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The Inhibitory Effect of *Conocarpus Lancifolius* Leaf Extract on Protease Produced by Clinical *Pseudomonas Aeruginosa* Isolate

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Abstract

The study was aimed at inhibiting the protease produced by *Pseudomonas aeruginosa* using an 80% alcoholic extract of *Conocarpus lancifolius* leaves. A total of 146 isolates of *P. aeruginosa* that were isolated and identified by microscopic and biochemical tests were 51 isolates submitted to primary and secondary screening techniques in order to choose the qualified *P. aeruginosa* isolate for protease synthesis. Among these isolates, forty-seven isolates showed hydrolysis zones on skim milk media (primary screening); six isolates were chosen for secondary screening. The result revealed that *P. aeruginosa* P51 had the highest ability to produce the enzyme, with a specific activity of 15.9 U/mg protein. In addition, the study included extracting the leaves of C. lancifolius using 80% ethanolic alcohol and conducting the GC-MC assay and the HPLC assay of the plant extract. The results revealed a significantly decreased specific activity of protease from 15.9 to 1.2 U/mg after treatment with 0.8 µg/ml of alcoholic extract of *C. lancifolius* leaves.

Keywords: *Conocarpus lancifolius*, Protease, Plant extract, inhibitory activity.

التأثير المثبط لمستخلص أوراق الكونكاربوس على البروتييز المنتج بوساطة عزلة زائفة زنجارية سريرية

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الخلاصة

هدفت الدراسة الحالية الى تثبيط البروتييز المنتج من بكتيريا الزائفة الزنجارية باستعمال المستخلص الكحولي لأوراق الكونكاربوس. أذ عزل 146 عزلة من بكتيريا الزوائف الزنجارية وشخصت بوساطة اختبارات مجهرية وكيموحيوية أذ اختير 51عزلة بعد التشخيص ثم خضعت لعمليات غربلة اولية وثانوية لاختيار العزلة الاكفأ في انتاج الانزيم. اظهرت 47 عزلة من بين العزلات منطقة تحلل في وسط حليب الفرز (غربلة اولية)، واختيرت ست عزلات للغربلة الثانوية، واظهرت النتائج انتخاب العزلة P51 *P.* الزوائف الزنجارية كونها الاكثر قابلية في انتاج الانزيم اذ بلغت الفعالية النوعية 15.9 وحدة/ملغم بروتين. بالإضافة الى ذلك، شملت الدراسة استخلاص أوراق نبات الكونكاربوس باستخدام 80% من الكحول الإيثانولي وعمل فحص GC-MC وفحص HPLC للمستخلص النباتي . كشفت النتائج وجود انخفاض معنوي في الفعالية النوعية أنزيم البروتييز من 15.9الى 1.2 وحدة/ملغم بعد معاملته في 0.8 مل من المستخلص الخام الكحولي لأوراق *C. lancifolius*.

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1. Introduction

The microorganisms produce several product types depending on their metabolic activities and the types of substrates consumed. Many of these products are considered naturally produced poisons that may be released into the medium of growth, known as exotoxins, or may accumulate inside the cells of the host, which are called endotoxins [1]. These bacteria have many virulence factors that increase their pathogenicity, including protease, hemolysin, and the formation of biofilm [2].

Researchers reported that *p. aeruginosa* is considered an opportunistic pathogenic bacterium that has the ability to cause mortality and morbidity in immunocompromised patients or patients with underlying medical conditions such as skin infections, respiratory tract infections, urinary tract infections, and nosocomial infections. In addition, these pathogenic bacteria often have the ability to resist common disinfectants and antibiotics [1], [3].

Protease is an extracellular enzyme that is released from *P. aeruginosa* and plays a vital role in its pathogenesis [4]. This enzyme hydrolyzes the peptide bonds in proteins to form polypeptides, or free amino acids. Proteases can cause many harmful effects, such as tissue necrosis and bleeding [5].

Many protease inhibitors have been obtained and identified from different types of plants [6]. These inhibitors contain phytoactive compounds that possess both preventive and curative characteristics. An example of plants that contain inhibitors of protease are *Conocarpus* trees, which are utilized in some countries for the treatment of many infections, such as syphilis, ulcers, skin, orchitis, hemorrhage, catarrh, fever, diarrhea, conjugative inflammatory, flu, anemia, and diabetes [7]–[9].

