

RESEARCH ARTICLE

Antioxidant activity and cytotoxicity of the edible mushroom, *Pleurotus cystidiosus* against Hep-2 carcinoma cells

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Abstract: This study was undertaken to evaluate the antioxidant activity and cytotoxicity of *Pleurotus cystidiosus*, an edible mushroom, against Hep-2 cancer cells. Fresh *P. cystidiosus* mushroom was extracted with acetone (fraction A). Fraction A was extracted into hexane, dichloromethane and ethyl acetate successively and the remaining fraction was labeled as "A4". Fraction A4 was further separated into three fractions, A4-1, A4-2 and A4-3 using a reverse phase column. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and nitric oxide (NO) radical scavenging activity assays were used to investigate the reducing power of the extracts. The DPPH-EC₅₀ of A4-2 and A4-3 were 0.81 and 0.82 mg/mL, respectively; NO - EC₅₀ of A4-2 and A4-3 were 0.87 and 0.61 mg/mL, respectively. The results on cytotoxic effects based on MTT and LDH assays have shown that the same two extracts to have the highest activity. These results were reinforced by the cell morphological changes observed using an inverted fluorescence microscope of the treated cells. Cells incubated with the highest dose (5 mg/mL) of A4-2 and A4-3 showed cell morphological changes such as cellular swelling, irregular cell shapes, condensed cytoplasm and vacuolar areas. Hence it can be concluded that the extract of *P. cystidiosus* has antioxidative activity as well as cytotoxicity against Hep-2 cancer cells. It can also be speculated that the use of whole mushroom as a medicinal food may bring about health benefits.

Keywords: Abalone, DPPH radical scavenging activity, LDH activity, MTT, mushroom.

INTRODUCTION

The study of mushrooms during the last few decades has resulted in many beneficial products such as notable mycopharmaceuticals, myconutriceuticals and mycocosmeceuticals. Literature reveals that *Pleurotus* species commonly known as oyster mushrooms also possess many biological activities including promising

anticancer and antioxidant activities (Wasser, 2002).

Cancer has become the second largest cause of death in humans, and has led to many research efforts and clinical studies to find potent cancer drugs (Daba & Ezeronye, 2003). Treatments by chemotherapeutic agents, surgery and radiation have not been fully effective in control or prevention of many cancers (Moongkarndi *et al.*, 2004). Medicinal mushrooms have been tested against several major cancers involving stomach, lung, liver and colon (Gu & Sivam, 2006).

Approximately 200 species of mushrooms have been found to markedly inhibit tumor growth, but most of the compounds responsible for their activity have not yet been identified (Wasser & Weis, 1999). Six of the polysaccharides of mushroom-origin that have been investigated with human cancers include lentinan, schizophyllan, active hexose correlated compounds (AHCC), maitake D-fraction, polysaccharide-K and polysaccharide-P (Daba & Ezeronye, 2003). In a recent pilot study it has been reported that the administration of DNA isolated from the *Pleurotus ostreatus* fruit body, significantly increases the life span of mice with solid Ehrlich carcinoma (Shlyakhovenko *et al.*, 2006).

The antioxidative activity of phytochemicals also plays an important role in anti-carcinogenic and anti-mutagenic activities (Padma *et al.*, 2007). Various compounds of mushroom origin and extracts have shown radical scavenging activities with anticancer properties. Pleuran (β -1,3-D glucan) isolated from *Pleurotus ostreatus* fibre complex has stimulated the activity of the antioxidant enzymes such as superoxide dismutase and glutathione peroxidase activity in rat liver and has

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reduced the glutathione levels significantly in rat colon (Bobek & Galbavy, 2001). In several other studies *Ganoderma lucidum*, *Phellinus rimosus*, *Pleurotus florida* and *Pleurotus pulmonaris* have been observed to possess profound antioxidant and antitumor activities (Ajith & Janardhanan, 2007).

There are no reports on the antioxidative activity and cytotoxicity of *Pleurotus cystidiosus* species, which is commonly referred to as abalone. *P. cystidiosus* mushroom is large and fleshy, and it grows on tree trunks or stumps in shelf-like layers. The pileus is shell-shaped and dark grayish brown in colour and the stipe is dark brown (Dube, 1996).

