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ORIGINAL PAPER

Chemical constituents and their bioactivities from truffle *Hysterangium inflatum*

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Abstract

Hysterangium inflatum is a truffle that grows naturally in the roots of *Eucalyptus* sp. and is distributed along the Mediterranean and Aegean coasts. Chemical investigation of the *H. inflatum* enabled to isolate of a new cerebroside, hysteroside (**1**), and seven known compounds namely, psyllic acid (**2**), brassicasterol (**3**), ergosterol (**4**), ergosterol D (**5**), ergosterol peroxide (**6**), ergosta-7,9,22-triene-3-O-β-D-glucoside (**7**) and mannitol (**8**). IR, NMR, MS techniques were used for structural elucidation and supported with literature data. Antioxidant, cholinesterase, urease, and tyrosinase inhibitory and cytotoxic activities on MCF-7 breast, H-1299 lung cancer cells, and murine fibroblast (L929) non-cancerous cells of extracts and isolated compounds from *H. inflatum* were analyzed. All the isolated compounds, except compounds **2** and **8**, displayed considerable cytotoxic activities against MCF-7 and H-1299 cancer cells. Compounds **1** (IC_{50}: 18.11 µg/mL) and **5** (IC_{50}: 24.93 µg/mL) were the most effective against MCF-7, while compounds **6** (IC_{50}: 27.61 µg/mL), **1** (IC_{50}: 36.20 µg/mL) and **5** (IC_{50}: 38.62 µg/mL) showed most potent toxicity against H-1299 and the extracts and compounds have no toxic effect on L929. Among the extracts, the methanol extract displayed the best antioxidant activity in all assays. Compound **1** exhibited highest enzyme inhibition activities with value of 58.71%, 52.84%, 45.37%, and 35.63%, against urease, tyrosinase, butrylcholinesterase (BChE), and acetylcholinesterase (AChE) enzymes at 100 µg/mL concentration, among the isolated compounds. These results support that *H. inflatum* is a steroid-rich truffle and might be a potential source, especially for ergostane type steroids.

Keywords *Hysterangium inflatum* · Truffle · Cytotoxicity · Bioactive compounds · Hysteroside · Biological activity

Introduction

Mushrooms are structures formed in the soil as a result of the condensation of the micelle networks that develop in a scattered way. Mushrooms have an important place in the world in terms of nutrition with their low-calorie content, rich protein, vitamin, mineral, and essential fatty acids [1, 2]. Also, since the existence of mankind, mushrooms have been consumed as food or used for medicinal purposes in folk medicine [3, 4]. In addition to mushrooms known as an immunomodulator and antitumor agent, scientific researches on various biological activities such as antiviral, antimicrobial, antioxidant, anticholinesterase, antimutagenic, antihypertension, anti-inflammatory, anticancer, antitumor, and immunomodulatory have increased in recent years [4–10].

Unlike other mushrooms, truffles complete all stages of development underground. Truffles known as ectomycorrhizal mushrooms and form an ectomycorrhizal partnership with the roots of trees such as oak, nut, pine, linden [1, 5]. Truffles are a complex family that mainly includes the genera *Tuber*, *Picoa, Tirmania*, and *Terfezia*, spread in barren and semi-arid areas covering the Mediterranean basin. Additionally, some species of Truffle have been found in North America, Japan, South Africa, and China, [5, 11–13]. Truffles have been consumed as food since ancient times, due to their unique flavor, smell, and aroma [14, 15]. Although

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the aromas of the truffles are sharp, they have an odor that resembles the scents of cheese, garlic and vanilla. They have a highly respected place in the culinary world with their nicknames such as "underground gold", and "diamond of the kitchen" [16]. Truffles include protein, unsaturated fatty acids, dietary fiber, ash, essential amino acids, vitamin D, and metal-mineral content, as well as volatile organic compounds such as aldehyde, alcohol, ketone, organic acid, and phenolic compounds [11, 15]. Truffles are used as a therapeutic agent in addition to being consumed as food worldwide. In vitro and in vivo studies have been determined that truffles have antioxidant, anti-inflammatory, antimicrobial, antimutagenic, and aphrodisiac activities, and contain bioactive compounds such as phenolic, polysaccharide, triterpene, and steroids [5, 17–21]. Despite increasing research in recent years, studies on the biological activities and chemical composition of edible truffles are limited, and more research is needed to determine the potential bioactive properties of truffles.

Hysterangium inflatum is a type of truffle that grows on the roots of eucalyptus trees. It has a strong and fragrant smell like eucalyptus. To date, no research has been published on the biological activities and chemical compositions of *H. inflatum*. Our aim was to investigate the characterization of chemical constituents of *H. inflatum* with antioxidant, cytotoxic, anticholinesterase, anti-urease and anti-tyrosinase activities. For this purpose, hexane, chloroform, acetone, methanol, water extract, and eight compounds (**1**–**8**) used for determining biological activities.

