

## Comparative Antimicrobial Activity of Tea Tree Oil (Melaleuca Oil) and Common Topical Antimicrobials against Bacteria Associated With Wound and Topical Infections

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### Abstract

Tea Tree Oil (TTO) is a popular herbal antimicrobial for topical application against many microbes. This study was conducted to determine a spectrum of antimicrobial activity of TTO against bacteria often associated with topical infections and wound infection in human and animals. A total of 550 strains of bacteria and one strain of *Candida albicans* were tested for their sensitivity to TTO and eight antibiotics including polymyxin B sulfate, gentamicin, nitrofurantoin, tetracycline, chloramphenicol, co-trimoxazole, ciprofloxacin, and novobiocin. Gentamicin was the most effective antibiotic followed by chloramphenicol, ciprofloxacin, nitrofurantoin and polymyxin B inhibiting 87.1%, 84.8%, 76.8%, 75% and 72.8% strains, respectively. Tea tree oil (at 1  $\mu$ L/ mL) could inhibit the growth of 20.5% strains. Except all strains of *Streptobacillus*, *Sphingomonas*, *Cytophaga* and *Brahmella*, 71.4% *Brucella*, 60% *Bordetella* and 53.1% *Aeromonas* species (46.9%), only a few strains of other genera were sensitive to TTO. Only 20.5% strains were sensitive to TTO and multiple drug resistance (MDR) was positively correlated to their resistance to TTO, as 50%, 25%, 12%, 6% and 5% of the strains resistant to 0, 1-2, 3-4, 5-6 and 7-8 antimicrobial drugs, respectively were sensitive to TTO. Sensitivity of bacteria to TTO was positively correlated ( $p, \leq 0.05$ ) with their sensitivity to novobiocin ( $r, 0.24$ ), tetracycline ( $r, 0.22$ ), gentamicin ( $r, 0.21$ ), ciprofloxacin ( $r, 0.17$ ), nitrofurantoin ( $r, 0.16$ ), and chloramphenicol ( $r, 0.14$ ) while correlation was insignificant ( $p, >0.05$ ) with sensitivity to co-trimoxazole ( $r, 0.10$ ) and polymyxin B ( $r, 0.12$ ). Minimum inhibitory concentration (MIC) of TTO varied from 0.001% to  $>0.512\%$  (v/v) for different strains. The study revealed that TTO is a broad-spectrum antimicrobial active on 26 out of 44 genera of bacteria is a less promising antimicrobial than antibiotics on MDR strains. The study concluded that resistance to TTO, antibiotics and other antimicrobials in bacteria of clinical origin go hand in hand.

**Keywords:** Wound infection; TTO; MDR; Drug resistance; *Staphylococcus*; *Streptococcus*; *Escherichia coli*; *Pseudomonas*; *Serratia*

### Introduction

Tea Tree (*Melaleuca alternifolia*) Oil (TTO) or melaleuca oil is known since long for many medicinal uses [1]. It is a volatile essential oil obtained through steam distillation of the leaves and terminal branches of *M. alternifolia*. It is available globally and is used for its antimicrobial properties [2]. It is toxic on oral consumption and for injection but can be used for topical applications [3] however; studies revealed that TTO is not genotoxic *in vitro* in mammalian cells [4]. It is incorporated as the active ingredient in many topical formulations used for the treatment of cutaneous infections for controlling dandruff, acne, lice, herpes, and other skin infections [5]. After an elaborate study on 800 TTO samples [6] about 100 compounds are identified in TTO. Antibacterial activity of TTO has been reviewed [1,2] and is reported to be broad-spectrum inhibiting bacteria of more than 20 genera. The minimum inhibitory concentration (MIC) of TTO for most of the susceptible bacteria has been reported ranging from 0.003% (v/v) for *Prevotella intermedia* [7,8] and maximum  $>8\%$  (v/v) for *Enterococcus faecalis* strains [9]. A recent study on ATCC reference and clinical strains [10] of different bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *E. faecalis*, *Salmonella Enteritidis*,

*S. Typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*) reported MIC of TTO  $\leq 0.5\%$  (v/v) for all *P. aeruginosa* strains (4%, v/v).

Tea tree oil is bacteriostatic in low concentration but bactericidal at higher concentrations. Though exact mechanism of action is yet to be lucid it is hypothesized and proved to some extent [11,12] that TTO act through increasing permeability of liposomal systems causing lysis and the loss of membrane integrity and manifested by the leakage of ions and the inhibition of respiration and ultimately death of the bacterium [13].

In early studies on the antimicrobial activity of TTO, it was hypothesized to be more active against antibiotic-resistant bacteria [14] thus attracted considerable interest. Thereafter, several studies were conducted but only on a limited number of strains specifically using mupirocin-resistant and methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and of other bacteria [15-17]. Most of the studies concluded an insignificant difference in TTO sensitivity of antibiotic-resistant and sensitive strains [1]. Resistance to TTO in clinical isolates has not yet been reported and resistance of bacteria to conventional antibiotics has not been correlated with susceptibility to TTO [1], suggesting that cross-resistance does not occur. Tea tree oil is seen as important alternatives as a topical antimicrobial for antibiotics. Search for "alternatives to antibiotics" is identified as one of the most important goals to combat the emerging antibiotic resistant pathogens [18]. It is often claimed that TTO can replace antibiotics at least for topical applications [1,19]. The present study aimed to test the

sensitivity of variety and number of the bacterial strain, isolated from clinical cases with the potential to cause a wound or cutaneous infections, to TTO and simultaneously to common use topical antimicrobials. Data was analyzed for correlation and association between TTO sensitivity and antimicrobial sensitivity and multi-drug resistance (MDR) in different bacteria of diverse origin.

