Original Article

Antibacterial activity of *Syzygium aromaticum* (clove) against uropathogens producing ESBL, MBL, and AmpC beta-lactamase: Are we close to getting a new antibacterial agent?

Sameer S. Faujdar[1], Dakshina Bisht[1], Amisha Sharma[2]

[1]Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, [2]Department of Microbiology, Maharishi Markandeshwar Medical College and Hospital, Kumarhatti, Solan, Himachal Pradesh, India

Abstract

Introduction: The present study was done to access the antibacterial activity of clove (*Syzygium aromaticum*) against extended-spectrum beta-lactamase (ESBL), metallo-beta-lactamase (MBL), and AmpC beta-lactamase-producing gram-negative bacteria causing urinary tract infection. **Methods:** A total of 221 gram-negative uropathogens were isolated and screened for beta-lactamase (ESBL, MBL, and AmpC) production and further tested against ethanolic extract of clove (*S. aromaticum*) for its antibacterial activity. **Results:** Clove was effective against all gram-negative isolates but the best antibacterial activity was shown against *Proteus* species with 19 mm zone of inhibition, 0.39 mg/ml minimum inhibitory concentration (MIC) and 0.19 mg/ml minimum bactericidal concentration (MBC).**Conclusions:** Clove extract showed different antibacterial potential against all gram-negative uropathogens. Clove activity for particular strain was found to be similar between isolates producing beta-lactamase and non beta-lactamase.

Keywords: Extended-spectrum β-lactamases, metallo-beta-lactamase, and AmpC beta-lactamase, *Syzygium aromaticum* (clove), urinary tract infection, uropathogens

Introduction

There are many infectious diseases that occur during a lifetime. One of these is urinary tract infection (UTI), which is experienced by approximately 10% of population and in some cases can lead to morbidity in patients if not treated on time. UTI is caused by many different microorganisms (uropathogens) which include viruses, fungi, and bacteria but the major microorganism responsible for causing UTI in 95% cases is the bacteria.[1,2] Antibiotic resistance against these bacteria causing UTI has been reported by many authors from developed and developing countries. This rapid spread of resistance especially toward beta-lactam antibiotics is a global threat as it possesses a therapeutic challenge which is mediated by different beta-lactamases enzymes such as extended-spectrum beta-lactamase (ESBL), metallo-beta-lactamases (MBLs), and AmpC beta-lactamase. Therefore, it has led to limited choice of antibiotics due to the continuous emergence of these enzymes. Hence, it has become utmost important to find out new antibacterial agents.[3,4] Due to the emergence

Address for correspondence: Dr. Dakshina Bisht, Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh - 201 009, India.
E-mail: dakshinabisht@gmail.com

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of resistance pattern currently, medicinal plant extracts have gained interest because of their known antimicrobial nature. Medicinal plants are the richest bioresource of drugs for traditional systems of medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs.[5] Many spices around the world have been used for several medicinal purposes and as food preservatives, and out of those *Syzygium aromaticum* (clove) is widely used as it has got anti-inflammatory, antimicrobial, antithrombotic, antioxidant, antimutagenic, and anti-ulcerogenic properties.[6] Considering the importance of clove as an antibacterial agent, the present study was designed to analyze the antibacterial potential of clove against ESBL, MBL, and AmpC Beta-lactamase producing gram-negative uropathogens.

Material and Methods

This study was conducted in the Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad and Department of Microbiology, M. M. Medical College and Hospital, Solan. Approval from the Institutional Ethical Committee was obtained F. No. SU/2017/683 (16) on 26/05/2017. All the urine specimens of clinically suspected patients of UTI were sent to the microbiology laboratory from different clinical departments and processed further. All samples were cultured on the blood agar, cystine-lactose-electrolyte deficient (CLED) agar, and MacConkey agar and were incubated further at 37°C for 18 h.[7] More than 10^5 cfu/mL colony count for urine specimens was considered as significant bacteriuria for UTI. Bacterial identification for positive urine cultures was performed using standard microbiological tests and was further processed for antibiotic susceptibility testing using Kirby–Bauer technique on Mueller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (CLSI) protocol.[8,9]

ESBL, MBL, and AmpC beta-lactamase detection of all gram-negative uropathogens were performed using phenotypic methods to CLSI guidelines.

