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Syzigium cumini and *Mangifera indica* Seed Extracts: *In Vitro* Assessment for Antibacterial Activity Alone and in Combination with Antibiotics against Clinical Bacteria

Malay Kumar Das and Shyamapada Mandal*

Department of Zoology, University of Gour Banga, India

***Corresponding author**: Shyamapada Mandal, Laboratory of Microbiology and Experimental Medicine, Department of Zoology, University of Gour Banga, Malda-732103, India, Tel: 91-9831279239; E-mail: samtropmed@gmail.com

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Abstract

Objective: To determine the antibacterial activity of *Syzigium cumini* (Family: Myrtaceae) and *Mangifera indica* (Family: Anacardiaeceae) seed extracts, alone and in combination with some conventionally used antibiotics, against clinical isolates of *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*.

Method: The antibacterial activity of ethanolic *S. cumini* seed extract (SSE) and *M. indica* seed extract (MSE), at different concentrations, were determined by disk diffusion. The combined activity with antibiotics of the extracts (SSE and MSE) was determined against the test isolates. The ZDI (zone diameter of inhibition) values for the agents (alone and in combination) were recorded, and growth inhibitory indices (GIIs) were calculated.

Result: The bacterial isolates were multidrug resistant, against which the SSE and MSE had excellent activity; ZDIs of SSE and MSE for *E. coli* and *K. Pneumoniae* ranged 8 - 20 mm, whereas for *Staph. aureus* ZDIs were 8 - 18 mm. The extracts (SSE and MSE) in combination with trimethoprim and vancomycin showed synergistic effect against all the test bacteria (GIIS: 0.53–1.0). The extracts, combined with ampicillin, ciprofloxacin and methicillin, had mixed interaction: synergistic (GIIs: 0.53–1.0) as well as antagonistic (GIIs: 0.37–0.47) against the test strains.

Conclusion: The plant extracts (SSE and MSE), having broad antibacterial activity alone, and synergistic interaction in combination with antibiotics against the human pathogenic bacteria, might be useful in preparing non-antibiotic as well as combined treatment regimen against bacterial infection to humans.

Keywords: Clinical bacteria; Antibiotic; Plant extract; Antibacterial activity; Synergy; Zone diameter of inhibition; Growth inhibitory index; Phytoconstituents

Introduction

The chemical based synthetic or semi-synthetic antimicrobials are lifesaving from several microbial infections following the discovery of penicillin; however, in the current antibiotic ages, there is an increasing emergence of multidrug-, extensively drug- and pan-drug- resistances among the microbial pathogens including bacteria [1], due to the growing demands as well as the indiscriminate usage of such antimicrobial agents [2]. This condition, along with the unnecessary side effects of the agents because of their rampant and non-judicious application, necessitated the discovery of new alternative therapeutics against various pathogenic infections [3]. Considering the fact, the researchers focused their studies on the antibacterial property of various indigenous plants [4-6], in order to get remedy from life-threatening infection with multidrug resistant bacteria including *Escherichia coli* (*E. coli), Klebsiella pneumoniae* (*K. pneumoniae*) and *Staphylococcus aureus* (*Staph. aureus*) [7-9].

The antibacterial potential of some indigenous plant extracts have been reported previously from our part of the globe [4-6]. The two popular seasonal fruits in many of the tropics such as India comprise *Syzygium cumini L.* (*S. cumini*; Family: Myrtaceae; Bengali vernacular name: 'Jaam') and *Mangifera indica* (*M. indica*; Family: Anacardiaeceae; Bengali vernacular name: 'Aam') that possess diverse medicinal properties and biological activities counting antibacterial efficacy [10-14]. The presence of multiple bioactive substances in different extracts of varied parts of both the plants has been demonstrated by several researchers worldwide. The *M. indica* seed kernel extract has been reported to exert growth inhibitory action on multidrug resistant (MDR) *Staph. aureus* and *E. coli* [15,16]. The ethyl acetate extract of *S. cumini* seed had antibacterial activity against Gram-positive as well as Gram-negative bacteria [17].

