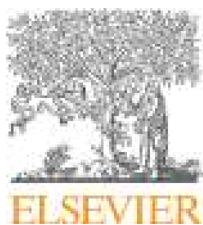

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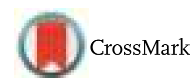
Original article

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

(BD Pharmingen). Briefly, 2×10^6 log phase promastigotes were incubated with IC_{50} concentration of zerumbone in triplicate for 24 h and 48 h respectively. They were washed twice with cold PBS and resuspended in 1× binding buffer at a concentration of 1×10^6 mL^{-1}. 100 µL of the samples was transferred to a fresh tube and Annexin V-PE (5 µL), 7-AAD (5 µL) were added, incubated for 15 min at RT in the dark. 400 µL binding buffer was added and cells were acquired in a flow cytometer (BD FACSVerse™, BD Biosciences, USA) and analyzed using Flowing 2.5 version software.[5,7]

Estimation of reactive oxygen species

In *Leishmania*, oxidative stress has been suggested to be responsible for the apoptotic process.[5,6] To estimate the level of ROS, the cell permeant probe H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) was used and analyzed by flow cytometer as described previously.[5] H_2DCFDA is a non-fluorescent dye which is converted into a fluorescent DCF (2′,7′-dichlorofluorescein) in the presence of proper oxidants inside the cells. Promastigotes were treated with IC_{50} concentration of zerumbone and the induction of ROS had been estimated at 1 h, 3 h, 5 h and 12 h by incubating with H_2DCFDA (20 µM) at room temperature for 20 min in dark. H_2O_2 has been used for positive control.[6]

Detection of chromatin condensation and cytoplasmic lipid droplet accumulation

The chromatin condensation and lipid accumulation in zerumbone-treated promastigotes were detected under confocal microscope after staining with DAPI (2 µg/mL) and Nile Red (10 µg/mL) as described earlier.[5–8] Images were obtained using an Olympus confocal laser scanning microscope (Model: IX81) and analyzed by Olympus fluoview version 3.0 viewer software.

Measurement of total lipid peroxidation

As elevation of ROS is related with the increase in lipid peroxides, we were keen to check the state of lipid peroxidation in treated promastigotes. *L. donovani* promastigotes (10^7) were treated with IC_{50} concentration of zerumbone for 1 h, 3 h and 5 h respectively. The cell-pellet was dissolved in 2 mL of 15% SDS-PBS solution. The fluorescence intensities of the total fluorescent lipid peroxidation products were measured with excitation at 360 nm and emission at 430 nm and expressed as relative fluorescence units with respect to quinine sulfate (1 mg/mL in 0.5 M H_2SO_4).[6]

Detection of the change in morphology by scanning electron microscopy

Control and treated promastigotes (2×10^6 cells) were fixed with 2.5% gluteraldehyde (Sigma Aldrich), dehydrated in ethanol, critical point-dried in CO_2, mounted on stubs, sputtered with a thin gold layer[5,6] and observed under a scanning electron microscope (Model: ZEISS EVO-MA 10).

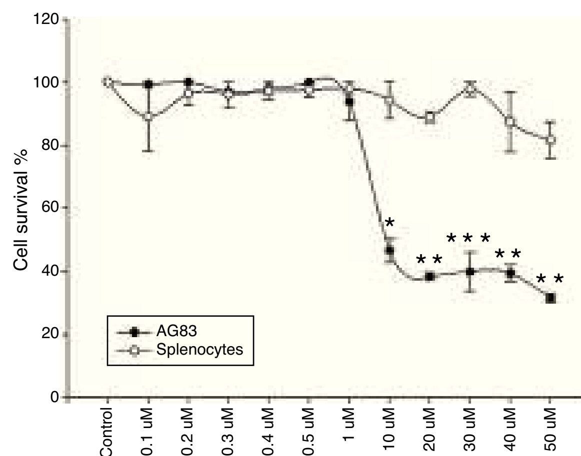

Fig. 1 – Anti-proliferative effect of zerumbone against *L. donovani*-promastigotes and PHA induced murine splenocytes with different concentrations of zerumbone (0.1–50 µM) as determined by MTT at 48 h and 96 h respectively. Each point corresponds to the mean ± SD of at least three experiments. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak post hoc test (*$p < 0.004$, **$p < 0.001$, ***$p < 0.01$ vs control).

