

In Vitro Antibacterial Activity of three Indian Spices Against Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Objective: To explore the *in vitro* antibacterial activity of ethanolic extracts of cinnamon (*Cinnamomum zeylanicum*; CIN), clove (*Syzygium aromaticum*, CLV) and cumin (*Cuminum cyminum*, CMN) against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA), from Kolkata, India.

Methods: The CIN, CLV and CMN were tested for their antibacterial activity against MRSA by *in vitro* methods. Minimum inhibitory concentration (MIC) values of the three extracts were determined, and time-kill studies were performed in order to investigate the bactericidal activity of the extracts (at the MIC level) for the isolates. The killing efficacy of the extracts was determined at various concentrations.

Results: The zone diameter of inhibition (ZDI) obtained due to CIN, CLV and CMN ranged between 22-27 mm, 19-23 mm and 9-15 mm, respectively; while the MICs, for the isolates, were in the range of 64-256, 64-512 and 128-512 µg/ml, respectively. When tested for their MIC levels; the CIN and CLV were found to be bactericidal after 6 hrs of incubation, while CMN showed bactericidal activity after 24 hrs. However, when tested at various concentrations; CIN, CLV and CMN displayed bactericidal activity against *S. aureus*, after 24 hrs of incubation, at 200, 200 and 300 µg/ml, respectively.

Conclusion: The *C. zeylanicum* and *S. aromaticum* showed the strongest *in vitro* antibacterial activity followed by *C. cyminum* against MRSA, and such findings could be considered a valuable support in the treatment of infection and may contribute to the development of potential antimicrobial agents for inclusion in anti- *S. aureus* regimens.

Keywords: MRSA; Zone diameter of inhibition; Minimum inhibitory concentration; Bactericidal activity; Spices.

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Introduction

Antibiotic therapy in recent years has faced difficulties due to the rapid emergence of multidrug resistance among bacteria causing several life threatening infections, and this in turn, making the future management of infectious diseases uncertain. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for potential antimicrobial activity, and the plant extracts were found to have potential against microorganisms.^{1,2} The spices that are generally used as food additives in order to provide taste, smell, and color also exhibited antibacterial activity, as has been documented in the literature. Agaoglu et al.³ reported the antibacterial activity of different spices including *Cinnamomum zeylanicum*, *Syzygium aromaticum* and *Cuminum cyminum*, in terms of the zone diameter of inhibition (ZDI), against gram-negative and gram-positive bacteria including *Staphylococcus aureus*. Khan et al.⁴ reported that spices including *C. zeylanicum* and *S. aromaticum* can be used against multidrug resistant (MDR) microbes causing nosocomial and community acquired infections such as *S. aureus*. Nanasombat and Lohasupthawee reported inhibitory activity of *C. zeylanicum*, *S. aromaticum* and *C. cyminum*, based on the ZDI and minimum inhibitory concentration (MIC) values against various enteric bacteria.⁵

Antibacterial activities of some medicinal plants such as *Althaea officinalis*, *Mentha longifolia*, *Melissa officinalis* and *Rosa damascene* against methicillin-resistant *S. aureus* (MRSA) have been reported in terms of MICs.⁶ But the bactericidal activity of *C. zeylanicum*, *S. aromaticum* and *C. cyminum* has not been documented against *S. aureus*, which is implicated in diseases like wound infections, abscesses of the muscle, and urogenital tract infections. Moreover, the increasing emergence of nosocomial isolates of *S. aureus* exhibiting resistance not only to methicillin but also to other anti-*S. aureus* antibiotics,^{7,8} led to therapeutic problems, and hence searching the source for new, safe and effective alternative treatment regimen against MRSA infections is imperative in order to control its spread.

In the present study, time-kill experiments with the ethanolic extracts of the stem bark of cinnamon (*Cinnamomum zeylanicum*; CIN), flower bud and stalk of clove (*Syzygium aromaticum*, CLV) and seed of cumin (*Cuminum cyminum*, CMN), were carried out in order to determine their bactericidal effect on MRSA. The ZDI and MIC values of the test extracts for the isolates were also determined.

