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ANTIBACTERIAL ACTIVITY OF *Mangifera indica* SEED EXTRACTS COMBINED WITH COMMON ANTIBIOTICS AGAINST MULTIDRUG-RESISTANT *Pseudomonas aeruginosa* AND *Acinetobacter baumannii* ISOLATES

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Abstract

In this project, we employed ethanolic (EMI) and aqueous (AMI) extracts of mango (*Mangifera indica* L., Anacardiaceae) fruit seeds as a modulator of antibiotic resistance against multidrug-resistant (MDR) *Pseudomonas aeruginosa* and *Acinetobacter baumannii* to evaluate natural compounds isolated from by-products or waste of edible plants. We also investigated the effect of these extracts alone and in combination with standard classes of antibiotics in the desired strains. *M. indica* seeds were processed and exploited using ethanol and water. The minimum inhibitory concentrations (MICs) of clinical isolates were examined against EMI and AMI extracts, followed by seven antibiotics of ceftazidime, ciprofloxacin, penicillin, amikacin, meropenem, ampicillin, and colistin. The checkerboard method evaluated the synergistic action between mango kernel extract (EMI) and seven antibiotics. EMI extract significantly revealed antimicrobial properties against MDR *A. baumannii* and *P. aeruginosa* with synergistic effects with the applied antibiotics. The considerable antibacterial efficacy of ethanolic extract of *M. indica* seeds can have great curative value as antibacterial drugs against infections caused by MDR *P.aeruginosa* and *A. baumannii*.

Keywords: *Acinetobacter baumannii*. Antimicrobial activity. *Mangifera indica.* Multidrug resistance. *Pseudomonas aeruginosa*.

1. Introduction

In recent years, the emergence of antibiotic-resistant bacteria as a worldwide concern has increased (Tacconelli et al. 2018; Jahangiri et al. 2021). Multi-drug resistant gram-negative bacilli (MDR-GNBs) like *P. aeruginosa* and *A. baumannii* have been described lately by World Health Organization (WHO) as a severe health threat (Delgado-Valverde et al. 2020; Jahangiri et al. 2021). This problem can lead to delay or failure of antimicrobial therapy and an increase in mortality (Safaei et al. 2017; Abd El-Baky et al. 2020) due to the resistance of these strains to first-line anti-gram-negative antibiotics, like fluoroquinolones and β-lactams (El-Mokhtar and Hetta 2018; Abd El-Baky et al. 2020). In this regard, some resistance mechanisms in *A. baumannii* and *P. aeruginosa* are efflux pumps, drug-inactivating enzymes, and changes in the membrane or target site (Livermore 2002; Bonomo and Szabo 2006; Zavascki et al. 2010). However, there is still no consensus among the medical society on the best definition for MDR bacteria (Falagas et al. 2006; Paterson and Doi 2007; Falagas and Karageorgopoulos 2008; Hirsch and Tam

2010). Most published studies have described resistance to three or more drugs, mainly aminoglycosides, carbapenems, cephalosporins, and fluoroquinolones, as multi-drug resistance (Gales et al. 2001). The WHO has released a priority pathogens list that instantly needs to produce new drugs, such as carbapenem-resistant *P. aeruginosa* and *A. baumannii* (WHO 2017; Delgado-Valverde et al. *2020).*

A. baumannii is a gram-negative bacillus, defined as non-fermenting, aerobic, non-motile, non-fastidious, catalase-positive, oxidase-negative bacteria (Perez et al. 2007; Montefour et al. 2008; Howard et al. 2012). Although it has been believed that *A. baumannii* was sensitive to most antibiotics previously, the pathogen has become resistant to nearly all first-line antibiotics nowadays (Howard et al. 2012). Indeed, MDR *A. baumannii* has become a cause of nosocomial, community-acquired, and healthcare-associated infections (Peleg et al. 2008; Howard et al. 2012; Barrasa-Villar et al. 2017). This pathogen is related to meningitis, pneumonia, bacteremia, septicemia, and surgical, skin, and urinary tract infections (UTIs) (Shin and Park 2017; Mustapha et al. 2018; Kara et al. 2019). Since treating MDR *A. baumannii* infections is challenging, tigecycline and polymyxins have been prescribed as the final therapeutic option (Ls and Weinstein 2008; Arroyo et al. 2009). However, tigecycline and polymyxins-resistant strains of *A. baumannii* have also been reported by scientists (Li et al. 2006; Park et al. 2009).

