# Anticancer PSP and phenolic compounds in *Lentinus squarrosulus* and *Lentinus polychrous*

JongrakAttarat*[a], Rapee Thamisak[a]
[a]Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand 65000

*rakae19@hotmail.com phone +66-55964639 fax +66-55964770

## ABSTRACT

At present, free radicals were originated from environment, stress and food. The free radicals are major issues in aging, diabetes, hypercholesterolemia and especially in tumor promotion. Many bioactive compounds in mushrooms such as peptides, proteins, vitamins and phenolic compound were served as antioxidant and anticancer activity for disposal free radicals.An anticancer PSP (Polysaccharide peptide) was detected in protein extracts from *Lentinus squarrosulus* and *Lentinus polychrous* at 9.87 ± 0.03, 0.41 ± 0.03 μg/g protein, respectively. Anti-oxidant activity of ethanolic mushroom extracts was identified by DPPH and FRAP. Percentage of inhibition onto scavenging activity by DPPH assay was higher than 70% in ethanolic extract of both *Lentinus squarrosulus* and *Lentinus polychrous*. In FRAP assay, micromolar trolox equivalent of *Lentinus squarrosulus* showed higher than and *Lentinus polychrous*. Catechin and tannic acid were major group of phenolic compounds in *Lentinus polychrous* via LC-MS. In summary, PSP, catechin and tannic acid from *Lentinus squarrosulus* and *Lentinus polychrous* may be effective natural product for clinical trial in patients because this edible mushrooms are nontoxic and also as functional food for human health.

**Keywords:** Anticancer, Mushrooms, *Lentinus squarrosulus*, *Lentinus polychrous,* Anti-oxidant assay, Catechin, PSP, polyphenol compounds

# 1. INTRODUCTION

Bioactive proteins isolated from more than 30 mushroom species have shown antitumor, antiviral, antimicrobial, antioxidant and immunomodulatory action such as lectins, fungal immunomodulatory proteins (FIP), laccases, protease, ribonuclease, proteoglycans, PSP (polysaccharopeptide) and other proteins [1-2]. PSP extracted fromTurkey tail (known as *Coriolus versicolor* or Yun-zhi) has a molecular mass of approximately 100 kDa [3]. PSP contains $\alpha$-1,4 and $\beta$-1,3 glucosidic linkages in their polysaccharide moieties. Of the *C. versicolor*-derived therapeutics extracts, polysaccharopeptides are commercially the best established. The polysaccharopeptides obtained from *C. versicolor*, known as *C. versicolor* polysaccharides, is a complicated protein-bound polysaccharide extracted from its mycelium, or fruiting body. PSP significantly improved the quality of life, provided substantial pain relief, and enhanced immune status in 70-97% of patients with cancers of the stomach, esophagus, liver, lung, ovary, and cervix [4]. Presently, PSP is considered as a potential candidate for drug development in treatment and prevention of human cancer because of its immunological properties as well as its ability to distinguish cancerous cells from normal cells. Besides bioactive proteins, mushrooms have become rich sources of natural antioxidant compounds such as phenolic compounds, tocopherols, ascorbic acid, and carotenoids. There are many phenolic compounds found in mushrooms such as benzoic acid, gallic acid, catechin, tannic acid, caffeic acid, and resveratrol [5]. Many phenolic compounds have been reported to process potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities to a greater or lesser extent. The aim of this study is to quantitate PSP and phenolic compounds in local cultivated edible mushrooms and then verify the antioxidative activity *in vitro*. *L. squarrosulus* was outstanding mushroom here in antioxidant and anticancer properties.

# 2. MATERIALS AND METHODS

**Chemicals**

All chemicals are from Sigma (Sigma Chemical Co., St. Louis, MO) unless otherwise noted.

