



Fatty Acid Composition and Antioxidant Activity of *Tricholoma Imbricatum* and *T. Focale*

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ARTICLE INFO

Research Article

Received 28 April 2017
Accepted 13 June 2017

Keywords:

Tricholoma focale
Tricholoma imbricatum
Antioxidant activity
GC-MS
Fatty acid composition

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ABSTRACT

Edible mushrooms are one of the important food source because of their tastes, and having high concentration of fatty acids and the other nutrients. In this study, fatty acid composition and antioxidant properties of *Tricholoma imbricatum* and *T. focale* were determined. The fatty acids were analysed by GC, and GC-MS while the antioxidant activity was tested using five complimentary methods; namely, β -carotene-linoleic acid, DPPH scavenging, ABTS scavenging, CUPRAC and metal chelating assays. Eight fatty acids were determined in hexane extract, and oleic acid was the major fatty acid with 46.4% and 35.0%, respectively. Palmitic acid (12.8%, and 5.12%) and linoleic acid (28.2% and 31.0%) were also detected in high amounts. In DPPH scavenging and ABTS⁺ scavenging assays, the methanol extract of *T. imbricatum* (IC₅₀: 0.12±0.01 mg/mL for both assay) showed better antioxidant activity than those of *T. focale* (IC₅₀>0.8±0.01, and IC₅₀: 0.21±0.01 mg/mL). All extracts of *T. focale* exhibited good activity in β -carotene-linoleic acid assay while only the hexane extract of *T. imbricatum* showed activity. Both mushroom indicated moderate in cupric reducing power. Since the mushrooms are nontoxic and edible, both can be used in food industry as preservatives.

DOI: <https://doi.org/10.24925/turjaf.v5i9.1080-1085.1291>

Introduction

Anatolia has rich flora on naturally growing edible and medicinal mushrooms. Many researches showed that most of the wild mushroom are not well recognized by the local peoples and only a few of them are introduced to dietary habits (Turkoglu et al., 2006, 2008, 2009; Türkoglu, 2008). No doubt that suitable ecological parameters resulted in highly diverse species during spring and autumn besides the species which can be seen at any season (Işıloğlu and Öder, 1995).

Recently, the researchers have interested the mushrooms due to their biologically active compounds. Triterpenes, steroids, flavonoids, phenolics, polysaccharides and polysaccharides-protein complexes indicating anticancer, antiviral, antioxidant and immunomodulatory activities were isolated from the mushrooms (El Enshasy and Hatti-Kaul, 2013)

Some of the *Tricholoma* species were studied particularly in Eastern Asian countries due to their special tastes. The most famous member of *Tricholoma* genus is *T. matsutake*, on which scientist have focused because of its medicinal properties and commercial potential. The various phytochemicals were isolated from the *T. matsutake* exhibiting anti-inflammatory, antioxidant and anticancer activities (Ding and Hou, 2012; Taofiq et al., 2016; You et al., 2013). Besides this species other *Tricholoma* species have been also attracted (Chen et al.,

2017; Hassan et al., 2015; Sadi et al., 2015). Having the similar aim, we have performed complimentary antioxidant activity tests in addition to fatty acid analysis of *T. imbricatum* and *T. focale* to increase the knowledge. Up to date, a few studies have been performed on *T. imbricatum* and *T. focale*. Previously, only metal composition of *T. focale* was studied. As for *T. imbricatum*, beside metal composition study and mero terpenoids were isolated (Dogan et al., 2012; Tel et al., 2011; Zhang et al., 2009). However, there has been no study on the fatty acid composition and the antioxidant activity of the *T. focale* and *T. imbricatum*. The goal of the study is to compare the fatty acid composition and the antioxidant activity of the both *Tricholoma* species with those of synthetic antioxidant commercially used in food industry.

In despite of having such commercial potential, nutritional composition has not been worked. Fatty acid composition and antioxidant capacity of this mushroom will be introduced to literature for the first time

Materials and Methods

All chemicals were analytical grade. Antioxidant tests were determined spectrophotometrically using 96-well plate Elisa reader (SpectraMax 340PC³⁸⁴, Molecular

Devices inc, USA). Fatty acid analysis was performed using GC-MS (gas chromatography mass spectrometry) (Varian Saturn 2100, USA).

