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Study of the inhibitory effect of cinnamon oil extract on *Pseudomonas putida* isolated from cattle respiratory infection

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ABSTRACT

Background and aim: *Pseudomonas putida* is a non-capsulated, rod-shaped, oxidative, gram-negative Bacterium. The aim of the study was to isolate the pathogenic P. putida from a cow with respiratory infection and to evaluate pharmacodynamics indices of cinnamon oil against isolated bacteria in order to determine its the in vitro antibacterial efficacy for the first time in Iraq.

Materials and methods: divided into two parts: first part involved the isolation and identification of pathogenic *P*. putida from cow with respiratory infection, swab samples were cultured on different media and examined microscopically.

Results: The isolates were identified based on the production of pyoverdine dye on nutrient agar, beta-hemolysis on blood agar and pale-mucoid on MacConkey agar, the identification was further confirmed using the VITEK 2 system. The second part evaluated the in vitro antibacterial activity of cinnamon oil against bacterium, the minimum inhibitory concentration was about (0.5 mg/mL) and minimum bactericidal concentration was approximately (0.1 mg/L), the sensitivity test demonstrated increasing inhibition zones with higher concentration of extract. Time-kill experiments revealed a complete lysis after 60 minutes, while 50% of bacterial cells were lysed with 10 minutes at 4× MIC.

Conclusions: intracellular components were released in a concentration- and time-dependent manner by UV spectrophotometry indicating membrane disruption.

Introduction

Pseudomonas putida is a non-capsulated, rod-shaped, oxidative, gram-negative Bacterium that is found in the environment and belongs to the wide group of fluorescent Pseudomonas species. This bacterium produces a wide range of metabolic enzymes, permitting the species to adjust to various environments, such as those connected with soil and waters[1] *P. putida* reproduces the variability of its natural environment through its opportunistic and undemanding nutritional capaciti es, rapid development, resilience to oxidative stress and hazardous chemical challenges, and other traits. Began in the 1960s when P. putida's capacity for xenobiotic biodegradation was discovered [2]. This bacterium may occur as an opportunistic condition in stressed or immunocompromised animals and its characterized clinically by fever, cough, tachypnea, mucopurulent nasal discharge, and reduced milk production [3]. Because they have efflux pumps, the majority of Pseudomonas species are naturally resistant to a number of antibiotics, including beta-lactams which they contribute significantly to antibiotic resistance and are engaged in the harmful chemicals' outflow from bacterial cells [4,5]. Due to antibiotic resistance, it was necessary to improve other strategies to fight infections caused by microorganism and one of these strategies is herbs. As a health-promoting substance, cinnamon has been used to treat conditions like inflammation, gastrointestinal disorders, and urinary tract infections [6,7]. Cinnamon's anti-microbial properties, especially its antibacterial activity, suggest yet another possible medical application. The specific portion of the plant from which cinnamon essential oils are derived determines their chemical makeup. Cinnamaldehyde makes up the majority of the ingredients in bark essential oil, ranging from 90% to 62%–73%, depending on the extraction method [8]. Several studies have highlighted that cinnamon essential oil possesses significant inhibitory effects against respiratory pathogens, including Streptococcus pneumoniae and Staphylococcus aureus, through disruption of cell membranes and leakage of intracellular components [9]. Furthermore, its major bioactive compound, cinnamaldehyde, has been shown to interfere with bacterial biofilm formation and enhance host respiratory defense mechanisms [10]. These findings suggest that cinnamon oil could be a promising natural alternative for controlling bacterial respiratory infections, particularly in animals where antimicrobial resistance is a growing concern. Therefore, the study's objective was to isolate the pathogenic P. putida from infected cows and to evaluate, for the first time in Iraq, the in vitro antibacterial activity of cinnamon oil extracts against this pathogen. Specifically, the study aimed to [11]. characterize the isolated bacteria using culture, microscopy and VITEK 2 identification system; [12]. determine the minimum inhibitory concentration and minimum bactericidal concentration of cinnamon oil; [7] assess the sensitivity of *P. Putida* to different concentrations of oil using diffusion assess; and [13] analyze the pharmacodynamics effect of oil through time-kill studies and measurement of cellular materials release.

