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Antibacterial activity of Libyan *Juniperus phoenicea* L. leaves extracts against common nosocomial pathogens

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Plant extract

ABSTRACT

In ancient times, botanical extracts were essential complementary method for microbial control. This study has been carried out to assess the antibacterial activities of methanol, acetone, and aqueous leaf extracts of Libyan *Juniperus phoenicea* L. against multidrug-resistant (MDR) clinical isolates (*Staphylococcus aureus*, *S. haemolyticus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*) using the agar well diffusion method. Based on the inhibition zone's diameter or appearance, the tested MDR bacteria were identified as susceptible, intermediate, or resistant using the standard criteria. The current study's findings showed that the concentration, type of solvent and bacterial species had a significant impact on the effectiveness of the plant extracts. Results of the study revealed that the methanol and acetone extracts demonstrated moderate to excellent antibacterial properties against all tested bacteria at all predefined concentrations (25, 50, 75, and 100%), with the zone of inhibition ranging from 15.66 to 27.66 mm. Among the tested solvents, the aqueous extract of *J. phoenicea was* the least effective against the clinical bacterial isolates. Further, the plant's leaf extracts were more effective against Gram-positive bacteria than Gram-negative bacterial pathogens. Most importantly, neither the aqueous extract nor the standard antibiotics inhibited *P. aeruginosa*, while the methanol and acetone extracts displayed remarkable inhibition zones against all tested bacteria. Consequently, the plant extracts (acetone and methanol) in this study may provide insightful information about the potential use of *J. phoenicea* leaves as a natural antibacterial agent, which could be used to combat antibiotic-resistant bacteria.

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1 Introduction

In recent decades, the prevalence of infections caused by multidrug-resistant (MDR) microbes has become a problem for global health. Among the most common MDR bacteria, *Staphylococcus aureus*, *S. haemolyticus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* are some fundamentally pathogenic bacteria that cause many nosocomial infectious diseases (Mehta and Kumari 1997; De Champs et al. 2000; EL-Mahmood 2009; Hoseini Alfatemi et al. 2014; Amenu 2014; Czekaj et al. 2015). Plant products exhibit strong antimicrobial properties, making them a compelling alternative route for treating and preventing infections caused by bacteria, especially in cases where traditional chemical antibiotics are ineffective or unavailable. Additionally, plant-based therapies may have fewer side effects and lower toxicity, making them much more reliable and promising avenues for future research and development than synthetic drugs (Akinduti et al. 2022; Akinyemi et al. 2005). In addition, plant products are widely available and affordable, making them accessible to a large population in developed and developing countries. Thereby, the use of plant products as therapeutic agents has the potential to provide a cost-effective and sustainable solutions to combat infectious diseases.

Juniperus phoenicea L., also known as Phoenician juniper, belongs to the family Cupressaceae and is an evergreen plant usually grown as a shrub or a tree in the Mediterranean basin (Fouad et al. 2011; Abu-Darwish et al. 2014). This plant species abundantly grows in the eastern part of Libya and is widely used in traditional medicine by Libyan people to treat various ailments (Aljaiyash et al. 2014; Al Groshi et al. 2018). Aqueous extracts of *J. phoenicea* leaves are used to treat diarrhea, gout, and anorexia in Libya (Qnais et al. 2005), nephrotoxicity and hepatotoxicity in Egypt (Ali et al. 2010). The aqueous extract also improves liver and kidney functions. The traditional Moroccan healers used *J. phoenicea* leaves powder to treat diuretics, diabetes, diarrhea, rheumatism, and hypoglycemia (Abu-Darwish et al. 2014). These researchers have also reported that the leaves of this plant are the fundamental source of various active ingredients like phenolic, lipid and mineral compounds. Radical scavenging activity of ethyl acetate and methanolic extracts of *J. phoenicea* leaves has been reported by Medini et al. (2013) in Tunisia. Although many previous studies have established the various medicinal and antimicrobial properties of J. phoenicea, the antibacterial activity of plant leaves collected from the Sidi Emhamad forest (Alabyar, Libya) against MDR bacteria has not yet been studied. Therefore, the current study has been conducted to assess the antibacterial efficacy of the *J. phoenicea* leaf extract against four multidrug-resistant bacteria (*Staphylococcus aureus*, *S. haemolyticus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*), which pose a high threat to human health. The findings of this investigation could potentially lead to the development of new and effective antibacterial agents which could be used to combat antibiotic-resistant bacteria.

