

CUPRAC assay-guided profiling of antioxidant compounds in methanol extract of *Lentinus squarrosulus* Mont. mycelium

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ABSTRACT

A cupric reducing antioxidant capacity (CUPRAC)-guided purification approach was performed on a methanol extract of *Lentinus squarrosulus* (LsqMeOH) by using reversed phase-high performance liquid chromatography. Using reversed phase-high performance liquid chromatography, three fractions were separated arbitrarily named FR1, FR2 and FR3. Results showed that FR2 exhibited the highest antioxidant activity in CUPRAC assay (A_{450} , 0.86) but not significantly different from LsqMeOH (A_{450} , 0.84). FR1 and FR3 showed much lower absorbance, with values (A_{450} , 0.21) and (A_{450} , 0.36) respectively at 1 mg ml⁻¹. The most active fraction (F3) was further subjected to LC-MS/MS to obtain its detailed chemical profile. Uridine, ganoderic acid derivative, and flavonoids were the first time being found in *L. squarrosulus* antioxidative fractions. The present results indicate that the fraction extracts of *L. squarrosulus* possess antioxidant properties and can be used as free radical inhibitors. Therefore, this research suggested the potentials of *L. squarrosulus* as a source of antioxidant extract to be used in food industries (functional food).

Key words: medicinal mushroom; active compounds; *Lentinus squarrosulus*

IZVLEČEK

CUPRAC TEST ZA LOČITEV ANTIOKSIDACIJSKIH SPOJIN V METANOLNEM IZVLČEKU MICELIJA GLIVE *Lentinus squarrosulus* Mont.

Izveden je bil CUPRAC ločitveni postopek metanolnega izvlečka micelija glive *Lentinus squarrosulus* (LsqMeOH) z uporabo vzratne visoko zmogljive tekočinske kromatografije, kar je dalo tri frakcije, poimenovane FR1, FR2 in FR3. Rezultati so pokazali, da je največjo antioksidacijsko aktivnost v CUPRAC preiskusu pokazala frakcija FR2 (A_{450} , 0.86), vendar ne značilno različno od LsqMeOH (A_{450} , 0.84). Frakciji FR1 in FR3 sta imeli veliko manjšo absorbanco z vrednostmi A_{450} , 0.21 in A_{450} , 0.36 pri 1 mg ml⁻¹. Antioksidacijsko najaktivnejša frakcija (F3) je bila v nadaljevanju analizirana z LC-MS/MS za pridobitev podrobnejše kemijske sestave. Uridin, derivat ganodermične kisline in flavonoidi so bili prvič najdeni v antioksidacijskih izvlečkih glive *L. squarrosulus*. Pridobljeni rezultati kažejo, da imajo frakcijski izvlečki glive *L. squarrosulus* antioksidacijske lastnosti in, da bi lahko bili uporabljeni kot lovilci prostih radikalov. Raziskava nakazuje, da je lahko gliva *L. squarrosulus* vir za antioksidacijske izvlečke za uporabo v prehranski industriji pri pripravi funkcionalne hrane.

Ključne besede: medicinske gobe; aktivne sestavine; *Lentinus squarrosulus*

1 INTRODUCTION

Oxidative stress is involved in the pathogenesis of lifestyle-related diseases, including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and malignancies (Yoshikawa and Naito, 2002). Reiter reported the increased oxidative damage observed

during aging is probably due to the deficiency of antioxidants (Reiter, 1995). Even though enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase and catalase or nonenzymatic antioxidants such as vitamin E (α -tocopherol), vitamin

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C (ascorbic acid), thiol antioxidants (glutathione), carotenoids and flavonoid compounds are useful for protection against free-radical damage they are still insufficient to prevent damage entirely (Rahman, 2007). Therefore, external sources of antioxidants from the diet are needed to compensate this unbalance situation. In most countries, strict governmental rules regarding the use of synthetic antioxidants has been imposed due to safety concerns associated with the toxicity studies (Wanasundara and Shahidi, 2005). Thus, exploring for natural antioxidant is necessary.

