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RESEARCH ARTICLE

EVALUATING THE SYNERGISTIC ACTIVITY OF *ROSMARINUS OFFICINALIS* EXTRACTS WITH ANTIBIOTICS AGAINST MDR BACTERIA

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Abstract

Background: The rise of antimicrobial resistance and multidrug-resistant bacteria is growing global threat, particularly *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, has become a critical challenge in clinical microbiology and pharmacotherapy and even be untreatable with conventional antibiotics. Exploring plant-derived antimicrobial offers promising complementary strategies.
Objective: This study evaluates the antibacterial activity of ethanolic and aqueous extracts of *Rosmarinus officinalis* and investigate the synergistic effect of the high effective concentration of ethanolic and aqueous extracts in combination with ceftazidime, cefoperazone and gentamycin against *P. aeruginosa*, and *K. pneumonia*e.
Method: The dried leaves of rosemary were macerated in 96% ethanol and water to prepare ethanolic and aqueous extract and phytochemical screening was conducted to identify active constituents. Clinical MDR isolates were tested using agar well diffusion method. For synergy, ceftazidime, cefoperazone and gentamycin discs were immersed in 100% ethanolic and aqueous extracts separately and tested against MDR strains.
Results: Ethanolic extract exhibited dose dependent antibacterial activity with maximum zones of inhibition at 100mg/ml: *P. aeruginosa* (7.50±0.50 mm), *K. pneumoniae* (6.00±0.20 mm). Aqueous extract showed negligible activity. Synergistic testing revealed enhanced inhibition zones when antibiotic discs were pre-treated with ethanolic extract. While aqueous extract showed decreasing in the inhibition zone.
Conclusions: The 100 mg/ml ethanolic extract of *R. officinalis* enhances the efficacy of cefoperazone and gentamicin and no effect on ceftazidime. These findings indicate that rosemary extract could serve as potential adjunct in overcoming antibiotic resistance.
Keywords: Ceftazidime, cefoperazone, gentamicin, *Klebsiella pneumonia*, MDR, *Pseudomonas aeruginosa*, *Rosemary officinalis*, Yemen.

INTRODUCTION

Pseudomonas aeruginosa and *Klebsiella pneumoniae* may be considered among the most serious opportunistic bacteria that can develop multiple resistance mechanisms among multidrug-resistant (MDR) pathogens these mechanisms include formation of efflux pump, biofilm and β-lactamase production[1-3]. Due to the ability of these bacteria to develop multiple resistance mechanisms, they are considered a major public health threat worldwide. They are commonly associated with serious infections, particularly in hospitals, leading to reducing effectiveness commonly used antibiotic or even making them ineffective[4,5].
In the process of searching for new alternatives or adjunct treatments, medicinal plants have gathered so much attention due to their rich supply of bioactive compounds. *Rosmarinus officinalis* L. from the *Lamiaceae* family, commonly known as rosemary, has been used for centuries in traditional medicine as an antimicrobial, antioxidant, and anti-inflammatory[6-9]. The bioactive compounds in rosemary, such as flavonoids, rosmarinic acid, carnosic acid, and essential oils such as camphor and α-pinene, are responsible for its pharmacological activity[7,10,11].
Globally, studies have demonstrated that rosemary possess broad-spectrum antibacterial activity. As an example[12], reported that rosemary essential oil showed significant inhibition against clinical isolates of *K. pneumoniae* and *P. aeruginosa*. Similarly[13], noticed a synergistic enhancement when conventional antibiotics like gentamicin and cefoperazone were combined with rosemary extract. Ethanolic extracts, in particular, showed superior activity due to their ability to extract

more phenolic compounds unlike aqueous extracts[14,15]. By using such a combination of plant extracts and antibiotics against MDR strains is an important thing in restoring the potency of these antibiotics as this study spotlighted[16].

