Antibiotics and *Conocarpus erectus* extract work synergistically against the multidrug-resistant *Pseudomonas aeruginosa*

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Abstract:

The health benefits of antibiotics have been compromised in recent decades due to the rise of drug-resistant microbes, rendering many commonly used antibiotics increasingly ineffective and potentially hazardous. For treating Pseudomonas aeruginosa infections, evidence suggests that combined antibiotic therapy may be more effective than monotherapy.

This study aims to evaluate the antibiofilm and bactericidal effects of Conocarpus erectus plant extracts (CEP) against Pseudomonas aeruginosa, isolated from various hospitals and exhibiting multidrug resistance. Between September 2021 and January 2022, clinical specimens from 150 patients were collected. Out of these, 83 isolates grew on cetrimide culture medium after thorough cultivation on selective media, while the rest were identified using the VITEK 2 compact system.

Results revealed high resistance rates to Ceftriaxone (92.7%) and Amoxicillin-Clavulanic acid (89.2%), with Trimethoprim showing a resistance rate of 79.5%. To investigate the antibacterial properties of CEP, ten multidrug-resistant isolates were selected. Methanolic and aqueous extracts were prepared via Soxhlet extraction and maceration. Radical scavenging activity tests indicated that methanolic and aqueous CEP extracts (96.4% and 94.1% at 10mg/ml) outperformed artificial antioxidants like BHT (93.1%) and vitamin C (97.2%). The total phenolic content for aqueous and methanolic extracts was recorded at 51.6 mg/g and 65.6 mg/g, respectively. The methanolic extract exhibited superior bactericidal activity compared to the aqueous extract at a concentration of 100 mg/ml, with minimum inhibitory concentrations ranging from 8 to 32 mg/ml for methanolic CEP, while aqueous CEP ranged from 128 to 256 mg/ml. Checkerboard analysis revealed synergistic effects between methanolic CEP and Cefepime against several isolates.

Keywords: *Conocarpus erectus*, multidrug resistance, *combined Pseudomonas aeruginosa*, synergism, antibacterial activity.

Introduction

The inappropriate and incorrect consumption of existing antimicrobial medications has been recognized as the growing problem of bacteria developing resistance to these drugs (1,2). Multiple antibiotic resistances, in addition to single drug resistance, are characteristics of clinically significant microorganisms (3,4). P. aeruginosa is an opportunistic bacterium that primarily affects cases receiving treatment in surgery, intensive care, and burn wards (5,6). It can also cause infections in immune-compromised persons. The placenta, respiratory tract, soft tissues, skin, and urinary tract are the most commonly affected organs by P. aeruginosa infections; burns, infection of surgical wounds, and pressure ulcers show the highest risk (7,8). *Conocarpus erectus L. is a* tropical plant that is widely distributed and a member of the Combretaceae family (9). It is used to treat fever, edema, gonorrhea, orchitis, syphilis, and other inflammatory diseases in traditional medicine (10). Antioxidants have been detected in several extracts taken from various plant tissues (11). One of the most essential approaches for defeating organisms resistant to several drugs is the use of antibacterial combinations, or treatments using two or more antibacterial agents (12,14). The checkerboard procedure is one of the approaches used to assess how well two antimicrobial combinations may work together. A methodology akin to that used for defining the minimum inhibitory concentration (MIC) is applied in the checkerboard method, or "Fractional inhibitory concentration (FIC) method".

Materials and methods

- **Plant collection:** The leafy portions of the plant had been gathered from nearby Iraqi markets, and the Division of Biology specialist at the Baghdad University, College of Science classified them as Conocarpus erectus L. After being cleaned with water, permitted to dry at 37°C, and then powdered with a grinder, the leaves were kept cold until further examination.
- **Aqueous extract preparation:** The technique of N'Guessan, JD. et al. was followed to prepare the aqueous CEP (15).
- **Methanolic extract preparation:** Using the Soxhlet equipment, the methanolic CEP was made following the American Association of Cereal Chemists (1984) (16).
- **Antioxidant activity analysis (DPPH assay):** As per the Ogunmoyole T et al, technique (17), an analysis was considered to determine the antioxidant influence of both aqueous and methanolic CE. 0.1 gram of CEP was placed in distilled water (DW) to yield 50 µl of various concentrations (0.312, 0.625, 1.25, 2.5, 5, and 10) mg/ml. Then, these were combined with five milliliters of newly made 0.004% of "2,2-diphenyl-1-picrylhydrazyl (DPPH)" in methanol. The working solution was then made to contain 10 mg/ml by completing the volume to 10 ml. The CEP was made in serial two-time dilutions to yield concentrations ranging from 50 to 1.625 mg/ml. Next, the absorbance of every dilution has been measured at 517 nm after 30 minutes. As positive controls, vitamin C, and butylated hydroxytoluene (BHT) were employed. Every test was run in triplicate. The following formula was used to determine the DPPH radical scavenging ability or % DPPH reduction:

Reduction% = (Abs DPPH – Abs Dil.) /Abs DPPH x 100

Absorption of DPPH = typical absorption of the 2,2-diphenyl-1-picrylhydrazyl solution

Absorption of Dil. = typical absorption of the 3 absorption measures of each dilution

Microsoft Excel was used to create a visual using the acquired values. The image was used to determine each extract's EC50 or the active concentration of the CEP or compound at which 50% of DPPH was lowered.

