MATERIALS AND METHODS
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1. Collection and Preservation

In this study, fungi were collected during field trips carried out around different places of Kashmir province in different seasons between the years 2003-2005. Periodic field trips were made to collect mushroom flora covering a wide range of habitats. The collection included both edible and inedible species. During the field trips local people were consulted to elicit the necessary information regarding vernacular names, edibility, medicinal use and other economic uses of mushrooms. Detailed field notes covering habit, habitat, time of collection, date of collection and morphological characteristics of the macrofungi found in various localities were recorded and the samples were photographed to aid in later identification of mushrooms including cap details, gill details, stipe details, other accessory details, distribution etc. were recorded on spot as an aid in identification. Fresh and undamaged specimens were preferred for further detailed examination in the laboratory. Spore prints were taken on paper or on glass slides to study the color of the spores, shape of the gills and pores and attachment of gills to the stipe (Kuo, 2001; Kuo, 2004). The fungi were identified with the help of relevant literature (Pacioni and Linecott, 1981; Pegler and Spooner, 1992; Purkayastha and Chandra, 1983). Identification was also confirmed from M. Kuo (www.MushroomExpert.com) and Index Fungorum. Expert advice was also taken from Dr. T. N. Kaul former Deputy Director and Head Regional Research Laboratory Jammu and Kashmir, Srinagar (CSIR). The authenticity of identified specimens was checked by referring the recent monographs and through comparison with authentic herbarium specimens at Regional Research Laboratory (CSIR) Srinagar. Further, update valid names of the species and genera were incorporated as much as possible (www.indexfungorum.org). Some of the specimens were preserved as museum specimen and are deposited in the Mycology section, regional Research Laboratory (CSIR) Srinagar.

1.1. Spore prints

The spore prints were prepared following the method of Kuo (2001)

Procedure

The spore print of the mushroom was obtained both on glass slide and spore print paper (black and white). A mushroom with its cap fully opened and gills exposed was taken and
with a sharp sterilized blade the stem was cut off as close to the gills as possible. The fertile surface of the cap was placed on a clean sheet of paper or on a sheet of glass that has been swabbed with alcohol, or on two or four microscopic glass slides and covered with bell jar or clean inverted bowl to prevent drying of the cap and intrusion of foreign organisms. For the larger specimen section of the cap was used. This apparatus (mushroom cap and bell jar) was allowed to stand for 24 hours. A good spore print was visible after 1 to 24 hours depending on the mushroom species. In case the spore print was light or absent the cap was lightly tapped with the flat surface of spatula to shake loose many spores. The jar was removed and cap was lifted slowly. The spore print formed made the pattern of gills in case of agaricoids since the spores fell directly down except Ascus fungi in which spores are ejected outwards. The print was covered immediately after removing the cap to prevent contamination.

1.2. Spore slides

The spore slides were prepared following the method given by Kuo (2004).

Procedure

With the help of clean and dry razor blade spore dust was scraped off from spore print. Spore dust was then tapped onto a clean slide. Drop of Melzer’s reagent and coverslip was placed on the slide. Spores were observed under microscope and noted whether they are amyloid (Turns bluish black in iodine) Inamyloid (No color change with iodine) and dextrinoid (Turns reddish brown in iodine)

Preparation of reagents

The reagent was prepared by the method of Kuo (2004)

Melzer’s reagent

Water: 22.0 g
Chloral hydrate: 20.0 g
Iodine crystals: 0.5 g
Potassium iodide (KI): 1.5 g

1.3. Preparation of Museum specimens
Collected samples were preserved in 4% formaldehyde as museum specimens.

**Preparation of reagent**

The fresh mushroom samples were preserved for museum following the method by Watling in *Introduction to Mushroom Science (Systematics)* by T. N. Kaul (1997).

**Formaldehyde solution**

- Methylated spirit (industrial): 550 ml
- 40% formaline (industrial): 50 ml
- Glycerol (commercial glycerine): 50 ml
- Water (Distilled water): 350 ml

Pure cultures of different mushrooms were prepared and maintained on PDA (Potato Dextrose Agar) slants and deposited in Regional Research Laboratory, Srinagar. Isolation was done from the deep tissues of stipe near attachment to cap. Mushroom specimens were also dried (shade dried) and stored in Ziplock plastic bag (Kuo, 2003) for museum as well as analysis for chemical constituents depending upon the availability of the samples collected.

