Original Article

Imperatorin a furocoumarin inhibits periplasmic Cu-Zn SOD of Shigella dysenteriae their by modulates its resistance towards phagocytosis during host pathogen interaction

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1. Introduction

Shigella are invasive bacteria that cause dysentery, a disease of considerable public health importance, particularly in the developing world, where it is often fatal for young children. Bacterial invasion of the colonic epithelium leads to severe inflammation together with bacterial dissemination generates abscesses and ulcerations [1,2]. This tissue damage accounts for the clinical manifestation of dysentery, which is a severe form of bloody diarrhoea [3]. Factors such as the 220 kbp plasmid, invasive plasmid antigens, lipopolysaccharides and outer membrane proteins contribute to its virulence. Apart from these, antioxidant enzymes play a vital role in protecting Shigella from the oxidative burst in epithelial cells and macrophages and live free in the cell’s cytoplasm [4]. Microbial antioxidant enzymes such as superoxides dismutases and catalases form part of the defence mechanism [5]. The activities of these enzymes have been shown to be induced when bacteria shift from the anaerobic environment of the host intestine to an aerobic environment during invasion when an instantaneous oxidative burst is confronted. In some Vibrio sp. of marine expression of SOD is constitute which helps bacteria to survive in the hostile environment of deleterious reactive oxygen intermediates [6,7].

The four main classes of antioxidants that have been identified in the bacterial system based on location, metallic co-factor and functional importance are:

- cytosolic Mn-SOD [8];
- FeSOD [9] which defend the bacterial cells against free radicals generated within the cell by bacterial metabolism;
- the Ni-SOD which was recently identified in Streptomyces coelicolor [10];
- periplasmic CuZnSOD which protects the periplasmic and membrane constituents from exogenous superoxide [11,12].

The emergence of multiple drug resistance in Shigella and inability to produce a vaccine even after 100 years of its discovery due to high antigenic variation has necessitated a search for
alternative therapeutic strategies. There is a growing interest in traditionally used medicinal plants, which produce a variety of compounds having therapeutic properties [13–15]. One such medicinal plant used since ancient time to cure diarrhea is Aegle marmelos.

A. marmelos Correa, commonly known as Bael, belongs to the family Rutaceae. Its stem, bark, root, leaves and fruits have medicinal value, and it has a long tradition as an herbal medicine. The medicinal properties of this plant have been described in the Ayurveda. In fact, as per Charaka (1500 B.C.), no drug has been longer or better known or appreciated by the inhabitants of India than the bael.

A. marmelos fruit is rich in two furocoumarins; psoralen and imperatorin, extract of fruit has been used for treating a stomachic and also used in treatment of diarrhea, dysentery and stomachalgie (Dikshit and Dutt, 1932). In this study, we exemplifies, decrease in virulence of Shigella dysenteriae grown in presence imperatorin, due to their vulnerability towards phagocytosis during its infection. This was facilitated by the inhibition of periplasmic copper, zinc, SOD of S. dysenteriae, which neutralizes the radical toxicity generated during host pathogen interaction.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Clinical isolates of S. dysenteriae were obtained from Dr. Mary V Jesudasan Head, Department of Microbiology, Christian Medical College, Vellore, India. Bacterial strains showing positive virulence by modified Sereny’s test [16] were chosen for the studies. A single colony from Luria-Bertani agar plate was inoculated in Luria-Bertani broth and incubated for 18 hrs at 37 °C, to grow for 24 hrs at ambient temperature for 24 hrs, and the plates were washed to remove non-adherent cells before infection, and new medium without antibiotics was added. The cells were infected Intracellular bacteria viability, host cell viability and TEM analysis of infected them to vacuum liquid chromatography on silica gel. Imperatorin fraction was subjected to HPLC and UV-Visible spectral analysis as described by Liu et al. 2004 [17]. The minimum inhibitory concentration of both AEAM and isolated imperatorin was determined by the 2-fold broth macrodilution method according to the National Committee for Clinical Laboratory Standards (1997) [18].