**Preparation of protein extract in mushrooms**

Fruiting bodies of edible mushrooms, *Lentinus polychrous* and *Lentinus squarrosulus* were collected from farm in Chiang Mai province. Those fruiting bodies were dried at 50°C and ground into powder and stored at -50°C until using for protein preparation and the phenolic compounds assay. Approximately 10 g of the mushroom powder was prepared by homogenizing in hot water and 0.15 N NaCl. The homogenized samples were then precipitated with 40-80% $(NH_4)_2SO_4$ saturation. Fractions extracted were resuspended in 50 mM Tris-HCl, pH 7.5, and dialyzed overnight against the same buffer. The resulting solution was added with protease inhibitor cocktail (AMRESCO LLC, OH 44139, USA) and collected at -20°C in order to quantitate protein concentration, PSP levels, and treat Cholangiocarcinoma cell lines. Protein concentration of the extract was determined by Bradford assay with BSA (Bovine serum albumin) as standard.

**Development of ELISA to quantify PSP concentration in edible mushrooms**

To quantify PSP in fruiting bodies, ELISA was performed following the procedure described previously in quantitation of vitellin and vitellogenin in ovary and hemolymph of prawns. Briefly, PSP and proteins extracted from fruiting bodies were coated on 96 well plates at room temperature. Anti-PSP antibody specific to commercial PSP (JHS Natural Products, OR 97402, USA) was used as a primary antibody at a dilution of 1:500. The enzyme reaction was carried out by *o*-phenylenediamine after an addition of anti-rabbit IgG-HRP (dilution 1:5,000). PSP concentrations in the samples were calculated on the basis of PSP concentrations according to a standard curve. The sensitivity of the assay for immunoreactive PSP was from 1-100 ng per assay.

**Quantitation of phenolic compounds**

Ten grams of dried mushrooms were incubated in 100 ml solvent [absolute ethanol and HCl acid media (1% v/v)] at room temperature on a shaker at 150 rpm for 24h and then centrifuged at 5,000 rpm for 10 minutes. The solution was filtered through a 0.45 μm membrane filter and then the residue was re-extracted. An Agilent 1100 HPLC system equipped with a quaternary pump, refrigerated autosampler, column heater and withDAD, FLD and MS ion trap detectors was used. Forphenolic analysis, a 150 x 4.6 mm Zorbax SB C18 column wasused at a flow rate of 1 ml/min and gradient elution of 0–5 min B 100% constant, 5–10 min A 0–20%, 10–20 min A 20% constant, 20–60 min A 20–40% (buffer A: 10 mM formic acid pH 3.5 with$NH_4OH$; Buffer B: 100 % methanol with 5 mM ammonium formate). Column temperature was 40°C and UV detectionwas at 270, 330, 350 and 370 nm. Compound identification was confirmed by injection of authentic standard. Compounds were quantitated by the external

standard method using authentic standards, gallic, tannic acid, catechin, rutin, isoquercetin, eriodictyol, quercetin, hydroguinin, kaempferol, and apiginin.

**Preparation of ethanolic mushroom extract**

Five grams of dried mushrooms were extracted by stirring with 100 ml of ethanol at 25 °C for 24 hours and filtering through Whatman No.4 filter paper. The residue was then extracted with addition of 100 ml of ethanol. The combined ethanolic extracts were then rotary evaporated at 40°C to dryness. The dried extract was resuspended in ethanol and stored at 4°°C for further use in DPPH, FRAP and total phenolic compound assays.

**DPPH assay**

The DPPH (2,2-diphenylpicrylhydrazyl)assay was used to determine free radical scavenging activity of mushroom extracts. Each mushroom extract in ethanol was mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm.

**FRAP assay**

The FRAP (Ferric reducing-antioxidant power assay) was done according to Benzie and Strain with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM$FeCl_3$ .$6H_2O$ solution. The fresh working solution wasprepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. Mushroom extracts were allowed to react with FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm absorbance. The standard curve was linear between 25 and 800 mM trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E). Results are expressed in µM TE (trolox equivalent antioxidant capacity) per gram of dried mushroom. Additional dilutions were needed if the FRAPvalue measured was over the linear range of the standard curve.