Materials and methods

Mushroom material

H. inflatum was collected from Muğla-Köyceğiz, Turkey in December 2016. The voucher specimen has been deposited at the Research and Application Center for Mushrooms, Muğla Sıtkı Koçman University (Fungarium no: MT1648).

Spectral measurements

Characterization of isolated compounds were carried out by FT-IR, ESI-MS, EI-MS, 1D, and 2D-NMR spectroscopy techniques. IR spectra were recorded on a Thermo Scientific one Nicolet IS10 FT-IR spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded at 600 MHz and 125 MHz, respectively on Agilent-600-MHz instruments connected with cooled cryoprobes probe for HSQC, HMBC, and COSY. EI–MS spectra was performed on a JEOL MS route resolution. SpectraMax^{340}PC, Molecular Devices, USA was used as a 96-well microplate reader to test bioactivities. Softmax PRO v5.2 software was used to measure and calculate of the bioactivity activity studies.

Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh, Merck). Thin layer chromatography (TLC) was performed using silica gel 60 F254 and RP-18 F254S plates. Normal phase (JAIGEL-SIL), and C_{18} (JAIGEL-ODS) preparative columns were used in Recycling preparative HPLC coupled with UV and RI detectors (Japan Analytical Industry Co. Ltd.). $CeSO_4$ chemical reagent, UV-254 and 366-nm light were used to identify the TLC spots.

Extraction and isolation

Dried and grounded *H. inflatum* (200 g) was extracted with *n*-hexane, chloroform, acetone, and methanol respectively, at room conditions for 24 h and three times. Then, the mushroom residue was extracted with hot distilled water (80 °C). Organic solvents were removed by rotary evaporator under vacuum. The aqueous part was allowed to freeze (− 20 °C) and the solvent (water) was removed using a freeze-dryer. Thus, five different extracts were obtained: hexane (2 g), chloroform (4 g), acetone (11 g), methanol (25 g), and water extracts (8 g).

The methanol extract (HIM) was chromatographed over silica gel column with hexane-dichloromethane, dichloromethane-acetone, acetone-methanol, and methanol solvent systems resulting fifteen fractions. Fraction 7 (HIM-7) was separated with column chromatography and eluted with dichloromethane: methanol (9:1) solvent system, and five subfractions were achieved. Subfraction 3 (HIM-7-3) was purified by preparative Recycle HPLC with normal phase silica column with dichloromethane: methanol (9:1) to obtain compound **1**. Fraction 6 (HIM-6) was re-chromatographed using silica gel column chromatography with hexane: ethyl acetate (1:1) and purified compound **2**. Fraction 4 (HIM-4) was subjected to silica gel column chromatography with hexane: ethyl acetate to obtain twelve sub-fractions. Subfraction 2 (HIM-4-2) was separated by preparative TLC with elution of hexane: ethyl acetate (9:1) solvent system to give compound **3**. Subfraction 6 (HIM-4-6) was afforded to Recycle HPLC with hexane: ethyl acetate (7:3) solvent system to isolate compound **4**. Fraction 7 (HIM-7) was re-chromatographed using silica gel column with elution of hexane: ethyl acetate to give seventeen sub-fractions. Compound **5** was isolated from subfraction 6 (HIM-7-6) by silica gel column chromatography with hexane: ethyl acetate (8:2) solvent system and compound **6** was obtained by preparative TLC using hexane: ethyl acetate (7:3) solvent system. Fraction 10 (HIM-10) was re-chromatographed using a silica gel column with dichloromethane: methanol (9:1) solvent system

and obtained eight sub-fractions. Subfraction 5 (HIM-10-5) was separated Recycle HPLC with elution of dichloromethane: methanol (9:1) solvent system by using normal phase silica column to give compound **7**. Fraction 13 (HIM-13) was separated on Recycle HPLC with methanol: water (7:3) solvent system on preparative C_{18} column to obtain compound **8**. As a result of extraction and isolation studies, eight compounds namely, 1-O-β-D-glucopyranosyl-(2S,3S,4E,9E)-2-[(2R)-2-hydroxyeicosanoylamino]-tetradec-4,9-diene-9-methyl-3-ol (hysteroside) (**1**), psyllic acid (**2**), brassicasterol (**3**), ergosterol (**4**), ergosterol D (**5**), ergosterol peroxide (**6**), ergosta-7,9,22-triene-3-O-β-D-glucoside (**7**) and mannitol (**8**) were obtained from *H. inflatum*.

Methanolysis of 1

Compound **1** was heated with 5% HCl solution in methanol at 70 °C for 12 h in a sealed flask. The reaction was checked by TLC analysis. After the reaction is complete, the liquid mixture was extracted with n-hexane and the *n*-hexane part was separated and concentrated for further analysis by GC–MS [22].

