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ANTIOXIDANT AND ANTITYROSINASE ACTIVITIES OF MILKY WHITE MUSHROOM

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Abstract

It was intended to investigate the biological activities of different solvent extracts of the fruiting bodies of *Calocybe indica* (P & C). The bioactive compounds were extracted using acetone, methanol, and hot water. The antioxidant activities of three different extracts at 0.5-20.0 mg/ml of *C. indica* on β-carotene-linoleic acid ranged from 65.83 - 92.56, 62.79 - 93.06 and 61.42 - 92.12%, respectively. The highest (2.825) reducing power inhibition was recorded in methanolic extract, while the lowest (2.332) was in hot water at the concentration of 8 mg/ml. The free radicle scavenging activity was more pronounced in methanolic extract. Chelating effect of *C. indica* was significantly strong as compared to positive control. The xanthine oxidase and tyrosinase inhibitory activity of the acetone, methanol, and hot water extracts of *C. indica* increased with increasing concentration. Thirteen phenolic compounds i.e. biochanin-A, caffeic acid, chlorogenic acid, ferulic acid, formononetin, gallic acid, hesperetin, homogentisic acid, naringenin, naringin, protocatechuic acid, resveratrol and vanillin were detected from acetonitrile-hydrochloric acid extract. The result indicated that the maximum and minimum concentrations were recorded for gallic acid (29 µg/g) and formononetin (10 µg/g). Thus, it could be suggested that *C. indica* has the potential to be used as antioxidant and tyrosinase protection system of the human body against oxidative damage and other complications.

Introduction

Calocybe indica (P & C), known as milky white mushroom, is popular in Bangladesh. It is prefered for its attractive white fruiting bodies as well as unique taste and flavor. On the other hand, low cost materials like agricultural wastes can be used as substrate for the production of this mushroom (Amin *et al.* 2010). Milky white mushrooms are rich in protein, edible fiber and minerals but lipid content is low. It is reported as therapeutic food as well as for preventing hypertension and hypercholesterolemia (Alam *et al.* 2008).

Edible mushrooms are good source of antioxidants that help to reduce oxidative damage in human body. Polyphenolic compounds have aptitude to scavenge free radicals by single-electron transfer. Thus, it is recognized as an excellent antioxidant (Diplock *et al*. 1998).

Xanthine oxidase (XO) is responsible for gout through the secretion of uric acid. Mushrooms have XO inhibitory actions with lesser side effects compared to uricosuric and anti-inflammatory agents. Thus, edible mushrooms are the source of such potential compounds (Zhou *et al*. 2001). In human physiology, melanin formatiom may be influenced by antioxidation, tyrosinase inhibition, and hormonal activities, etc. (Alam *et al.* 2012). Tyrosinase not only incorporates menalin production but also might be responsible for malignant cancer. Again, in whitening enhancement is due to the inhibition of tyrosinase activities (Sugimoto *et al*. 2004, Alam *et al.* 2011). Consequently, mushroom tyrosinase is popular among researchers due to its easy availability. In

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spite of the medicinal potential of *C. indica* has not been studied for its beneficial biochemical components. In addition, antioxidant activities, xanthine oxidase and and tyrosinase inhibitory properties of this mushroom are not available. Hence, the present investigation was undertaken to appraise the antioxidant, phenolic compound, xanthine oxidase activities and tyrosinase inhibitory effects with three different extracts of milky white mushroom.

Materials and Methods

Fresh fruiting bodies of *Calocybe indica* were collected from National Mushroom Development and Extension Centre (NAMDEC), Savar, Dhaka, Bangladesh. The fruiting bodies were dried through hot air at 40°C for 48 hrs and pulverized. Five g of each powdered sample were mixed with 100 ml of 60% aqueous acetone and 80% aqueous methanol stirring at 150 rpm for 24 hrs at 25°C to obtain acetone and methanol extracts. The extracts were filtered through two layers of Whatman No. 1 filter paper. To get a hot water extract the same quantity of sample was boiled at 100°C for 3 hrs with 100 ml deionized distilled water. The mixture was cooled at room temperature and filtered. The residues were then extracted with two additional 100 ml aliquots of acetone, methanol, and deionized water, as described above. Then combined extracts were evaporated with a rotary evaporator (Eyela, Saitama, Japan) at 40°C, and the remaining solvent was removed with a freeze-drier (Optizen, Daejeon, Korea). The yield from the 60% acetone, 80% methanol and hot water extraction of *C. indica* was 24.84, 26.44 and 20.56% (w/w), respectively.