Materials and Methods

Preparation of Culture Media

The culture media used in this experiment included brain heart infusion, nutrient agar, MacConkey agar and blood agar. Each of these culture media was prepared and autoclaved for 15 minutes at 121°C (15 Ibs/inch2) in accordance with the manufacturer's instructions. The prepared media then poured into sterile petri dishes and stored at 4°C until use. To verify sterilization, the media were Incubated for 24 hours at 37°C [14].

Isolation and Identification of Pseudomonas putida

Culturing of Sample

The swab from sputum was incubated for 24 hours at 37 °Cin test tube containing 5 ml of brain heart infusion broth. A loopful of the bacterial suspension was then streaked on nutrient agar, blood agar, and MacConkey agar to facilitate identification [15].

Microscopic Examination

A smear was prepared on a microscope slide using a colony obtained with a sterile loop from blood agar. The smear was fixed and stained using gram stain, then examined under a light microscope lens at 100x oil immersion [16].

Identification system VITEK 2

This system based on advanced colorimetric technology, is considered the next generation of the gold standard in microbial identification. All procedures were performed according to the manufacturer’s instructions (Biomerieux) [13].

Plant Material

The plant used in this study was obtained from a local market in Baghdad. A total of 100g of plant materıal was used. The extraction process

was carried out at the college of Veterinary Medicine / Department of Physiology, Biochemistry and Pharmacology [17].

Preparation of Cinnamon oil against Bacteria

Thirty-five grams of dried and ground cinnamon bark were extracted using a Soxhlet apparatus with 250 mL of ethanol as the solvent. The extraction was carried out for 10 hours at the boiling temperature of ethanol (approximately 78°C). After completion of the extraction process, the obtained extract was concentrated under reduced pressure at 50°C using a rotary evaporator to remove the solvent. The concentrated extract was then collected and stored appropriately for further analysis [12].

Preparation of Standard Inoculum of Bacteria

Colonies from 24-hour cultured media were taken to prepare the inoculum suspension. The colonies were suspended in a sterile 0.9% aqueous solution of NaCl. The turbidity was adjusted by comparing it in to 0.5 McFarland Standard equivalent to 1x108 CFU/mL [11].

Estimation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Of Cinnamon oil

Minimum inhibitory concentration is defined as a lowest concentration of essential oil at which no visible bacterial growth occurs. bacterial. Whereas Minimum Bactericidal concentration is characterized as a lowest concentration required to kill the bacterium. MIC and MBC were determined following the method described by [1], with slight modification, and based on [8]. A serial two-fold dilution was performed to prepare cinnamon oil concentrations ranging from 6 to 0.0312 mg/ml, a serial two-fold dilution was performed). Finally, 50 µl of bacterial suspensions (1×108CFU/mL) was added to each tube. All tubes were incubated at 37°C for 24 hours. To determine the MBC, 50 µL from each tube showing no visible bacteria was sub cultured on to nutrient agar and incubated again at 37°C for 24 hours. Each test tube was performed in duplicate to ensure reproducibility. The MIC and MBC assays were conducted in the pharmacology laboratory.

Sensitivity Test

The agar diffusion method was used to measure the antibacterial activity of cinnamon essential oil with a few minor adjustments. Equal amounts of the 20 ml MacConkey agar were added to each sanitized Petri dish, after ten minutes of setting to solidify the agar, 1 milliliter of a bacterial suspension (108 CFU/mL) was applied to the agar's surface. Sterilized blank of whatman paper discs of 6 mm diameter were saturated with cinnamon oil of varying concentrations 0.25, 0.5, 1, 2, 4 and 6 mg/mL. The discs were air-dried at room temperature before placement on the agar surface, and allowed to diffuse in agar at room temperature for two hours. Followed by, the plates incubated for 24 hours at 37 Co. For every concentration of extract, two replicates were done, and the diameter of the zone of inhibition was measured using a Vernier caliper to assess the plant extract's activity [18].

Effect of cinnamon oil on release of cellular materials

A suspension of 1×108 cells/mL of *Pseudomonas putida* has been used throughout the experiment. The release of cellular materials from the Pseudomonas suspension treated with cinnamon oil was measured by detecting ultraviolet (UV)-absorbing intracellular components, which serve as an indicator of cellular lysis. The release of these materials was evaluated based on two variables: time-dependent and concentration-dependent lysis. Both variables were assessed by measuring the absorbance at 260 nm using a UV spectrophotometer.

