Iraqi Journal of Biotechnology, 2025, Vol. 24, No. 1, 103-112

Evaluation Antibacterial Activity of Quercetin Against XDR - *Pseudomonas aeruginosa*

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Received: February 12, 2024 / Accepted: April 14 2024 / Published: March 5, 2025

Abstract*:* *Pseudomonas aeruginosa* is an opportunistic bacteria that is commonly linked to nosocomial infections and demonstrates resistance to many antibiotics. Thus, many groups of secondary metabolites from plants have been used in a number of investigations conducted in the past few years. According to reports, quercetin possesses pharmacological properties that include antibacterial and antioxidant, actions. The aim of the research was to study antoxidant and antibacterial activity of quercetin against XDR-resist *pseudomonas aeruginosa*. isolates of *pseudomonas aeruginosa* from different clinical sources was collected , laboratory diagnosis include morphological and biochemical testing, confirmed with molecular identification by *16rRNA* detection. Susceptibility test was applied to detect bacterial resistance to druge. The total phenolic content and antioxidant activity of quercetin was determined using the Folin-Ciocalteu method, and DPPH assay. The antibacterial activity against p*seudomonas*. *aeruginosa* was measured by determining MIC value using microtiter plate method. The results of this study shows that from 53 isolates. Ten (19%) of the bacterial isolates are resistant to more than five antibiotic classes, which are referred to as XDR. Total phenolic content and antioxidant activity of quercetin demonstrating that quercetin was superior than the synthetic antioxidant, and was rich in phenolic compound ,the antibacterial activity against p*seudomonas*. *aeruginosa* It was conducted that No impact of quercetin on growth of XDR-*pseudomonas aeruginosa.*

Keywords: Quercetin , phenolic content, *Pseudomonas aeruginosa*, antioxidant activity, MIC value.

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Introduction

Quercetin is a significant phytochemical that is a member of the polyphenolic flavonoid family. It may be found in a variety of fruits, vegetables, and drinks as well as in flowers, leaves, seeds, and other plant materials (1). Due to its powerful antioxidant activity, quercetin is a bioactive molecule that is frequently employed in traditional Chinese medicine and herbal treatments, quercetin has also been demonstrated to impede the development of a number of drug-resistant bacteria Additionally, quercetin is classified as GRAS (Generally Recognized as Safe) (2). Much study has been conducted recently on quercetin's antioxidant qualities, including how it affects glutathione (GSH), enzyme function, signaling pathways, and reactive oxygen species (ROS) caused by toxicological and environmental factors, and inhibit the development of (bacteria, fungi, and viruses) by altered membrane permeability, suppression of protein and nucleic acid synthesis, down regulation of virulence factor expression, and prevention of biofilm

formation (3). Around the world, pseudomonas aeruginosa is the cause of 10–15% of nosocomial infections. Owing to the species' natural resistance, treating these illnesses can occasionally be challenging (4). The bacteria *P. aeruginosa* is considered a major pathogen and is primarily linked to human illnesses. The bacteria is often the source of nosocomial infections and illnesses in immunocompromised people; infections brought on by the bacteria are especially concerning in patients with severe infections, growth disorders, cystic fibrosis, and (AIDS) (5). However, new techniques are required to stop the development of bacterial resistance since bacteria may change swiftly (6).

The aim of study was evaluate the antibacterial activity of quercetin against XDR-*pseudomonas aeruginosa.*

Materials and methods

Collection and identification of *pseudomonas aeruginosa*

Samples from different clinical sources were obtained Incuding wound exudate, urine, ear discharge, burn exudate, and CSF aspirate. isolates were cultured by selective media blood agar, MacConkey agar, andCetrimide Agar) morphological of colony was observed and biochemical tests with VITEK-2 system was measured, confirmed the identification with molecular detection by P.C.R.

Molecular study of *pseudomonas. aeruginosa*

Extraction of genomic DNA

DNA was extracted Using (iNtron®, Korea) Mini Kit.

The primers used in this study for *16rRNA* gene were indicate in the (Table 1):

Table (1): Primers sequences of *16rRNA* gene

Primername		Sequence (5´-3´)	Productionsize	Reference
16S rRNA	**F**	GGGGGATCTTCG GACCTCA	956 bp	(7)
	R	TCCTTAGAGTGC CCACCCG		

This step was carried out by adding 12.5 µl from OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1 µl 10 pmol/µl from each primer and 7.5 µl of free-nuclease water, the reaction done under the optimal PCR conditions for the gene as shown in (Table 2).

Table (2): PCR conditions for the*16rRNA* genS.

