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Research Article

IN VITRO AND *IN VIVO* ANTI-INFLAMMATORY ACTIVITY OF WHOLE PLANT METHANOLIC EXTRACT OF *MUKIA MADERASPATANA* (L.) M.ROEM. (CUCURBITACEAE)

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ABSTRACT

The study was aimed to evaluate the *in vitro* and *in vivo* anti-inflammatory activities of whole plant methanolic extract of the medicinal plant, *Mukia maderaspatana*. The extract was screened for *in vitro* anti-inflammatory activity by inhibition of protein denaturation and proteinase activity and *in vivo* anti-inflammatory activity through carrageenan induced paw edema in rat model and enzymic antioxidant status in serum. The extract showed significant activity against protein denaturation and proteinase inhibition at 400µg/mL (65.71% and 62.19% respectively). Similarly, the reduction of paw edema in carrageenan induced rat after 4hrs from the time of treatment with 200µg/Kg b. wt. and 400µg/Kg b. wt. were 34.29% and 54.29% respectively which are comparable to that of the effect of standard, the indomehacin. The inhibition of activity of enzymic antioxidant *viz*, SOD, CAT GPx and GST was significantly reduced in the animals treated with the methanolic plant extract. The lipid peroxidase and nitric oxide levels were also significantly decreased in the experimental rats as the influence of methanolic extract of *M. maderaspatana.*

Keywords: *Mukia maderaspatana,* Whole plant methanolic extract, *In vitro* and *In vivo* anti-inflammatory activities.

INTRODUCTION

Medicinal plants are an important source of new chemical substances with potential therapeutic effects[1]. The World Health Organization (WHO) is now actively encouraging developed countries to use herbal medicines which they have traditionally used for centuries[2]. Plants in the family, Cucurbitaceae have been well reported already for their antiinflammatory, analgesic, antioxidant or anti-proliferative activities[3,4,5,6,7,8].

Mukia maderaspatana is a locally used medicinal plant in Coimbatore and Tirupur districts of Tamil Nadu, belonging to the family, Cucurbitaceae, generally distributed in the low hills of Western Ghats, India. The plant is bitter, sweet, refrigerant, carminative, aperients, vulnerary, sudorific, expectorant, anodyne and tonic. It is useful in vitiated conditions of pitta, burning sensation, dyspepsia, flatulence, ulcers, cough, asthma etc[9]. Squeezed plant is applied to treat scabies of animals[10]. The root is chewed for 15 minutes to relieve toothaches. A decoction of the root is masticated for flatulence and is also applied to treat toothaches. Leaf juice is boiled and given for constipation and gas troubles, expectorant and astringent. Decoctions of leaves of this plant have been used by siddha practitioners in Tamil Nadu for the treatment of hypertension.

As there is no work on anti-inflammatory properties in this species, the present study was focused on to investigate the *in vitro* and *in vivo* anti-inflammatory potential of the whole plant extract of *M. maderaspatana*.

MATERIALS AND METHODS

Collection and preparation of plant material

The whole plant material of the study species, *M. maderaspatana* was collected from Gobichettipalayam, Tamil Nadu, India. It was chopped to small pieces, shade dried and coarsely powered. The powdered whole plant (50g) was extracted in soxhlet apparatus for two days with methanol (250ml). The extract was concentrated by evaporate the solvent for extractive yield.

Chemicals

Ethylenediaminetetraacetic acid (EDTA), phosphate buffer, tricarboxylic acid (TCA), glacial acetic acid, hydrochloric acid, phosphate buffered saline, trypsin, tris - HCl buffer, casein, perchloric acid, indomethacin, carrageenan, diethyl ether, pyrogallol, coomassie Brilliant Blue G-250, ethanol, phosphoric acid, dichromate, acetic acid, hydrogen peroxide, chromic acid, chromic acetate, potassium dichromate, dithionitrobenzoic acid (DTNB), sodium azide, glutathione, phosphate solution, sodium citrate, 1–chloro–2,4dinitrobenzene (CDNB), sulfhydryl, thiobarbituric acid (TBA), zinc sulphate, sulfanilamide, N–1- naphthylethylenediamine dihydrochloride, phosphoric acid and sodium nitrite were the chemicals used in the study.