Methods

A total of 12 clinical isolates of *S. aureus* (7 from throat swab, 3 from pus and 2 from skin swab) showing resistance to methicillin by disk diffusion technique were considered for the present study, and the *S. aureus* ATCC 25923 strain was used as the control.

The Mueller-Hinton broth (MHB; for subculturing the strains, inocula preparation and performing time-kill experiments) and Mueller-Hinton agar (MHA; for the determination of ZDIs and MICs), obtained from Hi-Media (India), were used in the study. The bacterial inocula were prepared as 10^4 CFU/spot, 10^8 CFU/ml and 5×10^5 CFU/ml, respectively for agar dilution, agar diffusion and time-kill studies, following the previously described methodology.²

Three Indian spices (Table 1), in dried forms, were purchased

Table 1: Details of the spices used in the study.

Spices	Family	Local name	Part used	Abbreviation (ethanolic extract)
Cumin (<i>Cuminum cyminum</i>)	Umbelliferae	Jeera	Fruit	CMN
Clove (<i>Syzygium aromaticum</i>)	Myrtaceae	Labanga	Flower stalk and bud	CLV
Cinnamon (<i>Cinnamomum zeylanicum</i>)	Lauraceae	Darchini	Stem bark	CIN

Agar diffusion technique, as described by Nas,¹⁰ was followed in order to determine the ZDIs, using an inoculum of 10^8 CFU spread on MHA plates. Each of the inoculated plates was divided into four equal sectors, and the extracts, 20 μ l (equivalent to 200 μ g) each, were dropped onto the sectors marked previously with CIN, CLV and CMN; onto the final sector 20 μ l of 50% ethanol was used for control. The plates were then incubated at 35°C for 24 hrs, and after that period, ZDIs produced due to the action of the extracts were recorded for all the isolates. The control sectors results no ZDI to ZDI of 6 mm for the test bacterial isolates, and therefore, the sensitivity to the plant extracts for the MRSA isolates were considered with ZDI \geq 7 mm, which has also been considered by earlier authors.^{1,11}

The determination of MICs of the extracts was conducted following agar dilution technique, using 10^4 CFU /spot as the inoculum and the concentrations of the extracts used were 512, 256, 128, 64 and 32 μ g/ml; the other details are described elsewhere.²

Bacterial killing studies were conducted for the *S. aureus* TSB3 strain that had MIC 256 μ g/ml for each of the extracts CMN, CLV and CIN, in MHB with an initial inoculum of approximately 5×10^5 CFU/ml, following the earlier published protocol.² In this method, viable cell counts were determined at 0, 3, 6 and 24 hrs, in the presence of each of the extracts at concentration of 256 μ g/ml ($1 \times$ MIC). To determine the effect of varied concentrations of the extracts (25-300 μ g/ml) on bacterial density (CFU/ml), the bacterial suspension (5×10^5 CFU/ml) in MHB was incubated for 24 hrs at 35°C. The $\geq 3 \log_{10}$ decrease in the inoculum after 24 hrs

from the local market in Kolkata, India, and used in the current study. The ethanolic extracts of the three spices were prepared following the published protocol,² with slight modification. Briefly, 50 mg of each of the spices, after grinding into small granules or dusts, were soaked in 50 ml of 95% ethanol for 48 hrs, with vigorous shaking at 15 min interval initially for 6 hrs, followed by shaking at 2 hr intervals. Afterwards, the mixtures were filtered, and the filtrates were vaporized to dryness, and weighed in order to determine the % yield of the extracts, following the formula: % yield = (weight of extract / weight of ground plant material) \times 100.⁹ The stock solutions of the crude ethanolic extracts were prepared by diluting the dried extracts: CIN, CLV and CMN, with 50% ethanol to obtain the final concentration of 10 mg/ml for each.

of incubation was considered as the bactericidal activity.¹²

The χ^2 -test was employed to compare the antibacterial activities of CIN, CLV and CMN, based on the bacterial growth (in terms of CFU/ml) at different concentrations and at different time intervals. A *p* value of <0.05 was considered significant.

