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Evaluation of nutritional value and dietary chemicals of winter truffles (*Tuber brumale*) from north of Iran

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Abstract: This study aimed to evaluate nutritional value and identify the mycochemical composition of winter truffles (*Tuber brumale*) with two different flesh colors (dark and bright) that were found in the Hyrcanian forests (Iran). The; carbohydrates, protein, and fat content were 11.3 mg. g^{-1} dry weight, 12.53%, and 2.41% in dark winter truffle and 12.64 mg. g^{-1} DW, 11.06%, and 2.4% in bright type, respectively. Although the results of GC-MS analysis showed that fatty acids and esterified fatty acids are the most abundant in methanolic extracts, ergosterol was the dominant compound in methanolic extracts, which was quantified 15.88 and 12.89% in dark and bright types, respectively. The most identified compounds in n-hexane extracts were alkanes. Also, the amount of ergosterol, as the dominant compound in n-hexane extracts, was quantified 32.41 and 24.3% in dark and bright winter truffles, respectively. In the methanolic extract of dark *T. brumale*, the Phenol, 2-methyl-5-(1-methyl ethyl) or carvacrol were measured 0.25%. The 2,4-Di-tert-butylphenol was one of the phenolic compounds which were discovered at 0.54% in n-hexane extract of bright winter truffle. Also, squalene as a natural triterpene was identified in methanolic and n-hexane extracts of both types of *T. brumale*. The most concentration of squalene (1.91%) was recorded in n-hexane extract of dark *T. brumale*.

Key words: Antioxidant, Ergosterol, Phenol, Squalene, True truffle, *Tuber brumale*

INTRODUCTION

Truffles (*Tuber* spp.) are classified in the fungi kingdom. They are known as a hypogeous Ascomycota that can produce valuable fruiting bodies after establishing a mycorrhizal symbiosis with certain vascular plants, including gymnosperms and angiosperms (Harki et al. 2006, Strojnik et al. 2020). These edible fungi contain different medicinal substances crucial for human health because of their anti-inflammatory, antioxidant, antimicrobial, anti-mutation, and anticancer properties (Yan et al. 2017). Some species of truffles are considered among the most expensive foods in the world, so their usages has been limited as a flavoring recently (Pacioni et al. 2014). Volatile Organic compounds (VOCs), including a mixture of alcohols, ketones, aldehydes, alongside aromatic and sulfuric compounds, are responsible for the unique odor of truffles (Vahdatzadeh & Splivallo 2018).

Truffles release some VOCs during their life cycle to interact with particular organisms. Following spore germination, fungus hypha searches for the root of host plants as long as the establishing a symbiosis relationship and forming an ectomycorrhiza. Fruiting bodies of truffles can be produced a couple of years after the establishment of symbiosis relationship, and this part of fungi can help ascospores spread by animals. Therefore, adult ascocarps diffuse intense aroma to attract different animals. Two compounds of C_2H_4 and 1-octen-3-ol have a signaling role for making a symbiosis relationship with host plants, and sulfuric volatiles are essential to attract animals like dogs and hogs (Splivallo et al. 2011).

A specific odor of truffles is partially familiar in different species, although some compounds are identified as exclusive for one species or limited in a few truffle species. For example, 2- methylbutanal, 3-methylbutanal, 2- methylbutan-1-ol, and oct-1-en-3-ol were detected in most of the truffle species, but 2,4-dithiapentane can be just found in *T. magnatum* (Vahdatzadeh & Splivallo 2018, Strojnik et al. 2020).

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Numerous studies were carried out to identify VOCs of Périgord truffle (*T. melanosporum*) (Mauriello et al. 2004). VOCs, were identified in the mycelium and peridium of *T. brochii* (Bellesia et al. 2001). Evaluation, of VOCs of winter truffle (*T. brumale*) indicated that all Dimethylsulfide, butanone, 2-methylbutanal, 1-methylpropyl formate, 2-methylpropanal, and 1,4-dimethoxybenzene were detected in the high level (Mauriello et al. 2004). Another study showed that 2-methyl-1-propanol, 1-octen-3-ol, 3-Methylbutanal, 2-Methylbutanal, and Dimethylsulfide are main sulfuric, alcoholic and aldehydic substances in *T. brumale* (Splivallo et al. 2011). Also, some crucial compounds like 1-methoxy-3-methylbenzene, 1,4- dimethoxybenzene, oct-1-en-3-ol, octan-3-one, and butan-2-yl formate were reported as the main aromatic compounds responsible for the odor of *T. brumale* (Strojnik et al. 2020).

In addition to volatile compounds, truffles have considerable importance for low fat and high protein (Harki et al. 2006). Also, some reports demonstrated the high antioxidant property of truffles in blocking free radicals thank their high magnitude of polyphenols, flavonoids, and sterols (Villares et al. 2012, Tang et al. 2012). Based, on molecular analysis, around 180 various truffle species exist throughout the world (Bonito et al. 2013), and 30 of them are being traded in different markets (Vahdatzadeh & Splivallo 2018).

The history of scientific identification and research on true truffles in Iran is not so long, and the first report of a species of *Tuber* genus related to Merényi et al. (2014) that among the evaluation of phylogeny and phylogeography of *Tuber brumale*'s different populations, reported *T. brumale* from Iran. Jamali (2016) identified *Tuber aestivum* in Kermanshah province. Recently, Puliga et al. (2021) have reported seven species of true truffles (*T. aestivum, T. borchii, T. brumale, T. macrosporum, T. rufum, T. lucidum, T. excavatum, and T. fulgens*) from northern parts of Iran. Their research showed that unique ecological properties of Hyrcanian forests, provide a suitable situation for true truffles species growth.

In another research, Puliga et al. (2021) reported a new species of Tuber genus (*T. iranicum*) as an endemic species from the north of Iran. Our probing in the Hezarjerib region, Behshahr city of Mazandaran, Iran, was yielded to find winter truffle (*T. brumale*). So; this study targeted identifying the most critical biochemical compounds of winter truffle harvested in this district.

