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Evaluation of the Antioxidant Activity of Ethanol Extract from *Terfezia claveryi* Harvested from Anbar Desert

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Abstract

Desert truffles are edible fungi that grow wild in various areas worldwide and are seasonal. The objective of the research is to illustrate the efficacy of the alcoholic extract of *Terfezia claveryi* as an antioxidant after diagnosing truffle species by polymerase chain reaction (PCR), antioxidant activities were determined by (DPPH) 2,2-diphenyl-1-picrylhydrazyl and (FRAP) ferric ion-reducing antioxidant power, then compared with positive controls. The antioxidant activity of ethanol extract was evaluated using *T. claveryi* ascocarps which were harvested from Anbar desert, Iraq. The ethanol extract exhibited the highest DPPH radical-scavenging activity compared to ascorbic acid, with IC50 values of 6 µg/ml and 1 µg/ml, respectively. The FRAP assay revealed no significant reducing power at a concentration of 0.16 µg/ml (1.859±0.003), while significant effects were noted at concentrations of 0.64, 0.32, and 0.08 µg/ml. In addition, catalase and peroxidase enzymes have been thoroughly investigated for their antioxidant properties. This study reveals that *T. claveryi* contains catalase and peroxidase enzymes at concentrations of 0.862 U/mg and 6000 U/mg, respectively. The results indicated that *T. claveryi* possesses antioxidant properties and may be useful in treating of disorders caused by free radicals.

Keywords: *Terfezia claveryi*, DPPH, FRAP, free-radical scavenging activity.

تقييم الفعاليه الضد تأكسديه للمستخلص الايثانولي ل *Terfezia claveryi* التي تم حصادها من صحراء الانبار

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الخلاصه

الكمأة الصحراوية هي فطريات موسميه صالحة للأكل تنمو برية في مناطق مختلفة من العالم. الهدف من البحث هو توضيح فعالية المستخلص الكحولي لـ *Terfezia claveryi* كمضاد للأكسدة بعد تشخيص الكمأة بواسطة تفاعل البوليميراز المتسلسل (PCR)، تم تحديد نشاط مضادات الأكسدة بواسطة (DPPH) 2,2-diphenyl-1-picrylhydrazyl و (FRAP) قوة مضادات الأكسدة المختزلة لأيون الحديديك ومن ثم مقارنتها بالمركبات القياسيه. تم تقييم النشاط المضاد للأكسدة لمستخلص الإيثانول باستخدام الاجسام الثمريه لفطر *T. claveryi* التي تم حصادها من صحراء الأنبار في العراق. كانت قيم IC50 DPPH للمستخلص

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الكحولي وحمض الأسكوربيك (6) ميكروجرام / مل و (1) ميكروجرام / مل على التوالي. أشار اختبار FRAP إلى أنه لايوجد فرق معنوي عند تركيز 0.16 ميكروجرام/مل (1.859 ± 0.003)، في حين وجدت اعلى الفروق المعنويه عند التراكيز (0.64 ,0.32 و0.08) ميكروجرام/مل. بالإضافة إلى ذلك، تمت دراسة كل من انزيم الكاتالايز والبيروكسيديز على نطاق واسع كمضادات للأكسدة. في هذه الدراسة تحتوي *T. claveryi* على إنزيمات الكاتالايز والبيروكسيديز (0.862) وحدة/ملجم و(6000) وحدة/ملجم على التوالي. اشارت النتائج الى انه *T. claveryi* لها خصائص مضادة للأكسدة وقد تكون مفيدة في علاج الاضطرابات الناجمة عن الجذور الحرة.