Anti-proliferative activity on intracellular amastigotes

Peritoneal macrophages were isolated from thioglycolate (i.p., 4% (w/v), 1.0 mL/mouse) elicited peritoneal lavage of 6–8 weeks old male BALB/c mice as per the guidelines of institutional animal ethics committee of West Bengal State University. Cells

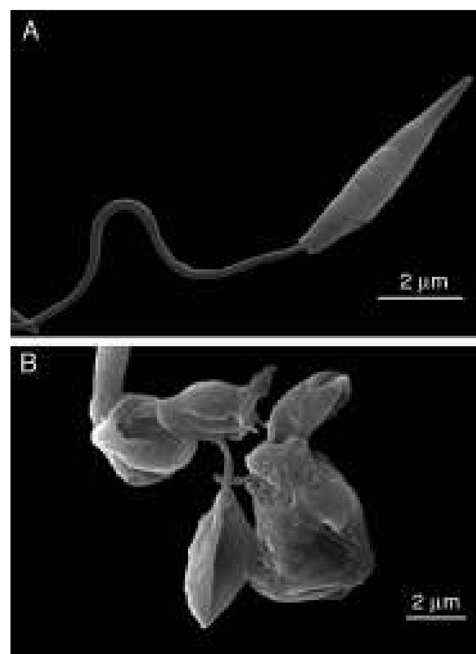

Fig. 2 – Scanning electron microscopy was performed to determine severe alterations in the morphology of zerumbone treated *L. donovani*-promastigotes in comparison to control culture. Images are representative profile of at least three independent experiments.

