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Anti-inflammatory efficacy of the rhizome of *Zingiber zerumbet* – an *in vitro* study using THP1 cell line

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Abstract

Inflammation arises as a protective mechanism but persistent inflammation is irritating, harmful and can result in chronic diseases. *Zingiber zerumbet* is a perennial herb belonging to the family Zingiberaceae. Anti-inflammatory effect of the methanolic extract of the rhizome of *Zingiber zerumbet* collected from the district of Kottayam, Kerala was studied using THP1 cell line. The cell line was induced with lipopolysaccharide and the inhibitory effect of the extract on the activity of cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase was tested. The extract possessed the ability to inhibit cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase induced by lipopolysaccharide. This showed multiple mode of action of the extract in executing anti-inflammatory effect. Hence the methanolic extract of the rhizome of *Zingiber zerumbet* can be used to develop drugs for various allergic and inflammatory disorders in traditional as well as modern medicine.

Keywords: cyclooxygenase, lipoxygenase, myeloperoxidase, nitric oxide synthase

Introduction

Zingiber zerumbet is a perennial herb belonging to the family Zingiberaceae. The plant is found throughout India and grows naturally in damp areas and shaded parts of hill slopes [1]. The rhizome of *Zingiber zerumbet* has been used as spice as well as traditional medicine [2]. *Zingiber zerumbet* is used to treat sores, swelling, worm infestation and loss of appetite. Rhizome is used in traditional medicine for anti-inflammation [3]. The rhizome has been reported to possess antiproliferative activity [4], antinociceptive activity and antipyretic activity [5], antitumour activity [6], antiallergic activity [7] and antiplatelet aggregation activity [8]. The rhizome has been reported to possess anti-inflammatory activity in carrageenan-induced paw edema test and cotton-pellet-induced granuloma test [9]. In the present study we have investigated the cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase inhibiting properties of the rhizome of *Zingiber zerumbet*.

Inflammation is the healing response of living tissues to infection, injury or any other irritation [10]. Inflammation rise as a protective or defence mechanism but its persistence may result in chronic inflammation and associated diseases like arthritis, atherosclerosis and cancer. Inflammation occurs through a number of mediators such as prostaglandins, leukotrienes, histamine, nitric oxide, cytokines, bradykinin, serotonin etc whose production is triggered by infection, injury or irritation [11]. Agents that can inhibit the production or block the action of these mediators can act as anti-inflammatory agents. They can be used to control chronic inflammation and associated illness. Inhibition of cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase activities is a commonly used method for the analysis of anti-inflammatory activity of test materials. In this study, inhibitory effect of methanolic extract of the rhizome of *Zingiber zerumbet* on the activity of cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase was tested using human monocytic cell line THP1.

Materials and methods

Collection of plant material

Rhizome of *Zingiber zerumbet* was collected from the district of Kottayam, Kerala. The rhizome was washed, cut into pieces, shade dried and powdered in kitchen blender. The plant was authenticated by a plant taxonomist from the Department of Botany, St. Thomas College, Pala, Kerala, India. A voucher specimen (SBSBRL20) has been maintained with the author's institute.

Solvent extraction

The powdered rhizome was extracted with methanol at room temperature in an orbital shaker for 7 days. The extract was filtered, dried and stored at 4 °C until use.

Chemicals

THP1 cell line was purchased from NCCS, Pune. Arachidonic acid, Sodium linoleate, Glutathione, Thiobarbituric acid, Diclofenac sodium, Guaiacol and Hydrogen peroxide were purchased from Sigma – Aldrich, USA. RPMI 1640 was purchased from HIMEDIA Laboratories, Mumbai. Haemoglobin standard was from Erba Mannheim, Germany. All other chemicals used were of analytical grade.