2. **Preliminary phytochemical and elemental analysis**

Seven edible mushrooms species viz, *Coprinus atramentarius*, *Coprinus comatus*, *Ramaria Formosa*, *Lentinus tigrinus*, *Morchella vulgaris*, *Pleurotus ostreatus* and *Helvella acetabulata*. Fully matured mushroom samples were collected and wiped off any adhering dust and soil. Samples were first washed in running tap water followed by distilled water. After complete cleaning, each sample was either shade dried or oven dried at 40-105°C for 24 hrs. Dried samples were homogenized and stored in polyethylene zip lock bags and labelled prior to analysis.

Three Mushrooms (same mushroom species but collected from two different sites) were separately analysed for the variation in their chemical composition.
2.1. Preliminary Phytochemical analysis

The preliminary phytochemical screening was carried out using different mushroom extracts following the standard methods (Ali, 1998) for the detection of alkaloids, carbohydrates, glycosides, phenolic compounds, tannins and saponins.

2.1.1. Test for alkaloids

i) Dragendorff’s test

To the extract (2ml), 4-8 drops of reagent were added; orange red color obtained indicates the presence of alkaloids.

ii) Wagner’s test

In 2ml of extract, few drops of reagent were added; reddish-brown color is obtained, indicating the presence of alkaloids.

Preparation of reagents

Dragendorff’s reagent

Bismuth nitratrate (Bi(NO₃)₃) 8gm dissolved in 2ml, HNO₂ + 27.2gm of potassium iodide (KI) in 50 ml of water. Two solutions were mixed and allowed to stand. When KNO₃ crystallizes out supernatant was taken and made upto 100ml with DW.

Wagner’s reagent

1.27g of iodine and 2g of potassium iodide was dissolved in 5ml of water and volume was made 100ml with dw.

2.1.2. Test for Carbohydrates

i) Molisch’s test

To about 2ml of test residue in test tube of Molisch’s reagent and concentrated H₂SO₄ was added from the side of the test tube formation of reddish brown ring at junction indicated presence of carbohydrate.
ii) Fehling’s solution

1 ml of fehling’s solution A and B was added to samples and heated on water bath. Presence of brick red precipitates indicate the presence of carbohydrate.

Preparation of reagents

Molisch’s reagent

10 gm of α-napthol in 100 ml of 95% alcohol

2.1.3. Test for Saponins

Aqueous extract of sample was shaken vigorously in a test tube with a drop of sodium bicarbonate solution and left for three minutes. Honeycomb like froth is formed indicating the presence of saponins.

2.1.4. Test for phenolic compounds and Tannins

Ferric chloride test

The extracts were taken in water and warmed; to this 2 ml of ferric chloride solution was added and observed for the formation of green and blue color indicating the presence of phenolic compounds and tannins.

Lead acetate solution

To the extract (2 ml) lead solution was added and observed for the formation of precipitates.

Preparation of reagent

Ferric chloride solution

5% w/v solution of ferric chloride in 90% alcohol.

Lead solution

10% w/v of Basic lead acetate in distilled water.
2.1.5. Test for glycosides

To the sample extract dilute HCL and FeCl₂ was added and heated for two minutes followed by chloroform extraction. Chloroform layer was separated from the extract and Ammonia was added. Reddish color was formed indicating the presence of Glycosides.

2.2. Biochemical analysis

2.2.1. Estimation of soluble protein content

The total soluble protein content of different mushroom samples was estimated following the method of Bradford (1976).

Preparation of reagents

a) Extraction buffer

0.1 M phosphate buffer (pH 7.2) was used as extraction buffer. The solution of potassium dihydrogen phosphate (KH₂PO₄) and dipotassium hydrogen phosphate (K₂HPO₄) was prepared in the following manner:

Solution A

1.3 g of KH₂PO₄ was dissolved in DDW and the volume was made to 50 ml.

