Research Article

Received: 25 December 2012 Revised: 18 February 2013 Accepted article published: 4 March 2013 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.6121

Nutrient composition, antioxidant properties, and anti-proliferative activity of *Lignosus rhinocerus* Cooke sclerotium

Yeannie HY Yap,[a]* NgetHong Tan,[a] ShinYee Fung,[a] Azlina A Aziz,[a] ChonSeng Tan[b] and SzuTing Ng[c]

Abstract

BACKGROUND: *Lignosus rhinocerus* (tiger milk mushroom) is an important medicinal mushroom used in Southeast Asia and China, and its sclerotium can be developed into functional food/nutraceuticals. The nutrient composition, antioxidant properties, and anti-proliferative activity of wild type and a cultivated strain of *L. rhinocerus* sclerotia were investigated.

RESULTS: The sclerotial powder has high carbohydrate but low fat content. Interestingly, the cultivated strain contains higher amounts of protein and water-soluble substances than the wild type. Phenolic content of hot-water, cold-water, and methanol extracts of the sclerotial powders ranged from 19.32 to 29.42 mg gallic acid equivalents g^{-1} extract, while the ferric reducing antioxidant power values ranged from 0.006 to 0.016 mmol min^{-1} g^{-1} extract. The DPPH•, $ABTS^{•+}$, and superoxide anion radical scavenging activities of the extracts ranged from 0.52 to 1.12, 0.05 to 0.20, and −0.98 to 11.23 mmol Trolox equivalents g^{-1} extract, respectively. Both strains exhibited strong superoxide anion radical scavenging activity comparable to rutin. The cold-water extracts exhibited anti-proliferative activity against human breast carcinoma (MCF-7) cells, with IC_{50} values of 206 μg mL^{-1} and 90 μg mL^{-1} for the wild type and cultivated strains, respectively.

CONCLUSION: The cultivated *L. rhinocerus* sclerotium has the potential to be developed into functional food/nutraceuticals.

Keywords: nutritional composition; phenolic content; antioxidant activity; anti-proliferative activity; *Lignosus rhinocerus*; tiger milk mushroom

INTRODUCTION

Mushrooms are perceived as a form of delicacy in most cultures, particularly for their special aroma and texture as well as for medicinal purposes. *Lignosus rhinocerus* (synonym: *Lignosus rhinocerotis* (Cooke) Ryvarden) is one of the most highly valued medicinal mushrooms by the natives of Malaysia.[1] The mushroom is known locally as 'cendawan susu rimau' (tiger milk mushroom) and the sclerotium is the part of the mushroom with medicinal value. It is used to treat a variety of diseases, including cancer, cough and asthma, and as a general tonic.[2] Current scientific literature has documented a number of bioactive properties of the sclerotium of *L. rhinocerus* such as enhancement of immunomodulatory activity and anti-proliferative effect against the human breast carcinoma (MCF-7) and lung carcinoma (A549).[3,4]

For many years, efforts to cultivate the mushroom have not been successful, and it can only be collected from the jungle. Since the existence of this mushroom in the jungle is always solitary, the collection of the sclerotium is a tedious process; as a result, *L. rhinocerus* sclerotium is not only costly but its supply is also limited, which probably accounts for the fact that the biopharmacological properties of the mushroom have not been well investigated.

Recently, Tan[5] reported successful cultivation of the mushroom with good yield, thus overcoming the cost and supply problem, and opening up the possibility of commercializing this mushroom and its extracts as functional food and nutraceuticals. An evaluation on the subacute toxicity of the sclerotial powder of both wild type and cultivated *L. rhinocerus* in Sprague Dawley rats indicated that the 'no observable adverse effect level' (NOAEL) dose is higher than 1 g kg^{-1}.[6]

Moreover, there are limited literatures reporting on the nutrient composition of *L. rhinocerus* sclerotium, though it has been reported that the main components of the dry matter were mostly carbohydrate, consisting of insoluble dietary fiber and non-starch polysaccharides, with low lipid content.[7,8] Also, little is known about the antioxidant properties of the *L. rhinocerus* sclerotium. Thus, in this report, we examined the antioxidant activities of hot-water, cold-water, and methanol extracts (HWE, CWE and ME, respectively) of sclerotial powder of the wild type and cultivated

* *Correspondence to: Yeannie HY Yap, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. E-mail: yean_ny_nie@yahoo.com*

a *Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

b *Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia*

c *Ligno Biotech Sdn. Bhd., 43300, Balakong Jaya, Selangor, Malaysia*

L. rhinocerus, as well as their anti-proliferative activity, nutrient composition, and amino acid content.

EXPERIMENTAL

Materials

Wild type and cultivated sclerotia of *L. rhinocerus* (termed WT Rhino and TM02, respectively) were provided by Ligno Biotech Sdn. Bhd. (Selangor, Malaysia). The wild type sclerotia were collected from Cameron Highlands, Pahang, Malaysia, whereas cultivated sclerotia TM02 was the product of Ligno Biotech Sdn. Bhd. The fungus was identified by the internal transcribed spacer regions of ribosomal RNA.[9] Human breast adenocarcinoma (MCF-7) cells was purchased from the American Type Culture Collection (ATCC, VA, USA) and grown in RPMI-1640 culture medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, MO, USA). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

The reagents (analytical grade), chemicals, polyphenolic standards (gallic acid, quercetin, and rutin) were purchased from Sigma Aldrich unless otherwise specified. MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Merck & Co. (Whitehouse Station, NJ, USA). Methanol was purchased from Friendemann Schmidt (Parkwood, WA, AU). Water used was of Millipore quality.