In this context, this study aimed to examine the ability of an alcoholic crude extract of *C. lancifolius* leaves to inhibit the protease production of clinical *P. aeruginosa* isolates.

2. Material and method

2.1 Isolation and identification of *Pseudomonas aeruginosa*

146 samples were collected from patients suffering from various infections, including males and females of several age groups, obtained from different teaching hospitals in Baghdad city. These samples were cultivated on MacConkey agar, blood agar, Pseudomonas agar, and cetrimide agar. For identification, some biochemical tests and microscopic examinations were used [10]. The identified *P. aeruginosa* isolates were prepared for screening experiments.

2.2 Primary (qualitative screening) of *P. aeruginosa* isolates for protease production

51 isolates of *P. aeruginosa* were primarily screened using skim milk medium to find the best ones that produced protease [11].

2.3 Secondary screening (Quantitative screening) of *P. aeruginosa* for protease production

Six isolates were subjected to quantitative screening. A 0.5 ml (1×10^6 cfu/ml) of overnight activated bacterial isolate was incubated in 25 ml of tryptic soya broth at 37 ˚C for 24 h at pH 8. After incubation, the crude enzyme was centrifuged at 3500 rpm for 20 min. After that, the specific activity, protein concentration, and enzyme activity were determined in the supernatant [11].

2.4 Protease activity assay

A 1.8 ml 1% casein solution was prepared; after that, tubes were placed in a water bath at 50 ^{0}C for 5 min. A 0.2 ml supernatant was incubated with a casein solution at 37 ^{0}C for 30 min.

2 ml of 5% trichloroacetic acid (TCA) was added to stop the reaction, and then the mixture was centrifuged at 6000 rpm for 15 min. The control was prepared by mixing 0.2 ml of enzymatic solution, 1.8 ml of 1% casein solution, and 3 ml of 5% trichloroacetic acid. The absorbance of the supernatant was measured at 280 nm in a spectrophotometer. The activity of enzymes was calculated using the formula [12]:

$$Enzymatic\ activity\ \left(\frac{U}{ml}\right) = \frac{\text{Absorbency at 280 nm}}{0.2 \times 30 \times 0.01}$$

Where 30 was the reaction time (in minutes), 0.2 was the amount of enzyme solution added in ml, and 0.01 ml was the amount of enzyme needed to raise the absorbance by 0.01 per minute under test conditions.
Protein concentration was calculated according to Bradford [13].

2.5 Preparation of *C. lancifolius* leaves

Fresh leaves of *C. lancifolius* from 32 trees were collected from variable regions of Baghdad, Iraq. Firstly, *Conocarpus* leaves were washed with tap water several times to clean them from dirt, clay, and dust. After that, they were washed with distilled water. The crude extract was prepared based on [14], with some modifications. The leaves of the plant were left to dry at room temperature, then crushed and kept in bags. Identification and classification of plants were performed in the biology department of the College of Science for Women at Baghdad University.

2.6 Preparation of alcoholic extract of plant leaves

100 g of the dried, crushed leaves of *C. lancifolius* were placed in a 2L flask, and then 1L of 80% ethanol was added while mixing to obtain a homogenous mixture. The mixture was placed in a shaker incubator for 6 hours at room temperature. The extract was filtered, then dried by spray drying at 67 ºC.

2.7 Analysis of the plant extract compounds using high-performance liquid chromatography

HPLC (Shimadzu, Japan) was utilized for the separation, identification, and quantification of alcoholic plant extract compounds, according to [15].

2.8 Gas Chromatography/ Mass Spectrometry (GC/MS)

The component identification was achieved by gas chromatography-mass spectroscopy analysis using a Shimadzu GC-MS analyzer system in the Iraqi Ministry of Science and Technology, Baghdad. Helium in the apparatus was used as a carrier gas, and 8 µL of *C. lancifolius* extract were injected into the GC-MS system using a microsyringe after being treated with siloxan to increase the thermal stability of the extract compounds. After a chromatogram was done for organic compounds, the results of the organic compounds appeared as peaks, and these compounds were identified in the program software of the apparatus. Then they were assigned based on the comparison of retention time and mass spectra fragmentation patterns for each compound with those stored in the computer library database. Compounds were identified by comparing their spectra to those of the NIST08.LIB mass spectral libraries.

