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Synergistic Effect of *Conocarpus erectus* Extract and some Antibiotics against Multi-Drug Resistant *Pseudomonas aeruginosa*

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Abstract: Over the past few decades, the health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to the emergence of drug-resistant bacteria. The clinical use of a combination of antibiotic therapy for *Pseudomonas aeruginosa* infections is probably more effective than monotherapy. The present study aims to estimate the antibacterial and antibiofilm activity of *Conocarpus erectus* leaves extracts against multi-drug resistant *P. aeruginosa* isolated from different hospitals in Baghdad city. One hundred fifty different clinical specimens were collected from patients from September 2021 to January 2022. All samples were cultured on specific and differential media, only 83 isolates were able to grow on cetrimide agar and at 42˚C, and then the VITEK 2 compact system was dependent to complete the identification. The results showed that the high resistance of the isolates was to the two antibiotics Ceftriaxone and Amoxicillin-Clavulanic acid with a percentage of (92.7%) and (89.2%) respectively, followed by Trimethoprim with a resistance rate of (79.5%). Ten isolates with multi-drug resistance are selected to evaluate the antibacterial activity of plant extracts and the combination between *Conocarpus erectus* extract and antibiotics. Maceration and Soxhlet apparatus were used to prepare the methanolic and aqueous extracts. The results of the radical scavenging ability showed that the methanolic and aqueous extracts (96.44 and 94.13%) in 10 mg/ml respectively, were more than the artificial antioxidant (BHT) which was 93.11% and the approach with the vitamin C which was 97.20%. The results of the total phenolic content were observed at 51.58 and 65.60 mg/g in 5 mg/ml for the aqueous and methanolic extracts respectively. The antibacterial activity of *C. erectus* leaves extracts showed that the methanolic extract was more effective than the aqueous extract at a concentration of 100 mg/ml. The results of the minimal inhibitory concentration (MIC) of the methanolic extract against *P. aeruginosa* were between 8-32 mg\ml. While the MIC values of the aqueous extract were 128-256 mg\ml. The synergistic activity between *C. erectus* methanolic extract and antibiotics against multidrug-resistant *P. aeruginosa* was assessed using the checkerboard analysis technique. The methanolic extract showed a synergistic effect with Cefepime against six isolates (FICI: ≤0.5), and an additive effect against four isolates (FICI: (≥ 0.5–1.0). Furthermore, a synergistic effect with Ceftriaxone against seven isolates and additive interaction was found against three isolates.

Keywords: *C. erectus, P. aeruginosa*, antibacterial activity, multidrug resistance, Synergistic therapy.

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Introduction

The increasing phenomenon of the acquisition of resistance among microorganisms to antimicrobial drugs is attributed to the indiscriminate and improper use of current antimicrobial drugs (1). Clinically important bacteria are characterized not only by single drug resistance but also by multiple antibiotic resistances (2). *P. aeruginosa* is an opportunistic pathogen that can cause infections in immune-

compromised individuals, most commonly in patients treated in intensive care, surgery, and burn units. *P. aeruginosa* infections most frequently involve the respiratory tract, placenta, urinary system, skin, and soft tissues; the most significant risk is associated with the infection of postoperative wounds, burns and pressure ulcers (3, 4). *Conocarpus erectus* L. belongs to the Combretaceae family and is widely distributed in the tropics (5). In folk medicine, it is used to treat infections and inflammatory conditions such as syphilis, gonorrhoea, orchitis, fever and oedema (6). Several extracts obtained from different tissues of this plant have shown antioxidants (7). Treatment with antibacterial combinations, using two or more antibacterial agents is one of the most important strategies to overcome multidrug-resistant organisms (8, 9). Among the techniques employed in the evaluation of the combination of two antimicrobials potentially exhibiting synergism is the checkerboard technique. The checkerboard or fractional inhibitory concentration (FIC) technique employs a methodology similar to that utilized for the determination of the minimum inhibitory concentration (MIC). The combination is said to have a synergistic effect if there is a 4-fold reduction in the MIC of each antimicrobial agent tested alone (10). The present study aims to estimate the antibacterial and antibiofilm activity of *Conocarpus erectus* leaves extracts against multi-drug resistant *P. aeruginosa* isolated from different hospitals in Baghdad city.