Preparation and extraction of the sclerotia

Freeze-dried sclerotia were ground into powder and passed through a 0.2 mm sieve. For hot-water extraction, the sclerotial powder was extracted with water at 95–100 °C for 2 h. Cold-water extraction was performed by 24 h stirring at 4 °C. For methanol extraction, the sclerotial powder was suspended in methanol at room temperature with 24 h stirring. All extractions were performed with a mass:volume ratio of 1:20 (g mL^{-1}). After the extraction, the mixture was filtered through Whatman No. 1 filter paper. HWE and CWE were freeze dried, while ME was evaporated to dryness using a rotary evaporator at 37 °C. HWE and CWE were redissolved in water prior to analysis, while ME was subsequently redissolved in 10% dimethyl sulfoxide (DMSO). Extracts were kept at −20 °C for long-term storage. To examine the possible interference of DMSO with the results, we have included DMSO controls throughout the study. Our results showed that this does not affect the final outcome, presumably because the final concentration of DMSO used was very low (<0.1% in all assays performed).

MTT cytotoxicity assay

The anti-proliferative activity of the extracts was examined using MTT assay. MCF-7 cells were seeded in 96-well plates at 1×10^3 cells per well and allowed to adhere overnight prior to treatment with *L. rhinocerus* extracts at various concentrations. Following 72 h incubation, 20 μL of 5 mg mL^{-1} MTT solution in phosphate-buffered saline (PBS) was added to each well. The plate was then incubated for 4 h at 37 °C until purple formazan crystals developed. All the solutions were then aspirated and 200 μL DMSO was added in order to dissolve the attached formazan crystals. Following 10–30 min incubation in the dark, absorbance was read at 570 nm. Half-maximal inhibitory concentration (IC_{50}) value was determined from the percentage cell viability against the extract concentration curve.

Total phenolic content analysis

Total phenolic content of the extracts was determined according to the Folin–Ciocalteu method.[10] Folin–Ciocalteu reagent (500 μL, 1:10) was added to 10 μL sample. After 5 min, 350 μL of 0.115 mg mL^{-1} Na_2CO_3 was added to the mixture, which was further incubated for 2 h in the dark before absorbance readings were taken at 765 nm. Gallic acid with different concentrations (10–200 μg mL^{-1}) was used as standard for construction of the calibration curve. Results were expressed as mg gallic acid equivalents (GAE).

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing activity of the extracts was determined according to the FRAP assay described by Benzie and Strain.[11] FRAP reagent was freshly prepared by mixing 300 mmol L^{-1} acetate buffer, 10 mmol L^{-1} TPTZ solution (2,4,6-tripyridyl-*s*-triazine) in 40 mmol L^{-1} HCl and 20 mmol L^{-1} $FeCl_3.6H_2O$ in a ratio of 10:1:1. Following incubation of FRAP reagent at 37 °C, 30 μL extract or standard and 90 μL water were added to 900 μL FRAP reagent. Absorbance readings, which were taken at 593 nm, were recorded at each 15 s interval, for 4 min upon the addition of FRAP reagent. $FeSO_4$ solution with different concentrations (200–1000 μmol L^{-1}) was used as standard for construction of the calibration curve. Quercetin and rutin were used as positive controls.

DPPH• radical scavenging assay

DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the mushroom extracts were measured according to Cos *et al.*[12] Mushroom extract (150 μL) was added to 900 μL of 0.04 mg mL^{-1} DPPH• solution in methanol. Reaction mixtures were vortex-mixed and incubated in the dark for 20 min before absorbance readings were taken at 517 nm. Trolox with different concentrations (25–125 μg mL^{-1}) was used as standard for construction of the calibration curve. Inhibition of DPPH• radical was calculated according to the following equation:

$$\text{DPPH}^{\bullet}\ \text{inhibition}\ (\%) = \frac{\text{OD of control at 0 } \mu\text{g mL}^{-1} - \text{OD of standard or extract}}{\text{OD of control at 0 } \mu\text{g mL}^{-1}}$$

The IC_{50} values (concentration in μg mL^{-1} that caused 50% inhibition of DPPH• radicals) of the extracts was also estimated from the dose–effect curves by linear regression analysis via Microsoft Excel® 2010 (Microsoft Corp., Redmond, WA, USA). Quercetin and rutin were used as positive controls.

ABTS•+ radical scavenging assay

The 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+) radical scavenging activity of the extracts was determined according to Re *et al.*[13] with slight modifications. ABTS solution (7 mmol L^{-1}) in water was reacted with 2.45 mmol L^{-1} potassium persulfate for 12–16 h in the dark to allow ABTS•+ radical generation. Absorbance of the reactant was then adjusted to 0.700 ± 0.02 at 734 nm. Diluted ABTS•+ solution (1 mL) was added to 10 μL extract or standard followed by 15 min incubation in the dark before absorbance readings were taken. Trolox with different concentrations (400–2000 μmol L^{-1}) was used as standard for construction of the calibration curve. Quercetin and rutin were used as positive controls.

Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity was determined according to Siddhuraju and Becker[14] with slight modifications. To a 96-well plate, a total reaction mixture volume of 200 μL containing the extract at the desired concentration, 468 µmol L^{-1} NADH, 150 µmol L^{-1} nitroblue tetrazolium, and 60 µmol L^{-1} phenazine methosulfate – all in 0.1 mol L^{-1} phosphate buffer, pH 7.4 – were added in that sequence at a ratio of 1:1:1:1. The resulting reaction mixture was incubated for 10 min at 25 °C in the dark before absorbance readings were taken at 570 nm. Trolox at different concentrations (500–2000 µg mL^{-1}) was used as standard for construction of the calibration curve. Quercetin and rutin were used as positive controls.

Cell-based superoxide anion radical scavenging assay

Cellular superoxide anion radical scavenging ability of the extracts was further investigated by determining their effects on MCF-7 cell viability upon introduction of an enzymatic oxy-radical generating system via the xanthine/xanthine oxidase reaction. The method used was modified from Beyer and Melzig[15] and Mimnaugh *et al*.[16] In a 96-well plate, cells grown in monolayer were exposed to 0.625 mU mL^{-1} xanthine oxidase and 0.5 mmol L^{-1} xanthine for a duration of 60 min in the presence of *L. rhinocerus* extract at concentrations ranging from 2 to 125 µg mL^{-1}. The reaction mixtures were then aspirated and the cells were cultivated in fresh RPMI-1640 medium containing 10% FBS for a further 72 h prior to cell viability assessment. Cell viability was determined by MTT assay. Quercetin and rutin were used as positive control. Negative control (PBS instead of the extracts) experiments were carried out in the same manner. The effect of *L. rhinocerus* extracts on the viability of the MCF-7 cells in relation to the negative control was then determined.

