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Tests of Antibiotic Properties of Algerian Desert Truffle against Bacteria and Fungi

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Abstract: In the present paper, the ethyl acetate extract from the fruiting bodies of *Tirmania pinoyi* (Maire) was obtained by Soxhlet extraction. Six fractions were separated from this extract using two chromatographic methods. All these fractions were submitted to antimicrobial activity against four clinically important bacteria *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* ATCC6538, *Pseudomonas aeruginosa* ATCC14028, *Escherichia coli* ATCC25922 and one pathogenic fungus *Candida albicans* ATCC10231. The *in vitro* antimicrobial activity was performed by agar disc diffusion method. The fractions with the greatest antimicrobial activity were fractions 02 and 06 which inhibited growth of both Gram negative and Gram positive bacteria and had significant antifungal activity against *Candida albicans*. The present study validates the folk use of the boiled truffle water-extract and indicates that it could be effective potential candidates for the development of new strategies to treat bacterial or fungal infections.

Key words: Agar diffusion assay, *Tirmania pinoyi*, antimicrobial activity, chromatographic methods, Soxhlet extraction.

1. Introduction

Tirmania and *Terfezia*, so-called desert truffles, are hypogeous ascomycota fungi (they form their fruitbodies below ground). Ecologically, they are mycorrhizal, forming mutually beneficial associations with the roots of host plants, especially with *Helianthemum* species [1]. Taxonomically, *Tirmania* and *Terfezia* were shown to belong to the Pezizaceae rather than to the distinctly hypogeous Terfeziaceae family, which was therefore abolished [2]. These truffles are edible and their geographical distribution is limited to arid and semi-arid lands, mostly in countries around the Mediterranean basin: (Turkey, Italy, Spain, Portugal, France, and Hungary), North Africa (Tunisia, Algeria, and Egypt) and the Middle East (Saudi Arabia, Kuwait, Iraq, Iran, Lebanon, Syria, and Jordon). In addition, some desert truffle species have been found in the Kalahari Desert [3-5], in North America [6], Australia [7, 8] and China [9].

Several studies on their chemical composition have shown that the dry matter (about 20% by weight) consists of: 20-27% protein, some 85% of which is digestible by humans; 3-7.5% fat, including unsaturated as well as saturated fatty acids; 7-13% crude fiber; close to 60% carbohydrates; and appreciable amounts (2-5%) of ascorbic acid [10-16].

Desert truffles have been used as traditional medicine in Arabia for over two millennia without known toxic harmful effects to its users [17]. As a fungal drug remedy, a boiled truffle water-extract is highly recommended by Bedouins for the treatment of one of the most common eye diseases at that time, e.g., trachoma [18]. 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 man's care) and their water is a cure for the eye".

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Rougieux (1963) was the first researcher to investigate the biological activity of desert truffle extracts on bacterial growth *in vitro*. He reported that the extract of *Terfezia boudieri* Chatin had a slightly inhibitory for *Staphylococcus aureus* [19].

Al-Marzooky (1981) [20] has tested the biological activity of desert truffle extracts on bacterial growth *in vitro*. He reported that all aqueous, polar and non-polar extracts of *Terfezia claveryi* exhibited good antibacterial activity against broad spectrum of the tested bacterial species, in particular, the causal organism of trachoma *Chlamydia trachomatis*. Consequently, preliminary results of a pilot clinical study conducted by Al-Marzooky (1981) [20] on *Terfezia claveryi* aqueous sterilized extracts for the treatment of trachoma infected patients proved useful but required longer time compared with standard eye antibiotic treatments.

Moreover, Chellal and Lukasova (1995) [21] have reported that antibiotics extracted from the desert truffles *Terfezia* and *Tirmania* spp. proved effective against bacteria. The most important bacteriostatic activity against Gram (+) bacteria including *Bacillus subtilis*, *Streptococcus pyogenes* and *Staphylococcus aureus* was indicated by methanolic extract at 45 °C from *Terfezia*. Finally, they noticed that extracts of *Tirmania* were less bacteriostatic than those of *Terfezia.*

Furthermore, Janakat et al. (2004) [22] have studied the antimicrobial activity of aqueous and methanolic extracts from *Terfezia claveryi* against *Staphylococcus aureus in vitro*. They showed that 5% of aqueous extract inhibited the growth of *S. aureus* by 66.4%, however, the methanolic extract did not cause a significant growth inhibition. In 2005, Janakat et al. [23] have investigated the antimicrobial activity of aqueous and methanolic extracts from *Terfezia claveryi* against *Pseudomonas aeruginosa in vitro*. Five percent of aqueous extract inhibited the growth of *P. aeruginosa* by 40.9%, while methanolic extract was ineffective.

