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ORIGINAL PAPER

Comparative nutritional and mycochemical contents, biological activities and LC/MS screening of tuber from new recipe cultivation technique with wild type tuber of tiger's milk mushroom of species *Lignosus rhinocerus*

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Abstract
Tiger's milk mushroom is known for its valuable medicinal properties, especially the tuber part. However, wild tuber is very hard to obtain as it grows underground. This study first aimed to cultivate tiger's milk mushroom tuber through a cultivation technique, and second to compare nutritional and mycochemical contents, antioxidant and cytotoxic activities and compound screening of the cultivated tuber with the wild tuber. Results showed an increase in carbohydrate content by 45.81% and protein content by 123.68% in the cultivated tuber while fat content reduced by 13.04%. Cultivated tuber also showed an increase of up to 64.21% for total flavonoid-like compounds and 62.51% of total β-D-glucan compared to the wild tuber. The antioxidant activity of cultivated tuber and wild tuber was 760 and 840 μg mL^{-1}, respectively. The cytotoxic activity of boiled water extract of cultivated tuber against a human lung cancer cell line (A549) was 65.50 ± 2.12 μg mL^{-1} and against a human breast cancer cell line (MCF7) was 19.35 ± 0.11 μg mL^{-1}. β-D-glucan extract from the purification of boiled water extract of cultivated tuber showed cytotoxic activity at 57.78 ± 2.29 μg mL^{-1} against A549 and 33.50 ± 1.41 μg mL^{-1} against MCF7. However, the β-glucan extract from wild tuber did not show a cytotoxic effect against either the A549 or MCF7 cell lines. Also, neither of the extracts from cultivated tuber and wild tuber showed an effect against a normal cell line (MRC5). Compound profiling through by liquid chromatography mass spectrometry (LC/MS) showed the appearance of new compounds in the cultivated tuber. In conclusion, our cultivated tuber of tiger's milk mushroom using a new recipe cultivation technique showed improved nutrient and bioactive compound contents, and antioxidant and cytotoxic activities compared to the wild tuber. Further investigations are required to obtain a better quality of cultivated tuber.

Keywords Cytotoxic activity · Fungal chromatography · Mushroom cultivation

Introduction

Edible mushrooms are considered important functional foods due to their high protein and low-fat contents (Khatun et al. 2012; Al-Obaidi 2016). Many bioactive compounds with potential biological properties have been identified in mushrooms, ranging from low molecular weight compounds such as quinones, flavonoid-like compounds, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes and steroids, to high molecular weight compounds such as homo- and heteropolysaccharides, glycoproteins, glycopeptides, proteins and RNA–protein complexes (Ferreira et al. 2010). These bioactive compounds have been shown to act as immune system enhancers and carry anticancer, antiviral, antioxidant and anti-hypercholesterolemia properties (Khatun et al. 2012). Long chain polysaccharides, especially β-D-glucan s such as lentinan, krestin, schizophyllan and lectin, have been found to be the most potent anticancer agents in mushrooms and have been evaluated against various cancer cell lines such as lung, breast and stomach cancer (Ferreira et al. 2010; Khatun et al. 2012; Patel and Goyal 2012).

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Tiger's milk mushroom, *Lignosus rhinocerus*, is a medicinal mushroom that has been widely used in Southeast Asia and southern China for medicinal purposes (Lai et al. 2013). The tuber part of this mushroom has been scientifically proven to have medicinal effects, such as in acute asthma treatment (Johnathan et al. 2016), as an anti-proliferative agent against cancer cells (Lee et al. 2012), and antimicrobial (Shopana et al. 2012), antioxidant (Ruiz-Rodriguez et al. 2009; Lau et al. 2014), and immune-modulating activities (Wong et al. 2011; Liu et al. 2016). It is usually consumed as a drink through boiling the sliced tuber (Yap et al. 2014). There are increasing demands for tiger's milk mushroom, either as a functional food or for nutraceutical purposes, as it is high in fibre, carbohydrate and protein yet low in fat (Yap et al. 2013). Obtaining a sufficient supply of the mushroom is still a challenge as it is a seasonal-based mushroom and there are difficulties with regard to the identification of optimal growth locations (Lee et al. 2011; Yap et al. 2014). In view of this, the objective of this study was to cultivate the tuber of this valuable edible mushroom and compare between cultivated and wild tiger's milk mushroom tuber for phytochemical content, including nutritional, total flavonoid-like compounds and β-D-glucan s content, biological activities specific to antioxidant and cytotoxicity, and compound profiling by LC/MS spectrometry approaches.

Materials and methods

Collection of wild tuber of tiger's milk mushroom

The wild tuber of tiger's milk mushroom *L. rhinocerus* was collected from Lata Iskandar Pahang, Malaysia (4.3245°N, 101.3324°E). The wild tuber was transported to the cell biology laboratory, Agro-biotechnology Institute Malaysia for further analysis.

