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Comparative antibacterial activity of clove extract against *Pseudomonas aeruginosa*

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Abstract
Pseudomonas aeruginosa is an opportunistic pathogen belonging to the γ-proteobacteria family, known to cause pneumonia linked with ventilator use and nosocomial infections. With the increasing prevalence of antibiotic-resistant bacteria, there is a pressing need to identify alternatives to conventional antibiotics. Plant-derived substances (PDSs) offer potential not only as antibacterial agents but also as modulators of antibiotic resistance. In this study, diffusion assay using disc agar, and the minimum inhibitory concentration (MIC) assay of each imipenem, amoxicillin-clavulanic acid, and clove extract was determined. The clove extract was analyzed via the UHPLC/MS, and the checkerboard assay was employed to evaluate the potential synergistic effects of combining clove extract with both antibiotics. The combination of clove extract to each antibiotic led to a significant reduction in their respective MICs. Moreover, each antibiotic exhibited synergistic effects on the fractional inhibitory concentration value (FIC) of clove extract. The analysis identified seventeen components in the clove extract, predominantly flavonoids and phenolic compounds. The antibacterial efficacy of the *Syzygium aromaticum Myrtaceae* extract against *P. aeruginosa* indicated its potential as a promising antibacterial agent capable of enhancing the effectiveness of existing medications.

Keywords Clove extract, *P. aeruginosa*, Antibiotics, Synergism, Analysis

Background

Since the mid-twentieth century, the world has been challenged with emerging and reemerging infectious diseases caused by bacteria. Emerging bacterial pathogens may include those that cause cholera, tuberculosis, gonorrhea, pertussis, and pneumococcal disease [1]. The gram-negative aerobic rod-shaped bacterium *Pseudomonas aeruginosa* is a member of the γ-Proteobacteria [2]. *P. aeruginosa* cells are straight or slightly curved, rod-shaped, 1.5 ± 3 µm in length and 0.5–0.7 µm in width [3]. Factors contributing to the pathogenicity of *P. aeruginosa* may include resistance to antibiotics, various virulence factors, and adaptation to a wide range of environments [3]. While *P. aeruginosa* rarely causes illnesses in healthy people, it can lead to severe infections in immunocompromised patients and those with cystic fibrosis, associated with high mortality and morbidity [4]. Additionally, *P. aeruginosa* is an opportunistic pathogen and may cause nosocomial infections and ventilator-associated pneumonia [5].

Antibiotics have transformed medicine and saved countless lives since their discovery [6]. Yet their origins are now overshadowed by the growing crises of antibiotic resistance [7]. Antimicrobial resistance is becoming

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burdensome for health communities and patients. Bacteria have the ability to become resistant to one or more antibiotics, complicating the treatment of common infections [8]. The Centers for Disease Control and Prevention (CDC); reports that annually in the United States there are 2.8 million cases of antibiotic-resistant infections, which lead to approximately 35,000 deaths [9]. This issue is exacerbated by factors such as misuse, overuse, and extensive use, all of which contribute to the significant increase in antimicrobial resistance [10]. Additionally, the inappropriate and irrational prescription of antibiotics has led to the development of antibiotic resistance in many bacterial species [11]. In light of this, there is an increasing need to identify alternative approaches for the prevention and treatment of microbial infections. Plant-derived substances (PDSs), such as alkaloids, tannins, and polyphenols, have shown potential as alternatives to antimicrobials and serve as modifiers of antibiotic resistance [11]. This potential is rooted in the traditional use of various medicinal plants for treating infections [12]. Research has highlighted significant antibacterial activities in plants such as *Allium sativum* L., *Artemisia absinthium* L., *Berberis lycium* Royale, *Curcuma longa* L., *Nerium oleander* L., *Swertia chirata* L., and *Adiantum capillus-veneris* L., all of which have been used traditionally for infection treatment [13].

