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ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS EXTRACTED FROM MEDICINAL PLANTS AGAINST MULTI-DRUG RESISTANT *STAPHYLOCOCCUS AUREUS*

S. A. Raja[1*], M. Ashraf[1], A. A. Anjum[2], A. Javeed[1], T. Ijaz[3] and A. Attiq[1]

[1]Department of Pharmacology, and Toxicology, [2]Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, [3]Microbiology Diagnostic lab, King Edward Medical University, Lahore.
[*]Corresponding Author: sarwat.ali@lmdc.edu.pk

ABSTRACT

Multi-drug resistant *Staphylococcus aureus* has been a major contributor to treatment failure in the last decade in Pakistan and indigenous plants focused for screening of new therapeutic options. Antibacterial properties of indigenous medicinal plants essential oils were determined against MDR *S. aureus* isolates from patients, associated healthcare workers and healthy community. Antibacterial activity was determined by agar well diffusion assay. Minimum inhibitory concentrations (MIC) of essential oils exhibiting activity against selected MDR *S. aureus* were calculated using Micro broth dilution method. Five plants essential oils *Carum copticum*, *Anethum sowa*, *Cuminum cyminum*, *Myristica fragrans* and *Zingiber officinale* were tested for antibacterial activity against selected MDR *S. aureus* isolates from three sampling groups. Out of five essential oils, *Carum copticum* was found to be the most effective followed by *Zingiber officinale* and *Cuminum cyminum*. Upon qualitative analysis of five essential oils, *Carum copticum, Cuminum cyminum and Zingiber officinale* essential oils showed zones of inhibition greater than the standards Vancomycin and Linezolid. *Anethum sowa* and *Myristica fragrans* essential oils showed no activity against MDR *S. aureus*. The mean MIC values obtained for *Carum copticum* against selected MDR *S. aureus* isolates from patients, healthcare workers and community were 4.2µl, 1.7 µl and 3.0 µl, respectively. Mean MIC value for *Zingiber officinale* were 10.8 µl, 3.5 µl and 6.8 µl and for *Cuminum cyminum* were 43.8 µl, 23.8 µl and 40 µl. The MIC value of *Carum copticum* essential oil was least against MDR *S. aureus* isolates and considered most effective plant oil.

Keywords: Multiple drug resistant *Staphylococcus aureus*, Post-operative wounds, Healthcare workers, Healthy volunteers from Community, Essential oils, Agar well diffusion assay, Minimum inhibitory concentration.

INTRODUCTION

Emergence of highly resistant bacteria has seriously affected the impact to combat infections by use of current antibiotics (Simoes *et al*., 2010). Clinical infections due to Methicillin resistant *Staphylococcus aureus* with decreased susceptibility to Vancomycin are increasing worldwide (Sakoulas and Moellering, 2008). Linezolid was alternative as compared to Vancomycin more effective choice of treatment (Kaleem *et al*., 2011). Serious complications have been reported with Linezolid on long term use in replacement for Vancomycin (Spellberg *et al*., 2004). It resulted in screening for novel and better molecules for adequate management of patients presented with multiple drug resistant infections and to handle the load of such pathogens in future (Harvey, 2008). Due to emergence of multiple drug resistant pathogens the clinical effectiveness of many current antibiotics has declined posing a serious threat as options available are scarce (Sangeetha *et al*., 2011). The problem of growing resistance requires to surge for new treatment options and to explore the medicinal plants for their bioactive molecules with antimicrobial properties. These include alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids and gums which are known to be physiologically active. This is the most important motivational aspect that has always encouraged researchers to explore for new antimicrobial substances from medicinal plants being one of the largest reservoirs to be quest (Rios and Recio, 2005).

Essential oils are gaining attention having high potential as antimicrobials with minimum risk of resistance (Prabuseenivasan *et al*., 2006). These are the secondary metabolites used as defensive agents by the plants and have been known through centuries for their beneficial effects *i.e.* antibacterial, antifungal, antiviral, insecticidal and antioxidant (Burt, 2004; Schafer and Wink, 2009). Essential oils have shown potential results as antimicrobials against clinical isolates as well as food-borne pathogens upon in vitro testing (Burt, 2004).

