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**Title:** Biodiversity of *Astraeus asiaticus*, a wild indigenous edible mushroom, in the forests of Bankura district, West Bengal and its antioxidant property

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**Abstract** Three forests, Beliature, Gangajal-ghati (G-ghati), and Joyur, were surveyed for mushroom collection and biodiversity. Mushrooms in the rhizospheric zone of some trees, such as *Shorea robusta*, *Petrocarpus marsupium*, *Terminalia bellirica*, and *Madhuca indica*, were identified at the molecular level as *Asterius asiaticus*. Thereafter, the ecological diversity of this mushroom was determined in the forests. The diversity indices of *Shorea robusta* in the Beliature, Joypur, and Gangajal-ghati forests were 2.303, 2.178, and 2.36, respectively. Notably, the diversity index of *Madhuca indica* in the Beliature and Joypur forests was nearly the same as that in the Gangajal-ghati forest, with a value of 2.29. The total phenolics contents of the hot water, acetone, and hexane extracts of this mushroom were  $6.8 \pm 0.15$ ,  $3.95 \pm 0.15$ , and  $2.16 \pm 0.26$  mg GAE/g, respectively, and the total flavonoid contents were  $2.03 \pm 0.12$ ,  $1.65 \pm 0.2$ , and  $1.01 \pm 0.08$  mg QE/g, respectively. The ascorbic acid contents in the hot water, acetone, and hexane extracts were low. The antioxidant activity of the extracts was determined using the DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay. The IC<sub>50</sub> values of the hot water, acetone, and hexane extracts were  $42.54 \pm 1.25c$   $\mu$ g/ml,  $54.06 \pm 1.50b$   $\mu$ g/ml, and  $82.97 \pm 1.58a$   $\mu$ g/ml respectively while that of the synthetic antioxidant, BHA (butylated hydroxyanisole), was  $32.41 \pm 1.26d$   $\mu$ g/ml. Overall, the hot water extract of this mushroom had the highest antioxidant content and displayed the best radical scavenging power.

**Keywords** Mushroom; Biodiversity; PCR; Antioxidant; *Astraeus asiaticus*

**Running title:** *Astraeus asiaticus* mushroom- its biodiversity and antioxidant property

## 1. Introduction

Mushrooms, owing to their medicinal and food values, have been historically viewed as an amicable part of human society and have at times superseded plant and animal-procured products. Mushrooms are recognized as functional foods with nutritional value, structural quality, and acceptability (Greeshma et al., 2018; Mwangi et al., 2022; Pinto et al., 2020). Moreover, mushrooms undergo remarkable adjustments on imminent days for adaptation as smooth-running therapeutic foods. The consumption of mushrooms in Asian countries, such as India and Japan, is increasing at a significant rate. Based on data from Research and Markets (2021) (<https://www.researchandmarkets.com/>), the value of the mushroom cultivation market was 16.7 billion \$ (USD) in 2020, and is projected to reach 20.4 billion \$ (USD) by 2025 (Landínez-Torres et al., 2021). Palatable mushrooms are low-calorie, low-fat food adjuncts with moderate amounts of proteins, carbohydrates, vitamins, minerals, amino acids, and dietary fiber (Johnsy et al., 2011). Mushrooms also exhibit antibacterial, antifungal, antiviral, antiparasitic, antioxidant, anti-inflammatory, antiproliferative, anticancer, anti-HIV, hypocholesterolemic, antidiabetic, and anticoagulant activities (Ghosh et al., 2020a; Ghosh et al., 2020b; Manimaran et al., 2021). Recently, Singh and Varshney (2020) estimated the food value of *Astraeus hygrometricus*, which was found to contain protein (16.02%), total carbohydrates (55.76%), fat (3.5%), monounsaturated oleic acid (4.59%), ash (3.8%), energy (159.5 kcal), pro-vitamin D2 (ergosterol) (1.09 mg/g), and dietary fiber (39.78%). Among the total free amino acids (8.20 mg/g), 7 essential amino acids (3.9 mg/g), including tyrosine (0.98) and leucine (0.92), were found. These researchers also found a high amount of selenium in *A. hygrometricus*. In this work, we opted to focus on the mushroom, *Astraeus* sp., owing to its food values of *Astraeus* sp. and its prominent use as a restorative drug owing to its antidiabetic, anticancer, hepatoprotective, anti-inflammatory, and cardioprotective activities (Phadannok et al., 2020). The primary aim of this study was to reveal the biodiversity of *Astraeus asiaticus* via morphological, biochemical, and molecular research in different woodlands or forests of the Bankura district, a red lateritic zone in West Bengal. Mycorrhizae provide nutrients between the roots of the host plant and fungi in the soil (Malajczuk et al., 1982). Biodiversity is a very important "natural capital." Different varieties, species, genera, and their complex interactions enable a functional ecosystem and productive economies (World Bank, 2022). Although wild mushrooms and fungi are an integral part of a given ecosystem, a study of their diversity and type has not been performed, particularly in tropical countries, such as India. As a result, a knowledge gap exists. Wild edible mushrooms act as natural resources for rural people and provide nutrition (Meena et al., 2020). Accordingly, an investigation of the biodiversity of wild edible mushroom and the consumption of this remarkable natural resource is of great economic importance. *Astraeus* species

are economically important as they are collected by people for consumption and sale in commercial markets (Manna & Roy, 2014). Bankura is recognized for its great abundance as residents can earn income by selling this wild edible mushroom. Thus, this mushroom is of great socioeconomic importance in the rural areas of West Bengal in India. *A. asiaticus* is a promising edible mushroom; however, its therapeutic value has not been explored. Therefore, in this study, the molecular characteristics, biodiversity, mycochemistry, antioxidant content, and antioxidant activity of this mushroom were determined to enable future research on its medicinal importance.

## 2. Material and Methods

### 2.1. Survey of habitat and sample collection

A survey on mushrooms was conducted in the Beliatare, Joypur, and Gangajal-ghati forest areas in Bankura. Joypur and Beliatare in the Bankura district are located in the southwest part of West Bengal and is associated with native extremities owing to the Damodar and Dwarakeswar rivers, which indicate the depletion of both forest range areas. The Damodar and Dwarakeswar rivers are almost parallel toward the southeast, as the general slope of the district runs from northwest to southeast. A periodic survey was conducted in the preferred area for the collection of *A. asiaticus* during the rainy season from mid-July to August 2019.

The habitat of mushrooms from the collection zone was noted, and photographs were captured. Soil samples from a rooting depth of 30 cm were collected from 15 zones (60 quadrats) of three forests. The samples were transported to the laboratory, and processed for soil chemical parameter analysis following the standard method (Iwabuchi et al, 1994, Das, 2021).

### 2.2. Identification of the mushroom species

Mushroom (basidioma) morphology was examined macroscopically and its size, shape, texture, color, and habit were determined, according to the pioneering work of Phosri et al. (2007). Biochemical tests were performed by dispensing a drop of chemicals (3% aqueous solution, 10% solution of potassium hydroxide (KOH), 10% solution of FeSO<sub>4</sub>, and vanillin) independently and directly on the tissue of freshly collected mushrooms, and observing the color changes immediately and after 4–5 min (Khaund & Joshi, 2014; Petrini et al., 2009).

The mushroom basidioma anatomy was observed under a compound microscope (Model No. Olympus CX31) after sectioning and staining with lactophenol cotton blue. Images were captured using a camera attached to a microscope.

