Original Article

Antibacterial efficacy of different combinations of clove, eucalyptus, ginger, and selected antibiotics against clinical isolates of *Pseudomonas aeruginosa*

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Abstract

Background: Nosocomial infections caused by multidrug-resistant *Pseudomonas aeruginosa* are commonly treated with conventional antibiotic which may lead to some serious side effects in the patients. Conventionally, medicinal plants, including clove, eucalyptus, and ginger, are used for the safe and effective treatment of several diseases. **Aims and objectives:** The aim and objective of this study is to evaluate the combined antibacterial efficacy of medicinal plants (clove, eucalyptus, and ginger) and selected antibiotic and also combined efficacy of different plants extracts against clinical isolates of *P. aeruginosa*. **Materials and methods:** A total of seven clinical isolates and one reference strain (PA01) of *P. aeruginosa* were included in this study. The antibacterial activity of crude methanol extracts of medicinal plants and selected antibiotics was screened using well-diffusion assay and their minimum inhibitory concentration (MIC) was determined by the microdilution method. Combined efficacy of ceftazidime and plant extracts was tested using standard checkerboard method and different plant extracts were evaluated using broth macrodilution method. **Results:** All of the seven clinical isolates of *P. aeruginosa* showed multidrug resistance pattern and were found highly sensitive to ciprofloxacin followed by ceftazidime and gentamicin. Clove exhibited better antibacterial activity as compared to eucalyptus and ginger. Synergistic interaction was found between ceftazidime and plants extracts against reference PA01 and clinical isolate 2. Highest two-fold reduction in MIC was found in the combination of clove-ginger against reference PA01 and clinical isolate 3. **Conclusion:** The selected medicinal plants are highly efficient for enhancing the antibacterial activity of antibiotic.

Keywords: Antibacterial activity, medicinal plants, minimum inhibitory concentration, nosocomial infections

Introduction

Infectious diseases are the major cause of death globally. Antibiotics are commonly used for the treatment of bacterial infections, but the increasing emergence of microbial resistance against the available antibiotics has becomes a serious issue for the health care.[1] *Pseudomonas aeruginosa* is a classical opportunistic Gram-negative bacteria and a major causative agent of nosocomial or hospital acquired infection. *P. aeruginosa* can resist a large number of antibiotics commonly used for the treatment and its infection is generally treated with β–lactam antibiotics (piperacillin/tazobactam, ceftolozane/tazobactam, ceftazidime, cefepime, or a carbapenem) in the combination of aminoglycoside/fluoroquinolones.[2] Ceftazidime, commonly used antibacterial agent for effective treatment of *P. aeruginosa* infection, is responsible for inhibition of bacterial cell wall.[3] Because of increasing resistance against the available antibiotics, an alternative approach is necessary for the treatment of bacterial infection and bioactive molecules of natural plants may be a solution of existing problems.[4,5] Medicinal plants are abounded with broad range of ptytoconstituents such as tannins, terpenoids, flavonoids, and alkaloids, and their antibacterial activity have been investigated in several studies. The higher molecular

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diversity and variability of phytoconstituents in natural plant extract provides the vision to explore them for the health benefits.[6]

Eucalyptus (*Eucalyptus globulus* Labill., family: *Myrtaceae*) has been used in the treatment of pulmonary infection and exhibit the antibacterial, anti-inflammatory, antiviral, antifungal, anticancer and antioxidant properties.[7-10] Ginger (*Zingiber officinale* Roscoe, family: *Zingiberaceae*) is traditional domestic remedy and commonly used for the treatment of cough, cold, asthma, nausea, loss of appetite and heart palpitation. Ginger exhibited antibacterial activity and may also be used as antipyretic, anticoagulation and anti-inflammatory agent.[11-13] Clove (*Syzygium aromaticum* [L.] Merr. and L. M. Perry, family: *Myrtaceae*) buds have been traditionally used for effective treatment of digestive problem, gastrointestinal spasm, nausea, vomiting, and as an antibacterial, antioxidant, anti-inflammatory (due to high flavonoid content) and antidiabetic agent.[14,15] Antibacterial activity of clove oil against *P. aeruginosa* has been previously reported.[16] The aim of the present study was to evaluate the combined antibacterial efficacy of crude methanol extracts of medicinal plants (clove, eucalyptus, and ginger) and selected antibiotics against clinical isolates of *P. aeruginosa* and to investigate the possible reduction in the dose/minimum inhibitory concentration (MIC) of antibiotics using plant extracts as combination agents. Combined antibacterial efficacy of plant extracts was also screened against *P. aeruginosa*.

