J Nat Med (2016) 70:769–779
DOI 10.1007/s11418-016-1013-1

ORIGINAL PAPER

Antioxidative activities of 62 wild mushrooms from Nepal and the phenolic profile of some selected species

Sonam Tamrakar[1] · Hai Bang Tran[1] · Marina Nishida[1] · Satoru Kaifuchi[1] · Hiroto Suhara[2] · Katsumi Doi[1] · Katsuya Fukami[3] · Gopal Prasad Parajuli[4] · Kuniyoshi Shimizu[1]

Received: 17 November 2015 / Accepted: 21 May 2016 / Published online: 4 June 2016

Abstract Mushrooms have garnered immense popularity for their nutritional as well as medicinal values. The therapeutic potential of mushrooms in Nepal, a country well known for its biodiversity and natural medicinal resources, remains largely unstudied. Therefore, this study attempts to unveil the antioxidative properties of Nepalese wild mushrooms. Sixty-two wild mushroom samples were collected from several forests in different parts of Nepal. Ethanol and water extracts of the dried samples were tested for their antioxidative activities using total phenolic content (TPC), oxygen radical absorbance capacity (ORAC) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and reducing power (RP) assays. Ethanol extracts of samples belonging to the order Hymenochaetales showed significantly high activity in all the assays. *Inonotus clemensiae* had an exceptionally high TPC of 643.2 mg gallic acid equivalent (GAE)/g extract and also exhibited the lowest EC_{50} values in DPPH (0.081 mg/mL), ABTS (0.409 mg/mL), and $EC_{0.5}$ value in reducing power (RP; 0.031 mg/mL) assays. High-performance liquid chromatography (HPLC) analysis of the top ten samples with the highest TPC was done to identify the phenolic compounds in the extracts, followed by liquid chromatography–mass spectrometry (LC–MS) analysis for some unknown compounds. These findings highlight the very strong antioxidative activity of Nepalese mushrooms, and paves the way for further research to explore their economic potential.

Keywords Nepal · Wild mushrooms · Antioxidants · TPC · HPLC

Introduction

Free radicals such as the reactive oxygen species (ROS) are formed as a byproduct of normal metabolic processes, such as electron transport chain reactions [1]. Under normal conditions, a fine balance is maintained between the production of the ROS and its elimination by the antioxidative system of the body [2]. However, certain conditions, such as excessive exercise [3], chronic inflammation, exposure to pollutants, and other xenobiotic substances, cause a disturbance in this balance [4], leading to a condition commonly known as oxidative stress. Oxidative stress can have detrimental effects on cellular lipids, proteins, and DNA, consequently, leading to a number of diseases, such as diabetes, Alzheimer's, cancer, and other cardiovascular and neurological diseases [5].

Dietary antioxidants can play an important role to mitigate the unfavorable effects of oxidative stress [6]. Moreover, due to the ambiguity in the use of synthetic antioxidants such as BHA and BHT for being potentially

Electronic supplementary material The online version of this article (doi:10.1007/s11418-016-1013-1) contains supplementary material, which is available to authorized users.

✉ Kuniyoshi Shimizu
shimizu@agr.kyushu-u.ac.jp

[1] Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

[2] Miyazaki Prefectural Wood Utilization Research Center, Miyazaki 885-0037, Japan

[3] Material Management Center, Kyushu University, Fukuoka 821-8581, Japan

[4] Plant Pathology Division, Nepal Agriculture Research Council, Khumaltar, Lalitpur, P.O. Box 3605, Kathmandu, Nepal

carcinogenic [7], the prominence of the natural antioxidants from food sources has been heightened even further.

One of the potential sources of dietary antioxidants is mushrooms. Mushrooms have been valued not only for their nutritional properties [8, 9] but also for various medicinal benefits [10–12]. In recent years, much research has been directed towards elucidating the therapeutic capabilities of a wide variety of mushrooms, antioxidative properties being one of the most important among them.

Nepal, a country nestled between India and China, has wide variations in geographic elevations and climatic conditions [13]. As a result, it is extremely rich in biodiversity. This diversity is also represented in the mushrooms found in different parts of the country [14]. However, apart from the consumption of a few species, and some traditional medicinal uses [15], the therapeutic value of Nepalese mushrooms remains largely unexplored. The present study aims to unveil the antioxidative potential of the wild mushrooms of Nepal, to promote their usage as therapeutic agents or nutraceuticals.

Materials and methods

Mushroom samples

Mushrooms which developed basidiomata were collected from several forests in different parts of Nepal. In this study, 62 samples were investigated. The botanical origin, location, and habitat of the samples are provided as supplementary data in Table S1. The pictures of the dried samples are also provided as supplementary data in Fig. S1.

Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium carbonate, and the standards used for high-performance liquid chromatography (HPLC) analysis were obtained from Wako Pure Chemical Industries (Japan). Fluorescein and potassium persulfate were purchased from Sigma-Aldrich Co. (USA), and Folin–Ciocalteu's reagent from Merck (Germany). All the reagents used had a minimum purity of 95 %. The solvents acetonitrile, ethanol, and methanol were of analytical grade and were obtained from Wako Pure Chemical Industries (Japan).

Sample identification

The samples were identified on the basis of morphological features and/or genetic analysis. The dried fungal materials were mounted in 3 % (w/v) KOH or Melzer's reagent and measurements of spore, basidia, cystidia, and other tissue features were made for each specimen using a Nikon Eclipse E600 stereomicroscope. For genetic analysis, the DNA was extracted following the procedure described by Hosaka and Castellano [16], with slight modifications. The internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using the primers ITS1 and ITS4B [17, 18]. Amplifications were performed with KOD-Plus-Neo polymerase (Toyobo Co. Ltd., Japan). The PCR products were purified using the illustra ExoProStar kit (GE Healthcare, UK). Both PCR reactions and purification steps were performed in accordance with the manufacturer's protocol. The purified samples were sequenced by Hokkaido System Science Co., Ltd. The sequences were subjected to a basic local alignment search tool (BLAST) search via the National Center for Biotechnology Information (NCBI). The ITS sequence of the samples that were successfully sequenced were deposited in the DNA Data Bank of Japan (DDBJ). The scientific names and their taxonomic positions used in the present study were described in accordance with the descriptions of the MycoBank Database.

Sample preparation

The samples were firstly air-dried and then dried in an air-ventilated oven at 35 °C for 10 h, followed by at 45 °C for 1 h. The dried samples were ground to a fine powder and extracted separately in ethanol and water. The extractions were performed by the shake flask method in an orbital shaker at 200 rpm, for 24 h at room temperature, and then filtered. Water extracts were freeze-dried and the ethanol extracts were rotary evaporated at 45 °C, and reduced pressure. The extract yield was calculated as the percentage of dried extract obtained from the dry weight of ground mushroom used for extraction. The dried extracts were used for all the analyses.