MATERIALS AND METHODS

Truffle samples

Winter truffles were collected from HezarJerib region in 36° 37́ 11.6″ latitude and 53° 43́ 8″ longitude, Behshahr, Mazandaran, Iran (Voucher number: Sanru-100-01). Then, all of the harvested truffles were transferred to Sari Agricultural Sciences and Natural Resources University for technical evaluation.Winter truffles that had two different tissue colors (dark and bright) were cleaned carefully and then stored in vacuum packages at 4 ˚C (Fig. 1). To evaluate the dietary value of truffles, some characteristics, including total protein, total soluble carbohydrate, and total fat, were assessed. Also, all samples were extracted with two solvents comprising n-hexane and methanol. Then, total antioxidant activity, total Phenol, and total flavonoids were measured. These; extracts were analyzed by their chemical compounds using GC-MS.

Morphological and molecular identification of winter truffles ascocarps:

Morphological identification of truffles ascocarps was made to evaluate asci and ascospores width, length, and shape. The data and obversions were investigated based on Dimitrova and Gyosheva's (2008) descriptions for *T. brumale*'s morphological properties. For molecular characterization, DNA was isolated from the selected ascocarps, based on Bonito et al. (2013). The nuclear loci used for molecular identification were ribosomal internal transcribed spacer (*ITS*), and the PCR was amplified using ITS5/ITS4 primer pair (white et al. 1990). Finally, the sequences were deposited in GeneBank (Table 1).

Standards and reagents

Ethanol 99.9%, methanol 99.9%, n-hexane ACS grade, hydrochloric acid 37%, sodium carbonate, aluminum chloride, sodium hydroxide, copper (II) sulfate pentahydrate, sodium sulfate, and boric acid were purchased from Merk (Germany). Sulfuric acid 99.9%, 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), Folin-Ciocalteu reagent, gallic acid, and glucose were purchased from Sigma-Aldrich (USA). Potassium acetate and anthrone were supplied from Bio Basic (Canada). Petroleum ether was purchased from ASD (Iran), and methyl red was bought from Neutron (Iran).

Preparation of methanol and n-hexane extract

At first, ascocarps of truffles were washed carefully, and their pridium were removed (Strojnik et al. 2020). Ascocarp tissue was cut as thin slices to be dried in the oven (manufactured by MEMMERT company, Germany) at 37 ˚C for 48 hours. Dried slices of ascocarp were grinded and converted to fine truffle powder. Exactly, 1 g of truffle powder was extracted by 10 ml of methanol and n-hexane from the glassy flask, at 27 ± 1˚C, shaking 100 rpm, for 72 hours. The harvested extracts were kept in the dark place at 4˚C till using.

Gas chromatography-mass spectrometry (GC-MS) analysis

A 7890 Gas Chromatograph system coupled with a 5975C mass-selective detector (MS) (Agilent Technologies, USA) equipped with an HP-5MS capillary column (30m*0.25mm*0.25µm) was used for the separation and identification of extracted compounds.

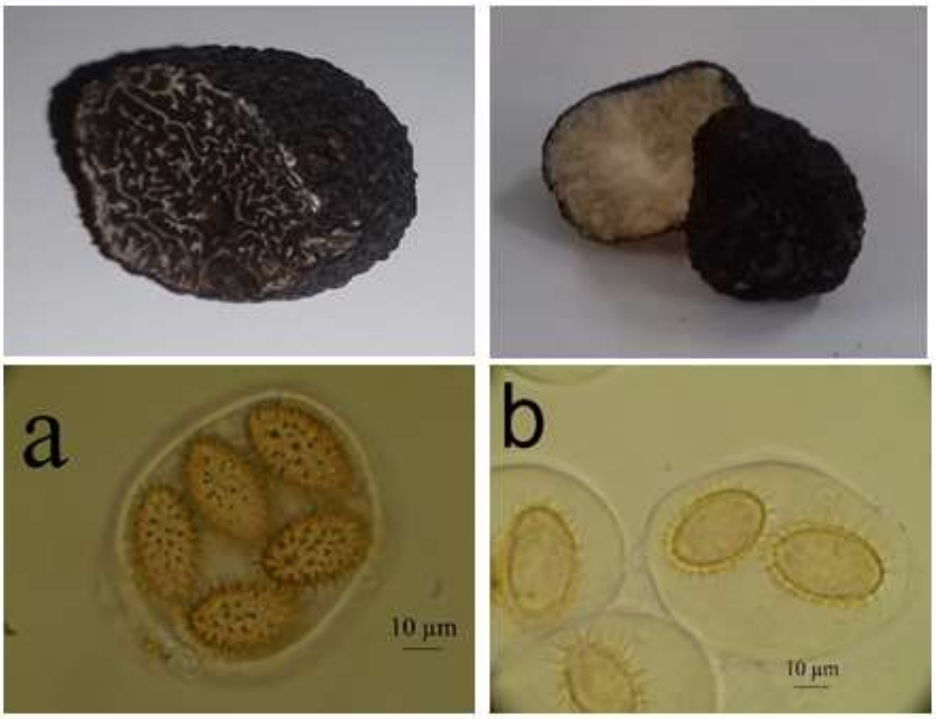

Fig. 1. Ascocarps; of winter truffles (*Tuber brumale*) with two different flesh colors (dark and bright) were collected from Hezarjerib Behshahr, Iran. a: Asci and ascospores of dark flesh *T. brumale*, b: Asci and ascospores of bright flesh *T. brumale***Fig. 1.** Ascocarps; of winter truffles (*Tuber brumale*) with two different flesh colors (dark and bright) were collected from Hezarjerib Behshahr, Iran. a: Asci and ascospores of dark flesh *T. brumale*, b: Asci and ascospores of bright flesh *T. brumale*

Table 1. Identification of truffles ascocarps according to the molecular method.

Source	**Isolate name**	**Sequences length (bp)**	**Accession number**	**Blast match**	
				Species	**Accession number**
Truffles ascocarp (white flesh)	culture20	859	MT495424	*T. brumale*	OL672491
Truffles ascocarp (dark flesh)	Culture32	869	MT495426	*T. brumale*	OL672491

The injector temperature was 260° C. The temperature of the oven was 50 ° C for 1 min, then rose to 180°C with 10°C.min^{-1} ratios and was held for 1 min. The run time in this step was 15min. After that, the temperature increased to 280°C with the ratio of 5°C.min^{-1} and holding for 5min. Also, the run time in this step was 40min. The carrier gas (He) flow rate was 1 ml/min. The electron impact (EI) ion energy was 70 eV, and the ion source temperature was 230°C; the chromatograms were obtained by recording the total ion currents in the range of 50–450 *m/z*.