Antioxidant activity of each extract against β-carotene-linoleic acid of *C. indica* in four different concentrations i.e. 0.5, 2.0, 8.0 and 20.0 mg/ml was determined according to the procedure described by Alam *et al.* (2012). Butylated hydroxytoluene (BHT) and α-tocopherol (TOC) were used as positive controls at 0.5 mg/ml and a blank consisting of 0.5 ml methanol. The mixture was measured by spectrophotometer (Optizen) at 490 nm.

Four different concentrations such as 1, 2, 4, and 8 mg/ml of acetone, methanol and hot water extracts of *C. indica* were used for the determination of reducing power according to the method of Gülçin *et al.* (2003). BHT and TOC were used as positive controls.

For the determination of DPPH radical scavenging activity, 0.125, 0.25, 0.5, 1.0, and 2.0 mg ml^{-1} concentrations of acetone, methanol, and hot water extracts of *C. indica* were used according to the method of Cuendet *et al.* (1997). The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. BHT, TOC and L-ascorbic acid were used as positive controls.

Five different concentrations i.e. 0.63, 0.125, 0.250, 0.5 and 1.0 mg/ml of acetone, methanol and hot water extract of *C. indica* were used for the determination of chelating effect according to the method of Dinis *et al.* (1994). The absorbance of the solution was measured by spectrophotometer at 562 nm. using BHT and TOC as positive controls.

Xanthine oxidase (XO) inhibitory activity in four different concentrations i.e. 0.5, 1.0, 2.0, 4.0, and 8.0 mg/ml of acetone, methanol, and hot water extracts from the fruiting bodies of *C. indica* was assayed spectrophotometrically under aerobic conditions according to the methods of Alam *et al.* (2012). Allopurinol was used as positive control, which was known as inhibitor of XO.

Tyrosinase inhibition activity in 0.125, 0.250, 0.5, and 1.0 mg/ml concentration of acetone, methanol, and hot water extracts from the fruiting bodies of *C. indica* were measured by dopachrome method with L-DOPA as the substrate (Masuda *et al*. 2005). L-ascorbic acid and kojic acid were used as positive controls.

Two g powders of milky white mushrooms were extracted with 10 ml of acetonitrile and 2 ml of 0.1N hydrochloric acid. Sample preparation for the phenolic compound analysis followed the method of Kim *et al*. (2008). Then 20 µl filtrate was loaded onto an Agilent-1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a 250 nm × 4.6

mm i.d., 5 µm, YMC-Pack ODS AM (YMC Co. Ltd., Kyoto, Japan) column. Fifteen standard compounds such as biochanin-A, caffeic acid, (+) catechin, chlorogenic acid, ferulic acid, formononetin, gallic acid, hesperetin, homogentisic acid, naringenin, naringin, protocatechuic acid, pyrogallol, resveratrol and vanillin were used as standards. Detection was performed with a diode array detector at a wavelength of 280 nm.

The data from all experimental findings have been expressed as means ± standard deviations (Sd). Inter group differences were analyzed by one-way analysis of variance followed by DMRT. Differences were considered as significant at $p \leq 0.05$.

Results and Discussion

β-carotene-linoleic acid from acetone, methanol and hot water extracts from the fruiting bodies of *Calocybe indica* ranged 65.83 - 92.56%, 62.79 - 93.06% and 61.42 - 92.12%, respectively (Table 1). Similar results were recorded from the edible mushrooms, *Leucopaxillus giganteus*, *Sarcodon imbricatus* and *Agaricus arvensis* (Barros *et al*. 2007) and their antioxidant activities were 61.4, 54.3 and 46.7% at 5 mg/ml, while antioxidant activity of positive control, tertiary butylhydroquinone (TBHQ) reached 82.2% at 2 mg/ml in methalone extract. The results suggested that the antioxidant activity on β-carotene-linoleic acid from the fruiting bodies of *C. indica* was highly promising as compared to those mentioned above. It is clear that the mushroom extracts reduced the β-carotene linoleate free radical and other free radicals, which is likely the result of volatile solvent extracts from several commercial and medicinal mushrooms (Mau *et al*. 2004).

Table 1. Antioxidant activity against β-carotene-linoleic acid at different concentrations of various extracts from the fruiting bodies of *Calocybe indica*.

