Journal of Natural Remedies
DOI: 10.18311/jnr/2026/54461

INFORMATICS JOURNALS
Research Article
eISSN: 2320-3358 | Vol 26(5), 2026 | pp. 1303-1314

Safety Assessment and Analgesic Anti-inflammatory Efficacy of *Machilus macrantha* Root Extract: *In Vitro* and *In Vivo* Studies

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Abstract

Background: In the management of pain and inflammation, the use of chemically derived anti-inflammatory and analgesics has limitations, which supports the use of plant-based phytoconstituents. In *Ayurveda*, the metabolites of *Machilus macrantha* root extract are less pharmacologically explored. **Aim:** Evaluate the phytochemicals, antioxidant, anti-inflammatory, and analgesic effects of aqueous root extract of *M. macrantha* by *in vitro/in vivo* models to validate traditional uses and assess it as a safer natural alternative. **Objectives:** Phytochemical screening, *in vitro* antioxidant assays, *in vivo* anti-inflammatory (carrageenan oedema), *in vivo* analgesic (writhing/hot plate) and therapeutic efficacy. **Methods:** The aqueous root extract of *M. macrantha* was evaluated for qualitative phytochemical tests and antioxidant potential. The screening of anti-inflammatories using an *in vitro* denaturation assay and carrageenan paw oedema in rats (180-200 g body weight) with a dose of 100, 200 and 400 mg/kg p.o. diclofenac sodium 10 mg/kg reference. The analgesic efficacy was assessed by acetic acid writhing and the hot plate method in rats (n=6), and statistical analysis was performed by ANOVA followed by Dunnett's test with a significance of p-value $p<0.05$. **Results:** The phytochemical investigations reveal the presence of good phenolic content in the aqueous root extract of *M. macrantha*, and the availability of good phytoconstituents. No observation of clinical signs of toxicity at a dose of 2000mg/kg body weight, with stronger H_2O_2 scavenging activity of the aqueous extract at 100µg/ml, was found to be good, with 54.77% and 62.83% at 100 in the reducing power assay. The maximum decrease in protein denaturation (86.38 ± 2.31%) was observed at 100 µg/ml concentration, with the highest writhing inhibition of 33.42% at a 400mg/kg dose of root extract than another dose. The analgesiometer hot plate animal latency was significantly higher at the 400mg/kg dose than at lower doses. **Conclusions:** The notable phytoconstituents in the root extract of *M. macrantha* include stigmasterol, β-sitosterol, Machigline and Machiline, flavonoids, and polyphenols, known to prevent activation of the cycloxygenase-2 enzyme responsible for analgesic and anti-inflammatory properties.

Major Findings: The aqueous root extract of *M. macrantha* aqueous root extract demonstrated significant antioxidant, analgesic, and anti-inflammatory activities, attributed to the presence of phytoconstituents. The extract showed strong H_2O_2 scavenging activity, maximum inhibition of protein denaturation (86.38 ± 2.31%), significant reduction in carrageenan-induced paw oedema, and dose-dependent analgesic activity in hot plate and writhing models, without producing toxicity at 2000 mg/kg.

Keywords: Analgesic and Anti-inflammatory, *In Vivo* and *In Vitro* Model, *Machilus macrantha* Root Extract, Phytoconstituents, Protein Denaturation

Abbreviation: STD: Standard, MMAE: *Machilus macrantha* Aqueous Extract, AEME: *Machilus macrantha* Methanolic Extract, AECE: *Machilus macrantha* Chloroform Extract, AEEE: *Machilus macrantha* Ether Extract, %INH: Percentage Inhibition, ABS: Absorbance, PV: Paw Volume.

(Article Chronicle: Article Received on: 20.01.2026; Revised on: 16.03.2026; Accepted on: 15.04.2026)