Present study was conducted to evaluate *in-vitro* antibacterial activity against multi-drug resistant *Staphylococcus aureus* isolates from patients, health-care staff members and community.

MATERIALS AND METHODS

Antibacterial activity of five selected medicinal plants essential oils was evaluated against multi-drug resistant *Staphylococcal aureus* isolates from patients, health-care staff members and community. Five medicinal plants from lot available in Pakistan were selected for screening of constituents having anti-bacterial activity against selected MDR *S. aureus* isolates. Plant parts were purchased from the local market and identified by a botanist from Dr. Sultan Ahmed Choudhary Herbarium Government College University.

Recovery of Essential Oils: Essential oils were recovered by steam distillation using an industrial plant for large scale extraction installed according to Guenther (1948). It consisted of a steam generator, a distillation flask, a flask head, a water-cooled double walled condenser and a receiver. The material was crushed and charged to the distillation flask without delay and distilled with steam. The condensates were collected in the receiver which was kept cool by flow of cold water in a retrograde fashion. Essential oils recovered were measured and yields of oils from plants calculated.

Antibacterial Activity of Essential oils: Antibacterial activity of plants essential oils was determined by an agar well diffusion method as described by Shan *et al*. (2007). Briefly, Muller Hinton agar plates were prepared. Wells were punched with gel puncher and sealed using melted agar. MDR *S. aureus* culture having OD equivalent to 0.5Mcfarland unit was spread over media surface. Plant essential oils such as *Myristica fragrans*, *Cuminum cyminum*, *Carum copticum*, *Zingiber officinale* and *Anethum sowa* were poured in the wells (50µl). Plates were incubated at 37°C for 24hrs. Activity was determined by measuring diameter of zone of inhibitions. All tests were performed in triplicate. Plant essential oils showing clear zones of inhibition greater than the standards Vancomycin and Linezolid were selected for calculation of minimum inhibitory concentration.

Minimum Inhibitory Concentration: The minimum inhibitory concentration was determined using micro broth dilution method (Lalitha, 2004). Serial two fold dilutions of *Carum copticum* essential oil in Muller Hinton broth prepared were 10 – 0.019µl, for *Zingiber officinale* 20 – 0.039µl and *Cuminum cyminum* 100 – 0.195µl. Test organisms inoculated at a concentration of $2x10^6$ CFU/ml in a 96 well micro titration plate. Well # 12 of micro plate was used as negative control having only media and well # 11 was taken as positive control by adding media and bacterial suspension. The wells Optical density (OD) values were recorded by ELISA reader at 524nm. Post 24 hours incubation at 37°C, the OD values again recorded. The lowest concentration showing inhibition of growth by decrease in OD value was considered the MIC of essential oils against test organisms. Mean MIC values were compared statistically by one way ANOVA followed by Duncan's Multiple Range post hoc test using SPSS version 16.0

RESULTS

Medicinal plants selected for essential oils were confirmed by Sultan Ahmad Herbarium Govt. college university and assigned voucher numbers GC. Herb. Bot. 2417 for *Cuminum cyminum* L, GC. Herb. Bot. 2418 for *Anethum sowa* L., GC. Herb. Bot. 2419 for *Myristica fragrans* L., GC. Herb. Bot. 2420 for *Carum copticum* L. and GC. Herb. Bot. 2421 for *Zingiber officinale* L. (Fig.1). Essential oils were recovered by hydro distillation (Fig.1) from five selected plants. The yield of essential oil from each plant was calculated (Table 1). Maximum yield was obtained in case of *Carum copticum* (1.9%), followed by *Cuminum cyminum* (1.8%), *Myristica fragrans* (1.2%) and *Anethum sowa* (0.7%). Minimum essential oil was obtained from *Zingiber officinale* (0.2%).