Studies on the antibacterial effects of PDSs using clinical isolates indicated that their crude extracts can serve as viable source of resistance modifying factors [14], and enhance the efficacy of antibiotics against multidrug-resistant bacteria [15–18]. These phytochemicals of PDSs have well-documented antibacterial properties [19], they can alter protein-protein interactions within cells, modulate host immune responses, and interfere with bacterial signal transduction and division, thereby enhancing apoptosis [20]. A study by Haroun et al., (2016) demonstrated synergistic effect between *Thymbra spicata* L. extract and antibiotics as ampicillin, cefotaxime, amikacin, and ciprofloxacin against multidrug-resistant strains of *Staphylococcus aureus* and *Klebsiella pneumonia*. The research revealed that the greatest synergistic enhancement was observed with cefotaxime against *S. aureus* strains [21]. Cai et al., (2016) demonstrated that baicalein exhibited synergistic activity when combined with cefotaxime against certain extended-spectrum β-lactamase-positive *Klebsiella pneumonia* strains. This synergy was attributed to the inhibition of CTX-M-1 mRNA expression. The CTX-M-1 gene encodes an extended-spectrum β-lactamase (ESBL) enzyme that confers resistance by hydrolyzing a broad spectrum of β-lactamase antibiotics [22]. In another study, the crude extract from the bark of *Hypericum roeperianum* enhanced the activity of oxacillin, doxycycline, and tetracycline by up to 64-fold against the multidrug-resistant Enterobacter aerogenes EA-CM64 strain [23]. Fatema et al., (2020) demonstrated that methanol extracts of *Salvia chorassanica* and *Artemisia khorassanica* significantly enhanced the efficacy of amikacin and imipenem against multidrug-resistant isolates of Acinetobacter [24]. Finally, combinations of methanolic extracts from *Withania somnifera* and *Catharanthus roseus* with ceftazidime, trimethoprim, and chloramphenicol demonstrated significant synergistic activity against multidrug-resistant isolates of *Salmonella typhi* [25].

Numerous studies on *Syzygium aromaticum Myrtaceae* extracts has been shown to have inhibitory effects on both gram-positive and gram-negative bacteria. Clove essential oil inhibited *Escherichia coli* and *Klebsiella*, two bacteria that produce β-lactamase [26], and extended-spectrum beta-lactamase, metallo-beta-lactamase, and AmpC beta-lactamase-producing gram-negative uropathogens [27]. Moreover, clove oil-based nanoemulsion exhibit antibacterial properties against *Staphylococcus aureus* [28]. The major active ingredient of clove essential oil is eugenol, a phenolic compound, which is responsible for its antimicrobial activity by acting on cell membrane phospholipids and denaturing proteins [29].

The purpose of this study was to evaluate the antibacterial effectiveness of clove extract against *P. aeruginosa* and to assess any potential synergistic effects of clove extract when used in combination with other antibiotics.

Methods and materials

Plant material

Clove (*Syzygium aromaticum* L.) was chosen based on ethnopharmacological studies and is known for its use as an antiseptic and antibacterial plant. Clove was purchased from the local market of Jubaiha–Amman in April 2022 and was determined by comparison to the verified sample at the herbarium of the Faculty of Science and Faculty of Agriculture at The University of Jordan. Clove pods were washed thoroughly with tap water followed by distilled water, air dried, and then further dried in an oven. A fine powder was created by grinding the dried plant material, which was subsequently stored in a dry place at room temperature for further use. A Soxhlet apparatus was used for the extraction procedure; the ground clove bud samples were weighed, and 12.5 g from each cycle was added to the main chamber, which was equipped with an extraction thimble. The extraction chamber was inserted into a 250 ml reflux flask with 100 ml of absolute ethanol for approximately 24 h. The temperature of the apparatus was set at 70–80 °C. After Soxhlet extraction, the extracts were concentrated using a rotary vacuum evaporator at 40 °C. The extracts yielded 42% (w/w) dark brown extract. The stock residues of clove extract were dissolved in 95% ethanol and diluted with the same solvent to a concentration range

of 4000 –7.8 μg/ml. The antibacterial properties of the extract against *Pseudomonas aeruginosa* (ATCC 9027) were subsequently examined.

Bacterial strain and growth conditions

The *Pseudomonas aeruginosa* (ATCC 9027) strain was used for this study. This strain was cultured on Mueller-Hinton agar (Biolab, Budapest, Hungary) and incubated at 37 °C for 24 h until the appearance of visible colonies. This strain was subsequently grown in Mueller-Hinton broth, and its growth was checked by observing changes in turbidity. Aliquots of the turbid medium were then mixed with 15% glycerol and stored at -70 °C until use [30].

Antimicrobial susceptibility testing

The disc agar diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [31] was performed, and the following antimicrobial discs (Oxoid Ltd., Basingstoke, England) were used for antimicrobial susceptibility testing: imipenem (10 μg), amoxicillin/clavulanic acid (30 μg), and blank discs impregnated with clove extract.