1. Introduction

Antioxidants are a large group of chemical substances that can help protect the body's cells from the damaging effects of reactive oxygen species (ROS) and free radicals [1]. Antioxidants that inhibit the oxidation of other molecules can alleviate the damage induced by ROS. They have the ability to donate electrons, neutralizing radical production and diminishing additional harm from free-radical activities. However, respiration and many processes produce countless free radicals that can adversely affect the organism, leading to functional impairment and potentially death [2]. Oxidation is an essential metabolic process in aerobic organisms that is necessary for energy production. The unregulated metabolic cascade following oxidation leads to the generation of free radicals. However, pathological conditions that are thought to be brought on by a condition that upsets cellular homeostasis and causes the breakdown of molecules, including proteins, DNA, carbohydrates, and lipids, include atherosclerosis, rheumatoid arthritis, cancer, and age-related degenerative events [3]. Due to their unpaired electrons, free radicals are electrically charged molecules that seek out and take electrons from other materials in an attempt to neutralize themselves, but in the process, a new free radical is created, which sets off a chain reaction. However, antioxidants have the ability to stabilize or deactivate free radicals before they harm cells; thousands of free radical reactions can happen within seconds before free radicals are killed [4]. Truffle refers to the edible hypogeous fruiting bodies of mycorrhizal ascomycetes that were harvested in wild areas for hundreds of years. The edible component of the truffle consists of an ascoma that forms underground and progressively ascends to the surface, resulting in soil swelling and eventual breaking. However, the basal section is made up of many branching hyphae that surround the host root (nombril), desert truffles, which are classified under the genus *Tirmania* and *Terfezia,* which are members of the Pezizaceae family are found in arid and semiarid regions of the Mediterranean basin and the Middle East [5]. Truffles are a significant source of proteins, fats, carbs, fiber, ash, lipids, unsaturated fatty acids, and several minerals for human nutrition. However, due to its anti-inflammatory, anti-mutagenic, and antioxidant properties, truffle oil contains a variety of volatile organic molecules, such as alcohols, aromatics, terpenes, alkanes, alkenes, esters, aldehydes, ketones, and some sulfuric compounds, these compounds are used as food and medicinal agents [6]. *Terfezia claveryi* is one of the many edible truffle species that are known locally as Aljabah because of their smooth, thick crust and dark brown hue [7]. Polymerase chain reaction (PCR), one of the molecular identification techniques, was applied in many studies to confirm the diagnosis by detecting the ITS gene [8]. By comparing sequences from fungus DNA with databases already in existence and housed in public sequence libraries, such as the DNA Data Bank of Japan (DDBJ), the National Center for Biotechnology Information (NCBI), and the European Molecular Biology Laboratory (EMBL), species identification has been accomplished [9]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is a popular way to measure antioxidant activity based on a single electron or hydrogen atom transfer. It is predicated on a persistent organic radical that, when encountering antioxidants, changes from purple to yellow. Another

technique to assess antioxidant activity based on single electron transfer is the ferric reducing antioxidant power (FRAP), which quantifies the amount of iron that antioxidants can reduce from Fe (III) to Fe (II) [10]. Among the many edible desert truffle varieties, our research focuses on dark brown color truffles from the genus *T. claveryi*. The aim of this study was to illustrate the efficacy of the ethanolic extract of *T. claveryi* as an antioxidant after macroscopic analysis and polymerase chain reaction (PCR).

2. Materials and Methods

2.1 Samples of Truffles

This study included the fruiting bodies of the Iraqi desert truffle *T. claveryi,* which were obtained from the western region from February to March 2024. To eliminate contaminants, the ascocarps were washed with distilled water, cut into slices, dried at 30-35°C until constant weight, and then ground into powder. The crushed truffles were stored at room temperature in a dry place until needed.

2.2 Identification Truffles by Polymerase Chain Reaction (PCR)

2.2.1 DNA extraction procedure

The genomic DNA of truffles was extracted with the Gene aid™ Taiwan Extraction protocol in accordance with the manufacturer's instructions. DNA concentration values were detected by a quantus fluorometer (Promega, USA) and then refrigerated at -20 °C.

2.2.2 PCR and sequencing

The PCR reaction was performed in a reaction volume of 25 µL, which included 10 µL of Taq® Master Mix (Promega / USA) 1 µL of each universal primers; the forward primer's sequence (18S28S) is 5`-ACCTTGTGTGGAACCTCTGG-3` and the reverse primer's sequence (18S28S) is 5`-TACAAAGCATGCTGCCCATA-3` with annealing temperature (58C°), 8 µL of nuclease-free water, and 5 µl on concentration < 250 ng of DNA [11]. The PCR program was performed with 35 cycles, as in Table 1. The amplified DNA fragments were subjected to 1% agarose gel electrophoresis at 70V for 1 hr. Following electrophoresis, the gel was exposed to UV light using a UV Trans-illuminator to visualize DNA bands before being photographed.