Effect of cinnamonoil concentration

Twelve test tubes containing a suspension of 1×108 cells/mL of bacteria were collected by centrifugation for 15 minutes at 400 rpm. Each tube's pellet was twice rinsed with 5 milliliters of DMSO (pH 7.4) before being re-suspended in the same volume of DMSO. Six equal groups, each consisting of two tubes, were created from the twelve tubes and handled as follows:

Group A : (positive control): *Pseudomonas putida* suspension with DMSO
Group B : *Pseudomonas putida* suspension with -DMSO containing 0.25MIC cinnamon oil extract
Group C : *Pseudomonas putida* suspension with -DMSO containing 0. 5MIC cinnamon oil extract
Group D : *Pseudomonas putida* suspension with -DMSO containing 1.0MIC cinnamon oil extract
Group E : Pseudomonas putidasuspension with -DMSO containing 2.0MIC cinnamon oil extract
Group F : Pseudomonas putidasuspension with -DMSO containing 4.0MIC cinnamon oil extract

The cells in each group were centrifuged for two minutes at 12,000 rpm after all test tubes had been cultured for two hours at 37°C. A UV

spectrophotometer was then used to measure the supernatant's absorbance at 260 nm.

Effect of time-kill

A 1×108 cell/ml bacteria suspension was collected from forty-four test tubes by centrifugation at 400 rpm for fifteen minutes. Five milliliters of DMSO (pH 7.4) were used to wash the pellet in each tube twice before it was re-suspended in the same volume of DMSO. Four groups of forty-four tubes were divided and treated as follows:

Group A: (positive control): Two tubes pseudomonasputida suspension with DMSO

Group B: Fourteen test tubes of *Pseudomonas putida* suspension with DMSO containing 1.0MIC cinnamon oil extract

Group C: Fourteen test tubes of *Pseudomonas putida* suspension with DMSO containing 2.0MIC cinnamon oil extract

Group D: Fourteen test tubes of pseudomonas putidasuspension with DMSO containing 4.0MIC cinnamon oil extract.

All test tubes were incubated for 0, 10, 20,30, 40,60, and 90 min. at 37Co following this time, cells of each groups were centrifuged at 12,000 rpm for two min. Then the absorbance of the supernatant was determined using a UV spectrophotometer at 260nm.

Statistical analysis

Data were analyzed using the Statistical analysis system – SAS (2012) and expressed as mean ±SEM. One –way ANOVA was performed to determine differences among treatments, followed by the Least significant differences (LSD) test for mean separation. Statistical analysis was set at $P \leq 0.01$. in tables and figures, different superscript letters indicate significant differences between means.

Results

Isolation and Identification of Pseudomonas Putida

The bacterial colonies on nutrient agar appeared circular smooth convex greenish coloration (Figure 1A), while on Mac Conkey agar appeared pale -mucoid colonies as (Figure 1B) with the presence of beta hemolysis on blood agar. (Figure 1C). The microscopic characteristics showed gram negative rod colonies as (Figure 1D). The result of VITEK revealed the presence of *Pseudomonas putida* with 94% probability. The identification process took 8.47 hours, and the result was finalized on February 7,2022.

