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Effect of *Rhizophora* Root Extracts on Wound Healing and Yeast-Induced Pyrexia in Rats

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Abstract

Mangrove plants are part of tropical marine ecosystems of coastal regions of Kerala and their parts are widely used as folklore medicines. Root extracts of *Rhizophora mucronata* and *Rhizophora apiculata* from Cochin backwater area were screened for antipyretic and wound healing properties. Methanolic aqueous root extracts of *R. mucronata* and *R. apiculata* were tested at 460 and 535 mg kg^{-1} concentrations respectively, in male albino rats. In both cases, bioactivities were compared with that of standard drug and a control group. Among the two species analyzed, maximum activity was observed in the methanolic root extracts of *R. apiculata* followed by *R. mucronata*. It is postulated that antipyretic and wound healing effects are caused by inhibition of prostaglandin synthesis and decrease in the cytokine levels. Phytochemical screening of residues revealed the presence of carbohydrates, alkaloids, tannins, flavonoids, saponins and glycosides, which may account for the observed pharmaceutical effects in the *Rhizophora* species studied.

Keywords: Antipyretic, excision wound, mangroves, *Rhizophora mucronata*, *Rhizophora apiculata*

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Introduction

Natural products have long been recognized as an important source of therapeutically effective medicines (Singh et al., 2008). India is blessed with diverse flora and fauna, many of which find abode in forests and swamps along the coastal regions (Selvam, 2003). Tropical marine ecosystems of coastal regions of India include lagoons, mangrove swamps, sandy and rocky shores and open sea front. The mangrove vegetation, an important coastal ecosystem associated with tidal mudflats and backwater systems, contributes to about 4662.56 km^2 in India (MoEF, 2012). The major mangrove zones of Kerala include Vallapattanam area in Kannur and Puthuvypene area in Ernakulam. *Rhizophora apiculata*, *Rhizophora mucronata*, *Bruguiera gymnorrhiza*, *Avicenia officinalis*, *Acanthus ilicifolius*, *Sonneratia caseolaris*, *Sonneratia apetala* and *Kandelia candal* are the major mangrove plants located in these regions of which *R. apiculata* and *R. mucronata* predominate (KSCSTE, 2008).

R. apiculata and *R. mucronata* are evergreen trees that grow upto 25-30 m height and 70 cm diameter, with numerous branching and arching stilt roots (Jayasurya et al., 2005). Barks of red mangroves (*Rhizophora mangle)* are effective against hemoptysis in pulmonary tuberculosis owing to its haemostatic properties (Roig, 1998). The leaves of *R. mucronata* serve as an astringent and are a common folk remedy for angina, haemorrhage, diabetes and dysentery. Antimicrobial properties (Caceres et al., 1991; Melchor et al., 2001), wound healing, including efficiency in healing of open surgical wounds (Fernandez *et al*., 2002) and gastric antiulcer properties (Sanchez Perera et al., 2001) have been observed in aqueous extracts of mangroves. It has been reported that alkaline extract from the leaves of *R. apiculata* successfully inhibited HIV replication and HIV-induced cytopathic effects (Premanathan et al., 1999).

Though reports are available to support the ethnic use of the stem, leaves and fruits of the *Rhizophora* species, there are limited reports on the efficacy of *Rhizophora* root extracts on pyrexia and wound healing properties. There have been some reports on

the use of roots by the Indo-Chinese for angina and haemorrhage (Perry, 1980). Aqueous extracts of *Rhizophora mangle* (Marrero et al., 2006) bark contain tannins, epicathechin, cathechin, gallic acid, ellagic acid, chlorogenic acid, fatty acids and carbohydrates and these compounds in turn could be responsible for the faster healing and regeneration of lost tissues by multiple mechanisms (Nayak et al., 2007). There have been no reports on wound healing and antipyretic effects of methanolic root extracts of *Rhizophora* species. The present study was conducted to evaluate the pharmacological significance of methanolic root extracts of *R. apiculata* and *R. mucronata*, collected from the Cochin backwaters, on yeast-induced pyrexia and excision wound healing in rats.