From previous studies, it is apparent that mushrooms have great potential as a source of novel compounds (Deiana et al., 2004; Lee and Yun, 2006; Chen et al., 2012). For instance, ergothionine was isolated from *Pleurotus citrinopileatus* Singer, *P. ostreatus* (Jacq. ex Fr.) P.Kumm and *Pleurotus salmoneostramineus* L.Vass., lovastatin from *Pleurotus ostreatus* and *Agaricus bisporus* (J.E.Lange) Imbach (Chen et al., 2012) and schizophyllan from *Schizophyllum commune* Fries (Bae et al., 2004). Some of these compounds have been developed as drugs as well as an ingredient in nutraceuticals. Mycelia of mushrooms serve as a convenient alternative to fruiting bodies due to the shorter incubation time, uniform and ease for higher production of biomass (Lee et al., 2004; Halpern, 2007). Moreover, the mycelial biomass can be produced in large quantities by using submerged fermentation technique.

Lentinus squarrosulus Mont. was amongst the commonly encountered Polyporales in Peninsular

Malaysia (Bolhassan et al., 2012). The nutrient content of *L. squarrosulus* mycelia has been analyzed. The authors (Omar et al., 2011) reported that the mycelia of *L. squarrosulus* is high on protein (57.6 %) and low on total fat (0.5 %) content, are rich in magnesium (0.4 %), potassium (3.8 %) and vitamin B₃ (0.2 %). Furthermore, they also investigated on anti-ulcerogenic activity of *L. squarrosulus* mycelia extract and proven that this extract was able to prevent and heal ulcer that is associated with the attenuation of proinflammatory cytokines IL-1 β and the inhibition of NF- κ B in ulcerated rats.

The cupric reducing antioxidant capacity (CUPRAC) is based on utilizing the copper (II)-neocuproine reagent as the chromogenic oxidant. This assay is based on the redox reaction with antioxidants producing the cuprous-neocuproine chelate (Cu(I)-Nc) stable complex showing maximum light absorption wavelength at 450 nm (Apak et al., 2004). As yet, no research has been established on the purification and identification of antioxidant compounds from *L. squarrosulus* mycelium grown by liquid fermentation. Hence, the objective of this study is to carry out CUPRAC-assay fractionation of antioxidant compounds from methanol extract of *L. squarrosulus* (LsqMeOH) mycelium using reversed-phase high-performance liquid chromatography (RP-HPLC). Confirmatory analysis using liquid chromatography (LC) combined with tandem (MS/MS) mass spectrometry approach is used to identify antioxidant compounds.

2 MATERIALS AND METHODS

2.1 Sample preparation

Mycelial culture of *L. squarrosulus* (KUM 50016) was obtained from Mycology Laboratory, Institute of Biological Sciences, University of Malaya and maintained on Glucose-Yeast-Malt-Peptone (GYMP) agar consisting of MgSO₄·7H₂O (0.40 g l⁻¹), KH₂PO₄, (0.40 g l⁻¹) K₂HPO₄, (0.40 g l⁻¹) glucose (4.00 g l⁻¹), peptone, (2.00 g l⁻¹) malt-extract (2.00 g l⁻¹), agar (7.20 g l⁻¹). Seven days old *L. squarrosulus* mycelium grown on GYMP agar media at 25 ± 2 °C was used as inoculum. Ten 7 mm diameter plugs of *L. squarrosulus* mycelium were inoculated into the 500 ml Erlenmeyer flasks containing 100 ml GYMP liquid medium and then incubated for 14 days at 25 ± 2 °C under static conditions. After 14 days, mycelial biomass obtained were harvested and freeze-dried before proceeded with extraction process. Dried mycelial biomass of *L. squarrosulus* was extracted by soaking in methanol for two days at room temperature. The filtrate was

concentrated under reduced pressure using a rotary evaporator (BüchiRotavapor R-114, Flawil, Switzerland) under 45 °C.

2.2 Fractionation of *Lentinus squarrosulus* methanol extract by RP-HPLC

Distilled water was pumped to remove air bubbles before use. All eluents except water were passed through a 0.2 μ m cellulose nitrate membrane (Whatman No. 1, Maidstone, England) filtration apparatus immediately before use. Distilled water was passed through a 0.45 μ m cellulose membrane filtration. The methanol extract of *L. squarrosulus* was applied onto an analytical reverse phase (250 mm × 4.6 mm, 5 μ m) C18 ODS Hypersil column (Thermo Fisher Scientific, MA, USA) and analyzed by LC UV/VIS photodiode array detector SPD-M10 AVP (Shimadzu, Kyoto, Japan). The mobile phase consisted of 100 % distilled water (solvent A) and 100 % acetonitrile (Merck, Darmstadt,

Germany) (solvent B) at a flow rate of 3.5 ml.min⁻¹. The gradient system started at 0.01 min (40 % B), 3.00 min (60 % B), 4.00 min (80 % B), 7.00 min (10 % B) and stopped at 8.00 min. These various eluted fractions were collected and each fraction was assayed for CUPRAC.