Materials and methods

Collection of plant materials and extract preparation

The fresh buds of clove (*S. aromaticum*), leaves of eucalyptus (*E. globulus*) and ginger rhizomes (*Z. officinale*) were collected and authenticated from Central Council for Research in Ayurvedic Sciences-Regional Ayurveda Research Institute, Jhansi (Uttar Pradesh), India. Conventional maceration method was used for the extraction of plant. Ten grams of dried plant powder was soaked in methanol (100%) and subjected for shaking using magnetic stirrer at the room temperature for three successive days. The crude extract was filtered through Whatman filter paper No. 1 and filtrate was centrifuged at 3000 rpm for 10 min. The supernatant was evaporated and concentrated with rotary evaporator. Prepared crude methanol extracts of eucalyptus, ginger, and clove were preserved at 4°C in air tight bottle until further experiments.

Antibiotics

Antibiotics, i.e. trimethoprim, ciprofloxacin, pefloxacin, chloramphenicol, tetracycline, gentamicin, kanamicin, ceftazidime, ampicillin and amoxycillin were purchased commercially.

Collection of bacterial strains and growth conditions

All of the clinical isolates of *P. aeruginosa* were collected from Department of Microbiology, Sarojini Naidu Medical College, Agra (Uttar Pradesh), India. Reference strain PA01 of *P. aeruginosa* was collected from Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh (Uttar Pradesh), India. All bacterial strains were maintained by subculture on Luria agar aerobically for 24 h at 37°C.

Screening of antibacterial activity of plant extracts and antibiotics (as plant/antibiotic monotherapy)

According to Clinical and Laboratory Standards Institute (CLSI) guidelines, overnight incubated bacterial culture (at 37°C) was suspended in normal saline solution (0.85%) and its turbidity was adjusted with 0.5 McFarland standard to standardize the final inoculum size (10^8 CFU/ml).[17]

Mueller Hinton agar plate was prepared with the standardized bacterial suspension and the wells of 6–8 mm diameter were made using the sterile cork borer. The plant extracts were reconstituted at the testing concentration (500 mg/ml) in DMSO (dimethyl sulfoxide) and their 50 µl were aseptically poured in the well and plate were incubated at 35 (±2°C) for 18–20 h. The diameter of zone of inhibition (mm) was measured with standard scale to determine the antibacterial activity of plant extracts. The ciprofloxacin and DMSO were used as positive and negative control, respectively. Similar process was used to screen the antibacterial activity of antibiotics for initial screening. 30 µl (30 µg) from stock solution (01 mg/ml) of each antibiotic were aseptically poured in the well, and the diameter zone of inhibition (mm) was measured. Clinical isolates were screened and selected for the study of combined efficacy on the basis of their drug resistance pattern against tested antibiotics.

Determination of minimum inhibitory concentration of antibiotics and plant extracts

The MIC was determined by the microdilution method using 96 well microtiter plate according to the guidelines of CLSI[17] with slight modification. Two-fold serial dilution of plant extracts stock solutions was made ranging from 500 mg/ml to 0.48 mg/ml in Mueller Hinton broth. 50 µl of standardized and diluted bacterial suspension (microbial inoculums containing 10^6 CFU/ml) was inoculated in each well aseptically and plates were incubated at 35°C for 18–20 h. After incubation as indicator solution 50 µl of 0.1% solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC) were added in each well and plates were further incubated at the room temperature for 30–45 min for the determination of bacterial growth. The MIC was the lowest concentration of plant extract at which the reduction of tetrazolium dye to red/pink formazan was not observed after incubation.

