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EVALUATION OF ANTIOXIDANT, ANTI-INFLAMMATION AND CHEMICAL COMPOSITION OF METHANOLIC EXTRACT OF *AMOMUM MASTICATORIUM* THWAITES (ZINGIBERACEAE)

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ABSTRACT: The anti-inflammatory and antioxidant activities of the methanol extract of *Amomum masticatorium* rhizome were observed in various experimental models. Phytochemical constituents like phenols and flavonoids were also estimated. The antioxidant activity was evaluated by DPPH and super oxide radical scavenging assays. The extract showed significant antioxidant activity. The *in vitro* anti-inflammatory activity of the extract was investigated by various methods *viz*. albumin denaturation, membrane stabilization and proteinase inhibitory assays. Results showed that, the extract exhibited significant anti-inflammatory activity by inhibiting the heat induced albumin denaturation with IC_{50} value 34.15 ± 0.404µg/ml, effectively stabilized RBC membrane with IC_{50} value of 51.47 ± 1.56µg/ml and significantly inhibited proteinase activity with IC_{50} value of 52.8 ± 0.61µg/ml. *In vivo* activity was evaluated in carrageenan and formalin induced paw edema test in mice and compared with that of standard drug diclofenac. In both the models the extract showed significant reduction in edema. The extract at 200mg/kg b. wt showed significant reduction in carrageenan induced paw edema at 3rd hour and chronic edema on 6th day. From the results, it is concluded that, methanol extract of *Amomum masticatorium* rhizome exhibited significant anti-inflammatory and antioxidant activity, which may be due to the presence of active phytochemicals in it.

INTRODUCTION: Inflammation is a protective response of cell which involves several mechanisms and complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair. Inflammation results in the release of endogenous mediators like histamine, serotonin, bradykinin and prostaglandin [1].

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It plays a major role in most chronic illnesses like neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases [2]. Sometimes inflammation may lead to events that are quite serious and become chronic, like occurrence of rheumatoid arthritis [3]. Many of the anti-inflammatory drugs available have many side effects which impose a growing concern for more natural alternatives. *Amomum masticatorium* of Zingiberaceae is found as under growth in the evergreen forest at high altitudes. It has slender stoloniferous rhizomes. The rhizome of this plant is aromatic and carminative. It is used to chew with betel in Sri Lanka [4].

Several species of *Amomum* has traditional medicinal uses. Some are prescribed for the treatment of indigestion, vomiting, abdominal pains and rectal diseases. They are also ingredients of Ayurvedic and Unani medicines. A closely related species *Amomum subulatum* have reported to posses valuable properties like allopathic, analgesic, anti-inflammatory, antimicrobial, anti-oxidant, antiulcer, cardio-adaptogen and hypolipidaemic activities [5].

MATERIALS AND METHODS:

Plant material: The plant material was collected from Wagamon, Idukki district, Kerala, India. The material was herbarised (CALI 123766) and deposited at CALI, University of Calicut. Rhizome of the plant was chopped into small pieces, shade dried and powdered. The powdered material was run in soxhlet apparatus for 6 hrs with methanol for extraction. The extract was evaporated in vacuum to completely remove the methanolic content to get a final thick extract.

Determination of Phytochemical Constituents:

Total Phenolic Content: The total phenolic content in the extract was determined by Folin-Ciocalteu assay [6]. Gallic acid was used as standard. The content of phenolic compounds of the sample was expressed as gallic acid equivalents in mg per gram dry weight (mg GAE/g DW). All the samples were analyzed in triplicates.

Determination of Total Flavonoid Content: The $AlCl_3$ method [6] was used for the determination of total flavonoid content of the methanolic plant extract. Quercetin was used as the standard. Total flavonoid content was expressed as quercetin equivalents in mg per gram dry weight (mg QE/g DW). All the determinations were performed in triplicates.

