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BACTERICIDAL VERSUS BACTERIOSTATIC ACTIVITY OF ORIGANUM VULGARE AND ANDROGRAPHIS PANICULATA EXTRACTS AGAINST STREPTOCOCCUS SPP.: AN IN VITRO MBC/MIC RATIO-BASED ASSESSMENT

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ABSTRACT

Background and Objective: There is a growing emergence of antibiotic resistance bacteria and there is a need to search for new antimicrobial agents from natural sources. The present study was designed to evaluate and compare the antibacterial activity of ethanolic leaf extracts of Origanum vulgare and Andrographis paniculata against clinical isolates of Streptococcus spp. at concentrations of 50, 100, 200 and 400 mg/mL and compare their efficacy with standard reference antibiotics (Cloxacillin 30 µg, Penicillin 6 µg, Amoxicillin 25 µg and Ciprofloxacin 5 µg).

Methods: Antibacterial activity was evaluated by agar well diffusion method and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated by broth microdilution method following NCCLS standards. To determine the nature of the antimicrobial effect as bactericidal or bacteriostatic, the MBC/MIC ratio was determined.

Results: Both plant extracts showed concentration dependent antibacterial activity against Streptococcus spp. The inhibition zone of O. vulgare ethanolic extract (13.73–44.89 mm) was substantially higher than that of A. paniculata (10.18–18.72 mm) at all tested concentrations ($p < 0.05$). At the highest concentration (400 mg/mL) O. vulgare extract was more effective than that of Cloxacillin and Ciprofloxacin i.e. 44.89 ± 0.92 mm vs 38.12 ± 0.65 mm and 38.47 ± 0.17 mm. For O. vulgare, the MIC and MBC values were 0.156 and 0.624 mg/mL (MBC/MIC ratio = 4.0, bactericidal), respectively, while for A. paniculata, the MIC and MBC values were 0.624 and 5.0 mg/mL (MBC/MIC ratio = 8.0, bacteriostatic), respectively.

Conclusion: *The ethanolic extract of O. vulgare displayed a higher bactericidal effect on Streptococcus spp. compared to A. paniculata and several conventional antibiotics, showing its potential as a natural antibacterial agent. Further phytochemical characterisation and in vivo research are justified*

Keywords: *Origanum vulgare, Andrographis paniculata, Streptococcus, antibacterial activity, MIC, MBC, agar well diffusion, plant extract, antimicrobial resistance.*