2 Materials and Methods

2.1 Plant Material

The leaves of *J. phoenicea* have been collected from one individual tree located in SidiEmhamad forest (Al-abyar city), which is roughly 60 km from the city of Benghazi (Libya) with geographical coordinates of 32° 11′ 20″ N, 20° 35′ 48″ E (Figure 1a & 1b). The collected plant samples were taxonomically identified by the expert taxonomist of the Department of Botany, Faculty of Science, University of Benghazi, Libya. The identified plant specimen was assigned voucher specimen no. 1-20582 and preserved in the herbarium of the Botany Department, Faculty of Science, University of Benghazi, Libya.

2.2 Preparation of Plants Extract

The collected plant leaves were gently washed with running tap water, rinsed with distilled water, and then allowed to dry in the shade for two weeks at room temperature. The home mixture and

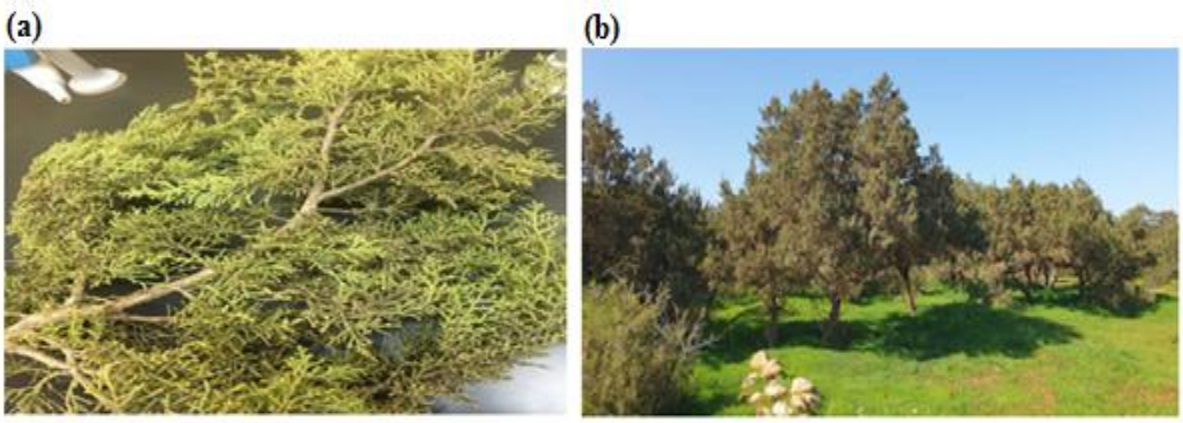

Figure 1 (a) leaves of *J. phoenicea* and (b) *J. phoenicea* trees at SidiEmhamad forest, Alabyar city, Libya.

grinder ground dried leaves and thirty grams of plant powder was mixed with 300 mL of methanol, acetone, and aqueous solutions separately overnight and then filtered through Whatman No.1 filter paper. The filtrated samples were then evaporated and dried under reduced pressure using a rotatory evaporator with a water bath temperature of 40°C for methanol and acetone, while the aqueous solvent was extracted at 100°C for 30 min. Four concentrations of each extract, i.e. 25, 50, 75, and 100% (v/v), were used to test their antibacterial activities. The obtained extract was stored at 4˚C in the refrigerator until the antibacterial test. All experiments were repeated in triplicate.