2.2. Preparation of aqueous extract of A. marmelos (AEAM)

Fruits of A. marmelos were collected from Vaniyambadi, Tamil Nadu, India. The fruits were shade dried, seeds were removed and fruits were ground mechanically. 500 g of powdered fruit were extracted with 1 liter of water for 12 hrs. The resulting extract was filtered using Whatman No. 1 filter paper. The filtrate was evaporated in vacuum to give a residue (Yield 2.98%). Further to isolate imperatorin, the filtrate was mixed with halogenated solvent in the ratio of 1:4 and incubated at ambient temperature for 24 hrs, which allows transfer of imperatorin to non polar solvent, subjecting them to vacuum liquid chromatography on silica gel. Imperatorin fraction was subjected to HPLC and UV-Visible spectral analysis as described by Liu et al. (2004) [17]. The minimum inhibitory concentration of both AEAM and isolated imperatorin was determined by the 2-fold broth macrodilution method according to the National Committee for Clinical Laboratory Standards (1997) [18].

2.3. Animals

The following animals were used for ileal loop inoculation of bacterial cells Wistar strain male albino rats weighing 120–150 g were obtained from Tanuvas-Lamu, Madhavaram, Chennai. Animals were fed normal pelleted diet and water, ad libitum. All the experiments were carried out as per the guidelines provided by the Institutional Animals Ethics Committee (IAEC) (IAEC No.01/015/06), CPCSEA No. 360/01/a CPCSEA.

2.4. Rat ileal loop ligation assay

Experimental shigellosis by Shigella species was carried out according to Arm et al. (1965) [19]. To assess the fluid accumulation and dilation of intestinal loops in rats, Male Wistar rats (body weight 100–120 g) were fasted for 24 hrs before being anaesthetized with Sodium pentothal (60 mg/kg body weight). After making a small incision in the abdominal region, inocula of 109 CFU in 0.5 ml of PBS, pH 7.4, were injected into ligated ileal loops in ileo caecal junction and the rats were allowed to live for 8 hrs. Histological analysis and intracellular bacterial count of ileal loop sample was performed according to Zychlinsky et al. 1996 [20].

2.5. Isolation and culture of human monocytes and bacterial infection

Monocytes were isolated from peripheral blood of healthy donors [21]. The heparinised blood was mixed with an equal volume (40 ml) of RPMI medium and layered on Ficoll-Hypaque at a ratio of 2:1 (Hi-Media) in 15-ml centrifuge tubes and spun for 20 min at 1500 rpm The layer containing the peripheral blood mononuclear cells was collected, resuspended in 20 ml of phosphate buffer saline (PBS), and recentrifuged for 10 min at 1500 rpm. After two washes in PBS, the cells were resuspended at a density of 5 × 10^6 cells/ml in RPMI medium containing 10% fetal bovine serum (FBS). Monocytes incubated in the same medium for only 1 day were used as the source of 1-day-old monocytes. Monocytes were suspended in fresh medium in either 12-well culture plates, or 6-well culture plates at a concentration of 10^6 cells/ml of medium containing 10% FBS in an atmosphere containing 5% CO2. Cells were termed monocytes if they were used within 24 hrs after isolation. These cells were used for bacterial infection. The plates were washed to remove non-adherent cells before infection, and new medium without antibiotics was added. The cells were infected Intracellular bacterial viability, host cell viability and TEM analysis of infected PBMC were done as described by Fernandez-Prada et al. 1998. Generation of reactive oxygen species (ROS) was evaluated by using dichlorofluorescein hydrate (DCFH) as a probe, according to Lebel et al. 1992 [22].

2.6. DNA fragmentation on agarose gels

DNA fragmentation was assessed as described by Mangan and Wahl, 1991 [23]. After incubation, cells were centrifuged (200 × g, 5 min, 5 °C), washed once in ice-cold PBS, and lysed. Low molecular weight DNA was isolated from the 13,000 × g supernatants from lysed cells and quantified by the diphenylamine reaction. DNA fragments from 5 × 10^6 cells were characterized by electrophoresis in 2% agarose.