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1. Introduction

Inflammation is a crucial physiological protective response of tissues of the vessel to detrimental effects, including pathogenic microbes, damaged cells, chemicals, and tissue irritants[1]. Inflammation has an important role in the pathophysiological process for a variety of illnesses, including tissue inflammation, the development of cancer, autoimmune disorders, diseases of the cardiovascular and neurological systems, which can also be exacerbated by inflammation[2]. The minimization of the release of inflammatory mediators, tissue irritants, and making the favourable conditions for body tissue healing, by inactivating or inhibiting the invasive organisms and factors. The blood cells that migrate are attracted to and accumulate in tissue by releasing chemical mediators, known as chemoattractants[3]. A flush, flare, and wheal with the physiological processes comprise the triple reaction of inflammation. Increased permeability in vascular tissues, physiological alterations in blood flow, and leucocyte activation and migration with the generation of Reactive Oxygen Species (ROS) and inflammatory agents, such as PGs, LTs, and Platelet-Activating Factors (PAF), triggered by phospholipase A2, cyclooxygenases, and lipoxygenases, are all part of the inflammatory process[4]. Atherosclerotic plaques, artery diseases, diabetes mellitus, obesity, cancer, and other inflammatory disorders are just a few of the many illnesses that are linked to inflammation. Macrophages become activated during inflammation when they detect molecules associated with pathogens (PAMPs) or Damage-related Molecular Patterns (DAMPs). Nitric Oxide (NO) and early inflammatory mediators such as cytokines, IL-1, IL-6, and TNF-α, as well as inducible nitric oxide synthase (iNOS), are produced when the transcription factor NF-κB is activated[5]. Physiological pain is a reaction to unpleasant stimuli by pathophysiology in living tissues. The physiology of pain has developed into a complicated process that is challenging to explain. Generally speaking, pain is an unpleasant emotional and sensory experience that may cause inflammation and tissue damage. Noxious stimuli like chemical irritation, mechanical harm, or excessively high or low temperatures can elicit nociceptive pain, also known as intrinsic pain. Neuropathic pain is also brought on by injury to nerve tissue in both nervous systems[6].

The use of herbal-based phytoconstituents in the prevention and minimisation of inflammation and pain is an effective and safer approach compared to the chemical-based system of medicine. The herbal plant *M. macrantha* (Nees.) of the Lauraceae family is a big and tallest tree that appears in tropical areas of Maharashtra, Karnataka, Tamil Nadu and Kerala, along with in the hills of the Himalayas in the parts of Sikkim, Arunachal Pradesh, Uttarakhand, and the Western and Eastern Ghats[7]. *M. macrantha* is an indigenous large-flowered bay tree, commonly known as Indian Persia, native to India. Their worldwide geographical distribution ranges from the Western Peninsula of India, Sri Lanka, Myanmar, and China (Western Yunnan)[8]. This herbal plant is considered an *Ayurveda*-based remedy for treating rheumatoid arthritis, tuberculosis, and respiratory diseases. The role of secondary phytoconstituents is to have cooling effects and is useful in burning sensations of the body and painful micturition. In India, it is commonly found in the moist and dense forests of the Western Ghats and the coastal parts[9]. The plant of *M. macrantha* is widely used in Asian ethno-pharmacology for medicinal benefits, including rheumatism, asthma, analgesic and anti-inflammatory, antioxidant, and antimicrobial roles. The preliminary phytochemical screening of aqueous root extract of the plant reveals the presence of secondary phytoconstituents like phenols, flavonoids, tannins, alkaloids, steroids, carbohydrates, and saponins, which provide a basis for a notable role in minimising inflammation and preventing pain[7]. The variety of medicinal plants from the Lauraceae family has been widely screened and reported in previous studies for their insecticidal and anthelmintic properties (Figure 1). However, the further benefits of *M. macrantha*, roots remain underexplored, particularly its phytoconstituents from the root and stem parts of the herbal plant[10]. This research work explored therapeutic benefits and pharmacological profile of the unexplored root part of the herb *M. macrantha* for the screening of analgesic and anti-inflammatory efficacy for the prevention and minimisation of biological pain stimuli[11].

2. Materials and Methods

2.1 Drugs, Chemicals and Reagents

The drug diclofenac sodium was used as a standard in the experimentation; other chemicals and reagents

were used for this research study of analytical grade. The organic solvents used for extraction processes were of analytical grade and purchased from Sisco Research Laboratory, India. The diclofenac sodium was supplied by Amoli Organics Pvt. Ltd, Batch no. DS/2102/0080A. The inflammation was induced by using chemical carrageenan (Sigma Aldrich, Germany), which was obtained as a gift sample from Rajashri Shahu College of Pharmacy, Buldhana, Maharashtra, India.

2.2 Collection of Plants and Authentication

The plant *M. macrantha* root part was collected from the Konkan region of Maharashtra, India, in the month of November 2022. The roots were collected and cleaned using running water. Then, after the roots were shade-dried, and stored in a dry place for the research study. The plant *M. macrantha* root part was authenticated by taxonomist Dr K. Madhava Chetty (IAAT:337), Assistant Professor, Department of Botany, Shri Venkateshwara University, Tirupati, India. A plant specimen was issued with a voucher number (0596) and filed for further use.

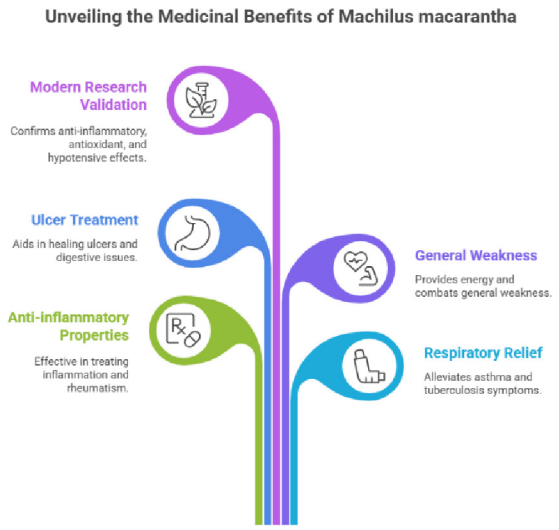


Figure 1. Medicinal importance and uses of *Machilus macrantha*.

