



Research Article

EVALUATION OF ANTI-INFLAMMATORY POTENTIAL OF ETHANOLIC EXTRACT OF THE LEAVES OF RHIZOPHORA MUCRONATA, A SUNDERBAN MANGROVE

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ABSTRACT

Objective: Biochemically unique mangrove plants are recognized worldwide for valuable medicinal actions. Experimental investigations of these pharmacological activities are needed for elucidation of cost effective new therapeutic compound with better efficacy and less toxicity. Present study evaluated the *in-vitro* and *in-vivo* anti-inflammatory activity of the ethanolic extract of the leaves of Sunderban mangrove, *Rhizophora mucronata* Lam.

Method: The leaves were collected from Sunderban, identified and extracted with ethanol. The phytochemical analysis of the extract, preliminary antioxidant studies were done and impact of the extract (RME) on nitric oxide production was assessed in lipopolysaccharide (LPS) stimulated RAW264.7 macrophage cell line. The anti-inflammatory activity was studied in Carrageenan induced acute and Freund's adjuvant induced chronic inflammatory experimental animal model.

Result: Present study showed that RME significantly scavenged free radicals and significantly inhibited nitrite accumulation in cell line. Quercetin is an important flavonoid present in the extract. The extract reduced the rodent paw edema in acute and chronic inflammation models. In the chronic study, the extract in 100mg/kg dosage orally markedly reduced (44.44%) paw edema than control, compared to the standard Diclofenac sodium (33.33%).

Conclusion: Study revealed the significant free radical scavenging and anti-inflammatory activity of the ethanolic extract of *Rhizophora mucronata* Lam. leaves. Further studies including isolation of active principle will assess the mechanisms involved in the observed activities.

Keywords: Inflammation, antioxidant, mangrove, Rhizophora.

INTRODUCTION

Inflammation is a complex cellular interaction process, which may be developed in response to any infectious, traumatic, toxic or autoimmune injuries.^[1] Chronic inflammatory conditions are widely recognized as the root of severe

diseases, from heart disease to diabetes and cancer.^[2]

Modern therapeutic approach for treating inflammation includes steroidal and non-steroidal medication (NSAIDs).^[3]

Though NSAIDs are the most prescribed and most commonly used over the counter (OTC) drugs for treating pain and

inflammation, but all NSAIDs can cause serious side-effects like duodenal ulcers, gastrointestinal bleeding, kidney failure, heart attacks, strokes etc. These harmful adverse effects of NSAIDs are accountable for many cases of hospitalizations and deaths every year.^[4] Because of these morbidity, incurring healthcare costs and side effect profiles of the commercially accessible synthetic medications, there is always a need for search of natural compounds with better efficacy and less toxicity. Research is in progress worldwide for the pharmacological analysis of numerous plants utilized in the traditional system of medicine for treatment of various chronic ailments. World Health Organization (WHO) has estimated that more than 75% of the world's total population depends on herbal or plant based medication for their primary healthcare needs.^[5]

Mangrove forests are a special type of vegetation found within the coastal regions of the tropical and subtropical parts of the world. Like other terrestrial plants, many mangrove plants have ethnopharmacological relevance and have been exploited by the native individuals for remedies for different ailments. They produce biochemically distinctive wide array of novel natural product, recognized worldwide for beneficial activity in many pathological conditions like inflammation, hypertension, diabetes, gastroduodenal ulcers, neurological disorders, liver diseases etc.^[6,7]

Rhizophora mucronata (Rhizophoraceae) is a typical mangrove plant, occurs on the coasts of the Indian Ocean and also the West-Pacific and abundant in the Gangetic mangrove forest within the Sunderban, West Bengal, India. It is a very popular medicinal plant that has been therapeutically utilized in the treatment of various diseases. In South Asian countries including India, the various parts like root, bark, leaves, fruit and flowers of the plant are used as ethnomedicine for treating diabetes, diarrhea, hepatitis, inflammation, wounds and ulcers, etc.^[7] To further substantiate and elucidate the medicinal activity and to validate the ancient ethno medicinal uses of this plants, experimental screening method is vital to validate the effectiveness and also to determine the active component of the herbal products, Present study was conducted to evaluate the preliminary *in-vivo* anti-inflammatory activity of the ethanolic extract of the leaves of Sunderban mangrove, *Rhizophora mucronata* Lam.

MATERIALS & METHODS

Materials

Ethanol (Merck), methanol (Merck), toluene (Merck), ethyl acetate (Merck), formic acid (Merck), acetic acid (Merck), acetonitrile (Merck), RPMI 1640 (Gibco), L-glutamine (SRL), penicillin, streptomycin, fetal bovine serum (Gibco), 3-(4,5-dimethylthiozol-2-il)-2,5-dipheniltetrazoliumbromide/MTT (SRL), dimethyl sulfoxide (SRL), lipopolysaccharide (Sigma), sodium chloride (Merck), potassium chloride (Merck), di-sodium hydrogen phosphate (Merck), potassium di-hydrogen phosphate (Merck), 2, 2- Diphenyl-1-picrylhydrazyl/DPPH (Sigma), sodium pyrophosphate (Merck), phenazine methosulphate (SRL), nitroblue tetrazolium (Himedia), nicotinamide adenine dinucleotide (reduced) disodium salt (SRL), Carrageenan (Sigma), Freund's complete adjuvant (Sigma), diclofenac sodium tablet (Cipla). Chemicals and reagents are of analytical grade.

Test sample

Ethanolic extract of *Rhizophora mucronata* Lam. leaves (RME)

Cell line

RAW264.7 murine macrophage cell line

Animals

In-vivo experiments were carried out on Wistar albino rats weighing 110-150gm. The animals were housed under conditions of $22 \pm 1^\circ\text{C}$, $50 \pm 10\%$ humidity and 12 hours light and 12 hours dark cycle. The animals received a diet of food pellets (fortified with minerals and vitamins) purchased from authenticated vendor and water ad libitum. The animal experiments were conducted in accordance with the accepted principles for laboratory animal use and care (CPCSEA) after getting clearance from Institutional Animal Ethics Committee of R. G. Kar Medical College, Kolkata (RKC/IAEC/13/17/1 dated 17.11.2013).

