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Egyptian truffles as a source of antimicrobial and antioxidant agents

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Abstract:

Edible desert truffles are obligate symbiotic macro hypogenous ascomycetes fruit bodies. These are growing in depth between 5 and 10 cm. These consider a miracle of nature and an unexploited source of therapeutic compounds with antimicrobial, antioxidant, and wonderful food, especially for Bedouins. The current study investigated the activity of premature and mature ethyl acetate truffle extracts (white and Red) collected from the Western Egyptian Desert as antimicrobial and antioxidant. This study evaluated in vitro the efficacy of antimicrobial activity of organic truffles extract, and their effect on various pathogens (Gram-negative, Gram-positive bacteria, filamentous fungi, and yeast) by using agar well diffusion. TEM micrographs had been done for the most effective crude extracts. Furthermore, the activity of DPPH scavenging was studied for both mature truffles. Both truffles extracts had antibacterial activity more than antifungal activity. The selected extracts exhibited an inhibitory effect on the cell wall and protoplasm of pathogens. Terfezia sp. had antioxidant activity more than Tirmania sp. This investigation concluded that the truffle extracts could be considered a promising antibiotic and antioxidant drug in near future.

Keywords: *Tirmania sp.; Terfezia sp.*; antimicrobial activity; antioxidant; pathogenic microorganisms.

1. Introduction

The desert is sometimes associated with a sterile environment that is unsuitable for life. However, it contains a number of arid-adapted plant and animal species **[1].** In this environment, desert truffles from the species *Tirmania* and *Terfezia*, are known as manna or kama'a **[2].**

Desert truffles are edible ascomycetes fungi that are hypogeous, mycorrhizal, and indigenous in the semi-arid and arid Mediterranean region, North Africa, and the Middle East

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[3, 4, 5], as well as Central and Southern Europe and various parts of Asia. *Tirmania* and *Terfezia* belong to the families *Pezizaceae* and *Terfeziaceae*, respectively, in the order Pezizales **[6].**

They are strictly dependent on other organisms to complete their lifecycle and live in a symbiotic relationship with the roots of various Helianthemum species (family of Cistaceae) **[7, 8].** Unless they are devoured by insects or mammals, fruiting bodies do not disseminate their spores. They employ volatile signals to govern their interactions with other organisms during their life cycle **[9].**

Truffles may be regarded as a nutrient source for Bedouin and nomad diets in dry places during harsh winter conditions **[10].** Truffles are high in anthocyanins, oligosaccharides, flavonoids, carotenoids, pheromones, and steroids, among other bioactive chemical elements. Truffles have anti-oxidant, anti-mutagenic, anticarcinogenic, anti-microbial, anti-viral, immune-modulating, anti-inflammatory, anti-depressant, and sedative bioactive constituents **[11,12]**. The aim of this study was to test *in vitro* the antagonistic human pathogenic bacteria and fungi as well as the antioxidant activity of ethyl acetate white and red truffles extracts.

2. Material and methods

2.1. Truffles sampling

White and red truffles were harvested by manual digging from Al-Hammam City in Marsa Matrouh in the Western Egyptian Desert (latitude 30°44'43.19"N and longitude 28°54'13.16" E), Egypt **(Fig. 1)**. These were collected from the area during the pre-maturing and maturing stages in January and March 2019, respectively. To avoid the risk of samples storage was unwashed and transferred to the lab/for further analysis.

Morphological identification was based on ascomata characteristics and microscopic examination of spores and asci for each species **[13].** The Regional Center for Mycology and Biotechnology in Cairo, Egypt, confirmed the findings using an image analyzer microscope (Olympus XP40, Germany, magnification power X-100).