- **Estimation of the total phenolic composition:** The "Folin-Ciocalteu technique", as described by a prior study (18), was applied to quantify the total phenolic composition spectrophotometrically in both methanolic and aqueous CEP. 0.4 ml of every specimen was mixed with two milliliters of the diluted "Folin-Ciocalteu reagent" and 1.6 milliliters of a solution of sodium carbonate 7.5%. The amount of DW was added to get the total size down to five ml. After the tubes were wrapped in parafilm to leave half an hour to stand at 37°C, the absorbance at 760 nanometers was calculated using a spectrophotometer. Gallic acid served as a standardization reference curve to determine the overall phenolic composition. The outcomes were expressed as gallic acid (mg) equivalent /gm of dry weight.
- **Bacterial isolation:** 150 distinct samples were obtained from cases admitted to multiple Baghdad major hospitals. The primary method used to cultivate the collected specimens was streaking on nutrient agar. For twenty-four hours, plates were aerobically incubated at 37°C. The VITEK-2 System is used for identifying diagnoses.
- **Antibiotic susceptibility analysis:** To conduct the antibiotic susceptibility assessment for 14 various antibiotics, the Kirby-Bauer method has been used, as detailed elsewhere (19). One or two isolated bacterial colonies from the initial culture were chosen, and they were added to test tubes containing four milliliters of isotonic saline to establish bacterial suspension with reasonable turbidity in comparison to the standard turbidness solution. The latter is roughly equivalent to 1.5x108 CFU/cc. The part of the bacterial solution was relocated to the Mueller-Hinton culture medium by a sterilized cotton swab, and it was spread out gently and uniformly before being left for ten minutes. After that, the antimicrobic discs were positioned over the culture agar and firmly pressed to make sure they made contact with the agar using sterile forceps. Subsequently, the plates were turned over before 18 to 24 hours of incubation at 37ºC. With a metric ruler, the inhibition zones that formed around the discs were calculated in mm, as stated by the "Clinical Laboratories Standards Institute" (20).
- **Analyze the antibacterial properties of *C. erectus* extracts**

❖ **Disc diffusion technique:** The disc diffusion system was used following the standard procedure (21) to estimate the bactericidal effect of the methanolic and aqueous CEP extract. Using a sterile swab, the bacteriological cultures (added to 0.5 McFarland standard) were utilized to equally inject Muller Hinton culture plates. After 15 minutes of drying, the plates were used for the sensitivity test. To reach an ultimate concentration of 400 mg/ml, 0.4 g of the CEP extract was liquified in one ml of DW to create the stock solution of CEP. Next, the stock solution has been thinned to yield 200 and 100 mg/ml concentrations of CEP. Six-millimeter sterile blank discs were impregnated with 20 microliters of each dilution. A disc of DW served as the negative control. Before each disc was put on the Mueller Hinton agar surface, it had completely dried. For 18 to 24 hours, the culture plates were incubated at room temperature. The caliber of the inhibitory zone surrounding the culture discs was calculated to assess the antibacterial activity following the incubation. To guarantee dependability, the test was conducted three times.

❖ **Measuring the Minimum Inhibitory Concentration (MIC) of extracts from C. erectus**: The 96-well microtiter plate was utilized to calculate the MIC of the CEP using

the "Broth microdilution method". CEP was made as a working solution at 256 as well as 512 mg/ml in broth. Sequential 2-time dilutions of the CEP were made on the culture media to achieve the concentrations of 128 and 256 mg/ml of the aqueous and methanolic CEP extract, individually. 200 µl of both extracts of CE were made and added to the initial wells in row A. The broth contained 100 µl in rows B through H of the columns. Two serial dilutions were carefully made using a micropipette down the columns (from row A-H). The process was continued up to the final row (H) while the last 100 microliters were thrown away. First, 100µl was taken out of the beginning amounts in row A and transported to a following row with the 100 microliters broth, appropriately mixed. As a result, the final volume in each test well containing the CEP is now 100 microliters, except for the column that included 200 microliters of broth to control sterilization. All the wells excluding the negative control received 100 microliters of the 1×106 CFU/ml bacterial inoculant. For 18 to 20 hours, microtiter plates hatched at 37 °C. Following the incubation period, each well received 20 µl of resazurin stain, which was added and given a half-hour incubation period to monitor any color changes. The lowest CEP concentrations at which the color of the resazurin broth assay did not shift from blue to pink were identified visually in broth micro-dilutions as the MIC (22).

❖ **Combination between Antibiotics and Plant Extract:** The combination effect was examined using a modification approach. The cultures of bacteria were performed at 37˚C in a nutrient broth medium that was kept sterile. Standardized inoculums of 1.5×106 CFU/ml of each bacterium were added to the surface of sterile Muller Hinton culture medium after 4 hours of development, and uniform distribution of inoculums was achieved using a sterile cotton swab. A few minutes later, the surface of infected Muller Hinton agar plates was covered with an antibiotic filter paper disc, five mm diameter, soaked with 20µL of (400, 200, 100 mg/ml) extract concentrations of methanolic CEP. For 24 hours, the culture medium was left then at room temperature. Measuring and comparing the calibers of cleared zones with those of the antibiotic and methanolic CEP alone was done. Three copies of each test solution were preserved (23).

❖ **Checkerboard assay to assess the synergistic effect:** The microdilution technique of checkerboard broth was utilized to ascertain the synergy between the antibiotics and CEP. Each constituent was put into the sterilized 96-well microtiter plate well in 50 µl aliquots. 2-time sequential dilutions of the CEP extract and the antibiotic were made for each combination verified. A 100µl bacterial inoculum (1×106 CFU/ml) was transported to all wells, except a negative control. The microtiter plate was kept for 24 hours at ambient temperature. Afterward, 20 microliters of resazurin dye were placed on every well, to be kept for an additional 30 minutes. Fractional inhibitory concentration (FIC) index (FICI) computation is frequently done in conjunction with the checkerboard technique. The FIC was produced by the lowermost levels of CEP and antibiotics; this mixture prevented the dye from changing color. The ensuing formula was applied to detect the FIC measure for all agents separately:

FICI = ΣFIC = FIC (plant extract) + FIC (antibiotic)

Whereas the FIC of the CEP is equal to the FIC of the CEP in the mixture divided by the MIC of the CEP extract alone, then the FIC (antibiotic) is equal to the MIC of the antibiotic in the mixture divided by the MIC of the antibiotic alone.

The interactions were categorized as antagonistic (ΣFIC > 4.0), additive (≥ 0.5–1.0), different (≥1.0–≤4.0), and synergistic (≤0.5), (24).

❖ Ethical consideration

Ethical considerations for this study include obtaining informed consent from all patients whose clinical specimens were collected. Participants must be fully aware of the study's purpose, procedures, potential risks, and benefits to make an informed decision about their involvement. The research team must ensure patient confidentiality by anonymizing data and securely storing personal health information to protect participants' privacy. Permission for conducting the study was likely obtained from relevant institutional review boards or ethics committees at Baghdad University and the hospitals involved, ensuring compliance with ethical standards in research involving human subjects. This process includes a thorough review of the study's design to guarantee that it adheres to ethical guidelines and protects the rights of participants.