METHODS

Collection and preparation of Test Sample

Rhizophora mucronata leaves were collected from the mangrove area of Sunderban, West Bengal in the month of October 2013. The leaves were identified from Botanical Survey of India, Howrah, West Bengal as *Rhizophora mucronata* Lam. (CNH/55/2013/Tech.II/19 dated 02.12.2013). The leaves were shed dried in room temperature and pulverized with a mechanical grinder. These were then extracted with ethanol in Soxhlet apparatus.

The solvent was evaporated in room temperature and the dried extract was kept in a tight container.

Phytochemistry

UV spectral analysis of *Rhizophora mucronata* leaves

Studies showed that both the fresh juice and ethanolic extract of *Rhizophora mucronata* Lam. leaves contain flavonoids, polyphenols, glycosides, terpenoids and good amount of tannins.^[8,9] In present study the UV spectral analysis of the extract was done. The powdered *R. mucronata* leaves was dissolved in hydro-ethanol (20:80) for 24 hour and filtered through Whatmann No. 1 filter paper. The filtrate was diluted at the concentration of 100µg/ml and 500 µg/ml and was examined under ultraviolet and visible light for spectral analysis. The extracts were scanned in the wavelength ranging from 280-600λ using UV-VIS Spectrophotometer (LMSP-UV 1000 B, Labman Instruments, India) and the characteristic peaks were detected. Each and every analysis was repeated four times for the spectrum confirmation.^[10]

High performance thin layer chromatography (HPTLC) analysis

The hydro-ethanolic (20:80) extract of *R. mucronata* leaves was dissolved in methanol and filtered by milipore sample filtration unit. The sample was prepared in a concentration of 1mg/ml and was spotted in the form of bands with on a precoated silica gel plates (Merck, 60F254, 20 x 20 cm) using Camag Linomat 5 applicator. The plates developed in a solvent system (toluene : ethyl acetate : formic acid = 4.5:3:0.2) for 30 min. Quercetin (3,4,5-5,7,3',4'-tetrahydroxy flavonol) was used to as bio-marker. The densitometric scanning was performed on Camag TLC Scanner 3 at absorbance 280 nm (D2 lamp) and operated by multi level winCATS planar chromatography manager.^[8,11]

High Performance Liquid Chromatography (HPLC) analysis with ethanol extract

The hydro-ethanolic (20:80) extract was dissolved in the mobile phases. After filtering through a filter paper and a 0.45 mm membrane filter (Millipore), the extract was injected into HPLC. The phenolic acids and flavonoids were analysed using Dionex Ultimate 3000 liquid chromatography (Germany) with photo Diode Array UV detector. Chromatographic analysis was carried out using a Acclaim™ 120 C18 column (5µm particle size, i.d. 4.6x250 mm) at

28°C. The solvent A (1% aq. acetic acid solution) and solvent B (acetonitrile) were used as mobile phase. Flow rate was 0.7 ml/min. 20µl sample was injected and the detection wavelength was 310 nm.^[12]

Free radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants. The antioxidant studies were evaluated with the ethanolic extract of *Rhizophora mucronata* leaves, various concentrations were prepared by dissolving in 80% methanol.^[13] DPPH solution (0.002% w/v) was prepared in methanol. 1ml of various concentrations of extracts were mixed with 1ml DPPH solution, colour factor for each concentration were also prepared without DPPH. Reactions mixtures were incubated in dark for 30mins at room temperature. Then absorbance was measured at 517 nm. The percentage inhibition was calculated for each concentration of RME, compared with the control and the IC50 value was determined. Every analysis was repeated four times.

Percentage inhibition was calculated as= $\frac{\{(\text{absorbance of blank} - (\text{absorbance of sample} - \text{colour factor}))\}}{(\text{absorbance of blank})} \times 100$

Superoxide radical scavenging activity

The superoxide radical scavenging activity of the ethanolic extract of *Rhizophora mucronata* leaves was performed following the method of Kakkar et al. with minor modifications.^[14] Briefly, 1.2 ml of sodium pyrophosphate buffer (0.052 mM; pH 8.3), 0.3 ml of 80% hydro-methanolic extract of *Rhizophora mucronata* leaves with different concentrations, 0.1 ml of 186µM phenazine methosulphate (PMS) and 1ml of distilled water were added to each reaction mixture. Thereafter 0.3ml of 300 µM nitroblue tetrazolium (NBT) and 0.2 ml of nicotinamide adenine dinucleotide (reduced) disodium salt i.e. NADH (780 µM) was added to start the reaction. The reaction mixtures were incubated in dark at room temperature for 90 seconds and reactions were stopped by adding 1 ml of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560 nm. The percentage inhibition was calculated for each concentration of RME,

compared with the control and the IC50 value was determined. Every analysis was repeated four times.

Percentage inhibition = $\frac{[(\text{absorbance of blank} - \text{absorbance of sample}) / (\text{absorbance of blank})] \times 100}{1}$

In-vitro anti-inflammatory study in RAW264.7 cell line

Macrophages are important components of the mammalian immune system, and they play a key role by providing an immediate defense against foreign agents prior to leukocyte migration and production of various pro-inflammatory mediators including the short-lived free radical NO. Lipopolysaccharide (LPS), a component from the cell walls of gram-negative bacteria is one of the most powerful activators of macrophages and involves the production of pro-inflammatory cytokines. Therefore, inhibition of NO production in LPS stimulated RAW 264.7 cells is one of the possible ways to screen various anti-inflammatory drugs.^[15]

Cell cytotoxicity study by MTT assay

The in-vitro cell culture studies were done with RAW264.7 murine macrophage. The cell line was purchased from National facility for animal tissue and cell culture, Pune. For cytotoxicity study RAW264.7 murine macrophage cell in a log phase were seeded in 96 well tissue culture plate 100µl cell suspension per well, at a concentration of 1x10⁵ cells/ml and allowed to adhere for 24hrs and then the medium was replaced with fresh medium. The ethanolic extract of *Rhizophora mucronata* leaves (RME) was dissolved in sterile phosphate buffer saline (PBS) to prepare 1mg/ml stock solution, sterilized by using membrane filter and from that sample was added to cells in different concentrations of 25, 50, 100, 200, and 400µg/mL for 24hrs and kept at 37°C in a humidified atmosphere containing 5% CO₂ in air. Thereafter, 20µl of MTT (5mg/ml in PBS as stock solution) was added to each well and incubated for another 4hrs. The MTT assay is colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. DMSO (dimethyl sulfoxide) 100µl was added to dissolve the insoluble purple formazan product into a colored solution. The absorbance was taken at 570nm by micro plate manager (Reader type: Model 680 XR Bio-Rad laboratories Inc).^[15,16] Every analysis was repeated four times.

