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Antioxidant Activity of Crude Polysaccharides from Edible Fresh and Dry Mushroom Fruiting Bodies of *Lentinus* sp. Strain RJ-2

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Abstract: Crude polysaccharides of mature fresh (FB) and dried fruiting bodies (DB) of the edible mushroom, *Lentinus* sp. strain RJ-2 were evaluated for their antioxidant properties. The crude polysaccharides yields in FB and DB were 115.84 and 93.66 mg g^{-1} dry weight mushroom, respectively. Trolox equivalent values in scavenging abilities of both crude polysaccharides against both 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals as well as reducing properties were in similar ranges. The crude polysaccharides contained protein, reducing sugar and phenol compounds. The results from Fourier transform infrared (FTIR) spectroscopy and Thin Layer Chromatography (TLC) suggested that the crude polysaccharide contained a monosaccharide with six carbon atoms in a pyranose ring and mannose are proposed to be the majority. This study suggested that the crude polysaccharides from *Lentinus* sp. mushrooms could potentially be used as natural antioxidants.

Key words: Antioxidant, crude polysaccharide, *Lentinus* sp., radical scavenging activity, reducing power

INTRODUCTION

Free radicals are classified as highly reactive molecules having one or more unpaired electrons, which are able to damage numerous biological substances, including DNA, protein and lipid membranes (Wu and Hansen, 2008; Tsai *et al*., 2007) leading to various diseases and disorders, such as cancer, cardiovascular diseases, impaired immune system, atherosclerosis and aging (Xu *et al*., 2009; Wu and Hansen, 2008). Molecules that are able to scavenge free radicals and provide protection against diseases caused by them are called antioxidants. Several synthetic antioxidants are being used currently in the preservation of foods, i.e., butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylated hydroxyquinone (TBHQ), but they could promote tumor formation (Cheung *et al*., 2003). Naturally occurring antioxidant substances from fruits and vegetables have been well known, which included: ascorbic acid, tocopherol, β-carotene, flavonoids and other phenol compounds (Wu and Hansen, 2008; Xu *et al*., 2009).

Polysaccharides exhibit varied bio-activities such as antitumor, anticancer, antiviral, antibacterial, antifungal, anticoagulant and immunological activities (Xu *et al*., 2009; Shanmugam *et al*., 2008). They are widely found in all domains of organisms including animals, plants and microorganisms as well as mushrooms. Apart from being a delicious and nutritious food component, the medicinal properties are a continuous issue. Most commonly studied mushroom species are Ganodermataceae, *Ganoderma* sp. such as Reishi, *G. lucidum* (Eo *et al*., 1999; Jia *et al*., 2009; YouGuo *et al*., 2009; Liu *et al*., 2010), *Ganoderma atrum* (Chen *et al*., 2008), *G. tsugae* (Mau *et al*., 2005; Tseng *et al*., 2008) as well as Shiitake Lentinula edodes (Maeda and Chihara, 1973; Surenjav *et al*., 2006; Wu and Hansen, 2008; Yu *et al*., 2009; Shen *et al*., 2009). To date bioactive polysaccharides have been reported from several other mushroom species, for example, *Grifola frondosa* (Lee *et al*., 2003), *Phellinus linteus* (Hwang *et al*., 2003), *Pleurotus tuber-regium* (Zhang *et al*., 2004, 2006), *Coriolus versicolor*, *Inonotus obliquus* (Kim *et al*., 2006), *Cordyceps militaris* (Yu *et al*., 2007; Dong and Yao, 2008), *P. ostreatus* (Tong *et al*., 2009) and *Russula virescens* (Sun *et al*., 2010). Polysaccharides from certain mushroom species have now become commercial pharmaceutical products, such as, Lentinan from *L. edodes* and *G. lucidum* polysaccharide.

