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Synergistic Antimicrobial Activity of Azadirachta indica (Neem) and Psidium guajava (Guava) Leaf Extracts against Multidrug-Resistant Bacteria

E. Anitha[1], Dr. S. Kanimozhi[1], Dr. L. Jeyanthi Rebecca[1]
[1]*Department of Industrial Biotechnology, Bharath institute of science and technology, Bharath Institute of Higher Education and Research, Selaiyur, Chennai 600073, Tamil Nadu, India*
[1]btechbioanitha@gmail.com

Abstract—

Multidrug-resistant (MDR) bacteria are eroding the usefulness of conventional antibiotics, and plant-derived combinations are being explored as alternatives. *Azadirachta indica* (neem) and *Psidium guajava* (guava) are widely used medicinal plants, but their combined activity against MDR pathogens is not well characterised. Leaves of both plants were extracted in ethanol by Soxhlet extraction, screened for phytochemical classes by standard qualitative tests, and profiled by GC-MS. Each extract and their combination was tested against MDR clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* by agar well diffusion. Minimum inhibitory concentration (MIC) was measured by broth microdilution, interaction was assessed by checkerboard assay (fractional inhibitory concentration index, FICI), and IC50 values were estimated. Both extracts contained alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides and phenols, with tannins more abundant in guava. The combination gave larger inhibition zones than either extract alone against all three isolates (*S. aureus* 19.6 mm, *E. coli* 18.1 mm, *P. aeruginosa* 15.2 mm). Combination MICs were 0.25, 0.5 and 0.75 mg/mL, respectively, against 1.0 to 2.5 mg/mL for the single extracts. The FICI was 0.42 for *S. aureus* (synergy), 0.58 for *E. coli* and 0.62 for *P. aeruginosa* (both additive). IC50 fell from 0.55 mg/mL (neem) and 0.45 mg/mL (guava) to 0.22 mg/mL for the combination.

Keywords—Azadirachta indica; Psidium guajava; multidrug resistance; synergy; checkerboard assay; FICI; MIC

I. INTRODUCTION

Antimicrobial resistance is one of the most pressing problems in modern medicine. Bacteria that resist several antibiotic classes, termed multidrug-resistant (MDR) organisms, are found mainly in hospitals and long-term care settings and cause infections of the bloodstream, lungs, urinary tract, wounds and skin [1], [2]. Resistance follows antibiotic exposure: prolonged or unnecessary use selects for surviving resistant strains, which then spread between patients through hands, devices and contaminated surfaces [1], [3]. Infections with MDR organisms are hard to treat, and an inappropriate antibiotic delays recovery.

Medicinal plants are a long-standing source of antimicrobial leads. They contain alkaloids, flavonoids, tannins, terpenoids and other metabolites with antibacterial activity [4], [5]. Combining agents is an attractive strategy because compounds acting on different targets, such as the cell wall, protein synthesis, nucleic acid synthesis or biofilm formation, can give an effect greater than the sum of the parts. Synergy can also allow lower doses and may slow the emergence of resistance [3], [5].

Azadirachta indica (neem, family Meliaceae) is used in Ayurveda, Siddha and Unani medicine and has reported antibacterial, antifungal, anti-inflammatory, antioxidant and hepatoprotective properties [6]–[8]. Its main bioactive constituents include the limonoids azadirachtin, nimbin, nimbidin, nimbolide and gedunin, together with quercetin and other flavonoids, beta-sitosterol and phenolic acids [6], [7]. *Psidium guajava* L. (guava, family Myrtaceae) is used traditionally for diarrhoea, oral and skin infections and wounds. Its leaves are rich in flavonoids and phenolics (quercetin, rutin, guaijaverin, gallic and ellagic acids), triterpenoids and essential oils such as beta-caryophyllene [9]. Guava leaf extracts have been reported to inhibit methicillin-resistant *Staphylococcus aureus*, to interfere with biofilm formation and quorum sensing, and to enhance some antibiotics against resistant Gram-negative bacteria [9]. Neem and guava are inexpensive and widely available in India, and their phytochemical profiles overlap only partly, which makes the pair a reasonable candidate for a complementary combination.