Nitric oxide assay with lipopolisaccharide (LPS) induction in RAW264.7 cell line

The cells were harvested and seeded in a 96 well plate with 1x10⁵ cells per well and allowed to adhere for 24hrs. After that the medium was replaced with fresh medium containing 1µg/ml of lipopolisaccharide (Sigma) for sensitization. The ethanolic extract of *Rhizophora mucronata* leaves (RME) was added to cells in different concentrations 25, 50, 100, 200, and 400 µg/mL from 1mg/ml stock solution. The plate was then incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air for 24hrs. Thereafter Griess reagent was added to the plate and purple colour was formed. Absorbance was measured at 570nm. The accumulation of nitrite in the culture supernatant was directly proportional with the NO production and measured using Griess reagent.^[15,16] Every analysis was repeated four times. Percentage inhibition = $\frac{(A-B)}{(A-C)} \times 100$ [A= absorbance of LPS+cell, B= absorbance of LPS+cell+sample, C= absorbance of only cell]

In-vivo anti-inflammatory study

Carrageenan induced acute inflammatory model

Acute anti-inflammatory study was performed in Carrageenan induced rat paw edema model. Male Wistar rats weighing 110gm-150gm was taken and were divided into six groups.

Group I: Negative control/ Normal control

Group II: Positive control/ Inflammatory control (distilled water 0.1ml/100gm orally).

Group III: Standard drug diclofenac sodium (10mg/kg body weight orally)

Group IV: Ethanol extract of *Rhizophora mucronata* leaf (RME) 50 mg/kg b.w. orally

Group V: Ethanol extract of *Rhizophora mucronata* leaf (RME) 100 mg/kg b.w. orally

Group VI: Ethanol extract of *Rhizophora mucronata* leaf (RME) 200 mg/kg b.w. orally

Initial paw volumes of the rats were measured on 0 hour by using plethysmometer and the drug treatment was done as above. After 30mins, 0.1ml/kg body weight Carrageenan from a 10mg/ml solution was injected into the sub-plantar region of the left hind paw of all the rats.^[17] Thereafter the paw volume of each rat was measured in every 1hr interval for the next 4 hours. The initial and final volume of the left hind paw of each rat was calculated, the differences in

volume were measured and the % inhibition was calculated for each group compared with the control.

Percentage inhibition = $[(\text{final paw volume of control} - \text{final paw volume of sample}) / (\text{final paw volume of control})] \times 100$

Freund's adjuvant induced chronic inflammation model

Rats were divided into five groups of 6 animals in each group. On day 0, 0.1 ml of Freund's complete adjuvant was injected into the plantar pad of each rat of group II to group V. Group I served as negative control or untreated normal rats, group II is the positive control received 0.1ml/100gm of distilled water orally, group III received 10mg/kg diclofenac sodium orally and rats of group IV and V received the two effective dose of ethanol extract of *Rhizophora mucronata* leaves (RME) found in acute study i.e. 100 mg/kg and 200 mg/kg b.w. orally respectively for 28 consecutive days. Body weight, paw volume for each group was measured using plethysmometer on day-0 before administration of adjuvant and on day 1, 7th, 14th, 21st and 28th day after drug treatment. The percentage inhibition was thereby calculated after the end of the study. On 28th day of the experiment, all rats were sacrificed after blood collection. The inflamed paws of the rats were cut from the ankle joint, the tissues were fixed in 10% formalin and histopathological examination was done. Sections were stained with hematoxylin and eosin (H&E) and examined under the light microscope.^[17,18]

Percentage inhibition = $[(\text{paw volume of control on 28th day} - \text{paw volume of sample on 28th day}) / (\text{paw volume of control on 28th day})] \times 100$

STATISTICAL ANALYSIS

The data were represented as mean \pm SEM. Measured parameters were compared among different groups using one way ANOVA followed by Dunnett's Post hoc test. All analyses were done by SPSS software and significance level was accepted at $p < 0.05$.

RESULT

Phytochemistry

UV spectral analysis of hydro-ethanolic extract of *Rhizophora mucronata* leaves

The qualitative UV-VIS spectrum profile of *Rhizophora mucronata* ethanolic extract was selected at wavelength from 190 to 700 nm. The ethanolic extract in two different concentrations 100 μ g/ml and 500 μ g/ml showed the highest

peak at same wavelength 360nm and the overall absorption pattern was same (Figure 1).

High performance thin layer chromatography (HPTLC) analysis

HPTLC analysis with the hydro-alcoholic extract of *Rhizophora mucronata* leaves (RME) was done and the bands were compared with quercetin (flavonoids) (Figure 2). The presence of Quercetin was observed in the RME.

High performance liquid chromatography (HPLC) analysis

The HPLC analysis also showed presence of quercetin (625.10 μ g/g dry leaves) in *R. mucronata* leaves. The other compound detected was kaempferol (109.2 μ g/g dry leaves). The relative area of chromatogram for quercetin and kaempferol was 85.75% and 14.25% respectively (Figure 3).

Free radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl(DPPH) radical scavenging activity

The ethanol extracts of *R. mucronata* leaves significantly inhibited the DPPH radical in a dose dependant manner (Figure 4). RME exhibited a concentration dependant inhibition of the free radical. The IC₅₀ values of aqueous ethanolic extract were found to be 127.5 μ g/ml. The results indicate that the ethanol extract processes potent DPPH scavenging activities.

Superoxide radical scavenging activity

The plant extract RME in concentrations of 50 to 1000 μ g/ml showed the superoxide radical scavenging activity ranged from 13.8% to 82.7% (Figure 5). The plant extract exhibited a dose dependant superoxide radical scavenging activity (IC₅₀=327 μ g/ml).

In-vitro anti-inflammatory activity in RAW264.7 cell line

Cell cytotoxicity study by MTT Assay

The effect of the ethanolic extract *Rhizophora mucronata* leaves (25, 50, 100, 200 and 400 μ g/ml) on viability of un-stimulated RAW264.7 cells was tested using the MTT assay. Treatment with RME had no significant inhibition of cell growth, indicating these doses were nontoxic to RAW264.7 cells.

Nitric oxide (NO) assay with lipopolisaccharide (LPS) induced RAW264.7 cell line

In the present study, the ethanolic extract of *Rhizophora mucronata* leaves were evaluated for the inhibition of NO production in the LPS-stimulated RAW264.7 cells. The RAW264.7 cells were activated by LPS.