Determination of anti-inflammatory effect on human monocytic cell line THP1

RPMI 1640 supplemented with 1.5% sodium bicarbonate, 10% heat inactivated FBS and the antibiotics penicillin and streptomycin was used as the media for culturing the cell line. The cells were grown till 60% confluency and stimulated with lipopolysaccharide. Stimulated cells were then treated with different concentrations of the extract – 6.25 µg, 12.5 µg, 25 µg, 50 µg, 100 µg and 200 µg for 24 hours. Cells were collected by spinning at 6000 rpm for 10 minutes and 200 µL of cell lysis buffer (1M TrisHCl, 0.25M EDTA, 2M NaCl, 0.5% Triton X-100) was added. Incubated for 30 minutes at 4 °C and assays were done with suspension of lysed pellet.

Cyclooxygenase assay (COX)

COX activity was measured using TBARS assay. The assay mixture contained Tris-HCl buffer (100 mM, pH 8), glutathione (5 mM), haemoglobin (5 µM) and the cell lysate. To this arachidonic acid (200 µM) was added and incubated at 37 °C for 20 minutes. Reaction was terminated by adding 0.2 mL of 10% TCA (in 1N HCl) and added 0.2 mL of thiobarbituric acid (1%). Then it was heated in a boiling water bath for 20 minutes, cooled and centrifuged. OD of supernatant was measured at 532 nm. Percentage inhibition was calculated as follows,

$$\frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

Diclofenac sodium was used as the reference drug. IC₅₀ value was calculated by plotting percentage inhibition against concentration.

5-Lipoxygenase assay (LOX)

The assay mixture contained 2.75 mL of Tris-HCl buffer (50 mM, pH 7.4), 0.2 mL of sodium linoleate and 50 µL of cell suspension. Increase in optical density due to the formation of conjugate double bonds in the product linoleic acid hydroperoxide was measured [12]. Percentage inhibition was calculated as follows,

$$\frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

Diclofenac sodium was used as the reference drug. IC₅₀ value was calculated by plotting percentage inhibition against concentration.

Myeloperoxidase assay (MPO)

In presence of H₂O₂, myeloperoxidase oxidise guaiacol into tetraguaiacol whose absorbance can be measured at 460 nm.

Cell suspension was mixed with 0.5% solution of HTAB in potassium phosphate buffer (50 mM, pH 6). After freeze thawing (3 times) the samples were centrifuged at 2000g for 30 minutes at 4 °C [13, 14].

Cellular nitrite levels

In inflammatory conditions nitric oxide is produced by inducible nitric oxide synthase which gets converted to nitrite and nitrate. Cellular nitrite level is a measure of nitric oxide produced and hence the activity of inducible nitric oxide synthase. To 0.5 mL of cell suspension, 0.1mM of sulphosalicylic acid was added and vortexed. It was then centrifuged at 5000 rpm for 15 minutes. To 200 µL of protein free supernatant, 30 µL of 10% NaOH and 300 µL of Tris-HCl buffer (100 mM, pH 8) were added and mixed well. Then added 530 µL of Griess reagent and incubated in dark for 10-15 minutes. Griess reagent will convert nitrite into a deep purple azo compound whose absorbance can be measured at 540 nm [15, 16].

Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curve. Percentage inhibition was calculated as follows,

$$\frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

Diclofenac sodium was used as the reference drug. IC₅₀ value was calculated by plotting percentage inhibition against concentration.

Results

Cyclooxygenase assay

The methanolic extract of the rhizome of *Zingiber zerumbet* (MRZZ) exhibited good inhibition of cyclooxygenase activity in a dose dependent manner. The inhibitory effect of the extract at different concentrations is depicted in Figure 1. IC₅₀ value of cyclooxygenase inhibition was 25µg and 4µg respectively for the extract and the reference drug diclofenac sodium (D.sod).

