of pain and support the traditional use of this plant in different painful conditions.

Keywords: *Malus pumila* Mill, Rosaceae, Antioxidant activity, Phytochemical screening, Anti-nociceptive activity

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INTRODUCTION

Pain is an essential sensation that plays a vital role as a body's natural defence system by alerting us to possible tissue injury while nociception is described as the neural processes of encoding and processing noxious stimuli that usually leads to pain. The process mentioned above is initiated by specialized peripheral sensory neurons (nociceptors) that are activated by noxious stimuli (i.e., mechanical, thermal, and chemical stimuli) due to tissue injury and damage, and these nociceptors are usually found in the cutaneous tissues, bone, muscle, connective tissues, vessels and viscera. These stimuli are transduced into electrical impulses (action potentials) that are transmitted predominantly through Aδ- and C-fibre nociceptors (primary afferent neurons) into the dorsal horn of the spinal cord[1]. A variety of excitatory neurotransmitters are released by the primary afferent neurons, such as excitatory amino acids, protons, peptides, lipids and cytokines, and others, which act on their specific receptors and ion channels, to activate the second order neurons of the spinal dorsal horn. Once activated, the action potentials are then ascended to the thalamus and cerebral cortex through spinothalamic or other tracts that lead to perception of pain. Pain can be modulated by various analgesic drugs that suppress pain signals by acting in various mechanisms on the peripheral (PNS) and central nervous system[2]. However, various adverse effects have been reported with these agents. For instance, nonsteroidal anti-inflammatory drugs may cause gastrointestinal irritation and/or bleeding, decreased platelet aggregation (leads to prolong bleeding time), kidney damage, edema, bone marrow suppression, rashes, as well as anorexia. Opioids, on the other hand, may also lead to constipation, dizziness, nausea, respiratory depression, sedation, vomiting, as well as physical dependence and tolerance with the most being constipation and nausea. Hence, there is an imperative need to discover new therapies that are more effective and safe with lesser or no

side effects. For instance, natural product-based medications, particularly plant-derived, are believed to be a valuable source of chemical substances that promise to have a good potential therapeutic applicability[3]. Apple (*Malus pumila* Mill) is the fruit of malus plant. It belongs to the family Rosaceae and has been widely cultivated in various parts of the world for centuries. At present, the annual global apple production is about 70 million tons, thus making apple the third largest consumed fruit next to bananas and oranges[4]. Apples contain a relatively high concentration of polyphenolics and their consumption has been linked with improved health due to their effectiveness in several chronic diseases[5, 6]. Many review articles are available on apple products to claim beneficial effects on cardiovascular disease, cancer, pulmonary function and age-related cognitive decline[7,8]. Apples are composed of different tissue types (peel, cortex, core and seed) and each tissue type contains a different composition of phytochemicals[9]. Unpeeled fruits possess higher contents of bioactive compounds as compared to peel ones[10]. Moreover, the polyphenolic content of apple peel extract is six times higher than that of the fresh extract. The apple fruit pulp contains mainly catechin, phloretin glycoside, procyanidins and caffeic acid whereas the peel possesses all these compounds and has flavonoids such as anthocyanins, quercetin glycosides and cyanidin glycoside in addition, which are absent in the pulp[11]. There are reports of beneficial effects of apple peel phytochemicals against a variety of experimentally induced pathological conditions[12]. It is reported to have an inhibitory effect on low density lipoprotein (LDL) oxidation[13], anti-proliferative property[14], depletion of reactive oxygen species (ROS) generation during stress conditions[15], antihypertensive activity[16], a-glucosidase inhibitory property[17] and protective property against damaged mitochondria and DNA[18]. Apple peel has also been reported to regulate metastasis[19]. The oral administration of apple extracts has been shown to inhibit AP-1 transactivation which involves signal transduction of MAP kinase, thus inhibiting cancer formation[20]. In addition, the intake of apple polyphenols is inversely proportional to coronary atherosclerosis by inhibition of lipid peroxidation[21]. This study was aimed at bioscreening the methanolic leaf extracts of *M. pumila* for antioxidant and antinociceptive activity in mice models, as a preliminary step towards development of a more efficacious plant-derived antinociceptive agent.

MATERIALS AND METHODS

Plant material

Leaves of *M. pumila* were collected from Himalaya region (Uttarakhand), India. The sample was identified by senior Botanist Dr. Zia-Ul-Hassan, Professor and head department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *M. pumila is* 112/Bot/Saf/18.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction

Dried pulverized leaves of *M. 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. For each solvent, soxhlation was continued till no colour was observed in siphon tube. For confirmation of exhausted plant marc (i.e. completion of extraction), colorless solvent was collected from siphon tube and completion of extraction was confirmed by absence of any residual solvent, The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts[22].

Phytochemical screening

The crude methanolic extract of *M. pumila* was qualitatively tested for the detection of alkaloids, flavonoids, saponins, tannins, glycosides, carbohydrates, reducing sugars, proteins, glucosides, terpenoids, and steroids following standard procedures[23].