Recently, in another study, Al laith (2010) [24] investigated the antioxidant capacities of *Tirmania nivea* from Bahrein, Iran, Morocco and Saudia Arabia. These truffles showed varying degree of antioxidant antiradical activities based on four analytical methods: FRAP (ferric reducing ability), DPPH (2, 2-Diphenyl-1-Picrylhydrazy), deoxyribose and nitric oxide. He found that the Iranian truffles possessed the highest DPPH values (30.6% ± 13%), the strongest nitric oxide radical scavenging activity (EC_{50} = 102 mg/mL) as well as highest percent of inhibition of deoxyribose breakage (91%). Both Bahraini and Saudi truffles possessed the highest FRAP values (18.62 mmol/100g and 18.06 mmol/100g, respectively) [24].

Janakat and Nassar (2010) [25] evaluated the hepatoprotective activity of the desert truffle (*Terfezia claveryi*) in the rat with different solvent extracts (water, methanolic and petroleum ether), using a potent hepatotoxin carbon tetrachloride (CCl_4) in comparison with the hepatoprotective activity of a reference plant Nigella sativa. The authors found that the aqueous extract of *T. claveryi* demonstrated a very powerfull hepatoprotective activity against CCl_4.

The present study is as an attempt to find an alternative antimicrobial preparation from natural desert truffles (*Tirmania pinoyi*). In our search we established antimicrobial activity of different fractions extracted from fruiting bodies of *T. pinoyi* against bacterial and fungal reference strains.

2. Material and Methods

2.1 Fungal Material

Fresh ascocarps of *T. pinoyi* were purchased from the local market of El Bayed (Algeria) in January 2009 (Fig. 1). The specimens were authenticated by Professor Fortas from the Laboratory of Biology of Microorganisms and Biotechnology, Faculty of Sciences, University of Oran, Algeria. Asci and ascospores of *T. pinoyi* were studied under electronic microscope.

Fig. 1 Fresh ascocarps of *T. pinoyi*.

2.2 Soxhlet Extraction

The fruiting bodies of *T. pinoyi* were cut into pieces, sun dried and powdered in a grinder and then 100 g of the powdered fruiting bodies were extracted with 600 mL of ethyl acetate by Soxhlet extractor. The solvent was heated to reflux. The procedure was run for 1 h to facilitate at least three cycles of Soxhlet extraction. The crude extract was concentrated in vacuum using a rotary evaporator and then weighed to calculate the yield of the extracts and stored at 4 °C until required. Before extraction procedure, all glassware was thoroughly washed and dried at 150 °C for 3 h to avoid any organic contamination [26, 27]

2.3 Separation of the Compounds

We subjected the crude ethyl acetate extract to CC (column chromatography) and TLC (thin layer chromatography) to separate different compounds. 3 g of the crude extract was chromatographed over silica gel $60GF_{254}$ using petroleum ether and increasing concentration of ethyl acetate in ether petroleum as eluents, and then each fraction collected was analyzed by the thin layer chromatography using a 1:6 petroleum ether: ethyl acetate mixture as the mobile phase. Six bands were identified by *p*-anisaldehyde [27].

2.4 Assay Microorganisms

In vitro antimicrobial activity of six fractions extracted from fruiting bodies of *T. pinoyi* was determined against the following panel microorganisms: *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* ATCC6538, *Pseudomonas aeruginosa* ATCC14028, *Escherichia coli* ATCC25922 and *Candida albicans* ATCC10231. All microorganisms were obtained from IPA (Pasteur Institute of Algeria). Microorganisms were maintained at 4 °C on nutrient agar slants (for bacteria), Sabouraud slants (for yeast).