2.3 Cupric ion reducing antioxidant capacity (CUPRAC)

The Cupric ion reducing antioxidant capacity (CUPRAC) was determined according to the method of Apak et al. (2004) based on the principle of utilizing copper (II)-neocuproine reagent as the chromogenic oxidizing agent. In this assay, 1 ml of extracts were mixed with 1 ml of Copper (II), 1 ml of ammonium acetate (NH₄Ac) buffer solution (1 mM, pH 7.0) and 1 ml of neocuproine (7.5 mM). The reaction mixture (total of 4 ml) then was mixed thoroughly and allowed to stand for 30 minutes before the absorbance was measured at 450 nm against a reagent blank. The assay was carried out in triplicate and the results expressed as means values ± standard deviations.

2.4 Profiling of chemical constituent by LC-MS/MS

The constituents of the most active fraction were analyzed by HPLC tandem mass spectrometry (LC-MS/MS). This procedure was done on an AB Sciex 3200QTrap® LC-MS/MS (Applied Biosystems, Toronto, Canada) triple quadrupole mass spectrometer equipped with a Turbospray Ionization source. HPLC separation was carried out on a Phenomenex Aqua C18 column (5 µm, 50 mm × 2.0 mm i.d.; Phenomenex, Torrance, California, US). A mobile phase gradient of

water with 0.1 % formic acid, 5 mM ammonium formate as eluent A and acetonitrile with 0.1 % formic acid and 5 mM ammonium formate as eluent B. Samples were diluted with 1.0 ml methanol and then filtered through 0.22 µm nylon filter. Injection volume was 20 µl. A gradient program was used as follows: 10 % A to 90 % B from 0.01 min to 8.00 min, held for 3.00 min and back to 10 % A in 0.10 min and re-equilibrated for 4.00 min, the flow rate was changing between 250 ml min⁻¹ to 400 ml min⁻¹. The negative ion mode for MS/MS analysis was selected. Data analysis was carried out by using AB Sciex Analyst software and Advanced Chemistry Development, Inc (ACD/Labs, Toronto, Canada) software. Identification of constituents present in FR2 was performed by comparing the retention time and MS spectra of the peaks in the samples with those authentic reference samples or data reported in the literature.

2.5 Statistical analysis

For each antioxidant fraction (FR1, FR2 and FR3), assays were carried out in triplicates. The results were expressed as mean values and standard deviation (SD) or standard errors (SE). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test. Comparisons were carried out to detect significant difference ($p < 0.05$) between the mean values that had more than two groups. This treatment was carried out using SPSS v.16.0 software. The ANOVA results were classified using letters (different letters mean significant differences among results).

3 RESULTS AND DISCUSSION

3.1 CUPRAC analysis of RP-HPLC isolates

Separation by RP-HPLC resulted in three fractions collected at retention times of 2.8, 4.9 and 6.5 min. The profile obtained at the detection wavelength of 254 nm. The gradient system started at 0.01 min (40 % B), 3.00 min (60 % B), 4.00 min (80 % B), 7.00 min (10 % B) and stopped at 8.00 min. Figure 1 shows the absorbance values of fraction 1 (FR1), fraction 2 (FR2), fraction 3 (FR3) and the crude extract (LsqMeOH). CUPRAC activity revealed FR1 and FR3 having lower absorbance than FR2 and LsqMeOH. From the result obtained, FR2

exhibited the highest absorbance of (A_{450} , 0.86) but not significantly different ($p < 0.05$) from LsqMeOH with an absorbance of (A_{450} , 0.84). FR1 and FR3 showed much lower absorbance, with values (A_{450} , 0.21) and (A_{450} , 0.36) respectively. The antioxidant capacities showed that the LsqMeOH (crude) was higher than its active fraction (FR1 and FR3) and comparable high to FR2. FR2 was subjected to LC-MS/MS analyses to profile the compounds present. Table 1 shows the masses of peaks and ion fragment productions detected by the LC-MS/MS analysis of this extracts.