Total antioxidant activity

For evaluation of the antioxidant activity of samples, the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used based on the method used by (Yan et al. 2009). To determine the free radical-scavenging activity of the extracts, specific concentrations of each extract were added, at an equal volume, to the methanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. Finally, total antioxidant activity was expressed as the inhibition percentage of DPPH.

Determination of total phenol content

Folin-Ciocalteau method was used to measure total phenolic contents (Yan et al. 2009). Briefly, 0.5 ml of extracts were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min, and aqueous sodium carbonate (4 ml, 1 M) was then added. The mixture was left at ambient temperature for 15 min, and the phenols were determined by spectrophotometer at 765 nm. Total phenol contents were calculated by referring to the standard curve and finally expressed in gallic acid equivalent (mg. g^{-1} of dry mass).

Determination of total flavonoid content

To determine flavonoid content, the aluminum chloride method was used based on the approach explained by (Yan et al. 2017). In summary, 0.5 ml solution of each extract was separately mixed with 1.5 ml of methanol, 0.1 mL of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and then kept at ambient temperature for 30 minutes. The absorbance of the reaction mixture was read at 415 nm with a spectrophotometer. Finally, total flavonoid contents were calculated as mg quercetin per gr dry matter, referring to a calibration curve.

Dietary value

Total protein and fat: Total protein percentage of samples was determined by the macro-Kjeldahl method using N×4.38 equation. The fat percentage of truffles was measured by a Soxhlet extractor with petroleum ether as solvent (Yan et al. 2017).

Total soluble carbohydrates were determined based on The protocol developed by (McCready et al. 1950). Briefly; 150 mg of anthrone was solved in 100 ml of diluted sulfuric acid (76 ml of sulfuric acid was diluted by 38 ml distilled water). Then, 100 µl of ethanolic extract of samples was added 3 ml by anthrone solution. The mixture was placed in the hot water bath for 20 min, and finally, absorbance was recorded at 620 nm. After; obtaining a standard curve, using the determined concentrations of glucose, the content of sugar was expressed as µg per gr dry matter.

Statistical analyzes

All obtained data was subjected to MS Excel then analyzed by SAS, version 9.1. In the end, the comparison of means was carried out using the Duncan test. Also, all graphs were drawn using Excel software.

RESULTS AND DISCUSSION

Dietary value Macronutrients

The results showed no statistically significant differences between the two colors of winter truffle in carbohydrate, protein, and fat contents. Carbohydrates concentration from dark and bright truffles were 11.3 and 12.64 mg. gr^{-1}, respectively (Fig. 2). The amount of protein in the dark and white truffles was recorded at 12.53, and 11.06% (Fig. 2) and fat content was 2.41, and 2.4 %, respectively (Fig. 2). Saltarelli et al. (2008) point out that protein content of *T. magnatum*, *T. borchii*, *T. melanosporum*, and *T. aestivum*, collected in the central part of Italy were 24, 13.1, 8.7, and 11% that our truffles had more protein content than two species of aestivum and melanosporum but less than two other species. That; article released 2.23, 3.59, 1.77 and, 5.56 mg total carbohydrate per 100 gr dry mater of those four truffles that *T. brumale* in our experiment had more carbohydrate than all of them. Protein; and fat content of *T. aestivum* harvested from Hungary were 19.11 and 2.21%, respectively (Kruzselyi & Vetter 2013). Therefore, in comparison with our data, *T. brumale* had less protein and more fat than Hungarian *T. aestivum*. Yan et al. (2017) evaluated three species of truffles, including *T. latisporum*, *T. psuedohimalayense*, and *T. subglobosum* and reported the protein contents of these species 14.64, 14.28 and 10.96%, respectively. So recorded protein content in our study was more than *T. subglobosum* and less than two others. They also reported the fat content of these truffles 2.4, 2.25, and 2.23% respectively (Yan et al. 2017). Obtained fat content in our studies was near *T. latisporum*.

Antioxidant properties

Total phenol content of methanolic extract of *T. brumale* was 0.469 in bright and 0.471 (mg gallic acid. g dry $weight^{-1}$) in dark ones (Fig. 3), but these figures in n-hexane extract were 0.481 and 0.342 (mg GA. g DW^{-1}) respectively (Fig. 3). For flavonoid content, methanolic extract of bright and dark truffles were 0.0183 and 0.00724 (mg quercetin. g DW^{-1}), however, in n-hexane extract, 0.01934 and 0.0227 (mg Q. g DW^{-1}) were recorded (Fig. 3). Observed differences between two kinds of tested truffles were statistically significant in phenol and flavonoid contents of n-hexane extract, and flavonoid in the methanolic extract at 0.05, 0.01, and 0.05 percent of probability level.