In the matter of Yemen, medicinal plants still play an important role in traditional medicine, but the scientific investigations remain limited. A previous study reported modest antibacterial activity of rosemary essential oil from Yemeni sources specially against Gram-negative bacteria[17], another study proved that Yemeni aromatic plants, including rosemary are phytochemically rich, confirming the presence of antimicrobial constituents[18]. Former ethnopharmacological surveys in a study confirmed that several Yemeni medicinal plants, including those from the *Lamiaceae* family have antibacterial potential[19]. Even though these studies highlighted the antimicrobial activity of Yemeni medicinal plants, they did not directly examine rosemary extract in synergy[20]. Additionally another study confirmed the safety and bioactivity of Yemeni rosemary oil in cytotoxic and enzyme inhibition assays[21]. Given the rising resistance and the under-explored potential of locally available medicinal plants, this study investigates the antibacterial activity of ethanolic, and aqueous extracts of *R. officinalis* collected in Yemen and evaluates the synergistic effect of 100 mg/ml ethanolic and aqueous extract when combined with gentamicin, cefoperazone, and ceftazidime against clinical MDR isolates of *P. aeruginosa* and *K. pneumoniae*.

MATERIALS AND METHODS

Plant collection and identification:

R. officinalis leaves were purchased from Al-Mujahid spices in Dhamar city, Yemen, and identified by a botanist, Dr. Aref Izzedine, who is an expert in pharmacognosy at Al-Saeeda University and Taiz University, washed with distilled water, shade-dried, and powdered.

Extraction procedure:

Total 150 g of powdered leaves were macerated in 96% ethanol and distilled water separately for 48h with shaking, then filtered with Whitman paper, evaporated by rotary evaporator at 50°C and 90 round per minute (RPM) and stored[22-24].

$$\text{Yield (\%)} = \frac{\text{Weight of dry extract}}{\text{Weight of dry plant powder}} \text{x100}$$

The % yield for ethanolic and aqueous extract was 6.6%, 4.6% respectively.

Phytochemical screening:

standard qualitative tests for saponins, tannins, phenols, resins, alkaloids, and triterpenoids were performed using established methods[25].

Preparation of extract concentrations:

The Stock solution of Rosemary (100 mg/ml) was prepared in dimethyl sulfoxide (DMSO), then serial dilutions to 50, 25, and 12.5 mg/ml were performed.

Bacterial strains:

Bacterial isolates of *K. pneumoniae* and *P. aeruginosa* were obtained from clinical isolates stored at Al-Dobai Laboratories in Dhamar city, Yemen, and identified by the lab according to standard microbiological methods.

Antibacterial Testing:

It was performed using agar well diffusion method on Muller-Hinton agar[26,27]. Bacterial suspensions of *P. aeruginosa* and *K. pneumonia* were standardized to a 0.5 McFarland standard and swabbed onto agar plates. Wells of (6 mm) were filled with 50 µl of each extract concentration, with DMSO as negative control and antibiotic discs as positive control[27,28]. Plates were incubated at 37ºC for 24 hours then the zones of inhibition were measured in millimeter.

Synergy testing:

Synergistic activity was assessed by immersing antibiotic discs (ceftazidime, cefoperazone, and gentamicin) in the highest effective concentration of *R. officinalis* extracts for 10 minutes before placement in inoculated bacterial plates. Plates were incubated at 37ºC for 24 hours and inhibition zones were measured in millimeter. The results were compared to the zone of antibiotic alone. This method follows the disc immersion (potentiation) technique as described in a previous study[29].

Statistical analysis:

Data obtained from antibacterial and synergistic activity experiments were analyzed using Microsoft Excel and IBM SPSS Statistics version 27. Descriptive statistics, including the mean and standard deviation (±SD) were calculated for inhibition zone diameters at each concentration. Results were triplicated and represented as mean and ±SD.

RESULTS

Qualitative phytochemical screening represented that the ethanolic extract of *R. officinalis* contained alkaloids, tannins, steroids, saponin, resins, and phenols. The aqueous extract also showed the presence of alkaloids, tannins, steroids, saponin, and resins but lacked detectable levels of phenols. The greater phytochemical diversity in the ethanolic extract might be the reason to its superior antibacterial activity.

P. aeruginosa and *K. pneumoniae* exhibit multidrug-resistant patterns. *P. aeruginosa* was resistant to ceftazidime, gentamicin, cefoperazone, piperacillin, amikacin, and ceftriaxone while remaining sensitive to imipenem and intermediate to levofloxacin. Similarly, *K. pneumoniae* showed resistance to ceftazidime, cefoperazone, piperacillin, and ceftriaxone, intermediate sensitivity to gentamicin, amikacin, imipenem, and levofloxacin (Table 1, Figure 1).

