

Synergistic antimicrobial potential of *Ziziphus jujuba* and *Ziziphus oenoplia* extracts combined with ciprofloxacin against multidrug-resistant strains

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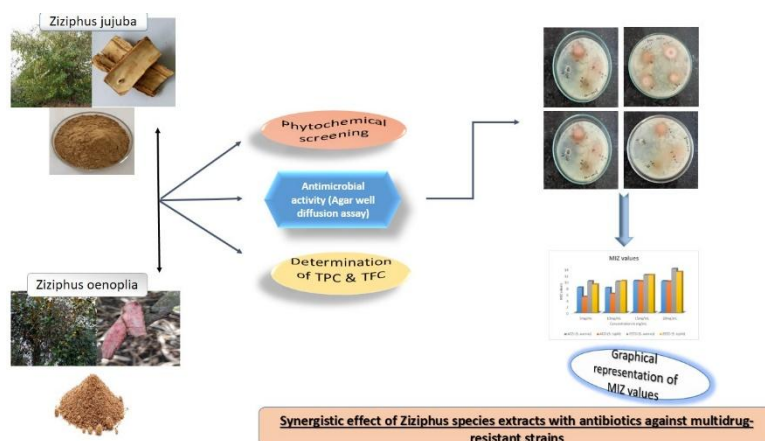
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Abstract

Background: The combined use of herbal medicines and first-line antimicrobial agents, to which many pathogens have developed resistance, is considered a practical strategy for managing polymicrobial infections and limiting the emergence of antimicrobial-resistant strains. **Objective:** The emergence of antimicrobial resistance has increased interest in plant-derived compounds as adjuncts to conventional antibiotics. This study evaluated the in-vitro antimicrobial activity of *Ziziphus* species extracts and their synergistic interaction with Ciprofloxacin against multidrug-resistant bacteria. **Methods:** Ethanolic extracts of *Ziziphus jujuba* and *Ziziphus oenoplia* were tested against *Staphylococcus aureus* (MTCC-7443), *Bacillus subtilis* (MTCC-121), *Salmonella typhi* (MTCC-733), and *Pseudomonas aeruginosa* (MTCC-1036). Antimicrobial activity was evaluated using inhibition zone and minimum inhibitory concentration (MIC) assays. **Results:** The extracts exhibited varying degrees of antimicrobial activity against all tested strains. EEZO showed the highest activity against *S. typhi* with an MIC value of $65.81 \pm 0.02 \mu\text{g/mL}$, while AEZO exhibited the lowest activity against *S. aureus* (MIC: $28.97 \pm 0.021 \mu\text{g/mL}$). The combination of EEZO with Ciprofloxacin produced enhanced inhibitory activity against *S. typhi* and *S. aureus*, demonstrating synergistic interactions. **Conclusion:** *Ziziphus* species extracts demonstrated notable antimicrobial activity and enhanced the efficacy of Ciprofloxacin against multidrug-resistant bacterial strains.



Keywords: *Ziziphus jujuba* Mill., *Ziziphus oenoplia*, antimicrobial, synergistic, total flavonoid and phenolics

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1. Introduction

Multidrug-resistant (MDR) bacterial infections have become more common due to antimicrobial resistance (AMR), a significant worldwide health problem that has decreased the efficacy of many conventional medicines. The hunt for substitute antibacterial agents derived from natural sources has increased as a result. Alkaloids, flavonoids, tannins, phenolics, and saponins are among the bioactive phytochemicals found in medicinal plants that are known to have strong antibacterial action via a variety of mechanisms [1-3]. Additionally, through synergistic interactions, plant-derived chemicals may improve the therapeutic potential of already available antibiotics against infections that are resistant to them.

There are around 170 species in the genus *Ziziphus*, which are found in tropical and subtropical areas [4]. Among them, *Ziziphus jujuba* and *Ziziphus oenoplia*

have long been used to treat inflammatory conditions, ulcers, wounds, diarrhoea, and microbiological infections [6-8]. According to phytochemical research, these species include flavonoids, phenolics, cyclopeptide alkaloids, betulinic acid, quercetin, kaempferol, and other bioactive components linked to antioxidant and antibacterial properties [6-8].