Table 1: Percentage inhibition of Nitric oxide (NO) production after treatment with ethanolic extract of *Rhizophora mucronata* leaves (RME) in LPS-stimulated RAW264.7 cells

Concentration (g/ml)	Inhibition of NO production (in %)
RME 25 g/ml	40%
RME 50 g/ml	58.33% *
RME 100 g/ml	78.33% *
RME 200 g/ml	96.66% *
RME 400 g/ml	110% *

Data are mean \pm S.E.M (n=4). The data were analyzed by one way ANOVA followed by Dunnett test, * denotes level of significance $p < 0.05$ RME- *Rhizophora mucronata* Lam. leaf ethanolic extract

Table 2: *In-vivo* anti-inflammatory activity of the ethanolic extract of *Rhizophora mucronata* leaves in Carrageenan induced rat paw edema model

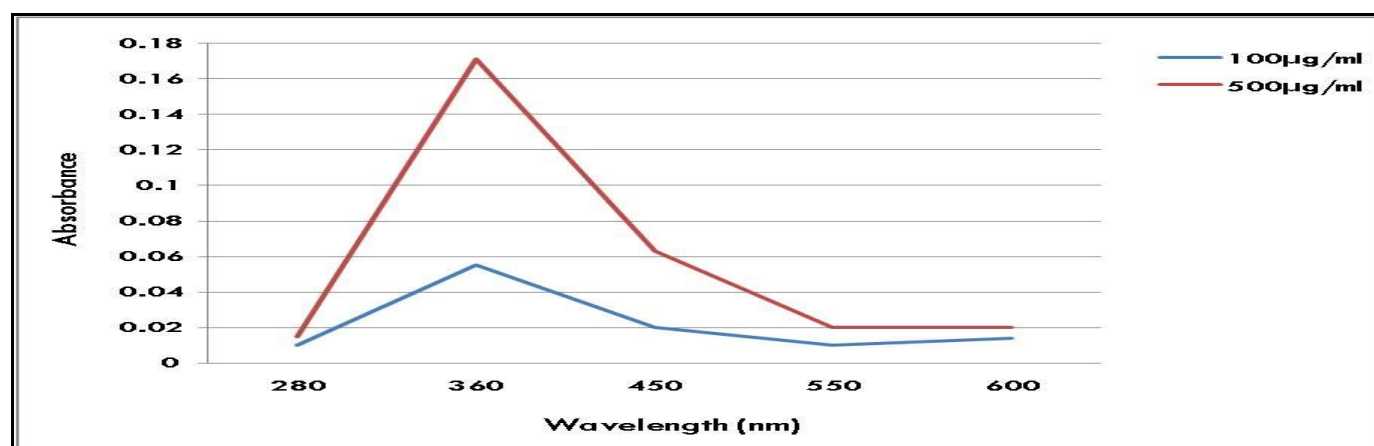
Groups	Time (in hours)				
	0 hour	1 hour	2 hour	3 hour	4 hour
Negative control	2 \pm 0	2 \pm 0	2 \pm 0	2 \pm 0	2 \pm 0
Positive control	2 \pm 0	4 \pm 0.5	4.5 \pm 0.33	4.33 \pm 0.17	4.33 \pm 0.25
Diclofenac sodium 10mg/kg	2 \pm 0	3.67 \pm 0.25	3.17 \pm 0.33 *	2.67 \pm 0.29 *	2.5 \pm 0.33 *
RME 50mg/kg	2 \pm 0	3.67 \pm 0.5	3.5 \pm 0.25	3.83 \pm 0.44	3.83 \pm 0.25
RME 100mg/kg	2 \pm 0	3.67 \pm 0.33	3.33 \pm 0.29	3.42 \pm 0.33	3.33 \pm 0.5
RME 200mg/kg	2 \pm 0	4.33 \pm 0.25	4.33 \pm 0.25	4 \pm 0	3.7 \pm 0.33

Data are mean \pm S.E.M (n=6). The data were analyzed by one way ANOVA followed by Dunnett test, * denotes level of significance $p < 0.05$ RME- *Rhizophora mucronata* Lam. leaf ethanolic extract

Table 3: Body weight distribution profile of different groups in Freund's adjuvant induced chronic inflammatory model

Groups	0 th day	15 th day	28 th day
Negative control	125 \pm 0	135 \pm 0.38	146 \pm 0.39
Positive control	137.5 \pm 0.43	128 \pm 0.49	115 \pm 0
Diclofenac sodium (10mg/kg)	130 \pm 0.39	120 \pm 0.46	120 \pm 0.41
RME 100mg/kg	123.33 \pm 0.53	135 \pm 0.46	138.33 \pm 0.37
RME 200mg/kg	132.5 \pm 0.39	145 \pm 0.42	147.5 \pm 0.37

Data are mean \pm S.E.M (n=6). The data were analyzed by one way ANOVA followed by Dunnett test, $p < 0.05$ RME- *Rhizophora mucronata* Lam. leaf ethanolic extract

**Figure 1:** UV spectral analysis of two different concentrations of ethanolic extract of *Rhizophora mucronata* leaves in different wavelength

