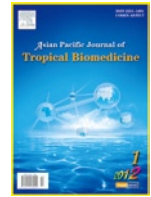




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Identification and determination of antioxidant constituents of bioluminescent mushroom

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ABSTRACT

Objective: To promote the sustainable use of wild mushrooms as a means to improve the mushroom identity and extent the knowledge on bioactive compounds of bioluminescent mushroom. **Methods:** The mushroom was identified by morphological, anatomical observations and preliminary phytochemical screening of mushroom extracts. Antioxidant activity was identified by DPPH as spray reagent after separating the compounds by thin layer chromatography (TLC). Dot-blot assay, determination of radical scavenging activity, total phenol and flavonoid contents by spectrophotometry were also carried out. **Results:** The bioluminescent mushroom was identified as *Omphalotus nidiformis* (*O. nidiformis*), which is the first report from Kerala. The preliminary phytochemical results showed the occurrence of active compounds such as phenol, flavonoid, alkaloid, terpenoid and saponins. The total phenolic content of the methanol extract was (1.901 ± 0.011) mg gallic acid equivalent/g of extract. The total flavonoid content of the *O. nidiformis* was estimated as 0.29 mg quercetin equivalent/g of dried methanol extract. The dot-blot assay and TLC–DPPH screening method indicated the presence of antioxidant compounds. Radical scavenging activity of the mushroom (IC_{50} 450 μ g/mL) was also observed. **Conclusions:** The present study identified the material as *O. nidiformis* (Berk.) and it contains secondary metabolites with high antioxidant activity.

1. Introduction

The higher basidiomycetes, especially mushrooms are unlimited source of biologically active agents with therapeutical use. It is not surprising that mushrooms are a source of many biologically active compounds because mushrooms manage to grow in darkness and highly competitive environments and protect themselves from hordes of attacking microbes by developing natural protective substances. Modern scientific studies on medicinal mushrooms have expanded exponentially during the last decades. Scientific explanation on the functions of mushroom derived compounds in human system are increasingly being established. In addition to a dietary as well as medicinal point of view, higher fungi may provide potent beneficial effects on human health either directly as

antioxidant or through prevention of alterations underlying major pathological states such as cancer, diabetes, neurodegenerative diseases, cardiovascular diseases and metabolic syndrome[1].

These antioxidants present in mushrooms are of great interest as possible protective agents to help the human body to reduce oxidative damage without any interference. Now they are recognized as functional foods and as a source of physiologically beneficial components. Mushrooms are reported to boost heart health; lower the risk of cancer; promote immune function; ward off viruses, bacteria, and fungi; reduce inflammation; combat allergies; and help to balance blood sugar levels and support the body's detoxification mechanism[2]. Mushrooms have shown the ability to accumulate a variety of secondary metabolites including phenolic compounds, polypeptides, terpenes, steroids, etc. Mushroom phenolics have been found to be an excellent antioxidant and synergist[2]. Furthermore, several companies are developing capsules from combinations of mushrooms, and these capsules, although expensive, are health beneficial, including fighting against cancer[3].

Aim of this study is to promote the sustainable use of wild

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mushrooms as a means to improve the mushroom identity and extent the knowledge on bioactive compounds of bioluminescent mushroom. It will be useful for evaluating substances of interest produced by these mushroom such as bioactive compounds and enzymes. No report on the antioxidant activities of bioluminescent mushroom was available. This present study include the identification of bioluminescent mushroom by analyzing diagnostic features and determination of total phenol and flavonoid. Antioxidant activity was assessed by dot–blot assay and free radical scavenging activity. This is the first report on the bioactive compounds of bioluminescent mushroom collected from Kerala, India.

2. Material and methods

2.1. Sampling site and collection of sample

The specimen of the investigated species was collected from Thiruvananthapuram district, southern part of Kerala, India, during the period of May 2010–June 2011. They were collected using paper bags and packed loosely with provision of aeration. Field data sheets were maintained. The collected material was transported to the laboratory at Department of Botany, Kariavattom. All the macro and micro characters including habit and morphological factors were recorded. The specimen cited in the article was kept in the herbarium of Department of Botany, University of Kerala, Kariavattom, and Coll. No: KUBH–5800.