Total phenolic content

The total phenolic content (TPC) was determined by the Folin–Ciocalteu method [19], with some minor modifications. In a 1.5-mL microfuge tube, 50 μL of the sample solution or standard or sample solvent (blank) and 100 μL of 10 % Folin–Ciocalteu reagent was mixed thoroughly. After 2–3 min, 400 μL of 7.5 % Na_2CO_3 was added and vortexed for a few seconds. The reaction mixture was incubated at room temperature for 60–90 min and then centrifuged at 6000 rpm for 2 min. Two hundred microliters of the supernatant was transferred to the respective wells in a 96-well plate and the absorbance was read at 765 nm using a Molecular Devices FlexStation 3

Microplate Reader. Gallic acid was used as the standard and the results are expressed as mg gallic acid equivalent (GAE)/g extract.

Free radical scavenging assay

ORAC assay

The oxygen radical absorbance capacity (ORAC) assay measures the fluorescence degradation of the fluorescein compound due to the peroxyl radicals generated by the heat treatment of AAPH solution. Antioxidants protect the fluorescein from this oxidative degradation. The method described by Ou et al. was followed, with some minor modifications [20]. The extracts were dissolved in 75 mM phosphate buffer (pH 7.4). In a 96-well plate, 20 μL of the sample solution, phosphate buffer (blank), and trolox (standard) solutions were added into the respective wells. Two hundred microliters of fluorescein solution was then added to each well and the plate was incubated at 37 °C for 10 min. After incubation, 75 μL of prewarmed AAPH solution was added and the fluorescence degradation was measured over a period of 90 min at 30-s intervals using a Molecular Devices FlexStation 3 Microplate Reader. The excitation and emission wavelengths were 485 and 535 nm, respectively. After the fluorescence degradation, the values for the area under the curve (AUC) were obtained from the SoftMax® Pro Data Acquisition and Analysis Software. The AUC value of the blank was reduced from that of the samples as well as the standards. Standard curve was prepared using 0–25 μmol trolox solutions. The results are expressed as μmol trolox equivalent (TE)/g extract.

DPPH assay

The ability of the samples to scavenge the DPPH radicals was determined using the method described by Miliauskas et al. [21], with some minor modifications. A 1-mL portion of DPPH solution (60 μM), freshly prepared in methanol, was mixed with 33 μL of the methanolic sample solution or methanol (blank). A sample concentration of 2.5 mg/mL was used for the ethanol extracts and 2 mg/mL was used for the water extracts. The reaction mixture was then incubated at 37 °C for 20 min, in dark conditions. The decolorization was monitored by checking the absorbance using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan) at 515 nm. The inhibition percentage was calculated using the following equation:

$$\text{Inhibition } \% = [(A_b - A_s)/A_b] \times 100 \quad (1)$$

where A_b and A_s are the absorbances of the reaction mixtures containing the blank and the samples, respectively.

ABTS assay

The free radical scavenging ability of the mushroom extracts was also tested using the ABTS assay, following the method described by Zhu et al. [22]. The ABTS radical cation ($ABTS^{+}$) was generated by reacting 5 mL of aqueous ABTS solution (7 mM) with 88 μL of potassium persulfate ($K_2S_2O_8$), followed by incubation for 12–16 h at room temperature in dark conditions. The working solution was then prepared by adjusting the absorbance at 0.7 ± 0.02 at 734 nm. A 1-mL portion of the working solution was mixed thoroughly with 10 μL of the sample solution or sample solvent (blank). A sample concentration of 2 mg extract/mL was used for ethanol extracts and 5 mg/mL was used for water extracts. After 4 min of incubation at 30 °C, the absorbance was read at 734 nm. The percentage of inhibition was calculated by the formula in Eq. (1).

Reducing power assay

The reducing power (RP) of the extracts were tested by the method described by Oyaizu [23], with minor modifications. In a 1.5-mL microfuge tube, 100 μL of the sample solution or sample solvent (blank) was mixed with 100 μL of phosphate buffer (200 mM; pH 6.6) and 100 μL of 1 % potassium ferricyanide solution. A sample concentration of 1 mg/mL was used for both ethanol and water extracts. The reaction mixture was placed in a water bath at 50 °C for 20 min, followed by rapid cooling in an ice bath and addition of 100 μL of trichloroacetic acid solution (10 %). It was then centrifuged at 3000 rpm for 10 min. A 100-μL portion of the supernatant was added to the respective wells in the 96-well plate and mixed with 100 μL of ultrapure Milli-Q water and 20 μL of 0.1 % $FeCl_3$ solution. It was then incubated at room temperature in dark conditions for 10 min, after which the absorbance was measured at 700 nm using a Molecular Devices FlexStation 3 Microplate Reader. The RP is expressed as the absorbance reading.

Indicators for antioxidative activity (EC_{50} values for DPPH and ABTS, $EC_{0.5}$ for RP)

The top ten samples with the highest TPC were selected for the determination of the EC_{50} values for the DPPH and ABTS assays and $EC_{0.5}$ for the RP assay. The sample concentration resulting in 50 % inhibition in the DPPH and ABTS assays and 0.5 absorbance value in the RP assay were considered as EC_{50} values and $EC_{0.5}$, respectively [24]. The inhibition percentage for the DPPH and ABTS assays and the absorbance for the RP assay were plotted against various sample concentrations, and the equations

thus obtained were used to calculate the EC_{50} and $EC_{0.5}$ values.

Statistical analysis

All the assays were conducted at least three times, and the results are expressed as mean ± standard deviation. Significant differences between sample groups, grouped on the basis of their taxonomic order, were analyzed by the Kruskal–Wallis test, followed by the Dunn–Bonferroni test. Correlation between the TPC and the antioxidative assays were calculated by Spearman's rank correlation. The statistical analyses were performed using SPSS Statistics version 23. *p*-Values less than 0.05 were considered statistically significant.