Production of starter seeds (liquid spawn)

The wild tuber was peeled off under sterile conditions to get 1 mm^3 samples of the inner tissue. The tissue was transferred onto potato dextrose agar and incubated in a growth chamber at 23 °C for 3 weeks. The produced mycelium was inoculated into a mixture of liquid medium containing yeast extract, glucose, peptone, dipotassium hydrogen phosphate (K_2HPO_4), magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$) and potassium dihydrogen phosphate (KH_2PO_4), followed by incubation at 23 °C for 14 days under orbital agitation at 150 rpm to produce liquid spawn (Rashid et al. 2016).

Development of cultivated tuber

The liquid spawn was homogenised at 5000 rpm for 5 min. The process of cultivation was performed according to Rashid et al. (2016). A substrate bag with a total weight of 500 g was prepared using a combination of 89% sawdust, 1% gypsum, and 10% rice bran; this was prepared in 80% humidity. The substrate bag was sterilised at 121 °C for 30 min before being inoculated with 10 mL liquid spawn of tiger's milk mushroom and incubated in the dark at room temperature for 3–4 weeks. The spawned substrate block (fully covered with mycelium) was then buried at a depth of 2 cm in a casing soil comprising of top soil and lump soil (1:1), 1% gypsum and water to provide 50–70% humidity. Cultivated tuber of tiger's milk mushroom was harvested after 3 months of incubation in the dark at a range of 28–32 °C.

Extraction process

The outer layer of both cultivated and wild tuber was cleaned and peeled off, and the inner parts were cut into small pieces prior to freeze-drying. The dried tuber was homogenised through a mesh with a pore size of 0.05 mm. Boiled water extract was obtained from the dried powder tuber according to the method by Han et al. (2009) with slight modifications. Dried tuber powder was soaked in Milli-Q water (1:15 sample to water) at 150 rpm at high temperatures (95–100 °C) for 2 h. The extract was centrifuged at 3000 rpm for 10 min and the residue was re-extracted following the same steps. The supernatant was then freeze-dried.

Proximate analysis for nutritional content

Proximate composition analysis was performed according to the standard method of the Association of Official Analytical Chemists (AOAC 2003) to determine the total carbohydrate, fat and crude protein compositions.

For total fat: 1 g of dried tuber was extracted using the Soxhlet apparatus with the receiving beaker filled with petroleum ether. The crude fat content (%) was determined by the following formula:

$$Total\ fat\ (\%) = \frac{Weight\ of\ extract}{Weight\ of\ dried\ tuber} \times 100\%$$

For crude protein: the Kjeldahl method was used to determine protein content in the tuber samples. 1 g of tuber was heated with sulphuric acid containing a digestion mixture, followed by the addition of sodium hydroxide and titration with hydrochloric acid.

For carbohydrate content: 2 g of dried tuber were extracted with 80% ethanol in the Soxhlet extractor for 2 h. Reducing and total sugars were determined by the ferricyanide method. Reducing sugar was analysed by mixing 2 mL of the extract and 8 mL of ferricyanide reagent, while total sugar was analysed by hydrolysing 25 mL of extract with 5 mL concentrated hydrochloric acid before mixing with ferricyanide reagent at the ratio listed above. Both were read at 380 nm using glucose as a standard. Starch content was analysed by refluxing 200 mg of the ethanol extract residue with 0.7 M HCl for 2.5 h. The collected acid hydrolysate was neutralised, diluted to 500 mL with distilled water and analysed as reducing sugar by the ferricyanide method at 380 nm.

Total β-D-glucan content

β-D-Glucan content was determined from the net percentage of total glucan content and α-D-glucan content using the commercial Mushroom and Yeast β-D-glucan Assay Kit (McCleary and Draga 2004). Total glucan was extracted by heating 100 mg of the boiled water extract in 1.5 mL of 37% hydrochloric acid at 30 °C for 45 min with short stirring every 15 min. Water was added and boiled at 100 °C for 2 h. The mixture was left to cool prior to the addition of 10 mL of 2 M potassium hydroxide, followed by 0.2 M sodium acetate buffer (pH 5.0) up to 100 mL. The supernatant was collected by centrifugation at 1500×*g* for 10 min. Then, 0.1 mL of supernatant was incubated with 0.1 mL of highly purified enzyme A (*exo*-1,3-β-glucanase + β-glucosidase) at 40 °C for 60 min. β-D-glucan was collected by mixing 100 mg of the boiled water extract with 2 mL of 2 N potassium hydroxide for 20 min on ice, followed by the immediate addition of 8 mL of 1.2 M sodium acetate buffer (pH 3.8) and 0.2 mL of enzyme B (amyloglucosidase + invertase). The mixture was incubated at 40 °C for 30 min and centrifuged at 1500×*g* for 10 min. Then, 0.1 mL of supernatant was incubated with 0.1 mL of sodium acetate buffer (200 mM, pH 5.0). Total glucan and α-D-glucan solution (0.1 mL of each) were incubated with 3 mL of GOPOD reagent at 40 °C for 20 min. Concentrations of total glucan and α-D-glucan were determined by UV–Vis spectrophotometer at 510 nm against reagent blank (0.2 mL of 0.2 M sodium acetate). The reagent blank and β-D-glucose standard (0.1 mL of 1 mg mL^{-1} D-glucose standard + 0.1 mL of 0.2 M sodium acetate buffer) were also incubated with GOPOD reagent under identical conditions. The β-D-glucan content (%) was calculated as described below:

