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Antioxidative Properties of an Extract of *Hygrocybe conica*, a Wild Edible Mushroom

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ABSTRACT

Introduction: *Hygrocybe conica* (HC), a wild mushroom commonly consumed by the indigenous people (*Orang Asli)* in Peninsular Malaysia, was assessed for its antioxidant content. **Methods**: The HC mushroom was extracted using distilled water and the crude extract partitioned using different solvents and open column chromatography to evaluate its potential antioxidant properties. The mushroom extract was partitioned using liquid-liquid extraction into the hexane (F1), chloroform (F2), butanol (F3) and formic acid (F4) fractions. Based on solvent polarity, the water extract of the mushroom was fractionated into non-polar (FI), semi-polar (FII), and polar fractions (FIII) using open column chromatography. Antioxidant capacities were determined using DPPH, ABTS, and ferric reducing antioxidant power (FRAP) assays while Folin-Ciocalteu reagent assay was used to determine total phenolic content (TPC). **Results:** The HC extract had the highest TPC and DPPH scavenging capacity compared to its extract fractions. TE values (ABTS assay) of F2 and F4 were not significantly higher than the HC extract. Among the extract fractions of different polarities, FIII had the highest antioxidant capacities (DPPH and FRAP) compared to FI and FII while FRAP values of these fractions were not significantly lower than the FRAP value of HC extract. The HC extract had significantly lower antioxidant capacity than antioxidant standards (ascorbic acid and BHA). Tannic acid as the main bioactive component in HC mushroom was detected using HPLC method. The presence of phenolics in HC extract was also confirmed using TLC. **Conclusion**: Due to the presence of potent phenolic components, the mycelia of HC could be consumed for potential antioxidative benefits.

Key words: Antioxidant capacity, *Hygrocybe conica*, tannic acid, total phenolic content

INTRODUCTION

Over 90 different groups of indigenous people (*Orang Asli*) living in Peninsular Malaysia have been using wild mushrooms for food, medicine and spiritual purposes (Lee & Chang, 2007; Lee, Chang & Noraswati, 2009). Mushrooms have been reported to be part of the human diet for centuries (Wong & Chye, 2009). More than 2000 species of mushrooms exist in nature, but only 22 species have been intensively cultivated for

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commercial purposes in specific environmental conditions (Manzi, Aguzzi & Pizzoferrato, 2001). Besides the high protein content (19×35% protein) (Chang & Buswell, 1996), mushrooms are rich in vitamins, specifically thiamine, riboflavin, ascorbic acid, and vitamin D_2 as well as minerals (Mattila *et al.*, 2001). Mushrooms also have a high phytochemicals content and these bioactives in mushroom have been claimed to have anti-tumor, anti-bacterial, anti-viral, and haematological properties (Turkoglu *et al.*, 2007). Besides phenolic compounds, mushrooms accumulate a variety of secondary metabolites such as polyketides, terpenes, and steroid that may have functional characteristics (Yang, Lin & Mau, 2002). Previous studies have also reported that mushrooms are therapeutic foods that are useful for prevention of hypertension, hypercholesterolemia, and cancers (Manzi *et al.*, 2001). In addition, wild edible mushrooms are traditionally used in various Asian countries as medicine due to their pharmacological characteristics (Isildak *et al.*, 2004). Although there is an increasing trend towards wild mushroom consumption, the mushroom does not constitute routine diet for humans (Wong & Chye, 2009).

Some species of mushrooms such as *Auricularia* sp., *Cantharellus* sp., *Clavulina* sp., *Hygrocybe conica*, *Lentinus sajor-caju*, and *L. squarrosulus* have been reported to be poisonous species by the indigenous people in Malaysia (Lee *et al.*, 2009). However, some of these mushrooms are edible as the mushrooms are consumed by indigenous people in certain villages. *Hygrocybe conica* also known as Witch's Hat or Conical Waxy Cap (Arora, 1986) falls into the category of ectomycorrhizal species (Mueller *et al.*, 2006). It is considered a saprophyte that is mainly found in grassland and damp places (usually in woods) (Oster, 2008). A majority of *Hygrocybe* mushrooms grow in semi-fixed dunes, dry sites, and well-drained soil (Newton *et al.*, 2003). It is bright in colour but a colour change is observed when it matures, with the colour changing from bright yellow to orange, red, and finally a dark red black (Arora, 1986). The cap of *H. conica* is known to be approximately 1-5 cm in diameter and blunt to sharply conical. It expands with age, but usually remains a pointed umbo of smooth surface. The striated or twisted stalk of *H. conica* is about 4-20 cm long, 0.3-1.5 cm thick. *H. conica* mushroom has been consumed as food rather than for its medicinal properties by the indigenous people in Peninsular Malaysia.