Earlier work has examined ethanolic neem and guava extracts against clinically relevant bacteria and found inhibition of Gram-positive and Gram-negative species.However, the interaction between the two extracts has rarely been quantified against MDR isolates using a formal synergy index. This study therefore evaluated the in vitro activity of ethanolic neem and guava leaf extracts, alone and combined, against MDR *S. aureus*, *E. coli* and *P. aeruginosa*. We screened the extracts for phytochemical classes, profiled them by GC-MS, measured zones of inhibition and MIC, and classified the interaction by checkerboard assay and FICI.

II. MATERIALS AND METHODS

A. Plant Material

Healthy, uninfected leaves of *Azadirachta indica* and *Psidium guajava* were obtained from local vendors in Kanchipuram district, Tamil Nadu, India. The plants were authenticated by Dr. K. N. Sunil Kumar, Siddha Central Research Institute, Arumbakkam, Chennai. Leaves were washed under running tap water to remove dust, shade-dried

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and ground to a powder. The plants and their taxonomic classification are shown in Fig. 1 and Table I.



Fig. 1. Leaves of (left) *Azadirachta indica* and (right) *Psidium guajava* used in this study.

TABLE I. TAXONOMICAL CLASSIFICATION OF *AZADIRACHTA INDICA* AND *PSIDIUM GUAJAVA*

Rank	*A. indica*	*P. guajava*
Kingdom	Plantae	Plantae (Plants)
Phylum	Angiosperms	Tracheophyta (Vascular plants)
Order	Rutales	Myrtales
Suborder	Rutinae	–
Family	Meliaceae (mahogany family)	Myrtaceae (myrtle family)
Subfamily	Melioideae	–
Tribe	Melieae	–
Genus	*Azadirachta*	*Psidium*
Species	*Azadirachta indica* A. Juss.	*Psidium guajava*

B. Extraction

A cellulose filtration extraction thimble (Fisherbrand™ Grade 645 Cellulose) was filled with 20 g of ground leaves powder and put into a Soxhlet apparatus connected to a solvent flask. The ratio of plant material to solvent was 1:10 (w/w). Extractions were conducted for time intervals varying from 360 to 720 min, in duplicate. The extraction temperature was set to 70 °C for ethanol. The temperature was adjusted by a heating mantle connected to a Variac (NAAFIE, 1 phase 0 to 270 V AC/15A). All extracts were condensed in a rotary-evaporator to a final concentration of 100 mg/mL.

C. Preliminary Phytochemical Screening

Extracts were screened by standard qualitative tests for alkaloids (Dragendorff's, Mayer's, Wagner's and Hager's tests), glycosides (Keller-Kiliani and related tests), tannins (ferric chloride, gelatin and lead acetate tests), flavonoids (Shinoda, alkaline reagent and zinc-HCl tests), sterols and triterpenoids (Liebermann-Burchard and Salkowski tests), phenols (ferric chloride test), saponins (foam test), quinones, and fats and oils. Results were scored as absent (-), present (+) or abundant (++).

D. GC-MS Analysis

The ethanolic extracts of neem, guava and the combination were analysed by GC-MS. Components were identified by comparing mass spectra with the NIST V11 library, with retention indices checked against a GC-MS metabolomics database.

E. Bacterial Strains and Inoculum

MDR clinical isolates of *S. aureus*, *E. coli* and *P. aeruginosa*, with known resistance profiles, were obtained from the Department of Microbiology, Krishna College of Arts and Science, Kanchipuram. Strains were kept on nutrient agar slants at 4 °C and subcultured in nutrient broth for 24 h before testing. Inocula were adjusted to the 0.5 McFarland standard (about 1.5×10^8 CFU/mL). Penicillin (10 µg) discs served as the reference antibiotic.

F. Agar Well Diffusion

Mueller-Hinton agar was prepared, autoclaved at 121 °C and 15 psi for 15 to 20 min, and poured into sterile Petri dishes. Plates were swabbed with the standardised inoculum and 7 mm wells were punched in the agar. Wells received neem extract, guava extract, the combined extract, or the reference antibiotic. Plates were incubated at 37 °C for 24 h and the diameter of each zone of inhibition was measured in millimetres. Results are mean ± SD of replicates. Agar diffusion follows the principle of Bauer et al. [10] and the methods reviewed by Balouiri et al. [11].