2.5 Antimicrobial Assay

The antimicrobial assay was performed by agar disc diffusion method [28].

2.5.1 Antibacterial Assay

Each bacterial strain tested was streaked onto nutrient agar medium to obtain isolated colonies. After incubation at 37 °C overnight, we selected four or five well-isolated colonies and transferred the growth to a tube of sterile nutrient broth (10 mL) and then incubated at 37 °C for 24 h. After that the bacterial suspension was agitated on a vortex mixer immediately then compared to the 0.5 McFarland standard (10^8 CFU/mL) to adjust the turbidity of the inoculum suspension. 15 mL of the molten Mueller Hinton Agar was poured evenly into Petri plate (9 cm diameter). Using a sterile pipette, 1 mL of the 24 h test bacterial broth culture (1×10^6 CFU/mL) was spread over the surface of the dried agar plates using a sterile glass spreader, allowed to absorb in the agar for 10 min and the plates dried, inverted, at 37 °C for approximately 30 min until the bacterial overlay had dried on the surface. Sterile filter discs (6 mm diameter) were impregnated with 20 µL of each fraction of the crude extract of *T. pinoyi*, initially dissolved in ethyl acetate which was previously tested for antimicrobial activity against all test bacteria and fungi and found to have no antimicrobial activity, air-dried and three filter paper discs were taken and placed equidistantly onto the surface of one Petri plate. Negative controls using 20 µL of ethyl acetate prepared for each assay. Inhibition zones around filter discs were measured after incubation periods of 24 h-48 h, and then the mean values were calculated. All experiments were

carried out in triplicate [29].

2.5.2 Antifungal Assay

In vitro antifungal activity was tested against *Candida albicans* which was determined as described previously in Section 2.5.1 except we have used Sabouraud agar instead of nutrient agar and Mueller Hinton Agar. After the agar solidified, 20 µL of different fractions of the crude extract of *T. pinoyi* dissolved in ethyl acetate were added to filter paper disks (6 mm diameter.) which were placed on the agar surface after solvent evaporation. Negative controls using 20 µL of ethyl acetate were prepared for each assay. After 48 h of incubation at 25 °C, the inhibition zones from the centre of the disc to the inner margin of the surrounding fungal growth was measured in millimetres and recorded. All tests were done in triplicate [29].

3. Results and Discussion

In this study, the identity of *T. pinoyi* was confirmed using electronic microscope. As shown in Fig. 2, this desert truffle has spherical spores, the asci contain 4-8 spores at maturity, and have ellipsoid form.

The yield of ethyl acetate extract of the powdered fruiting bodies of *T. pinoyi* was 4%. In total, six fractions were separated from the crude ethyl acetate extract of *T. pinoyi* by column chromatography and thin layer chromatography.

These fractions were screened for antimicrobial activity against two Gram-positive bacterial species (*S. aureus* and *E. feacalis*), two Gram-negative bacterial species (*E. coli* and *P. aeruginosa*) and one yeast species

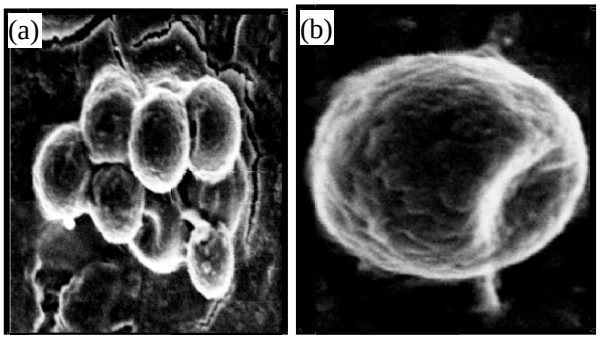

Fig. 2 (a): Ascus with ascospores of *T. pinoyi* (GR 1500); (b): Spore of *T. pinoyi* (GR 5000).

(*C. albicans*). The control treatment (ethyl acetate) had no inhibitory effect on any of the test microorganisms. The results of this screen were summarised in Table 1.

The results of the agar diffusion assay showed remarkable antimicrobial activity for the fractions 2 and 6; however the rest of fractions yielded small or no inhibition zones. We found that these fractions (i.e. 2 and 6) inhibited the growth of both bacteria and fungi.