2.7. Detection of superoxide dismutase activity

S. dysenteriae grown in presence and absence of AEAM were pelleted by centrifugation (2500 × g, 10 min, 4 °C). The supernatant was discarded, and the bacterial pellet was then suspended in 50 mM potassium phosphate (KPi) buffer, pH 7.8 containing 10−4 M EDTA and 0.2-mg/ml lysozyme. Bacteria were sonicated and debris [24]. Cell extracts were subjected to 12% PAGE under non-denaturing conditions at neutral pH. Prior to the application of the sample, a current was applied to each gel in the presence of Tris (187.5 mM) and EDTA (1 mM) to remove radicals such as ammonia persulfate. Bacterial extracts were then separated by electrophoresis in the presence of tris (50 mM), glycine (300 mM), and EDTA (1.8 mM) at constant current (40 mA). Wells were loaded with equal concentration of proteins, which were quantified by the method of Lowry et al. 1951 [25]. SOD activity was demonstrated as an achromatic zone in a blue background after photoactivation of riboflavin and reaction with nitroblue tetrazolium as described by Sun et al. 1988 [26].
2.8. Plasmid isolation

Isolation of Plasmid DNA was done using plasmid mini preparation kit obtained from GeNiTM, Bangalore according to the manufacturer’s instruction. Plasmids were detected by electrophoresis in 0.8% agarose gel containing 0.5 μg of ethidium bromide per ml and photographed under UV-light illumination [27].

2.9. Isolation of RNA

Bacterial total RNA was isolated according to Raja et al., 2008 [28]. Exponentially growing bacterial cell reaching the OD of 1.0 at 540 nm was harvested by centrifugation (5 min, 3800 g, 4°C). For preparation of total RNA, the phenol–guanidinium thiocyanate based Tri Reagent (GeNiTM, Bangalore) was used. To 10⁶ bacterial cells 1 ml of Tri reagent was added and lysed by repetitive pipetting and allowed to stand for 5 min followed by addition of 200 μl of chloroform for phase separation. Vigorously vortexed for 15 s and allowed to stand for 15 min followed by centrifugation at 12000 × g for 15 min at 4°C. The upper aqueous layer containing RNA, lipopolysaccharide and proteoglycans was transferred to a fresh sterile DEPC treated microfuge tube. To this, 250 μl of ice-cold isopropanol and 250 μl 0.8 M sodium citrate and 1.8 M sodium chloride were added, gently mixed and allowed to stand for 10 min and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol in DEPC treated water and again centrifuged at 14,000 × g for 10 min and stored at −80°C to get total RNA. This pellet was dissolved in 25 μl of sterile RNase free water by heating at 55°C for 20 min and stored at −20°C until use.

2.10. RT-PCR

To synthesize cDNA, a reverse transcription reaction solution containing the following reagents: 1.0 μg total RNA in RNase/ DNase-free water, and 1.5 μl random hexamer (d(T)6 primer (GeNiTM, Bangalore) were incubated for 10 min at 72°C and chilled immediately. To this, 5.0 μl premixed 10 mM dNTP solution (GeNiTM, Bangalore), 3.0 μl 10X M-MLV reverse transcriptase buffer (GeNiTM, Bangalore), 1.0 μl (200 units/μl) M-MLV reverse transcriptase (GeNiTM, Bangalore), were added and made up to 50 μl using sterile RNase/DNase-free water.

To amplify the cDNA, polymerase chain reaction (PCR) ready mix (GeNiTM, Bangalore) was used according to manufacturer’s instruction. All PCR samples were denatured at 94°C for 5 min prior to cycling and were extended for 10 min at 72°C following cycling. The PCR assay using primers was performed for 39 cycles at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s. Primer sequences for 16 s rRNA (Left 5’cagccacactggaactgaga 3’ and Right 5’gttagccggtgcttcttctg 3’) with product size 204 bp and sodC (Left 5’tctgcgccctactttacc 3’ and Right 5’gtgactgtaactgctgctgctcgc 3’) with product size 210 bp were designed using primer3 software available free on http://fokker.-wi.mit.edu/primer3/input.htm and nucleic acid sequence was accessed from http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val = NC_007606. The primers were purchased from Integrated DNA technologies, USA.

2.11. Docking Studies

Autodock 4.0 and Insight II were used to study interaction of imperatorin with Cu-Zn SOD of Escherichia coli (1eso). Due unavailability of Protein Data Base (PDB) structure of Cu-Zn SOD of Shigella, we used Protein Data Base (PDB) structure of Cu-Zn SOD of E. coli after analyzing the sequence similarities by Clustal W. Preliminary studies showed that autodock is a robust approach with good docking accuracy and reliability in the docking of 1eso. However, problems associated with Zn, Ca were found, causing a substantial error in the prediction of binding affinities with Insight, henceforth investigated further with autodock 4.0. Potential problems were associated with Insight for Zn, Ca, which were considered as limitation has been overcome by Autodock4.0 successfully. Zinc parameters were optimized including zinc radius; well depth and zinc charges were performed utilizing 1eso with imperatorin with good docking in Autodock 4.0. The following zinc parameters were considered for optimization:

- zinc radius 0.87 Å;
- well depth 0.35 kcal/mol;
- zinc charges +0.95 e.