$$\beta\text{-}D\text{-}glucan\ content\ \% = (Total\ glucan\ content\ \%) - (\alpha\text{-}D\text{-}glucan\ content\ \%)$$

$$\beta\text{-}D\text{-}glucan\ (\%) = [Abs\_TG \times F/W \times 90] - [Abs\_\alpha G \times F/W \times 9.27]$$

Abs_TG: absorbance value of total glucan in extract, Abs_αG: absorbance value of α-D-glucan in extract

$$F = \frac{100}{\text{Absorbance value of D-glucose standard}}$$

W = sample weight

Total flavonoid-like content

The dried boiled water extract was dissolved in Milli-Q water at 1 mg mL^{-1}. Total flavonoid-like content was determined by using total flavonoid assay as reported by Marinova et al. (2005). The extract solution (1 mL) was mixed with 4 mL of Milli-Q water, followed by 0.3 mL of 5% $NaNO_2$ (w/v). The mixture was left for 5 min. Immediately, 0.3 mL of 10% $AlCl_3$ (w/v) was added. After 6 min of incubation, 2 mL of 1 M NaOH (4% w/v) was added and the volume was made up to 10 mL with Milli-Q water. Absorbance was measured against the quercetin standard curve at 510 nm using a UV–Vis spectrophotometer. Solvent was used as a blank. The whole process was performed in the dark. The total flavonoid-like content was expressed in mg of quercetin equivalent per gram of extract as calculated below:

$$Total\ flavonoid\text{-}like\ content\ (\text{mg g}^{-1}) = \frac{Extract\ concentration \times dilution\ factor \times extract\ volume}{Sample\ weight}$$

Antioxidant activity using DPPH radical scavenging method

Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay according to Sahu et al. (2013). The dried boiled water extract was prepared at a concentration of 1 mg mL^{-1} by dissolving in Milli-Q water. A series of two-times dilution was done to obtain final extract concentrations of 62.5, 125, 250, 500 and 1000 µg mL^{-1}. Then, 1.5 mL of each extract concentration was mixed with 0.1 mM of DPPH and incubated in the dark at room temperature for 30 min. The mixture was read at 517 nm using a UV–Vis spectrophotometer. The same assay procedure was applied to the control (water). Water and DPPH reagent was used as a blank. IC_{50} of DPPH Radical scavenging activity of each extract was determined as shown below:

$$Antioxidant\ activity\ (\%) = \frac{Absorbance\ control - absorbance\ sample}{Absorbance\ control} \times 100\%$$

Isolation of β-D-glucan

The β-D-glucan extract was prepared according to the method of Jamil et al. (2013) with some modification. Dried tuber powder was added to Milli-Q water at a ratio

of 1:15 and boiled (95–100 °C) for 2 h. The process was repeated twice. Supernatant was collected by centrifugation at 3000 rpm for 10 min and mixed with four volumes of 96% ethanol followed by incubation for overnight at 4 °C. The mixture was centrifuged at 10,000 rpm for 10 min. The accumulated precipitates were collected and freeze-dried.

Cell lines

American Type Culture Collection (ATCC) human lung cancer cell line (A549), human breast cancer cell line (MCF7) and human normal cell line (MRC5) were maintained in RPMI 1640 complete culture media enriched with 10% heat inactivated foetal bovine serum, 1% penicillin–streptomycin and 0.1% fungizone.

Cytotoxicity activity by MTT assay

Cytotoxicity assay was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method as described by Rahman et al. (2011) with some modifications. Here, 100 µL of 1×10^4 cells mL^{-1} of monolayer cells were seeded into 96 well plates. The cells were incubated in 5% CO_2 at 37 °C for 24 h. All of the media were removed and replaced with another 100 µL of fresh complete culture media. Each dried extract (direct boiled and β-D-glucan extracts) was prepared at 1 mg mL^{-1} by using complete culture media and filtered through a 0.22 µm membrane filter under aseptic conditions. After that, 100 µL of extract was pipetted into the three seeded wells of the first row. Two-times serial dilution was performed directly onto the plate to obtain the final extract concentrations of 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg mL^{-1}. The cells were incubated for 72 h under 5% CO_2 at 37 °C. Then, 20 µL of 5 mg mL^{-1} MTT solution were added. After incubation for 4 h, 100 µL DMSO was added to each well to dissolve any purple formazan crystal produced. The dissolved solutions were read at 590 nm using a microplate reader. Cell viability (%) was calculated as below:

$$\textit{Cell viability}\ (\%) = \frac{\textit{Absorbance sample} - \textit{absorbance blank}}{\textit{Absorbance control} - \textit{absorbance blank}} \times 100\%$$