In relation to the antioxidant properties of *H. conica* mushroom, there is still a lack of scientific information on its antioxidant potential. Activity-guided fractionation is commonly used for determination of potential phytochemical antioxidants in plant extracts (Cakir *et al.*, 2003). Most of the phytochemicals are polar while some are non-polar. Phenolic compounds are semi-polar phytochemicals and this includes phenolic acids and flavonoids (Xiao, Zhou & Ressom, 2012). The types of solvents used for extraction are essential for partition or fractionation of certain phenolic compounds with specific polarity. For fat extraction, hexane is typically used as a non-polar solvent (Barron & Hanahan, 1958). Non-polar compounds that are fat soluble can be fractionated by hexane. A similar principal is applied to polar and semi-polar phenolic compounds. Therefore, this study aimed to determine potential antioxidants in *H. conica* extract based on different polarities. The extracts were also partitioned and fractionated using selected extraction solvents. Antioxidant capacity and potential phenolics compounds were also determined for the crude extract and extract fractions of *H. conica* mushroom.

METHODS

Sample preparation and extraction

Wild edible mushroom, *H. conica* (HC) were purchased from local markets in Kota

Kinabalu, Sabah, Malaysia. The samples were washed, air dried and then dried in an oven at 45°C for 24 h, ground into fine powder using a miller with 0.5 mm mesh size, followed by vacuum packing into a nylon-linear low density polyethylene film prior to analysis. The samples were extracted with distilled water and the mixture was shaken at 60°C for 4 h using a water bath shaker (150 rpm) (Yim *et al.*, 2011). Optimal levels of antioxidant capacity were obtained by re-extracting the respective residue using a similar procedure. The extracts were combined, centrifuged, and the supernatants were then concentrated to dryness using a rotary evaporator (BUCHI, Switzerland) at 45°C.

Extract fractionation

The dried extract of HC fractionated based on the method described by Kumaran & Karunakaran (2007) with some modifications. The extract was dissolved in formic acid and fractionated using n-hexane (KOFA Chemicals, China). Hexane in the mixture (hexane/formic acid, 1:1) was separated using a separating funnel. Formic acid was purchased from BASF-YPC Co., Ltd. (Nanjing, China). The formic acid extract was further fractionated using equal volumes of chloroform and n-butanol (Merck, Malaysia). All these steps were repeated three times. Finally, the solvents (hexane, chloroform, butanol, and formic acid) of all fractions were removed using a rotary evaporator. The water content of the formic acid fraction was removed by freeze-drying. Hexane, chloroform, butanol, and formic acid fractions were labeled as F1, F2, F3, and F4, respectively.

The crude water extract of HC was further fractionated based on different polarity using open column chromatography with silica gel 60 (230-400 mesh) as stationary phase. The solvents used were hexane, ethyl acetate, and methanol (KOFA Chemicals, China) and the extract fractions were labelled as FI, FII, and FIII, respectively. All the aliquots eluted from the column were determined for the presence of phenolics in HC extract using thin layer chromatography (TLC) (Kumaran & Karunakaran, 2007; Tabata *et al.*, 2008). The solvent of each fraction was removed using a rotary evaporator. The HC extract and all its extract fractions were determined for total phenolic content and antioxidant capacity, as well as identification of tannic acid using high-performance liquid chromatography (HPLC).

