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Evaluation of Antibacterial and Synergistic Effects of *Vitex agnus-castus* Leaves Extract on Multidrug-Resistant *Pseudomonas aeruginosa* Isolates

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Abstract: *Pseudomonas aeruginosa* is a common bacterium that causes diseases that are problematic to treat, its pervasiveness and contribution to increased morbidity and mortality have shown the need for developing and applying natural alternative antimicrobial medicines to control MDR bacteria. The aim of the study, the efficacy of *Vitex agnus-castus* leaf extract was experienced against MDR *P. aeruginosa*. and examining this extract's synergistic relationship with drugs to which the target microorganisms were resistant. Sixteen isolates of *P. aeruginosa* were obtained from Iraqi patients admitted to the Al Kindy Teaching Hospital in Baghdad. After testing for antibiotic vulnerability on every isolate, it was found that 7 (43.75%) of the isolates were MDR. After *V. agnus* leaves were extracted alcoholically, active compounds were discovered by GC-MS analysis of the *Vitex agnus* extract. Using the microdilution broth method, the Minimum Inhibitory Concentration (MIC) of methanolic V. Agnus extract was 512 µg/mL. The antibacterial activity of plant active components that prevent bacterial growth and decrease resistance to most tested antibiotics, with the highest effect toward Ciprofloxacin and Meropenem (P-value=0.0001), may be mirrored in the antibacterial activity of the *V. agnus* extract (at MIC value) when studied as an antibacterial agent against MDR isolates. There was a synergistic effect of combined *V. agnus* extract and antibiotics against *P. aeruginosa*. This was estimating by the increase in diameters of fold for the inhibition zones of each antibiotic disk after a combination with *Vitex* extract. The maximum increase in the fold was 330% when Meropenem and *V. agnus* extract were combined. Combining *Vitex* extract with antibiotics has the potential to be beneficial as a therapeutic agent for treating bacteria and in the fight against drug-resistant *P. aeruginosa*.

Keywords: *V. agnus* , *P. aeruginosa*, MDR, synergistic

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Introduction

The necessity for new antibacterial classes is emphasized by the global concern over the rise of bacterial resistance to currently existing antibiotics. Compounds from natural sources can be employed to make novel chemical scaffolds for the development of antibiotics. Natural-source chemicals are less costly, have fewer side effects, originate from renewable resources, and are more broadly accepted than synthesized drugs because of their prolonged history of usage. Synergistic interactions between antibiotics that target different goals are the basis for

many contemporary drugs (1). Plant antimicrobials have been discovered to be synergistic enhancers, meaning that even though they may not have any antimicrobial qualities on their own when taken in combination with conventional medications, they improve the effects of such medications. Combination therapy can be applied to achieve synergistic antimicrobial efficacy, lessen toxicity, stop resistant mutants from evolving, and extend the antibacterial range (2). For patients with aggressive infections that are challenging to treat, such as those caused by multi-resistant species or those who do not respond to conventional treatment, it may be an option for monotherapy(3). Because they are more economical to produce and have fewer adverse effects than chemical medications, medicinal plants are applied all over the world. A well-liked herb for therapeutic purposes is *Vitex agnus-castus* L. With 250 genera spread over the globe, it is regarded as the largest genus in the Verbenaceae family (4). *V. agnus-castus* is a well-known herb. *V. agnus-castus* is a medication used to treat menopause symptoms. *V. agnus*, also recognized by frequent other local names, including chaste tree, chaste berry, monk's pepper, and Kaf Maryem(5). It was selected for this study due to its abundance in the surrounding environment and its status as a common therapeutic plant in traditional medicine. Several investigations focused on a perspective examination of *V. agnus-castus* L.'s health-promoting properties. and its potential as a nutraceutical. This plant displays a wide range of advantageous health-promoting characteristics, such as anti-inflammatory, cytotoxic, immunomodulatory, antimutagenic, antimicrobial, antifungal, antinociceptive, opioidergic, antiepileptic, and antioxidant qualities (6,7).

Materials and methods

Study subjects

This search involved sixteen *P. aeruginosa* isolates from different infections that were gathered from Al Kindy Teaching Hospital in Baghdad over more than five months (October 2022 to February 2023).