Mushroom Collection

Mushroom samples were collected in December 2012. The vouchers of the mushrooms were kept in the herbarium of Mugla Sitki Kocman University, Department of Biology with the herbarium number CK-02460 and CK-01181 for *T. focale* and *T. imbricatum*, respectively.

Extraction

Air dried mushroom samples (100 g) were grinded into small pieces, and extracted with hexane, acetone and methanol, successively at room temperature for 24 h x 7. The extracts of *T. focale* and *T. imbricatum* were yielded for hexane extracts 1.15 and 1.11 g, and for acetone extract 2.14 and 2.51 g, and for methanol extract 12.06 and 15.10 g, respectively. The hexane extracts were further used for fatty acid analysis. Hexane extracts were methylated using BF₃:Methanol complex to analyze their fatty acid methyl esters (FAMES) according to Ozturk et al. (2014).

Fatty Acid Composition Analysis with GC-FID

GC-FID analysis was conducted on a Shimadzu GC-17 AAF, W3, 230W equipped with DB-1 capillary column (ID: 0.25 mm, film: 0.25 µm; length: 30 m). Carrier gas was He at flow rate of 1.3 ml/min, column temperature program started with 100°C for 5 min, then increased to 238°C with the rate of 3°C/min and held at 238°C for 9 min. The injector and detector temperature was 250 and 280°C, respectively. Determination of percentage concentration of FAMES was performed via fatty acid standards using CLASS GC10 computer software (Tel et al., 2013).

Fatty Acid Analysis with GC-MS

GC-MS analysis was conducted on an ion trap analyzer Varian Saturn 2100 T equipped with electron ionization and nonpolar DB-1 capillary column (ID: 0.25 mm, film: 0.25 µm; length: 30 m). Carrier gas was He at flow rate of 1.3 ml/min, column temperature program started with 100°C for 5 min, then increased to 238°C with the rate of 3°C/min and held at 238°C for 9 min. 70 eV was used for electron ionization. The injector, transferline and detector temperature was 220, 290 and 240°C, respectively. Determination of respected FAMES was performed via Nist/Wiley 2005 library searches and fatty acid standards comparison (Altuntaş et al., 2016).

Antioxidant Activity Tests

Lipid peroxidation Activity (β-carotene-linoleic acid assay): β-carotene-linoleic acid assay was used for determining the lipid peroxidation inhibition activity. Same method was used without any modification which was described, previously (Duru et al., 2012). Briefly, 1 mg β-carotene solved in 2 mL chloroform and then 400 mg Tween 40 and 50 µl of linoleic acid were added to solution. After evaporation of chloroform the residue was

diluted with 100 ml of oxygenated water. Freshly prepared β-carotene-linoleic mixture (160 µL) was added to the 40 µL sample. After two hour of incubation time at 50°C, the absorbance was read at 470 nm using 96-well plate Microplate Reader. BHA and α-tocopherol were selected as standard antioxidants. β-carotene bleaching rate was calculated according the formula given in below:

$$R = \frac{\ln a/b}{t}$$

where, ln=natural logharithm, a=initial absorbance, b=final absorbance, t=incubation time

Percent inhibition was calculated with the following formula:

$$\% \text{ inhibition} = \frac{R_{\text{blank}} - R_{\text{sample}}}{R_{\text{blank}}} \times 100$$

DPPH radical scavenging assay: DPPH radical scavenging activity was determined according to method described previously (Blois, 1958) with modifications. Freshly prepared free DPPH radical (160 µL) was added to 40 µL sample. The mixture was incubated in dark for 30 min, and then the absorbance was recorded at 517 nm with microplate reader. BHA and α-tocopherol were selected as standard antioxidants. Scavenging activity was calculated as % inhibition using following equation.