Materials and Methods

The roots of *Rhizophora mucronata* (Lam.) and *Rhizophora apiculata* (Blume) were collected from backwaters of Cochin, India and were identified with the help of the Captain Srinivasamurthi Drug Research Institute for Ayurveda, Arumbakkam, Chennai, India. All the reagents used for the study were of analytical grade obtained from Merck (Darmstadt, Germany). Paracetamol and neosporin sulphate salt were purchased from Glaxo Smith Kline Ltd. (Bangalore, India)

The collected mangrove roots of *R. mucronata* and *R. apiculata* were cleaned, cut into small pieces, shade dried, pulverized and subjected to extraction. The powdered material (200 g) was mixed with 4 × 2500 ml methanol and left for one week at room temperature, stirring the mixture at 6 h intervals with a sterile glass rod. The extract was filtered and the clear filtrate was evaporated to dryness under reduced pressure. The yields of the methanolic extracts were *R. mucronata*, 17.6 % and *R. apiculata*, 15.2 %. The extracts were reconstituted in a mixture of dimethyl sulfoxide (DMSO) and double-distilled water (2:3, v/v) prior to animal experiments.

The presence of secondary metabolites such as carbohydrates, alkaloids, tannins, flavonoids, saponins and glycosides in the extracts was confirmed by performing qualitative tests using standard procedures (Sofowora, 1993). Wistar strain male Albino rats (180-200 g) and Swiss mice (35-40 g) were used for the experiments. They were housed individually in polypropylene cages under hygienic conditions and were provided food and water *ad libitum*. The animals were maintained on a 12:12 h light:dark photoperiod under standard conditions of temperature and ventilation. The experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and with the approval of the Institutional Animal Ethics Committee (IAEC). Healthy male Swiss mice were randomly divided into groups of six animals. They were starved for 16 h prior to the administration of the test suspension. The control group received water containing 40% DMSO administered orally by gavage. A single dose of methanolic root extracts of *R. mucronata* and *R. apiculata* suspended in DMSO:water (2:3, v/v), was administered orally at concentrations ranging from 200 - 4000 mg kg^{-1} (Obici et al., 2008). The animals were observed for a week, the number of survivors was counted and the optimum average dosage was determined using Karber's Arithmetical method (Turner, 1965).

For the excision wound study, healthy Wistar strain male Albino rats were used. The laboratory acclimatized animals were anaesthetized with ether and an excision wound was created according to Morton & Malone (1972). A full thickness of the excision wound of circular area 300 mm and 2 mm depth was created on the dorsal thoracic region 5 cm away from the ears and the wound was left open (Diwan et al., 1982). The animals were maintained individually in separate cages. They were randomly divided into four groups of 6 each and treated as follows: Group 1- control animals which received DMSO : water (2:3, v/v), Group 2 - standard with application of neosporin sulphate salt 5% (w/w), Group 3- extracts of *R. mucronata* at 460 mg kg^{-1}, Group 4- extracts of *R. apiculata* at 535 mg kg^{-1}. The extracts were formulated as an ointment as described by Hukkeri et al. (2006) and 500 mg of the prepared ointment was applied on the wound once daily, till complete wound healing or up to the 21st post-operative day, whichever was earlier. Wound contraction was studied according to a modified method of Nayak et al. (2006) by tracing the raw wound area on a plotting paper every third day until wounds were completely healed. Except the extracts under study, no topical or systematic therapy was given to animals. Animals showing infection/deterioration of wounds were excluded from the study and replaced with other animals.

Antipyretic activity of the extracts was measured by slightly modifying the method described by Adams

et al.(1968). Rats were starved overnight with water *ad libitum* before the experiments. Pyrexia was induced by subcutaneously injecting 20% (w/v) brewer's yeast suspension in saline solution (10 ml kg^{-1}) into the animal's dorsum region. Rectal temperature of each rat was measured at 19th h after injection using a thermometer. Only rats that showed an increase in temperature of at least 0.7°C were employed for the experiments. *R. mucronata* and *R. apiculata* root extracts were administered orally by gavage and the temperature was measured at 0, 1, 2, 3 and 4 h after drug administration. Paracetamol IP (100 mg kg^{-1}) was used as standard for comparison of antipyretic activity and all control animals received 40% DMSO.

All data were expressed as mean ± SD and were analysed statistically by the one-way ANOVA using Duncan's test with the level of significance set at $P<0.05$. Statistical software, SPSS for Windows version 16, was employed for analyses.

Results and Discussion

Preliminary phytochemical screening of methanolic root extracts of *R. mucronata* and *R. apiculata* showed the presence of triterpenoids, flavonoids, alkaloids, tannins, saponins and glycosides, carbohydrates and amino acids (Table 1). Quantitative estimation of these compounds in the above mangrove species were studied by Asha et al. (In press). Maria et al. (2000) reported that *R. mangle* bark aqueous extracts contained 54% tannins; epicathechin, cathechin, gallic acid, ellagic acid, chlorogenic acid and fatty acids while carbohydrates contributed 17%.