By using the above parameters, it showed improvement in both docking accuracy at the zinc binding site and the prediction of binding free energies.

3. Results

3.1. Minimum inhibitory concentration of aqueous extract of A. marmelos

In the serial dilution experiment, the numbers of colonies (CFU/ml) were gradually decreased with increase in AEAM concentration, and the minimum inhibitory concentration of the extract was found to be 300 μg/ml. The concentration below this i.e. 200 μg/ml was used for further study.

3.2. Rat ileal loop Assay

Infection with AEAM treated S. dysenteriae are almost normal architecture of the colon (Fig. 1c) when compared with wild S. dysenteriae which caused shortening of villus, edema of villi, ulceration and inflammatory infiltration of hemorrhagic exudates in the luminal surface of the mucosa. (Fig. 1b) Uninfected loop and loop with AEAM alone injected showed normal architecture (Fig. 1a and d). Intracellular bacterial count of ileal loop was decreased by 46 percentile in case of AEAM treated S. dysenteriae infected loop when compared to wild S. dysenteriae infection. (Fig. 1e).

3.3. UV-visible spectral and HPLC analysis of imperatorin isolated form aqueous extract of A. marmelos

Further, analysis the active compound present in AEAM, the extract was subjected to isolation process and isolated of fraction was subjected to UV-visible spectral and HPLC analysis. UV spectrum of isolated fraction showed three absorption maxima at 219, 254 and 302 nm (Fig. 1f), which shows the presence of a furocoumarin imperatorin which was also confirmed by HPLC which shows a peak at 254 nm and its retention time at 14.5 min (Fig. 1g). MIC of isolated imperatorin was found to be 60 μg/ml; concentration below this i.e. 50 μg/ml was used for further analysis.

3.4. Intracellular survival of wild and imperatorin treated S. dysenteriae within PBMC

The infected PBMC were lysed, and the intracellular bacteria were recovered by plating on LB plates. The results depicted in Fig. 2a are representative of six experiments that were carried out with this protocol. CFU recovered from PBMCs infected with imperatorin treated S. dysenteriae at 3 and 6 hrs post infection is decreased when compared to wild S. dysenteriae, which showed a two fold increase in the number of intracellular bacteria.
Fig. 1. Histology of rat ileal loop and Intracellular bacterial count in infected rat ileal loop. a: control – showing normal architecture of rat intestinal mucosa; b: loop infected with *Shigella dysenteriae* showing shortening of villus, edema of villi, ulceration and inflammatory infiltration of hemorrhagic exudates in the luminal surface of the mucosa; c: loop infected with AEAM treated *S. dysenteriae* showing normal architecture with minimal inflammatory lesions; d: loop incubated with AEAM alone showing normal architecture; e: intracellular bacterial count in infected rat ileal loop. Intracellular bacterial count of ileal loop was decreased by 46% in case of AEAM treated *S. dysenteriae* infected loop when compared to wild *S. dysenteriae* infection; f and g: UV-visible spectral and HPLC analysis of isolated imperatorin aqueous extract of *Aegle marmelo*; f: shows UV spectrum of AEAM with three absorption peak at 219, 254 and 302 nm; g: HPLC which shows a peak at 254 nm (retention time – 14.51 min).
3.5. Cytotoxicity of PBMC infected with wild and imperatorin treated *Shigella dysenteriae*

At various time intervals after infection, PBMCs were recovered, washed with PBS and stained with 0.01% trypan blue counted in Neubauer hemocytometer by placing under light microscope. Viable cells will appear transparent while dead cells will appear blue. PBMCs infected with *Shigella dysenteriae* grown in absence of imperatorin showed a steady decrease in viability (48.7 and 12.1% at 3rd and 6th hour post infection, respectively) when compared with imperatorin treated PBMCs, which showed high viable cell count (78.1 and 63.1% at 3rd and 6th hour post infection, respectively) (Fig. 2b).