2.3 Preparation of Plant Extracts

The coarse powder of *M. macrantha* root, 300g, was extracted by the Soxhlet extraction for 72 hours at a temperature of 65-70 °C to get the complete extract[12]. The plant extract was collected, filtered, and dried using a rotating evaporator (Figure 2). The collected extract was kept in a hot air oven at 40°C for drying. After drying, the % yield was calculated and stored in a container (air-tight) for further study[13].

2.4 Phytochemical Screening

Identifying bioactive phytocomponents with therapeutic potential, such as antioxidant properties, pain-relieving, antimicrobial, anti-inflammatory, liver-protective, and cytotoxic properties, is made easier by phytochemical analysis of plant roots. Among all the extracts, an aqueous extract of *M. macrantha* root shows the presence of bioactive phytocomponents like alkaloids, flavonoids, phenols, terpenoids, tannins, and steroids[8,10].

2.5 Phenolic Content (PC)

The phenolic content of M. *macrantha* Aqueous Extract (MMAE) was calculated by the Folin–Ciocalteu colorimetric method described by Singleton and Rossi with slight variations[14]. The root extracts were reacted

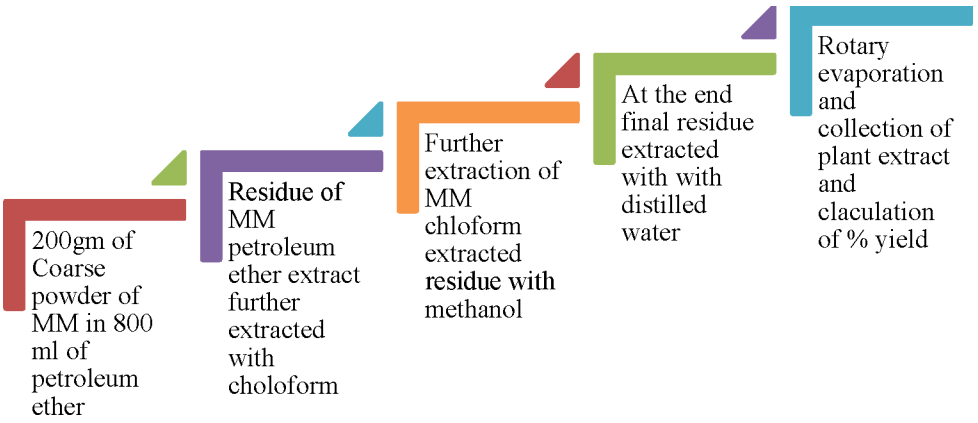


Figure 2. Stepwise extraction and collection of extractive residues by the Soxhlet extraction.

with the reagent of Folin–Ciocalteu and Na_2CO_3, which turned into colour, and the absorbance values of the extract solution were observed. The absorbance of the *M. macrantha* root extract and the standard gallic acid was analysed for phenolic content of the root extract using a standard calibration curve[14]. Gallic Acid (GA) equivalents (mg GAE) per hundred grams of sample (mg GAE/100 g sample) were used to express the overall phenolic content of the plant extract[15].

2.6 *In vitro* Antioxidants Assay[16]

2.6.1 H_2O_2 Scavenging Assay

The H_2O_2 scavenging activity of MMAE was determined by measuring the reduction of H_2O_2[17]. The volume was made up to 2 mL using 50 mM sodium phosphate buffer (pH 7.4) and 0.4 mL of test solution (MMAE), which includes 0.6 mL of 40 mM H_2O_2 solution. After 40 minutes of incubating at 30°C, the absorbance at 230 nm was measured[18]. The % inhibition of the H_2O_2 assay was calculated as follows.

Percentage (%) of H_2O_2 radical scavenging assay = $[(A_0\text{-}A_1)/A_0]$ x 100.

where A_0: control absorbance, and A_1: test sample absorbance

2.6.2 Reducing Power Assay

The assay of *M. macrantha* aqueous extracts (MMAE) and ascorbic acid was completed in accordance with the protocol outlined by the Oyaizu method[19]. An identical volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide (2.5 ml) was added to concentrations of MMAE ranging from 10 to 100 µg/ml. Following a 20-minute incubation period at 50°C, 2.5 ml of 1% trichloroacetic acid (1%) was incorporated, and the resulting solution was centrifuged at 5000 rpm for 10 minutes. After adding 0.1% FeCl3 to distilled water, the solution's absorbance at 700 nm was observed. Increased reducing power was shown by a high absorbance reading in the solution[20].

