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Original article

Thymol disrupts the membrane integrity of *Salmonella* ser. *typhimurium* in vitro and recovers infected macrophages from oxidative stress in an ex vivo model

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Abstract

Salmonella is a common bacterial enteropathogen responsible for many deaths every year. In the present study, we evaluated the mechanism of action of thymol against *Salmonella* ser. *typhimurium*, as well as its potential to induce intracellular killing and recovery from oxidative stress in macrophages. The minimum inhibitory concentration (MIC) of thymol against *S. typhimurium* was found to be 750 mg/l, and the CFU count decreased in a time-dependent manner. Excessive release of cellular materials and potassium ion also occurred in a time-dependent manner. Scanning electron microscopy showed disruption of membrane integrity. Intracellular killing capacity of macrophages was enhanced upon thymol treatment compared to control untreated cells. Thymol significantly reduced production of nitric oxide in a time-dependent manner, as well as the glutathione level. Disruption of membrane integrity was confirmed as the principle mechanism of action of thymol against *S. typhimurium*. Further, its potent role in inducing intracellular killing of *S. typhimurium* and recovery from oxidative stress in macrophages suggests that thymol can be applied as a naturally occurring drug against *S. typhimurium* in place of synthetic drugs.

Keywords: Thymol; *S. typhimurium*; Membrane damage; Intracellular killing; Oxidative stress

1. Introduction

Salmonella species are Gram-negative flagellated bacilli belonging to the Enterobacteriaceae family and are facultative intracellular pathogens. A variety of infectious diseases are caused by *Salmonella* spp., with gastroenteritis among the most common. Approximately 93.8 million cases of gastroenteritis occur each year worldwide, and among these, 80.3 million cases are food-borne [1]. The emerging problem of drug resistance has become a major obstacle in development of drugs against *Salmonella*. The most common route of *Salmonella* infection is through ingestion of contaminated foods or by animals infected with *Salmonella*. Other methods of transmission include mishandling of pets infected with *Salmonella*. Another very common route of *Salmonella* infection is hand-to-hand and mouth-to-mouth contact, especially in young children. The incubation period of *Salmonella* in the intestinal tissue is considered to be 5 days [1].

The antimicrobial properties of plant essential oils and their component compounds have been widely examined and reviewed [2] in terms of their effectiveness against numerous bacteria, viruses, fungi, parasites and insects [3]. A number of antibacterial agents derived from natural essential oils have been introduced [4,5]. Some of these supposedly interrupt the drug resistance mechanism of bacteria [6]. Among these, thymol (2-isopropyl-5-methylphenol) is abundant in a plant named *Thymus ciliates* and is reported to have better activity than other antimicrobial compounds [7] due to its effectiveness against both Gram-positive and Gram-negative bacteria [8].

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Thymol belongs to a group of compounds called monoterpenes, which are derived from isoprene hydrocarbons formed by the attachment of two or more isoprene molecules [9]. The chemical structure of thymol is hydrophobic, which suggests its capacity to permeabilize the cell membrane [9]. Several reports exploring the mechanisms of action of phenolic compounds have indicated that they mainly disrupt bacterial cell membranes, resulting in leakage of intracellular materials required for normal metabolism and survival [10,11].

Macrophages are the major cells involved in the first line of defense against a variety of extraneous invaders such as bacteria. Phagocytosis is the mechanism through which macrophages trigger the intracellular killing of pathogens. On the other hand, bacteria have various escape mechanisms, including alteration of phagocytosis, adaptation to phagolysosome fusion (*Salmonella* species) and inhibition of fusion between phagosomes and lysosomes in the case of *Listeria* and *Shigella* spp [12]. These alterations result in recurrent infection and failure of the complete treatment, especially for drugs that poorly penetrate cells [13].

Induction of oxidative stress is the most well-known mechanism by which macrophages interact with bacteria and their intracellular peptides, but persistent oxidative stress can lead to impaired macrophage function as well as inflammation through activation of the NF-κB pathway [14]. Therefore, drugs that not only kill intracellular bacteria but also recover macrophages from oxidative stress and inflammation can be very effective in treating bacterial infections.