In the acute toxicity trial, no deaths were observed for doses (oral administration by gavage) of up to 3500 mg kg^{-1} body weight for the methanolic root extracts of *R. apiculata* and *R. mucronata* in mice. Optimum dose of 460 and 535 mg kg^{-1} body weight were fixed for *R. mucronata* and *R. apiculata* extracts respectively, based on Karber's arithmetic method for determination of LD_{50} values. No changes in body weights were observed at any of the dosage levels.

Significant ($P< 0.05$) wound healing activity was observed in animals treated with the root extracts of *R. mucronata* and *R. apiculata* compared to the placebo control animals (Table 2). Moreover, the wound healing activity of these extracts was comparable to that of neosporin sulphate, which was administered as the reference drug in experimental rats. The contraction of excision wound was promoted from the 3rd day of treatment till the 12th day. Both the root extracts showed a significant ($P< 0.05$) reduction in wound area and a shorter period of epithelization compared to the control treated animals. On the 3rd day of post wound treatment, *R. mucronata* and *R. apiculata* extracts showed 59 and 52% reduction in wound area respectively, compared to only 20% in the control group. Maximum wound contraction occurred on the 6th day for rats treated with the root extracts and the standard reference drug wherein a 20% increment in the wound healing ability was observed. On the 12th day of post wound treatment, *R. mucronata* and *R. apiculata* extracts showed 93 and 96% wound contraction respectively, while only 78% of wound healing was achieved in the control animals. The control animals achieved 97% wound healing in 21 days wherein no scar or residual matter remained behind. The animals treated with neosporin sulphate, at a dose of 100 mg kg^{-1} body weight,

Table 1. Phytochemical screening of methanolic root extracts of *Rhizophora apiculata* and *Rhizophora mucronata*.

Phytochemical ingredient	Test method	Result	
		Rhizophora apiculata	*Rhizophora mucronata*
Triterpenoids	Libermannbuchard	+	+
Flavonoids	Shinoda's test	+	+
Alkaloids	Hager's test	+	+
Tannins	Swain test	+	+
Saponins and glycosides	Froth test	+	+
Amino acids	Ninhydrin test	+	+
Carbohydrates	Molisch test	+	+

Table 2. Mean percentage closure of excision wound area on rats treated with methanolic root extracts of *Rhizophora apiculata* and *Rhizophora mucronata*.

Day	Control	Neosporin sulphate	*R. apiculata*	*R. mucronata*
3rd	20.03 ± 0.49[a]	63.98 ± 6.06[c]	52.89 ± 4.93[b]	59.13 ± 3.03[b]
6th	43.03 ± 0.72[a]	83.05 ± 6.67[c]	79.68 ± 1.91[b]	73.83 ± 9.82[b]
9th	67.47 ± 2.56[a]	89.46 ± 2.44[b]	89.40 ± 5.14[b]	88.92 ± 1.94[b]
12th	78.14 ± 2.19[a]	95.26 ± 1.54[b]	96.33 ± 1.19[c]	93.44 ±2.28[b]

Values are expressed as Mean ± SD (n = 6 animals per group)

Values with different superscripts are significantly different compared to the control group ($P< 0.05$)

showed 64% reduction in wound area on the 3rd, 89% on the 9th and 97% healing on the 12th day, of the post excision-wound treatment.

In the excision wound study, it was observed that rats treated with methanolic root extracts showed better and faster healing as compared to the untreated group. A significant difference ($P<0.05$) in the wound healing process was observed on the third and sixth days of post wound treatment for the groups treated with root extracts of *R. apiculata*, *R. mucronata* and standard drug (neosporin sulphate) in comparison to the untreated control. Rats treated with *R. apiculata* root extracts showed faster healing than those treated with the reference drug.

In the excision wound model, the maximum wound contraction for rats treated with the root extracts as well as the standard reference drug could be accounted to the presence of triterpenoids, phenolic compounds and flavonoids in the extracts which counteract the effects of inflammation by lowering the level of inflammatory mediators. Triterpenoids and flavonoids possess potent anti-oxidative effects and anti-inflammatory properties (Corsi et al., 1994, 1995).