GC-MS analysis of FAME from compound 1

FAME from compound **1** was performed in GC-MS [column oven temperature was held at 100–250 °C (rate of temperature increase 5 °C/min]. The GC-MS analysis result was given methyl 2-hydroxyeicosanoate, $t_R = 45.52$, EI-MS m/z: 342 $[M]^+$.

Antioxidant activity

Antioxidant activities of the extracts and compounds from *H. inflatum* were investigated in five parallel test systems. Lipid peroxidation inhibition activity was performed by β-carotene-linoleic acid test system, free radical and cation radical scavenging activities by $DPPH^{\bullet}$ and $ABTS^{\cdot+}$ assays, metal chelating activity on Fe^{2+}, and cupric reducing antioxidant capacity (CUPRAC) were used to determine antioxidant activities of extracts and isolated compounds [23]. The results of antioxidant activity were stated as IC_{50} values and inhibition percentage (%) at 200 µg/mL concentration. $A_{0.50}$ which equals to the concentration giving 0.50 absorbance for CUPRAC assay.

Cytotoxic activity

Cytotoxic activities of various extracts and compounds of *H. inflatum* were measured by MTT assay against lung cancer (H1299), breast cancer (MCF-7), and murine fibroblast (L929) non-cancerous cells according to our recent publication [6]. The results of MTT assay were read by 96-well microliter plates at 540 nm. Doxorubicin was used as a reference compound for comparison of cytotoxic activity. Results were expressed as IC_{50} values which was calculated by the sigmoidal plot of the inhibition rate (%) versus the log concentration (µg/mL) for the isolated compounds.

Determination of enzyme inhibitory activity

Cholinesterase inhibitory activity was screened against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes spectrophotometrically according to Ellman's method with modifications [24, 25]. The anti-urease activity was carried out as reported in our previous study [25]. Anti-tyrosinase activity was screened by spectrophotometrically by mushroom tyrosinase enzyme and L-Dopa used as the substrate according to the protocols previously reported [26]. Galantamine, kojic acid, and thiourea were used as standards. Enzyme inhibitory activity results of the extracts and compounds from *H. inflatum* were given as percentage inhibition (%) at 100 µg/mL concentration.

Statistical analysis

The bioactivity results were presented as the average of triplicate analysis. The results were recorded as the mean ± S.E. The one-way ANOVA was carried out to assess the antioxidant and enzyme inhibitory activities. Student's *t* test determined significant differences, at the 95% confidential level ($p < 0.05$) using statistical SPSS software version 22.0.

Results and discussion

Identification of compounds

A new compound (**1**) and seven known compounds (**2–8**) isolated from the methanol extract of *H. inflatum*. Totally, one cerebroside, one fatty acid, five steroid and one sugar alcohol were purified and their structures were elucidated according to IR, 1D-NMR, 2D-NMR and MS techniques. Compounds **2–8** were identified as, psyllic acid [27], brassicasterol [28], ergosterol [29], ergosterol D [30], ergosterol peroxide [6], ergosta-7,9,22-triene-3-O-β-D-glucoside [31],

and mannitol [32] respectively, on the basis of their spectroscopic fully compatible with the literature (Fig. 1).

Compound **1** was obtained as a white amorphous powder. The molecular formula was assigned as $C_{41}H_{77}NO_9$ with the ESI-HR-TOF-MS which showed the protonated molecular ion peak $[M+H]^+$ at *m/z* 728.5668 (calcd for $C_{41}H_{78}NO_9$, 728.5676) (Fig. S1, S2). The IR spectrum demonstrated absorption bands at 3650 and 3418 cm^{-1} for hydroxy and amide groups, 1546 cm^{-1} for olefinic group and 1634 cm^{-1} amide carbonyl group. The ^{1}H-NMR spectrum of **1** (Table 1) showed three methyls at δ_H 0.89 (6 H, t, *J* = 7.0 Hz, H-14, H-20′), 1.56 (3 H, s, H-15), two oxygenated methylene at δ_H 3.70 (1 H, dd, *J* = 4.2; 10.8 Hz, H-1a), 4.11 (1 H, dd, *J* = 4.2; 10.8 Hz, H-1b), two oxymethines at δ_H 3.98 (1 H, m, H-2′), 3.94 (1 H, dd, *J* = 7.8; 9.0 Hz, H-3), a downfield signal at δ_H 5.20 (1 H, m, H-2), three olefinic protons δ_H 5.47 (1 H, m, H-4), 5.73 (1 H, m, H-5) and 5.14 (1 H, m, H-10), a long chain methylene protons δ_H 1.22–1.75 (Fig S3). Also, the signals for glucose moiety at δ_H 4.25 (1 H, d, *J* = 7.8 Hz), 4.13 (1 H, m), 3.34 (1 H, m), 3.26 (2 H, dd, *J* = 2.4; 4.8 Hz), 3.66 (1 H, dd, *J* = 9.6; 6.6 Hz) and 3.87 (1 H, d, *J* = 10.8 Hz) proposed that compound **1** could be glycosphingolipid [22, 33].