Thin layer chromatography

Thin-layer chromatography (TLC) was performed on silica gel plate (Merck, Darmstadt, Germany), where an aliquot of each eluate (25 mL) was spotted with a developing solvent system of ethyl acetate/water/formic acid (9:1:2, v/v/v). For confirmation of the presence of phenolics in HC extract, bioautographic reagent of 0.04% methanolic DPPH was sprayed on the TLC plate. The plate was allowed to stand at room temperature in the dark until de-coloured spots appeared on the purple background (DPPH radical scavenging activity) indicating the presence of phenolic compound. Retention factor (R_f) value of each spot was calculated.

Determination of total phenolic content (TPC)

TPC in HC extract and its extract fractions were determined using Folin-Ciocalteu reagent (FCR) based on the method described by Turkoglu *et al.* (2007) with slight modification. Briefly, 4 mL of FCR (Merck, Darmstadt, Germany) was added to 1 mL of sample and allowed to stand for 3 min. This was followed by the addition of 5 mL of 7.5% sodium carbonate (Fisher Scientific, Malaysia) to the mixture. The mixture was shaken vigorously and allowed to stand for 30 min. A sample blank was prepared by replacing the sample with deionised water. Absorbance of the reacting mixture was read

at 765 nm using a spectrophotometer (Secomam, Ales Gard, France). TPCs of the HC extract and its extract fractions were expressed as gallic acid equivalents (mg GAE/100 g) in triplicate using gallic acid (Acros Organics, USA) (0.2–25 µg/mL) with an equation of $y=0.0165x-0.0003$ ($R^2=0.997$).

High performance liquid chromatography (HPLC)

HPLC analysis was performed using a Chromolith RP-18e column, 100 × 4.6 mm ID, 5 µm particle size (Merck, Darmstadt, Germany). HPLC 1200 system (Agilent Technologies, CA, USA) was equipped with a quaternary pump solvent delivery system and a diode-array detector. Mobile phase used was deionised water and methanol (Merck, Darmstadt, Germany) at a ratio of 1:9. The mobile phase was filtered under vacuum using 0.45 µm pores nylon filter membrane. Solutions of the samples and standards were filtered using 0.45 µm syringe filters (Jet Biofil, Guangzhou, China) before injecting into the HPLC system. The injection volume and the extract concentration were 20 µL and 2 mg/mL, respectively. The column was eluted at a flow rate of 0.5 mL/min, with the elution being detected at 217 nm. The peaks detected on HPLC chromatogram were identified for phenolic compounds by comparing with the retention time and UV absorption spectra of phenolics standard (tannic acid). Tannic acid standard (Sigma-Aldrich, Malaysia) was used for the spiking test.

DPPH radical scavenging assay

DPPH radical scavenging capacities of HC extract and its extract fractions were determined based on the modified method of Sharma & Baht (2009). Briefly, 2 mL of sample (100 µg/mL) was added to 500 µl of ethanolic DPPH solution (Sigma-Aldrich, Malaysia) (final concentration of 0.2 mM). The mixture was vortexed using a vortex mixer (VTX-3000L, LMS, Tokyo, Japan) and placed in dark for 30 min at room temperature. Absorbance of the mixture was measured using a spectrophotometer at 517 nm against ethanol blank, with distilled water being used as a negative control. Ascorbic acid and butylated hydroxyanisole (BHA) (Sigma-Aldrich, Malaysia) were used for comparison. To calculate EC_{50} value of the sample (mg/mL), percentage of DPPH scavenging capacity was plotted against natural logarithm (Ln) of sample concentrations. EC_{50} is defined as the ability to reduce the initial DPPH concentration by 50%. The antioxidant capacities of HC extract and its extract fractions were calculated based on an equation as follows:

DPPH radical antioxidant capacity (%)

$$= \left(1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right) \times 100$$