In the present study, keratinization, epithelization, fibrosis, collagenation and neovascularisation could have occurred during the healing process and the anti-inflammatory mediators would have accelerated the process of healing (Charde et al., 2006). The action of the extracts might be through promotion of keratinization, fibrosis, collagen formation and neovascularisation, resulting in better healing compared to the control group. All healing markers seemed to be activated resulting in a high wound healing activity of the tested formulations.

A significant ($P< 0.05$) reduction in body temperature was observed when rats were treated with root extracts of *R. apiculata* and *R. mucronata* at doses of 460 and 535 mg kg^{-1} (Fig.1) respectively. They showed an antipyretic behavior similar to the standard reference drug paracetamol, at 100 mg kg^{-1}, upto two h of its administration. Between the two *Rhizophora* extracts, maximum antipyretic effect was observed in rats treated with the root extracts of *R. apiculata* followed by *R. mucronata*. Pyrexia or fever is a frequent medicalsymptom that describes an increase in internal body temperature to levels that are above the normal body temperature *viz.*, above 36.8 ± 0.7°C or 98.2 ± 1.3°F. Fever is most accurately characterized as a temporary elevation in the body's thermoregulatory set-point, usually by about 1-2°C.

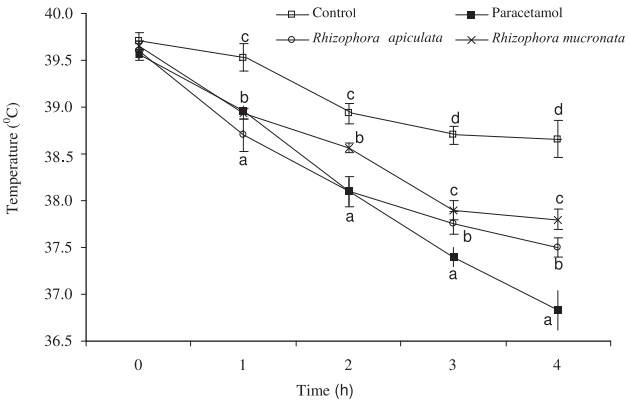

Fig. 1. Antipyretic effect of methanolic root extracts of *Rhizophora apiculata* and *Rhizophora mucronata on* yeast - induced pyrexia in rats.

Values are expressed as Mean ± SD (n = 6 animals per group)

Values with different superscripts are significantly different compared to the control group ($P< 0.05$)

In the yeast-induced pyrexia, maximum antipyretic effect was observed in rats treated with the root extracts of *R. apiculata*, wherein a 1.0 degree decline in temperature was observed within one h of its administration. *R. apiculata* and *R. mucronata* root extracts showed an antipyretic effect similar to the standard reference drug paracetamol, at a dose of 100 mg kg^{-1} bodyweight, up to two h of its administration. The result seems to support the view that the root extracts of *R. apiculata* and R. *mucronata* have some influence on prostaglandin biosynthesis because prostaglandins are regulators of body temperature (Morimoto et al., 1991).

Temperature is regulated in the hypothalamus, in response to prostaglandin E_2 (PGE_2)(Morimoto et al., 1991). PGE_2 release, in turn, comes from a trigger, a pyrogen (substance that induces fever). The hypothalamus generates a response back to the body, making it increase the temperature set-point. The endogenous pyrogens such as interleukin 1 (IL-1), interleukin 6 (IL-6), and the tumor necrosis factor-alpha (TNFá) are a part of the innate immune system, produced by phagocytic cells and cause the increase in the thermoregulatory set-point in the hypothalamus (Brydon et al., 2006). These cytokine factors are released into general circulation where they migrate to the circumventricular organs of the brain, where the blood-brain barrier is reduced. The cytokine factors bind with endothelial receptors on vessel walls or interact with local microglial cells. When these cytokine factors bind, they activate the arachidonic acid pathway, PGE_2 release comes from the arachidonic acid pathway. This pathway (as it relates to fever), is mediated by the enzymes, phospholipase A_2 (PLA_2), cyclooxygenase-2 (COX-2) and prostaglandin E_2 synthase (Gotlieb, 2008). These enzymes ultimately mediate the synthesis and release of PGE_2.

In conclusion, the root extracts of *R. apiculata* and *R. mucronata* possess wound healing and antipyretic effects. The role of extracts in reducing fever and healing wounds might be due to the action of their flavonoids and other phenolic compounds on endogenous pyrogens or on the arachidonic pathway and the mode of action is to be explored further.

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