2.7 *In Vitro* Anti-inflammatory Assay

2.7.1 Protein Denaturation Assay (Egg Albumin)

The MMAE were assessed for *in vitro* anti-inflammatory property with the use of 0.2 mL of freshly extracted albumin from eggs, 2.8 mL of Phosphate-Buffered Solution (PBS) with a pH of 6.4, and 0.6 mL of plant extract in varying concentrations diluted in a 0.2% DMSO solvent, which makes up the aqueous extract solutions utilised in this experiment[21]. The reaction solution included MMAE concentrations from 10 to 100 µg/mL. The egg albumin denatured process was started by heating the sample mixtures in a water bath at 70°C for 20 minutes after incubating at 37°C for 10 minutes. The solution mixture was cooled before the absorbance at 660 nm was obtained[22]. 2.8 mL of phosphate buffer, 0.6 mL of 0.2% DMSO, and 0.2 mL of recently extracted egg albumin made up the experiment's control sample. Standard diclofenac sodium was employed as a standard reference in the present investigation[23]. The following formula was used to calculate the percentage inhibition of egg albumin (protein) denaturation, which represents the anti-inflammatory action of MMAE root extract:

% Inhibition = (As/Ac -1) × 100 (As = absorbance of sample, Ac = absorbance of control).

2.8 Pharmacological Screening

2.8.1 IAEC Approval

This research protocol of a preclinical study was reviewed and approved by the IAEC of Loknete Dr J. D. Pawar College of Pharmacy, Manur, Kalwan, constituted as per guidelines of CCSEA, Government of India, with CCSEA registration number no. reg.1929/PO/Re/S/16/CPCSEA. The protocol follows the CCSEA and OECD guidelines[24] for the entire preclinical research work. This animal study protocol was sanctioned and approved by IAEC with protocol number LNJDP/IAEC/2024-25/02 for the research study.

2.8.2 Grouping and Housing of Experimental Animals

Healthy young (4–6-week-old) Wistar strain rats weighing between 180 and 200g, respectively, were used for the experimental study. The animals were obtained from a registered breeder and supplier, LACSMI BIOFARMS Pvt. Ltd., Pasaydan, Survey No. 28/3/21 Samarth Colony, Vishal Nagar, Jagtap Dairy, Pimple Nilakh, Pune 411027 Maharashtra, India. The animals were kept in the animal house facility of Loknete Dr J. D. Pawar College of Pharmacy, Manur, Kalwan, Dist. Nashik. All rats were housed with 12 12-hour day and a 12-hour dark cycle, with a temperature of 22-25 °C and a humidity of 54 ± 2 % under standard laboratory

conditions. All the animals were kept in well-ventilated polypropylene cages and provided with standard diet pellets and free access to water. The research study was conducted as per the IAEC and CCSEA guidelines[24].

2.8.3 Toxicity Study[25]

The acute toxicity of the plant extract was determined as per OECD guideline 425[26]. The random selection of female rats (n=3) was preferred as female rats are more sensitive than males for the evaluation of a toxicity study. The animals were kept fasting with restricted food for 12 h before the administration of the experimental drug dose. The *M. macrantha* aqueous root extract at a dose of 2000 mg/kg was administered orally to the test group animals[27]. The animals were observed for signs of clinical toxicity and other clinical signs were observed for 30 mins, 1 hr, 3hrs, 6 hrs, 24 hours and continued for 48 hrs, and consecutively for 14 days. The toxicity signs observed showed no clinical signs of toxicity in the animals, no deaths (mortality), and the selected dose of plant root extract was considered a safer dose for the preclinical study.

2.8.4 Grouping and Dosing of Animals[28]

The young healthy albino rats (males and females) were randomly selected and divided into five groups, each group of six rats.

- Negative control: Normal distilled water, orally
- Positive control: 10mg/kg body weight standard diclofenac sodium
- Test group MMAE 100mg/kg body weight
- Test group MMAE: 200mg/kg body weight
- Test group MMAE: 400mg/kg body weight
- These doses are appropriate for the investigation, according to the basic tests. An identical amount of physiological saline was given to the untreated group.

2.9 Analgesic Activity

2.9.1 Analgesiometer Model[29]

The analgesic benefits of MMAE were evaluated using Eddy's hot plate method; the animals were procured in the laboratory for study and kept on a metallic plate (Hot Plate) at a temperature of 55±1 °C. The rats' responses on the hot plate, observed for paw licking, jumping from the plate, and escape response, were key elements that were considered as a reaction time. The animals were fasted overnight (12 h) before the study and were administered the aqueous extract, vehicle, or standard drug (diclofenac sodium) as per the grouping of animals[25]. Each animal (rat) was placed on an analgesiometer with a cut-off time of 15 sec to prevent any paw injuries[30]. The animal's response time was observed, and the time for paw licking and jump response from the hot plate. The reaction times were measured at intervals of 0, 30, 60, 90, and 120 minutes, and the % increase in reaction time after administrations of test drugs was calculated using the given formula.