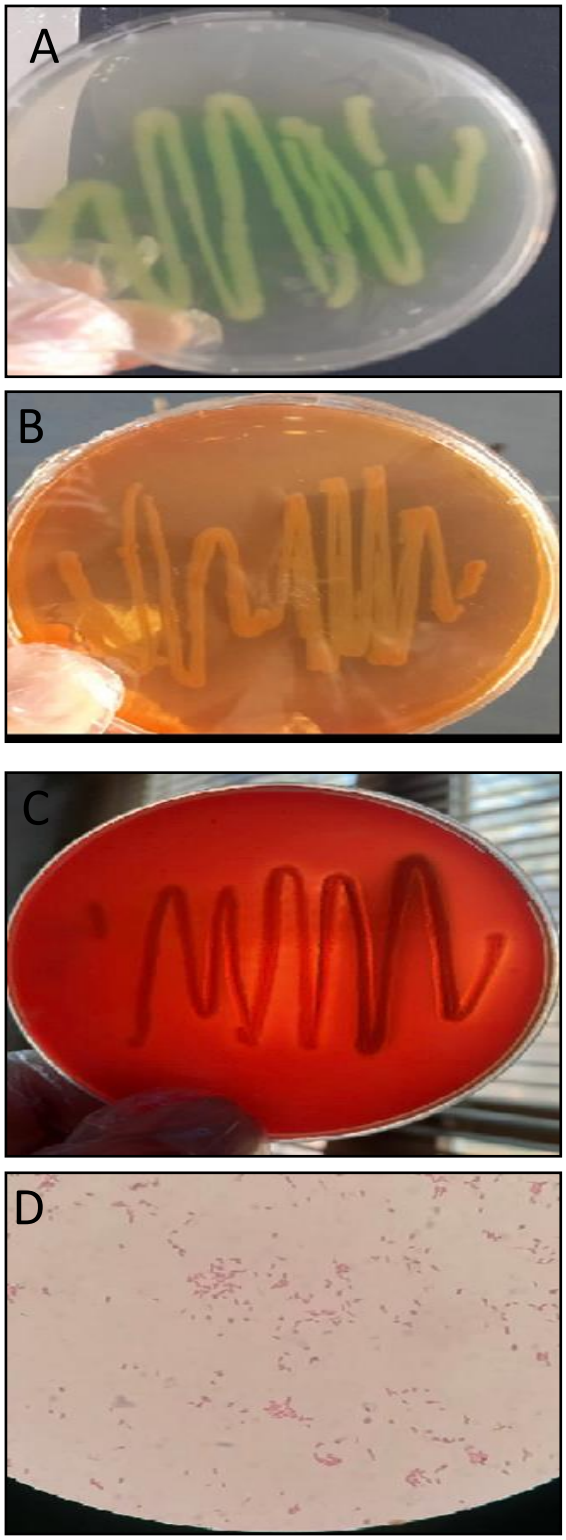

Figure 1. A. *Pseudomonus putida* showed green color colonies on Nutrient agar.
B. *Pseudomonas putida* showed pale color colonies on Mac Conkey agar.
C. *Pseudomonas putida* produced beta hemolysis on blood agar.
D. Microscopic appearance of *Pseudomonas putida* showed rod shape Colonies.

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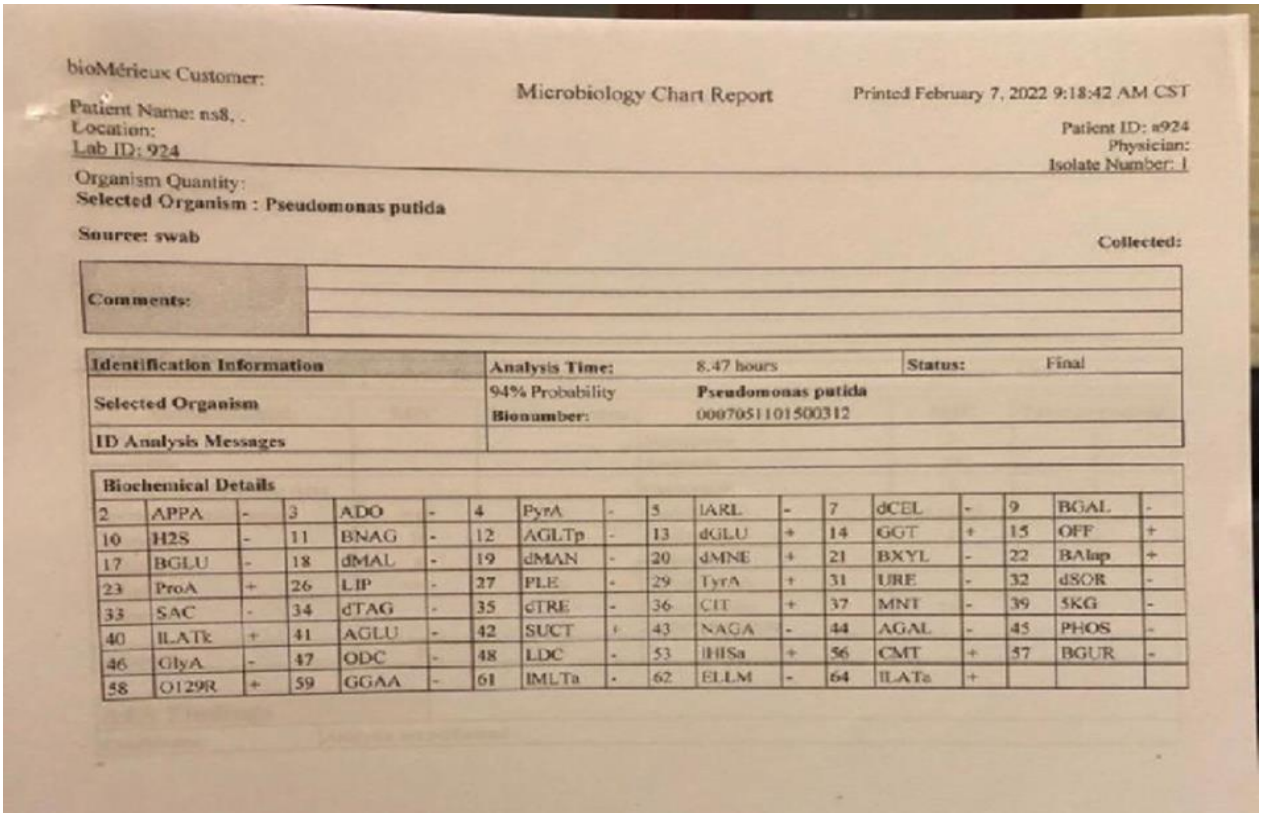