The antibacterial activity of various plant extracts in combination with antibiotics has also been reported by the earlier authors. Strong synergy was observed between *Ocimum sanctum* leaf extract and chloramphenicol (CM) and trimethoprim (TM) against *Salmonell typhi* [6]. Aqil et al. [18] reported synergism between tetracycline (TE) and a number of crude plant extracts against *S. aureus*. The studies carried out by Mandal et al. [5] demonstrated synergism between amoxicillin and *Emblica officinalis* and *Nymphae odorata* extracts against *Staph. aureus*. Several investigations have been done on the antibacterial activity of different parts of *S. cumini* and *M. indica* including the seeds; however, scanty report is available on their activity in combination with antibiotics against human pathogenic bacteria [19]. The current study, therefore, prompted us to ascertain the potential antibacterial activity of *S. cumini* and *M. indica* seed extracts alone and in combination with some conventionally used antibiotics

against Gram-negative (*E. coli* and *K. pneumoniae*) and Gram-positive (*Staph. aureus*) clinical bacterial isolates; the bioactive phytocomponents of both the seeds were evaluated qualitatively.

Materials and Methods

Bacterial strains

The bacterial strains used in the study included *E. coli* (n=2), *K. pneumoniae* (n=2), and *Staph. aureus* (n=2) procured from various clinical samples. The control strains used were *E. coli* ATCC 25922 and *Staph. aureus* ATCC 29213.

Plant materials and extract preparation

The matured *S. cumini* and *M. indica* fruits were collected from Rasakhowa village of Uttar Dinajpur district, West Bengal state, India, and the seeds were taken out, washed, sun dried and grinded using an electrical grinder. The seed granules, 25 g each of the two seed types, were dissolved in 75 ml of ethanol for 48 h with shaking at regular intervals. The liquid seed extract was filtered with Whatman No.1 filter paper following filtration through a sterile cheese cloth, and stored in refrigerator at 4°C for further use. The concentration of each of the extracts in the stock solutions was 0.33 mg/µl.

Antibiotic susceptibility test

The antibiotic susceptibility test was performed by Kirby-Bauer disk diffusion method, in nutrient agar plates, following the NCCLS guidelines, as has been mentioned by Jorgensen and Turnidge [20]. The antibiotic discs (Hi-media, India) used in the study were ampicillin (AM;10 µg/disc), TM (5 µg/disc) and ciprofloxacin (CP; 5 µg/disc) for gram-negative bacteria, and methicillin (MET; 5 µg/disc) and vancomycin (VA; 30 µg/disc), in addition to the above three, for gram-positive bacteria. The inoculated plates with impregnated antibiotic discs were incubated at 35°C for 24 h, and the ZDI (zone diameter of inhibition) values were recorded.

Antibacterial activity of plant extract

The antibacterial activity of *S. cumini* seed extract (SSE) and *M. indica* seed extract (MSE) was tested following the disc diffusion method. For this purpose, sterile nutrient agar plates were prepared, which with young broth culture of test bacteria were inoculated by swabbing. The sterilized paper disc (6 mm diameter, prepared from Whatman's No. 1 filter paper) placed on the surface of the inoculated agar were soaked with plant extracts, SSE or MSE, alone. The extract concentrations used were 3.3, 6.6, 9.9, 13.2 and 16.5 mg per disc. The ZDIs were recorded and interpreted according to the CLSI criteria [21], and as mentioned elsewhere by Mandal et al. [5], for resistance and sensitivity.

Combined antibacterial activity of plant extract and antibiotic

For combined antibacterial activity, on nutrient agar plates swabbed with test bacteria, antibiotic discs, such as AM (10 µg/disc), TM (5 µg/disc) and CP (5 µg/disc) for gram-negative bacteria, and AM (10 µg/disc), TM (5 µg/disc), CP (5 µg/disc), MET (5 µg/disc) and VA (30 µg/disc) for gram-positive bacteria were placed, on which 20 µl (6.6 mg) of the extract (SSE/MSE) was dropped, soaked and dried properly for 30 min at room temperature. The interaction between the two agents was considered synergistic, additive or antagonistic as per the criteria reported by Mandal et al. [5].