Materials and methods

- **Collection of plant:** Plant leaves were collected from the local Iraqi markets, identified as (*Conocarpus erectus L*) by the specialist, Department of Biology, College of Science, University of Baghdad. The leaves were washed with water and dried at room temperature, and ground using a grinder, then stored at 4^{o}C for further analysis.
- **Preparation of aqueous extract:** The aqueous extract was prepared according to (11).
- **Preparation of methanolic extract:** The methanolic extract was prepared according to (12) by using Soxhlet apparatus.
- **Evaluation of the antioxidant activity (DPPH assay):** According to (13), the antioxidant activity of the prepared *C. erectus* methanolic and aqueous leaves extracts was conducted. Five ml of a freshly prepared 0.004 % of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was mixed with 50 µl of different concentrations (0.312, 0.625, 1.25, 2.5, 5 and 10) mg/ml, these were prepared by dissolving 0.1 gram of the *C. erectus* extract in distilled water. Then, the volume was completed into 10 ml to make the working solution 10 mg/ml. Serial two-fold dilutions of the *C. erectus* extract were prepared to make the concentrations 50-1.625 mg/ml. The absorbance of each dilution, after 30 minutes, was measured at 517 nm. Butylated hydroxytoluene (BHT) and vitamin C were used as positive control. All tests were performed in triplicate. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated as:

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

Whereby: **Abs DPPH** = average absorption of the DPPH solution, **Abs Dil.** = average absorption of the three absorption

values of each dilution. With the obtained values, a graphic was made using Microsoft Excel. The EC_{50} of each extract (effective concentration of extract or compound at which reduced 50% of DPPH) was taken from the graphic.

- **Determination of total phenolic content: T**otal phenolic content of methanolic and aqueous extracts was determined spectrophotometrically using the Folin-Ciocalteu method described by (14). 0.4 ml of each sample was mixed with 2.0 ml of the Folin- Ciocalteu reagent (diluted 10 times), and 1.6 ml of 7.5% sodium carbonate solution. The total volume was adjusted to 5 ml by adding distilled water. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was read at 760 nm spectrometrically. The total phenolic content was calculated from a calibration standard curve of gallic acid and the results were given as mg gallic acid equivalent per gram of dry weight.
- **Isolation of bacteria:** One hundred fifty different clinical specimens were collected from patients referring to several hospitals in Baghdad. The collected specimens were primarily cultured by streaking on Nutrient agar. Plates were incubated aerobically at 37°C for 24 hours. The emphasize diagnoses using VITEK-2 System.
- **Antibiotic susceptibility test:** Kirby-Bauer's method was followed as described by (15), to carry out the antibiotic susceptibility test for 14 different antibiotics. The bacterial suspension was prepared by picking 1-2 isolated colonies of bacteria from the original culture and introducing them into a test tube containing 4 ml of normal saline to produce a bacterial suspension of moderate turbidity compared with the standard turbidity solution. This approximately equals $1.5x10^8$ CFU/ml. By a sterile cotton swab, a portion of bacterial suspension was transferred and carefully and evenly spread on Mueller- Hinton agar medium, and then it was left for 10 min. Thereafter the antimicrobial discs were placed on the agar with sterile forceps pressed firmly to ensure contact with the agar. Later the plates were inverted and incubated at 37ºC for 18-24 hours. Inhibition zones developed around the discs were measured by a millimetre (mm) using a metric ruler according to Clinical Laboratories Standards Institute (16).
- **Study the antibacterial activity of *Conocarpus erectus* extracts**
  - **Disc diffusion method:** According to the standard method by (17), the disc diffusion method for antibacterial activity was carried out to assess the presence of antibacterial activities of the *C. erectus* methanolic and aqueous extracts. The bacterial culture (adjusted to 0.5 McFarland standard), was used to inoculate Muller Hinton agar plates evenly using a sterile swab. The plates were dried for 15 minutes and then used for the sensitivity test. A stock solution of plant extract was prepared by dissolving 0.4 g of the extracts with 1 ml distilled water to produce a final concentration of 400 mg/ml. The stock solution was then diluted to concentrations of 200 and 100 mg/ml of extract. 20 μl of each dilution was impregnated into sterile blank discs 6mm in diameter. Distilled water disc was

used as a negative control. All discs were fully dried before being placed on the Mueller Hinton agar surface. The plates were incubated at 37°C for 18 to 24 hrs. After the incubation, the antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the discs. The test was repeated three times to ensure reliability.