ESBL-producing isolates were screened in accordance with the zone of inhibition of ≤25 mm for ceftriaxone and ≤22 mm for ceftazidime using disc-diffusion method which was further confirmed by cephalosporin/clavulanate combination disks method.[10]

Phenotypic confirmatory test for ESBL production by cephalosporin/clavulanate combination disks method:

All isolates were inoculated in peptone water and adjusted to 0.5 McFarland unit then isolates were swabbed on to MHA. A 30 μg disk of ceftazidime and 30/10 μg disk of ceftazidime-clavulanic acid were placed on the same plate by keeping a minimum distance of 30 mm between them. Plates were further incubated for overnight at 37°C. Zone size of more than 5 mm around ceftazidime-clavulanic disk compared to ceftazidime disk alone was considered positive for ESBL production. Control strains *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used for the procedure.[10]

Screening for AmpC beta-lactamases

AmpC production by the isolates was screened by a disc-diffusion method using cefoxitin disk and the zone size of <18 mm was considered possible producer. It was further confirmed using cefoxitin-cloxacillin double-disc synergy test.[11]

Phenotypic AmpC confirmatory test by cefoxitin-cloxacillin double-disc synergy test:

30 μg disk of cefoxitin and the combination of cefoxitin and 200 μg of cloxacillin were used for this study. All strains of 0.5 McFarland unit were inoculated on MHA and further kept for overnight incubation at 37°C. Equal or more than 4 mm zone size difference between both the disks was indicative of AmpC production.[11]

Table 1: Distribution of uropathogens

Uropathogens	Total (221)
Escherichia coli	100
Klebsiella pneumoniae	37
Pseudomonas aeruginosa	25
Enterobacter species	20
Citrobacter species	17
Acinetobacter baumannii	12
Proteus species	10

Table 2: Distribution of *E. coli* isolates

ESBL-producing strains	AmpC-producing strains	Non (ESBL, AmpC, and MBL)-producing strains
32	18	50

Table 3: Antibacterial activity of clove (*Syzygium aromaticum*) against *E. coli*

E. coli: 100	Average zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
ESBL-producing strains: 32	17	15	11	8	7	0.39	0.19
AmpC-producing strains: 18	17	14	11	9	7	0.39	0.19
Non (ESBL, AmpC, and MBL) producing strains: 50	17	15	12	9	6	0.39	0.19

Screening for metallo-beta-lactamase

All the clinical isolates showing resistance to imipenem disk were considered positive for MBL screening and were further subjected to confirmation by a combined disk test.[12]

Phenotypic confirmatory test by imipenem-ethylene diamine tetraacetic acid (EDTA).

Combined disk test

MBL-screened isolates were swabbed on MHA and two disks of imipenem (10 μg) and imipenem with 10 μL of an EDTA solution were placed on the same plate, further incubated for overnight at 37°C. A zone of inhibition ≥7 mm around with the imipenem-EDTA disk compared to imipenem disk alone indicated MBL production.[12]

Collection and certification of medicinal plants

Clove (*S. aromaticum*) was obtained and certified (UHF herbarium no. 13632) from the Department of Forestry, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India.

Preparation of plant extract

The plant parts (buds of *S. aromaticum*) were separated, washed, and dried in shade. The plant extract was prepared with 800 g of dry plant powder soaked in 2.5 L of 70% ethanol, for 8–10 days, and stirred every 10 h using a sterilized glass rod. At the end of extraction, it was passed through Whatman filter paper no. 1 (Whatman Ltd., England). This ethanoic filtrate was concentrated using water bath at 40°C till the sticky semisolid mass was obtained and then was stored at 4°C for further use. The crude extract was prepared by dissolving known amount of the dry extract in dimethyl sulfoxide (DMSO) to have a stock solution of 100 mg/ml concentration.[13]