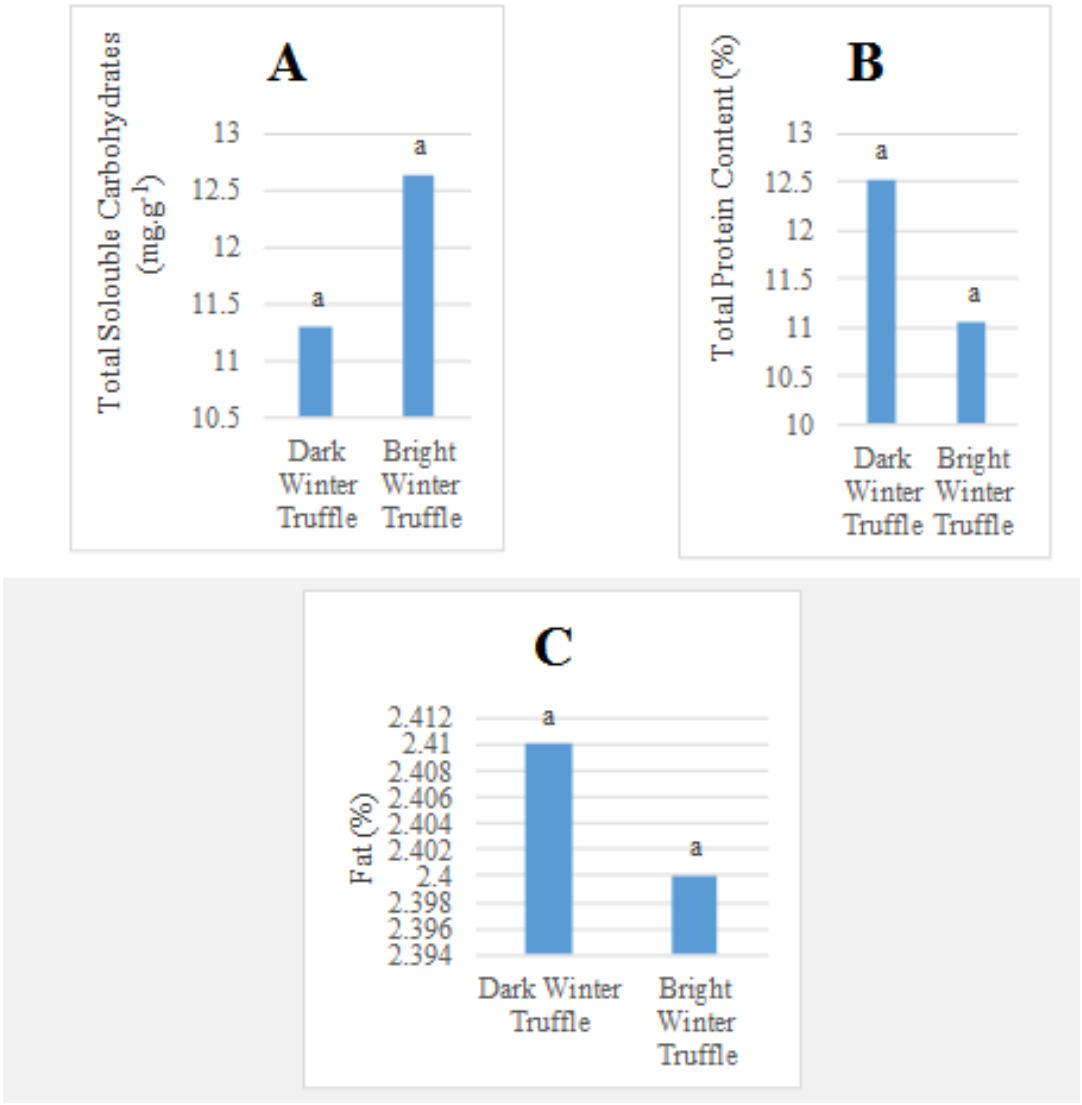

Fig. 2. A: Total soluble carbohydrate, B: Total protein content, C: Total fat content in *T. brumale* collected from Hezarjerib Behshahr, Iran

Total antioxidant activity in the methanolic extract was 74.17 and 70.82% of free radical inhibition for dark and bright truffles, respectively (Fig. 3), but this trait was 73.79 and 73.27% in n-hexane extract (Fig. 3). The observed differences in total antioxidant activity were not statistically significant, neither between the solvents nor the two kinds of truffles.

Based on (Yan *et al*., 2017), who tested various species of truffles, the highest content of Phenol (735.01 mg of GAE/100 g extract) and flavonoid (1355.43 mg of Rutin/100 g extract) were observed in *T. pseudohimalayense* and *T. subglobosum*, respectively.