The MIC of selected antibiotics was determined with the similar protocol as described above at two-fold serial dilution of stock concentration (01 mg/ml) ranging from 1000 µg/ml to 0.97 µg/ml.

Determination of combined efficacy of antibiotic and plant extracts (as antibiotic-plant combination therapy)

Combined efficacy of ceftazidime and methanol extracts of eucalyptus, ginger, and clove was tested using standard checkerboard method against four selected clinical isolates and one reference strain PA01. The fractional inhibitory

concentration (FIC) and FIC index (FICI) was calculated as follows.[18,19]

FIC (antibiotic) = MIC of antibiotic in combination/MIC of antibiotic alone

FIC (plant extract) = MIC of plant extract in combination/MIC of plant extract alone

FICI = FIC (antibiotic) + FIC (plant extract)

Synergistic effect (synergism) is traditionally been defined as an FICI of ≤0.5 while additive effect as a FICI of >0.5 but of ≤4.0 and antagonism as a FICI of >4.0.

Determination of combined efficacy of different plant extracts (as plant–plant combination therapy)

Different plant combinations (1:1) were prepared and subjected to evaluate their combined efficacy using broth macrodilution method according to the CLSI guidelines.[17] Plant extracts were combined at one-fold higher dilution from their screened MIC and further serially diluted to determine their combined MIC. TTC (0.1%) was used as indicator solution for the determination of bacterial growth.

The combined MIC of plants extract combination was the lowest concentration at which the reduction of tetrazolium dye to red/pink formazan was not observed after incubation.

Statistical analysis

Experiments for the measurement of inhibition zone with different extracts were performed in triplicate and mean ± standard deviations were calculated. One-way analysis of variance (ANOVA) was used to determine the statistical significance (*P* value) among the tested extracts producing inhibition zone in a single strain of *P. aeruginosa.* Moreover, *t*-test was applied to determine the significant difference among the reference PA01 and clinical isolates. One-way ANOVA and *t*-test were performed in IBM SPSS statistics 20 (Version 20.0. Armonk, NY, USA: IBM Corporation).

Results

Antibacterial activity of antibiotics and plant extracts

All of the clinical isolates were found highly resistant to pefloxacin, ampicillin, amoxicillin, and trimethoprim showing no zone of inhibition and highly sensitive to ciprofloxacin followed by ceftazidime and gentamicin. Clinical isolates showed multidrug resistant pattern as per the characteristic of *P. aeruginosa* [Figure 1]. Among the plant extracts, clove showed higher growth inhibitory activity against all of tested strains in comparison of eucalyptus and ginger. Clove showed significantly higher inhibition zone against reference strain PA01 (20.06 ± 0.57; $P < 0.05$), clinical isolate 2 (17.6 ± 0.57; $P < 0.01$) and clinical isolate 4 (17.3 ± 0.57; $P < 0.01$) [Table 1].

Minimum inhibitory concentration of selected antibiotics and plant extracts

MIC of effective antibiotics, for example, ciprofloxacin, gentamicin, and ceftazidime were determine using microdilution method against four clinical isolates (showing higher multidrug resistant pattern) and reference strain PA01. MIC of ciprofloxacin was <0.97 µg/ml for all of the selected strains. The MIC of gentamicin and ceftazidime was in ranged from 3.9 µg/ml to 15.62 µg/ml and 15.62 µg/ml to 31.25 µg/ml, respectively, against the selected clinical isolates (clinical isolates 2, 3, 4 and 5) along with reference strain PA01 [Figure 2]. Among plants extract, MIC of clove extract was found of lower ranged from 3.9 mg/ml to 15.62 mg/ml in comparison of eucalyptus (7.81 mg/ml to 62.52 mg/ml) and ginger extract (7.81 mg/ml to 31.25 mg/ml) [Figure 3].