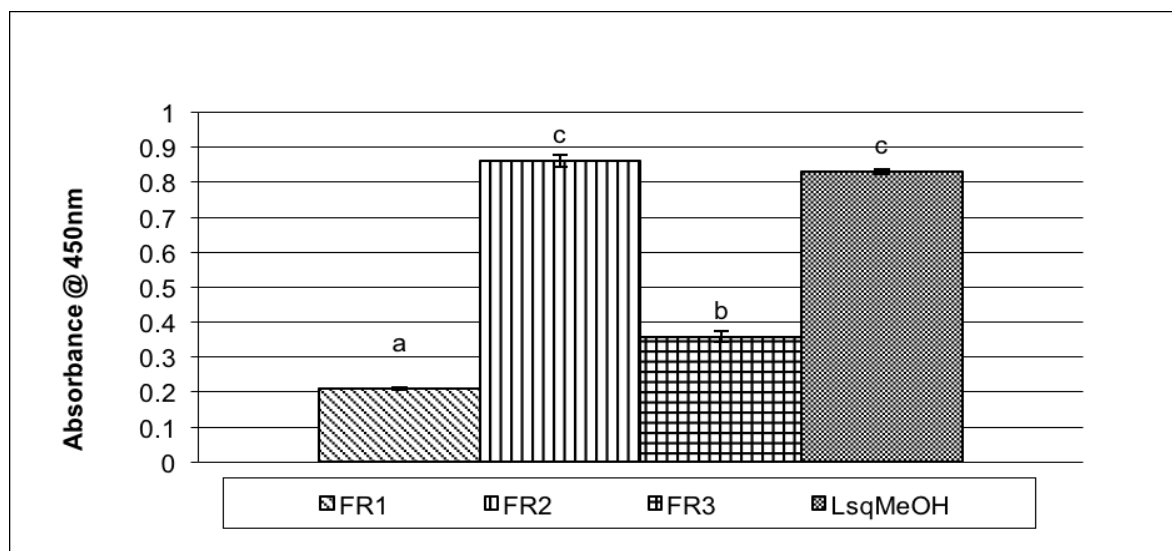


Figure 1: Reducing power of fraction RP-HPLC (FR1 to FR3) and LsqMeOH extracts on cupric ion. Concentration tested at 1mg ml^{-1} . Each value is expressed as mean \pm standard deviation ($n = 3$). Average values with different letters are significant at $p < 0.05$

3.2 Profiling of selected potent antioxidative extract constituent by LC-MS/MS

From the LC-MS/MS data obtained (Table 1), Peak 1 was identified as uridine with mass fragments at m/z (mass to charge ratio) 225, 200 and 140. From the fragmentation scheme (Figure 2), uridine produces m/z ion at 200 with the loss of $-C_2H_3O$ (-43) due to hemolytic cleavage at the side chain. Further fragment ions unique to uridine were also observed at m/z 225 formed by a loss of a H_2O molecule and at m/z 140 due to further hydrogen rearrangements and hemolytic

cleavage. Uridine is known to be one of the four basic components of ribonucleic acid (RNA) and it plays a role in the glycolysis pathway of galactose (Berg et al., 2002). Nucleosides including adenosine, cytidine, uridine, guanosine, inosine and thymidine had been isolated from *Cordyceps* (Li et al., 2006). A study by Yu et al. (2006) showed that uridine has antioxidant activities. They reported that uridine from *Cordyceps sinensis* (Berk.) Sacc. and *Cordyceps militaris* (L.) Fr. extracts have Trolox Equivalent Antioxidant Capacity value of $2.0 \pm 0.9 \mu\text{g/ml}$.

Table 1: Masses of peaks and ion fragments productions detected by LC-MS/MS of FR2

Peak no.	Rt (min)	MW	MS, M/Z [M-H]-	Fragment	Detected compound
1	0.80	244	243	225, 200, 140	Uridine
2	1.28	228	227	183, 181, 155, 140, 127, 82	Phenolic acid
3	1.93	299	298	254, 236, 223, 193, 179, 167	Phenolic acid
4	2.25	242	241	197, 168, 141, 130, 82	Phenolic acid
5	3.21	276	275	231, 147, 127, 109	Unknown
6	3.69	498	497	451, 433	Ganoderic acid derivative
7	4.17	725	724	724, 678	Unknown
8	8.50	312	311	293, 225, 183	Flavonoids
9	8.82	326	325	225, 197, 183	Flavonoids
10	9.14	340	339	293, 197, 183	Flavonoids
11	9.62	874	873	647, 607	Unknown