Antibacterial activities of plant extracts

Antibacterial activity of ethanolic clove extract was carried out by disc-diffusion method. The turbidity of the culture was adjusted to 0.5 McFarland standards. Culture suspensions were inoculated on MHA so as to obtain a lawn culture. Sterile paper discs (6 mm, HiMedia, Mumbai) were impregnated with 20 μl of the different concentrations (100, 50, 25, 12.5, and 6.25 mg/ml) of plant extracts and were placed on the inoculated agar. For negative control, discs impregnated with 20 μL of 70% ethanol were placed at the center of inoculated MHA. Culture plates were incubated at 37°C for 24 h. After incubation period, the zones of inhibition were measured.[14]

Table 4: Distribution of *K. pneumoniae* isolates

ESBL-producing strains	AmpC-producing strains	Non (ESBL, AmpC, and MBL)-producing strains
14	07	16

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The lowest concentration of the antibacterial agent at which there is no visible growth seen is considered as minimum inhibitory concentration (MIC). The broth microdilution method was used for the estimation of MIC. Microplate (96 polystyrene well) was used for the preparation of different concentrations of clove extract (100 mg/ml to 6.25 mg/ml) by serial dilution. The final concentration of each strain suspension was adjusted to 5×10^5 CFU/ml with 10 μL aliquot of bacterial suspension in supplemented MH broth, in a final volume of 100 μL. Positive growth control, negative controls, and color control (wells containing only extracts) were also prepared. The plates were covered with a sterile plate sealer, carefully mixed and incubated at 37°C for 24 h. Bacterial growth was indicated by the turbidity, relative to the negative and positive controls. The minimum bactericidal concentration (MBC) was obtained by subculturing from each well of microplate onto a nutrient agar plate. The well containing the lowest concentration of the extract that failed to show growth, on subculture was considered as MBC for that test strain.[15,16]

Results

The selection of *S. aromaticum* for this study was based on ethnobotanical data on the traditional use of it in the treatment of bacterial diseases. Antibacterial potential of ethanolic extract of *S. aromaticum* was tested against a total of 221 gram-negative uropathogens [Table 1].

Antibacterial activity of *Syzygium aromaticum* against *E. coli*

Of the 100 *E. coli* isolates, 32 (32%) were ESBL producers, 18 (18%) were AmpC producers and 50 were non (ESBL, AmpC, and MBL) producing strains [Table 2].

S. aromaticum was tested against all *E. coli* isolates for zone of inhibition, MIC, and MBC. The maximum zone of inhibition (17 mm) was shown at 100 mg/ml concentration and minimum zone of inhibition (6 mm) was shown at 6.25 mg/ml. MIC was found to be 0.39 mg/ml, and MBC was 0.19 mg/ml for all *E. coli* [Table 3].

Table 5: Antibacterial activity of clove (*S. aromaticum*) against *K. pneumoniae*

K. pneumoniae: 37	Average zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
ESBL producing strains: 14	16	13	11	10	10	0.78	0.39
AmpC producing strains: 07	15	12	10	10	09	0.78	0.39
Non (ESBL, AmpC, and MBL) producing strains: 16	16	13	11	10	09	0.78	0.39

Antibacterial activity of *S. aromaticum* against *Klebsiella pneumoniae*

Of the total 37 *K. pneumoniae* isolates, 14 (38%) were ESBL producers, 07 (19%) were AmpC producers and 16 (43%) were non (ESBL, AmpC, and MBL) producing strains [Table 4].

All *K. pneumoniae* isolates were tested against *S. aromaticum* and maximum zone of inhibitions (16 mm) was shown at 100 mg/ml concentration and minimum zone of inhibitions (09 mm) was shown at 6.25 mg/ml. MIC was 0.78 mg/ml and MBC was 0.39 mg/ml for all *K. pneumoniae* isolates [Table 5].

Antibacterial activity of *S. aromaticum* against *Enterobacter* species

Of the 20 *Enterobacter* species isolated, 4 (20%) were ESBL producers and 16 (80%) were non (ESBL, AmpC, and MBL) producing strains [Table 6].

S. aromaticum showed maximum zone of inhibition (17 mm) at 100 mg/ml concentration and minimum zone of inhibition (08 mm) at 6.25 mg/ml. MIC was 0.78 mg/ml and MBC was 0.39 mg/ml for all *Enterobacter* isolates [Table 7].