2.2. Microscopic studies

For microscopic structures analysis cross section of lamellae was stained by lacto phenol cotton blue and then observed under Image Analyzer (Olympus–BX51TF, Japan) and hand drawings were also made. Based on the morphology and anatomy the specimen identification was done.

2.3. Preparation of extract

The mushrooms were shade dried and powdered in an electric mixer grinder. The powdered mushroom sample was extracted using methanol as a solvent by soxhlet apparatus. After extraction the extract was evaporated to dryness under reduced pressure in a rotary evaporator.

2.4. Phytochemical screening

Methanolic extract of bioluminescent mushroom was subjected to preliminary phytochemical screening for the identification of various classes of active chemical constituents using the methodology of Harborne[4].

2.5. Determination of total phenolic content

The amount of phenol compounds in the methanol extract of *Omphalotus nidiformis* (*O. nidiformis*) was determined

using folin ciocalteu reagent, according to the modified method[5]. The sample extract (three replica of 0.5 mL each) solution (1 mg/mL), was added to 2.5 mL of 10% Folin–Ciocalteu reagent and 2 mL of NaCO₃ (2% w/v). The resulting mixture was incubated at 45°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/VIS spectrophotometer. Results were expressed as milligrams of gallic acid (0–0.5 mg/mL) dissolved in distilled water.

2.6. Determination of total flavonoid content

Aluminum chloride colorimetric method was used for flavonoid determination. Dried methanol extracted sample (1 mg/1 mL, was mixed with 3 mL methanol,) was taken in test tubes and, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water were added and all remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm by spectrophotometer. The content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution (0–0.8 mg/mL) in distilled water. The concentration of flavonoid was expressed in term of mg/mL[5].

2.7. Rapid screening of antioxidant by dot–blot and DPPH staining

Each diluted sample was carefully loaded onto a 20 cm × 20 cm thin layer chromatography (TLC) plate (silica gel 60; Merck) and allowed to dry (3 min). Drops of each sample were loaded, in order to increase concentration (200–700 µg/mL), along the row. The sheet bearing the dry spots was placed upside down for 10 s in a 0.05% 2, 2–diphenyl–1–picrylhydrazyl (DPPH) solution. Then the excess of solution was removed with a tissue paper and the layer was dried with a hair–dryer blowing cold air. Stained silica layer revealed a purple background with yellow spots at the location where radical–scavenging capacity presented. The intensity of the yellow color depends upon the amount and nature of radical scavenge present in the sample[6].

2.8. Thin layer chromatography analysis for antioxidant constituents

About 2 µg of extracts of mushroom was loaded on TLC plates (Merck, 20 cm×20 cm). The plates were developed in hexane: methanol: acetic acid (3:9:2) to separate various constituents of the extracts. The developed plates were air dried and observed under visible, long and short UV light. After this examination, the antioxidant constituents were analyzed by DPPH technique[7,8]. For this 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plates and incubated for 10 min at room temperature. The active antioxidant constituents of the mushroom extracts were detected as yellow spots produced via reduction of DPPH by resolved bands against purple background on the TLC plates, the R_f value of the bands were also determined.

2. 9. Determination of DPPH radical scavenging activity

DPPH radical scavenging activity of the extract was measured by the method described by Barros *et al*[9]. For this, different concentrations of extract and ascorbic acid (standard) were prepared with methanol (Sigma–Aldrich) as the test solutions. One mL of each prepared concentrations were placed into test tubes and 0.5 mL of 1 mM DPPH solution in methanol was added. The test tubes were incubated for 15 min and the absorbance was read at 517 nm. A blank solution consisted of DPPH dissolved in same amount of methanol. The DPPH radical scavenging activity percentage was calculated by using the following formula.

$$\text{DPPH radical scavenging activity (\%)} = \frac{[A_{\text{control}} - A_{\text{extract}}] / A_{\text{control}}}{\times 100}$$

Where A_{control} is the absorbance of a DPPH solution without extract, A_{extract} is the absorbance of the tested extract. All measurements were performed in triplicate. The results were expressed as mean values and standard deviation (SD). The results were analyzed using one way analysis of variance (ANOVA).

3. Results

3.1. Morphological characters of mushroom

Mushroom fruiting bodies were observed in cluster, gray brown color when young later it became white (Figure 1). At high temperature the cap color was fade. It was funnel shaped to oyster shaped in appearance, some time with distinguishing morphology of bird nest shape. Annulus and veil were absent. Mushroom fluoresced light (Figure 2) under dark condition.