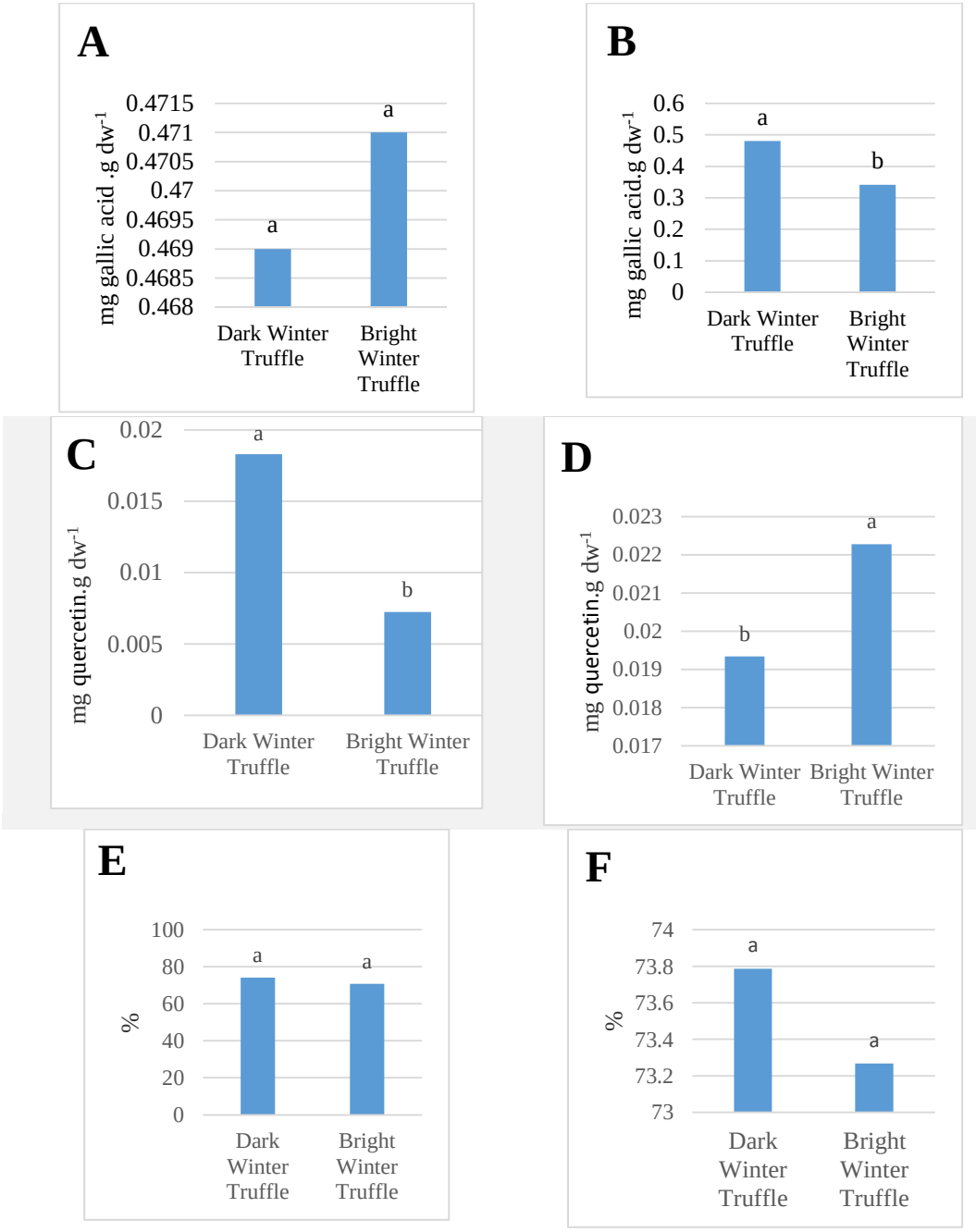

Fig. 3. A: Total phenol content in methanolic extract (ME), B: Total phenol content in n-hexane extract (HE), C: Total flavonoid content in ME, D: Total flavonoid content in HE, E: Total antioxidant activity in ME, F: Total antioxidant activity in n-hexane extract of *T. brumale* collected from Hezarjerib Behshahr, Iran.

Gas chromatography analysis

Methanolic extracts

Results of GC analysis of *T. brumale* methanolic extract identified twenty-two compounds in dark truffle, including thirteen fatty acids and esterified fatty acids, three sterols, one alkane, one alcohol, one phenol, one unsaturated aldehyde, and one triterpene (Table 2). In contrast, the number of identified compounds in bright truffle was twenty, including twelve fatty acids, four sterols, one phenol, one cycloalkene, one alkane, and one triterpene (Table 3). Among fatty acids and esterified fatty acids in dark winter truffle, such as Methyl 10-trans,12-cis-octadecadienoate, 9,12-Octadecadienoic acid, Hexadecanoic acid methyl ester, and Linoelaidic acid were dominant compounds with 10.29, 5.89, 4.92,and

4.2%, respectively (Table 2). Although, in bright truffle, Methyl 10-trans, 12-cis-octadecadienoate with 7.64% was the highest fatty acid, its content was less than dark one (Table 3).

The second abundant fatty acid in bright truffle with 6.47% was Linoelaidic acid which was more than the dark truffle. In the following, Hexadecanoic acid, methyl ester, and 9-Octadecenoic acid (Z)-, methyl ester with 4.03 and 3.7% respectively were in the highest amount (Table 3).

Based on (Omer et al. 1994), *Tirmania nivea* had three compounds comprising Hexadecanoic acid, ll,14-Eicosadienoic Acid methyl ester, and Octadecanoic acid in amounts of 49.11, 18.8, and 18.8% as the most quantitatively essential substances in this edible fungus. Also, other reported compounds in *Tirmania nivea* include Tetradecanoic acid and Pentadecanoic acid, both of which are categorized in the fatty acid groups (Omer et al. 1994). Based on (Yan et al. 2017), Linoleic acid and Oleic acid were introduced as the primary fatty acids of three tested truffle species (T. *latisporum, T. subglobosum*, and *T. pseudohimalayense*).

Ergosterol, with 15.88%, was the dominant compound among the identified sterols in methanolic extract of dark winter truffle (Table 2). In addition to ergosterol, two other substances including Ergosta-5,22-dien-3-ol (6.30%) and Campesterol (1.22%) were identified (Table 2). In contrast, in bright winter truffle, ergosterol (12.89%), 5,22-dien-3-ol (8.28%), Ergosta-7,22-dien-3-ol (5.71%) and Campesterol (1.92%) were identified that Ergosta-7,22-dien-3-ol was only detected in methanolic extract of bright winter truffle (Table 3).

Ergosterol which was identified as the dominant compound of methanolic extract in both dark and bright winter truffles is an essential substance from different aspects. On the one hand, ergosterol can be converted to vitamin D_2 precursor following exposure to ultra violate radiation (280-320 nm). On the other hand, ergosterol and its derivations contain a broad range of health improvement factors like antioxidant activity, anti-inflammatory, and anti-hyperlipidemia (Villares et al. 2012).

Another compound that was found in methanolic extract of bright winter truffle was an unsaturated aldehyde, 9,17-Octadecadienal, (Z)-, with 0.82% quantity (Table 2). This compound was reported in methanolic extract of *Lentinus squarrosulus* previously (Adeoye-Isijola et al. 2018).

Other identified compounds in methanolic extract of bright winter truffle were Dianhydromannitol and Undecane (Table 2). Dianhydromannitol, as alcohol is produced from mannitol during a metabolic pathway (Wiggins, 1949). It is well established that glucose, mannitol, trehalose, and their derivatives are common carbohydrates in edible mushroom tissue, although their amount in the dry matter may not be very much (El Enshasy et al. 2013).

In the methanolic extract of winter truffles, two compounds, including squalene, and Phenol, 2-methyl-5-(1-methylethyl), were measured 0.63 and 0.25% in dark type (Table 2), and 0.65 and 0.43% in bright one (Table 3). Squalene is a natural triterpene and an intermediate substance in the cholesterol biosynthesis pathway.

According to different studies, squalene has a direct and indirect roles in cancer prevention and anticancer effects (Reddy & Couvreur 2009). This compound was reported in *Terfezia claveryi* collected from west Iraq, at 10.09% (Dahham et al. 2018). Phenol, 2-methyl-5-(1-methylethyl), or carvacrol, which has antimicrobial, anticancer, and antioxidant properties, is a monoterpenoid that can be found in large quantities in *Origanum vulgare*, *Thymus vulgaris*, and *Citrus aurantium* bergamia (Sharifi-Rad et al. 2018).

n-Hexane extracts

Gas chromatography analysis of n-hexane extract showed that twenty-six different compounds exist in dark winter truffle, including sixteen alkanes, three sterols, two iodo alkane, two alkatrienes, one esterified fatty acid, one unsaturated aldehyde, and one triterpene (Table 4). In n-hexane extract of bright winter truffle, thirty compounds were identified that comprised of eighteen alkanes, three sterols, three iodo alkanes, one fatty amide, one esterified fatty acid, one phenol, one unsaturated aldehyde, one alkatrienes and one triterpene (Table 5).