ABTS radical inhibition capacity

The procedure used for ABTS assay was previously developed by Re *et al.* (1999) with some modifications. Briefly, 5 mL of 7 mM ABTS (Calbiochem, Germany) was reacted with 88 µL of 140 mM potassium persulphate (Mallinckrodt, Japan). The ABTS radical cation was generated by storing in dark for 16×18 h at room temperature. To obtain an absorbance of 0.70±0.05 that measured at 734 nm, 1 mL of the ABTS stock solution was subsequently diluted with 70 mL of ethanol. An aliquot of 100 µL sample was added with 1 mL of the ABTS reagent and vortexed. Absorbance of the mixture was measured at 734 nm against a blank after 6 min. Ascorbic acid and BHA were used for comparison. To obtain an EC_{50} value (mg/mL) for each of the sample, percentage of ABTS radical inhibition capacity was plotted against natural logarithm (Ln) of sample concentration. Inhibitions of HC extract and its extract fractions were determined based on the equation as follows:

ABTS radical antioxidant capacity (%)

$$= \left(1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right) \times 100$$

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed based on a modified method of Mohammadzadeh *et al.* (2007). TPTZ [2,4,6-tri(2 pyridyl)-s-triazine] (Fluka, Switzerland) at a concentration of 10 mM in 40 mM HCl (Thermo Fisher Scientific, Malaysia) was mixed with 20 mM iron (III) chloride anhydrous (Acros Organics, USA) and 0.3 M sodium acetate buffer (pH 3.6) at a ratio of 10:1:1 (v/v/v) for preparation of FRAP reagent. Sodium acetate was obtained from Merck (Malaysia). The freshly prepared FRAP reagent was incubated in a water bath at 37°C before addition of the sample. Briefly, 3 mL of the FRAP reagent was added with 100 μL of sample, and the mixture was vortexed and incubated for 4 min. Absorbance of the mixture was read against a blank at 593 nm using a spectrophotometer. Ascorbic acid and BHA were used for comparison. FRAP values of HC extract and its extract fractions were expressed as Trolox equivalent (mg TE/ 100 g). The calibration equation of Trolox at concentrations of 5–100 μg/mL was $y=0.0057x-0.0214$ ($R^2=0.998$).

Statistical analysis

Data are presented as mean ± standard deviation and analysed using SPSS statistical software Version 21.0 (SPSS Inc., Illinois, USA). All data were analysed based on one-way analysis of variance (ANOVA) and Bonferronis' test was used for post-hoc comparison. Differences between the means at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Total phenolic content and identification of potential phenolic compounds in HC mushroom

Total phenolic content (TPC) of HC extract and the solvent fractions (hexane-F1, chloroform-F2, butanol-F3, and formic acid-F4) are tabulated in Table 1. The results showed that HC extract had the highest TPC, followed by F3, F2, F4, and F1 fractions. Among the fractions, a high TPC in F3 had contributed to the high antioxidant capacity. F1 of the HC extract had the lowest TPC as it was partitioned using hexane. The low TPC in F1 also contributed to the lowest antioxidant capacity.

Table 1. Antioxidant capacities of HC extract and its fractions partitioned with different solvents

Sample	*DPPH*		*ABTS*		*FRAP (mg TE/100 g)*	*TPC (mg GAE/100 g)*
	% AC	*EC_{50}*	*% AC*	*EC_{50}*		
HC extract	19.12±1.81[c]	2.1	ND	ND	6.45±0.23[c]	2.65±0.04
F1	4.80±2.11[g]	ND	2.05±0.79[f]	ND	4.98±0.24[c]	0.26±0.01
F2	7.64±0.51[f]	ND	4.50±0.62[e]	ND	7.35±0.23[c]	1.93±0.07
F3	16.48±0.83[d]	2.56	14.96±0.35[c]	8.08	5.16±1.18[c]	2.27±0.12
F4	12.92±2.39[e]	ND	5.90±0.42[d]	ND	6.60±0.31[c]	0.54±0.07
Ascorbic acid	68.82±1.56[a]	0.02	74.51±0.63[b]	0.06	534.58±29.10[b]	-
BHA	61.57±2.16[b]	0.01	98.83±0.11[a]	0.004	794.58±18.20[a]	-

[a] Each value is presented as mean ± standard deviation (n = 3).
[b] EC_{50} (mg/mL): the effective concentration of the antioxidant capacity (AC) at which the DPPH/ABTS radicals were scavenged by 50%.
[c] EC_{50} values for some of the sample were not determined (ND) due to the sample not being able to give $\geq$50% of AC.
[d] Different superscript lower case letters within each column (a-f) denote significant differences ($p<0.05$).
[e] F1: hexane fraction; F2: chloroform fraction; F3: butanol fraction; F4: formic acid fraction; ND: not determined