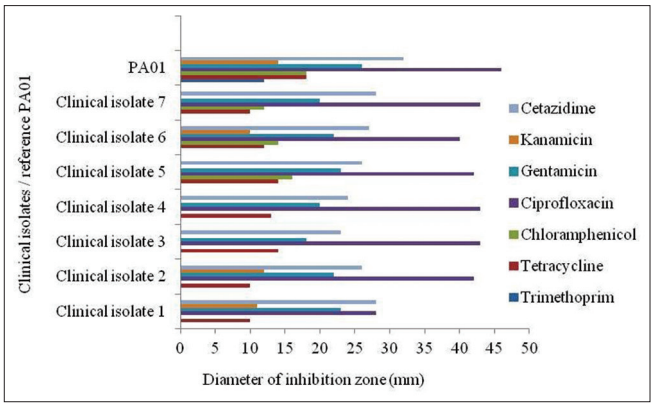

Figure 1: Antibacterial activity (zone of inhibition) of different antibiotics against clinical isolates and reference PA01 of *Pseudomonas aeruginosa*

Combined efficacy of selected antibiotic and plant extracts

All of the plant extracts (eucalyptus, ginger, and clove) showed synergistic effect with ceftazidime against PA01 and clinical isolate 2 (FICI: ≤0.5) while surprisingly additive effect were found against clinical isolate 3 (FICI: >0.5 but of ≤4.0) [Table 2]. Antagonistic activity was not found in any combination against *P. aeruginosa*.

Combined efficacy of different plant extracts combinations

Among plant combinations, maximum two-fold reduction in individual MIC was found in combination of clove and ginger extract against clinical isolate 3 and PA01. Ginger and eucalyptus extract were not found effective for the reduction of their individual MIC as combination against clinical isolate 2 and clinical isolate 4 [Table 3].

Comparative statistical analysis of reference PA01 and clinical isolates in response to different plants methanol extracts

All extracts showed significantly higher inhibitory activity ($P < 0.05$, *t*-test) against reference strain PA01 than clinical MDR isolates 2, 3, 4 [Figure 4a-c].

Discussion

P. aeruginosa is a Gram-negative and opportunistic bacteria and one of the common causative agent of nosocomial infection with an increased prevalence of multidrug resistance against

Table 1: Diameter of inhibition zone (mm) of different methanol extracts against clinical isolates and reference PA01 of *Pseudomonas aeruginosa*

Clinical isolates and reference strain/plant extracts	Clinical isolate 1	Clinical isolate 2	Clinical isolate 3	Clinical isolate 4	Clinical isolate 5	Clinical isolate 6	Clinical isolate 7	PA01
Eucalyptus	13.6±1.52	15.3±0.57	13.0±1.00	15.6±0.57	13.6±1.52	14.3±1.5	14.0±1.00	17.6±0.57
Ginger	13.3±0.57	12.6±1.15	13.3±0.57	12.6±1.15	13.3±0.57	15.0±2.00	13.6±1.15	17.0±1.00
Clove	16.3±0.57	17.6±0.57	15.3±1.15	17.3±0.57	16.6±0.57	17.0±1.00	16.6±0.57	20.6±0.57
P	<0.05	<0.01	<0.05	<0.01	<0.05	NS	<0.05	<0.05

P value corresponds to one-way ANOVA, mean±SD. NS: Not significant, SD: Standard deviation

Table 2: Fractional inhibitory concentration and fractional inhibitory concentration index of ceftazidime and different plants methanol extracts combination against selected clinical isolates and reference PA01 of *Pseudomonas aeruginosa*