HPLC analysis

The HPLC analysis was done for the top ten samples with the highest TPC, using the 1220 infinity LC system, Agilent Technologies, equipped with a diode array detector, and fitted with a YMC-Triart C18 column (250 mm × 4.6 mm i.d., 5 μm particle size). The method described by Kim et al. [25] was followed, after some modifications. The solvent system comprised of water with 0.15 % formic acid as solvent A and acetonitrile with 0.15 % formic acid as solvent B. The analysis was carried out at room temperature with a flow rate of 1 mL/min. The gradient flow of the mobile phase was set as: 0–12 min, 5–15 % B; 12–18 min, 15–17 % B; 18–20 min, 17–20 % B; 20–35 min, 20–25 % B; 35–40 min, 25–40 % B; 40–60 min, 40–42 % B; 60–68 min, 42–90 % B; 68–70 min, 90–100 % B; 70–72 min, 100 % B; 72–75 min, 100–5 % B; and 75–85 min, 5 % B. The preferred wavelength of detection was 280 nm and the UV–vis spectra were recorded from 190 to 400 nm. The peaks obtained from the ethanol extracts of mushrooms were compared to the chromatogram of 21 standard phenolic compounds with respect to the retention time and UV–vis spectra. Phenolic compounds present in extracts were quantified by the preparation of standard curves for each standard compound.

LC–MS analysis

Liquid chromatography–mass spectrometry (LC–MS) analysis was done to elucidate the unknown compounds from the HPLC analysis. LCMS-IT-TOF, Shimadzu, Tokyo was used for the analysis. All the chromatographic conditions were the same as for the HPLC analysis, apart from the flow rate, which was reduced to 0.5 mL/min to maintain pressure within permissible limits of the device. For MS, the ESI source was used in positive and negative ionization mode with *m*/*z* values of 100–2000 for MS and 50–1500 for MS/MS. A probe voltage of ±4.5 kV, nebulizer gas flow of 1.5 L/min, CDL temperature of 200 °C, and heat block temperature of 200 °C were used.

Results and discussion

Sample identification

The mushroom samples were identified to the species or genus level on the basis of morphological and/or genetic analyses. The scientific names of the samples are listed in Table 1, along with the extract yield and the antioxidative activities. Samples belonging to the same species can be differentiated by their sample numbers (SN). The DDBJ accession number of genetically identified samples are provided in Table S1.

Antioxidative activities

Ethanol and water extracts of the 62 samples were tested for their antioxidative activity by using four kinds of assays based on the radical scavenging and reducing capabilities. The samples were grouped into four groups, based on their taxonomic order: Hymenochaetales, Polyporales, Agaricales, and Others. Since the majority of the samples belonged to the first three groups, the few remaining samples were grouped as "Others". The water extracts showed a much weaker antioxidative activity than the ethanol extracts. So, only the results for the ethanol extracts have been shown in Table 1. The results for the antioxidative activity of the water extracts are provided as supplementary data in Table S2.

Total phenolic content

The TPC was measured using the Folin–Ciocalteu method. Samples in the Hymenochaetales group showed a significantly greater phenolic content compared to the other three groups. However, *Oxyporus* sp. (SN 13) was a notable exception in this group, with a TPC value of only 7.9 mg GAE/g extract. *Oxyporus* sp. is the only sample that does not belong to the Hymenochaetaceae family, within this order. Although there is a lack of detailed research into this mushroom, the major compounds are reported to be sterols and triterpenes in *Oxyporus populinus* [26]. The highest phenolic content was seen for *Inonotus clemensiae* (SN 1), with 643.2 mg GAE/g extract. The TPC of *Inonotus clemensiae* was higher than that reported for the ethanol extract of *Inonotus obliquus* from Thailand, with 590.87 mg GAE/g extract [27], which is one of the highest phenolic contents reported for mushrooms. The *Ganoderma* species exhibited the highest TPC values within the

Table 1 Antioxidative activities and percentage yield of the ethanol extracts of 62 wild mushrooms of Nepal