In dark winter truffles, decane (12.4%), heneicosane (2.72%), and eicosane (2.30%) were dominant alkanes (Table 4). Main alkanes in bright winter truffle were similar to detected alkanes in dark winter truffle, but their quantities were different, so decane, heneicosane, and eicosane were observed 90.09%, 1.37%, and 1.25%, respectively (Table 5). (Bellesia *et al*. 1996) revealed that decane is an identified alkane in *T. magnatum* collected from the central region of Italy. Also, this compound was reported in other truffle species, including *T. excavatum*, *T. borchii*, and *T. brumale* (Mauriello et al. 2004).

Table 2. Analysis of methanolic extract of dark winter truffle by gas chromatography

Compounds	Amount (%)	Time (min)	Score (%)	Chemical Formula
Alkanes				
Undecane	0.36	7.494	81	C11H24
Alcohols				
Dianhydromannitol	0.75	9.253	86	C6H10O
Phenols				
Phenol, 2-methyl-5-(1-methylethyl)-	0.25	10.487	93	C10H14O
Fatty acids and esterified fatty acids				
Hexadecanoic acid, methyl ester	4.92	18.974	98	C17H34O2
n-Hexadecanoic acid	3.92	19.588	99	C16H32O2
Methyl 10-trans,12-cis-octadecadienoate	10.29	21.772	99	C19H34O2
9-Octadecenoic acid (Z)-, methyl ester	5.36	21.865	99	C19H36O2
8-Octadecenoic acid, methyl ester	1.49	21.962	99	C19H36O2
Methyl stearate	1.95	22.297	98	C19H38O2
9,12-Octadecadienoic acid (Z,Z)-	5.89	22.456	99	C18H32O2
Oleic Acid	3.24	22.535	98	C18H34O2
Octadecanoic acid	0.73	22.884	99	C18H36O2
9,12-Octadecadienoic acid, methyl ester	0.42	23.289	98	C19H34O2
Eicosanoic acid, methyl ester	0.22	25.538	91	C21H42O2
Linoelaidic acid	4.2	30.98	95	C18H32O2
Tetracosanoic acid, methyl ester	0.25	31.557	94	C25H50O2
Alkatrienes				
1,8,11-Heptadecatriene, (Z,Z)-	0.22	27.037	83	C17H30
Sterols				
Ergosta-5,22-dien-3-ol, (3.beta.,22E,24S)-	6.30	37.730	87	C28H46O
Ergosterol	15.88	38.465	81	C28H44O
Campesterol	1.22	38.805	74	C28H48O
Unsaturated aldehydes				
9,17-Octadecadienal, (Z)-	0.82	31.101	95	C18H32O
Triterpenes				
Squalene	0.63	32.916	90	$C_{30}H_{50}$

Table 3. Analysis of methanolic extract of bright winter truffle by gas chromatography

Compounds	Amount (%)	Time (min)	Score (%)	Chemical Formula
Phenols				
Phenol, 2-methyl-5-(1-methylethyl)-	0.43	10.487	94	$C_{10}H_{14}O$
Fatty acids and esterified fatty acids				
Hexadecanoic acid, methyl ester	4.03	18.974	98	$C_{17}H_{34}O_2$
n-Hexadecanoic acid	3.66	19.593	99	$C_{16}H_{32}O_2$
Methyl 10-trans,12-cis-octadecadienoate	7.64	21.772	99	$C_{19}H_{34}O_2$
9-Octadecenoic acid (Z)-, methyl ester	3.70	21.865	99	$C_{19}H_{36}O_2$
8-Octadecenoic acid, methyl ester	0.96	21.962	99	$C_{19}H_{36}O_2$
Methyl stearate	1.40	22.279	99	$C_{19}H_{38}O_2$
9,12-Octadecadienoic acid (Z,Z)-	3.45	22.451	98	$C_{18}H_{32}O_2$
Octadecanoic acid	0.50	22.880	97	$C_{18}H_{36}O_2$
Eicosanoic acid, methyl ester	0.16	25.538	87	$C_{21}H_{42}O_2$
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	0.64	28.354	70	$C_{19}H_{38}O_4$
Linoelaidic acid	6.47	30.984	95	$C_{18}H_{32}O_2$
Tetracosanoic acid, methyl ester	0.37	31.562	81	$C_{25}H_{50}O_2$
Cycloalkenes				
Cyclooctene, 3-ethenyl-	0.16	27.037	70	$C_{10}H_{16}O$
Sterols				
Ergosta-7,22-dien-3-ol, (3.beta.,5.alpha.,22E)-	5.72	30.877	89	$C_{30}H_{48}O_2$
Ergosta-5,22-dien-3-ol, (3.beta.,22E,24S)-	8.28	37.739	93	$C_{28}H_{46}O$
Ergosterol	12.89	38.470	93	$C_{28}H_{44}O$
Campesterol	1.91	38.810	90	$C_{28}H_{48}O$
Alkynes				
7-Pentadecyne	0.29	30.584	94	$C_{15}H_{28}$
Triterpenes				
Squalene	0.65	32.916	90	$C_{30}H_{50}$

Table 4. Analysis of n-hexane extract of dark winter truffle by gas chromatography

Compounds	Amount (%)	Time (min)	Score (%)	Chemical Formula
Alkanes				
Nonane, 3-methyl-	1.27	5.543	86	$C_{10}H_{22}$
Decane	12.40	5.981	91	$C_{10}H_{22}$
Decane, 2,3,5-trimethyl-	0.51	8.965	78	$C_{13}H_{28}$
Hexadecane	0.91	14.132	97	$C_{16}H_{34}$
Heptadecane	0.33	15.636	83	$C_{17}H_{36}$
Heptadecane, 8-methyl-	1.64	16.953	90	$C_{18}H_{38}$
2-methyloctacosane	0.82	18.946	72	$C_{29}H_{60}$
Eicosane	2.30	20.165	97	$C_{20}H_{42}$
Heptacosane	0.69	22.446	90	$C_{27}H_{56}$
Octacosane	0.75	23.126	86	$C_{28}H_{58}$
Heneicosane	2.72	23.452	91	$C_{21}H_{44}$
Pentacosane	0.68	25.877	86	$C_{25}H_{52}$
Tetracosane	3.58	26.645	94	$C_{24}H_{50}$
10-Methylnonadecane	2.23	29.657	91	$C_{20}H_{42}$
Hentriacontane	0.49	29.727	86	$C_{31}H_{64}$
Eicosane, 9-octyl-	1.64	32.493	87	$C_{28}H_{58}$
Iodo compounds				
Dotriacontane, 1-iodo-	0.40	16.269	90	$C_{32}H_{65}I$
Eicosane, 1-iodo-	0.43	32.730	86	$C_{20}H_{41}I$
Fatty acids and esterified fatty acids				
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	0.55	21.762	99	$C_{18}H_{32}O_2$
Sterols				
Ergosta-5,22-dien-3-ol, (3.beta.,22E,24S)-	12.59	37.730	93	$C_{28}H_{46}O$
Ergosterol	32.41	38.465	90	$C_{28}H_{44}O$
Campesterol	2.17	38.810	70	$C_{28}H_{48}O$
Alkatrienes				
E,Z-1,3,12-Nonadecatriene	2.08	27.04	93	$C_{19}H_{34}$
1,8,11-Heptadecatriene, (Z,Z)-	0.25	27.767	81	$C_{17}H_{30}$
Unsaturated aldehydes				
9,17-Octadecadienal, (Z)-	0.71	27.116	97	$C_{18}H_{32}O$
Triterpenes				
Squalene	1.91	32.916	91	$C_{30}H_{50}$