2.3 Collection of bacterial strains

The antibacterial activity of three leaves extracts of *J. phoenicea* was tested against the two Gram-positive (*Staphylococcus aureus* "SA" and *S. haemolyticus* "SH") and two Gram-negative bacteria (*Pseudomonas aeruginosa* "PA", and *Proteus mirabilis* "PM"). Selected bacterial isolates were obtained from the Benghazi Children's Hospital, Libya and were identified by standard methods in El-Jala Teaching Hospital and confirmed by Phoenix (Alhadad et al. 2021).

2.4 Determination of Antibacterial Activity

The antibacterial assay was performed on Muller-Hinton agar (MHA) using the agar well diffusion method according to Debalke et al. (2018) with some required modifications. Each bacterial suspension was calibrated to a turbidity standard of 0.5 McFarland (10^8 CFU/mL). The MHA plates were inoculated by spreading the bacterial inoculum across the agar surface using a sterile swab. After that, five wells with a diameter of 8 mm were cut using a sterile cork borer. Four predefined concentrations of particular plant extract solutions (25, 50, 75, and 100% v/v) and 100 µL of each solvent was filled in the prepared wells. The negative control was placed at the centre of each plate with absolute methanol, dimethylsulfoxide (DMSO), and distilled water for methanol, acetone, and aqueous solutions, respectively. While the reference antibiotic discs of levofloxacin (5µg), amoxicillin (25µg), and ampicillin (10µg) were used as positive controls and put in separate plates. The plates were allowed to diffuse for 20 min at room temperature and then incubated for 24 h at 37°C. The inhibition zone formed around each well (including the well's diameter) was measured in millimetres using a ruler and compared with the standard discs and negative controls.

The sizes of the inhibitory zones for the plant extracts and the tested antibiotics were classified as susceptible, intermediate or resistant based on the criteria outlined in the Clinical and Laboratory Standards Institute (CLSI) breakpoint system (CLSI 2020).

2.5 Statistical Analysis

The statistical analysis was conducted using an ANOVA followed by a *post-hoc* Tukey HSD test in GraphPad Prism version 9.4.1 (681). Differences between means were considered statistically significant at a p-value< 0.05. All obtained results were presented as mean ± standard error (SEM) in triplicate.

3 Results and Discussion

Due to the lack of new antimicrobial agents being discovered and developed, MDR bacteria are currently the most significant threat to human health and are typically associated with nosocomial infections (van Duin and Paterson 2020). In this investigation, the antibacterial efficacy of Libyan *J. phoenicea* leaf extracts was assessed against the four MDR bacteria, i.e. *Staphylococcus aureus*, *S. haemolyticus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* by using methanol, acetone, and aqueous extracts. To ensure higher accuracy and improved antimicrobial susceptibility performance, the Clinical Laboratory Standards Institute (CLSI 2020) testing was used to interpret the findings of the current research (susceptible, intermediate, or resistant).

Results presented in Table 1 and Figure 2 revealed the inhibitory effects of three plant extracts and selected common antibiotics against the four selected clinical MDR isolates. Concerning the negative controls, the results of this study exhibited that none of the tested bacteria had any inhibition zones (Figure 2). All the studied extracts exhibited concentration-dependent antibacterial activity against all tested clinical bacterial isolates.

Findings of the present study showed that methanol and acetone extracts of *J. phoenicea* had demonstrated moderate to high antibacterial activity against all tested clinical isolates (zones of inhibition ranging from 15.66 to 27.66 mm) at all studied concentrations and *S. aureus* and *S.haemolyticus* were found most susceptible to these extracts (Table 1 and Figure 3a-c). These results are in agreement with previous investigations, which illustrated that methanol and acetone extracts showed moderate to high antibacterial activities toward the growth of pathogenic bacteria when compared to aqueous extracts (El-mahmood et al. 2008; Dewangan et al. 2010; Al-Daihan et al. 2013; Atwaa et al. 2022; Borges et al. 2020; Prakash 2023).