Although Ciprofloxacin is a broad-spectrum antibiotic that is frequently used to treat both Gram-positive and Gram-negative bacterial infections, its therapeutic effectiveness has been hampered by the advent of ciprofloxacin-resistant pathogens. The synergistic antibacterial activity of *Ziziphus jujuba* and *Ziziphus oenoplia* extracts in conjunction with ciprofloxacin against MDR infections is not well understood, despite the fact that several research have examined the antimicrobial qualities of medicinal plants. In order to assess the physicochemical characteristics, total phenolic and flavonoid contents, and *in vitro* antimicrobial activity of aqueous and ethanolic extracts of these species, as well as their synergistic interaction with Ciprofloxacin against specific multidrug-resistant bacterial strains, the current study was conducted.

2. Results and discussion

2.1. Physicochemical analysis

The physicochemical parameters of powdered stem bark of *Ziziphus jujuba* and *Ziziphus oenoplia* are presented in Tables 1 and 2. The analysis was performed in triplicate (n = 3), and results are expressed as mean ± standard deviation (SD). Total ash and acid-insoluble ash values were within acceptable pharmacognostic limits, indicating low contamination with inorganic and siliceous materials. The higher water-soluble extractive values observed in both species suggest the abundance of polar phytoconstituents such as phenolics and flavonoids.

The differences in extractive values between aqueous and ethanolic solvents indicate solvent-dependent extraction efficiency (Table 4). Similar observations have been reported in previous studies on medicinal plants rich in phenolic compounds, where polar solvents showed enhanced extraction of bioactive metabolites. These physicochemical parameters may therefore serve as reference standards for quality control and authentication of the investigated plant materials.

Table 1: Physicochemical constant values of *Z. jujuba* and *Z. oenoplia*

Plant materials	Total ash (%)	Acid insoluble ash (%)
<i>Z. jujuba</i>	8.7±0.216	0.6±0.085
<i>Z. oenoplia</i>	9.0±0.108	0.67±0.065
Plant materials	Total ash (%)	Acid insoluble ash (%)

Ash values and moisture content are presented as mean ± SD

Table 2: Extractive values of *Z. jujuba* and *Z. oenoplia*

Plant materials	Extractive values (%)			
	Chloroform extractive value	Ethyl acetate extractive value	Water extractive value	Ethanol extractive value
<i>Z. jujuba</i>	2.2±0.092	2.4±0.085	7±0.096	8.7±0.057
<i>Z. oenoplia</i>	1.3±0.050	1.4±0.057	8.8±0.098	4.4±0.04

Extractive values are mentioned as mean ± SD

2.2. Phytochemical screening

The qualitative phytochemical screening of the aqueous and alcoholic stem bark extract of *Z. jujuba* and *Z. oenoplia* showed the presence of carbohydrates, flavonoids, alkaloids, tannins, and phenolics except steroids in EEZJ (Ethanolic Extract *Z. jujuba*) and EEZO (Ethanolic Extract of *Z. oenoplia*); saponin glycosides, amino acids in AEZJ (Aqueous Extract of *Z. jujuba*) and AEZO (Aqueous Extract of *Z. oenoplia*) as depicted in Table 3.

Table 3. Phytochemical analysis of aqueous and alcoholic extracts of *Z. jujuba* and *Z. oenoplia*

Chemical Constituents	Phytochemical tests	EEZJ	EEZO	AEZJ	AEZO
Carbohydrates	Molish's test	+	+	+	+
	a) Fehling's test	+	+	+	+
Reducing sugars	b) Benedict's test	+	+	+	+
	Proteins	Biuret test	-	-	-
Amino acid	Ninhydrin test	-	-	+	+
	a) Salkowski reaction	+	+	-	-
Steroids	b) Liebermann-Burchard's reaction	-	-	-	-
	c) Lieberman's reaction	-	-	-	-
	Saponin glycosides	Foam test	-	-	+
Flavonoids	a) Shinoda test	+	+	+	+
	b) Lead acetate test	+	+	+	+
	c) Sodium hydroxide test	+	+	+	+
Alkaloids	a) Mayer's test	+	+	+	+
	b) Wagner's test	+	+	+	+
	a) FeCl ₃ solution	+	+	+	+
	b) Gelatine solution	+	+	-	-
Tannins and phenols	c) Potassium dichromate	-	-	+	+
	d) Dilute Iodine solution	+	+	+	+

