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Enhancing chloramphenicol and trimethoprim *in vitro* activity by *Ocimum sanctum* Linn. (Lamiaceae) leaf extract against *Salmonella* enterica serovar Typhi

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ABSTRACT

Objective: To evaluate the antibacterial activity of *Ocimum sanctum (O. sanctum)* leaf extract, alone, and in combination with chloramphenicol (C) and trimethoprim (Tm) against *Salmonella enterica* serovar Typhi (*S. typhi*). **Methods:** The antibacterial activity of ethanolic extract of tulsi, *O. sanctum*, leaf (TLE; 500 μg) for 23 *S. typhi* isolates was determined following agar diffusion. The C (30 μg) and Tm (5 μg) activity alone and in combination with TLE (250 μg) was determined by disk diffusion. The zone diameter of inhibition (ZDI) for the agents was recorded, and growth inhibitory indices (GIIs) were calculated. **Results:** The *S. typhi* isolates (*n*=23), which were resistant to both C (ZDI 6 mm) and Tm (ZDI 6 mm), had TLE (500 μg) ZDIs 16-24 mm. The ZDIs of C and Tm were increased up to 15-21 mm and 17-23 mm, respectively, when TLE (250 μg) was added to the C and Tm discs. The GIIs ranged 0.789-1.235 and 0.894-1.352, due to combined activity against *S. typhi* isolates, of C and TLE and Tm and TLE, respectively. **Conclusions:** The data suggest that TLE, in combination with C and Tm, had synergistic activity for *S. typhi* isolates, and hence *O. sanctum* is potential in combating *S. typhi* drug resistance, as well promising in the development of non-antibiotic drug for *S. typhi* infection.

1. Introduction

Typhoid fever, caused by *Salmonella enterica* serotype Typhi (*S. typhi*), is an infectious disease of global distribution, and it remains an important worldwide cause of morbidity and mortality[1]. The emergence of multidrug-resistant (MDR) *S. typhi* showing resistance to ampicillin (A), chloramphenicol (C), trimethoprim-sulfamethoxazole (Tm-Smz) and fluoroquinolones, created therapeutic problem, and in turn the outbreaks of typhoid fever[2,3]. In this situation, the expanded-spectrum cephalosporins (parenteral as well as oral) were found effective, plus azithromycin could become the preferred drug of choice[4,5]. But these agents are expensive for routine use in developing countries including India[6]. Moreover, resistance to the cephalosporins like ceftriaxone has been reported[7]. This situation necessitated the introduction of new treatment regimen, and traditional medical practice has been known in many parts of the world[8-10]; about 80% of the world population depends on traditional medicine for its primary health care needs. Tulsi [*Ocimum sanctum* (*O. sanctum*) Linn., Lamiaceae], the holy basil, is described as medicinal plant in ancient literature, and in Ayurveda the plant has been well documented for its therapeutic potentials. The *O. sanctum* essential oil has been known to administer against asthma, bronchitis, sinus infections, constipation, nausea, vomiting and cramp[11].

The antibacterial activity of *O. sanctum* is also known. The aqueous extract of *O. sanctum* showed growth inhibitory effect on *Klesbiella*, *Proteus*, *Escherichia coli* (*E. coli*), and *Staphylococcus aureus* (*S. aureus*); while its alcoholic extract had similar effect on *Vibrio cholerae* (*V. cholerae*)[12]. The *O. sanctum* alcoholic extract was found to be active against MDR *S. aureus* showing resistance to common β-lactam antibiotics[13]. Rahman *et al*[14] reported antibacterial activity of *O. sanctum* leaf methanol extract against gram positive and gram negative bacterial pathogens, but they did not document the zone diameter of inhibition (ZDI) of

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the extract for *S. typhi*. We reported earlier the antibacterial activity of different plant extracts excluding *O. sanctum* against various pathogenic bacteria including *S. typhi*[9,10].

The synergistic antibacterial activity of plant extract in combination with antibiotics has been reported earlier. *In vitro* synergistic interaction of crude extracts from *Camellia sinensis, Lawsonia inermis, Punica granatum, Terminalia chebula* and *Terminalia belerica* was detected with tetracycline (T) against *S. aureus*; moreover, the *C. sinensis* extract showed synergism with A[13]. We for the first time reported synergistic anti-*S. aureus* activity of amoxicillin in combination with *Emblica officinalis* and *Nimphae odorata* extracts[10]. No report has been documented, at least from our part of the globe, the antibacterial actvity of *O. sanctum*, against *S. typhi*, alone or in combination with antibiotic. Herein, an *in vitro* study has been designed to ascertain the potential antibacterial activity of *O. sanctum*, and the possible synergistic action in combination with C and Tm against the clinical *S. typhi* isolates.