Preparation and Characterization of *Vitex agnus* leaves extract

The *V. agnus* leaves were purchased at neighborhood markets in Baghdad, and the botanical herbarium verified the species by classifying it at the University of Baghdad's College of Science for Women. The surface of the fresh *V. agnus* leaves has been repeatedly cleaned with distilled water to get rid of any dust particles. With certain adjustments, the extracting of *V. agnus* leaves was carried out utilizing the soxhlet system and hexane and methanol as the solvents, respectively, following(8). A firmly closed funnel-shaped filter paper was filled with 100 gm of powdered leaves, and it was then placed in the soxhlet system. 500 ml of solvent was added after that, and it was left for 24 hours. at room temperature. To obtain a dark brown crude extract, the extract was concentrated using a rotary evaporator with a water bath set at 40˚C. Until they were used, the crude extracts were stored in sterile containers at 4˚C. Furthermore, an alcoholic extract of the plant was made using a gas chromatography-integrated mass

spectrometer (GC-MS) apparatus to recognize the active components.

Antibiotic susceptibility test for detection multi-drug resistance (MDR) isolates

The resistance of bacterial isolates to eleven antibiotics was estimated using the Kirby-Bauer method in 1966 and subsequently reported by CLSI in 2023. Six antibiotic categories were among the studied antibiotics: 1. Carbapenem [Imipenem (IPM, 10 µg) and Meropenem (MEM, 10 µg)] 2. Aminoglycoside [Amikacin (AK, 30 µg) and Gentamicin (CN,10 µg)], 3. Flouroquinolones [Levofloxacin(LEV, 5µg) and Ciprofloxacin (CIP,5µg)], 4. Cephalosporin [Ceftazidime (CAZ, 30 µg)], 5. Monobactam [Aztreonam (ATM, 30 µg)] and 6. Beta-lactamase inhibitor [Piperacillin (PIP, 100 ug), Piperacillin-Tazobactam (P/T, 100/10 µg) and Ticarcillin-Clavulanate (TCC, 75/10 µg)]. *P. aeruginosa* which exhibits resistance to multiple antimicrobial agents in three or more antimicrobial classes is referred to as having a multidrug-resistant (MDR) phenotype.

Minimum Inhibitory Concentration (MIC) determination of *V. agnus* extract

Multi-drug bacterial isolates that were resistant to over 80% of the tested antibiotics were chosen for the current study. Another study explored the lowest inhibitory concentration (MIC) of the methanolic leaf extract of *V. agnus* extract against isolates of MDR *P. aeruginosa* (9). To engender a concentration, range from 8 to 8192 µg/ml, a stock solution (10000 µg/ml) of *Vitex* extract was consecutively diluted in 96-well microtiter plates using Mueller Hinton broth. Each bacterial culture (1.5×10^5 CFU/ml) was set overnight, and 10 µl of the culture was added to each well. A microtiter plate was incubated at 37°C for 24 hours. Afterward, each well received 5 µl (6.75 mg/ml) of resazurin dye (Thermo Fisher, USA), and they were incubated for an extra 4 hours at 37°C. The color transition from purple to pink was examined. The Minimum Inhibitory Concentration (MIC) was distinct as the lowest concentration at which the color continued unaltered.

Antibiotic susceptibility test after treatment isolates with *V. agnus* extract

Antibiotics were also verified against MDR *P. aeruginosa* utilizing the disc diffusion method in combination with *V. agnus* extract. A test tube containing 3ml of nutritional broth was infected with 1ml of bacterial inoculum suspension (1.5×10^8 CFU / ml) and 1ml of *Vitex* extract (at MIC value) was added. They were spread out onto Mueller Hinton agar plates after being incubated in a tube for an hour at 37°C. After incubation, the whole diameters of the inhibition zones were measured using antibiotic discs that had been positioned on the surface of infected agar plates.

Synergistic effect of antibiotic and *V. agnus* leaf extract combinations

The potent methanolic plant extract against *P. aeruginosa* that is resistant to many drugs was studied utilizing the diffusion method of discs. To assess the synergistic antibacterial action, *Vitex* extract was joined with 11 antibiotics. On Mueller Hinton agar plates, the suspension of bacterial isolates was dispersed with a turbidity of 0.5

McFarland. The antibiotic discs were located on the surface of the infected agar plate after being individually soaked in 20 µL of *Vitex* extract (at the MIC value) and let dry. Plates were incubated overnight at 37 °C. The formula used to calculate the fold increase (F.I) = (b-a)/a x100, where a is the antibiotic's inhibition zone alone and b is the antibiotic's inhibition zone plus plant extract, was used to determine the diameter of each antibiotic's inhibition zone after it was joint with *Vitex* extract(10).