Nutrients and amino acid composition analysis

The chemical compositions of WT Rhino and TM02 sclerotial powder were analyzed. Energy, crude protein, sugars, and fat content were determined based by AOAC procedures.[17] The protein conversion factor used was 6.25. Total carbohydrates were calculated by difference while mineral concentrations were determined according to US Environmental Protection Agency method.[18] Amino acids composition of the sclerotial powder was determined using high-performance liquid chromatography (HPLC)-based amino acid analyzer according to the method established by Hewlett-Packard's AminoQuant.[19] Amino acid scores of the essential amino acids were calculated using the FAO reference pattern as 100.[20]

Statistical analysis

Results were expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance followed by the LSD post hoc test for multiple comparisons was used to compare mean values. A *P*-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Nutrient and mineral composition

The major constituents of *L. rhinocerus* sclerotia were carbohydrates, mostly composed of fiber and starch as the sugar (monosaccharides and disaccharides) content was very low, and $<1\%$ fat (Table 1). Protein content of the sclerotium of cultivar TM02 was at least 3.6 times higher compared to the wild type, WT Rhino. Dietary minerals are vital for overall mental and physical well-being. Generally, *L. rhinocerus* sclerotia showed high content of potassium and magnesium, with modest amounts of calcium and sodium. In comparison to WT Rhino, TM02 has higher mineral content. The higher K/Na ratio in TM02 (23.09) as opposed to WT Rhino (15.55) makes them favorable as a part of dietary intake with defined electrolytic balance.

Table 1. Proximate composition and mineral content of wild type and cultivated *L. rhinocerus* sclerotial powder

Composition	WT Rhino	TM02
Energy (kcal kg^{-1} dry weight)	21.80 ± 1.20	32.19 ± 0.71
Proximate composition (g kg^{-1} dry weight)		
Carbohydrate	8.84 ± 0.35	7.76 ± 0.04
Total sugar	0.07 ± 0.04	0.30 ± 0.10
Protein	0.38 ± 0.03	1.38 ± 0.02
Total fat	0.03 ± 0.01	0.08 ± 0.00
Mineral (mg kg^{-1} dry weight)		
Calcium, Ca	0.37 ± 0.17	1.93 ± 0.60
Potassium, K	13.22 ± 0.56	20.32 ± 2.53
Sodium, Na	0.85 ± 0.07	0.88 ± 0.09
Magnesium, Mg	7.58 ± 0.37	14.79 ± 0.31

WT Rhino and TM02 are the wild type and cultivated strain of *L. rhinocerus*. Each value represents mean ± SEM of triplicate measurements.

Table 2 shows the amino acid composition and amino acid scores of both the WT Rhino and TM02 sclerotial powders. Total essential amino acid content of TM02 (0.839 g kg^{-1} dry weight) is much higher than WT Rhino (0.207 g kg^{-1} dry weight). While these data are consistent with the difference in protein content of the cultivated and wild type strains using nitrogen determination, the amino acid content values also indicate that the protein content calculated from nitrogen determination may be overestimated, as a substantial amount of nitrogen in the sclerotial powder may be attributable to non-protein nitrogen compounds. The amino acid content of TM02 is also much higher than other common mushrooms, as reported by Lee *et al*. [21] – *Agrocybe chaxingu*, *Pleurotus ostreatus* and *Flammulina velutipes* – which ranged from 0.166 to 0.270 g kg^{-1} dry weight. Amino acid scores of all the essential amino acids (except for lysine) exceed the FAO reference.[20] The lysine scores of only 40 and 50 for WT Rhino and TM02 respectively are comparable to the proteins of the three mushrooms as mentioned above (ranging from 49 to 75). Thus lysine appears to be the limiting essential amino acid of mushroom proteins. Nevertheless, the higher essential amino acid content in TM02 sclerotium suggests that it is a good source of dietary protein.

Yield of extractions and phenolic content

The yields of cultivar TM02 HWE, CWE, and ME were substantially higher than in WT Rhino (Table 3). Hot-water extraction of the sclerotial powder of TM02 yielded the highest quantity of extract and was 50 times higher than WT Rhino, followed by cold-water and methanol extraction. The high yields of water extracts compared to ME in TM02 showed that it contained more water-soluble substances of high polarity.

Table 2. Amino acid compositions wild type and cultivated *L. rhinocerus* sclerotial powder

	Amino acid content					
	g kg^{-1} dry weight		g kg^{-1} protein		Amino acid score	
Amino acid	WT Rhino	TM02	WT Rhino	TM02	WT Rhino	TM02
Aspartate	0.043	0.187	104.1	112.0	—	—
Glutamate	0.040	0.232	96.9	138.9	—	—
Serine	0.028	0.085	67.8	50.9	----	—
Histidine*	0.010	0.034	24.2	20.4	142	120
Glycine	0.026	0.089	63.0	53.3	—	—
Threonine*	0.027	0.091	65.4	54.5	164	136
Alanine	0.030	0.114	72.6	68.3	—	—
Arginine*	0.042	0.204	101.7	122.2	—	—
Tyrosine	0.014	0.065	33.9	38.9	—	—
Cystine	0.023	0.042	55.7	25.1	—	—
Valine*	0.029	0.114	70.2	68.3	140	137
Methionine*	0.009	0.039	21.8	23.4	221**	139**
Phenylalanine*	0.022	0.085	53.3	50.9	145***	150***
Isoleucine*	0.022	0.093	53.3	55.7	133	139
Leucine*	0.037	0.133	89.6	79.6	128	114
Lysine*	0.009	0.046	21.8	27.5	40	50
Proline	0.002	0.017	4.8	10.2	—	—

WT Rhino and TM02 are the wild type and cultivated strain of *L. rhinocerus*. Value for amino acid content is the mean of duplicate measurements. Amino acid scores of the essential amino acid were calculated using FAO reference patterns.[20]
*Essential amino acid;
**amino acid score for sulfur amino acids;
***amino acid score for phenylalanine + tyrosine.

