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Antioxidant and *in vitro* anti-inflammatory activities of *Mimusops elengi* leaves

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ABSTRACT

Objective: To assess the antioxidant and *in vitro* anti-inflammatory activities of the alcoholic extract of *Mimusops elengi* L (*M. elengi*) leaves. **Methods:** *In vitro* antioxidant activity was evaluated for peroxynitrite, superoxide and hypochlorous acid scavenging activity. Total phenolic content also determined. Inhibition of protein denaturation and HRBC (Human Red Blood Cell) membrane stabilization method was evaluated for anti-inflammatory activity. **Results:** The leave extract of *M. elengi* exhibited dose dependent free radical scavenging property in peroxynitrite, superoxide and hypochlorous acid models and the IC_{50} value were found to be (205.53 ± 2.30), (60.5±2.3), (202.4±5.3) μg/mL respectively. Total phenolic content was found to be 97.3 μg/mg of extract. The maximum membrane stabilization of *M. elengi* L was found to be (73.85±0.80)% at a dose of 1 000 μg/0.5 mL and that of protein denaturation was found to be 86.23% at a dose of 250 μg/mL with regards to standards in the anti-inflammatory activity. **Conclusion:** From the result it can conclude that *M. elengi* extract show good antioxidant and *in vitro* anti -inflammatory activities.

1. Introduction

Free radical is defined as unstable, highly reactive atom or molecule possessing unpaired electrons, which induces free radical damage. Reactive oxygen species (ROS) are widely believed to be involved in the etiology of many diseases such as ageing, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts and including inflammation as indicated by the signs of oxidative stress[1]. Inflammation is our body's natural reaction to invasion by an infectious agent, burn, toxin or physical, chemical or traumatic damage[2]. One purpose of inflammation is to protect the site of an injury.

Mimusops elengi (*M. elengi*) Linn. commonly known as Bakul belongs to the family Sapotaceae. It is a small to large evergreen tree found all over the different parts of India, Andaman Islands, Burma, Pakistan, Thailand and parts of Northern Australia. Several triterpenoids, steroids, steroidal glycosides, flavonoids and alkaloids have been reported from this species[3]. Phytochemical review shows the presence of taraxerol, taraxerone, ursolic acid, betulinic acid, V-spinosterol, W-sitosterol, lupeol, alkaloid isoretronecyl tiglate and mixture of triterpenoid saponins in the bark and hentriacontane, carotene and lupeol in the leaves, heartwood and roots of *M. elengi*[4]. The bark of *M. elengi* has been used for antioxidant activity[5], antibacterial[6], antiulcer[7], anti-inflammatory, analgesic and antipyretic activity[8] and leave of this plant used as antioxidant[9] and antidiabetic activity[10]. In the traditional use, leaves boiled and applied to the head as a cold compress for headache and juice of the leaves squeezed into the eye for sore eyes, together with bark of this plant has many therapeutic uses such as cardiotonic, alexipharmic, stomachic, anthelmintic and astringent[11]. The wide use of traditional medicine and plants still have represent a large source of natural anti-oxidants that might serve as leads for the development of the novel drugs. This plant has been used externally for rheumatism and pain by the local people of salipur, Cuttack districts in Odisa. However the leave part of this plant is not scientifically explored for its anti-inflammatory activity. Hence an effort has been made here to investigate the methanol extract of *M. elengi* of leave for its antioxidant and *in vitro* anti-inflammatory activity.

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2. Materials and methods

2.1. Collection and extraction of plant material

The fresh leaves of *M. elengi* was collected from Cuttack (District of Odisha) and identified by Dr Mondal, taxonomist at Botanical survey of India, West Bengal. The voucher specimen [CNH/1-1/05/2011/Tech-II] was deposited in Pharm. department herbarium for future reference. Before use, it was ensured that the leaves were free from contamination, sand and no microbial growth. The leaves were shade dried and was made into coarse powder using commercial blender. The powdered leave material was macerated with methanol (1:1 weight/volume) at room temperature for 4 d with occasional shaking, followed by remaceration for 3 d. After filtration, the filtrate was evaporated at 30 ℃ under reduced pressure in a rotary evaporator (Buchi R-210). The dry extract was kept in a refrigerator until use. Preliminary phytochemical analysis[12] revealed the presence of true alkaloids, triterpenes, steroids, flavonoids and saponins in *M. elengi* extract.

2.2. Drugs and chemicals

The chemicals used were Bovine serum albumin (BSA), Folin Ciocalteau (FC) reagent, Ascorbic acid, Diclofenac sodium was purchased from Sigma-Aldrich. All other chemicals and reagents used were of highest analytical grade.

