Evaluation of antibacterial activity of aqueous and methanolic extracts of the truffle Terfezia claveryi against Pseudomonas aeruginosa

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ABSTRACT

Objective: To investigate the antibacterial activities of aqueous and methanolic extracts, as well as, partially purified proteins extracted from *Terfezia claveryi* aqueous, against *Pseudomonas aeruginosa (P. aeruginosa)*.

Methods: Five percent of the aqueous and methanolic extracts were added to growth medium of *P. aeruginosa*. The extract that caused growth inhibition (aqueous) was then partially purified using ammonium sulfate precipitation, gel chromatography and ion exchange chromatography. Antibacterial activities of the obtained fractions were assessed using agar-well diffusion test, and then all the results were compared with reference antibiotics. Excremental procedures were performed at the Department of Nutrition and Food Technology and the animal house of Jordan University of Science and Technology, Jordan during the year 2000.

Results: Five percent aqueous extract inhibited the growth of *P. aeruginosa* by 40.9%, while methanolic extract was ineffective. Partial purification of the aqueous extract using ammonium sulfate precipitation revealed that antimicrobial activity was within the second pellet (25-45%). This fraction was then subjected to gel permeation chromatography using Sephadex G-25. Peak one, of the 2 peaks obtained, possessed higher antimicrobial activity. Peak one was then subjected to ion exchange chromatography using DEAE Sephadex. Only peak one, of the 3 peaks obtained, showed a slight antimicrobial activity. Relative antimicrobial activities of these fractions were found to be superior to most of reference antibiotics used for comparison.

Conclusion: Aqueous extract of the truffle *Terfezia claveryi* contains a potent antimicrobial agent that is protein in nature and may be used in the treatment of eye infections caused by *P. aeruginosa*.

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Truffles grow naturally in special localities in the deserts of the Middle East. They grow during late winter and early spring in the years where rainfall is sufficient and the area encounters thunder storms.[1] Truffles are considered to be one of the oldest foodstuffs known for their nutritional value specially when compared with meat and fish.[2,3] Truffle extract is used as nourishing and invigorating preparation for convalescents in Mediterranean countries.[4] Furthermore, truffles aqueous extract is used for the treatment of eye ailments in Iraq, Saudi Arabia, Eastern Badia of Jordan and in the Negev Desert.[5] This practice developed the following recommendations of the Prophet Mohammad (peace be upon him) whom was reported to have said: "Truffles are from man (as they grow naturally without mans care) and their water is a cure for the eye".[6] Among Gram-negative bacteria that infect the eye, *Pseudomonas aeruginosa (P. aeruginosa)* is the most encountered species. Many eye infections such as bacterial conjunctivitis and blepharitis are caused by *P.*

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aeruginosa.[7] Numerous antibacterial preparations are used to treat eye infections such as chloramphenicol, fusidic acid, fluoroquinolones, neomycin, oxacillin, ticarcillin, tobramycin, and aminoglycosides.[8] Most of these antibiotics have serious side effects.[9-15] With the increasing resistance of many microorganisms and the serious side effects of the currently used antibiotics, this study comes as an attempt to find a new antibacterial preparation from truffles that may be used for the treatment of eye infections.

Methods.

Fungal material. Brown Iraqi truffles were purchased from the local market during spring of 1999. Specimens were authenticated as *Terfezia claveryi* by Professor Khalid Hameed from the Department of Plant Production, Jordan University of Science and Technology.

Preparation of aqueous and methanolic extracts. Fifty grams of fresh truffle ascocarps were cut into small pieces and soaked in 1:3 distilled water (w/v) for 24 hours. Then, the mixture was homogenized in a house hold blender for one minute at full speed. The homogenate was then filtered through double layer of cheese cloth and centrifuged at 3000xg for 10 minutes at 4°C. The supernatant was then filtered through sterile filter (0.45 µm) and kept at -20°C for later use. The supernatant is referred to as aqueous extract.[16] The same protocol was used for methanolic extraction but the sample was soaked in 95% methanol. After centrifugation, methanol was evaporated using a steam of air at room temperature. The sample was then re-suspended to its original volume with distilled water. This was filtered through sterile filter (0.45 µm) and kept at -20°C for later use. The supernatant is referred to as methanolic extract.[16]