Cycle No.	Step	Temperature	Time
1	Initial Denaturation	94 ºC	5 min.
38x	Denaturation	94 ºC	30 sec.
	Annealing	57 ºC	45 sec.
	Extension	72 ºC	45 sec.
1	Final Extension	72 ºC	7 min.

Gel electrophoresis of amplified *16Rrna* (956 bp), from *P. aeruginosa* using conventional PCR. Agarose 2% stained with Ethidium bromide dye DNA ladder 100-1500 bp and visualized on a UV transilluminator was used.

Antibiotic susceptibility test

The antibiotics were used for susceptibility in the current study were provided from Himedia (India) are:

Amikacin (30µg), Tobramycin (10µg), Ceftazidime (30µg), Cefepime (30µg), Colistin (0.5µg), Aztreonam (30µg), Piperacillin (100µg), Imipenem (10µg), Meropenem (10µg), Ciprofloxacin (5µg), Levofloxacin (5µg). The result was read by measuring the diameter of the inhibitory zone in millimeters, the results were compared to the National Community for Clinical Laboratory Standard results.

Determination of total phenolic content

Quercetin were used in the current study were provided by (Chemical point company) (Germeny). Using the Folin-Ciocalteu technique, which was described by Jayaprakasha et al. (8), the total phenolic content of quercetin was ascertained spectrophotometrically. Each sample was combined with 2.0 ml of the Folin-Ciocalteu reagent (10 times diluted), 1.6 ml of 7.5% sodium carbonate solution, and 0.4 ml of each. The total capacity was increased to 5 milliliters by adding distilled water. After the tubes were covered in parafilm and allowed to stand at room temperature for half an hour, At 760 nm, the wavelength of absorption was calculated. The results were given as the gallic acid equivalent in milligrams per gram of dry weight.

Evaluation of the antioxidant activity (DPPH assay)

In order to evaluate the antioxidant activity of quercetin, the radical scavenging activity was determined according to the method of Ogunmoyole et al. (9). After dissolving 0.01 grams of quercetin in D.M.S.O., The finished volume was complete into 10 ml to create a working solution 1 mg/ml. A series of two-fold dilutions resulted in concentrations of the quercetin ranging from 1 to 0.0156 mg/ml. Then 100 micro liters of each concentrations mg/ml were added to five milliliters of the freshly created 0.004% 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. After 30 minutes, the absorbance of each dilution was measured at 518 nm. A positive control was performed using vitamin C and butylated hydroxytoluene (BHT). Every test was run in triplicate. The formula used to determine the % DPPH decrease(or DPPH radical scavenging capability) is% 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.

Study the antibacterial activity of quercetin

The (MIC) of quercetin was calculated using the 96-well microtiter plate and the broth microdilution technique. The workable solution was created at concentrations of 64, 32, and 16 mg/ml. The first wells in row A were filled with 200µl of the prepared quercetin. Columns B through H contained 100 µl of the broth only.

Micropipette-based double serial dilutions were performed methodically down the columns (starting with rows A–H). From the initial concentrations in rows A and B, 100 µl were taken out. The 100 µl of broth was correctly mixed and moved to the following row before the process was repeated all the way to row H, where the last 100 l was

dumped. With the exception of the column, which contained 200 µl of the broth that functioned as a sterility control, this reduces the total volume in all of the test wells containing the quercetin to 100 µl. All of the wells except the negative control received 100 µl of the 1 $\times 10^6$ CFU/ ml bacterial inoculum. Microtiter plates were incubated for 18 to 20 hours at 37°C. To check for any color changes, 20 µl of resazurin dye was added to each well and incubated for 30 minutes. The Minimum Inhibitory Concentrations in broth microdilutions were determined visually to be the lowest concentrations of quercetin at which no color shift from blue to pink occurred in the resazurin broth test (10).

Statistical analysis

The impact of various factors on research parameters was determined using the SAS (2018) software (11).

Results and discussion

P. aeruginosa samples were obtained from different clinical soureces originally identified using conventional technique and VITEK-2, then its confirmed with P.C.R, *P.aeruginosa* growing on the selective medium (MacConkey agar and Cetrimide agar).

On blood agar, *P. aeruginosa* often exhibits beta hemolysis and a blue or green pigment, all isolates were grown on MacConkey appeared as small and pale colonies resulting from Bacteria did not ferment lactose , in another hand *P. aeruginosa* differs from other *Pseudomonas* species grow in Cetrimide agar with a greenish-yellow color, confirming that *P. aeruginosa* sp. is capable of withstanding cetrimide , Cetrimide inhibits bacterial growth except *Pseudomonas aeruginosa* and enhances fluorescein and pyocyanin pigment production that gives greenish-yellow color (12). The biochemical test with VITEK2 system was used and gives positive result with high confident.