% Increase Reaction time = (latency (test) – latency (control)) / (latency (test)) × 100

2.9.2 Writhing Test[30]

The writhing due to the acetic acid test method was performed to assess the analgesic effectiveness of the aqueous extract of *M. macrantha* root. The randomly selected animals of each group, with 6 rats given plant extract (100, 200, and 400 mg/kg b.w. orally) and control treatments at selected doses. The standard drug was given as diclofenac sodium (10 mg/kg, orally) to the standard group of rats. A post-30-minute administration of the test drug and i.p. injection of acetic acid (0.7%, 10 ml/kg) was observed for contractions or writhing movements of the stomach. The stomach contractions or number of writhings were counted at a 5-minute interval for each group for 10 minutes[31]. The number of abdominal contractions (writhes) noted for the test groups and the standard drug group at different doses was counted and compared with the control group of experimental animals.

2.9.3 Anti-inflammatory Activity

2.9.3.1 Carrageenan-Induced Paw Oedema[32]

In this inflammation animal model, carrageenan (1% w/v in normal saline, 0.05mL) was injected into the right back leg of the rats 1 hour before. The doses of plant root extract, the standard diclofenac drug, and water (control group) were administered by oral route[33]. The inflammatory swelling in the paw due to carrageenan administration and the thickness of the paw of each group of animals were observed and measured using a Digital Plethysmometer (Orchid Pvt Ltd) at 0, 15, 30, 45, 60, and 120-minute intervals after administration of carrageenan. The volume and swelling occupied

by the paw in the test group of animals, in relation to the control group of animals, were measured and calculated.

2.10 Statistical Analysis

InStat Graph Pad software version 10.2.3 was used to perform the statistical analysis of the *in vivo* animal study. One-way ANOVA and Dunnett's test were used to determine the analysis and differences between the groups. The mean ± standard error of the mean (SEM) was used for presenting the findings. For all animal groups, p-values less than 0.05 have been considered statistically significant[34].

3. Results

3.1 Percentage Yield of Plant Extract

A plant material, *M. macrantha* root coarse powder, yielded 5.2% of extractive yield for aqueous extract. The extractive yield for aqueous extract is higher than for other solvents. Preliminary phytochemical screening is shown in Table 1.

3.2 Phenolic Content (PC)

The total phenolic content was calculated for all the extracts using the Singleton and Rossi method with the Folin-Ciocalteu method. The values were mentioned in Table 2 as mg of Gallic Acid Equivalent (GAE) per 100 g of the extracted sample. The measurement of absorbance was conducted at a wavelength of 725 nm. The analysis shows the highest phenolic content was in the aqueous root extract of *M. macrantha* compared to other extracts. This indicated that the presence of more phenolic compounds in the root extract could be responsible for analgesic and anti-inflammatory activity in rats. The data are expressed as mean ± Standard Deviation (SD). The statistical evaluation gives significantly higher phenolic contents ($p < 0.05$) compared to other extracts. These results demonstrate the presence of higher phenolic phytochemicals in the aqueous root extract than in other root extracts.

3.3 *In Vitro* Antioxidant Assay

The *in vitro* antioxidant efficacy of plant root extracts of *M. macrantha* was concentration-dependent (10–100 µg/ml) in the hydrogen peroxide assay method, shown in Tables 3 and 4. The aqueous extract of *M. macrantha* root depicted strong H_2O_2 scavenging activity (IC_{50} 86.13 µg/ml), whereas the methanolic extract exhibited an IC_{50} value of 91.78 µg/ml. The other extracts of the plant exhibited concentration-dependent free radical scavenging activity. The scavenging ability of the aqueous extract was found to be good (54.77%). At a concentration of 100 µg/ml, the antioxidant activity of ascorbic acid was 79.02%, while that of methanolic extracts was 47.62%. The aqueous extract has the most antioxidant activity, while the methanolic extract has the lowest. Tables 3 and 4 show the relative antioxidant activity of the root extracts of *M. macrantha* and the standard ascorbic acid in terms of IC_{50} (µg/ml) and scavenging activity at 100 µg/ml. Significant variations ($p < 0.05$) between the methanolic extracts of the tested animals were found in the antioxidant activity. Methanolic and aqueous plant extracts differed significantly ($p < 0.05$), according to the results. The strongest antioxidant has the lowest IC_{50} value since IC_{50} is inversely related to antioxidant capacity. In contrast with other extracts and the usual medication, our findings demonstrated the aqueous extract's strong antioxidant activity.