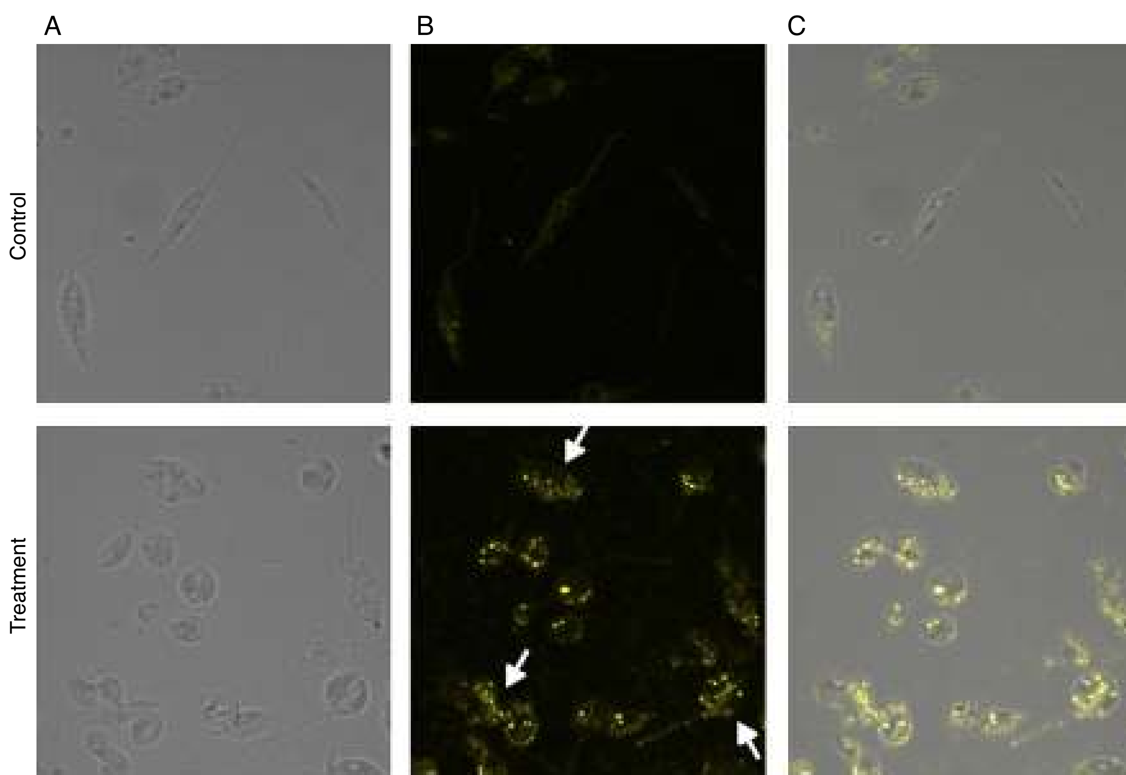

Fig. 3 – Accumulation of cytosolic lipid in zerumbone-treated *L. donovani* AG83 promastigotes as evident by nile red staining (magnification: 100×, zoom: 3.4×). (A) Phase contrast; (B) fluorescence; (C) phase contrast–fluorescence merge. Zerumbone treatment significantly increased lipid droplets accumulation (white arrows) in the cytosol in comparison to the untreated cells. Images are representative profile of at least three independent experiments.

were allowed to adhere in 8-chambered slides (10^5 cells per well) in complete RPMI-1640 at 37 °C in 5% CO_2 environment for 4 h. The subsequent steps of washing (3× PBS) were performed to move out the nonadherent cells and granulocytes and then the cultures were continued for another 48 h without any manipulation.[9,10] Adhered resting macrophages were infected with stationary phase of promastigotes (1:10), incubated for 6 h, washed (2× PBS) to remove the uningested promastigotes and cultured overnight in complete RPMI-1640 at 37 °C in 5% CO_2 environment. Cells were washed (3×) and incubated for additional 48 h in the presence or absence of graded concentrations of zerumbone. Prechilled methanol-fixed cells were stained with Giemsa, and examined under phase contrast microscope. At least 400 macrophages were examined for each set. Anti-leishmanial activity was determined by calculating the number of amastigotes per 100 macrophages.[9,11]

Statistical analyses

Statistical analyses for all experiments were performed by one-way ANOVA followed by post hoc Holm–Sidak test with the program Sigma Plot.

Results

Analysis of phytochemicals

The yield of essential oil was 0.12%. GC–MS analyses of the essential oil led to the identification of ten major compounds accounting for the 98.4% of the oil. Zerumbone (75.2%), α-caryophyllene (7.1%), camphene (5.1%), eucalyptol (2.4%), and camphor (3.0%) were the major components of the oil were identified in oil samples by Kovat analysis and comparison of mass spectra with those reported in the NIST mass spectra database (Supplementary Fig. 1). Compounds were quantified by performing area percentage calculations based on the total combined FID area. The percentage of a peak is a percentage relative to all other constituents integrated in the FID chromatogram. The differences in chemical composition of essential oil of the present study and previous research may be because of the geographic and climatic factors, chemo types, drying conditions and mode of distillation. Zerumbone was isolated in pure form and its structure was confirmed by ^{1}H NMR, ^{13}C NMR, DEPT, HR-ESIMS and comparison with literature data.