- **Determination of minimum inhibitory concentration (MIC) of *C. erectus* extracts**: The broth microdilution method was used to determine the (MIC) of the *C. erectus* extracts using the 96-well microtiter plate. The working solution of the plant extracts was prepared at 256 and 512 mg/ml in broth and serial two-fold dilutions of extract were prepared directly on the plate to make the concentrations 128-1 and 256-1 mg/ml for methanolic and aqueous extracts respectively. 200 µl of the prepared *C. erectus* methanolic and aqueous extracts were introduced into the first wells in row A. Rows B-H in columns had 100 µl of the broth alone. Twofold serial dilutions using a micropipette were done systematically down the columns (from rows A-H). 100 µl was removed from the starting concentrations in row A and transferred to the next row with the 100µl broth, properly mixed, and the procedure was repeated up to the last row (H) where the last 100µl was discarded. This brings the final volume in all the test wells with the extracts to 100 µl except the column which had 200 µl of the broth that served as sterility control. 100µl of the 1×10^6 CFU/ ml bacterial inoculum was transferred into all the wells except the negative control . Microtiter plates were incubated at 37oC for 18-20 hrs. After incubation, 20 µl of resazurin dye was added to all the wells and incubated for 30 minutes to observe any color changes. The Minimum Inhibitory Concentrations were determined visually in broth micro dilutions as the lowest concentrations of the extracts at which no color changed from blue to pink in the resazurin broth assay (18).
- **Combination between Plant extract and antibiotics:** A modification procedure was followed to investigate the combination effect. The bacterial cultures were grown in a sterile nutrient broth medium at 37˚C. After 4 h of growth, standardized inoculums of each bacterium, 1.5×10^6 CFU/ml, were introduced onto the surface of sterile Muller Hinton agar plates and a sterile cotton swab was used for even distribution of inoculums. After a few minutes, the antibiotic filter paper disk of 5 mm in diameter impregnated with 20 µL of concentrations (100, 200 and 400 mg/ml) of methanolic extract was placed on the surface of inoculated Muller Hinton agar plates. The plates were incubated at 37˚C for 24 h. The diameters of cleared zones were measured and compared with that of the antibiotic and methanolic extract alone. For each test solution, three replicates were maintained (19).

❖ **Evaluation of the synergistic effect (checkerboard assay):** The checkerboard broth microdilution method was used for the determination of synergy between the antibiotics and plant extracts. Two-fold serial dilutions of the antibiotic and two-fold serial dilutions of the plant extracts were prepared for every combination tested and 50 µl aliquots of each component were placed into the wells of the sterile 96-well microtiter plate. 100µl of the bacterial inoculum (1×10^6 CFU/ ml) was transferred into all the wells except the negative control. After incubation of microtiter plates at 37 ˚C for 24 hr, 20 µl of resazurin dye was added to each well and further incubated for 30 minutes. The checkerboard method is often combined with the calculation of fractional inhibitory concentration (FIC) index (FICI). The FIC was derived from the lowest concentration of antibiotic and plant extracts combination showing no color change of resazurin dye. FIC value for each agent was calculated using the formula:

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

Where: **FIC (antibiotic)** = MIC of antibiotic in combination/ MIC of antibiotics alone, **FIC (extract)** = MIC of extract in combination/ MIC of extract alone

The interactions were classified as being **synergistic** for ΣFIC values of ≤0.5, **additive** (≥ 0.5–1.0), **indifferent** (≥1.0–≤4.0) or **antagonistic** (ΣFIC > 4.0) (20).

Results and discussion

DPPH assay: Relatively stable DPPH radical had been used widely to test the ability of compounds to act as free radical scavengers or hydrogen donors, in addition, this capability was used to evaluate antioxidant activity (21). In this study, the radical scavenging ability of extracts was checked using DPPH as a free radical and it was noted that the scavenging activity increased gradually with extract concentrations. Furthermore, the results showed that the methanolic and aqueous extracts (96.44% and 94.13%) respectively in 10 mg/ml were more than artificial antioxidant (BHT) which was 93.11% and approach with the natural antioxidant (vitamin C) which was 97.20% as shown in (Table 1).