For scanning electron microscopy (SEM), the samples were transferred to a Petri dish and immersed in 10 mL of water to rehydrate the tissues around the spores. Thereafter, the spore samples were incubated in the following ethanol series with 2% glutaraldehyde for 15 min each: 30, 50, 70, 80, 90, 95, and 100%. The samples were centrifuged, and the supernatant was collected. The sample was then placed in a Critical Point Dryer (CPD) and mounted. Finally, spores were prepared for SEM observation. The samples were coated with gold using a sputter coater. A focused image of the sample was used as the starting point under a scanning electron microscope. The magnification of the image was increased to a level close to the maximum, and the image was re-focused. A high-resolution SEM image was captured using an attached camera. All data or characters from macroscopic, microscopic, and biochemical reactions were compared with standard keys of mushrooms (Jordon, 1999; Pacioni, 1981; Phsori, 2007; Purkayastha & Chandra, 1985), and phenotypic mushroom identification was conducted.

For molecular identification of mushroom species, DNA was isolated from fresh basidioma using the modified hexadecyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). In brief, 100 mg sample was frozen in liquid nitrogen and ground using 500  $\mu$ L lysis buffer [CTAB 3% (pH 7.5), 100 mM Tris HCl, 20 mM EDTA (pH 7.5) 1.4 M NaCl<sub>2</sub>, 0.2% polyvinyl pyrrolidone] and micro pestle in Eppendorf tubes. The sample was then incubated at 64-65 °C for 1 h in a water bath and vortexed at 10-min intervals.  $\beta$ -mercaptoethanol (20  $\mu$ g/mL) was added to the tube, which was then incubated at 37 °C for 45 min. The sample was combined and centrifuged at 8000 rpm for 10 min at 4 °C using a cooling centrifuge. A total of 500  $\mu$ L of chloroform: isoamyl Alcohol (24:1) was added to the solution, which was vortexed under chilled conditions. The solution was centrifuged at 12000 rpm for 10 min at 4 °C in a cooling centrifuge. The supernatant was collected in fresh Eppendorf tubes. A total of 500  $\mu$ L of ice-chilled isopropyl alcohol (double volume) and 3 M Sodium acetate (1/10<sup>th</sup> volume) were added to the solution. Cold centrifugation was then performed at 12,000 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was collected (containing DNA), dehydrated with 70% ethanol, and left to dry under laminar airflow. Thereafter, the pellet was resuspended in 50-100  $\mu$ L of TE buffer, and stored at 4 °C overnight. Isolated DNA was purified using a HiMedia Purification Kit. The purified DNA was electrophoresed via electrophoresis using 0.8% agarose gel at 100 volts for 2-3 h. DNA bands were observed using a UV transilluminator.

The ITS1–5.8S- ITS2 marker zone for the rRNA of isolated gDNA was amplified using a PCR (polymerase chain reaction) protocol modified from Gardes & Bruns (1993), with primers ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5' TCCTCCGCT TATTGATATGC3'). In brief, PCR was conducted in a 50- $\mu$ L reaction volume, which contained 1 ng/ $\mu$ L DNA template, approximately 1  $\mu$ L; sterile distilled water, 38  $\mu$ L; 10 $\times$  Taq polymerase buffer with MgCl, 25  $\mu$ L; 10 mM dNTP mixes, 3  $\mu$ L; template DNA, 1  $\mu$ L; ITS1 forward primer, 1  $\mu$ L; and Taq DNA polymerase, 1  $\mu$ L. The following PCR cycling program was employed: initial denaturation at 96 °C for 2 min followed by 35 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The final extension at 72 °C for 10 min was terminated at the end of the amplification. The PCR product was sent to the SCI GENOME Laboratory, Kerala, for sequencing. The obtained sequence was compared with other related sequences using a BLAST search in GenBank (NCBI) (Ghosh et al., 2022; Podder & Ghosh, 2019) and submitted to GenBank, NCBI (Baltimore, USA).

### 2.3. Biodiversity analysis and mushroom extraction in different solvents

#### Biodiversity analysis

Fifteen study zones were selected from three forests in the Bankura district. Thus, 60 quadrats were used in this study. For mushroom biodiversity, quadrats (50 m  $\times$  50 m) were randomly selected and the mushroom diversity was determined. Mushroom diversity was analyzed using the Shannon Weaver Biodiversity Index (H) (Shannon and Weaver 1962):  $\hat{H} = -\sum(n_i/N) \log_e(n_i/N)$ , where  $n_i$  is the individual number of species  $i$  and  $N$  is the sum of  $n_i$ .

#### Mushroom extraction in different solvents

Mushroom extraction was carried out using the most polar solvent (hot water), semipolar solvent (acetone), and nonpolar solvent (hexane). The collected basidiomata were washed, dried, and crushed into powder using a grinder machine. Thereafter, 10 g of powder was poured separately into hot water, acetone, and hexane in a 1:10 ratio. For the hot water extract (HWE), a mixture of mushroom powder and water was boiled at 100 °C for 2 h in a water bath (Chen et al., 2016). However, for the acetone and hexane extracts, the mixture of mushroom powder and solvent was shaken on a shaker at 150 rpm for 1 h and then it was placed in an incubator which was set to 38 °C for 72 h. Each extract was filtered through Whatman No. 4 filter paper and Whatman No.1 filter paper. The filtrates were centrifuged at

3000 rpm for 15 min, concentrated through evaporation using a rotary evaporator at 50 °C, and lyophilized into powder using a lyophilizer (Figure 1). The extracts were stored at –20 °C for further use.

#### 2.4. Qualitative tests of different solvent extracts of mushroom *fruit body* for mycochemicals

##### Flavonoid

Briefly, 0.2 g of extract was dissolved in 4 mL of distilled water in a test tube. Thereafter, diluted sodium hydroxide (1 M) was added to the mixture, resulting in a yellow solution that turned colorless following the addition of a few drops of dilute acid (10% hydrochloric acid), confirming the presence of flavonoids (Hossain et al., 2013).

##### Terpenoids

Approximately 0.2 g of extract was dissolved in a mixture of 2 mL of chloroform and 3 mL of concentrated sulfuric acid. A reddish-brown color eventually appeared, indicating the presence of terpenoids (Mujeeb et al., 2014).

##### Cardiac glycosides

Briefly, 0.2 g of the extract was combined with 2 mL of glacial acetic acid and one drop of FeCl<sub>3</sub>. This mixture was then added to 1 mL concentrated H<sub>2</sub>SO<sub>4</sub>. When the upper layer and the interface between the two layers turned bluish-green and reddish-brown, respectively, this indicated the presence of cardiac glycosides (Auwal et al., 2014).

##### Steroids

The crude extract (0.2 g) was treated with a few drops of acetic acid anhydride in a test tube. Concentrated H<sub>2</sub>SO<sub>4</sub> was carefully added dropwise using the inner wall of the test tube. The presence of a brown ring on the surrounding surface indicated the presence of steroids (Gul et al., 2017)

##### Saponins

The extract (0.2 g) was mixed with 5 mL distilled water in a test tube. The solution was shaken vigorously for approximately 5 min, and then incubated for 30 min. The formation of a honey comb froth indicated the presence of saponin (Auwal et al., 2014).

### Alkaloid

Briefly, 0.2 g crude extract was weighed in a separate test tube and warmed in 2% sulfuric acid for 2 min. In a separate test tube, the solution was mixed with a few drops of Dragendroff reagent. The presence of an orange red precipitate indicated the presence of alkaloid (Auwal et al., 2014).

### Carbohydrate

The extract (0.2 g) was dissolved in water (2 mL). Thereafter, 2-3 drops of Molisch's reagent [3.75 g of  $\alpha$ -naphthol in 25 mL ethanol (99%)] were added to the solution for the formation of a layer without shaking the test tube. Thereafter, a drop of concentrated sulfuric acid (99%) was added to the mixture. The presence of a purple color, which indicates the presence of carbohydrates, was determined at the interface (Auwal et al., 2014).

### Phlobatannins

Briefly, 0.2 g extract was dissolved in 2 mL of water and then added to 3 mL of dilute HCl (10%). A red precipitate indicated the presence of phlobatannins (Auwal et al., 2014).