The ^{13}C-NMR spectrum showed characteristic signals amide carbonyl group at δ_C 177.2 (C-1′) and four olefinic carbons at δ_C 131.1 (C-4), 134.7 (C-5), 136.8 (C-9) and 124.8 (C-10) suggesting the presence of two double bonds in compound **1** (Fig S4). Also, ^{13}C-NMR spectrum displayed methine linked to the amide N-atom at δ_C 54.6 (C-2), a downfield signal due to an oxymethylene seemed at δ_C 69.7 (C-1), oxymethine carbons at δ_C 74.9 (C-3) and 73.1 (C-2′), three methyl carbons at δ_C 14.5 (C-14

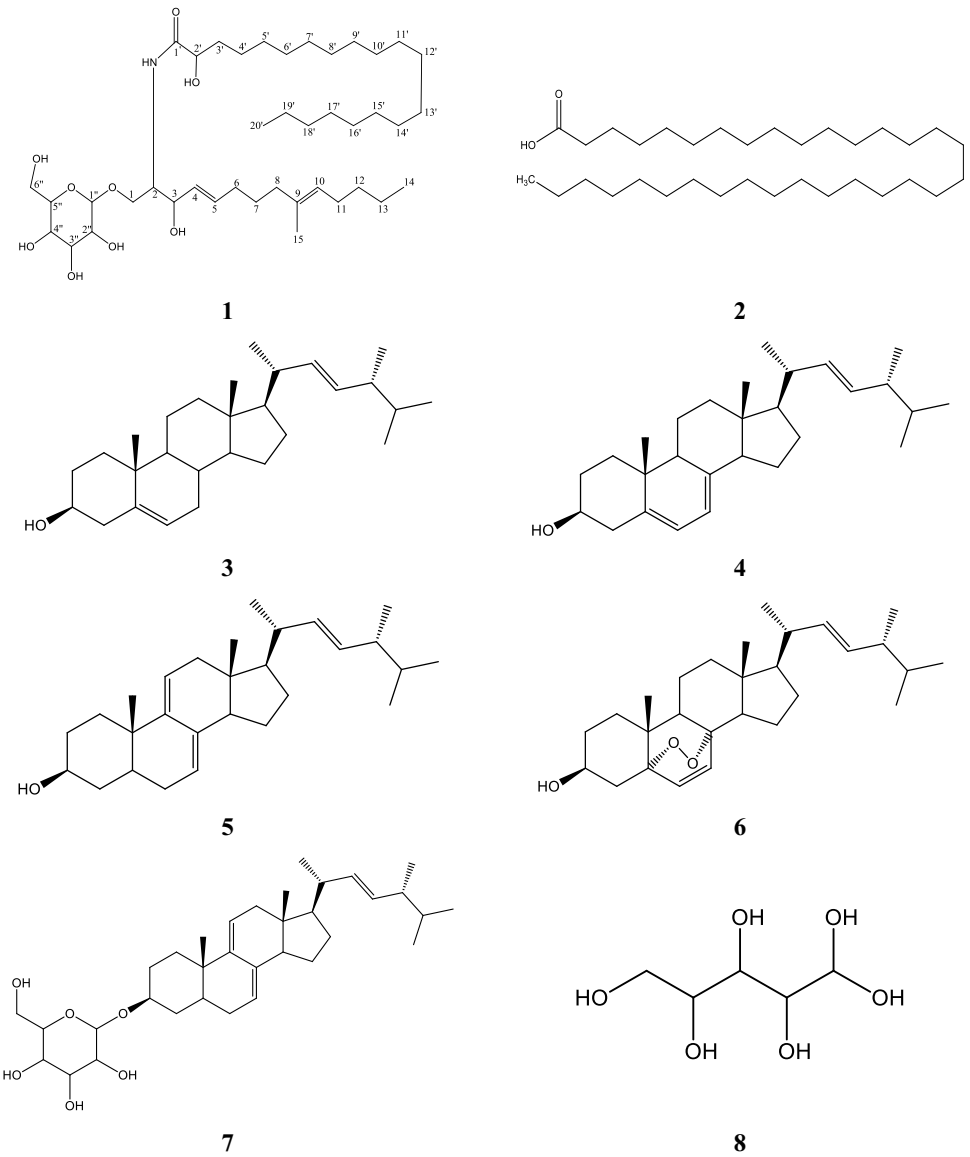

Fig. 1 Chemical structures of the isolated compounds from *H. inflatum*

Table 1 The ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of compound **1**