Statistical Analysis

The SAS (2018) program was utilized to determine the impact of different variables on the study parameters. To compare means significantly, the Least Significant Difference (LSD) test (Analysis of Variation, or ANOVA) was employed. The study employed the Chi-square test to assess the significance of the difference between the percentage (0.05 and 0.01 probability) and the assessed correlation coefficient between the variables.

Results and discussion

GC-MS analysis: the outcomes of GC-Ms exposed that Phytol (C20H40O), Phenol (C6H5OH), Vitamin E (C29H50O2), 4,5-Dichloro-1,3-dioxolan-2-one (C3H2Cl2O3), Squalene (C30H50), and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy (C6H8O4) are just a few of the phytochemical components (> 50 chemical compounds) that are rich in methanolic extract. These findings agree with earlier research by (11) and (12), which confirmed that the extract of *V. agnus* contained a diversity of chemical components. These substances fall into several chemical classes, including glycosides, phenolic compounds, essential oils, and flavonoids. Variations in the genotypes of *V. agnus* may be the cause of the difference in the number of phytochemical substances.

Antibiotic susceptibility

In this search, we screened the antibiotics action against 16 bacterial isolates before assessing the methanolic *Vitex* extract's antibacterial activity; the consequences are exposed in (Figure 1). Imipenem 12 (75%) and Piperacillin 11 (68.75%), Gentamicin 10 (62.5%), Levofloxacin and Aztreonam 9 (56.25%), Meropenem and ceftazidime 8 (50%) and Ciprofloxacin, Amikacin, and Piperacillin/ Tazobactam 7 (43.7%) and Ticarcillin/Clavulanate 6 (37.5%) isolates were found to have the highest levels of resistance (Figure 2). Seven isolates (43.75%) were predicted to be multidrug-resistant (MDR) based on the CDC's classification of MDR, which is defined as an isolate that is resistant to at least one antibiotic in three or more medication classes. One of the principal medical challenges facing the world today is the growing prevalence of antibiotic resistance in Gram-negative bacteria, especially in *P.aeruginosa* (14). The lack of progress in the past several decades in the clinical development of novel antibiotics for Gram-negative bacteria worsens the situation (15). In summary, since their debut, antibiotics have been used to treat a wide range of bacterial diseases. However, due to widespread use, antibiotic-resistant strains have emerged, posing a threat to world health. To address the issue of drug-resistant bacteria emergence, new

resolutions must be originated (16). The patient groups that were examined in this study. These results defined the CML patients' response to TKI according to European Leukemia Net 2020 (11).