2.3. Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the reference method[13] with slight modification. Briefly 1 mL (1 mg) of extract, 45 mL of distilled water and 1 mL FC were mixed in a conical flask and was shaken for 30 min. Then 3 mL of Na_2CO_3 (2%) were added to the mixture and shaken for 2 h at room temperature. The absorbance was measured at 760 nm against distilled water as blank. Total phenolic content was calculated according to the following formula.

Absorbance at 760 nm = 0.001× Pyrocatechol (μg) + 0.003 3

2.4. Antioxidant activity

2.4.1. Peroxynitrite scavenging

The scavenging activity was measured by using an Evans Blue bleaching assay. 1 mL reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM evans blue, different concentration of extract or standard ascorbic acid and 1 mM freshly prepared peroxynitrite. The absorbance was measured after 30 min of incubation at 25 ℃. The concentration of $ONOO^-$ was measured spectrophotometrically at 302 nm (€ = 1 670 $M^{-1}\bullet cm^{-1}$). The percentage scavenging of $ONOO^-$ was calculated by using the formula[14].

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.4.2. Superoxide radical scavenging

Reaction mixture contains 1 mL of nitroblue tetrazolium (NBT, 156 μM), 1 mL of reduced nicotinamide adenine dinucleotide (NADH, 468 μM) and 3 mL of the test solution or standard ascorbic acid at different concentrations. The reaction was initiated by adding 100 μL of phenazine methosulphate (PMS, 60 μM) and incubated at 25 ℃ for 5 min. All the solutions were prepared in 100 mM phosphate buffer (pH 7.4). Then absorbance was measured at 560 nm and the percentage inhibition was calculated by using the above mentioned formula.

2.4.3. Hypochlorous acid scavenging

Hypochlorous acid (HOCl) was freshly prepared adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 $M^{-1}\bullet cm^{-1}$[15]. The reaction mixture contained, 1.5 mM of HOCl and different concentration of the extract or standard ascorbic acid and incubated for 1 h at 37 ℃. After that taurine (30 mM) was added and incubate again 30 min at 37 ℃ followed by the addition of thionitro benzoic acid (TNB). Absorbance was measured at 412 nm against blank and % scavenging was calculated according to the standard formula.

2.5. In vitro anti-inflammatory activity

2.5.1. Inhibition of protein denaturation method

Test solution (0.5 mL) consist of 0.45 mL of BSA (5% w/v aqueous solution) and 0.05 mL of test solution (250 μg/mL).

Test control solution (0.5 mL) consists of 0.45 mL of BSA (5% w/v aqueous solution) and 0.05 mL of distilled water.

Product control solution (0.5 mL) consists of 0.45 mL of distilled water and 0.05 mL of test solution (250 μg/mL).

Standard solution (0.5 mL) consists of 0.45 mL of BSA (5%w/v aqueous solution) and 0.05 mL of diclofenac sodium (250 μg/mL).

All the above solutions were adjusted to pH 6.3 using 1N hydrochloric acid. The samples were incubated at 37 ℃ for 20 min and the temperature was increased to keep the samples at 57 ℃ for 3 min. After cooling, 2.5 mL of phosphate buffer saline was added to the above solutions. The absorbance was measured using UV Visible spectrophotometer at 416 nm[16]. The percentage inhibition of protein denaturation was calculated as,

$$\text{Percent Inhibition} = 100 - \frac{\text{O.D of test solution} - \text{O.D of product control}}{\text{O.D of test control}} \times 100$$

The control represents 100% protein denaturation. The results were compared with diclofenac sodium (250 μg/mL).

2.5.2. HRBC membrane stabilization method

The principle concerned in this method is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. Blood was collected (2 mL) from healthy volunteers and was mixed with equal volume of sterilized Alsevers solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl in distilled water) and centrifuged at 3 000 rpm. The packed cells were washed with isosaline

solution and a 10% v/v suspension was prepared with normal saline and kept at 4 ℃ undisturbed before use. Different concentrations of *M. elengi* extract (50, 100, 200, 500 and 1 000 μg/0.5 mL) in normal saline, diclofenac sodium as standard (50, 100, 200, 500 and 1 000 μg/0.5 mL) and control (distilled water instead of hypo saline to produce 100% hemolysis) were separately mixed with 1 mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of 10% HRBC suspension was added to prepared. All the assay mixtures were incubated at 37 ℃ for 30 min and centrifuged at 3 000 rpm for 20 min and hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm[17]. The percentage of HRBC membrane stabilization or protection was calculated by using the following formula

$$\text{Percentage Stabilization} = \frac{\text{Absorbance of control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

2.6. Statistical analysis

The experimental data were expressed as mean ± SEM. The inhibitory concentrations 50% (IC_{50}) was calculated by plotting the data in the graph as concentration versus percentage inhibition using Graph Pad Prism software, version 5.