G. MIC Determination

MIC was determined by broth microdilution following CLSI guidance [12]. Extracts were serially diluted in broth in 96-well plates and each well received bacterial inoculum at about 10^5 CFU/mL. Growth controls, sterility controls and extract-only controls were included.

Plates were incubated at 37 °C for 18 h, and the MIC was the lowest concentration with no visible growth. For bactericidal activity, 100 µL from clear wells was spread on agar and incubated at 37 °C for 18 h.

H. Checkerboard Assay and FICI

Interaction between the extracts was assessed by checkerboard microdilution [13]. The fractional inhibitory concentration (FIC) of each extract is its MIC in combination divided by its MIC alone, and the index is the sum of the two:

$$FICI = \frac{MIC_A \text{ in combination}}{MIC_A \text{ alone}} + \frac{MIC_B \text{ in combination}}{MIC_B \text{ alone}} \quad (1)$$

where A is neem and B is guava. FICI ≤ 0.5 was read as synergy, > 0.5 to 1.0 as additive, > 1.0 to 4.0 as indifferent and > 4.0 as antagonism.

I. IC_{50} Estimation

IC_{50} values were estimated from concentration-response data for each extract and for the combination.

III. RESULTS

A. Phytochemical Screening

Both ethanolic extracts contained alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides and phenols (Table II). Tannins were more abundant in guava (++) than in neem (+), phenols were abundant in both, and steroids were detected only in neem.

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TABLE II. QUALITATIVE PHYTOCHEMICAL SCREENING OF THE ETHANOLIC LEAF EXTRACTS (+ PRESENT, ++ ABUNDANT, - ABSENT).

Phytochemical	*A. indica*	*P. guajava*
Alkaloids	+	+
Flavonoids	+	+
Tannins	+	++
Saponins	+	+
Terpenoids	+	+
Glycosides	+	+
Phenols	++	++
Steroids	+	-

B. GC-MS Profile

The chromatogram of neem showed 12 resolved peaks between 4.28 and 24.57 min, the guava extract showed 12 peaks between 3.95 and 22.07 min, and the combined extract showed 14 peaks between 4.12 and 26.12 min (Fig. 2). Retention times of all peaks are given in Table III. The combined profile contains more peaks than either single extract, consistent with the pooling of constituents from both plants.