Peaks 2, 3, and 4 had masses corresponding to phenolic acid group of compounds. This is due to the fact that all these peaks have a common loss of carbon dioxide molecule ($-\text{CO}_2$, - 44), which is characteristic of carboxylic-based compounds. Phenolic acid received considerable attention in the literature, specifically because of their biological and physiological importance. In a study of phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species, Barros et al. (2009) reported that various phenolic acids such as protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids, and two vanillic acid isomers were detected. *p*-Hydroxybenzoic acid exhibited in the majority of the samples tested and also being the most abundant compound in *Agaricus silvicola* (Vittad.) Peck (238.7 mg kg⁻¹). In the same study, *Ramaria botrytis* (Pers.) Ricken showed the highest phenolic acids concentrations (356.7 mg.kg⁻¹) due to the significant contribution of protocatechuic acid (342.7 mg kg⁻¹).

A type of ganoderic acid derivative was also determined from the FR2 extract. This finding contradicts Xu et al. (2010) who claimed that *Ganoderma* spp. are the only known producer for ganoderic acid. Peak number 6 (Table 1) had a mass corresponding to ganoderic acid [M-H]⁻ ion at *m/z* 497 produced a fragment ion with *m/z* 451 and 433. When applied to a collision energy spread

of 35eV (+/-15), the ganoderic acid compound produces ion *m/z* 451 formed by sequential loss of a water molecule ($-\text{H}_2\text{O}$, -18), followed by carbon dioxide ($-\text{CO}_2$, -44) followed by re-arrangement process at the side chain, as shown in the schematic diagram below (Figure 3). A further loss of an H₂O molecule formed the next fragment ion at *m/z* 433. Ganoderic acid was proven to exhibit significant pharmacological activities by means of anti-histamine, anti-hypercholesterolemic, antibacterial and capacity to scavenge free radicals (Kohda et al., 1985; Komoda et al., 1989; Zhu et al., 1999; Wang and Liu, 2008). Other research has implicated that ganoderic acid DM isolated from *Ganoderma lucidum* (Curtis) P. Karst. inhibits cell proliferation and colony formation in MCF7, human breast cancer cells (Wu et al., 2012). Triterpene, a type of ganoderic acid, from *G. lucidum* was antiviral, specifically towards HIV (El-Mekkawy et al., 1998). Terpene and polysaccharide fractions were obtained from *G. lucidum* in a study on antioxidative activity by bioassay guided isolation (Zhu et al., 1999). Results showed that terpene fraction possesses the highest activity at 40 µg ml⁻¹ against iron (II)-ascorbic acid induced lipid peroxidation, chemical isolation of the terpene fraction resulted in the detection of ganoderic acid A, B, C and D, lucidenic acid B and ganodermantriol.