Sample	Concentration (mg/ml)			
	0.5	2.0	8.0	20.0
Acetone extract	65.83 ± 0.24	85.94 ± 0.43	89.02 ± 0.32	92.56 ± 0.41
Methanol extract	62.79 ± 0.19	80.25 ± 0.24	89.12 ± 0.21	93.06 ± 0.24
Hot water extract	61.42 ± 0.45	54.92 ± 0.36	83.62 ± 0.23	92.12 ± 0.19
BHT	95.21 ± 0.17	-	-	-
TOC	96.02 ± 0.18	-	-	-

A value expressed as means ± Sd (n = 3). -, not analyzed, BHT- butylated hydroxytoluene and TOC - α-tocopherol.

Table 2 shows the result of reducing power inhibition of *C. indica* in three different extracts at selective concentrations. The maximum reducing power inhibition (2.825) was recorded in methanol extract, while lowest (2.332) was observed in hot water extract at 8.0 mg/ml concentration. The results indicated that the reducing power inhibition of milky white mushroom is very low as compared to positive control, where the reducing power of BHT and TOC at 1.0 mg/ml were 3.21and 2.16.

According to the report of Lee *et al*. (2007) the reducing power of three edible mushrooms, *Hypsizygus marmoreus*, *Agricus bisporus* and *Pleurotus ferulae* were 0.99, 0.76 and 0.70 at 5 mg/ml concentration. Consequently, the reducing power of *C. indica* was significantly higher and effective than the above mentioned mushrooms. Generally, the reducing properties are associated with the presence of reductones that exert antioxidant by breaking the free radical chain donating a hydrogen atom (Barros *et al*. 2007, Alam *et al*. 2012).

Table 2. Reducing power of different extracts from the fruiting bodies of Calocybe indica.

Sample	Concentration (mg/ml)			
	1.0	2.0	4.0	8.0
Acetone extract	0.622 ± 0.03	1.125 ± 0.02	1.581 ± 0.05	2.628 ± 0.06
Methanol extract	0.654 ± 0.04	1.318 ± 0.15	1.610 ± 0.15	2.825 ± 0.03
Hot water extract	0.515 ± 0.02	1.012 ± 0.02	1.310 ± 0.04	2.332 ± 0.08
BHT	3.212 ± 0.49	-	-	-
TOC	2.162 ± 0.32	-	-	-

A value expressed as means ± Sd (n = 3). - Not analyzed, BHT- Butylated hydroxytoluene and TOC- α-tocopherol.

DPPH radical scavenging activity of acetone, methanol and hot water extracts from the fruiting bodies of *C. indica* ranged 33.71 - 91.75, 31.75 - 87.46, and 25.41 - 83.55%, respectively (Fig. 1). The results indicated that DPPH scavenging activity of each extract was increased with increasing concentration. The acetone, methanol, and hot water extracts of *C. indica* showed good, moderate, and poor activities, respectively at the concentration tested. BHT, TOC and L-ascorbic were used as positive controls which showed excellent scavenging activities ranging from 85.25 - 98.74, 67.37 - 97.78, and 96.74 - 98.23%, respectively at the concentration of 0.125 - 2.0 mg/ml.

Ethanolic extract from the fruiting bodies of *H. marmoreus* and *A. bisporus* on DPPH radicals showed the scavenging activities that ranged 46.6 - 68.4% at 5 mg/ml (Lee *et al.* 2007). The scavenging activities both cold and hot water extracts of the fruiting bodies and mycelia at 20 mg/ml were 20.7 - 52.3 and 37.6 - 48.3, respectively. It revealed that the fruiting body of *C. indica* had more scavenging properties than those of the above mentioned edible mushrooms. Peroxy radicals are the major propagators of the autoxidation chain of fat, might have reacted by various extracts with free radical and thereby terminating the chain reaction (Frankel 1991). The natural antioxidant also showed the termination of free radical reaction.

