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Study the effect of Ethanolic extracts of *Tirmania nivea* against some antibiotic- resistant bacteria isolated from burns

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Abstract

This study shows that ethanol extracted from the (*Tirmania nivea*) has antibacterial activity against multidrug-resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are common in cases of contaminated burns in the Al-Kindi Teaching Hospital. The effectiveness of the extract was evaluated by the well diffusion method using different concentrations (25, 50and 100 mg /ml. The results indicated a variation in inhibition levels, *Pseudomonas aeruginosa* showed an inhibition zone ranging from 2.667 to 9.0 mm, compared to a control in which no inhibition was shown. Similarly, the results of *Staphylococcus aureus* showed an inhibition zone of at least 8.667 mm and a maximum of 20.667 mm, compared to the control (ethanol), it was noted that *Pseudomonas aeruginosa* showed higher resistance compared to *Staphylococcus aureus* that showed higher inhibition zone. Biochemical analysis was carried out using Gas Chromatography - Mass spectrometry (GC-MS) to detect highly active compounds in the ethanolic extract.

Keywords: *Tirmania nivea, Staphylococcus aureus, Pseudomonas aeruginosa,* antibacterial activity.

دراسة تأثيرالمستخلصات الإيثانولية للفطر *Tirmania nivea* ضد بعض البكتريا المقاومة للمضادات الحيوية المعزولة من الحروق

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الخلاصة

اظهرت الدراسة الحالية، أن المستخلص كحول الاثيلي ل *Tirmania nivea* اظهر نشاطًا مضادًا للجراثيم بشكل كبير ضد بكتيريا الزائفة الزنجارية *Pseudomonas aeuroginosa* وبكتيريا المكورات العنقودية الذهبية *Staphylococcus aureus* متعددة المقاومة للمضادات الحيوية ، والتي توجد عادة في مرضى الحروق الملوثة في مستشفى الكندي التعليمي. تم تقييم فعالية المستخلص باستخدام طريقة الانتشار بالحفر well diffution method بتراكيز 25، 50، و100 ملغم/مل. أشارت النتائج إلى أن المستخلص أظهر مستويات متفاوتة من التثبيط، اذ أظهرت *Pseudomonas aeruginosa* أدنى منطقة تثبيط بلغت 2.667 ملم وحد أقصى 9.0 ملم بالمقارنة مع مجموعة السيطرة(الايثانول) الذي لم يظهر اي منطقة تثبيط. وبالمثل، أظهرت المكورات العنقودية الذهبية منطقة تثبيط دنيا قدرها 8.667 ملم وحد أقصى 20.667 ملم

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وبالمقارنة مع مجموعة السيطرة(الايثانول) الذي لم يظهر اي منطقة تثبيط . والجدير بالذكر أن الزائفة الزنجارية أظهرت مقاومة أعلى مقارنة بالمكورات العنقودية الذهبية،و التي أظهرت أعلى منطقة تثبيط. كشف التحليل الكيميائي باستخدام جهاز كروماتوغرافيا الغاز – قياس الطيف الكتلي (GC–MS) عن وجود مركبات نشطة قوية في المستخلص الإثيلي.