In addition, P. aeruginosa is a gram-negative, non-fermentative (Livermore 2002; Grossi and Gasperina 2006; Dijkshoorn et al. 2007) and opportunistic bacterium found in soil, water, plants, and hospital environment (Goli et al. 2016; Farhan et al. 2019; Sheikh et al. 2019). It is associated with UTIs, pneumonia, burns, wounds, surgical infections, and sepsis in intensive care units (ICUs) (Weinstein et al. 2005; Gad et al. 2007). Generally, when MDR isolates of *P. aeruginosa* cause infections, the mortality and morbidity of these infections increase (Zavascki et al. 2006; Zavascki et al. 2010).

It is worth mentioning that both of these bacteria are among ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter*) (Zuniga-Moya et al. 2020) and significant nosocomial pathogens (Zavascki et al. 2010). Furthermore, the problem of drug resistance in *P. aeruginosa* and *A. baumannii* limits their treatment of infections. So, it is a challenge for physicians (Boucher et al. 2009; Akbari et al. 2019). Influential factors in pathogenicity and resistance to antibiotics include the presence of lipopolysaccharide and lipid A, stress response mechanism, the ability to adapt to the environment, genetic exchanges, the ability to receive mobile genetic elements, such as plasmids, transposons, and integrons, and the formation of biofilm, which is the most critical factor in the virulence and high antibiotic resistance of these bacteria (Longo et al. 2014). On the other hand, several genes are effective in biofilm formation in *A. baumannii* and *P. aeruginosa*, including *bap* (Biofilm associated protein) gene, csuA, B, C, D, E, A/B operon, pgaA, B, C, D operon genes, *ompA* (Outer membrane protein) gene, and *luxI* gene (Kodori et al. 2017). The expansion of new antibacterial elements to control diseases of *A. baumannii* and *P. aeruginosa* has been slow (Peck et al. 2012; Kara et al. 2019), Indeed, no helpful commercial antibiotics have been recommended so far (Ahmad et al. 2016; Chatterjee et al. 2016; Lee et al. 2017; Merakou et al. 2018). Therefore, there is a need for research on effective treatment options without side effects (El-Gied et al. 2012; Jahangiri et al. 2021). Natural resources are more admissible to consumers, unlike chemically synthesized antimicrobial factors. As a result, new element production from natural resources is one of the research fields (Kabuki et al. 2000). Moreover, combination therapy may help overcome resistance (Bergen et al. 2011; Ly et al. 2015).

Mango fruits (*M. indica* L., Anacardiaceae), which belong to the genus *Mangifera* and the family Anacardiaceae (Arulselvi et al. 2010; Donga et al. 2020; Donga and Chanda 2021), grow in tropical and subtropical regions (Kabuki et al. 2000; Jahurul et al. 2015). Each part of a mango tree, like flowers, leaves, roots, seeds, barks, peels, and fleshes of the fruit, has numerous pharmaceutical properties and is traditionally used to cure several diseases and pains (Donga et al. 2020; Jahurul et al. 2015). Also, it has anti-diabetic, anticancer, anti-ulcer, anti-inflammatory, anti-hemorrhagic, antibacterial, antiviral, anti-parasite, antifungal, and antiseptic properties. It has been reported that mango can cure diarrhea, colic, bronchitis, cough, hypertension, rheumatism, toothache, leucorrhoea, anemia, abscesses, tumor, insomnia, anthrax, bacillosis, and tetanus (Arulselvi et al. 2010; Shah et al. 2010; Donga and Chanda 2021). Moreover, the peels and seeds are the central part of mango processing (Jahurul et al. 2015). Various extracts of the seeds, barks, and leaves of *M. indica* are active against human pathogens (Rajan et al.

2015). Since *M. indica* is commonly available and cheap, it would be valuable to evaluate its antimicrobial effect (Sairam et al. 2003).

This research aims to assess the antibacterial activity of ethanolic and aqueous extracts of *M. indica* seeds against MDR *P. aeruginosa* and *A. baumannii*. Then, we evaluate the impact of ethanolic extract of *M. indica* seeds combined with standard classes of antibiotics used to treat these infections.

