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# Antimicrobial, Antioxidant and Cytotoxic Properties of *Hypsizygus tessulatus* Cultivated in Bangladesh

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## ABSTRACT

The aim of the present study was to evaluate certain biological properties of *Hypsizygus tessulatus*. Metabolic extract of submerged culture and mycelia mats of *H. tessulatus* mycelium was taken for investigating cytotoxic and antimicrobial activity using brine shrimp lethality bioassay and disc diffusion technique, respectively. Ethyl acetate extract showed higher antibacterial activity than chloroform extract against the Gram positive and Gram negative bacteria. The average zone of inhibition for ethyl acetate extract was in the range of 17-22 mm. Ethanol extract of mycelia mats of *H. tessulatus* showed significant antioxidant property using 1,1-diphenyl-2-pecrylhydrazyl (DPPH) scavenging assay along with total phenol content demonstrated moderate activity with $IC_{50}$ value of 105 µg $mL^{-1}$ and 36.45±1.754 g/100 g, respectively. The total antioxidant capacity of *H. tessulatus* was 442.61 mg equivalents of ascorbic acid. In brine shrimp lethality bioassay, $LC_{50}$ values obtained from the best fit line slope were 7.36 and 10.63 µg $mL^{-1}$ for ethyl acetate and chloroform extract, respectively. The study reveals that *H. tessulatus* mushroom has potential antimicrobial effect with moderate anticancer and antioxidant activity.

**Key words:** Shimeji, *Hypsizygus tessulatus*, antioxidant, *Hypsizygus marmoreus*, antimicrobial activity

## INTRODUCTION

Mushrooms have become attractive as a functional food and as a source for the development of drugs and nutraceuticals (Barros *et al*., 2007). Mushroom cultivation presents an economically important biotechnological industry that has expanded markedly all over the world in the past few decades. Major medicinal properties attributed to mushrooms include anticancer, antibiotic, antiviral activities, immune response stimulating effects and blood lipid lowering effects (Bobek and Galbavy, 2001). The add-value arising from mushrooms are bioactive materials which lead to an increase in its consumption and therefore, stimulate the commercialization of edible species. Mushrooms also have been used extensively in traditional medicine for curing various types of diseases such as viral infection, bacterial infection, cancer, tumor, inflammation, cardiovascular diseases (Benedict and Brady, 1972; Iwalokun *et al*., 2007). Both fruiting body and mycelium of different mushrooms contain different compounds such as terpenoids, steroids, polyphenol,

polyketides, polyglucan, flavonoids, alkaloids, polysaccharides and dietary fibers which exert several pharmacological activities (Breene, 1990). They are the rich source of antioxidants, antibiotics and antineuplastic activity (Akyuz and Kirbag, 2009).

*Hypsizygus tessulatus* which is commonly known as Shimeji mushroom contains glucans, niacin, vitamin B and D. Glucans are effective for cholesterol control, immunity, constipation and weight loss. Niacin is effective for insomnia, mental health and blood circulation. Vitamin B aids in immunity and weight loss, while vitamin D is deemed to be effective against osteoporosis and anti-aging (Ikekawa, 2001). The previous name of this mushroom was *Hypsizygus marmoreus* and it also contain different biologically active compounds like polysaccharides, l-ergothioneine, sterols, ergosterol - provitamin $D_2$ which have different medicinal uses (Tsuchida *et al.*, 1995; Saitoh *et al.*, 1997; Matsuzawa *et al.*, 1998). *Hypsizygus tessulatus* showed anti-platelet activity which may involved in the suppression of intracellular calcium mobilization and integrin αIIbβ3 activation (Park *et al.*, 2011). *Hypsizygus* is a small genus of fungi that are widely distributed in north temperate regions. The mushroom also has been reported to have beneficial effects on skin conditions. *Hypsizygus tessulatus* is one of the valued species of traditional medicine. *Hypsizygus tessulatus* is a small mushroom with light brown or white umbrella- shaped caps. They are firm and crunchy in texture and they have a delicate mild, sweet flavor (Waites *et al.*, 2001).