This study aimed to uncover the mechanism of action of thymol against *S. typhimurium* as well as to determine its role in intracellular killing. We further analyzed the potential of thymol to recover macrophages from oxidative stress induced by *S. typhimurium*.

2. Materials and methods

2.1. Bacterial strain and chemicals

The bacterial strain *Salmonella enterica serovar typhimurium* wild-type, ATCC (14028), was used in the present study. All chemicals were used as supplied. Thymol, with a purity of 99.5%, was purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in 5% dimethyl sulfoxide (Sigma–Aldrich). Phosphate-buffered saline (PBS) with 5% dimethyl sulfoxide was used as a negative control for all experiments.

2.2. Minimum inhibitory concentration (MIC)

Active culture of *S. typhimurium* for MIC determination was prepared by transferring a loopful of cells from stock cultures into fresh LB medium, followed by incubation at 37 °C for 24 h. After the incubation period, culture was diluted with LB broth to achieve an optical density of 10^7 CFU/ml at 600 nm using a UV/Vis Spectrophotometer (Optizen 2120 UV). Dilutions of thymol were prepared in 96-well microplates to a final concentration ranging from 0 to 1000 mg/l in LB broth medium. Finally, 20 µl of inoculum of the bacterial strain (10^7 CFU/ml) was inoculated into the microplate and the tests were performed in a volume of 200 µl. The plates were incubated at 37 °C for 24 h. The lowest concentration of the test samples showing no visual growth of the tested organisms after macroscopic evaluation was determined as the minimum inhibitory concentration (MIC), which was expressed in mg/l.

2.3. Determination of bacterial killing efficacy by thymol

Viable count method was used for the bacterial killing assay according to time [2]. Briefly, all tubes containing fresh *S. typhimurium* culture in LB broth medium were inoculated with the MIC of thymol in LB broth and kept at 37 °C. At 0, 20, 40, 60, 80 and 120 min post-inoculation, culture was plated onto *Salmonella*-specific chromogenic medium (*Salmonella* Agar Base, Oxoid) with appropriate dilutions, followed by incubation at 37 °C for 24 h. Colonies were counted after the incubation period. The control was inoculated without thymol under the same experimental conditions as mentioned above.

2.4. Assay of potassium ion efflux

The concentration of free potassium ions in the bacterial suspension was measured after exposure to thymol (MIC) in 0.1% sterile peptone water for 0, 30, 60 and 120 min. The test was performed by a photometric procedure using a Kalium/Potassium kit (Quantofix, GmbH, Wiesbaden, Germany). A control flask without thymol was tested similarly and the results were expressed as the amount of extracellular free potassium (mmol/l) in the growth media at each interval of incubation.

2.5. Release of cellular materials

Measurement of cellular release materials (DNA) from *S. typhimurium* cells was carried out by inoculating the log phase of culture into 0.1% sterile peptone water with thymol (MIC) and without thymol as a control; tubes were incubated at 37 °C. Every 0, 30, 60 and 120 min of the incubation period, 1 ml of broth was transferred to an Eppendorf tube, which was centrifuged at 3500 rpm. The absorbance of the supernatant was then measured at 260 nm in an Optizen UV/Vis spectrophotometer. Results were expressed in the form of optical density recorded at each time interval.

2.6. Scanning electron microscopy (SEM) detection

To determine the efficacy of thymol on membrane integrity of *S. typhimurium*, SEM was performed on cells treated (90 min) with thymol (MIC) as well as control without treatment. SEM was performed as described previously with slight modifications [15]. The bacterial samples were washed gently with 50 mM phosphate buffer solution (pH 7.3) and fixed with 2.5 g/100 ml of glutaraldehyde and 1 g/100 ml of osmic acid solution. The specimen was dehydrated using sequential exposure to