## Materials and Methods

Tea tree oil: Pure TTO (CAS Number 68647-73-4) purchased from Sigma-Aldrich (USA) was used in the study for determining its antibacterial activity.

Bacterial strains: A total of 550 strains of bacteria, belonging to 44 genera (Table 1) and one *Candida albicans* strain, isolated from clinical

samples of patient (Table 2) buffaloes (45), cattle (111), deer (16), dogs (87), elephants (4), fish (12), goats (5), human (39), mithun (*Bos frontalis*, 10), pigs (76), poultry (4), swamp buffaloes (38), and tigers (12) including 11 reference strains available in glycerol stocks at Epidemiology Laboratory of ICAR-Indian Veterinary Research Institute, Izatnagar were revived and maintained on brain heart infusion agar (BD Difco) slants throughout the study at 4-8°C in refrigerator [20]. The reference strains included *Enterobacter agglomerans* (Ravi), *Salmonella enterica* ssp. *enterica* serovar *Abortusequi* (E-155 and E-156), *Escherichia coli* (E 382), *Staphylococcus aureus* (Cocci-7), *Streptococcus milleri* (SFB), *Brucella abortus* strain 19 (S-19 and S-19B), *Brucella abortus* (S-99), *Listeria monocytogenes* (MTCC-839), and *Salmonella enterica* ssp. *enterica* serovar *Gallinarum* (E-79).

Genus of bacteria (strains tested in the study)	Species of bacteria tested and number of strains tested for sensitivity to TTO and other antimicrobials	Tested for MIC	MIC range in nL/mL	Strains with minimum and maximum MIC
<i>Achromobacter</i> (1)	<i>A. xylosoxidans</i> 1	1	>5120	<i>A. xylosoxidans</i>
<i>Acinetobacter</i> (8)	<i>A. bowmanni</i> 1, <i>A. haemolyticus</i> 1, <i>A. Iwoffii</i> 5, <i>A. schindleri</i> 1	1	10	<i>A. Iwoffii</i>
<i>Actinobacillus</i> (2)	<i>A. actinomycetecomitans</i> 1, <i>A. equeli</i> 1	0	0	
<i>Actinomyces</i> (1)	<i>A. pyogenes</i> 1	0	0	
<i>Aerococcus</i> (2)	<i>A. sanguinicola</i> 2	0	0	
<i>Aeromonas</i> (32)	<i>A. bestiarum</i> 6, <i>A. caviae</i> 2, <i>A. eucranophila</i> 1, <i>A. hydrophila</i> 6, <i>A. media</i> 8, <i>A. salmonicida</i> 2, <i>A. schubertii</i> 2, <i>A. sobria</i> 2, <i>A. veronii</i> 2	11	10->5120	<i>A. salmonicida</i> 10 and <i>A. schubertii</i> >5120
<i>Agrobacterium</i> (1)	<i>A. tumefaciens</i> 1	0	0	
<i>Alkaligenes</i> (8)	<i>A. denitrificans</i> 4, <i>A. faecalis</i> 4	0	0	
<i>Bacillus</i> (23)	<i>B. alvei</i> 1, <i>B. cereus</i> 3, <i>B. firmus</i> 2, <i>B. mycoides</i> 8, <i>B. pentothenicus</i> 1, <i>B. polymyxa</i> 4, <i>B. sphaericus</i> 1, <i>B. steariothermophilus</i> 1, <i>B. subtilis</i> 2	14	10->5120	<i>B. steariothermophilus</i> 10, <i>B. polymyxa</i> 10->5120
<i>Bordetella</i> (5)	<i>B. bronchiseptica</i> 5	2	2560->5120	<i>B. bronchiseptica</i>
<i>Brahmnelia</i> (1)	<i>B. cuniculi</i> 1			
<i>Brucella</i> (21)	<i>B. abortus</i> 20, <i>M. melitensis</i> 1	5	10->5120	<i>B. abortus</i> 10->5120, <i>B. melitensis</i> 2560
<i>Budvicia</i> (1)	<i>B. aquatic</i> 1	0	0	
<i>Burkholderia</i> (4)	<i>B. pseudomallei</i> 4	1	>5120	<i>B. pseudomallei</i>
<i>Candida</i> (1)	<i>C. albicans</i> 1	1	>5120	<i>C. albicans</i>
<i>Citrobacter</i> (5)	<i>C. freundii</i> 5	0	0	
<i>Cytophaga</i> (1)	<i>C. columnaris</i> 1	1	40	<i>C. columnaris</i>
<i>Dermatophilus</i> (2)	<i>D. congolensis</i> 2	2	40	<i>D. congolensis</i>
<i>Edwardsiella</i> (5)	<i>E. tarda</i> 4, <i>E. hoshiniae</i> 1	4	640->5120	<i>E. hoshiniae</i> >5120, <i>E. tarda</i> 640->5120
<i>Enterobacter</i> (26)	<i>E. agglomerans</i> 22, <i>E. amnigenus</i> 1, <i>E. canerogenus</i> 1, <i>E. gregoviae</i> 1, <i>E. intermedius</i> 1	1	10	<i>E. agglomerans</i>
<i>Enterococcus</i> (9)	<i>E. avium</i> 1, <i>E. durans</i> 1, <i>E. faecalis</i> 3, <i>E. raffinosus</i> 2, <i>E. solitarius</i> 2	1	640	<i>E. faecalis</i>
<i>Erwinia</i> (10)	<i>E. amylovora</i> 4, <i>E. carotovora</i> 2, <i>E. chrysanthemi</i> 3, <i>E. tracheiphila</i> 1	2	640->5120	<i>E. carotovora</i> 640, <i>E. chrysanthemii</i> >5120