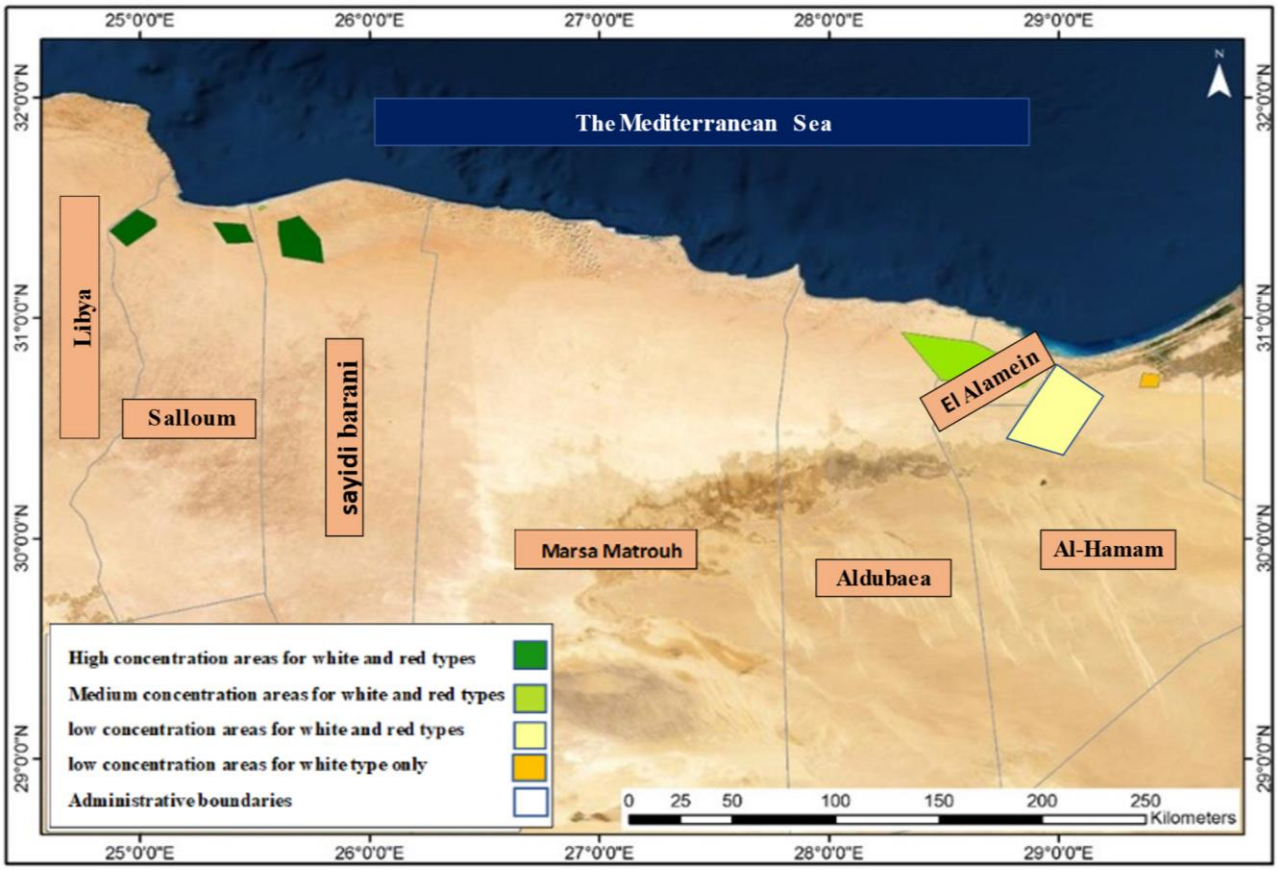

Figure 1: A map of the region of collection of Egyptian truffles.

2.2. Extraction by ethyl acetate

Fresh fruiting bodies were cleaned and cut into small pieces to obtain the crude truffles extracts (premature and mature), grinded in a blender with ethyl acetate (7g/100ml), and suspended overnight at 4 °C then centrifuged (4000 rpm,4^{o}C for 20 minutes) to remove insoluble material. The supernatant was evaporated from the organic matter solvent [14]. For subsequent usage, all of the semi-solid extracts were lyophilized and kept at 4°C.

