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Induction of apoptosis by zerumbone isolated from *Zingiber zerumbet* (L.) Smith in protozoan parasite *Leishmania donovani* due to oxidative stress

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ABSTRACT

In the present context of emergence of resistance aligned with the conventional anti-leishmanial drugs and occasional treatment failure compelled us to continue the search for replaceable therapeutic leads against *Leishmania* infection. Various ginger spices of the Zingiberaceae family are widely used as spices, flavouring agents, and medicines in South-east Asia because of their unique flavour as well as due to their medicinal properties. Zerumbone, a natural component of *Zingiber zerumbet* (L.) Smith, has been studied for its pharmacological potential as antiulcer, antioxidant, anticancer, and antimicrobial. In this study, we have shown that zerumbone could induce ROS mediated apoptosis in *Leishmania donovani* promastigotes and also found effective in reducing intracellular amastigotes in infected-macrophages. We emphasized the potential of zerumbone to be employed in the development of new therapeutic drugs against *L. donovani* infection and provided the basis for future research on the application of transitional medicinal plants.

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Introduction

Leishmaniasis, a parasitic disease caused by protozoa of the genus *Leishmania*, affects more than 12 million people worldwide. Treatment of leishmaniasis is based on pentavalent antimonials, drugs developed more than 50 years ago that are toxic and prone to drug resistance. Several drug screening of natural compounds have been successful in discovering novel compounds for treating some parasitic diseases. Extracts obtained from plants, as well as pure compounds including terpenoids, flavonoids (quercetin, rotenone) have been reported to possess significant antiprotozoal activities. Plants and natural products remain as the ideal resource in search for drug discovery because of their unique structural diversity and promising long term safety records.[1] *Zingiber zerumbet* (L.) Smith (*awapuhi*), also known as shampoo ginger (Malay = *lempoyang*) or pinecone ginger is a vigorous species of the ginger family with leafy stems growing to about 1.2 m (3.9 ft) tall. It is found in many tropical countries. The rhizomes of *Z. zerumbet* have been used as food flavouring and appetizers in various cuisines while the rhizome extracts have been used in herbal medicine. In Hawaii, the fresh rhizomes were used as medicine for indigestion and other ailments. For a toothache or a cavity, the cooked and softened '*awapuhi*' rhizome was pressed into the hollow and left for as long as was needed. To ease a stomach ache, the ground and strained rhizome material is mixed with water and drunk. Zerumbone was identified as a monocyclic sesquiterpene moiety [2,6,10-cy-cloundecatrien-1-one, 2,6,9,9-tetramethyl-,(E,E,E)-] of the essential component in rhizomes of *Z. zerumbet* (L.) Smith, shows a variety of physiological effects e.g. anti-cancer, HIV inhibitory, anti-inflammatory, anti-viral effects.[2] Recently, our neighbouring group indicated the anti-leishmanial effect of essential oil and zerumbone from *Z. zerumbet* (L.) Smith against *Leishmania donovani* promastigotes.[3] In this study, we have shown that zerumbone could induce apoptosis by disrupting oxidative axis and also effectively inhibited the intracellular amastigotes, pathogenic stage of the parasite in mammalian host.

Materials and methods

Extraction of essential oil and Purification of zerumbone

The plant materials were collected from Manipur, North-East India, 920 m from sea level, longitude 93°58″ and latitude 24°44″ in March, 2012. The plant was identified by the taxonomist of the institute and had given the accession number as IBSD/Z-42-23. Fresh rhizomes were collected and washed thoroughly with tap water. These were cut into 5–6 mm slices and put into the Clevenger type oil extractor. Oil samples were analyzed by GC-FID on a Agilent 5975 C inert XL MSD. The oil was dried over anhydrous sodium sulphate and stored at 4 ± 2 °C. The oil was analyzed by GC–MS on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. The GC was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, with film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 °C, column temperature, 60–240 °C at 3 °C/min, then held at 240 °C for 5 min; carrier gas, He; injection volume, 1 μL (splitless). The MS mass ranged from 40 to 650 *m/z*, filament delay of 3 min, target TIC of 20,000, a prescan ionization time of 100 μs, an ion trap temperature of 150 °C, manifold temperature of 60 °C, and a transfer line temperature of 170 °C. The constituents of the oil were identified using retention times, Kovats indices and mass spectra. Confirmed integrated peaks were then used for the percentage of each chemical constituent present in the essential oil. Kovats indices were calculated using the equation: $KI(x) = 100[(\log RT(x) - \log Pz)/(\log RT(Pz+1) - \log RT(Pz)]$, where $RT(Pz) \leq RT(x) \leq RT(Pz+1)$, and P4, …, P25 are *n* paraffins.[3,4]

Parasites maintenance and viability assay

The anti-proliferative effect of zerumbone was estimated on *L. donovani* AG83 (MHOM/IN/1983/AG83) as per the guidelines of biosafety committee of West Bengal State University. Promastigotes were transformed from splenic intracellular amastigotes of infected BALB/c mice in complete M199 medium (Invitrogen) supplemented with 1% penicillin–streptomycin (Invitrogen) and 10% FCS (GIBCO) at requisite temperature (22 °C). To estimate the percentage of inhibition, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micro method was used. Briefly, promastigotes cultures were incubated with or without (control) increasing concentrations of zerumbone (0.1–50 μM) for 48 h in a 96-well flat-bottom plate (200 μL per well; BD Falcon) in complete M199 medium. After 48 h of incubation at 22 °C, MTT (10 mg/mL, 10 μL per well) was added to each well and the plates were incubated for another 4 h at 37 °C. The reaction was then stopped with acidic isopropanol (0.4 mL 10 N HCl in 100 mL isopropanol, 100 μL per well), and the absorbance was measured at 595 nm.[5] The 50% inhibitory concentration of zerumbone had been determined from the plot of percent inhibition against increasing concentrations. Cytotoxic effect was also evaluated on PHA (5 μg/mL) stimulated murine splenocytes (1×10^6 cells per well) cells without (control) or with increasing concentrations of zerumbone (0.1–50 μM).

Analysis of cell cycle progression in L. donovani promastigotes

2.5×10^6 cells/mL exponential phase *L. donovani* AG83 promastigotes were incubated for 24 h and 48 h respectively in complete M199 medium in the presence or absence of 50% inhibitory concentration of zerumbone on promastigotes at 22 °C. After washing with 1× PBS, the cells were fixed in 45% ethanol (diluted in 1× PBS), treated with 500 μg/mL RNAse A and then suspended in 0.5 M sodium citrate containing 69 μM PI.[6] Acquisition was performed using a flow cytometer (BD FACSVerse™, BD Biosciences, USA) and the data were analyzed using Flowing software 2.5.

Externalization of phosphatidyl serine

In order to study the apoptosis inducing capacity of zerumbone in promastigotes, the treated cells were stained with Annexin V-PE and 7-AAD as per manufacturer's instruction