3.2. Microscopic characters of *O. nidiformis*

Pileus size was 4–5 cm, infundibuliform, margin striate, involutes thin undulating like an oyster, fleshy and soft. Lamellae were long–decurrent, crowd, white colored and having smooth edge. Number of gills was 14 cm. Stems attached centrally or acentrally to the cap. Stipe was 0.7–1 cm in length. We made the hand sections for the microscopic studies. Cross section showed basidia with two spores. Large numbers of sterile chelocystidia were found to be attached to the hymenium. They were hyaline and irregular or globose in shape (Figure 3). Presence of white colored spores was confirmed by spore print. Spores were spheroid and smooth. Considering morphological similarity

to the oyster mushroom, it was previously considered a member of *Pleurotus* and described under the names *Pleurotus nidiformis* or *Pleurotus lampas* and later it was placed under the genus *Omphalotus*[10–12]. Based on these macro and micro characters the present mushroom identity was confirmed as *O. nidiformis* (Berk.)



Figure 1. Mushroom fruiting bodies on coconut tree base.

3.3. Preliminary phytochemical screening

The preliminary phytochemical analysis showed the presence of active compounds such as phenol, flavonoid, alkaloid, terpenoid and saponins in *O. nidiformis* (Table 1).

3.4. Total phenol and flavonoid content

The total phenolic content of the methanol extract was (1.901 ± 0.011) mg gallic acid equivalent/g of extract. The total flavonoid content of the *O. nidiformis* was estimated by using aluminium chloride colorimetric technique and found to be 0.29 mg quercetin equivalent/g of dried methanol extract.

3.5. Dot–blot assay for antioxidant

The dot–blot assay method was typically based on the inhibition of the accumulation of oxidized products. The results of dot–blot assay showed yellow coloured spot when sprayed with DPPH solution (Figure 4). The higher

Table 1. Results of preliminary phytochemical screening of methanol extract of *O. nidiformis*.

Solvents	Phenolic compounds	Flavonoids	Alkaloids	Steroids	Terpenoids	Anthraquinone	Saponins
Petroleum ether	–	–	+++	++	++	–	–
Chloroform	+	+	++	++	++	–	–
Methanol	++	+	–	++	+	–	–
Water	++	+	+	–	–	–	++

concentration (500–700 μ g) showed higher scavenging activity.

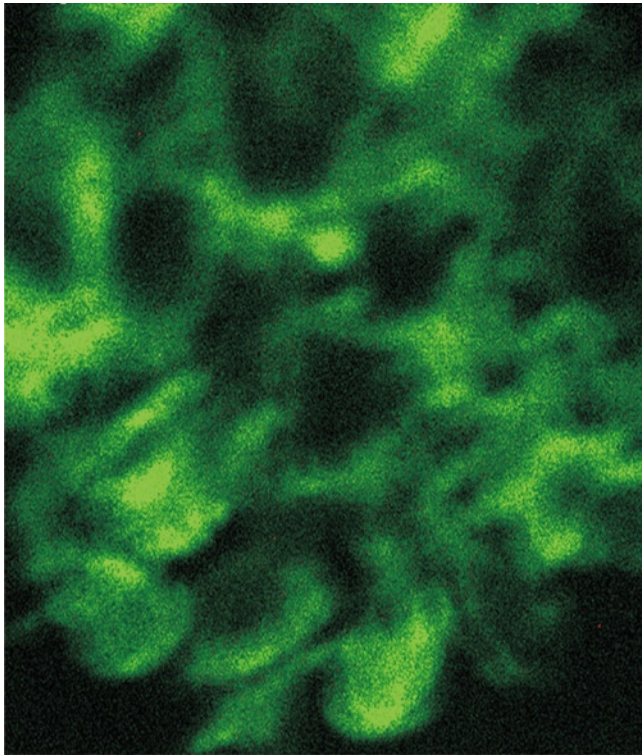


Figure 2. Green fluorescence of *O. nidiformis*.