Primary screening of antimicrobial activity

Imipenem and amoxicillin/clavulanic acid in the form of a dry powder from the Hikma pharmaceutical industry were freshly dissolved in sterile distilled water. Imipenem was prepared ata final concentration of 5 mg/ml, and amoxicillin/clavulanic acid was used at a final concentration of 6 mg/ml. The stock solutions were diluted with distilled water until the desired concentrations were reached. The antimicrobial activity of clove extract and both antibiotics was tested using the disc diffusion method [32] and agar well diffusion method [33]. The disc diffusion method was performed as follows: A suspension of *P. aeruginosa* equivalent to 0.5 McFarland standard was prepared from an overnight culture and spread evenly on the surface of Mueller–Hinton agar plates. Sterile blank disks (6 mm) were impregnated with 30 μl of clove extract, imipenem, and amoxicillin/clavulanic acid, and the disks were then placed and pressed gently on plates and incubated for 24 h at 37 °C. The antibacterial activity of the clove extract and the antibiotics was assessed by measuring the diameter of the inhibition zone around the discs, and the results are reported in mm. The agar well diffusion method was performed as follows: Mueller–Hinton agar plates were steaked evenly with a *P. aeruginosa* suspension equivalent to the 0.5 McFarland standard. Wells of 8 mm were made in the agar, and the clove extract and antibiotic solutions were poured into the wells. The plates were kept upright in an incubator at 37 °C until absorption of the solutions, and the plates were inverted and kept in the same incubator for 24 h. The antibacterial activity of the clove extract and the antibiotics was evaluated by measuring the diameter of the inhibition zone around the wells, and the results are reported in mm.

Determination of minimum inhibitory concentrations (MICs)

The microdilution method was used to determine the MIC of each antibiotic and clove extract [34]. A 96-well microplate was used; the first well was filled with 180 μl of Muller-Hinton broth (MHB), and another 100 μl of MHB was added to wells 2 to 10. Then, 20 μl of prepared clove extract was added to the first well, and a serial dilution was performed to obtain a concentration range of 1000–7.81 μ/ml. Each well was then inoculated with 100 μl of a suspension of *P. aeruginosa* (ATCC 9027) that matched the turbidity of the 0.5 McFarland standard. The previous steps were also performed to dilute imipenem and amoxicillin/clavulanic acid to a concentration range of 1000–0.976 μg/ml. The MICs were determined visually and confirmed by measuring the optical density of the wells at a wavelength of 650 nm using a Gen5™ microplate readerThe MIC was recorded as the lowest concentration that can visibly inhibit the growth of *P. aeruginosa* (ATCC 9027). Imipenem served as the positive control. The MICs of clove extract and antibiotics were determined from independent triplicate assays.

Determination of the synergistic effect between clove extract and antibiotics

A checkerboard assay was carried out to determine the possible synergistic effect of clove extract combined with imipenem and amoxicillin/clavulanic acid [35]. After incubation for 24 h at 37 °C, the fractional inhibitory concentrations (FICs) were calculated as follows:

$$FIC = (\text{MIC of drug A in combination}/\text{MIC of drug A alone}) + (\text{MIC of drug B in combination}/\text{MIC of drug B alone})$$

The FICs were interpreted as ≤ 0.5 synergistic, 0.5–1 additive, 1–4 indifferent and ≥ 4 antagonistic [36, 37].

UHPLC/MS analysis

UHPLC/MS analysis of the methanol extract (clove extract was dissolved in methanol) was performed using an Elute UHPLC coupled to a Bruker impact II QTOFMS. Chromatographic separation was performed using a Bruker solo 2.0_C-18 UHPLC column (100 mm × 2.1 mm × 2.0 μm) at a flow rate of 0.51 mL/min and a column temperature of 40 °C.

Stock solutions were prepared by dissolving our extract sample in 2.0 ml of DMSO, and then the volume was adjusted to 50 ml with acetonitrile. The prepared sample

Fig. 1 Zone of inhibition results of imipenem, amoxicillin + clavulanic acid and clove extract

was then centrifuged at 4000 rpm for 2.0 min, and 1.0 ml was removed from the sample and transferred to an autosampler. An injection volume of 3.0 μl was used for the diluted clove extract. All reagents used (acetonitrile, methanol, water, and formic acid) were of LC/MS grade. Available chemical compounds were identified according to their retention times and mass fragmentation patterns using available standard data.