1. INTRODUCTION

Pathogenic bacteria can quickly become resistant to many classes of antibiotics, posing a significant risk to global public health due to bacterial illnesses [1-3]. The indiscriminate and excessive use of antibiotics has favoured the emergence of natural and acquired resistance mechanisms, resulting to treatment failures globally [4]. Antimicrobial resistance (AMR) is a major global public health problem. The World Health Organization has designated AMR as one of the top 10 dangers to global health. Traditional medicinal herbs have been utilised for ages in many cultures to cure different diseases including microbial infections [5,6]. Bioactive chemicals of plants are a possible source of new antimicrobial drugs that may work through processes different from traditional antibiotics, and hence bypass known resistance mechanisms. Recent studies have been dedicated to the evaluation of plant extracts regarding their direct antibacterial properties and their capacity to influence drug resistance in bacteria [7,8]. Many plants have been evaluated in vitro against pathogenic bacteria. Several of them have shown strong action against both Gram positive and Gram negative pathogens at low MIC values [9]. Origanum vulgare L. (Lamiaceae) known as oregano, Spanish marjoram or wild marjoram, is one of the most widely distributed species of the genus Origanum and is extensively used as culinary spice and in traditional medicine [10]. O. vulgare is a perennial herbaceous plant up to 100 cm tall with erect often scarlet stems with branched upper parts. Its essential oil is rich in monoterpenic phenols, mainly thymol and carvacrol, and other terpenoids, such as myrcene, γ-terpinene, and p-cymene [11,12]. The plant is usually collected during early flowering stage in order to maximise the essential oil content [13,14]. Oregano essential oil (OEO) and oregano extracts (OEX) have well established antimicrobial, expectorant and cholagogue characteristics and are traditionally used for oral hygiene, digestive support and the treatment of inflammation of upper respiratory tract [15,16]. Their positive effects on the function of the digestive system, especially in the case of pathological inflammation conditions, have been proven, which justifies their use as natural preservatives for food [17]. OEO and its main phenolic components, thymol and carvacrol, have been found to inhibit the formation of Streptococcus mutans biofilm and induce bacterial autolysis [18]. Among the most investigated antibacterial phenolics are thymol and carvacrol, which show significant activity both individually and synergistically [19]. These chemicals have been proved to be effective against a variety of pathogenic organisms such as Escherichia coli, Listeria monocytogenes and Salmonella enterica serovar Typhimurium [20]. Synergistic interactions of carvacrol-thymol combinations with conventional antibiotics (azithromycin, minocycline, tigecycline, clarithromycin) and antifungal drugs against Pythium insidiosum have also been reported [21]. Andrographis paniculata (Burm. f.) Wall. ex Nees (Acanthaceae) is a well known medicinal plant in traditional Asian medicine especially in India, Malaysia and Thailand popularly called the "King of Bitters". Its major bioactive constituents include diterpenes (e.g. andrographolide and neoandrographolide), diterpenoids, glycosides and flavonoids with various biological activities including antioxidant and antibacterial properties [22]. Ethanolic crude extracts of A. paniculata have been shown to suppress the development of Staphylococcus aureus [23] and disrupt biofilm formation of Pseudomonas aeruginosa [24]. Both plants have been reported to have antibacterial characteristics, however comparative research on their relative efficiency against Streptococcus spp. are scarce. Considering the clinical importance of streptococcal infections and the developing resistance to antibiotics, a good reason exists for the search for natural alternatives. The present study aimed to: (1) evaluate and compare the antibacterial activity of ethanolic leaf extracts of O. vulgare and A. paniculata against clinical isolates of Streptococcus spp.; (2) determine MIC and MBC of each extract; (3) classify the nature of their antimicrobial action (bactericidal vs. bacteriostatic) through MBC/MIC ratio analysis; and (4) compare the efficacy of both extracts with standard reference antibiotics.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Authentication

Leaves of Origanum vulgare and Andrographis paniculata were gathered from local herbal markets in Al-Diwaniyah, Iraq. The plant materials were authenticated at the Department of Clinical Laboratory Science, College of Pharmacy, University of Al-Qadisiyah by means of morphological and organoleptic features. Voucher specimens were placed in the College of Pharmacy herbarium [voucher numbers: OV-QU-2024-01 and AP-QU-2024-02]. Leaves were inspected for damage and contamination, air-dried at room temperature in the shade for 7 d, then processed.

2.2 Preparation of Plant Extracts

The dried leaves were ground to a coarse powder by a mechanical blender. The extraction was carried out by putting 100 g of the corresponding powdered material in a Soxhlet apparatus with 500 mL of 70% (v/v) ethanol and extraction at 50 °C for 48 h. The temperature of 50 °C was chosen to maximise the extraction efficiency and minimise the heat destruction of thermolabile bioactive chemicals [25]. The resultant extracts were filtered using Whatman No. 1 filter paper and concentrated using rotary vacuum evaporator at 40 °C under reduced pressure. The % yield and weights of the dried crude extracts were obtained. The extracts were stored in amber glass vials at 4 °C until use. The dried extracts were reconstituted with 20% (v/v) ethanol to make the working solutions at 50, 100, 200 and 400 mg/mL.