Table 3. Yield and total phenolic content of water and methanol extracts of *L. rhinocerus*

		Yield	Total phenolic content	
	Solvent extract	g kg^{-1} dry weight	mg GAE g^{-1} extract[a]	mg GAE g^{-1} DW[b]
WT Rhino	HWE	4	29.42 ± 0.26c	0.12 ± 0.00c
	CWE	8	28.38 ± 0.08d	0.23 ± 0.00c
	ME	7	19.32 ± 0.15e	0.14 ± 0.00c
TM02	HWE	200	23.35 ± 0.75f	4.67 ± 0.15d
	CWE	100	28.23 ± 0.50d	2.82 ± 0.05e
	ME	27	27.47 ± 0.38 g	0.74 ± 0.01f

WT Rhino and TM02 are the wild type and cultivated strain of *L. rhinocerus*. Hot-water extract (HWE): sclerotial powder was extracted with hot water at 95–100 °C for 2 h. Cold-water extract (CWE): sclerotial powder was extracted with cold water at 4 °C for 24 h. Methanol extract (ME): sclerotial powder was extracted with methanol at room temperature for 24 h. All extractions were performed with a mass:volume ratio of 1:20 (g mL^{-1}).
[a] Total phenolic content is expressed as mg gallic acid equivalents (GAE) in 1 g extract ± SD ($n = 3$).
[b] Total phenolic content is expressed as mg gallic acid equivalents (GAE) in 1 g dry weight (DW) mushroom ± SD ($n = 3$).
Means in the same column with different letters (c–g) are significantly different ($P < 0.05$).

Phenolics are secondary metabolites that are ubiquitously found in plants including mushroom and usually exhibit high antioxidant activities. The total phenolic content of the various extracts of *L. rhinocerus* is shown in Table 3. In terms of mg GAE g^{-1} sclerotial powder dry weight, the amount of phenolic compounds in HWE of TM02 was highest, possibly due to their high extraction yield, followed by CWE and ME of TM02. Although the concentration of phenolics in the extracts of WT Rhino was generally comparable to TM02 in terms of mg GAE g^{-1} extract, when expressed in terms of mg GAE g^{-1} of sclerotial powder the phenolic content was very low (≤0.23 mg GAE g^{-1} dry weight), possibly due to the low yield of the extracts.

Antioxidant activity of *L. rhinocerus* extracts

To assess the antioxidant activities of the mushroom extracts, the ability of antioxidants in the extracts to reduce ferric ions and scavenge free radicals was measured. Data on these activities are more relevant than knowledge on chemical composition of the antioxidants (phenolics and other secondary metabolites) as far as the nutritional benefits of the antioxidants are concerned, as protective effects of the antioxidants in health are due to their ability to scavenge free radicals.[22] The free radical scavenging activity is expressed as Trolox equivalent antioxidant capacity (TEAC).

FRAP assay

Antioxidant activity in *L. rhinocerus* extracts is determined based on their ability to reduce ferric tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous complex (Fe^{2+}-TPTZ). Table 4 shows the ferric reducing power of *L. rhinocerus* extracts expressed as initial rate. All mushroom extracts exhibited relatively low FRAP value in comparison to the positive controls quercetin and rutin in the first 4 min. The ferric reducing activities of the mushroom extracts follow a slow kinetic mechanism with a steady increment throughout the assay. This is in agreement with a study on some wild (*Agaricus* spp., *Boletus* spp., and *Macrolepiota* spp.) and commercial (*Agaricus bisporus* white strain and portabella: *A. bisporus* brown strain) mushrooms where the reaction was not totally completed even after 30 min.[23]

Antioxidant activity of all the mushroom extracts follows a similar pattern to the phenolic content (mg GAE g^{-1} extract) except for the ME of both wild type and cultivar TM02. The higher FRAP value in the ME of *L. rhinocerus* compared to both HWE and CWE despite their phenolic content suggests the presence of other less polar components such as tocopherols and flavonoids, which might also contribute to their reducing/electron-donating ability.

DPPH• radical scavenging activity

The DPPH• radical scavenging activity of all the *L. rhinocerus* sclerotial extracts at concentrations ranging from 1 to 16 mg mL^{-1} shows a concentration-dependent DPPH• radical scavenging activity up to a concentration of 8 mg mL^{-1}, after which the reactions slow down significantly. In terms of TEAC, the DPPH• radical scavenging activity of all the extracts is much lower than that of the positive controls quercetin and rutin (Table 4). DPPH• radical scavenging capacity of the extracts decreased in an order similar to the FRAP assay: ME > HWE > CWE for WT Rhino and ME > CWE > HWE for cultivated TM02. This again suggests that ME may contain different types of reducing compounds, besides the phenolics, which have a high capacity to scavenge DPPH• radicals.[24] Based on the concentration-scavenging activity response curves, the IC_{50} values of the various extracts of WT Rhino

Table 4. Ferric reducing activity, radical scavenging activity, and relative antioxidant index (AI) of *L. rhinocerus*

	Solvent extract	FRAP value	Trolox equivalent antioxidant capacity (TEAC, mmol TE g^{-1} extract) DPPH•	ABTS•+	Superoxide anion	AI value
WT Rhino	HWE	0.012 ± 0.00a	0.81 ± 0.06a	0.12 ± 0.01a	11.23 ± 0.12a	30.19
	CWE	0.007 ± 0.00a	0.55 ± 0.03b	0.12 ± 0.02a	9.09 ± 0.04b	24.18
	ME	0.016 ± 0.00a	1.12 ± 0.05c	0.18 ± 0.00b	8.48 ± 0.12c	26.52
TM02	HWE	0.006 ± 0.00a	0.52 ± 0.07b	0.05 ± 0.02c	8.00 ± 0.24d	20.78
	CWE	0.008 ± 0.00a	0.89 ± 0.02a	0.20 ± 0.01b	9.90 ± 0.09e	28.68
	ME	0.015 ± 0.00a	1.01 ± 0.09d	0.20 ± 0.00b	−0.98 ± 0.15f	5.54
Quercetin		3.38 ± 0.15b	4.98 ± 0.01e	2.00 ± 0.00d	11.43 ± 0.10a	100.00
Rutin		0.57 ± 0.04c	4.87 ± 0.01f	1.64 ± 0.00e	9.62 ± 0.07 g	70.22