Table (1): Radical scavenging activity of *Conocarpus erectus* extracts

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 activity is expressed as an Effective Concentration (EC_{50}). The half maximal Effective Concentration (EC_{50}) often refers to the

concentration of a drug, toxicant, or antibody which induces a response halfway between the baseline and maximum after a specified exposure time and is commonly used as a measure of the potency of a drug (22). The radical scavenging capacity (EC_{50}) of the Vitamin C and the methanolic extract was found to be (0.1 and 0.2) mg/ml respectively, this result was more effective than BHT and aqueous extract which was 0.6 for each as shown in (Figure 1). The effectiveness of antioxidant properties is inversely correlated with EC_{50} values. (23) reported that if the EC_{50} value of an extract is less than 10 mg/ml, it indicates that the extract is an effective antioxidant. The EC_{50} value of *Conocarpus erectus* extracts was less than 10 mg /ml; this indicates that the extracts were an effective antioxidant. The antioxidant profile of *C. erectus* is also related to the phenolic compounds present in the plant (24). As well as, (7) showed that the plant had a high content of phytochemicals and can be used as an efficient natural antioxidant. Moreover, the ethanolic and ethyl acetate extracts fractions of *C. erectus* presented good antioxidant activities and were able to sequester approximately 71% of the DPPH free radical (25). Furthermore, a study by (26) showed that the methanolic extract of *C. erectus* fruit part presented the highest antioxidant activity, flowed by the three other methanolic extracts of flowers, stems and leaves.

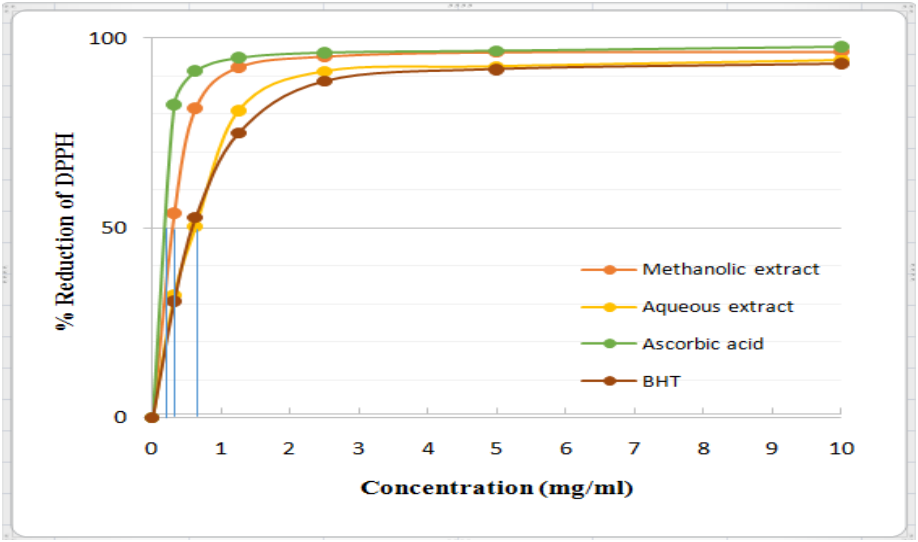


Figure (1): EC_{50} of *Conocarpus erectus* leaves extracts.

Total phenolic content of *Conocarpus erectus* extracts

The total phenolic content of the *C. erectus* extracts was evaluated by using Follin-Ciocalteu´s reagent. Numerous phenolic compounds have been studied for their biological properties and benefits to human health, polyphenols are secondary metabolites of plant origin that are synthesized from L-phenylalanine or L-tyrosine through the phenylpropanoid pathway (27). Phenolic compounds are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their

abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals (28). Phenolic compounds detected in different *C. erectus* extracts have been described to exhibit antioxidant, antibacterial, antifungal and antiviral activities, as well as act in the activation of the immune system (25). The results of the total phenolic content in the *C. erectus* extracts were observed at 16.42, 36.39, and 51.58 mg/g in 1.25, 2.5 and 5 mg/ml respectively for aqueous extract, while in the methanolic extract, the total phenolics content was 21.43, 43.76, and 65.60 mg/g in the same concentrations respectively as shown in (Table 2). The result of this study was in agreement with (29) who found that the amounts of phenolic compounds in *C. erectus* methanolic extract were much higher than in the case of the less polar solvents. Furthermore, the analysis of the extraction yield of three organic extracts of *C. erectus* leaves showed higher phenolic compound amounts of methanolic extract followed by ethyl acetate and hexane fractions (25).