2.3 Bacterial Strains and Culture Conditions

Clinical isolates of Streptococcus spp. were collected from the Microbiology Laboratory of Al-Diwaniyah Teaching Hospital, Al-Diwaniyah, Iraq. The isolates were collected from clinical specimens and identified by standard microbiological procedures including Gram staining, catalase test (negative), colony morphology on blood agar (beta-hemolytic) and biochemical testing. Isolates were subcultured on blood agar and incubated at 37 °C for 24 h to obtain fresh colonies Stock cultures were grown on nutrient agar slants at 4 °C. Bacterial suspensions were produced in sterile normal saline (0.85% NaCl) for experimental use and adjusted to a turbidity comparable to 0.5 McFarland standard (~1.5 × 10^8 CFU/mL) [21].

2.4 Antibacterial Activity Assay (Agar Well Diffusion)

The antibacterial activity was determined by the agar well diffusion method as described before [26]. Standardised bacterial suspensions were inoculated on Mueller–Hinton agar (MHA) plates using sterile cotton swabs to uniformly distribute the germs on the surface to generate a lawn. After 5 min at room temperature to allow the plates to dry, wells of 6 mm diameter were aseptically punched in the agar using sterile glass pipettes. Each well was loaded with 100 µL of the corresponding concentration of plant extract (50, 100, 200 or 400 mg/mL). The plates were held at room temperature for 30 min to enable pre-diffusion of the extracts in the agar and incubated at 37 °C for 24 h. Zones of growth inhibition were measured in millimetres with a digital calliper. The solvent control, 100 µL of 20% ethanol (v/v) was added to negative control wells. The positive controls were standard antibiotic discs, i.e. Cloxacillin (30 µg), Penicillin (6 µg), Amoxicillin (25 µg) and Ciprofloxacin (5 µg). All experiments were performed in duplicate.

2.5 Determination of MIC and MBC

The minimum inhibitory concentration (MIC) was established by the broth microdilution method according to the standards of the National Committee for Clinical Laboratory Standards (NCCLS). Serial two fold dilutions of each plant extract in Mueller-Hinton broth (MHB) were made to the final concentrations of 0.039 to 40 mg/mL. Bacterial inocula were produced from fresh overnight cultures diluted 1:10 in MHB and adjusted to ~105 CFU/mL. In each well of a sterile 96-well microtiter plate, 100 µL of diluted extract and 100 µL of bacterial suspension were added. Growth control wells (broth + bacterium, no extract) and sterility control wells (broth + extract, no bacteria) were included. Plates were incubated for 20 h at 37 °C. The lowest concentration of extract at which no observable growth of bacteria was seen was considered as the minimum inhibitory concentration (MIC).

The minimum bactericidal concentration (MBC) was determined by subculturing 10 µL aliquots from wells at and above the MIC concentration onto fresh nutrient agar plates. The plates were incubated at 37 °C for 24 hours. The MBC was defined as the lowest concentration that killed ≥99.9% of the original inoculum, evidenced by no colony growth on the subculture plates [26].

2.6 Determination of Bactericidal or Bacteriostatic Nature

The nature of antimicrobial activity (bactericidal vs. bacteriostatic) was assessed by calculating the MBC/MIC ratio. An MBC/MIC ratio of ≤4 was classified as bactericidal activity, while a ratio of >4 was classified as bacteriostatic activity, in accordance with established criteria [26].

2.7 Statistical Analysis

All the tests were carried out in triplicates and data were expressed as mean ± standard error (SE). SPSS software (version 10) was used for statistical analysis. Differences between groups were assessed using a one-way analysis of variance (ANOVA) with a least significant difference (LSD) post hoc test. Differences were judged statistically significant when p ≤ 0.05.