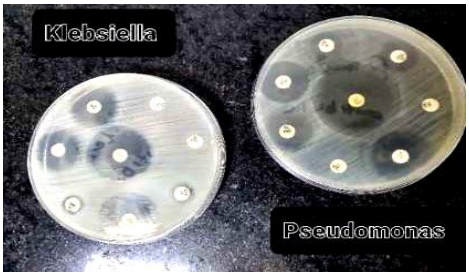

Figure 1: Antibiotic-sensitivity tests against *P. aeruginosa* and *K. pneumonia.*

Table 1: Antibiotic-sensitivity tests against *P. aeruginosa* and *K. pneumoniae*.

Antibiotic	*P. aeruginosa*		*K. pneumoniae*	
	Inhibition Zone (mm)	Result	Inhibition Zone (mm)	Result
Ceftazidime (CTZ)	4.67±0.58	R	6.00±0.00	R
Gentamicin (GNT)	9.67±0.58	R	12.47±0.58	M
Cefoperazone(CPZ)	12.67±0.58	R	7.33±1.15	R
Imipenem (IMP)	19±1.00	S	17.00±1.00	M
Piperacillin (PI)	4.33±0.58	R	4.33±0.58	R
Amikacin (AK)	12.00±1.00	R	12.33±0.58	M
Levofloxacin (LE)	14.33±0.58	M	14.00±1.00	M
Ceftriaxone (CTR)	7.33±0.58	R	5.00±0.00	R

S= Sensitive, M= Moderate, R= Resistance

Table 2: Antibacterial activity of ethanolic and aqueous extracts of *R. Officinalis* against *P. aeruginosa* and *K. pneumoniae*.

Bacteria	Ethanolic extract		Aqueous extract	
	Concentration	Inhibition zone (mm)	Concentration	Inhibition zone (mm)
P. aeruginosa	100 mg	7.67±0.58	100 mg	1.33±0.58
	50 mg	6.33±0.58	75 mg	1.00±0.00
	25 mg	6.33±0.58	50 mg	0.00±0.00
	12.5 mg	5.00±0.00	25 mg	0.00±0.00
K. pneumoniae	100 mg	6.00±0.00	100 mg	1.67±0.58
	50 mg	5.50±0.50	75 mg	0.33±0.58
	25 mg	5.30±0.00	50 mg	0.00±0.00
	12.5 mg	5.00±0.00	25 mg	0.00±0.00

The ethanolic extract of *R. officinalis* demonstrated moderate antibacterial activity against both *P. aeruginosa* and *K. pneumoniae* with inhibition zones ranging from 4.67±0.58 to 7.67±0.58 mm and 4.87±0.32 to 6.00±0.00 mm, respectively, depending on the concentration tested. In contrast, the aqueous extract exhibits minimal to no antibacterial activity with inhibition zones ranging from 0.00±0.00 to 1.67±0.58 mm against both bacteria (Table 2, Figure 2, Figure 3). Due to low activity observed with aqueous extracts and lower concentrations of the ethanolic extract, only the ethanolic and aqueous extracts at concentration of 100 mg/ml were used for the synergy study. The ethanolic extract showed a slight increase in the inhibition zones when combined with antibiotics against *P. aeruginosa* increasing cefoperazone from 12.67±0.58 mm to 13.00±1.00 mm and gentamicin from 9.67±0.58 mm to 11.00±0.00 mm. a more notable increase was observed against *K. pneumonia* with cefoperazone increasing from 7.33±1.15 mm to 10.00 ±1.00 mm and gentamicin from 12.47±0.58 mm to 16.67±0.58 mm when combined with the ethanolic extract. This indicates a synergistic effect, particularly with gentamicin against *K. pneumoniae*. But when combined with ceftazidime, the opposite thing happens, and the inhibition zone decreases from 4.67±0.58 mm to 0.67±0.29 mm against *P. aeruginosa* and from 6.00±0.00 mm to 0.00±0.00 mm against *K. pneumonia* (Table 3, Figure 4).