Combinations/clinical isolates and reference strain	Clove + ceftazidime			Ginger + ceftazidime			Eucalyptus + ceftazidime		
	FIC (P)	FIC (A)	FICI	FIC (P)	FIC (A)	FICI	FIC (P)	FIC (A)	FICI
Clinical isolate 2	0.24	0.12	0.36 S	0.25	0.25	0.5S	0.25	0.25	0.5 S
Clinical isolate 3	0.5	0.5	1.0 A	0.5	0.5	1 A	0.5	0.49	0.9 A
Clinical isolate 4	0.24	0.12	0.36 S	0.5	0.5	1 A	0.5	0.25	0.75 A
PA01	0.24	0.12	0.36 S	0.24	0.12	0.36 S	0.24	0.25	0.49 S

S: Synergistic effect (FIC index: ≤0.5), A: Additive effect (FIC index >0.5 but ≤4.0). FIC: Fractional inhibitory concentration, FICI: FIC index

Table 3: Minimum inhibitory concentration (mg/ml) of methanol extracts of plants alone and in combinations against selected clinical isolates and reference PA01 of *Pseudomonas aeruginosa*

Clinical isolates	MIC (mg/ml) of plant extracts alone			MIC (mg/ml) of individual plant extracts in different combination (1:1)		
	Clove	Ginger	Eucalyptus	Clove+ ginger	Clove + eucalyptus	Ginger + eucalyptus
Clinical isolate 2	3.9	15.6	15.6	1.9/7.8[a]	1.9/7.8[a]	15.6/15.6[c]
Clinical isolate 3	15.6	31.25	62.5	3.9/7.8[b]	7.8/31.2[a]	15.6/31.2[a]
Clinical isolate 4	7.8	31.2	31.2	3.9/15.6[a]	3.9/15.6[a]	31.2/31.2[c]
PA01	3.9	7.81	7.81	0.95/1.9[b]	1.9/3.9[a]	3.9/3.9[a]

[a]One fold reduction in MIC, [b]Two-fold reduction in MIC, [c]No reduction in MIC. MIC: Minimum inhibitory concentration

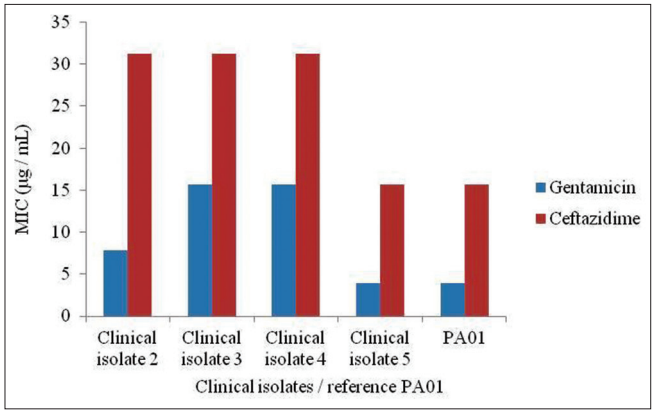

Figure 2: Minimum inhibitory concentration of selected antibiotics against selected clinical isolates and reference PA01 of *Pseudomonas aeruginosa*

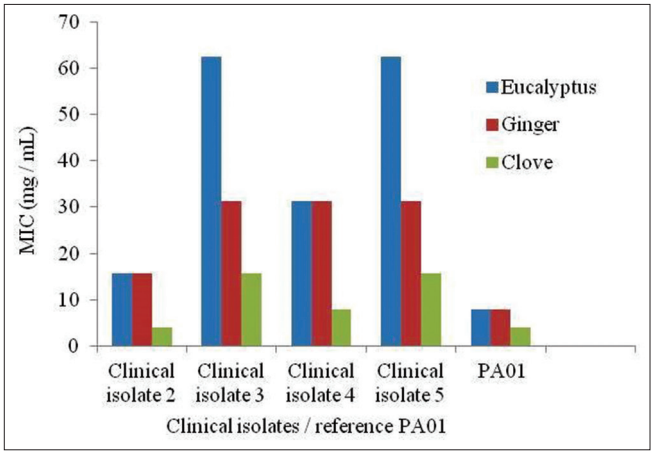

Figure 3: Minimum inhibitory concentration of different plants methanol extracts against selected clinical isolates and reference PA01 of *Pseudomonas aeruginosa*