Antioxidant Potential: The antioxidant property of the extract was determined by superoxide radical scavenging by the NBT reduction method [7]. The optical density was measured at 560 nm and percentage inhibition was evaluated by comparing the absorbance value of the control and samples. DPPH radical scavenging potential was measured by the method proposed by Coruh *et al*., 2007 [8]. DPPH has an absorption peak at 515 nm which disappears on reduction by an antioxidant compound. Extract was added to 1.5ml of freshly prepared DPPH solution and absorbance measured at 515 nm 20 min after the reaction was started. The percentage inhibition was calculated by comparing with that of the control.

In vitro Anti-inflammation:

Inhibition of Albumin Denaturation: Inhibition of albumin denaturation was studied according to methods previously described [9, 10]. The reaction mixture containing test extracts of different concentrations (50µg, 100µg and 200µg/ml) and 5% aqueous solution of bovine albumin fraction were incubated at 37 °C for 20 min and then heated to 57 °C for 20 min. After cooling the samples, the turbidity was measured at 660 nm. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was then calculated. Diclofenac was used as the standard.

Proteinase Inhibition: The potential for inhibiting proteinase was evaluated according to the method of Oyedepo *et al*., (1995) [11]. The reaction mixture (2ml) with 0.06mg trypsin, 1ml 20mM Tris HCl buffer (pH 7.4) and 1ml test sample of different concentrations (50 – 200µg/ml) was incubated at 37 °C for 5 min. Then 1ml of 0.8% (w/v) casein was added and was incubated for an additional 20 min. 2ml of 70% perchloric acid was added to stop the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Membrane Stabilization: Preparation of Red Blood cells (RBCs) suspension was done according to the standard protocol [12]. The blood sample was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

Heat Induced Haemolysis: The reaction mixture consisted of 1ml test sample of different concentrations (50 - 250µg/ml) and 1ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube [13]. Diclofenac was used as standard drug. The centrifuge tubes

were incubated in water bath at 56 °C for 30 min. The tubes were then cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was read at 560 nm. The experiments were performed in triplicates. The percentage of inhibition of haemolysis was calculated.

***In vivo* Anti - inflammation:**

Animals: Young male swiss albino mice were purchased from small animal breeding station, Kerala Agriculture University, Mannuthy, Kerala, India. They were maintained in well ventilated cages under controlled temperature and provided with 12 h light/dark cycle. The experimental protocol was approved by the Institutional Animal Ethical Committee.

Carrageenan Induced Acute Inflammation: Male swiss albino mice were used for the experimental study. The animals were divided into four groups with five animals in each group. Group I was treated with carrageenan alone and was taken as the control. Group II was treated with standard drug diclofenac (10mg/ kg/ b. wt). Group III and IV were treated with different doses of the extract (50mg / kg and 200 mg/kg b. wt). The extract was administered orally for five consecutive days. One hour after the fifth dose of the extract, acute inflammation was induced in the right hind paw of the mice by sub plantar injection of 0.02ml freshly prepared 0.1% carrageenan in normal saline [14]. Paw edema volume was measured using vernier callipers. Reading was taken every 1 hr up to 6 hr, and finally reading at 24 hr was also taken.

Formalin Induced Chronic Inflammation: Male swiss albino mice were divided into four groups with five animals in each group. Group I was treated as the control. Group II was treated with standard drug diclofenac (10mg/ kg/ b. wt). Group III and IV were treated with different concentrations of the extract (50mg / kg and 200mg/kg b. wt). The extract was administered orally for five consecutive days. One hour after the fifth dose of the extract, chronic inflammation was induced in the right hind paw of the mice by sub plantar injection of 0.02ml of 2% formalin [15]. Paw edema thickness was measured using vernier callipers. Reading was taken for six consecutive days.

RESULTS AND DISCUSSION:

Phytochemical Constituents:

Total Phenol Content: The total phenolic content of *A. masticatorium* was determined by Folin-Ciocalteu assay [16]. A standard caliberation curve for gallic acid was determined for a range of concentrations 20-120 mg/ml (**Fig. 1**). Total phenolic content of the extract was found to be 89.54 ± 4.67mg GAE/g dry weight of the extract.