The inhibition efficiency of the aqueous extract was also observed against the selected clinical bacterial isolates, but it varied from moderate to less or no inhibition. At the higher concentrations (50-100%), the aqueous extract exhibited moderate antibacterial activity, with the inhibition zones ranging from 15.66 to 17.00 mm against *S. aureus*, 15.33 to 15.66 mm towards *S. haemolyticus*, and 15.00 mm against *P. mirabilis* at a concentration of 100% only. However, at the lowest concentration (25%), all the

Table 1 Antibacterial activity of *J. phoenicea* leaves extracts and standard drugs against clinical bacterial isolates

Extract/ Antibiotic	Concentration (%)	Diameter of inhibition zone (mm)			
		Gram-positive bacteria		Gram-negative bacteria	
		SA	SH	PA	PM
Aqueous	25	14.66±0.88 (R)	13.33±0.33 (R)	-	10.33±0.33 (R)
	50	15.66±0.33 (I)	15.33±0.33 (I)	-	12.33±0.33 (R)
	75	17.00±0.00 (I)	15.66±0.33 (I)	-	13.66±0.33 (R)
	100	17.00±0.00 (I)	15.66±0.33 (I)	-	15.00±0.00 (I)
Acetone	25	21.66±0.33 (S)	23.00±0.57 (S)	17.00±0.00 (I)	16.33±0.33 (I)
	50	23.66±0.33 (S)	23.33±0.66 (S)	18.33±0.66 (I)	17.00±0.57 (I)
	75	24.66±0.33 (S)	24.33±0.33 (S)	19.33±0.33 (I)	18.33±0.66 (I)
	100	25.66±0.66 (S)	27.00±0.57 (S)	20.33±0.88 (S)	19.66±0.33 (I)
Methanol	25	23.33±0.88 (S)	19.33±1.20 (I)	16.66±0.33 (I)	15.66±0.66 (I)
	50	25.00±0.00 (S)	21.33±0.66 (S)	19.66±0.33 (I)	18.00±1.00 (I)
	75	26.33±0.88 (S)	23.33±0.33 (S)	22.33±0.33 (S)	19.00±0.57 (I)
	100	27.66±0.66 (S)	24.00±0.00 (S)	23.66±0.33 (S)	19.66±1.20 (I)
Levofloxacin (5µg)		34.33±0.33 (S)	33.00±0.57 (S)	-	36.33±0.33 (S)
Ampicillin (10µg)		15.33±0.33 (R)	23.00±0.00 (R)	-	-
Amoxicillin (25µg)		15.67±1.85 (R)	11.00±0.57 (R)	-	-

Here - = No zone of inhibition detected; results are presented as mean values ± standard error (SE) (N=3); Clinical breakpoints, as recommended by CLSI guideline, are denoted by the susceptible (S), intermediate (I), and resistant (R); SA; *Staphylococcus aureus*, SM; *Staphylococcus haemolyticus*, PA; *Pseudomonas aeruginosa*, and PM; *Proteus mirabilis*