SN	Order Scientific name (% yield)	TPC mg GAE/g	ORAC μM TE/g	DPPH[A] Inhibition %	ABTS[B] Inhibition %	RP[C] Absorbance 700 nm
	Hymenochaetales	c	bc	df	bc	ce
1	*Inonotus clemensiae* (16.89)	643.2 ± 21.32	31,966.9 ± 198.6	83.2 ± 0.06	92.2 ± 0.05	1.73 ± 0.03
2	*Inonotus cuticularis* (5.19)	102.8 ± 1.73	13,228.5 ± 125.5	82.3 ± 0.17	39.8 ± 1.25	0.88 ± 0.01
3	*Inonotus* sp. (1.73)	155.7 ± 14.55	6015.9 ± 90.2	89.9 ± 0.11	18.7 ± 0.21	1.00 ± 0.01
4	*Inonotus* sp. (1.90)	58.1 ± 1.09	1917.9 ± 27.3	60.3 ± 0.25	18.4 ± 0.43	0.58 ± 0.00
5	*Cyclomyces setiporus* (1.91)	423.7 ± 19.65	11,017.9 ± 401.3	86.1 ± 0.11	83.3 ± 0.79	1.64 ± 0.03
6	*Cyclomyces setiporus* (0.64)	245.0 ± 2.73	6570.9 ± 169.7	85.3 ± 0.17	67.3 ± 1.14	1.77 ± 0.11
7	*Cyclomyces setiporus* (1.96)	204.5 ± 0.10	2952.3 ± 71.6	88.0 ± 0.07	70.9 ± 0.58	2.01 ± 0.10
8	*Cyclomyces setiporus* (1.11)	155.3 ± 8.73	9988.7 ± 664.5	85.1 ± 0.09	75.2 ± 0.65	2.20 ± 0.06
9	*Cyclomyces setiporus* (1.20)	145.0 ± 17.07	7548.79 ± 261.17	86.1 ± 0.10	75.5 ± 0.87	1.97 ± 0.14
10	*Phellinus* sp. (1.25)	116.8 ± 5.63	4219.9 ± 71.9	87.4 ± 0.38	46.8 ± 0.56	0.75 ± 0.02
11	*Phellinus* sp. (2.10)	66.0 ± 1.77	1829.5 ± 56.6	52.1 ± 1.87	8.6 ± 0.18	0.54 ± 0.07
12	*Phellinus adamantinus* (0.72)	91.0 ± 2.24	2470.1 ± 389.3	77.2 ± 0.57	17.6 ± 0.16	0.84 ± 0.01
13	*Oxyporus* sp. (6.14)	7.9 ± 0.48	328.7 ± 14.7	2.3 ± 0.11	–	0.11 ± 0.00
	Polyporales	ad	a	be	a	ad
14	*Ganoderma* sp. (6.89)	124.2 ± 4.59	3546.6 ± 212.8	90.9 ± 0.47	63.2 ± 0.45	1.22 ± 0.04
15	*Ganoderma* sp. (1.39)	61.1 ± 1.05	2640.2 ± 40	64.4 ± 0.89	20.0 ± 0.98	0.81 ± 0.00
16	*Ganoderma lingzhi* (4.90)	63.1 ± 1.13	1450.8 ± 50.3	56.7 ± 0.52	20.5 ± 0.41	0.43 ± 0.01
17	*Ganoderma lingzhi* (3.43)	41.2 ± 0.36	936.7 ± 7.9	26.0 ± 0.11	12.8 ± 0.23	0.43 ± 0.01
18	*Ganoderma endochroum* (3.01)	50.2 ± 0.06	1730.4 ± 26.54	40.5 ± 0.90	14.2 ± 0.58	0.43 ± 0.01
19	*Ganoderma multipileum* (2.19)	39.5 ± 0.84	2035.7 ± 65.7	35.0 ± 1.19	6.0 ± 0.26	0.32 ± 0.00
20	*Amauroderma calcigenum* (2.53)	61.8 ± 0.07	1795.4 ± 28.3	46.8 ± 0.53	15.4 ± 0.68	0.60 ± 0.01
21	*Microporus xanthopus* (1.08)	14.5 ± 0.33	562.9 ± 38.4	7.0 ± 0.39	1.2 ± 0.25	0.15 ± 0.00
22	*Microporus xanthopus* (1.11)	13.3 ± 0.30	196.7 ± 11.9	6.8 ± 0.43	1.5 ± 0.45	0.14 ± 0.01
23	*Fomes fomentarius* (4.26)	32.4 ± 2.89	144.6 ± 29.2	50.8 ± 1.16	24.1 ± 0.05	0.59 ± 0.01
24	*Trichaptum abietinum* (1.22)	9.5 ± 0.34	782.5 ± 19.9	65.8 ± 0.58	–	0.10 ± 0.00
25	*Trichaptum biforme* (1.23)	5.7 ± 0.60	221.9 ± 41.0	4.0 ± 0.18	1.6 ± 0.29	0.12 ± 0.00
26	*Bjerkandera adusta* (2.63)	51.6 ± 2.39	2042.0 ± 180.7	48.3 ± 1.03	9.7 ± 0.08	0.33 ± 0.01
27	*Abortiporus biennis* (2.93)	20.7 ± 0.34	698.5 ± 24.6	11.4 ± 0.14	2.8 ± 0.37	0.18 ± 0.00
28	*Phlebia tremellosa* (2.23)	19.7 ± 1.17	649.3 ± 38.3	17.6 ± 0.10	6.1 ± 0.37	0.21 ± 0.05
29	*Mycorrhaphium* sp.(0.79)	8.3 ± 0.23	360.6 ± 56.4	2.9 ± 0.16	–	0.15 ± 0.00
30	*Antrodiella zonata* (1.05)	7.5 ± 0.12	201.3 ± 27.0	3.3 ± 0.38	–	0.13 ± 0.00
31	*Antrodiella zonata* (1.61)	13.8 ± 0.35	441.0 ± 30.6	6.6 ± 0.48	–	0.13 ± 0.00
32	*Antrodiella zonata* (3.49)	13.6 ± 0.32	679.9 ± 51.9	5.0 ± 0.07	1.5 ± 0.44	0.18 ± 0.00
33	*Laetiporus versisporus* (4.53)	6.6 ± 0.25	413.7 ± 46.6	2.0 ± 0.18	1.8 ± 0.53	0.16 ± 0.00
34	*Laetiporus versisporus* (7.36)	8.6 ± 0.50	478 ± 36.8	4.8 ± 0.18	–	0.13 ± 0.00
35	*Laetiporus montanus* (5.68)	8.4 ± 0.21	167.8 ± 14.0	3.8 ± 0.18	–	0.17 ± 0.00
36	*Grifola frondosa* (4.95)	4.1 ± 0.61	193.9 ± 37.606	2.9 ± 0.03	–	0.14 ± 0.00
37	*Polyporus arcularius* (5.28)	4.3 ± 0.26	383.3 ± 31.4	9.2 ± 0.10	1.0 ± 0.08	0.12 ± 0.00
38	*Lentinus* sp. (4.68)	3.2 ± 0.79	119.1 ± 1.9	4.2 ± 0.04	–	0.13 ± 0.00
	Agaricales	a	a	a	a	a
39	*Marasmius* sp. (8.75)	23.6 ± 0.99	647.5 ± 11.0	11.5 ± 0.10	5.1 ± 0.10	0.26 ± 0.00
40	*Marasmius mavium* (8.16)	3.3 ± 0.12	309.3 ± 46.7	2.9 ± 0.26	1.6 ± 0.44	0.11 ± 0.00
41	*Pholiota nameko* (5.02)	12.5 ± 0.49	1107.8 ± 91.5	4.3 ± 0.13	–	0.10 ± 0.00
42	*Pholiota nameko* (7.75)	1.6 ± 0.20	177.7 ± 17.4	1.7 ± 0.15	–	0.14 ± 0.00
43	*Gymnopus* sp. (4.41)	11.8 ± 0.42	182.3 ± 21.0	3.3 ± 0.15	0.3 ± 1.17	0.21 ± 0.01
44	*Pleurotus ostreatus* (4.16)	8.9 ± 0.54	945.9 ± 37.4	3.4 ± 0.03	1.2 ± 0.25	0.12 ± 0.00