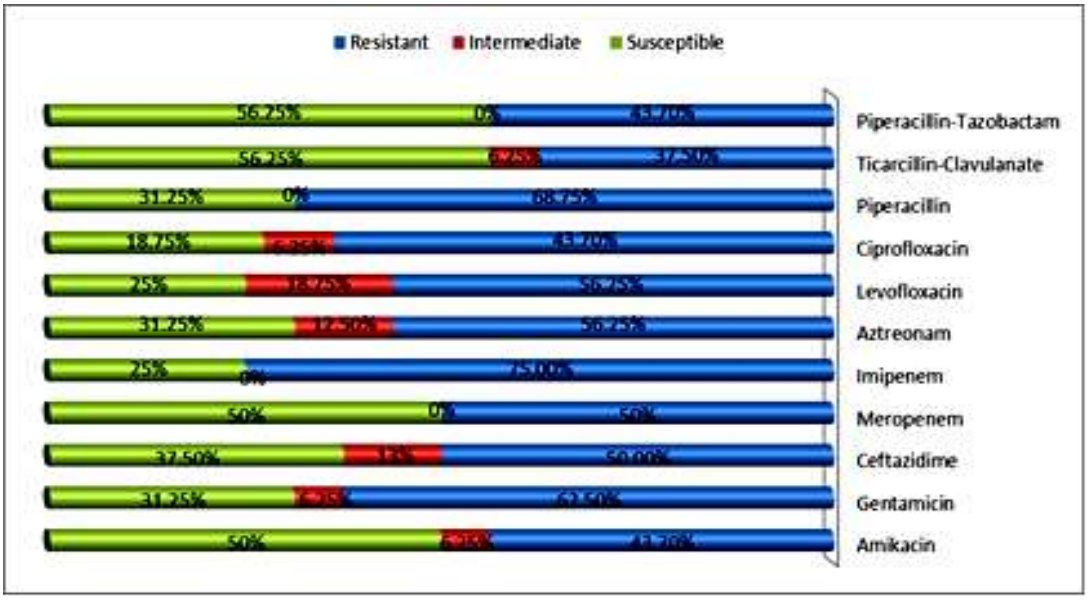

Figure (1): Susceptibility of *P. aeruginosa* isolates to 11 antibiotics.

Four multidrug-resistant bacterial isolates that were resistant to over 80% of the tested drugs were elected for the current examination. Methanolic *Vitex* extract was useful for bacterial strains to judge the extract's antibacterial effectiveness.

MIC results of *V. agnus* extract

The MIC was established using the microdilution method. The MIC value of *V. agnus* methanol extract against MDR *P. aeruginosa* was exposed to be 512 µg/ml. The *Vitex* agnus-castus plant is regarded as one of the noteworthy medicinal plants that have drawn important interest and investigation from scholars(17). The antimicrobial efficacy of *V. agnus* was surveyed in numerous studies by(18,19). The extract from the leaves of the plant confirmed an inhibitory effect against both Gram-negative and Gram-positive bacteria (18,20,21). The existence of active phytochemical compounds like flavonoids, tannins, essential oils, terpenes, and glycosides is what gives *V. agnus*-castus extract its antibacterial properties (18,21). Otherwise, it could be because phenols contain hydroxyl groups (-OH), which can form hydrogen bonds with water molecules in bacteria to disrupt the cell's essential functions(21).

Results of antibiotic susceptibility test after treatment isolates with *V. agnus* extract

Before and after treating isolates with *V. agnus* extract, an antibiotic susceptibility test against MDR *P. aeruginosa* was conducted employing the disc diffusion method. The antibacterial activity of plant active components that constrain bacterial growth and alter it from antibiotic resistance to sensitivity was found to be extremely effective in reducing P.

aurgenosa's resistance to Meropenem and Ciprofloxacin, followed by Aztreonam, Imipenem, and Piperacillin-Tazobactam. Significant differences after treatment with *Vitex* extract However, no antibacterial activity of the extract was found toward Ceftazidime, Ticarcillin-Clavulanate, Levofloxacin, and Gentamicin (Table 1 and Figure 2).

Table (1): The results of antibiotic susceptibility before and after treatment of isolates with *V. agnus* extract.

Antibiotic	Symbol	CLSI,2023			Inhibition zone diameter (mm)		P-value
		S	I	R	Before treatment	After treatment	
Amikacin	AK	≥ 17	15-16	≤ 14	18	28	0.0255 *
Ceftazidime	CAZ	≥ 18	15-17	≤ 14	0	0	NS
Meropenem	MEM	≥ 19	16-18	≤ 15	0	32	0.0001 **
Piperacillin	PIP	≥ 22	18-21	≤ 17	0	0	NS
Levofloxacin	LEV	≥ 22	15-21	≤ 14	22	27	0.248 NS
Ticarcillin-Clavulanate	TCC	≥ 24	16-23	≤ 15	14	17	0.502 NS
Aztreonam	ATM	≥ 22	16-21	≤ 15	16	30	0.0063 **
Imipenem	IPM	≥ 19	16-18	≤ 15	21	35	0.0071 **
Gentamicin	CN	≥ 15	13-14	≤ 12	11	12	0.902 NS
Piperacillin-Tazobactam	PIT	≥ 22	18-21	≤ 17	17	30	0.0076 **
Ciprofloxacin	CIP	≥ 25	19-24	≤ 18	0	30	0.0001 **
P-value					0.0001 **	0.0001 **	---

NS: Non-significant; **S:**Sensetive; **I:** Intermediate; **R:**Resistance.