The isolates was confirmed with P.C.R and gel electrophoresis detecting the size of amplicons as indicate in (Figure 1).

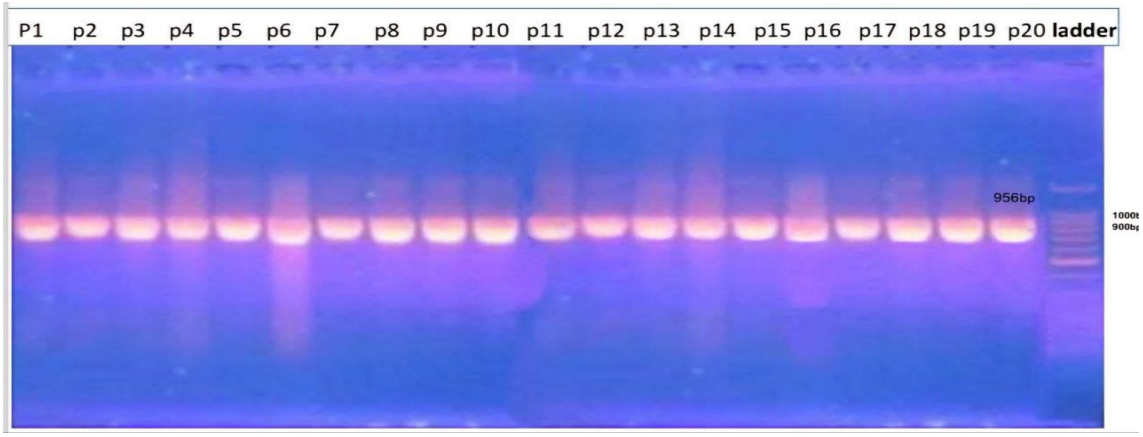

Figure (1): Amplicons of *16S rRNA* gene (956bp) fragments afterelectrophoresis on agarose gel (2%) for 80 min at 85V/cm.

These PCR techniques were shown to be useful in reliably identifying *P. aeruginosa* isolates that had not been correctly identified by phenotypic analysis (7).

The total (*Pseudomonas aeruginosa*) accounts 53 isolates from different clinical sources including (burn, C.S.F, ear discharge, wound, and UTI). Burns make up 40% of the isolates.

Pseudomonas aeruginosa is unique pathogens that colonize burn wounds, Wound infections, also known as burn wounds, are thought to be one of the most common causes of serious problems globally. Burn wounds are defined as the destruction of the protective layer of skin that normally prevents bacterial invasion, making them the most common source of sepsis (13).

Antibiotic susceptibility results

In the current investigation, 53 isolates of *P. aeruginosa* were tested for 11antibiotic. The study revealed that the majority of the isolates were resistant to two or more of the antibiotics examined.

Overuse of antibiotics causes *P. aeruginosa* to become increasingly resistant to a variety of antibiotics, which in turn causes an accumulation of antibiotic resistance, antibiotic cross-resistance, and the emergence of multidrug-resistant (MDR) forms of P. aeruginosa (14).

In the current study, ten extensively drug-resistant *P. aeruginosa* isolates were employed, as indicated in (Table 3).

Table (3): Results of antibiotic susceptibility for isolates with the greatest resistance.

	Sourses of isolates	PRL	FEP	CAZ	IPM	MEM	CN	ToB	AK	CIP	LEV	ATM	Percentage of resistance
P1	Burn	R	R	R	R	R	R	R	R	R	R	R	100%
P2	Burn	R	R	R	R	R	S	R	R	R	R	R	90%
P3	Burn	R	R	R	R	R	S	R	R	R	R	R	90%
P4	Ear swab	R	R	R	R	R	S	R	R	R	R	R	90%
P5	Burn	R	R	R	R	R	S	R	R	S	R	R	81%
P6	Burn	R	R	R	R	R	S	R	R	S	R	R	81%
P7	Burn	R	R	R	R	R	S	R	R	S	S	R	72%
P8	Burn	R	R	R	R	R	S	R	R	S	S	R	72%
P9	Burn	R	R	R	R	R	S	R	R	R	S	S	72%
P10	Urine	R	R	R	S	S	S	R	R	R	R	R	72%

Antioxidant activity of quercetin

1.Total phenolic content of quercetin

Using Follin-Ciocalteu's reagent, the quercetin's overall phenolic content was assessed. Polyphenols are secondary metabolites of plant origin that are produced from L-phenylalanine or L-tyrosine via the phenylpropanoid pathway, and they're a portion of the phenolic substances that have been extensively studied for their biological effects and possible benefits to human health (15).