Results and discussion

Pseudomonas aeruginosa is a multidrug-resistant pathogen that has the potential to develop resistance to various antibiotics [38]. Different antibiotics are being used to treat infections caused by this pathogen. In this study, two classes of antibiotics, amoxicillin/clavulanic acid® and imipenem®, were chosen as reference standards to evaluate the potential antibacterial activity of clove extract. The sensitivity of *P. aeruginosa* to clove extract, imipenem, and amoxicillin/clavulanic acid was initially evaluated by the disk diffusion method and agar well diffusion method. As shown in Fig. 1, imipenem had the largest zone of inhibition (41 mm). The zone of inhibition of amoxicillin/clavulanic acid could not be measured, indicating complete resistance of *P. aeruginosa* (ATCC 9027) to this combination of antibiotics. The clove extract showed a 19 mm zone of inhibition. Our results are consistent with those of a previous study showing that clinically isolated *P. aeruginosa* strains were resistant to amoxicillin/clavulanic acid after at least two weeks of treatment [39]. Both *P. aeruginosa* and *Enterobacteriaceae* strains are known for their ability to deactivate

Table 1 Zone of inhibition, MIC, and FIC values of the tested products

Tested product	Zone of inhibition (mm)	MIC (μg/ml)	IC_{50} (mg/ml)	R^2
Imipenem	41	1.95	1.435383	0.9165
Amoxicillin + clavulanic acid	NA	1000	2.198891	0.9184
Clove extract	19	31.25	1.856382	0.8918

MIC = minimum inhibitory concentration, NA = not significant, FIC = fractional inhibitory concentration, (*) = synergistic effect. The R^2 and IC_{50} were calculated using an Excel spreadsheet

β-lactam antibiotics via efflux pumps and the production of metallo-β-lactamases [40]. The sensitivity of *P. aeruginosa* to imipenem in our study was expected and correlated with the results of a previous study [41]. The MIC of the clove extract was determined and compared with the MICs of amoxicillin/clavulanic acid and imipenem using the microdilution method. The MIC and FIC values of these products are presented in Table 1.

When comparing the percentage of inhibition for clove extract to amoxicillin/clavulanic acid as shown in Fig. 2, a remarkable antimicrobial effect of clove extract which is comparable to that of imipenem could be seen. This result is supported by Ahmad et al., study [42]. This high percentage of inhibition may be explained by the presence of various bioactive compounds that act synergistically to disrupt bacterial cell wall, thereby inhibiting biofilm formation [43].

According to the MIC results, imipenem showed greater potency against *P. aeruginosa* (MIC of 1.95 μg/ml) than the clove extract or amoxicillin/clavulanic acid. The MICs of both antibiotics decreased significantly when both antibiotics were combined with clove extract. The MIC of imipenem decreased from 1.95 μg/ml to 0.48 μg/ml, and the MIC of clove extract decreased from 31.29 μg/ml to 1.95 μg/ml; consequently, the calculated FIC for their combination was 0.31, indicating possible synergistic activity. Moreover, the MIC of amoxicillin/clavulanic acid was 1000 μg/ml and sharply decreased to 7.81 μg/ml when combined with clove extract. This decrease indicates an improvement in the inhibitory effect of amoxicillin/clavulanic acid when combined with clove extract, as reflected in the calculated FIC value of 0.26, which also indicates a synergistic effect. This valuable finding may explain the possible antibacterial activity that could be achieved by clove extract, which inhibits microbial growth and disturbs microbial defense mechanisms, thus enhancing the effects of both antibiotics (Table 2).

A previous study showed that clove extract can intersperse the virulence factors expressed by *Pseudomonas aeruginosa* without affecting the growth of the pathogen [44], which may explain the mechanism by which the