Table 1. Preliminary phytochemical screening of *M. macrantha* root extracts

Sr. No.	Phytoconstituents	Petroleum ether	Chloroform	Methanol	Aqueous
1	Alkaloids	+	+	+	+
2	Steroids	+	+	-	-
3	Tannins	-	-	+	+
4	Phenols	-	-	+	+
5	Flavonoids	-	-	+	+
6	Carbohydrates	-	-	+	+
7	Oils and fats	+	+	-	-
8	Terpenoids	+	+	-	-

3.4 Protein Denaturation Assay (Egg Albumin)

The results indicated a diminution of denaturation of proteins that is influenced by the dose of test extract in the presence of *M. macrantha* at all concentrations tested, with maximum decrease in protein denaturation (86.38 ± 2.31%), at 100 µg/ml concentration, as shown in Table 5 and Figure 3.

3.5 Analgesic Activity by the Hot Plate Method

Significant and increased response times were observed in rats exposed to a hot-plate thermal stimulus, followed by the test drug treatment and the standard analgesic agent, Diclofenac Sodium. Based on previous research findings, *M. macrantha* root contains various secondary phytochemicals, including stigmasterol, β-sitosterol, caryophyllene, squalene, flavonoids, and polyphenols. The constituents are known to prevent activation of the Cycloxygenase-2 enzyme. By this, *M. macrantha* root extract was found to have dose-dependent analgesic activity, as depicted in the given Table 6.

Table 2. Phenolic content of *M. macrantha*

Sr. No.	Extract	TPC (mg GAE/g)
1	Petroleum ether	31.58 ± 0.02
2	Chloroform	49.21 ± 1.02
3	Methanol	54.37 ± 0.14
4	Aqueous extract	62.43 ± 1.06*

* Indicates statistically significantly higher than other extracts ($p < 0.05$).

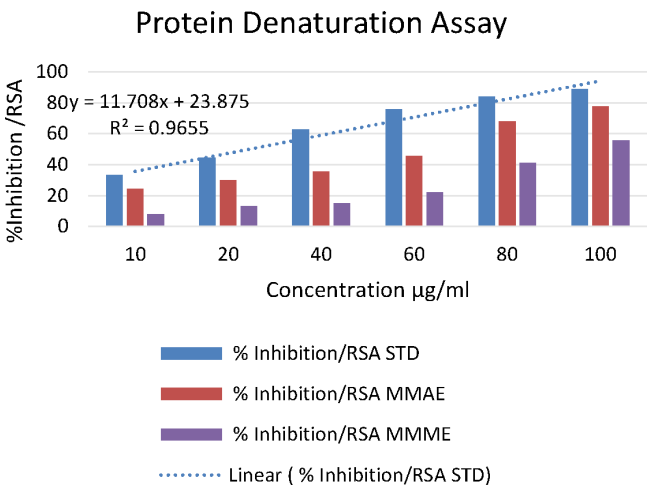


Figure 3. Standard calibration curve of protein denaturation assay.

Table 3. Observations of hydrogen peroxide assay of MMAE

Sr. No.	Conc. (µg/ml)	Absorbance Std Ascorbic acid (230 nm)	Absorbance of MMAE (Plant extract)	% Inhibition/RSA
1	10	0.97	0.735	24.58
2	20	0.85	0.612	28.27
3	40	0.78	0.514	34.87
4	60	0.72	0.426	40.94
5	80	0.65	0.364	44.18
6	100	0.44	0.202	54.77

Table 4. Observations of reducing power assay of MMAE

S. No.	Conc. (µg/ml)	Absorbance Std Ascorbic acid (700 nm)	Absorbance of MMAE (Plant extract)	% Inhibition/RSA
1	10	0.78	0.61	21.79
2	20	0.87	0.67	22.98
3	40	1.21	0.78	35.53
4	60	1.64	0.89	45.73
5	80	1.86	0.94	49.46
6	100	2.61	0.97	62.83

Table 5. Percentage inhibition of protein denaturation assay

Sr. No.	Conc. (µg/ml)	% Inhibition				
		STD	MMPE	MMCE	MMME	MMAE
1	10	33.14±3.24	26.34±1.22	30.2±1.19	7.89±1.13	24.44±2.11
2	20	44.57± 5.43	30.21± 4.14	35.07± 3.22	13.33± 5.21	41.58± 2.12
3	40	62.69± 3.67	35.55± 1.61	48.11± 3.32	15.22± 4.07	59.56± 1.41
4	60	75.85± 4.28	45.55± 4.01	68.43± 4.01	22.21± 1.16	73.48± 3.21
5	80	84.14± 2.21	67.78± 4.33	77.49± 1.42	41.11± 2.35	80.75± 1.31
6	100	88.72± 2.71	77.78± 3.47	84.46± 2.42	55.52± 4.31	86.38± 2.31

The values are shown as mean ± SEM (standard error of the mean) with n=6.