Table 1: The optimum condition (PCR) of detection of gene

No.	Phase	Temperature °C	Time	No. of cycle
1	Initial Denaturation	94	5 mints	1
2	Denaturation	94	40 second	
3	Annealing	58	40 second	35
4	Extension	72	40 second	
5	Final extension	72	10 mints	1

2.3 Preparation of alcoholic extract

In order to determine the antioxidant properties, truffle extracts were made from dried *T. claveryi* using 95% ethanol. The dried sample (10) g was extracted using 100 ml of solvent at room temperature on a shaker at 150 rpm for 24 hours, and it was filtered through No. 4 Whatman filter paper. The residue was then extracted twice, and the filtrates were combined, according to Al-Atassi *et al.,* [12]. The extract was nearly dried in a rotary evaporator at (40)°C for 2 hours, and then the extract was stored at (-20)°C.

2.4 DPPH (1, 1- diphenyl-2-picryl hydrazyl radical) assay

DPPH˙ scavenging activity of the extracts was estimated according to [10], truffle extract was added in an equivalent proportion to an ethanolic solution of DPPH (10) µM at several concentrations (0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 10) µg/ml. The UV/visible spectrophotometer was used to measure the absorbance value at 517 nm following a 15-minute incubation period at room temperature. The formula was utilized to determine the percentage (%) of inhibition of the DPPH radical:

Percentage inhibition % = [(Acontrol _ Asample] / Acontrol] × 100

A control: Absorbance of the DPPH solution alone

A sample: Absorbance of the sample

2.5 Reducing Power

The reducing power was determined according to the method of [10]. A mixture of (2.5) ml of truffle extracts, (2.5) ml of (200) mM sodium phosphate buffer pH (6.6), and (2.5) ml of (1%) potassium ferricyanide was incubated at (50)°C for 20 minutes. Subsequently, 2.5 ml of 10% trichloroacetic acid was added, the mixture was centrifuged for 10 minutes, the top layer was removed, 5 ml of it was combined with 5 ml of D.W. and 1 ml of (0.1%) ferric chloride, and the absorbance was determined at 700 nm. A blank solution contains all of the reagents without extracts, and a greater absorbance means a higher reduction power.

2.6 Assay of antioxidant enzymes activity

2.6.1. Catalase

Catalase activity was assayed according to the method of [13]. Briefly, the assay was done by preparing 1 ml of H_2O_2 (0.019) M, 1.95 ml of phosphate buffer (0.05) M, pH 7.0, and 0.05 ml of truffle extracts were used to create a mixture that had a final volume of 3 ml. All components were present in the control, except for the substrate; a wavelength change was recorded at 240 nm, and catalase activity was assessed in U/mg protein.

2.6.2. peroxidase

Peroxidase activity was assayed according to the method of De Vargas *et al.,* [14], containing (50) mM sodium phosphate buffer pH (7.0), (50) mM guaiacol, (10) mM H_2O_2 and truffles extract. The peroxidase activity was determined by the increase in absorbance at (470) nm due to guaiacol oxidation.

2.7 Statistical Analysis

In order to evaluate *T. claveryi*, using analysis of variance to explain the differences between means, using least significant differences (LSD) at $p \leq 0.05$. Further that using t-test to explain the differences between control and alcoholic extract for *T. claveryi*, expressed that as (mean ± SEM). Means with stars indicate significant differences between means.

3. Results

3.1 Morphological diagnosis of Terfezia claveryi

Macroscopic truffle identification based on ascocarp morphological traits identifies *T. claveryi* species (black truffle), as shown in Figure 1.