1. Introduction

Desert truffles are a seasonal and important edible fungus that grows in many countries around the world [1]. They are especially famous in areas like the Middle East, the Mediterranean Basin, the African Kalahari, North Africa and the Australian Outback[2]. In the arid and semi-arid regions of the Mediterranean basin and the Middle East, "these fungi are classified under the genera" *Tirmania* "and" *Terfezia* " within the family Pisizaceae and are called "desert truffles [3]. Truffles, belong to the genus and family tuber, and the order Pezizales, are found within six pezizales families, including glazellaceae, dicinaceae, morchellaceae, helvelaceae, tuber, pezizaceae, and peronemaceae[4]. Of the deficient fungal species, desert truffles (*Terfezia*, *Tirmania*, and Piqua), and forest truffles (tuber) received great attention[5]. They usually take an irregular spherical shape, and slightly spongy texture. They also show colors such as pale, sandy, brown, gray, and white, and range in size from(1 to 7) cm and have no characteristic odour [6]. According to Shavit, desert truffles do not contain any toxic compounds [7]. These fungi provide health advantages because of their bioactive compounds, including phenolics, flavonoids, vitamins, β-glucans, and sterols such as ergosterol and brassicasterol [8]. *Tirmania nivea* (Pezizaceae) is a desert truffle discovered in arid and semiarid alkaline soils in numerous Middle Eastern and Mediterranean countries [9]. *Tirmania* species are commercially valuable edible mushrooms with considerable socio-economic importance [10]. A burn, an acute trauma caused by heat, affects both local and systemic aspects of living organisms, including humans, leading to short- and long-term consequences [11]. Among in-patient cases, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*

were the most frequently encountered bacterial microbes in burn wounds [12]. Iraqi truffles (*Terfezia* sp. and *Tirmania* sp.) showed significant inhibitory activity against pathogenic bacteria such as *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *Escherichia coli*, *Proteus* sp., *Enterobacter* sp., and *Aeromonas* sp., as well as fungi like *Fusarium* sp., *Aspergillus niger*, and *Aspergillus terreus*, owing to their phenolic compounds content [13]. *P. aeruginosa* quickly develops antimicrobial resistance, making medical treatment challenging. It is often found in patients and hospital environments, leading to nosocomial infections in burn patients[14]. *S. aureus* is becoming more acknowledged as a significant pathogen because of increased in antibiotic resistance [15]. In the realm of antimicrobial research, natural sources have gained attention for their potential in combating multidrug-resistant bacteria. One study explored the green synthesis of silver nanoparticles using the tree oyster mushroom *Pleurotus ostreatus*, known for its medicinal properties [16]. Their study focused on the inhibitory activity of these nanoparticles against pathogenic bacteria. A critical health concern is the rising bacterial resistance to existing antibiotics, which highlights the urgent need to discover new bactericidal agents. Consequently, there is a growing demand for the development of agents and strategies to address these challenges, now more pressing than ever[17] .This study aims to evaluate the inhibitory effects of ethanolic extracts from *Tirmania nivea* against multidrug-resistant bacteria, potentially offering a solution to combat antibiotic resistance.

2.Materials and Methods

2.1Collection and identification of *T. nivea*

Fresh ascocarps of *T. nivea* were purchased from the local market in Baghdad,Iraq. Identification of truffle according to morphological features (White color with cracked skin)[18] .DNA has been used in the classification and identification of truffle, using polymerase chain reactions (PCR), a PCR-based diagnostic method was developed using species-specific primer pairs designed from the sequences of the internal transcribed spacer regions (ITS1 and ITS2) [19].

2.2DNA extraction

The fungi, identified based on morphological characteristics as *Tirmania nivea*, had their genomic DNA extracted using the Geneaid^{TM} DNA isolation kit (Geneaid, Taiwan) following the manufacturer's protocol.

2.3 PCR amplification

The primer pair [20] for *T. nivea,* ITS1 F `5-CTCAAGCTATGCATCCAACG-3` and ITS2 R `5-GCATTTCACTGCGTTCTTCA-3` with an amplicon size of 359 bp was used for identification. The PCR amplification reaction mixture for the specific diagnosis gene (*T.nivea*) was prepared in a total volume of 25 µl. It contained of 10 µl of GoTaq® Green Master Mix (2X), (Geneaid, Taiwan), 5 µl of DNA, 1 µl of each primer and 8 µl of nuclease free water, added to achieve a final volume of 25 µl.

Table 1: The optimum condition of detection

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	940C for 5 minutes
Denaturation	35cycle	940C for 40 min
Annealing		580C for 40 min
Extension		720C for 40 min
Final Extension	1 cycle	720 C for 10 min.