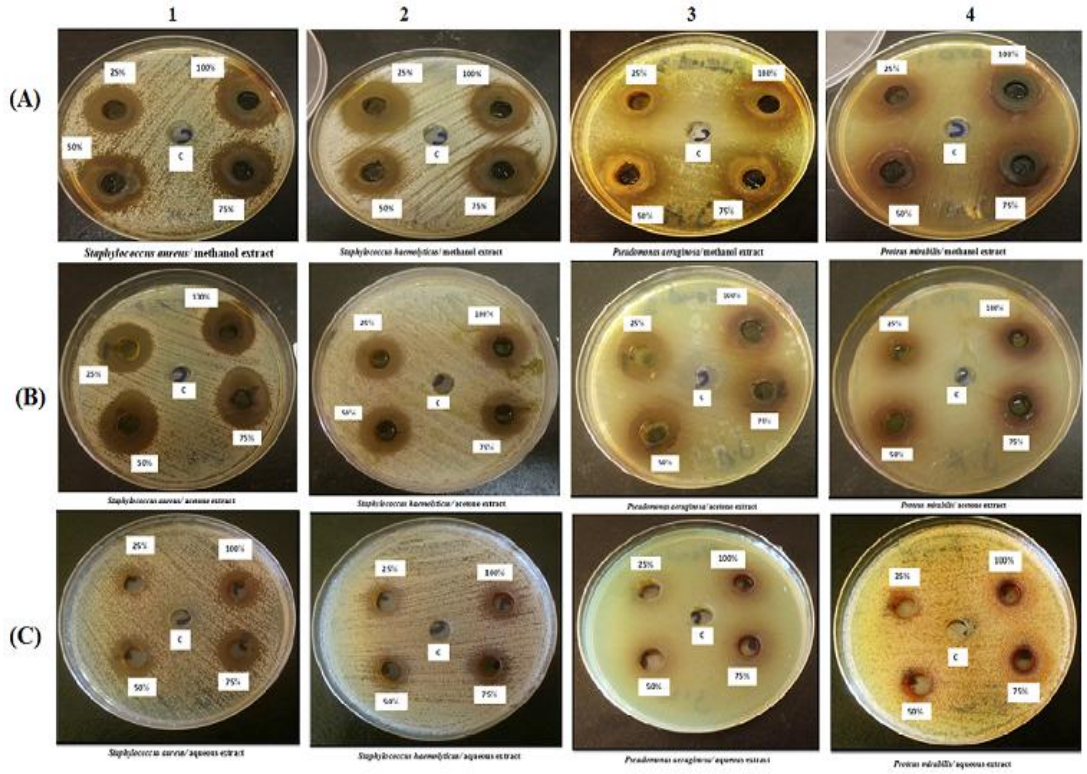

Figure 2 Antibacterial activity of three leaves extracts of *J. phoenicea* by agar well diffusion method against four pathogenic bacteria; A (methanol extract), B (acetone extract), and C (aqueous extract); 1 *S. aureus*, 2 *S. haemolyticus*, 3 *P. aeruginosa*, and 4 *P. mirabilis*