2. Material and Methods

Bacterial samples and antibiotics

Our study included eight strains of MDR *A. baumannii* and nine strains of MDR *P. aeruginosa.* These bacterial samples were isolated from the patients of Ghaem Hospital and Emam Reza Hospital of Mashhad University of Medical Sciences. The species accuracy of these seventeen isolates was previously confirmed in the hospitals using the polymerase chain reaction (PCR) test. We also defined antibiotic susceptibility by the disk diffusion technique based on guidelines from the Clinical and Laboratory Standards Institute (CLSI-2020) (Bhaumik et al. 2022). We employed antibiotic discs, including ceftazidime (CAZ), ciprofloxacin (CIP), penicillin (PEN), amikacin (AMK), meropenem (MEM), ampicillin (AMP), cefepime (CPM), co-trimoxazole (SXT), imipenem (IPM) and colistin (CST).

Extraction of Mango seeds

The mango fruit was bought from a fruit shop and washed with water. After breaking, the seeds were separated, shade-dried at room temperature, powdered, and exposed to the maceration procedure with ethanol and water individually. After taking a certain amount of powdered mango seeds, the raw extract was provided by adding water and ethanol. Then, the EMI and AMI extracts of *M. indica* seeds were dried at a 55-60 ◦C temperature by a rotary evaporator and stored. Storage solutions of aqueous and ethanolic extracts with 75,000 µg/ml concentrations were prepared and stored.

Determination of minimum inhibitory concentration (MIC)

The MICs of seventeen isolates were performed against AMI and EMI seed extracts, further as seven antibiotics of ceftazidime, ciprofloxacin, penicillin, amikacin, meropenem, ampicillin, and colistin in 96-well microplates with 100 µl of Brain Heart Infusion (BHI) broth included 0.01% of Triphenyl-2,3,5-tetrazolium chloride indicator as described by the CLSI recommendation (Wiegand et al. 2008). First, 100 µl of the initial concentration of each antimicrobial agent (256 µg/ml for antibiotics and 128 µg/ml for mango extracts) was added to the first well of each column. Then, serial two-fold dilutions were prepared directly for each antimicrobial agent in a 96-well microplate. Bacterial suspensions were prepared in BHI broth until the culture reached the turbidity equal to that of 0.5 McFarland standard (1×10^8 CFU ml^{-1}) and then diluted 1:200 in BHI broth for the test. 100 µl of inoculum was added to each microplate well (5×10^4 CFU/well). The microplate was covered with sterile plastic bags to prevent and reduce evaporation and then incubated for 18-24 hours at 37 °C. Sterile BHI and bacterial suspension in BHI were put into columns 12 and 11 wells as a negative and positive control, respectively. After that, we scanned the microplate. If the bacteria grow, they will turn red. If the bacteria do not increase, there will be no discoloration, which means their growth has been inhibited. Accordingly, the MIC was the lowest antimicrobial agent concentration that inhibited 90% of bacterial growth.

Checkerboard assay

A checkerboard assay was performed to determine the antimicrobial effects of aqueous and ethanolic extracts of *M. indica* seeds combined with seven antibiotics of ceftazidime, ciprofloxacin, colistin, amikacin, meropenem, ampicillin, and penicillin against *P. aeruginosa* and *A. baumannii* in 96-well microplates. For assessing the combined effect of S and L antimicrobial factors (S: antibiotic and L:

antimicrobial extract) on bacterial isolates in this assay, first of all, 50 µl of BHI broth containing 10^6 CFU ml^{-1} of bacterial sample and 0.01% of Triphenyl-2,3,5-tetrazolium chloride indicator was added to each well of 96-well microplate. Then, 50 µl of S (antibiotic) with the concentration of 4MIC was added to the first well of row A, and 50 µl of it with the attention of 2MIC was added to other wells in row A. Subsequently, serial two-fold dilutions were prepared directly in B-H row wells. After that, 50 µl of L (antimicrobial extract) with a concentration of 2MIC was added to all wells of the first column. Then, serial two-fold dilutions were prepared in all wells of columns 2 to 10. Sterile BHI and bacterial suspension in BHI were put into columns 12 and 11 wells as a negative and positive control, respectively. Then, the plates were incubated at 37 °C for 18-24 hours and scanned to check for microbial growth. If the bacteria grows, it will be marked with red color at the bottom of the well. The fractional inhibitory concentration index (FICI) was calculated for each combination separately. The FICI is expressed as FICI = FICS + FICL, where FICS is the MIC of S in combination/MIC of S alone, and FICL is the MIC of L in combination/MIC of L alone. FICI ≤ 0.5, 0.5 < FICI ≤ 1.0, 1.0 < FICI ≤ 4.0, and FICI > 4.0 were interpreted as synergy, additive, indifferent, and antagonism, respectively.