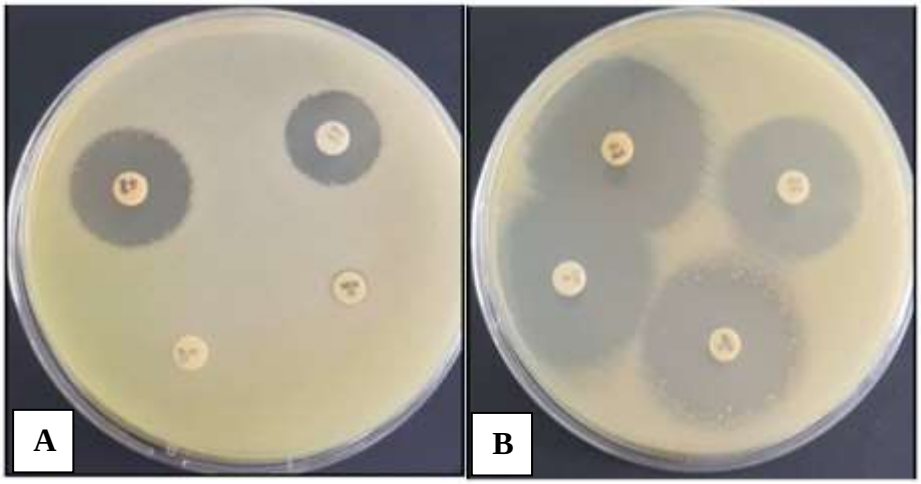

Figure (2): The effect of the antibiotics (A) before treatment of isolates, (B) after treatment of isolates with *V. agnus* extract on the growth of bacteria.

Synergistic effect of antibiotic and *V. agnus*

The synergistic impact of *Vitex* extract blended with antibiotics was also examined by measuring the diameter of the inhibition zone and assessing the increase in diameters of fold for each antibiotic disk's inhibition

zone after grouping with Vitex extract. Compared to antibiotics alone, the mixture of antibiotics and Vitex extract confirmed a synergistic effect on the current study's antibacterial activity against the tested MDR clinical strains.

The Vitex–antibiotic combination was effective against the MDR isolates. The antibacterial effectiveness of the antibiotic amplified synergistically with Vitex against *P. aeruginosa* after mingling the antibiotic disks with Vitex extract, as evidenced by the results that showed an increased effect of most antibiotics by growing the diameters of the inhibitory zone against isolates rather than the inhibition zone diameter for antibiotic alone (Table 2 and Figure 3). The formation of complexes between antibiotics and vitex is suggested as a potential mechanism explaining their synergistic effects. One possible mechanism to explain the synergistic effects of vitex and antibiotics is the development of complexes between the two substances. When these antibiotics were mixed with Vitex extract, the diameter of the inhibitory zone did not change in the case of Amikacin (additive action). However, when other antibiotics were added to Vitex, there was a rise in the diameter of the inhibitory zone (a synergistic effect) and the bacteria developed susceptible to antibiotics instead of resistant. The maximum fold increase (F.1) observed was 330% when Meropenem and *V. agnus* extract were combined.

Table (2): Synergistic antimicrobial activity of *V. agnus* extract in combination with different antibiotics.

Antibiotics	Symbol	Concentration	CLSI,2023			Inhibition zone diameter (mm)		F. I. %
		µg/Disk	S	I	R	Antibiotic Alone	Antibiotic + Vitex extract	
Amikacin	AK	30 ug	≥ 17	15-16	≤ 14	18	18	0
Ceftazidime	CAZ	30 ug	≥ 18	15-17	≤ 14	6	15	150
Meropenem	MEM	10 ug	≥ 19	16-18	≤ 15	6	26	330
Piperacillin	PIP	100 ug	≥ 22	18-21	≤ 17	6	17	183.3
Levofloxacin	LEV	5 ug	≥ 22	15-21	≤ 14	22	28	27
Ticarcillin-Clavulanate	TCC	75/10 ug	≥ 24	16-23	≤ 15	14	27	90
Aztreonam	ATM	30 ug	≥ 22	16-21	≤ 15	16	34	112.5
Imipenem	IPM	10 ug	≥ 19	16-18	≤ 15	21	28	33.3
Gentamicin	CN	10 ug	≥ 15	13-14	≤ 12	11	15	36.3
Piperacillin-Tazobactam	P/T	100/10	≥ 22	18-21	≤ 17	17	30	76.5
Ciprofloxacin	CIP	5 ug	≥ 25	19-24	≤ 18	6	25	316.6
						0.0001 **	0.0001 **	

F.I.: fold increase, F = ((b − a)/a) × 100; Note: To calculate the fold increase in the absence of bacterial growth inhibition zones, the disc diameter (6 mm) was used.