commonly used antibiotics.[20] The increasing resistance in bacteria has become a global problem that reduces the effect of conventional drugs.[21] Drug development based on the plant component may be a solution of this serious problem of drug resistance.[22] The present study was focused to evaluate the combined antibacterial efficacy of plants and antibiotics and also the possible reduction of MIC of antibiotics commonly used for controlling the pseudomonas nososcomial infection. The interaction between the plant extracts (clove, eucalyptus, ginger) and ceftazidime were evaluated and had significant reduction in MIC of ceftazidime. In the present study, the antibacterial activity of selected antibiotics was

screened and ciprofloxacin was found most effective which was similar to the finding of Kapoor and Murphy.[23] The methanol extract of clove exhibited higher zone of inhibition against reference strain PA01, clinical isolate 2 and clinical isolate 4. Almost similar activity of clove was reported by Pandey and Singh, Mostafa *et al*.[24,25] In the present study, methanol extract of ginger showed significant antibacterial activity and in accordance with Gull *et al*.[26] Antibacterial activity of eucalyptus showed similarity with findings of Khatoon *et al*.,[27] Singh *et al*.[28] but surprisingly higher than reported by Pereira *et al*.(2014).[29] This difference may be due to the variability in geographical conditions and active phycoconstituents of eucalyptus and also method of extract preparation. During comparative analysis, the reference strain PA01 was found highly sensitive to all extracts in comparison to other clinical MDR isolates (e.g.,; Clinical isolates 2, 3, 4) [Figure 4a-c]. It may be due to no any previous exposure of PA01 to extracts which justify that routine or previous exposure of microorganism to drugs (including plant extracts) may lead chances of emergence of resistance in microorganism. However, contrary to the result of the present study, considerably higher MIC of gentamicin was reported by Reda *et al*.[30] The MIC of ceftazidime was found within the range reported by Rains *et al.*, Tunney and Scott.[31,32] Ceftazidime inhibits the synthesis of peptidoglycan layer in bacterial cell wall that causes bacterial growth inhibition and cell lysis. The reported common side effects of ceftazidime are rashes on skin, transient eosinophilia, fever, diarrhea and reversible elevation of liver function tests, changed renal function (in 1.2% of patients). Different plant extracts exhibit varied antimicrobial activity may be due to the presence of different phytoconstituents including phenolic, terpenoides, tannins and alkaloid compounds and each phytoconstituents exhibit their unique and complicated mode of action for antimicrobial attributes.[33,34] The one specific and exact mechanism for antibacterial activity of plant extracts/phytoconstituents has not been established, but the presence of different groups of compounds targeting shape and structure of cell, intracellular critical molecules, ions and other different possible active cell sites may be responsible for antimicrobial properties.[35,36] High phenolic compound in the clove methanol extract has been previously reported.[37] The active phenolic compound may be one of the reasons of higher antibacterial activity of clove extract which cause change in permeability of membrane, blocking of efflux pump and protein denaturation of microorganism.[38,39] Obviously, further screening of phytoconstituent of clove is required for confirmation. The synergistic interaction between the different antibiotics and crude plant extracts against different microorganism has been previously reported.[40-42] Clove showed the better interaction with ceftazidime in comparison to eucalyptus and ginger which indicate that the phenolic components synergistically interact with other antimicrobial compound.