Table 6. Results of analgesic activity by the analgesiometer

Animal Groups	Observation time in minutes				
	Reaction time (in sec) after 30 min	Reaction time (in sec) after 60 min	Reaction time (in sec) after 90 min	Reaction time (in sec) after 120 min	Reaction time (in sec) after 180 min
Control	11.83±0.600	12.66±0.42	12.5±0.67	15.16±0.47	17.16±0.79
Diclofenac 10 mg/kg	17.33±0.49*	15.5±0.619*	22.66±0.843*	24.83±0.600*	26.16±0.40*
MMAE 100mg/kg	13.66±066	14.83±0.477	16.66±0.55	20.33±0.80*	22.66±0.714*
MMAE 200mg/kg	11.83±0.654^{ns}	15.16±0.65^{ns}	18.33±0.66^{ns}	22.16±0.65^{ns}	23.66±0.71^{ns}
MMAE 400mg/kg	12.83±0.477	17±0.730	20.33±0.802*	24.16±0.477*	25.83±0.401*

The values are shown as mean ± SEM (standard error of the mean) with n=6. The statistical analysis was performed using InStat Software version. 10.2.3, applying One-Way ANOVA followed by Dunnett's test. The P value (P<0.05) was considered significant compared to the control group of animals.

Table 7. Results of the writhing test in animals

Animal Groups	No. of writings (Mean ± SEM)	Percentage Inhibition
Control	57.83±1.75	100
Diclofenac 10mg/kg	22.16±1.07***	61.67
MMAE 100mg/kg	57.24±1.29^{ns}	1.44
MMAE 200mg/kg	46.33±1.62*	19.88
MMAE 400mg/kg	38.5±1.87**	33.42

The values are shown as mean ± SEM (standard error of the mean) with n=6. The statistical analysis was performed using InStat Software version. 10.2.3, applying One-Way ANOVA followed by Dunnett's test. The P value (P<0.05) was considered significant compared to the control group of animals.

3.6 Writhing Test (Acetic Acid)

In the acetic acid test, there was a significant reduction in the writhing response after *M. macrantha* aqueous root extract treatment (57.24±1.29, 46.33±162, and 28.14±1.47 in 100, 200, and 400 mg/kg MMAE vs the control group animals), shown in Table 7. Notably, the analgesic activity observed with 400 mg/kg closely approached that of Diclofenac sodium (22.16±1.07). Furthermore, other doses of the plant also exhibited a dose-dependent effect on the writhing response. As per previous findings, the study of the analgesic activity of other extracts of the plant. The presence of some

Table 8. Observations of carrageenan-induced rat hind paw oedema

Animal Groups	Time Interval										
	0 hr	1hr		2hr		3hr		4hr		5hr	
	PV	PV	% INH	PV	% INH	POV	% INH	PV	% INH	POV	% INH
Diclofenac 10mg/kg	0.85 ± 0.03	0.85 ± 0.03	27.89	0.93 ± 0.08 ***	51.29	0.88 ± 0.04 **	62.82	0.78 ± 0.07	70.11	0.72 ± 0.05	72.45
MMAE 100mg/kg	0.962 ± 0.04	1.373 ± 0.08	6.80	1.306 ± 0.15 *	32.12	1.179 ± 0.20 *	49.14	1.030 ± 0.16	58.96	1.030 ± 0.16	61.50
MMAE 200mg/kg	0.922 ± 0.03	1.212 ± 0.09	17.68	1.221 ± 0.13 **	36.78	1.094 ± 0.24 *	53.41	1.012 ± 0.08 **	64.14	0.817 ± 0.10	67.16
MMAE 400mg/kg	0.972 ± 0.06	1.212 ± 0.04 *	17.68	1.268 ± 0.11 **	34.71	1.012 ± 0.17 *	56.83	0.911 ± 0.14 **	67.33	0.717 ± 0.08	70.18

The values are shown as mean ± SEM (standard error of the mean) with n=6. The statistical analysis was performed using InStat Software version. 10.2.3, applying One-Way ANOVA followed by Dunnett's test. The P value (P<0.05) was considered significant compared to the control group of animals.

phytoconstituents, like Machigline and Machiline, showed anti-nociceptive responses in both peripheral and central areas. Nociceptive reactions, neuropathic pain, and anti-inflammatory reactions originate from the peripherally active sensory afferent neurons.

3.7 Carrageenan-induced Paw Oedema

The carrageenan administered to the hind paw of rats revealed a significant elevation in paw volume, compared to the negative control group. The use of the *M. macrantha* plant extract shows a significant volume of rat paw compared to the control groups of rats over time. Similarly, administration of the standard drug, diclofenac sodium, also depicted a significant reduction of paw oedema compared to the control group of rats. The higher dose of aqueous extract shows a more significant action as compared to the lower dose of plant extracts (Table 8). Some of the stigmasterol, β-sitosterol, Machigline and Machiline, flavonoids, tannins, and polyphenols were seen in *M. macrantha* root extract and revealed anti-inflammatory activity[35].