Table 1 continued

SN	Order Scientific name (% yield)	TPC mg GAE/g	ORAC μM TE/g	DPPH[A] Inhibition %	ABTS[B] Inhibition %	RP[C] Absorbance 700 nm
45	*Pleurotus ostreatus* (4.84)	8.2 ± 0.89	386.0 ± 8.7	2.7 ± 0.15	–	0.11 ± 0.00
46	*Pleurotus ostreatus* (3.66)	8.1 ± 0.23	694 ± 17.4	2.3 ± 0.09	1.9 ± 0.42	0.13 ± 0.01
47	*Pleurotus ostreatus* (4.94)	5.6 ± 0.19	309.9 ± 27.9	2.0 ± 0.81	–	0.12 ± 0.01
48	*Lentinula edodes* (3.98)	4.3 ± 0.04	428.8 ± 11.9	2.0 ± 0.38	–	0.14 ± 0.00
49	*Lentinula edodes* (2.94)	6.2 ± 0.63	98.8 ± 45.3	3.0 ± 0.10	1.7 ± 0.39	0.13 ± 0.00
50	*Inocybe* sp. (6.08)	6.0 ± 0.26	343.5 ± 19.1	2.5 ± 0.50	1.8 ± 0.44	0.11 ± 0.00
51	*Panellus edulis* (13.23)	6.1 ± 0.52	556.4 ± 44.9	2.4 ± 0.24	0.7 ± 0.26	0.09 ± 0.00
52	*Mucidula mucida* (5.19)	0.4 ± 0.42	943.15 ± 43.6	0.3 ± 0.39	0.9 ± 0.22	0.12 ± 0.00
	Others	bd	cef	ac	ac	bde
53	*Pseudomerulius curtisii* (14.61)	131.6 ± 0.48	11,204.9 ± 414.9	89.0 ± 0.29	53.0 ± 0.34	1.10 ± 0.07
54	*Xylobolus princeps* (2.80)	126.6 ± 6.62	1290.4 ± 16.4	83.3 ± 0.25	47.8 ± 0.62	1.19 ± 0.12
55	*Xylobolus princeps* (2.65)	97.7 ± 2.31	2949.3 ± 265.1	85.1 ± 0.01	56.7 ± 0.56	1.02 ± 0.05
56	*Xylobolus princeps* (2.56)	94.4 ± 1.32	2349.5 ± 340.53	86.3 ± 0.22	54.3 ± 0.61	1.19 ± 0.01
57	*Xylobolus princeps* (3.67)	57.9 ± 5.29	257.65 ± 29.4	86.3 ± 0.08	44.1 ± 0.11	0.50 ± 0.02
58	*Engleromyces goetzii* (6.54)	30.6 ± 0.14	4666.6 ± 81.88	30.5 ± 0.51	7.8 ± 0.38	0.31 ± 0.03
59	*Neolentinus lepideus* (5.41)	22.7 ± 1.57	631.3 ± 38.3	9.7 ± 0.60	2.1 ± 0.05	0.27 ± 0.04
60	*Lactarius* sp. (3.60)	11.7 ± 0.64	209.0 ± 16.6	7.4 ± 0.42	0.2 ± 0.19	0.14 ± 0.00
61	*Russula brevipes* (7.67)	9.9 ± 2.74	41.4 ± 31.5	3.6 ± 0.19	–	0.13 ± 0.01
62	*Cantharellus ferruginascens* (4.08)	6.3 ± 0.09	170.1 ± 30.8	3.1 ± 0.09	–	0.13 ± 0.00

The values are expressed as "mean ± standard deviation" and $n = 3$. – means that the activity was not detected at the tested concentration. Differences between letters in each column means that there is a statistical difference between groups at a significance level of $p < 0.05$

[A–C] Sample concentrations of 2.5, 2, and 1 mg/mL were used for the respective assays

Polyporales group. The lowest phenolic content was seen in Agaricales, with *Mucidula mucida* (SN 52) showing the lowest TPC value of 0.4 mg GAE/g extract. Some samples in the Others group, such as *Pseudomerulius curtisii* (SN 53) and *Xylobolus princeps* (SN 54), showed a relatively high phenolic content of 131.6 mg GAE/g extract and 126.6 mg GAE/g extract, respectively.

The major antioxidant compounds found in mushrooms belong to the phenolic group [28]. Reducing agents like ascorbic acid are sometimes known to contribute to the seemingly elevated values [29]. However, total phenolics continue to thrive to be good indicators of the antioxidative activity.

Free radical scavenging assay

The radical scavenging capacity of the extracts was studied using the ORAC, DPPH, and ABTS assays. Again, the Hymenochaetales group showed a much higher ORAC activity compared to Polyporales and Agaricales. The highest ORAC value was shown by *Inonotus clemensiae* (SN 1) (31,966.9 μM TE/g extract), which outperformed the previously reported highest ORAC value [30] for *Inonotus andersonii* (21,015 μM TE/g extract). Significant differences between groups were not seen for Polyporales, Agaricales, and Others. In the Others group, *Pseudomerulius curtisii* (SN 53) showed a very high ORAC value of 11,204.9 μM TE/g extract. The ORAC assay is one of the few assays that monitors the free radical scavenging from the time of sample addition, throughout regular intervals, until the completion of the reaction. This enables complete assessment of the reaction, providing information about the inhibition time as well as degree of inhibition [31].

The Hymenochaetales group also outperformed the rest of the groups in the DPPH and ABTS assays. However, *Oxyporus* sp. (SN 13) remained a notable exception within the group, with negligible DPPH and ABTS radical scavenging activity. In the Polyporales group, several *Ganoderma* species showed high DPPH inhibition percentage, with *Ganoderma* sp. (SN 14) showing the highest inhibition percentage of 90.9 %. Agaricales showed the least DPPH inhibition. In the Others group, a very high DPPH inhibition percentage was seen in *Pseudomerulius curtisii* (SN 53) (89.0 %) and *Xylobolus princeps* (SN 54–57) (83.3–86.3 %).

In the case of the ABTS assay, SN 1 *Inonotus clemensiae* showed the highest inhibition percentage of 92.2 %. However, other *Inonotus* sp. could not show comparable activity. Also, considerable variations were seen among the

Ganoderma sp. of the Polyporales group (6 %–63.2 %). In the Others group, high ABTS activity was observed for *Pseudomerulius curtisii* (SN 53) (53.0 %) and *Xylobolus princeps* (SN 54–57) (44.1–56.7 %).

The DPPH and ABTS assays are known for its reproducibility, ease of application, and low cost [5]. However, attempts to check the activity at maximum dissolved concentration were hindered due to sample color interference, especially for highly pigmented samples. Although reports for the phenomenon of color interference with regard to mushrooms could not be found, it has been reported for plant extracts [32, 33], especially for DPPH assay. While performing these assays for extracts with unknown composition, it has been recommended to avoid using sample concentrations greatly exceeding the concentrations of DPPH and ABTS solutions [34].

Reducing power

The ability of the extracts to reduce the ferricyanide complex to its ferrous form was measured by the RP or sodium nitroprusside assay. Significant differences were seen between the Hymenochaetales, Polyporales, and Agaricales groups. In the Hymenochaetales group, *Cyclomyces setiporus* (SN 5–9) along with *Inonotus clemensiae* (SN 1) showed the highest RP values (1.64–2.20). Among Polyporales, *Ganoderma* sp. (SN 14) exhibited the highest RP value (1.22). Also, in the Others group, *Pseudomerulius curtisii* (SN 53) and *Xylobolus princeps* (SN 54–56) showed relatively high RP values (1.02–1.19). A very low variation was seen in the RP values among all samples. This could be due to the lower sensitivity of the method compared to other antioxidative assays. The RP of mushrooms has been attributed to their ability to donate hydrogen atoms, thereby pacifying the free radicals [35].

Correlation between TPC and the antioxidative assays

In order to illustrate the relationship between the antioxidative activity and the phenolic content, the antioxidative assays were correlated with the TPC values for each group, using the Spearman's rank correlation. Table 2 shows the correlation coefficient between TPC and each of the antioxidative assays for all four groups. Significant correlations were observed for all the assays in the Hymenochaetales, Polyporales, and Others groups. The highest degree of correlation was seen in the Others group, followed by Polyporales and Hymenochaetales. However, in Agaricales, only DPPH and TPC could correlate significantly. The major bioactive compounds in Agaricales are known to be ergosterol, lectin, terpenes, and β-glucans [36]. Also, it must be considered that the translation of the phenolic compounds into antioxidative activity is dependent on several factors, such as the availability of hydroxyl group, and the synergistic, additive, or antagonistic activities in the sample matrix [37].