2.9 Determination of MIC and MBC for plant extract and tested antibiotics

The MIC and MBC of both plant extracts and tested antibiotics (amoxicillin and nalidixic acid) were estimated using the microtiter method. The first step was adding 100µl sterile Mueller-Hinton broth to each well. The second step was the preparation of work solution from stock solution (256 or 100 µ g/ml for plant extract and antibiotic, respectively). A 100 µl from each well in the first column (column 1) were taken by a multi-channel pipette and placed into

the wells of the second column (column 2), which already contained 100 µl sterile Mueller-Hinson broths (in the first step). Then 100µl from each well in the second column (column 2) were taken by a multi-channel pipette and placed into the wells of the third column (column 3). The last step was adding 100µl bacterial inoculum with a concentration of 1*106 cfu/ml to all wells. All plates were placed in an incubator under aerobic conditions at 37 °C for 24 hours. Finally, the plates were read by an ELASA reader on wavelength 630 nm to observe growth in wells, and the least concentration of antibiotic that has been able to inhibit bacterial growth has been considered (MIC) [16]. While the maximum bactericidal concentrations (MBC) of Conocarpus extract against P. aeruginosa were determined as follows: A loopful from wells with no virtual growth (from step MIC) was incubated in fresh nutrient agar at 37 °C for 24 hours. MBC of medical extract or antibiotic is defined as the lowest concentration resulting in negative growth or only a single colony; this test was done based on [17]. This test of MIC and MBC was done in triplicate for all samples. Also, MIC and MBC were exposed at µg/ml.

2.10 Enzyme Inhibition

A constant ratio of enzyme extract was added to several ratios of Conocarpus extract (0.1, 0.2, 0.4, 0.6, 0.8, 1 ml) of 10% w/v of plant crude extract, and then 1.8 ml of 1% casein solution were added. This solution was mixed and incubated at 37 ºC for 10 min. 2 ml of 5% trichloroacetic acid was added to stop the reaction. The enzymatic specific activity of protease was assayed, and the procedure was repeated without the addition of plant extract [18]. The enzyme inhibition was calculated by using this formula [3]:

Inhibition ratio (%) = $(C-T)/C \times 100$

where C represents control (enzymatic activity without treatment with plants). T represents enzymes treated with plants.

3. Results and Discussion

3.1 Isolation and identification of *P. aeruginosa*

The collected 146 samples were cultivated on Blood and MacConkey agar. The results revealed that the samples displayed pale colonies on MacConkey agar, which are non-lactose ferment bacteria [19]. These samples were given beta homolysis on blood agar. Then, these isolates were re-cultured in *Pseudomonas* agar as a selective medium for the genus *Pseudomonas*. The results showed that the 51 isolates have the ability to grow in this medium**.** These isolates were re-cultured in cetrimide agar to confirm the diagnosis. Where *P. aeruginosa* differs from other *Pseudomonas* species by growth on the selective medium (Cetrimide agar) [20], all isolates were grown on this medium as greenish-yellow colonies, which confirmed *P. aeruginosa* due to their ability to resist Cetrimide material, which is considered toxic material for other bacteria, resulting in releasing nitrogen and phosphorus from bacterial cells more than *Pseudomonas* sp.

3.2 Primary screening

Protease production experiments Table 1 showed that 47/51 (92.2%) of *P. aeruginosa* isolates were able to produce protease in skimmed milk agar (1%), where these results were indicated by the zone lysis around the colony in varying degrees ranging from 6 to 35 mm. This variance in zone lysis may be due to the diversity of isolate sources and culture conditions, while 4/51 (7.8%) of isolates didn't show any zone lysis around their colony. These results agree with Onal *et al*. (2015), who found that most clinical isolates represent 93% of those that were able to produce protease [21]. Protease is considered one of the important virulence factors, and it plays a role in the lysis of tissue proteins such as elastin and collagen and helps bacteria invade infected tissues, especially in people with burns. It also acts to protect bacteria from body defenses [22].

Table 1: Production of protease by *P. aeruginosa* isolates in skimmed milk agar at 37 ^{o}C for 24 h and PH 7 according to inhibition zone of growth

No. of Isolates	Clear zone of Protease Production (mm)	No. of Isolates	Clear zone of Protease Production (mm)	No. of Isolates	Clear zone of Protease Production (mm)
P1	19	P18	13	P35	No clear zone
P2	15	P19	16	P36	15
P3	13	P20	11	P37	16
P4	21	P21	10	P38	14
P5	16	P22	23	P39	13
P6	6	P23	14	P40	10
P7	15	P24	11	P41	17
P8	18	P25	14	P42	15
P9	14	P26	8	P43	15
P10	15	P27	13	P44	12
P11	No clear zone	P28	16	P45	11
P12	16	P29	15	P46	No clear zone
P13	18	P30	11	P47	No clear zone
P14	17	P31	21	P48	11
P15	15	P32	12	P49	15
P16	16	P33	14	P50	12
P17	17	P34	20	P51	35

P1- 51 : *P. aeruginosa* isolates.