Apart from well studied *L. edodes*, rarely has any other species in this genus been reported, unlike the genus Ganoderma in which several species have been the subjects of studies. One edible mushroom, *Leninus* sp., is commonly consumed in the Northeast and North of

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Thailand. The mushroom is cultivatable with high potential to be a good mushroom product for the market. However, there has not been any scientific research about the many beneficial aspects on this mushroom including polysaccharide antioxidants. Therefore, in the present study, we report the antioxidant activity of crude polysaccharides from hot water extraction of *Lentinus* sp. strain RJ-2 and a study of certain properties, including total phenol content, amount of protein, total carbohydrate content and reducing sugar as well as proposed monosaccharide composition.

MATERIALS AND METHODS

Chemicals and apparatus: Absolute ethanol, ferric chlorine hexahydrate, ferrous sulfate heptahydrate and sodium chloride were purchased from Merck (Germany). Methanol, Sodium Dodecyl Sulphate (SDS) and sulfuric acid were from BDH (England). Bromophenol blue, Folin-Ciocalteu's reagent, phenol crystal and sodium carbonate anhydrous were obtained from Carlo erba (France). 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2,2-Diphenyl-1-picryhydrazyl (DPPH) were products of Sigma (Germany). Coomassie Brilliant Blue R250, 3,5-Dinitrosalicylic acid (DNS), gallic acid, N,N'-Methylenebisacrylamide, N,N,N',N'-Tetramethylenediamine and 2,4,6-tripyridyl-s-triazine, were purchased from Fluka (Germany). Protein assay dye reagents were a product of Bio-Rad (USA). Ammonium persulfate and acrylamide were obtained from Sigma (USA). All chemicals are of analytical grade.

Mushroom samples: Fresh fruiting bodies of *Lentinus* sp. strain RJ-2 were obtained from Rujira Mushroom Farm in Ka La Sin province (Northeast, Thailand) during October 2009. Some mushrooms were left to completely dry at room temperature. The dry weight was calculated gravimetrically. All of experiments were done at the Proteins and Enzymes Technology Research Unit and the Central Instrumental Center, Faculty of Science, Mahasarakham University.

Crude polysaccharides preparation: The fresh and dried fruiting bodies of the mushrooms were boiled for 4 h. After centrifugation at 6,000 rpm for 30 min, the supernatant had the crude polysaccharide extracted with 4 volumes of absolute ethanol (4:1 v/v) at 4°C overnight as described by Lee *et al.* (2003). After centrifugation at 6,000 rpm for 20 min, the pellet was collected and washed with absolute ethanol and centrifuged (ethanol washing step was repeated twice) and left to completely dry at room temperature. The crude polysaccharide was re-dissolved (50 mg mL^{-1}) in distilled water for further study.

Antioxidant activity assay

$ABTS^{\bullet+}$ radical scavenging activity: The $ABTS^{+\bullet}$ radical used in this work was prepared with the laccase reaction as previously described (Khammuang and Sarnthima, 2008). Before antioxidant activity assay, laccase was removed from the ABTS radicals stock by centrifugation in selection membrane, molecular weight cut off of 10 kDa and was diluted in distilled water to give an initial absorbance (A_{734}) at 0.7. $ABTS^{+\bullet}$ scavenging activity was modified from Thaipong and others (Thaipong *et al.*, 2006). An aliquot of each sample (20 μL) was added to 980 μL of $ABTS^{+\bullet}$ radical dilute solution. The absorbance was measured after 30 min of incubation at 734 nm by a spectrophotometer. Total antioxidant capacity was calculated relative to the reactivity of Trolox under the parallel experiment and the result was expressed as μmol Trolox/g sample.

$DPPH^{\bullet}$ radical scavenging: $DPPH^{\bullet}$ scavenging activity was measured by the modified method of Thaipong and co-workers (Thaipong *et al.*, 2006). $DPPH^{\bullet}$ radical stock solution in methanol (2.4 mg mL^{-1}) was diluted in methanol to give the initial A_{515} at 0.7. An aliquot of each sample (20 μL) was added to 980 μL of $DPPH^{\bullet}$ radical solution. The absorbance was measured at its maximum absorption (λ_{max} 515 nm) after 30 min of incubation at room temperature. Total antioxidant capacity was also calculated relative to the reactivity of Trolox under the parallel experiment and the results were reported as μmol Trolox/g sample.