Solution B

1.70 g of KH₂PO₄ was dissolved in double distilled water (DDW) and the volume was made to 100 ml. Solution A and B were mixed in an appropriate ratio to adjust the pH at 7.2 with the help of a pH meter. 1.0 g PVP was added to 100 ml of this buffer.

b) 10% (w/v) TCA

10 g of TCA was dissolved in doubled distilled water to a final volume of 100 ml.

c) 0.1N NaOH
0.4 g of sodium hydroxide (NaOH) pellets was dissolved in double distilled water to make a final volume of 100 ml.

d) Bradford’s reagent

50 ml 90% ethanol was mixed to 100 ml of ortho-phosphoric acid (85%). Its volume was made up to 1 L and 100 mg of Coomassie Brilliant Blue (G) dye was added to it which was stirred well on a magnetic stirrer in dark covered volumetric flask. The solution was then filtered through Whatman filter paper no.1 and stored in dark conditions. Resultant reagent was called Bradford’s reagent. The final concentrations of components in the reagent were 0.01% Coomassic brilliant blue G-250 (w/v), 4.75% ethanol (w/v) and 8.5% O-phosphoric acid (w/v).

Procedure

Extraction of total soluble protein

0.1 g of dried sample was homogenized in 1.5 ml of 0.1M phosphate buffer (extraction buffer) at below 4°C with the help of a mortar and pestle, pre cooled and kept in ice during the process of homogenization. The homogenate was transferred to a 2 ml eppendorf and centrifuged at 5000 rpm for 10 min at 4°C. An equal amount of chilled 10% TCA was added to 1 ml of the supernatant, which was again centrifuged at 3300 rpm for 10 min. The supernatant was discarded and the pellet left was washed with acetone. It was then dissolved in 1ml of 0.1 N NaOH.

Estimation of total soluble protein

To 0.1 ml of aliquot, 5 ml of Bradford’s reagent was added and vortex mixed. The tubes were kept for 10 min for optimal color development. The absorbance was then recorded at 595 nm on a Beckman spectrophotometer (DU 640, Fullerton, USA). The soluble protein concentrations were quantified with the help of a standard curve prepared from the standard of Bovine Albumin Serum (BSA) from Sigma, USA. The protein content was expressed in mg g⁻¹ dw.

2.2.2. Estimation of total soluble sugar
The soluble sugar was estimated by the method of Dey, (1990).

**Preparation of reagent**

5% Phenol

Phenol solution was prepared by mixing 5g of phenol in 100ml of DDW.

**Procedure**

0.1 g material was kept in 10 ml of alcohol for 1 hour at 60°C in incubator. The extract was then decanted into a 25 ml volumetric flask and the residue re-extracted. Final volume was made up to 25 ml by adding alcohol. One ml of aliquot was transferred to a thick walled test tube and 1.0 ml of 5% phenol was added to it and mixed thoroughly. Five ml of analytical grade sulphuric acid was then added to it and mixed thoroughly by vertical agitation with a glass rod. For exothermic reaction the test tube were cooled in the air. Absorbance was recorded at 485 nm on Perkin Elmer UV/VIS Spectrometer Lambda Bio 20 (Switzerland).

The corresponding concentration was determined against a standard curve prepared by using a glucose solution.

2.2.3. Estimation of total Amino acid content

Lee and Takahashi’s method (1996) was use for the estimation of soluble amino acids.

**Preparation of Reagents**

a) Citrate buffer (0.5 M, pH 5.5)

0.5 M citric solution was prepared by dissolving 52.339 g citric acid in DDW and final volume was made 500 ml. 0.5 M sodium citrate solution was prepared by dissolving 73.53 g of sodium citrate in DDW and final volume was made to 500 ml. Two components of the buffer were mixed in an appropriate amount to maintain the pH of buffer 5.5 that was monitored with the pH meter.

b) 55% glycerol
It was prepared by mixing 55 ml of glycerol and 45 ml of DDW

C) 1.0% ninhydrin solution

Ninhydrin solution was prepared by dissolving 1.0 g of ninhydrin in 0.5 M citrate buffer to a final volume of 100 ml.

Procedure

0.1 g of material was ground in 5.0 ml of absolute ethanol with the help of mortar and pestle and transferred to the centrifuge tubes. It was then centrifuged at 5000 rpm for 10 minutes at 4°C. After that supernatant was taken in a test tube and alcohol was evaporated by incubating the test tubes at 80°C for 1 hr in a water bath. Pellet was dissolved in 10 ml of 0.5 M citrate buffer (pH 5.5).