Results and Discussion

DPPH examination: The radicals of DPPH were extensively utilized for evaluating a compound's ability to serve as an H2 donor or a free-radical scavenger, and also to test its antioxidant properties (25). In this study, the CEP's ability to scavenge radicals was tested with DPPH as free radicals. It was found that as CEP concentrations rose, so did the scavenging ability. Moreover, the study results displayed that the aqueous and methanol CEP extracts (96.44% and 94.13%, respectively) performed better at 10 mg/ml than the BHT had a rating of 93.11%, and they were on par with the natural antioxidant (vitamin C), as revealed in Table 1.

Table (1): Radical scavenging properties of extracts from Conocarpus erectus

Concentration (mg/ml)	Aqueous extract	Methanolic extract	BHT	Vit. C	LSD value
0.312	32.25 ±0.13 F c	53.36 ±0.11 E b	30.18 ±0.08 F d	82.44 ±0.23 E a	1.58 **
0.625	50.78 ±0.18 E d	81.20 ±0.13 D b	52.15 ±0.11 E c	91.42 ±0.12 D a	1.07 **
1.25	80.72 ±0.13 D b	92.48 ±0.21 C a	75.43 ±0.29 D c	94.26 ±0.02 C a	2.32 **
2.5	91.61 ±0.16 C c	95.64 ±0.14 B a	88.61 ±0.14 C d	96.81 ±0.01 B a	1.29 **
5	92.83 ±0.11 B c	96.16 ±0.03 A a	91.69 ±0.11 B c	96.93 ±0.02 AB a	1.62 **
10	94.13 ±0.02 A b	96.44 ±0.08 A a	93.11 ±0.13 A b	97.20 ±0.06 A a	1.07 **
LSD value	0.417 **	0.398 **	0.497 **	0.342 **	---
Means with different big letters in the same column and small letters in the same row are significantly different. ** (P≤0.01).					

The antioxidant action is displayed as an "Effective Concentration (EC50)". The greatest EC50, which is the level of a toxicant, medication, or antibody that stimulates a reaction midway between the initial baseline and the maximum levels after a specific exposure duration, is a widely applied method to assess a drug's effectiveness (26). The methanolic CEP and vitamin C's radical scavenging capacities (EC50) were shown to be 0.1 and 0.2 mg/ml, separately, as shown in Figure 1. These outcomes were determined to be more efficacious than the 0.6 results obtained from BHT and the aqueous CEP. There is an inverse relationship between the effectiveness of antioxidant properties and EC50 values. A CEP is deemed effective as an antioxidant if its EC50 measure is below 10 mg/ml, per data that has been published (27). Also, the antioxidant property of CEP is influenced by the existing phenol constituents (28). It was reported that the plant's high phytochemical constituents make it useful as a natural antioxidant (29, 30). Furthermore, both ethyl acetate and ethanolic CEP extracts also demonstrated significant antioxidative activity and can sequester around 71% of the free radicals of DPPH (31). Additionally, a study found that the methanol CEP fruit portion had higher antioxidant activity than the other three extracts of leaves, stems, and flowers (32).

The overall proportion of phenols in CEP extracts

The overall phenol amount of the CEP extract was calculated with "Follin-Ciocalteu Reagent". Polyphenols are metabolites derived secondarily from plants that were produced from L-tyrosine or L-phenylalanine via the phenyl propanoic mechanism. A wide range of phenolic complex compounds have been investigated for their organic characteristics and potential health advantages (33). Phenolic compounds' bioactivities as radical terminators and antioxidant agents might be linked to their capacity to chelate metallic elements, deactivate lipoxygenase, and scavenge free radicals (34). It has been reported that phenolic chemicals found in several CEPs have antiviral, antibacterial, antifungal, and antioxidant properties in addition to activating the immunity system (31). The aqueous CEP showed total phenolic content values of 16.42, 36.39, and 51.58 mg/g in 1.25, 2.5, and 5 mg/ml, individually. In contrast, the methanolic CEP showed total phenolic values of 21.43, 43.76, and 65.60 mg/g in identical amounts, individually (Table 2). The results of the current investigation have concurred with another study (35) that discovered that the methanolic of CEP contained much more phenolic chemicals than the less polar solvents. The study also revealed that the methanolic CEP had greater phenolic compounds among the three organic extracts made from CEP leaves, with hexane and ethyl acetate contents.

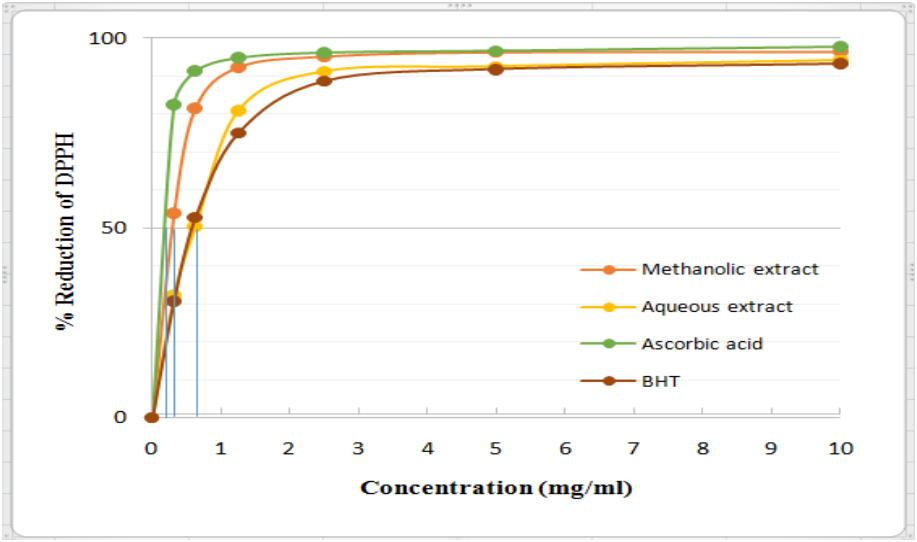

Figure 1: EC50 of extracts from leaves of Conocarpus erectus

Table 2: Conocarpus erectus extracts' total phenolic composition

Concentration (mg\ml)	Methanolic extract (mg\g)	Aqueous extract (mg\g)	LSD value
1.25	21.43 ±0.13 C a	16.42 ±0.05 C b	0.802 **
2.5	43.76 ±0.12 B a	36.39 ±0.01 B b	0.977 **
5	65.60 ±0.13 A	51.58 ±0.11 A	0.852 **
LSD value	0.445 **	0.236 **	---
Means with different big letters in the same column and small letters in the same row are significantly different. ** (P≤0.01).			