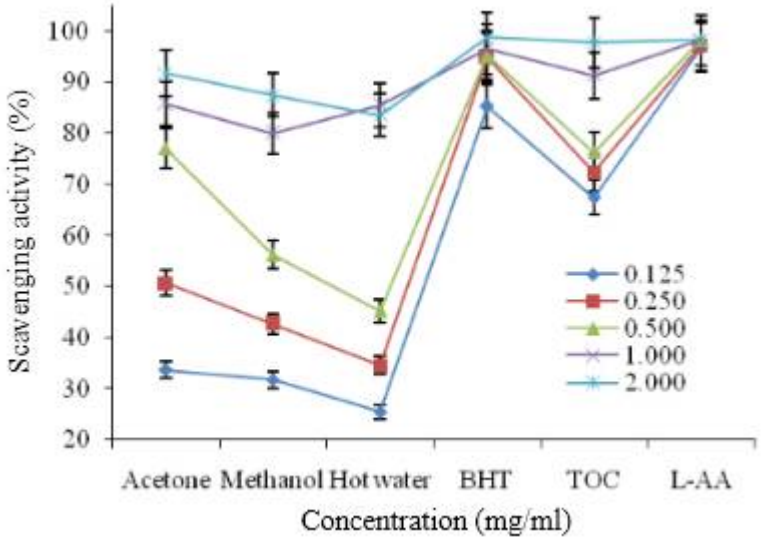

Fig. 1. Scavenging activity of various extracts from the fruiting bodies of *Calocybe indica*. A value expressed as means ± Sd (n = 3). BHT- butylated hydroxytoluene, TOC- α-tocopherol and L-AA-L-ascorbic acid.

The chelating effect on ferros ions of acetone, methanol and hot water extracts from the fruiting bodies of *C. indica* at five different concentrations have been presented in Fig. 2. BHT and TOC were used as positive control for the investigation of ferrous ions. The results showed that the chelating aptitude of the tested extracts was increased with increasing concentration. The maximum chelating activity on ferrous ions (87.10%) was recorded in methanol extract, while the lowest effect (81.55%) was observed in TOC and hot water extract at the concentration of 1.0 mg/ml.

Chelating ability 42.6 and 45.8% were found in hot water extracts from the fruiting bodies of *G. tsugae* and *A. cylindracea* at the concentration of 20 mg/ml (Tsai *et al*. 2006). It refered that *C. indica* in ferrous ions had the same chelating abilities as that of *H. marmoreus*, while it showed more effectiveness than that of *G. tsugae* and *A. cylindracea*. Chelating agents may act as secondary antioxidants as they reduce the redox potential and thereby stabilizing the oxidized form of the metal ions. The various extracts from the fruiting bodies of *C. indica* has high ferrous ions chelating abilities. Therefore, it would be beneficial because ferrous ions are the most effective pro-oxidants in food system.

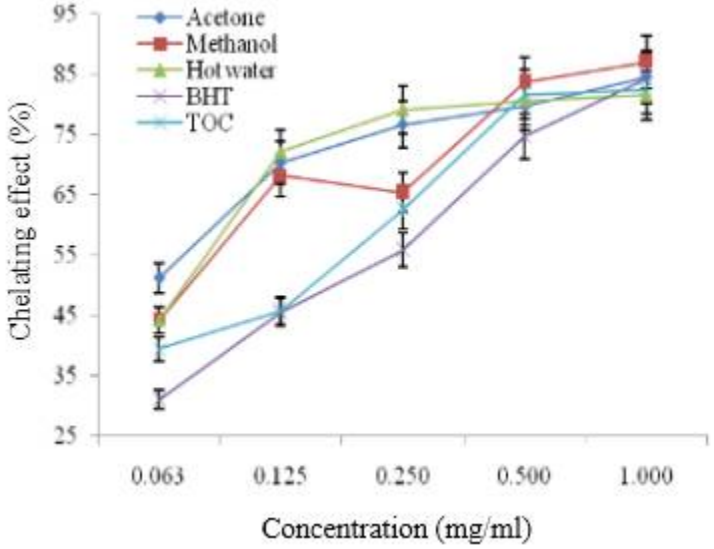

Fig. 2. Chelating effect of various extracts from the fruiting bodies of *Calocybe indica*. A value expressed as means ± Sd (n = 3). BHT- butylated hydroxytoluene and TOC-α-tocopherol.

Thirteen phenolic compounds such as biochanin-A, caffeic acid, chlorogenic acid, ferulic acid, formononetin, gallic acid, hesperetin, homogentisic acid, naringenin, naringin, protocatechuic acid, resveratrol and vanillin were detected from the fruiting bodies of *C. indica* (Fig. 3). Among the total concentration (225 µg/g) of phenolic compound, the highest and lowest concentrations were recorded for gallic acid (29 µg/g) and formononetin (10 µg/g), respectively. Similar findings were observed in some edible mushrooms (Kim *et al*. 2008) in which total concentration of phenolic compounds was 174 µg/g. Phenolic compounds were present in different edible mushrooms in varying numbers ranging from 3 - 15 and among them gallic acid is the most common. Thus, phenolic compounds would be used as an important indicator of antioxidant potentiality. There is a close relationship between antioxidant activity and phenolic content (Yoon *et al*. 2011). High levels of phenolic compounds were detected from the various edible and medicinal mushrooms

that composed of one or more aromatic rings bearing one or more hydroxyl groups and exhibit extensive free radical-scavenging activities as hydrogen donors or electron-donating agents, and metal ion-chelating properties.