Results

The agar dilution test results are represented in Fig. 1. All the *S. aureus* isolates were found to be sensitive to the crude ethanolic extracts of the three spices tested. The isolates had ZDI 22-27 mm, 19-23 mm and 9-15 mm against CIN, CLV and CMN, respectively.

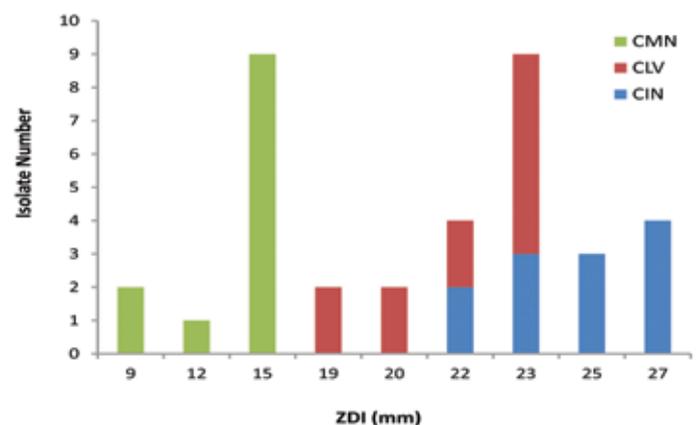


Figure 1: Zone diameter of inhibition (ZDI) of three spices for MRSA isolates. CIN= *Cinnamomum zeylanicum*; CLV= *Syzygium aromaticum*; CMN= *Cuminum cyminum*.

The MICs of CIN, CLV and CMN for the 12 *S. aureus* isolates (Fig. 2) ranged as follows; 64-256, 64-512 and 128-512 µg/ml, respectively. Most of the isolates; 8 (66.67%) and 9 (75%) had CIN and CMN MICs 256 µg/ml and 512 µg/ml, respectively, while 9 (75%) showed CLV MICs in between 256 µg/ml and 512 µg/ml. Moreover, 3 (25%) and 2 (16.67%) isolates had MIC of 64 µg/ml, respectively to CIN and CLV.

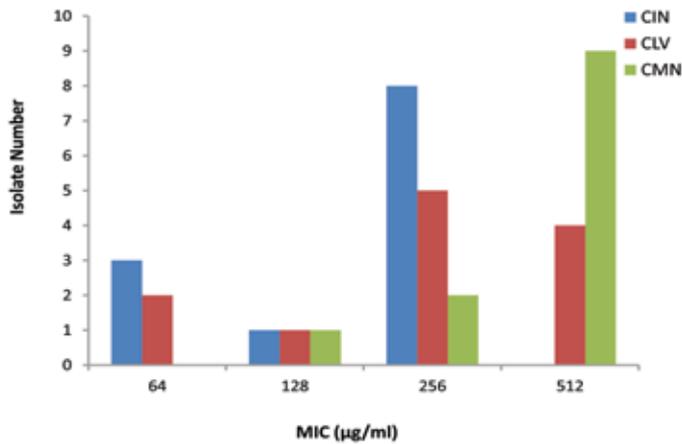


Figure 2: Minimum inhibitory concentration (MIC) of three spices for MRSA isolates. CIN= *Cinnamomum zeylanicum*, CLV= *Syzygium aromaticum*, CMN= *Cuminum cyminum*.

Fig. 3 depicts the killing activities of various concentrations (25-300 µg/ml) of CIN, CLV and CMN against *S. aureus*. At the lowest concentration (25 µg/ml), CIN, CLV and CMN did not affect the bacterial growth, and at this concentration the bacterial density increased up to 6.102, 6.3 and 6.254 log₁₀ CFU/ml, respectively. The CIN and CLV at 50 µg/ml inhibited the growth of *S. aureus*, while the CMN began to show a growth inhibitory effect at 100 µg/ml. The CIN and CLV showed bactericidal activity at concentration 200 µg/ml against *S. aureus* reducing 4.508 log₁₀ CFU/ml and 3.978 log₁₀ CFU/ml, respectively; CMN exhibited bactericidal effects at 300 µg/ml, leaving 0.59 log₁₀ CFU/ml, after 24 hrs.