<i>Escherichia</i> (108)	<i>E. coli</i> 103, <i>E. fergusonii</i> 4, <i>E. vulneris</i> 1	11	10->5120	<i>E. coli</i> 10->5120, <i>E. fergusonii</i> 5120
<i>Hafnia</i> (3)	<i>H. alvei</i> 3	0	0	
<i>Klebsiella</i> (21)	<i>K. oxytoca</i> 3, <i>K. pneumoniae</i> 18	1	320	
<i>Lemnorea</i> (1)	<i>L. ghrimontii</i> 1	0	0	
<i>Listeria</i> (1)	<i>L. monocytogenes</i> 1	1	5120	<i>L. monocytogenes</i>
<i>Micrococcus</i> (7)	<i>M. varians</i> 7	1	2560	<i>M. varians</i>
<i>Moraxella</i> (13)	<i>M. atlantae</i> 4, <i>M. canis</i> 2, <i>M. non liquifaciens</i> 2, <i>M. osloensis</i> 3, <i>M. phenylpyruvica</i> 2	5	640->5120	<i>M. nonliquifaciens</i> 1280->5120, <i>M. osloensis</i> 640-5120
<i>Morganella</i> (1)	<i>M. morganii</i> 1	1	>5120	<i>M. morganii</i>
<i>Pasteurella</i> (8)	<i>P. canis</i> 3, <i>P. multocida</i> 5	6	10->5120	<i>P. canis</i> 10, <i>P. multocida</i> 10->5120
<i>Plesiomonas</i> (5)	<i>P. shigelloides</i> 5	0	0	
<i>Pragia</i> (1)	<i>P. fontium</i> 1	0	0	
<i>Proteus</i> (22)	<i>P. mirabilis</i> 17, <i>P. penneri</i> 2, <i>P. vulgaris</i> 3	3	320->5120	<i>P. mirabilis</i> 320-2560, <i>P. penneri</i> >5120
<i>Providencia</i> (1)	<i>P. stuartii</i> 1	0	0	
<i>Pseudomonas</i> (49)	<i>P. aeruginosa</i> 29, <i>P. alcaligenes</i> 1, <i>P. fluorescens</i> 6, <i>P. paucibacillus</i> 5, <i>P. pseudoalcaligenes</i> 7, <i>P. vesicularis</i> 1	8	10->5120	<i>P. vesicularis</i> 10, <i>P. fluorescens</i> , <i>P. aeruginosa</i> >5120
<i>Raoultella</i> (6)	<i>R. terrigena</i> 6	3	5120->5120	<i>R. terrigena</i>
<i>Salmonella</i> (11)	<i>S. Gallinarum</i> 1, <i>S. Abortusequi</i> 2, <i>S. Adelaide</i> 1, <i>S. Kentucky</i> 3, <i>S. Typhimurium</i> 4	7	160->5120	<i>Serovar Gallinarum</i> 1280, <i>Abortusequi</i> 1280, <i>Typhimurium</i> 160->5120
<i>Serratia</i> (4)	<i>S. marcescens</i> 1, <i>S. odorifera</i> 3	3	1280	<i>S. marcescens</i> , <i>S. odorifera</i>
<i>Sphingomonas</i> (1)	<i>S. echinoides</i> 1	1	320	<i>S. echinoides</i>
<i>Staphylococcus</i> (59)	<i>S. aureus</i> 11, <i>S. auricularis</i> 2, <i>S. capitis</i> 6, <i>S. carnosus</i> 3, <i>S. caseolyticus</i> 1, <i>S. chromogenes</i> 4, <i>S. epidermidis</i> 2, <i>S. felis</i> 2, <i>S. haemolyticus</i> 7, <i>S. hominis</i> 1, <i>S. hyicus</i> 3, <i>S. intermedius</i> 8, <i>S. lentus</i> 4, <i>S. lugdunensis</i> 1, <i>S. schleiferi</i> 2, <i>S. warneri</i> 2	14	10->5120	<i>S. aureus</i> 20->5120, <i>S. haemolyticus</i> 10->5120
<i>Streptobacillus</i> (1)	<i>S. moniliformis</i> 1	1	10	
<i>Streptococcus</i> (50)	<i>S. adjacens</i> 1, <i>S. agalactiae</i> 2, <i>S. bovis</i> 7, <i>S. canis</i> 1, <i>S. defectivus</i> 2, <i>S. dysgalactiae</i> 1, <i>S. equi</i> 8, <i>S. faecalis</i> 1, <i>S. milleri</i> 12, <i>S. porcinus</i> 2, <i>S. pyogenes</i> 12, <i>S. suis</i> 1	16	10->5120	<i>S. equi</i> 10, <i>S. canis</i> >5120
<i>Vibrio</i> (6)	<i>V. alginolyticus</i> 2, <i>V. anguillarum</i> 2, <i>V. choleare</i> 1, <i>V. mimicus</i> 1	1	>5120	<i>V. choleare</i>
<i>Xenorhabdus</i> (3)	<i>X. bovienii</i> 1, <i>X. poinarii</i> 2	0	0	
Total	About 128 species of 45 genera 551	131	10->5120	