2.4 Agarose gel glectrophoresis of DNA

All PCR products were separated through 1.5% agarose gel electrophoresis, and visualized under ultraviolet light (302nm) following staining with ethidium bromide, the process took time about 80 minutes

2.5 Preparation of alcoholic extracts of truffle

For ethanol extraction, 5 grams of dried truffle powder were mixed with 50 ml of 70% ethanol, and macerated at room temperature, for 24 hours with shaking. The solvent was evaporated under vacuum, and any remaining solvent was removed by flushing with nitrogen [21].

2.6 Bacterial isolates

The bacterial isolates were isolated from the burns patients at Al-kindi Teaching Hospital. The bacteria were diagnosed and an antibiotic sensitivity test were done by the VITEK2 compact system to determine the most resistant bacteria present in the burns. Two resistant strains of bacteria were obtained: the Gram positive bacteria, *S.aureus* and the Gram negative bacteria *P.aeroginosa.*

2.7 Well diffusion method

A specific weight of *T.nivea* ethanolic extract was dissolved in distilled water to prepare different concentrations (25, 50, and 100) mg/ml, the antibacterial activity of the edible truffle extracts was evaluated using the agar well diffusion method ,one milliliter of bacteria was equally distributed throughout the Muller-Hinton agar surface, and any extra was removed using a micropipette. 100 ml of ethanolic extracts of the truffle *T. nivea* in concentrations (25,50 and 100 gm/ml) were placed in wells that were six millimeters in diameter and punched into the agar. To allow for the diffusion of any antimicrobial metabolites, plates were first maintained at 40C for at least two hours. After that, they were incubated at 370 C for twenty-four hours.. Every experiment was run in triplicate. The antibacterial activity was evaluated based on the zone of inhibition (ZOI) [22

2.8 Statistical analysis

A form of difference analysis (ANOVA) was used .It was used to determine the average and significant differences . And to compare the percentages. The Chi-Square was heavily used in this study, P-values equal to or less than 0.01 were considered to be of high statistical significance.

3.Results

3.1 Identification of *T.nivea*

The fungi diagnosed based on the morphological characteristic[23] was identified as *T.nivea* and the identification was confirmed by PCR analysis, as shown in Figure 1