Antibiotic susceptibility testing

In the current study, the disc diffusion method has been employed to test the antibiotic susceptibility of 83 P. aeruginosa isolates using ten different antibiotics: ticarcillin (TC), amikacin (AK), cefepime (CFP), ciprofloxacin (CIP), imipenem (IM1), colistin (CS), ceftriaxon (CRO), amoxicillin-clavulanic (AUG), trimethoprim (TMP), and tobramycin (TOB). According to Table 3, the data demonstrated that the isolates of *P. aeruginosa* had considerable resistance to AUG and CRO, with proportions of 89.2% & 92.7%, individually. Trimethoprim had the lowest resisting degree, at 79.5%. Either their genetic material has undergone mutations, they have gained this ability naturally, or genes have been transferred horizontally (36). *P. aeruginosa* is an opportunistic bacterium that can infect people, particularly those with weakened immune systems. Bacteria with drug resistance are accountable for rising expenses, longer hospitalizations, and death (37, 38). Ten P. aeruginosa isolates that were multi-drug resistant were selected for this investigation, (Table 4).

The antimicrobial properties of Conocarpus erectus

Disk diffusion technique

Using the isolates of *P. aeruginosa*, the disk-diffusion technique was utilized to evaluate the antibacterial influences of CEP. As shown in Table 5, 6 the outcomes indicated that the methanolic extract outperformed the aqueous CEP extract at a concentration of a hundred mg/ml with high statistical variations (P≤0.01) among the concentrations. Statistical tests were conducted between different concentrations for each type of CE. The results displayed that the methanolic CEP had the best activity on *P. aeruginosa* with multidrug-resistant, No. 5 and 6 reveal inhibitory zones of 22.67 as well as 22.33 mm at 400 mg/ml, individually. The aqueous CEP had the best activity on multidrug-resistant P. aeruginosa No. 10, and inhibitory zones of 15.33 mm at an equal concentration. Phenolic substances have anti-inflammatory and anti-proliferative properties (39), both antiviral and antimicrobial (40). These substances affect the microbial cell by changing its permeability, harming the cell membrane, and interrupting the mechanism that produces energy, all of which ultimately result in cell death (41). The findings of this investigation were consistent with those that were printed earlier (42), which displayed that the methanolic CEP had a bactericidal effect against *Staphylococcus aureus* and *P. aeruginosa*, exhibiting inhibitory zones of 21 and 18 mm, respectively. It was proved that multidrug-resistant *S. aureus* isolated from dermal wounds could be inhibited by aqueous CEP for up to 10 mm (31).

Table (3): Antibiotic susceptibility examination of 83 *Pseudomonas aeruginosa*

Antibiotics	Resistance No. / (%)	Sensitive No. / (%)
CIP	31 (37.3%)	52 (62.6%)
TOB	33 (39.7%)	50 (60.2%)
TC	36 (43.3%)	47 (56.6%)
IMI	41 (49.3%)	42 (50.6%)
CEP	44 (53.0%)	39 (46.9%)
CS	46 (55.4%)	37 (44.5%)
AK	53 (63.8%)	30 (36.1%)
TMP	66 (79.5%)	17 (20.4%)
AUG	74 (89.2%)	9 (10.8%)
CRO	77 (92.7%)	6 (7.2%)

(**IM1):** Imipenem**,** (**TMP):** Trimethoprim**, (AUG):** Amoxicillin-Clavulanic acid**, (CFP):** Cefepime**,** (**CRO):** Ceftriaxon, (**CIP):** Ciprofloxacin**,** (**AK):** Amikacin**,** (**TOB):** Tobramycin**,** (**TC):** Ticarcillin**,** (**CS):** Colistin**.**

Table 4: Ten MDR *P. aeruginosa* isolates tested for antibiotic susceptibility

No.	Number of Isolate	IMI	CEP	TMP	TOB	AK	CIP	TC	CS	AUG	CRO	Percentage of Resistance
P_1	P_7	R	R	R	R	R	S	R	R	R	R	90 %
P_2	P_{10}	R	R	R	R	R	S	R	R	R	R	90 %
P_3	P_{20}	R	R	R	R	R	R	R	R	R	R	100 %
P_4	P_{32}	R	R	R	R	R	R	R	R	R	R	100 %
P_5	P_{37}	R	R	R	R	R	S	R	S	R	R	80 %
P_6	P_{48}	R	R	R	R	R	S	R	S	R	R	80 %
P_7	P_{56}	R	R	R	R	R	R	R	R	R	R	100 %
P_8	P_{58}	R	R	R	R	R	S	R	R	R	R	90 %
P_9	P_{65}	R	R	R	R	R	S	R	R	R	R	90 %
P_{10}	P_{82}	R	R	R	R	R	R	R	R	R	R	100 %

(***P***): *P. aeruginosa,* (**IM1**): Imipenem**,** (**CFP**): Cefepime**,** (**TMP**): Trimethoprim**,** (**TOB**): Tobramycin**,** (**AK**): Amikacin**,** (**CIP**): Ciprofloxacin**,** (**TC**): Ticarcillin**,** (**CS**): Colistin**,** (**AUG**): Amoxicillin-Clavulanic acid**,** (**CRO**): Ceftriaxone.