### Phenol

Briefly, 0.2 g of extract was dissolved in 2 mL of distilled water. The solution was then treated with two drops of 5% FeCl<sub>3</sub>. The production of a deep blue color indicated the presence of phenol (Talukdar et al., 2017).

### Tannin

The extract (0.2 g) was dissolved in 4 ml of distilled water, heated in a water bath (50 °C), and filtered. Two drops of 1% FeCl<sub>3</sub> were added to the filtrate. The formation of a dark green solution indicated the presence of tannins (Fransina et al., 2019; Usman et al., 2009).

## 2.5. Divergent biochemical assay for antioxidant content and antioxidant potentiality

### Antioxidant content

#### Total phenolics content assay

The total phenolic content (TPC) of various solvent extracts was measured using the Folin–Ciocalteu reagent method (Mc Donald et al., 2001). Briefly, 0.1 mL Folin-reagent Ciocalteu's (0.5 N) reagent and 0.5 mL extract were mixed and incubated for 15 min at room temperature. Saturated sodium

carbonate solution (2.5 mL) was then added to the mixture, which was then incubated at room temperature for 30 min. The absorbance was measured at 760 nm. The results are expressed as mg of quercetin equivalent (QEs) per g of mushroom dry weight and are presented as mean  $\pm$  SD (standard deviation) of triplicate experiments.

#### Total flavonoid content (TFC) assay

The TFC of the extract was evaluated using a standard guideline suggested by Meda et al. (2005). Briefly, equal amounts of 2% AlCl<sub>3</sub> (prepared in absolute methanol) and the extracts (1 mg/mL) were incubated in a glass tube at room temperature for 10 min. Thereafter, the absorbance was measured at 415 nm. The results are expressed as mg of quercetin equivalent (QEs) per g of mushroom dry weight and are presented as mean  $\pm$  SD of triplicate experiments.

#### Total ascorbic acid content assay

The total ascorbic acid content (TAAC) was determined using the Folin-Ciocalteu reagent method proposed by Jagota and Dani (1982). Briefly, 0.5 mL extract solution (1 g extract in 1 mL ethyl acetate) was quickly mixed with 0.8 mL of 10% trichloroacetic acid and refrigerated for 5 min before centrifugation at 3000 rpm for 5 min. Thereafter, 0.2 mL was retrieved from the mixture and mixed with 1.8 mL distilled water to a final volume of 2 mL. Finally, 0.2 mL of diluted Folin-Ciocalteu (1:9) was added to the diluted extract mixture. After a 10 min incubation at room temperature (27 °C), the absorbance at 760 nm was measured. Finally, the results are expressed as milligrams of ascorbic acid equivalents (AAEs) per gram of mushroom extract, with the mean standard deviation (SD) of three replicates.

#### Antioxidant activity assay

##### DPPH Assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (RSA) was determined using a modified method described by Bondet et al. (1997). Ten different concentrations (10-100  $\mu$ g/mL) were prepared from the mushroom extract, and 900  $\mu$ L DPPH (0.1 M) was added to each. The DPPH solution was prepared by adding 2 mg DPPH powder to 50 mL ethanol (0.1 M). After 2 h of incubation in the dark, the sample was subjected to a spectrophotometric study at a wavelength of 517 nm, and the absorbance was recorded. The percentage of DPPH reduction was calculated using the following formula: % DPPH reduction or % of inhibition =  $\frac{Ac-As}{Ac} \times 100$

Where Ac is the absorbance of the solvent (control) and As is the absorbance of the sample.

Finally, the IC<sub>50</sub> (50% of inhibition) value was calculated.

### 3 Results

#### 3.1 Habitat study and soil analysis

The collected mushrooms were rhizospheric and ectomycorrhizal under the roots of *Shorea robusta*, *Modhuca indica*, *Petrocarpus marsupium*, and *Terminalia bellrica*. When immature, the mushrooms were subterranean but when they were fully matured, they exposed by breaking the soil surface. The soil pH of the Beliatore, Gangal-ghati (G-ghati) forest was 6.3, and that of the Joypur forests was 6.5, 6.3, and 6.2, respectively. The soil analysis revealed that the organic carbon content of the collected soils ranged from 0.82 to 0.93%. The carbon content of Beliatore soil samples was 0.93%, while that of the G-ghati soil sample was 0.87%, and that of the Joypur soil sample was 0.82%. The electrical conductivity (EC) of the three forest soil samples ranged from 81.58 to 86.72  $\mu$ S. The value was 86.72  $\mu$ S in the Beliatore forest, 84.29  $\mu$ S in the G-ghati Forest, and 81.58  $\mu$ S in the Joypur Forest. The available nitrogen content of the Beliatore forest sample was 220 kg/ha, while that of G-ghati, and Joypur forest sample was 140 kg/ha and 180 kg/ha, respectively. The available phosphorus content in the forest soils of the Bankura district ranged from 72.67 to 74.34 kg/ha, with values of 74.34 kg/ha, 73.54 kg/ha, and 72.67 kg/ha in the Beliatore, G-ghati, and Joypur forests, respectively. In the study area, available potassium ranged from 215.7 to 218.4 kg/ha, indicating a high potassium content in the three-forest soil sample of Bankura district. The available potassium contents in the three forests (Beliatore, G-ghati and Joypur) were 215.7, 214.2, and 218.4 kg/ha respectively and the N:P: K ratios in these forests were 3:1:3, 2:1:3, and 2:1:3, respectively (Table 1).

#### 3.2. Identification of mushroom

##### Phenotypical identification

The fruiting season for this mushroom is May-August. The basidioma of this species was globose and smooth, with an unexpanded diameter of 10-16 x 8-14 mm and expanded diameter of 14-23 x 8-12 mm. The gleba was initially white; however, when the spores matured, the gleba and its granulate outer peridium turned purplish chestnut. The exoperidium was slightly viscous and smooth, and the

color was white, covering the endoperidium (Figure 2). The endoperidium is a thin membranous layer of tissue, enclosing the spore mass in a 20–30 mm diameter sac, nearly round, with an irregularly shaped ostiole at the top, surface felty rough, whitish, and gray to brown color. Basidiospore mass was whitish when young, and black and powdery at maturity. The spores were reddish-brown, roughly spherical with minute warts, measuring 7.5–11 µm in diameter. The mycelial layer consisted of branched hyphae that were 4–7 µm in diameter. The hyphae of the fibrous layer were 6–9 µm in diameter and branched, and the collenchyma-type layer contained branched hyphae 3–4 µm in diameter (Figure 3). Scanning electron micrographs provided a clear three-dimensional image and stereoscopic view of the mushroom spore and hyphal structures. Ornamentation on the spore surface and hyphal structure were clearly observed (Figure 4). The basidiospores showed extensive, dense, and rounded spine, and were globose, ranging from 8.65–15.3 mm diameter. Other features included ornamentation, purplish chestnut color, very densely ornamented with rounded, narrow, tapered, occasionally coalescent, spines 0.9–1.50 mm long.

In the present biochemical identification study, no color change was observed when 10% KOH, 4% HNO<sub>3</sub>, Phenol, Vanillin, and 10% H<sub>2</sub>SO<sub>4</sub> were employed (Table 2). The color change profiles generated for each species of wild edible mushrooms in the present study served as an additional tool to identify similar mushrooms.

The collected mushroom was identified as *Astraeus* sp. based on the macroscopic and microscopic characteristics, biochemical reactions, and comparison with the standard keys of mushrooms (Jordon, 1999; Pacioni, 1981; Phosri et al., 2007; Purkayastha & Chandra, 1985).

#### Molecular Identification of Fungal Species

The mushroom sample was identified as *Astraeus asiaticus* using BLAST in the NCBI database based on query coverage (98.0%) and identity score (83.05%) (Figure 5) of sequence homology search. The nucleotide sequence of the ITS zone was submitted to Genbank, NCBI (Baltimore, USA) and published under the Accession no MZ931288.1. The strain name is “Aa\_skg.”