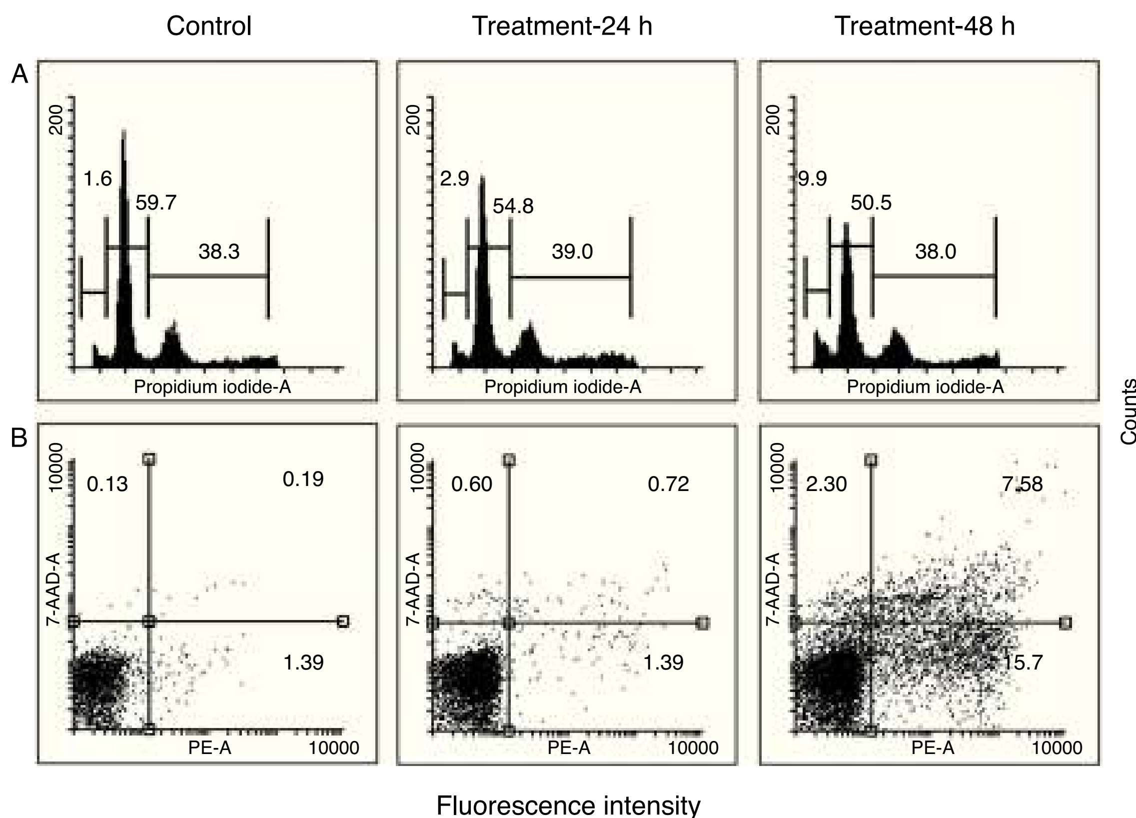

Fig. 4 – (A) Cell cycle progression in zerumbone treated promastigotes was assessed flow cytometrically by Propidium Iodide staining. Zerumbone induced cell death in promastigotes at 48 h by increasing the proportion of sub G_0–G_1 cells in comparison to control culture. Data are representative of at least three independent experiments. (B) Zerumbone caused the externalization of phosphatidyl serine as estimated by Annexin V and 7-AAD incorporation assay. The proportions of cells at early apoptotic (Annexin V+ 7AAD−) and late apoptotic phase (Annexin V+ 7AAD+) were increased time dependently following zerumbone treatment. Values represent the percentage of positive cells at the respective quadrants. Data are representative of three independent experiments.

Zerumbone inhibited the proliferation of L. donovani promastigotes

Zerumbone was found to inhibit the growth of *Leishmania* promastigotes dose dependently, *in vitro*. At a concentration of 10 μM, zerumbone inhibited the growth of *L. donovani* AG83 promastigotes approximately by 53.43%. Interestingly, the 50% inhibitory concentration of zerumbone (9.36 μM) could only inhibit the proliferation of PHA induced murine splenocytes by 5.75% even at 96 h (Fig. 1).

Zerumbone induced morphological alterations in L. donovani promastigotes

The treated promastigotes appeared rounded with loss of flagella with porous cell membrane (Fig. 2B) in comparison to the flagellated and slender promastigotes of the control culture (Fig. 2A).

Zerumbone caused lipid accumulation in L. donovani promastigotes

Another prominent effect resulting from the treatment of the promastigotes with zerumbone was the accumulation of lipid droplets in the cytoplasm (Fig. 3B and C), probably resulting from the accumulation of lipid precursors due to the drastic alteration of the sterol content in the parasite membrane. The alteration in lipid contents as evidenced from the deposition of lipid in the cytosol might also be correlated with plasma membrane integrity, leading to apoptosis.

Zerumbone altered the cell cycle progression and induced externalization of phosphatidyl serine in L. donovani promastigotes

At first, we have shown that the cell cycle progression of promastigotes was arrested at the sub-G_0/G_1 time dependently. At 24 h, the proportion of cells in the sub-G_0/G_1 was found only 2.9% in comparison to 1.6% of the control culture. Zerumbone further increased sub-G_0/G_1 cells from 1.6% (untreated) to 9.9% (treated) at 48 h accompanied by a decrease in the number of cells in G_0/G_1 from 59.7% to 50.5% (Fig. 4A).