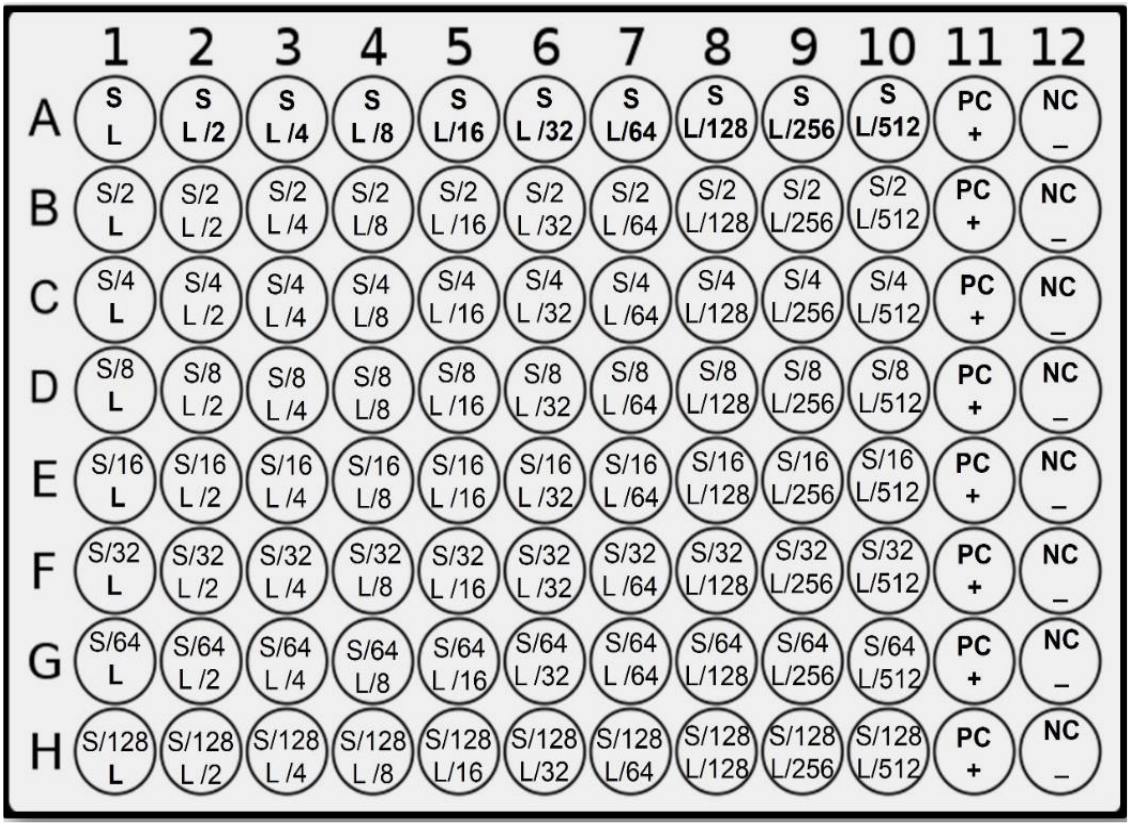

Figure 1. 96-well microplate containing serial dilutions of S and L antimicrobial factors (S: antibiotics and L: antimicrobial extract) to determine their combined effect by the checkerboard assay.

3. Results

The MIC results of ethanolic and aqueous extracts of *M. indica* seeds against drug-resistant bacterial strains of *P. aeruginosa* and *A. baumannii* are prepared in Table 1. According to these results, the ethanolic extract of *M. indica* seeds had an antimicrobial effect facing MDR *P. aeruginosa* and *A. baumannii*. However, the aqueous extract of *M. indica* seeds had no antimicrobial effect against these bacteria. Also, combining EMI extract with the standard antibiotics (ceftazidime, ciprofloxacin, penicillin, amikacin, meropenem, ampicillin, and colistin) showed synergistic effects to reduce the growth of the studied strains, demonstrated in Table 2.

