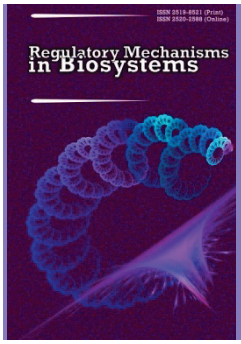

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Detection of IMP and VIM genes and antibacterial activity of some plant extracts in carbapenem-resistant *Pseudomonas aeruginosa* isolated from burn infections

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Carbapenem-resistant *Pseudomonas aeruginosa* has emerged as a deadly pathogenic agent globally, responsible for the majority of morbidity and fatalities in burn patients. Genes of carbapenemase are considered to be among the most prevalent means of resistance in *P. aeruginosa* to carbapenem. In the present study, the existence of metallo-beta-lactamase (MBL) genes of the VIM and IMP in carbapenem-resistant *P. aeruginosa* isolated from burn patients were determined and also the antibacterial effects of five ethanolic plant extracts were investigated. Twenty carbapenem-resistant *P. aeruginosa* isolates were isolated from burn infections. The disc diffusion test was used for examination of antibacterial susceptibility. Subsequently, MBL were identified by the imipenem-EDTA combined disc test and PCR. This was followed by evaluation of the plant extract and MIC determination by the well-diffusion method. The results revealed that among 20 *P. aeruginosa* isolates, 17 (85%) were imipenem-resistant. Among them, 11 (64.7%) isolates appeared positive for the IMP-EDTA combined disc test. The VIM gene was detected positively in seven (41.2%) isolates. Not a single isolate had the IMP gene. None of the imipenem-sensitive isolates harbored the IMP or VIM genes. Evaluation of ethanolic extract of *Rhus coriaria*, *Punica granatum*, *Thymus vulgaris*, *Syzygium aromaticum* and *Curcuma longa* revealed possibly effective though variable efficacy against the tested *P. aeruginosa* isolates. *Rhus coriaria* extract was the most efficient and exhibited bactericidal and bacteriostatic activities with MIC's of 3.6 mg/mL while *Punica granatum* and *Syzygium aromaticum* showed MIC which reached 4.5 mg/mL. These plant extracts which were demonstrated to be potentially efficient could be utilized as natural alternative treatment to eradicate carbapenem-resistant *P. aeruginosa*.

Keywords: carbapenemase; *Rhus coriaria*; *Punica granatum*; *Thymus vulgaris*; *Syzygium aromaticum*; *Curcuma longa*.

Introduction

Carbapenems, such as meropenem (MEM) and imipenem (IPM), are the most effective anti-pseudomonal therapies. They are often used as a last resort in patients with multi-β-lactam-resistant *Pseudomonas* infections (Kazeminezhad et al., 2017). Carbapenems are considered to be the last effective line of defense against *Pseudomonas aeruginosa*; hence, the emergence of carbapenem-resistant *P. aeruginosa* has lately confused treatment options (Al Bahrani et al., 2024). Evidence indicates that individuals infected with carbapenem-resistant bacteria have a higher risk of mortality and morbidity than those infected with susceptible infections (Nordmann & Poirel, 2019). Carbapenemases can cause resistance to carbapenems and other β-lactam medicines, including novel β-lactam-β-lactamase inhibitors (Reyes et al., 2023). *Pseudomonas aeruginosa* may produce metallo-β-lactamases (MBL) that hydrolyze all carbapenems except aztreonam, which contributes to resistance to carbapenem antibiotics (Doosti et al., 2013; Das, 2023). Therefore, their action is hindered by chelators such as ethylenediaminetetraacetic acid (EDTA) and others. Tazobactam, sulbactam, and clavulanic acid, which are often used to block beta-lactamase enzymes, are ineffective against MBLs (Shirani et al., 2016). Carbapenemases are classified into three out of four β-lactamase classes (Ambler A, B, and D) based on their hydrolytic processes at active sites. Class A and D carbapenemases are known as serine carbapenemases because they contain serine (amino acid) at the site of action (serine-dependent), while class B carbapenemases contain zinc (zinc-dependent) and are commonly referred to as metallo-β-lactamases (Kateete et al.2016). Class B metallo-β-lactamases are plasmid-encoded (or chromosomal) and the most prevalent enzymes, primarily belonging to the IMP, VIM, NDM, SPM, GIM, AIM, DIM, and SIM families (Tsakris et al., 2006; Anvarinejad et al., 2014). The identification of metallo-beta-lactamase (MBL)-generating *P. aeruginosa* is essential for preventing the increase of resistant strains as well as creating novel treatment plans and preventative strategies to manage bacterial infection in burn patients (Anvarinejad et al., 2014). The elevated use / misuse of antibiotic doses is mainly contributing to the occurrence of antimicrobial resistance (Alam et al., 2022). The development of antibiotic resistance and associated toxicity problems restrict the use of these medications, resulting in a revival in phytotherapy research (Radji et al., 2013). Because of the common development of resistance during single-drug therapy treatment of patients with *P. aeruginosa*, natural plant extracts have been identified as a promising way for dealing with and overcoming related infections and resistance mechanisms (Ahmed et al., 2021).

