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ORIGINAL ARTICLE

Physicochemical Properties, Nutritional Composition, and Phylogenic Analysis of Black Truffles Grown in Fars Province, Iran

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ABSTRACT

Background: Among all edible mushrooms, truffles are the most expensive ones. This study assessed nutritional properties and phylogenic characteristics of black truffles grown in two regions of southern Iran.

Methods: In this experimental study, the samples were collected from two towns of Firuzabad and Sarvestan in Fars Province, southern Iran. They were analyzed in terms of chemical properties (carbohydrate, protein, reducing sugar, antioxidant, fat, minerals, and ash) according to the Association of Official Analytical Chemists (AOAC) procedures. The sequence alignment and tree were determined using Molecular Evolutionary Genetics Analysis (MEGA7) software. The Internal Transcribed Spacer (ITS) region of rDNA of the two truffles was amplified using ITS1 and ITS4 primers and were sequenced. The phylogenic analysis was conducted using Nucleotide Basic Local Alignment Search Tool (BLAST) in the GeneBank (NCBI).

Results: Specimens for the two regions were very similar in composition. The samples of Firuzabad and Sarvestan contained 63.5 and 66% carbohydrate, 13.06 and 12.93% protein, 5.81 and 5.69% fat, 5.16 and 5.05% ash, 2.05 and 1.72% reducing sugar based on dry weight, respectively. The truffles belonged to the species of *Terfezia claveryi* and the IST sequences of the truffles of the two areas were similar.

Conclusion: The Iranian black truffles were shown to be a good source of carbohydrate, protein, and minerals. These truffles in comparison to other mushroom had more antioxidant activity considering Ferric Reducing Antioxidant Power (FRAP) values of 21.57 and 23.54 mmol per 100 g on dry-weight base. The genome sequences of truffles for the two cities were identical.

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Introduction

Mushrooms due to their particular flavor, nutritional properties and unique texture in many cultures are one of the most popular foods that are useful for human health (1, 2). Among all the edible mushrooms, truffles are the most expensive ones. Truffles are the fruiting bodies of the complex family of *hypogeous* fungi, mainly containing species of

the genera *Balsamia, Picoa, Terfezia, Tirmania,* and *Tuber*. Because of the physical characteristics, truffles are easy to distinguish; they have no stalk, no gills, and its mycelium grows underground (1).

They have been mainly found in the East European countries and the Middle East region. However, some truffle species have been found in South Africa, North America, Japan, and China. Truffles have various textures and colors (white, brown, and black). Desert truffles are the nutritious food and have a considerable amount of carbohydrates, protein, fatty acids, minerals, antioxidants and other nutritional or biological components (3-5). The nutritional and sensorial properties of this valuable mushroom vary depending on the species (6). So far, two species of the black truffles belonging to the genus *Terfezia* and one species of the white color truffles belonging to the genus *Tirmania* have been found in Iran (7).

Terfezia claveryi is a dark brown color truffle, locally called *Donbalan* in Fars Province, Iran. It grows in several regions of north and south of Iran that typically emerges in the deserts after the rainy period between February and April. Like other herbal products, the type and amount of the macronutrients and micronutrients of the species of truffle vary depending on the geographical location and climate in which it grows (7). The aim of this paper was to determine physicochemical properties and nutritional composition of black truffles (*T. claveryi*) in two towns of Fars province and also study the phylogenic relationships between these truffles and the other species in the world.

Materials and Methods

In this experimental study, fresh black truffles were collected from cracks in the soil near the host plants from eight different locations in two towns of Firuzabad and Sarvestan of Fars Province, southern Iran during the month of April 2017. All 16 specimens were brushed to remove the residue sludge and soil, rinsed with tap water to clean their surface and then rinsed with distilled water and stored in the freezer at -20°C until the assays were performed. The samples were analyzed for moisture, protein, fat, ash, carbohydrate, and reducing sugar content using Association of Official Analytical Chemists (AOAC) procedures (8).