**Table 1:** Minimum inhibitory concentration (MIC) of tea tree oil (TTO) for strains of different bacteria of clinical importance.

Antimicrobial sensitivity assay for TTO and antimicrobials: All the 551 strains were tested in triplicate for their sensitivity to TTO and 8 other antimicrobials using disc diffusion assay [21,22] on Mueller-Hinton agar (MHA, BD Difco) or 5% bovine serum supplemented MHA for slow growing and/ or fastidious bacteria (*Streptococcus*, *Brucella*, *Bordetella*, *Brahmnella*, *Pasteurella*, *Listeria* and *Micrococcus* species strains). Test plates were incubated at 37°C aerobically for 24 h

except for *Brucella* (incubated in 5% CO<sub>2</sub> enriched environment, at 37°C for 48 h). Discs of TTO were made to contain 2 µL of oil using sterile 6 mm discs cut from Whatman filter paper no.3, discs of 8 of the topically applicable antibiotics including polymyxin B sulfate (50 IU), gentamicin (30 µg), nitrofurantoin (300 µg), tetracycline (30 µg), chloramphenicol (25 µg), co-trimoxazole (25 µg), ciprofloxacin (10 µg) and novobiocin (5 µg) were procured from BD Difco. The maximum

growth inhibition zones (in mm) measured from the triplicate tests were recorded for each strain and entered into the Excel 2010 worksheet.

Source	N	Number of bacteria resistant out of total number of bacteria tested									MDR strains
		TTO	PB	G	Nit	T	C	Cot	Cip	Nb	
Buffalo	48	38	10	6	5	5	4	11	8	38	10
Cattle	111	91	17	9	36	31	14	36	11	93	36
Deer	16	13	3	0	2	7	0	9	9	14	12
Dog	87	68	31	17	24	29	18	43	29	48	35
Elephant	4	4	0	0	0	0	0	0	0	4	0
Fish	12	10	4	0	5	0	3	4	0	12	5
Goat	5	5	2	0	0	1	0	0	0	3	0
Horse	78	66	34	13	22	31	19	23	24	65	44
Human	39	34	12	10	9	19	7	21	17	32	21
Mithun	10	4	0	0	1	0	6	0	0	3	0
Pig	76	57	24	11	18	30	10	41	19	59	38
Poultry	4	3	2	0	2	2	0	0	0	4	2
Reference	11	6	1	0	1	1	0	1	1	4	1
Swamp buffalo	38	29	8	1	11	3	1	2	0	36	6
Tiger	12	10	2	4	2	12	2	7	10	12	12
Total resistant (n=551)		438	150	71	138	171	84	198	128	427	218
% Resistant		79.5	27.2	12.9	25.0	31.0	15.2	35.9	23.2	77.5	39.6

**Table 2:** Source of bacteria and their resistance to different antimicrobials and tea tree oil. TTO, tea tree oil; PB, polymyxin B sulphate; G, gentamicin; Nit, nitrofurantoin; T, tetracycline; C, chloramphenicol; Cot, cotrimoxazole; Cip, ciprofloxacin; Nb, novobiocin, MDR, resistance to three of more antibiotics.

Determination of minimum inhibitory concentration (MIC) of TTO: Considering the findings of a recent study [10] that MIC of TTO for most of the bacteria is  $\leq 0.5\%$  (v/v) a total of 130 strains of 31 genera (Table 1) were tested to determine MIC of TTO in triplicate using agar well dilution method [21] using TTO dilutions to achieve 10 to 5120 nL of TTO/mL of medium. The growth medium and incubation procedures were the same as described for disc diffusion assay. After filling the wells (with 50  $\mu$ L of two-fold dilutions to contain 5120 nL to 10 nL/well) with the desired concentrations of TTO diluted in dimethyl sulfoxide (DMSO), plates were incubated without inversion for 1h and then inverted for the required period of incubation. In each plate, one well filled with only DMSO was kept as control.

Statistical analysis: To determine a correlation between sensitivity (zone of growth inhibition in mm) of test strains to TTO and other 8 antimicrobials correlation coefficient was calculated using MS Office Excel-7. To estimate the association between the sensitivity of bacteria of different types and of different sources to TTO and other antimicrobials, the  $\chi^2$  test was performed in MS Office Excel-2007. The statistical analysis to compare in the  $\chi^2$  test was done for those bacterial genera or sources only where the number (n) of strains tested was  $\geq 10$ .