## 3. RESULTS

In Figure 1, PSP level was quantitated in different edible lentinus mushrooms using indirect ELISA. In one gram of protein concentration in three mushrooms, PSP level in *L. squarrosulus* was significantly higher than *L. polychrous*. As previously reported, PSP was produced from *C. versicolor* mushrooms harvested in the wild or cultivated commercially, or from mycelial growth of *C. versicolor* in submerge fermentation [3]. PSP also enhanced the cytotoxicity of certain S-phase targeted-drugs, such as doxorubicin, etoposide, camptothecin and cyclophosphamide, on human cancer [6]. In addition, PSP has shown a chemopreventive effect on prostate cancer via the targeting of prostate cancer stem cell-like populations. Immunoreactive protein of anti-commercial PSP antibody using ELISA may be PSP as reported in *C. versicolor.*

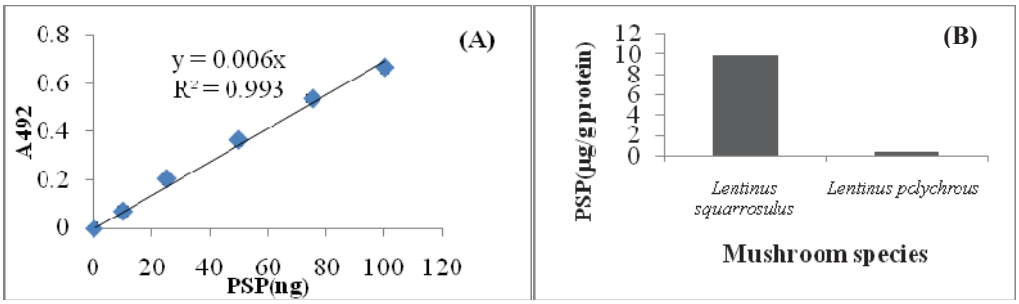


Figure 1. Detection of PSP concentration with indirect ELISA in three mushrooms species, *L. squarrosulus* and *L. polychrous*. (A) Calibration curve of PSP standard. (B) Concentration of PSP (mg/g of protein) two mushroom species. Error bars indicate mean±standard deviation (n = 3).

Table 1. Antioxidant activity of ethanolic extract from mushrooms

| Mushrooms | DPPH assay (%Inhibition) | FRAP assay (Trolox equivalent) |
|---|---|---|
| *L. squarrosulus* | 80.13 ± 0.002 | 117.27 ± 0.009 |
| *L. polychrous* | 73.13 ± 0.008 | 20.91 ± 0.013 |

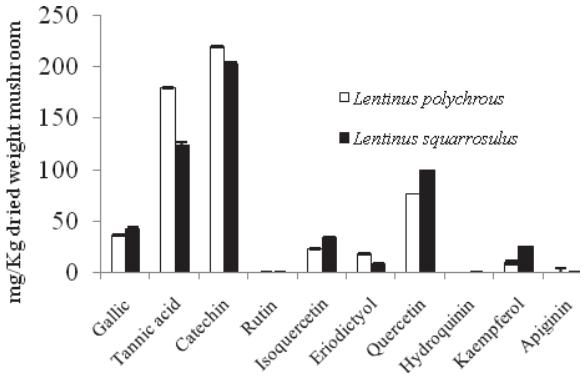


Figure 2. Concentration of phenolic compounds in three mushrooms, *L. squarrosulus* and *L. polychrous*. Catechin and tannic were the major phenolic compound in three lentinus mushrooms. Error bars indicate mean ± standard deviation (n=3).