Clove may be involved in membrane disruption or blocking of efflux pump due to high phenolic content and ceftazidime in cell wall degradation activity due to high affinity for PBP-3 (penicillin binding protein-3). This combined and

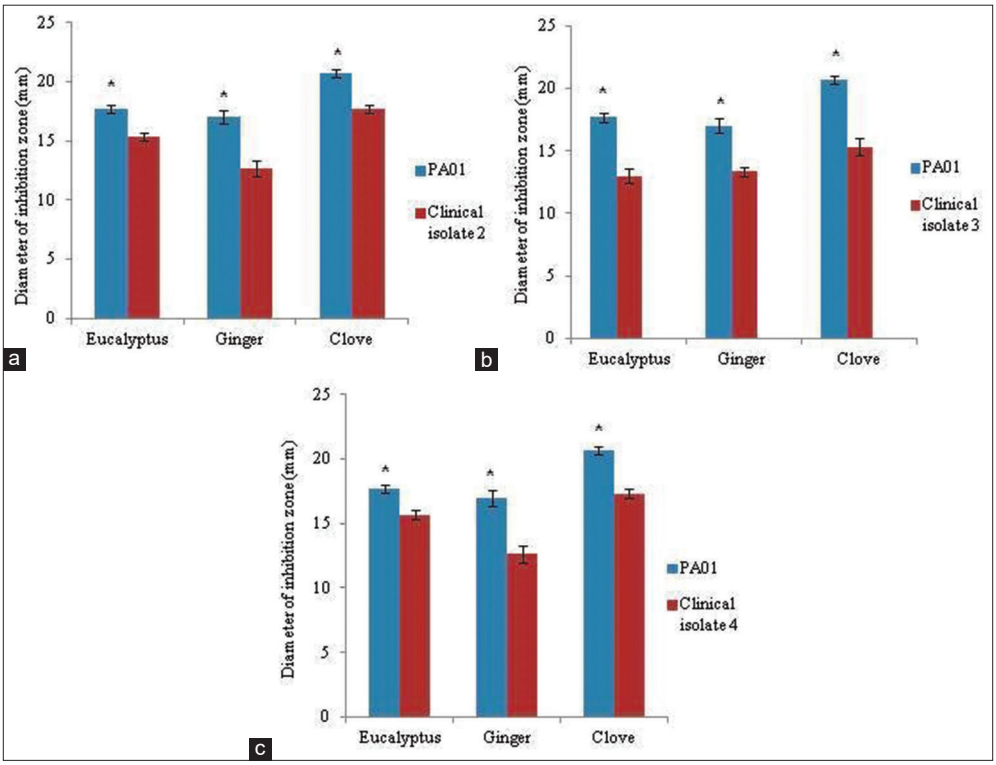

Figure 4: (a-c) Comparative analysis of reference PA01 and clinical isolates in response to different plants methanol extracts. Each bar represents the mean value of three independent replicates for inhibition zone and error bar showed standard error. * Represents significant difference at $P < 0.05$ (*t*-test)

simultaneous activity of clove and ceftazidime at their concern active site may be responsible for significant reduction in MIC of ceftazidime. However, molecular investigations are essential for further elucidation. In the present study, the growth inhibitory activity of different combinations of clove, eucalyptus, and ginger was analyzed against *P. aeruginosa* and most of the combinations were found effective in terms of reduction of individual MIC. These combinations have the potential to be an effective and safe substitute of plant and antibiotic combination therapy or antibiotic monotherapy. The results of the present study showed that dose of antibiotics may be reduced in combination of plant extracts consequently along with the reduced dose of antibiotic chances of developing resistance in microorganism may also be decreased. The present study reveals that growth inhibitory activity of antibiotic-plant combinations and plant-plant combinations are isolate specific and single combination may not be effective against all of isolates.

Conclusion

The present study scientifically validates the efficacy of medicinal plants, i.e., clove, eucalyptus, and ginger for enhancing the antibacterial activity of antibiotic. The findings of present study support that different plant combinations should also be examined along with the plant-antibiotic combinations for the treatment of microbial infection with minimum/no side effect and decreasing the emergence of antibiotic resistance.

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Conflicts of interest

There are no conflicts of interest.

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