Out of five plants essential oils tested for antibacterial activity against multiple drug resistance (MDR) *Staphylococcus aureus* isolates; *Carum copticum* was found to be the most effective with the higher zone of inhibition diameters (Table 2). The essential oils with the zones of inhibition higher than commercial standards of Vancomycin and Linezolid were considered effective. Upon comparison in relation to both the commercial standards overall highest activity against MDR *S. aureus* isolates was of *Carum copticum* (88.4% & 78.2%), followed by *Cuminum cyminum* (67.9% & 30.7%) and *Zingiber officinale* (39.7% & 21.8%). Essential oil of *Anethum sowa* was found less effective with overall activity of 24.4 percent as compared to vancomycin and 11.5 percent to linezolid. *Myristica fragrans* was found least effective with overall antibacterial activity of 8.9 and 3.8 percent upon comparison to both vancomycin and linezolid. The results of all five essential oils in comparison with the commercial standards for antibacterial activity against MDR *S. aureus* isolates in all three sampling groups are summarized at table (3). Representative plates of well diffusion test showing zones of inhibition against MRSA isolates from the three sampling groups are presented as figure (2).

The mean MIC values of *Carum copticum*, *Cuminum cyminum* and *Zingiber officinale* against MDR *S. aureus* isolates from patients were 4.2±3.0 µl /ml, 43.8±11.1µl /ml and 10.8±3.4 µl/ml respectively. For MDR *S. aureus* isolates from health care workers mean MIC values of *Carum copticum*, *Cuminum cyminum* and *Zingiber officinale* were 1.7±0.6 µl /ml, 23.8±3.9 µl /ml and 3.5±1.3 µl /ml respectively. For MDR *S. aureus* isolates from community mean MIC values of *Carum copticum*, *Cuminum cyminum* and *Zingiber officinale* were 3.0±2.5 µl /ml, 40±12.6 µl /ml and 6.8±2.8 µl /ml respectively. On comparison by statistical analysis significant difference was observed among mean MIC values of essential oils of three tested plants ($p \leq 0.05$).

Table 1. Yield of essential oils from selected indigenous medicinal plants

Plant Name	Part Used	Total Quantity of Plant part used	Total Quantity Obtained	Yield of essential oils (ml/g)	Percentage Yield (v/w)
Myristica fragrans L. (Joiphal)	Seed	10kg	120ml	0.012ml/g	1.2%
Cumimum cyminum L.(Sufaid zeera)	Seed	11kg	195ml	0.018ml/g	1.8%
Carum copticum L. (Ajwine)	Seed	9kg	171ml	0.019ml/g	1.9%
Zingiber officinale L. (Sund)	Rhizome	64kg	100ml	0.002ml/g	0.2%
Anethum sowa L. (Sowy)	Seed	13kg	95ml	0.007ml/g	0.7%

Note: Names in brackets are the local names in urdu

Table 2. Mean diameters of zones of inhibition of selected indigenous medicinal plants essential oils

Sr. #	Plants Essential Oils	Patients (n=22)	Health-Care Workers (n=36)	Community (n=20)	Total (n=78)
1.	*Myristica fragrans* (Joiphal)	13.5±1.3	20±0	20.2±8.1	17.9±3.1
2.	*Cuminum cyminum* (Sufaid Zeera)	23.3±9.9	23.3±6.6	29.3±6.7	25.3±2.8
3.	*Carum copticum* (Ajwine)	33.7±9.8	36.1±6.3	37.3±7.3	35.7±1.5
4.	*Zingiber officinale* (Sund)	21.42±9.8	27.3±9.3	24.8±13.7	24.5±2.4
5.	*Anethum sowa* (Sowy)	17±5.7	18.7±8.1	24.7±7.8	20.1±3.3

Note: mean diameter of zones of inhibition are in mm

Table 3. Activity of indigenous plants essential oils in relation to vancomycin and linezolid against MDR *Staphylococcus aureus*