## Results

Of the 550 bacterial strains belonging to more than 124 species of 44 genera and (Table 1) 20.5% were sensitive to TTO. The only strain of *C. albicans* of human origin was resistant TTO. Of the bacteria tested, 397 and 153 strains were of Gram-positive (GPB, of 47 species of 10 genera) and Gram-negative bacteria (GNB, of more than 77 species of 34 genera), respectively. They could further be divided into oxidase positive (186) and oxidase negative (364) bacteria. Of the total tested strains 31, 155, 122 and 242 strains were of oxidase positive GPB (OPGPB), oxidase positive GNB (OPGNB), oxidase negative GPB (ONGPB) and oxidase negative GNB (ONGNB), respectively. Of the tested strains of OPGPB, OPGNB, ONGPB and ONGNB, 71%, 65.8%, 84.4% and 86.8%, respectively were resistant to TTO having no zone of inhibition around TTO discs in any of the three repeats of the test. There was no significant difference in sensitivity of GPB and GNB (p, 0.4) strains to TTO. But oxidase positive strains were significantly more often (p, <0.001) sensitive to TTO than oxidase negative strains. More precisely, ONGNBs were significantly more often resistant to TTO than OPGPBs (p, 0.02), OPGNBs (p, <0.01) but no significant difference was observed with strains of ONGPBs (p, >0.5). However,

more of OPGNBs were sensitive to TTO than ONGPBs ( $p, <0.01$ ). Reference laboratory strains were more often sensitive to TTO than isolates from clinical samples either of human ( $p, 0.02$ ) or of animal ( $p, 0.04$ ) origin.

Of the tested microbes, 20.5%, 27.2%, 12.9%, 25%, 31%, 15.2%, 35.9%, 23.2%, and 77.5% were resistant to TTO, polymyxin B, gentamicin, nitrofurantoin, tetracycline, chloramphenicol, co-trimoxazole, ciprofloxacin and novobiocin, respectively (Table 3). Multiple drug resistance (resistance to three or more antibiotics, MDR) was present in 39.6% strains. Zone of growth inhibition by TTO was positively correlated ( $p, \leq 0.05$ ) with the zone of growth inhibition by novobiocin ( $r, 0.24$ ), tetracycline ( $r, 0.22$ ), gentamicin ( $r, 0.21$ ), ciprofloxacin ( $r, 0.17$ ), nitrofurantoin ( $r, 0.16$ ), and chloramphenicol ( $r, 0.14$ ) discs. However, the correlation was insignificant among TTO sensitivity and sensitivity to co-trimoxazole ( $r, 0.10$ ) and polymyxin B ( $r, 0.12$ ). Sensitivity to TTO had significant ( $p, \leq 0.005$ ) negative correlation ( $r, -0.23$ ) with MDR. All the bacterial strains having resistance to any one or more of the antibiotics were significantly more

often ( $p, <0.01$ ) resistant to TTO than those 32 strains sensitive to all the antimicrobials tested. Almost half of the strains sensitive to all the 8 antimicrobials were also sensitive to TTO, while only 25%, 12%, 6% and 5% of those resistant to 1-2, 3-4, 5-6 and 7-8 antimicrobial drugs respectively were sensitive to TTO.

Of the strains of 44 genera of bacteria, none of the 47 strains of 18 genera was sensitive to TTO (Table 3) including strains of some common bacteria associated with topical and wound infections viz., strains of *Acinetobacter*, *Actinobacillus*, *Actinomyces*, *Dermatophilus*, *Pasteurella* and *Serratia* spp. On another hand, all the four strains, one each of *Streptobacillus*, *Sphingomonas*, *Cytophaga* and *Brahmnella* species were sensitive to TTO. More than 50% of the strains of other bacteria tested were resistant to TTO except *Brucella* (28.6%), *Bordetella* (40%) and *Aeromonas* (46.9%) strains. The TTO was only little effective on the strains of the most common wound-infecting bacteria and bacteria causing topical skin infections including strains of *Staphylococcus* (19.9%), *Streptococcus* (22%), *Pseudomonas* (14.3%), *Escherichia coli* (8.3%) and *Klebsiella* (4.8%) species.