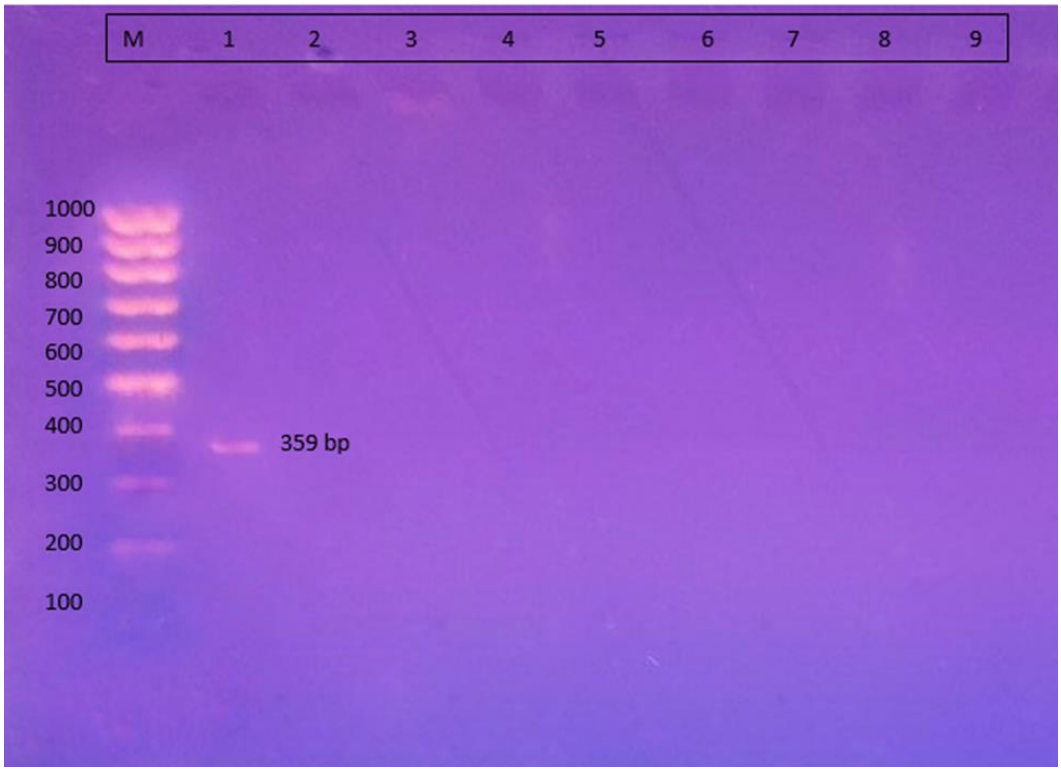

Figure 1: Agarose gel electrophoresis (1.5%) of PCR product showing the amplification of (*T.nivea*) with 359 bp and M corresponds to the DNA Marker (100 bp)

3.2 Gas Chromatography-Mass Spectrometry (GC-MS) technique

To find more powerful and safer anti-bacterial compounds and the well-known therapeutic potential of plant-based compounds, the crude ethanol recovered from the *T.nivea* by

extraction technique was examined by the Gas Chromatography-Mass Spectrometry (GC-MS) technique.GC MS is used to study the fruiting bodies of *T. nivea*. As, per studies these fruiting bodies are rich in compounds known for their inflammatory properties. These compounds include resveratrol, margaric, naringenin, oleic and lauric acids [24]. The characteristic aroma of truffle fruiting bodies comes from sulfurs, esters, alcohols, aldehydes and ketones. Their therapeutic benefits mainly lie in their inflammatory, hepatoprotective, antiviral and antibacterial properties. Phytosterols, phenolics, N arachidonoyl ethanolamine (anandamide) and steroidal glycosides are substances, for these therapeutic effects [25]. This study found that crude extracts include important compounds such as hexanal, dodecane, phenol, palmitate, linolelaidate, oleate, and stearate. 4-hydroxyphenylacetic acid, heptadecane, 1-eicosene, hexa-decanoic acid-methyl ester, 7-pentadecyne, 2,4-bis(1,1-dimethylethyl), Table 2 includes medical and industrial substances such 9,12-octadecadienoic acid (Z,Z)-, heptadecane, 1-octadecene, 5-hydroxymaltol, 11-dodecen-1-ol trifluoroacetate, and others [26].

Table 2: The active compounds of *T.nivea* by the GC-Mass Technology

NO	Constituents	Retention Time	Peak Area %
1	Benzene Benzene Benzene	5.333	1.88
2	Propylamine, N, N,2,2-tetramethyl-, N-oxide Butane, 2,2'-this 4-hydroxyphenylacetic acid	6.711	34.33
3	d-Arabinose, cyclic 1,2-ethanediol mercaptan, tetraacetate .alpha.-D-Galactopyranoside, methyl l 3,6-anhydrous-6- Oxa-3,9-dithiaundecane-1,11-diol	6.836	1.06
4	2,8-Didehydro-noradamantan-9-ol 4-Nonyn-3-ol, 2,2-diethoxy 2-Mercaptothiazole	6.894	1.94
5	Toluene Toluene Benzene, methyl 2,4-bis(1,1-dimethylethyl)	7.322	19.62
6	3-Hexanone 3-Hexanone 3-Hexanone	7.654	0.74
7	N-[Dimethylaminomethyl]aziridine 9,12-Octadecadienoic acid (Z,Z)- 2-Hexanone 2-Hexanone	7.774	0.89
8	Oxirane, butyl 3-Buten-2-ol, 2-methyl 3-Buten-2-ol, 2-methyl	8.059	1.08
9	Benzene, 1,3-dimethyl Benzene, 1,3-dimethyl Benzene, 1,3-dimethyl	10.237	0.66
10	2-Aminocyclopentane-1-carboxylic acid 3,4,5-Trimethyldihydrofuran-2-one Hexanal	10.466	0.87
11	Benzene, 1,2-dimethyl Benzene, 1,3-dimethyl o-Xylene	10.574	0.70