Table 5: Methanolic extract from Conocarpus erectus exhibits antibacterial activity against clinical isolates of P. aeruginosa

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	8.66 ±0.33 c	10.33 ±0.33 b	16.33 ±0.66 a	2.07 **
P_2	9.00 ±0.57 c	12.33 ±0.33 b	15.66 ±0.67 a	1.64 **
P_3	7.66 ±0.33 c	10.33 ±0.66 b	15.33 ±0.66 a	1.91 **
P_4	7.33 ±0.33 c	13.66 ±0.33 b	17.66 ±0.33 a	1.78 **
P_5	9.33 ±0.33 c	12.66 ±0.33 b	22.67 ±0.66 a	2.17 **
P_6	9.66 ±0.33 c	13.66 ±0.33 b	22.33 ±0.66 a	2.35 **
P_7	7.33 ±0.33 c	11.66 ±0.33 b	15.33 ±0.33 a	1.67 **
P_8	9.66 ±0.33 c	12.33 ±0.33 b	16.66 ±0.33 a	1.46 **
P_9	8.66 ±0.33 c	12.33 ±0.33 b	15.66 ±0.33 a	1.41 **
P_{10}	7.33 ±0.33 c	12.66 ±0.66 b	18.33 ±0.33 a	2.15 **
Means with different small letters in the same row are significantly different. ** ($P \leq 0.01$).				

P: *Pseudomonas aeruginosa*

Table (6): The antibacterial effect of an aqueous C. erectus extract on clinical isolates of P. aeruginosa

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	0 ±0 b	0 ±0 b	14.33 ±0.33 a	1.03 **
P_2	0 ±0 b	0 ±0 b	12.66 ±0.33 a	0.773 **
P_3	0 ±0 c	9.33 ±0.33 b	11.66 ±033 a	0.858 **
P_4	0 ±0 c	7.66 ±0.33 b	10.66 ±0.33 a	0.803 **
P_5	0 ±0 b	0 ±0 b	11.66 ±0.33 a	0.739 **
P_6	0 ±0 b	0 ±0 b	10.33 ±0.33 a	0.760 **
P_7	0 ±0 b	0 ±0 b	10.66 ±0.66 a	0.745 **
P_8	0 ±0 b	0 ±0 b	10.66 ±0.33 a	0.745 **
P_9	0 ±0 b	0 ±0 b	14.66 ±0.33 a	1.15 **
P_{10}	0 ±0 b	0 ±0 b	15.33 ±0.33 a	1.09 **
Means with different small letters in the same row are significantly different. ** ($P \leq 0.01$).				

P: *Pseudomonas aeruginosa*

Determination of the extracts *of C. erectus*

The MIC of the CEP was determined with the 96-well microtiter plates and the broth microdilution method. The antimicrobial MIC for P. aeruginosa has been ascertained by a technique that makes use of the oxidation-reduction colorimetric indicator resazurin. Resazurin is visible to the unaided eye and is blue when oxidized. It turns red when reduced by live cells, and the MIC could be found even without a spectrophotometer (43). The MIC result demonstrated that the methanolic CEP outperformed the aqueous CEP in its ability to inhibit P. aeruginosa. Tables 7, Figures 2 A, and B demonstrate that methanolic CEP extract has a MIC of 8–32 mg/ml, whereas that of the aqueous CEP extract was about 128–256 mg/ml.

Phenolic chemicals are present in Conocarpus erectus. Higher herbaceous and woody plants are frequently reported to have tannins, which are water-soluble polyphenols (44). According to preceding reports (45), CE has both bactericidal and bacteriostatic properties (46) revealing the effects of methanolic CEP extract, fruit, trunk, and flowers on gram-negative bacteria, *K. pneumoniae*, *S. aureus*, and *S. Typhimurium*. The results indicated that tannic acid extracted from CEP is mostly behind the bactericidal activity of CEP. *P. aeruginosa* demonstrated multidrug resistance to widely used drugs in this investigation. The quantity of phenolic substances (Gallic acid, Catechin, Kempferol, Quercetin, Taxifolate, besides Rutin) in extracts from CEP appears to be associated with the antibacterial action. Any reducing molecules that bind with ATP, which represents oxidizing substances, and then prevent using ATP as a fuel source, causing poor energy inside the germ cell, reducing its efficacy and destroying it. Alternatively, the chemicals in phenolics carry hydrogen ions. These substances enter the bacterial cell and obstruct the metabolism of the cell. Additionally, they connect to the cell's active sites of the enzymes and attempt to seal them off so the basic materials can no longer be bound to them (3, 47). It was exposed from earlier research on methanolic and aqueous that the methanolic CEP extracts worked better than the aqueous forms. For these reasons, these extracts have been selected for additional research.

The combination of antibiotics and CEP extracts

The combined impact of five conventional antibiotics and CEP methanol toward multidrug-resistant *P. aeruginosa* was evaluated using the disc diffusion technique. As indicated in Tables (8, 9, 10, 11, and 12), the combination drug's inhibitory zone proved to be more effective than that of each methanolic CEP and antibiotic by themselves. There is a promise for the creation of new antimicrobial treatments and the treatment of several illnesses brought on by microbes when therapeutic extracts and well-recognized antibiotics are combined (48).

Table (6): C. erectus aqueous extract's (mg/ml) antibacterial efficacy against P. aeruginosa clinical isolates

Isolate	Aqueous CEP extracts	Methanolic CEP extracts
	MIC	MIC
P_1	256	16
P_2	256	16
P_3	128	32
P_4	128	32
P_5	256	8
P_6	256	8
P_7	256	32
P_8	256	16
P_9	256	16
P_{10}	256	32

(P): *P. aeruginosa* isolate

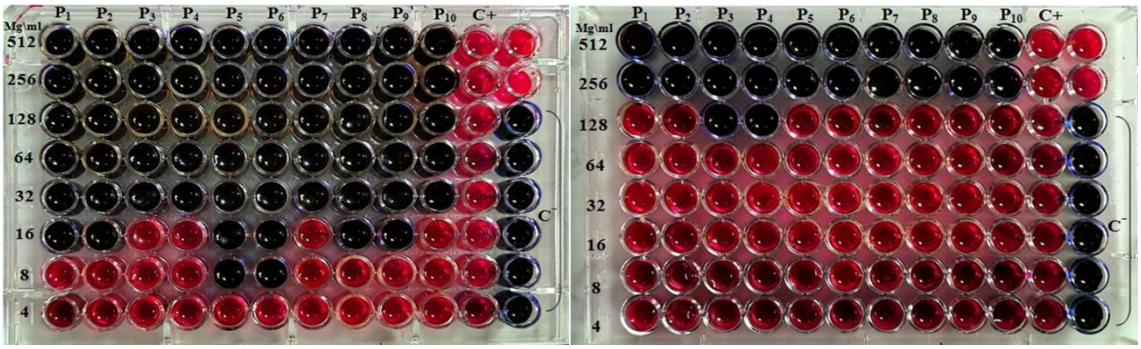

Figure 2: (A) MIC of methanolic leaf extract and (B) aqueous leaf extract from Conocarpus erectus against P. aeruginosa