Growth inhibitory index

The growth inhibitory indices (GIIs) were calculated following the formula: [ZDI in combination/(total of ZDIs of the two agents in single action)] [5], in order to corroborate the synergistic activity (as has been defined in terms of increment of ZDI) of the antibiotics in combination with the plant extract. The synergistic, additive or antagonistic activities, if any, in between the two of the antimicrobial agents were defined with GIIs >0.5, 0.5 and <0.5, respectively [5].

Qualitative analysis for bioactive compounds

The specific qualitative tests were performed for the presence of bioactive compounds viz., phenol, quinone, flavonoids, steroids, terpenoids and glycosides in both of the test plant extracts (SSE and MSE), following the protocol of Radhakrishnan et al. [22].

Results

The antibiotic susceptibility test results for the bacterial isolates is depicted in Figure 1**,** for the test isolates AM, TR and CP had ZDIs 6–22, 6–28, and 6–30 mm, respectively, while the gram-positive bacteria had sensitivity to VA (ZDIs: 16–17 mm) and resistance to MET (ZDI: 6 mm).

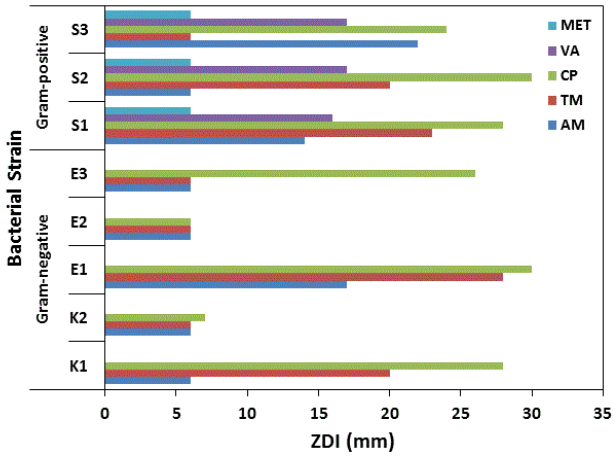

Figure 1: Antibiotic susceptibility test results for Gram-positive and Gram-negative bacterial isolates. AM: Ampicillin; TM: Trimethoprim; CP: Ciprofloxacin; VA: Vancomycin; MET: Methicillin; K1: *K. Pneumoniae* LMEM401; K2: *K. Pneumoniae* LMEM402; E1: *E. Coli* ATCC-25922; E2: *E. Coli* LMEM201; E3: *E. Coli* LMEM202; S1: *Staph. aureus* ATCC-29213; S2: *Staph. aureus* LMEM301; S3: *Staph. aureus* LMEM302. ZDI: zone diameter of inhibition.

The antibacterial activities of different concentrations SSE and MSE against the test bacteria are depicted in Figure 2 and Figure 3**;** the ZDIs of SSE and MSE for the gram-negative bacteria ranged 8-12 mm and 10-20 mm, respectively, whereas for *Staph. aureus* ZDIs ranged 8-15 mm and 12-18 mm, respectively.