The present study investigated the antioxidant and cytotoxic activity of *P. cystidiosus* against Hep-2 cancer cell line and the most active *P. cystidiosus* fraction responsible for those activities.

METHODS AND MATERIALS

Chemicals: Eagle's minimum essential medium (EMEM), foetal bovine serum (FBS), antibiotics (penicillin/streptomycin), trypsin (from porcine pancreas), ethylenediamine tetraacetic acid (EDTA), 3,4,5-(dimethylthiazol-2-yl)2-5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA), and camptothecin were purchased from Sigma Chemicals Co., Ltd. (USA). L-Glutamine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), polyethylene glycol tert-octylphenyl ether (Triton X-100) solution (1 %), and dimethyl sulfoxide were purchased from Fluka Chemie GmbH (Switzerland). Ascorbic acid, sodium nitroprusside (SNP) and ethanol were purchased from BDH Chemicals Ltd. (England). All the other chemicals were obtained in the purest form available commercially. The lactate dehydrogenase (LDH) enzyme assay and the total protein kits were purchased from Randox Laboratories Ltd. (UK).

Cell culture: A Hep-2 cell line was obtained from the Medical Research Institute, Colombo 8. The cells were cultured in EMEM growth media supplemented with 10 % FBS, MEM (1 %), L-glutamine (3 %), 50 IU/mL penicillin and 50 µg/mL streptomycin. The pH of the growth media was adjusted to physiological pH (7.4) using 7.5 % sodium bicarbonate. The cells were maintained at 37 °C in a 5 % CO₂ atmosphere with 95% humidity.

Preparation of *P. cystidiosus* mushroom extracts/fractions: Extraction and fractionation of *P. cystidiosus* mushroom was carried out using the procedure of

Vasudewa *et al.* (2008). This procedure was very successful, giving a fraction with potent anti nociceptive activity. The summary of the procedure is as follows.

Fresh *P. cystidiosus* mushroom was extracted into acetone and the extract was labelled as "A". Fraction A was extracted successively into hexane (A1), dichloromethane (A2) and ethyl acetate (A3) and the remaining fraction was labelled as "A4". After the removal of all the solvents, fraction A4 (1g) was loaded onto a reverse phase column and three bulk fractions; A4-1, A4-2 and A4-3 were eluted with 100 mL each of 7:3, 1:1 and 3:7 water:methanol solution mixtures (Menikpurage *et al.*, 2009). All extracts/fractions were subjected to rotary evaporation followed by freeze drying. In all cytotoxicity experiments, a negative control without *P. cystidiosus* extract/fraction and a positive control with 5 mM camptothecin (25 µL) were used.

Determination of reducing power:

a) DPPH free radical scavenging activity: The antioxidant activity of *P. cystidiosus* mushroom extracts/fractions was assayed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging method (Blois, 1958) with slight modifications.

A concentration series (1.0, 2.0, 5.0, 10.0, 20.0, 30.0 and 40.0 mg/mL) of water soluble samples were prepared using freeze dried *P. cystidiosus* extracts/fractions. Each solution of *P. cystidiosus* extract/fraction (50 µL) was added to a solution of DPPH reagent in ethanol (100 µM; 950 µL). The mixtures were incubated at room temperature for 30 min in the dark and the absorbance of the resulting solutions were measured at 517 nm. Controls were prepared using 50 µL de-ionized water and 950 µL DPPH reagent (A_{control}). Different concentrations of ascorbic acid (2.5–75 µg/mL) were used as the positive controls. The radical scavenging activity (%) was obtained using the following equation.

$$\text{Radical scavenging activity (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100 \% \quad \dots(1)$$

b) Nitric oxide radical scavenging activity: Nitric oxide generated from sodium nitroprusside (SNP) was measured using Griess reagent (1 % sulfanilamide and 0.1 % naphthylenediamine in 2 % H₃PO₄) according to Green *et al.*, (1982) and Sumanont *et al.*, (2004). SNP (100 mM) was prepared by dissolving SNP in phosphate buffered saline (PBS) at pH 7.4. The reaction mixture (2 mL) containing the mushroom extract/fraction (1 mL),

100 mM SNP (0.2 mL) and PBS (0.8 mL) was incubated at 25 °C for 3 h. A control experiment was conducted using 100 mM SNP (0.2 mL) and PBS (1.8 mL). After the incubation period, the sample (1 mL) was mixed with Griess reagent (1 mL) and the absorbance of the pink coloured azo dye was measured at 540 nm. The final concentrations of the extract/fraction in these solutions were 0.25–3.0 mg/mL. To exclude the interference of the other compounds in the extract/fractions with the absorbance value of nitrite detection, the absorbance of extract/fractions (1.0 mL) and PBS (1.0 mL) was also measured. The difference in the aforementioned absorbance values was used in the calculation of nitric oxide radical scavenging activity (A_{sample}) (equation 1).