3. Result

3.1. Total phenol contents

Total phenolic content was determined using the Folin-Ciocalteau (FC) reagent and it was calculated as 97.3 μg/mg of *M. elengi* extract which is accounted for its free radical as well as antioxidant activity.

3.2. Antioxidant activity

3.2.1. Peroxynitrite scavenging

The peroxynitrite scavenging activity was found (Figure 1) in a dose dependent manner as compared to standard ascorbic acid. The IC_{50} values (Figure 4) were found to be (205.53 ± 2.30) μg/mL and (56.88 ± 2.32) μg/mL for *M. elengi* extract and standard respectively.

3.2.2. Superoxide radical scavenging

Superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The increase in inhibition capability indicates the extract has good superoxide radicals scavenging activity as compared to standard ascorbic acid (Figure 2). The IC_{50} values of *M. elengi* extract and ascorbic acid were found to be (60.5±3.5) μg/mL and (8.61 ± 1.30) μg/mL (Figure 4) respectively.

3.2.3. Hypochlorous acid scavenging

Dose-dependent hypochlorous acid scavenging activity of *M. elengi* extract and standard ascorbic acid was found in this study (Figure 3). The IC_{50} values (Figure 4) of extract and standard were found to be (202.4 ± 5.3) μg/mL and (150.47 ± 3.23) μg/mL respectively.

Table 1.
Effect of alcoholic extract of *M. elengi* on HRBC membrane stabilization (mean±SEM).

Sl. No	Concentration (μg/mL)	*M. elengi* extract stabilization (%)	Diclofenac sodium stabilization (%)
1	50	62.20±1.20	78.41±0.20
2	100	65.33±0.40	81.21±0.40
3	200	61.62±1.20	82.65±0.30
4	500	68.56±1.30	84.75±0.10
5	1 000	73.85±0.80	94.23±0.50

Values represent the mean of triplicates.

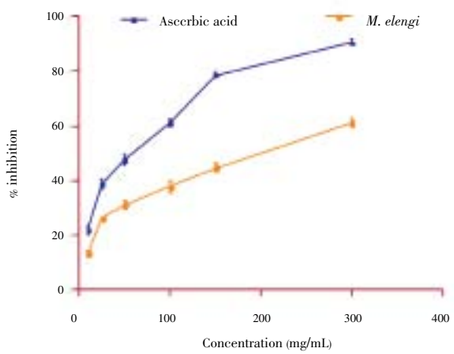

Figure 1. Peroxynitrite anion scavenging of alcoholic extract of *M. elengi* and standard Ascorbic acid. The data represent the percentage of peroxynitrite anion inhibition. Each point represents the values obtained from three experiments, performed in triplicate (mean ± SEM).

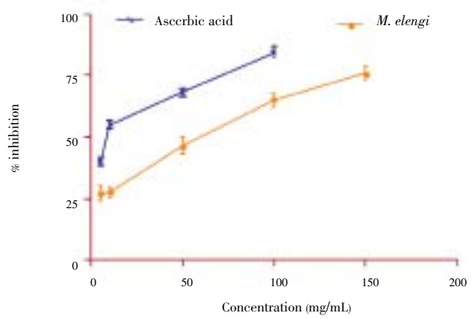

Figure 2. Superoxide radical scavenging of alcoholic extract of *M. elengi* and standard Ascorbic acid. The data represent the percentage superoxide inhibition. Each point represents the values obtained from three experiments, performed in triplicate (mean ± SEM).

3.4. In vitro anti-inflammatory activity

3.4.1. Inhibition of protein denaturation method

The *M. elengi* exhibited maximum inhibition of protein denaturation of (86.23±0.8)% at 250 μg/mL and its effect was compared with the standard anti inflammation drug, diclofenac sodium showed the maximum inhibition (94.22± 0.30)% at the same concentration.

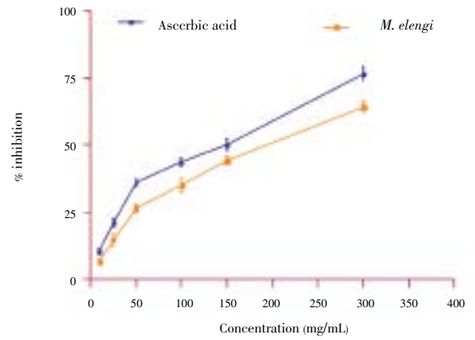

Figure 3. Hypochlorous acid scavenging of alcoholic extract of *M. elengi* and standard Ascorbic acid. The data represent the percentage of HOCl inhibition. Each point represents the values obtained from three experiments, performed in triplicate (mean ± SEM).