Sr. #	Plants Essential Oils	Patients (n=22)	Health-Care Workers (n=36)	Community (n=20)	Total (n=78)
	% Sensitivity in relation to vancomycin				
1.	*Myristica fragrans* (Jaiphal)	2(9%)	2(6%)	3(15%)	7(8.9%)
2.	*Cuminum cyminum* (Sufaid Zeera)	12(55%)	23(64%)	18(90%)	53(67.9%)
3.	*Carum copticum* (Ajwine)	18(82%)	35(97%)	16(80%)	69(88.4%)
4.	*Zingiber officinale* (Sund)	5(23%)	14(39%)	12(60%)	31(39.7%)
5.	*Anethum sowa* (Sowy)	5(23%)	8(22%)	6(17%)	19(24.4%)
	% Sensitivity in relation to linezolid				
1.	*Myristica fragrans* (Jaiphal)	2(9%)	0(0%)	1(5%)	3(3.8%)
2.	*Cuminum cyminum* (Sufaid Zeera)	6(27%)	7(19%)	11(55%)	24(30.7%)
3.	*Carum copticum* (Ajwine)	15(68%)	30(83%)	16(80%)	61(78.2%)
4.	*Zingiber officinale* (Sund)	3(14%)	7(19%)	7(35%)	17(21.8%)
5.	*Anethum sowa* (Sowy)	4(18%)	2(6%)	3(15%)	9(11.5%)

*% Sensitivity = MRSA Isolates in three sampling groups with the zones of inhibition more than the commercial standards

Table 4. Minimum inhibitory concentrations of selected plants essential oils against MRSA isolates from patients, health care workers and community.

Isolates	Patients			Health care workers			Community		
	Carum copticum µl/ml	*Cuminum cyminum* µl/ml	*Zingiber officinale* µl/ml	*Carum copticum* µl/ml	*Cuminum cyminum* µl/ml	*Zingiber officinale* µl/ml	*Carum copticum* µl/ml	*Cuminum cyminum* µl/ml	*Zingiber officinale* µl/ml
1.	0.625	25	10	1.25	25	2.5	0.625	50	5
2.	5	25	10	1.25	25	2.5	1.25	25	10
3.	10	25	10	1.25	25	2.5	1.25	25	5
4.	2.5	25	20	2.5	25	2.5	0.625	50	10
5.	5	25	10	2.5	25	5.0	0.625	50	5

6.	5	50	10	1.25	25	2.5	10	50	5
7.	2.5	50	10	2.5	25	5.0	5	50	10
8.	10	50	10	1.25	25	5.0	1.25	50	5
9.	0.625	50	10	2.5	25	5.0	5	25	2.5
10.	1.25	50	5	1.25	25	2.5	1.25	25	10
11.	10	50	10	1.25	12.5	2.5	1.25	25	5
12.	0.625	50	10	1.25	25	2.5	1.25	25	5
13.	5	50	10	1.25	25	2.5	2.5	25	5
14.	5	50	10	2.5	25	5.0	5	25	10
15.	5	50	10	2.5	25	2.5	1.25	50	10
16.	2.5	50	20	1.25	25	2.5	5	50	10
17.	2.5	50	10	1.25	25	5.0	5	50	5
18.	1.25	50	10	1.25	25	5.0	5	50	10
19.	5	50	10	1.25	25	5.0	2.5	50	5
20.	5	50	10	2.5	12.5	2.5	5	50	2.5
MIC Range	0.625-10	25-50	5-20	1.25-2.5	12.5-25	2.5-5.0	0.625-10	25-50	2.5-10
Mean±S.D	4.2 ± 3.0^{a}	43.8 ± 11.1^{b}	10.8 ± 3.4^{c}	1.68 ± 0.6^{a}	23.8 ± 3.9^{b}	3.5 ± 1.3^{c}	3.0 ± 2.5^{a}	40 ± 12.6^{b}	6.8 ± 2.8^{c}