12	p-Xylene o-Xylene Benzene, 1,2-dimethyl	11.477	0.61
13	2-Pinene 2-Pinene (1R)-2,6,6-Trimethylbicyclo kept-2-ene	13.180	2.61
14	3-Hexanol 3-Hexanol (CAS) 3-Hexanol	13.666	9.23
15	2-Butene, 2,3-dimethyl 2-Pentene, 3-methyl-, (E) 1-Butene, 2,3-dimethyl	14.083	4.73
16	2-Oxabicyclo[2.2.2]octane, 1,3,3-t dimethyl Eucalyptol 2-Oxabicyclo[2.2.2]octane, dimethyl	17.775	0.80
17	11-Hexadecenoic acid, 15-methyl, methyl ester 11-Hexadecenoic acid, 15-methyl-, methyl ester 4-DEUTERIO-4-HYDROXY-CYCLOPENTENE	19.399	0.55
18	Bicyclo[2.2.1]heptan-2-one, 1,7,7trimethyl, Bicyclo[2.2.1]heptan-2-one, 1,7,7trimethyl	23.496	1.50
19	Undecenal 3-Hexene, 2,2,5,5-tetramethyl 2-Dodecenal	33.378	3.78
20	2-Dodecenal 2-Dodecenal 2-Methylene cyclopentanol	33.395	2.81
21	2-Propenoic acid, 3-phenyl-, methyl ester 2-Propenoic acid, 3-phenyl-, methyl ester 2-Propenoic acid, 3-phenyl-, methyl ester	34.498	0.34
22	"2,6-bis (1,1-dimethyl ethyl)-4-met ethyl-phenol "2,6-bis (1,1-dimethyl ethyl)-4-met ethyl-phenol "2,6-bis (1,1-dimethyl ethyl)-4-met ethyl-phenol	39.391	0.81
23	3-Heptadecene 8-Heptadecene 8-Heptadecene methyl stearate	46.089	2.85
24	8-Heptadecene 3-Heptadecene, (cis)-2-nonadecene	46.301	1.25
25	ICOSANE Heptadecane Eicosane	46.929	3.24
26	Eicosane Eicosane Eicosane	50.547	1.12