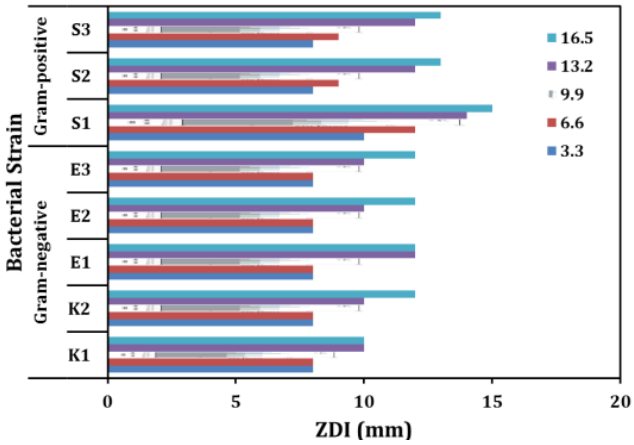

Figure 2: Antibacterial activity of *Syzygium cumini* seed ethanolic extract (SSE) against bacterial isolates. K1: *K. pneumoniae* LMEM 401; K2: *K. pneumoniae* LMEM 402; E1: *E. coli* ATCC 25922; E2: *E. coli* LMEM 201; E3: *E. coli* LMEM 202; S1: *Staph. aureus* ATCC 29213; S2: *Staph. aureus* LMEM 301; S3: *Staph. aureus* LMEM 302. The digits within the figure indicate the concentrations (mg/disc) of SSE used. ZDI: zone diameter of inhibition.

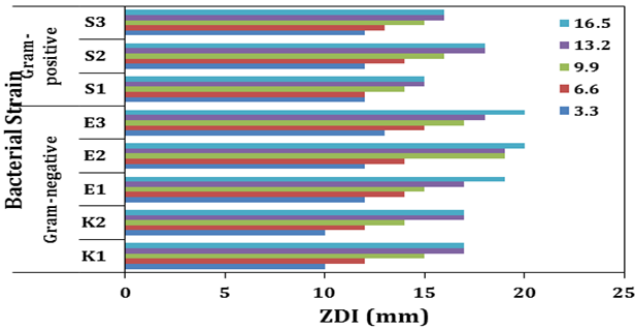

Figure 3: Antibacterial activity of *Mangifera indica* seed ethanolic extract (MSE) against bacterial isolates. K1: *K. pneumoniae* LMEM 401, K2: *K. pneumoniae* LMEM 402, E1*: E. coli* ATCC 25922, E2: *E. coli* LMEM 201, E3: *E. coli* LMEM 202, S1: *Staph. aureus* ATCC 29213, S2: *Staph. aureus* LMEM 301, S3: *Staph. aureus* LMEM 302. The digits within the figure indicate the concentrations (mg/disc) of SSE used. ZDI: zone diameter of inhibition.

The antibacterial activities of SSE and MSE in combination with the test antibiotics are represented in Figure 4. For the gram-positive bacteria, the ZDIs from SSE-antibiotic (VA, MET, AM, TM and CP) combination ranged 15–17, 6-11, 13–18, 8-22 and 14–32 mm, respectively, while from MSE-antibiotic (VA, MET, AM, TM and CP) combination had ZDIs 16–18, 12–14, 14–22, 12–24 and 14–24 mm, respectively. For gram-negative test bacteria, the ZDIs of AM, TM and CP in combination with SSE ranged 8–18, 8–26 and 8-26 mm, respectively, while in combination with MSE, ZDIs ranged 12 – 14, 12 – 24 and 11–28 mm, respectively.