bioMérieux Customer:

Microbiology Chart Report

Printed February 7, 2022 9:18:42 AM CST

Patient Name: ns8, .
Location:
Lab ID: 924

Patient ID: n924
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas putida

Source: swab

Collected:

Comments:	

Identification Information	**Analysis Time:**	8.47 hours	**Status:**	Final
Selected Organism	94% Probability	**Pseudomonas putida**		
	Bionumber:	0007051101500312		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	+			

Figure 2. *Pseudomonas putida* on VITEK 2 system.

Extraction of Cınnamon oil

The barks' powder yield percentage was 42.9% as showed in (Figure 3), after the barks were extracted with 96% ethanol. The extract had a bright yellow color and smelled like cinnamon oil. This was achieved using the equation:

Percentage yield of the extract = weight of extract (gram) / weight of cinnamon barks powder (gram) × 100.

= 15 gram / 35 grams x100= 42.9%

Figure 3. Cinnamon oil extract

Pharmacodynamics Analysis

Determination of MIC and MBC of Cinnamon Bark Extract

The results showed that the concentration 0.5 mg/mL of cinnamon oil was effective to avoid P. putida from growing, this concentration of cinnamon oil was displayed to have a positive value that inhibit the growth of bacteria while 0.1 mg/mL killed *P.putida* so it was considered as the (MBC) that tested in micro-dilution assay as shown in the (Figure 4).

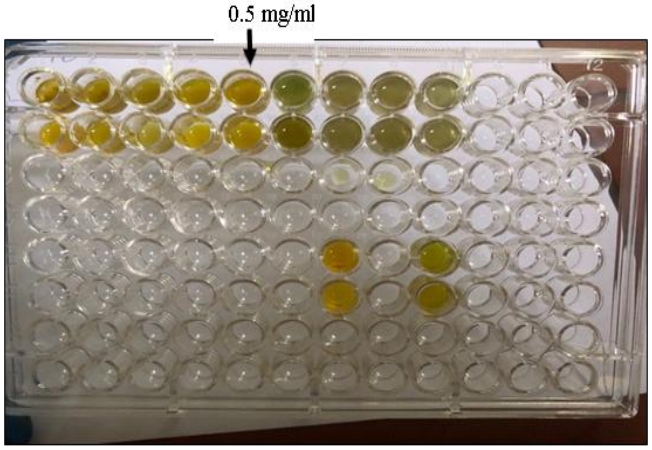

Figure 4. Minimum inhibitory concentration of cinnamon oil, against *P.putida* Isolates {yellow (no growth) green (growth)}.

Sensitivity Test

In the agar diffusion assay, different cinnamon oil extract concentrationswere used. Different zones of inhibition against P. putida were elicited by these concentrations. The size of the inhibitory zones varied as a result of using different concentrations of extract, and they grew proportionally as the concentration of cinnamon oil increased (Figure 5).