TPC of the HC extract that was fractionated based on different polarities (FI-FIII) using open column chromato-graphy is shown in Table 2. The non-polar extract fraction (FI) had the lowest TPC (0.99 mg/ 100 g) while TPC in the polar extract fraction (FIII) was the highest (1.64 mg/100 g) among all the extract fractions. In this study, most of the phenolic compounds in HC mushroom were polar to semi-polar as the mushroom contained low fat level. Although the HC extract had TPC of 2.65 mg GAE in 100 g fresh mushroom, identification of potential phenolic compounds had to be performed using HPLC.

The presence of phenolic compounds in the HC extract fractions (FI-FIII) were also determined using TLC method. The extract fractions obtained from the open column chromatographic method were spotted on TLC plates, developed using the developing solvents, and examined under UV lamp at 254 nm and 365 nm. As shown in Table 3, the spots on TLC plate could be seen as blue, green, and yellow spots. One major spot was observed under UV light for each fraction, where the spot from FI was seen as a blue spot at both UV wavelengths. At a wavelength of 254 nm, the spots from FII and FIII were seen as yellow spots, while at 356 nm, the spots from both of the extract fractions were green spots. The TLC plates, sprayed with DPPH solution, confirmed the existence of reducing agents (phenolic compounds). Ribeiro *et al.* (2006) also reported the presence of phenolic

Table 2. Antioxidant capacities of HC extract and its extracts fraction based on different polarities

Sample	*DPPH*		*FRAP*	*TPC*
	% AC	*EC_{50} (mg/mL)*	*(mg TE/100 g)*	*(mg GAE/100 g)*
HC extract	19.12±1.81[d]	2.10	6.45±0.23[c]	2.65±0.04
FI	8.84±2.33[e]	10.3	2.51±0.52[d]	0.99±0.07
FII	5.40±3.26[f]	6.23	3.24±0.88[d]	1.26±0.05
FIII	6.54±3.11[ef]	9.30	4.02±0.30[d]	1.64±0.04
Ascorbic acid	68.82±1.56[b]	0.02	534.58±29.10[b]	-
BHA	61.57±2.16[c]	0.01	794.58±18.20[a]	-

Each value was presented as mean ± standard deviation (n=3). Different superscript lower case letters within each column (a-f) denote significant differences ($p<0.05$). FI: non-polar fraction; FII: semi-polar fraction; FIII: polar fraction.

Table 3. Thin layer chromatography analysis of HC extract fractions

Fraction	*Qualitative Analysis*				
	Distance travelled (mm)	*R_f*	*UV wavelength (nm)*	*Appearance colour*	*DPPH spray*
FI	75	0.68	254	Blue	+
	75	0.68	356	Blue	+
FII	81	0.74	254	Yellow	+
	81	0.74	356	Green	+
FIII	75	0.68	254	Yellow	+
	75	0.68	356	Green	+

[a] FI: non-polar fraction; FII: semi-polar fraction; FIII: polar fraction; +: Presence of radical scavenging agent.

compounds in the mushroom species studied.

Identification of phenolic compounds using TLC is not an accurate technique since TLC separation of phenolic compounds is ambiguous. The R_f values of the spots were 0.68 for FI and FIII while 0.74 for FII. Therefore, HPLC separation of phenolic compounds was performed. HLPC chromatogram of FI showed the presence of two major peaks, with peak 1 being identified as tannic acid. The retention time of tannic acid (peak 1) was 3.0±0.06 min while for peak 2, it was 12.1±0.03 min. Based on previous literature, peak 2 was tentatively identified as muscaflavin (Terradas & Wyler, 1991). Confirmation of this compound needs to be performed using a more advanced technique. For FI, the peak area of peak 2 was larger than the peak area of peak 1. As FI was fractionated from HC extract using hexane, the compounds obtained in the extract fraction were mostly semi-polar to non-polar compounds. Therefore, the peak area of peak 1 was smaller than the peak area of peak 2 as tannic acid (peak 1) is a more polar compound. Muscaflavin (peak 2), which was tentatively detected in the HC extract is a non-polar compound. A high amount of this compound detected is possibly due to the extract being fractioned using hexane. As the solvent polarity increases, the peak area of peak 2 decreases. The methanolic extract fraction of HC extract (FIII) had peak 2 as minor compound, but the peak areas of peak 2 for FI and FII were greater than FIII. Besides, another small peak formed about 1.5 min after peak 1 was tentatively identified as one of the secodopa compounds in wild mushrooms (Terradas & Wyler, 1991). This compound can be considered as a non-polar organic compound as it was only detected in the hexane fraction (FI). Muscaflavin and other secodopas derivatives could also serve as potential antioxidants and in the inhibition of oxidative stress.