Ferric Reducing Antioxidant Power (FRAP): The FRAP assay was done as previously described by Vasco *et al.* (2008) and Soong and Barlow (2004). The fresh FRAP reagent was prepared daily by mixing 10 volumes of acetate buffer (300 mM, pH 3.6), 1 volume of TPTZ (2,4,6-tripyridyl-*s*-triazine) solution (10 mM TPTZ in 40 mM HCl) and 1 volume of $FeCl_3 \cdot 6H_2O$ solution (20 mM). The reagent was warmed to 37 °C and then an aliquot of each sample (20 μL) was added to 980 μL of FRAP reagent. The change in absorbance was measured after 90 min at 593 nm. Aqueous solution of $FeSO_4 \cdot 7H_2O$ was used for the calibration curve and reducing power was reported as μmol Trolox/g sample.

Properties of crude polysaccharides

Determination of total carbohydrate content: The carbohydrate contents were determined by the slightly modified Phenol-sulfuric acid method according to Masuko and co-workers (Masuko *et al.*, 2005). Fifty microliters of crude polysaccharide solution was mixed with 150 μL of concentrated sulfuric acid and immediately with 30 μL of 5% phenol and then the reaction mixture was kept at 90°C for 5 min. The absorbance of the mixture after

cooling to room temperature was measured at 490 nm. The total carbohydrate content was calculated using a standard curve of D-glucose.

Determination of reducing sugar: The reducing sugar was determined by the modified method of Miller (1959). Briefly, 0.5 mL of 1% 3,5-Dinitrosalicylic acid (DNS) was added to each aliquot sample (20 μL) and adjusted up to 5 mL with distilled water. The mixture was boiled in boiling water for 5 min and cooled to room temperature. Distilled water (2.5 mL) was added to the mixture and the absorbance was measured at 540 nm. The total reducing sugar was calculated with D-glucose as a standard. Total polysaccharide was the subtraction of reducing sugar from total carbohydrates.

Determination of total phenol content: The crude polysaccharide was estimated from the total phenol content determined by the Folin-Ciocalteu colorimetric method based on the procedure described by Singleton and Rossi (1965) with some modifications. Briefly, 0.5 mL of Folin-Ciocalteu reagent (10x dilution) was mixed with each crude polysaccharide sample (0.5 mL). After 3 min, 0.5 mL of 35% (w/v) Na_2CO_3 was added and then the mixture was brought up to 5 mL with distilled water. The absorbance of the mixture was read at 725 nm after being kept in the dark for 90 min. The quantification was determined based on a standard curve of gallic acid. The total amount of phenol contents was expressed as GAE (mg g^{-1} sample).

Determination of protein content and protein pattern analysis: The total protein content was measured by the Bradford method (Bradford, 1976). An aliquot of sample (10 μL) was mixed with 200 μL of Bio-Rad Protein Assay kit and brought up to 1 mL with distilled water. After 5 min standing at room temperature, the absorbance was measured at 595 nm and the protein content was calculated using a Bovine Serum Albumin (BSA) as standard.

Protein profile analysis of each polysaccharide fraction was performed by a 15% separating Tris-tricine SDS-PAGE gel from the slightly modified method of Schagger and von Jagow (1987). To the gel an initial voltage of 30 V and then a constant voltage at 200 V was applied. The protein bands in the gel were visualized by silver staining.

Infrared spectroscopy (FTIR) and Thin Layer Chromatography (TLC): Crude polysaccharides were subjected to FTIR spectroscopy at 400-4000 cm^{-1}. Dried samples were made into a pellet with KBr powder.