Cytotoxic activity:

a) MTT assay:

Hep-2 cells (1×10^5 cells/well) were seeded in 24 well plates (NUNC, Denmark) and cultured over-night. The cells were treated with 6 different concentrations of 5 different *P. cystidiosus* mushroom extracts prepared in culture medium; namely, fractions A, A4, A4-1, A4-2 and A4-3 (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL) and incubated in a CO₂ incubator at 37 °C for 24 h. Culture media without the extracts were used as negative controls. The supernatant was replaced with MEM (0.5 mL) followed by sterile 5 mg/mL MTT (50 µL) and the cells were incubated (37 °C for 4 h) in the dark. The supernatant was removed carefully and the formed formazan crystals were fully dissolved in 700 µL of 0.05 M hydrochloric acid in isopropyl alcohol. It was centrifuged at 5000 x g for 5 min. The absorbance was measured at 570 nm (Fotakis & Timbrell, 2006) and the percentage cell viability was calculated using the following equation.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the negative sample}} \times 100 \%$$

...(2)

b) Lactate dehydrogenase (LDH) activity:

The LDH assay was performed (Fotakis & Timbrell, 2006) by measuring the reduction of pyruvate to lactate by lactate dehydrogenase. Hep-2 cells (2×10^5 cells/well) were seeded in 12 well plates (NUNC, Denmark) and left to adhere to the plastic plates over-night in humidified atmosphere in a carbon dioxide incubator (5 % CO₂) at 37 °C. Monolayers were treated with different concentrations of the *P. cystidiosus* extract A, fractions A4, A4-1, A4-2 and A4-3 (1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL) prepared in culture media and incubated in

a CO₂ incubator at 37 °C for 24 h. The supernatant was separated out and the cells were disrupted with 0.1 % triton X-100 (2 mL). LDH activity of the supernatant and the lysate was measured using the LDH assay kit.

Griess nitrite assay: The nitric oxide content in the supernatant obtained from the LDH assay was estimated using the Griess nitrite assay (Green *et al.*, 1982). The culture supernatant (300 µL) obtained in the LDH assay was incubated with Griess reagent (300 µL) at room temperature for 10 min. The absorbance was measured at 540 nm. The nitrite content was calculated based on a NaNO₂ standard curve.

Estimation of protein content: The protein content in the cell lysate obtained in the LDH assay was determined using Randox total protein kit.

Determination of reduced glutathione: The reduced glutathione (GSH) content in the cell lysate obtained in the LDH assay was also determined using the methods given in Weisburg *et al.* (2004) and Padma *et al.* (2007) with slight modifications. The proteins of lysate (500 µL) were precipitated with 2.5 % sulfosalicylic acid and centrifuged. To 500 µL of the clear supernatant, PBS (250 µL) and 5,5'-dithiobis-2-nitrobenzoic acid (50 µL) were added and the absorbance was measured immediately at 412 nm. A series of reduced glutathione standards (0.5 – 3 µg/mL) treated in a similar manner were also run to determine the reduced glutathione content in cell lysate.

c) Morphological determination:

The morphological changes of cells after the treatment with *P. cystidiosus* extract A, fractions A4, A4-1, A4-2 and A4-3 (1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL) over 24 h were detected by microscopic examination of cells. Photographs of morphological changes were compared with negative and positive controls.

In all cytotoxicity experiments, a negative control without the *P. cystidiosus* extract/fraction and a positive control with 5 mM camptothecin (25 µL) were used.

Statistical analysis: All experiments were performed in triplicate. The effective concentration of sample required to scavenge radicals by 50 % (EC₅₀) was obtained by linear regression analysis using the linear segment of the dose response curve plotted with radical scavenging activity (%) vs concentration of the mushroom extract/fraction. Graphical data were presented as mean ± standard error of the mean (SEM).