3.6. Levels of free radicals in PBMC

Fig. 2c shows the levels of free radicals in PBMC lysate. Levels of free radicals were increased significantly in both wild and imperatorin treated *Shigella* infected PBMC when compared to control.

3.7. Electron microscopy of PBMC

Transmission electron microscopy of control, wild and imperatorin treated *S. dysenteriae* infected PBMCs was carried out to observe detailed nuclear and cellular ultrastructure of PBMC and to analyze the fate of intracellular bacteria (Fig. 3). PBMC infected with wild bacteria showed many bacteria enclosed within small and large phagocytic vacuoles even after 6 hrs of infection. It was clear that PBMCs internalized more than one bacterium, suggesting that multiple uptake events occurred per mononucleocyte. It was also evident that internalized bacterial cells were dividing, indicating either that these bacteria cells were internalized as such or that the bacteria after internalization were actively multiplying within the confines of the phagosome. The nuclei were swollen, the chromatin was dispersed, cytoplasmic structures were obliterated, and the plasma membrane was ruptured in PBMC, whereas the double membrane of the gram-negative bacterium and the surrounding vacuolar membrane were clearly visible (Fig. 3b). In imperatorin treated *S. dysenteriae* infected cells, bacteria were found inside the phagocytic vacuoles, had only a partially preserved double membrane and more severe bacterial destruction was observed corresponding to lethal injury to bacteria (Fig. 3c). Uninfected PBMCs showed normal architecture (Fig. 3a).

3.8. DNA fragmentation assays of PBMCs

DNA isolated from PBMCs infected with imperatorin treated *S. dysenteriae* showed low levels of chromatin cleavage or ladder-like pattern on agarose gels at 6 hrs post infection (lane 3) (Fig. 3d) when compared to PBMC infected with wild *S. dysenteriae* (lane 2). PBMCs incubated with imperatorin alone (50 μg/ml) were used to study the effect of imperatorin and uninfected PBMCs which served as control, did not show the characteristic ladder-like pattern indicative of apoptosis (lane 4 and 1, respectively).

3.9. Superoxide dismutase activity

Activity of isoforms of SOD such as Mn SOD, hybrid SOD, Fe SOD and Cu-Zn SOD were found in *S. dysenteriae* grown in absence of imperatorin, whereas activity Cu-Zn SOD was not found in *S. dysenteriae* grown in presence imperatorin, while the activities of the other isoforms were not affected (Fig. 4a).

3.10. Plasmid profile

The plasmid DNA profiles of wild and imperatorin treated *Shigella* species are shown in Fig. 4b. The plasmid profile did not show any significant difference in imperatorin treated *Shigella* when compared with the wild type.

3.11. Expression analysis of sodC and 16 s rRNA

RT-PCR was used to determine the expression levels of the sodC, which codes for Cu-Zn SOD. sodC did not show any significant change in expression between *Shigella* grown in presence and absence of imperatorin. 16 s rRNA was used as internal control (Fig. 4c).

3.12. Docking studies

Due unavailability of Protein Data Base (PDB) structure of Cu-Zn SOD of *Shigella*, we used Protein Data Base (PDB) structure of Cu-Zn SOD of *E. coli* after analyzing the sequence similarities by Clustal W. Multiple Sequence Alignments done with CLUSTAL 2.0.8 sequence
1. *E. coli* (1eso) and sequence2. *S. dysenteriae* from reference sequence NC_007606 with GeneID: 3795781, showed a Score: 98% similarity (Table 1).

Docking of imperatorin with Cu-Zn SOD showed specific binding in all the ten frames with binding energy of –8.03 with least inhibition constant of 1.3 µM (Table 2a). It also revealed that imperatorin interacts with Cu-Zn SOD in the active site with a hydrogen bond distance of 2.22 inferring strong binding (Fig. 4d and f). Table 2b shows interaction of imperatorin with particular amino acid of Cu-Zn SOD with their bond length.