#### Phylogenetic analysis

The phylogenetic tree (Figure 6) revealed that the test organism was closely related to *Astraeus asiaticus*. Thus, the wild mushroom was concluded to be a member of *Astraeus asiaticus*.

### 3.3. Biodiversity Analysis

The mushroom is terrestrial, grows under various trees, and is widely distributed in the three forests of Bankura district, West Bengal, India. *Astraeus asiaticus* can establish a symbiotic relationship with a wide range of trees. This ectomycorrhizal species forms a symbiotic relationship with trees, such as *Shorea robusta*, *Madhuca indica*, *Petrocarpus marsupium*, and *Terminalia bellrica*. In our study, the abundance of basidioma in this species was found to be associated with the roots of *Shorea robusta*; however, in the case of *Madhuca indica*, a moderate number of this species was found. Very poor or low numbers of this species were found in association with the roots of *Terminalia bellrica* and *Petrocarpus marsupium*. Further, no mushroom basidioma was found in association with *Eucalyptus globulus* and *Acacia auriculiformis* trees (Table 3).

The ecological diversity of *Shorea robusta* and *Madhuca indica* in the 60 quadrats of 15 zones of three forests was calculated using the Shannon diversity index formula.

The diversity index of *Shorea robusta* was 2.303 in the Beliature forest, 2.178 in the Joypur forest, and 2.36 in the Gangajal–ghati forest (Table 4). Further, the diversity index of *Madhuca indica* was 2.25 in the Beliature forest, 2.26 in the Joypur forest, and 2.29 in the Gangajal–ghati forest (Table 5).

#### 3.4. Mushroom extraction, mycochemistry, antioxidant content, and potentiality study

##### Mushroom extraction

The HWE had the highest extraction yield ( $0.47 \pm 0.01\%$ ) for *A. asiaticus* (Table 6). The polarity index of water is 10.2. Accordingly, water dissolves many polar and ionic solutes, such as proteins, water soluble carbohydrates, and mineral salts. In contrast, the nonpolar solvent, n-hexane, only dissolves nonpolar compounds, such as lipids, as its polarity index is 0.1, resulting in  $0.32 \pm 0.01\%$  crude product (HE). Acetone is a dipolar solvent; thus, it has an intermediate relative polarity. The polarity index of acetone is 5.1, resulting in a crude product, acetone extract (AE) of  $0.42 \pm 0.02\%$ . The yields of HWE and AE were similar ( $P=0.005$ ); however, their yields differed from that of HE based on Duncan multiple test analysis (Table 6).

##### Mycochemical screening using a qualitative test

Based on the mycochemical screening results (Table 7), the active ingredients in the HWE were flavonoids, cardiac glycosides, saponins, alkaloids, carbohydrates, and phenols. For acetone, the active ingredients were flavonoids, cardiac glycosides, saponins, alkaloids, and phenols; and for the hexane extract, the active ingredients were terpenoids, cardiac glycosides, saponins, alkaloids, and tannins. Phlobatannins and steroids were not detected in any sample. Major active

ingredient metabolites, such as flavonoids, alkaloids, phenols, and carbohydrates, were found in the polar HWE, while terpenoids were found in the nonpolar hexane extract.

#### Antioxidant content

##### Total phenolics, flavonoid, and ascorbic acid content

The total phenolics contents of the hot water, acetone, and hexane extracts were  $6.8 \pm 0.15$ ,  $3.95 \pm 0.15$ , and  $2.16 \pm 0.26$  mg GAE/g, respectively, and the TFCs were  $2.03 \pm 0.12$ ,  $1.65 \pm 0.2$ , and  $1.01 \pm 0.08$  mg QE/g, respectively. Figure 7 shows the ascorbic acid concentration in the mushroom extracts. The ascorbic acid content in the hot water, acetone, and hexane extracts was low ( $0.167 \pm 0.03$ ,  $0.14 \pm 0.04$ , and  $0.11 \pm 0.005$  mg AAE/g, respectively).

##### Antioxidant potential or scavenging activity based on the DPPH method

DPPH was used to evaluate the RSA of the mushroom decoction. DPPH is a stable free radical that exhibits idiosyncratic sorption at 517 nm. The absorption of these radicals decreases as antioxidants provide protons. As a measure of the magnitude of radical scavenging, a decrease in absorbance was confirmed. The free radical-scavenging capabilities of the extracts were calculated using the DPPH assay. A method by which antioxidants prevent lipid oxidation is free radical scavenging. The distillate method for scavenging DPPH free radicals can be used to assess the antioxidant activity of individual chemicals or extracts in a specific situation.

Figure 8 shows the DPPH RSA of all solvent extracts of this mushroom. The  $IC_{50}$  (50% of inhibition) values for the HWE, acetone extract, and nonpolar hexane extract were  $42.54 \pm 1.25$   $\mu\text{g/mL}$ ,  $54.06 \pm 1.50$   $\mu\text{g/mL}$ , and  $82.97 \pm 1.58$   $\mu\text{g/mL}$ , while that of synthetic antioxidant BH was  $32.41 \pm 1.26$   $\mu\text{g/mL}$ .

## 4. Discussion and Conclusions

Wild edible mushrooms, which serve as a good economic source for underprivileged residents of Bankura district, W.B., were collected to perform phenotypic and molecular identification studies and determine their diversity. This mushroom has been consumed as a good food in this area and adjoining districts. According to the literature, this genus of mushroom is distributed worldwide, especially in Africa, Asia, Australia, Europe, Mexico, North America, and South America (Pavithra et al., 2015). A literature survey revealed that *Astraeus hygrometricus*, *A. asiaticus*, *A. morgani*, *A. sirindhorniae*,

*A. odoratus*, *A. pteridis*, *A. smithii*, *A. telleriae*, *A. koreanus*, and *A. thailandicus* have been discovered globally (Verma et al., 2019). In this study, both phenotypic and molecular identification of this mushroom were successfully performed. The relevance of nucleic acids as molecular markers applies to fungal taxonomy. PCR is an established habitual tool for field mycology. For example, internal transcribed spacer (ITS) regions are amplified by PCR and subsequently sequenced for molecular phylogenetic analysis of diverse mushroom species. The DNA sequences obtained via PCR are used for population genetics and biodiversity research on mushroom species (Ghosh et al., 2022, Izumitsu et al., 2012). In this study, both phenotypical and molecular (ITS1-5.8s-ITS-2 zone of rDNA) identification revealed that this mushroom belonged to the species, *Astraeus asiaticus*, which was first identified in this district of West Bengal, India. *Astraeus asiaticus* is a new species of star-shaped gasteroid fungus that differs morphologically from other genera of star-shaped fungi. This genus does not possess distinct peristome or columella. Further, the spores of this genus are larger than those of *Geastrum*, and its capillitial hyphae are longer and more branched (Phosri et al., 2014). Phosri et al. (2007) identified this species at the morphological and molecular levels. Our phenotypical and molecular results align with the findings of Phosri et al. (2007). Recently, Topno and Srivastava (2021) collected puff balls from Chota Nagpur Plateau (Jharkhand). These puff balls were phenotypically and molecularly (ITSs markers) similar to *Astraeus asiaticus*. Similarly, Vishal et al. (2021) collected two earth star mushrooms from Sal forest of the Porahat forest division of Jharkhand state, India and identified these mushrooms *A. asiaticus* and *A. odoratus* based on molecular studies (ITSs marker). From the GENBANK database (NCBI, Baltimore, USA), we identified five accession Nos for *Astraeus* sp. from 2008 to 2020: AJ629895.1 for *A. hygrometricus* from West Bengal, India in 2008; MN257431.1 and MT611066.1 for *A. asiaticus* from our neighboring state, Jharkhand, India in 2019 and 2020; and MN262679.1 and KJ847767.1 for *Astraeus odoratus* in 2019 and 2016.