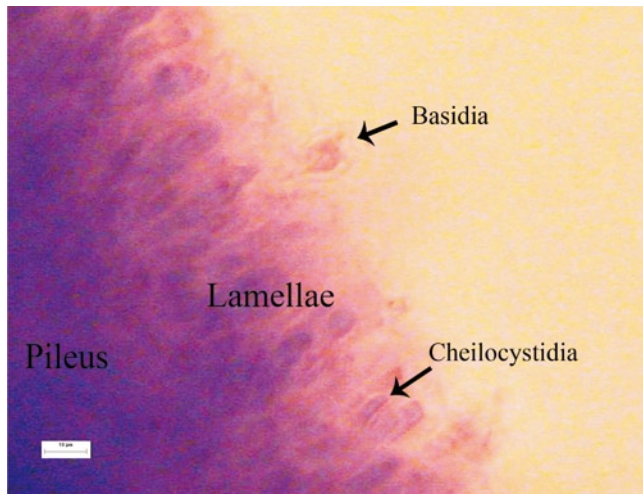


Figure 3. Cross section of mushroom fruiting body.

3.6. TLC analysis for antioxidant constituents

The plates TLC were developed in hexane: methanol: acetic acid (3:9:2) and sprayed with 0.05% DPPH reagent. It gave four antioxidant constituent in the mushroom extract with Rf value of 0.39, 0.49, 0.67 and 0.77. The eluted compounds showed yellow color corresponding with antioxidant behavior (Figure 5). Under long UV the eluted spots showed blue, blue green and yellow fluorescence.

3.7. DPPH radical scavenging activity of *O. nidiformis*

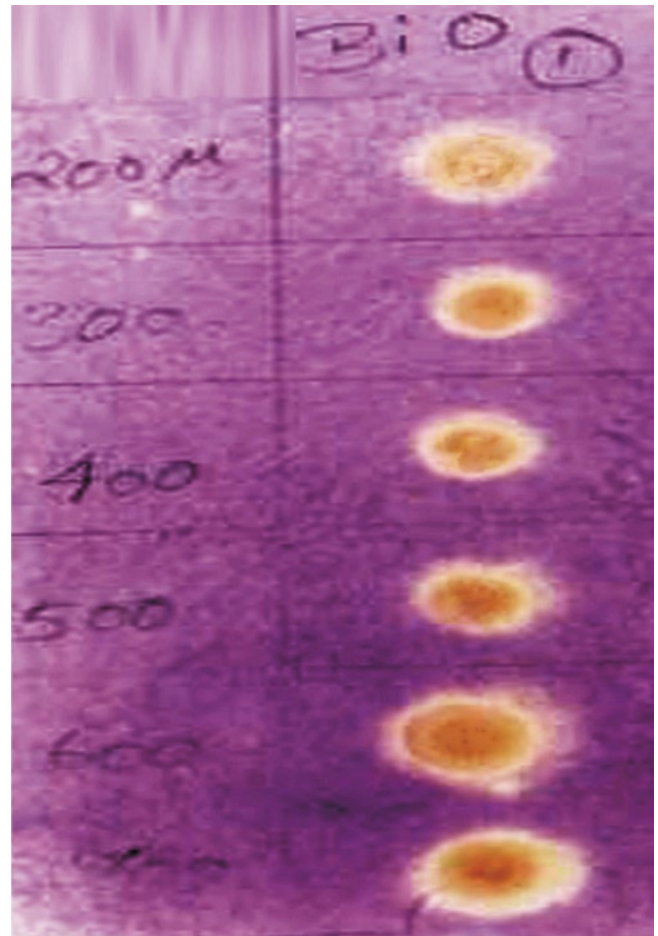


Figure 4. Dot blot assay.