2.3. Antimicrobial activity of truffles extracts

The agar well diffusion method was used to assess the antimicrobial activity of extracts from the two edible truffles, whereas the inhibition of pathogenic growth was measured (mm). The extracts were filtered and sterilized using a 0.45-micro membrane filter and had a final concentration of 20 mg/ml. A sterile cork borer was used to make small wells (10 mm in diameter) in agar plates. A 100 µl of each extract was loaded into the different wells in plates inoculated with Gram-positive bacteria (*Staphylococcus sciuri* strain MH491952.1

and *Bacillus cereus* strain 151007-R3-K09, Gram-negative bacteria (*Escherichia coli* Strain E11 KY780346.1 and *Pseudomonas aeruginosa* strain Kasamber5), local fungi (*Penicillium* sp. and *Aspergillus flavus*), and yeast *(Candida albicans* and *candida glebrata*). All bacterial and yeast plates were incubated for 24 hours at 37 ºC while filamentous fungi were incubated for 48 hours at 28 ºC. All of the tests were done in triplicate and the results were recorded **[15, 16].**

2.4. Microdilution technique for determining antibacterial activity

Each truffle extract in the stock solutions was produced in PBS: DMSO at a concentration of mg/mL (1:1). The minimum inhibitory concentration (MIC) of an antifungal or antibacterial is defined as the lowest concentration that inhibits the microorganism's development. The most potent extract was investigated to determine its MIC against *Staphylococcus sciuri and Pseudomonas aeruginosa*. stock dilutions (0.02 mg/ml) were examined by the agar diffusion method. A control was without truffle extract. The tested bacterial pathogens were cultured overnight in nutrient broth at 37°C and the fungal pathogens were incubated at 30 ºC 48 hours on Malt extract broth **[17,18]**.

2.5. Transmission Electron Microscopy (TEM)

TEM photography was performed on the same species of MIC experiment. One mm^3 block of the tested pathogens as control and others treated with EHP compound were fixed and dehydrated then frozen on an ultramicrotome (Leica ultracut S), ultrathin sections were cut to a thickness of 70–90 nm with a diamond knife and put on copper grids. They were dyed with uranyl acetate and lead citrate, coated with plasma–polymerized naphthalene support film and examined at the Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt, in a JEM 12000EX TEM (Jeol, Tokyo) at 80kv **[19]**.

2.6. DPPH Radical Scavenging Activity

A freshly made (0.004% w/v) methanol solution of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and kept in the dark at 10 ºC. The mature truffle extracts were dissolved in methanol. A UV-visible spectrophotometer was used to take instantaneous absorbance values (at 515 nm) (Milton Roy, Spectronic 1201). The absorbance of the DPPH radical (control) and the reference chemical ascorbic acid were also measured. All of the tests were repeated three times and the results were averaged. The DPPH radical's percentage inhibition (PI) was estimated using the formula:

[(AC- AT)/ AC x 100] = PI (1)

where AC is the control's absorbance at t = 0 minutes and AT is the sample's absorbance plus DPPH at t = 16 minutes **[20,21]**.

Statistical analysis

One-way analysis of variance (ANOVA 1) was used. All tests were performed three times and represented as a mean ± standard deviation, at $p<0.001$ indicating statistical significance [22].

3. Results

3.1. Truffles morphology

The morphology of white and red truffles was subglobose. The white type had a larger size (3-11 cm) than the red type (2-7 cm). The peridium was light brown to yellowish and brown for white and red truffles, respectively **(Fig. 2)**. According to morphological features, the white truffle was *Tirmania* sp. while the red type was *Terfezia sp*.

Figure 2: Morphological characteristics of white and red truffles; where (a) in pre-maturing (b) and maturing stages.

3.2. The antimicrobial activity of the truffles extracts against the pathogenic bacterial and fungal strains

The antimicrobial activities of two species of truffle extracts were screened *in vitro* by agar well diffusion method against eight strains of pathogenic bacteria, fungi, and yeast, and showed highly significant differences in antimicrobial activity against these pathogens ***(p<0.001)***. All the extracts from fresh truffles were examined and summarized in **Table 1**.

These results confirmed the antimicrobial activity of desert truffles; this was evidenced by the clear zone of inhibition.