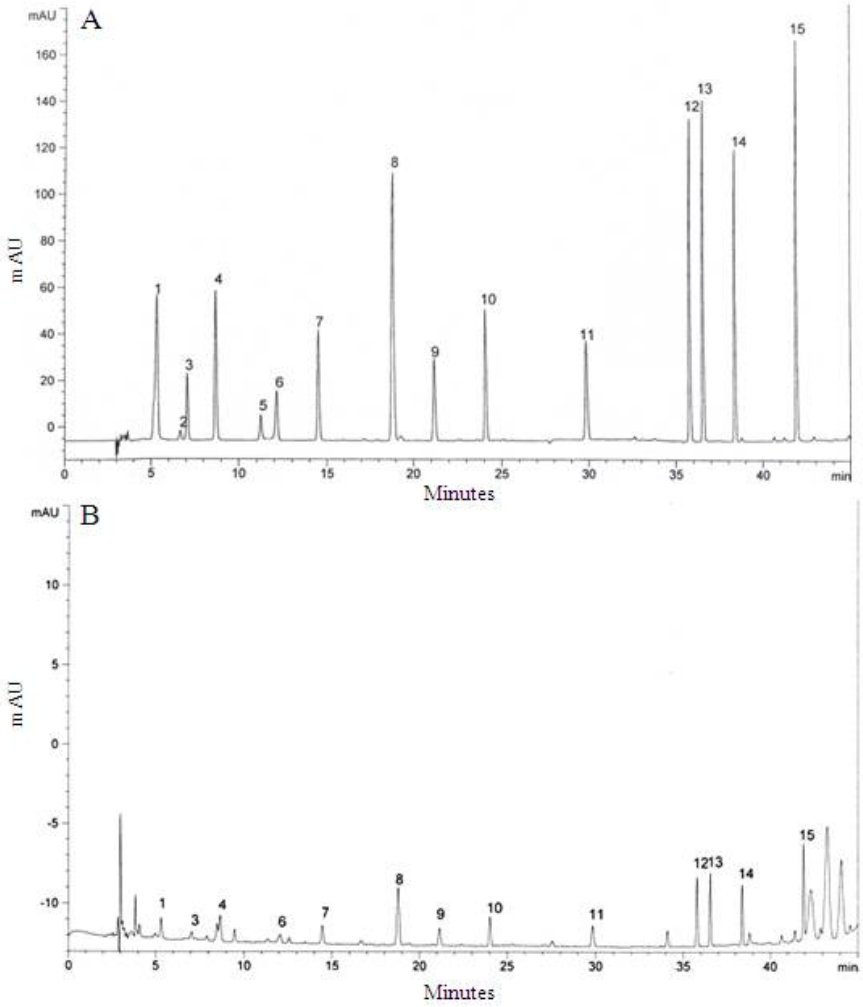

Fig. 3. High performance liquid chromatography of phenolic compounds. A - Standard mixture of 15 phenolic compounds, B - *Calocybe indica* extract. 1 - gallic acid, 2 - pyrogallol, 3 - homogentisic acid, 4 - protocatechuic acid, 5 - (+) catechin, 6 - chlorogenic acid, 7 - caffeic acid, 8 - vanillin, 9 - ferulic acid, 10 - naringin, 11 - resveratrol, 12 - naringenin, 13 - hesperetin, 14 - formononetin and 15 - biochanin-A.

Xanthine oxidase inhibitory activities of acetone, methanol and hot water extracts from the fruiting bodies of *C. indica* ranged 3.25 - 46.38, 2.76 - 46.09 and 1.78 - 45.03%, respectively at the concentration of 0.5 - 8.0 mg/ml. However, allopurinol showed the excellent xanthine oxidase inhibitory activity of 92.31 - 94.58% at the same concentrations. The results indicated that xanthine oxidase inhibitory activities of each extract increased with increasing concentration (Fig. 4). It revealed that the acetone and methanol extracts showed good results, while hot water extract revealed average activities at the tested concentration. Flavonoids are a group of polyphenolic

compounds that possess xanthine oxidase inhibitory properties (Costantino *et al*. 1992). Thus, phenolic and flavonoid type of compounds in the extract would have contribution towards xanthine oxidase inhibition. Therefore, at the higher doses of extract, XO would be significantly inhibited.