(C^+): Control positive (Bacteria + Media), **(P):** *P. aeruginosa* isolate, **(C^-):** Control negative (Media only)

Phenolic chemicals are present in Conocarpus erectus. Higher herbaceous and woody plants are frequently reported to have tannins, which are water-soluble polyphenols (44). According to preceding reports (45), CE has both bactericidal and bacteriostatic properties (46) revealing the effects of methanolic CEP extract, fruit, trunk, and flowers on gram-negative bacteria, *K. pneumoniae*, *S. aureus*, and *S. Typhimurium*. The results indicated that tannic acid extracted from CEP is mostly behind the bactericidal activity of CEP. *P. aeruginosa* demonstrated multidrug resistance to widely used drugs in this investigation. The quantity of phenolic substances (Gallic acid, Catechin, Kempferol, Quercetin, Taxifolate, besides Rutin) in extracts from CEP appears to be associated with the antibacterial action. Any reducing molecules that bind with ATP, which represents oxidizing substances, and then prevent using ATP as a fuel source, causing poor energy inside the germ cell, reducing its efficacy and destroying it. Alternatively, the chemicals in phenolics carry hydrogen ions. These substances enter the bacterial cell and obstruct the metabolism of the cell. Additionally, they connect to the cell's active sites of the enzymes and attempt to seal them off so the basic materials can no longer be bound to them (3, 47). It was exposed from earlier research on methanolic and aqueous that the methanolic CEP extracts worked better than the aqueous forms. For these reasons, these extracts have been selected for additional research.

The combination of antibiotics and CEP extracts

The combined impact of five conventional antibiotics and CEP methanol toward multidrug-resistant *P. aeruginosa* was evaluated using the disc diffusion technique. As indicated in Tables (8, 9, 10, 11, and 12), the combination drug's inhibitory zone proved to be more effective than that of each methanolic CEP and antibiotic by themselves. There is a promise for the creation of new antimicrobial treatments and the treatment of several illnesses brought on by microbes when therapeutic extracts and well-recognized antibiotics are combined (48).

Table 8: The combined impact of Amikacin and methanolic plant extract from Conocarpus erectus on Pseudomonas aeruginosa

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	11.33 ±0.33 c	13.33 ±0.33 b	18.66 ±0.66 a	1.38 **
P_2	10.33 ±0.33 c	12.33 ±0.33 b	19.66 ±0.66 a	1.42 **
P_3	12.00 ±0.57 b	13.33 ±0.33 b	18.66 ±0.66 a	1.39 **
P_4	10.33 ±0.33 c	12.33 ±0.33 b	17.33 ±0.33 a	1.41 **
P_5	12.33 ±0.33 b	13.33 ±0.33 b	21.33 ±0.33 a	1.57 **
P_6	11.00 ±0.57 c	13.33 ±0.33 b	21.33 ±0.33 a	1.25 **
P_7	12.33 ±0.33 b	13.66 ±0.66 b	19.66 ±0.66 a	1.37 **
P_8	11.66 ±0.66 c	13.33 ±0.33 b	20.00 ±1.00 a	1.43 **
P_9	11.33 ±0.33 c	13.33 ±0.33 b	18.33 ±0.33 a	1.50 **
P_{10}	11.33 ±0.33 c	13.66 ±0.66 b	18.66 ±0.66 a	1.36 **
Means with different small letters in the same row are significantly different. ** (P≤0.01).				

P: *Pseudomonas aeruginosa*

Table (9): Combined effect of CEP methanolic extract and AUG on *Pseudomonas aeruginosa*

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	12.33 ±0.33 c	16.33 ±0.33 b	20.33 ±0.33 a	1.74 **
P_2	15.33 ±0.33 c	19.33 ±0.33 b	21.66 ±0.33 a	1.37 **
P_3	12.33 ±0.33 c	18.33 ±0.33 b	21.66 ±0.66 a	2.06 **
P_4	11.33 ±0.33 c	13.33 ±0.33 b	18.33 ±0.33 a	1.56 **
P_5	13.33 ±0.33 c	17.33 ±0.33 b	27.66 ±0.33 a	1.93 **
P_6	16.33 ±0.33 c	18.66 ±0.33 b	28.66 ±0.33 a	1.41 **
P_7	11.33 ±0.33 c	14.33 ±0.33 b	21.33 ±0.33 a	1.83 **
P_8	12.33 ±0.33 c	14.33 ±0.33 b	21.33 ±0.33 a	1.38 **
P_9	11.33 ±0.33 c	16.33 ±0.33 b	21.66 ±0.33 a	1.82 **
P_{10}	15.33 ±0.33 c	18.33 ±0.33 b	21.66 ±0.33 a	1.57 **
Means with different small letters in the same row are significantly different. ** (P≤0.01).				

P: *Pseudomonas aeruginosa*

Table (11): Combined effect of CEP methanolic extract and Ceftriaxone on Pseudomonas aeruginosa

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	14.33 ±0.33 c	18.66 ±0.33 b	23.66 ±0.33 a	1.15 **
P_2	17.33 ±0.33 c	24.33 ±0.33 b	29.33 ±0.33 a	1.96 **
P_3	15.33 ±0.33 c	22.33 ±0.33 b	28.66 ±0.33 a	2.05 **
P_4	12.33 ±0.33 c	15.33 ±0.33 b	19.33 ±0.33 a	1.37 **
P_5	18.66 ±0.33 c	25.33 ±0.33 b	32.33 ±0.33 a	2.44 **
P_6	16.66 ±0.33 c	23.33 ±0.33 b	32.66 ±0.33 a	1.81 **
P_7	19.33 ±0.33 c	24.33 ±0.33 b	30.33 ±0.33 a	1.52 **
P_8	14.33 ±0.33 c	18.33 ±033 b	23.33 ±0.33 a	1.29 **
P_9	13.33 ±0.33 c	18.33 ±0.33 b	23.33 ±0.33 a	1.40 **
P_{10}	17.33 ±0.33 c	27.33 ±0.33 b	31.00 ±0.57 a	1.87 **
Means with different small letters in the same row are significantly different. ** (P≤0.01).				