The fraction 2 showed significant inhibitory activity against Gram positive bacteria (Fig. 3) especially *Enterococcus faecalis* ATCC6538 and Gram negative bacteria (Fig. 4) especially *Escherichia coli* ATCC25922.

The fraction 6 showed also a good inhibitory activity against Gram positive bacteria (Fig. 5) and Gram negative bacteria (Fig. 6).

Among tested fungi species, the growth of *Candida albicans* was markedly inhibited (diameter of inhibition zone was 20 mm) by these fractions (i.e. 2 and 6) (Figs. 7 and 8).

Table 1 Strains used and mean inhibition zones in disc diffusion assays with the six fractions separated f om the crude extract of *T. pinoyi*.

Species	Inhibition zone (mm)					
	F1	F2	F3	F4	F5	F6
Gram positive bacteria						
Staphylococcus aureus ATCC6538	6	15	0	0	7	13
Enterococcus faecalis ATCC6538	0	18	6	0	0	6
Gram negative bacteria						
Escherichia coli ATCC25922	0	16	0	0	0	8
Pseudomonas aeruginosa ATCC14028	0	13	0	7	0	14
Yeast-like fungi						
Candida albicans ATCC10231	7	20	0	0	0	16

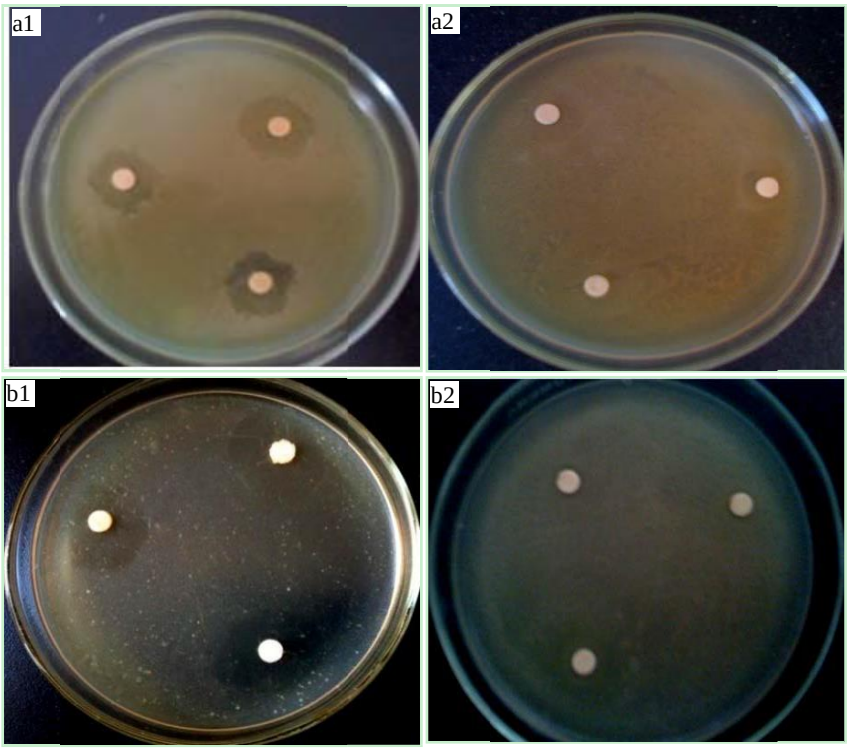

Fig. 3 Inhibition growth of Gram positive bacteria in the presence of the fraction 2 and their negative controls.
a1: *Enterococcus faecalis* ATCC6538; a2: Negative control; b1: *Staphylococcus aureus* ATCC6538; b2: Negative control.