Genus (Strains tested)	Percent of strains resistant to the antimicrobials									MDR strains
	TTO	PB	G	Nit	T	C	Cot	Cip	Nb	
<i>Achromobacter</i> (1)	1	0	0	0	0	0	0	0	0	0
<i>Acinetobacter</i> (8)	6	3	1	5	1	1	2	0	7	3
<i>Actinobacillus</i> (2)	2	0	0	1	0	0	1	0	2	0
<i>Actinomyces</i> (1)	1	0	0	0	0	0	1	0	1	0
<i>Aerococcus</i> (2)	2	0	0	1	0	0	0	1	1	0
<i>Aeromonas</i> (32)	15	2	1	2	2	0	6	1	31	3
<i>Agrobacterium</i> (1)	1	0	0	0	1	0	1	0	1	1
<i>Alkaligenes</i> (8)	7	0	0	4	2	0	4	0	8	3
<i>Bacillus</i> (23)	15	4	1	3	0	7	7	2	10	4
<i>Bordetella</i> (5)	2	0	0	4	0	0	0	0	5	0
<i>Brahmnella</i> (1)	0	1	0	0	0	0	0	0	1	0
<i>Brucella</i> (21)	6	0	1	3	1	2	5	2	12	2
<i>Budvicia</i> (1)	1	0	0	1	0	0	0	0	1	0
<i>Burkholderia</i> (4)	3	4	0	4	0	4	0	0	4	4
<i>Candia</i> (1)	1	1	1	1	1	1	1	1	1	1
<i>Citrobacter</i> (5)	4	2	0	0	1	0	0	0	5	0
<i>Cytophaga</i> (1)	0	0	0	1	0	0	1	0	1	1
<i>Dermatophilus</i> (2)	2	1	0	0	0	0	0	0	2	0
<i>Edwardsiella</i> (5)	4	0	1	0	1	0	1	0	5	1
<i>Enterobacter</i> (26)	21	8	0	8	11	4	6	4	25	11
<i>Enterococcus</i> (9)	9	6	7	2	4	4	5	5	7	7
<i>Erwinia</i> (10)	7	2	0	1	3	0	2	1	10	4
<i>Escherichia</i> (108)	99	18	17	12	56	11	50	43	108	56

<i>Hafnia</i> (3)	3	0	0	1	1	0	1	1	3	1
<i>Klebsiella</i> (21)	20	6	1	10	7	0	4	6	19	12
<i>Leminorella</i> (1)	1	0	0	0	0	0	0	0	1	0
<i>Listeria</i> (1)	1	0	0	0	0	0	0	0	0	0
<i>Micrococcus</i> (7)	6	3	2	0	0	1	1	1	4	2
<i>Moraxella</i> (13)	7	2	0	3	1	0	4	0	11	1
<i>Morganella</i> (1)	1	0	0	1	0	0	1	0	1	1
<i>Pasteurella</i> (8)	8	0	0	0	1	0	4	0	6	0
<i>Plesiomonas</i> (5)	5	0	0	0	0	0	1	0	5	0
<i>Pragia</i> (1)	1	0	0	0	0	0	0	0	1	0
<i>Proteus</i> (22)	20	19	4	17	18	8	12	8	21	19
<i>Providencia</i> (1)	1	1	0	1	1	0	0	0	1	1
<i>Pseudomonas</i> (49)	42	7	6	36	27	31	31	10	44	35
<i>Raoultella</i> (6)	5	1	1	3	2	1	1	1	6	2
<i>Salmonella</i> (11)	6	0	0	0	3	0	0	3	10	3
<i>Serratia</i> (4)	4	0	0	0	0	0	0	0	4	0
<i>Sphingomonas</i> (1)	0	0	0	1	0	0	0	0	0	0
<i>Staphylococcus</i> (59)	52	19	16	2	17	4	24	27	21	24
<i>Streptobacillus</i> (1)	0	1	0	0	0	0	1	0	0	0
<i>Streptococcus</i> (50)	39	36	11	9	9	5	20	11	13	15
<i>Vibrio</i> (6)	4	3	0	1	0	0	0	0	5	1
<i>Xenorhabdus</i> (3)	3	0	0	0	0	0	0	0	3	0
Total (551)	438	150	71	138	171	84	198	128	427	218
% Resistant	79.5	27.2	12.9	25.0	31.0	15.2	35.9	23.2	77.5	39.6

**Table 3:** Tea tree oil and Antimicrobial drug resistance in bacteria isolated from clinical cases. TTO, tea tree oil; PB, polymyxin B sulphate; G, gentamicin; Nit, nitrofurantoin; T, tetracycline; C, chloramphenicol; Cot, cotrimoxazole; Cip, ciprofloxacin; Nb, novobiocin, MDR, resistance to three or more antibiotics.

On testing MIC of TTO on 130 strains of 31 genera of bacteria, the MIC was detected minimum (10 nL/mL, 0.001%, v/v) for strains of *Acinetobacter lwoffii*, *Aeromonas salmonicida*, *Bacillus stearothermophilus*, *Enterobacter agglomerans*, *Escherichia coli*, *Pasteurella canis*, *P. multocida*, *Pseudomonas vesicularis*, *Staphylococcus haemolyticus*, *Streptobacillus moniliformis*, and *Streptococcus equi*. The highest MIC of TTO (>0.512%, v/v) was observed in strains of *Achromobacter xyloxydans*, *Aeromonas schubertii*, *Bacillus polymyxa*, *Bordetella bronchiseptica*, *Brucella abortus*, *Edwardsiella tarda*, *E. hoshiniae*, *Erwinia chrysanthemii*, *E. coli*, *E. fergusonii*, *Listeria monocytogenes*, *Moraxella nonliquifaciens*, *M. osloensis*, *Morganella morganii*, *Pasteurella multocida*, *Proteus penneri*, *Pseudomonas aeruginosa*, *Raoultella terrigena*, *Salmonella Typhimurium*, *Staphylococcus aureus*, *Streptococcus canis* and *Vibrio cholera*. There were strains of the same bacterial species having TTO

MIC at both of the extremes viz., *B. polymyxa*, *E. coli*, and *P. multocida*.