RESULTS

Determination of reducing power

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of *P. cystidiosus* extract A, fractions A4, A4-1, A4-2 and A4-3 was dose dependent and increased as the dose increased. The EC_{50} values of extract A, fractions A4, A4-1, A4-2 and A4-3 obtained were 1.12, 1.13, 0.87, 0.81 and 0.82 mg/mL, respectively (Figure 1). Ascorbic acid was used as the standard antioxidant and the EC_{50} value obtained for ascorbic acid was 0.044 mg/mL.

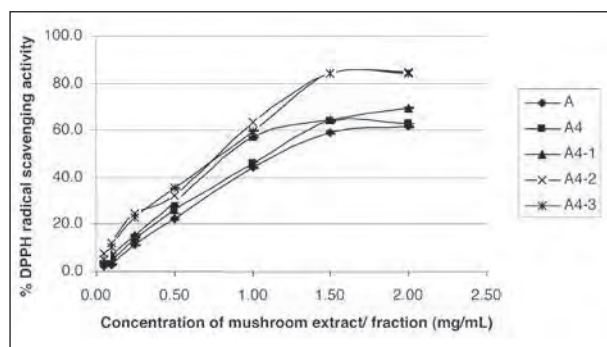


Figure 1: Percentage DPPH radical scavenging activity of *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3

Nitric oxide radical scavenging activity:

SNP in aqueous medium at physiological pH, spontaneously generates nitric oxide; it interacts with

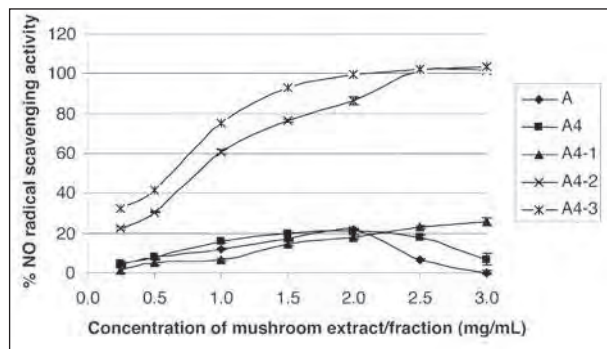


Figure 2: Nitric oxide radical scavenging activity (%) of *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3

oxygen to produce nitrite ions, which were estimated using Griess reagent. EC_{50} values for *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3 from the nitric oxide radical scavenging activity assay were 4.81, 3.82, 5.38, 0.87 and 0.61 mg/mL, respectively (Figure 2).

Cytotoxic activity

MTT assay

MTT assay was used to determine the cell viability of Hep-2 cancer cells. The percentage cell viability in the presence of *P. cystidiosus* samples A, A4, A4-1, A4-2 and A4-3 decreased as shown in Figure 3 and dose dependent declines in cell viability were observed in all the fractions except A4-1. The percentage cell viability (mean \pm SEM) of the positive control, camptothecin was 43.7 ± 2.0 %.

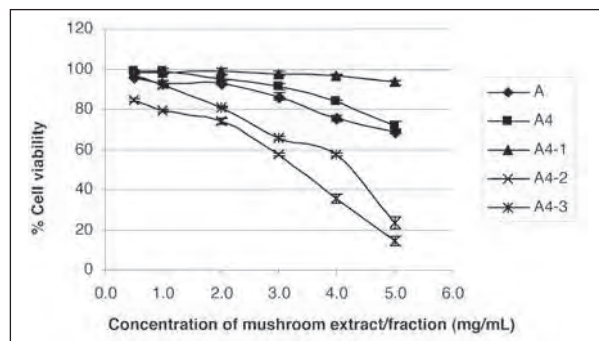


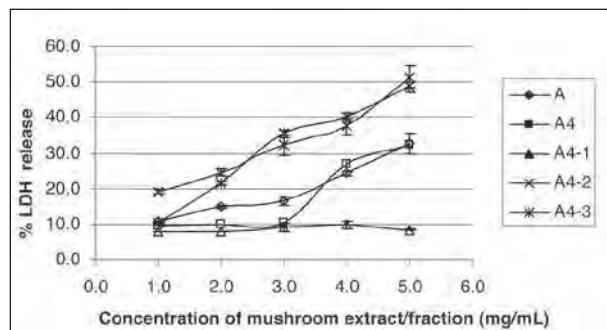
Figure 3: Percentage cell viability of Hep-2 cells induced by *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3 in MTT assay