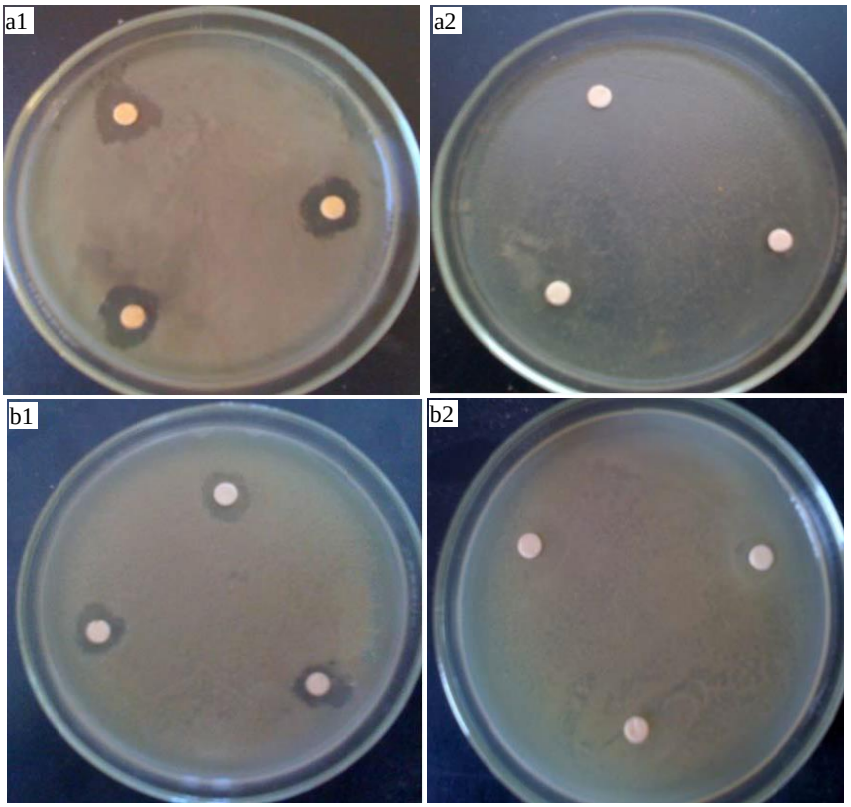

Fig. 4 Inhibition growth of Gram negative bacteria in the presence of the fraction 2 and their negative controls.
a1: *Escherichia coli* ATCC25922; a2: Negative control; b1: *Pseudomonas aeruginosa* ATCC14028; b2: Negative control.

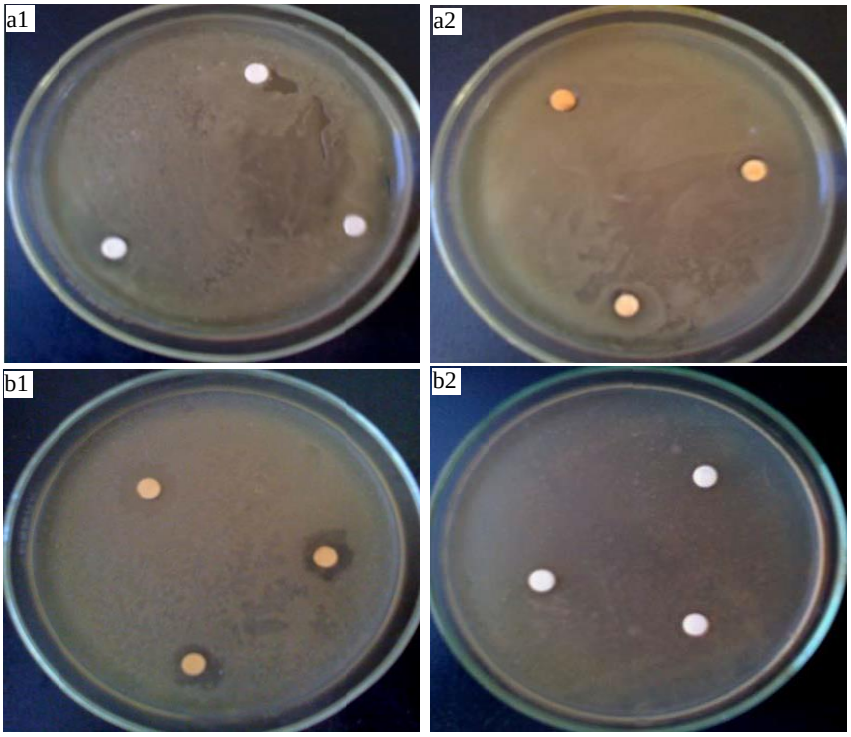

Fig. 5 Inhibition growth of Gram positive bacteria in the presence of the fraction 6 and their negative controls.
a1: *Enterococcus faecalis* ATCC6538; a2: Negative control; b1*: Staphylococcus aureus* ATCC6538; b2: Negative control.