SEM studies of spores and hyphae were performed according to the findings of Phosri et al. (2007). The study of biodiversity of this species of mushroom as ectomycorrhizan in this work in W.B. was first time work but in other states of India, diversity work has been done to some extent in other species (*A. hygrometricus*) (Topno and Srivastava, 2021; Semwal et al., 2014; Verma et al., 2017; Vishal et al., 2021). After a distribution search of *Astraeus* species in India, this genus was found in the provinces of Himachal Pradesh, Uttar Pradesh, Uttarakhand, Madhya Pradesh, Chhattisgarh, Punjab, Karnataka, Kerala, Jharkhand, and Odisha, and in our state (Pavithra et al., 2015; Phosri et al., 2004; Pyasi et al., 2013; Semwal et al., 2014; Topno & Srivastava, 2021; Vishal et al., 2021). Some Asian varieties of *A. hygrometricus* are reported to differ from those which are found in North America and are recognized as new species, such as *A. asiaticus* and *A. odoratus* (Mortimer et al., 2012). In the present case, the *Astraeus* species was identified as *A. asiaticus*.

In our study, the plants supporting this mushroom were *Shorea robusta*, *Madhuca indica*, *Petrocarpus marsupium*, and *Terminalia belerica*. Such finding is supported by other researchers (Harley et al., 1997; Verma et al., 2017). The ectomycorrhizal association of *Astraeus* with the roots of members of Pinaceae, Betulaceae, Fagaceae, Ericaceae, and Dipterocarpaceae has been reported (Wilson et al., 2012). The diversity index obtained in our study indicated that the ecological diversity of this mushroom was similar in the three forests in Bankura district, W.B. By analyzing the soil quality of these three forests, they were found to be more or less similar in respect to NPK and pH status, aligning with the results of another study (Das, 2021). Mushrooms contain high amounts of antioxidant compounds (Chun et al., 2021; Shaffiqueet et al., 2021; Wani et al., 2010). The antioxidant activity of *A. hygrometricus* was demonstrated previously (Mandal et al., 2015, Pavithra et al., 2016). Compounds, such as phenolic compounds, flavonoids, and ascorbic acids, have been found in other *Astraeus* species (*Astraeus odoratus* and *Astraeus pteridis*) (Arpha et al., 2012; Isaka et al., 2016; Lai et al., 2012; Mallick et al., 2016; Pimjuk et al., 2015; Rahi & Malik, 2016; Stanikunaite et al., 2008). In this study, the different parameters of the mycochemistry study of *A. asiaticus* were successfully analyzed based on the total phenolic, flavonoid, ascorbic acid, and antioxidant contents, and the free RSA of this mushroom. In our experiment, all extracts of this mushroom had a high amount of phenolic compounds; however, the HWE had the best results. Similarly, this HWE had the highest antioxidant activity among the extracts. The antioxidant properties depend on phenolic compounds (Velioglu et al., 1998; Xu, 2013). The dietary intake of a substantial amount of phenolic compounds is important as they play the role of free radical terminators that reduce the incidence of many diseases, such as atherosclerosis, cancer, and heart diseases (Allothman et al. 2009; Xu, 2013). The antioxidant activity of many wild mushrooms is positively correlated with total phenolics (Lo & Cheung, 2005; Xu, 2013). Phenolic compounds have redox properties and antioxidant properties. As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration can be used as a basis for rapid screening of antioxidant activity. Recently, Bouyahya et al. (2022) reviewed the role of phenolic compounds in improving the chemosensitivity of anticancer drugs. This study first time has compared the antioxidant activity of polar, semipolar, and nonpolar extracts of *Astraeus asiaticus*. So, mycochemical analyses should be performed to identify the active phenolic and flavonoid components. Ascorbic acid plays an important role as a protective antioxidant. In this study, the ascorbic acid contents in the hot water, acetone, and hexane extracts were  $0.167 \pm 0.03$ ,  $0.14 \pm 0.04$ , and  $0.11 \pm 0.005$  AAEs/g, respectively, aligning with previous results (Mau et al, 2002). The highest ascorbic acid content of  $0.167 \pm 0.03$  AAEs/g, which was obtained using the HWE, might account for the better results found for their antioxidant activity. The HWE of *A. asiaticus* displayed potential scavenging activity ( $IC_{50} = 42.54 \pm 1.25$   $\mu$ g/mL), which is comparable to that of the standard

antioxidant, BHA. In general, the lower the IC<sub>50</sub> value, the higher the antioxidant property. Accordingly, the HWE of *Astraeus asiaticus* was found to exhibit antioxidant attributes. These findings indicate that the HWE of mushroom species has a conspicuous effect on scavenging free radicals. Pavitra et al. (2016) reported that the antioxidant activity of *A. hygrometricus* is concentration-dependent and does not decrease after cooking. Herein, the HWE of *A. asiaticus* had the best antioxidant activity, which aligns with previous findings (Pavitra et al., 2016). Antioxidants are important compounds that help increase the resistance of the body to free radicals. Mycochemistry revealed that this mushroom was the source of some volatile compounds, such as 1-octanol, 1-octen-3-ol, and 1-octen-3-one, which had a "mushroom-like, earthy, and pungent odor that was evident as an oily and moss-like smell upon opening the caps." During the cooking of mushroom samples, furfural, benzaldehyde, cyclohexenone, and furanyl compounds are emitted (Mortimer et al., 2012; Verma et al., 2017). Recently, Isaka et al. (2017) isolated 17 known and seven new lanostane triterpenoids, such as astraeusins, 26-epi-artabotryol C1, and 26-epiastrasiaone, from *A. asiaticus*. To our knowledge, the current study is the first to assess the antioxidant content and activity of this edible mushroom species, and its biodiversity in the forests of Bankura district.

Based on our findings, the species of *Astraeus* in the forest of the Bankura district was *A. asiaticus*. Further, this species was mycorrhizally associated with *Shorea robusta* and *Madhuca indica*. Based on the ecological diversity of this mushroom, *A. asiaticus* was associated with the above-mentioned specific plants. The mycochemistry of this mushroom revealed good quality and content of antioxidants, including phenolic compounds, flavonoids, and ascorbic acid, and good antioxidant activity (scavenging of free radicals). Therefore, this mushroom is a good source of valuable nutrients, including antioxidants, scientifically validating our eating habits. This work may fill the knowledge gap, to some extent, related to this wild edible mushroom (gift of nature), and encourages further assessments.

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### **Authors' contributions**

SKG designed the study and wrote and revised the manuscript. KP performed the experimental work, literature survey, and statistical analysis.

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#### Tables:

**Table 1** Mean values for the soil physico-chemical parameters and the N, P, and K ratios of the soil samples collected at selected quadrants of the Forests in Bankura district.

Name of the district	Name of the forests	Soil pH	Electrical Conductivity ( $\mu\text{S}$ )	Carbon (%) Organic	Nitrogen Kg/ha	Phosphorus Kg/ha	Potassium Kg/ha	N:P:K
Bankura	Beliatore	6.5	86.72	0.93	220	74.34	215.7	3:1:3
	Gangajal-ghati(G-ghati)	6.3	84.29	0.87	140	73.54	214.2	2:1:3
	Joypur	6.2	81.58	0.82	180	72.67	218.4	2:1:3

**Table 2** Color change profile based on the fruiting bodies of *Astraeus asiaticus*.

Test	Colour changed observed
10% KOH	None
4% HNO <sub>3</sub>	None
Phenol	None
Vanilin	None
10% H <sub>2</sub> SO <sub>4</sub>	None

**Table 3** Association of *Astraeus asiaticus* with different trees.

Trees	Fruit body association with trees
<i>Shorea robusta</i>	+++
<i>Petrocarpus marsupium</i>	+
<i>Terminalia bellrica</i>	+
<i>Acacia auriculiformis</i>	-
<i>Modhuca indica</i>	++
<i>Eucalyptus globulus</i>	-

Note: - indicates no association or mushroom fruit body, + indicates presence of a lower number of mushroom fruit bodies or association, ++ indicates a moderate number of fruit bodies, and +++ indicates an abundance of mushroom fruiting bodies.