LC/MS Q-ToF profiling

Analysis was performed using an Agilent 1290 RRLC (Rapid Resolution Liquid Chromatography) series equipped with 6550 iFunnel LC/MS Q-ToF System (Agilent Technologies Inc., U.S.). Boiled water extract was prepared at 1 mg mL^{-1} and filtered through a 0.2 µm membrane filter. Samples were run according to the method described by Jamil et al. (2013) with some modifications. Mobile phases (A) 100% water and (B) 100% acetonitrile were used at a gradient of 5% B (0.5 min), 5–30% B (8.5 min); 30–95% B (9 min), 95% B (3 min) and 95–5% B (7 min). A sample at an injection volume of 5 µL was flowed through a 3 µm × 2.1 mm × 100 mm carbon column (Thermo-Scientific) at a flow rate of 0.1 mL min^{-1} for a total running time of 27 min. The mass spectrometry analyses were set under negative ion mode under the following conditions: gas temperature: 290 °C; gas flow: 11 L min^{-1}; nebuliser pressure (N_2): 35 psig; sheath gas: 11 L min^{-1} at 350 °C; capillary voltage: 3500 V; nozzle voltage: 1000 V; and fragmentor voltage: 175 V. Clean purified water was used as a blank.

Statistical analysis

All analysis was expressed as mean ± standard deviation of independent triplicates and analysed by using Statistical Package for Social Sciences (SPSS version 20.0).

Results

Sample preparation and extraction

Tuber from the cultivation technique was harvested after 3 months in casing soil containing a mixture of top soil and loam soil at a ratio of 1:1. From our patented report on the cultivation technique of *L. rhinocerus* (Patent no: WO2016076702 A3), the average fresh weight of the cultivated tuber was 45.533 ± 19.392 g with 50% moisture content. In comparison, the average weight of wild tuber collected in Lata Iskandar Pahang was 31.213 ± 10.185 g with 80% moisture content. Extraction was done by stirring in Milli-Q water for 2 h starting from room temperature until boiling point was reached. The freeze-dried form of collected extracts was used for the determination of nutritional content, total flavonoid-like content and total β-D-glucan content and also antioxidant and cytotoxic activities. The extracts were also analysed through mass spectrometry for compound profiling.

Nutritional content in the cultivated and wild tuber

The nutritional content of cultivated and wild tubers was investigated by proximate analysis. Results obtained were reported on a dry weight basis (mg g^{-1}). Both tubers were high in carbohydrate and protein, and low in fat (Table 1). However, the nutritional content in the cultivated tuber was better than that in the wild tuber, as the cultivated tuber contained higher carbohydrate and protein contents yet a lower fat content. This showed that the nutritional content of the cultivated tuber had improved by 123.68 and 45.81% for

Table 1 Nutrition content of cultivated tuber and wild tuber of tiger's milk mushroom

	Wild tuber	Cultivated tuber
Total carbohydrate (mg g^{-1})	513.0	748.0
Total protein (mg g^{-1})	30.4	68.0
Total fat (mg g^{-1})	4.6	4.0

Table 2 Total flavonoid and β-D-glucan content of cultivated tuber and wild tuber of tiger's milk mushroom

	Wild tuber	Cultivated tuber
Total flavonoid content (mg g^{-1})	110.84 ± 5.89	136.92 ± 9.06
Total β-D-glucan content (% w/w)	40.52 ± 2.41	63.51 ± 10.92

protein and carbohydrate, respectively, while the fat content had reduced by 13.04% through this cultivation technique.

Total flavonoid-like and β-D-glucan content

Total flavonoid-like content was expressed as quercetin equivalent, while total β-D-glucan content was determined based on the number of monosaccharides with β-linkage. Both results revealed that our cultivation technique successfully improved the bioactive contents of the tuber. According to the data shown in Table 2, the total flavonoid content of the cultivated tuber was 64.21% higher than the wild tuber. The cultivation technique also increased the total β-D-glucan content of the tuber to 62.51% as the cultivated tuber showed 63.51 ± 10.92% w/w total β-D-glucan content, which was equivalent to 0.64 g g^{-1}.

Antioxidant activity

The antioxidant activity of the boiled water extract of either cultivated tuber or wild tuber was assayed by DPPH scavenging activity. Compounds with antioxidant properties reacted with DPPH free radicals by donating hydrogen to produce a more stable DPPH molecule (Biswas et al. 2010). The antioxidant activity was obtained by determining the concentration of extracts at 50% scavenging activity (IC_{50}) through a graph of percentage of DPPH scavenging activity versus extract concentration. As shown in Fig. 1a, 50% of DPPH activity was inhibited by the cultivated tuber at an extract concentration of 760 μg mL^{-1}. However, a higher concentration (840 μg mL^{-1}) of wild tuber extract was required to achieve the same effect.