Antibacterial activity of *S. aromaticum* against *Citrobacter* species

Of the 17 *Citrobacter* species, 5 (29%) were ESBL producers and 12 (71%) were non (ESBL, AmpC, and MBL) producing strains [Table 8].

The maximum zone of inhibition (18 mm) was observed at 100 mg/ml concentration and minimum zone of inhibition (08 mm) at 6.25 mg/ml. MIC was 0.39 mg/ml and MBC was 0.19 mg/ml for all *Citrobacter* isolates [Table 9].

Antibacterial activity of *S. aromaticum* against *Proteus* species

Of the10 *Proteus* species, 03 (30%) were ESBL producers and 07 (70%) were non (ESBL, AmpC, and MBL) producing strains [Table 10].

S. aromaticum revealed maximum zone of inhibition (19 mm) at 100 mg/ml concentration and minimum zone of inhibition (09 mm) at 6.25 mg/ml. MIC was 0.39 mg/ml and MBC was 0.19 mg/ml for all *Proteus* isolates [Table 11].

Table 6: Distribution of *Enterobacter* species

ESBL-producing strains	Non (ESBL, AmpC, and MBL)-producing strains
04	16

Antibacterial activity of *S. aromaticum* against *Pseudomonas aeruginosa*

Of the 25 *Pseudomonas aeruginosa* isolates, 07 (28%) isolates were MBL producers and rest 18 (72%) were Non (ESBL, AmpC, and MBL) producing strains [Table 12].

S. aromaticum revealed maximum zone of inhibition (14 mm) at 100 mg/ml concentration and minimum zone of inhibition (09 mm) at 6.25 mg/ml. MIC was 1.56 mg/ml and MBC was 0.78 mg/ml for all *P. aeruginosa* [Table 13].

Antibacterial activity of *S. aromaticum* against *Acinetobacter baumannii*

Of the 12 *Acinetobacter baumannii* isolates, 04 (33%) were MBL producers and 08 (67%) were non (ESBL, AmpC, and MBL) producing strains [Table 14].

S. aromaticum showed maximum zone of inhibition (18 mm) at 100 mg/ml concentration and minimum zone of inhibition (09 mm) at 6.25 mg/ml. MIC was 0.78 mg/ml and MBC was 0.39 mg/ml for all *A. baumannii* isolates [Table 15].

Discussion

Infections caused by beta-lactamase-producing bacteria continue to pose serious health problems in the world and particularly in developing countries. The use of medicinal plant extracts is nowadays essential in the search for new active antibacterial biomolecules against antibiotics. Clove extract acts through the presence of potentially bioactive components such as eugenol (2-methoxy-4-(2-propenyl) phenol), glycosides, flavonoids, saponins and tannins, and essential oils. These bioactive components have shown several bioactivities like antipyretic, antispasmodic, anticarcinogenic, inhibition of 5-LOX enzyme activity in human polymorphonuclear leukocytes cells, antioxidant, protection against peroxynitrite-mediated tyrosine nitration and lipid peroxidation, antifungal activity of essential oil, antimicrobial, and, antibacterial. Hence, *in vitro* evaluation of antibacterial activity of clove was screened against seven bacterial strains: *E. coli, K. pneumoniae, Enterobacter* species, *Citrobacter* species, *Proteus* species, *P. aeruginosa,* and *A. baumannii.*

Our study showed *a* range of 6–17 mm zone of inhibitions against *E. coli* at different concentrations of clove. Mostafa

Table 7: Antibacterial activity of clove (*S. aromaticum*) against *Enterobacter* species

Enterobacter species: 20	Average zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
ESBL-producing strains: 4	17	15	12	10	08	0.78	0.39
Non (ESBL, AmpC, and MBL)-producing strains: 16	16	15	12	09	08	0.78	0.39

et al.[17] reported a 12 mm zone of inhibition at 15 mg/ml, however, the same zone of inhibitions came at 25 mg/ml in our study. Other studies also revealed the zone of inhibition of *E. coli* ranging from 17–19 mm against clove which are in accordance with our study.[18-20] Clove demonstrated its best antibacterial activity against *E. coli* and *Proteus* species as they had maximum inhibition zones and minimum MIC and MBC values [Figures 1 and 2]. MIC and MBC for *E. coli* turned out to be 0.39 and 0.19 mg/ml, respectively whereas in a study done by Noumedem *et al.*[21] they reported 0.51–1.02 mg/ml MIC. Another study by Pundir *et al.* reported 10 mg/ml MIC value, which was higher compared to the present study against *E. coli*.[22]