P: *Pseudomonas aeruginosa*

Table (10): Combined action of cefepime and CEP methanolic extract on Pseudomonas aeruginosa

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	11.33 ±0.33 c	14.66 ±0.33 b	18.33 ±0.33 a	1.26 **
P_2	13.33 ±0.33 c	18.33 ±0.33 b	22.66 ±0.66 a	1.33 **
P_3	15.33 ±0.33 c	19.33 ±0.33 b	23.33 ±0.33 a	1.09 **
P_4	13.33 ±0.33 c	16.66 ±0.33 b	22.66 ±0.66 a	1.41 **
P_5	15.66 ±0.33 c	18.66 ±033 b	29.66 ±o.66 a	1.58 **
P_6	15.33 ±0.33 c	21.33 ±0.33 b	30.66 ±0.88 a	1.19 **
P_7	11.66 ±0.33 c	15.33 ±0.33 b	23.66 ±0.66 a	1.25 **
P_8	13.33 ±0.33 c	16.33 ±033 b	21.66 ±0.33 a	1.02 **
P_9	16.33 ±0.33	19.66 ±0.33 b	24.33 ±0.33 a	1.37 **
P_{10}	15.33 ±0.33	18.66 ±0.33 b	24.66 ±0.66 a	1.18 **
Means with different small letters in the same row are significantly different. ** (P≤0.01).				

P: *Pseudomonas aeruginosa*

Table (12): Imipenem and C. erectus methanolic extract combined action against Pseudomonas aeruginosa

Isolates	100 mg/ml	200 mg/ml	400 mg/ml	LSD value
P_1	11.33 ±0.33 c	16.33 ±0.33 b	20.33 ±0.33 a	1.63 **
P_2	13.33 ±0.33 c	17.33 ±0.33 b	21.33 ±0.33 a	1.38 **
P_3	12.33 ±0.33 c	18.33 ±0.33 b	22.33 ±0.33 a	1.40 **
P_4	11.33 ±0.33 c	14.33 ±0.33 b	19.33 ±0.33 a	1.52 **
P_5	15.33 ±1.33 b	16.33 ±0.33 b	24.66 ±0.33 a	2.19 **
P_6	14.33 ±0.33 c	20.33 ±0.33 b	25.33 ±0.33 a	1.94 **
P_7	14.33 ±0.33 c	18.33 ±0.33 b	23.33 ±0.33 a	1.47 **
P_8	12.33 ±0.33 c	16.33 ±0.33 b	21.33 ±0.33 a	1.72 **
P_9	12.33 ±0.33 c	15.33 ±0.33 b	22.33 ±0.33 a	1.66 **
P_{10}	16.33 ±0.33 c	18.33 ±0.33 b	20.33 ±0.33 a	1.78 **
Means with different small letters in the same row are significantly different. ** ($P \leq 0.01$).				

P: *Pseudomonas aeruginosa*

Phenolic compounds are the primary phytochemicals in the methanolic CEP. These complexes presumably intercalated in the lipid bilayer to impede the development of the bacterial cell (49). The pharmaceutical sector (medical herbs) has a strong possibility to be a source of natural medications, which can be used to tackle the threat presented by resisting antibiotics because there aren't many new antimicrobials on the market. It was reported that because herbal antimicrobials have strong antimicrobic activities and might be administered separately or combined with other antibiotics to address the existing antibiotic resistance contest, they may find application in the pharmaceutical industry (50). Additionally, showed that medicinal herbs are a very effective way to boost medicines' antibacterial activity (51).

Antibacterial synergistic activity of antibiotics and plant extract

Using the checkerboard analysis technique, the synergistic in vitro effects between methanolic extracts of CEP and drugs toward multidrug-resistant *P. aeruginosa* were evaluated. Cefepime and ceftriaxone's respective MICs were assessed, as indicated in Table 13 and Figures 3A and B. Table 14 and Figures 4A demonstrate that the methanolic CEP exhibited an additive impact towards four isolates of *pseudomonas aeruginosa* (FICI>0.5–1.0) and a synergistic activity with Cefepime antibiotic against six isolates (FICI≤0.5). Additionally, Figure 4B and Table 15 show that Ceftriaxone had a synergistic activity against seven P. aeruginosa isolates and an additive effect towards three bacterial isolates. The use of antibiotics in conjunction with the methanolic CEP caused a 2 to 5-time decrease in each MIC. Few antimicrobial medications are successful in treating MDR bacterial infections. Therefore, it is useful to identify substances that increase the antibiotics' antimicrobial effectiveness against these microorganisms. One novel strategy for addressing the issue of bacterial resistance is the potential of CEP to work in concert with antibiotics (52). Numerous scientific investigations have demonstrated the synergistic in vitro effect of leaf-active substances against multidrug-resistant

microorganisms. The advancement in synergy research makes it more likely to develop novel plant-based antibacterial medicines for treating illnesses (53). The synergistic relationship between two substances, wherein one substance improves the influence of the others. Also, collectively they perform more proficiently than as separate items, inspiring several researchers to evaluate the implication of the synergistic activity of herbal mixtures and antibiotics conventionally used (24).