This study aims to investigate the prevalence of metallo-beta-lactamase among *P. aeruginosa* collected from burn infections, utilizing both phenotypic and genotypic methods, and evaluate some ethanolic plant extracts to determine antibacterial activity as a new source of drugs and an alternative treatment approach.

Materials and methods

Pseudomonas aeruginosa isolates. Twenty *P. aeruginosa* isolates were used in the current study, isolated from burn infections and identified

previously phenotypically and molecularly in the Biology Department, Science College, Mosul University, Iraq.

Antibiotic sensitivity test. Mueller Hinton Agar (MHA) plates were used in the disc diffusion approach for antibiotic sensitivity, and the results were explained in accordance with the guidelines provided by the Clinical Laboratory Standards Institute (CLSI, 2023). Discs of antibiotic were used as follows: imipenem (IPM 10 μg), meropenem (MEM 10 μg), aztronam (ATM 30 μg), ceftazidime (CAZ 30 μg), ceftriaxone (CRO 10 μg), cefepime (FEP 10 μg), pipracillin (PRL 100 μg), amikacin (AK 10 μg), tobramycin (TOB 10 μg), ciprofloxacin (CIP 10 μg), levofloxacin (LEV 5 μg), rifampin (RA 5 μg).

Detection of MBL activity phenotypically. Using the IMP-EDTA combined disc tests (IMP-EDTA CDT), all strains resistant to (IPM 10 μg) (size of zone ≤15 mm according to CLSI recommendations, 2023) were subjected to test on MHA by disc diffusion approach to determine MBL action. To clarify this briefly, the test organism was seeded on an MHA plate after an overnight culture was adjusted with 0.5 McFarland, that is equivalent to the concentration of the suspension of bacteria 1.5×10^8 CFU/mL. On the plate, two discs of imipenem (10 μg) were positioned five centimeters apart. One of the discs received 10 μL of a 0.5 M EDTA solution. For 16–18 hours, the plates were incubated at 35 °C. After comparing the inhibitory zones of each disc, test isolates that revealed a size of zone of ≥7 mm for the IMP-EDTA disc in comparison to the single imipenem disc were identified as MBL producers (Joji et al., 2019).