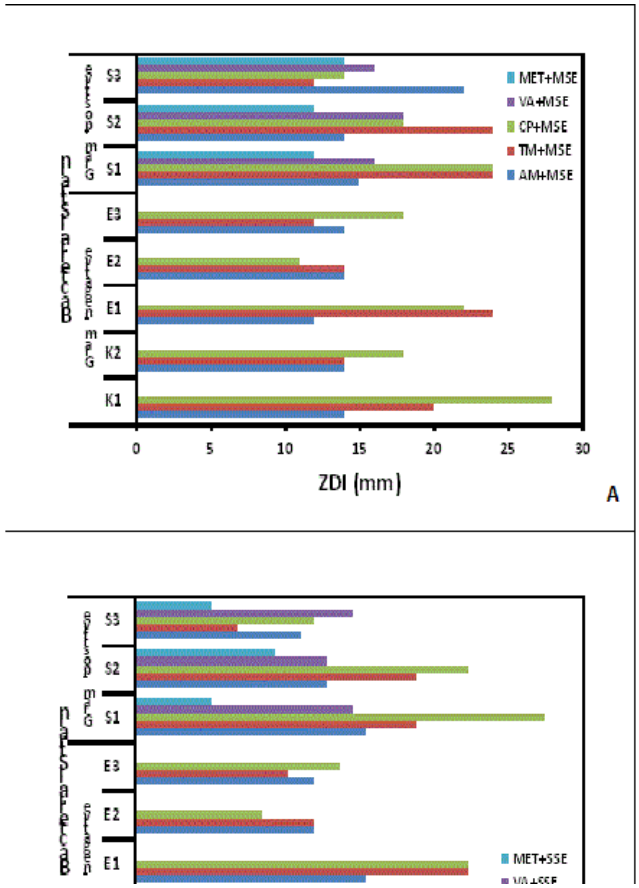

Figure 4: Antibacterial activity of (A) MSE: Mangifera indica seed extract and (B) SSE: Syzigium cumini seed extract, in combination with antibiotics against bacterial isolates. AM: ampicillin; TM: Trimethoprim; CP: Ciprofloxacin; VA: Vancomycin; MET: Methicillin; K1: *K. pneumoniae* LMEM401; K2: *K. pneumoniae* LMEM 402; E1: *E. coli* ATCC 25922; E2: *E. coli* LMEM 201; E3: *E. coli* LMEM 202; S1: *Staph. aureus* ATCC-29213); S2: *Staph. aureus* LMEM 301; S3: *Staph. aureus* LMEM 302; ZDI: zone diameter of inhibition.

The GIIs for the test bacterial isolates are presented in Table 1. The extracts in combination with TM and VA had synergistic effect against the test bacteria with GIIs 0.53–1.0; the extracts in combination with AM, CP and MET, had mixed interaction: synergistic (GIIs: 0.53 –1.0) as well as antagonistic (GIIs: 0.37–0.47).

Extract	GII					Bacterial strain
	Antibiotics (µg/disc)					
	AM (10)	TM (5)	CP (5)	VA (30)	MET (5)	
SSE	0.72	0.72	0.68	*	*	*E. coli* ATCC 25922
	1	1	0.71	*	*	*E. coli* LMEM 201
	1	0.85	0.47	*	*	*E. coli* LMEM 202

	0.57	0.71	0.61	*	*	*K. pneumoniae* LMEM 401
	0.71	0.57	0.53	*	*	*K. pneumoniae* LMEM 402
	0.69	0.62	0.8	0.60	0.39	*Staph. aureus* ATCC 29213
	1	0.75	0.66	0.57	0.73	*Staph. aureus* LMEM 301
	0.41	0.53	0.42	0.65	0.4	*Staph. aureus* LMEM 302
MSE	0.38	0.57	0.5	*	*	*E. coli* ATCC 25922
	0.7	0.7	0.55	*	*	*E. coli* LMEM 201
	0.66	0.57	0.43	*	*	*E. coli* LMEM 202
	0.77	0.62	0.7	*	*	*K. pneumoniae* LMEM 401
	0.77	0.77	0.94	*	*	*K. pneumoniae* LMEM 402
	0.57	0.68	0.6	0.57	0.66	*Staph. aureus* ATCC 29213
	0.7	0.7	0.4	0.58	0.6	*Staph. aureus* LMEM 301
	0.62	0.63	0.37	0.53	0.73	*Staph. aureus* LMEM 302

Table 1: Growth inhibitory index (GII) values from the combined action of antibiotics and plant seed extracts against bacterial strains.* *M. indica and S. cumini*

The bioactive compounds that have been detected in SSE and MSE are depicted in Table 2. Among the six qualitative tests performed, the MSE showed positive result in five tests whereas the SSE showed positive result in four tests.