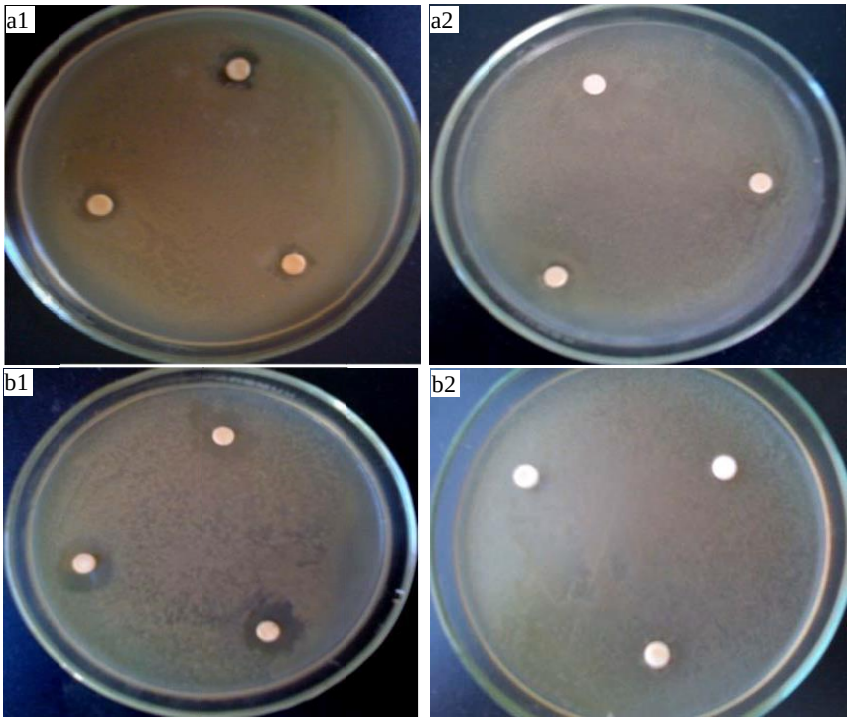

Fig. 6 Inhibition growth of Gram negative bacteria in the presence of the fraction 6 and their negative controls.
a1: *Escherichia coli* ATCC25922; a2: Negative control; b1: *Pseudomonas aeruginosa* ATCC14028; b2: Negative control.

The results of the antibacterial activity against Gram positive bacteria come in agreement with the findings of Chellal and Lukasova (1995) [21] who found out that ethyl acetate extract of *Tirmania* inhibited the growth of Gram positive bacteria. Moreover, the antibacterial activity of aqueous extract

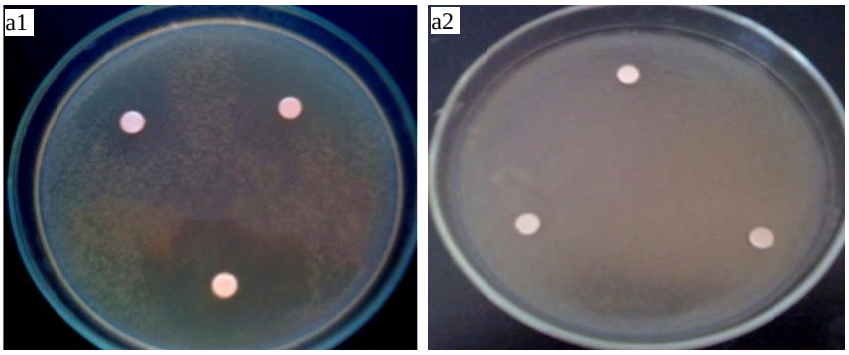

Fig. 7 Inhibition growth of *Candida albicans* ATCC10231 in the presence of the fraction 2 and negative control.
a1: *Candida albicans* ATCC10231; a2: Negative control.