Lactate dehydrogenase (LDH) activity

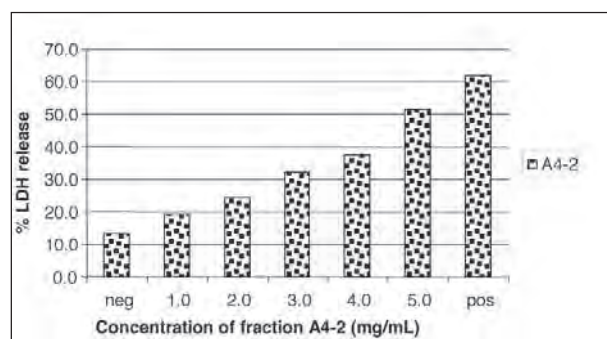
The NADH remaining in the mixture was used to calculate the lactate dehydrogenase released from Hep-2 cells. A dose dependent increase of LDH release (Figure 4) was observed with A, A4, A4-1, A4-2 and A4-3. Mean \pm SEM LDH release percentage of the positive control was 57.7 ± 3.9 %.

Griess nitrite assay

The nitrite level of A4-2 and A4-3 fractions treated cell supernatant obtained from the LDH assay resulted in dose independent decreases compared to the negative control (Figure 5). The nitrite levels of the other fractions were not dose dependent.



(a)



(b)

Figure 4: (a) Percentage of LDH release, which is induced by the exposure to *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3 (b) Percentage of LDH release at different concentrations of A4-2 fraction of *P. cystidiosus*

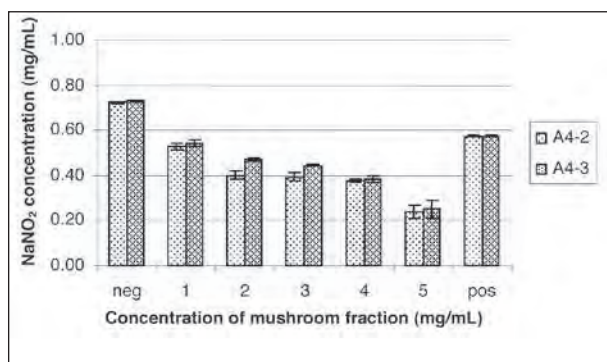


Figure 5: NaNO₂ content (mg/mL) in the supernatant after treating with *P. cystidiosus* fractions A4-2 and A4-3

Protein content

A dose dependent decrease in protein synthesis could be observed for A4, A4-2 and A4-3 fractions (Figure 6).

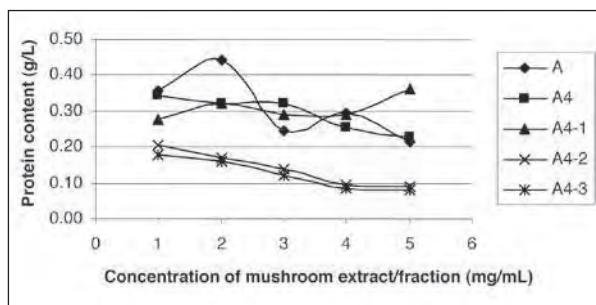


Figure 6: Protein content of lysate after treating with *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3

Reduced glutathione

Glutathione content of lysate after treating with *P. cystidiosus* extract A and fractions A4, A4-1, A4-2 and A4-3 did not show any dose dependent relationship (data not given).

Cell morphological changes

The Hep-2 cells treated with *P. cystidiosus* A, A4, A4-1, A4-2 and A4-3 preparations were observed using an inverted fluorescence microscope (Figure 7). The untreated cells appeared elongated in shape and were attached smoothly to the culture surface, forming a monolayer. The cells treated with *P. cystidiosus* A, A4, A4-2 and A4-3 had lost their integrity and appeared rounded. The cells treated with A4-2 and A4-3 showed higher numbers of dead cells with the increase in concentration.