$$\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

ABTS cation radical scavenging assay: ABTS radical inhibition activity was evaluated according to the method described by Re et al (Re et al., 1999; Sabudak et al., 2013). ABTS cation radical was produced via the reaction of ABTS and potassium persulfate. After having the cation radical it was diluted until reaching the absorbance value within the range of 0.65-0.70 absorbance value at 734 nm. Prior to final reading 160 µl radical solution and 40 µl sample solution were mixed and incubated for 10 min. BHA and α-tocopherol were selected as standard antioxidants. Scavenging activity was calculated with the following formula:

$$\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

Metal chelating activity assay: Metal chelation ability of the extracts were tested using Ferrin-Fe²⁺ method (Decker and Welch, 1990). Extract solutions were prepared in different concentrations. 80 µL of extract solution was mixed with 40 µL 0.2 mM FeCl₂, then the 40 µL 0.5 mM ferrin solution was added and left for 10 min. Spectrophotometric measurement was carried out at 593 nm. Inhibitory results were given calculated according to following formula:

$$\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

CUPRAC (Cupric Reducing Antioxidant Capacity):

Cupric reducing capacity was measured according to the method described by Apak et al., (Apak et al., 2004) with minor modifications (Öztürk et al., 2011). To the 40 µL sample, 60 µL 1M ammonium acetate buffer (pH:7.0), 40 µL of 7.49 mM neocuprine, 50 µL of 10 mM CuCl₂. Results were expressed as absorbance at 450 nm. BHA and α-tocopherol were selected as standard antioxidants.

Results and Discussion**Fatty acid Analysis**

The fatty analyses of the mushroom species were performed using GC, and GC-MS instruments. Table 1 shows the fatty acid composition of the mushrooms. Oleic acid was the major fatty acid in *T. imbricatum* and *T. focale* with the 46.3 and 34.9% concentrations. Palmitic, linoleic, and stearic acids were also major components in both mushrooms. In addition, 8,11-Eicosadienoic acid (9.21%) was also in a good amount in *T. focale*. However, it is in low quantity in *T. imbricatum*. Pentadecanoic, heptadecanoic, and arachidonic acids were also detected. *T. imbricatum* and *T. focale* possessed high unsaturation level with 77.0% and 78.0%, respectively. The Oleic acid and linoleic acid ratio which represents the

chemotaxonomic significance were calculated as 1.64 and 1.12, respectively. Our results were in good agreement according major fatty acids concentrations with those reported literatures on *T. portentosum* and *T. terreum* (Díez and Alvarez, 2001), *T. portentosum* (Barros et al., 2007) and *T. myomyces* (Ergönül et al., 2012).

Antioxidant Activity Results

Antioxidant activity was tested using five complimentary assays due to the chemical complexity of the extracts. One test may lead to scattered results. Therefore, the antioxidant activity was performed according various tests having different mechanisms. The antioxidant activity results were given in Table 2, Figure 1 and 2.

In β-carotene-linoleic acid assay, the antioxidants give H radicals to the media which stops the peroxidation of linoleic acid. In this assay, the hydrogen radicals also scavenge singlet oxygen which lead to peroxidation of linoleic acid. Figure 1 shows the lipid peroxidation inhibitory activity of the mushroom extracts by the β-carotene-linoleic acid assay. All extracts exhibited good activity demonstrating IC₅₀ values lower than 0.05 mg/mL except hexane extract of *T. imbricatum* (IC₅₀: 0.29 ± 0.01 mg/mL).

Table 1 Fatty acid composition of *Tricholama* species.^a

No	Compounds	<i>T. imbricatum</i>	<i>T. focale</i>
		% Conc.	% Conc.
1	Pentadecanoic acid (C _{15:0})	0.82 ± 0.01	0.86 ± 0.01
2	Palmitic acid (C _{16:0})	12.8 ± 0.01	5.12 ± 0.01
3	Heptadecanoic acid (C _{17:0})	0.06 ± 0.01	0.26 ± 0.01
4	Linoleic acid (C _{18:2})	28.2 ± 0.06	31.0 ± 0.05
5	Oleic acid (C _{18:1})	46.3 ± 0.09	34.9 ± 0.05
6	Stearic acid (C _{18:0})	5.12 ± 0.01	4.77 ± 0.01
7	Arachidonic acid (C _{20:4})	1.56 ± 0.01	2.79 ± 0.01
8	8,11-Eicosadienoic acid (C _{20:2})	0.80 ± 0.01	9.21 ± 0.01
	Saturated	18.8	11.0
	Unsaturated	77.0	78.0
	Oleic acid/linoleic acid	1.64	1.12
	Total identified	95.8	89.1