Table (2): Total phenolic content of *Conocarpus erectus* extracts

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 test

In the present study, the antibiotic susceptibility test of the 83 isolates of *P. aeruginosa* was performed on 10 antibiotics represented by Amikacin (AK), Cefepime (CFP), Ticarcillin (TC), Ciprofloxacin (CIP), Colistin (CS), Imipenem (IM1), Ceftriaxone (CRO), Trimethoprim (TMP), Amoxicillin-Clavulanic (AUG), and Tobramycin (TOB) using the disc diffusion method. The results showed that the high resistance of the *P. aeruginosa* isolates was to the two antibiotics CRO and AUG with a percentage of (92.7%) and (89.2%) respectively, followed by TMP with a resistance rate of (79.5%) as shown in (Table 3). This ability is either normal or acquired through mutations in their genetic material or through the horizontal transference of genes (30). *P. aeruginosa* is an opportunistic pathogen causing infections, especially in immune-compromised patients. Drug resistance bacteria are responsible for increased cost, length of hospital stay and mortality (31). In the present study, ten isolates of *P. aeruginosa* have been chosen which were multi-drug resistant as shown in (Table 4).

Table (3): Antibiotic susceptibility test of eighty-three *P. aeruginosa*

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

(*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 (4): Antibiotic susceptibility test of ten MDR *P. aeruginosa*

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.

Antibacterial activity of *Conocarpus erectus*

Disk diffusion method

The antibacterial activity of *C. erectus* leaves extracts was evaluated by the disk-diffusion method on *P. aeruginosa* clinical isolates. The result showed that the methanolic extract was more effective than aqueous extract in concentration of 100 mg/ml with significant differences ($P \leq 0.01$) between concentrations, as seen in (Tables 5 and 6). For each type of *C. erectus* extracts, statistical tests were performed between different concentrations, for methanolic extract, the best effect was seen on multidrug-resistant *p. aeruginosa* No. 5 and 6 with inhibition zone 22.67 and 22.33 mm in 400 mg/ml respectively, while the best effect of the aqueous extract was seen on multidrug-resistant *p. aeruginosa* No. 10 with inhibition zone 15.33 mm in the same concentration. Phenolic compounds exert anti-inflammatory and anti-proliferative (32), antimicrobial and antiviral (33). These compounds act on the microbial cell altering the cellular permeability, damaging the cytoplasmic membrane and interfering with the energy generation system, finally leading to cell death (34). The results of this study were similar to (5) which showed the antimicrobial effects of

Conocarpus erectus methanolic leaves extract exerted antimicrobial activity against *staphylococcus aureus* and *pseudomonas aeruginosa* with inhibition zone 21 and 18 mm respectively. (25) Showed that the aqueous extract of the leaves can form an inhibition zone of 10 mm for multidrug-resistant *staph. aureus* isolated from cutaneous wounds.

Table (5): Antibacterial activity of *Conocarpus erectus* methanolic extract on *P. aeruginosa* clinical isolates

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≤0.01).				

P: *Pseudomonas aeruginosa*

Table (6): Antibacterial activity of *Conocarpus erectus* aqueous extract on *P. aeruginosa* clinical isolates

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≤0.01).				