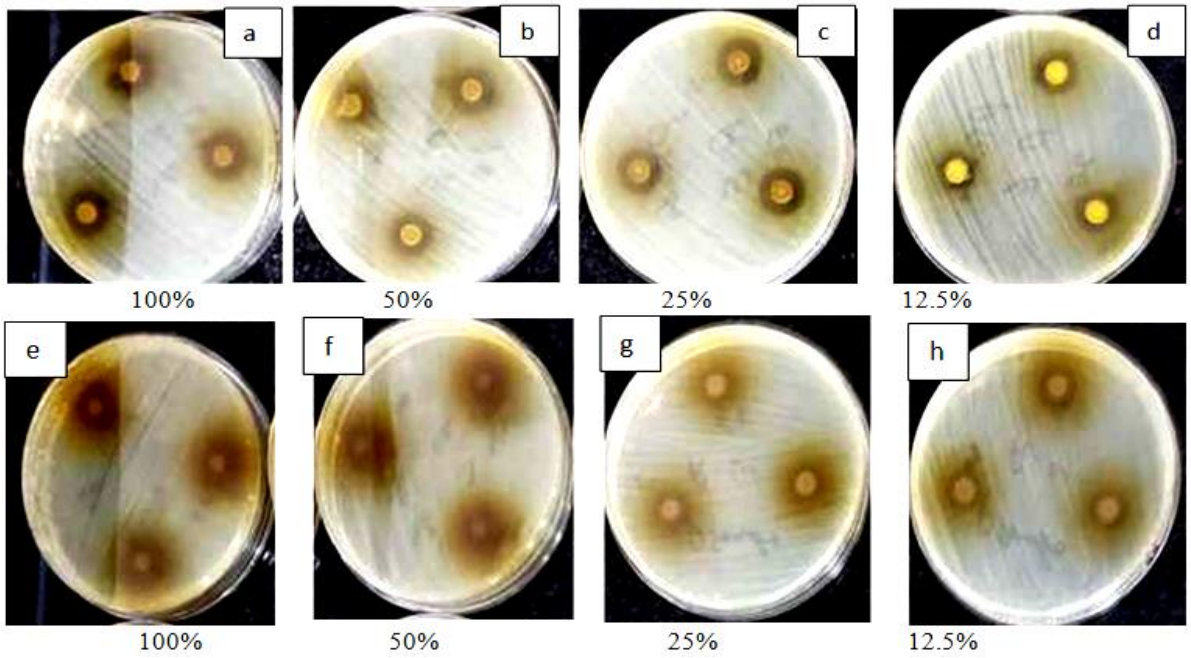

Figure 2: Antibacterial activity of ethanolic extracts (a, b, c, and d) and aqueous extracts (e, f, g, and h) of *R. officinalis* against *P. aeruginosa* at different concentration.