Anti-inflammatory activity

In vitro anti-inflammatory activity

Inhibition of protein denaturation

The reaction mixture (0.5 ml) was prepared by combining 0.45mL of bovine serum albumin (5 % aqueous solution) and 0.05 ml of sample extract (200 and 400 µg/mL of final volume). The pH was adjusted to 6.3 using a small amount of 1 N HCl. The samples were incubated at 37ºC for 20 minutes and then heated at 57ºC for 3 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm[11]. For control tests, 0.05 ml distilled water was used instead of extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percent inhibition} = \frac{100 - (\text{OD of test} - \text{OD of product control})}{\text{OD of control}} \text{ X } 100 \quad \ldots(1)$$

The control represents 100 % protein denaturation.

Proteinase inhibitory action

The reaction mixture (2 ml) was prepared by combining 0.06 mg trypsin, 1 ml of 25 mM tris–HCl buffer (pH 7.4) and 1 ml of methanol extract (200 and 400 µg/ml of final volume). The mixtures were incubated at 37º C for 5 minutes and then 1 ml of 0.8% (w/v) casein was added. The mixtures were incubated further for 20 min. Two ml of 70% (v/v) perchloric acid was added to terminate the reaction and the cloudy suspension was centrifuged[12]. The absorbance of the supernatant was measured spectroscopically at 280 nm against the buffer blank. The percentage of inhibition was calculated by using formula (1).

In vivo anti-inflammatory activity

Animals

Healthy male albino rats of wistar strain (120-150g) were obtained from the Small Animal Breeding Station, Mannuthy, Thrissur, Kerala, India and were maintained under standard environmental conditions (22–28ºC, 60–70% relative humidity and 12-h dark:12-h

light cycle). They were fed with standard rat feed (M/S Hindustan Lever Ltd., Bangalore, India) and *ad libitum*. Before performing the experiment, the ethical clearance was obtained from Institutional Animal Ethics Committee.

Carrageenan-induced paw edema

The anti-inflammatory activity of methanol extract of *M. maderaspatana* was investigated in carrageenan induced inflammatory model[13]. For the experiment, the male wistar rats were divided into five groups. The animals were fasted overnight prior to the start of the experiment. The I and II groups received distilled water, while the III group was treated with indomethacin (10 mg/Kg p.o.). The IV and V groups were administered with the methanol extract of *M. maderaspatana* at the concentration of 200 and 400 mg/Kg per day p.o. respectively. After one hour of the treatment, acute inflammation was produced to the Group II – V by the sub plantar administration of 0.1 ml of 1 % carrageenan (CGN) in the right hind paw of the rats. The thickness (mm) of the paw was measured immediately at 30 min interval by using vernier caliper for 4 hrs after the carrageenan injection.

The percentage inhibition of edema was calculated by using the following formula:

$$\frac{V_c - V_t}{V_c} \text{ X } 100$$

Where,

V_c = The average increase in paw volume of induced rats.

V_t = The average increase in paw volume after the administration of test and standard drug.

At the end of experimental regimen, all the animals were subjected to mild diethyl ether anesthesia. Blood was then collected by cardiac puncture and allowed to clot for 20–30 min and centrifuged in a refrigerated centrifuge (4ºC) at 3000 rpm for 10 min. Fresh serum samples were used to estimate total protein, superoxide dismutase, catalase, glutathione peroxidase, glutathione – S – transferase, lipid peroxide and nitric oxide.

Estimation of protein

Serum sample of 0.2 ml was mixed with 5 ml of alkaline copper reagent and kept for 10 min. Then added with 0.5 ml of diluted Folin Ciocalteau reagent, mixed well and incubated at room temperature for 30 min. The blue colour developed was read at 660nm in spectrophotometer. To the blank, 0.2 ml of water and 0.2 mg of bovine serum albumin were added instead of enzyme extract and treated as control[14]. Concentration of the enzyme specific activity was expressed as mg/dL.