The history of mushroom cultivation is not so long in Bangladesh; however, mushroom consumption is increasing rapidly in this country due to cheap source of dietary protein, carbohydrate, vitamins and minerals. Several species of mushroom are being cultivated in Bangladesh among which some are of medicinal importance (Amin and Alam, 2008). These are reishi, enoki, oyster, shiitake, shimeji, milky, beech and nameko etc. (Imtiaj and Rahman, 2008). The aim of this study was to evaluate the antibacterial, cytotoxic and antioxidant properties of *Hypsizygus tessulatus* different extract both from submerged culture and mycelia mat.

## MATERIALS AND METHODS

**Mushroom:** *Hypsizygus tessulatus* were collected from National Mushroom Development and Extension Centre, Dhaka. The mushroom was obtained as potato dextrose agar slants. This mushroom was reported by the supplier to grow at 25°C. The species was maintained on agar slants at 4°C and sub-cultured every twelve weeks according to the supplier's recommendation.

**Subculture and media composition:** Three mycological media were used to evaluate the optimum growth of the organism. The organism was sub-cultured on potato dextrose agar or broth (PDA/PDB), malt-yeast extract (MY) and glucose-yeast extract peptone (GPY) media. Potato dextrose and glucose-yeast extract peptone media were sterilised at 121°C for 15 min whereas, malt-yeast extract medium was sterilised at 115°C for 10 min. All these media were purchased from Oxiod and Difco, UK.

**Collection of study materials:** The *Hypsizygus tessulatus* was culture in potato dextrose broth (PDB) medium, as maximum growth was observed in this medium (data not shown). The submerged culture was divided into two parts after 10 days of incubation at 28°C. The mycelia which formed a mat like structure was separated by filtration (Whatman No.1) and both parts were collected for further study. The isolated mats were air dried in an oven at 40°C before analysis and pulverized to fine powder by using motor and pestle. Dried mat (40 g) was extracted by stirring with 300 mL of ethanol at 30°C at 150 rpm for 24 h and filtering through Whatman No. 1 filter

paper. The residue was then, extracted with two additional 300 mL of ethanol as described above. The combined ethanolic extract was then rotary evaporated at 40°C to dryness.

The culture filtrate (200 mL) from submerged culture was transferred to a separating funnel (500 mL) and extracted with chloroform and ethyl acetate, respectively. The extracts thus obtained were evaporated under reduced pressure in a rotary evaporator at 45°C to dryness and stored at 4°C for further use. These extracts were used for antimicrobial and cytotoxicity study on the other hand, mycelia mat extracts were used for antioxidant study. The reason for using two different parts for separate biological activity was to observe if both metabolite and mushroom itself is active in biological activity.

**Antibacterial and antifungal screening:** Both antibacterial and antifungal activities of chloroform and ethyl acetate extract were observed by disc diffusion assay (Bauer *et al*., 1966; Barry, 1976). A total of four Gram positive and three Gram negative bacteria (Table 1) were used in this antimicrobial screening. Chloroform and ethyl acetate extract (400 and 500 μg disc$^{-1}$) were prepared by dissolving with chloroform and ethyl acetate, respectively. To compare the antibacterial activity, kanamycin (30 μg disc$^{-1}$) was used as standard antibiotic. As a negative control, blank discs impregnated with solvents followed by drying off were used. The antifungal activity of the chloroform and ethyl acetate extract were tested against four pathogenic fungi (*Candida albicans*, *Aspergillus niger*, *Aspergillus flavus* and *Epidermophyton floccosum*) at a concentration of 400 μg disc$^{-1}$. Potato Dextrose Agar (PDA) media used for this purpose. The activity was determined after 72 h of incubation at room temperature (37°C). Nystatin was used as standard at a concentration of 30 μg disc$^{-1}$.

**Total phenolic content:** Total phenolic content of *Hypsizygus tessulatus* extract was measured by the method of Skerget *et al*. (2005). In this method, Folin-Ciocalteu reagent acts as an oxidizing agent and gallic acid as a standard. Briefly, 0.5 mL of extract solution (2 mg mL$^{-1}$) in water was taken and 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 mL of sodium carbonate (7.5% w/v) solution were added. The mixture was then incubated for 20 min at room temperature and after that period absorbance was measured at 760 nm. Total phenolics were quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100 μg mL$^{-1}$). The phenolic contents of the sample were expressed as gm of GAE (gallic acid equivalent)/100 gm of the dried extract.

