SUPPLEMENTARY MATERIAL

A new fatty acid ester from an edible mushroom *Rhizopogon luteolus*

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Abstract

Phytochemical investigation of the *Rhizopogon luteolus* Fr. led to the isolation of one new fatty acid ester, 3-hydroxy-2,4-dimethylheptacosyl acetate (1) together with two known compounds tetracosanoic acid (2), and ergosterol (3). 1D, and 2D NMR, and MS techniques were used for structural elucidation. Phenolic and fatty acid compositions were identified using HPLC-DAD, and GC-MSD, respectively. Fumaric acid was the major phenolic acid whereas linoleic, stearic and oleic acids were the most abundant fatty acids. Antioxidant and anticholinesterase activities of the extracts and compounds (1-3) were tested spectrophotometrically. Among the extracts, hexane extract showed the highest activity in all tests, particularly in β-carotene-linoleic acid assay (IC\(_{50}\): 16.65±1.12 µg/mL). Furthermore, compound 3 exhibited higher antioxidant and anticholinesterase activities. The study indicates that *R. luteolus* can be used in food, cosmetic and pharmaceutical industries.

Keywords: *Rhizopogon luteolus*; fatty acid; ergosterol; phenolic compounds; antioxidant activity; anticholinesterase activity
Experimental

Mushroom Material

*Rhizopogon luteolus* Fr. was collected from Muğla, Turkey in December 2012 and identified by Dr. Aziz Türkoğlu. A voucher specimen was deposited in the Fungarium of the Department of Biology, Mugla Sitki Koçman University (code: AT 1831).

Spectral measurements and chemicals used

Purified compounds were analysed by FAB-MS and EI-MS, 1D-, 2D-NMR and FTIR spectroscopy. EI-MS spectra were obtained on a JEOL MS Route resolution, NMR spectra were on Bruker Avance AV-500-MHz and 600-MHz instruments coupled with cooled cryoprobes probe for \(^1\)H- and \(^13\)C- NMR including DEPT, HSQC, HMQC, and COSY on a Bruker Microsoft Q Spectrometer. The phenolic composition analysis was carried out using a Shimadzu 20AT series high performance liquid chromatograph (HPLC, Shimadzu Coperation, Japan) while fatty acid analysis were on a Shimadzu GC-17 AAF, V3, 230 V series gas chromatography (Japan), and Varian Saturn 2100 GC-MS (USA). Bioactivity studies were determined using a 96-well microplate reader (SpectraMax 

Column chromatography, and thin-layer chromatography (TLC) were performed on silica gel (Kieselgel 60, 70-230 mesh, Merck). Silica gel 60 F\(_{254}\) plates and RP-18 F\(_{254}\)S, respectively. TLC spots were detected under UV-254-nm light, and visualized by CeSO\(_4\) solution whenever needed.

Ethylenediaminetetraacetic acid (EDTA), ferrous chloride, copper (II) chloride and ammonium acetate were obtained from E. Merck (Darmstadt, Germany). Polyoxyethylene sorbitan monopalmitate (Tween-40), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5′′-disulfonic acid disodium salt (Ferene), β-carotene, linoleic acid, 2,2′-azino bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), α-tocopherol, neocuproine, butylated hydroxyl anisole (BHA), acetylcholinesterase from electric eels (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg, Sigma), acetyltiophiocholine iodide, butyrylcholinesterase, from horse serum (BChE, EC 3.1.1.8, 11.4 U/mg, Sigma), butyrylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), galantamine, gallic acid (≥99%), fumaric acid (≥99%), protocatechuic acid (97%), catechin hydrate (≥98%), p-hydroxybenzoic acid (99%), 6,7-dihydroxy coumarin (98%), caffeic acid (≥98%), vanillin (99%), 2,4-dihydroxy benzoic acid (98%), p-coumaric acid (≥98%), ferulic acid (99%), coumarin (≥99%), trans-2-hydroxycinnamic acid (99%), ellagic acid (≥98%), rosmarinic acid (≥98%) and trans-cinnamic acid (99%) were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were in analytical grade.