Detection of metallo-beta-lactamase genes of VIM and IMP by polermerase chain reaction. Following the manufacturer's instructions, conventional PCR testing was performed on each isolate to determine if the VIM and IMP genes were present. Table 1 lists the primer sequences specific to the VIM and IMP that were employed in this investigation. All of the total DNA of the bacterial isolates was extracted using the Geneaid DNA extraction kit, then kept at –20 °C until it was processed further. The PCR reaction was performed in a total volume of 25 μL, consisted of 12.5 μL GoTaq Green Master Mix (Promega), 1 μL of the forward and reverse primers, 3 μL of the DNA template, and finally 7.5 μL nuclease-free water to complete the volume. The optimum condition was one cycle at 95 °C for 5 min as initial denaturation. 35 cycles at 95 °C for 30 s were completed for denaturation, at 53 °C for 30 s for annealing, and at 72 °C for 30 s for extension, followed by one cycle at 72 °C for 10 min for final extension. To detect the amplicon bands, gel electrophoresis was carried out with 5 μL of each product and 100 bp of ladder separated on an agarose gel made by 1.5% of agarose. Amplicons were investigated after being observed using a UV transilluminator.

Table 1
Primers for VIM and IMP genes

Gene	Sequence of primer 5'–3'	Size of amplicon, bp	Reference
VIM	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG	390	Joji et al. (2019)
IMP	F- GAAGGCGTTTATGTTCATAC R-GTAAGTTTCAAGAGTGATGC	587	Goundarzi et al. (2023)

Preparation of plant extract. Plant materials for the five plant species used in this study (Table 2) were obtained from the local market in Mosul. The received plants were thoroughly cleansed, decontaminated, washed with distilled water, and then dried in the shade. Dried plant material of each species was crushed into a fine powder that could pass through a 100-mm sieve. To get a clear filtrate, 50 g of the finely ground powder was allowed to soak in 200 mL of ethanol with shaking for 24 hours, purified through eight layers of gauze, centrifuged at 3000 rpm for 5 minutes, and filtered again using Whatman filter paper No. 41. The filtrate solutions were evaporated and dehydrated at 45 °C in a cooled incubator (Gallenkamp). The extract outputs were measured and placed in tiny vials in the refrigerator at 5 °C (Mostafa et al., 2018).

Determination of the antimicrobial activity by Well diffusion susceptibility method. The bacterial suspensions were prepared by comparison with standard tube (McFarland number 0.5) containing 1.5×10^8 CFU/mL. Bacterial suspension was streaked into Mueller-Hinton agar plates. Cotton swabs were dipped into a screw tube containing bacterial suspension and spread over the surface of the plates, after which the plates were allowed to dry for 5–15 minutes at the ambient temperature. Several wells (8 mm in diameter) were made into the medium of agar with a sterile cork borer and then plant extracts in 24 μL volumes containing 7.2, 9.6, 12.0 mg for thyme and turmeric, sumac, pomegranate and clove respectively were poured into the wells, An DMSO (24 μL per well) was also poured into one well as negative control, then allowed to incubate at 37 °C for 24 hrs and zones of inhibition were measured in mm (Bakht et al., 2011; Al-Ahbabi et al., 2016).

Table 2
Data of the plant species used

Species	Families	Local names	Common names	Plant part utilized
Rhus coriaria L.	Anacardiaceae	sumack	sumac	fruit
Syzygium aromaticum (L.) Merr. & L.M.Perry	Myrtaceae	koronfil	clove	flower
Punica granatum L.	Lythraceae	romman	pomegranate	peel
Curcuma longa L.	Zingiberaceae	kurkum	turmeric	rhizome
Thymus vulgaris L.	Lamiaceae	za'ater	thyme	leave

Determination of the minimum inhibitory concentration of the ethanolic extracts (MIC). MIC is described as the lowest concentration of the antimicrobial substance that inhibits the growth of microbes after 24 hours of incubation. The most efficient plant extract which showed a strong antibacterial activity at 12.0 and 9.5 mg/mL was used to detect their MIC by well-diffusion method. Several concentrations of the efficient plant extract (2.25, 4.5, 6.0 and 12.0 mg/mL) were prepared separately for *P. granatum* and *S. aromaticum*, and (1.8, 3.6, 4.8 and 9.6 mg/mL) prepared for *R. coriaria* DMSO used as diluent.