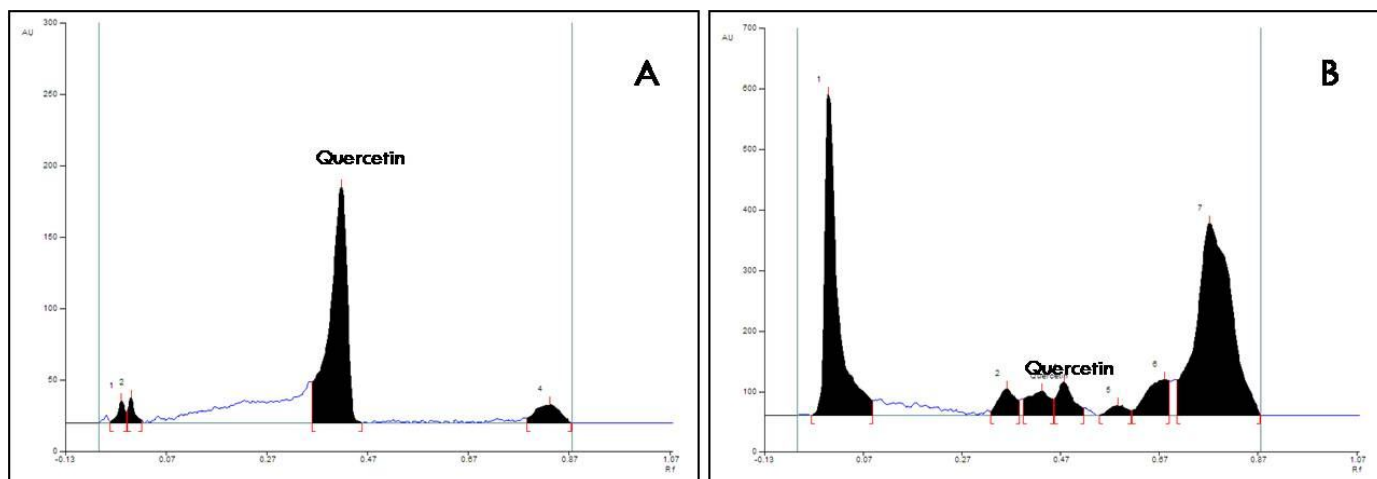


Figure 2: HPTLC chromatogram of flavonoid standard quercetin [A] and hydro-alcoholic extract of *Rhizophora mucronata* Lam. leaves [B] showing different peaks of phytoconstituents

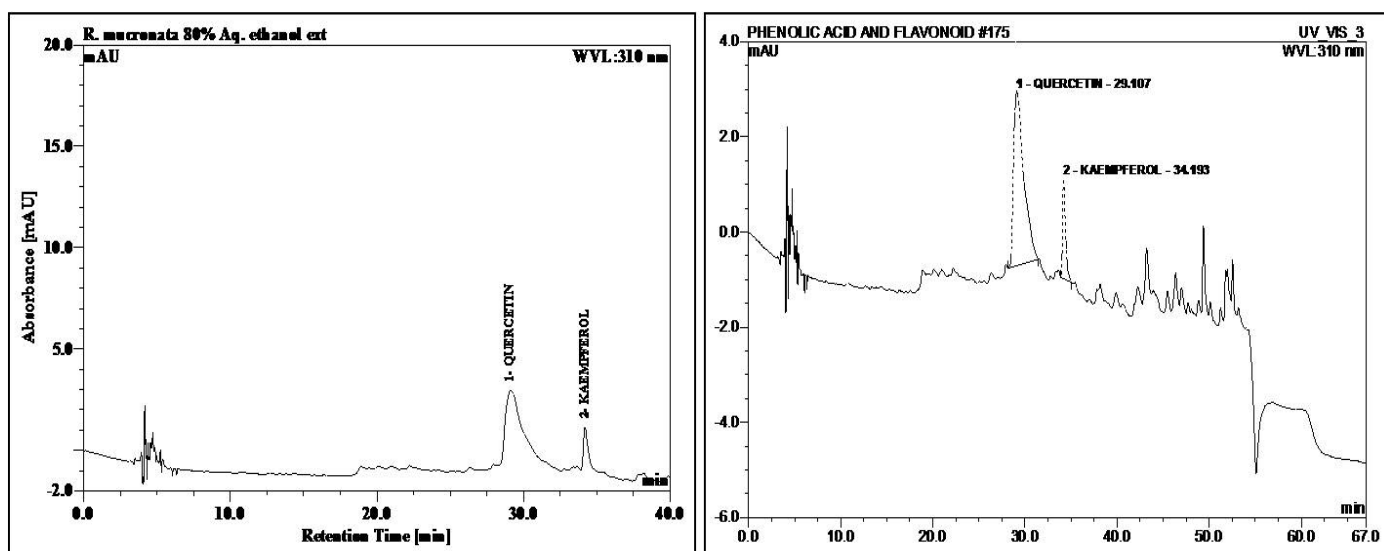


Figure 3: HPLC analysis of 80% ethanolic extract of *Rhizophora mucronata* Lam. leaves

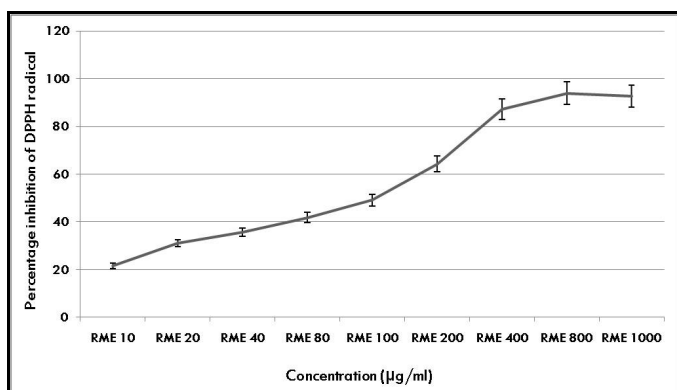


Figure 4: DPPH scavenging activity of ethanolic extract of *Rhizophora mucronata* Lam. leaves
Data are mean \pm S.E.M (n=4). The data were analyzed by one way ANOVA followed by Dunnett test, $p < 0.05$
RME- *Rhizophora mucronata* Lam. leaf ethanolic extract

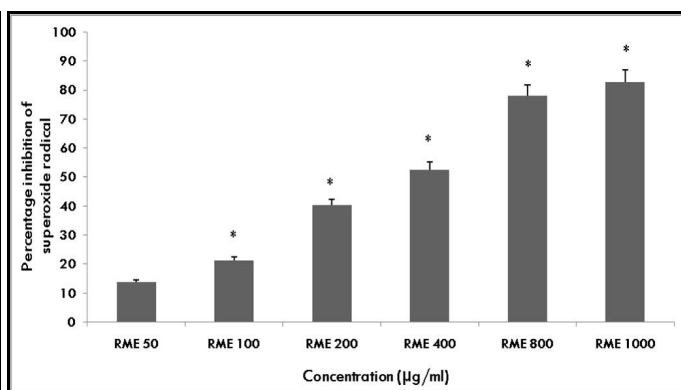


Figure 5: Superoxide radical scavenging activity of ethanolic extract of *Rhizophora mucronata* leaves
Data are mean \pm S.E.M (n=4). The data were analyzed by one way ANOVA followed by Dunnett test, * denotes level of significance $p < 0.05$
RME- *Rhizophora mucronata* Lam. leaf ethanolic extract