WT Rhino and TM02 are the wild type and cultivated strain of *Lignosus rhinocerus*. Antioxidant activities are expressed as mean ± SD ($n = 3$) for each extract. FRAP value is represented in mmol min^{-1} g^{-1} extract. Means in the same column with different letters (a–g) are significantly different ($P < 0.05$). HWE, hot-water extract; CWE, cold-water extract; ME, methanol extract.

were estimated to be 2.4, 3.3, and 4.4 mg mL^{-1} for ME, HWE, and CWE, respectively; whereas for the cultivated TM02 IC_{50} values were 3.1, 3.2, and 4.7 mg mL^{-1}, respectively, for ME, CWE, and HWE. The IC_{50} values of *L. rhinocerus* extracts were on a par or even lower than most medicinal, edible mushroom species, including lingzhi mushroom (*Ganoderma lucidum*, IC_{50} value 5.28 mg mL^{-1}), shiitake (*Lentinus edodes*, IC_{50} value 19.09 mg mL^{-1}), and king oyster mushroom (*Pleurotus eryngii*, IC_{50} value 15.42 mg mL^{-1}).[25]

ABTS•+ radical scavenging activity

ABTS•+ is a chromophore produced by reacting ABTS and potassium persulfate; the addition of antioxidant to the pre-formed radical cation will reduce it to ABTS in a concentration-dependent manner. The inhibition of the ABTS•+ radicals was dose-dependent, where most but not all mushroom extracts were able to inhibit 50% of ABTS•+ radical at 16 mg mL^{-1}, except for TM02-HWE. Table 4 shows the ABTS•+ radical scavenging activity of the *L. rhinocerus* extracts, expressed in terms of mmol Trolox equivalents g^{-1} extract. The values range from 0.05 to 0.20, where a higher value indicates a more potent radical scavenging effect. The ABTS•+ scavenging potential of WT Rhino shows a profile very similar to their DPPH• scavenging activity, where ME exhibited the highest scavenging activity (0.18 ± 0.00 mmol Trolox equivalents g^{-1} extract) despite its low phenolic content (mg GAE g^{-1} extract) compared to the water extracts. However, for cultivated TM02, ABTS•+ scavenging activities for CWE and ME are comparable.

Superoxide anion radical scavenging activity

In contrast to the FRAP, DPPH• and ABTS•+ assays, all the *L. rhinocerus* sclerotial extracts exhibited potent dose-dependent superoxide anion radical scavenging activities at low concentrations (Fig. 1). The activities of the extracts increased progressively with concentration up to approximately 125 µg mL^{-1}, after which there was a leveling off. TM02-ME, however, showed reduced inhibition of superoxide anion at concentrations above 100 µg mL^{-1} and essentially lost all its superoxide anion radical scavenging activity at 900 µg mL^{-1}; and at 1 mg mL^{-1} it actually stimulated superoxide anion radical generation. A study with marine macroalgae also reported reduced inhibition of superoxide anion at higher concentrations.[26]

Table 4 reports the superoxide anion radical scavenging activity of the *L. rhinocerus* sclerotial extracts expressed in terms of mmol Trolox equivalents g^{-1} extract. The data show that, except for the

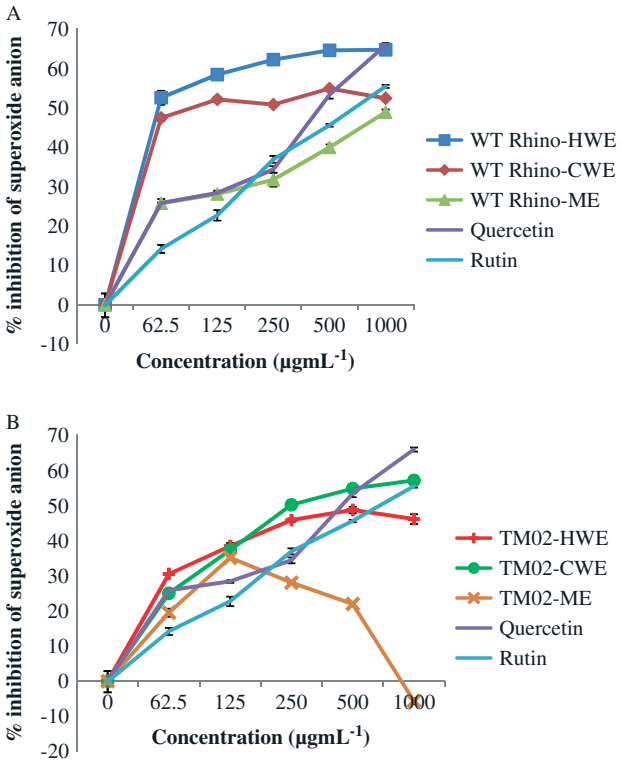

Figure 1. Superoxide anion radical scavenging activity of *L. rhinocerus* sclerotial extracts. The radical scavenging activity of positive controls (quercetin and rutin) together with the extracts of both wild type and cultivated strain TM02 are expressed as percentage inhibition (values are mean ± SD, $n = 3$). (A) Wild type *L. rhinocerus* (WT Rhino); (B) Cultivated *L. rhinocerus* TM02 extracts. ME, methanol extract; HWE, hot-water extract; CWE, cold-water extract.