Table 1. MIC values of *M. indica* seed extracts and antibiotics against MDR *P. aeruginosa* and *A. baumannii.*

Bacterial Strains	MIC (µg/mL)								
	EMI	AMI	PEN	CST	CAZ	AMK	MEM	AMP	CIP
AB-MDR1	8	>128	≥256	0.5	128	128	64	≥256	≥256
AB-MDR2	8	>128	≥256	64	128	128	128	≥256	≥256
AB-MDR3	8	>128	≥256	2	≥256	≥256	16	≥256	≥256
AB-MDR4	8	>128	≥256	2	128	128	32	≥256	≥256
AB-MDR5	32	>128	≥256	8	≥256	128	16	≥256	≥256
AB-MDR6	64	>128	≥256	32	≥256	≥256	32	≥256	≥256
AB-MDR7	16	>128	≥256	1	≥256	128	128	≥256	≥256
AB-MDR8	64	>128	≥256	≥256	≥256	≥256	128	≥256	≥256
PA-MDR1	64	>128	≥256	1	≥256	8	≥256	≥256	≥256
PA-MDR2	64	>128	≥256	0.12	≥256	32	≥256	≥256	≥256
PA-MDR3	64	>128	≥256	2	≥256	64	≥256	≥256	≥256
PA-MDR4	64	>128	≥256	0.25	≥256	16	≥256	≥256	≥256
PA-MDR5	64	>128	≥256	1	≥256	32	≥256	≥256	≥256
PA-MDR6	32	>128	≥256	2	≥256	≥256	≥256	≥256	≥256
PA-MDR7	16	>128	≥256	32	≥256	32	≥256	≥256	≥256
PA-MDR8	16	>128	≥256	4	≥256	8	≥256	≥256	≥256
PA-MDR9	16	>128	≥256	1	≥256	8	≥256	≥256	≥256

AB, *A. baumannii*; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; PA, *P.aeruginosa*; EMI, ethanolic extract of *M. indica;* AMI, aqueous extract of *M. indica*; PEN, penicillin; CST, colistin; CAZ, ceftazidime; AMK, amikacin; MEM, meropenem; AMP, ampicillin; CIP, ciprofloxacin.

Table 2. Synergistic activities of *M. indica* seed extracts and antibiotic combinations against MDR *A. baumannii* and *P. aeruginosa.*

Combination	Bacterial strains	ΣFIC	Drug interaction
EMI + CIP	PA-MDR	0.271	synergism
EMI + PEN	PA-MDR	0.358	synergism
EMI + MEM	PA-MDR	0.199	synergism
EMI + CST	PA-MDR	0.410	synergism
EMI + CAZ	AB-MDR	0.374	synergism
EMI + AMP	AB-MDR	0.216	synergism
EMI + AMK	AB-MDR	0.483	synergism
EMI + MEM	AB-MDR	0.192	synergism
EMI + CIP	AB-MDR	0.500	synergism

AB, *A. baumannii*; MDR, multidrug-resistant; ΣFIC, The total fractional inhibitory concentration; PA, *P.aeruginosa*; EMI, ethanolic extract of *M. indica*; PEN, penicillin; CST, colistin; CAZ, ceftazidime; AMK, amikacin; MEM, meropenem; AMP, ampicillin; CIP, ciprofloxacin.

4. Discussion

In the past, when antibiotics were sufficiently responsive, infections caused by microbial pathogens, such as *P. aeruginosa* and *A. baumannii*, were not considered a critical threat to human health. Nevertheless, with the quick spread of antibiotic-resistant bacterial strains in recent years, existing antibiotics' inadequate efficacy and side effects have become a crisis (Exner et al. 2017). This study examined two strategies to defy antibiotic-resistant bacteria (MDR *P. aeruginosa* and *A. baumannii*). Initially, aqueous and ethanolic extracts of *M. indica* seeds were used as new antimicrobial candidates. Then, we considered the combined effect of ethanolic mango seed extracts and current antibiotics to find possible synergistic activities.

There are some valuable discoveries from the former studies, including the the antibacterial activity of mango kernel extract (MKE) against *Staphylococcus*, *Escherichia*, *Klebsiella*, *Salmonella*, *Listeria*, *Clostridium*, *Bacillus*, *Aeromonas*, *Campylobacter*, *Yersinia*, *Vibrio* (Kabuki et al. 2000), methanolic (MMI) and AMI seed extracts against *Streptococcus*, *Klebsiella*, *Escherichia*, and *Proteus* (Sairam et al. 2003), seed extracts of mango against *Staphylococcus*, *Streptococcus*, *Yersinia*, *Salmonella*, *Shigella, Escherichia*, *Pseudomonas*, *Bacillus*, *Mycobacterium*, *Nocardia*, *Listeria*, *Citrobacter*, *Enterobacter* (El-Gied et al. 2012), aqueous and alcoholic extracts of mango stalk bark against *staphylococcus*, *Klebsiella*, *Escherichia*,

Pseudomonas, and *Bacillus* (Arulselvi et al. 2010), mango seed extracts to combat *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Escherichia*, and *Klebsiella* (Shabani and Sayadi 2014).