TLC of each crude polysaccharide was performed after the crude polysaccharides (2 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 mL) at 110°C for 4 h (Pramanik *et al*., 2007) and TFA was removed by evaporation. The samples and standard monosaccharides were analyzed on the same TLC aluminum sheet pre-coated with silica (Silica gel 60 F254, Merck, Germany). The developing agent was a mixture of acetonitrile (MeCN), ethyl acetate (EtOAc), 1-propanol and water (85:20:20:15, v/v) (Han and Robyt, 1998). The monosaccharides were visualized on the plate after dipping into 10% sulfuric acid in ethanol and heating until they appeared as a dark spots. D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose were used as standard monosaccharides.

RESULTS

The fresh Fruiting Bodies (FB) and dry fruiting bodies (DB) of the studied mushroom yielded crude polysaccharides of 115.84 and 93.66 mg g^{-1} dry weight of mushroom (or 11.58 and 9.37%, respectively) (data not shown). The antioxidant activities of the crude polysaccharides from mushrooms of *Lentinus* sp. were evaluated by means of Trolox Equivalent Antioxidant Capacity (TEAC) calculated from $ABTS^{\bullet+}$, $DPPH^{\bullet}$ scavenging and FRAP assays. The analyzed crude polysaccharides of FB and DB showed similar antioxidant capacities according to the three assays (Fig. 1).

The concentration of the reference compound, Trolox (vitamin E analog), that was able to scavenge 50% of ABTS radicals (50% inhibition concentration, IC_{50}) was 0.016 μM or 4.00±0.03 μg mL^{-1}. The crude polysaccharides from FB and DB, of the

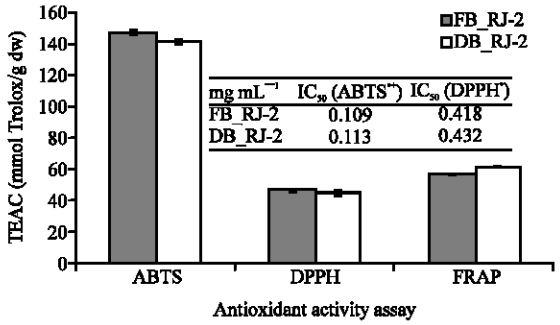

Fig. 1: The antioxidant capacity of crude polysaccharides measured as Trolox equivalents assay (μmol Trolox/g dry weight extract). Error bars indicate mean±standard deviation (n = 3). In set table shows concentration of Trolox for 50% ABTS and DPPH radical scavenging (IC_{50}) of the crude polysaccharide extracts. The IC_{50} for both radicals were 4.00±0.03 and 4.82±0.06 μg mL^{-1}, respectively

Table 1: The yield of crude polysaccharides, amount of proteins, total carbohydrate, total polysaccharides, reducing sugar and total phenol content of crude polysaccharides of *Lentinus* sp. strain RJ-2 (n = 3)

Crude polysaccharide	Protein	Total Carbohydrate	Total Polysaccharide	Reducing sugar	Total phenol content (mgGAE/g dw)
	----------(mg g^{-1} dw)----------				
FB_RJ-2	31.54±1.47	503.9±44.8	476.8±43.8	27.14±1.01	53.08±1.45
DB_RJ-2	53.22±2.49	450.6±57.4	424.9±58.5	25.63±1.14	51.29±1.45

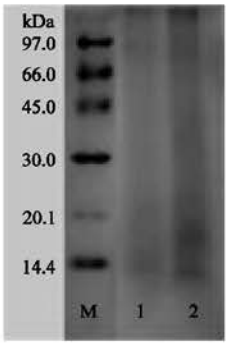

Fig. 2: Tris-tricine SDS-PAGE (15%) of crude polysaccharides from *Lentinus* sp. strain RJ-2. M, molecular weight markers; 1, fresh fruiting bodies PS; 2, dried fruiting bodies PS

studied mushroom, had their IC_{50} values as 0.109 and 0.113 mg mL^{-1} respectively (Fig. 1, in set table). There was some extent of phenol compound in the crude polysaccharide samples as shown in Table 1. The total phenol content as determined by Folin-Ciocalteu method in the crude PS samples were around 50 mgGAE/g dw extract. The total carbohydrate, total polysaccharide, reducing sugar content as well as total phenol content in both crude polysaccharides were similar (Table 1). Dried fruiting bodies yielded slightly lower components, except for protein content when compared to the fresh fruiting bodies.