ME of TM02, all other extracts exhibited very strong superoxide anion radical scavenging activities, with potency comparable to that of quercetin and rutin. This is of great significance as superoxide anion is generated during energy metabolism in cells and is a very harmful radical to cellular components,[27] thus indicating that *L. rhinocerus* may be beneficial in preventing oxidative stress induced by superoxide anion radicals. The strong superoxide anion radical scavenging activity could be attributed to the presence of soluble polysaccharides such as β-glucan, which

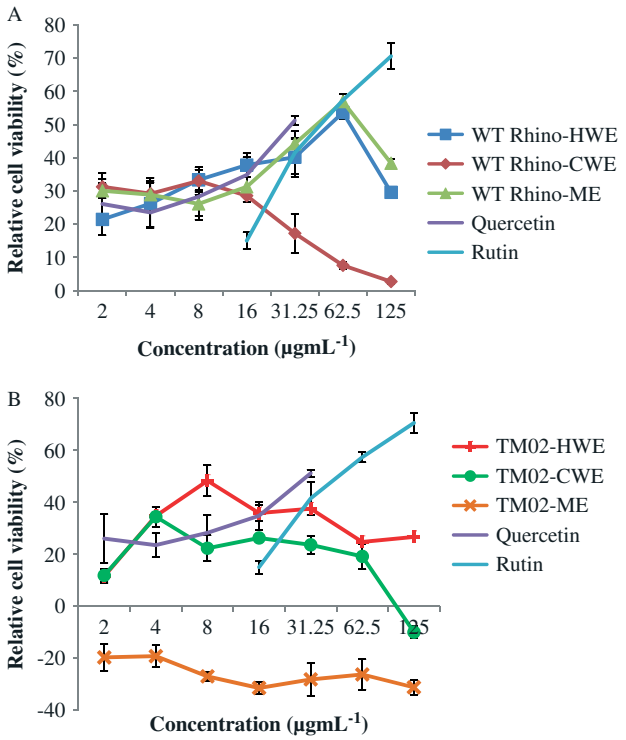

Figure 2. Superoxide anion radical scavenging activity of *L. rhinocerus* extracts in a cell-based system. Xanthine/xanthine oxidase was used to generate superoxide anion radical in MCF-7 cells and the ability of various extracts to protect against the generated superoxide was examined by measuring relative cell viability (values are mean ± SD, $n = 3$). (A) Wild type *L. rhinocerus* (WT Rhino); (B) cultivated *L. rhinocerus* TM02 extracts, together with positive controls (quercetin and rutin). ME, methanol extract; HWE, hot water extract; CWE, cold-water extract.

is more likely to be present in the water extracts of *L. rhinocerus*.[28] Such scavenging activities had also been reported on some antitumor polysaccharide extracts of a few important medicinal mushroom species such as *G. lucidum*, *Polyporus umbelatus* and *Tricholoma lobayense*, indicating that *L. rhinocerus* extracts may be used for cancer prevention as well.[29]

Inhibition of superoxide anion radical in cell-based xanthine/xanthine oxidase assay

The superoxide anion radical scavenging activity of *L. rhinocerus* extracts was further examined using cell-based xanthine/xanthine oxidase assay. In this assay, the enzymatic conversion of xanthine to urate generates both superoxide anion (mainly) and hydrogen peroxide. The xanthine/xanthine oxidase system with/without the presence of PBS causes approximately 75–85% cytoxicity in MCF-7 cells, whereas xanthine and xanthine oxidase on their own were not toxic at the concentration applied. The effects of *L. rhinocerus* extracts and the positive controls quercetin and rutin on the viability of MCF-7 cells in relation to the negative control are shown in Fig. 2.

Rutin and quercetin alone do not exhibit cytotoxic effects on the MCF-7 cells at 500 and 31.25 μg mL^{-1} with cell viability of 96.3% and 107.4% (non-significant fluctuation), respectively. The ability of rutin to scavenge superoxide anion is proportional to its concentration, where the EC_{50} value (the concentration which inhibits 50% of the xanthine/xanthine oxidase-induced toxicity) was estimated to be 47 μg mL^{-1}. At lower concentrations, ranging from 1 to 16 μg mL^{-1}, the scavenging ability of quercetin was not significant, probably due to the trivial differences between each concentration applied. An estimation of the EC_{50} value of quercetin is 30 μg mL^{-1}, which is much higher compared to Beyer's studies (EC_{50} value = 1 μg mL^{-1}) using ECV-304, a presumptive endothelial cell line,[15] indicating a cell-dependency inhibition. In our study, the cell viability at 1 μg mL^{-1} is 35%. The estimated EC_{50} value in the cell-based assay of both quercetin and rutin is much lower compared to the superoxide anion radical scavenging assay, where their IC_{50} (concentration in μg mL^{-1} that caused 50% inhibition of superoxide anion radicals) were 440 and 720 μg mL^{-1}, respectively.

All of the *L. rhinocerus* extracts in the tested concentrations demonstrate no toxicity, at least during the incubation period in which the MCF-7 cells were exposed to the extracts. As the scavenging ability of most of the *L. rhinocerus* extracts does not reach 50%, their EC_{50} values could not be determined, except for HWE and ME of WT Rhino, with EC_{50} values of 55 and 46 μg mL^{-1} respectively, where the latter was comparable to that of rutin. The maximal scavenging ability of WT Rhino-CWE was 33.04% at 8 μg mL^{-1}, after which there was a loss in scavenging ability. Maximal inhibition of TM02 was 48.31% for HWE and 34.46% for CWE, at concentrations of 8 and 4 μg mL^{-1} respectively. Although the TM02 extracts did not reach 50% inhibition of the cells, overall their extracts seem to achieve maximal inhibition at lower concentrations compared to WT Rhino, except for TM02-ME, which showed negative inhibition. Both TM02-HWE and TM02-CWE also lost their superoxide anion scavenging activities at higher concentrations. Negative inhibition could be due to complete loss of scavenging ability and thus promoting the cells to be more susceptible to the xanthine/xanthine oxidase-induced toxicity, most likely due to the concentration-dependent biphasic effect as demonstrated in the *in vitro* superoxide anion radical scavenging assay. It is noteworthy that the loss of scavenging activity in the cell-based assay occurred much earlier compared to the *in vitro* chemical assay for all the WT Rhino and TM02 extracts.