3. RESULTS

3.1 Antibacterial Activity by Agar Well Diffusion

Both the ethanolic extracts showed concentration dependent antibacterial activity against Streptococcus spp (Table 01, Figure 01). The inhibitory zone diameters considerably increased with the increase in the concentration of the extract (p < 0.05). For both plants, indicating a dose–response relationship. *O. vulgare* ethanolic extract demonstrated markedly superior activity compared to *A. paniculata* at all tested concentrations. At 50, 100, 200, and 400 mg/mL, *O. vulgare* produced inhibition zones of 13.73 ± 0.25, 19.82 ± 0.47, 26.75 ± 0.71, and 44.89 ± 0.92 mm, respectively. In contrast, *A. paniculata* yielded inhibition zones of 10.18 ± 0.52, 14.22 ± 0.24, 15.24 ± 0.65, and 18.72 ± 1.25 mm at corresponding concentrations. The negative control (20% ethanol) showed no zone of inhibition (0 ± 0 mm), demonstrating that the antibacterial effects reported were due to the bioactive ingredients of the plant and not the solvent carrier.

Table 01: Zone of inhibition caused by *O. vulgare* and *A. paniculata* ethanolic extracts against *Streptococcus* spp.

Drug Substance	Concentration (mg/mL)	Inhibition Zone (mm)
O. vulgare extract	50	13.73 ± 0.25 ^{A}
	100	19.82 ± 0.47 ^{B}
	200	26.75 ± 0.71 ^{C}
	400	44.89 ± 0.92 ^{D}
A. paniculata extract	50	10.18 ± 0.52 ^{E}
	100	14.22 ± 0.24 ^{A}
	200	15.24 ± 0.65 ^{A}
	400	18.72 ± 1.25 ^{B}
Diluted ethanol	50	0 ± 0 ^{F}
Standard antibiotics	Cloxacillin (30 µg)	38.12 ± 0.65 ^{G}
	Penicillin (6 µg)	15.84 ± 0.29 ^{A}
	Amoxicillin (25 µg)	18.78 ± 0.48 ^{B}
	Ciprofloxacin (5 µg)	38.47 ± 0.17 ^{G}

Values expressed as mean ± SE (n = 3). Sample volume: 100 µL. Inhibition zone diameter in mm (well diameter 6 mm) after overnight incubation at 37 °C. Similar superscript letters indicate non-significant differences;

different letters indicate significant differences (LSD, p ≤ 0.05).

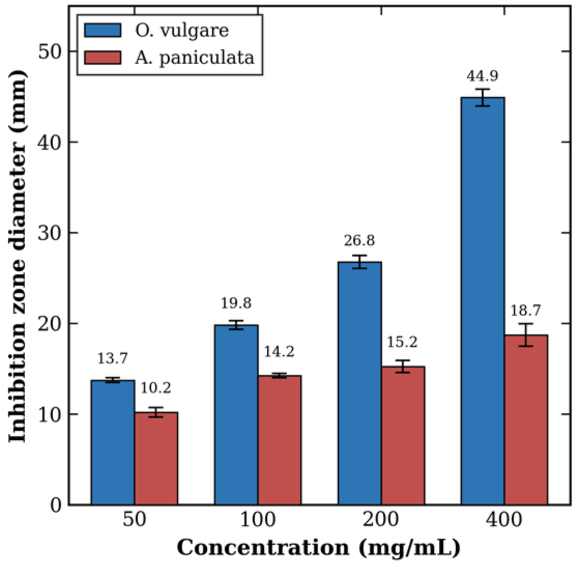

Figure 01: Comparative antibacterial activity of O. vulgare and A. paniculata ethanolic extracts at different concentrations against Streptococcus spp. Error bars represent standard error (n = 3).