Antioxidant capacity of HC extract and extract fractions

Antioxidant capacity of HC extract and its extract fractions were determined based on three electron transfer reaction assays. DPPH radical scavenging capacity, ABTS radical inhibition capacity, and FRAP values of HC extract and its solvent fractions (F1-F4) are shown in Table 1 while the antioxidant capacities of HC extract fractions obtained based on different polarity (FI-FIII) are tabulated in Table 2. EC_{50} values of the HC extract and extract fractions calculated from DPPH and ABTS assays are also shown in Table 1.

The results showed that HC extract had the highest DPPH radical scavenging capacity (19.12% antioxidant capacity, AC) compared to the solvent fractions (<16.5% AC). The percentages of AC among HC extracts and its solvent fractions were significantly different ($p<0.05$). Their percentages of AC were in the order of HC extract > F3 (butanol fraction) > F4 (formic acid fraction) > F2 (chloroform fraction) > F1 (hexane fraction). F1 had the lowest scavenging capacity, possibly due to the hexane functioning as an extraction solvent. The significant differences in AC among the samples show that different extraction solvents used are able to extract a wide range of phytochemicals in HC mushroom. On the other hand, applying open column chromatography in the fractionation of HC extract based on different solvent polarities, showed the hexane fraction (FI) to have the highest DPPH radical antioxidant capacity (8.84%) as compared to the other extract fractions, while FII (ethyl acetate fraction) had the lowest percentage of AC (5.4%). As compared to the hexane fraction (F1) that was partitioned based on liquid-liquid extraction, the percentage of AC for F1 (4.8%) was almost two times lower than the percentage of AC for FI.