Results

Bacterial strains were collected from burn infections. The antimicrobial susceptibility test of twenty *P. aeruginosa* isolates to 12 various antibiotics revealed that from 20 *P. aeruginosa* isolates, 17 (85%) were resistant to imipenem. 95% of the strains were meropenem, levofloxacin and ciprofloxacin resistant. All the isolates were resistant to eight antibiotics (100%), which is shown in Figures 1 and 2.

To investigate whether resistance to imipenem is produced by MBL synthesis or different mechanisms, the 17 isolates were tested using the IMP-EDTA CDT. During phenotyping, it was shown that 11 (64.7%) of the 17 strains were positive for MBL. Figure 3 shows an example.

Genotyping of all 20 bacterial isolates was implemented to detect the existence of the IMP and VIM genes. In Figure 4, the results show that among 17 isolates resistant to imipenem, 7 (41.2%) strains carried the VIM gene, and no strain carried the IMP gene. None of the isolates sensitive to imipenem harbored these genes.

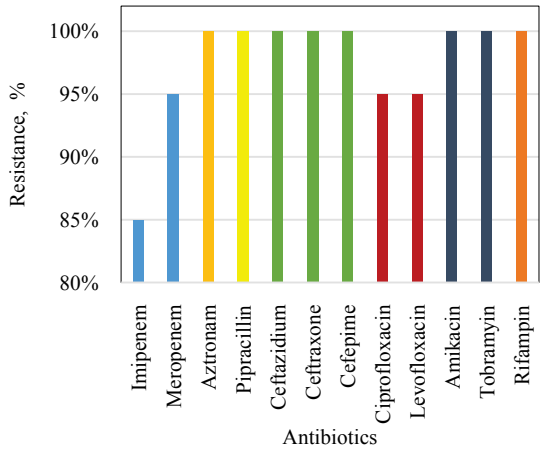

Fig. 1. Antibiotics resistance pattern of twenty *P. aeruginosa* isolates to 12 antibiotics

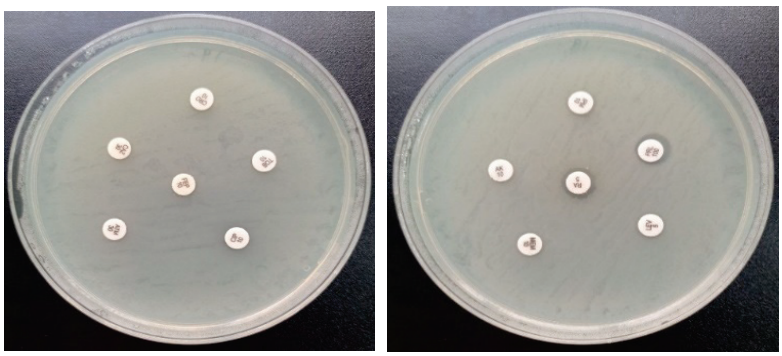

Fig. 2. Pattern of antibiotics resistance of *P. aeruginosa* isolate to 12 antibiotics

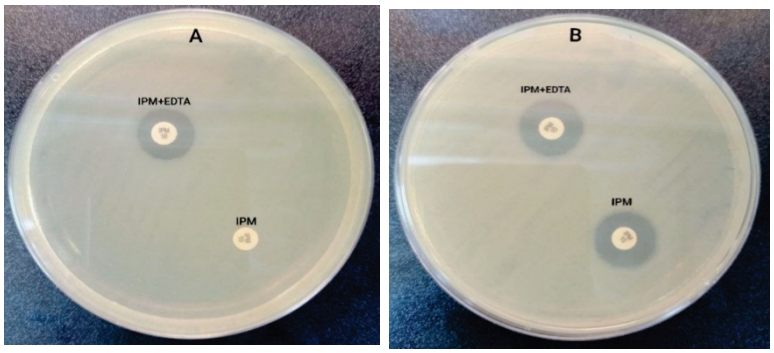

Fig. 3. IMP-EDTA CDT exhibiting isolate A as MBL producer and isolate B as non-MBL producer