A significant step of apoptosis is the translocation of phosphatidyl serine from the inner to the outer leaflet of the plasma membrane.[12] The externalization of phosphatidyl serine residues was observed in 1.39% (early apoptotic; Annexin V only) and in 0.19% (late apoptotic; Annexin V+ 7AAD+) of

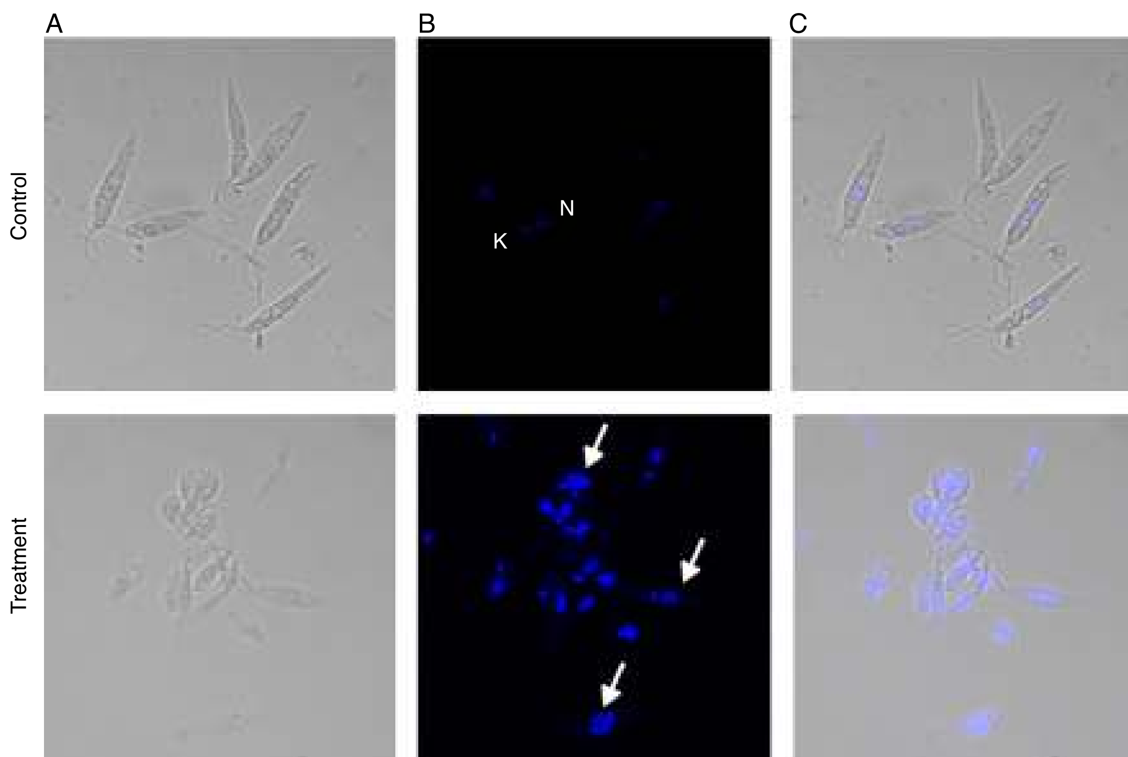

Fig. 5 – Zerumbone induced DNA condensation as visualized after DAPI staining at 48 h. Images were taken using an Olympus fluoview confocal microscope (Model: IX81) and analyzed by Olympus fluoview ver.3.0 viewer software (magnification: 100×, zoom: 3.4×). (A) Phase contrast; (B) fluorescence; (C) phase contrast–fluorescence merge. In untreated promastigotes, the nuclei (K: kinetoplast; N: nucleus) appeared as distinct blue structure, whereas zerumbone treated promastigotes showed blebbed nuclei and condensed chromatin material (white arrow). Images are representative profile of at least three experiments.

untreated promastigotes at 48 h. After the treatment with zerumbone (IC_{50} concentration) for 48 h, the percentage of early as well as late apoptotic cells was increased significantly with respect to untreated cells. The percentage of Annexin V positive cells (early apoptotic) increased to 15.7% at 48 h. The percentage of Annexin V positive 7AAD stained cells (late apoptotic) increased to 7.58% at 48 h with respect to the untreated cells (Fig. 4B).