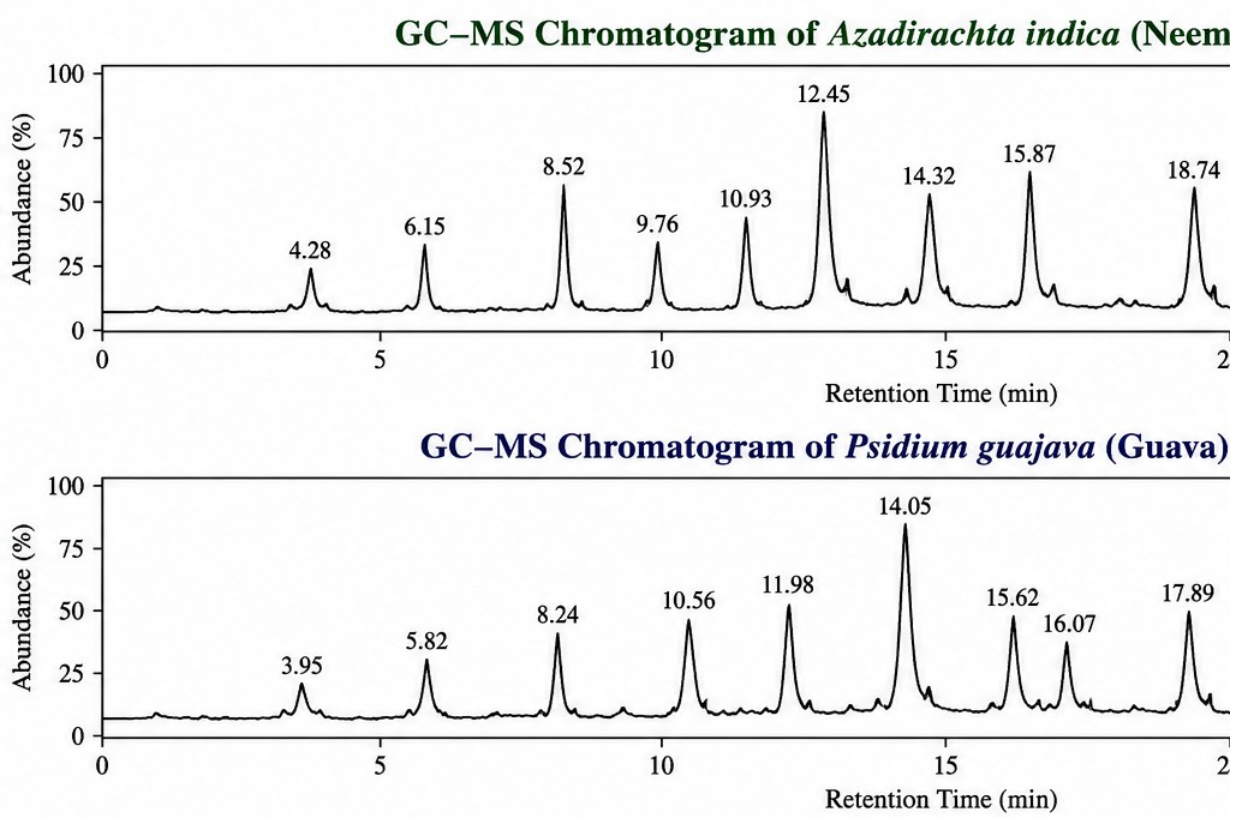

Fig. 2. GC–MS chromatograms of the ethanolic leaf extracts of (top) *Azadirachta indica*, (middle) *Psidium guajava* and (bottom) the combined neem–guava extract.

TABLE III. GC–MS RETENTION TIMES (RT, MIN) OF THE RESOLVED PEAKS

Peak No.	*A. indica*	*P. guajava*	Combination
1	4.28	3.95	4.12
2	6.15	5.82	5.96
3	8.52	8.24	7.84
4	9.76	10.56	9.21
5	10.93	11.98	10.48
6	12.45	14.05	11.93
7	14.32	15.62	13.67
8	15.87	16.07	15.14
9	18.74	17.89	16.82
10	20.18	19.64	18.39
11	22.43	21.15	20.57
12	24.57	22.07	22.84

C. Zones of Inhibition

All three extract preparations inhibited all three MDR isolates (Table IV). Guava gave slightly larger zones than neem for every organism. The combination gave the largest zones throughout, exceeding the better single extract by 5.6 mm for *E. coli*, 5.8 mm for *S. aureus* and 4.6 mm for *P. aeruginosa*. *S. aureus* was the most susceptible isolate and *P. aeruginosa* the least.

TABLE IV. ZONES OF INHIBITION (MM, MEAN ± SD) AGAINST MDR ISOLATES.

MDR organism	*A. indica*	*P. guajava*	Combination
Escherichia coli	11.2 ± 0.4	12.5 ± 0.5	18.1 ± 0.5
Staphylococcus aureus	12.4 ± 0.5	13.8 ± 0.4	19.6 ± 0.6
Pseudomonas aeruginosa	9.8 ± 0.3	10.6 ± 0.4	15.2 ± 0.4

D. Minimum Inhibitory Concentration

The combination lowered the MIC for every isolate (Table V). For *S. aureus* the MIC fell from 1.5 mg/mL (neem) and 1.0 mg/mL (guava) to 0.25 mg/mL. For *E. coli* it fell from 2.0 and 1.5 mg/mL to 0.5 mg/mL, and for *P. aeruginosa* from 2.5 and 2.0 mg/mL to 0.75 mg/mL.

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TABLE V. MIC (MG/ML) BY BROTH MICRODILUTION.