Position	^{13}C-NMR	^{1}H [δ, mult, J (Hz)]
1a	69.7	3.70 (*dd*, J = 4.2; 10.8 Hz)
1b		4.11 (*dd*, J = 4.2; 10.8 Hz)
2	54.6	5.20 (*m*)
3	74.9	3.94 (*dd*, J = 7.8; 9.0 Hz)
4	131.1	5.47 (*m*)
5	134.7	5.73 (*m*)
6	33.8	2.06 (*m*)
7	28.7	2.06 (*m*)
8	40.9	1.97 (*m*)
9	136.8	–
10	124.8	5.14 (*m*)
11a	26.2	1.28 (*m*)
11b		1.39 (*m*)
12	33.3	1.28 (*m*)
13	23.8	1.28 (*m*)
14	14.5	0.89 (*t*, J = 7.0 Hz)
15	16.1	1.56 (*s*)
1′	177.2	–
2′	73.1	3.98 (*m*)
3′a	35.89	1.54 (*m*)
3′b		1.70 (*m*)
4′–19′	23.8–33.6	1.26–1.75 (*m*)
20′	14.5	0.89 (*t*, J = 7.2 Hz)
1″	104.7	4.25 (*d*, J = 7.8 Hz)
2″	72.9	4.13 (*m*)
3″	77.8	3.34 (*m*)
4″	71.5	3.26 (*dd*, J = 2.4; 4.8 Hz)
5″	77.9	3.26 (*dd*, J = 2.4; 4.8 Hz)
6″a	62.7	3.66 (*dd*, J = 9.6; 6.6 Hz)
6″b		3.87 (*d*, J = 10.8 Hz)

and C-20′), 16.1 (C-15) and sugar moiety were observed at δ_C 104.7 (C-1″), 72.9 (C-2″), 77.8 (C-3″), 71.5 (C-4″), 77.9 (C-5″) and 62.7 (C-6″) which are characteristic of a *β*-glucopyranoside unit. Compound **1** having two carbon-carbon double bond and two oxymethine protons in its structure which considered as a glycosphingolipid. In the HMBC spectrum the carbon signal at 104.7 ppm showed correlation the β-D-glucopyranosyl anomeric proton at δ_H 4.25 (1H, d, J = 7.8 Hz), further confirming the α-orientation of proton of the glucoside unit and the β-configuration in the glucoside moiety (Fig S6). 2D-NMR techniques like COSY and HMBC were used

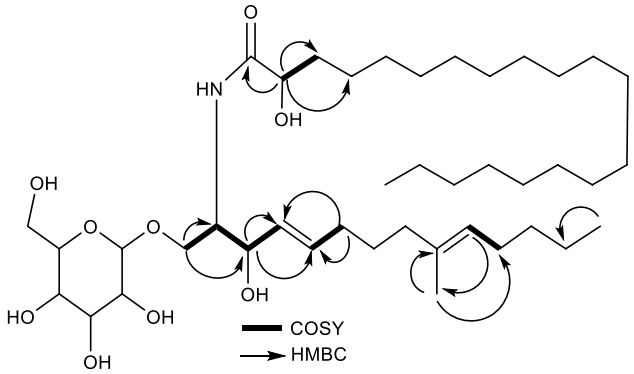

Fig. 2 Important COSY and HMBC correlations of compound **1**

Table 2 Cytotoxic activity of the extracts and isolated compounds of *H. inflatum*

IC_{50} (μg/mL)			
	MCF-7	H-1299	L929
Extracts			
Hexane	NT	NT	NT
Chloroform	NT	NT	NT
Acetone	NT	NT	NT
Methanol	82.57 ± 1.13	> 200	> 200
Water	141.21 ± 1.54	> 200	> 200
Compounds			
1	18.11 ± 0.21	36.20 ± 0.10	> 200
2	> 200	> 200	NT
3	38.08 ± 0.75	63.85 ± 0.12	> 200
4	30.91 ± 0.15	47.15 ± 0.31	> 200
5	24.93 ± 0.80	38.62 ± 0.46	> 200
6	41.07 ± 0.81	27.61 ± 0.18	> 200
7	63.47 ± 0.29	88.24 ± 0.75	> 200
8	> 200	> 200	NT
Standard			
Doxorubicin	0.93 ± 0.05	1.25 ± 0.08	NT

IC_{50} values represent the means ± SE of three parallel sample measurements ($p < 0.05$)

NT not tested

to confirm the substitutions at several positions and long chain hydrocarbon skeleton. Similarly, location of glucose moiety was confirmed with HMBC (Fig S6, S7). Anomeric proton (H-1″) showed correlation C-1, C-2″ and C-3″ in HMBC spectrum. (Fig. 2). According to all spectral data, previously undescribed cerebroside named, hysteroside (**1**), could be characterized

as 1-*O*-β-D-glucopyranosyl-(2S,3S,4E,9E)-2-[(2R)-2-hydroxyeicosanoylamino]-tetradec-4,9-diene-9-methyl-3-ol (Fig. 1).