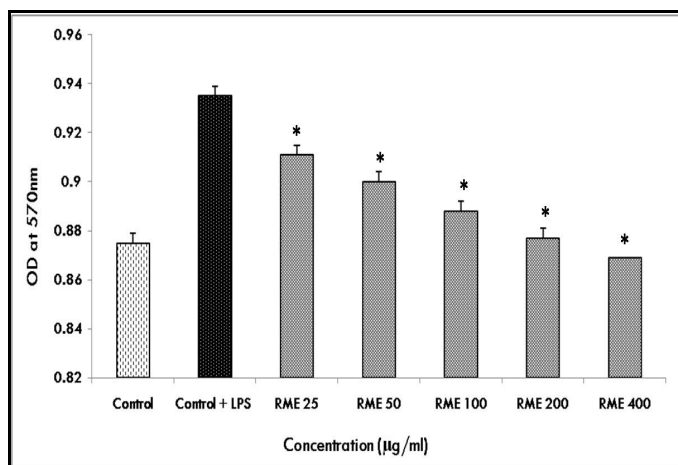


Figure 6: Effects of the ethanolic extract of *Rhizophora mucronata* leaves (RME) on NO production in LPS-stimulated RAW264.7 cells

Data are mean ± S.E.M (n=4). The data were analyzed by one way ANOVA followed by Dunnett test, * denotes level of significance p < 0.05

RME- *Rhizophora mucronata* Lam. leaf ethanolic extract

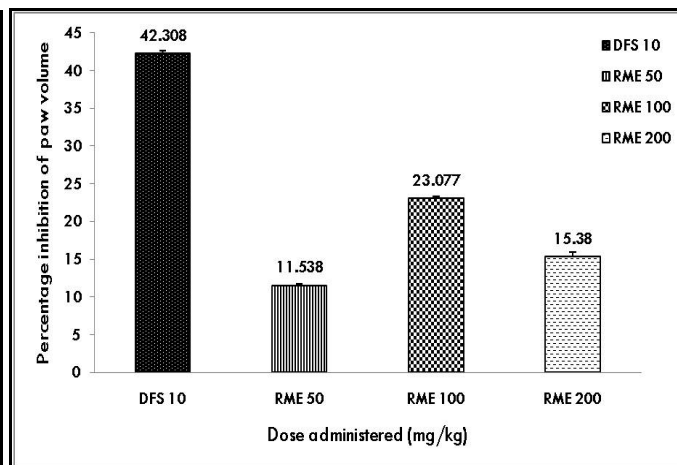


Figure 7: Reduction in rat paw volume (in%) on the 4th hour of the Carrageenan induced inflammatory model.

Data are mean ± S.E.M (n=6). The data were analyzed by one way ANOVA followed by Dunnett test, * denotes level of significance p < 0.05 All data were compared with the positive control (untreated inflammatory control) group. RME- *Rhizophora mucronata* Lam leaf ethanolic extract, DFS- Diclofenac sodium

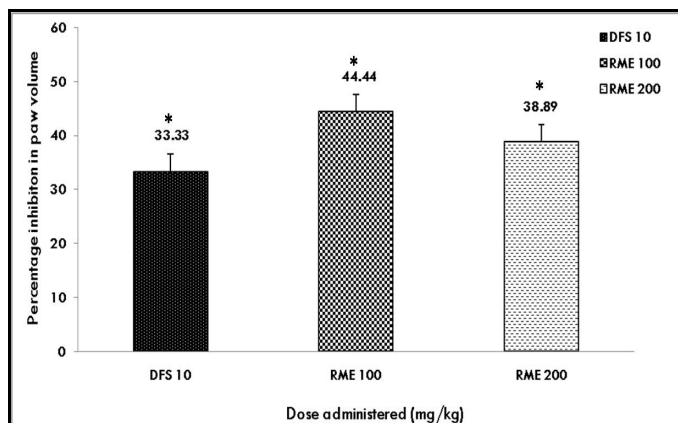


Figure 8: Reduction in rat paw volume (in %) on 28th day of Freund's adjuvant induced chronic inflammation model

Data are mean ± S.E.M (n=6). The data were analyzed by one way ANOVA followed by Dunnett test, * denotes level of significance p < 0.05 All data were compared with the positive control (untreated inflammatory control) group.

RME- *Rhizophora mucronata* Lam. leaf ethanolic extract, DFS- Diclofenac sodium

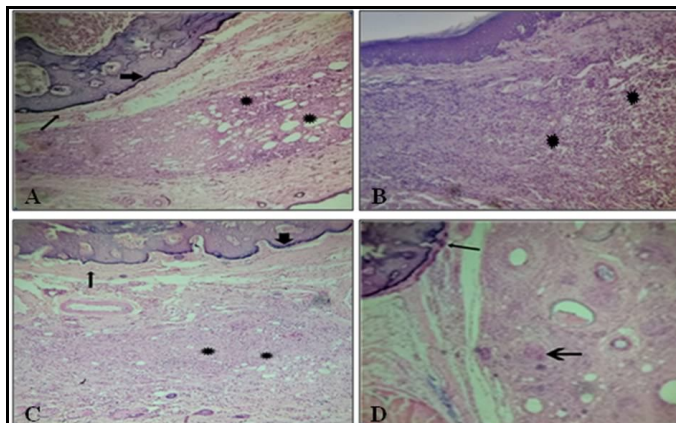


Figure 9: Histological sections of paw tissues of Inflammatory control rats [A], Diclofenac sodium 10mg/kg treated rats [B], RME 100mg/kg dose treated rats [C] and RME 200mg/kg dose treated rats [D]. Sections were stained with Hematoxylin and Eosin, magnification=40x.

The nitrite accumulation in the cells increased due to the LPS treatment. Different concentrations (25, 50, 100, 200, and 400 µg/mL) of the extract significantly inhibited (P<0.05) the nitrite accumulation in LPS-stimulated RAW264.7 cells, in

a concentration dependent manner (Figure 6, Table 1). The IC50 value of the ethanolic extract (RME) was 34.28µg/mL.

In-vivo anti-inflammatory study

Carrageenan induced acute inflammatory model

Carrageenan was injected in the sub-plantar region of the rat paws, which induced edema. The edema in the untreated inflammatory control rats persisted for even after 4 hours of the Carrageenan administration. The standard drug diclofenac sodium significantly decreased the inflammation at every hour interval till 4 hours. The ethanol extract of *Rhizophora mucronata* leaf (RME) at the different doses of 100 mg/kg and 200mg/kg body weight orally showed reduction in paw edema by 23.077% and 14.615% respectively than the inflammatory control group of rats (Figure 7). The extract at 50mg/kg body weight dose orally didn't show any significant anti-inflammatory activity even after 4 hours (Table 2).