Determination of EC_{50} and $EC_{0.5}$ values

To further clarify the activity of the mushroom samples showing the highest TPC, the EC_{50} values (for the DPPH and ABTS assays) and $EC_{0.5}$ values (for the RP assay) were determined. Table 3 shows the EC_{50} and $EC_{0.5}$ values expressed as sample concentration in mg/mL for the DPPH and ABTS and the RP assays, respectively. The lower $EC_{50}/EC_{0.5}$ values indicate stronger antioxidative activity. *Inonotus clemensiae* (SN 1) required the least sample concentration to obtain the EC_{50} values for both the DPPH and ABTS assays, as well as $EC_{0.5}$ value in the RP assay, with 0.081 mg/mL for the DPPH assay, 0.409 mg/mL for the ABTS assay, and 0.031 mg/mL for the RP assay.

All of the antioxidative assays tested revealed a very high activity for the Hymenochaetales group. Although commonly studied species such as *Inonotus obliquus* have been extensively reported for their antioxidative and other biological activities, the antioxidative activities of *Inonotus clemensiae* and *Cyclomyces setiporus* have not been reported previously. Within the Polyporales group, samples of *Ganoderma* species exhibited a relatively higher activity than other members. The Others group contained a mixture of samples from different orders. However, the antioxidative assays and the TPC correlated well in this group. Also, *Pseudomerulius curtisii* (SN 53) and *Xylobolus princeps* (SN 54–56) consistently showed high activity for almost all of the assays tested. Parallels can be observed between the pattern of antioxidative activity in the previous study of the antioxidative activities of 29 wild mushroom samples of Nepal [30], where *Inonotus* sp., *Phellinus* sp., and *Ganoderma* sp. had the most predominant antioxidative activity. These findings elucidate the presence of high antioxidative activity in previously unreported mushroom species in Nepal.

HPLC analysis

In order to identify and quantify the phenolic compounds present in the ethanol extracts of the top ten samples with the highest TPC values, HPLC analysis was performed. Twenty-one phenolic compounds, reported to be commonly found in mushrooms [28] (5-sulfosalicylic acid, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, chlorogenic acid, (+)-catechin, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, vanillin, rutin, *p*-coumaric acid, ferulic acid, veratric acid, naringin, benzoic acid, abscisic acid,

Table 2 Spearman's rank correlation coefficients for the total phenolic content (TPC) and the antioxidant assays oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and reducing power (RP)

Order	TPC–ORAC	TPC–DPPH	TPC–ABTS	TPC–RP
Hymenochaetales	0.731**	0.674*	0.852**	0.753**
Polyporales	0.812**	0.821**	0.786**	0.851**
Agaricales	0.327	0.854**	0.165	0.102
Others	0.806**	0.888**	0.900**	0.927**

*$p < 0.05$, **$p < 0.01$

Table 3 EC_{50} values of the DPPH and ABTS assays, and $EC_{0.5}$ values of the RP assay for ten samples with the highest TPC values

SN	Scientific name	DPPH (EC_{50})	ABTS (EC_{50})	RP ($EC_{0.5}$)
1	*Inonotus clemensiae*	0.08 ± 0.004	0.40 ± 0.012	0.03 ± 0.003
5	*Cyclomyces setiporus*	0.14 ± 0.003	0.71 ± 0.012	0.07 ± 0.030
6	*Cyclomyces setiporus*	0.20 ± 0.008	0.84 ± 0.016	0.17 ± 0.041
7	*Cyclomyces setiporus*	0.29 ± 0.041	1.19 ± 0.045	0.13 ± 0.061
3	*Inonotus* sp.	0.66 ± 0.035	1.24 ± 0.016	0.30 ± 0.256
8	*Cyclomyces setiporus*	0.15 ± 0.002	0.87 ± 0.003	0.10 ± 0.033
9	*Cyclomyces setiporus*	0.18 ± 0.013	0.92 ± 0.007	0.11 ± 0.074
53	*Pseudomerulius curtisii*	0.73 ± 0.004	2.03 ± 0.024	0.38 ± 0.021
54	*Xylobolus princeps*	0.57 ± 0.006	2.21 ± 0.041	0.64 ± 0.339
14	*Ganoderma* sp.	0.47 ± 0.011	1.52 ± 0.015	0.26 ± 0.026

EC_{50} is the sample concentration required for 50 % inhibition in the DPPH and ABTS assays, and $EC_{0.5}$ is the sample concentration required for 0.5 absorbance units (700 nm) in the RP assay. The results are expressed as sample concentration in mg/mL

quercetin, *trans*-cinnamic acid, naringenin, and kaempferol), were used as standards. Table 4 shows a list of the standard compounds, along with their retention time and the amount present in the respective samples. Also, the HPLC chromatograms of the standards and the samples are provided as supplementary data in Fig. S2.

The ten selected samples showed the presence of 12 out of 21 standard phenolic compounds. Flavonoids such as (+)-catechin, naringin, quercetin, naringenin, and kaempferol, along with some other phenolic compounds commonly found in mushrooms, such as *p*-hydroxybenzoic acid, benzoic acid, caffeic acid, and ferulic acid, were not detected in any of the samples. Also, the presence of some unknown compounds (UC1–4) were found to be in high abundance, as indicated by the peak intensity in HPLC chromatograms. The presence of such major unknown compounds in the respective samples and their retention times are provided in Table 3. The compound UC3 was detected in four of the tested extracts, SN 3, 6, 8, and 9. Although SN 5, 6, 7, 8, and 9 belonged to the same species, *Cyclomyces setiporus*, the HPLC chromatograms of only SN 8 and 9 were similar. The possible reason for this variation may be traced back to the origin of the samples. As can be seen in supplementary Table S1, SN 5, 6, and 7 were collected from different locations, whereas SN 5, 8, and 9 were collected from the same location. Yet, the chromatogram of SN 5 was quite different from those of SN 8 and 9. Interestingly, SN 5 was collected from the wood of a living tree, unlike SN 8 and 9, which were collected from the wood of dead trees. From these results, we can speculate that the environmental factors have a profound effect on the chemical composition of the wild mushrooms. Additionally, other factors such as growth stage [38], time of collection, and abiotic stress [39] must also be considered.