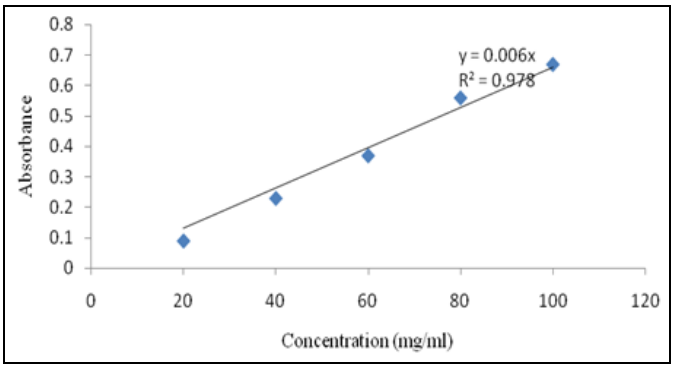

FIG. 1: STANDARD CALIBRATION CURVE OF GALLIC ACID

Total Flavonoid Content: Total flavonoid content was determined by $AlCl_3$ method. Standard calibration curve of quercetin for a range of concentrations 20-100mg/ml was determined (**Fig. 2**). Total flavonoid content of the extract was found to be 58.28 ± 3.32mg QE/g dry weight of the extract.

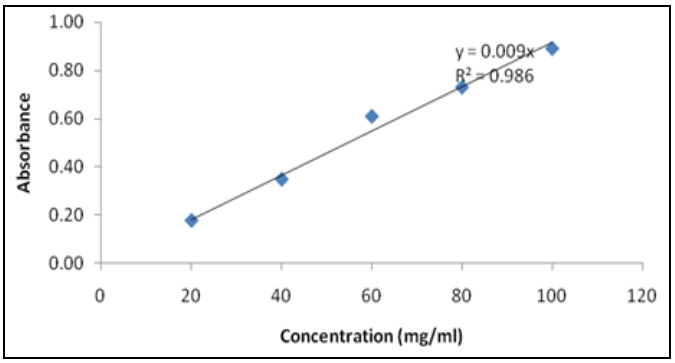

FIG. 2: STANDARD CALIBRATION CURVE OF QUERCETIN

The total phenolic and flavonoid content of the extract was found to be in considerable high amounts. Flavonoids are used in the treatment of many common diseases. Their ability to inhibit specific enzymes, simulate some hormones and neurotransmitters, and scavenge free radicals is already proven [17]. Phenolic compounds are also reported to have several biological properties, including antioxidant activity [18].

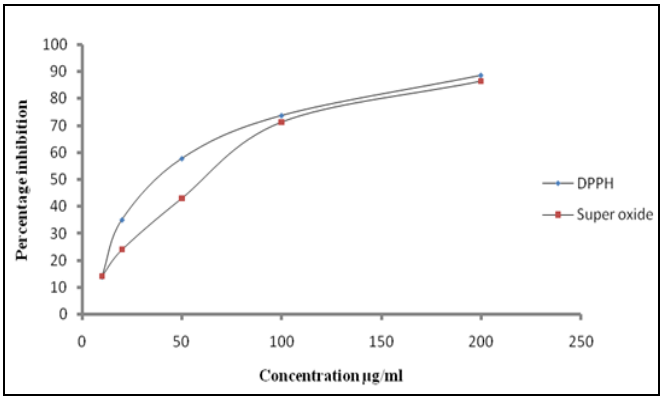

FIG. 3: GRAPH SHOWING ANTIOXIDANT ACTIVITY OF THE EXTRACT

Antioxidant: The methanolic rhizome extract has significant radical scavenging activity which exhibits its high antioxidant prospective. The IC_{50} values are given in **Table 1**.

It shows a dose dependent increase in the activity in both DPPH and superoxide radical scavenging assays (**Fig. 3**). Antioxidants have many health benefits and are considered to be important nutraceuticals [19]. The antioxidant potential of the plant extract is mainly from the flavonoids which contain hydroxyl functional groups [20].