Protein patterns were identified on Tris-tricine SDS-PAGE gels stained with Coomassie brilliant blue R250, with the numerous protein bands being visualized on a polyacrylamide gel. Both crude polysaccharides had similar major protein bands, which had molecular weights lower than 20.1 kDa and around 66.0-97.0 kDa (Fig. 2).

The IR spectra of crude polysaccharides are shown in Fig. 3a and b. The strong absorption in the range of 1200-1000 cm^{-1} suggested the stretching vibration of the pyranose ring. The broad band around 3400-3370 cm^{-1} is the characteristic absorption of the hydroxyl group and the weak band at 2928 cm^{-1} is C-H stretching vibration. The absorption at 1654 and 1560 cm^{-1} showed the feathering of protein, which corresponding to the carbonyl bond of an amide group and bending vibration of the N-H bond, respectively. The C-H bond in α-configuration has an absorption peak nearby 844 cm^{-1} while the C-H bond in β-configuration has an

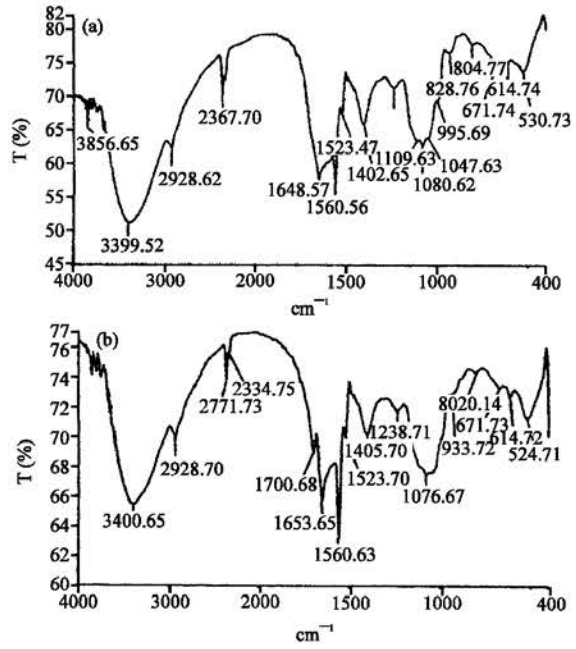

Fig. 3: FTIR spectrum of (a) FB and (b) DB of crude polysaccharides from *Lentinus* sp. strain RJ-2

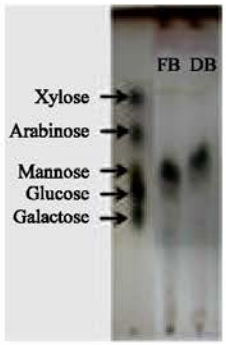

Fig. 4: TLC chromatogram of mixed standard monosaccharide and crude polysaccharides from *Lentinus* sp. strain RJ-2. FB: crude polysaccharide from fresh fruiting bodies; DB: crude polysaccharide from dried fruiting bodies

absorption peak nearby 891 cm^{-1}, but these spectra in this study cannot be clearly identified α-configuration or β-configuration.

The TLC results (Fig. 4) indicated that these crude polysaccharides consisted of simple monosaccharide of

six carbon atoms. According to the retention factor (R_f) which showed that the crude polysaccharides of FB and DB of *Lentinus* sp. had mannose (R_f 0.50±0.01) as the predominant monosaccharide.