As most of the phytochemicals in HC mushroom are water soluble (due to low fat

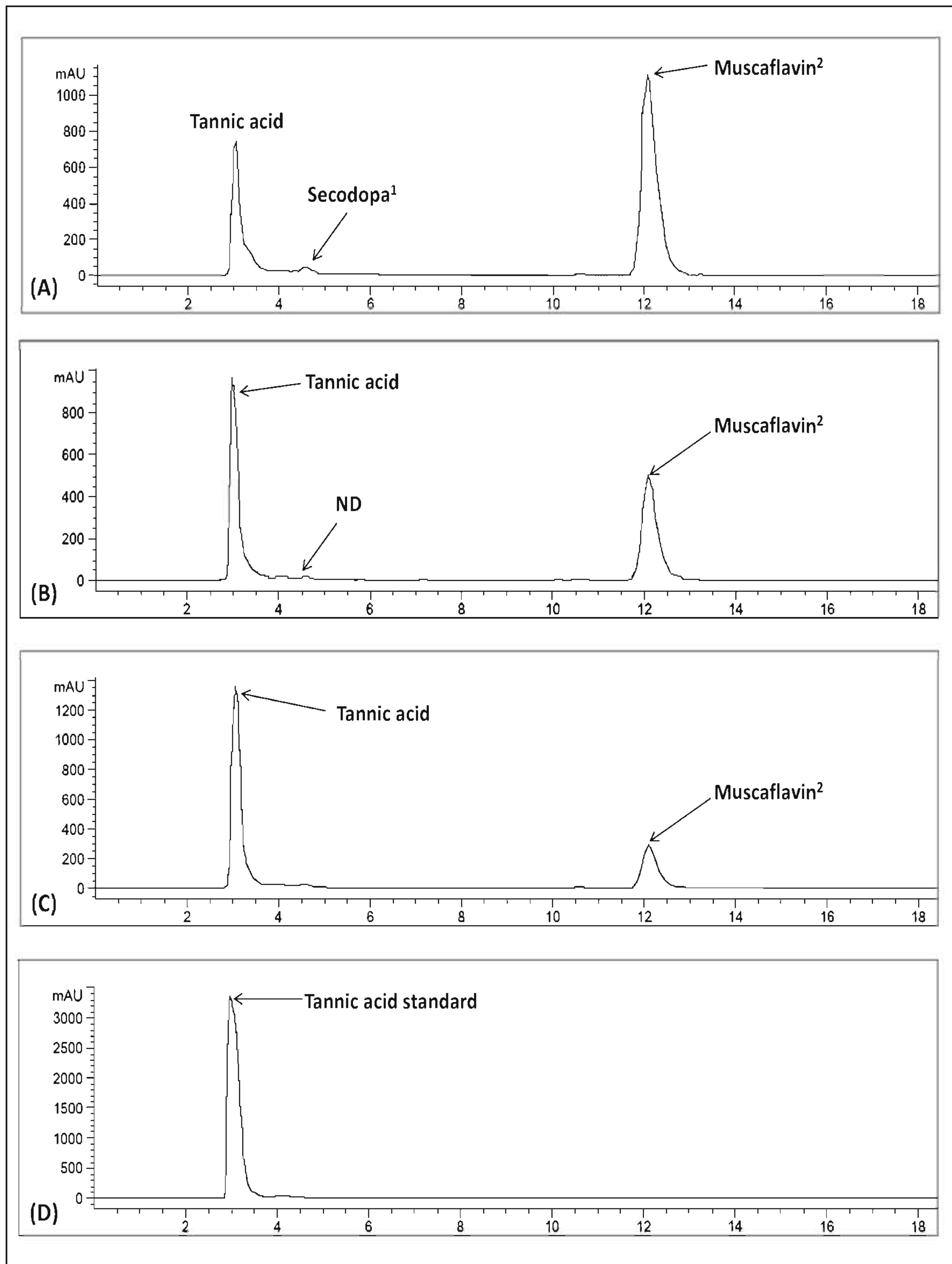

Figure 1. HPLC chromatograms of HC extract fractions (A) FI, (B) FII, (C) FIII, and (D) tannic acid standard analysed at 217 nm.

[a] Superscript letters ([1,2]) denote the compounds were tentatively identified based on the HPLC results obtained from HC mushroom naturally grown in a meadow in Switzerland.

[b] ND: not detected; tannic acid (peak 1); muscaflavin (peak 2).

content), hexane fraction of the extract should contain a small amount of antioxidants. F3 (butanol fraction) had higher AC% maybe due to tannic acid being a major compound in most of the mushrooms and a small amount of other potential antioxidants such as secodopa (Terradas & Wyler, 1991) which is less polar (Stintzing & Schliemann, 2007). These compounds are highly soluble in alcohol. Therefore, the finding of this study indicates that HC mushroom contained mostly semi-polar phenolic compounds. The HC extract and its extract fractions had significantly lower ($p<0.05$) percentages of DPPH radical antioxidant capacity than the antioxidant standards (ascorbic acid and BHA). EC_{50} values of HC extract and its solvent fractions were higher than 1 mg/mL while EC_{50} of the antioxidant standards were less than 0.02 mg/mL. Some of the solvent fractions were not able to scavenge up to 50% of DPPH radical. Similarly, EC_{50} values of the extract fractions obtained based on different solvent polarities were higher than 1 mg/mL. This indicates that the bioactive compounds in HC extract and its extract fractions are not strong scavenging agents as the scavenging ability of the HC extract was about 20% compared to similar concentrations of ascorbic acid, which had >60% of scavenging ability.