Plant Extract	Phytoconstituents					
	Quinone	Phenol	Steroids	Terpenoids	Glycosides	Flavonoids
SSE	+	–	+	+	+	–
MSE	+	–	+	+	+	+

Table 2: Phytoconstituents present in the test plant extracts; MSE: Ethanolic *M. indica* seed extract; SSE: Ethanolic *S. cumini* seed extract; +: positive; – : negative

Discussion

The scientific studies carried out by the researchers on indigenous medicinal plants with traditional knowledge of affectivity against various illnesses might provide valuable justification for utilizing those as the natural sources of new antimicrobial agents of non-antibiotic types with the trace or no side effects [23]. The *S. cumini* leaf ethanolic extract and seed aqueous extract had high antimicrobial activity for several bacterial strains, as has been reported by Prabhakaran et al. [24]. As per the report of Kothari et al. [25], the *S. cumini* methanolic seed extract had more antibacterial activity than that of the ethanolic extract. Banerjee and Narendhirakannan [26] demonstrated the antibacterial activity of *S. cumini* ethanolic seed extract (250 mg/ml) having ZDIs 18 – 22 mm against Gram-negative bacteria and 20–23 mm against Gram-positive bacteria. Prakash et al. [27] reported that the kernel extract (10%) of Bangapalli variety of *M. indica* possessed the capacity to inhibit 100 % bacterial growth, while the Senthura variety kernel extracts (10%) showed partial growth inhibitory action against the test bacteria. Sahrawat et al. [28] showed that *M. indica* leaf benzene extract had growth inhibitory action against *Pseudomonas fluorescens* showing resistance to multiple antibiotics. Sahu et al. [29] reported that the acetone extract of mango kernel had top ZDI against *Aeromonas hydrophila*, whereas the ethanolic and methanolic extracts had ZDIs 14 - 20 mm for *A. hydrophila*. As per the report of Jaiswal [30], the ethanolic *M. indica* extract has been found to be effective against *S. typhi, Staph. aureus, K. pneumoniae* and *Pseudomonas aeruginosa* ZDIs 10 – 14 mm. In the current study, both the plant extracts (SSE and MSE) showed growth inhibitory activity against clinical isolates of *Staph. aureus, E. coli, K. pneumoniae*, and both SSE and MSE had concentration dependent antibacterial activity. The ZDIs noted against *Staph. aureus* were 10 mm and 12 mm due to the action 3.3 mg of SSE and MSE, respectively, and 15 mm and 18 mm, respectively, at 16.5 mg, while for gram-negative test bacteria the respective ZDIs were 8 mm and 13 mm, at 3.3 mg, and 12 mm and 20 mm at 16.5 mg extracts concentrations (SSE and MSE). The S. cumini methanolic seed and pulp extracts had dose-response association in showing antibacterial activities against *E. coli* and *S. typhi* [31]. Vaghasiya et al. [32] documented the potent antibacterial activity of *M. indica* seed methanolic extract at concentrations 0.6–1.2 mg/ml, while El-Gied et al. [33] reported its action at the higher concentrations: 1.25–5 mg/ml. Kaur et al. [34] reported that the crude methanolic mango seed kernel extract at a concentration of 100 mg/ml had potential antimicrobial activity against MRSA (ZDIs: 9–21 mm) and *E. coli* (ZDIs: 15–18 mm) compared to *Vibrio vulnificus* (ZDIs: 7–20 mm). The *S. cumini* extracts had dose-response relationship of antibacterial activity against *E. coli* and *S. typhi,* and the extracts, therefore, have been considered as useful source a safe and novel antibacterial compounds [31].

Bacterial strains, under the selective pressure of antibiotics, develop inevitable capacity to acquire resistance to antibiotics, and as such the clinical efficacy of previously effective first-line antibiotics results shifting of antibiotic treatment regimen to second-line or third-line of choice, which are often more expensive and with side effects [35]. Based upon the fact the capacity of the plant crude extracts to augment the activity of antibiotics has been studied. Using plant materials, Darwish et al. [36] demonstrated an improvement in the efficacy of gentamycin (GM) and chloramphenicol (CM) against *Staph. aureus*. Mandal et al. [5] reported synergistic anti-*Staph. aureus* activity of amoxicillin in combination with *E. officinalis* seed and *N. odorata* stamen extracts. In the present investigation, the antibacterial activity of AM, TM and CP, against Gram-negative bacteria (*E. coli* and *K. pneumoniae*), and VA and MET along with the above antibiotics, against *Staph. aureus* were assessed for possible synergistic interaction in combination with SSE and MSE. The CP-MSE and CP-SSE combinations had antagonistic interaction against the clinical isolates of *E. coli* and *Staph. aureus*, and AM-MSE and AM-SSE combinations against *E. coli* ATCC 25922 standard and *Staph. aureus* clinical strains with GIIs 0.37–0.47; AM-SSE and MET-SSE also had antagonism against *Staph. aureus* (GIIs: 0.39–0.41). However, most of the combinations had synergistic interactions against the bacterial strains tested (GIIs: 0.53–1.0). de Oliveira et al. [19] recorded the mango peel ethanol extract MIC as ≥ 2.048 mg/ml for Staph. aureus strains, and the extract, at 0.512 mg/ml, displayed synergistic interaction with tetracycline and erythromycin having four-fold reduction in the MICs