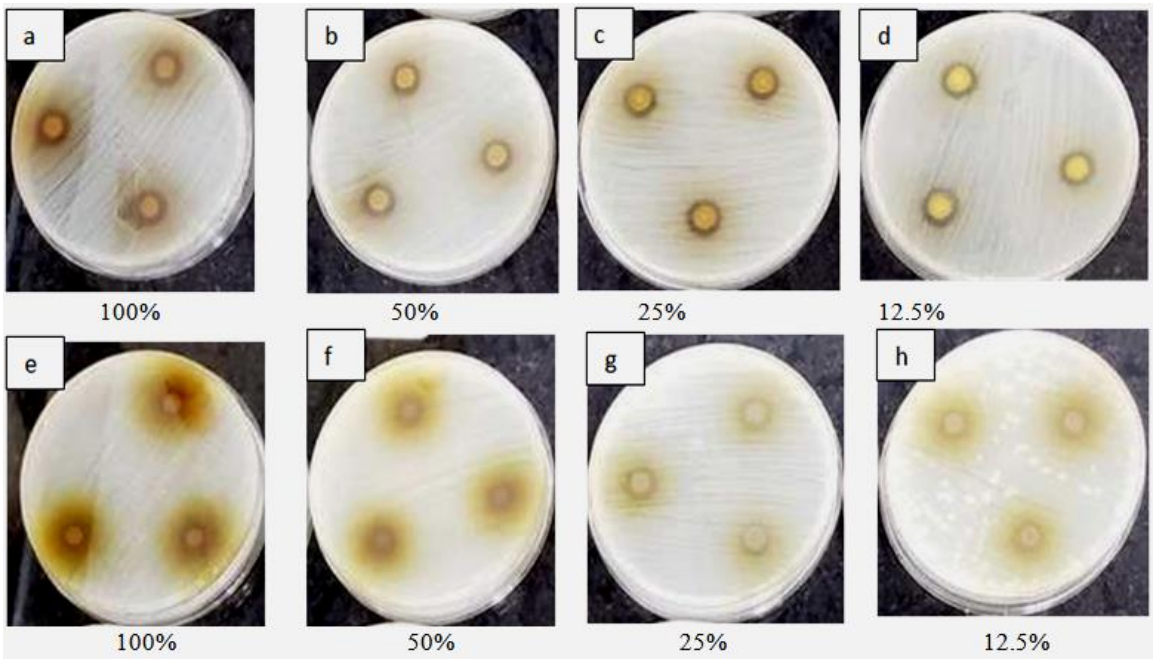

Figure 3: Antibacterial activity of ethanolic extracts (a, b, c, and d) and aqueous extracts (e, f, g, and h) *R. officinalis* against *K. pneumonia* at different concentration.

Table 3: Synergistic activity of ethanolic and aqueous extracts of *R. officinalis* with Ceftazidime, Cefoperazone and Gentamicin against *P. aeruginosa,* and *K. pneumoniae*.

	Item tested	*P. aeruginosa* inhibition Zone (mm)	*K. pneumoniae* Inhibition Zone (mm)
Antibiotics	CPZ	12.67±0.58	7.33±1.15
	GNT	9.67±0.58	12.47±0.58
	CTZ	4.67±0.58	6.00±0.00
Ethanolic extract	*R. officinalis* (100mg)	7.67±0.58	6.00±0.00
	R. officinalis +CPZ	13.00±1.00	10.00 ±1.00
	R. officinalis +GNT	11.00±0.00	16.67±0.58
	R. officinalis +CTZ	0.67±0.29	0.00±0.00
Aqueous extract	*R. officinalis* (100mg)	1.33±0.58	1.67±0.58
	R. officinalis +CPZ	0.00±0.00	0.00±0.00
	R. officinalis +GNT	0.00±0.00	0.00±0.00
	R. officinalis +CTZ	0.00±0.00	0.00±0.00

CPZ= Cefoperazone, GNT= Gentamicin, CTZ= Ceftazidime.

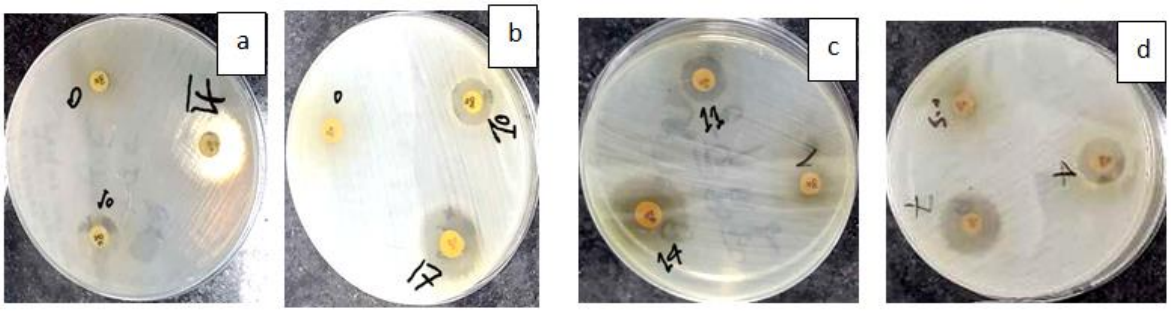

Figure 4: Synergistic test of ethanolic extract of *R. officinalis* against *K. pneumonia* (a, b) and *P. aeruginosa* (c, d).

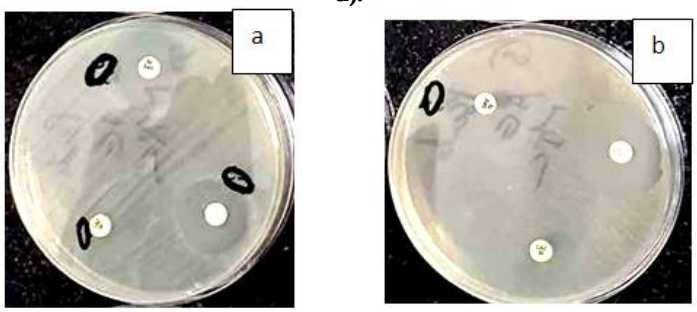

Figure 5: Synergistic test of aqueous extract of *R. officinalis* against *K. pneumonia* (a) and *P. aeruginosa* (b).