3.3 Secondary screening

Six isolates have been chosen for secondary screening to select the highest protease-producing isolate. The results showed that the specific activity of protease produced by these isolates ranged between 7.5 and 15.9 U/mg (Table 2). Genckal and Tari *et al*. (2006) referred to the fact that the differences in enzyme production for each isolate might be because of the isolation source and conditions, and the variation in codes of genes results in variation in enzyme synthesis [23]. Evaluation of protease activity depends on the enzyme's ability for casein degradation; for that, casein was used as a medium for the detection of protease produced by *P. aeruginosa*. The method for measuring the activity of protease based on the productivity of casein is one of the most widely used for determining the efficacy of the enzyme in bacterial culture [24]. Depending on the results obtained from the study, the *P. aeruginosa* P51 isolate showed the best efficiency for the production of the protease enzyme.

Table 2: Specific activities of protease produced by *pseudomonas aeruginosa* after 24hr of incubation at 37 ^{o}C, pH 8, in tryptic soya broth

Number of p. aeruginosa isolate	Specific activity(U/mg)
p1	7.5
P4	13.1
P22	9.6
P31	8.2
P34	12.8
P51	15.9

3.4 Crude extraction of C. lancifolius leaves

After treating 100 g of the dried, crushed leaves of *C. lancifolius* with 80% ethanol for 6 hours, the extract was filtered, then dried by spray drying at 67 °C. 14 g of green, dried extract was collected and stored in a dark vial until use.

3.5 High-performance liquid chromatography (HPLC)

Eight different compounds in the alcoholic extract of *C. lancifolius* leaves were detected (p-coumaric acid, sinapic acid, catechin, hydroxybenzoic acid, tannic acid, vanillic acid, caffeic acid, and gallic acid). Figure 1 shows the number of peaks; each peak refers to one compound.

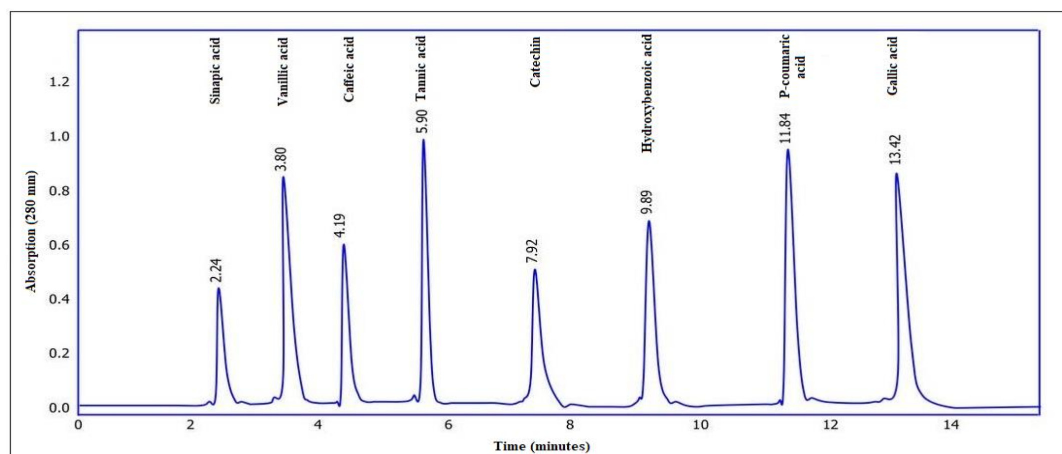

Figure 1: HPLC chromatogram of an ethanolic extract of *C. lancifolius*

After analysis of the data in Figure 1, the concentration of each compound was defeminized as shown in Table 3.