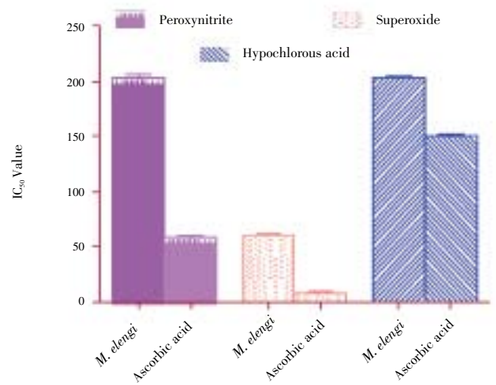

Figure 4. The IC_{50} values of *M. elengi* extract and Ascorbic acid (standard) in peroxynitrite, superoxide and hypochlorous acid are (205.53 ± 2.30) μg/mL and (56.88 ± 2.32) μg/mL, (60.5 ± 3.5) μg/mL and (8.61 ± 1.30) μg/mL, (202.4 ± 5.3) μg/mL and (150.40 ± 3.23) μg/mL respectively. The results are mean ± SEM of three experiments.

3.4.2. In vitro anti-inflammatory activity

In the *in vitro* anti-inflammatory activity screening it was observed that *M. elengi* extract showed significant activity when compared to the standard diclofenac sodium. The percentage of stabilization was found to be (73.85±0.80)% and (94.23±0.50)% at concentration of 1 000 μg/mL of *M. elengi* extract and standard respectively. The activity of the extracts were concentration dependent, with the increasing concentration the activity is also increased. These results may be attributes due to the presence of phenolic content, good antioxidant properties.

4. Discussion

The data presented in this study demonstrate that *M. elengi* L extract possess antioxidant and *in vitro* anti-inflammatory activity. Indeed, *M. elengi* extract scavenged peroxynitrite, superoxide and hypochlorous acid in a concentrated dependent manner.

Peroxynitrite ($ONOO^-$) is relatively stable and toxic compound compared to other free radicals but once protonated it forms the highly reactive peroxynitrite acid (ONOOH). Generation of excess ONOO- leads to oxidative damage and tissue injury[18]. According to the present results, the *M. elengi* extract inhibits evans blue bleaching by scavenging peroxynitrite.

The most important ROS produced by the inflammatory cells is superoxide, hydrogen peroxide, hydroxyl radical and hypochlorous acid. Membrane bound NADPH oxidase reduce the molecular oxygen to produce the superoxide anions which intern converted to hydrogen peroxide and hydroxyl radical as well as hypochlorous acid by using different enzymatic reaction in our body[19]. In this present study, *M. elengi* extract exhibited concentration-dependent increasing of superoxide radical scavenging activity.

At sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of another harmful HOCl which have the ability to react with the taurine and the resultant compound decrease the color of TNB[20], *M. elengi* extract showed dose dependent inhibition of HOCl.

These observed antioxidant activities suggest that the alcoholic extract *M. elengi* extracts was found to possess concentration dependent scavenging activity on peroxynitrite, superoxide and hypochlorous acid. It could exert protective effects also *in vivo* against oxidative and free radical injuries occurring in different pathological conditions, which may be potentially responsible for its anti-inflammatory activity.

The presence of high phenolic content in the *M. elengi* extract has been observed by FC reagent test and also from phytochemical test, it was also found as a good source of flavonoids and triterpenes. Therefore the anti-inflammatory activity of *M. elengi* extract of seems to be due to the phenolic compounds in it[21].

Denaturation of protein is one the cause of inflammation. The production of auto antigens in inflammation disease may be due to *in vivo* denaturation of protein. The mechanism of denaturation possibly involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding[22]. From the result, it can be stated that the extracts of *M. elengi* was capable of controlling the production of auto antigen and thereby it inhibit the denaturation of proteins and its effect was compared with the standard drug diclofenac sodium.

M. elengi L. leaves extracts exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the liposomal membrane[23] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases,

which cause further tissue inflammation and damage upon extra cellular release[24]. Though the exact mechanism of the membrane stabilization by the extract is not known yet; hypotonicity-induced hemolysis may arise from shrinkage of the cells due to osmotic loss of intracellular electrolyte and fluid components or interaction with membrane proteins[25,26]. The extract may inhibit the processes, which may stimulate or enhance the efflux of these intracellular components. On the basis of the above results it can be concluded that the *M. elengi* extracts have an anti-inflammatory activity.

The present investigation has shown that the alcoholic extract of *M. elengi* exhibited concentration dependent free radical scavenging activity. These activities due to strong occurrence of phenolic compounds such as flavonoids, tannins, terpenoids, phenols and saponins.This antioxidant effect may be responsible for *in vitro* anti-inflammatory activity of the alcoholic extract of *M. elengi*. Studies are in progress in order to isolate and identify some active compounds which might be responsible the activity, also to understand the exact mechanism of action in relation to the observed anti-inflammatory activity.

Conflict of interest statement

We declare that we have no conflict of interest

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