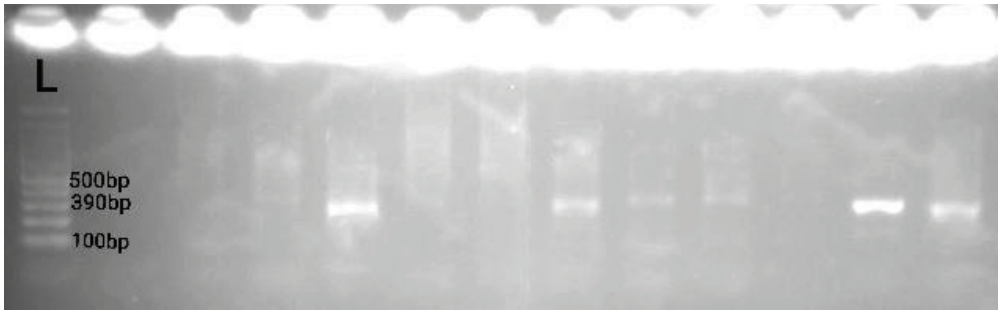

Fig. 4. Gel electrophoresis showed PCR products: whereas some lanes revealed bands with amplicon size equal 390 bp (VIM gene), some lanes without band for VIM gene, lane ladder contains a 100 bp

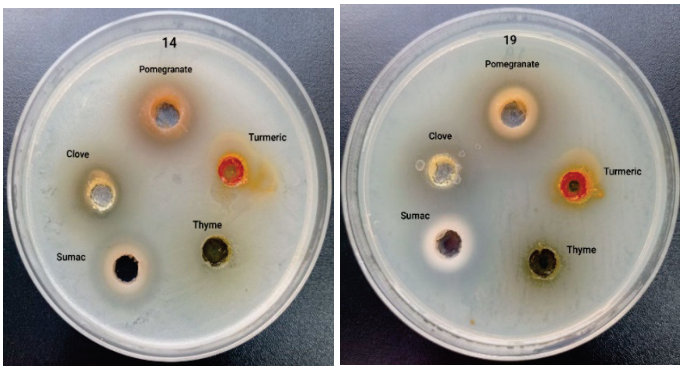

Fig. 5. Growth inhibition of two bacterial isolates initiated by ethanolic plant extracts clove, pomegranate, sumac, turmeric and thyme

Some plant species were studied to determine their antimicrobial capability against carbapenem-resistant *P. aeruginosa*, as shown in Figure 5. The findings demonstrated that all plant extracts have the ability to inhibit the microbial replication of carbapenem-resistant *P. aeruginosa* with variable efficacy.

Discussion

Our study showed the prevalence of multidrug-resistant (MDR) phenotype in 100% of the isolates of *P. aeruginosa* obtained from burn infections. Emergence of resistance to carbapenem in *P. aeruginosa* has become a serious threat. Resistance can develop via a variety of strategies, including the biosynthesis of carbapenemase enzymes, reduced permeability of the bacterial cell wall, raised efflux pump activity, modifications of outer membrane porins, and target-specific mutations that impair affinity to carbapenems (Gashaw et al., 2024). Periodic antimicrobial resistance monitoring in *P. aeruginosa* is essential for knowing the current activity level of commonly administered antipseudomonal medicines. In the current research, carbapenems were among the least active drugs investigated, with just 5% and 15% of isolates sensitive to meropenem and imipenem, respectively. Several recent investigations have found imipenem to

be particularly active against *P. aeruginosa* (Abdeta et al., 2023; Hafiz et al., 2023).