Cytotoxic activity

The cytotoxic activity against human lung cancer cell line (A549) and human breast cancer cell line (MCF7) was assessed using the boiled water extract and β-D-glucan extract from cultivated and wild tubers. A normal human cell line, MRC5, was also treated as a reference to determine any lethal effect on normal cells. Boiled water extract of both tubers was added to each cell line for 72 h, which was previously determined to be the optimum incubation time for boiled water extract of tiger's milk mushroom. The results obtained were based on the colour intensity of MTT reagent. The cytotoxic activity (IC_{50}) of cultivated and wild tuber extracts to inhibit 50% of cell viability was determined using a graph of cell viability versus extract concentration. As shown in Fig. 1, cytotoxic activity of boiled water extract from the cultivated and wild tubers against A549 was mild (Malek et al. 2011). The cytotoxic activity of the water extract from the cultivated tuber (IC_{50} at 65.50 ± 2.12 μg mL^{-1}) was slightly higher than that of the wild tuber (67.50 ± 1.15 μg mL^{-1}). Meanwhile, both samples showed an effect against MCF7 with IC_{50} values of 19.35 ± 0.11 and 19.13 ± 0.35 μg mL^{-1} for the cultivated and wild tubers, respectively (Fig. 1c). Purification of the boiled water extract to obtain β-D-glucan extract by a precipitation process using ethanol gave a cytotoxic activity for the cultivated tuber of 57.78 ± 2.29 μg mL^{-1} against A549 and 33.50 ± 1.41 μg mL^{-1} against MCF7 (Fig. 1b, c). Meanwhile, the β-D-glucan extract of the wild tuber did not have any cytotoxic effect against either cancer cell line. However, neither the boiled water nor β-D-glucan extracts from either tuber had a lethal effect on the human normal cell line (MRC5) as the extracts did not inhibit 50% of cell viability up to an extract concentration of 100 μg mL^{-1} (Fig. 1d).

Spectrometry analysis by LC/MS-QToF

Spectrometry analysis on boiled water extract of both cultivated and wild tuber was performed using liquid chromatography mass spectrometry-quodropole time of flight (LC/MS-QToF). Figure 2 shows the LC/MS-QToF profiles of both extracts under optimised conditions. The chromatogram pattern shows the presence of several potential compounds at different intensities. It was observed that most peaks and compounds in the boiled water extract of wild tuber (Fig. 2a, c) were successfully maintained in the boiled water extract from cultivated tuber (Fig. 2b, d). However, there were several peaks that were notably different between the two samples; these are labelled Point 1, 2, 3 and 4 (Fig. 2c, d). At Point 1, the peak intensity in the chromatogram of the cultivated tuber was higher compared to wild tuber (Fig. 2a, b); this was due to the presence of a larger compound peak (p3) in the cultivated tuber compared to the wild tuber, as