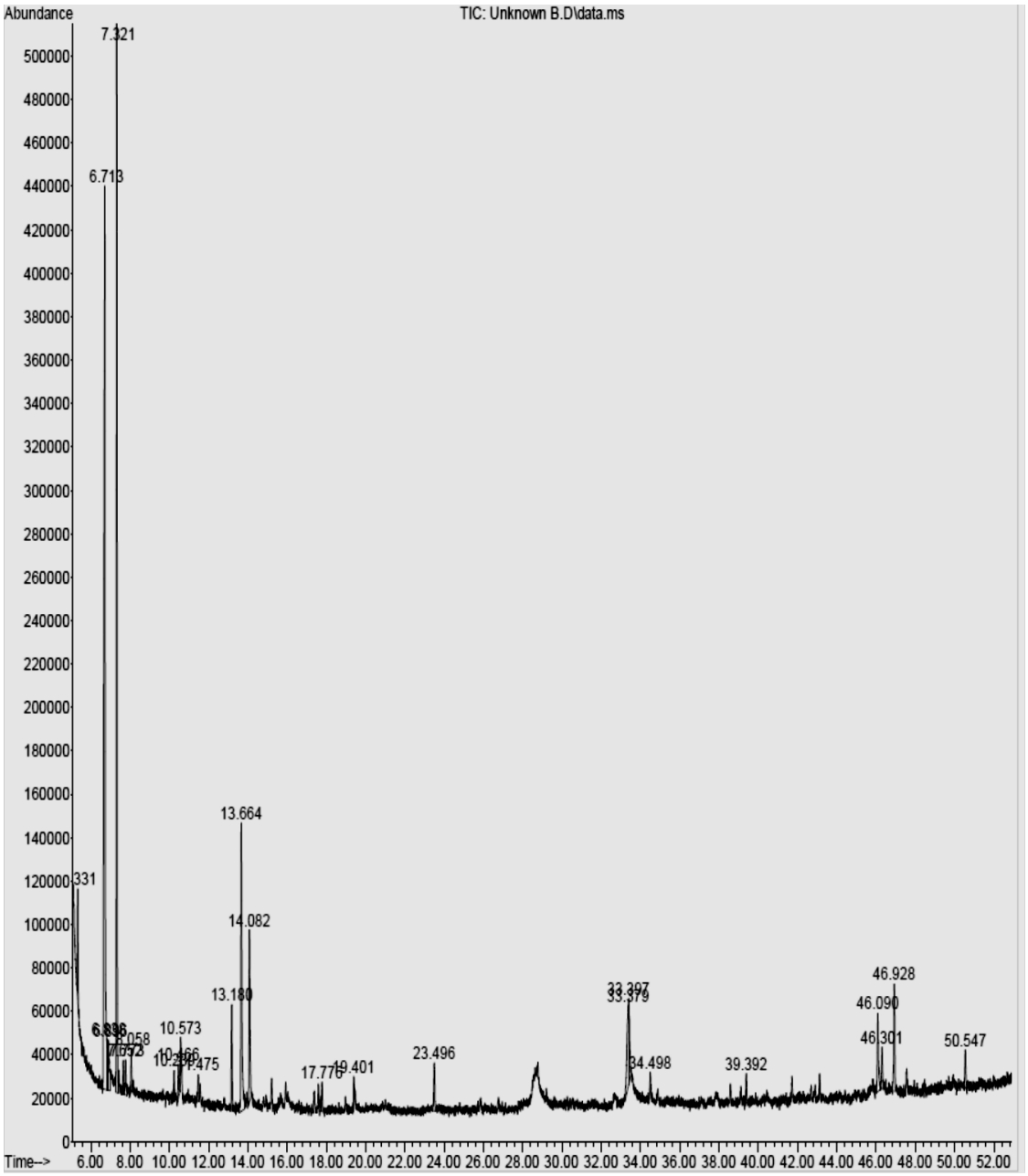

Figure 2: The detection of the active compounds by the GC-Mass technology

3.3 Bacterial isolates

A study was carried out on 66 samples that were gathered between November 2023 and March 2024, with 58 positive samples and 8 negative samples of varying ages in both sexes. The Bacteria were isolated from the burn patients at Al-kindi Teaching Hospital. The bacteria were identified, and the most resistant strains found in the burns were identified using an antibiotic sensitivity test conducted using the VITEK2 compact system. *P.aeroginosa* is resistant to the antibiotics : Cefoxitin, Linezolid, Benzylpenicillin, Teicoplanin, Oxacillin, Vancomycin, Gentamicin, Tetracycline, Ciprofloxacin, Tigecycline, Moxifloxacin, Fusidic Acid, Inducible Clindamycin, Erythromycin, Trimethoprim, Sulfamethoxazole, Clindamycin. *Staphylococcus aureus* is resistant to the following antibiotics: Cefoxitin, Oxacilin,

Ciprofloxacin, Gentamicin, Moxifloxacin, Inducible Clindamycin, Erythromycin, Teicoplanin, Vancomycin, Fusidic Acid, Trimethoprim, Sulfamethoxazole, Gentamicin while it was sensitive for Tetracycline, Tigecycline.