Zerumbone caused DNA condensation in L. donovani promastigotes

DAPI was used to measure DNA condensation in promastigotes. DAPI staining showed discrete nuclei (blue spots) in untreated promastigotes whereas the treated promastigotes were observed to have blebbed nuclei and condensed chromatin material (Fig. 5B and C).

Zerumbone induced the oxidative stress in L. donovani promastigotes

We found that 50% inhibitory concentration of zerumbone against promastigotes could increase the level of ROS time dependently resulting in oxidative damage of the promastigotes (Fig 6A). The mean fluorescence intensity (MFI) of ROS generation in treated promastigotes increased time dependently in comparison to control culture (Fig. 6A).

Zerumbone increased the level of lipid peroxidation in L. donovani promastigotes

Zerumbone elevated the level of lipid peroxides in a time dependent manner after 1 h of treatment and reached to maximum level at 12 h [control vs treatment – 492.14 vs 696.55] (Fig. 6B).

Zerumbone inhibited the intracellular amastigotes in infected macrophages

Zerumbone was found effective against intracellular amastigotes and the 50% inhibitory concentration was estimated with the treatment of 5 μM of zerumbone at 48 h (Fig. 7).

Discussion

Zerumbone is a naturally occurring dietary compound, present in many natural foods consumed today. The compound derived from several plant species of the Zingiberaceae family that has been found to possess multiple biomedical properties, such as antiproliferative, antioxidant,

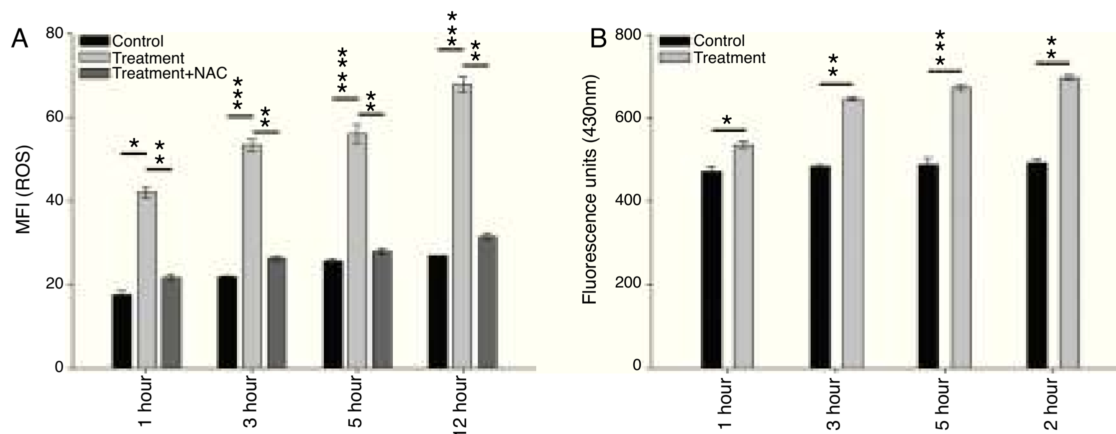

Fig. 6 – (A) Zerumbone induced ROS production in *L. donovani* AG83 promastigotes. ROS generation in treated promastigotes has been measured using H_2DCFDA at 1, 3, 5 and 12 h. Treatment of promastigotes with IC_{50} concentration of zerumbone revealed an elevation of intracellular ROS time dependently. However, pretreatment of promastigotes with the antioxidant NAC before treatment with zerumbone abrogated ROS generation in each time point. Each point corresponds to the mean ± SD of at least three experiments. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak post hoc test ($^{*}p < 0.004$, $^{***}p < 0.002$, $^{****}p < 0.005$ vs control; $^{**}p < 0.001$ vs treatment). (B) Zerumbone increased the level of lipid peroxidation time dependently. The total fluorescent lipid peroxidation products was quantified with excitation at 360 nm and emission at 430 nm and expressed as relative fluorescence units with respect to quinine sulfate (1 mg/mL in 0.5 M H_2SO4) by a spectrofluorometer at 1, 3 and 5 h. Each point corresponds to the mean ± SD of at least three experiments in duplicate. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak post hoc test ($^{*}p < 0.04$, $^{**}p < 0.002$, $^{***}p < 0.005$ vs control).