Where: + means present and – means absent

2.3. Total flavonoid contents

The total flavonoid content (TFC) of the extracts was quantified using the aluminium chloride colorimetric method and expressed as mg quercetin equivalents (QE)/g dry extract. The calibration curve of quercetin demonstrated good linearity within the tested concentration range ($R^2 = 0.9983$), indicating the reliability of the analytical method. All measurements were performed in triplicate to ensure reproducibility.

Among the investigated extracts, AEZJ exhibited the highest flavonoid content (12.5 ± 0.03 mg QE/g), whereas AEZO showed the lowest value. The higher flavonoid content in AEZJ may contribute to its comparatively enhanced antibacterial activity, as flavonoids are known to disrupt bacterial membranes, inhibit nucleic acid synthesis, and interfere with microbial enzyme systems [9]. The observed variation in flavonoid content between aqueous and ethanolic extracts suggests that solvent polarity significantly

influences phytochemical extraction efficiency. Similar findings have been reported for other medicinal plants possessing antimicrobial potential (Table 4 and Figure 1).

Table 4: Percentage yield, TPC and TFC of ethanolic and aqueous extracts of *Ziziphus* species

Extracts	TPC in mg GAE/gm	TFC in mg QE/gm	% yield
EEZJ	16.03±0.042	5.65±0.040	8.5±0.044
AEZJ	17.12±0.025	12.5±0.03	7.6±0.106
EEZO	16.67±0.153	10.94±0.025	4.5±0.140
AEZO	10.86±0.045	4.38±0.04	8.7±0.252

Here, TFC, TPC and % yield was mentioned as mean ± SD

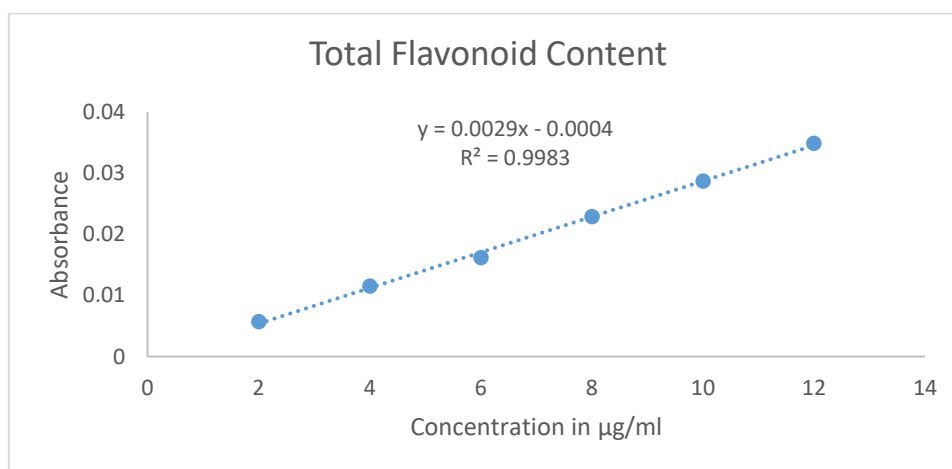


Figure 1: Standard calibration curve of quercetin

2.4. Total phenolic contents

The total phenolic content (TPC) was estimated using the Folin–Ciocalteu method and expressed as mg gallic acid equivalents (GAE)/g dry extract. The gallic acid calibration curve exhibited excellent linearity ($R^2 = 0.998$), confirming the suitability of the method for quantitative estimation. Each concentration was analysed in triplicate, and mean absorbance values were used for calibration (Table 4 and Figure 2). Among the tested extracts, AEZJ showed the highest phenolic content, while AEZO exhibited the lowest. Phenolic compounds are well known for their antimicrobial and antioxidant properties, particularly through membrane disruption, metal chelation, and inhibition of microbial enzymes. The higher TPC observed in AEZJ may therefore explain its comparatively stronger antibacterial activity. A positive correlation between phenolic/flavonoid content and antimicrobial activity has also been reported in previous studies involving medicinal plants rich in polyphenolic constituents.