^a Values are mean ± SEM of three parallel measurements. P<0.05.

Table 2 Antioxidant activity of mushroom extracts by the DPPH[•] radical scavenging, ABTS^{•+} radical scavenging, and metal chelating assays.^a

Mushroom	Extracts	DPPH [•] assay	ABTS ^{•+} assay	Metal chelating assay
		IC ₅₀ (mg/mL)	IC ₅₀ (mg/mL)	IC ₅₀ (mg/mL)
<i>T. focale</i>	Hexane	>0.8 ± 0.01	>0.8 ± 0.02	0.58 ± 0.02
	Acetone	>0.8 ± 0.02	0.40 ± 0.01	>0.8 ± 0.03
	Methanol	>0.8 ± 0.02	0.21 ± 0.01	>0.8 ± 0.02
<i>T. imbricatum</i>	Hexane	>0.8 ± 0.01	>0.8 ± 0.03	0.46 ± 0.01
	Acetone	0.54 ± 0.02	0.53 ± 0.02	>0.8 ± 0.03
	Methanol	0.12 ± 0.02	0.12 ± 0.01	0.54 ± 0.01
Standard antioxidants	BHA	0.045 ± 0.0001	0.007 ± 0.0004	NT
	α-Tocopherol	0.007 ± 0.0002	0.004 ± 0.0005	NT
	EDTA	NT	NT	0.006 ± 0.0002