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging potential of the plant extract was determined using the method described by Jayaprakasha et al[24] with little modification. A solution of hydrogen peroxide (20mM) was prepared in phosphate buffer saline (PBS, pH 7.4). Different concentrations of the extract (20to100µg/ml) in water (1ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min the absorbance was measured at 230 nm against a blank solution that contained hydrogen peroxide solution without the extract. Ascorbic acid was used as positive control. The percentage of H_2O_2 scavenging of the plant extract was calculated as follows:

% scavenged [H_2O_2] =

[(Abs control – Abs sample) / Abs control] × 100

Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee. All animals were procured and housed in animal house maintained under standard hygienic conditions. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No. PBRI/IAEC/PN- 1803.

Acute oral toxicity

Acute toxicity study of the prepared leaves extracts of *M. pumila* was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423[25] the animals were fasted for 4 h, but allowed free access to water throughout. Nulliparous healthy female mice were used for this study. 3 animals per step were 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 behavior pattern changes in skin and eye, respiration, 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. Acute toxicity was determined as per reported method[26].

Antinociceptive activity test

Formalin test

The method was used as narrating by Santos and Calixto and Santos et al [27,28] with minor modification. The animals were arranged into four groups (n = 6). The control group received deionized water orally at the volume of 0.1 ml/mouse 30 min before the experiments. Twenty microliters of 2.5% formalin (in deionized water, subplantar) was injected subcutaneously into the right hind paw 1 h after *M. pumila* extract treatment (200 and 400 mg/kg, p.o.) and 15 min after injection of Morphine (10 mg/kg, i.p.) of the mice. The time spent licking and biting the injected paw was measured as an indicator of pain response. Responses were measured for 5 min subsequent to formalin injection (first phase, neurogenic) and 15-30 min after formalin injection (second phase, inflammatory). Antinociceptive activity was calculated as the percentage inhibition of licking time.

Tail immersion test

The tail immersion test is based upon the observation that morphine-like drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice. This method was used to evaluate the central mechanism of analgesic activity. Here, the painful reactions in animals were produced by the thermal incentive that is dipping by the tip of the tail in hot water[29]. Mice were divided into four groups consisting of six mice in each group. According to the procedure, 5 cm of the tail of mice pretreated with morphine (10 mg/kg, i.p.) or *M. pumila* extract (200 and 400 mg/kg, p.o.) were immersed in warm water kept constant at 54 ±1°C. The latency between tail submersion and deflection of the tail was recorded. A latency period of the 20s was maintained to avoid tail tissue damage in mice. The latency period of the tail-withdrawal response was taken as the indicator of antinociception and was determined at 0, 30, 60, 90, and 120 min after the administration of the morphine and *M. pumila* extract.

RESULTS AND DISCUSSION

The crude extracts so obtained after the soxhlation process, extract were further concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield calculated by the formula was found to be 0.41 % (by petroleum ether) and 9.43 % (by methanol). Phytochemical analysis of methanolic extract of *M. pumila* leaves showed the presence of carbohydrate, flavonoids, phenolics, tannin, saponins, triterpenoids Table 1.

Table1 Phytochemical analysis of methanolic leaves extract of *M. pumila*

S. No.	Constituents	*M. pumila*
1.	**Alkaloids** Hager's test	 -ve
2.	**Flavonoids** Lead acetate Alkaline test	 +ve +ve
3.	**Phenolics** $FeCl_3$	 +ve
4.	**Proteins and amino acids** Xanthoproteic test	 +ve
5.	**Carbohydrates** Fehling's test	 +ve
6.	**Saponins** Foam test	 -ve
7.	**Diterpins** Copper acetate test	 +ve
8.	**Glycosides** Legal's test	 +ve
9.	**Tannin and phenolic** Lead acetate test	 +ve
10	**Carbohydrates** Fehling's test	 +ve

As shown in Table 2, *M. pumila* leaf extract demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 17.95, 38.06µg/ml. Scavenging activity of H_2O_2 by the extract may be attributed to their phenolics, which can donate electrons to H_2O_2 thereby neutralizing it into water[30].

Table 2 % Inhibition of ascorbic acid and methanolic extract of *M. pumila* using hydrogen peroxide scavenging assay method

S. No.	Concentration (µg/ml)	Ascorbic acid % Inhibition	*M. pumila* % Inhibition
1.	20	51.47808	44.34251
2.	40	56.88073	49.43935
3.	60	62.69113	58.10398
4.	80	70.33639	64.8318
5.	100	77.47197	69.72477
	IC_{50}	17.95	38.06

Acute oral toxicity was calculated at four different concentrations 5mg/kg, 50mg/kg, 300 mg/kg and 2000 mg/kg. Observations were performed in groups of three and no mortality was observed Table 3.