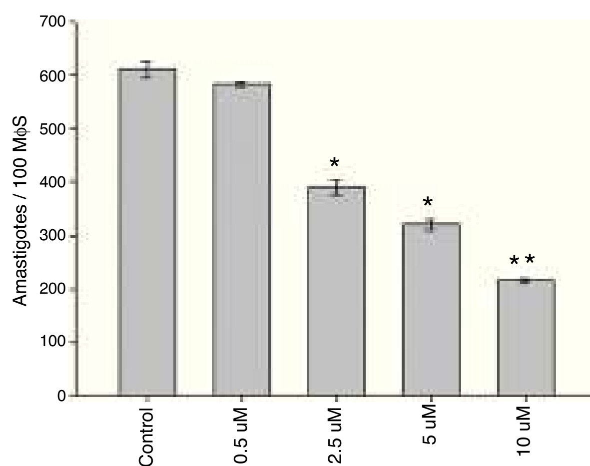

Fig. 7 – Zerumbone inhibited the proliferation of intracellular amastigotes with an IC_{50} of only 5 μM. Thioglycolate-elicited peritoneal macrophages of BALB/c mice were infected *in vitro* with *L. donovani* AG83 promastigotes. After established infection, cells were incubated with or without (control) graded concentrations (0.5–10 μM) of zerumbone at 37 °C in 5% CO_2 environment for the determination of anti-leishmanial activity on intracellular amastigotes. Each point corresponds to the mean ± SD of at least three experiments in duplicate. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak post hoc test ($^{*}p < 0.001$, $^{**}p < 0.002$ vs infected control).

anti-inflammatory, and anticancer activities.[2] However, evidence of efficacy is sparse against protozoan infection to support therapeutic claims to identify future uses against *L. donovani* infection. In the present study we successfully analyzed the nature of zerumbone-mediated cell death in *L. donovani* promastigotes and the possible key cellular mediators involved in the death cascade. Our initial observation that the zerumbone was effective against promastigotes but substantially non-toxic towards murine splenocytes (Fig. 1) made us curious for further in depth study. Morphological structure as observed through SEM has also authenticated the cytotoxic nature of zerumbone against *Leishmania* promastigotes (Fig. 2B and C) which can be correlated with the unnatural lipid accumulation on treatment (Fig. 3B and C). Interestingly, we found that zerumbone could increase the sub-G_0/G_1 (dead cells) up to 9.9% from 1.6% as in control promastigotes (Fig. 4A), concomitantly caused the externalization of phosphatidyl serine (Fig. 4B) in promastigote plasma membrane which is a crucial step in the process of apoptosis.[10] However, recently an interesting study raised the question on relevance of Annexin V binding assay in detecting apoptosis in *Leishmania* as they showed that the promastigotes lack phosphatidyl serine and Annexin V can also bind other lipids, including phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol.[13] Accordingly, we enquired the effect of zerumbone on *Leishmania* chromatin condensation and nuclear blebbing, the hallmark of apoptosis. The debate regarding the induction of apoptosis by zerumbone has been resolved and further confirmed by the DNA condensation in promastigotes (Fig. 5B and C). Looking into the mechanism, we found that the oxidative stress (Fig. 6A) followed by an increase

in the level of lipid peroxidation (Fig. 6B) in zerumbone-treated promastigotes might involve the alteration of mitochondrial membrane potential leading to apoptosis.[14] The ultimate conviction came true when we found that zerumbone inhibited the clinically important morphs of *L. donovani* in mammalian host, the intracellular amastigotes in macrophages (Fig. 7). In conclusion, our findings indicate that zerumbone induced ROS-mediated apoptosis in *L. donovani* promastigotes and further pharmacological studies on this particular anti-leishmanial efficacy, *in vivo*, against *L. donovani* infection appear promising.

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Ethical approval

Approved.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bjid.2015.10.002.

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