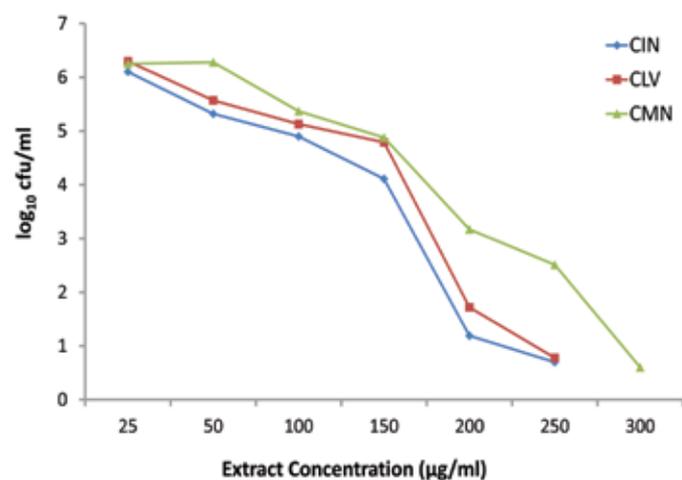


Figure 3: Effect of various concentrations of CIN (*Cinnamomum zeylanicum*), CLV (*Syzygium aromaticum*) and CMN (*Cuminum cyminum*) on growth of MRSA.

The time dependent killing effect of CIN, CLV and CMN at concentration 256 µg/ml (1 × MIC) on *S. aureus* strain is depicted in Fig. 4. In the presence of 256 µg/ml of CIN, CLV and CMN; the viable cell counts were decreased after 3 hrs by up to 3.27, 3.61 and 3.97 log₁₀ CFU/ml, respectively. The CIN and CLV had bactericidal effects at 6 hrs, when the viable cell counts decreased to 2.12 and 2.39 log₁₀ CFU/ml, respectively. However, the CMN showed bactericidal effect on *S. aureus* after 24 hrs of incubation, decreasing the viable cell count to 4.218 log₁₀ CFU/ml.

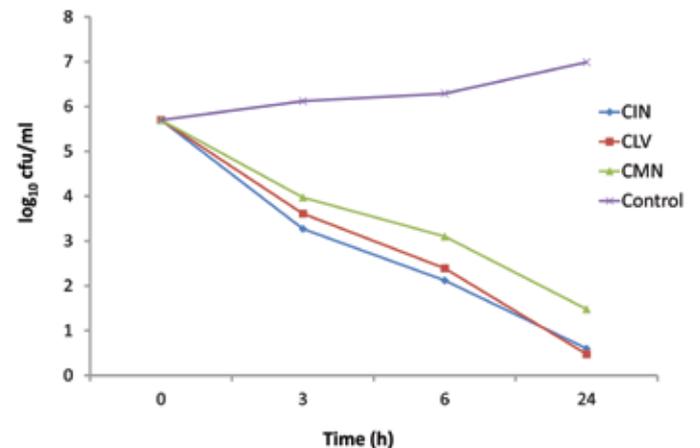


Figure 4: Kill kinetics of CIN (*Cinnamomum zeylanicum*), CLV (*Syzygium aromaticum*) and CMN (*Cuminum cyminum*) against MRSA.