In another research, the existence of *n*-undecane in *T. magnatum* was reported, and 1-decane was observed exclusively in samples collected from San Miniato district of Italy (Vita et al. 2018). Other alkanes, and their derivatives like Hexane, Pentane, 2-Thiabutane, Heptane, Octane and 2-Thiahexane were found in *T. magnatum*, *T. aestivum*, and *T. melanosporum* (Pacioni et al. 2014). It was reported that hexacosane and heptacosane are two essential substances as aromatic compounds of *T. mesentericum* (Mauriello et al. 2004).

Some alkanes like eicosane, heneicosan, and pentacosan with 0.87, 0.55, and 0.51%, were identified in *Tirmania nivea* (Omer et al. 1994). Undecane is considered as a pheromone compound that has a role in insect sexual attraction (Hillery & Fell 2000).

The role of heneicosane as a pheromone was well established in *Reticulitermes flavipes* (Funaro et al. 2018), which can show the importance of these aromatic constituents for insect attraction and then truffle spore dispersion.

In both kinds of winter truffles, not only was ergosterol the main sterol, but also it was the dominant constituent in n-hexane extract. In methanolic extract of dark winter truffle, ergosterol with 32.41%, Ergosta-5,22-dien-3-ol, (3. beta.,22E,24S)- with 12.59% and Campesterol with 2.17% were identified (Table 4). These three sterols in bright winter truffle were quantified 24.3, 13.78, and 2.44%, respectively (Table 5).

Peer-to-peer comparison of sterols between methanolic and n-hexane extracts showed that all sterols were higher in the n-hexane extract. In a case study on sterols of various Tuber species, it was found that ergosterol in three species, including *T. sinense*, *T. aestivum*, and *T. indicum*, were reported 643.5, 1403.3 and 762.5 $\mu g.g^{-1}$, respectively, however amounts for campesterol were recorded 111.0, 154.9, and 117.5 $\mu g.g^{-1}$ (Tang et al. 2012). In another research, total ergosterol in *T. melanosporum*, *T. aestivum* and *T. indicum* were 1.9, 1.86, and 1.37 mg. gr^{-1} dry matter, respectively. In these contents, 1.8, 1.51 and 1.8 mg. gr^{-1} were detected as free ergosterol and the rest were ergosterol ester (Villares et al. 2012).

In n-hexane extract of dark winter truffle, E, Z-1,3,12-nonadecatriene (2.08%) and 1,8,11-heptadecatriene, (Z, Z)- (0.25%) were identified (Table 4) and n-hexane extract of bright winter truffle, 1,8,11-heptadecatriene, (Z, Z)- was reported (Table 5). It is claimed that E,Z-1,3,12-nonadecatriene 5,14-diol is an antimicrobial compound that was detected in a wild fungus, *Laetiporus sulphureus*, by (Younis et al. 2019).

Iodic compounds consisting of dotriacontane, 1-iodo- and eicosane, 1-iodo- were identified in dark winter truffle (Table 4), and tridecane, 1-iodo-, hexadecane, 1-iodo- and eicosane, 1-iodo- were found in bright winter truffle (Table 5). As quantity aspect, squalene in n-hexane extract with 1.91% in the dark and 1.54% in bright winter truffle, (Table 4 and 5) were more than methanolic extracts.

Observed esterified fatty acids in dark and bright winter truffles were 9,12-octadecadienoic acid (Z, Z)-, methyl ester, and hexanedioic acid, bis (2-Ethylhexyl) ester, respectively (Tables 4 and 5). Also, 9,17-Octadecadienal, (Z)- an Unsaturated aldehyde, was detected in n-hexane extract of both truffles (Tables 4 and 5).

In n-hexane extract of bright winter truffle, 9-Octadecenamide, (Z)- with 2.36% was isolated (Table 5), which was reported in *Lignosus rhinocerotis* previously (Lau et al. 2014).

The 2,4-Di-tert-butylphenol (0.54%), was one of the phenolic compounds discovered in bright winter truffle (Table 5). Not only this substance has antioxidant effects, but it is also considered as an anti-pathogens, especially *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium chrysogenum* (Varsha et al. 2015).

At least 169 bacterial species were mentioned in scientific articles which contained 2,4-Di-tert-butylphenol, based on (Zhao et al. 2020), in kingdom fungi, just eight families and eleven species contain this compound, including Agaricaceae (*Agaricus bisporus*), Bionectriaceae (*Gliomastix murorum*), Glomerellaceae (*Colletotrichum gloeosporioides*), Nectriaceae (*Fusarium tricinctum*), Omphalotaceae (*Lentinus edodes*), Polyporaceae (*Trametes suavelens*), Tremellaceae (*Cryptococcus albidus*) and Trichocomaceae (*Aspergillus terreus*, *Penicillium flavigenum*, and *Didymium iridis*) contain this compound.

CONCLUSION

The total fat content of winter truffles (*Tuber brumale*) was mediocre in comparison with other species of truffles. The total protein content of winter truffle was remarkably lower than *T. aestivum*, based on previously released data.