DISCUSSION

Mushrooms have recently become important for drug development. Many investigators have isolated and identified antitumor compounds of mushroom origin such as lentinan, β-glucans (Mizuno *et al.*, 2001), lucidenic acids (Hsu *et al.*, 2008), and ergosterol peroxide (Kobori *et al.*, 2007). The results obtained in this study shows that the edible mushroom *P. cystidiosus* is a rich source of antioxidants and has cytotoxic activities against Hep-2 carcinoma cells.

DPPH radical scavenging activity and nitric oxide radical scavenging activity were measured to investigate the reducing power of *P. cystidiosus*. DPPH radical scavenging assay has been widely used to measure

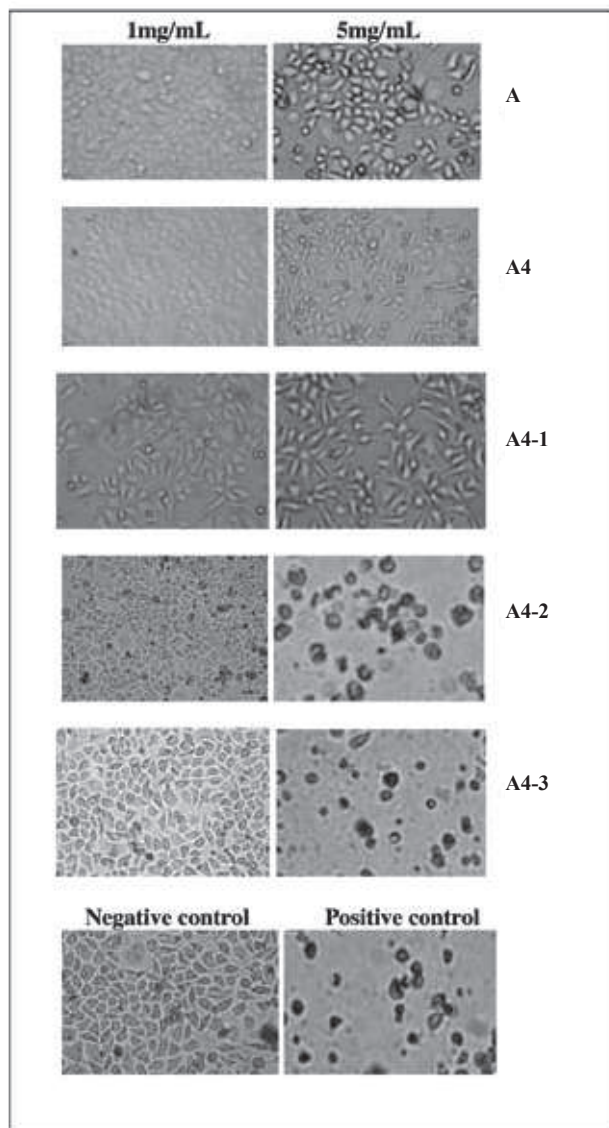


Figure 7: Morphological observation of Hep-2 cells after 24 hour exposure with acetone extract and derived subfractions of *P. cystidiosus* extracts. The cell morphology was observed using an inverted microscope at 200 \times magnification (Olympus IX 70). Cell death was indicated by cell shrinkage, cell disintegration and reduction in cell number. Positive control has the cells incubated with camptothecin (5 mM; 25 μ L).

radical scavenging activity. DPPH radicals in the presence of suitable reducing agents decrease the colour stoichiometrically, with the number of electrons consumed and can be measured spectrometrically (Govindarajan *et al.*, 2003). As shown in Figure 1, fractions A4-2 and A4-3 effectively scavenged DPPH

radicals with EC_{50} values of 0.81 and 0.82 mg/mL, respectively. The EC_{50} value of the standard ascorbic acid was 0.044 mg/mL.

Nitric oxide acts as a mediator of physiological processes such as neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity (Govindarajan *et al.*, 2003; Sumanont *et al.*, 2004). In the presence of nitrite ions formed by the oxidation of nitric oxide, sulfanilic acid is quantitatively converted to a diazonium salt under acidic conditions. Then the diazonium salt reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form the red pink azo dye, which can be spectrometrically measured at 540 nm. *P. cystidiosus* preparations facilitated nitric oxide scavenging activity in a dose dependent manner (Figure 2). As in the DPPH assay, fractions A4-2 and A4-3 showed high NO scavenging activities. The free radical scavenging activity and inhibition of nitric oxide production of *Trichoma matsutate* Sing. (pine mushroom) have been reported (Lim *et al.*, 2007). This supports the results obtained from our studies of DPPH and NO scavenging activities of *P. cystidiosus*.