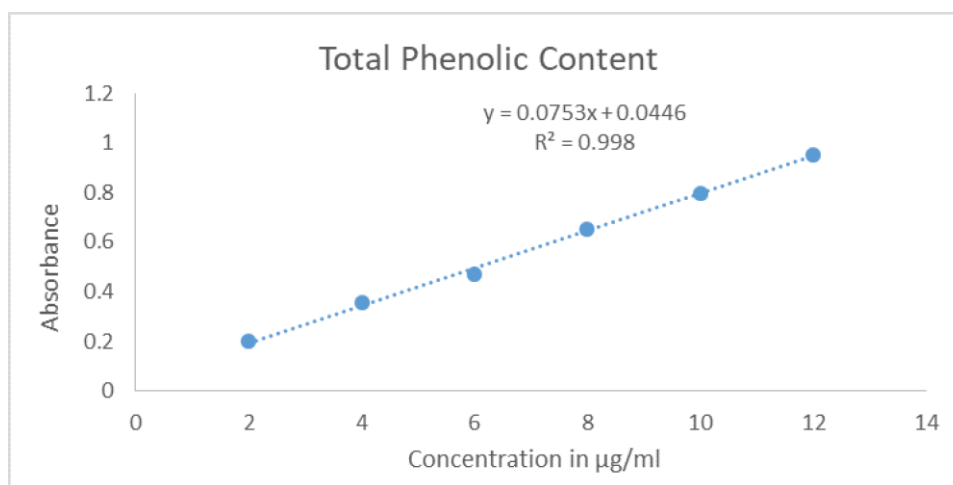


Figure 2: Standard calibration curve of gallic acid

2.5. Antimicrobial activity

The antibacterial activity of aqueous and ethanolic extracts of *Z. jujuba* and *Z. oenoplia* was evaluated against selected Gram-positive and Gram-negative bacterial strains using agar well diffusion and MIC methods. The experiments were conducted in triplicate, and results are expressed as mean ± SD.

Among the tested extracts, EEZO exhibited comparatively higher antibacterial activity against *Staphylococcus aureus*, whereas AEZJ showed moderate inhibition against *Salmonella typhi* and *Pseudomonas aeruginosa*. Variations in antibacterial activity among extracts may be attributed to differences in phytochemical composition and solvent extraction efficiency.