Determination of enzymic antioxidant status

Estimation of superoxide dismutase activity (SOD)

The reaction mixture consisted of 100 µl of the serum, 0.05 ml of pyrogallol solution (0.2 mM pyrogallol in 10 mM HCl) was added and made upto the volume, 2.5 ml with tris-HCl buffer (50 mM tris-HCl buffer of pH, 7.0). Absorbance was read at 420 nm against reagent blank[15]. A unit of enzyme was referred as the amount of the enzyme which inhibits the reaction by 50%. Specific activity was expressed as moles/min/mg of protein.

Estimation of catalase activity (CAT)

The reaction mixture (1.5 ml) contained, 1 ml of 0.01 M phosphate buffer (pH – 7.0), 0.1 ml of enzyme preparation and 0.4 ml of 2 M hydrogen peroxide was prepared. The reaction was stopped by adding 2.0 ml of dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid in the ratio of 1:3). The tubes were kept in a boiling water bath for 10 min and cooled[16]. The absorbance was spectrophotometrically measured at 620 nm. A system devoid of enzyme served as control. Activity of catalase was expressed as moles of hydrogen peroxide consumed/min/mg protein.

Estimation of Glutathione Peroxidase (GPx)

To 0.4 ml of phosphate buffer (pH – 7), 0.2 ml of 4.0 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.2 ml of 4.0 mM reduced glutathione and 0.1 ml of 2.5 mM H_2O_2 were added separately to two test tubes labeled as test (T) and control (C). The test tube of T was added with 0.2 ml of sample and the test tube of C was added with 0.2 ml of water. The contents were mixed well and incubated at 37 ºC for 10 min. The reaction was arrested with the addition of 0.5 ml of 10 % TCA. To determine the glutathione content, 1ml of supernatant was removed by centrifugation. To that supernatant, 3 ml of buffer and 0.5 ml of Ellman's reagent (19.8 mg DTNB in 1% sodium citrate) were added[17]. The absorbance was read at 412 nm. The activity was expressed in terms of µg of glutathione consumed/min/mg protein.

Determination of Glutathione – S- Transferase activity

To 1.0 ml of buffer, 0.1 ml of sample, 1.7 ml of water and 0.1 ml of CDNB were added and incubated at 37 ºC for 5 min. After incubation, 0.1 ml of reduced glutathione was added[18]. The increase in optical density of the enzyme was measured against that of the blank at 340 nm. The enzyme activity was calculated in terms of µmoles of CDNB conjugate formed/min/mg protein.

Estimation of lipid peroxidation (LPO) levels

0.1 ml of the serum was treated with 2.0 ml of TBA-TCA-HCl reagent (0.375 % TBA, 15% TCA and 0.025N HCL in 1:1:1 ratio) and it was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged at 1000 rpm for 10 minutes and the clear supernatant was taken for measurement[19]. The absorbance of chromophore was read at 535 nm against a reagent blank that contained no serum. TBARS (thiobarbituric acid reactive substances) was expressed as mole of MDA (malondialdehyde)/ mg protein.

Estimation of nitric oxide (NO) levels

Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulphate (300 mg/ml) to a final concentration of 15 mg/ml. After centrifugation at 10000 rpm for 5 min at room temperature, supernatant was collected[20]. 0.1 ml of supernatant was mixed with 0.1 ml of Griess reagent (1% sulfanilamide and 0.1 % N –naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) at room temperature for 5 min and absorbance was measured at 540 nm.