4. Discussions

This research protocol screened for analgesic and anti-inflammatory activity effects of *M. macrantha* aqueous root extract. The use of herbal plants for safer and economical treatment of pain and inflammation, together with the potential benefits of herbal medicines, would make considerable use to explore phytoconstituents against chemical-based analgesic and anti-inflammatory drugs, which are associated with minimal side effects and comparable efficacy[35]. *M. macrantha* is a traditionally used herbal plant for the treatment of various inflammatory and pain conditions. As of this writing, there have not been published scientific studies on the plant root's effectiveness to alleviate pain and inflammation. Therefore, to support its reported traditional medicinal benefits, it could be beneficial to do scientific screening on the pain-relieving and anti-inflammatory action of *M. macrantha* stem root extract in animal models[36].

The *in vivo* animal experimentation was performed using models of hot plate, acetic acid writhing, and carrageenan, used to examine the analgesic effects of *M. macrantha* aqueous root extract. When compared to the usual medication diclofenac and the animals in the negative control group, the application of different doses of *M. macrantha* extract significantly prolonged the reaction time on the hot plate ($P < 0.05$). The protein denaturation experiment showed a dose-dependent percentage inhibition, indicating the anti-inflammatory properties of *M. macrantha* phytoconstituents that guard against denaturation and prevent inflammation[37]. The mechanism for acetic acid developed writhing effect is due to increased production of PGs with the release of mediators such as histamine, serotonin, bradykinin, and substance P. The use of phytoconstituents-rich extract prevents the release of all these inflammatory mediators[38].

The carrageenan-induced rat paw oedema is another model to evaluate the anti-inflammatory effects of the extract. After 1 to 2 hr, carrageenan injection causes the development and release of bradykinin, 5-hydroxytryptamine, and histamine mediators for paw swelling with a rise in PG level[39].

In an effort to improve rats' sensation of pain in response to heat, centrally acting analgesics are frequently screened using the hotplate model. According to this paradigm, an analgesic activity is indicated by a behavioural reaction like jumping or paw licking. According to the current study, all doses of the plant extract tend to have analgesic effects starting at 30 minutes ($p < 0.05$). When contrasted with the positive and negative control groups of diclofenac, the percentage of elongation time following dosages of 100, 200, and 400 mg/kg showed the greatest effect at two hours, showing considerable nociceptive protection[40].

The findings and results of analgesic medications like diclofenac, the conventional medication, considerably reduced paw oedema, and the reduction was most considerable. This suggests that suppression of the mediators and PG production may be one of the potential anti-inflammatory actions of plant phytoconstituents[41,42].

The use of herbal plant-based phytoconstituents for analgesic and anti-inflammatory effects of *M. macrantha* is due to the abundance of secondary metabolites in the plant extract. The secondary phytochemicals like flavonoids, tannins, and saponins, play a crucial role in pain perception and anti-inflammatory[43,44] properties due to PG synthesis and inhibition of mediator release in inflammation, especially prostaglandin synthesis.

Furthermore, by lowering the expression of cytokines that trigger inflammation, especially interleukin 1 and interleukin 6, saponins, phenolic compounds, and terpenoids showed significant anti-inflammatory and analgesic effects.

The observations, results and animal screening outcomes, the plant root extract of *M. macrantha* secondary phytocomponents, like polyphenols, flavonoids, and terpenoid chemicals, may play a vital role in preventing and reducing inflammatory mediators and animal pain perception[45]. Similarly, in the denaturation assay, these phytoconstituents could be effective in the prevention of denaturation with *in vitro* antioxidant properties for the reduction of pain and inflammation by reducing the release of inflammatory mediators[46,47].

5. Conclusion

The results of this research work explicitly demonstrate the effective analgesic and anti-inflammatory potential of *M. macrantha* root extract, adding practical and experimental support for traditional claims as an herbal medicine for inflammation and pain. These observations may specify that the plant extract is effective in the prevention and minimisation of biological inflammatory mediators involved in inflammation. These results might be due to the availability of secondary phytochemical constituents, including polyphenols, flavonoids, alkaloids, saponins, and terpenoids. Our research findings provide the scientific documentation about the traditional medicinal uses of *M. macrantha* for the minimisation of pain and inflammation in Indian folk medicines.

6. References

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Author Contribution

Experimental research work, results and observations and manuscript preparation were performed by Priti C Patle. Sanjay B. Bhawar contributed to concept, experimentation and organization of observation tables. Aman B. upaganlawar assisted in experimental methodology, data collection and organization of figures. The final approval of the research work and critical revision of manuscript were done by Chandrashekhar D. Upasani. All authors have read and agreed to the published version of the manuscript.