Cytotoxic activity

Cytotoxic activities of various extracts and compounds against lung cancer (H1299), breast cancer (MCF-7), and murine fibroblast (L929) non-cancerous cells are given in Table 2. Among the extracts, methanol extract indicated the best cytotoxicity on the MCF-7 cell line with the IC_{50} value of 82.57 µg/mL and followed by the water extract (IC_{50}: 141.21 µg/mL). Compound **1** had the highest cytotoxic activity against MCF-7 with IC_{50}: 18.11 µg/ml, followed by compound **5** (IC_{50}: 24.93 µg/mL). Cytotoxic activities of the other compounds on MCF-7 cell line were decreased in the order of compounds **4** (IC_{50}: 30.91 µg/mL) > **3** (IC_{50}: 38.08 µg/mL) > **6** (IC_{50}: 41.07 µg/mL).

Even if, tested extracts exhibited low toxicity against H-1299, isolated compounds showed considerable activities. Compound **6** was found to be the most potent activity against H-1299 with the IC_{50} value of 27.61 µg/mL. Also, compounds **1** and **5** showed noticeable toxicities, with the IC_{50} values of 36.20 and 38.62 µg/mL, respectively. Additionally, extracts and isolated compounds have no cytotoxic effects against murine fibroblast cells (L929). It is the first study to investigation of cytotoxicity of extracts and isolated compounds **1**, **2**, and **5**.

Antioxidant activity

The results of antioxidant activity of various extracts and compounds summarized in Table 3. The best lipid peroxidation activity was observed in methanol extract of *H. inflatum* (27.12 µg/mL) followed by the water extract (40.33 µg/mL). Similarly, methanol extract displayed the strongest activities in ABTS, CUPRAC, and DPPH assays, with IC_{50} values of 209.1, 164.2, and 70.3 µg/mL, respectively. In the metal-chelating activity, the methanol extract (32.87% at 200 µg/mL) indicated the highest inhibitory activity among all the samples studied. None of samples has comparable activity with EDTA (Table 3).

Among the isolated compounds, compound **1** indicated the highest activity in all antioxidant tests and followed by

Table 3 Antioxidant activity of the extracts and isolated compounds of *H. inflatum* by β-carotene-linoleic acid, $DPPH^{\cdot}$, $ABTS^{\cdot+}$, CUPRAC and metal chelating assays