RESULTS AND DISCUSSION

***In vitro* anti-inflammatory activity**

Inhibition of protein denaturation and proteinase activity

The data on the effect of extract of *M. maderaspatana* on the inhibition of protein denaturation and proteinase activity are shown in Table 1. The extract showed significant protection against denaturation of protein and the percentage of inhibition was varied from 58.24% (in 200 µg/mL) to 65.71% (in 400 µg/mL). Denaturation of protein is one of the causes of inflammations[21]. Several anti-inflammatory drugs have shown dose dependent ability to inhibit theally induced protein denaturation[22]. Production of auto-antigens in certain inflammatory diseases may be due to *in vivo* denaturation of proteins. From the results of the present study, it is known that the extract of *M. maderaspatana* is capable of controlling the denaturation of proteins in inflammatory diseases effectively.

The extract showed significant inhibition against proteinase activity and the percentage of inhibition at the doses of 200 and 400 µg/ml of extract were determined to be 51.80% and 62.19% respectively. Proteinases enzymes have been implicated in inflammatory reactions. It was previously reported that leucocyte proteinases play an important role in the development of tissue damage during inflammatory reactions and proteinase inhibitors provided significant level of protection. The methanol extract of the study species, *M. maderaspatana* exhibited significantly high proteinase inhibitory activity. This finding justifies the usefulness of *M.maderaspatana* in the management and treatment of inflammation. Similar trend of inhibition of protein denaturation was observed in another Cucurbitaceae member, *Coccinia grandis*[23].

Table 1: Inhibitory effect of whole plant methanolic extract of *Mukia maderaspatana* on protein denaturation and proteinase activity

Sample concentration (µg/ml)	Inhibition of protein denaturation (%)	Proteinase inhibition (%)
200	58.24	51.80
400	65.71	62.19

***In vivo* anti-inflammatory activity**

Carrageenan induced paw edema

The results of the experiments of carrageenan induced rat paw edema model for anti-inflammatory activity of methanol extract of *M. maderaspatana* are presented in Table 2. The paw thickness of the normal rats was found to be 4.33 mm. The paw volume was increased from this thickness to 7.63 mm at 60 minutes and then to 6.42 mm at 120 minutes and to 5.61 mm at 180 minutes and then to 5.03 mm at 240 minutes in carrageenan induced rats (Group II). The increase in the paw thickness over a period of 4 hours indicates the release of first and second stage mediators of inflammation. Upon pretreatment of the animals with the methanol plant extract at 200 mg/kg b.wt. (Group IV) one hour prior to induction, the paw thickness was measured to be 4.56 mm, and 6.70 mm at 60 minutes, 6.26 mm at 120 minutes, 5.71 mm at 180 minutes and 5.02 at 240 minutes. For the Group V rats of pretreatment with the plant extract at 400 mg/kg b. wt. one hour prior to induction, the paw thickness was measured as 4.63 mm and 6.86 mm at 60 minutes, 6.09 mm at 120 minutes, 5.39 mm at 180 minutes and 4.95 mm at 240 minutes. In the standard drug pretreated groups (Group III), one hour prior to induction, the paw thickness was 4.48mm and 6.27 mm at 60 minutes, 5.61mm at 120 minutes, 4.92mm at 180 minutes and 4.59 mm at 240 minutes after induction. The increase in paw thickness upon carrageenan induction at 0.1 ml was found to be significantly reduced upon pretreatment of the animals with 400 mg/kg b.wt. of the extract in that, the thickness was considerably minimized upto 0.32mm when compared to Group II rats that were carageenan induced control group and it was comparable to that of the pretreated animals with a standard drug, indomethacin. This considerable decrease in paw thickness upon treatment of extract indicates the functional behaviour of the plant in terms of anti-inflammatory activity.

The development of edema in the paw of the rat after the injection of carrageenan is due to the release of certain inflammatory mediators, histamine, serotonin and prostaglandin like substances[24]. Significantly high anti-inflammatory activity of the extract of *M. maderaspatana* (400 mg/Kg b.wt.) determined in the present study may be due to the inhibition of such inflammatory mediators. A similar trend of observation was made in plants like *Trigonella foeum-graecum*[25], *Rosa damascena*[26] and *Leucas cephalotes*[27]. From the results it could be confirmed that the extract of *M. maderaspatana* at the dose, 400 mg/Kg b. wt. posses potent anti-inflammatory activity.