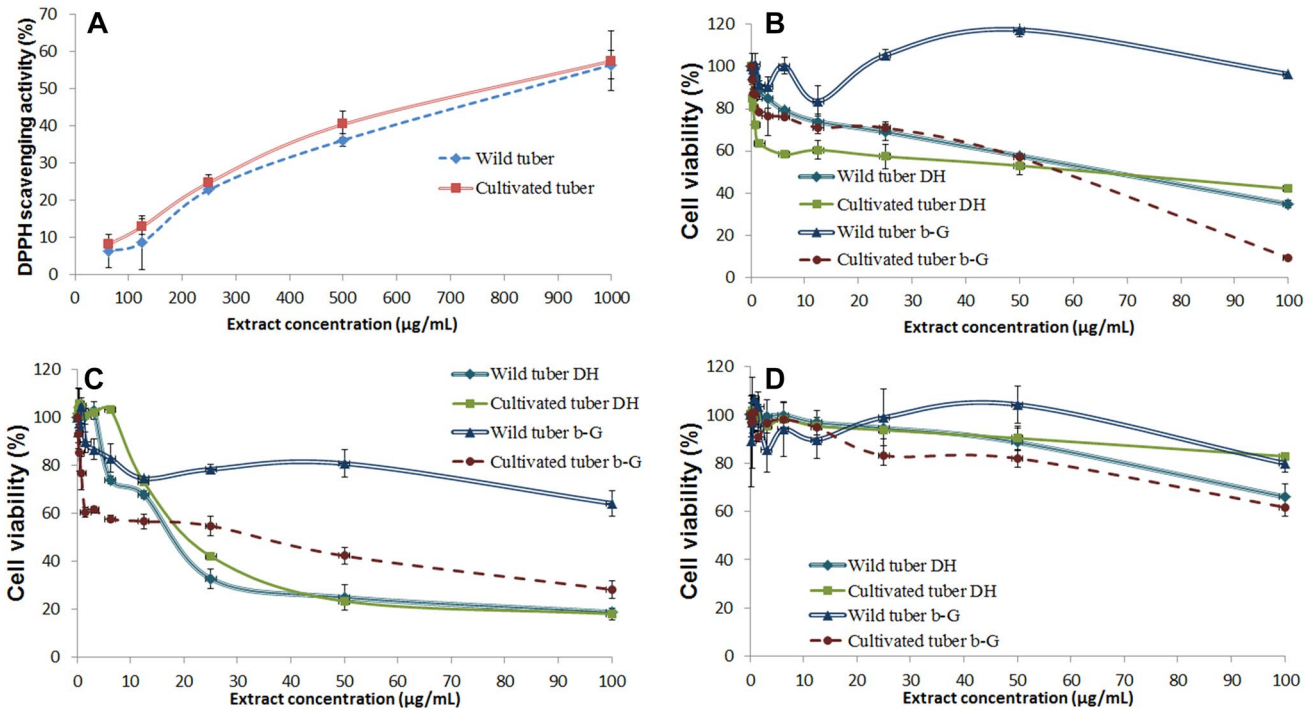

Fig. 1 Antioxidant and cytotoxic activities of cultivated and wild tuber of tiger's milk mushroom: **a** antioxidant activity of boiled water extract on DPPH free radical; **b** cytotoxic effect of boiled water and β-D-glucan extracts against A549; **c** cytotoxic effect of boiled water and β-D-glucan extracts against MCF7; **d** cytotoxic effect of boiled water and β-D-glucan extracts against MRC5. Error bar is representing the standard deviation for the mean of three replicates

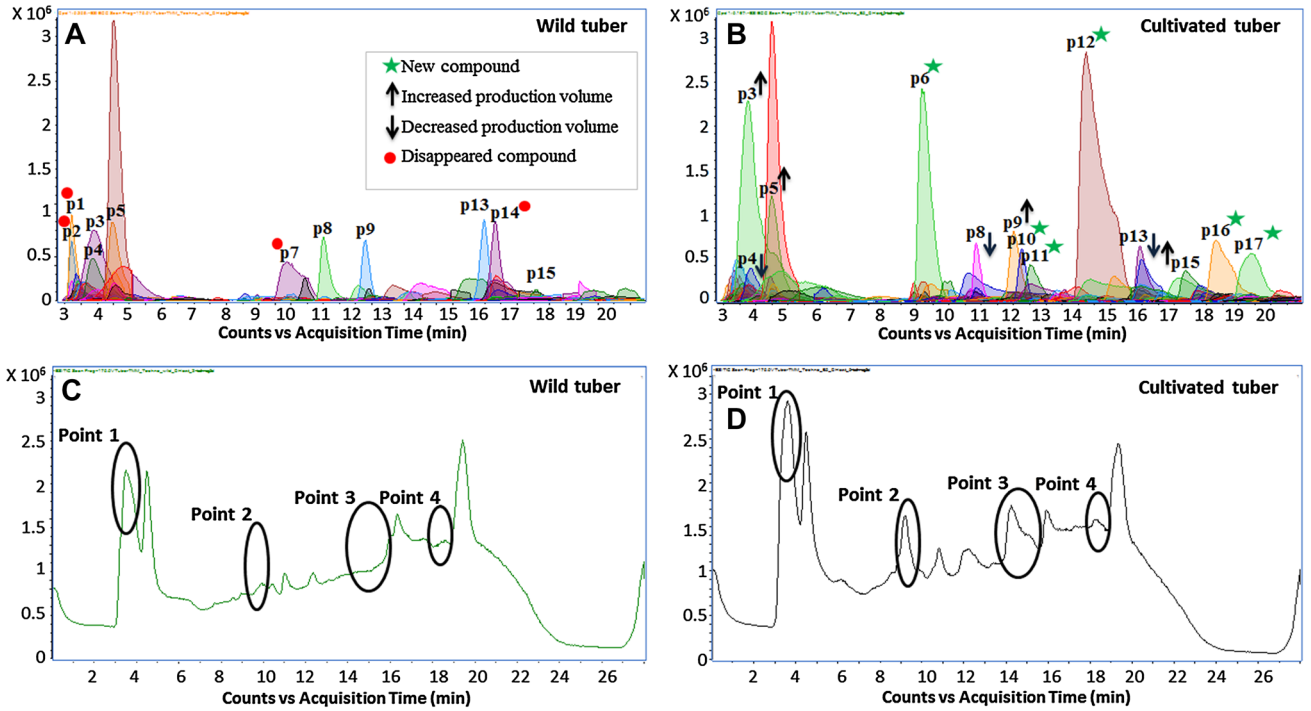

Fig. 2 LC/MS-QToF profiling of boiled water extracts from cultivated tuber and wild tuber: **a** chromatogram pattern of boiled water extract from wild tuber; **b** chromatogram pattern of boiled water extract from cultivated tuber; **c** extracted compounds in boiled water extract of wild tuber; **d** extracted compounds in boiled water extracts of cultivated tuber