TABLE 1: IC_{50} OF THE EXTRACT IN ANTIOXIDANT ASSAYS

Assay	IC_{50} value
Super oxide radical scavenging	32.59 ± 1.22
DPPH radical scavenging	46.0 ± 0.21

***In vitro* Anti-inflammation:** In all the three *in vitro* anti-inflammatory assays, the extract showed significant activity. The results are given in **Table 2** and **3**.

TABLE 2: PERCENTAGE INHIBITION OF THE EXTRACT IN VARIOUS ANTI-INFLAMMATORY ASSAYS

Treatment	Proteinase inhibition	Albumin denaturation	Membrane stabilisation
A. masticatorium 50µg/ml	31.56 ± 2.31[a]	39.51 ± 1.68[a]	33.86 ± 6.56[a]
A. masticatorium 100µg/ml	48.43 ± 5.84[b]	58.63 ± 2.22[b]	55.30 ± 7.15[b]
A. masticatorium 200µg/ml	92.33 ± 1.36[c]	89.06 ± 0.84[c]	91.72 ± 2.99[c]
Diclofenac 100µg/ml	90.21 ± 1.22[c]	91.83 ± 0.52[c]	94.40 ± 1.55[c]

Each value (mean ± S.E.) represents mean of three replicates. Means in a column followed by the same superscript letters are not significantly different ($p < 0.05$, one way ANOVA, DMR test).

TABLE 3: IC_{50} OF THE EXTRACT IN *IN VITRO* ANTI-INFLAMMATORY ASSAYS

Assay	IC_{50} value
Proteinase inhibition	52.80 ± 0.61
Albumin denaturation	34.15 ± 0.40
Membrane stabilisation	51.47 ± 1.56

The *in vitro* anti-inflammatory assays provide considerable evidence for the anti-inflammatory ability of the extract. Protein denaturation and inflammation are concomitantly related. Tissue damage during inflammatory reactions occurs due to the release of leukocytes proteinase [21]. Significant level of protection was provided by the extract which may have inhibited the activity of proteinase. The erythrocyte membrane is analogous to the lysosomal membrane [22, 23]. The extra cellular activity of lysosomal enzymes released during inflammation may lead to severe disorders. The extract might have stabilised lysosomal membrane thereby inhibiting the activity of these enzymes.

***In vivo* Anti-inflammation:** Carrageenan and formalin induced edema models are important assays for establishing the *in vivo* anti inflammatory activity of the extract.

Carrageenan Induced Inflammation: The extract significantly reduced inflammation in a dose dependent manner (**Fig. 4**). The extract inhibited carageenan induced edema formation by 51% at a concentration of 50mg/kg b. wt and by 68% at 200 mg/kg b. wt (**Table 4**). The extract showed significant inhibition starting at 3rd hour after the administration of the drug.