Catechin was the major phenolic compound in both *L. polychrous* and *L. squarrosulus*; the higher concentration was existent in *L. polychrous*. From results in Figure 2, the amount of catechin comprised over 200 mg/kg dried weight of the mushroom. The second and third amount of phenolic compound in ethanol extract of mushrooms were tannic and quercetin, respectively. Gallic, isoquercetin, kaempferol and eriodictyol level were less than 50 mg/kg dried weight of the mushroom and these phenolic contents were detected at the highest amount in *L. squarrosulus*. Rutin and hydroquinin were not detected in any ethanolic extracts of mushrooms in this study. Catechin is a group polyphenol of condensed tannins. Normally, cathechins are the main compounds in green tea [7]. Catechin served as powerful antioxidant against lipid peroxidation when phospholipid bilayers are exposed to aqueous oxygen radicals. Catechin prevented cancer of the liver, lung, breast and colon, and also suppresses cancer promotion. Besides anticancer activity, antibacterial, antiviral agent and anti-hyperglycemic action of catechin were also reported. A systematic review revealed that tannic acid or commercial product of tannin have also been reported to exert many physiological effects, such as accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses [8]. The antioxidant activities of mushroom extracts were estimated by means of trolox equivalent antioxidant capacity (TEAC) calculated from the DPPH and FRAP assay. At concentration of 50 mg/ml, scavenging effects of *L. squarrosulus*, *L. polychrous* and *L. edodes* were about 80, 73, and 30%, respectively, while scavenging activity of trolox by DPPH assay at 0.15 mg/ml was 88%. The scavenging effect of 50 mg/ml *L. squarrosulus* extract showed its highest value at 500 μM TE/g dried mushroom in FRAP assay; this data was correlated with the TPC and DPPH assays.

## 4. CONCLUSIONS

Some advantages of using mushrooms over plants as sources of bioactive compounds are that often the fruiting body can be produced in much less time and can be manipulated to produce optimal quantities of active compounds. In this study, it is the first time to detect PSP in protein extracts from fruiting body of *L. squarrosulus* and *L. polychrous* by ELISA. In addition, other bioactive proteins except PSP may also exist in *L. squarrosulus*. However, further studies are required to confirm that this is the same PSP as characterized in *C. versicolor*. Purification and characterization of the PSP of *L. squarrosulus* has been studied and this data will be reported soon in a different manuscript. A good antioxidant activity of *L. squarrosulus* extract and high phenolic content in *L. polycrous* will challenge us to prove its mechanism in cancer cell lines.

## ACKNOWLEDGEMENTS

This work was supported by grants from Naresuan university annual government statement of expenditure 2556 (NRCT) and Naresuan University annual income 2554.

## REFERENCES

1. Ng TB. 2004. Peptides and proteins from fungi. Peptide. 25, 1055-1073.
2. Xu X, Yan H, Chen J, Zhang X. 2011. Bioactive proteins from mushrooms. Biotechnol Adv. 29, 667-674.
3. Cui J, Chisti Y. 2003. Polysaccharopeptides of *Coriolus versicolor*: physiological activity, uses, and production. Biotechnol Adv. 21, 109-122.
4. Kidd PM. 2000. The use of mushroom glucans and proteoglycans in cancer treatment. Altern Med Rev. 5, 4-27.
5. Kim MY, Seguin P, Ahn JK, Kim JJ, Chun SC, Kim EH, Seo SH, Kang EY, Kim SL, Park YJ, Ro HM, Chung LM. 2008. Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. J Agric Food Chem. 56, 7265-7270.
6. Wan JM, Sit WH, Yang X, Jiang P, Wong LL. 2010. Polysaccharopeptides derived from *Coriolus versicolor* potentiate the S-phase specific cytotoxicity of Camptothecin (CPT) on human leukemia HL-60 cells. Chinese Med. 5, 16.
7. Sutherland BA, Rahman MA, Appleton I. 2006. Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. J Nutr. Biochem. 17, 291–306.
8. Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. 1998. Tannins and human health: a review. Crit Rev Food Sci Nutr. 38, 421–464.