For the mineral analysis, the freeze-dried sample (0.5 g) was weighed, while the cells were placed in microwave digestion equipment (Iran 2017), 3.5 mL of pure HNO_3 and 1.5 mL of H_2O_2 was added. After 80 minutes, the cells were removed and allowed to cool. Then deionized water was added to dissolve the ash. After filtration by Whatman filter paper No. 40, the clear solution was used for elemental analysis. An AA-6300 Shimadzu atomic absorption spectrophotometer (Shimadzu Italia, via G.B. Cassinis 7, 20139 Milano) was used for the determination of Cu, Fe, Na, K, Ca and Zn contents.

For determination of phosphorous content, 3-4 g of each sample was changed to ash in a muffle furnace (Model Ex.1200-4L, Exciton Co. LTD., Mashhad, Iran) at 500°C, overnight. The white ashes were mixed with 5 mL of hydrochloric acid (HCl 37%; Merck, Darmstadt, Germany) and boil-heated for 10 min on the hot plate, while the volume was kept constant by adding HCl during boiling. The digests were diluted to a final volume of 40 mL with deionized water in a beaker and boil-heated for 10 min. After adequate cooling, the solutions were filtered and made up to 100 mL with deionized water. Then, 4 mL of the solutions were mixed with 25 mL of molybdovanadate reagent (20 g L-1 ammonium molybdate, 1 g L-1 ammonium metavanadate, and 140 mL L-1 concentrated nitric acid). The final solution was diluted to a volume of 100 mL with deionized water followed by 10 min standing at room temperature. Finally, the absorbance was measured at 420 nm against a reagent blank. Potassium dihydrogen phosphate was used as a standard (9, 10).

The antioxidant properties of the truffles were conducted by Ferric Reducing Antioxidant Power (FRAP) assay. Preparation of the sample was performed according to the method by Al-Laith (2010) (3). To this aim, both types of truffles which were previously cleaned, they were dried at 65°C in an air-circulation oven to reach constant weight (for 3 days). The dried truffles were later pulverized. One gram of powdered truffles was extracted with 10 mL of phosphate buffer (7.1 mM) for 30 min using an Erlenmeyer shaker. Then, the suspension was centrifuged at 2000 g for 20 min. To prepare a reaction mixture, all reagents including acetate buffer (300mM, pH 3.6), $FeCl_3\_6H_2O$ (20 mM), and TPTZ (10 mM) were freshly provided and mixed in a ratio of 10:1:1. Thereafter, 0.1 mL of the clear extract was added to a flask containing 3 mL of reaction mixture, mixed and kept at room temperature. The absorbance was measured after incubation at 37°C for 8 min at 593 nm. Ascorbic acid was used as a positive control to generate the standard curve, and FRAP value was calculated relevant to the activity of ascorbic acid. Under these conditions, ascorbic acid had a relative activity of 2. The FRAP values were expressed as mmol per 100 g dry weight.

Genomic DNA was isolated from 150–200 mg of the inner gleba of the ascocarps using the EZNA Fungal DNA kit (Omega Bio-Tek, Doraville, GA, USA) based on the manufacturer's instructions. The Internal Transcribed Spacer (ITS) region

of the rDNA, including the 5.8S ribosomal gene, was amplified using the universal ITS1F and ITS4 primers (11). All PCR amplifications were carried out in a final volume of 25 µL containing 0.2 mM of each dNTP, 0.4 µM of each primer, 5.2 mM $MgCl_2$, 0.625X PCR buffer and 1.25 U of Taq DNA polymerase (Invitrogen, California, USA). PCR reactions were performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: an initial denaturalization step for 2 min at 94°C, 45 cycles consisted of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C, and a final extension at 72°C for 4 min. PCR products were purified using the EZNA Cycle-Pure kit (Omega Bio-Tek) according to the manufacturer's instructions. Clean PCR products were sequenced in both directions at the Molecular Biology Service (University of Murcia). The sequences generated in this study were deposited on GenBank (NCBI).