3.4 Antibacterial activity

The antibacterial activity of the extract against the two pathogenic bacteria is shown in Table (3). The extract from the desert truffle showed significant activity against *P. aeruginosa* and *S. aureus*[27]. The inhibition zones varied from 2.667 to 9.0 mm in compare with control 0.0 mm for *P. aeruginosa* and 8.667 to 20.667 mm in compare with control 0.0 mm for *S. aureus*. The minimum inhibitory concentration extract was 25 mg/ml, and the maximum inhibitory concentration of the extract was 100 mg/ml for the two bacteria species. The agar well diffusion method was utilized to assess the antimicrobial activity, and the extracts demonstrated significant effectiveness against both bacterial strains. The maximum inhibition zone was observed against *S.aureus*.

Table 3: Effect of concentrations of *Tirmania nivea* ethanol extract on *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Bacterial species	Inhibition zone(mm)				
	Control (D.W)	25 mg/ml	50 mg/ml	100 mg/ml	P value LSD value
Pseudomonas aeruginosa	0.00	2.667	4.667	9.00	0.0024 4.416
Staphylococcus aureus	0.00	8.667	17.00	20.667	

**** (P≤0.01)**

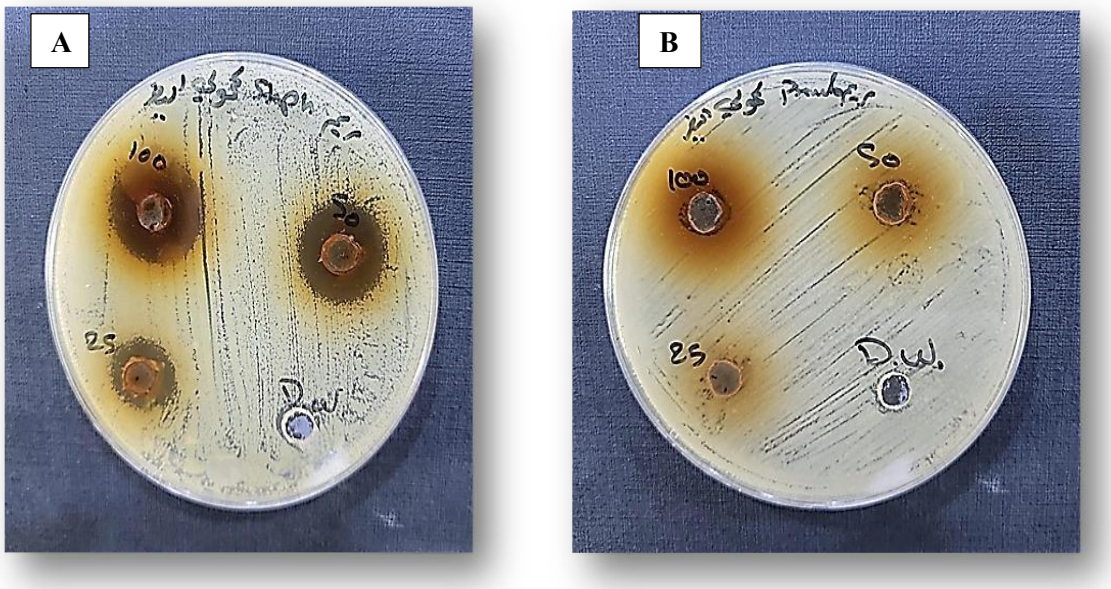

Figure 3: Inhibition zone of *Tirmania nivea* ethanol extract against multidrug-resistant bacteria.**(**A) *Staphylococcus aureus* (B) *Pseudomonas aeruginosa*