The robust antibacterial effect of *M. indica* extracts has previously been examined. In a study by Kabuki *et al*. (Kabuki et al. 2000), MKE showed significant antibacterial activity against both gram-negative and gram-positive strains. Their MIC values varied among species, similar to our results on MDR gram-negative bacteria (*P. aeruginosa and A. baumannii*). Moreover, Sairam *et al.* (2003) pointed out that AMI inhibited the growth of *Streptococcus aureus* and *Proteus vulgaris*. Still, contrary to our findings, EMI has a significant antimicrobial effect against *P. aeruginosa and A. baumannii*, yet the AMI does not affect the desired bacteria. In another investigation by El-Gied *et al.* (2012), the EMI and MMI seed extracts showed significant antimicrobial effects against nearly all tested bacteria with variable MICs, in agreement with our findings. However, the advantage of our study was working on the MDR strains. Also, Arulselvi *et al.* (Arulselvi et al. 2010) concluded that both extracts represented significant antimicrobial properties. Their studies determined that the aqueous extract has a better impact on inhibiting the growth of gram-positive bacteria than gram-negative bacteria. Likewise, the alcoholic extract showed a better result on gram-negative bacteria than gram-positive bacteria, which is similar to the effect of our alcoholic extract against gram-negative bacteria (*P. aeruginosa* and *A. baumannii*). Furthermore, Shabani *et al.* (2014) reported that AMI and EMI seed extracts had the strongest antimicrobial effects, which is similar to the antimicrobial results of our EMI seed extract (Shabani and Sayadi 2014).

In addition, combination therapy with using by-products of natural plants and antibiotics may help overcome the issue of drug resistance, which is possible by exploring the synergism of antimicrobial agents (Bergen et al. 2011; Ly et al. 2015). In this regard, Souto de Oliveira *et al.* (2011) considered tetracycline, erythromycin, and norfloxacin synergistic activity with EMI peel extract against *S. aureus*. Their study demonstrated that *M. indica* peel extract could assist antibiotics as a potential adjuvant, which increases the value of this mango by-product (Oliveira et al. 2011). The results of the synergistic effects of their study are similar to our work. In another study, Adikwu *et al.* (2010) analyzed the combined effects of the MMI extract of plant leaves called *Euphorbia hirta* and erythromycin on clinical strains of *S. aureus* using the checkerboard assay that the desired bacteria were sensitive to the extract. As a result, a synergistic effect was obtained by combining *E. hirta* and erythromycin against *S. aureus* (Adikwu et al. 2010). Although we examined the synergistic effect of more antibiotics, the combined result of their research shows similarity with our findings. *Adwan et al.* (2010) also assessed the combined effects of ethanolic extracts of *Rhus coriaria* seed, *Rosa damascene flower, Sacropoterium spinosum* seed, and several antibacterial drugs, such as penicillin G, oxytetracycline, cephalexin, and enrofloxacin against MDR *P. aeruginosa* that the synergy between antibiotics and *R. coriaria* significantly reduced MIC values (Adwan et al. 2010). Our findings revealed that the synergistic effect of these extracts was similar to the synergistic effect of mango extract.

We recommend that other researchers investigate the effect of mango seed extracts on other gram-negative and positive bacteria in future studies. Although we used the high antimicrobial properties of mango seed extract in this study, we suggest more comprehensive studies to evaluate the antimicrobial properties further.

5. Conclusions

Our study found that the ethanolic extract of *M. indica* seeds could have a favorable inhibitory effect on the growth of MDR strains of *P. aeruginosa* and *A. baumannii*. However, the aqueous extract of *M. indica* seeds could not defeat the MDR strains of *P.aeruginosa* and *A. baumannii*. Furthermore, our prepared extracts had a synergistic effect with conventional antibiotics. They were also cheap, without side effects, and commonly available. They can lead to a decrease in MIC results alone. Therefore, we can use them to treat diseases caused by MDR strains of *P. aeruginosa* and *A. baumannii* infections.

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BAFGHI, M.: conception and design, analysis and interpretation of data, drafting the article, critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

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