SUPPLEMENTARY MATERIAL

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

Gülşen Tel-Çayan^a, Akhtar Muhammad^{a,b}, Mehmet Emin Duru^{a,*} Mehmet Öztürk^a, Achyut Adhikari^b and Aziz Türkoğlu^c

^a Faculty of Sciences, Department of Chemistry, Muğla Sıtkı Koçman University, 48121 Muğla, Turkey

^b H.E.J Research Institute of Chemistry, ICCBS, University of Karachi, Karachi-75270, Pakistan

^c Faculty of Sciences, Department of Biology, Muğla Sıtkı Koçman University, 48121 Muğla, Turkey

*Corresponding author. E-mail: eminduru@mu.edu.tr

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 (IC₅₀: 16.65±1.12 μ g/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 Sıtkı 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. IR spectra were recorded on a Thermo Scientific one Nicolet IS10 FT-IR Spectrometer, NMR spectra were on Bruker Avance AV-500-MHz and 600-MHz instruments coupled with cooled cryoprobes probe for ^1H - 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 Cooperation, 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 (SpectraMax³⁴⁰PC, Molecular Devices, USA). The measurements and calculations of the activity results were evaluated using Softmax PRO v5.2 software.

Column chromatography, and thin-layer chromatography (TLC) were performed on silica gel (Kieselgel 60, 70-230 mesh, Merck), Silica gel 60 F₂₅₄ plates and RP-18 F₂₅₄S, respectively. TLC spots were detected under UV-254-nm light, and visualized by CeSO₄ 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), acetylthiocholine 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 ($\geq 99\%$), fumaric acid ($\geq 99\%$), protocatechuic acid (97%), catechin hydrate ($\geq 98\%$), p-hydroxybenzoic acid (99%), 6,7-dihydroxy coumarin (98%), caffeic acid ($\geq 98\%$), vanillin (99%), 2,4-dihydroxy benzoic acid (98%), p-coumaric acid ($\geq 98\%$), ferulic acid (99%), coumarin ($\geq 99\%$), trans-2-hydroxycinnamic acid (99%), ellagic acid ($\geq 98\%$), rosmarinic acid ($\geq 98\%$) 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 $\times$ 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/CHCl₃, CHCl₃/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*, Me - 2'), 1.13 (6 H, *t*, *J* = 6.0 Hz, 3', 4' -Me), 2.33 (1 H, *m*, H-2), 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). ¹³C NMR (125 MHz, CDCl₃) δ: 173.3 (C-1'), 70.6 (C-1), 34.4 (C-2), 65.1 (C-3), 33.3 (C-4), 34.0 (C-5), 26.7 (C-6), 29.1 (C-7), 29.2 (C-8), 29.3 (C-9), 29.4 (C-10), 29.5 (C-11), 29.4 (C-12), 29.4 (C-13), 29.4 (C-14), 29.4 (C-15), 29.4 (C-16), 29.4 (C-17), 29.4 (C-18), 29.5 (C-19), 29.5 (C-20), 29.5 (C-21), 29.5 (C-22), 29.7 (C-23), 29.7 (C-24), 31.9 (C-25), 22.7 (C-26), 14.1 (C-27), 20.1 (2'-Me), 17.3 (3'-Me), 17.4 (4'-Me).

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:

$$\text{Absorbance} = 0.0073[\text{pyrocatechol } (\mu\text{g})] - 0.1665 (r^2, 0.9976) \quad \text{eq.1}$$

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:

$$\text{Absorbance} = 0.082[\text{quercetin } (\mu\text{g})] - 0.0073 (r^2, 0.9998) \quad \text{eq.2}$$

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 (Chemsal 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 A_{0.50}, which corresponds to the concentration producing 0.500 absorbance for CUPRAC assay and inhibition percentage (%) at 200 μ g/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 $\pm$ 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

^aValues 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	<i>Rhizopogon luteolus</i> (%)
Pentadecanoic acid (C _{15:0})	0.31
Palmitoleic acid (C _{16:1})	5.8
Palmitic acid (C _{16:0})	6.6
Linoleic acid (C _{18:2})	45.8
Oleic acid (C _{18:1})	16.2
Stearic acid (C _{18:0})	23.7
Arachidonic acid (C _{20:4})	0.39
Tetracosanoic acid (C _{24:0})	1.2
Total saturation	31.8
Total unsaturation	68.2
Saturation/Unsaturation	0.47
L/O ^a	2.80

^aL/O: linoleic acid-oleic acid ratio.

Table S3: Antioxidant activity of the extracts and compounds of *R. luteolus* by the β -carotene-linoleic acid, DPPH[•], ABTS^{•+}, O₂^{•-}, CUPRAC and Metal Chelating assays.^a

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

^aValues represent the means $\pm$ S.E.M. of three parallel measurements ($p < 0.05$).^bReference compounds.

NT: not tested; BHA: Butylatedhydroxyl anisole; EDTA: Ethylenediaminetetraacetic acid

Table S4: Anticholinesterase activity of the extracts and compounds of *R. luteolus*^a

	AChE assay		BChE assay	
	Inhibition (%)	IC ₅₀	Inhibition (%)	IC ₅₀
	(at 200 µg/mL)	(µg/mL)	(at 200 µg/mL)	(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 ^b	65.01±1.92	4.31±0.03	76.90±0.20	12.29±0.06

^aValues represent the means ± S.E.M. of three parallel measurements (*p*<0.05).^bReference compounds.

Figure S1: Important HMBC interactions of compound **1**

Figure S2: ¹H NMR spectrum of compound **1** (CDCl₃, 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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