K. pneumoniae showed 9–16 mm inhibitory zones in our study. Sethi *et al.*[18] also reported 17 mm maximum zone of inhibition while Gupta *et al.*[19] reported 15 mm zone of *K. pneumoniae* against clove which was close to our study. Researchers from University of Dschang reported MIC 1.02 mg/ml which was in concordance to our study.[21] Sethi *et al.* and Nascimento *et al.* demonstrated high values of MIC against *K. pneumoniae* which is in contrast to present study.[18,23] Sharmeen *et al.* have also concluded that clove extract had significant antibacterial activity against *K. pneumoniae*.[24]

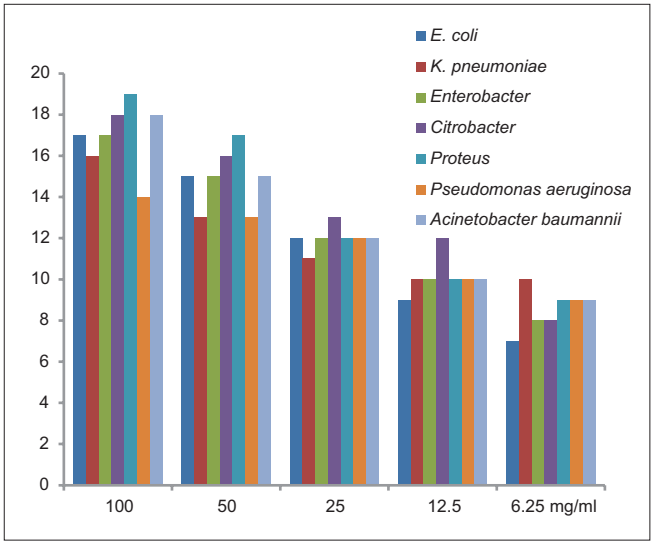

Figure 1: Maximum zone of inhibition (mm) of clove against uropathogens

Table 8: Distribution of *Citrobacter* species

ESBL-producing Strains	Non (ESBL, AmpC, and MBL)-producing strains
05	12

Although, very few studies have been done on antibacterial activity of clove against *Enterobacter* species, *Citrobacter* species, *Proteus* species, and *A. baumannii*. The present study revealed that all these clinical isolates responded good sensitivity to clove. Noumedem *et al.*'s study also reported significant antibacterial property against different *Enterobacter* strains which was quite similar to our results.[21] On the other hand, researchers from Brazil did not observe any antibacterial activity against *Enterobacter aerogenes*.[23] Sethi *et al.* stated that 100% concentration of clove showed best antibacterial activity (19 mm) against *Citrobacter* of the seven different pathogenic strains.[18] Clove demonstrated best activity against *Proteus* species in our study which was supported by studies done by other researchers from different countries.[18,23] A study done in Iraq showed 28 mm inhibition zone at 10% clove concentration whereas our study showed maximum 18 mm zone at 100 mg/ml concentration.[25]

Mostafa *et al.* applied different concentrations (1.25–15.0 mg/ml) against *P. aeruginosa* and they observed maximum zone of inhibition of 17.5 ± 0.35 mm at highest concentration.[17] Other authors from different countries, Sulieman *et al.* and Nascimento *et al.* also demonstrated range a of 7–20 mm inhibition zones at different concentrations which were in concordance to present study.[20,23] In contrast to our findings, Mehrotra *et al.* and Noumedem *et al.* reported MIC value of 0.025 mg/ml and 0.2 mg/ml, respectively.[21,26]