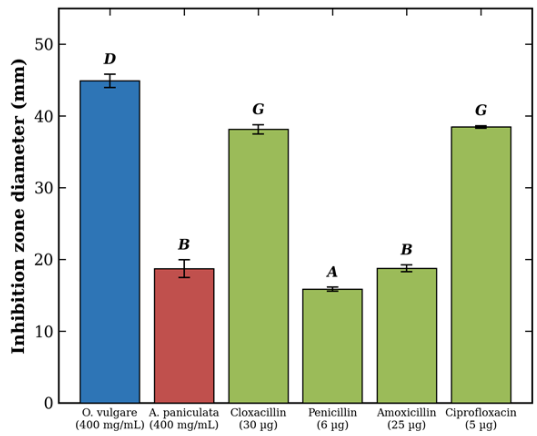

Figure 02: Comparison of antibacterial activity at the highest extract concentration (400 mg/mL) with standard reference antibiotics against Streptococcus spp. Letters denote statistical groups (LSD, p ≤ 0.05).

3.2 Minimum Inhibitory and Bactericidal oncentrations

Tables 02 and 3 show the MIC and MBC values of each extracts. MIC of O. vulgare ethanolic extract was 0.156 mg/mL and MBC was 0.624 mg/mL against Streptococcus spp. (Table 02). On the other hand, ethanolic extract of A. paniculata exhibited much greater MIC (0.624 mg/mL) and MBC (5.0 mg/mL) values (Table 03), reflecting reduced efficacy as compared to O. vulgare.

Table 02: MIC and MBC of *O. vulgare* ethanolic extract against *Streptococcus* spp.

0.039	0.078	0.156	0.312	0.624	1.248	2.5	5	10	20	40	MIC	MBC
–	–	+	+	++	++	++	++	++	++	++	0.156	0.624

– no inhibition; + MIC (lowest concentration inhibiting visible growth); ++ MBC (complete killing). Concentrations in mg/mL.

Table 03: MIC and MBC of *A. paniculata* ethanolic extract against *Streptococcus* spp.

0.039	0.078	0.156	0.312	0.624	1.248	2.5	5	10	20	40	MIC	MBC
–	–	–	–	+	+	+	++	++	++	++	0.624	5

– no inhibition; + MIC (lowest concentration inhibiting visible growth); ++ MBC (complete killing). Concentrations in mg/mL.

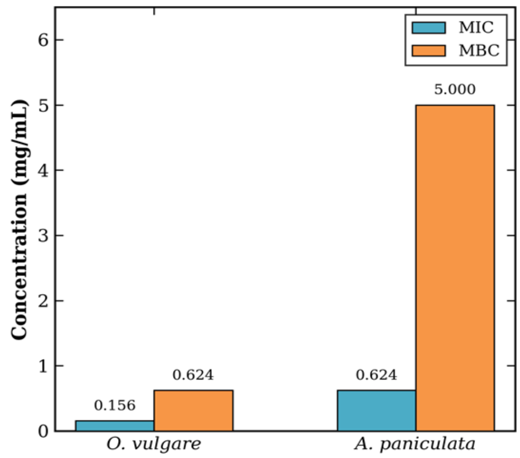

Figure 03: Comparison of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of O. vulgare and A. paniculata ethanolic extracts against Streptococcus spp.

3.3 MBC/MIC Ratio and Nature of Antimicrobial Action

Table 04: MBC/MIC ratio and classification of antimicrobial activity of plant extracts against *Streptococcus* spp.

Plant Extract	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC Ratio	Classification
O. vulgare	0.156	0.624	4.0	Bactericidal

A. paniculata	0.624	5.0	8.0	Bacteriostatic

MBC/MIC ratio ≤4 indicates bactericidal activity; MBC/MIC ratio >4 indicates bacteriostatic activity [26]. The MBC/MIC ratio study (Table 04) showed different mechanisms of antibacterial activity between the two extracts. The ethanolic extract of O. vulgare showed an MBC/MIC ratio of 4.0 (bactericidal activity against Streptococcus spp.). In contrast, the ethanolic extract of A. paniculata showed MBC/MIC ratio of 8.0 showing bacteriostatic action.