No significant difference in sensitivity of GPBs and GNBs (p, 0.4) to TTO was seen. But oxidase positive strains were significantly more often (p, <0.001) sensitive to TTO. Oxidase negative GNBs were significantly more often resistant to TTO than OPGPBs (p, 0.02), OPGNBs (p, <0.01) but the difference was insignificant with ONGPBs (p, >0.5). More of OPGNBs were sensitive to TTO than ONGPBs (p, <0.01). However, no difference between OPGPBs, ONGPBs (p, >0.08) was evident for TTO sensitivity.

Gram-positive bacteria were more often resistant to polymyxin B (p, <0.001), gentamicin (p, 0.001) and ciprofloxacin (p, <0.001) than GNBs but for nitrofurantoin, tetracycline and novobiocin the opposite were the observation (p, 0.001), and no significant difference in their sensitivity to chloramphenicol, co-trimoxazole and TTO (p, >0.4).

Bacterial strains of human origin were more often resistant to gentamicin, tetracycline, co-trimoxazole and ciprofloxacin ( $p, <0.02$ ) than strains of animal origin. Similarly, oxidase positive strains were more often resistant to nitrofurantoin ( $p, <0.01$ ) than oxidase negative strains but more often sensitive to polymyxin B, gentamicin, tetracycline and ciprofloxacin ( $p, <0.01$ ). In general, irrespective of Gram staining, oxidase positive strains were more often resistant to chloramphenicol ( $p, <0.03$ ) but less to ciprofloxacin ( $p, \leq 0.03$ ). For tetracycline, sensitivity didn't differ significantly among OPGPBs and ONGBPBs ( $p, 0.8$ ), but GNBs were more often ( $p, <0.01$ ) resistant to tetracycline irrespective of their oxidase activity. Oxidase activity and Gram reaction were significantly associated characters with resistance to polymyxin B ( $p, <0.01$ ).

Results summarized in table 3 revealed that novobiocin was the least effective antibiotic on GNB (~7%) but quite good on GPBs (>62%) with significant difference ( $p, <0.01$ ) among the two groups of bacteria. Among GNBs, *E. coli* (100%) strains were the most resistant ones followed by *aeromonads* (97%) and other *Enterobacteriaceae* members (90-96%). There was no significant difference in sensitivity pattern of different GPBs to novobiocin ( $p, >0.1$ ).

Gentamicin, though the most effective (87.1%) antibiotics on most of the bacterial strains were significantly less ( $p, <0.05$ ) effective on strains of *Staphylococcus* species strains than on other bacterial strains except those of *E. coli*, *Proteus*, *Pseudomonas* and *Streptococcus* species strains ( $p, >0.05$ ).

About 85% bacterial strains in the study were sensitive to chloramphenicol, strains of *Bacillus* (<70%), *Proteus* (<64%) and *Pseudomonas* (<34%) species were significantly ( $p < 0.03$ ) more resistant than strains of other bacteria.

Ciprofloxacin, the 3rd most effective antibiotic (~77%) after gentamicin and chloramphenicol, was significantly ( $p, <0.01$ ) more effective on GNBs (~80%) than GPBs (~69%). Among the GNBs, *Proteus* species (>36%) and *E. coli* (~40%) strains were often more commonly ( $p, <0.05$ ) resistant to ciprofloxacin.

Polymyxin B resistance was equally ( $p, 0.2$ ) more common in strains of *Proteus* ( $p, <0.001$ ) and *Streptococcus* spp. ( $p, <0.001$ ) than in strains of other bacteria, irrespective of their staining or oxidase characteristics. Polymyxin B was more effective on strains of *Brucella* spp. ( $p, <0.05$ ) than strains of other bacteria except *Moraxella*, *Pseudomonas* and *Salmonella* species ( $p, >0.06$ ) strains.

Although nitrofurantoin inhibited about 75 % strains of most of the bacteria, it was less effective on strains of *Proteus* (22.7%) and *Pseudomonas* (26.5%) than on strains of other bacteria ( $p, <0.04$ ). Next in nitrofurantoin resistance were strains of *Klebsiella* (52.4%) and *Enterobacter* (69.2%) species.

Though more than 30% bacterial strains were resistant to tetracycline, strains of *Aeromonas* ( $p, <0.05$ ), *Bacillus* ( $p, <0.01$ ), *Brucella* ( $p, <0.05$ ) and *Moraxella* ( $p, <0.03$ ) were more often sensitive than strains of other bacteria especially members of *Enterobacteriaceae* (>50% resistant).

*Salmonellae* were among the most sensitive strains (100%) to co-trimoxazole while significantly ( $p, <0.05$ ) less number of *Proteus* (44.5%), *Pseudomonas* (36.7%) and *Escherichia* (53.7%) and *staphylococci* and *streptococci* (~60%) strains were sensitive to co-trimoxazole.

## Discussion

Tea Tree oil is seen as potential oil to replace the use of antibiotics for topical use [19] and shown to inhibit many different types of bacteria [1,5,10]. However, in the present study on >500 strains of microbes of 45 genera, only 20.5% strains were sensitive to TTO, even less than the least effective antibiotic (novobiocin). The difference might be due to the fact that in the present study most of the strains included were of clinical origin while earlier studies are reported mostly on reference strains. In the present study too, reference strains were more often sensitive to TTO than those from clinical samples.