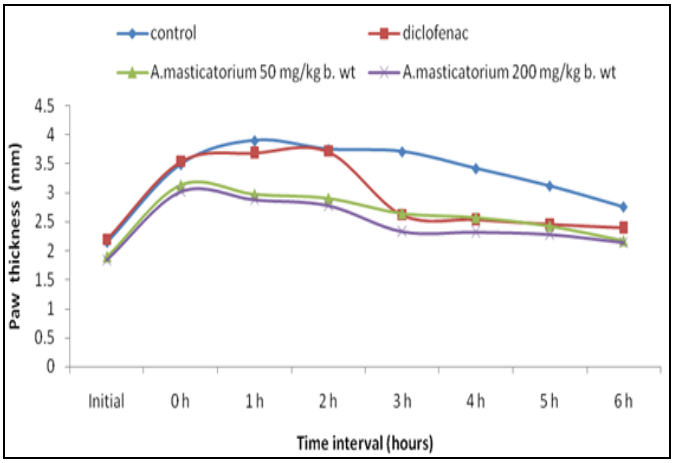

FIG. 4: GRAPH SHOWING THE EFFECT OF EXTRACT ON CARRAGEENEN INDUCED INFLAMMATION

TABLE 4: EFFECT OF THE EXTRACT ON CARRAGEENAN INDUCED PAW EDEMA IN MICE

Treatment groups	Initial paw edema thickness (mm)	Paw edema thickness at 3rd hour (mm)	Difference in thickness (mm)	% inhibition
Control	2.15 ± 0.01[b]	3.71 ± 0.03[c]	1.56 ± 0.04[b]	--
Diclofenac	2.20 ± 0.07[b]	2.62 ± 0.16[b]	0.04 ± 0.13[a]	73 ± 8.39[b]
A. masticatorium 50 mg/kg b. wt	1.89 ± 0.07[a]	2.64 ±0 .07[b]	0.75 ± 0.12[a]	51 ± 7.42[b]
A. masticatorium 200 mg/kg b. wt	1.84 ± 0.07[a]	2.33 ± 0.07[a]	0.49 ± 0.13[a]	68 ± 7.92[b]

Each value (mean ± S.E.) represents mean of five replicates. Means in a column followed by the same superscript letters are not significantly different ($p < 0.05$, one way ANOVA, DMR test).

Formalin Induced Inflammation: The extract also effectively inhibited formalin induced edema (**Fig. 5**). The extract at higher concentration showed 53.97% inhibition which was comparable with the effect of standard drug (**Table 5**).

Edema formation in the paw is the result of a synergism between various inflammatory mediators that increases vascular permeability and blood flow. The probable activity of the extract may be by the inhibition of inflammatory mediators such as histamine, serotonin and prostaglandin. Anti-inflammatory properties of plants are attributed to the presence of phenolics and flavonoids in them which is also responsible for their antioxidant properties. ROS is scavenged by the antioxidants thus decreasing the disorders.

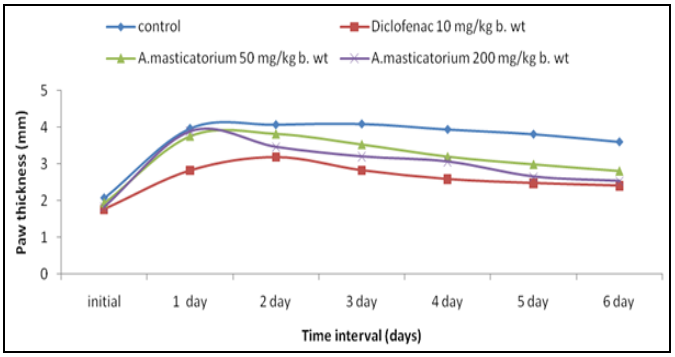

FIG. 5: GRAPH SHOWING THE EFFECT OF EXTRACT ON FORMALIN INDUCED INFLAMMATION

TABLE 5: EFFECT OF THE EXTRACT ON FORMALIN INDUCED PAW EDEMA IN MICE

Treatment groups Initial paw edema thickness (mm)		Paw edema thickness at 6th day (mm)	Difference in thickness (mm)	% inhibition
Control 2.08 ± 0.05[b]		3.61 ± 0.08[c]	1.53 ± 0.11[b]	---
Diclofenac 1.77 ± 0.06[a]		2.41 ± 0.08[a]	0.64 ± 0.11[a]	58.04 ± 6.95[a]
A. masticatorium 50 mg/kg b. wt	1.94 ± 0.04 [a,b]	2.81 ± 0.1[b]	0.87 ± 0.11[a]	43.01 ± 7.12[a]
A. masticatorium 200 mg/kg b. wt	1.84 ± .07[a]	2.54 ± 0.09[a]	0.70 ± 0.1[a]	53.97 ± 6.39[a]

Each value (mean ± S.E.) represents mean of three replicates. Means in a column followed by the same superscript letters are not significantly different ($p < 0.05$, one way ANOVA, DMR test).

CONCLUSION: The bioactivities of this species have been assessed for the first time. The extract has considerably high phenolic and flavonoid contenst. The therapeutic applications of flavonoids on inflammation have previously been reported [24]. Flavonoids and phenolics are also responsible for antioxidant activity. The anti-inflammatory property is highly related to the antioxidant capacity. This suggests that *A. mastcatorium* may be a potential source of chemical compounds which can be utilised in pharmaceutical industry.

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CONFLICT OF INTEREST: The authors declare that there are no conflicts of interest.

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