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**Fig. 3.** Transmission electron microscopy of PBMC infected with *Shigella dysenteriae*. PBMCs infected with wild and imperatorin treated *S. dysenteriae* were fixed after 6 hrs of incubation at 37 °C; a: shows normal architecture of uninfected PBMC. Both wild and imperatorin treated *S. dysenteriae* are seen clearly contained within vacuoles; b: multiplication of wild *S. dysenteriae* within phagosome is clearly seen; c: signs of degeneration of imperatorin treated *S. dysenteriae* are evident at 6 hrs. B: bacteria; N: nucleus; V: vacuoles. Bars: 1 µm; d: electrophoresis pattern of DNA fragments isolated from PBMCs. DNA from PBMCs infected with AEAM treated *S. dysenteriae* showed low levels of ladder-like pattern on 2% agarose gels at 6 hrs post infection (lanes 3) when compared to PBMC infected with wild *S. dysenteriae* (lanes 2). PBMCs incubated with imperatorin (50 µg/ml) were used to study the effect of imperatorin and uninfected PBMCs served as control, which did not show the characteristic ladder-like pattern indicative of apoptosis (lane 4 and 1).
4. Discussion

Infection of rat ileal loop with AEAM (200 μg/ml) treated *S. dysenteriae* showed almost normal architecture and decrease intracellular bacterial count when compared with *S. dysenteriae* infected loop. Further, the active principle present in AEAM is identified as furocumarin, imperatorin. Hence, the loss of virulence and decrease viable intracellular bacteria is attributed as imperatorin's effect. Thus, further study was carried by using imperatorin isolated from the AEAM, by treating *S. dysenteriae* at a concentration of 50 μg/ml.

The loss of virulence and decrease viable intracellular bacteria may be due to effect of imperatorin on 220 kbp plasmid, which encodes for Invasive plasmid antigens or on antioxidant enzymes of bacteria which negotiates with radical toxicity generated during host pathogen interaction [29,30]. Since 220 kb plasmids of *S. dysenteriae* play a vital role in invasion of bacteria, plasmid profile of the both wild and imperatorin treated *S. dysenteriae* was analysed. However, change in plasmid profile was not observed between wild and imperatorin treated *S. dysenteriae*.

In *Shigella* invasion, primarily pathogens need to survive the deleterious effect of resident macrophages cells that prevail in the

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**Fig. 4.** a: SOD profile of *Shigella dysenteriae* grown in presence and absence of imperatorin. Twelve percent native PAGE showed achromatic bands detected in *Shigella dysenteriae* grown in absence and presence of imperatorin (Lane 1 and 2, respectively). Significant differences were observed between *S. dysenteriae* grown in presence and absence of imperatorin. Cu-Zn SOD band was absent in *S. dysenteriae* grown in presence of imperatorin (Lane 2); b: Plasmid profile of *S. dysenteriae* grown in presence and absence of imperatorin. Five plasmids were detected in all *S. dysenteriae* grown in absence and presence of imperatorin [lane 1 and 2, respectively]. No significant differences were observed between bacteria grown in presence and absence of imperatorin; c: expression patterns of 16 s rRNA, and sodC of *S. dysenteriae* grown in absence and presence of imperatorin. Lanes 1 and 3 represent amplified product of 16 s rRNA of *S. dysenteriae* grown in absence and presence of imperatorin, respectively, showing almost equal expression. Lanes 4 and 5 represent amplified product of sodC of *S. dysenteriae* grown in absence and presence of imperatorin showing no significant difference between these lanes. Lane 1 is loaded with molecular weight marker; d and e: docking of imperatorin with leso. c: ligand imperatorin interacting with Cu-Zn SOD represented as surface level model. d: back bone structure representation of the interaction of imperatorin with Cu-Zn SOD at His 61.
The above results infer that the both sequences (1eso and Shigella) has strong similarity between them with a score of 98%.

<table>
<thead>
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<th>Table 1</th>
<th>Sequence analysis.</th>
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<td><strong>Sequences</strong></td>
<td><strong>Aligned</strong></td>
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<tr>
<td>seq1</td>
<td>ASEKVMENLTVSQG/VGSISGVTITETDKGEFSIPDJKALP</td>
</tr>
<tr>
<td>seq2</td>
<td>MKRFSLALALVATCGAQAEASEKVMENLTVSQG/VGSISGVTITETDKGEFSIPDJKALP</td>
</tr>
<tr>
<td>seq1</td>
<td>PGWGHFIHAKGQCPAT</td>
</tr>
<tr>
<td>seq2</td>
<td>KGKASAAEAGGHAIPNTGKREGPECAGHLGDLP</td>
</tr>
<tr>
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</tr>
<tr>
<td>seq2</td>
<td>DGDADIAVAPRLSDEIKDLAAMHVGGDNMSDQPK</td>
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CLUSTAL 2.0.8 Multiple Sequence Alignments. Sequence 1: Sodc of Escherichia coli (1eso) = 154 aa; sequence 2: Sodc of Shigella (GeneID: 3795781) = 173 aa.