Antioxidant activity									
	β-Carotene-linoleic acid assay		$DPPH^{\cdot}$ assay		$ABTS^{\cdot+}$ assay		CUPRAC assay		Metal chelating assay
	Inhibition (%) (at 200 µg/mL)	IC_{50} (µg/mL)	Inhibition (%) (at 200 µg/mL)	IC_{50} (µg/mL)	Inhibition (%) (at 200 µg/mL)	IC_{50} (µg/mL)	Absorbance (at 200 µg/mL)	$A_{0.50}$ (µg/mL)	Inhibition (%) (at 200 µg/mL)
Extracts									
Hexane	86.99 ± 1.73[a]	46.90 ± 1.21	9.08 ± 0.93[e]	> 200	18.77 ± 0.56[f]	> 200	0.44 ± 0.03[e]	> 200	10.24 ± 0.51[d]
Chloroform	58.85 ± 1.12[b]	164.7 ± 1.13	13.82 ± 0.89[e]	> 200	19.20 ± 0.27[f]	> 200	0.48 ± 0.04[e]	> 200	10.97 ± 0.38[d]
Acetone	57.98 ± 0.18[b]	140.3 ± 0.30	21.27 ± 0.15[d]	> 200	38.89 ± 1.09[e]	> 200	0.59 ± 0.03[d]	93.35 ± 0.01	12.46 ± 0.49[d]
Methanol	81.82 ± 0.84[a]	27.12 ± 1.30	48.96 ± 0.82[b]	209.1 ± 1.82	53.96 ± 0.34[c]	164.2 ± 1.33	0.63 ± 0.01[c]	70.31 ± 0.05	32.87 ± 1.54[b]
Water	68.30 ± 1.32[b]	40.33 ± 1.54	24.02 ± 0.79[d]	> 200	47.01 ± 1.28[d]	> 200	0.39 ± 0.01[f]	> 200	17.57 ± 0.59[c]
Compounds									
1	63.68 ± 1.26[b]	118.5 ± 0.57	55.60 ± 0.95[b]	143.1 ± 0.50	66.74 ± 0.56[b]	102.7 ± 0.74	0.65 ± 0.08[c]	138.4 ± 0.23	16.49 ± 0.76[c]
2	16.74 ± 0.86[e]	> 200	9.63 ± 0.78[e]	> 200	15.73 ± 1.18[f]	> 200	0.24 ± 0.06[g]	> 200	10.94 ± 0.66[d]
3	30.15 ± 1.04[d]	> 200	11.23 ± 0.84[e]	> 200	30.45 ± 0.80[e]	> 200	0.28 ± 0.02[g]	> 200	6.54 ± 0.35[e]
4	45.83 ± 1.46[c]	> 200	33.67 ± 0.89[c]	> 200	52.70 ± 1.26[c]	160.4 ± 1.05	0.54 ± 0.04[d]	167.6 ± 0.65	14.81 ± 0.90[c]
5	58.33 ± 0.75[b]	125.2 ± 1.24	51.76 ± 1.01[b]	182.5 ± 0.70	55.47 ± 0.96[c]	131.3 ± 0.90	0.61 ± 0.03[c]	140.9 ± 0.36	7.75 ± 0.42[e]
6	52.90 ± 0.86[c]	166.3 ± 1.70	38.24 ± 1.56[c]	> 200	51.95 ± 0.93[c]	157.6 ± 0.41	0.57 ± 0.06[d]	155.4 ± 0.38	12.46 ± 0.19[d]
7	21.42 ± 0.76[d]	> 200	5.53 ± 0.52[f]	> 200	13.06 ± 0.14[f]	> 200	0.36 ± 0.05[f]	> 200	5.43 ± 0.48[e]
8	6.28 ± 0.17[f]	> 200	5.23 ± 0.49[f]	> 200	9.14 ± 0.16[g]	> 200	0.25 ± 0.01[g]	> 200	5.85 ± 0.63[e]
Standards									
α-Tocopherol	90.51 ± 0.18[a]	2.10 ± 0.08	87.14 ± 0.28[a]	37.20 ± 0.41	85.83 ± 0.12[a]	38.51 ± 0.54	0.85 ± 0.02[b]	66.72 ± 0.81	NT
BHA	92.80 ± 0.02[a]	1.34 ± 0.04	88.60 ± 0.29[a]	19.80 ± 0.36	86.70 ± 0.10[a]	11.82 ± 0.09	2.47 ± 0.01[a]	24.40 ± 0.69	NT
EDTA	NT	NT	NT	NT	NT	NT	NT	NT	94.70 ± 0.60[a]

Different subscripts in the same row indicate significant difference ($p < 0.05$)

NT not tested

compound **6**. Compound **1** exhibited IC_{50} values of 118.5, 143.1, 102.7, and 138.4 µg/mL while compound **6** showed IC_{50} values of 166.3, 157.6, 155.4 µg/mL in β-carotene-linoleic acid, DPPH, ABTS, and CUPRAC assays, respectively. In metal chelating activity, all isolated compounds indicated low inhibitory activity (5.43–16.49% inhibitions at 200 µg/mL concentration) compared with EDTA (94.70% at 200 µg/mL concentration). Even if, antioxidant activities of some truffle species reported in the literature, there is no report on the antioxidant activities of extracts from *H. inflatum*. Additionally, this is the first study to determine the antioxidant activity of compounds **1**, **2**, **5**, and **7**.

Anticholinesterase activity

Alzheimer's disease (AD) is described by cognitive abilities and causing loss of memory. Inhibition of butyrylcholinesterase (BChE), and acetylcholinesterase (AChE) are the most accepted approach for the treatment of AD [34]. Anticholinesterase activities of various extracts and compounds from *H. inflatum* were determined against acetyl- and butyrylcholinesterase enzymes using the Ellman method (Table 4). Among the tested extracts, hexane extract showed the best inhibitory activity against AChE (32.80%) and BChE (44.61%) enzymes at 100 µg/mL concentration. Among the isolated compounds, compound **1** was found to be most active compound against both enzymes. Compound **1** showed 35.63 and 45.37% inhibitions for AChE and BChE, respectively. Compounds **5** and **3** exhibited moderate activity against BChE with value of 39.21 and 30.83% inhibitions, respectively at 100 µg/mL concentration, while compounds **5** and **3** showed 28.76 and 21.69% inhibitions against AChE at the same concentration. We investigated cholinesterase inhibitory activities of all extracts and compounds **1**, **2**, **5**, and **7** for the first time with this report.