DISCUSSION

In this work, fresh and dry fruiting bodies of *Lentinus* sp. (RJ-2) were extracted for crude polysaccharide by hot-water extraction as used previously in many reports (Lee *et al.,* 2003; Tsai *et al*., 2007; Tseng *et al*., 2008). The yield of polysaccharide from fruiting bodies from this work were clearly higher than those from fruiting bodies of other species such as *P. ostreatus*, 3.32% (Tong *et al.,* 2009); *R. virescens*, 1.94% (Sun *et al*., 2010) and *G. tsugae*, 1.5-1.7% (Tseng *et al.,* 2008). The crude polysaccharide yields from *Lentinus* sp. were in the same range as those from fruiting bodies, 6.2-9.9% as reported by Mau *et al.* (2005). High yields polysaccharides were extracted from both the natural and cultured mycelia of *Cordyceps sinensis*, which were 30.46 and 39.11%, respectively (Dong and Yao, 2008). Mushroom mycelia extraction for polysaccharides might be further studied for this mushroom.

Comparison of response to the antioxidant capacity value between the three assays; $ABTS^{\bullet+}$ assay exhibited the highest value followed by FRAP and finally $DPPH^{\bullet}$ assay. It was shown that ABTS radical scavenging activity of crude polysaccharides from this study is about 27-28 times less active than Trolox. This might be due to the action of different compounds as well as different mechanism which might take part such as phenol compounds. With regards to scavenging ability on ABTS radicals, IC_{50} values of the crude polysaccharides from the studied mushroom were less than 0.5 mg mL^{-1}, whereas the IC_{50} of crude polysaccharides from fruiting bodies of *L. polychrous* Lév. were 1.66 and 3.08 mg mL^{-1} for dried and fresh fruiting bodies, respectively (unpublished data). This result suggested that crude polysaccharides from different strains of *Lentinus* sp. have different antioxidant capacities. Combined with the results from Table 1, this suggested that those antioxidant activities might be due to both reducing sugars and phenol compounds presented in samples.

The antioxidant potential of *L. edodes* methanol extract measured as DPPH radical scavenging activity reported by Sasidharan *et al.* (2010) had an EC_{50} value of 4.4 mg mL^{-1}. The IC_{50} (~EC_{50}) values of the crude polysaccharides in our work is approximately 10 times lower than that value in their report, which indicate a stronger antioxidant activity in these mushroom extracts.

The reducing property of the crude PS might also be linked to the reducing sugar content in the samples. This present work showed that antioxidant activities of the crude polysaccharide extracts were far less than those of the reference phenol compound, Trolox. This indicates that radical scavenging activity might be roles held by different mechanisms or compounds. Purification and deproteinization of this crude polysaccharide might be explored in the future to elucidate the key bioactive material(s).

From the present study, total polysaccharides of *Lentinus* sp. (47.7, 42.5% for FB and DB, respectively) are far higher than those reported in *L. edodes*, 3.5-10.0% (Surenjav *et al*., 2006). In the same time, the values are in the same range as polysaccharides from the fruiting bodies of *G. lucidum,* approximately 57.91% (YouGuo *et al*., 2009). However, the total polysaccharide content from fruiting bodies of cultured *C. militaris* are higher than 95% (Yu *et al.,* 2007).

Slightly lower components were found in dried fruiting bodies, except for protein content when compared to the fresh fruiting bodies. This might be due to the difficulty of hot water to solubilize phenol compounds from the dead, dried sample or there might be some structural or chemical change during the drying process. From this work, Total phenol content values of crude polysaccharides are far higher than the value of polysaccharides isolated from the fruiting bodies of *G. lucidum* (YouGuo *et al.,* 2009), which was only about 1.53 mgGAE/g polysaccharides.