shown in Fig. 2c, d. New peaks appeared at Point 2, Point 3 and Point 4 in the cultivated tuber (Fig. 2b) due to the presence of compounds at peaks p6, p12 and p16, respectively (Fig. 2b, d). Peaks p10, p11 and p17 were also new compounds that were only present in the cultivated tuber. This cultivation technique also improved the production volume of some compounds of the tuber of tiger's milk mushroom, as showed by peaks p5, p9 and p15. However, peaks p4, p8 and p13 in the wild tuber chromatogram were at lower volumes compared to the cultivated tuber. Peaks p1, p2, p7 and p14 also disappeared in the tuber following this cultivation technique.

Discussion

Cultivation of tiger's milk mushroom involves a specific technique as they generate tubers during their life cycle. Tiger's milk mushroom tuber was cultivated successfully following the method developed by our team, with fresh and dry weights of the cultivated tuber being higher compared to the wild tuber. This may be due to the combination of top soil and loam soil used in the cultivation process. Top soil contains a high percentage of organic materials resulting from dead plant or organism decomposition, and can be found at approximately 2–12 inches from the soil surface. In contrast, loam soil has good water-holding capacity that can contribute to a high percentage of humidity (Rashid et al. 2016; Sher et al. 2010; Uddin et al. 2011; Olufokunbi and Chiejina 2013). The spawned substrate block was buried at a depth of 2 cm inside the casing soil for cultivation as it gained a higher tuber weight compared to tubers buried deeper. This could be due to the fact that better aeration resulted in better oxygen flow in comparison to the cultivation of substrate deeper than 2 cm. This was also shown in the study by Olufokunbi and Chiejina (2013), who reported that good aeration from soils was important to obtain higher fresh and dry weight of tiger's milk mushroom from species of *Pleurotus tuberregium*. In general terms, mushrooms are high in carbohydrate and protein yet low in fat. This is considered ideal for a healthy food requirement for adults (Valverde et al. 2015). From the nutrient content analysis, the protein and carbohydrate contents of the cultivated tuber were higher than the wild tuber, while the fat content was much lower. This may be due to the use of top soil in the cultivation process as it is rich in proteins and carbohydrates, which are an important source of nutrients and also promote water retention (Spence et al. 2013). The same finding was reported by Olufokunbi and Chiejina (2013), where higher amounts of protein were produced when using top soil for the cultivation of *P. tuberregium*. In a study by Lau et al. (2013), the carbohydrate and protein contents of cultivated tuber using their cultivation technique were 826 and 70.2 mg g^{-1}, respectively, whereas the fat content was 4.9 mg g^{-1} (Lau et al. 2013).

Polysaccharide and phenolic compounds have been found to be potent mushroom-derived antioxidants (Wang et al. 2013) and anticancer agents (Ferreira et al. 2010; Khatun et al. 2012). Different types of mushrooms may have different bioactive compound contents. Total flavonoid-like content and total β-D-glucan content were found to be up to 65% higher in the cultivated tubers than that in wild tubers. This may be related to the high nutritional content in the cultivated tuber that contributes to the production of higher bioactive compounds (Deduke et al. 2012). However, as reported in a study by Lau et al. (2013), the total β-D-glucan content in the cultivated tuber obtained through their cultivation technique was low (0.01 g g^{-1}), even though it contained high carbohydrate and protein contents. Higher total β-D-glucan content in our cultivated tuber may also be due to the use of loam soil in our cultivation technique that aided in retaining the required amount of water for tuber production. This was confirmed by Jirapa et al. (2016) who observed higher bioactive compound contents and biological activities in rice seed following greater water uptake (Jirapa et al. 2016). Mushroom age has also been reported to influence the production of bioactive compounds, as some mushrooms generate lower contents of bioactive compounds at more mature stages (Camelini et al. 2005; Barros et al. 2007). This may be due to the involvement of those compounds in the defence mechanism due to the aging process (Barros et al. 2007). As reported by Jamil et al. (2013), different concentrations of β-D-glucan was found in mushrooms such as *Lentinus edodes* (24.18% w/w), *Pleurotus ostreatus* (41.36% w/w), *Agaricus bisporus* (6.08% w/w) and *Termitomyces heimii* (21.17% w/w) (Jamil et al. 2013). Other types of mushrooms detected with β-D-glucan were *Ganoderma lucidum* (0.24 g g^{-1}) and *Grifola frondosa* (0.33 g g^{-1}) (Megazyme International Ireland Limited 2016). Total flavonoid content was also presented at different concentrations in different mushrooms, as determined in *Lentinus edodes* (3.75 mg g^{-1}), *Volvariella volvacea* (7.29 mg g^{-1}) and *Pleurotus sajor-caju* (2.29 mg g^{-1}) (Boonsong et al. 2016).