of antibiotics tested. The alcoholic extract of Thai mango seed kernel extract and the phenolics present in the extract improved and increased the antibacterial potentiality of penicillin against the clinical isolates of methicillin-resistant *Staph. aureus* with a 5-fold or more decrement in minimum inhibitory concentration [15]. Rakholiya et al. [37] reported the antibacterial potentiality of *M. indica* matured seed and leaf methanol extracts, which demonstrated synergistic interaction in combination with chloramphenicol and ceftazidime against *Pseudomonas* spp., including *P. aeruginosa*, suggesting the extracts as the valuable source of prospective adjuvant of antibiotics and food products in combating bacterial infection to humans as well as spoilage microorganisms. The current study has been the first to demonstrate the combined antibacterial activity between antibiotics and the plant seed extracts (MSE and SSE), from our part of the globe, which documents varied nature and strength of interaction, in terms of GIIs, between antibiotics and the test extracts; such interaction might be due to the presence of different bioactive components in SSE and MSE, and the variation in their mechanism of action against different test bacterial strains.

The presence of bioactive compounds in plant extracts specifies the therapeutic potentiality of herbs and spices. In the current study, the phyto-constituents were determined qualitatively in SSE and MSE that showed the presence of a variety of phytochemicals such as quinone, steroids, terpenoids and glycosides, while flavonoids were found only in MSE; no phenolics were detected in either of the test extracts. However, Abdalla et al. [16] detected phenolic compounds in mango kernel extract and recorded the presence of high levels of tannin and vanillin. The phytohemical tests revealed the existence of alkaloids, phenol, tannins and flavonoids in the ethanolic M. indica leaf extract [30]. Rajan et al. [38] reported that the antibacterial activity of mango kernel was plausibly attributed to tannins contained in the extracts. The antimicrobial activity of *S. cumini* might be due to the presence of diverse phytochemicals including tannins and phenolics, as has been reported by Sing et al. [39]. It has been demonstrated that the ethanolic *M. indica* seed kernel extract, possessing phytohemicals (alkaloids, phenol, tannins and flavonoids), had efficacy against *Bacillus subtilis* (ZDI: 10-22 mm), *Staph. aureus* (ZDI: 9-20 mm), *P. aeruginosa* (ZDI: 12-20 mm), and *E. coli* (ZDI: 13-18 mm) [40]. The *S. cumini* ethanolic leaf and seed extracts that demonstrated positive test results for the presence of phenols, flavonoids and tannins had inhibitory activity against Gram-positive (*Citrobacter freundii*, *Clostridium acetobutylicum*, *Streptococcus lactis*, and *Strepto. pyogenes*) as well as Gram-negative (*Vibrio cholerae, Enterococcus faecium* and *S. paratyphi*) bacterial strains [24]. The current study demonstrated the presence of various active phyto-compounds in both SSE and MSE, for which the extracts displayed excellent antibacterial activities against the bacterial strains causing life-threatening infection to humans. Therefore, the test plants might be useful in combating bacterial infection to humans, alone and in combination with some selected antibiotics; however, further studies (toxicity and pharmacokinetic) in the issues of safety aspect and dose determination are warranted [41-45].

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