LC–MS analysis

The major compounds present in some of the samples did not match with the standards used in the HPLC analysis. Therefore, LC–MS analysis was performed to elucidate the unknown compounds (UC1–4). The retention time and molecular ion *m*/*z* values are shown in Table 5. Although clear fragmentation patterns could not be obtained, the molecular ion peaks provided some hint for the tentative identification of the unknown compounds. High antioxidative activity of mushrooms belonging to *Inonotus* sp. have been largely attributed to styrylpyrone-class

Table 4 Retention time and quantification of standard phenolic compounds in the top ten samples with the highest TPC value

Peak no.	Phenolic compound	Rt (min)	SN 1	SN 5	SN 6	SN 7	SN 3	SN 8	SN 9	SN 53	SN 54	SN 14
1	5-Sulfosalicylic acid	6.3	–	–	–	–	–	–	–	–	16.1	–
2	Gallic acid	8.3	–	–	1.4	–	2.2	–	–	–	–	–
3	Pyrogallol	8.9	–	–	–	–	–	–	–	–	–	10.9
4	3,4-Dihydroxybenzoic acid	13.0	–	–	11.6	3.2	2.2	7.1	–	–	–	–
5	Chlorogenic acid	17.1	–	54.1	–	24.6	–	–	–	–	–	–
6	(+)-Catechin	17.4	–	–	–	–	–	–	–	–	–	–
7	*p*-Hydroxybenzoic acid	18.0	–	–	–	–	–	–	–	–	–	–
8	Vanillic acid	20.1	–	–	3.2	–	–	–	–	–	–	–
9	Caffeic acid	20.6	–	–	–	–	–	–	–	–	–	–
10	Vanillin	25.5	–	–	2.1	–	–	–	–	–	2.0	–
11	*p*-Coumaric acid	27.3	–	–	2.1	–	–	–	–	–	–	–
12	Rutin	28.0	–	–	–	–	–	23.1	–	–	–	–
13	Ferulic acid	29.5	–	–	–	–	–	–	–	–	–	–
14	Veratric acid	30.0	–	–	–	30.8	–	–	–	–	–	–
15	Naringin	33.1	–	–	–	–	–	–	–	–	–	–
16	Benzoic acid	34.0	–	–	–	–	–	–	–	–	–	–
17	Abscisic acid	42.3	–	–	–	2.6	–	–	–	–	–	–
18	Quercetin	44.4	–	–	–	–	–	–	–	–	–	–
19	*trans*-Cinnamic acid	44.7	–	–	–	–	–	–	–	30.9	–	–
20	Naringenin	47.7	–	–	–	–	–	–	–	–	–	–
21	Kaempferol	49.2	–	–	–	–	–	–	–	–	–	–
22	UC1	32.3	+	–	–	–	–	–	–	–	–	–
23	UC2	43.4	–	+	–	–	–	–	–	–	–	–
24	UC3	16.5	–	–	+	–	+	+	+	–	–	–
25	UC4	28.5					+					

+ indicates the presence of an unknown major compound in the respective sample, − indicates not detected. The values of phenolic compound in each sample is expressed as μg/mg extract

Rt retention time, *UC* unknown compound

Table 5 Retention time and *m*/*z* values of the molecular ions of the unknown compounds

Compound	Rt (min)	$[M + H]^+$	$[M - H]^-$
UC1	44.25	247.0477	245.0369
UC2	48.17	491.0598	489.0869
UC3	26.52	–	251.0259
UC4	38.67	303.0443	301.0024

Rt retention time, *UC* unknown compound, – not detected

compounds, such as hispidin, inoscavin, phelligridin, and others [40]. Therefore, it can be predicted that UC1 with an *m*/*z* value of 247.0477 in positive mode and 245.0369 in negative mode is hispidin (exact mass 246.0528 g/mol) [41]. The molecular ion *m*/*z* values of the rest of the unknown compounds were detected as follows: 491.0598 in positive mode and 489.0869 in negative mode for UC2, 251.0259 in negative mode for UC3, and 303.0443 in positive mode and 301.0024 in negative mode for UC4.

Conclusion

A wide range of antioxidative activities was observed among the various orders of wild mushrooms species of Nepal. Hymenochaetales was the most active group, which included the exceptionally potent species *Inonotus clemensiae*. It was found to exceed the antioxidative activity of even *Inonotus obliquus* (chaga), a mushroom highly revered for its strong antioxidative activity among food products. *Pseudomerulius curtisii* (SN 53), *Xylobolus princeps* (SN 5–56), and *Ganoderma* sp. (SN 14) emerged as other promising candidates with high antioxidative activity from the rest of the groups. The correlation between the phenolic content and the various antioxidative assays across the four groups suggest that the phenolic compounds are largely responsible for the antioxidative activity of the mushrooms. As evident from the HPLC and LC–MS analyses, the presence of high concentrations of particular phenolic compounds in some species and the

absence of several commonly found phenolic compounds indicates the uniqueness of the phenolic profile of Nepalese mushrooms. This could be a result of its exclusive habitat and growing conditions. Covering 62 samples from the forest areas of different geographic regions of Nepal, this study provides a deep insight into the potential of a largely untapped, yet rich, resource of wild mushrooms. Studies on other biological activities and detailed analysis of the active compounds shall further enhance our knowledge on the capability of these mushrooms and open up paths for their use as nutraceuticals.

Acknowledgments The authors would like to thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University for providing the FlexStation 3 Microplate Reader and the Center for Advanced Instrumental and Educational Supports, Faculty of Agriculture, Kyushu University for supporting the LCMS-IT-TOF analysis. We are thankful to the Nepal Agriculture Research Council for providing the mushroom samples. The support of the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) in the form of Ph.D. Scholarship is greatly acknowledged. This work was supported by KAKENHI grant no. 26304047.