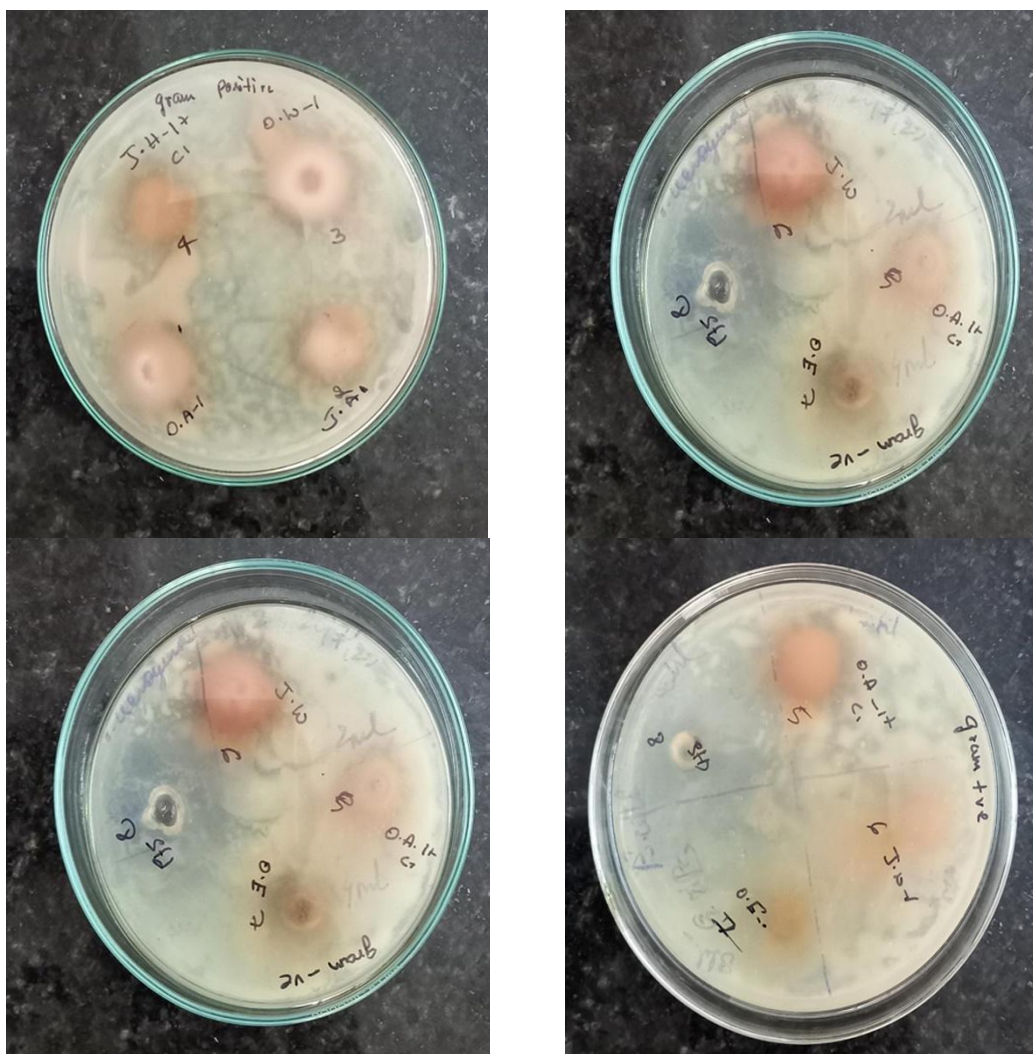
Although inhibition zone diameters provide preliminary evidence of antibacterial activity, MIC values offer a more accurate assessment of extract potency. In the present study, EEZO demonstrated the lowest MIC value against *S. typhi*, indicating stronger inhibitory activity compared with the other extracts. The observed differences between ZOI and MIC values may be associated with variations in compound diffusion rates in agar medium, which do not always directly correlate with antimicrobial potency (Table 5).

Previous reports have demonstrated that flavonoids, phenolics, and alkaloids present in medicinal plants can alter bacterial membrane permeability and interfere with intracellular metabolic pathways. The antibacterial activity observed in the present study may therefore be associated with the synergistic action of these phytoconstituents.

Table 5: Antimicrobial activity of aqueous and alcoholic extracts of *Z. jujuba* and *Z. oenoplia*

Extracts	Zone of Inhibition (ZOI) with Microorganisms (mm)				MIC (Minimum Inhibitory Concentration) values
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>S. typhi</i>	<i>P. aeruginosa</i>	
AEZO	4.1±0.208	6.1±0.152	4.2±0.265	5.1±0.115	-
EEZJ	6.2±0.153	6.2±0.208	7.2±0.2	6.2±0.265	-
AEZJ	6.2±0.153	6.1±0.153	6.2±0.2	6.2±0.265	28.97±0.021
EEZO	7.1±0.062	6.07±0.066	6.0±0.045	5.0±0.04	65.81±0.02
CIPROFLOXACIN	11±0.047	12±0.026	11±0.035	11±0.025	-

Values are represented as mean ± SD (n=3), one-way ANOVA along with post-hoc Tukey's test. If P<0.01, significant, p>0.01, not significant



Where: JW – aqueous extract of *Z. jujuba*, OA – alcoholic extract of *Z. oenoplia*, OW – aqueous extract of *Z. oenoplia*, JA – alcoholic extract of *Z. jujuba*, and Std – standard

2.5.1. Zone of Inhibition studies

The result of the present study demonstrated the antimicrobial activity of alcoholic and aqueous extracts of *Z. jujuba* and *Z. oenoplia* against selected gram-positive and gram-negative microorganisms. EEZO demonstrated the strongest antibacterial activity against *S. typhi*, as indicated by its comparatively lower MIC value.