The presence of flavonoid-like phenolic compounds (Gawron-Gzella et al. 2012; Biswas et al. 2010) and β-D-glucan (Khan et al. 2014) in both tubers has contributed to the antioxidant activity by the oxidation of DPPH through abstraction mechanisms of hydrogen or electron. Our cultivated tubers exhibited a better effect as an antioxidant compared to wild tubers as lower concentrations of the tuber extract of cultivated tubers was required to oxidise the DPPH. This may be due to the higher phenolic and β-D-glucan contents in the cultivated tuber. This was supported by Khatua et al. (2013), who found a positive correlation between total phenolic compounds and β-D-glucan content with mushroom antioxidant activity. The lower antioxidant

activity in wild tuber could also be due to the mature stage of the mushroom. The high maturation stage affects the antioxidant activity, which results in a decrease in the scavenging capability (Hussein et al. 2015). This result was in contrast to that of Yap et al. (2013), who showed lower antioxidant activity in the tuber from their cultivation technique (0.52 ± 0.07 mmol Trolox equivalents g^{-1} extract) compared to wild tuber (0.81 ± 0.06 mmol Trolox equivalents g^{-1} extract). However, the antioxidant activity of both cultivated and wild tubers was lower compared to the hot water extract from other types of mushrooms such as *Lentinus edodes* (220 µg mL^{-1}), *Volvariella volvacea* (250 µg mL^{-1}) and *Pleurotus sajor-caju* (600 µg mL^{-1}) (Boonsong et al. 2016).

The cytotoxic activity of the boiled water extracts from both cultivated and wild tubers against human cancer cell lines showed an active to a mild effect. There was no toxic effect of the extracts on the normal human cell line. However, purification of the boiled water extract changed the cytotoxic levels of the tubers, with the cultivated tuber showing a higher cytotoxic effect against A549 than against MCF7. This may be due to the presence of different polysaccharides with different chemical structures, molecular weights and branching patterns that affected its biological activity (Lemieszek and Rzeski 2012). The change in the extract components perhaps changed the cell sensitivity towards bioactive compounds. This was supported by Konarikova et al. (2015), who reported that the different cytotoxic effect of black tea extract against A549 and MCF7 was related to different sensitivities between both cell lines towards the extract. This could also explain the ineffectiveness of β-D-glucan extract from wild tuber against both cancer cell lines, as it may be related to the lower β-D-glucan polysaccharide content, which further reduced the sensitivity of the cancer cell line to that extract (Elisashvili 2012; Konarikova et al. 2015). The cytotoxic activity of tuber of tiger's milk mushroom from other cultivation techniques was also tested by other researchers. However, only the cold water extract of the cultivated tuber showed cytotoxic activity with IC_{50} values of 96.7 ± 14.5 µg mL^{-1} against MCF7 and 466.7 ± 43.7 µg mL^{-1} against A549 (Lee et al. 2012; Yap et al. 2013). There was no cytotoxic activity obtained from the hot water extract of both the cultivated and wild tubers (Yap et al. 2013).

Spectrometry approaches were used to determine any significant differences in metabolite production. Data analysis from LCMS-QToF showed that both samples were not very similar. Variations in the bioactive compounds between the tubers could be due to a number of reasons; for instance, certain secondary metabolites are only produced at specific stages of maturity (Eguchi et al. 2015). The wild tuber was several years old, since it was collected after the development of stalks and caps which take many years to grow. As the cultivated tuber was just 3 months old, it might not be mature enough to produce some compounds but would still be able to produce other compounds. The appearance of some new compounds might be due to nutrient sources from liquid spawn and substrate blocks used in the previous stage (Barrios-Gonzalez et al. 2003; Deduke et al. 2012; Vandermolen et al. 2013). As in plants, the production of metabolites could also be influenced by environmental factors such as growth location, temperature, humidity, rainfall, solar radiation and macro- and micronutrient contents in soil (Sampaio et al. 2016). However, the absence of such compounds did not affect the antioxidant and cytotoxic activities of cultivated mushrooms.

Conclusion

The applied improved cultivation technique was able to produce the tuber of tiger's milk mushroom *L. rhinocerus* in a higher yield. The cultivation technique applied in this research used a new recipe to produce tuber of *L. rhinocerus* tiger's milk mushroom at an average fresh weight of 45 g. This technique successfully improved the mushroom nutrition value and bioactive compound contents (specifically to phenolic compounds and β-D-glucan) and yielded better antioxidant and cytotoxic activities. Future work might focus on the molecular biology during mushroom production and the changes in gene expression and protein profiling using different "omics" approaches to understand the changes at the molecular level between cultivated and wild mushrooms to further improve the cultivation and production procedures.

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