2. Materials and methods

2.1. Bacterial strains

The MDR *S. typhi* (n=23), isolated from blood samples from enteric fever patients, who attended the Calcutta School of Tropical Medicine, India for treatment, were used in the present study; the *S. typhi* isolates were associated with the enteric fever outbreaks in and around Kolkata, India[15]. The control strain used was *E. coli* ATCC 25922.

2.2. Plant materials and extract preparation

The fresh leaves of tulsi (*O. sanctum*) were collected from Kallabera, a village of the district Purulia of the West Bengal state, India, and the extracts were prepared following the protocol mentioned earlier[10] using 50 g of the dried materials, and ethanol as the extractant. The tulsi leaf extract (TLE) obtained was stored at 4 ℃ in 50% ethanol (final concentration of 10 mg/mL), and was utilized within one week; the extracts were prepared freshly when needed.

2.3. Agar diffusion susceptibility

The antibacterial activity of TLE was assessed for 23 *S. typhi* isolates following agar diffusion technique. The test protocol has been described in our earlier publication[10]; briefly, the extract (500 μg, *i.e.*, 50 μL), alone was dropped on one of the two properly marked sectors, TLE, on Mueller-Hinton agar (MHA) plates, each of which was inoculated with 10^8 CFU. The second sector, on each of the plates, on which dropped 50 μL of 50% ethanol results no ZDI to ZDI of 6 mm for the test bacterial isolates, was used as the control. The sensitivity to the plant extract for the *S. typhi* strains were considered with ZDI ≥7 mm, which has also been considered by earlier authors[10].

2.4. Disk diffusion susceptibility

The susceptibility to C and Tm for the isolates was determined by disk diffusion method, using 30 μg C and 5 μg Tm disks (Hi-Media, Mumbai, India). To determine the combined effect of C and Tm with TLE, 250 μg TLE (25 μL) was added to 30 μg C and 5 μg Tm disks placed on the surface of MHA plate inoculated with 10^8 CFU. The antibacterial activity of TLE alone was also recorded using 250 μg (25 μL) of the extract.

2.5. Interpretation of the results

The antibacterial activity of the agents was expressed by measuring the ZDI due to the action of the plant extract, alone or in combination with C and Tm, and C and Tm alone, after 24 h incubation at 35 ℃. The effect between the two antimicrobial agents was considered synergistic by the increase of ZDI from combined action compared to the average ZDI obtained due to single action of the two components; when zone diameter was less in combination, the interaction is defined as antagonistic, and additive when there was no change in ZDI in combination[10].

The growth inhibitory indices (GIIs) were calculated following the formula: [ZDI in combination / (total of ZDIs of the two agents in single action)][10], in order to corroborate the synergistic activity (as has been defined in terms of increment of ZDI) of the antibiotics in combination with the plant extract. The synergistic, additive and antagonistic activities, if any, in between the two of the antimicrobial agents were defined with GIIs > 0.5, 0.5 and < 0.5, respectively[10].

2.6. Statistical analysis

The χ^2 test was employed in order to compare the antibacterial activity (in terms of ZDIs) of the antibiotics (C and Tm) alone and that of the antibiotic (C or Tm) plus TLE, against *S. typhi* isolates; a P value of ≤0.001 was considered significant.

3. Results

The antibacterial activity of TLE (500 μg), in terms of ZDI, for *S. typhi* isolates is depicted in Figure 1. The ZDIs for the isolates ranged 16-24 mm; most of the isolates (n=15; 65.22%) had ZDIs 17-21 mm.

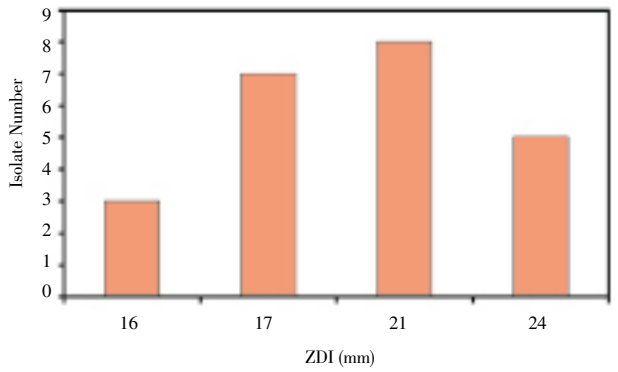

Figure 1. Zone diameter of inhibition (ZDI) of *O. sanctum* leaf ethanolic extract (TLE; 500 μg) for *S. typhi* isolates (*n*=23).