Freund's adjuvant induced chronic inflammation model

The two effective doses of RME i.e. 100 mg/kg and 200mg/kg body weight (orally) found in the acute anti-inflammatory study were further evaluated in the Freund's adjuvant induced chronic inflammation model. The standard drug Diclofenac sodium (10mg/kg body weight orally) as well as both dose of RME reduced the inflammation, decreased the paw volume and increased joint mobility. The test extract in 100mg/kg dose orally in rats potentially inhibited the paw edema (44.44%) as compared to inflammatory control after 28 days consecutive treatment. The percentage inhibition was found to be better in the extract treated groups than that of the groups treated with standard drug diclofenac sodium (33.33%). In the other group of rats treated with the 200mg/kg dose of RME, there was 38.89% reduction in paw volume after the end of the study (Figure 8).

Body weight change

The body weight in the normal group gradually and significantly increased during the experiment, while in the control group, a significant decrease in animals weight was observed from on day 0, then on day 14th and at the end of the study on day 28th. With the treatment of the animals by RME, the body weight of animal slowly increased. Changes in body weight were not significant in diclofenac treated group (Table 3).

Histopathological examination of rat paws

The figure 9 showed the histological sections of inflamed paw tissues of the rats of different study groups. The paw tissue of the inflammatory control rats [A] showed

inflammatory cells and vacuoles plenty in subcutaneous tissue (marked by black asterix). Synovial membrane thickened, fibrous with inflammatory cells (marked by black arrow). Irregularity and discontinuity were seen on cartilaginous surface (thick black arrow). In the diclofenac sodium 10mg/kg treated rat paw tissues [B] showed very less no. of inflammatory cells and vacuoles. In the sections of RME 100mg treated rat paws [C], the subcutaneous tissue showed less inflammatory cells and vacuoles (marked by black asterix). Synovial membrane showed less number of inflammatory cells (marked by black arrow). Cartilaginous surface were continuous, smoother (thick black arrow). RME 200mg rat paws [D], the subcutaneous tissue showed pannus formation (thin arrow). Synovial membrane showed few inflammatory cells and cartilaginous surface was smooth.

DISCUSSION

Prolonged inflammation contributes to the pathogenesis of many inflammatory illnesses. Oxidative stress to cellular part by free radicals is accepted to be connected with pathology of numerous ailments and conditions including different inflammatory conditions, ageing, diabetes, cardiovascular ailments and cancer. Antioxidants provide resistance against the oxidative damage by scavenging the free radicals through numerous mechanisms and along these avoid ailments.^[19] In the present study, ethanolic extract of *Rhizophora mucronata* leaves (RME) demonstrated promising antioxidant properties in decreasing the DPPH in a dose dependant way. It likewise quenches the superoxide radical, which is additionally an essential parameter to test antioxidant property of plant. Some other study also support the significant antioxidant activity of the methanolic leaf extract of *Rhizophora mucronata* using free radical scavenging, reducing power, and metal chelating activity.^[20] In the present study, presence of quercetin in the test extract is also a significant finding, which is a polyphenolic compound, a potent antioxidant agent that decreases the oxidative damage in body. A study from Tamilnadu, India revealed that in vitro studies on extract of *Rhizophora mucronata* leaves demonstrate the significant anti-inflammatory and anti-arthritic activity which might be due to the presence of active principles such as polyphenolic content, triterpenoids, alkaloids and flavanoids in it.^[21]

Presently RAW264.7, a murine macrophage cell line has been frequently used for the screening of anti-inflammatory drugs. It is well known that macrophages play a crucial function in the regulation of inflammation and the immune response with the aid of releasing inflammatory mediators such as pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6), nitric oxide (NO) and prostaglandin E2 (PGE2). Consequently, greater interest is now being paid to the improvement of new medicines as effective inhibitors of NO production with regards to the remedy of persistent or chronic inflammatory diseases.^[15,22] In the present study the ethanolic extract of leaves of *Rhizophora mucronata* Lam. (RME) showed the significant inhibition of NO production in LPS-stimulated RAW264.7 cells in a concentration dependent manner, indicating that presence of antioxidant molecules present in the leaf extract would be responsible for the inhibitory action.

Rhizophora mucronata Lam. leaves ethanolic extract (RME) was found to be non toxic in acute toxicity study in rats.^[9] In the present study, in in-vivo acute and chronic inflammation model the *Rhizophora mucronata* leaves ethanolic extract (RME) showed significant anti-inflammatory actions. RME showed maximum inhibition of edema formation with 100 & 200 mg/kg b.w doses at the end of 4 hour in the Carrageenan induced rat paw edema model. A study from Bangalore, India stated that, the methanol extract of *Rhizophora mucronata* bark administered to the rats resulted dose dependent inhibition in acute and chronic paw edema in 250 and 500 mg/kg doses orally.^[23] Another study reported the fractions of *Rhizophora mucronata* bark extracts at 10mg/kg dose showed anti inflammatory effect in carrageen induced paw edema and isolated active phytoconstituents were lupeol, quercetin and caffeic acid. Quercetin was an active principle identified for the anti-inflammatory properties.^[24] In the present study also the presence of quercetin in the leaf extract of the plant in a significant amount must be considered as one of the major phytoconstituents responsible for the potent action. But very few studies have been carried out till date to evaluate the anti-inflammatory action with the leaf extract of the plant and identification of the novel active principle is still in research stage.

Freund's adjuvant induced inflammatory model is the established model of rheumatoid arthritis, has been extensively used to preclinical screening of new anti-arthritic compounds and has successfully predicted activity in new therapeutics.^[25] In the present research, the test extract RME in 100mg/kg and 200mg/kg dose orally in rats potentially inhibited the paw edema (44.44% and 38.89% respectively), efficacy was even better than the standard drug diclofenac sodium (33.33%). The histopathological study of the paw of RME treated rats showed less number of inflammatory cells than the control. The body weight of the inflammatory control and standard drug treated group of rats decreased during the study period. But daily administration of RME maintains good health in all the rats throughout the study period than the control group of rats. It has been already reported that the ethanolic extract of *Rhizophora mucronata* (Sunderban mangrove) leaves possess potential peripheral analgesic action like non steroidal anti-inflammatory drugs (NSAIDs).^[8] The analgesic and anti-inflammatory activities of the ethanolic extract of *Rhizophora mucronata* leaves has been scientifically validated. It can be stated that the extract must be helpful for the treatment of inflammatory disorders.