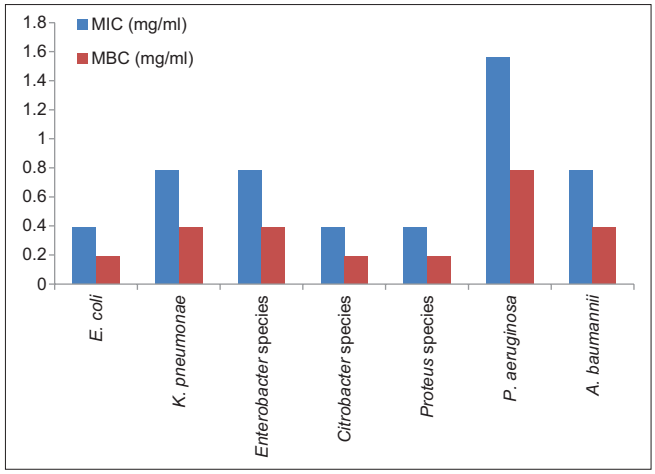

Figure 2: Average minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of clove against uropathogens

Table 9: Antibacterial activity of clove (*S. aromaticum*) against *Citrobacter* species

Citrobacter species: 17	Average zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
ESBL-producing strains: 05	18	15	12	12	08	0.39	0.19
Non (ESBL, AmpC, and MBL)-producing strains: 12	18	16	13	11	08	0.39	0.19

Medicinal plants and primary care physicians

The global prevalence of the use of medicinal plants or its product continues to rise as patients self-medicate with or without informing their physicians. In India, physicians generally do not ask the patients about having used herbal preparations while taking their history. But it is very important that primary care physicians must ask the patient about any prior use of medicinal plant or preparation especially when presenting with unusual signs and symptoms while prescribing allopathic medicine as they may cause side effects and adverse reactions. On the other hand, Traditional Medicines Programme by WHO encourages countries to identify aspects of traditional medicine from medicinal plants that provide safe and effective remedies and utilize these aspects in primary health care. Medicinal plants are one aspect of the traditional medicine, should be incorporated into primary health care because many individuals already use medicinal plants, they could be an effective way to alleviate problems caused by the high demand and limited availability of modern medicines in primary health care setting in developing countries. Before inclusion into national policies and protocols, medicinal plants must be studied at local levels for effectiveness.[27,28]

Conclusion

Thus, ethanolic extracts of clove at all concentrations were found to be effective against all clinical isolates and provided baseline information for the potential use of clove to treat bacterial infections. Hence, our study concluded that clove extracts can be used to develop new antimicrobial drug which is the need of the hour. However, further research is required for the identification and characterization of bioactive molecules present in the clove extract and their *in vivo* antibacterial activities against human pathogens.

Table 10: Distribution of *Proteus* species

ESBL-producing strains	Non (ESBL, AmpC, and MBL)-producing strains
03	07

Table 11: Antibacterial activity of clove (*S. aromaticum*) against *Proteus* species

Proteus species: 10	Average zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
ESBL-producing strains: 03	19	17	12	10	09	0.39	0.19
Non-ESBL-producing strains: 07	19	16	12	10	09	0.39	0.19

Table 12: Distribution of *P. aeruginosa*

MBL-producing strains	Non (ESBL, AmpC, and MBL)-producing strains
07	18

Table 13: Antibacterial activity of clove (*S. aromaticum*) against *P. aeruginosa*

P. aeruginosa: 25	Average zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
MBL-producing strains: 07	14	13	12	10	09	1.56	0.78
Non-MBL-producing strains: 18	14	13	11	10	09	1.56	0.78

Table 14: Distribution of *A. baumannii*

MBL-producing strains	Non (ESBL, AmpC, and MBL)-producing strains
04	08

Table 15: Antibacterial activity of clove (*S. aromaticum*) against *A. baumannii*

A. baumannii: 12	Zone of inhibition (mm) at different concentrations (mg/ml)					MIC (mg/ml)	MBC (mg/ml)
	100	50	25	12.5	6.25		
MBL-producing strains: 04	18	15	12	10	09	0.78	0.39
Non-MBL-producing strains: 08	18	15	11	10	09	0.78	0.39

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Conflicts of interest

There are no conflicts of interest.

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