4.Discussion

The results of the study show that 48.14% of the cases in which burn infections are spread by *P.aeruginosa* type, this high prevalence is due to the ability of bacteria to thrive and grow in hospital environments where the hospital environment enables them to survive for long periods, and it increases the risk of multiple drug-resistant nosocomial infections in patients

receiving treatment, the second common pathogen is *Staphylococcus aureus*, which is also one of the causes of repeated infections in hospitals and accounts for 28.77% of burn infections [28] experimental studies have shown that ethanol extracts of *T.nivea* possess a high ability to inhibit the growth of *S.aureus* bacteria, this indicates its potential as a natural antibacterial agent, All strains of *P. aeruginosa* and other Gram-negative bacteria include lipopolysaccharide(LPS), a lipid that is a major part of their outer membrane. LPS is one of the most important bacterial molecules because it can trigger an immune response and is readily available. It consists of a moiety, inner and outer core oligosaccharides, and the O-antigen. [29] *P.aeruginosa* is able to withstand oxidative stressors, dehydration, and the immune system thanks to exopolysaccharides, which are large sugar-based molecules found outside of cells [30]. Ethanolic extracts of *T. nivea* contain active components that have antibacterial qualities. These include breaking down and lysing cell walls, preventing the formation of biofilms, preventing the synthesis of cell walls, preventing the replication of microbial DNA, producing energy, and neutralizing bacterial toxins[31]. In addition, these compounds may prevent antibacterial resistance as well as synergetics to antibiotics, which can ultimately kill pathogenic organisms. The urgent need for innovative antibacterial strategies for the treatment of antibiotic resistance in infectious diseases is underlined by the formation of membranes that increase the resistance of bacteria to antibiotics .The treatment of biofilm-related infections is a difficult and significant biomechanical challenge in healthcare [32].As mentioned above, the extract contains multiple active compounds that can disrupt the formation of biofilms and thereby prevent microbial adhesion to cellular and inert surfaces, then inhibit microbial ability to adhere to inert surfaces [33]. The greatest inhibition zone targeted *S. aureus*, this might be due to the fact that Gram-positive bacteria's membranes, which are composed of a thick, uniform coating of peptidoglycane containing low-density lipid, are more permeable than those of Gram-negative bacteria. Gram-negative bacteria's membrane, on the other hand, is composed of an outer membrane rich in protein, lipopolysaccharides, and phospholipids, covering a slime layer of peptidoglycane. Therefore, in Gram-positive bacteria, the majority of antimicrobial agents have a high degree of effectiveness in penetrating and interacting with the cell membrane[34].According to the GC-MS analysis, fungal biomass and extracellular metabolites in the growth medium are industrial renewable resources of different biological compounds that may be used as antibacterial agents, the results showed that several important compounds, such as phenol, 2,4-bis(1,1-dimethylethyl), methyl palmitate, methyl linolelaidate, methyl oleate, methyl stearate, 1-eicosene, hexa-decanoic acid-methyl ester, 7-pentadecyne, and hexanal, dodecane, 4-hydroxyphenylacetic acid, heptadecane, 1-eicosene, hexa-decanoic acid-methyl ester, 7-pentadecyne, phenol, and heptadecane, 9,12-Octadecadienoic acid (Z,Z) .This result is in line with that of the other commercial and medicinally valuable chemicals, specifically 1-octadecene, 5-hydroxymaltol, and 11-dodecen-1-ol trifluoroace(35).

5.Conclusion

Tirmania nivea ethanolic extract is a good source of an effective therapeutic substance, *P. aeruginosa* which is resistant to antibiotics was used in the current study, the ethanolic extracts of *T. nivea* tested against *P. aeruginosa* gave an inhibition zone and in comparison with *Staphylococcus aureus* has resistance to all antibiotics except Tetracycline and Tigecycline and recorded a higher inhibition zone than *P.aeruginosa* when extract tested against it . When comparing the extracts with the antibiotics, this study found that the extract was more efficient in eliminating bacteria. This study is recommends the use of active substances extracted from the *T.nivea* because they are more efficient and cheaper. *T. nivea* has promising antibacterial properties against multidrug-resistant bacteria, particularly *P. aeruginosa* and *S.aureus*.

6.Conflict of Interest

The authors declare that they have no conflict of interest

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