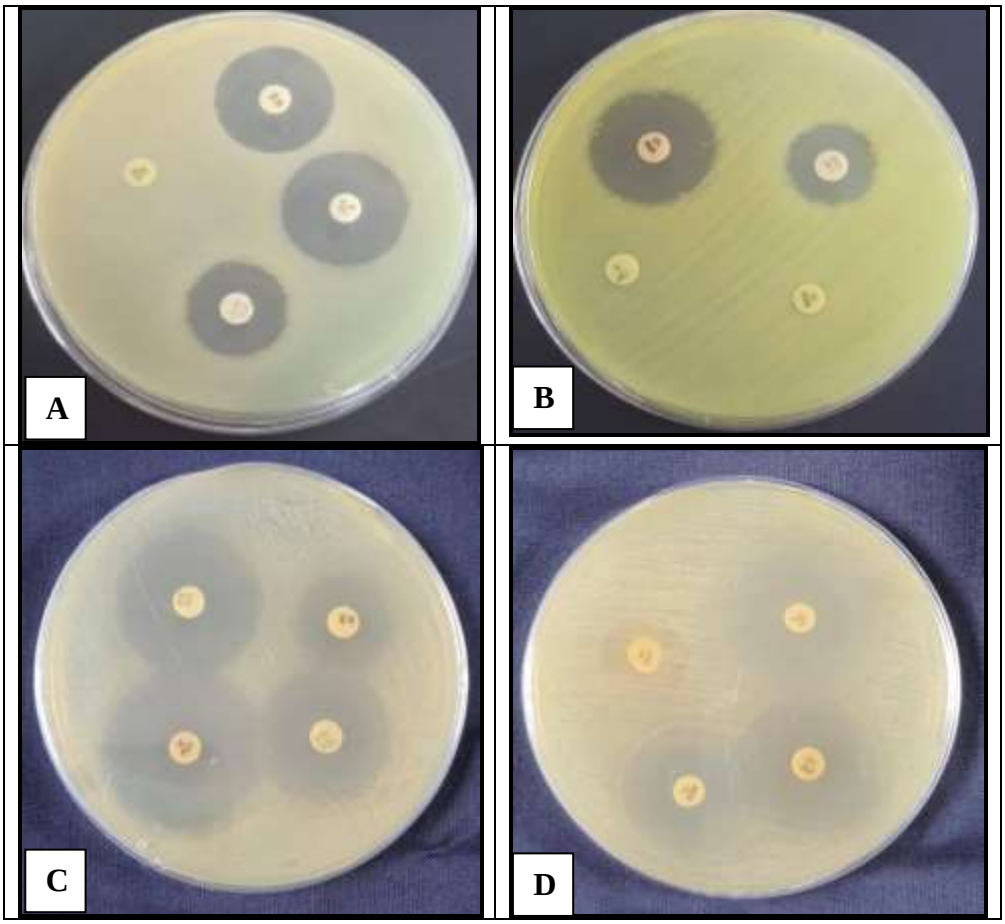

Figure (3): The effect of the (A) (B) antibiotic alone, (C) (D) antibiotics with *V. agnus* on the growth of MDR *P. aeruginosa* isolates.

The variations between the two experiment's outcomes when compared to the control are summarized in Table 4 and Figure 4. These findings might propose that the extract from Vitex contains naturally occurring inhibitors that block efflux pumps or operate in numerous ways. The synergistic interactions between plant extracts and antibiotics may be caused by suppression of drug efflux, increased permeability, inhibition of β-lactamase, and other modes of action, according to theories of Garvey *et al.* (22). Furthermore, it was noted by Kaur *et al.* (23) that natural compounds can have synergistic effects. These mixtures have the possibility to improve the efficiency of other antimicrobial drugs and serve as a treatment option for illnesses caused by multidrug-resistant microbes for which there is no feasible therapy.