The results showed that mature extracts were more potent than premature ones. The extract of premature *Tirmania* sp. affected *Staph. sciuri* (21mm) followed by *Ps. aeruginosa* (19 mm), whereas, the extract of mature *Tirmania* sp. affected all tested bacteria with inhibition zone in a range from 23 to 33 mm. *Staph. sciuri and Ps. aeruginosa* were sensitive to the extract of premature *Terfezia. sp.* with 23 and 20 mm inhibition zone, respectively, but in the maturing stage, the inhibition zones were 31, 30, 26, and 25 mm for *Ps. aeruginosa*, *E. coli*, *B. cereus*, and *Staph. sciuri*, respectively.

All the fungal species were inhibited by the extract of premature *Tirmania* sp. *C. glebrata* was the most sensitive with a 19 mm inhibition zone while *A. flavus* was the least sensitive with an 11mm inhibition zone. In contrast, the extract of mature *Tirmania* sp affected only *A. flavus* by 16 mm inhibition zone. the extract of mature *Terfezia sp.* affected all fungal species with 18, 15, 14, and 12 mm inhibition zone for *C. glebrata, C. albicans*, *Penicillium*, and *A. flavus*. But the pre-maturing stage didn't affect any fungal species.

Table1: Antimicrobial activity of ethyl acetate extract of fresh *Tirmania* sp. and *Terfezia* sp.

Inhibition zone (mm)									
Truffles		**G+ve bacteria**		**G-ve bacteria**		**Filamentous fungi**		**Yeast**	
		Staph.	***B.***	***E.***	***Ps.***	***Penc.***	***A.***	***C.***	***C.***
		sciuri	***cereus***	***coli***	***aeruginosa***		***flavus***	***albicans***	***glebrata***
Tirmania **sp.**	**Premature**	**21±0.81**	**-**	**-**	**19 ±0.97**	**17±0.74**	**11±1.02**	**15±0.81**	**19±0.78**
	Mature	**33±1.63**	**23±0.81**	**28 ±1.06**	**25±1.06**	**-**	**16±0.81**	**-**	**-**
Terfezia **sp.**	**Premature**	**23±1.22**	**-**	**-**	**20±0.89**	**-**	**-**	**-**	**-**
	Mature	**26±0.90**	**25±0.77**	**30±0.81**	**31±1.24**	**14±0.81**	**12±0.89**	**15±0.83**	**18±1.38**
	F-value	**93.00*** **	**1.156*** **	**1.686*** **	**90.75*** **	**489.5*** **	**187.667*** **	**421.5*** **	**685.50*** **

- Not recorded

***significant difference at $p<0.001$

3.3. MIC of the most active antimicrobial extract

The MIC was done for the crude extracts most affecting the pathogens. Where the extract of mature *Tirmania sp.* and *Terfezia Sp.* affected *Staph. sciuri* with a 5 µg/ml MIC and *Ps. aeruginosa* with a 2.5 µg/ml MIC, respectively.

MBC at the lowest doses was (10 and 5 g/ml) for *Staph. sciuri* and *Ps. aeruginosa*, respectively, indicating that these concentrations would prevent the organism from developing after subculture on antibiotic-free media.

3.4. Effect of truffles extracts on the morphological structure of the tested bacteria

TEM micrograph was performed on *Staph. sciuri* and *Ps. aeruginosa* because they were the most sensitive to the extracts. The cells of both pathogens became empty and the cell walls were ruptured **(Fig. 3A2, B2)** when compared with the same control species **(Fig.3A1 and B1)** indicating that the treatment with both mature truffle extracts had an effect on the cell division and reproduction.