A similar trend was observed for ABTS radical antioxidant capacity, where the EC_{50} values increased in the order of F3 < F4 < F2 < F1. In this study, the percentages of ABTS radical antioxidant capacity for HC extract and its extract fractions that were obtained based on different solvent polarities (FI×FIII) were not determined. The phytochemicals in HC extract had lesser ability to reduce ABTS radical than DPPH radical. However, antioxidant standards such as ascorbic acid and BHA have higher ability in scavenging ABTS radicals. The results showed that BHA possessed the strongest AC (98.8%) in reducing ABTS radical compared to ascorbic acid (74.5%). Similarly, EC_{50} values (ABTS assay) of HC extract and its extract fractions were higher than 1 mg/mL while the EC_{50} values of the antioxidant standards were lower. BHA had the lowest EC_{50} value (0.004 mg/mL) as compared to ascorbic acid (0.06 mg/mL). The possible explanation is that the hydroxyfuran structure of ascorbic acid does not donate an electron to the ABTS radical, but the hydroxyphenol structure of BHA can easily donate an electron to the ABTS radical. Therefore, BHA is a strong reducing agent. It is best to scavenge ABTS radical compared with DPPH radical. DPPH molecule has three benzene rings while ABTS molecule has two benzene rings. The structure of DPPH allows phenolic acid, or ascorbic acid to react better with the DPPH radical than larger compounds such as BHA or tannic acid (a major compound in mushroom).

The results obtained from FRAP assay showed that the TE values were not significantly different ($p \geq 0.05$) among the HC extract and its solvent fractions (Table 1). F2 showed a higher TE value than the HC extract. As shown in Table 2, the TE values of FI-FIII were generally lower than the TE values of F1-F4. Based on different solvent polarities, hexane fraction (FI) had the lowest TE value (2.51 mg TE/100 g), followed by ethyl acetate fraction (FII) and methanolic fraction (FIII), which were 3.24 and 4.02 mg TE/100 g, respectively. As the fractionation had been done using open column chromatography, the TE values of the extract fractions were lower than the extract fractions obtained from liquid-liquid partitioning. Therefore, fractionation of the extract using open column chromatography had caused a loss of phenolic compounds in the extract fraction. Based on the same concentrations of ascorbic acid and BHA (100 μg/mL), the two antioxidant standards had TE values about 100 times higher than the samples. It appears that synthetic antioxidants have extremely high reducing ability as compared to crude extract. The antioxidant standards also strongly reduced

the Fe^{3+} of TPTZ to Fe^{2+}. One of the possible explanations is the crude extract of HC mushroom containing a lower amount of phenolic compounds. Crude extract typically contains a mixture of matrices, including protein, carbohydrate, fat, and fibre. Most of these matrices are weak reducing agents. Fractionation using open column chromatography removed most of these matrices. Therefore, the crude extract showed lower TE values compared to some of its extract fractions.

Generally, crude plant extract contains a mixture of phytochemicals, sugars, and peptides. Sugar (Cao *et al.*, 2003) and amino acid (Tan, Lee & Wang, 2010) are weaker reducing agents than phenolic compounds. Partitioning of HC extract using different solvents has also caused a loss of a small amount of sugar and peptide in the fractions. Therefore, the HC extract has a TE value lower than the F2 and F4. Although there is a variation in antioxidant capacity for the different antioxidant assays studied, the use of these different antioxidant assays might be measuring different types of antioxidants present in different mushroom samples. Therefore, it is essential to perform more than one antioxidant assay in which these assays take into account the various mechanisms of action for the different reagents used (Huang, Ou & Prior, 2005). It is clear that different antioxidant assays demonstrate different antioxidant activities based on different mechanisms (Sah *et al.*, 2012).

CONCLUSION

The presence of tannic acid as one of the phenolic compounds in HC mushroom shows its potential as a functional food. The water soluble tannic acid as well as other semi-polar to non-polar antioxidants including secodopas, found in the HC extract, contribute to antioxidant capacity. Although the HC extract did not show a high antioxidant capacity, the mushroom is still a good source of natural antioxidants as it contains a moderate level of total phenolics, especially tannic acid and possibly other semi-polar phenolic compounds. It is suggested that the nutraceutical potential of tannic acid and secodopas in the HC extracts be further explored.

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