**Table 4** Shannon Weaver biodiversity index of the association between *Astraeus asiaticus* and *Shorea robusta*.

Forest	Zones	Quadrates in each zone	Total no of quadrates in the forest	Shannon Weaver biodiversity index of <i>A. asiaticus</i>
Beliatore	5	4	20	2.303
Joypur	5	4	20	2.178

<b>Gangajal – ghati</b>	5	4	20	2.36
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**Table 5** Shannon Weaver biodiversity index of the association between *Astraeus asiaticus* and *Modhuca indica*.

<b>Forest</b>	<b>Zones</b>	<b>Quadrate in each zone</b>	<b>Total no of quadrates in the forest</b>	<b>Shannon Weaver biodiversity index of <i>A. asiaticus</i></b>
<b>Beliatore</b>	5	4	20	2.25
<b>Joypur</b>	5	4	20	2.26
<b>Gangajal – ghati</b>	5	4	20	2.29

**Table 6** Percentage yield of the crude products using different solvents.

<b>Solvent name (Extract name)</b>	<b>Method of extraction</b>	<b>Ratio of solute and solvent</b>	<b>Initial weight of Mushroom powder(gm)</b>	<b>Final crude weight (gm)(±SD)</b>	<b>Percentage of yield (%) (±SD)</b>
<b>Hot water (HWE)</b>	<b>Dipping method</b>	1:10	500	2.26± 0.15	0.47± 0.01a
<b>Acetone (AE)</b>	<b>Dipping method</b>	1:10	500	2.1± 0.12	0.42± 0.01a
<b>Hexane (HE)</b>	<b>Dipping method</b>	1:10	500	1.52± 0.08	0.32± 0.02b

Note: Same letters in the same row indicate statistical similarity, while different letters indicate statistical difference (P=0.05) based on Duncan's multiple test.

**Table 7** Mycochemical screening of the polar (water), dipolar (acetone), and nonpolar (hexane) solvent extracts of *Astraeus asiaticus*.

<b>Mycocompound /class</b>	<b>Hot water extract (HWE)</b>	<b>Acetone extract (AE)</b>	<b>Hexane extract (HE)</b>
<b>Flavonoid</b>	+	+	-
<b>Terpenoid</b>	-	-	+
<b>Cardiac glycoside</b>	+	+	+
<b>Steroid</b>	-	-	-
<b>Saponins</b>	+	+	+
<b>Alkaloid</b>	+	+	+
<b>Tanins</b>	-	-	+
<b>Carbohydrate</b>	+	-	-
<b>Phenol</b>	+	+	-
<b>Phlobatannins</b>	-	-	-

Note: (-) Absence, (+) Presence.

## Figures

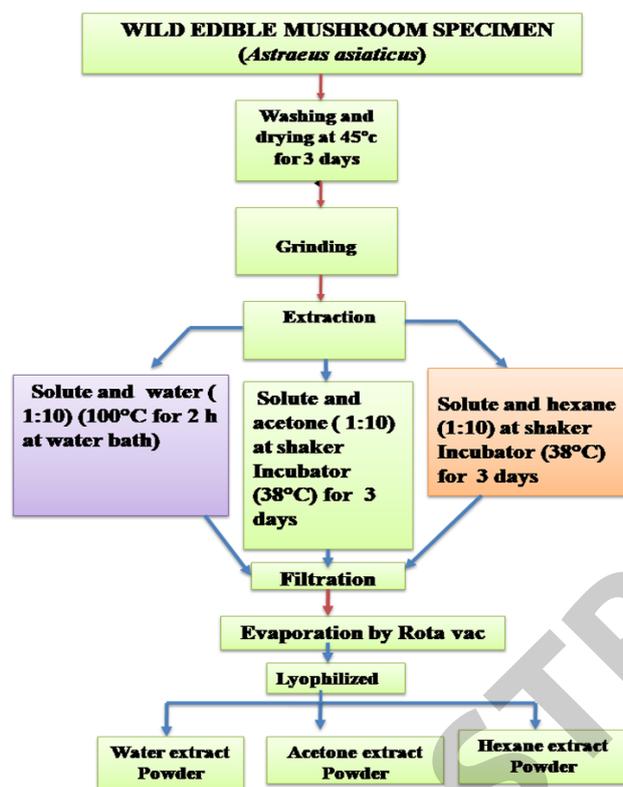
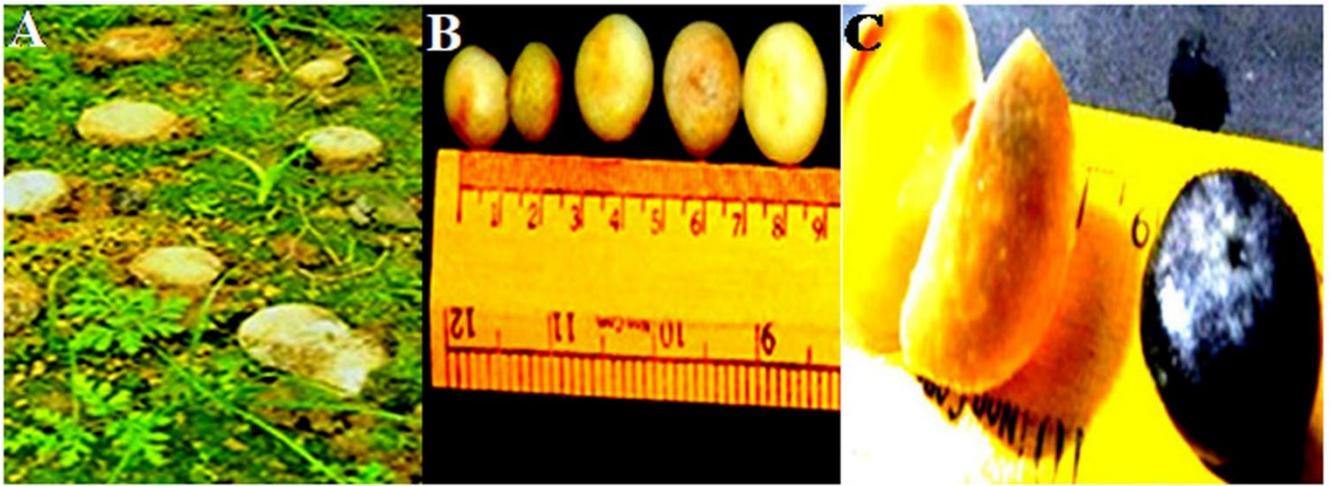
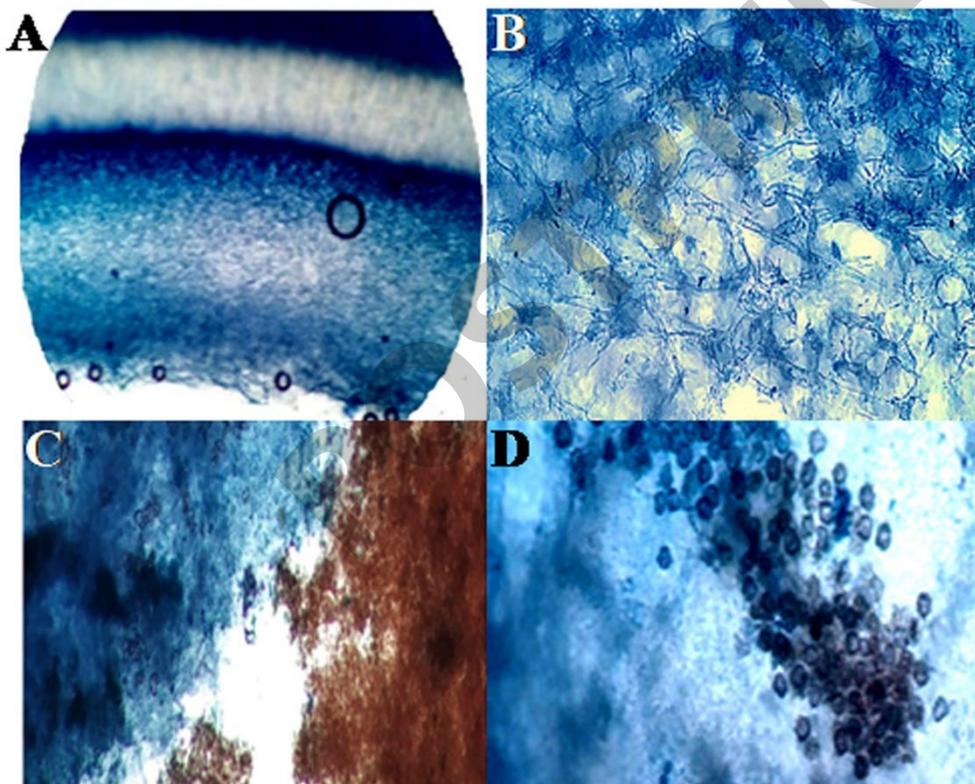