^a Values are mean ± SEM of three parallel measurements. P>0.05. NT: not tested

Lipid peroxidation inhibitory assay

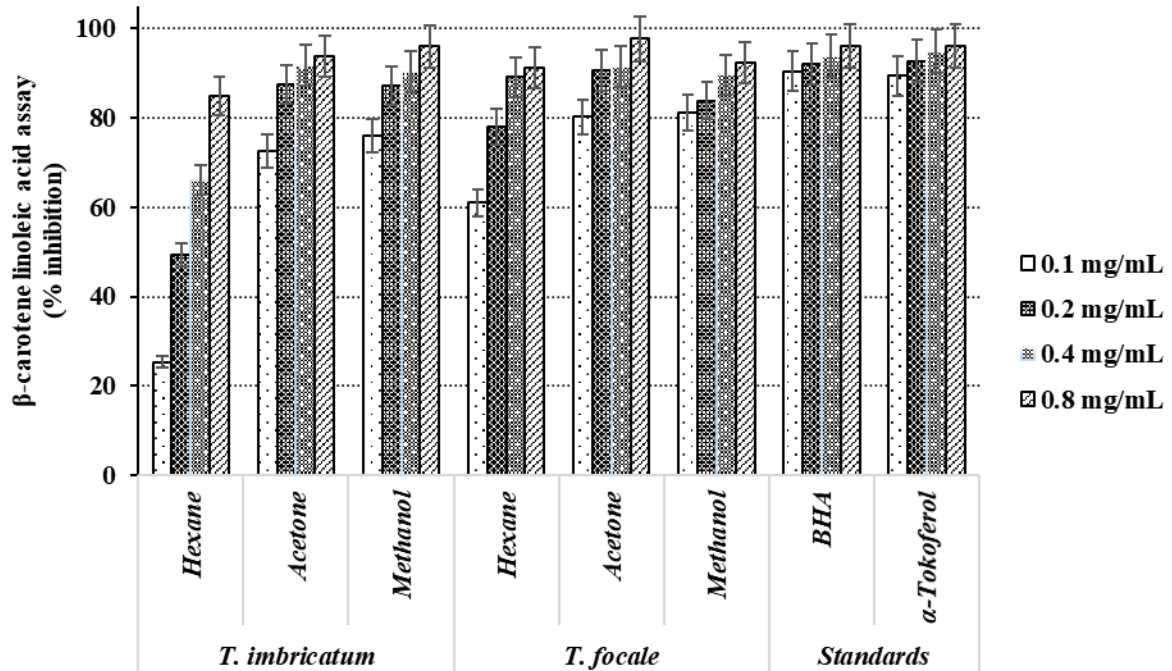


Figure 1 Lipid peroxidation inhibitory activity of the extracts of *T. focale*, *T. imbricatum* and standards by the β -carotene-linoleic acid assay, $P < 0.05$.

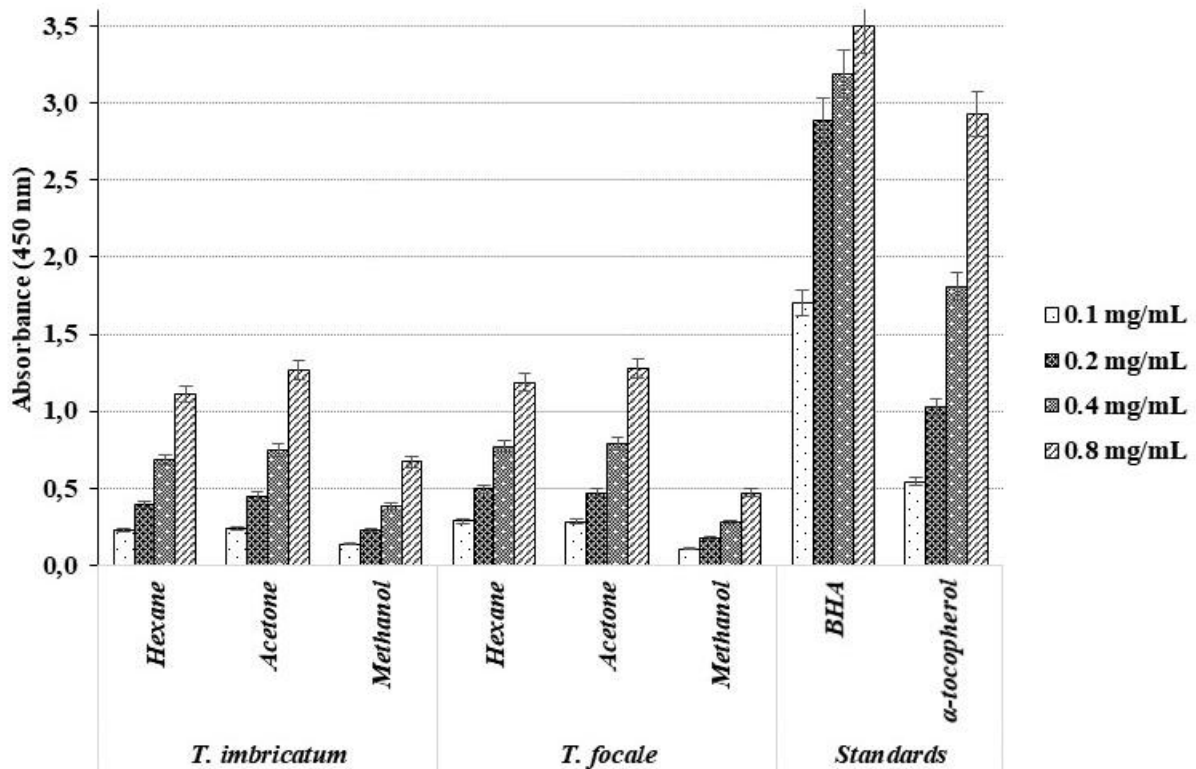


Figure 2 Cupric reducing Antioxidant Capacity (CUPRAC) of extracts of mushrooms using spectrophotometric detection of Cu^{2+} - Cu^+ transformation, $P < 0.05$.