Anti-urease activity

Urease is a nickel-dependent metalloenzyme and involved in the hydrolysis of urea into ammonia and carbon dioxide. Inhibition of urease has been considered as targets for the treatment of gastric and peptic ulcers [35]. Urease inhibitory activity was tested ammonia production using the indophenol method. Results were given as percentage inhibitions (%) at 100 µg/mL concentration (Table 4). Methanol extract demonstrated the most potent inhibitory activity (65.45%) when compared with thiourea (76.49%). Chloroform (37.19%) and acetone (34.17%) extracts showed moderate activity, while hexane (15.19%) and water (15.51%) extracts

Table 4 Cholinesterase, tyrosinase and urease inhibitory activities of the extracts and isolated compounds of *H. inflatum*

	Cholinesterase inhibitory activity		Urease inhibitory activity	Tyrosinase inhibitory activity
	AChE assay	BChE assay		
Extracts				
Hexane	32.80 ± 1.14^{b}	44.61 ± 1.15^{b}	15.19 ± 1.13^{d}	5.54 ± 0.49^{e}
Chloroform	29.59 ± 1.05^{c}	33.85 ± 1.63^{d}	37.19 ± 1.25^{c}	6.04 ± 0.25^{e}
Acetone	5.36 ± 0.96^{f}	16.22 ± 0.89^{f}	34.17 ± 0.15^{c}	11.63 ± 0.85^{d}
Methanol	6.89 ± 1.16^{f}	18.01 ± 0.59^{f}	65.45 ± 1.09^{b}	40.61 ± 0.97^{c}
Water	15.89 ± 0.12^{e}	37.09 ± 0.69^{c}	15.51 ± 0.98^{d}	6.43 ± 0.33^{e}
Compounds				
1	35.63 ± 0.46^{b}	45.37 ± 0.81^{b}	58.71 ± 0.85^{b}	52.84 ± 0.95^{b}
2	5.33 ± 0.17^{f}	8.46 ± 0.55^{h}	6.63 ± 0.22^{e}	5.48 ± 0.15^{e}
3	21.69 ± 0.55^{d}	30.83 ± 0.95^{d}	8.93 ± 0.29^{e}	14.22 ± 0.47^{d}
4	20.66 ± 0.98^{d}	24.74 ± 1.05^{e}	12.68 ± 0.91^{d}	13.98 ± 0.56^{d}
5	28.76 ± 0.54^{c}	39.21 ± 0.86^{c}	9.95 ± 0.13^{e}	12.27 ± 0.36^{d}
6	13.61 ± 0.42^{e}	25.57 ± 1.20^{e}	10.62 ± 0.54^{d}	15.78 ± 1.08^{d}
7	4.95 ± 0.12^{f}	13.66 ± 0.76^{g}	7.88 ± 0.62^{e}	5.96 ± 0.27^{e}
8	NA	NA	NA	NA
Standards				
Galantamine	77.69 ± 0.45^{a}	80.39 ± 0.17^{a}	NT	NT
Kojic acid	NT	NT	NT	71.50 ± 0.25^{a}
Thiourea	NT	NT	76.49 ± 0.30^{a}	NT

Inhibition % of 100 µg/mL concentration of the extracts and compounds. Different subscripts in the same row indicate significant difference ($p < 0.05$)

NA not active; *NT* not tested

showed low inhibition activity against urease. Compound **1** exhibited the highest urease enzyme inhibition activity with the value of 58.71%. Except for compound **1**, isolated compounds showed low activity against urease.

Anti-tyrosinase activity

Tyrosinase enzyme has a crucial role involved in melanin synthesis in mammals, plants, fungi, and bacteria. Tyrosinase inhibitors have become increasingly important in the medical and cosmetic industries to prevent hyperpigmentation [36]. The inhibitory effects of various extracts and compounds from *H. inflatum* on tyrosinase are presented in Table 4. Among the tested extracts, methanol extract of *H. inflatum* displayed the best tyrosinase inhibition activity with the value of 40.61% at 100 µg/mL concentration. Other tested extracts showed low inhibitory activity against tyrosinase. Compound **1** was found to be the most active (52.84%) against tyrosinase compared with isolated compounds (Table 4).

Conclusions

Purification studies of *H. inflatum* enabled to isolate and identify new cerebroside (**1**) and seven known (**2**–**8**) compounds. The cytotoxicity tests of compounds **1**, **2**, and **5** were determined for the first time. Except for compounds **2**, and **8**, all the isolated compounds showed significant activities against both MCF-7 and H-1299 cancer cell lines, leading to the belief that they could be a potential medical agent against these cancer cell lines. Also, with this research, the enzyme inhibition (cholinesterase, urease, and tyrosinase) activities of various extracts and compounds **1**, **2**, **5**, and **7** were determined for the first time. *H. inflatum* can be considered as a natural steroid source due to most of the isolated compounds have ergostane-type steroids. Investigating various biological activities of the related compounds *in vivo* conditions will be able to prove definitively that could be a functional food. As a consequence, the conviction that *H. inflatum* is a truffle with high medicinal value and needs to be cultivated and brought into the economy has emerged with this study.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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