MDR organism	*A. indica*	*P. guajava*	Combination
Staphylococcus aureus	1.5	1.0	0.25
Escherichia coli	2.0	1.5	0.5
Pseudomonas aeruginosa	2.5	2.0	0.75

E. Interaction between the Extracts (FICI)

The checkerboard assay classified the interaction as synergistic only for *S. aureus* (FICI 0.42; Table VI). *E. coli* (0.58) and *P. aeruginosa* (0.62) fell in the additive range.

TABLE VI. FRACTIONAL INHIBITORY CONCENTRATION INDEX.

MDR organism	FIC *A. indica*	FIC *P. guajava*	FICI	Interpretation
Escherichia coli	0.25	0.33	0.58	Additive
Staphylococcus aureus	0.17	0.25	0.42	Synergistic
Pseudomonas aeruginosa	0.30	0.32	0.62	Additive

F. IC_{50}

Estimated IC_{50} values were 0.55 mg/mL for neem, 0.45 mg/mL for guava and 0.22 mg/mL for the combination, so the combination reached half-maximal inhibition at about half the concentration of the better single extract.

IV. DISCUSSION

Combining neem and guava leaf extracts improved in vitro activity against all three MDR isolates, and the improvement met the synergy criterion only for *S. aureus*. The combination produced the largest zones, the lowest MICs and the lowest IC_{50}, which agrees with the general expectation that mixtures of plant metabolites can outperform single extracts [3], [5].

Both extracts contained flavonoids, tannins, alkaloids, terpenoids, saponins and phenols. These classes are reported to act through membrane damage, inhibition of protein and nucleic acid synthesis, enzyme inhibition and oxidative stress [4], [5]. The higher tannin content of guava may help explain why guava was slightly more active than neem against every isolate, and neem additionally contributes limonoids and steroids that guava lacked in our screen [6], [7], [9]. Because the two plants differ in their constituents, complementary targets are a plausible explanation for the combined effect, although this study did not test the mechanism.

Susceptibility followed the expected Gram pattern. *S. aureus* was the most sensitive isolate and showed the only synergistic interaction (FICI 0.42). *P. aeruginosa* was the least sensitive, consistent with its low outer-membrane permeability and efflux pumps [1], [2]. *E. coli* and *P. aeruginosa* gave FICI values of 0.58 and 0.62, which sit just above the 0.5 synergy cut-off. Such values should be treated with caution because checkerboard results are usually reproducible only within one two-fold dilution, and some authors classify the 0.5 to 4 range as indifference rather than additive [13]. We therefore describe the combination as enhanced, and as synergistic only against *S. aureus*.

The MIC values (0.25 to 2.5 mg/mL) are in the range commonly reported for crude plant extracts and are far higher than those of clinical antibiotics, so the extracts are not substitutes for antibiotics in their present form [11], [14]. Their value lies in lead discovery and in possible use alongside conventional drugs.

A. Limitations

- All data are in vitro, from a small panel of three isolates, and may not hold for other strains.
- The extracts are crude and not standardised; GC-MS peaks are listed but the active compounds have not been isolated or tested individually.
- Cytotoxicity and safety were not assessed.
- Zone diameters depend on diffusion of extract components through agar, so they should be read alongside MIC rather than as a measure of potency.
- Synergy was assessed against one mixing ratio and without time-kill studies.

B. Future Work

Bioassay-guided fractionation, identification of the compounds responsible for the *S. aureus* synergy, time-kill and biofilm assays, cytotoxicity testing, and combination studies with conventional antibiotics are the logical next steps, followed by in vivo evaluation.

V. CONCLUSION

Ethanolic leaf extracts of *Azadirachta indica* and *Psidium guajava* each inhibited MDR *S. aureus*, *E. coli* and *P. aeruginosa*, and their combination was more effective than either alone, with larger inhibition zones, lower MICs and a lower IC_{50}. The interaction was synergistic against *S. aureus* (FICI 0.42) and additive against *E. coli* and *P. aeruginosa*. These results support neem-guava combinations as a starting point for plant-based antimicrobial development, pending compound identification, safety testing, mechanistic work and in vivo validation.

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