Relative antioxidant index

Since different types of extracts of mushroom usually exhibit various potency of antioxidant and radical scavenging activities using different assays, Puttaraju *et al.*[30] proposed the use of relative antioxidant index (AI) to compare the overall antioxidant potential of the various extracts. In this method, the extracts were graded in a numerical scale, with quercetin arbitrarily assigned a value of 100. AI represents the average relative percentage of the antioxidant activity compared to quercetin obtained using different antioxidant assays. As in Table 4, using AI as a parameter and the data obtained from FRAP and the three radical scavenging assays (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and superoxide anion), WT Rhino-HWE was shown to have the highest AI, followed closely by the TM02-CWE, while TM02-ME had the lowest AI value, possibly due to its weak superoxide anion scavenging property and a tendency towards generating more radicals. Although the cultivated TM02 strain possessed lower antioxidant capacity in comparison with the WT Rhino, its high yield and phenolic content (mg GAE g^{-1} dry mushroom) together with its availability could still favor it as a potential dietary source of antioxidant nutrients over the wild type.

Anti-proliferative activity of *L. rhinocerus* water extracts

As the chemical composition (in terms of protein content and water-soluble substances) of the cultivated *L. rhinocerus* sclerotia differ from that of the wild type, it is of interest to examine

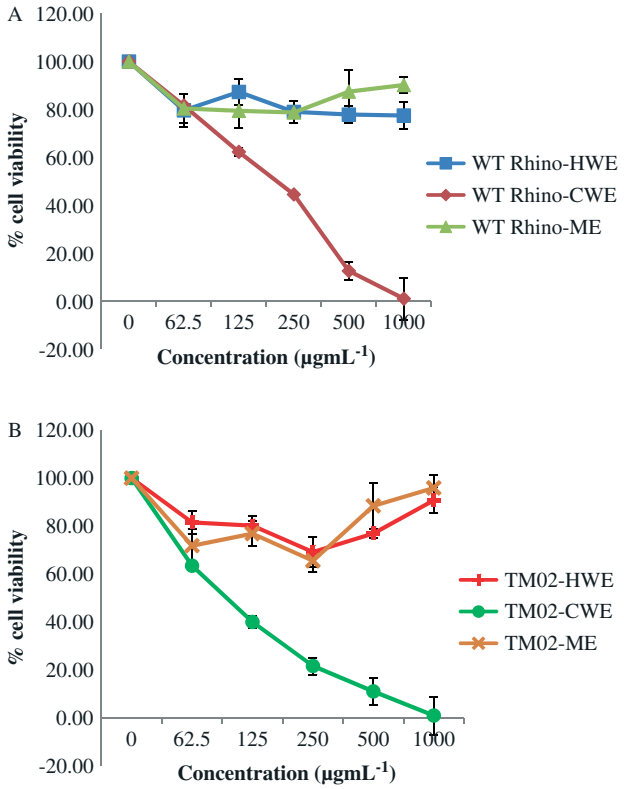

Figure 3. Anti-proliferative activity of *L. rhinocerus* sclerotial extracts. MCF-7 cells were treated with various concentrations of *L. rhinocerus* extracts. Effects of extracts on the viability of cells was examined by measuring relative cell viability using the MTT assay (values are as mean ± SD, $n = 3$). (A) Wild type *L. rhinocerus* (WT Rhino); (B) cultivated *L. rhinocerus* TM02 extracts. ME, methanol extract; HWE, hot-water extract; CWE, cold-water extract.

whether the cultivated strain exhibits similar bio-pharmacological activity. Traditionally, the sclerotium of the tiger milk mushroom is used to treat breast cancer. Lee *et al.*[4] demonstrated that CWE of the cultivated strain TM02 exhibited anti-proliferative activity against MCF-7 cells. In this study, we compared the anti-proliferative activity of both the wild type and cultivated strain of *L. rhinocerus* sclerotial powder against MCF-7 cells, using the MTT assay (Fig. 3). The HWE and ME of both WT Rhino and TM02 did not show significant anti-proliferative activity on the cells, with IC_{50} values $> 1000\ \mu g\ mL^{-1}$. The CWE of both strains of *L. rhinocerus* sclerotia, however, were able to induce cell death against MCF-7 in a dose-dependent manner. The IC_{50} value of WT Rhino-CWE and TM02-CWE were $206.3 \pm 9.46\ \mu g\ mL^{-1}$ and $90.0 \pm 8.16\ \mu g\ mL^{-1}$, respectively. The IC_{50} value of TM02-CWE is comparable to that reported by Lee *et al.*[4] and is even lower than that of the wild type. The nature of the anti-proliferative bioactive compound has yet to be fully investigated; however, it was reported to potentially be a high-molecular-weight protein–carbohydrate complex.[4] Thus our results show that, despite the differences in chemical composition, the sclerotium of the cultivated strain of *L. rhinocerus* exhibits more potent anti-proliferative activity than the wild type.

Comparison of the chemical composition and properties of cultivated and wild type *L. rhinocerus* sclerotia

Our results show that the sclerotia of the cultivated strain TM02 contain higher amounts of protein, water-soluble substances and antioxidant capacity, compared to that of the wild type. These differences are not entirely surprising. Wild type sclerotium is usually collected only after the formation of the cap, as prior to that the sclerotium is buried beneath the ground and hence not noticeable. On the other hand, sclerotium from the cultivated strain is harvested in the laboratory just before the formation of the stem and cap, and hence is at the earlier stage of maturation of the sclerotium. It is interesting to note that the sclerotium of the cultivated strain also exhibits stronger anti-proliferative activity against MCF-7 cells than the wild type.

CONCLUSIONS

The sclerotium of the cultivated strain is richer in its nutrient composition and with stronger antioxidant capacity and anti-proliferative activity against MCF-7 cells than the wild type. The strong superoxide anion radical scavenging activity, anti-proliferative activity and the high protein content suggest that the sclerotium of the *L. rhinocerus* cultivar TM02 can be a promising source of functional food and/or nutraceutical.