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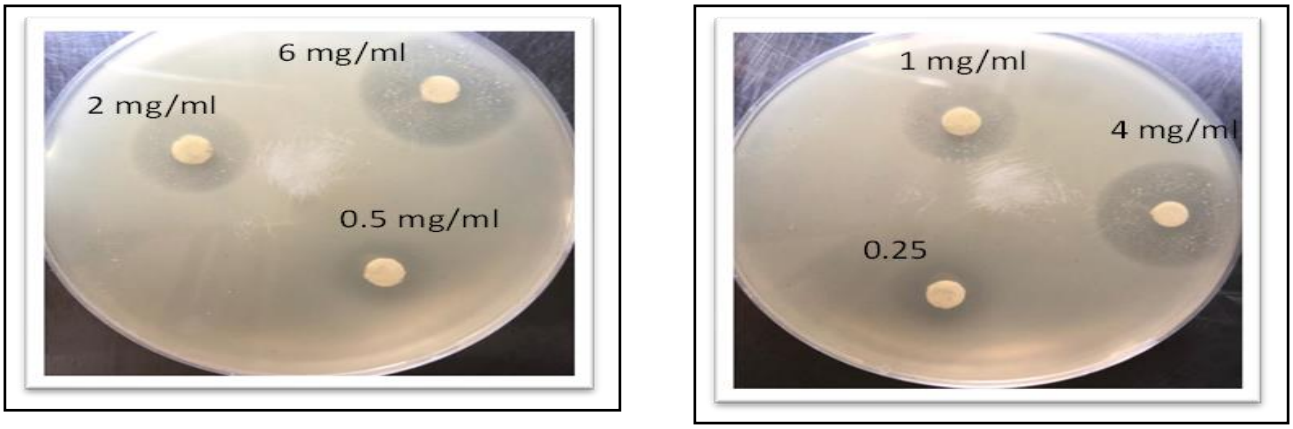

Figure 5. Sensitivity of Pseudomonas putida to different concentrations of cinnamon oil extract

Table 1. In Vitro antibacterial efficacy of cinnamon oil against P. putida at varying concentrations: evaluation by inhibition zone diameter

Zone inhibition (mm)						
Conc. mg/ml Groups	6	4	2	1	0.5	0.25
Cinnamon oil	18.0±0.5 A a	15.0±0.3 AB a	12.0±0.08 B a	11.0±0.05 BC a	9.0±0.03	8.0±0.01 C a
Dimethyl sulfoxide	0.00 A b	0.00 A b	0.00 A b	0.00 A b	0.00 A b	0.00 A b

Means with different big letters in the same row and small letters in the same column are significantly different, ** (P≤0.01).

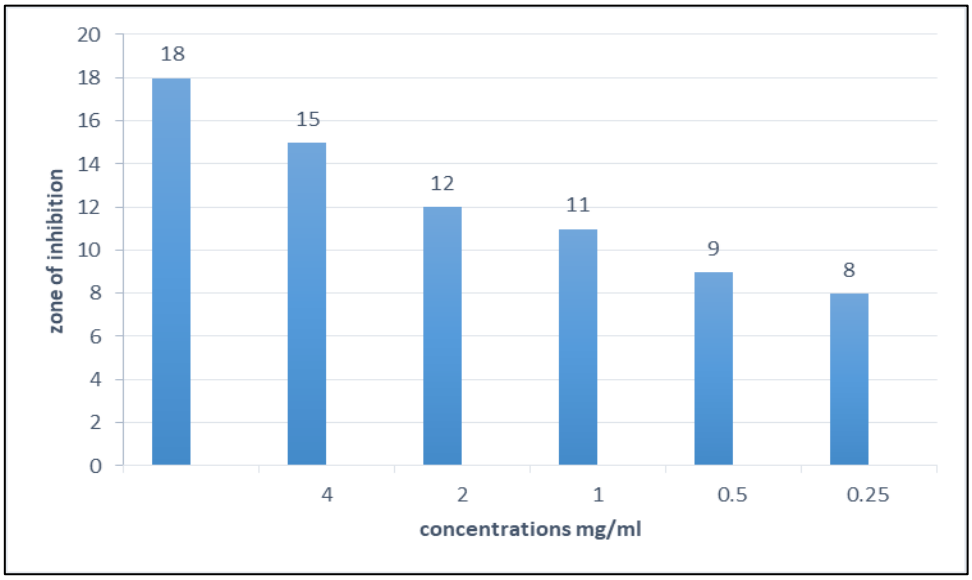

Figure 6: relationship between the concentration of cinnamon oil and mean diameter of zone of inhibition against *P. putid*a