Figure 2 shows the ZDIs, for the test isolates, of TLE (250 μg) and antibiotics alone, and TLE plus the antibiotic (C or Tm). The isolates were resistant to both the antibiotics, C and Tm (ZDI 6 mm), while ZDIs from the action of TLE ranged 11-13 mm; thus, the active most agent of the current study was TLE. The ZDIs due to the action of C plus TLE and Tm plus TLE ranged 15-21 mm and 17-23 mm, respectively. The GIIs for the *S. typhi* isolates are represented in Figure 3. The GIIs values were > 0.5, and ranged 0.789-1.235 for C-TLE combination and 0.894-1.352 for Tm-TLE combination.

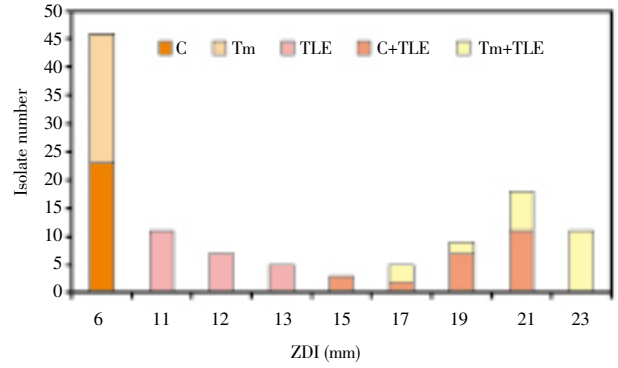

Figure 2. Zone diameter of inhibition (ZDI) of *O. sanctum* leaf ethanolic extract (TLE; 250 μg) alone and in combination with chloramphenicol (C) and trimethoprim (Tm) for *S. typhi* isolates (*n*=23).

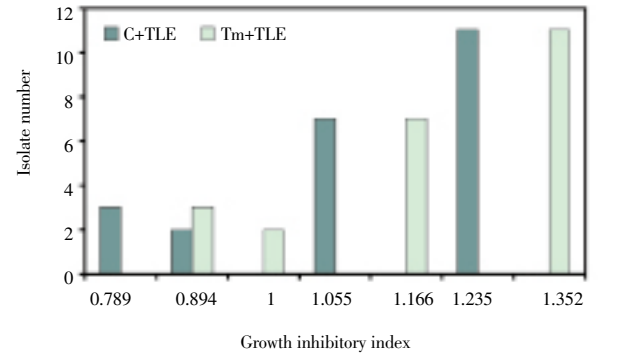

Figure 3. Growth inhibitory indices from combined action of *O. sanctum* leaf ethanolic extract (TLE) and antibiotics for *S. typhi* isolates (*n*=23). C = chloramphenicol, T = trimethoprim.

4. Discussion

Tulsi (*O. sanctum*) is abundantly available, easily accessible, economically feasible and culturally acceptable medicinal plant that exhibit less side-effect, and hence it can be recommended for long-term use. The important advantages of medicinal plants in various treatments include the safety besides their less expensiveness, efficacy and availability; the use of such plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases[16]. The *O. sanctum*, which in Sanskrit means "the incomparable one", is grown widely in India and is taken as the most sacred plant, and the whole plant is used as a source of remedy for a wide spectrum of diseases[17]. The use of this herb has been reported in Indian traditional system of medicine, and currently its application is receiving wide spread attention[18]. In this communication, TLE was found excellent against *S. typhi* associated with the enteric fever outbreaks in and around Kolkata, India.

The *O. sanctum* antibacterial activity has been reported earlier. The aqueous extract of *O. sanctum* showed wider ZDI for *Klebisella*, *E. coil*, *Proteus* and *S. aureus* while the alcoholic extract had ZDI for *V. cholerae*[11]. Mahmood *et al*[19] recorded higher antibacterial activity of *Ocimum* essential oil in gram positive bacteria compared to gram negative bacteria; however, Mishra and Mishra[20] found *O. sanctum* essential oil equally effective against both gram positive and gram negative bacteria. The ZDI was 17 mm due to the action of methanol extract of *O. sanctum* leaf against *S. aureus*[14]. The *O. sanctum* ZDI for *S. typhi* was 6 mm, as has been reported by Joshi *et al*[16]. The *O. sanctum* essential oil exhibited antibacterial activity against other enteric pathogens like *E. coli*, *P. aeruginosa* and *P. vulgaris* with ZDIs 10.5, 8.23 and 8.9 mm, respectively[21]. The *O. sanctum* showed significant antibacterial activity against human pathogenic bacteria like *E. coli*, *Klebsiella* sp., *Proteus mirabulus* (*P. mirabulus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *S. aureus* with maximum ZDI against *S. aureus* (20 and 41.5 mm), and minimum ZDI against *E. coli* (10.2 and 17.8 mm) using 5 and 10 μL of essential oil, respectively[19]. The current study demonstrates a concentration dependent activity of TLE against *S. typhi*; ZDIs ranged 11-13 mm and 16-24 mm, when TLE concentrations were 250 μg/mL and 500 μg/mL, respectively, and thus the isolates were found sensitive to the agent. The hexane extract of leaves of *O. sanctum* (eugenol) had antibacterial activity with a minimum inhibitory concentration (MIC) of 85-256 μg/mL against *Neisseria gonorrhoeae*[22]. The essential oil has been reported to show growth inhibitory effects of *Mycobacterium tuberculosis*; it has one tenth anti-tubercular potency of streptomycin and one-fourth that of isoniazid[23]. Goel[24] reported that *O. sanctum* leef extract effectively enhanced activation in macrophage and lymphocytes, depicted by the