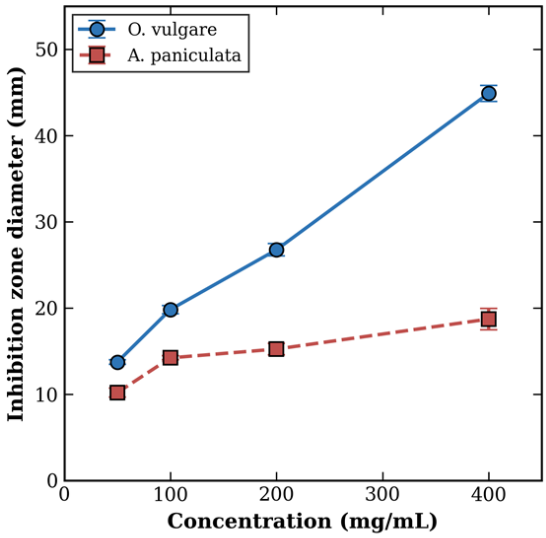

Figure04: Dose–response curves showing the concentration-dependent increase in inhibition zone diameter for O. vulgare (●) and A. paniculata (■) ethanolic extracts against Streptococcus spp.

4. DISCUSSION

The present work is aimed at comparative evaluation on antibacterial properties of O. vulgare and A. paniculata ethanolic leaf extracts against clinical isolates of Streptococcus spp. The results indicate that both extracts showed antibacterial activity, although O. vulgare was significantly more effective at all concentrations tested, with inhibition zones roughly 2.4 times greater than those of A. paniculata at the highest concentration (400 mg/mL).

The observed concentration dependent increase in antibacterial activity for both extracts (Figure04) is consistent with the idea that higher concentrations would offer larger quantities of bioactive phytochemicals at the site of bacterial interaction. The dose-response relationship has been widely reported for plant-derived antimicrobials and confirms the presence of concentration-sensitive active ingredients in both extracts [7,29].

Interestingly, the extract of O. vulgare (400 mg/mL) (44.89 ± 0.92 mm) was shown to be more effective than the four common antibiotics tested (Cloxacillin (38.12 ± 0.65 mm), and Ciprofloxacin (38.47 ± 0.17 mm)) (Figure 02). The significance of this result is that it reveals that extracts from plant sources can reach an antibacterial activity equal to or superior than conventional antibiotics under in vitro settings. Our results accord with the findings of Tao et al. [29] who showed the essential oil of O. vulgare has a strong antibacterial action against S. aureus and E. coli and Radu et al.

[30], who demonstrated its efficacy against oral pathogens including *Enterococcus faecalis* and *Candida albicans*. Similarly to Gwiazdowska et al. [32], the antibacterial and antibiofilm potential of supercritical extracts of O. vulgare was confirmed.

The better antibacterial activity of O. vulgare than A. paniculata may be due to the difference in phytochemical profiles. Thymol and carvacrol are monoterpenic phenols and the main antimicrobial compounds in O. vulgare [38]. They exhibit their antibacterial activity through several ways. These are breakdown of integrity of bacterial cell membrane by interaction with phospholipid bilayers, increase in membrane permeability and leaking of intracellular components such as ions, ATP and nucleic acids [41]. Additionally, thymol and carvacrol block bacterial enzymes that are necessary for energy metabolism and biosynthesis pathways [42]. The presence of the hydroxyl group on the phenolic ring is necessary for their antibacterial activity, because it allows the interaction with the hydrophilic head groups of membrane phospholipids [42]. Conversely, the bioactive compounds of A. paniculata, mainly andrographolide and its derivatives, display antibacterial activity via pathways that are potentially less membrane disrupting than those of the other substances. Andrographolide has been found to limit bacterial biofilm formation, interfere with bacterial quorum sensing and modify host immunological responses, rather than directly lyse bacterial cells [22, 24, 34]. This difference in mechanism could explain the bacteriostatic rather than bactericidal categorisation seen for A. paniculata in the present study (MBC/MIC ratio = 8.0).