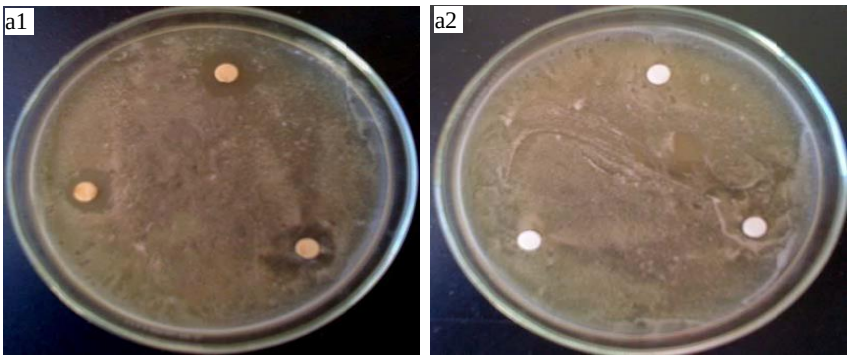

Fig. 8 Inhibition growth of *Candida albicans* ATCC10231 in the presence of the fraction 6 and negative control.
a1: *Candida albicans* ATCC10231; a2: Negative control.

of *Terfezia claveryi* against *Staphylococcus aureus* (diameter of inhibition zone was 8 mm) and *Pseudomonas aeruginosa* (diameter of inhibition zone was:10 mm) was reported by Jankat *et al*. (2004 and 2005) [23, 24]. It is possible to conclude that the fractions 2 and 6 separated from the ethyl acetate extract of *T. pinoyi* had a broad spectrum of activity against many bacterial species, as well as these fractions were more active against bacterial species as compared to fungal species.

4. Conclusion

In the present work, the antimicrobial activity of different fractions separated from the crude ethyl acetate extract of *T. pinoyi* tested against various microorganisms allow us to conclude that the fractions 2 and 6 exhibited antibacterial and antifungal activities that support traditional use of the extract of *T. pinoyi* in the treatment of some diseases. We confirm that the fractions 2 and 6 possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. In conclusion, it is obvious that we are dealing with a promising antibiotic, which needs to be characterized.

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References

[1] Z. Fortas, G. Chevalier, Effect of culture conditions on the mycorrhization of *Helianthemum guttatum* by three species of the genus of Terfez: *Terfezia* and *Tirmania* of Algeria, Can. J. Bot. 70 (1992) 2453-2460. (in French with English abstract)

[2] T. Laessoe, K. Hansen, Truffle trouble: What happened to

the Tuberales?, Mycological Research 111 (2007) 1075-1099.

[3] Y. Ferdman, S. Aviram, N. Roth-Bejerano, J.M. Trappe, V. Kagan-Zur, Phylogenetic studies of *Terfezia pfeilii* and *Choiromyces echinulatus* (Pezizales) support new genera for southern African truffles: Kalaharituber and Eremiomyces, Mycological Research 109 (2005) 237-245.

[4] W.F.O. Marasas, J.M. Trappe, Notes on southern African Tuberales, Bothalia 11 (1973) 139-141.

[5] J.M. Trappe, Use of truffles and false truffles around the world, in: M. Bencivenga, B. Granetti (Eds.), Proceedings, Atti del Secondo Congresso Internazionale sul Tartufo. Comunita Montana dei Monti Martini e del Serano, Spoleto, Italy, 1990, pp. 19-30.

[6] J.M. Trappe, M.A. Castellano, Keys to the genera of truffles (Ascomycetes), McIlvainea 10 (1991) 47-65.

[7] T. Lebel, M.A. Castellano, Australasian truffle-like fungi. IX. History and current trends in the study of the taxonomy of sequestrate macro fungi from Australia and New Zealand, Australian Systematic Botany 12 (1999) 803-817.

[8] J.M. Trappe, A.W. Claridge, D.L. Claridge, L. Liddle, Desert truffles of the Australian outback ecology, Ethnomycology and Taxonomy, Economic Botany 62 (3) (2009) 497-506.

[9] B.C. Zhang, Ascospore nuclear number and taxonomy of truffles, Micologiae Vegetazione Mediterranea 7 (1992) 39-42.

[10] L.G.J. Ackerman, P.J. Vanwyk, L.M. Du Plassis, Some aspects of the composition of the Kalhari truffle of Nabba, South Afr. Food Rev 2 (1975) 145-147.