Table 2: Changes in paw thickness in the control and experimental rats at 30 minutes time intervals for 4 hours.

Group	Initial paw thickness (mm)	Paw thickness after carrageenan induction				Increase in paw thickness (mm)	Inhibition (%)
		60th min	120th min	180th min	240th min		
I	4.45±0	4.45±0	4.45±0	4.45±0	4.45±0	0	-
II	4.33±0.12	7.63±0.89	6.42±0.62	5.61±1.04	5.03±0.15	0.7	-
III	4.48±0.17	6.27±0.27	5.61±0.25	4.92±0.48	4.59±0.1	0.11	84.29
IV	4.56±0.11	6.70±0.32	6.26±0.34	5.71±0.29	5.02±0.28	0.46	34.29
V	4.63±0.27	6.86±0.17	6.09±0.27	5.39±0.20	4.95±0.29	0.32	54.29

I – Control, II - Induced, III – Standard, IV – 200mg/kg/b.wt., V – 400mg/kg b.wt.

Determination of enzymic antioxidant status

Effect of *M. maderaspatana* extract on serum antioxidant

In the present study, activity of the enzymic antioxidants in the serum *viz.,* SOD, CAT, GPx and GST were found to be significantly ($p<0.05$) reduced in the inflammation induced animals, while it was elevated in the animals of treatment with methanol extract of *M. maderaspatana* (200 and 400 mg/Kg b. wt.) and the standard, indomethacin (Table 3). The activity of the enzyme, SOD was found to be decreased from 4.34 µmoles/min/mg protein in control animals to 2.53 µmoles/min/mg protein in carrageenan induced group of animals. In the pretreated animals with the extract at 200 and 400 mg/Kg b. wt., (Groups IV and V respectively), the activity went upto 3.08 and 3.42 µmoles/min/mg protein respectively. Similar trend of results were also observed for the animals of standard drug treatment (Group III). The activity of the catalase (CAT) enzyme was also found to be decreased from 18.49 µmoles/min/mg protein in control animals to 10.28 µmoles/min/mg protein in carrageenan induced group of animals. In the animals of treatment with the plant extract at 200 and 400 mg/Kg b. wt., (Groups IV and V respectively), the activity went upto 13.80 and 16.66 µmoles/min/mg protein respectively. Animals of standard drug treatment (Group III) also registered the same trend of CAT activity. In the induced group (Group II), the glutathione peroxidase (GPx) activity was found to be 8.50 µmoles/min/mg protein. Treatment with extract at 200 mg/Kg b. wt., (Group IV) resulted in the increase of activity to 11.69 µmoles/min/mg protein and in 400 mg/Kg b. wt., (Group V) it was increased to 16.12 µmoles/min/mg protein. The amount of glutathione-S- transferase (GST) was found to be 4.60 µmoles/min/mg protein in carrageenan induced rats (Group II), whereas in the rats treated with the extract at 200 and 400mg/ Kg b. wt., (Groups IV and V respectively) and the standard drug, indomethacin (Group III), the GST levels were found to be 5.93, 7.30 and 8.05 µmoles/min/mg protein respectively.

The antioxidant enzymes (SOD, CAT, GPx and GST) protect aerobic cells against oxygen toxicity and lipid peroxidation[28]. Generally these enzymes are found to be decreased in carrageenan induced animals be due to the production of high amount of free radicals[29]. The decrease in SOD activity leads to declined production of hydrogen peroxide and hence the catalase and GPx as the hydrogen peroxide is the substrate for the production of these two enzymes. On drug treatment, the activities of SOD, CAT, GPx and GST were brought to near normal levels, which may be attributed to the free radical scavenging activity of phytochemicals present in the drug[30]. This fact was at par with the results of the present study made with the rats of pretreatment whole plant methanolic extract of the study species, *M. maderaspatana.* Chang *et al.,* (2011)[31] also observed that the status of antioxidant level in the serum of carrageenan induced rat was brought to normal when it was pretreated with ethanol extract of the plant species, *Phellinus linteus.*

Table 3: Enzymic antioxidant status in the control and experimental rats.