Means carrying different superscripts differ significantly

a) ***Myristica fragrans*** **(Joiphal) Seeds**
b)

b) *Cumimum cyminum* (Sufaid Zeera) Seeds

(c) *Carum copticum* (Ajwine) Seeds

(d) *Zingiber officinale* (Sund) Rhizome

(e) *Anethum sowa* (Sowy) Seeds

(f) Hydro distillation plant

Figure 1: Indigenous medicinal plant parts selected for yield of essential oils

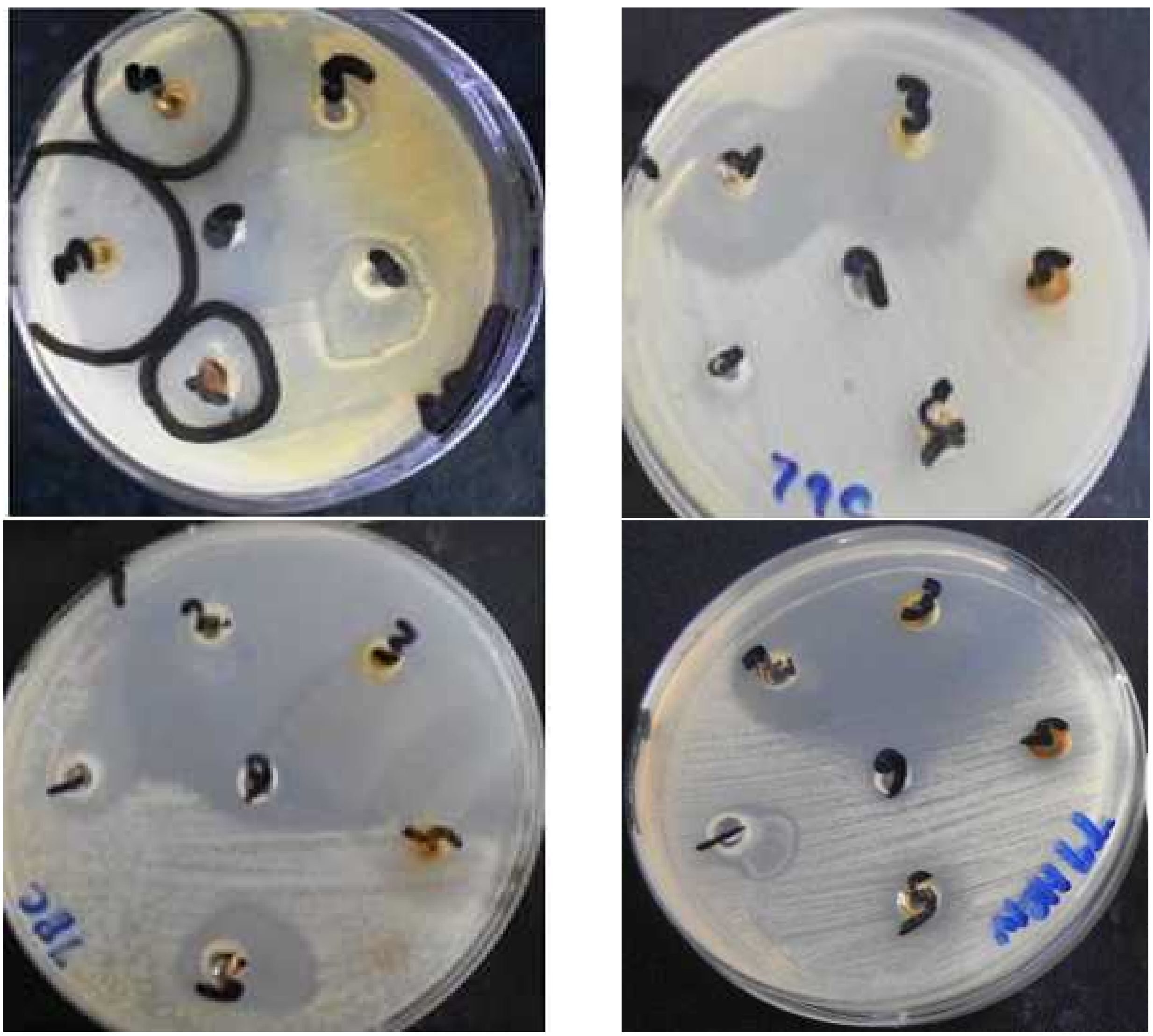

Figure 2. Representative plates of agar well diffusion test showing zones of inhibition