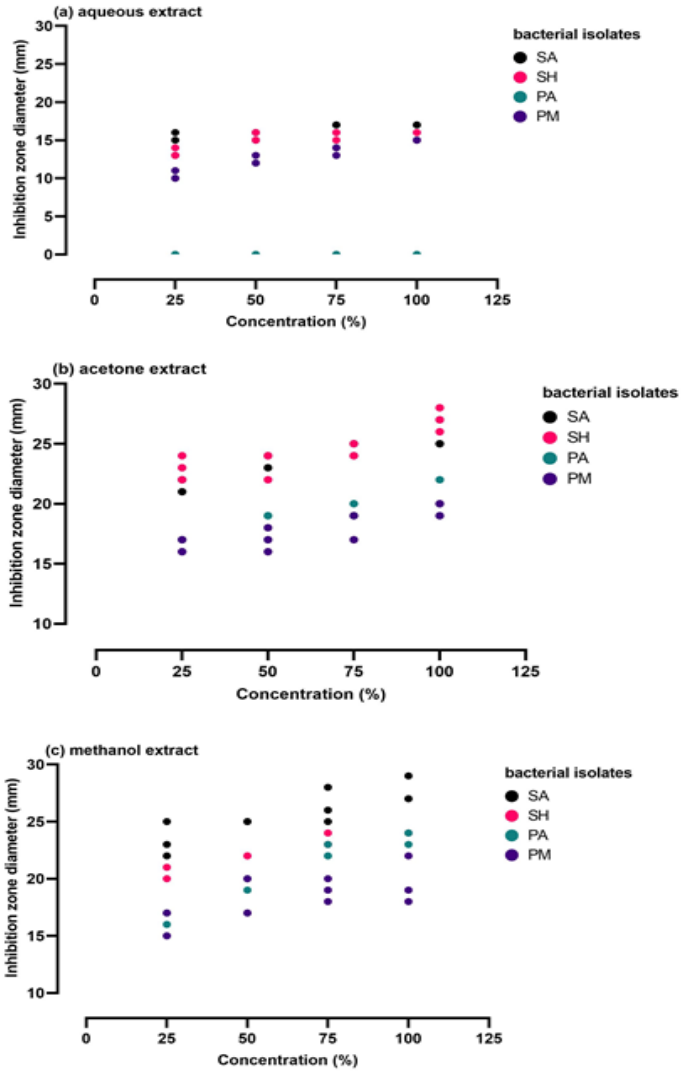

Figure 3 Effect of *J. phoenicea* plant aqueous extract, (a) acetone extract, (b) methanol extract, (c) on the growth of pathogenic bacteria; SA - *S. aureus*, SH - *S. haemolyticus*, PA - *P. aeruginosa*, and PM *P. mirabilis*

studied bacterial isolates were shown to be resistant to the aqueous extract, with a zone of inhibition diameter ranging between 0.00 to 14.66 mm. These findings are consistent with a previous study, which demonstrated that the antibacterial efficiency of an aqueous leaf extract of *Bidens plasa* was detected against different bacterial pathogens, such as *P. aeruginosa* and *S. aureus* at the highest concentrations only (Omotanwa et al. 2023).

On the other hand, Mohammed and Aziz (2023) have reported that the aqueous extract of *Peganum harmala* had the highest antibacterial activity against *Proteus mirabilis* at a concentration of 10%. However, the aqueous extract of *Cinnamomum zeylanicum* was ineffective against *Proteus mirabilis*. Prakash (2023) recently demonstrated that the aqueous extract of *Baccharoides anthelmintica* seed displayed potent activity against *Yersinia pestis* and *Listeria monocytogenes* and exhibited no inhibition against the bacterial species *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus*. These variations might be attributed to the concentration of the plant extracts, the kind of used plant material and species, as well as dependent on the bacterial strains.

Overall the results of the current study also revealed that the aqueous extract had the lowest antibacterial efficacy against all clinical isolates of bacteria. This could be because the aqueous extract was subjected to high temperatures (boiling), which can affect the bioactive components. The findings of this investigation were similar to the previous study, which reported that the chemical compositions of the aqueous extract of *Ocimum gratissimum* were significantly reduced under extraction temperature (100^{o} C). Still, these compounds increased at a

temperature of 110^{o} C (Onyebuchi and Kavaz 2020). Shehadi et al. (2014) revealed that the effect of high temperature (100^{o}C) on the activity of bioactive compounds (terpenoids, flavonoids, and phenolics) was not observed in the extractions of cloves and rosemary, but these components were not found in mint.

Conversely, El-mahmood et al. (2008) illustrated the antibacterial efficiency of the stem, bark, and leaf extracts of *Vitellaria paradoxa* was not affected by increasing the temperature (boiled water). Furthermore, other studies also demonstrated that the aqueous extracts of various plant species showed the highest activity against different strains of pathogenic bacteria, even at the heat temperature of 100^{o} C (Saeed and Tariq 2008; Atwaa et al. 2022). These results suggest that the effect of the extraction temperature (100^{o} C) on the bioactive compounds is significantly dependent upon the species of the plant.

The standard drug (Levofloxacin) was active against all the selected clinical bacterial isolates except for *P. aeruginosa*, with the inhibition zones ranging between 33.00-36.33 mm in diameter. In contrast, all the tested bacteria were resistant to Ampicillin and Amoxicillin (Table 1). Various previous studies have reported that *P. aeruginosa* displayed resistance to a wide variety of antimicrobials (Breidenstein et al. 2011; Mishra and Padhy 2013; Cole et al. 2014; Pang et al. 2019; Zgurskaya and Rybenkov 2020; Ahmed et al. 2021; Atwaa et al. 2022; Tabcheh et al. 2023). Interestingly, methanol and acetone extracts showed the highest remarkable inhibition of antibacterial activity against all the tested clinical bacterial isolates, including *P. aeruginosa*, while no inhibition was reported by either the antibiotic standards or aqueous extract on this bacterium. These results are supported by the findings of a previous study conducted by Alhadad et al. (2022).