Bacteria and growth conditions. *Pseudomonas aeruginosa* ATCC (PA 0303) was grown in 100 ml of brain-heart infusion broth, which was considered as a control. Experimental cocktails consisted of 95ml of the growth medium inoculated with *P. aeruginosa*, then 5ml of aqueous or methanolic extract was added. All media were incubated at 37°C for 24 hours. Growth was monitored by measuring the absorbance at 420 nm.[17]

Ammonium sulfate protein precipitation. The aqueous crude extract was fractionated by ammonium sulfate precipitation.[18] Ammonium sulfate powder was added slowly to the extract until the concentration reached up to 25%. The mixture was centrifuged at 3000xg for 20 minutes at 4°C. The pellet was reconstituted to its original volume using 0.05 M phosphate buffer, pH 7.2. The sample was then dialyzed using the same buffer for 24 hours at 4°C. Part of the dialyzed fraction was kept for antimicrobial activity test and the rest was further fractionated with 45-75% and 75-100% ammonium sulfate following the same procedure.

Gel chromatography. The 45-75% ammonium sulfate fraction was further purified using 2.2x 60cm Sephadex G-25 column.[19] Proteins were eluted with 0.05 M phosphate buffer, pH 7.2. Three ml fractions were collected and the absorbance at 280 nm was measured to detect protein peaks, using Jenway 6105- spectrophotometer.

Ion exchange chromatography. The peak that showed antimicrobial activity from the gel filtration step was further dialyzed against 0.05 M phosphate buffer, pH 8.0 overnight, then it was applied on DEAE anion exchanger (2.5 x 25cm) column. The unbound proteins were eluted with 0.05 M phosphate buffer, pH 7.0 until the optical density at 280 nm was zero. The bound proteins were eluted with increasing concentration of NaCl (0-1M) in phosphate buffer, pH 7.2. Four ml fractions were collected and the absorbance at 280 nm was measured using Jenway 6105- spectrophotometer.[16]

Agar well-diffusion test. Relative antimicrobial activity was determined using agar-well diffusion method.[20] *Pseudomonas aeruginosa* was spread onto the surface of brain-heart infusion agar with sterile swab (0.1 ml containg105 cell/ml). Six mm diameter wells were punched into the agar and filled with 0.1ml of the aqueous extract or protein fractions that showed antimicrobial activity. The antimicrobial activity was compared with reference antibiotics such as ciprodar, erythromycin, gentamycin, tetracyclin and chloramphenicol. The plates were then incubated overnight at 37°C. All experiments were carried out in triplicates. After 24 hours of incubation inhibition zones were measured. Control wells were filled with 0.05 M phosphate buffer, pH 7.2.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Minimal inhibitory concentration and MBC were determined using broth dilution method.[19] Serial dilution of peak one obtained from Sephadex G-25 ranged from 0-3 mg protein/ml nutrient broth. The protein concentration was determined using Lowry method.[21] Each vial was inoculated with 0.1 ml of *P. aeruginosa* containing 105 cells/ml. The control group consisted of extract-free broth inoculated with 0.1 ml of *P. aeruginosa*. The tubes were then incubated at 37°C for 24 hours.

Results.

Effect of aqueous and methanolic extracts on growth of P. aeruginosa. **Figure 1** shows the effect of 5% aqueous truffle extract on the growth of *P. aeruginosa*. This concentration inhibited the growth by 40.9% when compared with the control. However, methanolic extract did not inhibit the growth.

Inhibition zones determination. **Table 1** shows that protein fraction precipitated using 25-45% ammonium sulfate caused a 10mm inhibition zone,

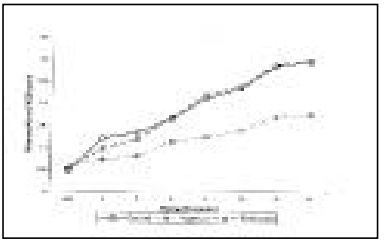

Figure 1 - Growth of *Pseudomonas aeruginosa* in the presence of 5% aqueous and methanolic *T. claveryi* extracts.