elevated serum concentration of TNF-α, IFN-λ and IL-2 cytokines, leading to induce a protective resistance against *Salmonella typhimurium* infection.

Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *O. sanctum*, is mainly responsible for the therapeutic potential of the plant[22,25]; the other important constituents include carvacrol, and ursolic acid[26]. Recent studies showed linalool[27,28], methyl eugenol[29] and methyl chavicol[27] as lead compounds in the composition of *O. sanctum* essential oil. Thus, it is reasonable to speculate that the antimicrobial activity of *O. sanctum* can be attributed to the single or combined action of these molecules; the wider ZDI (16-24 mm) of TLE against *S. typhi* in the current communication supported this view. The tulsi fixed oil showed good antibacterial activity against *Bacillus pumilus*, *P. aeruginosa* and *S. aureus*; the higher content of linolenic acid in the fixed oil could contribute towards its antibacterial activity[30].

Synergism between known antimicrobial agents and bioactive plant extracts is a novel concept and has currently been described against infectious diseases[31]. Interestingly enough the combination C/Tm-TLE administered against the *S. typhi* isolates augmented the efficiency of the two antibiotics under consideration in the present study. Darwish *et al*[32] demonstrated an improvement of the efficacy of gentamycin (G) and C against *S. aureus* by the use of plant materials. Adwan *et al*[33], using well-diffusion method, showed additive interaction between water extracts of plants (*Psidium guajava, Rosmarinus officinalis, Salvia fruticosa, Majorana syriaca, Ocimum basilucum, Syzygium aromaticum, Laurus nobilis* and *Rosa damascene*) and antimicrobial agents (oxytetracycline; Tx, G, penicillin G; PG, Smz and enrofloxacin; Rfx) against *S. aureus*, while using microdilution method synergistic effects of antibiotic-plant extracts combination was achieved with significant reduction in the MICs of the antibiotics against the test isolates. Synergy testing between plant extracts (*Rus coriaria, Sacropoterium spinosum* and *Rosa damascena*) and antibiotics (Tx, PG, cephalexin, Smz and Rfx) against MDR *P. aeruginosa* isolates, following microdilution method, demonstrated a decrease in the MICs of the test antimicrobial agents, which thus be useful in fighting emerging drug-resistance[34]. Ahmad and Aqil[35] found synergistic interaction between crude extracts of Indian medicinal plants and antibiotics (T and ciprofloxacin) against extended spectrum β-lactamase producing multidrug-resistant enteric bacteria. The Tx presented synergism with methanolic extract of *Thespesia populnea*; *S. typhi* and *Shigella boydii* shows higher synergism, which has been indicated by large ZDI (32-36 mm)[36]. The tea extract showed synergistic activity with C and other antibiotics like G, methicillin and nalidixic acid against enteric bacteria[37]. The *O. gratissimum* leaf ethanolic extract showed synergistic activity against *P. aeruginosa* *P. mirabilis* in combination with Cp, against *P. mirabilis* and *E. coli* in combination with A, and against *E. coli* in combination with septrin[38]. The strong synergy observed between the action of TLE and C/Tm in our study is a significant finding demonstrating the therapeutic potentials of this plant; the increased ZDI of C plus TLE (15-21 mm) and Tm plus TLE (17-23 mm), and the GIIs values > 0.5, 0.789-1.235 for C-TLE combination and 0.894-1.352 for Tm-TLE combination supported the fact mentioned above. The current findings are consistent with the earlier *in vitro* studies that documented synergistic effects with significant increase in the ZDI of the antibiotic due to combined action of amoxicillin with crude plant extracts against *S. aureus*[10]. Thus, the evidence of *in vitro* synergism suggests the possibility of concurrent use of cost effective conventional anti-typhoid antibiotics (like C and Tm), in combination with the plant extracts, in treating human infection due to MDR *S. typhi*, when the agents no longer effective during monotherapy. However, future research based on animal models, may resolve *in vivo* efficacy of *O. sanctum*, either alone, or in combination with antibiotics.

Conflict of interest statement

We declare that we have no conflict of interest.

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