Results of quercetin's total phenolic content were found at 50.79, 83.91, and 121.79 mg/g in 0.25, 0.5 and 1mg/ml respectively as shown in (Table 4).

Table (4): Effect of Concentration in Total phenolic content of Quercetin.

Concentration (mg\ml)	Mean ± SE of Quercetin (mg\g)
0.25	50.79 ±0.09 c
0.5	83.91 ±0.04 b
1	121.83 ±0.04 a
LSD value	0.217 *
* (P≤0.05).	

Phenolic compounds' bioactivities, which operate as free radical terminators, may be linked to their capacity to chelate metals, inhibit lipoxygenase, and scavenge free radicals, Phenolics' redox characteristics, which enable them to function as reducing agents, hydrogen donors, and singlet oxygen quenchers, are primarily responsible for their antioxidant action(16).

2. DPPH assay

The comparatively stable DPPH radical had been employed frequently to test substances' capacity to behave as hydrogen donors or free radical scavengers, and this capacity was also utilized to assess antioxidant activity (17). The capacity of quercetin to scavenge free radicals was tested in this work utilizing the free radical DPPH, and it was discovered that the scavenging activity steadily increased with quercetin concentrations.

Additionally, the results indicated that quercetin outperformed the artificial antioxidant (BHT) with 94.46% and 95.53%, which was 66.73%) and 88.76%. This was comparable to the natural antioxidant (vitamin C), which was 95.33% and 96.38% in 0.125 and 0.25 mg/ml, respectively, while there were no significant differences between the three in 0.5 and 1 mg/ml, as indicated in (Table 5).

Table (5): Radical scavenging activity of Quercetin

Conc. (mg/ml)	Mean ± SE			LSD value
	Quercetin %	BHT %	Vit. C %	
0.0156	31.92 ±0.05	13.74 ±0.12	34.03 ±0.05	4.397 *
0.0312	42.63 ±0.05	28.91 ±0.05	53.95 ±0.06	3.263 *
0.0625	75.60 ±0.02	41.75 ±0.20	82.17 ±0.05	4.157 *
0.125	94.46 ±0.05	66.73 ±0.31	95.33 ±0.22	4.045 *
0.25	95.53 ±0.21	88.76 ±0.10	96.38 ±0.24	4.291 *
0.5	96.14 ±0.43	95.20 ±0.18	97.02 ±0.10	2.367 NS
1	96.99 ±0.09	96.58 ±0.21	97.23 ±0.06	2.084 NS
LSD value	3.572 *	3.565 *	4.419 *	---
* (P≤0.05).				

Furthermore, an Effective Concentration (EC50) is used to represent the antioxidant activity. The half maximal Effective Concentration (EC50), which is frequently employed as a gauge of a drug's efficacy, is the amount of a medication, toxicant, or antibody that, after a specific amount of exposure time, results in a response that lies between the baseline and maximum (18). Vitamin C and quercetin were found to have radical scavenging capacities (EC50) of (0.1) mg/ml and (0.1) mg/ml, respectively. Compared to BHT, which was 0.5 for each, this outcome was more effective as seen in (Figure 2).

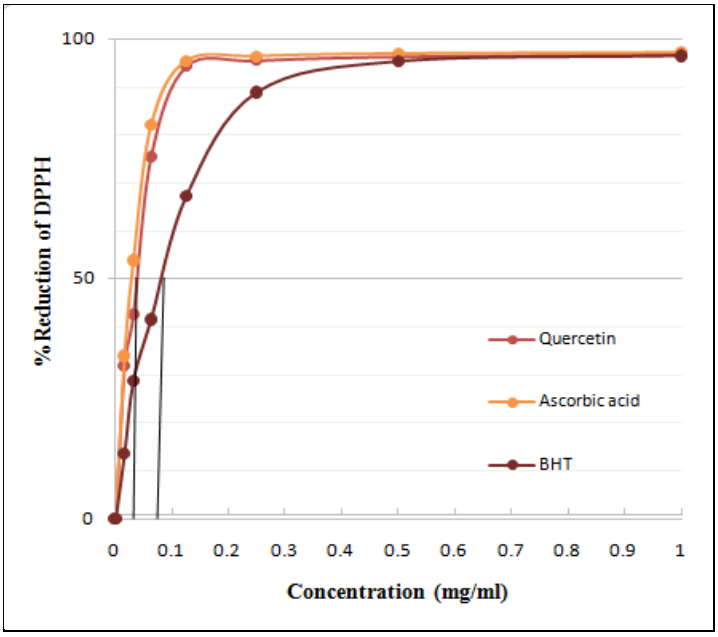

Figure (2): EC_{50} of Quercetin
EC_{50} = 0.1 (Vit. C and Quercetin)
EC_{50} = 0.5 (BHT)

The relationship between EC50 levels and antioxidant properties is inverse, a natural product is likely to be an efficient antioxidant if the EC50 value of flavonoid is less than 10 mg/ml. Its EC50 value was less than 0.4 mg/ml, demonstrating quercetin's potency as an antioxidant (19).