Multiple drug resistance (MDR) in bacterial strains and their sensitivity to TTO had strong ( $p, <0.005$ ) negative correlation ( $r, -0.023$ ), i.e., more the resistance to a number of antibiotics in bacteria more the chances of being them TTO resistant. In early studies, it was assumed that TTO was more active on antibiotic resistant bacteria, [14] which later on, were proved to be wrong assumptions [1]. The present study further proved the weakness of the present belief that resistance to antibiotic and sensitivity to TTO cannot be correlated [1,17] and thus the mechanism of bacterial resistance to TTO may not be similar to antibiotic drug resistance. The sensitivity of bacteria to TTO was strongly ( $p, \leq 0.05$ ) correlated with sensitivity to several antibiotics including novobiocin ( $r, 0.24$ ), tetracycline ( $r, 0.22$ ), gentamicin ( $r, 0.21$ ), ciprofloxacin ( $r, 0.17$ ), nitrofurantoin ( $r, 0.16$ ), and chloramphenicol ( $r, 0.14$ ). The strong correlation between TTO and antimicrobial sensitivity suggest that there may be a similarity in mechanism of action of antimicrobials and bacteria may also be employing a similar mechanism for defense against TTO as used against antibiotics. However, to prove the similarity between mechanism of action, and mechanism to resistance of TTO and antibiotics further studies are required. The variation observed from the earlier studies might be due to the inclusion of a large number of strains and clinical strains in the present study than in earlier studies on limited numbers and mostly the reference strains and diversity among the strains tested in different studies.

The MIC of TTO observed in the present study (0.001% to >0.512%, v/v) was apparently similar to earlier observations [7,9,10]. However, some important differences observed might be due to number of strains included in the study, and strain diversity, as Andrade and coworkers [10] reported that for most of the *Enterobacteriaceae* and *Enterococcus faecalis* strains MIC of TTO was  $\leq 0.5\%$  (v/v) while in the present study it ranged from 0.001% to >0.512%. In the same study [10], MIC of TTO for *P. aeruginosa* strains is reported >4%, in the present study too for all of the *P. aeruginosa* strains MIC was >0.512%. Another important observation was MIC of TTO (<0.5%) for *E. faecalis* strains, in earlier studies it is reported either in similar range (Andrade et al., 2016) or much higher [9], indicating that source of strain might be an important determinant for sensitivity of bacteria to TTO. In total, sensitivity to TTO among strains of human origin and strains of animal origin not differed significantly ( $p, 0.025$ ), but bacteria isolated from mithun (*Bos frontalis*) samples were significantly more often sensitive to TTO than in strains isolated from water buffalo ( $p, 0.01$ ), deer ( $p, 0.03$ ), dog ( $p, <0.01$ ), fish ( $p, 0.04$ ), horse ( $p, <0.01$ ), human (0, <0.01), pigs ( $p, 0.02$ ), swamp buffaloes ( $p, 0.03$ ) and tigers ( $p, 0.04$ ). The higher sensitivity of bacteria from mithuns might be due to no use of antibiotics in mithun due to their semi-domestic habitat and thus no resistance among bacteria of mithun origin [23].

Difference in sensitivity to TTO among strains of different origin observed in the present study further proved the same fact that origin

of strains might be an important determinant for determining its sensitivity to TTO as reported earlier for other herbal drugs [21,24-29]. Similar sensitivity pattern of bacteria of mithun origin for other antimicrobials as to TTO, i.e., more commonly sensitive to other antimicrobials than strains of other origin further suggested the similar mechanism of emergence of resistance to antibiotics and TTO or probably for other herbal drugs too [29]. However, significantly ( $p < 0.02$ ) more common occurrence of resistance to chloramphenicol in strains of mithun origin than in microbes of other origin is unexplainable with the present study as it is the drug not at all used in animals especially in mithuns [22]. Resistance to chloramphenicol in the strains with no history of exposure of the drug might be due to the maintenance and circulation of the resistance gene even in naïve population and needs more studies to understand the phenomenon. More common occurrence of resistance in bacterial strains of human origin to gentamicin, tetracycline, co-trimoxazole and ciprofloxacin ( $p < 0.02$ ) than in strains of animal origin, further proves that more and indiscriminate use of antibiotics might be associated with emergence of strains resistant to antibiotics [18,30-32]. Sensitivity of all the strains of Salmonella to cotrimoxazole might be an example to understand effect of discontinuation of an antimicrobial drug on getting rid of the emergence of antimicrobial drug resistance and support the policy advocating for controlling and minimizing the use of antimicrobials in therapeutics and for non-therapeutic purposes [18,30-32].

The study concludes that resistance to TTO, antibiotics and other antimicrobials in bacteria of clinical origin go hand in hand, and more hopes cannot be put on herbal antimicrobials to combat the threat of widespread antimicrobial drug resistant bacterial strains and emergence of more drug resistant strains. Though TTO inhibited the growth of wide range of bacteria proving it a broad spectrum antimicrobial potential, the study also revealed limitations of TTO as only one-fifth of clinical strains of bacteria were sensitive to it.

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