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Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane

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ABSTRACT

Aim of the study: To evaluate the antibacterial activity of eugenol and its mechanism of bactericidal action against *Salmonella typhi*.
Materials and methods: The antibacterial activity was checked by disc-diffusion method, MIC, MBC, time course assay and pH sensitivity assay. The chemo-attractant property of eugenol was verified by chemotaxis assay. The mode of action of eugenol was determined by crystal violet assay, measurement of release of 260 nm absorbing material, SDS-PAGE, FT-IR spectroscopy, AFM and SEM.
Results: Treatment with eugenol at their MIC (0.0125%) and MBC (0.025%) reduced the viability and resulted in complete inhibition of the organism. Eugenol inactivated *Salmonella typhi* within 60 min exposure. The chemo-attractant property of eugenol combined with the observed high antibacterial activity at alkaline pH favors the fact that the compound can work more efficiently when given *in vivo*. Eugenol increased the permeability of the membrane, as evidenced by crystal violet assay. The measurement of release of 260 nm absorbing intracellular materials, SDS-PAGE, SEM and AFM analysis confirmed the disruptive action of eugenol on cytoplasmic membrane. The deformation of macromolecules in the membrane, upon treatment with eugenol was verified by FT-IR spectroscopy.
Conclusion: The results suggest that the antibacterial activity of eugenol against *Salmonella typhi* is due to the interaction of eugenol on bacterial cell membrane.

1. Introduction

Salmonella typhi (*Salmonella enterica* subsp. *enterica* ser. *typhi*) is a human restricted pathogen that causes 21 million cases of typhoid fever and 200,000 deaths each year. The disease is endemic in many developing countries particularly the Indian subcontinent, Southeast Asia, Africa and Central America. Infection with *Salmonella typhi* usually results from ingestion of contaminated food and water (Stearns and Koella, 2008). *Salmonella* are Gram-negative motile rods and belongs to the family Enterobacteriaceae. Based on the serology *Salmonella* is classified into more than 2200 serovars. Fundamental for *Salmonella typhi* infectivity is its capacity to cross the mucosa of the distal ileum, as well as to survive and multiply within macrophages (Contreras et al., 1997). It makes the innate immune system ineffective by inhibiting the oxidative burst of leukocytes. Once the bacterium invades the blood stream, it causes severe damage in gut epithelial cells, which leads to gastroenteritis and salmonellosis (typhoid). Many antibiotics and drugs like ampicillin, chloramphenicol and fluoroquinolones like ciprofloxacin are active against *Salmonella*, but the strain has developed multiple resistance to the first-line antibiotics in many developing countries (Finch, 2003). There are reports from India that ciprofloxacin has begun to produce delayed clinical responses in enteric fever with gradual increase in MICs of ciprofloxacin and clinically quinolone-resistant typhoid fever (Nath et al., 2000). The pathogenic role of *Salmonella* infection in the development of human diseases and the impact of resistance on the clinical outcome stimulated the search for newer treatments and natural products could provide alternative therapies against salmonellosis. Natural compounds possess an excellent therapeutic potential without developing resistance in the causative organisms (Culafic et al., 2005).

Research in the past decade has focused on the antimicrobial activity of various plant oil extracts and their components in the field of medicine and therapeutics (Gill and Holly, 2006). More specifically, essential oils derived from aromatic medicinal plants have been reported to exhibit exceptionally good antimicrobial

Abbreviations: AFM, atomic force microscope; ATR, attenuated total reflection; CFU, colony forming unit; CGMA, Chemical Gradient Motility Agar; EDTA, ethylenediaminetetraacetic acid; FT-IR, Fourier transform infrared spectroscopy; MAM, motility agar medium; MAO, monoamine oxidase; MBC, minimum bactericidal concentration; MHA, Mueller Hinton Agar; MIC, minimum inhibitory concentration; MTCC, microbial type culture collection; PBS, phosphate buffer saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM, scanning electron microscope; TNTC, too numerous to count.

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effects against bacteria, yeasts, filamentous fungi, and viruses (Reichling et al., 2009).

Eugenol (4-allyl-2-methoxyphenol), is a naturally occurring phenol essential oil extracted from cloves, is known to be an antioxidant (Jirovetz et al., 2006; Ogata et al., 2000), a monoamine oxidase (MAO) inhibitor and known to have neuroprotective effects (Kabuto et al., 2007). In addition eugenol exhibits an excellent bactericidal activity against a wide range of organisms like *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* (Walsh et al., 2003) *Listeria monocytogenes* (Filgueiras and Vanetti, 2006). Previous studies suggested that the mode of antibacterial action of eugenol is through disruption of cytoplasmic membrane, which increases its non-specific permeability (Gill and Holly, 2006). Moreover, the hydrophobic nature of eugenol enables it to penetrate the lipopolysaccharide of the Gram-negative bacterial cell membrane and alters the cell structure, which subsequently results in the leakage of intracellular constituents (Burt, 2004). Recent evidences reveal that the hydroxyl group on eugenol is thought to get bind to proteins, preventing enzyme action in *Enterobacter aerogenes* (Burt, 2004).