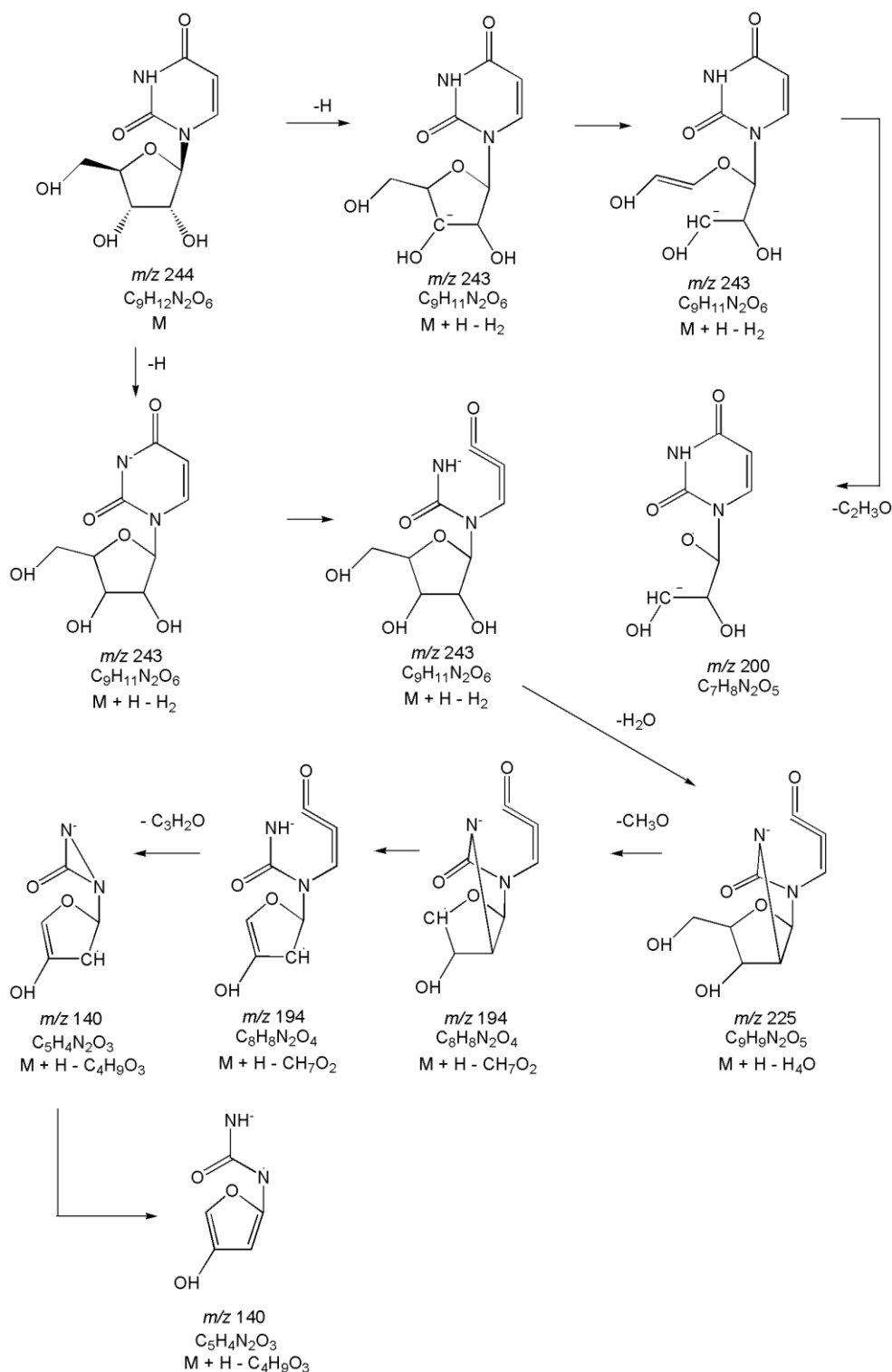


Figure 2: Uridine fragmentation pathway scheme

Peaks 8, 9 and 10 for FR2 (Table 1) have masses corresponding to flavonoid compounds. For peak 8 with m/z parent mass of 311 (M), the fragment ion observed was 293, which correspond to a loss of H₂O and other fragment ions were m/z 225 and 183. The fragment ion

at m/z 225 was most likely the loss of M-H-C₂H₂O-CO₂, and further loss of C₂H₂O that corresponds to the ion m/z 183. This fragmentation pathway usually corresponds to compounds from the class flavonoids, probably for apigenin, as reported by Wu et al. (2004)

which showed such a fragmentation pathway. Peaks 9 and 10, which correspond to m/z 325 and 339 respectively, also shared similar fingerprint fragment ions (Table 1). Both the masses differ by a mass of 14 sequentially. This showed most likely to be carbon and hydrogen rearrangements of CH_2 to form both compounds that are derivatives from compound Peak 8 (Table 1). Flavonoids can be subdivided into six main subclasses namely flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins. Other flavonoid groups, which quantitatively are in comparison, minor components of the diet, are dihydroflavonols, flavan-3, 4-diols, coumarins, chalcones, dihydrochalcones and auronones (Crozier et al., 2006). Methanol extracts from cultivated fruiting bodies of endophytic fungus *Xylaria* sp. YX-28, are rich in phenolics and flavonoids with 54.51 mg GAE g^{-1} dry

mass and 86.76 mg RE g^{-1} dry mass respectively (Liu et al., 2007). The total phenols and among them flavonoids were the major components found in the mushroom extract of *Lactarius deliciosus* (L.) Gray, *Sarcodon imbricatus* (L.) P. Karst., and *Tricholoma portentosum* (Fr.) Quèl (Barros et al., 2007). Flavonoids have been considered as important antioxidants for health and were reported to play an important role in the prevention of lipid peroxidation and cardiovascular disease (Peng and Kuo, 2003; Ding et al., 2006). Several unknown compounds were also detected in this study. Three unknown compounds for FR2 that were represented by peak numbers 5, 7 and 11 (Table 1). A match for the mass spectrum of unknown compounds could not be found from both the database and literature.