Parameter (µ moles/min /mg protein)	Group I (Control)	Group II (Induced)	Group III (Standard)	Group IV (200 mg/kg b.wt.)	Group V (400 mg/kg b.wt.)
SOD	4.34±0.38 d	2.53±0.20 a	3.68±0.25 c	3.08±0.16 b	3.42± 0.38 bc
CAT	18.49±0.45 e	10.28±0.58 a	17.45±0.68 d	13.80±0.39 b	16.66±0.50 c
GPx	17.79±0.38 d	8.50±0.22 a	16.48±0.35 c	11.69±0.45 b	16.12±0.49 c
GST	8.92±0.42 e	4.60±0.33 a	8.05±0.48 d	5.93±0.35 b	7.30±0.21 c

Values are expressed as mean±SD. n=6; Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT.

Effect of *M. maderaspatana* extract on serum lipid peroxides (LPO) and nitric oxide (NO) levels

The level of lipid peroxide of the induced rats (Group II) was found to be significantly increased to 30.91 µmoles/min/mg protein from the control, 13.13 µmoles/min/mg. On the other hand, the animals treated with the extract at two different doses *viz*., 200 and 400mg/Kg b. wt., (Groups IV and V respectively), the LPO level was found to be reduced significantly ($p<0.5$) upto 20.72 µmoles/min/mg protein and 17.15 µmoles/min/mg protein respectively and it was comparable to that of the standard drug treated rats of Group III (Table 4). The level of serum nitric oxide (NO) was 4.06 µmoles/min/mg proteins in the control rats. Nitric oxide was increased to 10.21 µmoles/min/mg protein in the carageenan induced rats of Group II, and in Groups III, IV and V rats which were treated respectively with the standard drug, indomethacin and 200 and 400 mg/kg b.wt doses of plant extract, the levels of NO were 5.25, 7.44 and 5.45 µmoles/min/mg protein respectively (Table 4).

The enzymes, lipid peroxide (LPO) and nitric oxide (NO) are used as a biomarker to show the index of oxidative stress, and causes cell membrane damage resulting in gradual loss of cell membrane fluidity, decreased the membrane potential and increased permeability to ions[32,33]. In view of this fact, significant decrease in LPO and NO levels in the animals treated with the methanol extract of *M. maderaspatana* confirmed the therapeutic potent of the study species. This report is in accordance with the study made by Tabassum *et al.* (2010)[34] for *Ocimum sanctum* and *Camellia sinensis,* and Chitra *et al*. (2010)[35] for *Strychnos nuxvomica*.

Table 4: Lipid peroxide and nitric oxide levels in the control and experimental rats.

Attribute	Group I (Control)	Group II (Induced)	Group III (Standard)	Group IV (200 mg/kg b.wt.)	Group V (400 mg/kg b.wt.)
LPO (n moles/mg protein)	13.13± 0.36 [a]	30.91±0.95 [d]	16.79±0.60 [b]	20.72±2.96 [c]	17.15±0.48 [b]
NO (µ moles/dl)	4.06±0.38 [a]	10.21±0.36 [d]	5.25±0.20 [b]	7.44±0.34 [c]	5.45±0.50 [b]

Values are expressed as mean±SD. n=6; Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT.

CONCLUSION

On basis of the results obtained in the present study, it can be concluded that the methanol extract of the species, *M. maderaspatana* had significant anti-inflammatory activities. The whole plant of this species is reported to have wide spectrum of alkaloids, flavonoids, saponins and terpenoids[36]. These bioactive compounds of the study plant possesses chemo preventive potential. Hence, the combined action of these active ingredients present in *M. maderaspatana* through their free radical scavenging activity and by inhibition of mediators of inflammation may be exerting the anti-inflammatory activity. Further clinical trials using human models are required to confirm the activities before going for commercialization of this species through obtaining appropriate and suitable drugs.

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