**DPPH radical scavenging assay:** The antioxidant activity of the extracts on the stable radical 1, 1-diphenyl;-2-picrylhydrazyl (DPPH) was determined by the method developed by Feresin *et al*. (2002). The 0.1 mL of mushroom ethanol extract, at various concentrations was added to 3 mL of a 0.004% methanol solution of DPPH and was allowed to stand for 30 min for the reaction to occur. The absorbance of the resulting solution was measured at 517 nm from this values the corresponding percentage of inhibitions were calculated by using the following equation:

$$(I\ \%) = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

Where, $A_{blank}$ is the absorbance of the control reaction (containing all reagents except the test sample) and $A_{sample}$ is the absorbance of sample/standard.

Extract concentration providing 50% inhibition ($IC_{50}$) was calculated from the graph plotted I% versus concentration curve. The free radical scavenging activity was measured for different concentrations of sample and compared with standard (Ascorbic acid).

**Determination of total antioxidant capacity (TAC):** The total antioxidant capacity was evaluated using the method described by Prieto *et al.* (1999). Ascorbic acid was used as the standard antioxidant drug. Three millilitter of the extract/standard drug (0.1, 0.3, 1 and 3 mg $mL^{-1}$) was placed in a test tube. The 0.3 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was then added and the resulting mixture was incubated at 95°C for 90 min. After the mixture has cooled to room temperature, the absorbance of each solution was measured in triplicate using the UV-visible spectrophotometer at 695 nm against a blank. The total antioxidant capacity was expressed as the number of gram equivalents of ascorbic acid.

**Cytotoxicity screening:** For cytotoxicity screening, DMSO solutions of the compounds were applied against *Artemia salina* for 24 h *in vivo* assays (Meyer *et al.*, 1982). The eggs of the brine shrimp, *Artemia salina*, were collected from a local aquarium shop and hatched for 48 h to mature shrimp called nauplii. The test samples were prepared by dissolving them in DMSO (not more 3.8% NaCl in water) to attain concentrations 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563 and 0.781 µg $mL^{-1}$. A vial containing 50 µL DMSO diluted to 5 mL was used as a control. Then 20 matured shrimps were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 h was counted. The findings were presented graphically by plotting log of concentration versus percentage of mortality of nauplii from which $LC_{50}$ was determined by extrapolation and compared with the positive control using vincristine sulphate. The assay was performed in duplicate and the result was calculated as an average of two determinations.

**Statistical analysis:** Three replicates of each sample were used for statistical analysis and the values were reported as Mean±SEM. Correlation analysis of free radical scavenging activity versus total phenolic content and cytotoxicity were carried out using the correlation and regression program.

## RESULTS AND DISCUSSION

*Hypsizigus tessulatus* has recently become a popular edible mushroom in Asia and has been cultivated by culture methods such as bottle culture and bag-culture. In the present study, we use submerged culture for growth instead of solid-state fermentation and as far as we concern this is the first report of biological activity of *Hypsizygus tessulatus* grown in submerged culture. This mushroom has been reported to exhibit many biological activities such as anti-tumor, antioxidant and antiproliferative activity when grown in solid culture.

The results of antibacterial activity are presented in Table 1. The fractions (chloroform and ethyl acetate) showed reasonable antibacterial activity against pathogenic bacteria. However, ethyl acetate was more active than chloroform extract against both Gram positive and Gram negative bacteria. The antimicrobial activity increases gradually as dose is increased (Table 1). Ethyl acetate extract (500 µg $disc^{-1}$) showed highest activity towards every pathogen especially *Shigella dysenteriae* (22 mm) and *Staphylococcus aureus* (21 mm) compare to standard. The chloroform fraction exhibited moderate activity towards most of the pathogen and the highest zone of