2.5.2. Combination effect of *Ziziphus* species with standard antibiotic

The combination of plant extracts with conventional antibiotics has emerged as a promising strategy to overcome multidrug resistance through synergistic enhancement of antibacterial activity. In the present study, AEZJ and EEZO were evaluated in combination with Ciprofloxacin against selected MDR bacterial strains. Among the tested combinations, EEZO combined with ciprofloxacin produced the greatest enhancement in antibacterial activity, particularly against *Salmonella typhi* and *Staphylococcus aureus*. The increase in inhibition zone diameter compared with ciprofloxacin alone suggests a synergistic interaction between phytoconstituents and the antibiotic. The synergistic activity may be attributed to multiple mechanisms, including increased bacterial membrane permeability, inhibition of efflux pumps, disruption of biofilm formation, or interference with bacterial resistance enzymes by flavonoids and phenolic compounds present in the extracts. Similar synergistic interactions between plant-derived phenolics and fluoroquinolone antibiotics have also been reported in earlier studies [10]. Furthermore, the combination approach did not exhibit any antagonistic effects under the tested experimental conditions, indicating its potential therapeutic significance [10].

Table 6: Combination effect of AEZJ and EEZO with standard antibiotic

Extracts	Concentration of Ciproflaxacin in mg/mL	ZOI with microorganisms (mm)	
		<i>S. aureus</i>	<i>S. typhi</i>
AEZJ (100mg/mL)	5	8.0±0.036 ^s	5.0±0.015 ^a
	10	7.9±0.025 ^s	6.0±0.026 ^a
	15	10.1±0.025 ^s	10.0±0.025 ^s
	20	10.0±0.067 ^s	9.9±0.067 ^s
EEZO (100mg/mL)	5	10.0±0.020 ^s	9.0±0.02 ^s
	10	9.9±0.015 ^s	10.1±0.176 ^s
	15	12.0±0.015 ^s	12.0±0.015 ^s
	20	14.0±0.025 ^s	13.0±0.021 ^s