Nucleotide Basic Local Alignment Search Tool (BLAST) was used to compare the sequences obtained in this study with other DNA sequences in GenBank (NCBI) to provisionally identify the specimens prior to phylogenic analyses. Before sequence alignment, a decrease redundancy was undertaken using the ExPASy database. The sequence alignment and tree were carried out applying the Molecular Evolutionary Genetics Analysis (MEGA, version 7.0) software following the default options. The sequence alignment was carried out with ClustalW and phylogeny tree based on the Neighbor Joining method, with bootstrap of 1000 replicas and Maximal Parsimony (MP) by bootstrap of 500 replicas (12, 13). The sequences from *Tirimania pinoyi* and *T. nivea* were chosen as out group.

SPSS software (version 12.1, SPSS, Chicago, IL, USA) was used for statistical analysis. The results found in the present study were reported as mean values (obtained from the three replications)±Standard Deviation (SD). One-way analysis of variance was used to evaluate differences between two regions. Significance between means was defined at $p<0.05$.

Results

The contents of the moisture, protein, ash, fat, carbohydrate, reducing sugar, and antioxidant activity of the samples were shown in Table 1. According to the results, truffles of the two towns were very similar in chemical components as no significant difference was observed between their chemical composition except for reducing sugar ($p>0.05$). The findings also showed that the truffles were good source of protein and carbohydrate. Data related to the mineral contents was summarized in Table 2. Both analyzed groups illustrated that the major mineral was potassium and followed by phosphorus, calcium, and sodium. Iron, zinc, copper, and manganese were found at the low levels, and selenium was present in trace amount in the all analyzed samples.

There were significant differences between the two groups in the content of Ca, Na, and Fe that may be due to difference between the soils of the two towns. The ITS region of the rDNA, including the 5.8S ribosomal gene, from black truffles of Firuzabad and Sarvestan using the universal ITS1F and ITS4 primers could be successfully amplified. The gene sequences were deposited in Gene Bank with accession number of MN583175 and MN583046 (Supplementary data 1).

Sequence analyses of the ITS-rDNA from the examined samples produced one tree based on the Neighbor Joining method, with bootstrap of 1000 replicas and MP. In the phylogeny, the 26 sequences were clearly distributed in three wells separated lines that matched the three described species, including *T. claveryi*, *T. boudieri* and *T. arenaria* (Figure 1). The results of this study demonstrated that the genome sequences of truffles of two towns were quite similar and they were also close to the truffles with accession numbers of MH084950 and MG845398 that both belonged to Iran (Table 3).

Table 1: Physicochemical and chemical composition of black truffles (*Terfezia claveryi*) grown in south of Iran (Fars Province, Iran).

Properties	Firuzabad (mean±SD)	Sarvestan (mean±SD)
Moisture%	77.46±0.56[a]	77.6±0.88[a]
Ash%	5.16±0.3[a]	5.05±0.54[a]
Fat%	5.81±0.74[a]	5.69±1.1[a]
Protein%	13.06±1.52[a]	12.93±2.05[a]
[c]FRAP value (mmol/100 g dry weight)	21.57±0.94[a]	23.84±0.77[a]
Carbohydrate%	63.51±2.93[a]	66.02±3.13[a]
Reducing sugar%	2.05±0.09[a]	1.72±0.08[b]

In both groups (n=8). The chemical compositions are expressed as percentage of dry matter. [c]Antioxidant activity is expressed as FRAP value (mmol/100 g dry weight). Means with different letters (a,b) within the same group are significantly different ($p<0.05$).

Table 2: Mineral levels (mg/kg dry weight) of black truffles (Terfezia claveryi) grown in south of Iran (Fars Province, Iran).

Minerals	Sarvestan (mean±SD)	Firuzabad (mean±SD)
K (mg/kg dry weight)	25869±11456.6[a]	33105±10831.4[a]
P (mg/kg dry weight)	6497.2±68.2[a]	6237.3±269.3[a]
Ca (mg/kg dry weight)	2976.6±605.5[a]	1604±310[b]
Na (mg/kg dry weight)	2188.2±252.6[a]	1826.6±307.9[b]
Zn (mg/kg dry weight)	55.6±6.3[a]	57.3±8.3[a]
Cu (mg/kg dry weight)	43.6±9.5[a]	49.8±5.2[a]
Fe (mg/kg dry weight)	148.9±54.1[a]	92.0±18[b]
Se (mg/kg dry weight)	ND	ND

In both groups (n=8). Means with different letters (a,b) within the same group are significantly different (p<0.05).