Extraction and isolation

Dried *R. luteolus* (500 g) was cut into small pieces and extracted four times with 15 L methanol, (4 × 15 L) at room temperature over a period of 15 days, filtered and evaporated under vacuum. The crude extract was dissolved in water and transferred to a separation funnel. n-Hexane, and ethyl acetate, fractions were obtained from the aqueous layer. Both fractions were subjected to column chromatography on silica gel, and eluted with hexane/CHCl\(_3\), CHCl\(_3\)/acetone, acetone/methanol, and methanol with increasing polarities that provided 75
fractions (Fr. 1-Fr. 75 hexane), and 49 sub-fractions (Fr. 1-Fr. 49 ethyl acetate). Hexane fraction 6, 7, and 8 were purified by silica gel column chromatography eluted with n-hexane:ethyl acetate (8:2) to give compound 1 (3.6 mg) and 2 (2.5 mg). Compound 3 (7.1 mg) was obtained from fraction 5 and 6 of ethyl acetate extract by repeated silica gel column chromatography using n-hexane:CHCl₃ (1:1).

**Compound 1**
Colourless gum; IR (cm⁻¹): –OH (3096), –C=O (2951, 1689). ¹H NMR (600MHz, CDCl₃): δ 2.08 (3 H, s, Me₂/Me), 1.13 (6 H, t, J = 6.0 Hz, 3'-/4'-Me), 2.33 (1 H, m, H₂), 4.04 (1 H, m, H-3), 2.34 (1H, m, H-4), 3.63 (1H, d, J = 6.0 Hz, H-1a), 3.55 (1H, d, J = 6.0 Hz, H-1b), 0.95 (3 H, d, J = 6.0 Hz, H-27), 1.59-1.62 (44 H, m, H-5, 26).

**Compound 3**
Compound 3: obtained as white needles. ¹³C NMR (125 MHz, CDCl₃+CD₃OD) δ: 38.2 (C-1), 31.5 (C-2), 70.0 (C-3), 40.3 (C-4), 141.2 (C-5), 119.4 (C-6), 116.1 (C-7), 139.8 (C-8), 46.1 (C-9), 36.9 (C-10), 20.9 (C-11), 28.1 (C-12), 42.7 (C-13), 54.4 (C-14), 22.8 (C-15), 38.9 (C-16), 55.6 (C-17), 11.9 (C-18), 16.1 (C-19), 40.3 (C-20), 20.9 (C-21), 131.8 (C-22), 135.5 (C-23), 42.7 (C-24), 17.4 (C-25), 32.9 (C-26), 19.5 (C-27), 19.7 (C-28).

**Analysis of phenolic compounds**
Phenolic profile was determined according to the method of Barros et al. (2009) with slight modification. All conditions were identical to those described in earlier publication (Tel-Çayan et al. 2015)

**Total phenolic and flavonoid content**
The phenolic content in all extracts were expressed as µg of pyrocatechol equivalents (PEs) determined with FCR according to the method of Slinkard & Singleton (1977). Phenolic contents were calculated according to equation 1 obtained from standard pyrocatechol graph:

\[ \text{Absorbance} = 0.0073[\text{pyrocatechol (µg)}] - 0.1665 (r², 0.9976) \quad \text{eq.1} \]

Measurement of extract flavonoid contents was based on the aluminium nitrate method (Park et al. 1997). Results are expressed as µg of quercetin equivalents. The flavonoid contents were calculated according to equation 2 obtained from the standard quercetin graph:

\[ \text{Absorbance} = 0.082[\text{quercetin (µg)}] - 0.0073 (r², 0.9998) \quad \text{eq.2} \]

**Fatty acids analysis**
Fatty acids were derivatized according to our reported method (Tel et al. 2013). Qualitative and quantitative analysis of the fatty acid esters were also performed by GC and GC/MSD as we reported earlier (Ozturk et al. 2014).
**Antioxidant activity**

Total antioxidant activity by β-carotene-linoleic acid test (Siebert et al. 2015), Free radical scavenging activity by the DPPH’ assay (Tu et al. 2015; Ullah et al. 2015), ABTS•+ cation radical scavenging activity (Tel et al. 2012), Superoxide anion radical scavenging activity (Öztürk et al. 2011), CUPRAC antioxidant activity (Chemsa et al. 2015), Metal chelating activity on Fe²⁺ (Tel et al. 2012) were determined according to the our reported procedures with slight modifications (Öztürk et al. 2011). The results are given as 50 % inhibition concentration (IC₅₀), and A₀.₅₀, which corresponds to the concentration producing 0.500 absorbance for CUPRAC assay and inhibition percentage (%) at 200 µg/mL concentration of the extracts and compounds.