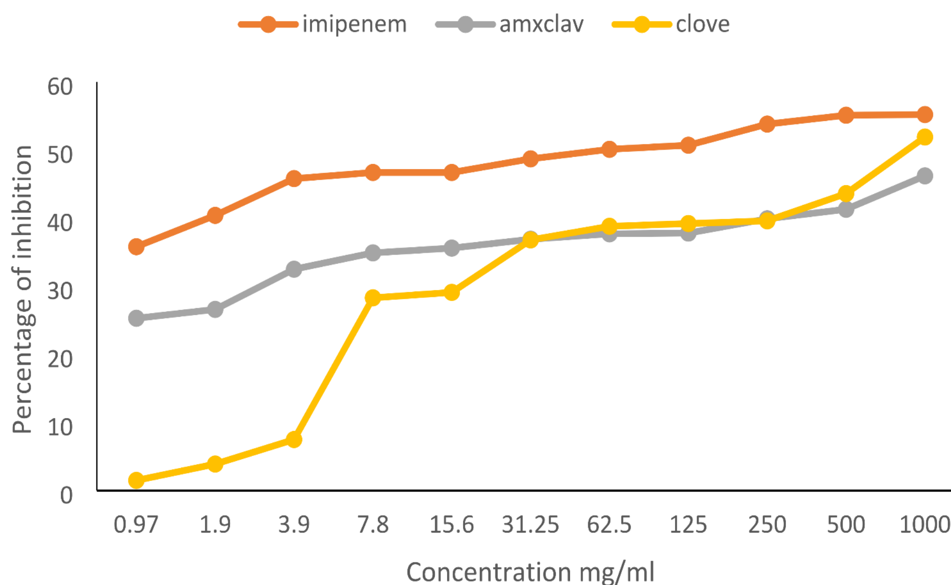

Fig. 2 Percentage of inhibition for imipenem, amoxicillin/clavulanic acid and clove extract

Table 2 Antimicrobial activity of clove extract in combination with Imipenem and Amoxicillin + clavulanic acid against *Pseudomonas aeruginosa* (ATCC 9027)

Tested products	MIC in combination (μg/ml)	FIC_C	FIC_I	FIC_A	FIC	Interpretation
Imipenem	0.48	---------	0.25	------	0.31	Synergistic
Amoxicillin + clavulanic acid	7.81	---------	------	0.008	0.26	Synergistic
Clove extract[a]	1.95	0.062	------	------	------	--------------
Clove extract[b]	7.81	0.25	------	------	------	--------------

FIC_C = fractional inhibitory concentration of clove extract, FIC_I = fractional inhibitory concentration of imipenem, FIC_A = fractional inhibitory concentration of amoxicillin + clavulanic acid, clove extract[a] = the extract combined with imipenem, clove extract[b] = the extract combined with amoxicillin + clavulanic acid

extract enhanced the antimicrobial activity of the antibiotics after combination. On the other hand, another study showed that clove extract may inhibit the growth of *Pseudomonas aeruginosa* because eugenol is the major bioactive compound [45], although it cannot inhibit swarming motility, which is a quorum sensing-controlled factor [46].

The examination of clove extract revealed 17 components, mostly flavonoids and phenolic compounds (Table 3). In plants, flavonoids play a significant role as antimicrobial agents. Plants have inherent immunity that consists of several layers of defense responses to halt the spread of disease. One of these defenses is the production of flavonoids [47]. Numerous plant-derived flavonoids have antibacterial properties that differ from those of prescription medications, making them potentially significant for improving antimicrobial treatment [48].

The greatest peak of robinetin was observed in our extract, indicating that, in accordance with the findings of a previous study, we may trust the antibacterial activity of clove extract for this composition. It was also suggested that the mechanism underlying the antibacterial activities of flavonoids involves flavonoid DNA intercalation, which prevents bacterial nucleic acid production. This assumption is supported by the results, which showed that DNA synthesis was predominantly inhibited by robinetin in *Proteus vulgaris*, whereas RNA synthesis was inhibited in *S. aureus* [49].

The antibacterial activity of quercetin (18.59%) and kaempferol (16.15%) has been well investigated, and their mechanisms of action have been described in various ways. These compounds possess antioxidant activity, and it was reported that the way they kill *E. coli* and *B. subtilis* is through an oxidative burst caused by the production of reactive oxygen species (ROS), which alters the permeability of the membrane and causes damage [50]. Additionally, they act as potent cell−cell signaling antagonists [51] and prevent FAS-I [52]. Quercetin has also been shown to inhibit the 3-hydroxyacyl-ACP dehydrase [53] of *Helicobacter pylori*, and another in silico study revealed that quercetin may target subunit B of DNA gyrases from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* [54].