Analysis of the MBC/MIC ratio gave further information into the nature of the antibacterial effect. The extract of O. vulgare showed a bactericidal mode of action (MBC/MIC = 4.0) which indicates that it can kill bacteria in concentrations near to the inhibitory level. This is clinically important since bactericidal drugs are usually chosen over static treatments in serious infections because they reduce the probability of the germs persisting and becoming resistant. On the other hand, A. paniculata showed bacteriostatic activity (MBC/MIC = 8.0), demonstrating that it can suppress bacterial growth, but a much greater dosage was necessary to produce a bactericidal effect. These observations are in agreement with the observations of

Hossain et al. [34], who found that A. paniculata extracts exhibited mostly growth-inhibitory rather than lethal action.

The results of A. paniculata extract in the present investigation are in agreement with the findings of Tejada-Muñoz et al. [33], and Sah et al. [36], who showed that A. paniculata extracts have antibacterial properties mainly against Gram-positive bacteria. Gram-positive bacteria were differentially susceptible to A. paniculata than Gram-negative bacteria. This may be attributed to changes in cell wall architecture, whereby the lack of an outer membrane in Gram-positive bacteria allows more penetration of hydrophilic diterpenoid chemicals [35].

5. STUDY LIMITATIONS

The present study has certain limitations that should be considered. Bacterial isolates were first identified to the genus level (Streptococcus spp.) by standard biochemical methods and molecular identification techniques (e.g., 16S rRNA gene sequencing) were not used for conclusive species-level identification. Second, no phytochemical screening or instrumental analysis (GC-MS, HPLC) was performed on the crude extracts, so the attribution of the antibacterial activity to specific compounds (thymol, carvacrol and andrographolide) is based on published literature, not on direct chemical characterisation of the extracts used in this study. Third, the investigation was confined to a single bacterial genus; the inclusion of other Gram-positive and Gram-negative species would have offered a more complete antimicrobial profile. Fourth, the two plant extracts were not investigated for synergistic interactions. Finally, the present investigation was solely in vitro; in vivo effectiveness, pharmacokinetics and toxicity studies are necessary before any therapeutic applications can be addressed. These limitations should be addressed by future studies including phytochemical characterisation, molecular bacterial identification, larger pathogen panels, time-kill kinetics and in vivo models.

6. CONCLUSION

The present investigation indicated that ethanolic leaf extracts of both O. vulgare and A. paniculata showed antibacterial activity against Streptococcus spp. in a concentration dependent manner. The extract of O. vulgare showed a considerably better effect than A. paniculata at all the concentrations tested and was better than the standard reference antibiotics (Cloxacillin and Ciprofloxacin) at the highest concentration (400 mg/mL). The MBC/MIC ratio analysis showed that the O. vulgare extract was bactericidal (ratio = 4.0) and the A. paniculata extract was bacteriostatic (ratio = 8.0). These results suggest the possibility of O. vulgare as a candidate for the development of natural antibacterial drugs. Further studies: Phytochemical profiling, bacterial identification at species level, time-kill tests and in vivo studies are suggested to pave the way for practical applications.

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8. AUTHOR CONTRIBUTIONS

Conceptualisation: Farah Razzaq Kbyeh Methodology: Farah Razzaq Kbyeh Investigation: Farah Razzaq Kbyeh Data curation: Farah Razzaq Kbyeh Formal analysis: Farah Razzaq Kbyeh Writing – first draft: Farah Razzaq Kbyeh Mohamed Abdul Rida Yaseen: Supervision, Resources, Validation, Writing - review & editing.

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10. CONFLICT OF INTEREST

The authors disclose no conflict of interest. The authors declare no competing financial or personal interests that may have seemed to affect the work presented in this study.

11. DATA AVAILABILITY

The data supporting the conclusions of this analysis are provided in the publication. Additional raw data are accessible from the relevant author on reasonable request.

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