Table 3
Antimicrobial test of ethanolic plant extracts against *P. aeruginosa* (inhibition zone in mm)

Isolate number	Ethanolic plant extract				
	pomegranate, 12 mg/mL	clove, 12 mg/mL	sumac, 9.6 mg/mL	turmeric, 7.2 mg/mL	thyme, 7.2 mg/mL
14	19	12	16	0	0
19	18	14	16	0	11

Table 4
MIC of the greatest efficient ethanolic plant extracts against *P. aeruginosa*

Plant extract	Concentration, mg/mL	Inhibition zone, mm
R. coriaria	1.8	0
	3.6	10
	4.8	12
	9.6	16
P. granatum	2.3	0
	4.5	11
	6.0	16
	12.0	19
S. aromaticum	2.3	0
	4.5	9
	6.0	10
	12.0	12

A prior study conducted in Iraq by Ronat et al. (2014) reported 23% imipenem resistance in *P. aeruginosa* (Ronat et al., 2014). But, our results revealed an increase in resistance to imipenem (85%). Also previous studies from Iraq and other countries reported variable rates of imipenem resistance to *P. aeruginosa* among patients. In Karbala, imipenem resistance was 58.3% (Shilba et al., 2015), in Duhok 60.6% (Polse et al., 2024), in Saudi Arabia 29.5% (Hafiz et al., 2023) and in Ethiopia 16.0% (Abdeta et al., 2023). Previous studies showed a similar trend from other countries to our study (Rodríguez-Martínez et al., 2009; Gill et al., 2016). The variation in the occurrence of imipenem resistant *P. aeruginosa* in various studies could be related to the type of isolates, type of hospitals, the studied population and geographical locations. Our recent investigation found a substantial prevalence of phenotypic carbapenem resistance. This rise in resistance might be related to the increased use of carbapenems in hospitals and inadequate infection control methods. Infections produced by these resistant strains significantly restrict treatment choices.

PCR evaluation is the important standard for MBL. However, it is not commonly employed in typical microbiology labs. This study employed phenotypic techniques like the IMP-EDTA combined disc test for MBL testing and then compared it with genotyping findings. IMP-EDTA CDT phenotyping revealed that 11 (64.7%) of the 17 isolates resistant to imipenem produced MBL. Similarly, a study in Iran detected MBL activity in 65 of 69 imipenem-resistant strains (Saderi et al., 2010). Also, a study found that 90% of imipenem-resistant isolates were positive for production of MBL (Moosavian & Rahimzadeh, 2015). Many factors contribute to these variations, including variability in strains from different places, the type of equipment and antibiotics used, the amount of EDTA/disk used, and mistakes committed by the individual doing the testing. However, we encourage utilizing the CD test to detect MBL-producing *P. aeruginosa* since it is easy to implement, can be quickly integrated into a clinical laboratory workflow, and is less costly than other methods (Sheikh et al., 2014).

Our genotyping results showed that among 17 imipenem resistant strains, 7 (41.2%) strains carried the VIM gene, and no strain carried the IMP gene. None of the imipenem-sensitive isolates harbored these genes, which is similar to a study in Bahrain where the VIM gene was found in 47.5% of strains (Joji et al., 2019). A study in Al-Najaf city detected (bla-VIM, bla-IMP) with the following percentages (10.4% and 22.9%, respectively) (Almayali & Al-Muhana, 2023). A study in Iran showed that 56.0% carried blaVIM and 24.3% possessed the blaIMP gene (Doosti et al., 2013). A study in Turkey showed the presence of VIM in one of the *P. aeruginosa* strains. None of the strains were positive for the IMP from 200 carbapenem-resistant *P. aeruginosa* isolates (Cayci et al., 2022), whereas a study in Saudi Arabia noted VIM in all 15 MBL carrying isolates (100%) (Tawfik et al., 2012). Geographical differences and variation in the use of antibiotics may explain variation in the occurrence and categories of these genes found in MBL-synthesizing bacteria (Joji et al., 2019). Also may be these genes are plasmid encoded.