Quercetin Acts as a Reactive Hydrogen Donor on GSH to Produce Antioxidant Effects by controlling the amount of glutathione GSH, quercetin can improve the body's ability to fight against free radicals. This is due to the fact that the body creates free radicals during metabolic processes, which result in genetic abnormalities and cell membrane damage; cause a number of illnesses, including diabetes, heart disease, and liver disease; and hasten the aging process of the body (20).

The International Union of Pure and Applied Chemistry identified quercetin as 3,30,40,5,7-pentahydroxyflavone after examining the structure of the compound this nomenclature denotes that quercetin has an OH group connected at positions 3, 5, 7, 30 and 40 , Quercetin's effects on glutathione (GSH), signal transduction pathways, reactive oxygen species (ROS), and enzyme activity are mostly indicative of its in vivo antioxidant mechanism. Low doses of quercetin's antioxidant activities exhibit concentration dependency, whereas high doses of the compound have the reverse effect (21).

Determination of the (MIC) of quercetin

Quercetin was assessed using the microbroth dilution technique, and To guarantee an accurate outcome, the experiment was conducted many times with varying variables and gradients in this test. the findings indicate that even at high concentrations8000 mcg, quercetin has no influence on bacterial growth as shows in (Figure3).

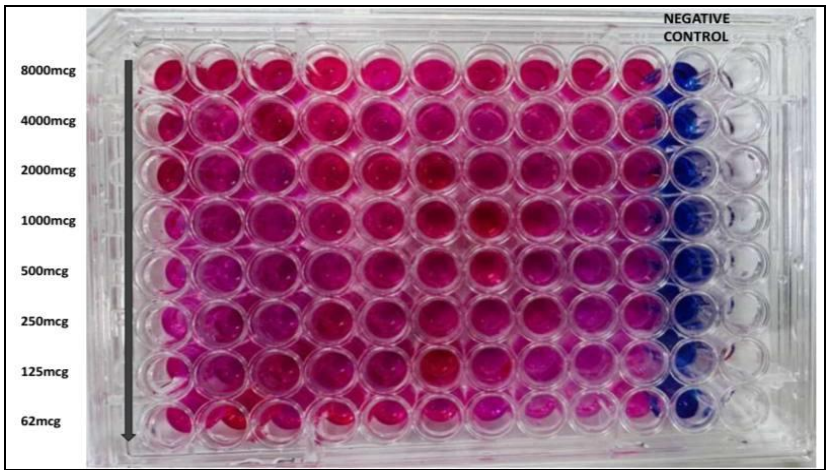

Figure (3): Antibacterial active determination of quercetin against *pseudomonas.aeruginosa*.

The results conflict with those of Jaisinghani, Renu. (22) who found quercetin inhibite *pseudomonas. aeruginosa* at 20 mcg/mL. However, our study confirms previous findings *Ouyang et al*., (2016) that quercetin has no effect on PAO1 growth as measured by MIC and growth curve analysis (23).

Another investigation found quercetin has no effect on the growth rate of pseudomonas comparison to an untreated instance of aeruginosa ,despite the fact that it inhibits both the production and expression of ExoS (24). Another study suggested that quercetin at the concentrations studied had no impact on growth of *P. aeruginosa* PAO1, and 20–100 μM of such a compound was used in the following experiments (25). P. aeruginosa are problematic. Limited permeability regarding the outer membrane(26), so quercetin faced difficult to enter the bacteria.One of the two major barriers in this test was Quercetin's limited solubility in aqueous solutions (27) , quercetin dissolves in 10% DMSO (21), in our study reveales that quercetin dissolve in only 100% DMSO not in diluted DMSO . The second obstacles faced in this study was quercetin poor stability, the quercetin participated in bottom of tube after dilution with broth. Two significant obstacles stand in the way of using quercetin as a medicine or diet supplement: its poor stability and limited solubility in aqueous solutions (28). DMSO has significant antibacterial activity against *pseudomonas. aeruginosa* so we have to diluted under 8-10 % , *p .aeruginosa* showed the least resistance to DMSO inhibition, with 90% of bacteria being suppressed in the presence of 8% DMSO (29).

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