The antioxidant activity of fruits and vegetables often correlates to its total phenolic content (Soong and Barlow, 2004; Vasco *et al*., 2008; Oboh, 2008). In this study, we found certain amounts of total phenol contents (Table 1), which suggested that phenol compounds might be also involved in antioxidant activity in *Lentinus* sp. mushrooms presented in the crude polysaccharides.

Protein patterns according to Tris-tricine SDS-PAGE showed similar major protein bands of both crude polysaccharides. The smear of the bands might be due to the natural viscosity of samples of polysaccharide and reducing sugar. Reducing sugar content could be related to the viscosity of the crude polysaccharide solution (Sachslehner *et al*., 2000). In this work, crude polysaccharides contain protein about 3.1-5.3%. The polysaccharide fractions of the same genus, *L. edodes* also contained protein ranging from 4.6-15.2% depending on strain (Surenjav *et al.,* 2006). Dong and Yao (2008) also reported that hot water extracts of both natural and cultured mycelia of *C. sinensis* contained proteins as well as polysaccharides. The exact proportion of glycan and protein should be further investigated in the purified fractions. Further study on the purification and structural

elucidation of polysaccharides from this mushroom are still needed in order to understand more about the biochemical properties and potential uses of these polysaccharide fractions. Other bioactive polysaccharides have been reported as polysaccharide-peptide complexes (Chan and Yeung, 2006; Eo *et al.*, 1999).

The IR spectra of crude polysaccharides had strong absorption in the range of 1200-1000 cm^{-1} suggested the stretching vibration of the pyranose ring (Liu and Wang, 2007; Chen *et al*., 2008). The broad band of the hydroxyl group was around 3400-3370 cm^{-1} and weak band of C-H stretching vibration at 2928 cm^{-1}. The similar absorption pattern of protein at 1654 and 1560 cm^{-1} which corresponding to the carbonyl bond of an amide group and bending vibration of the N-H bond, respectively (Chen *et al*., 2008). The C-H bond in α-configuration has an absorption peak nearby 844 cm^{-1} while the C-H bond in β-configuration has an absorption peak nearby 891 cm^{-1} (Liu and Wang, 2007). However, result spectra in our study cannot be clearly identified α-configuration or β-configuration.

The TLC results indicated that these crude polysaccharides of *Lentinus* sp. might consist of mannose according to R_f had mannose. On the other hand, the active fractionation polysaccharide from *Phellinus ribis* had only glucose as a component (Liu and Wang, 2007). From Fig. 4, spots of crude polysaccharides were not clear for saccharide identification. TLC is the principle technique of chromatography. Therefore, they still need further analysis by higher techniques such as High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC) for clear identification of monosaccharide composition.

Beta-glucan from the same genus, *L. edodes* named *Lentinan* has been reported as an antitumor polysaccharide by Maeda and Chihara (1973) as well as having other biological activities such as antioxidative stress and immunity activity (Yu *et al*., 2009), tumor-inhibitory effects (Shen *et al*., 2009) and an anti-fatigue effect. The crude polysaccharide from the *Lentinus* sp. RJ-2 in this study might not have β-glucan in its structure (according to the TLC result). More specific techniques such as Gas liquid chromatography-mass spectrometric (GLC-MS) analysis and NMR spectroscopy are required for monosaccharide analysis.

CONCLUSIONS

Fresh and dry fruiting bodies of *Lentinus* sp. mushroom yielded 47.7 and 42.5% crude polysaccharide extracts after hot water extraction. Both crude polysaccharides revealed similar antioxidant activities according to three assays ($ABTS^{\bullet+}$, $DPPH^{\bullet}$ scavenging activity and FRAP assay). The crude polysaccharides fractions also consisted of proteins and phenol compounds. The crude polysaccharides of the fruiting bodies of *Lentinus* sp. had potent antioxidant activity which clearly indicates the beneficial effects of mushroom polysaccharides as antioxidants. Further study in more detail about the structure of the polysaccharides and other biological activities such as antitumor and immune modulation as well as anti-inflammatory activities are of interest.

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