<table>
<thead>
<tr>
<th>Table 2b</th>
<th>Interaction of imperatorin with particular amino acids of Cu-Zn SOD with their bond length.</th>
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<tr>
<td>Binding residues</td>
<td>Hydrogen bonding</td>
</tr>
<tr>
<td>His 178</td>
<td>1.921</td>
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<tr>
<td>Asp 180</td>
<td>1.23</td>
</tr>
<tr>
<td>Tyr 182</td>
<td>1.69</td>
</tr>
<tr>
<td>His 61</td>
<td>2.22</td>
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Follicular dome, instead of entering straight into epithelial cells via their apical pole. Invasion of the villous epithelium thus becomes the second event in the chronology of intestinal invasion [31]. Survival of bacteria within monocytes and macrophages needs periplasmic Cu-Zn SOD, which neutralizes the oxygen toxicity created by host during pathogen invasion.

Most of the available data dealing with oxidative stress and bacteria have come from observations with gram-positive microorganisms. Such studies involved the use of noncharacterized mutants or isolates that produced lower quantities of catalase or superoxide dismutase. On the other hand, Salmonella typhimurium TnJO transposon mutants have been isolated on the basis of their hypersensitivity to killing by monocyte [32,33]. Hence effect on imperatorin on intracellular survival of bacteria in PBMCs was studied. Wild S. dysenteriae were less sensitive to PBMC killing whereas S. dysenteriae grown in presence of imperatorin were killed by PBMC more efficiently. This can be correlated to the levels of free radicals in PBMCs and TEM analysis of infected PBMCs.

Generally, Shigella infection induces apoptosis in monocytes previous reports have indicated that a time-dependent differentiation of human monocytes into macrophages in in vitro studies is an important factor affecting the mode of cell death occurring after Shigella infection [34]. S. dysenteriae grown in presence of imperatorin infected PBMCs showed less cytotoxicity when compared with wild S. dysenteriae, which induced apoptosis in monocytes. Cleavage of chromosomal DNA is a characteristic feature of apoptosis [35]. Biochemically, the DNA is broken down into segments that are multiples of 200 bp due to specific cleavage between nucleosomes. DNA isolated from PBMCs infected with imperatorin treated S. dysenteriae showed low levels of ladder-like pattern on 2% agarose gels at 6 hrs post infection when compared to PBMC infected with wild S. dysenteriae. These findings suggest that S. dysenteriae succumb to free radicals produced by the host during Shigella invasion might be due to lack of Cu-Zn SOD, hence effect of imperatorin on antioxidant enzyme SOD of Shigella were investigated further.
Comparison of SOD profiles of S. dysenteriae in the absence and in the presence of imperatorin showed absence of Cu-Zn SOD activity. S. dysenteriae in presence of imperatorin. Expression of sodC transcript was unaltered in S. dysenteriae grown in presence of imperatorin when compared with S. dysenteriae grown in absence of imperatorin showing loss of activity in independent of transcriptional regulation by imperatorin. Since 220 kbp plasmid of Shigella also play a role in regulating expression of this bacterial Cu-Zn SOD [30]. However involvement of plasmid is neglected as there was no significant difference observed in plasmid profile of S. dysenteriae in presence and absence of imperatorin. These results suggest that decrease in activity is due to inhibition of Bacterium Cu-Zn SOD by imperatorin. To study the mechanism of inhibition of bacterial Cu-Zn SOD, it is important to identify active compound of imperatorin with Cu-Zn SOD by imperatorin. To study the mechanism of inhibition of bacterial Cu-Zn SOD, it is important to identify active compound of imperatorin.

In conclusion, AEM can be used alternative therapeutic strategy to treat shigellosis, as imperatorin in AEAM inhibits periplasmic Cu-Zn SOD of S. dysenteriae, modulates its resistance towards phagocytosis during host pathogen interaction, where S. dysenteriae succumb to oxidative radical toxicity generated during host pathogen interaction.

Conflicts of interest

We declare that authors do not have conflict of interest.

Acknowledgements

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References