Morin (9.99%), another flavonoid present in our clove extract, may have contributed to the antibacterial activity and the synergistic effect when combined with the antibiotics used in this study. This assumption is supported by findings from a study indicating that morin inhibits *E.*

Table 3 Organic compounds identified in clove methanolic extract samples by UHPLC/MS analysis

Compound name	Classification	Chemical formula	RT (min)	AUC	Peak %	MW (Da)
Gentisic acid	Phenolic compound	$C_7H_6O_4$	2.02	15,974	2.95	153
Cinnamic Acid	Phenolic compound	$C_9H_8O_2$	8.03	408	0.08	147
Syringic acid	Phenolic compound	$C_9H_{10}O_5$	3.32	322	0.06	197
Scopoletin	Coumarin	$C_{10}H_8O_4$	4.29	248	0.05	191
7-Hydroxy-2'-methoxyflavone	Flavonoid	$C_{15}H_{10}O_6$	12.22	598	0.11	285
Kaempferol	Flavonoid	$C_{15}H_{10}O_6$	10.47	87,504	16.15	287
Morin	Flavonoid	$C_{15}H_{10}O_7$	7.49	79,148	9.99	303
Robinetin	Flavonoid	$C_{15}H_{10}O_7$	6	338,522	62.50	303
Quercetin	Flavonoid	$C_{15}H_{10}O_7$	8.87	100,682	18.59	303
Chlorogenic acid	Phenolic compound	$C_{16}H_{18}O_9$	3.87	92,254	17.04	353
Sakuranetin	Flavonoid	$C_{16}H_{14}O_5$	13.18	2444	0.5	285
Eupatilin	Flavonoid	$C_{18}H_{16}O_7$	12.66	726	0.13	343
Salvianolic acid A	Phenolic compound	$C_{26}H_{22}O_{10}$	9.33	606	0.11	493
Genistein	Flavonoid	$C_{27}H_{28}O_{16}$	6.27	926	0.17	607
Neohesperidin	Flavonoid	$C_{28}H_{34}O_{15}$	8.59	512	0.09	609
Acteoside	Phenolic compound	$C_{29}H_{36}O_{15}$	5.59	1480	0.27	623
Hederagenin	Triterpene	$C_{30}H_{48}O_4$	20.9	312,910	57.72	471

RT = retention time, AUC = area under the curve and MW (Da) = molecular weight in daltons

coli replicative helicases such as RecBCD helicase/nuclease and DnaB [55].

The chemical properties of phenolic compounds, notably their aromatic structure and numerous hydroxyl groups capable of donating electrons or hydrogen atoms to neutralize free radicals and other reactive oxygen species (ROS), are linked to their beneficial bioactivities [56]. Additionally, phenolic compounds exert antibacterial effects by altering virulence factors, genetic regulatory elements, the ability to disrupt biofilms, and the structure and function of membranes [57].

Interestingly, our findings against *P. aeruginosa*, an organism notorious for its multidrug resistance, suggests the potential antibacterial action of clove extract, either by itself or in combination with antibiotics. These findings might pave the way for additional research and use of clove extract or its components alongside lower dosages of antibiotics, hence reducing the adverse effects of antibiotic administration. Moreover, this combination might enhance the effectiveness of antimicrobial agents and retard the microorganism's adaptation to the antibiotics, especially when using a mixture of bioactive compounds not a single phytochemical. Further research is necessary to explore the antibacterial effect of clove extract and its combinations on other organisms, which could provide valuable insights for combating multidrug-resistant pathogens. It is worth mentioning that more studies are needed to investigate the antimicrobial effect of clove extract's components individually, and the feasibility of combining the extract with existing antibiotics in clinical trials to validate such combinations.

Conclusion

Clove extract is a promising antibacterial that can potentiate and enhance the effectiveness of other available antibiotics at lower doses and for a shorter duration of time, thereby decreasing their side effects. However, such combinations might also result in the continued use of antibiotics known to be ineffective against certain pathogens, potentially broadening the range of treatment regimens available. Further in vivo work is needed to investigate the effect of combining clove extract with antibiotics on biological system, beside targeted studies to understand the bioavailability of clove extract, as well as the pharmacokinetics and pharmacodynamics of such combination to predict the clinical outcomes and enhance the effectiveness.

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Author contributions
D. A principal investigator, making the experimental work and writing manuscript and J. A co-principal investigator, writing manuscript, proof reading manuscript and analyzing the data and W. M proof read the data and A. M extracting the plant.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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