Table 3: The phenolic compounds and their concentrations

Compound	Concentration (mg/ml)
P-coumaric acid	199
Sinopic acid	78.4
Catechin	84.5
Hydroxybenzoic acid	118.5
Tannic acid	203.8
Vanillic acid	101
Caffeic acid	73.9
Gallic acid	94

Most of the plants utilized in traditional medicine systems are polyphenol- and flavonoids-rich compounds for regulation of plant growth and as a source of phytochemicals applied to the health of humans and animals, such as catechin, rutin, genistein, quercetin, kaempferol, etc. These phytochemicals are utilized for the treatment of various diseases due to their great healing potential [26].

3.6 Analysis of GC-MS Results for *Cononcarpus lancifolius* extract

Gas chromatography and mass spectroscopy analysis of compounds for leaf extract of *C. lancifolius* appeared as 25 peaks; four peaks were side effects of silane treatment, which were cyclotetrasiloxane, octamethyl, cycloheptasiloxane, tetradecamethyl, bis(pentaamethylcyclotrisiloxy) tetramethyldisiloxane, octasiloxane, and cyclonasiloxane,

octadecamethyl. These compounds were created by the reaction of silane molecules with compound fragments such as methyl groups, oxygen, etc. Also, cis-13-octadecenoic acid, methyl ester, showed the biggest area percentage (22.40%), followed by indeno[2,1-c] pyridine -9-one, 3,5,7-trimethyl, with an area percentage of 15.60% in the extract, while 2-anthracenamine showed a lower area percentage (0.69%). Nine alkaloid compounds appeared in the GC-MS of the current study. While total phenols consisted of eight compounds, six were phenol derivatives and two were flavonoids. These results agree with those of thin layer chromatography (TLC) for the alcohol extract of *C. Lancifolius,* which was described by [27]. In addition, 1 terpenoid compound, 3 alcohol compounds, and 4 lipid fatty acids appeared in this chromatogram. Moreover, four amido amine compounds and an additional amido compound common with phenol appeared, which are chemical compounds with antimicrobial effects. As well, the mechanism of antibacterial activity for amido compounds is qualified by electrostatic interaction between the cationic amido compound and the anionic surface of the phosphate group of phospholipids in bacterial cells, which leads to disruption of the phospholipid bilayer, consequent cell lysis, and possible death [28].

In the current study, all of the compounds in the *C. lancifolius* extract showed up in the GC-MS and area percentages, as well as in their GC-MS results, which showed that lipid compounds were present in the aqueous extract. Whereas, lipids are insoluble in water [29]. However, the presence of three alcoholic compounds assists the solubility of lipids because alcohol molecules contain a hydroxyl group that has hydrogen bonding with water molecules and is called a hydrophilic group. On the other hand, a large portion of the remaining alcohol molecule is lipid-soluble, and it is called the hydrophobic group [30]. In contrast, phenols and alkaloids were present in the current aqueous extract due to the water-soluble characteristics of both phenols and alkaloids [31]. As well, the amido (amine) group has a hydrophilic feature, which is used by chemists to improve the water solubility of chemical compounds [32].

3.7 Estimation of MIC and MBC for alcoholic leaves extract and tested antibiotics

The results, as shown in Table 4, indicated that both amoxicillin and nalidixic acid have higher MIC (4 and 8 µg/ml) and MBC (16 and 16 µg/ml) values than alcoholic leaf extract (2 for MIC and 8 µg/ml for MBC).

Amoxicillin is an antibiotic utilized for the treatment of bacterial infections, including skin infections, pneumonia, strep throat, and middle ear infections [33]. However, the incidence of resistance in P. aeruginosa to amoxicillin is considered a major problem in treatment [34]. whereas nalidixic acid is an effective antibiotic against gram-negative and positive bacteria. At its low concentration, this antibiotic acts as a bacteriostatic, which inhibits bacterial growth, and its bactericidal effect is observed with higher concentrations. This antibiotic is utilized for the treatment of urinary tract infections [35].

Table 4: MIC and MBC of alcoholic plant extracts and antibiotics

	P. aeruginosa	**P51**
MIC (µg/ml)	**Plant Extract**	**2**
	Amoxicillin	**4**
	Nalidixic acid	**8**
MBC (µg/ml)	**Plant Extract**	**8**
	Amoxicillin	**16**
	Nalidixic acid	**16**