Table 1: Antibacterial activity of chloroform and ethyl acetate extract of *H. tessulatus* metabolite

| | Diameter of zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|
| Test organism | Ethyl acetate (400 μg disc$^{-1}$) | Ethyl acetate (500 μg disc$^{-1}$) | Chloroform (400 μg disc$^{-1}$) | Chloroform (500 μg disc$^{-1}$) | Kanamycin (30 μg disc$^{-1}$) |
| **Gram positive** | | | | | |
| *Staphylococcus aureus* | 17.0±0.3 | 21.0±0.1 | 8.0±0.7 | 9.0±0.5 | 27.0±0.4 |
| *Staphylococcus agalactiae* | 16.0±0.2 | 20.0±0.3 | 7.0±0.4 | 8.0±0.8 | 28.0±0.8 |
| *Bacillus cereus* | 17.0±0.1 | 18.0±0.7 | 8.0±0.3 | 9.0±1.3 | 28.0±1.5 |
| *Bacillus subtilis* | 18.0±0.3 | 19.0±1.2 | 10.0±0.1 | 12.0±1.5 | 27.0±1.2 |
| **Gram negative** | | | | | |
| *Pseudomonas aeruginosa* | 19.0±0.6 | 20.0±0.8 | 8.0±0.7 | 10.0±0.7 | 29.0±1.3 |
| *Escherichia coli* | 17.0±0.5 | 18.0±0.9 | 7.0±0.6 | 9.0±0.8 | 26.0±0.8 |
| *Shigella dysenteriae* | 20.0±0.4 | 22.0±1.5 | 8.0±0.9 | 11.0±0.1 | 25.0±0.9 |

Values are expressed as Mean±SEM (n = 3)

inhibition was observed against *Bacillus subtilis* (12 mm) and *Staphylococcus aureus* (11 mm) at a concentration of 500 μg disc$^{-1}$. There are several antimicrobial activity reported from different mushrooms (Hearst *et al*., 2009) however, the antibacterial activity of *Hypsizygus tessulatus* is obscure in literature and hence direct comparison was not possible. The extracts have no antifungal activity against the pathogenic fungi.

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centred free radicals and other reactive oxygen species that are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). Although, almost all organisms possess antioxidant defence and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1988). However, antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage.

The model of scavenging DPPH radical is a widely used method to evaluate the free radical scavenging activities of antioxidants. In the DPPH assay, the antioxidants are able to reduce the stable DPPH radical (purple) to the non-radical form DPPH-H (yellow). The DPPH scavenging activities of antioxidants are attributed to their hydrogen donating abilities. In case of antioxidant screening of ethanol extract of *Hypsizygus tessulatus* powdered mycelium has shown in Table 2. The $IC_{50}$ value, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period, is a parameter widely used to measure antioxidant activity; a smaller $IC_{50}$ value corresponds to a higher antioxidant activity of the plant extract. $IC_{50}$ value of the ethanol extract was 105.0 μg mL$^{-1}$. These values state that *H. tessulatus* has moderate DPPH scavenging activity compared to Ascorbic acid standard.

The content of the phenolic compounds in the ethanol extract was determined through a linear gallic acid standard curve ($y = 0.0008x + 0.2177$, $R^2 = 0.8388$). The higher the TPC value, the higher total phenolic content is present in the extract. The total phenolic content of the extract occurred 36.45±1.754 gm of GAE/100 gm of dried extract which might be the evidence of showing antioxidant property of that extract. Therefore, a positive correlation was seen between the total phenolic content and antioxidant activity. The key role of phenolic compounds as scavengers of free

radicals is emphasized in several reports (Komali *et al*., 1999). Poly-phenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Gulcin *et al*., 2003). This is in accordance with the fact that phenolic compounds have potent free radical scavenging activity (Suzuki *et al*., 1982; Velioglu *et al*., 1998). It is suggested that poly-phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily ingested from a diet rich in fruits and vegetables (Tanaka *et al*., 1998). The presence of several cytotoxic steroids (Zhao *et al*., 2010) and flavonoids in the *H. tessulatus* was established previously. This might be the cause of producing antioxidant effect of ethanol extract. The Total antioxidant capacity of *H. tessulatus* expressed as the mg of ascorbic acid/100 g of mushroom extract (mycelia mat) was determined by phosphomolybdenum assay. The value was found in ethanolic extract was 442.61 mg equivalents of ascorbic acid.