P: *Pseudomonas aeruginosa*

Determination of the (MIC) of the *Conocarpus erectus* extracts

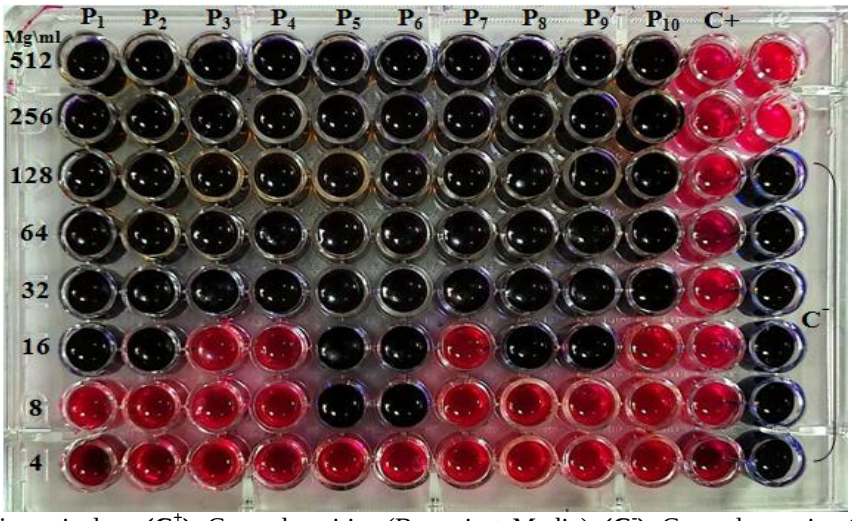
The broth microdilution method was used to determine the MIC of the

plant extracts using the 96-well microtiter plate. A method using the oxidation-reduction colourimetric indicator resazurin has determined the MIC of the antimicrobial agents against *P. aeruginosa*. Resazurin, which is blue in its oxidized state, turns pink when reduced by viable cells and can easily be detected with the naked eye, and the MIC is determined even without the aid of a spectrophotometer (35). The result of the MIC showed that the methanolic extract was more effective than the aqueous extract on *P. aeruginosa*. The MIC of the methanolic extract ranged between 8-32 mg\ml, while the MIC of the aqueous extract was 128 and 256 mg\ml, as shown in (Tables 7) and (Figures 2 and 3).

Table (7): MIC of *Conocarpus erectus* methanolic and aqueous extract on *P. aeruginosa*

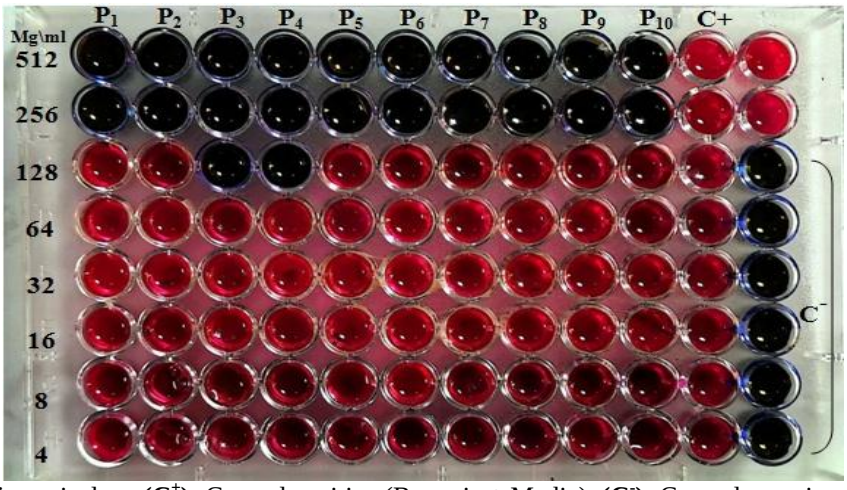
Isolate	Methanolic extract (mg/ml)	Aqueous extract (mg/ml)
	MIC	MIC
P_1	16	256
P_2	16	256
P_3	32	128
P_4	32	128
P_5	8	256
P_6	8	256
P_7	32	256
P_8	16	256
P_9	16	256
P_{10}	32	256

(P): *P. aeruginosa* isolate



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

Figure (2): MIC of *Conocarpus erectus* methanolic leaves extract on *P. aeruginosa.*



(P): *P. aeruginosa* isolate, **(C^+):** Control positive (Bacteria + Media), **(C^-):** Control negative (Media only)
Figure (3): MIC of *Conocarpus erectus* aqueous leaves extract on *P. aeruginosa*