3.8 Effect of *C. lancifolius* in the production of protease.

After determining the minimum inhibitory concentration (MIC) of *C. lancifolius*, sub-MICs were used to determine their effect on the production of protease, which is produced by *P. aeruginosa*. The results showed that *C. lancifolius* reduced protease production by observing low zone lysis around the colony of isolates after using the extract when cultured in skimmed milk agar (Figure 2). This may be due to the fact that extracts relatively inactivate the role of the enzyme in lysis protein due to the presence of active compounds within the extract that have a main role in disrupting the cell metabolism and reducing the bacterial virulence. Crude extracts of *C. lancifolius* have rich phytochemical contents, so the presence of these phytochemicals contributes to medicinal as well as physiological properties in the treatment of different ailments. Therefore, extracts from these plants could be seen as a good source for useful drugs. Furthermore, the polyherbal extract displays high antibacterial activity against gram-positive bacteria. *Staphylococcus aureus* and the gram-negative bacteria *P. aeruginosa*, *Escherichia coli,* and *Klebsiella pneumoniae* were resistant to commonly used antibiotics [36].

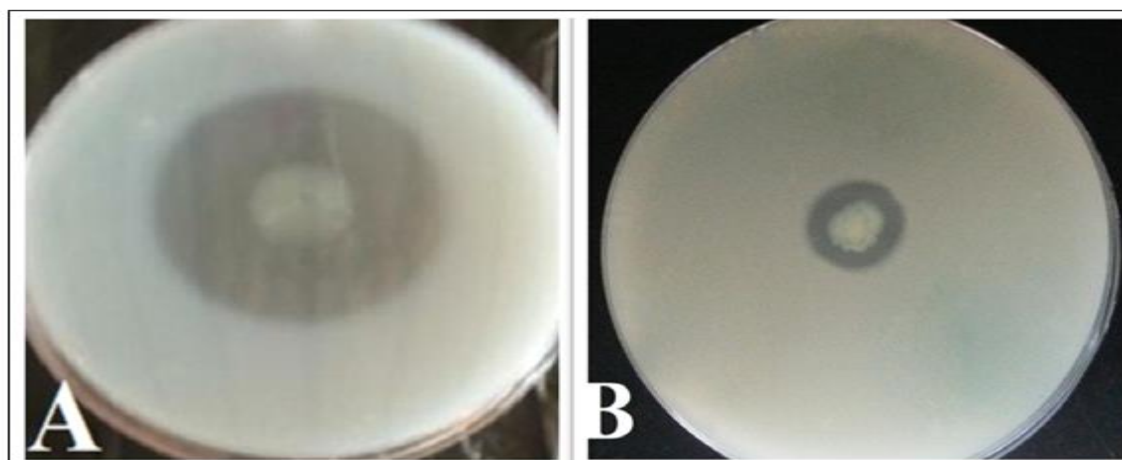

Figure 2: Effect of *C. lancifolius* leaf extract on protease production of *P. aeruginosa* isolate.
A: before use extract. B: after use extract

3.9 Inhibition of protease

A variable ratio (0.2, 0.3, 0.4, 0.8, and 1 ml) of the extracted *C. lancifolius* leaves was utilized as an inhibitor of the enzyme. The enzyme was assayed before and after treatment with the plant to determine the role of extracted *C. lancifolius* on its specific activity. The results in Table 5 determine that the best inhibitory ratio for protease-specific activity was 1:4 (0.2 ml of protease treated with 0.8 ml of plant extract). The specific activity decreased from 15.9 to 1.2 U/mg in the other concentrations. This inhibitory effect of *C. lancifolius* extract may be due to the plant's containing a wide variety of compounds that have been found to provide inhibitory compounds or a platform on which to synthesize active molecules [37].

Table 5: Inhibitory activity of *C. lancifolius* leaf extract on protease specific

NO	Enzyme:*C. lancifolius*	Enzyme specific activity (U/mg)	Control	Inhibitory ratio%
1	1:1	4.5	15.9	71.7%
2	1:2	4.0	15.9	74.9%
3	1:3	3.5	15.9	77.9%
4	1:4	1.2	15.9	92.5%
5	1:5	1.2	15.9	92.5%

5. Conclusion

The results of this study concluded that ethanolic extraction (80%) is a good method for the extraction of plants. Besides, the extract of *C. lancifolius* leaves is able to decrease the activity of *P. aeruginosa*-produced protease. Different techniques, including HLPC and GC-mass, are effective for the detection of plant compounds.

6. Ethical Clearance

The Biotechnology Department's local committee agreed to the experiments mentioned in this research. The study was undertaken by the University of Baghdad team.

7. Conflict of Interest

There are no conflicts of interest between the authors.

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