Figure 1: shows *Terfezia claveryi*

3.2 Molecular identification of Terfezia claveryi by Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was performed for the detection of the (18S28S) gene region in *T. claveryi* samples, showing a positive result for the 18S28S region with sizes 386 pb. Demonstrated that the 18S28S primer based PCR method had high sensitivity and specificity in *T. claveryi* detection, as shown in Figure 2.

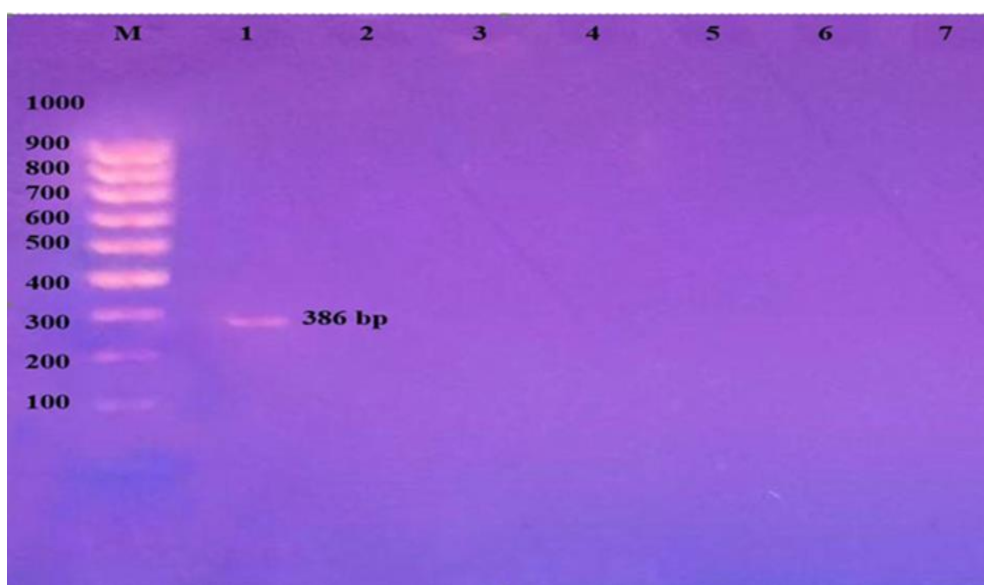

Figure 2: Product the band size Agarose gel electrophoresis of PCR after amplification, Marker (100 bp ladder Gene aid

3.3 Scavenging activity of DPPH radical

In order to show the *in vitro* antioxidant activity of *T. claveryi*, the DPPH free radical trapping technique was employed. The results of the DPPH radical scavenging activity are shown in Figure 3. In this study, the alcoholic extract has been identified in *T. claveryi* (61.789%), (40.227%), and (7.5234%) at (10), (2), and (0.2) µg/ml, respectively. In addition, *T. claveryi* has a high significance for all concentrations ($p = 0.001$), as shown in Table 2. This result indicates that the IC50 values of *T. claveryi* and ascorbic acid were (6 and 1 µg/ml), respectively. That *T. claveryi* has high antioxidant activity.

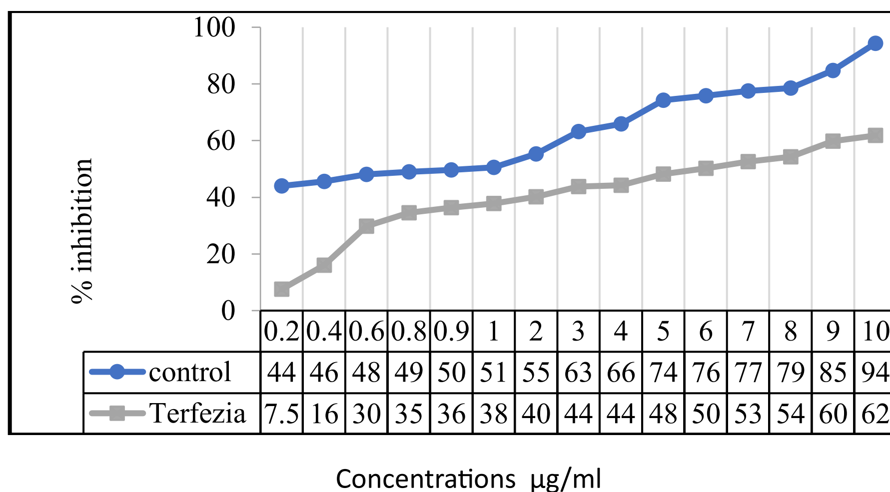

Figure 3: Shows DPPH radical scavenging effects of *Terfezia claveryi* compared with positive control