**Anticholinesterase activity**

Acetylcholinesterase (AChE)- and butyrylcholinesterase (BChE)-inhibitory activities were measured spectrophotometrically according to Ellman’s method (Ellman et al. 1961) with slight modifications (Ertaş et al. 2015). Galantamine was used as a reference compound. The results are given as the 50 % inhibition concentration (IC₅₀). The sample concentration providing 50 % enzyme inhibition (IC₅₀) was calculated from the graph of the enzyme-inhibition percentage against the sample concentration.

**Statistical analysis**

All data on the antioxidant and anticholinesterase activities were the averages of three parallel sample measurements. The data were recorded as the mean ± S.E.M. Significant differences between the means were determined by student’s t test, and p values <0.05 were regarded as significant.
Table S1: Composition of phenolic and organic acids in *Rhizopogon luteolus*

| Compounds                     | Retention time (min) | Composition (µg/g) |
|-------------------------------|-----------------------|-------------------|
| Gallic acid                   | 4.37                  | 1.50±0.002        |
| Fumaric acid                  | 5.59                  | 102±0.002         |
| Protocatechuic acid           | 6.87                  | 1.15±0.001        |
| Catechin hydrate              | 8.46                  | nd                |
| p-hydroxybenzoic acid         | 10.64                 | nd                |
| 6,7-dihydroxy coumarin        | 11.62                 | nd                |
| Caffeic acid                  | 13.13                 | nd                |
| Vanilin                       | 14.89                 | nd                |
| 2,4-dihydroxy benzoic acid    | 15.54                 | 0.61±0.001        |
| p-coumaric acid               | 18.74                 | nd                |
| Ferulic acid                  | 19.76                 | nd                |
| Coumarin                      | 20.96                 | 0.05±0.001        |
| trans-2-hydroxy cinnamic acid | 21.98                 | 0.12±0.002        |
| Ellagic acid                  | 22.54                 | nd                |
| Rosmarinic acid               | 23.61                 | nd                |
| trans-cinnamic acid           | 24.52                 | 1.08±0.002        |

*Values represent the means ± S.E.M. of three parallel measurements (p<0.05). n.d. Not detected

Table S2: The fatty acid composition (%) of *R. luteolus*.

| Fatty acids                     | *Rhizopogon luteolus* (%) |
|---------------------------------|---------------------------|
| Pentadecanoic acid (C₁₅:₀)      | 0.31                      |
| Palmitoleic acid (C₁₆:₁)        | 5.8                       |
| Palmitic acid (C₁₆:₀)           | 6.6                       |
| Linoleic acid (C₁₈:₂)           | **45.8**                  |
| Oleic acid (C₁₈:₁)              | **16.2**                  |
| Stearic acid (C₁₈:₀)            | **23.7**                  |
| Arachidonic acid (C₂₀:₄)        | 0.39                      |
| Tetracosanoic acid (C₂₄:₀)      | 1.2                       |
| Total saturation                | 31.8                      |
| Total unsaturation              | 68.2                      |
| Saturation/Unsaturation         | 0.47                      |
| L/O                             | **2.80**                  |

*L/O: linoleic acid-oleic acid ratio.*
Table S3: Antioxidant activity of the extracts and compounds of *R. luteolus* by the 
\[\beta\text{-carotene}-\text{linoleic acid, DPPH}^\bullet, \text{ABTS}^\bullet, \text{O}_2^\bullet, \text{CUPRAC and Metal Chelating assays}.^a