In DPPH and ABTS radical scavenging assays antioxidant transfers electron to the media. DPPH and ABTS assays have some differences in that their test media. DPPH assay test the lipophilic antioxidants while ABTS assay tests both lipophilic and hydrophilic compounds. Another superiority of ABTS is that it tests the antioxidant activity of the bulky compounds. Table 2 shows the DPPH radical and ABTS radical scavenging activities of the mushroom species. In DPPH assay, the methanol extract of *T. imbricatum* (IC₅₀: 0.12 ± 0.02 mg/mL) exhibited the best activity and followed by acetone extract of *T. imbricatum* (IC₅₀: 0.54 ± 0.02 mg/mL). Other extracts were inactive in DPPH assay. In ABTS assay, the acetone and methanol extracts of both mushrooms also indicated good activity. The methanol and acetone extracts of *T. imbricatum* demonstrated the similar IC₅₀ values as in DPPH assay. The IC₅₀ values of methanol and acetone extracts of *T. focale*, however, were 0.21 ± 0.01, and 0.40 ± 0.01 mg/mL. Ferreira et al (2007) previously tested DPPH scavenging activity of methanol extract of another wild edible mushroom *T. portentosum* and found IC₅₀ as 40.2 mg/mL and Sadi et al (2015) found IC₅₀ as 3.25 mg/mL for *T. fracticum* which was not even close to *T. imbricatum* extracts. Tel et al (2011) worked on wild mushroom species namely *T. fracticum*, *T. terreum* and *T. imbricatum* with different extracts our result were comparable according to ABTS test.

Metal chelating activity tests the secondary antioxidants in the media. Secondary antioxidants chelate with the metal ions which lead to radical generation via Fenton Reaction when reacted with oxidized molecules. Acceleration of radical by the ferrous state of iron is 10-fold higher than by the ferric state of iron. The metal chelating activity of the mushrooms were given in Table 2. The metal chelating ability of hexane extracts of *T. imbricatum* (IC₅₀: 0.46 ± 0.01 mg/mL) and *T. focale* (IC₅₀: 0.58 ± 0.02 mg/mL) and methanol extracts of *T. imbricatum* (IC₅₀: 0.54 ± 0.01 mg/mL) were higher than the other extracts. In a recent paper IC₅₀ was found 1.62 mg/mL for *T. terreum* (Sadi et al., 2016). Herein, possessing of metal chelation of methanol extract is very interesting in that this extract also scavenged radicals.

CUPRAC (Cupric reducing antioxidant Capacity) tests the electron power of the antioxidant. In this assay, antioxidant gives electron to the media. The significance difference is that this assay tests both lipophilic and hydrophilic antioxidants which can provide electron to the media. Figure 2 shows the CUPRAC assay of the mushrooms. In general, among the extracts the acetone extracts of the mushrooms exhibited higher activity, followed by hexane and methanol extracts. The activity increased with the increasing concentrations. Interestingly the methanol extracts indicated lower activity than the others. Previously, *Tricholoma* species were tested for cupric reducing power and the results were compatible with those in this study (Tel et al., 2011).

Conclusion

The fatty acid compositions of *T. focale* and *T. imbricatum* were studied for the first time. Moreover, the antioxidant activity of *T. focale* was also studied for the first time. In general methanol extracts showed better DPPH and ABTS radical scavenging activities. In CUPRAC assay, however, the acetone extract was the most active. Both mushroom have rich fatty acid composition with the unsaturation of 77% averagely. Since *T. focale* and *T. imbricatum* have been unreported nor toxic neither poisonous, the mushrooms could be considered as supplementary foods as well as food preservatives in the Food Industry. Both mushrooms preferably consumed due to unsaturated fatty acid and antioxidant properties.

Acknowledgment

The fatty acid analyses part of the study has been granted by the Mugla Sitki Kocman University Research Projects Coordination Office. Project Grant Number: 14/004. Remaining parts of the study has been granted by the Scientific and Technological Research Council of Turkey (TUBITAK) with the project number of 113R012.

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