In brine shrimp lethality bioassay, % mortality increased gradually with the increase in concentration of the test samples. Table 3 and 4 show the result of brine shrimp lethality testing of various extractives of *H. tessulatus* after 24 h of exposure to the samples and the positive control, vincristine sulphate (VS). The $LC_{50}$ values were found 7.36, 10.63, 0.52 μg $mL^{-1}$ for chloroform, ethyl acetate and vincristine sulphate, respectively. In comparison to positive control (vincristine sulphate), the cytotoxicity exhibited by chloroform extract was promising. Previous study reveals that *H. tessulatus* found to give a significant increase in life span when assayed using solid tumor. It has an inhibitory activity of spontaneous tumor metastasis (Saitoh *et al*., 1997). Another study stated that antioxidant activity of mice with tumor were significantly higher than non tumerogenic mice (Matsuzawa *et al*., 1998). Different types of polysaccharides has been reported from *H. tessulatus* species which contained glucose, xylose, galactose and they showed potent antitumor activity because such extracts induce the arrest of the cell cycle at the $G_0/G_1$ transition

Table 2: $IC_{50}$ value and total phenolic content of the sample

| Sample | Free radical scavenging activity ($IC_{50}$ μg $mL^{-1}$) | Total phenolic content (TPC) (g of GAE/100 g of dried extract) |
|---|---|---|
| Ascorbic acid | 5.25±0.21 | - |
| HT extract | 105.0±1.23 | 36.45±1.754 |

The values of TPC and $IC_{50}$ are expressed as Mean±SEM (n = 3)

Table 3: Effect of chloroform, ethyl acetate extract and positive control vincristine sulphate (VS) on brine shrimp nauplii

| Conc. (mg $mL^{-1}$) | Log C | %Mortality CH | %Mortality EA | $LC_{50}$ (μg $mL^{-1}$) CH | $LC_{50}$ (μg $mL^{-1}$) EA | VS Conc. (mg $mL^{-1}$) | VS Log C | VS %Mortality | VS $LC_{50}$ (μg $mL^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 400 | 2.602 | 100 | 100 | 7.36 | 10.63 | 40 | 1.602 | 100 | 0.52 |
| 200 | 2.301 | 100 | 100 | | | 20 | 1.301 | 100 | |
| 100 | 2 | 100 | 90 | | | 10 | 1 | 90 | |
| 50 | 1.699 | 90 | 80 | | | 5 | 0.698 | 90 | |
| 25 | 1.398 | 70 | 60 | | | 2.5 | 0.397 | 80 | |
| 12.5 | 1.097 | 50 | 50 | | | 1.25 | 0.096 | 70 | |
| 6.25 | 0.796 | 50 | 40 | | | 0.625 | -0.204 | 50 | |
| 3.125 | 0.495 | 40 | 30 | | | 0.3125 | -0.505 | 50 | |
| 1.563 | 0.194 | 20 | 20 | | | 0.15625 | -0.806 | 30 | |
| 0.781 | -0.107 | 10 | 10 | | | 0.078125 | -1.107 | 20 | |

CH stands for chloroform, EA stands for ethyl acetate, VS stands for vincristine sulphate

Table 4: The result of cytotoxic activity of chloroform and ethyl acetate extract on brine shrimp nauplii

| Sample | $LC_{50}$ ($\mu g\ mL^{-1}$) | Regression equation | $R^2$ |
|---|---|---|---|
| VS | 0.52 | Y = 33.256 X±58.740 | 0.9580 |
| CH | 7.36 | Y = 36.44 X±17.536 | 0.9537 |
| EA | 10.63 | Y = 36.24 X±12.787 | 0.9858 |

by means of induction of the expression of p53 and p21 proteins, which inhibit the cyclinkinase complexes (CDK) (Ikekawa *et al.*, 1992).

## CONCLUSION

Medicinal mushrooms used in the traditional medicine may be an interesting and largely unexplored source for the development of potential new compounds while the pragmatic medicinal properties have been attributed to mushrooms for thousands of years. But it is necessary to isolate the active principles and characterize their constituents for the benefit of human being. In conclusion, the metabolite and mycelia mat extracts of *H. tessulatus* demonstrated moderate antimicrobial, antioxidant activity, free radical scavenging activity as well as strong cytotoxic activity.

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