Table 1 - Antimicrobial activity of protein fractions purified from Truffle aqueous extract on *Pseudomonas aeruginosa.*

Purification methods	Inhibition zone (mm)
Ammonium sulfate	
0-25%	0
25-45%	10
45-75%	3
75-100%	0
Sephadex G-25	
Peak 1	8
Peak 2	3
DEAE Sephadex	
Peak 1	2
Peak 2	0
Peak 3	0

Numbers presented in this table represent the average of the 3 trials

Table 2 - Determination of relative antimicrobial activity* of different protein fractions purified from *T. claveryi* aqueous extract that showed antimicrobial activity against *Pseudomonas aeruginosa*.

Antibiotics	Aqueous crude extract (%)	$(NH_4)_2SO_4$ Pellet (25-45%) (%)	G-25 (Peak 1) (%)	DEAE (Peak 1) (%)
Cip 5	136	100	72	9
E15	1500	1100	800	100
GN	214	157	73	14
Tet	500	366	266	33
CPC	115	91	16	13

Relative antimicrobial activity = ([mean diameter of inhibition zone of active compound] 2/[mean diameter of inhibition zone of reference antibiotic] 2) x 100%. Cip - 5 mg ciprodar, GN - gentamycin, E15 - 15 mg erythromycin, Tet - 30 mg tetracycline, CPC - 5 mg/ml chloramphenicol, DEAE - DEAE Sephadex, G-25 - Sephadex G-25

which indicates that the nature of the antibacterial substance is protein like. Therefore, this fraction was subjected to gel filtration using Sephadex G-25. Two peaks were obtained from the gel filtration (data are not shown), peak one caused 8 mm inhibition zone, while peak 2 caused 3 mm inhibition zone (**Table 1**). When peak one was further subjected to ion-exchange chromatography using DEAE Sephadex, 3 peaks were obtained after elution with 0-1 M NaCl in phosphate buffer. Only peak one showed a slight antimicrobial activity against *P. aeruginosa* (2 mm, inhibitory zone), while other peaks did not show any antimicrobial activity (**Table 1**).

Relative antimicrobial activities. Fractions that showed antimicrobial activity against *P. aeruginosa*, as well as, the aqueous extract, were compared with reference antibiotics (**Table 2**). The antimicrobial activity of the aqueous extract, compared with the standards were; 136% of ciprodar, 1500% of erythromycin, 214% of gentamycin, 500% of tetracyclin and 115% of chloramphenicol (**Table 2**). The relative antimicrobial activity of (25-45%) ammonium sulfate fraction was 100% of ciprodar, 1100% of erythromycin, 157% of gentamycin, 366% of tetracyclin and 91% of chloramphenicol (**Table 2**). Peak one activity resulted from Sephadex G-25 was 72% of ciprodar, 800% of erythromycin, 73% of gentamycin, 266% of tetracyclin and 16% of chloramphenicol (**Table 2**). The relative antimicrobial activities of all reference antibiotics were higher than the antimicrobial activity of peak one obtained from DEAE Sephadex, except for erythromycin which was 100% of the antimicrobial activity of that peak (**Table 2**).

Determination of MIC and MBC. Minimal inhibitory concentration for peak one obtained from Sephadex G-25, which possessed the highest antimicrobial activity against *P. aeruginosa,* was found to be 1.6 mg/ml and MBC was 1.7 mg/ml.

Discussion. The antibacterial activity of aqueous extract of *T. claveryi* and the inactivity of methanolic extract was reported earlier against *Staphylococcus aureus*.[16] Moreover, this comes in agreement with the findings of an Algerian research group, who found out that methanolic extract of *Algerian Terfezia* (species was not reported) did not possess anti-microbial activity against *P. aeruginosa*.[22] Partial purification of the aqueous extract revealed that the active component has protein-like nature, because it is pricepitated using ammonium sulfate salt, which agrees with the findings of Chelal and Lukasova (1995),[22] who separated the crude extract via thin layer chromatography (TLC) and determined the protein content using Lowry method.

In conclusion, it is obvious that we are dealing with a promising peptide antibiotic, which needs to be characterized and sequenced. This can lead to the development of a new spectrum of antibiotics with minimal side effects.

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