CONCLUSION

The experimental data from the present study revealed that, the ethanolic extract of *Rhizophora mucronata* (Sunderban mangrove) leaves possesses anti-inflammatory activities, which might be regulated through inhibition of the nitro-oxidative stress and reduction of free radical generation. Further research on the fractionation and isolation of the active phytoconstituents and to study the mechanism of action are in progress.

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REFERENCES

1. Nathan C. (2002) Points of control in inflammation. *Nature*. 420: 846-52.
2. Kolattukudy PE, Niu J. (2012) Inflammation, endoplasmic reticulum stress, autophagy, and the

- monocyte chemoattractant protein-1/CCR2 pathway. *Circ Res.* 110: 174-89.
3. Brunton LL, Parker KL: "Goodman and Gilman's manual of pharmacology and therapeutics". New York: McGraw-Hill; 2008. p. 434-60.
 4. More SK, Lande AA, Jagdale PG, Adkar PP, Ambavade SD. (2013) Evaluation of anti-inflammatory activity of *Solanum xanthocarpum* Schrad and Wendl (Kaṇṭhakāri) extract in laboratory animals. *Anc Sci Life.* 32(4): 222-226.
 5. Sagnia B, Fedeli D, Casetti R, Montesano C, Falcioni G, Colizzi V. (2014) Antioxidant and Anti-Inflammatory Activities of Extracts from *Cassia alata*, *Eleusine indica*, *Eremomastax speciosa*, *Carica papaya* and *Polyscias fulva* Medicinal Plants Collected in Cameroon. *PLOS ONE.* 9(8): e103999.
 6. Shilpi JA et al. (2012) Antinociceptive, Anti-Inflammatory, and Antipyretic Activity of Mangrove Plants: A Mini Review. *Advances in Pharmacological Sciences.* 1-7.
 7. Bandaranayake WM. (2002) Bioactivities, bioactive compounds and chemical constituents of mangrove plants. *Wetlands Ecology and Management.* 10(6): 421-452.
 8. Ray M, Adhikari A, Sur TK, Mondal C, Pathak A, Das AK. (2014) Pharmacognostic and Anti-Hyperglycemic Evaluation of the Leaves of Sunderban Mangrove, *Rhizophora mucronata* L. *Pharmanest.* 5(5): 2289-2294.
 9. Adhikari A, Ray M, AK, Sur TK, Kanjilal S, Roy RK. (2016) Analgesic actions of Sunderban mangrove, *Rhizophora mucronata* L. leaves. *Journal of Medicinal Plants Studies* 4(3): 140-143.
 10. Santhanakrishnan D, Shankar SN, Chandrasekaran B. (2014) Studies of phytochemistry, spectroscopic characterization and antibacterial efficacy of *Salicornia brachiata*. *International J Pharmacy Pharmaceutical Sciences.* 6(6): 430-432.
 11. Rohini RM, Das AK. (2011) Determination of lupiol, beta-sitosterol and quercetin from ethyl acetate extract of *Rhizophora mucronata* bark by HPTLC technique. *Asian J Pharmaceut Clin Res.* 4: 103-105.
 12. Raut S, Khan S, Gaikwad R. (2014) Isolation and characterization of major phytoconstituents from the leaves of *Rhizophora mucronata* Lamk and *Acanthus ilicifolius* Linn. *Int. Res. J. of Science & Engineering.* 2(2): 51-59.
 13. Nehete J., Bhatia M., Narkhede M. (2010) In-vitro Evaluation of Antioxidant Activity and Phenolic Content of *Costus speciosus* (Koen) J.E. Sm. *Iranian Journal of Pharmaceutical Research.* 9(3): 271-277.
 14. Patro G, Bhattamisra SK, Mohanty BK, Sahoo HB. (2016) In vitro and In vivo Antioxidant Evaluation and Estimation of Total Phenolic, Flavonoidal Content of *Mimosa pudica* L. *Pharmacognosy Res.* 8(1): 22-28.
 15. Taira J, Nanbu H, Ueda K. (2009) Nitric oxide-scavenging compounds in *Agrimonia pilosa* Ledeb on LPS-induced RAW264.7 macrophages. *Food Chem.* 115: 1221-1227.
 16. Joo T et al. (2014) Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila* L. *Saudi Journal of Biological Sciences.* 21: 427-435.
 17. Mandal G, Chatterjee C, Chatterjee M. (2015) Evaluation of anti-inflammatory activity of methanolic extract of leaves of *Bougainvillea spectabilis* in experimental animal models. *Pharmacognosy Res.* 7(1): 18-22.
 18. Patwardhan S, Bodas K, Gundewar S. (2010) Coping with arthritis using safer herbal options. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2: 1-11.
 19. Gul et al. (2011) Evaluation of *Abelmoschus moschatus* extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using in vitro assays. *BMC Complementary and Alternative Medicine.* 11: 64.
 20. Suganthy N, Devi KP. (2016) In vitro antioxidant and anticholinesterase activities of *Rhizophora mucronata*. *Pharm Biol.* 54(1): 118-29.
 21. Kumari CS, Yasmin N, Hussain MR, Babuselvam M. (2015) In vitro Anti-Inflammatory and Anti-Arthritic Property of *Rhizophora mucronata* Leaves. 6(3): 482-485.
 22. Maroon JC, Bost JW, Maroon A. (2010) Natural anti-inflammatory agents for pain relief. *Surg Neurol Int.* 1(80): 1-15.
 23. Rohini RM, Das AK. (2009) A Comparative Evaluation of Analgesic and Anti-inflammatory Activities of *Rhizophora mucronata* Bark Extracts. *Pharmacologyonline.* 1: 780-791.
 24. Rohini RM, Das AK. (2010) Antidiarrheal and anti-inflammatory activities of Lupeol, Quercetin, β -Sitosterol, Adene-5-en-3-ol and Caffeic acid isolated from *Rhizophora mucronata* Bark. *Der Pharmacia Lettre.* 2(5): 95-101.
 25. Patel SS, Shah PV. (2013) Evaluation of anti-inflammatory potential of the multidrug herbomineral formulation in male Wistar rats against rheumatoid arthritis. *J Ayurveda Integr Med.* 4(2): 86-93.