Although the *in vitro* antimicrobial activity of eugenol against various pathogens has been reported earlier, very little is known about its activity and mode of action against *Salmonella typhi*. Therefore, in the present study the mode of bactericidal action of eugenol against *Salmonella typhi* was evaluated.

2. Materials and methods

2.1. Bacteria and culture conditions

Salmonella typhi 733 used in this study was obtained from MTCC. Stock cultures were frozen at −80 °C in glycerol. For experimental use *Salmonella typhi* culture was maintained on nutrient agar slopes at 4 °C and subcultured every 4 weeks.

2.2. Preparation of antimicrobial agent

Eugenol 99% (v/v) was supplied by Loba Chemie, India. Stock solution of 30% (v/v) eugenol was prepared in methanol and the concentrations required for the experiments were prepared from this stock solution.

2.3. Determination of antibacterial activity

The antibacterial activity of eugenol was evaluated by disc-diffusion method (Kim et al., 1995). The exponential phase cultures of *Salmonella typhi* were adjusted to the concentration of 1.02×10^9 CFU/ml and were swabbed on Mueller Hinton Agar (MHA) plates. Sterile paper discs (6 mm diameter) were loaded with 30 μl of different concentrations of eugenol (1%, v/v and 10%, v/v). The air-dried discs were placed on MHA and incubated at 37 °C overnight. Ciprofloxacin (500 ng/ml) was used as positive control. Since eugenol was dissolved in methanol, antibacterial activity was measured for methanol by loading 30 μl of methanol in the sterile disc. Zone of clearance surrounding the discs was measured using a transparent ruler and the diameter was recorded in mm.

2.4. Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was measured by broth dilution method using Mueller Hinton Broth (MHB) (Ogata et al., 2000). Overnight broth cultures of *Salmonella typhi* were adjusted to the concentration of 2.85×10^9 CFU/ml. Ninety μl of different concentrations of eugenol [0.006, 0.0125, 0.025, 0.05 and 0.1% (v/v) (Final concentration)] was placed in sterilized test tubes to which 900 μl of medium and 10 μl of the overnight broth cultures were added. Ciprofloxacin (500 ng/ml) was used as positive control. Control and methanol control was also prepared with overnight broth culture and overnight culture with methanol, respectively. The tubes were incubated at 37 °C for 18–24 h and OD_{600} values of the cultures were measured, and the lowest concentration that inhibited the bacterial growth was taken as the MIC; the determinations were performed in triplicates. All the MIC tubes (100 μl of culture from each tube) were then used for spreading on Muller Hinton Agar plates for colony counting. The concentration at which no growth was observed was determined as minimal bactericidal concentration (MBC).

2.5. Determination of rate of kill

The rate of kill of *Salmonella typhi* upon treatment with eugenol was evaluated as described earlier (Culafic et al., 2005). Overnight broth cultures of *Salmonella typhi* were adjusted to the concentration of 2.85×10^9 CFU/ml and were treated with 0.05% (v/v) of eugenol (4× MIC). Control tubes were also prepared without eugenol. Then 100 μl of sample was taken and plated on MHA plates at regular time intervals (0, 30 min, 1 h, and 2 h). The plates were incubated at 37 °C for 24 h and CFU was calculated. All the determinations were done in triplicates.

2.6. pH sensitivity assay

The effect of pH on antibacterial activity of eugenol was examined by pH sensitivity assay (Wu et al., 2008). Overnight broth cultures of *Salmonella typhi* with different pH range (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) were prepared using 0.1N HCl and 5 M NaOH and swabbed on MHA plates with the corresponding pH. The antibacterial activity was analyzed by disc-diffusion method. Ciprofloxacin (500 ng/ml) was used as positive control.

2.7. Chemotaxis assay

The chemotaxis activity of eugenol was evaluated by using Chemical Gradient Motility Agar method (CGMA) (Garg and Kanitkar, 2006). Motility agar medium (MAM), which contains 0.7% agar, was used for this assay. Once the agar gets solidified three long rectangular wells (8 cm × 8 mm) were made. The wells on the right and left side were loaded with eugenol [1% (v/v)] and ciprofloxacin (1 μg/ml), respectively. The plate was left undisturbed for 60 min. The well at the center was loaded with 100 μl of exponential phase culture. The plates were incubated at 37 °C for 24 h. Glucose (1%) was used as positive control, since it has an excellent chemo-attractant property.

2.8. Crystal violet assay

The alteration in membrane permeability was detected by crystal violet assay (Vaara and Vaara, 1981). Suspensions of *Salmonella typhi* were prepared in LB broth. Cells were harvested at 4500 × *g* for 5 min at 4 °C. The cells were washed twice and resuspended in PBS (pH-7.4). Eugenol [1 and 5% (v/v)] and ciprofloxacin (500 ng/ml) was added to the cell suspension and incubated at 37 °C for 30 min. Control samples were prepared similarly without treatment. The cells were harvested at 9300 × *g* for 5 min. After that the cells were resuspended in PBS containing 10 μg/ml of crystal violet. The cell suspension was then incubated for 10 min at 37 °C. The suspension was then centrifuged at 13,400 × *g* for 15 min and the OD_{590} of the supernatant was measured in HITACHI UV–VIS spectrophotometer.

The OD value of the crystal violet solution, which was originally used in the assay, was taken and it was considered as 100%. The