Cinnamon oil effect on release of cellular materials

Cinnamon oil concentration effect on cell lysis

In relation to the concentration of cinnamon oil used, Table 2 demonstrates a rise in the release of cellular components. Furthermore, the findings indicate that when P. putida was exposed to 1.0, 2.0, and 4 MIC, respectively, the release of cellular material was 55.7%, 96.2%, and 100%. According to this measurement, cinnamon oil weakens P. putida cell wall structure, which leads

to the subsequent leaking of intracellular components. Cells of P. putida were lysed with varying doses of cınnamon, and the release of UV-absorbing compounds at 260 nm was measured in order to determine the reason of cell death.

Table 2: Effect of cinnamon oil concentration on cell viability and release of 260 nm- absorbing materials from *P. putida*

Concentrations of cinnamon oil	Cellular material contents released at OD260 (%)
0	0
0.25xMIC	25.9
0.5xMIC	38.5
0.1 xMIC	55.7
0.2xMIC	96.2
0.4xMIC	100
LSD value	11.516

The effect of time-kill

Cell lysis was assessed at different intervals up to 90 minutes following treatment with 1x MIC, 2x MIC, and 4x MIC of cinnamon oil Table 3. 50% of the P. putida population was killed and the cellular materials were released at the median lethal time (LT50). 50% of the P.putida population must be killed by 4xMIC concentrations of cinnamon oil in 10 minutes, whereas 50% of the P. putida population must be killed by 2xMIC and 1xMIC concentrations in 20 and 60 minutes, respectively. There was a time-dependent effect for these three cinnamon oil concentrations. As the concentration of clove increases, the time required to kill the P. putida cells may decrease.

Table 3: Time -dependent release of cellular components from *p. putida* treated with varying concentrations of cinnamon oil

Time/min	**Absorbing of cellular material release**			**LSD value**
	4x MIC	**2x MIC**	**1x MIC**	
0	0	0	0	0.00 NS
10	50.1	33.8	22.9	5.90 *
20	83.9	52.7	36.8	8.98 *
40	96.1	84.5	42.9	8.85 **
60	100	95.1	59.0	9.08 **
90	100	100	90.0	7.40 **
LSD value	8.90 **	11.08 **	7.60 **	---
** (P≤0.01).				

Discussion

Colony morphology was identified on nutrient agar and MacConkey agar which are considered a selective and differential culture media which are used for isolation and identification of P. Putida. The bacterial colonies were pale on MacConkey agar according to [19]. While, on nutrient agar had green color colonies asa result of the releasing of pyoverdine dye [20]. The isolated bacteria on blood agar showed a beta hemolysis, which is considered the simplest method to confirm the hemolysin enzyme- producing virulence isolate of P. Putida according to [21]. The results indicated that the confirmation diagnosis with VITEK 2 is more beneficial, time saving and cost-effective than the conventional biochemical tests for pathogenic bacterial diagnosis [22] Since ethanol was a more effective solvent for extraction than water, it was utilized in the Soxhlet extraction procedure. Compared to other extraction solvents including methanol, hexane, and chloroform, ethanol proved less hazardous [23]. The Soxhlet extraction yield of cinnamon oil was 42.9%, which is consistent with earlier research showing that the Soxhlet extraction process yields a higher yield of extracted cinnamon oil than steam distillation [24]. This result arises from the fact that cinnamon oil dissolves more readily in

alcoholic solvent than in water [24]. The disc diffusion method results were validated by the MICs and MBCs, which were 0. 5 and 0.1 mg/ml, respectively, indicating the bacteria' sensitivity to the essential oil [25]. Through the diameter of the inhibitory zone that appeared, it was possible to observe that cinnamon extract had inhibitory effects on P. putida bacteria in this investigation. Since cinnamon extract exhibited antibacterial action across all concentration gradients, this finding aligns with other prior research that demonstrated cinnamon's antibacterial activity against various bacterial species [21]. The concentration of 6 mg/ml produced the biggest diameter of the inhibition zone, measuring 18 mm, while the concentration of 0.5 mg/ml produced the lowest diameter, measuring 8 mm. The diameter of the inhibitory zone increases as the concentration of cinnamon extract does, and this expansion is due to a greater variety of chemicals. Essential oils from spices and herbs, such as cinnamon, have impact on the structure of the cell envelope because their potent antibacterial properties can permeate the cell wall and damage the cytoplasmic membrane [26,27]. Higher amounts of cinnamon essential oil were associated with swollen cells and poor membrane structure. These alterations in bacterial cells could be the result of membrane lysis and transformation brought on by damage to the membrane's integrity and permeability from cinnamon essential oil indicated that cinnamon oil could destroythe bacterial cell membrane, resulting in cell lysis and death. As a result, the modifications may cause the inner cell components to disappear [28]. According to a recent study, the integrity of the cytoplasmic membrane may be impacted by the action of cinnamon essential oil. As a result, proteins and nucleic acids were liberated via the damaged membrane, as evidenced by the noticeably increased protein content in response to the administration of cinnamon essential oil [29,30].