Where: s – synergistic effect, a – antagonistic effect

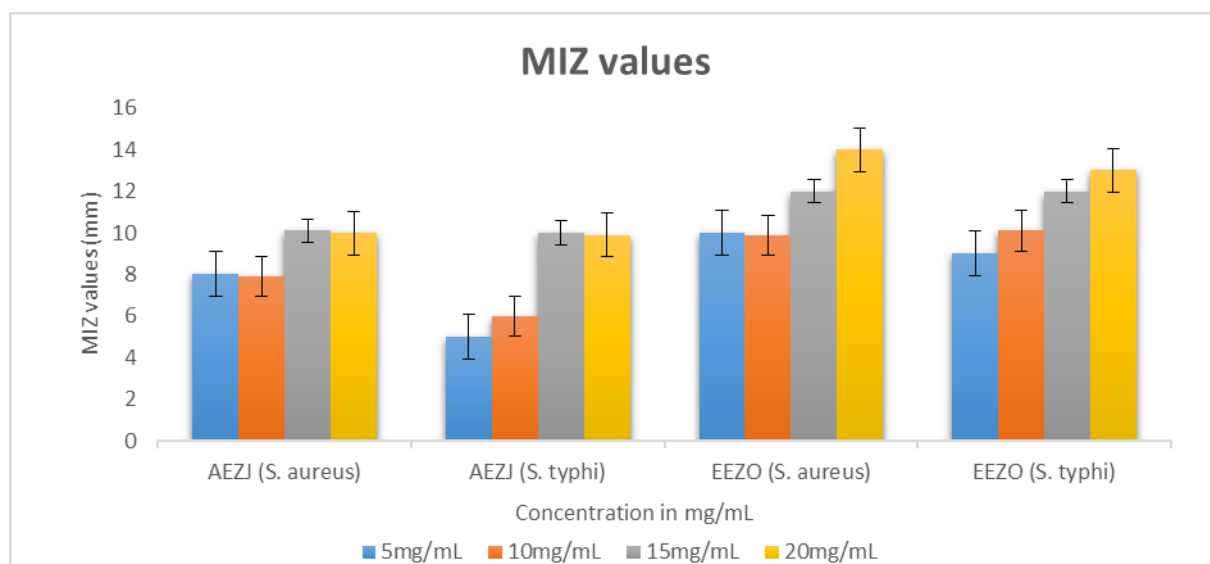


Figure 3: Comparative studies of combination effects of AEZJ and EEZO

3. Materials and methods

3.1. Collection of plant materials

Fresh stem barks of *Z. jujuba* were collected from local areas of Chitradurga district, Karnataka, India. Whereas *Z. oenoplia* plants were collected from herbal garden area of Davanagere University. Both the plants were identified and authenticated by Dr. Haleesh C, Department of Botany, Davanagere University, Davanagere district, Karnataka, India with the Voucher specimens HDUD. NO-457 and HDUD.NO-456.

3.2. Physicochemical analysis

Z. jujuba and *Z. oenoplia* stem barks were obtained, dried in the shade, and ground into a powder. According to Shukla et al. (2016f), the physicochemical characteristics of both powdered samples were assessed, including moisture content, total ash, acid-insoluble ash, water-soluble ash, and extractive values for alcohol and water [11].

3.3. Preparation of plant extracts

Dried stem bark powders of *Z. jujuba* and *Z. oenoplia* were extracted separately by maceration using distilled water and 80% ethanol according to the method described by Natheer et al. [12] with slight modifications. Briefly, 100 g of powdered plant material was soaked in 1000 mL of solvent for 72 h at room temperature with intermittent shaking. The extracts were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C. The concentrated extracts were dried, weighed to determine percentage yield, and stored at 4°C in airtight containers until further analysis.

3.4. Phytochemical investigation

The standard Harborne method was used to carry out phytochemical experiments to evaluate the concentration of secondary metabolites and to identify the constituents such as flavonoids, alkaloids, tannins, glycosides, saponins, phenols, carbohydrates, and proteins produced by plants [13].

3.5. Estimation of total flavonoids content (TFC)

Total flavonoid content was determined using the aluminium chloride colorimetric method described by Woisky and Salantino [14] with slight modifications. Quercetin was used as a reference standard. Briefly, 25 mg of each extract was dissolved in methanol and diluted to obtain concentrations of 2, 4, 6, 8, and 10 µg/mL. To 0.5 mL of each diluted solution, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate, and 2.8 mL of distilled water were added. The reaction mixture was incubated at room temperature for 30 min, and absorbance was measured at 415 nm with Shimadzu UV-visible spectrophotometer. A blank solution was also prepared in the same manner by excluding a sample. Total flavonoid content was expressed as milligrams of quercetin equivalent per gram (mg QE/g) of dried extract. A calibration curve of quercetin was prepared using mean absorbance values obtained from triplicate measurements.

3.6. Estimation of total phenolic content (TPC)

Total phenolic content was estimated Folin-Ciocalteu colorimetric method described by Genwali et al [15] with slight modifications. Briefly, 25 mg of each extract was dissolved in methanol and diluted to concentrations of 2, 4, 6, 8, and 10 µg/mL. To 0.5 mL diluted extract, 2.5 mL 10% Folin-Ciocalteu reagent was added followed by incubation for 5 min, subsequently, 2 mL of 7.5% Na₂CO₃ was added and, the sample was incubated at room temperature for 30 min. Absorbance was measured at 750 nm using UV-Visible spectrophotometer. All analyses were carried out in triplicate, and results were expressed as mg gallic acid equivalents per gram of dried extract (mg GAE/g). The gallic acid calibration curve was generated using mean absorbance values from triplicate determinations.