ACKNOWLEDGEMENTS

This study was supported by University of Malaya high impact research grant (HIRGA) J-20014-73807 and postgraduate research fund PV024/2012A from the University of Malaya, Kuala Lumpur, Malaysia.

REFERENCES

1 Chang YS and Lee SS, Utilisation of macrofungi species in Malaysia. *Fungal Divers* **15**:15–22 (2004).
2 Lee SS, Chang YS and Noraswati MNR, Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. *For Ecol Manage* **257**:2062–2065 (2009).
3 Lai CKM, Wong KH and Cheung PCK, Antiproliferative effects of sclerotial polysaccharides from *Polyporus rhinocerus* Cooke (Aphyllophoromycetideae) on different kinds of leukemic cells. *Int J Med Mushrooms* **10**:255–264 (2008).
4 Lee ML, Tan NH, Fung SY, Tan CS and Ng ST, The antiproliferative activity of sclerotia of *Lignosus rhinocerus* (Tiger Milk Mushroom). *Evid Based Complement Alternat Med* **2012**:1–5 (2011).
5 Tan CS, Setting-up pilot-plant for up-scaling production of 'Tiger-Milk'-mushroom as dietary functional food. in *Technical Report MOA TF0109M004*, Malaysia (2009).
6 Lee SS, Tan NH, Fung SY, Pailoor J and Sim SM, Evaluation of the sub-acute toxicity of the sclerotium of *Lignosus rhinocerus* (Cooke), the Tiger Milk mushroom. *J Ethnopharmacol* **138**:192–200 (2011).
7 Wong KH and Cheung PCK, Sclerotia: emerging functional food derived from mushrooms, in *Mushrooms as Functional Foods*, ed. by Cheung PCK. Wiley, Hoboken, NJ, pp. 111–146 (2008).
8 Wong KH, Cheung PCK and Wu JZ, Biochemical and microstructural characteristics of insoluble and soluble dietary fiber prepared from mushroom sclerotia of *Pleurotus tuber-regium*, *Polyporus rhinocerus*, and *Wolfiporia cocos*. *J Agric Food Chem* **51**:7197–7202 (2003).
9 Tan CS, Ng ST, Vikineswary S, Lo EP and Tee CS, Genetic markers for identification of a Malaysian medicinal mushroom *Lignosus rhinocerus* (Cendawan susu rimau). *ISHS Acta Hortic* **859**:161–167 (2010).
10 Singleton VL and Rossi JA, Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am J Enol Vitic* **16**:144–158 (1965).
11 Benzie IF and Strain JJ, The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Anal Biochem* **239**:70–76 (1996).
12 Cos P, Rajan P, Vedernikova I, Calomme M, Pieters L, Vlietinck AJ, Augustyns K, Haemers A and Vanden Berghe D, *In vitro* antioxidant profile of phenolic acid derivatives. *Free Radic Res* **36**:711–716 (2002).

13 Re R, Pellegrinni N, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorisation assay. *Free Radic Biol Med* **26**:1231–1237 (1999).
14 Siddhuraju P and Becker K, The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. *Food Chem* **101**: 10–19 (2007).
15 Beyer G and Melzig MF, Effects of propolis on hypoxanthine–xanthine oxidase-induced toxicity in cultivated human cells and on neutrophil elastase activity. *Biol Pharm Bull* **28**: 1183–1186 (2005).
16 Mimnaugh EG, Dusre L, Atwell J and Myers CE, Differential oxygen radical susceptibility of adriamycin-sensitive and –resistant MCF-7 human breast tumor cells. *Cancer Res* **49**:8–15 (1989).
17 AOAC, *Official Method of Analysis of AOAC International* (16th edn). Association of Official Analytical Communities, Arlington, VA (1995).
18 US Environmental Protection Agency, Test Method Collections. [Online]. Available: http://www.epa.gov/fem/methcollectns.htm [24 January 2012].
19 Hewlett-Packard, *HP AminoQuant Series II Operator's Handbook*. Waldbronn Analytica Division, Waldbronn, Germany (1990).
20 Seligson FH and Mackey LN, Variable predictions of protein quality by chemical score due to amino acid analysis and reference pattern. *J Nutr* **114**:682–691 (1984).
21 Lee KJ, Yun IJ, Kim KH, Lim SH, Ham HJ, Eum WS et al., Amino acid and fatty acid compositions of *Agrocybe chaxingu*, an edible mushroom. *J Food Compost Anal* **24**:175–178 (2011).
22 Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L and Byrne DW, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Compos Anal* **19**:669–675 (2006).
23 Alvarez-Parrilla E, de la Rosa LA, Martínez NR and González Aguilar GA, Total phenols and antioxidant activity of commercial and wild mushrooms from Chihuahua Mexico. *Ciência e Tecnologia de Alimentos* **5**:329–334 (2007).
24 Cheung LM, Cheung PCK and Ooi VEC, Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem* **81**:249–255 (2003).
25 Abdullah N, Ismail SM, Aminudin N, Shuib AS and Lau BF, Evaluation of selected culinary–medicinal mushrooms for antioxidant and ACE inhibitory activities. *Evid Based Complement Alternat Med* **2012**:1–12 (2011).
26 Zubia M, Robledo D and Freile-Pelegrin Y, Antioxidant activities in tropical marine macroalgae from the Yucatan Peninsula, Mexico. *J Appl Phycol* **19**:449–458 (2007).
27 Korycka-Dahl M and Richardson M, Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino acids. *J Dairy Sci* **61**:400–407 (1978).
28 Thetsrimuang C, Khammuang S and Sarnthima R, Antioxidant activity of crude polysaccharides from edible fresh and dry mushroom fruiting bodies of *Lentinus* sp. Strain RJ-2. *Int J Pharmacol* **7**:58–65 (2011).
29 Liu F, Ooi VE and Chang ST, Free radical scavenging activities of mushroom polysaccharide extracts. *Life Sci* **60**:763–771 (1997).
30 Puttaraju NG, Venkateshaiah SU, Dharmesh SM, Urs, SM and Somasundaram RJ, Antioxidant activity of indigenous edible mushrooms. *J Agric Food Chem* **54**:9764–9772 (2006).