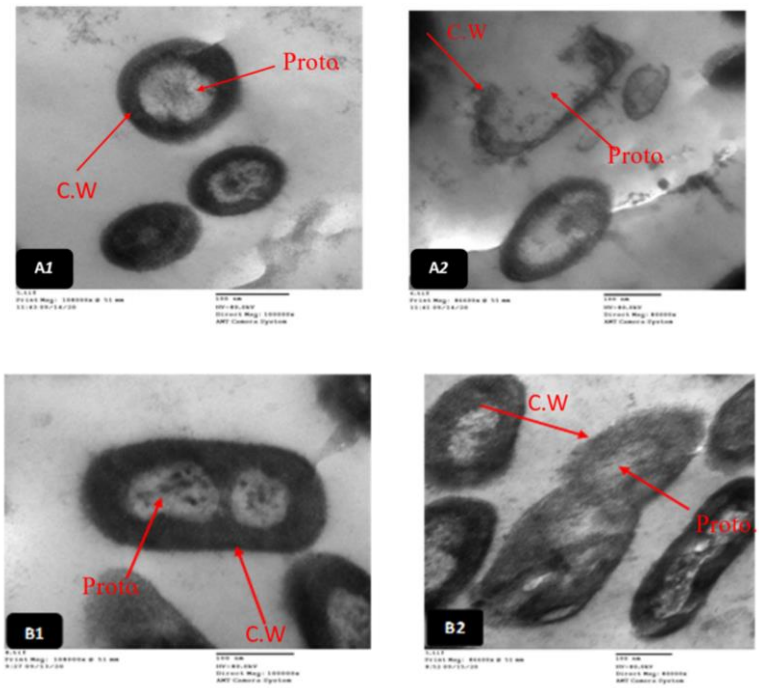

Figure 3: TEM micrographs of *Staph. sciuri* and *Ps. aeruginosa* cells; A1 & B1 untreated cells x100000, A2 & B2 treated cells x80000 with the extracts of mature *Tirmania sp.* and *Terfezia sp.*, respectively. C.W refers to the cell wall, Proto. refers to Protoplasm.

3.5. Evaluation of antioxidant activity using DPPH scavenging

DPPH is a free radical molecule that has been routinely utilized to evaluate natural product antioxidant capabilities. This approach is based on donating antioxidants to reduce DPPH (purple color) in solution, then forming the non-radical form DPPH-H in the reaction. The reduction of DPPH by the truffles extracts resulted in a loss of absorbance and was expressed as IC_{50} value (the extract concentration which reduced 50% of the initial DPPH concentration). It has been found that all the extracts of *Tirmania* sp. and *Terfezia* sp. had significant amounts of radical scavenging activity **(Tables 2)**. The IC_{50} of *Tirmania* and *Terfezia* extract was 287.2 and 191.8 ± 1 µg/ml, respectively. It indicated that *Terfezia sp.* extracts were more effective than *Tirmania sp.* extracts. While IC_{50} of ascorbic acid was 10.6 µg/ml which was more powerful than IC_{50} of the truffle extracts. These results showed significantly high differences between the values, which was $p<0.001$.

Table 2: Antioxidant activity of mature *Tirmania* sp. and *Terfezia* sp.

Sample conc. (µg/ml)	DPPH Scavenging antioxidant activity		
	***Tirmania* sp.**	***Terfezia* sp.**	**Ascorbic acid**
1280	**91.56±0.92**	**93.75±1.23**	**98.91±0.74**
640	**86.41±1.73**	**91.96±1.42**	**97.83±0.39**
320	**55.14±2.89**	**80.42±2.06**	**95.64±1.22**
160	**30.07±3.29**	**42.46±2.85**	**92.31±0.87**
80	**21.88±0.64**	**31.79±1.31**	**90.25±0.41**
40	**11.69±0.37**	**16.58±1.24**	**83.09±1.95**
20	**8.79±0.25**	**9.69±0.73**	**71.38±1.39**
10	**3.14±0.28**	**3.30±0.28**	**48.52±2.64**
0	**0**	**0**	**0**
F-value	**0.297*** **	**1.759*** **	**3.021*** **

***significant difference at p<0.001

4. Discussion

Truffles are spread all over the world, recorded in the Western Egyptian Desert as *Tirmania* spp and *Terfezia* spp. [23], which was confirmed in our study by morphological identification. Both types were different in size, color **(Fig. 2)**, and humidity due to various biotic and abiotic factors surrounding them.

Truffles had multiple nutritional and therapeutic importance. They had been shrouded with mystery since antiquity **[10]** from this point of view, our study had conducted the antimicrobial activity of truffles extracts, which proved their strength as anti-pathogens against all tested pathogens.