3.7. Microbial activity

3.7.1. Collection of the test samples

Four clinically isolated strains of Gram-positive and Gram-negative bacteria used in this study like *Staphylococcus aureus* (MTCC – 7443), *Bacillus subtilis* (MTCC – 121), *Salmonella typhi* (MTCC – 733), and *Pseudomonas aeruginosa* (MTCC – 1036) were collected from MTCC, Chandigarh, India for further study.

3.7.2. Antimicrobial susceptibility assay

The antibacterial activity of the extracts was evaluated using agar well diffusion method according to CLSI guidelines [16]. Briefly, bacterial suspensions were adjusted to 0.5 McFarland turbidity standard corresponding to 1×10^8 CFU/mL. Sterile Muller-Hinton agar plates were inoculated uniformly using sterile cotton swabs. Wells of 6 mm diameter were prepared aseptically using a sterile cork borer.

Different concentrations of plant extracts were introduced into the wells, while Ciprofloxacin was used as the positive control, and dimethyl sulfoxide (DMSO) as the negative control. Plates were allowed to diffuse at 4°C for 1 h and incubation at 37°C for 24 h. Antibacterial activity was determined by measuring the zone of inhibition (ZOI) in millimeters (mm). All experiments were performed in triplicate, and results were expressed mean \pm SD.

3.7.3. Minimum inhibitory concentration

MIC values were determined using the broth microdilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines [17]. Serial two-fold dilutions of each extract were prepared in Mueller-

Hinton broth to obtain concentrations ranging from 25 to 100 mg/mL. Equal volumes of standardized bacterial inoculum (approximately 1×10^8 CFU/mL) were added to each tube and incubated at 37°C for 24 h. Following incubation microbial growth was accessed visually and confirmed spectrophotometrically at 625 nm. The MIC was defined as the lowest concentration of extract showing no visible bacterial growth. All determinations were carried out in triplicate.

3.7.4. Synergistic interaction study

The synergistic interaction between plant extracts and Ciprofloxacin was preliminarily evaluated using the agar diffusion combination method. Briefly, fixed concentrations of extracts (100 mg/mL) were combined with varying concentrations of Ciprofloxacin (5, 10, 15, and 20 µg/mL), and antibacterial activity was assessed by measuring changes in inhibition zone diameter compared with Ciprofloxacin alone.

To improve methodological reliability, synergistic interactions were further interpreted according to the fractional inhibitory concentration (FIC) concept described in previous antimicrobial combination studies [18]. Synergistic activity was considered when the combination produced enhanced antibacterial activity compared with the individual agents alone, whereas antagonistic interaction referred to reduced activity after combination.

Although the checkerboard microdilution assay remains the gold standard for determining FIC indices, the present study used a preliminary diffusion-based combination approach to evaluate potential synergistic interactions.

3.8 Analysis

Values are expressed as mean \pm SD of three independent experiments (n = 3). Statistical significance for antibacterial and synergistic studies was analysed using one-way ANOVA followed by Tukey's post hoc test (p < 0.01).

4. Conclusion

The antibacterial activity of aqueous and ethanolic stem bark extracts of *Z. jujuba* and *Z. oenopia* was evaluated against four MTCC bacterial strains using the agar well diffusion method. The observed antibacterial activity may be associated with the presence of phenolic and flavonoid compounds identified during phytochemical screening. Among the tested extracts, EEZO and AEZJ exhibited comparatively higher inhibitory activity against *S. aureus* and *S. typhi*. Furthermore, combinations of these extracts with Ciprofloxacin demonstrated enhanced antibacterial activity compared with the individual treatments, suggesting a potential synergistic interaction. The improved activity may be attributed to phytoconstituents that enhance bacterial membrane permeability or interfere with resistance mechanisms, thereby increasing antibiotic susceptibility. These findings indicate that Ziziphus extracts may serve as promising adjuncts to conventional antibiotics against multidrug-resistant pathogens. However, the present study employed a preliminary diffusion-based synergistic evaluation rather than a standardized checkerboard assay; therefore, further mechanistic studies, FIC index determination, toxicity assessment, and *in vivo* investigations are required to validate the therapeutic potential of these combinations.

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