Conocarpus erectus contains phenolic compounds. Tannins are water-soluble polyphenols that are commonly found in higher herbaceous and woody plants (36). (33) and (37) reported that *C. erectus* possess both bacteriostatic and bactericidal activities. (38) suggested that tannins extracted from *C. erectus* are largely responsible for the antimicrobial activity of this plant when studied the methanolic extracts fractions of leaves, stem, fruit and flower on *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *P. aeruginosa*. In this study, *P. aeruginosa* showed multidrug-resistant to common antibiotics used. The antimicrobial activity seems to be related to the abundance of phenolic compounds (Catechin, Gallic acid, Quercetin, Kaempferol, Taxifolin acid, and Rutin) in *C. erectus* leaves extracts. The phenolic compounds penetrate the bacterial cell and interfere with cellular metabolism, they also bind in active sites with the enzymes inside the cell and work to close them and then make them unable to bind to the basic materials, so they metabolically inhibit the enzymes and then nullify their work, or the phenolic compounds act as hydrogen ion carriers, any reducing agents that bind with adenosine triphosphate, which represent oxidizing agents, and then stop the work of ATP as an energy source, making the energy weak inside the microbial cell, whether bacterial or fungal, reducing its effectiveness and killing it (39, 40). Through the previous experiments conducted on the *C. erectus* methanolic and aqueous extracts in this study, it was found that the methanolic extract was more effective than the aqueous extract. Therefore, the methanolic extract has been chosen for further experiments.

The combination between plant extract and antibiotics

The combined effect between *C. erectus* methanolic extract and five conventional antibiotics was evaluated using the disk diffusion method against multidrug-resistant *P. aeruginosa*. The result revealed that the inhibition zone for the combination drug was more

effective than each methanolic extract and antibiotic alone as shown in Tables (8, 9, 10, 11 and 12). (41) Indicated that the combination of medicinal plant extracts and known antibiotics offers significant potential for the development of novel antimicrobial therapies and the treatment of several diseases caused by microorganisms.

Table (8): Combination effect of *Conocarpus erectus* methanolic extract and Amikacin on *P. 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): Combination effect of *Conocarpus erectus* methanolic extract and Amoxicillin-Clavulanic acid on *P. 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 (10): Combination effect of *Conocarpus erectus* methanolic extract and Cefepime on *P. 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 (11): Combination effect of *Conocarpus erectus* methanolic extract and Ceftriaxone on *P. 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 (12): Combination effect of *Conocarpus erectus* methanolic extract and Imipenem on *P. 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*

The main phytochemicals in the *C. erectus* methanolic extract are phenolic compounds, which inhibited the growth of the bacterial cell, probably by intercalating in the lipid bilayer (42). With the relative absence of new antimicrobials in the market, the pharmaceutical industry (medicinal herbs) has a high potential for being a source of natural drugs that can be utilized to combat the menace created by antibiotic resistance. (43) mention that plant-based antimicrobials have the potential for use in the manufacturing of drugs, exhibiting a potent antimicrobial activity, plant-based antimicrobials, either alone or combined with antibiotics, can help in dealing with the present crisis of antibiotic resistance. Also, (44) revealed that medicinal plants are highly efficient for enhancing the antibacterial activity of antibiotics.

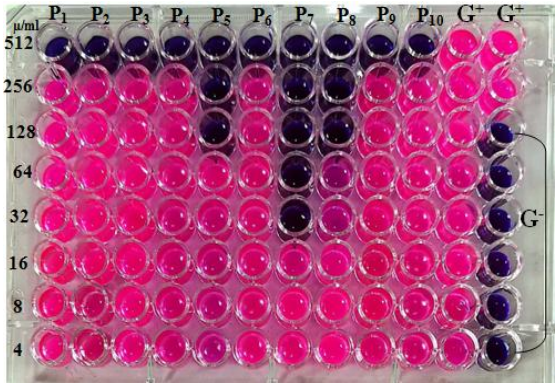
Synergistic antibacterial activity of plant extract and antibiotics

The *in vitro* synergistic activity between *C. erectus* methanolic extract and antibiotics against multidrug-resistant *p. aeruginosa* was assessed using the checkerboard analysis technique. The MIC of Cefepime and Ceftriaxone was evaluated individually as shown in (Table 13) (Figures 4 and 5). The methanolic extract showed a synergistic effect with Cefepime against six isolates of *p. aeruginosa* (FICI: ≤0.5), and an additive effect was found against four isolates (FICI: (≥ 0.5–1.0) (Table 14 and Figures 6). Furthermore, a synergistic effect with Ceftriaxone against seven isolates of *p. aeruginosa* and additive interaction was found against three isolates (Table 15 and Figures 7). A 2-to 5-fold reduction in individual MIC was found in a combination of *C. erectus* methanolic extract with antibiotics. Antimicrobial drugs effective for the treatment of infection with MDR bacteria are limited. Thus, it is valuable to find compounds that potentiate the antimicrobial activity of antibiotics on these bacteria. The ability of plant extracts to act synergistically with antibiotics is considered a new approach that helps in solving the problem of bacterial resistance (45). The *in vitro* synergistic activity of plant active compounds against multidrug-resistant bacteria has been widely shown by numerous scientific studies. This progress in synergy research enhances the possibility of designing new