Table (13): Minimal inhibitory concentrations of Ceftriaxone and Cefepime on *P. aeruginosa*

Isolate	Ceftriaxone (µg/ml)	Cefepime (µg/ml)
	MIC	MIC
P_1	256	512
P_2	256	512
P_3	512	512
P_4	512	512
P_5	512	128
P_6	512	512
P_7	512	32
P_8	512	128
P_9	256	512
P_{10}	512	512

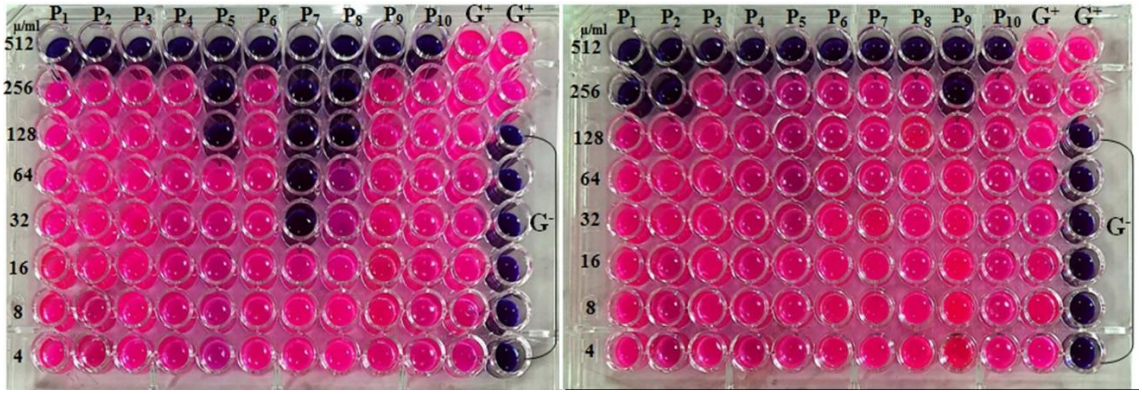

Figure 3: (A) MIC of Cefepime and (B) Cefatriaxone antibiotics on *Pseudomonas aeruginosa*
P: *Pseudomonas aeruginosa* isolate, **(C^+):** Positive Control (Bacteria + Media), **(C^-):** Negative control (Media only)

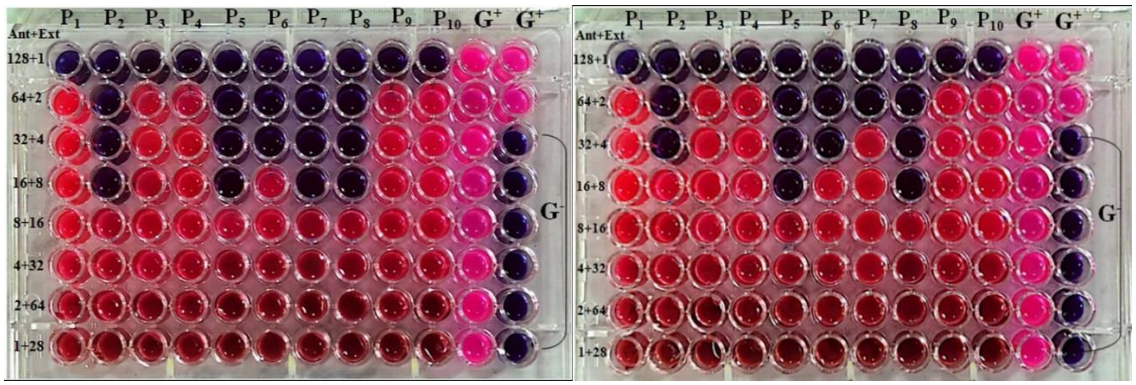

Figure 4: (A) Checkerboard method between Cefepime and methanolic and (B) Ceftriaxone and methanolic extracts on *Pseudomonas aeruginosa*

Table (14): Combined bactericidal activity of methanolic extract with cefepime against Pseudomonas aeruginosa

Isolates	MIC Anti (µg) / Ext (mg)		FIC Anti / Ext	FICI	Interaction
	Individual	Combination			
P_1	512/16	128/1	0.25/0.0625	0.3125	Synergism
P_2	512/16	16/8	0.312/0.5	0.53125	additive
P_3	512/32	128/1	0.25/0.3125	0.28125	Synergism
P_4	512/32	128/1	0.25/0.3125	0.28125	Synergism
P_5	128/8	16/2	0.125/0.25	0.375	Synergism
P_6	512/8	32/4	0.0625/0.5	0.5625	additive
P_7	32/32	16/8	0.5/0.25	0.75	additive
P_8	128/16	16/8	0.125/0.5	0. 625	additive
P_9	512/16	128/1	0.25/0.0625	0.3125	Synergism
P_{10}	512/32	128/1	0.25/0.03125	0.28125	Synergism

(P): *P. aeruginosa* isolate, **(ANT):** Antibiotic, **(EXT):** Methanolic Extract

Table (15): Combined bactericidal activity of methanolic CEP extract and Ceftriaxone against Pseudomonas aeruginosa

Isolates	MIC Anti (µg) / Ext (mg)		FIC Anti / Ext	FICI	Interaction
	Individual	Combination			
P_1	256/16	128/1	0. 5/0.0625	0.5625	additive
P_2	256/16	32/4	0.125/0.25	0.375	Synergism
P_3	512/32	128/1	0.25/0.3125	0.28125	Synergism
P_4	512/32	128/1	0.25/0.3125	0.28125	Synergism
P_5	128/8	16/2	0.125/0.25	0.375	Synergism
P_6	512/8	32/4	0.0625/0.5	0.5625	additive
P_7	512/32	64/2	0.125/0.0625	0.1875	Synergism
P_8	512/16	16/8	0.03125/0.5	0. 53125	additive
P_9	256/16	128/1	0.25/0.0625	0.3125	Synergism
P_{10}	512/32	128/1	0.25/0.03125	0.28125	Synergism

(P): *P. aeruginosa* isolate, **(ANT):** Antibiotic, **(EXT):** Methanolic Extract

Conclusion

Developing innovative plant-based antibacterial chemicals will surely be aided by an understanding of the synergistic bactericidal interactivities in medical plant-based combination treatments. The CEP antibacterial activity against P. aeruginosa, a multidrug-resistant pathogen, was improved and increased when combined with medications. Therefore, by lowering dosage concentration and utilizing their synergy with medicinal plants, antibiotics that cause side effects might be employed. Combining CEP with antibiotics broadens their spectrum and prevents resistance from growing.

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