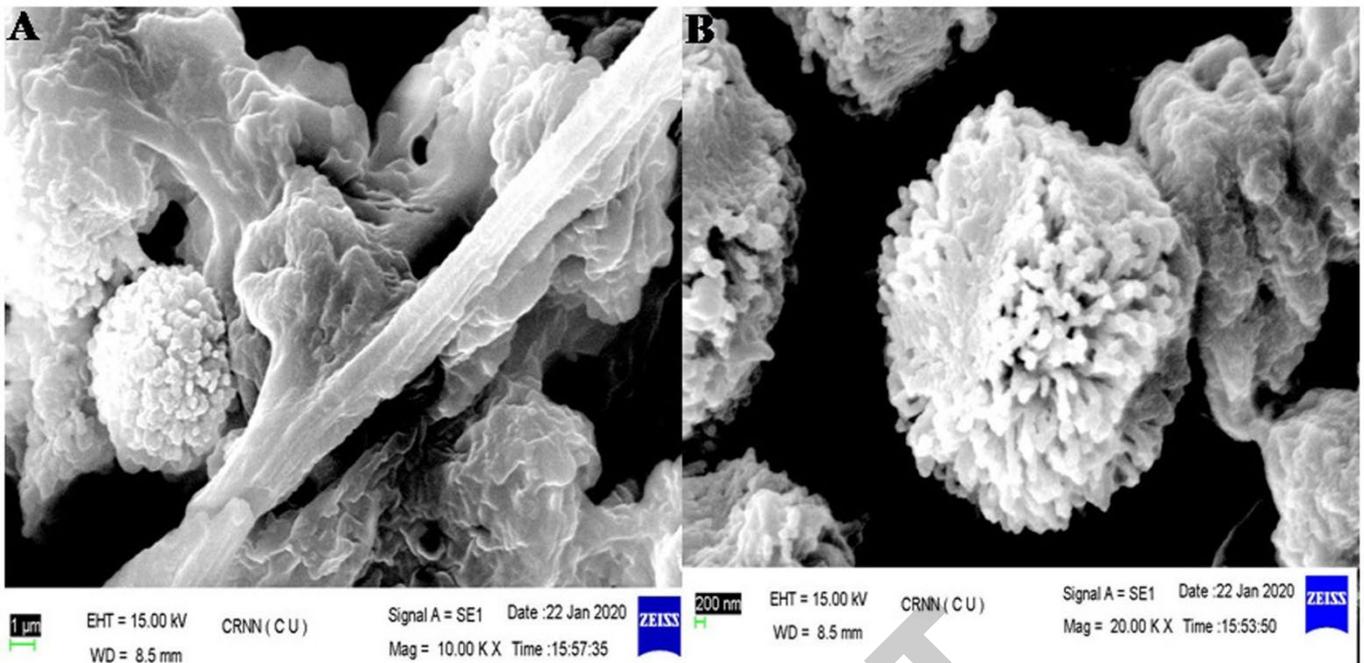
Figure 1 Design of the mushroom fruitbody extraction process in different solvent systems



**Figure 1** Images of *Astraeus asiaticus*. A. Young sporocarps collected from the field; B. Young sporocarp; C. Mature sporocarp.



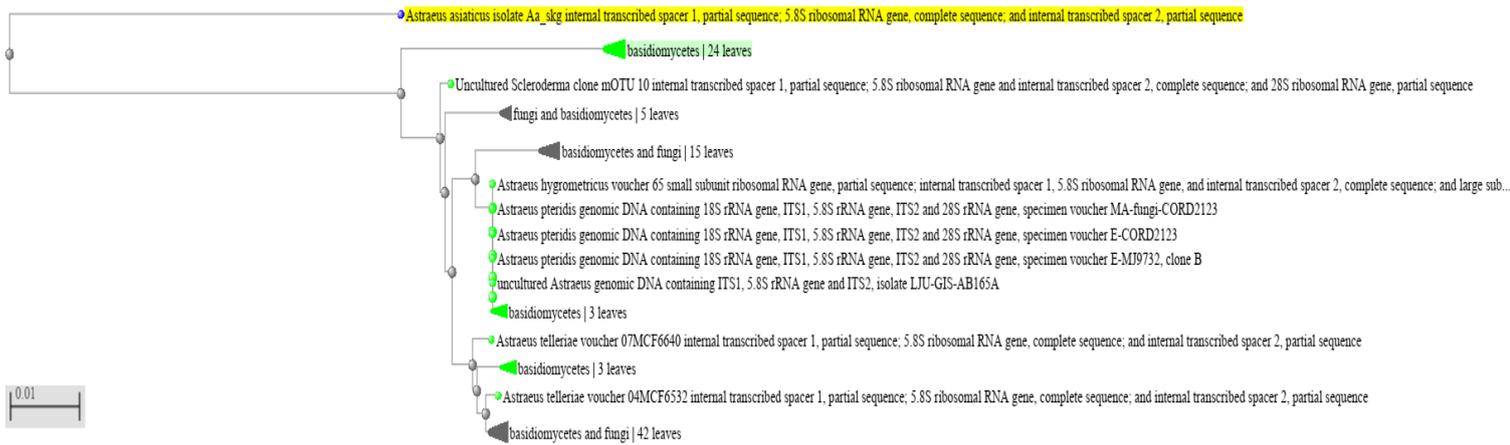
**Figure 3** Images of the anatomy of *Astraeus asiaticus* - A. Cross-section of the gleba; B. Cross-section showing the arrangement of long thick-walled septate hyphal cells; C. spore mass; D. spore mass with basidiospore (40X).



**Figure 4** Scanning electron micrograph. A. Scanning electron micrograph of the hyphae structure (bar= 1 µm); B. Scanning electron micrograph of mature basidiospores displaying dense, rounded spine in groups (bar = 200 nm).