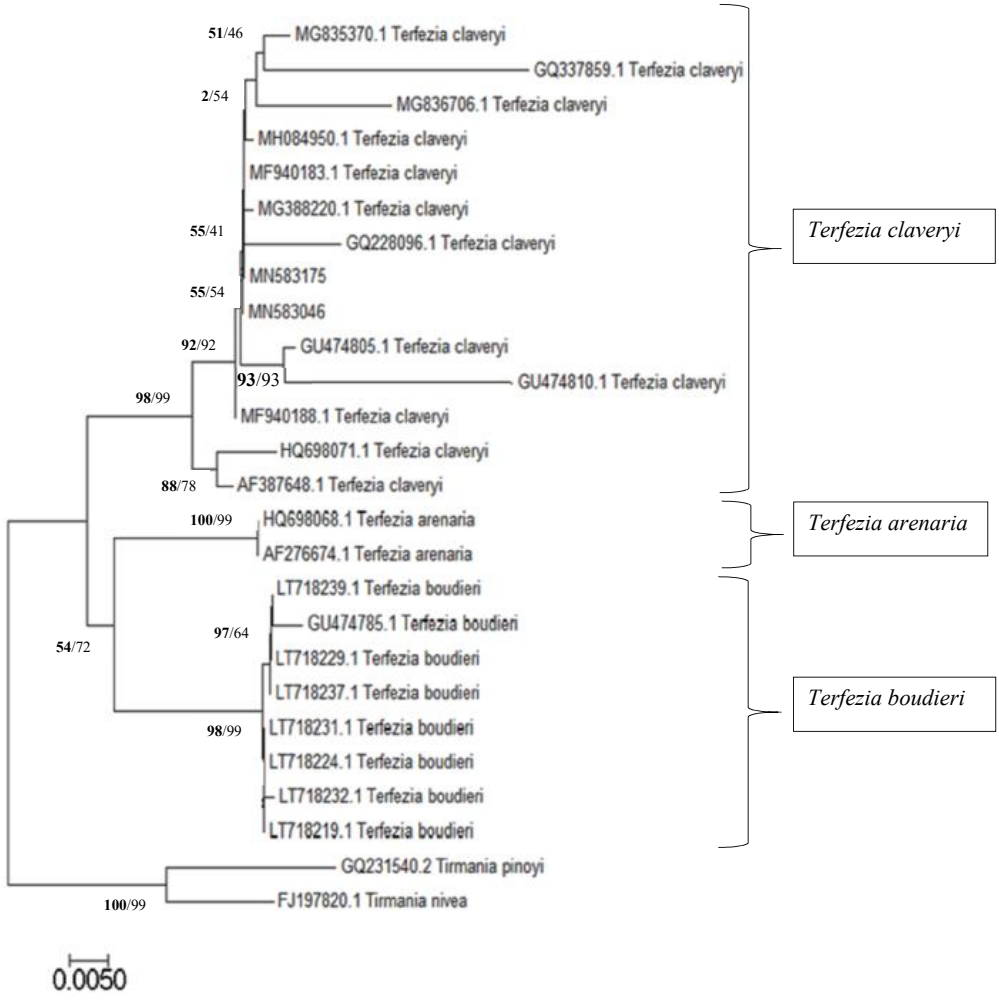

Figure 1: Neighbor-Joining phylogenetic tree. Numbers are percentages of 1000 bootstrapping replicates supporting the NJ tree presented here. Non-bold numbers are percentages of 500 bootstrapping replicates supporting the same node by the MP method.