Antibacterial activity of five ethnolic plants extract was evaluated against two isolates of carbapenem-resistant *P. aeruginosa* which have high resistance to all antibiotics used in our study using the well diffusion method. The results revealed that three plant extracts were potentially effective in suppressing microbial growth with variable potency, *R. coriaria*, *P. granatum* and *S. aromaticum* at concentration 9.6, 12.0, 12.0 mg/mL, respectively. *T. vulgaris* was found to suppress microbial growth in one strain at concentration 7.2 mg/mL so it can be used at higher concentration to achieve inhibitory effect against *P. aeruginosa*. A study in Iran revealed that ethanol extracts of *T. vulgaris* reached MIC values of 15.6 mg/mL (Ahmadi et al., 2022). Also, *C. longa* can be used at a higher concentration than 7.2 mg/mL to exert antibacterial activity, as shown by a study in Nepal where the plant exhibited MIC at 40 mg/mL (Suwal et al., 2021).

The MIC of the most effective plant extracts was used by well diffusion technique to estimate their bactericidal and bacteriostatic attributes. This method showed that there was a significant relationship between the concentration and growth inhibition zone diameter. The concentration effects of the efficient plant extracts (*R. coriaria*, *S. aromaticum* and *P. granatum*) are presented in Table 4. The inhibitory effect of *R. coriaria* extract began at 3.6 mg/mL with inhibition zone of 10 mm while extract of *P. granatum* and *S. aromaticum* suppressed bacterial growth at concentration of 4.5 mg/mL with inhibition zones of 11 and 9 mm respectively. Similarly, a study in Saudi Arabia showed bacteriostatic and bactericidal activities of *P. granatum* and *S. aromaticum* ethanolic extracts against *P. aeruginosa* with MICs which reached 2.5 and 5.0 mg/mL, respectively (Mostafa et al., 2018). A study in Iran revealed antibacterial activity of *R. coriaria* ethanolic extracts with MIC equal 8.0 mg/mL against MDR isolates of *P. aeruginosa* (Ahmed et al., 2022). In a Slovenian study on *P. aeruginosa* growth inhibition, the highest applied concentration (2.7 mg/mL) of ethanolic peel extract reduced growth by 87% (Kupnik et al., 2016).

According to a study in Karbala, Iraq, showed ethanolic *P. granatum* peel extract to be ineffective against *P. aeruginosa* (Al-Daamya et al., 2016). Therefore, the final concentation in the well would become 1.25 mg/mL according to Bakht et al. (2011). This concentration is low when tested against *P. aeruginosa*. However, a study in Iran showed that the MICs of pomegranate peel extracts were 12.5 mg/mL (Nozohour et al., 2018). According to another study Kupnik et al. (2021), *P. granatum* fruit has outstanding antioxidant capabilities, as well as anti-atherosclerotic, antihypertensive, anti-inflammatory, and anti-mutagenic characteristics, which promote wound healing.

The MIC values for *S. aromaticum* ethanolic extract in a study in Egypt showed ranged higher than our study from 10.0 to 21.3 mg/mL (Ahmed et al., 2021) while a study in India revealed that the MIC was 1.56 mg/mL (Faujdar et al., 2020). This variance in MIC might be caused by differences in phytochemical content throughout the plant, as well as geographical and environment-related factors that may influence the bioactivity level of secondary metabolites and the methodologies utilized in measurement.

Conclusion

Ethanolic extracts of pomegranate, sumac, and clove at the abovementioned concentrations, as well as thyme and turmeric at higher concentrations, were found to be effective against carbapenem-resistant *P. aeruginosa* and supplied baseline data for the possible treatment of bacterial infections. As a result of our research, we determined that these extracts can be utilized to generate novel antibacterial drugs, which are urgently needed. However, further study is required to identify and characterize the bioactive compounds observed in these extracts, as well as their *in vivo* antibacterial properties against human infections.

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