References

1. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84. doi:10.1016/j.biocel.2006.07.001
2. Carocho M, Ferreira ICFR (2013) A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem Toxicol 51:15–25. doi:10.1016/j.fct.2012.09.021
3. Powers SK, Nelson WB, Hudson MB (2011) Exercise-induced oxidative stress in humans: cause and consequences. Free Radic Biol Med 51:942–950. doi:10.1016/j.freeradbiomed.2010.12.009
4. Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 91:179–194. doi:10.1093/aob/mcf118
5. Moon JK, Shibamoto T (2009) Antioxidant assays for plant and food components. J Agric Food Chem 57:1655–1666. doi:10.1021/jf803537k
6. Lobo V, Patil A, Phatak A, Chandra N (2010) Free radicals, antioxidants and functional foods: impact on human health. Pharmacogn Rev 4:118–126. doi:10.4103/0973-7847.70902
7. Parke DV, Lewis DF (1992) Safety aspects of food preservatives. Food Addit Contam 9:561–577
8. Barros L, Cruz T, Baptista P, Estevinho LM, Ferreira IC (2008) Wild and commercial mushrooms as source of nutrients and nutraceuticals. Food Chem Toxicol 46:2742–2747. doi:10.1016/j.fct.2008.04.030
9. Kalač P (2013) A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. J Sci Food Agric 93:209–218. doi:10.1002/jsfa.5960
10. Wasser SP (2002) Review of medicinal mushrooms advances: good news from old allies. Herbal Gram 56:28–33
11. Ajith A, Janardhanan K (2007) Indian medicinal mushrooms as a source of antioxidant and antitumor agents. J Clin Biochem Nutr 40:157–162. doi:10.3164/jcbn.40.157
12. Lindequist U, Niedermeyer THJ, Jülich WD (2005) The pharmacological potential of mushrooms. Evid Based Complement Alternat Med 2:285–299. doi:10.1093/ecam/neh107
13. Vetaas OR, Grytnes JA (2002) Distribution of vascular plant species richness and endemic richness along the himalayan elevation gradient in Nepal. Glob Ecol Biogeogr 11:291–301. doi:10.1046/j.1466-822X.2002.00297.x
14. Adhikari MK (2012) Researches on the Nepalese mycoflora-2: checklist of macrofungi (mushrooms). Published by KS Adhikari, Kathmandu, Nepal. 20:1–84
15. Christensen M, Bhattarai S, Devkota S, Larsen HO (2008) Collection and use of wild edible fungi in Nepal. Econ Bot 62:12–23. doi:10.1007/s12231-007-9000-9
16. Hosaka K, Castellano MA (2008) Molecular phylogenetics of Geastrales with special emphasis on the position of *Sclerogaster*. Bull Natl Mus Nat Sci Ser B 34(4):161–173
17. White TJ, Bruns T, Lee SJ, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: InnisMA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols:a guide to methods and applications. AcademicPress, New York, pp 315–322
18. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118. doi:10.1111/J.1365-294x.1993.Tb00005.X
19. Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16:144–158 (citeulike-article-id:7170825)
20. Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK (2002) Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. J Agric Food Chem 50:3122–3128. doi:10.1021/jf0116606
21. Miliauskas G, Venskutonis PR, van Beek TA (2004) Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem 85:231–237. doi:10.1016/j.foodchem.2003.05.007
22. Zhu Q, Nakagawa T, Kishikawa A, Ohnuki K, Shimizu K (2015) In vitro bioactivities and phytochemical profile of various parts of the strawberry (*Fragaria* × *ananassa* var. Amaou). J Funct Foods 13:38–49. doi:10.1016/j.jff.2014.12.026
23. Oyaizu M (1986) Studies on products of browning reaction—antioxidative activities of products of browning reaction prepared from glucosamine. Japanese J Nutr Diet 44:307–315. doi:10.5264/eiyogakuzashi.44.307
24. Barros L, Ferreira MJ, Queirós B, Ferreira IC, Baptista P (2007) Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chem 103:413–419. doi:10.1016/j.foodchem.2006.07.038
25. Kim MY, Seguin P, Ahn JK et al (2008) Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. J Agric Food Chem 56:7265–7270. doi:10.1021/jf8008553
26. Asuquo JE, Etim EE (2011) Phytochemical and antinutrients evaluation of *Oxyporus populinus*. J Emerg Trends Eng Appl Sci 2:817–820
27. Glamočlija J, Ćirić A, Nikolić M et al (2015) Chemical characterization and biological activity of chaga (*Inonotus obliquus*), a medicinal "mushroom". J Ethnopharmacol 162:323–332. doi:10.1016/j.jep.2014.12.069
28. Ferreira ICFR, Barros L, Abreu RMV (2009) Antioxidants in wild mushrooms. Curr Med Chem 16:1543–1560. doi:10.2174/092986709787909587
29. Wong JY, Chye FY (2009) Antioxidant properties of selected tropical wild edible mushrooms. J Food Compos Anal 22:269–277. doi:10.1016/j.jfca.2008.11.021

30. Hai Bang T, Suhara H, Doi K et al (2014) Wild mushrooms in Nepal: some potential candidates as antioxidant and ACE-inhibition sources. Evid Based Complement Alternat Med 2014:195305. doi:10.1155/2014/195305
31. Ou B, Hampsch-Woodill M, Prior RL (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 49:4619–4626. doi:10.1021/jf010586o
32. Teow CC, Den Truong V, McFeeters RF, Thompson RL, Pecota KV, Yencho GC (2007) Antioxidant activities, phenolic and β-carotene contents of sweet potato genotypes with varying flesh colours. Food Chem 103:829–838. doi:10.1016/j.foodchem.2006.09.033
33. Arnao MB (2000) Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. Trends Food Sci Technol 11:419–421. doi:10.1016/S0924-2244(01)00027-9
34. Apak R, Gorinstein S, Böhm V et al (2013) Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure Appl Chem 85:957–998. doi:10.1351/PAC-REP-12-07-15
35. Ferreira ICFR, Baptista P, Vilas-Boas M, Barros L (2007) Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: individual cap and stipe activity. Food Chem 100:1511–1516. doi:10.1016/j.foodchem.2005.11.043
36. Novaes MRCG, Novaes LCG, Taveira VC (2007) Pharmacological effects of Agaricales fungi: a review of evidence. Rev Ciênc Méd Campinas 16:87–95
37. Jacobo-Velázquez DA, Cisneros-Zevallos L (2009) Correlations of antioxidant activity against phenolic content revisited: a new approach in data analysis for food and medicinal plants. J Food Sci 74:R107–R113. doi:10.1111/j.1750-3841.2009.01352.x
38. Barros L, Baptista P, Estevinho LM, Ferreira ICFR (2007) Effect of fruiting body maturity stage on chemical composition and antimicrobial activity of *Lactarius* sp. mushrooms. J Agric Food Chem 55:8766–8771. doi:10.1021/jf071435+
39. Sudheer S, Yeoh WK, Manickam S, Ali A (2016) Effect of ozone gas as an elicitor to enhance the bioactive compounds in *Ganoderma lucidum*. Postharvest Biol Technol 117:81–88. doi:10.1016/j.postharvbio.2016.01.014
40. Lee I-K, Yun B-S (2011) Styrylpyrone-class compounds from medicinal fungi *Phellinus* and *Inonotus* spp., and their medicinal importance. J Antibiot (Tokyo) 64:349–359. doi:10.1038/ja.2011.2
41. National Center for Biotechnology Information. Pub Chem Compound Database. Hispidin, CID = 54685921. Available online at: https://pubchem.ncbi.nlm.nih.gov/compound/54685921