Table (4): Comparing between two experiment's outcomes.

Antibiotics	Symbol	Inhibition zone diameter (mm)		P- value
		After treatment with *Vitex* extract	Antibiotic + *Vitex* extract	
Amikacin	AK	28	18	0.140 NS
Ceftazidime	CAZ	0	15	0.0001 **
Meropenem	MEM	32	26	0.430 NS
Piperacillin	PIP	0	17	0.0001 **
Levofloxacin	LEV	27	28	0.892 NS
Ticarcillin-Clavulanate	TCC	17	27	0.131 NS
Aztreonam	ATM	30	34	0.617 NS
Imipenem	IPM	35	28	0.377 NS
Gentamicin	CN	12	15	0.563 NS
Piperacillin-Tazobactam	PIT	30	30	1.00 NS
Ciprofloxacin	CIP	30	25	0.500 NS
P- value		0.0001 **	0.0001 **	---
** (P≤0.01), NS: Non-Significant.				

Antibiotic molecules with active groups like amino and hydroxyl groups can readily react with plant extract by chelation, which improves their diffusion and penetration through biofilm and their capacity to show antimicrobial activity against bacterial cells. This synergistic effect may be the outcome of this interaction.

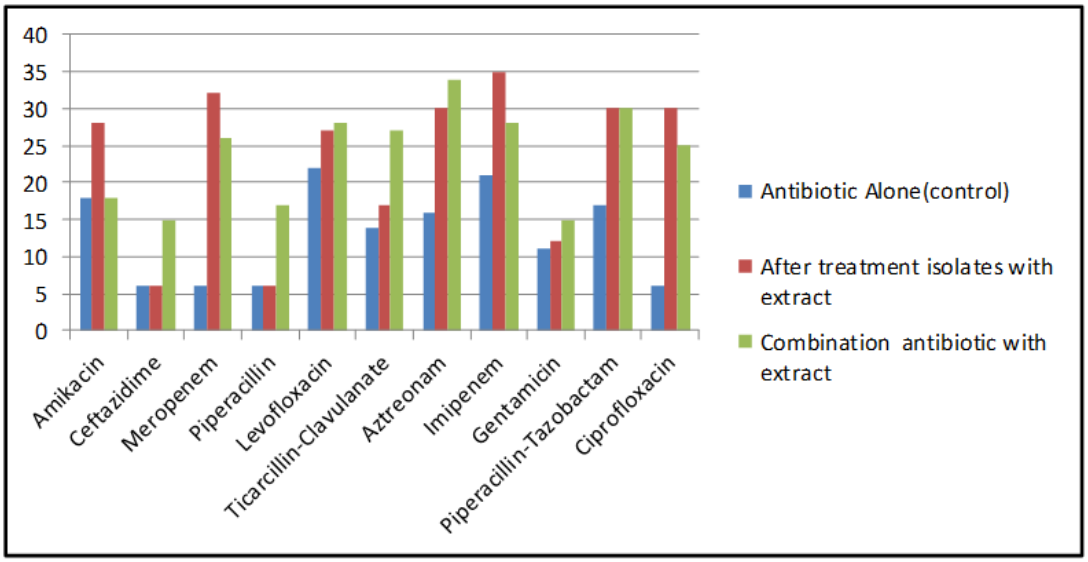

Figure (4): Results of antibiotic susceptibility test after treatment of isolates with *V. agnus* extract and in combination *V. agnus* extract with antibiotics in comparing with control.

Conclusion

In summary, the current study's consequences are encouraging. Vitex leaf extract confirmed important promise as an antibacterial agent against *P. aeruginosa*, a multidrug-resistant strain of bacteria, and it also confirmed synergistic effects when combined with medicines. When used against MDR *P. aeruginosa*, the antibiotic's antibacterial activity was improved by the action of vitex AB. It provided the chance to stop the appearance of bacterial resistance and reduce the negative consequences

of an overabundance of antibiotics. The antibiotic's antibacterial activity was improved by the action of vitex AB. It provided the chance to stop the appearance of bacterial resistance and reduce the negative consequences of an overabundance of antibiotics.

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