Discussion

The results related to dry matter of truffles was in agreement with those reported before (14, 15). However, some other studies found higher moisture content and lower dry matter for truffles of Middle Eastern countries (5, 16-22). The regions where the truffles were collected are hot semi-arid zones with low annually rainfall. Therefore, the differences observed in various studies are probably related to changes in climate and soil types (16). Protein content of the samples were obtained about 13% which is close to the findings reported by Alirezalu *et al.* (2016) for black truffles (*T. claveryi*) in northwestern of Iran (13-17%) (16).

However, Vahdani *et al.* (2017) reported protein content of 3.35% that was significantly less than our results for Iranian truffles in three areas of Fars province (15). Nonetheless, such differences also were observed for the protein content of samples belonging to the same countries in other studies (5, 20, 21). For example, the studies which evaluated the chemical composition of *T. claveryi* in Saudi Arabia

Table 3: Terfezia collections analyzed.

Accession	Species	Country
LT718224	*Terfezia boudieri*	Italy
GU474785	*T. boudieri*	Southern Tunisia
LT718229	*T. boudieri*	Italy
LT718237	*T. boudieri*	Italy
LT718232	*T. boudieri*	Italy
LT718219	*T. boudieri*	Italy
LT718239	*T. boudieri*	Italy
LT718231	*T. boudieri*	Italy
HQ698068	*T. arenaria*	Spain
AF276674	*T. arenaria*	Spain
HQ698071	*T. claveryi*	Spain
AF387648	*T. claveryi*	Spain
MF940188	*T. claveryi*	North Africa
MF940183	*T. claveryi*	North Africa
GQ228096	*T. claveryi*	Iran
GU474805	*T. claveryi*	Southern Tunisia
GU474810	*T. claveryi*	Southern Tunisia
MG836706	*T. claveryi*	Iran
MH084950	*T. claveryi*	Iran
MN583046	*T. claveryi*	Iran
MN583175	*T. claveryi*	Iran
MG388220	*T. claveryi*	Iran
MG835370	*T. claveryi*	Iran
GQ337859	*T. claveryi*	Iran

reported a protein range content of 16% to 27% (5, 20, 21). Authors who assessed protein content of some edible truffle species grown in Italy depending on the species reported a range of about 7.6 to 24% (23, 24).

The protein content of Iraqi truffles in various studies also revealed different values as Al-Kaisey *et al.* (1996) reported 17.62%; while in another study, Al-Shabibi *et al.* (1982) obtained a range of 24.38 to 27.26 % for the same species (25, 26). The differences in protein content of the analyzed samples may be related to the climate and location (15). Due to the abundance of sulfuric amino acids such as cysteine, methionine, and lysine reported in previous studies (21, 23) and the results reported by Sawaya *et al.* (1985) and Kagan-Zur and Roth-Bejerano (2008) which showed a digestibility of about 85% for the desert truffles (20, 21), it seems that the protein of the truffles have a higher quality compared to the legume protein, such as beans and peas (16, 23).

Regarding the fact that ash generally contains heavy metals and silica, the truffles containing lower ash contents are more healthy (16). The ash content of our samples were in close agreement with results reported by Hussain and Al-Ruqaie (1999), Sawaya *et al.* (1985), and Hamza *et al.* (2016) for Iraqi, Saudi, and Tunisia truffles (18, 19, 21). However, the values were much higher than 0.70 to 0.90% reported by Alirezalu *et al.* (2016) for truffles of Northern regions of Iran (16). The fresh truffles grown in Italy and Turkey also showed a low ash content of about 1-1.9% (17, 24).

The fat contents obtained in this study were higher than the results of previous studies. For example, one study reported a range from 3.5 to 5.5% for Iranian truffles, or results of another study for Iraqi truffles showed 3.9% crude fat content for the same truffle species (*T. claveryi*) (16, 20). However, Vahdani *et al.* (2017) reported a higher value for *T. claveryi* (8.9 g per 100 g dry matter) in Iran. Hamza *et al.* (2012) also found a similar result (about 8%) for *T. boudieri* (15, 18). The fat contents reported by previous studies ranged from 0.89 to 19.9% in different truffle species of *Terfezia spp.*, *Tirmania spp.* and *Picoa juniperi* (27, 28). Comparison of three popular Saudi Arabian truffles including one white truffle (*Zubaidi*) and two black desert truffles (*Kholeissi* and *Gibaah*) showed that the truffles' fat contents were different from 2.81 to 7.42% (21).