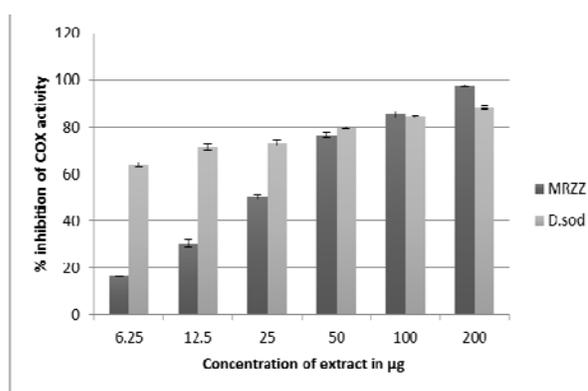


Fig 1: Percentage inhibition of cyclooxygenase activity by methanolic extract of the rhizome of *Zingiber zerumbet* and diclofenac sodium

Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

MRZZ exhibited a dose dependent increase in the inhibition of cyclooxygenase activity. At 50 and 100 µg, the inhibitory effect of extract was equal to that of the standard drug diclofenac sodium. At 200 µg the inhibitory effect was greater

than diclofenac sodium.

5-Lipoxygenase activity

MRZZ was found to possess good inhibitory effect against lipoxygenase activity. The inhibitory effect of the extract at different concentrations is shown in Figure 2. IC₅₀ value of lipoxygenase inhibition was 24µg and 5µg respectively for the extract and the reference drug diclofenac sodium.

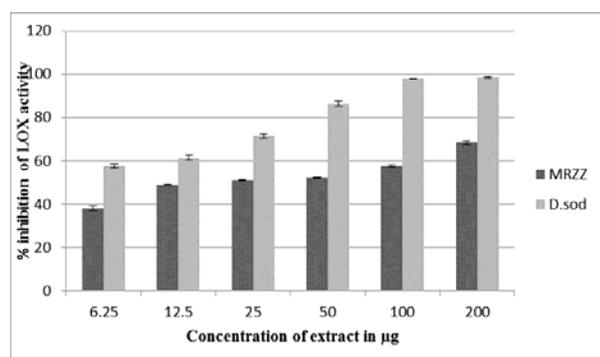


Fig 2: Percentage inhibition of lipoxygenase activity by methanolic extract of the rhizome of *Zingiber zerumbet* and diclofenac sodium

Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

MRZZ exhibited dose dependent increase in the inhibitory activity. The effect of the extract in inhibiting the activity of lipoxygenase was comparable to that of the reference drug diclofenac sodium, but less than it.

Myeloperoxidase activity (MPO)

MRZZ was highly effective in inhibiting Myeloperoxidase. The inhibitory effect of the extract at different concentrations is shown in Figure 3. IC₅₀ value of myeloperoxidase inhibition was 20µg for MRZZ. (IC₅₀ could not be determined for diclofenac sodium due to the very high activity at the tested concentrations).

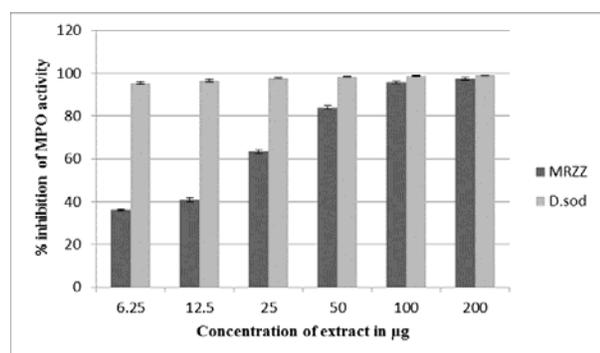


Fig 3: Percentage inhibition of myeloperoxidase activity by methanolic extract of the rhizome of *Zingiber zerumbet* and diclofenac sodium

Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

MRZZ exhibited dose dependent increase in the inhibition of myeloperoxidase activity. At higher concentrations inhibitory effect was very high and equal to reference drug.

Cellular nitrite level

MRZZ exhibited good percentage inhibition of nitrite formation. Decreased cellular nitrite level in presence of MRZZ reveals its ability to inhibit nitric oxide synthase and hence the production of nitric oxide. The inhibitory effect of

MRZZ at different concentrations is shown in Figure 4. IC₅₀ value of cellular nitrite level inhibition was 4µg and 6µg respectively for the extract and the reference drug diclofenac sodium.