[11] K.S. Al-Delaimy, Protein and amino acid composition of truffle, Journal of the Canadian Institute of Food Science and Technology 10 (1977) 221-222.

[12] M.M.A. A1-Shabibi, S.J. Toma, B.A. Haddad, Studies on Iraqi truffles, proximate analysis and characterization of lipids, Journal of the Canadian Institute of Food Science and Technology15 (3) (1982) 200-202.

[13] H.A. Bokhary, A.A. Suleiman, M.O. Basalah, The fatty acid components of the desert truffle "AlKamah" of Saudi Arabia, Journal of Food Protection 52 (1989) 668-669.

[14] M.A. Murcia, M. Martinez-Tome, A.M. Jimenez, A.M. Vera, M. Honrubia, P. Parras, Antioxidant activity of edible fungi (truffles and mushrooms) losses during industrial processing, Journal of Food Protection 65 (2002) 1614-1622.

[15] F.A. Sakri, Chemical composition and mineral content of Iraqi truffles, Iraqi. J. Sci. 30 (1989) 421-424.

[16] W.N. Sawaya, A. A1-Shahat, A. A1-Sogair, M. A1-Mahammad, Chemical composition and nutritive value of truffles of Saudi Arabia, J. Food Sci. 50 (1985) 450-453.

[17] A.N. Al-Rahmah, Truffle of Deserts and Jungles, King Saud University Publications, Riyadh, Saudi Arabia, 2001, p. 272. (in Arabic)

[18] H.A. Bokhary, Desert truffles 'Al-Kamah' of the Kingdom of Saudi Arabia, occurrence, identification and distribution, Arab Gulf Journal of Scientific Research B 5 (1987) 245-255.

[19] R. Rougieux, Ntibiotic and stimulating actions of the desert truffle (*Terfezia Boudieri* Chatin), Ann. Inst. Paris 105 (1963) 315-318.

[20] M.A. Al-Marzooky, Truffles in eye disease, in: Proceedings of the International Conference on Islamic Medicine, Kuwait, 1981, pp. 353-357.

[21] A. Chellal, E. Lukasova, Evidence for antibiotics in the two Algerian truffles *Terfezia* and *Tirmania*, Pharmazie 50 (1995) 228-229.

[22] S. Janakat, S. Al-Fakhiri, A.K. Sallal, A promising peptide antibiotic from *Terfezia claveryi* aqueous extract against *Staphylococcus aureus in vitro*, Phototherapy Research 18 (2004) 810-813.

[23] S. Janakat, S. Al-Fakhiri , A.K. Sallal, Evaluation of antibacterial activity of aqueous and methanolic extracts of the truffle *Terfezia claveryi* against *Pseudomonas aeruginosa*, Saudi Medical Journal 26 (2005) 952-955.

[24] A.A.A. Al-Laith, Antioxidant components and antioxidant/antiradical activities of desert truffle (*Tirmania nivea*) from various Middle Eastern origins, Journal of Food Composition and Analysis 23 (2010) 15-22.

[25] S. Janakat, M. Nassar, Hepatoprotective activity of desert truffle (*Terfezia claveryi*) in comparison with the effect of Nigella sativa in the rat, Pakistan Journal of Nutrition 9 (2010) 52-56.

[26] I. Karabegovic, M. Nikolova, D. Veli kovic, S. Stojievic, V. Veljkovic, M. Lazic, Comparison of antioxidant and antimicrobial activities of methanolic extracts of the *Artemisia* sp. recovered by different extraction techniques, Chinese Journal of Chemical Engineering 19 (3) (2011) 504-511.

[27] G. Tan, W. Lei, S. Juan, H. Cheng-lin, F. Li, Antioxidant activities of extract and fractions from *Tuber indicum* Cooke & Massee, Food Chemistry 127 (2011) 1634-1640.

[28] A.W. Bauer, W.M.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol. 45 (1966) 493-496.

[29] J.M. Wilkinson, M. Hipwell, T. Ryan, M.A. Cavanagh, Bioactivity of *Backhousia citriodora*, antibacterial and antifungal activity, Journal of Agriculture and Food Chemistry 51 (1) (2003) 76-81.