To determine the possibility of using *P. cystidiosus* as an anticancer agent, cytotoxic effects, cell viability and antiproliferative activity were assayed using MTT and LDH assays.

The MTT assay (Fotakis & Timbrell, 2006) is based on the ability of metabolically active cells to reduce MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to blue formazan compounds by mitochondrial dehydrogenases. The reduction of absorbance in samples treated with mushroom extract/fraction reflects the reduction of the cell viability in the aforementioned samples. The observed reduction in cell viability is due to the cytotoxicity of the mushroom extracts towards the cells. Fraction A4-1 had no effect on cytotoxic assays. The cytotoxic effect of *P. cystidiosus* extract A on cell population growth of Hep-2 cells increased with the concentration. Fraction A4-2 and A4-3 had high activities. The cytotoxicities of the samples were A4-2 > A4-3 > A4 > A in a dose dependent manner (Figure 3). The 50 % cell viability of fractions A4-2 and A4-3 were 3.1 mg/mL and 3.6 mg/mL, respectively. Lucidenic acids (A, B, C and N) isolated from *Ganoderma lucidium* have shown decreased cell population growth of HL-60 carcinoma cells assessed with MTT assay (Hsu *et al.*, 2008).

In the LDH assay, the reduction of pyruvic acid to lactic acid is determined by measuring the NADH remaining in the reaction mixture (Lopez *et al.*, 2003).

The LDH release increased with the concentration of extracts, except in fraction A4-1. The highest LDH release was observed in the samples treated with A4-2 (Figure 4). The percentage LDH release with 5 mg/mL extract were 51.1 ± 1.5 , 48.7 ± 2.9 , 32.7 ± 3.0 and 32.2 ± 2.5 % for A4-2, A4-3, A and A4, respectively.

Overproduction of NO can mediate toxic effects such as DNA fragmentation, cell damage and neuronal cell death (Dawson *et al.*, 1992; Forrester *et al.*, 1996). In this study, decrease in nitric oxide generation was observed in the supernatants of A4-2 and A4-3 treated samples (Figure 5). This augments the results obtained for NO radical scavenging activities (Figure 2) in establishing the fact that extracts A4-2 and A4-3 are effective in reducing NO radical generation as well as scavenging the NO generated.

A dose dependant decrease in total protein content (Figure 6) was observed for A4, A4-2 & A4-3 fractions and this too supports the results obtained from cytotoxic assays.

Cell morphological changes were observed for Hep-2 cells treated with extracts A, A4, A4-1, A4-2, A4-3 in order to strengthen the results obtained from MTT and LDH assays. Cells incubated with the highest doses of A4-2 and A4-3 showed cell morphological changes such as cellular swelling, irregular cell shapes, condensed cytoplasm and vacuolar areas.

GSH plays an important role in protection of cells against oxidative stress. It reduces H_2O_2 directly to water, forming oxidized glutathione (Padma *et al.*, 2007). However, the GSH levels recorded did not show any relationship to the extract concentrations.

Antiproliferation and the removal of malignant cells through apoptosis have become a positive strategy in cancer prevention. In addition, many studies have demonstrated that antioxidative compounds play an important role in cancer prevention (Hou, 2003). In this study, *P. cystidiosus* fractions A4-2 and A4-3 demonstrated high antioxidant activities and antiproliferative activities. Thus, *P. cystidiosus* has promising activity as an anticancer agent.

Our previous studies have shown that consumption of *P. cystidiosus* (Kudahewa *et al.*, 2008) or *P. ostreatus* (Vasudewa *et al.*, 2007) may be beneficial as a remedy for body aches and pains arising due to daily activities. This study shows that extracts of *P. cystidiosus* has antioxidant and anticancer properties. We conclude that

the mushroom *P. cystidiosus* may have disease preventing and curing properties beyond the basic function of providing nutrients.

Acknowledgement

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