Table 3 Acute oral toxicity

S. No.	Groups	Observations/Mortality
1.	5 mg/kg Bodyweight	0/3
2.	50 mg/kg Bodyweight	0/3
3.	300 mg/kg Bodyweight	0/3
4.	2000 mg/kg Bodyweight	0/3

The acute toxicity results showed that methanolic extracts of *M. pumila* was safe up to a dose of 2000 mg/kg body weight. Based on acute toxicity data, two different dosages 200 and 400 mg/kg (p.o.) were selected for in vivo anti-nociceptive activity. *M. pumila* leaf extract produced a dose-related inhibition of formalin induced nociception and caused significant inhibition of both neurogenic (0-5 min) and inflammatory (15-30 min) phases of formalin-induced licking test at the doses of 200 and 400 mg/kg when compared with control group (Deionized water) (Table 4). However, its antinociceptive effect was more pronounced in the second phase of this model of pain. Morphine (10 mg/kg, i.p.) significantly reduced formalin induced nociception in both phases ($p < 0.001$). In formalin-induced paw licking test *M. pumila* has shown the ability to affect both the early and late phase inflammatory effects of the formalin test, which implies the involvement of not only the central mechanism but also the peripheral antinociceptive activity of the extract. The early phase, classified as neurogenic pain, is an acute response observed immediately after the administration of formalin and is due to direct action of injected formalin on nociceptors. While the late phase, classified as an inflammatory pain, is a late response resulting from the inflammatory processes generated by the release of inflammatory mediators such as histamine, serotonin, prostaglandins and bradykinin, and activation of the neurons in the dorsal horns of the spinal cord[31]. Both phases have their own characteristics that can be used as tool to assess the antinociceptive potential as well as to elucidate the mechanisms of antinociception. The early phase represents a direct irritant effect of formalin on sensory fibers, while the late phase represents response secondary to the development of inflammatory process and the release of inflammatory mediators[32]. It has been reported that drugs acting centrally (i.e. narcotics/opioids) inhibit both phases of the formalin test while those acting peripherally (i.e. NSAIDs) inhibit only the late phase, respectively[33,34]. Therefore, the results shown by *M. pumila* suggest that the extract contains bioactive compound(s) with central and peripheral antinociceptive actions and additional anti-inflammatory activity[31]. The ability of *M. pumila* to inhibit chemically- and thermally-induced nociceptive processes tested in this study presents its potential to be used as an analgesic agent. The tail-immersion test results asserted significant antinociceptive effect ($p < 0.001$) compared with control, at the doses of 200 and 400mg/kg. The antinociceptive effect of 200 and 400 mg/kg of *M. pumila* leaf extract were comparable to that of the reference drug (Table 5). A significant antinociceptive effect was produced by morphine ($p < 0.001$) when compared with control group (Deionized water). Tail immersion model is considered as an acute pain model. The tail-withdrawal response of mice is predominantly considered to be selective for centrally acting analgesics, whereas the peripherally acting drugs are known to be inactive on such heat-induced pain response[35]. The significant increase ($p<0.05$) in tail-withdrawal time by the extract suggests the involvement of central mechanisms in its antinociceptive effect. Tail immersion monitors a spinal reflex involving μ2- and δ-opioid receptors[36]. Therefore, the results of the present study indicate that the central antinociceptive effect of *M. pumila* may be prominent on μ-opioid receptors.

Table 4 Antinociceptive effect of *M. pumila* extract and morphine in formalin-induced paw licking test

S.N0.	Treatment groups	Mean lick time (sec) ± SD	
		Early Phase	Late Phase
1	**Control**	102 ± 4.358	240.5±26.113
2	**Morphine**	28.5 ± 3.593	37.16±2.339
3	**Extract 200**	82.5 ± 4.958	132 ±3.696
4	**Extract 400**	35.66 ± 2.94	45.33±3.543

Table 5 Antinociceptive effect of *M. pumila* extract and morphine in tail immersion test

S.N0.	Treatment groups	Latency period (s) (% MPE) Pretreatment	60 min	90 min	120min	150 min
1	**Control**	1.33± 0.74	1.5± 0.5	1.66±0.74	1.25±0.901	1.8±0.786
2	**Morphine**	3.16±0.68	36.16± 4.41	40.16±4.59	41.33± 3.63	37.83± 3.23
3	**Extract 200**	0.816±0.81	8.5 ± 0.763	15.16± 2.26	20 ± 2.08	15± 1.41
4	**Extract 400**	1.83±0.37	16.33± 1.24	20.0 ± 1.29	35.83± 2.40	22.83± 1.34

CONCLUSIONS

M. Pumila has recently received some attention for its beneficial effects against several diseases. Our research study showed that the use of *M. Pumila* could effectively reduce the severity of pain. It can be concluded that *M. Pumila* possesses significant antinociceptive activity in both chemical and heat induced pain models in mice. The antinociceptive effect of *M. Pumila* is most likely mediated via inhibition of peripheral mediators and central inhibitory mechanisms. These results support the traditional use of this plant in different painful conditions. Further investigations are required to perceive the mechanisms of action of *M. Pumila* extract and to identify the active constituents that may be used as a lead compound for new drug development.

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