In contrast, Rukundo et al. (2023) investigated and found that the aqueous extract of ginger was effective against *P. aeruginosa* at a higher concentration (2g/ml). The current investigation revealed that Gram-negative bacteria (*P. aeruginosa* and *P. mirabilis*) were comparatively less susceptible to antibacterial agents than Gram-positive bacteria (*S. aureus* and *S. haemolyticus*). By contrast, Karuppiah and Mustaffa (2013) have demonstrated that the antibacterial activity of the leaves of *Musa* species was more efficient against the Gram-negative bacteria than the Gram-positive bacteria. On the other hand, many previous studies have revealed that juniper essential oils or extracts were less sensitive against Gram-negative bacteria than Gram-positive ones (Ennajar et al. 2009; EL-Mahmood 2009; Elmhdwi et al. 2015; Guedri et al. 2020). Hence, despite the presence of an outer membrane in Gram-negative bacteria, which acts as a permeability barrier for impeding the passage of drugs and preventing the antibiotic from reaching its binding site in the bacterial cell, the antibacterial activity was mainly dependent on the extraction method and the plant species (Ghai and Ghai 2018; Zgurskaya and Rybenkov 2020).

According to ANOVA results, the means of inhibition zones for all the concentrations of the plant extracts were significantly different compared to the negative control. Figure 4 showed that all tested concentrations showed no significant differences between the mean growth inhibition zones of methanol and acetone extracts. The reference antibiotic disc (Levofloxacin) had the highest mean

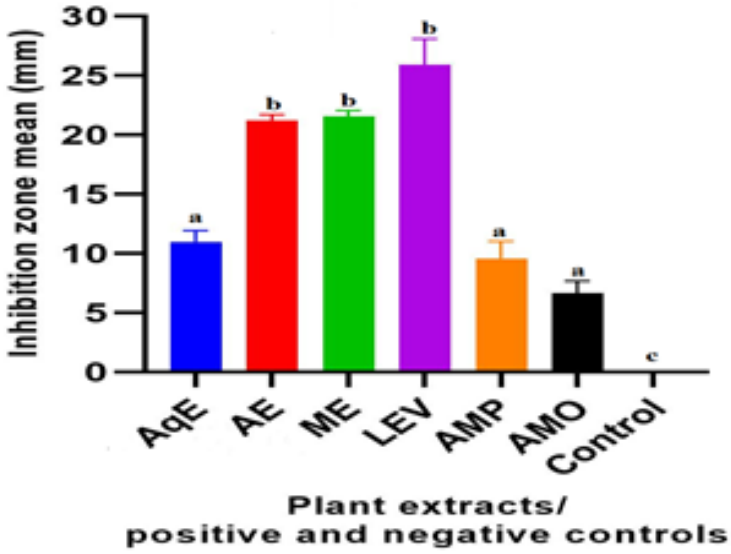

Figure 4 The average of inhibition zones of triplicates (n=3) ± standard error (SEM); AqE; aqueous extract, AE; acetone extract, ME; methanol extract; LEV; levofloxacin, AMP; ampicillin, AMO; amoxicillin (positive controls); Different letters indicate significant differences ($p< 0.01$) between mean values of inhibition zones for all the concentrations of the plant extracts compared with the positive and negative controls according to ANOVA followed by Tukey's HSD test

antibacterial activity. Hence, in this investigation, to prove and support the results of the plant extracts, further minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays should be performed for their antibacterial activity against the susceptible bacterial isolates.

Conclusion

The present study illustrated that the methanol and acetone extracts could be potential sources of antibacterial agents for developing novel drugs to combat infectious diseases. Further research is required to detect the active substances responsible for the observed antibacterial activity and to evaluate their potential as alternative remedies for bacterial infections. In addition, investigating the cytotoxicity of these extracts on human cells is necessary before considering their use in clinical settings.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgements

None

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