antibacterial agents of plant origin for the treatment of infections (46). Synergistic interaction between two agents, in which one agent enhances the effect of the other and together they act more efficiently than as individual agents, motivated many scientists to examine and assess the significance of synergistic acting of plant-derived compounds and traditional antibiotics (47,48,49).

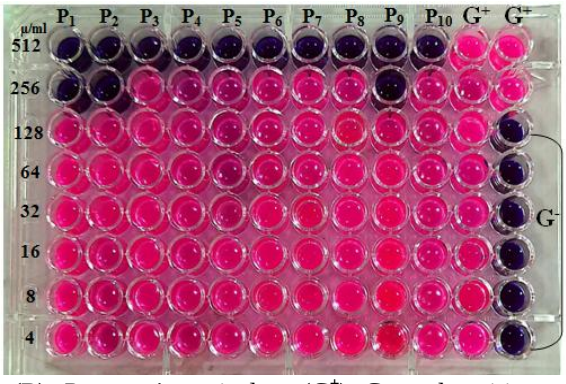
Table (13): MIC of Cefepime and Ceftriaxone on *P. aeruginosa*

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



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

Figure (4): MIC of Cefepime antibiotic on *P. aeruginosa*



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

Figure (5): MIC of Ceftriaxone antibiotic on *P. aeruginosa*

Table (14): Antibacterial interaction between Cefepime and methanolic extract combinations on *P. 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): Antibacterial interaction between Ceftriaxone and methanolic extract combinations on *P. 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

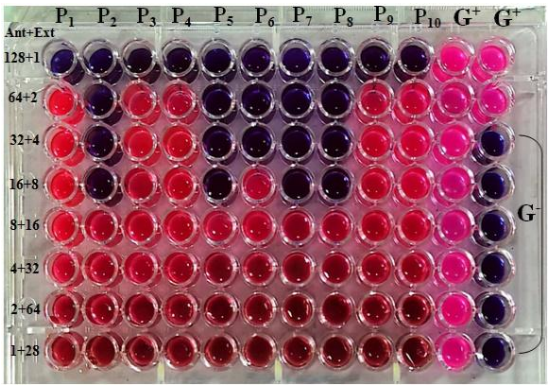


Figure (6): Checkerboard method between Cefepime and methanolic extract on *P. aeruginosa*

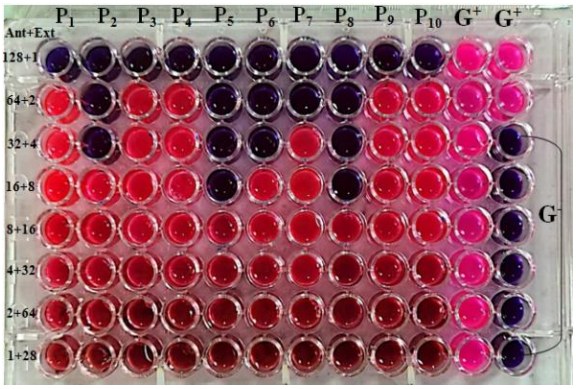


Figure (7): Checkerboard method between Ceftriaxone and methanolic extract on *P. aeruginosa*

Conclusion

Understanding the antibacterial synergistic interactions in medicinal plant-based combination therapies will undoubtedly provide important leads in the discovery of novel antibacterial resistance compounds from plants. Mixing *Conocarpus erectus* extracts with antibiotics enhanced and increase the antibacterial activity against multi-drug resistant *P. aeruginosa*. Therefore, the antibiotics that produce side effects can be used by reducing dose concentration and exploiting their synergy with medicinal plants. Mixing plant extracts with antimicrobials also increase the spectrum of antibiotics and avoids the development of resistance.

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