The significant differences in killing were found between CIN and CLV ($p < 0.05$), as well as between CIN and CMN ($p < 0.05$), when 200-250 µg/ml concentrations (bactericidal concentrations) were considered, as has been found in the first set of killing studies, (Fig. 3). In the second set of time-kill experiments; CIN, CLV and CMN at a concentration of 256 µg/ml (1 × MIC) showed significant killing activities ($p < 0.05$), both at 3 and 6 hrs. (Fig. 4)

Discussion

The antibacterial activity of *C. zeylanicum*, *S. aromaticum* and *C. cyminum*, in terms of ZDI, has been reported in earlier studies. Agaoglu et al.³ reported *C. zeylanicum* as the most effective spice against the microorganisms tested, and the most susceptible bacterial strain to this spice was *S. aureus* (ZDI: 32 mm); the ZDIs due to the action of *S. aromaticum* and *C. cyminum* against *S. aureus* were documented as 15 mm and 10 mm, respectively. Fabio et al.¹³ reported ZDIs of 24 and 18 mm, respectively for *C. zeylanicum* and *S. aromaticum* against *S. aureus*.

In the current study, the three ethanolic extracts (CIN, CLV and CMN) displayed excellent antibacterial activity against MRSA, since the all 12 (100%) isolates showed sensitivity to the extracts with ZDI >7 mm. The CIN exhibited the best anti-*S. aureus* activity producing ZDI ranging between 22 mm and 27 mm; most (n=7; 58.33%) of the isolates were highly sensitive to CIN (ZDI: 25-27 mm). By the agar diffusion method, the growth inhibitory activity was followed by the action of CLV with ZDI 19-

23 mm; 8 (66.67%) were the most sensitive isolates for which the ZDI were 22-23 mm. The isolates showed relatively less sensitivity to CMN; however 9 (75%) of the tested isolates had ZDI 15 mm. Thus, the three spice-extracts exhibited different degrees of growth inhibition against *S. aureus* isolates, and the ranking of anti-*S. aureus* activity of the extracts tested is CIN>CLV>CMN.

Based on the above findings, the three extracts were subjected to further evaluation of their effects on MRSA by MIC determination following agar dilution method, and the study results supported the establishment of similar ranking (CIN>CLV>CMN) of antibacterial activity, as has been mentioned above. The CMN exhibited the highest MIC values; the 9 (75%) isolates had maximum MICs of 512 µg/ml. The isolates though had CLV MIC range of 64-512 µg/ml; only 2 (16.67%) had MIC of 512 µg/ml.

Agar dilution test results revealed that *S. aureus* were most susceptible to CIN (MICs: 64-256 µg/ml), which showed the highest antibacterial activity in terms of ZDI obtained for the isolates. Abu-Shanab reported antibacterial activity in terms of MICs on ethanolic extracts of *M. longifolia* (MICs: 3.125-12.5 mg/ml), *M. officinalis* (MICs: 3.125-12.5 mg/ml) and *R. damascene* (MICs: 0.3951-0.78 mg/ml) against MRSA.⁶ Nanasombat determined the MICs of ethanolic extracts of various spices including *C. zeylanicum*, *S. aromaticum* and *C. cyminum* for a number of gram-negative enteric bacteria; and found *S. aromaticum* to be the most effective spice (MICs: 2.6-41.7 mg/ml).⁵ However, the earlier investigators did not determine MICs of *C. zeylanicum*, *S. aromaticum* and *C. cyminum* extracts against *S. aureus*.^{3,13} Prabuseenivasan et al. reported that *C. zeylanicum* oil showed maximum activity with MICs ranging from 0.8 to 3.2 mg/ml, followed by *S. aromaticum* oil with MICs between 1.6 mg/ml and 6.4 mg/ml against bacterial isolates including *S. aureus*.¹⁴

The *C. zeylanicum* bark is rich in cinnamaldehyde (50.5%), which has been proven to be active against many pathogenic gram-positive and gram-negative bacteria.^{15,16} Ali et al. reported cinnamaldehyde as the active agent to inhibit the growth of both antibiotic-sensitive and -resistant stains of *Helicobacter pylori*.¹⁷ It has been reported that *S. aromaticum* oil contains high (75%) eugenol, and the antibacterial activity of *S. aromaticum* is attributed to this compound.^{18,19} Another important antimicrobial compound is tannin, in *S. aromaticum*, which also aids the process of antimicrobial action. The antibacterial activity of *C. cyminum* essential oil is perhaps attributable to the high levels of cuminaldehyde (16.1%), the other main component includes *a*-pinene (11.4%).^{20,21}