DISCUSSION

An alarming rise in antibiotic-resistant mutants called super bugs is of great concern globally in medical practice and public health. In developing countries like Pakistan emergence of multiple drug resistant pathogens is a major contributor to treatment failure resulting in an overall increase in morbidity and mortality. Moreover their prevalence in the community imposes a serious threat towards public health. A lot of work is being carried out on metabolites of the medicinal plants for screening of sensitive and potent antimicrobial agents.

Antibacterial activity of essentials oils extracted from indigenous medicinal plants was evaluated against multi-drug resistant *S. aureus*. Highest essential oil yield obtained from *Carum copticum* was 1.9 percent v/w. Higher yields had been reported by Gilani *et al*. (2005) which was 2-3percent. Mohagheghzadeh *et al*. (2007) and Zarshenas *et al*. (2014) reported 2.6 and 2.2-4.8 percent v/w yields of the same plant. The reasons for lower yield of *Carum copticum* essential oil in present study might be the variation in cultivation conditions and prolonged storage of seeds. In case of *Cuminum cyminum* percent yield obtained was 1.8 v/w slightly lesser than those reported by Li and Jiang (2004) and El-Ghorab *et al*. (2010) which were 3.8 and 2.52 percents, respectively. The major contributor to inferior yield of essential oils especially from seeds is the drying of the seeds. From over dried seeds essential oil yield is comparatively lesser. Higher yield (1.2%) was obtained from *Myristica fragrans* than (0.047%) reported by Al-Jumaily and Al-Amiry (2012). In contrast yield recovered by Subarnas *et al.* (2010) was much higher (6.85%). Essential oil obtained in case of *Anethum sowa* was 0.7 percent lesser than those reported by Babri *et al*. (2012). Another important critical factor affecting the yield of essential oil is the method of extraction together with cultivation and storage conditions. Essential oil yield and quality is high in case of Supercritical method of extraction from conventional Clevenger type apparatus (Pourmortazavi and Hajimirsadeghi, 2007). In conventional methods overheating results in loss of volatile components. The percentage of essential oil obtained in case of *Zingiber officinale* was minimum (0.2%) comparable to Connell and Sutherland (1969). Higher yields reported by Jantan et. al. (2003), El-Ghorab (2010) which were 1.4 and 0.3 percents, respectively. Drying, grounding and flaking are three important determinants in essential oil yield by *Zingiber officinale*.

Overall in the present study the yield of essential oils was relatively lower than already reported. Many factors affect the yield like extent of reduction in particle size of plant part used *e.g.* flaking can enhance yield of essential oils (Sowbhagya *et al*., 2008), modifications in distillation methods (Wang *et al*., 2009) as better yield and quality can be achieved by Supercritical method. Other influential factors include environment and temperature conditions provided to the plants during growth like manure in the soil and water stress during growth of plants influence their essential oil yield (Ahmadian *et al*., 2011). Use of biofertilizer can enhance essential oil yield because of impact on available nitrogen for growth and nutrient assimilation (Singh *et al*., 2014).