Conclusions

In this study, P. Putida was ısolated from respiratory ınfected cow for the first tıme in Iraq, and it was demonstrated that cinnamon oil extract works well as an antibiotics agaınst pseudomonas putida. The essential oil of cinnamon, which is rich in various component, demonstrated a strong antibacterial effect with a minimum inhibitory concentration (MIC) of 0.25 mg/mL and minimum bactericidal Concentratıon 0.1 mg/mL. Thıs extract can be suggested as a successful chemotherapy alternative to treat bacterial infections and reduce the incidence of mortality from systemic ınfection. In veterinary medicine, these findings suggest that oil could be applied for the prevention and treatment of infections in livestock such as mastitis in ewes, respiratory infection in calves. Thereby, improving animal health and reducing reliance on synthetic drugs.

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Declaration of interests

The authors have no conflicts of interest to declare.

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Publication consent

The authors agree to publication

Data and material availability

All data analyzed during this study are included in this article.

Author contribution

The authors contributed equally to the article

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دراسة التأثير المثبط لمستخلص زيت القرفة على بكتيريا الزائفة البوتيدية المعزولة من عدوى الجهاز التنفسي للماشية

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الملخص

خلفية للبحث والهدف: بكتيريا *Pseudomonas putida* هي بكتيريا سالبة الغرام، غير محاطة بكبسولة، عصوية الشكل، مؤكسدة. هدفت هذه الدراسة إلى عزل سلالة *P. putida* الممرضة من بقرة مصابة بعدوى تنفسية، وتقييم المؤشرات الدوائية لزيت القرفة ضد البكتيريا المعزولة، وذلك لتحديد فعاليته المضادة للبكتيريا في المختبر لأول مرة في العراق. **المواد وطرائق العمل:** قُسّمت الدراسة إلى جزأين: تضمن الجزء الأول عزل وتحديد سلالة *P. putida* الممرضة من البقرة المصابة بعدوى تنفسية، حيث زُرعت عينات المسحات على أوساط مختلفة وفُحصت مجهريًا. **النتائج:** تم تحديد العزلات بناءً على إنتاج صبغة البايوفيردين على وسط أجار المغذيات، والتحلل الدموي بيتا على وسط أجار الدم، واللون المخاطي الشاحب على وسط أجار ماكونكي، وتم تأكيد التحديد باستخدام نظام VITEK 2. قيّم الجزء الثاني النشاط المضاد للبكتيريا لزيت القرفة في المختبر ضد البكتيريا، حيث بلغ الحد الأدنى للتركيز المثبط حوالي 0.5 ملغم/مل، والحد الأدنى للتركيز القاتل للبكتيريا حوالي 0.1 ملغم/لتر. وأظهر اختبار الحساسية زيادة في مناطق التثبيط مع زيادة تركيز المستخلص. وكشفت تجارب القتل الزمني عن تحلل كامل للبكتيريا بعد 60 دقيقة، بينما تحللت 50% من الخلايا البكتيرية خلال 10 دقائق عند تركيز يعادل 4 أضعاف الحد الأدنى للتركيز المثبط. **الاستنتاجات:** أظهر قياس الطيف الضوئي بالأشعة فوق البنفسجية إطلاق مكونات داخل الخلايا بطريقة تعتمد على التركيز والوقت، مما يشير إلى تمزق الغشاء.

الكلمات المفتاحية: اختبار حساسية *Pseudomonas puti* VITEK 2، زيت القرفة، الحد الأدنى للتركيز المثبط، اختبار الحساسية.