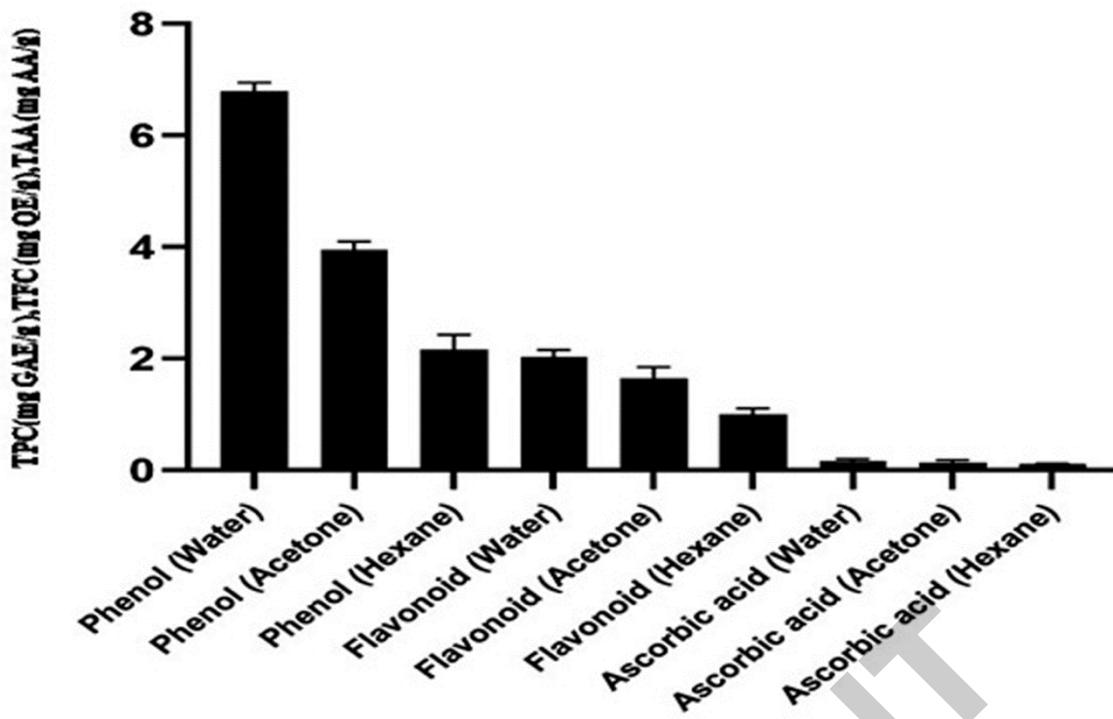
	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
✓	<a href="#">Astraeus asiaticus isolate RUG-a small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...</a>	<a href="#">Astraeus asiaticus</a>	514	514	98%	4e-141	83.05%	793	<a href="#">MN257431.1</a>
✓	<a href="#">Astraeus asiaticus isolate RMUAA28 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, c...</a>	<a href="#">Astraeus asiaticus</a>	514	514	98%	4e-141	83.05%	687	<a href="#">MW403858.1</a>
✓	<a href="#">Astraeus asiaticus genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: Aswan29</a>	<a href="#">Astraeus asiaticus</a>	514	514	98%	4e-141	83.05%	774	<a href="#">AB507404.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-64</a>	<a href="#">Astraeus asiaticus</a>	514	514	98%	4e-141	83.05%	687	<a href="#">AJ629400.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-54</a>	<a href="#">Astraeus asiaticus</a>	514	514	98%	4e-141	83.05%	687	<a href="#">AJ629397.1</a>
✓	<a href="#">Astraeus asiaticus isolate Arora02-121 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...</a>	<a href="#">Astraeus asiaticus</a>	512	512	95%	1e-140	83.54%	774	<a href="#">EU718089.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-9</a>	<a href="#">Astraeus asiaticus</a>	512	512	81%	1e-140	86.10%	686	<a href="#">AJ629381.1</a>
✓	<a href="#">Astraeus asiaticus 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-15</a>	<a href="#">Astraeus asiaticus</a>	510	510	81%	5e-140	86.10%	640	<a href="#">AJ629384.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-8</a>	<a href="#">Astraeus asiaticus</a>	508	508	98%	2e-139	82.88%	687	<a href="#">AJ629382.1</a>
✓	<a href="#">Astraeus asiaticus 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-44</a>	<a href="#">Astraeus asiaticus</a>	505	505	81%	2e-138	85.89%	642	<a href="#">AJ629395.1</a>
✓	<a href="#">Astraeus asiaticus E Wat28787 ITS region, from TYPE material</a>	<a href="#">Astraeus asiaticus</a>	503	503	98%	8e-138	82.71%	663	<a href="#">NR_119468.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain Astrae-23</a>	<a href="#">Astraeus asiaticus</a>	503	503	98%	8e-138	82.71%	687	<a href="#">AJ629389.1</a>
✓	<a href="#">Astraeus asiaticus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, speci...</a>	<a href="#">Astraeus asiaticus</a>	501	501	81%	3e-137	85.74%	714	<a href="#">AB453034.1</a>
✓	<a href="#">Astraeus asiaticus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS 2, 28S rRNA, partial and complete sequence, speci...</a>	<a href="#">Astraeus asiaticus</a>	501	501	98%	3e-137	82.65%	734	<a href="#">AB453028.1</a>
✓	<a href="#">Astraeus hygrometricus 5.8S rRNA gene, ITS1 (partial) and ITS2 (partial), strain ASTRAE-38</a>	<a href="#">Astraeus hygrom...</a>	499	499	66%	1e-136	89.95%	452	<a href="#">AJ629895.1</a>
✓	<a href="#">Astraeus asiaticus isolate Gel band a3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene,...</a>	<a href="#">Astraeus asiaticus</a>	497	497	65%	4e-136	90.13%	664	<a href="#">MT611066.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-17</a>	<a href="#">Astraeus asiaticus</a>	492	492	98%	2e-134	82.36%	686	<a href="#">AJ629385.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-13</a>	<a href="#">Astraeus asiaticus</a>	490	490	98%	6e-134	82.36%	685	<a href="#">AJ629383.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain Astrae-22</a>	<a href="#">Astraeus asiaticus</a>	486	486	98%	8e-133	82.19%	686	<a href="#">AJ629388.1</a>
✓	<a href="#">Astraeus asiaticus 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, strain ASTRAE-19</a>	<a href="#">Astraeus asiaticus</a>	479	479	98%	1e-130	81.85%	686	<a href="#">Feedback</a>

Figure 5 BLAST result of *Astraeus* sp. with the highest sequence similarity of *Astraeus asiaticus*.



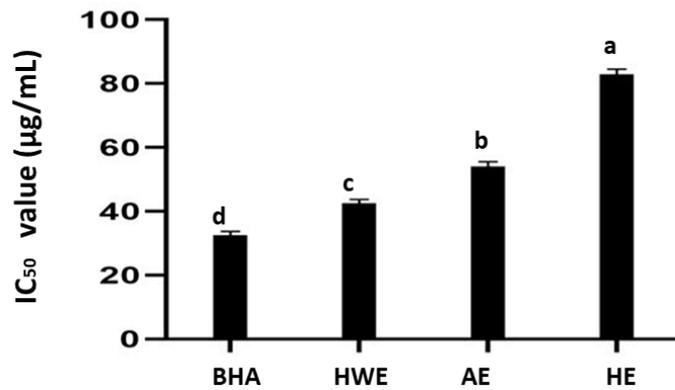
**Figure 6** Neighbor-joining phylogenetic tree showing the evolutionary relationship of the ITS gene sequences. The tree was constructed based on evolutionary distances, which revealed that the test organism is closely related to *Astraeus asiaticus* sp.

POST-PRINT



**Figure 7** Total phenol, flavonoid, and ascorbic acid contents of *Astraeus asiaticus* in the hot water, acetone, and hexane extracts.

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**Figure 8** IC<sub>50</sub> value of the different solvent extracts of *Astraeus asiaticus* ( $\pm$  SD is depicted at the top of data bar). Similar letters at the top of data bars indicate statistical similarity, while different letters indicate statistical difference ( $P=0.05$ ) based on Duncan's multiple range test.

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