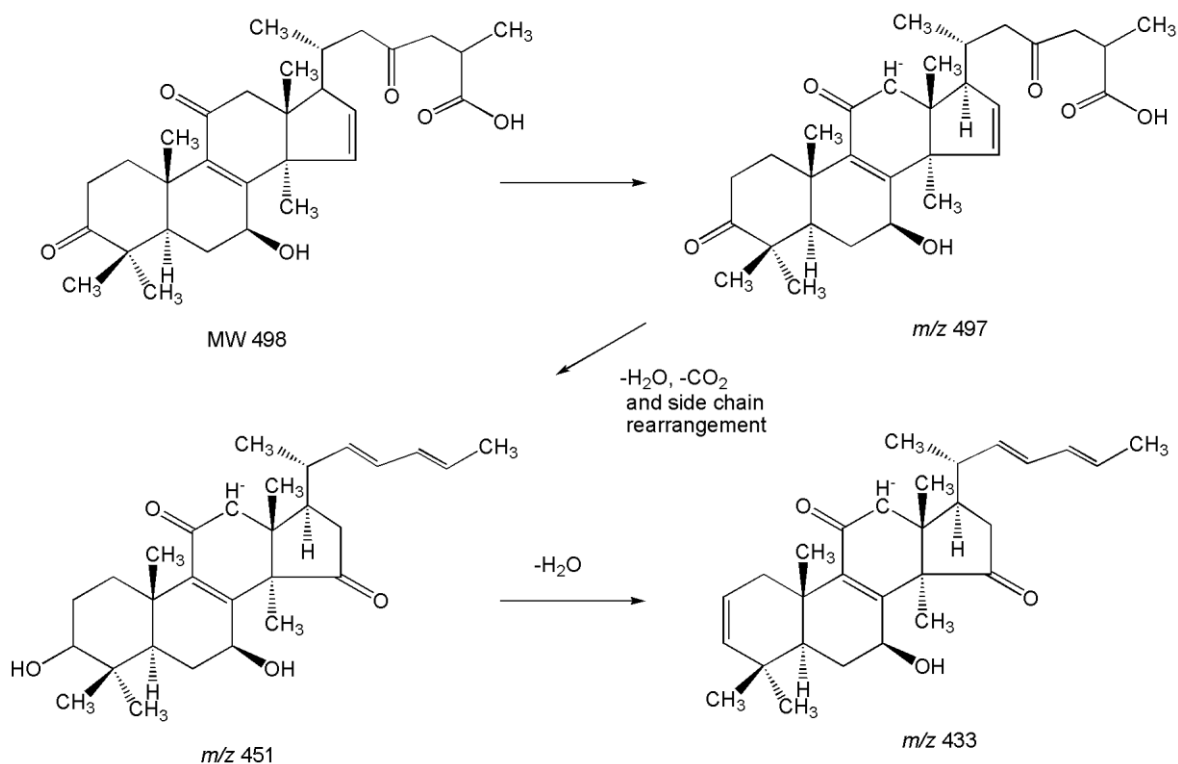


Figure 3: Ganoderic acid derivative fragmentation pathway scheme

4 CONCLUSIONS

Compounds contributing to antioxidant capacities from *Lentinus squarrosulus* Mont. are varied and between them in the complex system such as food sources and foodstuff there exist interactions and synergistic effect, therefore rather than a single compound it is preferred to use them as a whole. Chang and Buswell (2003) reported that the combination of several individual components contributed to the overall medicinal effect

of the mushroom-based nutraceutical products. In this study, the potent antioxidant fraction from *L. squarrosulus* has been standardized by HPLC to contain phenolic-based compound, ganoderic acid derivative, uridine and flavonoids. The use of this extract as nutraceutical ingredient must conform to this standardized component to ensure quality and potency. Furthermore, *L. squarrosulus* mycelial antioxidant

extract is non toxic based on our previous study in experimental rats. It contains absorbable antioxidants that enter the circulating plasma and cause a significant acute increase in plasma antioxidant capacity (Omar et

al., 2015). Therefore, this study validates the potential use of *L. squarrosulus* Mont. as an alternative source of natural antioxidant for nutraceuticals and pharmaceuticals industries.

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