Nanasombat and Lohasupthawee reported that carvone and carvacrol are also known to have inhibitory effects on bacterial strains.⁵ Here, we have not studied the antibacterial activity of the components within the extracts; however, the compounds mentioned above may be responsible for the anti-MRSA activity of the extracts, as has been investigated in our study, and the extracts exhibited broad-spectrum antibacterial activity against both gram-positive and gram negative strains.⁶

A system has been demonstrated earlier that shows the rate and

extent of bacterial killing (kill kinetics) providing more accurate description of antimicrobial activity than the MIC does, and it has also displayed better sensitivity trends to physicians than disk diffusion methods.^{2,22} Okemo et al.²³ investigated the rate and extent of bacterial killing with stem-bark extract of *Azadirachta indica* against *S. aureus*, and Mandal et al.² reported the results of time-kill experiments with *A. indica* seed extract against *Salmonella enterica* serovar Typhi. In the present report, two sets of time-kill experiments were performed for the first time, at least in this part of the globe, with three spice-extracts CIN, CLV and CMN against MRSA, using 5×10^5 CFU/ml of the initial inoculum. When increasing concentrations (25-300 µg/ml) of the extracts were used, no significant difference in killing was found among the extracts up to the concentration of 150 µg/ml after 24 hrs. Moreover, in the presence of the lowest concentration (25 µg/ml) of CIN, CLV and CMN; bacterial population increased to 6.102, 6.3 and 6.254 log₁₀ CFU/ml, respectively. Raising the concentration of the extracts increased the rate and amount of killing at 24 hrs; at the concentration of 200 µg/ml, CIN and CLV had bactericidal effect on MRSA, thus decreasing cell counts by 4.522 log₁₀ CFU/ml and 4.252 log₁₀ CFU/ml, respectively, and significant differences in killing were found between CIN and CLV ($p < 0.05$), and between CIN and CMN ($p < 0.05$).

The CMN exhibited bactericidal activity at a concentration of 300 µg/ml, while CIN and CLV, at this concentration, killed the whole bacterial population in 24 hrs. In another set of time-kill experiments; CIN, CLV and CMN at a concentration of 256 µg/ml (1×MIC) showed significant killing activity ($p < 0.05$) at 3 hrs, reducing viable cell counts up to 3.27, 3.61 and 3.97 log₁₀ CFU/ml, respectively, compared to the initial inocula, 5×10^5 CFU/ml (5.698 log₁₀ CFU/ml). At 6 hrs, CIN and CLV (both at 256 µg/ml) showed bactericidal effects against MRSA, decreasing the bacterial population to 2.12 and 2.39 log₁₀ CFU/ml, respectively; thus a significant difference in the viable cell counts ($p < 0.05$) were observed between the extracts (CIN and CLV) as well as between CLV and CMN (that reduced the population to 3.1 log₁₀ CFU/ml). The CMN (256 µg/ml) displayed bactericidal effect at 24 hrs with a reduction of bacterial population by 4.218 log₁₀ CFU/ml. As a result, it is interesting to note that for *S. aureus*; the killing with CIN, CLV and CMN was both dosage and time dependent, indicating that antimicrobial substances in the tested spices might affect the synthesis of the peptidoglycan layer of the cell wall and the mode of action of the spice extracts is cell wall related.^{2,23} This in turn implies a more rational basis for determining the optimal dosage for antimicrobial treatment regimens,²⁴ in order to combat the emergence and spread of antimicrobial resistance.

Conclusion

The degree of antibacterial activity of the spices tested can be represented in the following order: *C. zeylanicum* > *S. aromaticum* > *C. cyminum*; all the three spices were found to be excellent bactericidal agents. Thus, *C. zeylanicum*, *S. aromaticum*, *C. cyminum*

could be selected for use as potential anti- MRSA agents, which will also be of great benefit in combating antibiotic resistance of *S. aureus* causing serious human infections as found in the current study. However, further studies are required to determine the toxicity and proper dose selection, but before that; these agents can easily be utilized topically.

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