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Table 5. Analysis of n-hexane extract of bright winter truffle by gas chromatography

Compounds	Area%	Time (min)	Score	Chemical Formula
Alkanes				
Nonane, 3-methyl-	0.91	5.543	86	$C_{10}H_{22}$
Cyclopentane, 1-hexyl-3-methyl-	0.98	5.813	86	$C_{12}H_{24}$
Decane	9.09	5.976	91	$C_{10}H_{22}$
Decane, 2,3,5-trimethyl-	0.32	8.960	78	$C_{13}H_{28}$
Hexadecane	0.39	14.132	94	$C_{16}H_{34}$
Heptadecane, 8-methyl-	0.92	16.953	93	$C_{18}H_{38}$
2-methyloctacosane	1.01	18.946	78	$C_{29}H_{60}$
Eicosane	1.25	20.161	95	$C_{20}H_{42}$
Heptacosane	1.04	22.446	91	$C_{27}H_{56}$
Octacosane	1.03	23.126	80	$C_{28}H_{58}$
Heneicosane	1.37	23.452	91	$C_{21}H_{44}$
Heptadecane, 9-octyl-	0.97	25.877	90	$C_{25}H_{52}$
Pentacosane	0.73	29.131	86	$C_{25}H_{52}$
Tetracosane	1.07	29.658	90	$C_{24}H_{50}$
10-Methylnonadecane	0.42	31.096	83	$C_{20}H_{42}$
Hentriacontane	0.57	32.171	86	$C_{31}H_{64}$
Hexacosane	0.86	32.488	90	$C_{26}H_{54}$
Tetratetracontane	0.69	35.146	81	$C_{44}H_{90}$
Fatty amides				
9-Octadecenamide, (Z)-	2.36	32.255	93	$C_{18}H_{35}NO$
iodo compounds				
Tridecane, 1-iodo-	0.46	15.636	78	$C_{13}H_{27}I$
Hexadecane, 1-iodo-	0.64	29.732	86	$C_{16}H_{33}I$
Eicosane, 1-iodo-	0.59	32.725	86	$C_{20}H_{41}I$
Phenols				
2,4-Di-tert-butylphenol	0.54	13.164	96	$C_{14}H_{22}O$
Fatty acids and esterified fatty acids				
Hexanedioic acid, bis(2-ethylhexyl) ester	17.42	26.640	91	$C_{22}H_{42}O_4$
Sterols				
Ergosta-5,22-dien-3-ol, (3.beta.,22E,24S)-	13.78	37.730	95	$C_{28}H_{46}O$
Ergosterol	24.30	38.451	90	$C_{28}H_{44}O$
Campesterol	2.44	38.805	91	$C_{28}H_{48}O$
Unsaturated aldehydes				
9,17-Octadecadienal, (Z)-	0.81	27.111	98	$C_{18}H_{32}O$
Alkatrienes				
1,8,11-Heptadecatriene, (Z,Z)-	0.23	27.763	83	$C_{17}H_{30}$
Triterpenes				
Squalene	1.54	32.916	90	$C_{30}H_{50}$

Phenol and flavonoid contents of winter truffle were moderate magnitude among reported data for different truffles. It has been considered that the primary fatty acid of truffle is linoelaidic acid, but our analysis showed that in bright winter truffle Methyl 10-trans,12-cis-octadecadienoate was at the greatest extent. Ergosterol which can produce the precursor of vitamin D_2 was identified as the main sterol of winter truffle. In addition, detection of two important medicinal compounds, including squalene and carvacrol, in winter truffle, can be exciting and momentous. Also, finding 2,4-Di-tert-butylphenol in this fungus is considerable because of its vigorous antioxidant activity and anti-pathogen property.

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ارزیابی ارزش غذایی و ترکیبات شیمیایی ترافل‌های زمستانه (*Tuber brumale*) شمال ایران

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چکیده: این پژوهش به منظور بررسی ارزش غذایی و شناسایی ترکیبات مایکوشیمیایی ترافل‌های زمستانه (*Tuber brumale*)، با دو رنگ متفاوت بافت داخلی اندام خوراکی (تیره و روشن) که از جنگل‌های هیرکانی (ایران) جمع‌آوری گشتند، صورت گرفت. محتوی کربوهیدرات، پروتئین و چربی، به ترتیب: ۱۱/۳ میلی‌گرم بر گرم وزن خشک، ۱۲/۵۳٪ و ۲/۴۱٪ در ترافل زمستانه با بافت تیره، و ۱۲/۶۴ میلی‌گرم بر گرم وزن خشک، ۱۱/۰۶٪ و ۲/۴٪ در ترافل زمستانه با بافت روشن اندازه‌گیری شد. اگرچه نتایج گروماتوگرافی گازی نشان داد اسیدهای چرب و اسیدهای چرب استری فرآوان‌ترین ترکیبات شناسایی شده در عصارهای متانولی را شامل می‌شوند، اما ارگوسترول با مقادیر ۱۵/۸۸ و ۱۲/۸۹٪ به ترتیب در عصاره‌های متانولی ترافل زمستانه با بافت تیره و روشن، ترکیب غالب بود. بیشترین ترکیبات شناسایی شده در عصاره‌های ان-هگزانی نیز آلکان‌ها بودند. همچنین محتوی ارگسترول به عنوان ترکیب غالب عصاره ان-هگزانی نیز به ترتیب ۳۲/۴۱ و ۲۴/۳٪ در ترافل زمستانه با بافت تیره و روشن مشاهده شد. در عصاره متانولی *T. brumale* تیره، 2-methyl-5-(1-methylethyl) یا کارواکرول، با مقدار ۰/۲۵٪ اندازه‌گیری شد. در عصاره ان-هگزانی ترافل زمستانه با بافت روشن نیز، 2,4-Di-tert-butylphenol به عنوان یکی از اجزای فنولی با مقدار، ۰/۵۴٪ مشاهده شد. همچنین اسکوآلن نیز بعنوان یک تریترپن طبیعی در عصاره‌های متانولی و ان-هگزانی هر دو نوع ترافل زمستانه شناسایی شد. بیشترین محتوی اسکوآلن (۹۱/۰٪) نیز در عصاره ان- هگزانی ترافل زمستانه با بافت داخلی تیره اندازه‌گیری شد.

کلمات کلیدی: آنتی‌اکسیدان، ارگوسترول، فنول، اسکوآلن، ترافل‌های حقیقی، *Tuber brumale*

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