Our results found that the mature truffles extract had more potent antibacterial than in the pre-maturing stage due to the complete formation of therapeutic compounds in mature **[24, 25]**, *Staph. sciuri* was the most sensitive with *Tirmania* sp. extract (33mm diameter of inhibition zone, 5 and 10 µg/ml for MIC and MBC, respectively), but *Terfezia* sp. had a strong effect on *Ps. aeruginosa* (31 mm diameter of inhibition zone, 2.5, and 5 µg/ml for MIC and MBC, respectively). MIC values were lower than MBC values, indicating that the extracts were bacteriostatic at low concentrations but bactericidal at higher, antibacterial activity of desert truffles cited in the literature **[14]**.

This study found that *Tirmania* sp. and *Terfezia* sp. ethyl acetate extract possessed a very powerful antibacterial activity, confirmed by the TEM micrograph for *Staph. sciuri* and *Ps. aeruginosa* affected cells morphology, genetic material, and reproduction **(Fig. 3)**, which was proved in other studies by **Aldebasi *et al*., [26]**. *Staph. sciuri* and *Ps. aeruginosa* increased their risk by the emergence of resistance to multiple antibiotics [27]. Chemotherapeutic drugs, like all other medications, have side effects [28,29]. Fortunately, truffles grow naturally without chemicals, and this enhances the production of antibiotics that are safer for human health.

Chronic diseases such as aging, cancer, arthritis, autoimmune disorders, and cardiovascular disease are all linked to oxidative stress **[30]**. Truffles never use chemicals in their products, so everything is all-natural. Therefore, the antioxidant activity for two mature extracts was done and exhibited the highest DPPH for *Terfezia* sp. extract with IC_{50} = 191.8 ± 11 µg/ml followed by *Tirmania* sp. extract with an IC_{50}= 287.2 ± 16µg/ml **(Table 2)**. Similar findings were obtained from **Saad *et al*., [31]**.

5. Conclusion

This paper has clearly shown that *Tirmania* sp. and *Terfezia* sp exhibited a good potentiality as antimicrobial activity against various pathogens by ethyl acetate crude extracts. They had been capable of making both internal and external changes for both *Staph. sciuri* and *Ps. aeruginosa* cells confirmed by TEM micrograph. Also, the truffles extract had a radical

scavenging activity. Moreover, research on their bioactive components is necessary in order to employ them as potential medicinal agents, characterization, and purification.

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الملخص العربي

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الملخص العربي

تعتبرالكمأه الصحراوية الصالحة للأكل معجزة الطبيعة فهي تنمو علي بعد من 5-10 سم تحت سطح التربة ، وتعد مصدر للمركبات العلاجية كمضادات للميكروبات والاكسدة و مصدر غذاء لذيذ خاصة للبدو. والهدف من هذة الدراسة التحقق من نشاط النوع الأبيض (*Tirmania sp.*) والأحمر (*Terfezia sp.*) للكمأه الذي تم جمعة من الصحراء الغربية المصرية في مرحلة النضج وما قبل النضج كمضاد للميكروبات والأكسدة . فقد تم دراسة كفاءة المستخلص العضوي للكمأه كمضاد لمسببات الأمراض مثل البكتريا السالبة والموجبة لصبغة جرام وايضا للفطريات الخيطية والخمائر بطريقة الانتشار في الاجار وقد ثبت أن الكماه تعد مضاد للبكتريا أقوي من أنها مضادة للفطريات. وتم اجراء صور مجهرية باستخدام TEM لأكثر المستخلصات الخام فعالية علي الكائنات الممرضة من النوعين الأبيض والأحمر الناضجين ، وتبين أن المستخلص أثر علي نمو وتكاثر الكائنات الممرضة.

بالاضافة لذلك تم دراستها كمضادة للأكسدة باستخدام DPPH Radical Scavenging Activity وكان لها تاثير ملحوظ كمضاد للأكسدة. هذه الدراسة تدعم التأثير الفعال للكمأه التي يمكن اعتبارها كمضاد حيوي ومضاد للأكسدة في المستقبل القريب.