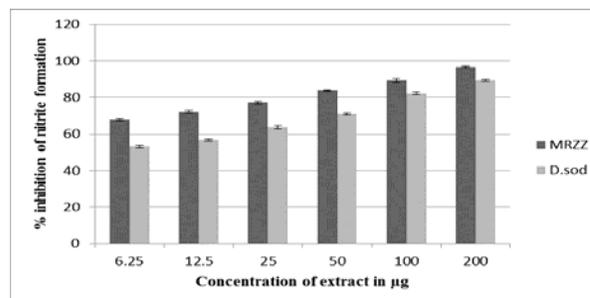


Fig 4: Percentage inhibition of cellular nitrite level by methanolic extract of the rhizome of *Zingiber zerumbet* and diclofenac sodium

Results are expressed as mean±SD (n=3), error bar indicating the standard deviation

MRZZ exhibited dose dependent increase in the inhibition of cellular nitrite level. Its activity was greater than the reference drug diclofenac sodium.

Discussion

Inflammation is a double edged sword. It arises as a healing process and is necessary to protect or recover the body from infections and injuries. But persistent inflammation is harmful, irritating and can cause chronic diseases. Prostaglandins and leukotrienes play key role in the mediation of inflammation. Both are synthesized from the polyunsaturated acid known as arachidonic acid. Prostaglandins are synthesized by the enzyme cyclooxygenase (cyclooxygenase pathway) and leukotrienes by lipoxygenase (lipoxygenase pathway). Leukotrienes are important cause of pathological symptoms in asthma.

The enzyme myeloperoxidase is present in neutrophils and monocytes. It catalyses formation of powerful oxidants from H₂O₂ formed during respiratory burst. Function of these oxidants is phagocytosis and destruction of microorganisms. But if released to outside of phagosome it can cause damage to adjacent tissue thus contributing to pathogenesis. Recently myeloperoxidase has been reported to be involved in pathological conditions like myocardial infarction [17], atherosclerosis [18], atrial fibrillation [19], transplant rejection [20] etc. It has been reported that in addition to inhibiting cyclooxygenase activity, anti-inflammatory effect of nonsteroidal drugs might also be due to antioxidant/free radical scavenger action against MPO system [21].

Nitric oxide is involved in different physiological processes like neurotransmission, vasodilation, blood pressure control, immunity processes and act as a mediator in inflammation. It is produced by the enzyme nitric oxide synthase (NOS). Constitutively expressed NOS produce nitric oxide under the physiological control of calcium-calmodulin system. Inducible NOS produce nitric oxide in inflammatory conditions [22].

In this study it was found that the methanolic extract of the rhizome of *Zingiber zerumbet* possessed the ability to inhibit cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase (induced by LPS). This showed that the extract has very good anti-inflammatory activity. Cyclooxygenase has two isoforms – COX 1 and COX 2. COX 1 is expressed under physiological conditions particularly in platelets, endothelium and kidneys. COX 2 plays an important role in inflammation and the pain associated with it. Hence selective inhibition of

COX 2 is desirable. In this study we have not investigated selective inhibition but it has been reported that dual inhibitors of COX and LOX have excellent gastrointestinal tract safety profile [23]. MRZZ inhibited both COX and LOX. If only cyclooxygenase is inhibited, this will result in shunting of arachidonic acid metabolism towards leukotriene pathway and production of leukotrienes will be increased. Hence dual inhibitors of COX and LOX can achieve optimal anti-inflammatory activity with lower side effects.

Conclusion

Inflammation is a very complex process mediated through wide variety of mechanisms. In this study methanolic extract of the rhizome of *Zingiber zerumbet* exhibited cyclooxygenase, 5-lipoxygenase, myeloperoxidase and nitric oxide synthase inhibiting property. This shows multiple mode of action of the extract in executing the anti-inflammatory effect. Thus the methanolic extract of the rhizome can be used to develop medicine for various inflammatory disorders either in the crude form or as a novel source of new drug for modern medicine after further studies.

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