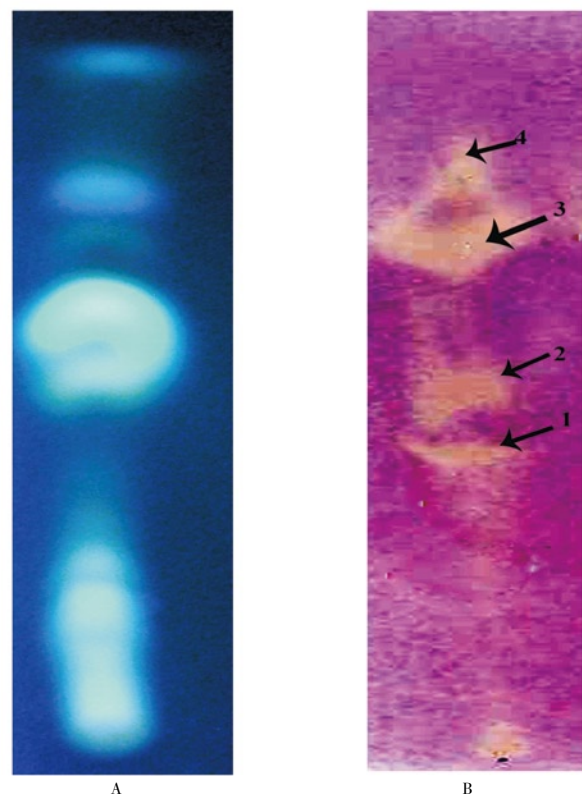


Figure 5. TLC analysis for antioxidant compounds. A: Plate under long UV; B: Plate after DPPH spray.

DPPH is a free radical and it gives strong absorption band at 517 nm in the visible region of electromagnetic radiation. As antioxidants donate protons to these radicals, the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging activity. The results were compared with that of ascorbic acid and the IC₅₀ value of the extract was found to be 450 µg/mL. Increase in DPPH free radical scavenging activity was observed with an increase in the concentration of *O. nidiformis* methanol extract (Figure 6).

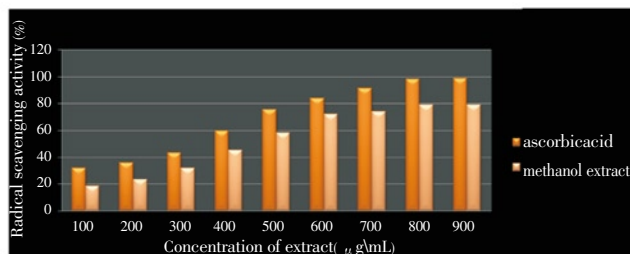


Figure 6. DPPH radical scavenging activity of methanolic extract of *O. nidiformis*.

4. Discussion

This reported mushroom, *O. nidiformis* is found in coconut tree base in the region of Thiruvananthapuram which is the capital city of Kerala. The mean maximum temperature is 34 °C and the mean minimum temperature is 21°C. The humidity is high and rises to about 90% during the monsoon season. This weather condition is conducive for the growth of *O. nidiformis*. The results of macro and microscopic characters, white spore print and fluorescent emission are confirming the mushroom identity. This is the first report from Kerala, and it was observed only in the mature coconut tree base. Concerning the edibility, the reports suggest that it is not lethal but cause discomfort to humans if eaten. Several species with similar bioluminescence properties occur worldwide, all of which are poisonous^[13].

Scientists reported that the bioactive compound from luminescent mushroom was used biologically to control plant diseases, especially to control root–knot nematode (*Meloidogyne incognita* Chitwood), which caused damage to the economic crops over 3 000 species in temperate and tropical areas^[13]. Recently scientists demonstrated that ethanol, cold and hot water extracts derived from *O. nidiformis*, *Cordyceps cranstounii* and *Cordyceps gunnii* have significant cytotoxic activity towards a variety of cancer cell lines^[14].

In this present study, preliminary phytochemical analysis revealed that the methanol extract of *O. nidiformis* contain phenol, flavonoid, steroid and terpenoid. The total phenolic content of the methanol extract was (1.901±0.011) mg gallic acid equivalent/g of extract which was higher than that reported for garlic extract (0.98 mg gallic acid equivalent/g of extract), but much lower than that reported for asparagus extract (26.95 mg gallic acid equivalent/g extract)^[5]. Phenolic compounds have attracted much interest recently because *in vitro* and *in vivo* studies suggest that they have

a variety of beneficial biological properties like anti-inflammatory, antitumor and antimicrobial activities^[2,3] studies have attributed that antioxidant properties are due to the presence of phenols and flavonoids^[15]. The key role of mushroom phenolic compounds as scavengers of free radicals is emphasized in several reports^[16–18]. Overall, *O. nidiformis* revealed better antioxidant properties (EC₅₀ values 450 µg/mL). The scavenging activity of methanolic extract of *O. nidiformis* was increased steadily with increasing concentrations. The high percentage of scavenging activity is an indication of high antioxidant activity. This suggests that the *O. nidiformis* extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical scavenging activity. The highest content of total phenols in the *O. nidiformis* extracts might account for the better results found in their antioxidant activity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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