Table 2: Shows DPPH radical scavenging effects of *Terfezia claveryi*

Conc. (µg/ml)	*T. claveryi*	Abs. ± SD *T. claveryi*
0.2	$p < 0.001$ → ***	7.523 ± 0.868
0.4	$p < 0.001$ → ***	16.044 ± 1.023
0.6	$p < 0.001$ → ***	29.842 ± 0.748
0.8	$p < 0.001$ → ***	34.557 ± 0.303
0.9	$p < 0.001$ → ***	36.285 ± 1.111
1	$p < 0.001$ → ***	37.797 ± 0.415
2	$p < 0.001$ → ***	40.227 ± 0.943
3	$p < 0.001$ → ***	43.754 ± 0.790
4	$p < 0.001$ → ***	44.240 ± 0.822
5	$p < 0.001$ → ***	48.236 ± 0.612
6	$p < 0.001$ → ***	50.252 ± 0.041
7	$p < 0.001$ → ***	52.628 ± 1.029
8	$p < 0.001$ → ***	54.230 ± 1.493
9	$p < 0.001$ → ***	59.791 ± 0.333
10	$p < 0.001$ → ***	61.789 ± 0.824

*** means high significant

I 3.4 Reducing power

Another widely reported chemical assay for antioxidant activity in crude food extract is the FRAP assay. *T. claveryi* exhibited no significant reducing power ($p = 1.000$) at a concentration (0.16) µg/ml (1.859±0.003); but there were high significant on other concentrations (0.64, 0.32, 0.08). It may be because of differences in condition. In this study, trolox has the lowest absorbance compared to ethanolic extract for *T. claveryi* at a concentration of (0.08) µg/ml Figure 4.

Table 3: Shows reducing power of *Terfezia claveryi*

Conc. (µg/ml)	*T. claveryi*	Abs. ± SD *T. claveryi*
0.64	$p < 0.001$ → ***	1.978±0.002
0.32	$p < 0.001$ → ***	1.918±0.004
0.16	$p = 1.000$ → ns	1.859±0.003
0.08	$p < 0.001$ → ***	1.732±0.004

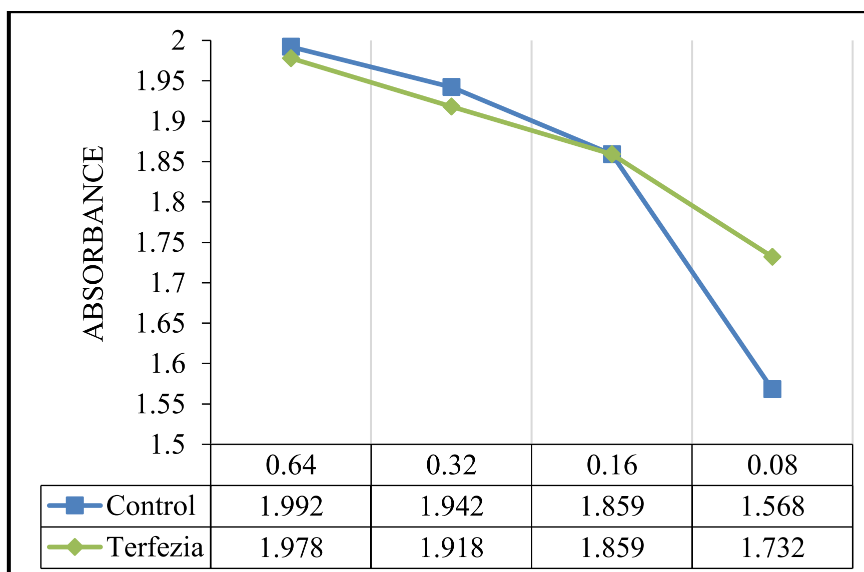

Figure 4: Shows reducing power of *Terfezia claveryi* compared with control (Trolox)

3.5 Catalase and peroxidase enzymes

In this study, it was proven that *T. claveryi* contains both catalase and peroxyase enzymes (0.862) U/mg and (6000) U/mg, respectively.