|                     | \[\beta\text{-carotene}-\text{linoleic acid assay} | DPPH\(^\bullet\) assay | ABTS\(^\bullet\) assay | \text{O}_2^\bullet \text{ assay} | CUPRAC assay | Metal Chelating assay |
|---------------------|--------------------------------------------------|------------------------|------------------------|-------------------------------|--------------|----------------------|
|                     | Inhibition (%) (at 200 µg/mL) | IC\(_{50}\)(µg/mL) | Inhibition (%) (at 200 µg/mL) | Inhibition (%) (at 200 µg/mL) | Absorbance (at 200 µg/mL) | Inhibition (%) (at 200 µg/mL) |
| Methanol            | 86.96±1.31                                    | 24.63±1.54             | 21.13±0.44             | 57.81±1.53             | 9.42±0.54          | 0.31±0.01              | 46.63±1.74              |
| Hexane              | 88.83±1.22                                    | 16.65±1.12             | 61.76±0.56             | 67.13±1.20             | 11.39±0.30          | 0.91±0.03              | 50.79±1.83              |
| Ethyl acetate       | 53.97±0.44                                    | 76.39±1.25             | 19.89±0.28             | 36.36±0.57             | 1.38±0.16           | 0.30±0.01              | 14.04±2.60              |
| Water               | 86.23±1.81                                    | 20.82±1.08             | 14.09±0.41             | 46.37±2.62             | 2.32±1.43           | 0.26±0.00              | 32.86±1.77              |
| Compound 1          | 5.52±1.80                                     | >250                   | 5.78±0.19              | 27.66±0.62             | 7.83±1.46           | 0.11±0.00              | 11.28±1.88              |
| 2                   | 8.45±1.69                                     | >250                   | 7.10±0.47              | 20.76±1.77             | 2.29±0.76           | 0.23±0.01              | 20.10±1.50              |
| 3                   | 35.00±1.49                                    | >250                   | 0.28±0.04              | 15.88±0.28             | 8.35±2.07           | 0.21±0.00              | 21.26±0.46              |
| BHA\(^b\)           | 91.78±1.26                                    | 1.34±0.04              | 87.13±0.09             | 91.50±0.20             | 91.78±1.26          | 2.47±0.01              | NT                     |
| \(\alpha\)-Tocopherol\(^b\) | 90.51±0.18                                   | 2.10±0.08              | 87.14±0.28             | 91.95±0.09             | 90.51±0.18          | 0.85±0.02              | NT                     |
| EDTA\(^b\)          | NT                                             | NT                     | NT                     | NT                     | NT                     | NT                     | 94.09±0.75              |

\(^a\)Values represent the means \[ S.E.M. of three parallel measurements (\(p<0.05\)).

\(^b\)Reference compounds.

NT: not tested; \(\text{BHA}\): Butylatedhydroxyl anisole; \(\text{EDTA}\): Ethylenediaminetetraacetic acid
Table S4: Anticholinesterase activity of the extracts and compounds of *R. luteolus*\(^a\)

|                | AChE assay |                | BChE assay |                |
|----------------|------------|----------------|------------|----------------|
|                | Inhibition (%) | IC\(_{50}\) (µg/mL) | Inhibition (%) | IC\(_{50}\) (µg/mL) |
| **Methanol**   | 63.12±0.45 | 153±1.26        | 14.66±1.50 | >250           |
| **Hexane**     | 65.16±0.34 | 114±1.09        | 44.55±0.21 | 224±2.07       |
| **Ethyl acetate** | 10.40±0.04 | >250            | 3.26±0.18  | >250           |
| **Water**      | 13.22±0.12 | >250            | 13.43±1.22 | >250           |
| **Compound 1** | 8.14±0.20  | >250            | 29.29±1.53 | >250           |
| 2              | 3.18±0.03  | >250            | 16.47±1.02 | >250           |
| 3              | 15.11±0.08 | >250            | 47.82±1.40 | 218±1.25       |
| **Galantamine**| 65.01±1.92 | 4.31±0.03       | 76.90±0.20 | 12.29±0.06     |

\(^a\)Values represent the means ± S.E.M. of three parallel measurements (\(p<0.05\)).

\(^b\)Reference compounds.
Figure S1: Important HMBC interactions of compound 1
Figure S2: $^1$H NMR spectrum of compound 1 (CDCl$_3$, 500 MHz)
**Figure S3:** $^{13}$C NMR spectrum of compound 1 (CDCl$_3$, 150 MHz)
Figure S4: DEPT135 spectrum of compound 1
**Figure S5:** HMBC spectrum of compound 1
Figure S6: HSQC spectrum of compound 1
Figure S7: The HPLC-DAD Chromatogram of *Rhizopogon luteolus*

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