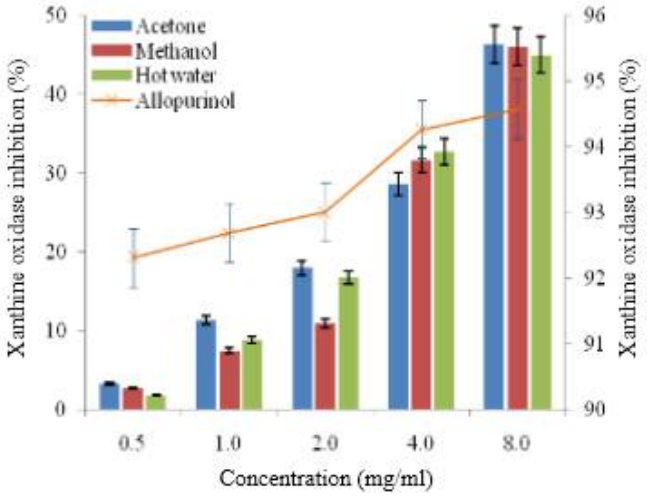

Fig. 4. Xanthine oxidase inhibition activity of various extracts from the fruting bodies of *Calocybe indica*. A value expressed as means ± Sd (n = 3).

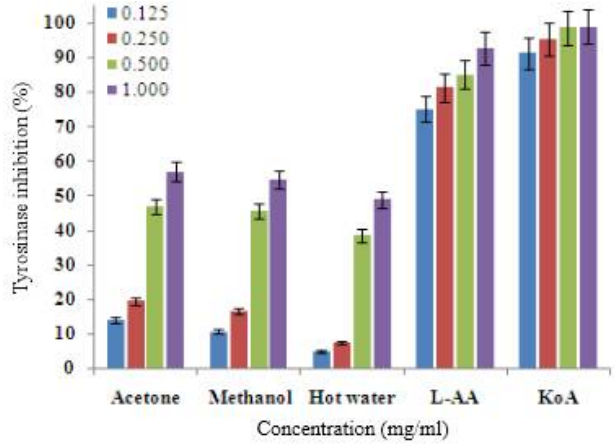

Fig. 5. Tyrosinase inhibition activity of various extracts from the fruiting bodies of *Calocybe indica*. A value expressed as means ± Sd (n = 3). L-AA- L-ascorbic acid and KoA- kojic acid.

Tyrosinase inhibitory activities of acetone, methanol, and hot water extracts from the fruiting bodies of *C. indica* ranged from13.90 - 56.87, 10.60 - 54.59 and 4.86 - 48.82%, respectively (Fig. 5). The results indicated that the tyrosinase inhibitory activities of each extracts were increased with increasing concentration and the acetone and methanol extracts showed good, while hot water showed moderate activities at the concentration tested. L-ascorbic acid and kojic acid used as positive control, showed the excellent tyrosinase inhibitory activities ranging from 75.12 - 92.74 and 91.23 - 99.00%, respectively at the concentration of 0.125 - 1.0 mg/ml.

The hydroxyl groups might be responsible for the inhibition of tyrosinase activities on the phenolic compounds of mushroom extracts that may form hydrogen bonds with an enzyme site leading to lower enzymatic activity. Some tyrosinase inhibitors bind to the tyrosinase active site through hydroxyl group, resulting in steric hindrance or altered conformation (Baek *et al*. 2008, Kim *et al*. 2008). Again, gallic acid acts as effective inhibitors of tyrosinase activity (Kubo *et al*. 2003, Momtaz *et al*. 2008). Moreover, the antioxidant activity may be one of the most important mechanisms for tyrosinase inhibitory activity. The experimental findings showed that *C. indica* have higher chelating effects than that of BHT and TOC on ferrous ions. Thirteen phenolic compounds were detected from the fruiting bodies of *C. indica*. The high phenolic content exhibited good antioxidant and antityrosinase activities. Therefore, the fruiting bodies of milky white mushroom may be suggested as a natural food source of antioxidants.

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