4. Discussion

Truffles were identified through morphological traits and exhibit a rough and solid texture, contrasting with the soft and delicate nature of other mushrooms, *T. claveryi* possesses a globose shape, lacks gills and a stalk, and features a wrinkled surface. [15, 12]. PCR is considered crucial for several procedures, including the principal diagnostic approach or its application in the earliest phases of diagnostic testing [16]. The results indicated that the increase in concentration correlates with an elevation in DPPH activity, consistent with the notion that free radical-scavenging activity is significantly affected by the sample's phenolic compounds [17]. In this study, IC50 was calculated using linear regression models correlating percentage inhibition (%I) with extract levels, and a lower IC50 value indicates greater antioxidant activity in a substance. While it is best to avoid comparing pure compounds and extracts because the former are purified compounds rather than combinations, whereas the concentration of each individual molecule is actually too low [18]. The current study examined the FRAP method that was used to assess the antioxidant activity of truffle ethanol extracts. It was an easy, quick test that was repeatable. Depending on the extract's power reduction, the yellow hue of the solution in this test turns either green or blue. Fe^{+3} ferricyanide complexes are reduced to generate Fe^{+2} ferrous when extracts containing reducing agents are present [12]. The highest power decrease is shown by the largest absorption; this result doesn't agree with Gouzi, H. *et al.,* they study antioxidant and

antiradical properties of methanolic extracts from Algerian wild edible desert truffles *Terfezia*, *Tirmania* and ascomycetes. However, they found truffles have the lowest reducing capacity, although their activities were less than those of positive controls [19]. Therefore, synthetic antioxidants trolox have better reducing ability compared to antioxidants from wild truffles, in order to reduce the harmful effects of ROS, antioxidant defense mechanisms are essential. One important enzyme antioxidant, catalase, breaks down hydrogen peroxide H_2O_2, a potentially dangerous by-product of the metabolism of cells into water and oxygen; this process stops oxidative damage and detoxifies H_2O_2 [20]. The crude extract of *T. claveryi* may function through various mechanisms, including antioxidant, antiradical, and antimicrobial activities; the antioxidant activity may be attributed to the potent constituents in truffles, which encompass a range of bioactive compounds, including organic acids and phenolic compounds such as gallic acid, fumaric acid, ferulic acid, vanillin, coumarin, alginic acid, trans-2-dihydroxycoumarin, catechin hydrate, 2,4-dihydroxybenzoic acid, and ellagic acid [21, 22]. Different fungal species utilize various enzymes as primary defenses against H_2O_2, which may necessitate diverse regulatory systems. This implies that the response of antioxidant enzymes to stressors may correlate with species-specific antioxidant system profiles [15].

5. Conclusions

Truffles, or terfess, are regarded as a luxury delicacy; their high cost accounts for their rarity. Where they grow naturally in the Iraq desert and other countries. According to basic nutritional principles, truffles are an excellent source of protein, fat, and minerals. In addition, *T. claveryi*, which grows underground and has a climate-specific effect, was most likely the cause of these chemical constituents such as carbohydrates, proteins, amino acids, fatty acids, vitamins, minerals, phenolics, and flavonoids, suggesting its potential as an antioxidant. Ethanolic extract demonstrated significant DPPH free radical scavenging activity and reducing power using the FRAP assay by contributing an electron or a hydrogen atom, indicating that they can function as antioxidants. The presence of the enzymes catalase and peroxidase in *T. claveryi* indicates their association with antioxidants. More in-depth research on truffles and fungi in general, which are abundant in Iraq's desert climate, should be done since they may have significant economic and health benefits.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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