In contrast, the aqueous extract increases the growth of both bacteria and decreases the antibiotic activity. For cefoperazone from 12.67±0.58 mm to 0.00±0.00 mm against *P. aeruginosa* and from 7.33±1.15 mm to 0.00±0.00 mm against *K. pneumoniae*. For gentamicin from 9.67±0.58 mm to 0.00±0.00 mm against *P. aeruginosa* and from 12.47±0.58 mm to 0.00±0.0 mm against *K. pneumoniae*. For ceftazidime, the inhibition zone decreased from 4.67±0.58 mm to 0.00±0.0 mm against *P. aeruginosa* and from 6.00±0.0 mm to 0.00±0.0 mm against *K. pneumonia* (Table 3, Figure 5).

Limitations of study

This study lacks the determination of MIC and MBC, restraining the precision of evaluation. Synergy testing of both extracts was performed only at 100% concentration using the disc immersion method without a confirmatory checkerboard assay. The study also lacks quantitative phytochemical tests such as GC-MS or HPLC and cytotoxicity tests, which are essential to ensuring the safety of the extract and evaluating the bioactivity of specific compound. Also, the number of isolates and lack of strain diversity restrict the generalizability of the study results.

DISCUSSION

This study evaluated the antibacterial effect of *R. officinalis* ethanolic and aqueous extracts against *P. aeruginosa* and *K. pneumoniae*. The ethanolic extract showed moderate antibacterial activity, while the aqueous extract exhibited minimal to no inhibition, particularly at lower concentrations. The findings are consistent with those of Abkhoo & Jahani *et al.*[30], who also reported strong antibacterial activity of ethanolic extract of *R. officinalis* against *E. coli* and *S. aureus*, with a greater inhibition zone when compared to aqueous preparation. Similarly, Manilal *et al.*[14], confirmed that the ethanolic extract of *R. officinalis* showed a higher antibacterial activity against MDR *K. pneumoniae* when compared to aqueous extract, strengthening the current observation. Also, Ali *et al.*[15], and Kabotso *et al.*[31], have the same results that reported a significant antibacterial activity of ethanolic preparations of Yemeni medicinal plants extracts. This may explain the higher efficacy observed with the ethanolic extract of rosemary in the current study.

The higher antibacterial activity of the ethanolic extract may be due to higher extraction of bioactive compounds, that is known for their multiple mechanisms to exert their antibacterial effects, such as protein denaturation and membrane disruption. Kabotso *et al.*[15], and Nieto *et al.*[20], emphasised that phenolics bioactive compounds of *R. officinalis*, particularly Rosmarinus acid, play a vital role in bacterial inhibition.

The synergy observed between the ethanolic extract and antibiotics (cefoperazone and gentamicin) may indicate a potential for combining plant extracts with standard antibiotics.

The combination of ethanolic extract and gentamicin shows modest enhancement against *P. aeruginosa*, while a greater increase in the inhibition zone was observed against *K. pneumoniae*. These results are aligned with those of Husein *et al.*[13], and Kafa *et al.*[32], who also reported that essential oils could enhance the activity of antibiotics against resistant strains .Ojeda-Sana *et al.*[33], demonstrated that the active constituents of rosemary, like carnosic acid and carnosol may facilitate the penetration of antibiotics into bacteria by disrupting its membrane.

CONCLUSIONS

The findings of this study showed that the ethanolic extract of *R. officinalis* has a higher antibacterial activity against multidrug-resistant *P. aeruginosa* and *K. pneumoniae* than aqueous extract that showed minimal to no effect. Remarkably, synergy is suggested between the combination of the ethanolic extract with gentamicin and cefoperazone, especially against *K. pneumoniae*, indicating that rosemary extract could be combined with conventional antibiotics to overcome multidrug-resistant bacteria.

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AUTHOR'S CONTRIBUTION

Khalil NHA: designed and performed the experiments, analyzed the data andwrote original draft, investigation. **Al-Lebathi HH:** developed methodology, carried out the phytochemical screening, performedthe antibacterial evaluations, manuscript review, and editing. Final manuscript was checked and approved by all authors.

DATA AVAILABILITY

Data will be made available on request.

CONFLICT OF INTEREST

None to declare.

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