The carbohydrate content of the samples was similar to the other studies (16, 17, 19, 29). Hamza *et al.* (2016) also reported a similar result for *T. boudieri* (18). Moreover, the reducing sugar contents exhibited a significant difference between reducing sugar contents of samples of the two regions ($p<0.05$). Alirezalu *et al.* (2016) also reported carbohydrate content of 1.33-1.67% (based on dry weight) for the Iranian black truffles (16). The antioxidant activity of the samples which were expressed in FRAP value

displayed a higher level compared to the results reported by Al-Laith *et al.* (2010) that showed the FRAP values of 18.62, 10.34, 14.62, and 18.06 mmol per 100 g (based on dry weight) for *T. nivea* grown in Bahrain, Iran, Morocco, and Saudi Arabia, respectively (3).

Some factors such as seasonal variations, climatic conditions, and geographical area affect the antioxidant content of plant and natural foods. Also antioxidant activity might be reduced during the consumption (3). The results related to the both analyzed groups showed that the major mineral was potassium and followed by phosphorus, calcium, and sodium. Iron, zinc, copper and manganese were found at low levels. Selenium was present in trace amount in the all analyzed samples. As Table 2 shows there were significant differences between two groups in contents of calcium, sodium, and iron. Levels of all three minerals in the samples of Sarvestan town were generally higher than Firuzabad town.

According to the results, 100 g fresh truffle of Sarvestan and Firuzabad towns can provide 9.5 and 17.74%, 39.36 and 40.36%, and 64.05 and 39.61% requirement daily intake of calcium, zinc, and iron, respectively. The obtained results were comparable with findings reported in previous studies (15, 16). Contents of sodium and phosphorus of our samples were almost similar to Iranian truffles which were investigated by Alirezalu *et al.* (2016) (16). However, the amount of potassium and calcium in our truffles were significantly higher than their findings. Singer *et al.* (1961) reported a range of 18.4-30.2% phosphorus for fresh truffles grown in Italy (24).

One study in Iraq also found phosphorus as the major element (19). Sawaya *et al.* (1985) also found potassium (1408 to 1734 mg /100g dry weight) followed by phosphorus (506 to 756 mg/100g dry weight) as the major elements in all types of the tested truffles in Saudi Arabia (21). One study which assessed nutritional value of two truffle species of *T. olbiensis* and *T. claveryi* showed that potassium (24254.54 and 19337.34 mg kg-1 dry weight) was higher than the level of other minerals followed by magnesium (1.017 and 998 mg kg-1 dry weight), phosphorus (837 and 469 mg kg-1 dry weight) and calcium (458 and 718 mg kg-1 dry weight) that were major elements, respectively (30).

Hamza *et al.* (2016) assessed the mineral content of *T. boudieri* in Southern Tunisia. According to their results, potassium was found to be the most prevalent element (1512.6 mg/100 g dry weight) followed by calcium, phosphorus, iron and magnesium, respectively (18). Dundar *et al.* (2012) also showed that in *T. boudieri* of Turkey, magnesium was the most abundant element (182.30 mg/100 g dry mass) and followed by potassium (102.6 mg/100 g), sodium (11.10 mg/100 g), and calcium (10.00 mg/100 g), respectively (17). Literature revealed that in comparison to the agriculture crops, mushroom contained higher level of heavy metals.

Conclusion

Sequence analysis and the phylogenic study showed that the collected truffles from two regions of Fars Province belonging to the species of *T. claveryi* were close to the truffles with accession numbers of MH084950 and MG845398 that both belonged to Iran. A comparison of nutrient values of two regions revealed that the truffles of both areas were very similar in composition. This study also showed that the truffles can be considered as a good source of protein, carbohydrate, minerals and to some extent antioxidants. Totally, our results were comparable with those reported previously and the differences are probably due to the variation in climate and also soil characteristics.

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Conflict of Interest

None declared.

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