Out of the five plants essential oils tested for antibacterial activity against MDR *S. aureus* isolates *Carum copticum* was found to be most effective followed by *Zingiber officinale* and *Cuminum cyminum* essential oils. Essential oils of *Anethum sowa* and *Myristica fragrans* were found least effective against MDR *S. aureus* isolates. Moreover in case of all three essential oils non-significant difference ($P>0.05$) was observed for MIC values against patient and community MDR *S. aureus*. *Carum copticum* has been reported to possess strong bactericidal activity against a number of resistant and virulent bacteria such as MRSA, pathogenic *E.coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterococcus faecalis*, *Streptococcus pyogenes* (Goudarzi *et al*., 2010; Zomorodian *et al*., 2011). Activity also reported against *Enterobacter aerogenes*, *Shigella dysenteriae*, *Shigella flexeneri*, *Serratia marcescens* (Mahboubi and Kazempour, 2011). MIC of *Carum copticum* tested against 55 bacteria was found to be less than 2µl/ml (Boskabady *et al*., 2014). Zomorodian *et al*. (2011) reported MIC against MRSA 3.56µl/ml and range 1-8µl/ml which exactly matches to the findings in the present study. Gourdazi *et al*. (2010) reported mean MIC 0.3 µl/ml which is lower than present study. The reason behind this variation is the difference in the proportion of various constituents in combination. Chemotype of *Carum copticum* with thymol as dominant constituent reported to possess better activity (Gourdazi *et al*., 2010). All these findings were regarding clinical isolates but no individual findings were found regarding the mean MIC of *Carum copticum* on MRSA carriers in the community and health care workers. Upon comparison with *Zingiber officinale* and *Cuminum cyminum* essential oil its activity was found superior (Oroojalian *et al*., 2010).

Zingiber officinale is known to possess antibacterial activity against many gram positive and gram negative bacteria (Gull *et al*., 2012). Higher activity found against *S. aureus*, *E.coli*, *B. subtilis* and *Enterococcus faecalis* (Sivananthan, 2013). Moderately activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was observed by Sivasothy *et al*. (2011). *Zingiber officinale* extracts were found superior than standard antibiotics like chloramphenicol, gentamicin against *S. aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Proteus vulgaris* (Suhad *et al*. 2012). In accord activities were observed by Mickiene *et al*. (2011) against *S. aureus* and Chao *et al*. (2008) against MRSA. *Zingiber officinale* found very effective against MDR *S. aureus* with MIC range of 2.5-20µl/ml for all three sampling groups in present study. Sasidharan and Menon (2010) reported MIC of *Zingiber officinale* essential oil against *Bacillus subtilis* and *Pseudomonas aeruginosa* in range of 5-10µg/ml. Similarly *Cuminum cyminum* essential oil reported to have antibacterial activity against many pathogenic bacteria such as *S. epidermidis*, *S. aureus, S. haemolyticus*, *Propionibacterium acnes*, *Corynebacterium diphtheriae*, *Erysipelothrix rhusiopathiae*, *Bacillus cereus*, *Clostridium tetani*, *Clostridium difficile*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* (Chaudhary *et al*., 2014). Warner *et al*. (2010) also reported its antibacterial activity against *S. aureus* except Pseudomonas. In current findings, out of three plants highest MIC values of *Cuminum cyminum* were much lower than other two essential oils in agreement with Mandal *et al*. (2011). The antibacterial activities of essential oils depend upon their composition and relative proportion of different constituents present. In *Carum copticum* the major constituents reported are monoterpenes Thymol, ɤ-Terpinene, p-cymene (Goudrazi *et al*. 2010) where as in *Zingiber officinale* sequeterpines like alpha Zingebrene , β-sesquiphallandrene, alpha curcumene (Nampoothiri *et al*., 2012). In *Cuminum cyminum* the dominant constituent present is Cumin aldehyde (Ravi *et al*., 2013). All these constituents are reported to possess antibacterial properties (Bassole and Juliani, 2012) as observed in current findings as well but one constituent may possess superior activity than the other and secondly the relative abundance and combination proportion with different constituents can be considered intricate factors responsible for the variation in response of different essential oils.

Conclusion: *Carum copticum*, *Cuminum cyminum and Zingiber officinale* essential oils can be considered as alternative treatment options with most promising activity exhibited by *Carum copticum* essential oil with lowest MIC values against MDR *S. aureus* isolates.

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