Assay mixture

To 0.5 ml of aliquot, 1.2 ml of 55% glycerol and 0.5 ml of 10% ninhydrin solution was added. The mixture was boiled for 20 minutes and volume was made up to 6 ml by adding citrate buffer. Absorbance was recorded at 570 nm on UV-vis Spectrophotometer (Model DU 640 B, Beckman, USA). Calibration curve was prepared from Glycine (Sigma) of different concentrations to calculate the amino acid content in different samples. The concentration was expressed in mg g⁻¹ dw.

2.2.4. Estimation of Vitamin C (ascorbic acid)

Estimation of reduced, oxidized and total ascorbate was done by the method of Law et al. (1983) with a slight modification.

Preparation of Reagents

a) Extraction mixture

0.1 M PO₄-buffer (pH 7.0) with 1 mM EDTA

b) 5M NaOH

It was prepared by dissolving 2 g of NaOH in 10 ml of double distilled water.
c) 150 mM PO₄-buffer (pH 7.4)

It was prepared from the buffer components as follows

**Solution A**

150 mM NaH₂PO₄ was prepared by dissolving 1.7 g in DDW and the volume was made up to 50 ml

**Solution B**

150 mM Na₂HPO₄ was prepared by dissolving 1.06 g of this chemical in DDW and the volume was made up to 50 ml. 150 mM PO₄-buffer (pH 7.4) was prepared from these two (A and B) components by mixing them in an appropriate ratio and adjusting pH to 7.4 with the help of a pH meter.

d) 2 mM MgCl₂

It was prepared by dissolving 19.9 mg MgCl₂ in 100 ml of Tris buffer 0.1 M (pH 8.0).

e) 10 mM Dithiothreitol (DTT)

It was prepared by dissolving 7.7 mg of DTT in 5 ml of double distilled water.

f) 0.5% (w/v) N-ethylmaleimide

It was prepared by dissolving 75 mg of the chemical DDW to a final volume of 15 ml.

g) 10% (w/v) TCA

It was prepared by dissolving 10 g of TCA in DDW and the volume was made up to 100 ml.

h) 44% (v/v) H₃PO₄

It was prepared by mixing 6.67 ml of H₃PO₄ in DDW and the volume was made up to 20 ml with DDW.

i) 70% (v/v) Ethanol
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It was prepared by mixing 70 ml of ethyl alcohol in DDW and the volume was made up to 20 ml.

j) 4% (w/v) Bipyridyl dye

It was prepared by dissolving 2 g of N’N-dimethyl bipyridyl dye in 50 ml of 70% ethyl alcohol.

k) 3% (w/v) FeCl₃

It was prepared by dissolving 0.75 g of FeCl₃ in DDW and the volume was made up to 25 ml with DDW.

Procedure

0.1 of material was ground in 2ml of extraction buffer and centrifuged at 10,000g for 10 min. The supernatant was collected and used for the assay immediately. 1ml supernatant was taken and mixed with 0.5ml TCA 10%. Store in ice for 5 min. add 0.5 ml of 0.1 M NaOH and mix. Centrifuge again at 5,000 rpm for 10 min. To 0.2ml supernatant add 0.1ml DTT (1mM), mix and keep for 15 min at room temperature. Add 0.1ml of 0.5% N-Ethylmaleimide. Vortex and incubate for 30 minutes at room temperature. Add 0.4ml of 10% TCA, 0.4ml 44% H₃PO₄, 0.4ml Bipyridyl (4% in 70% Ethanol) and 0.2ml FeCl₃ (3%). Vortex and incubate at 37°C for 60 minutes. Take O.D at 525 nm.

2.3 Elemental composition

Mushroom samples were analysed for assessing Nitrogen (N), Hydrogen (H) Sulphur (S) and Carbon (C) status along with Cadmium (Cd), Arsenic (As), Manganese (Mn), Iron (Fe), Zinc (Zn), Copper (Cu), Mercury, (Hg), Sodium (Na), Potassium (K) and Selenium (Se) accumulation in Fruiting bodies.

2.3.1. Estimation of Nitrogen (N), Sulphur (S), Hydrogen (H) and Carbon (C)

The N, H, S and C content (% dry weight) were analysed by packing the powder in tin boats after careful weighing (Balance) with the help of Elementar Analyse system GmbH (CHNS Analyser, Model Vario EL).
2.3.2. Estimation of Crude protein

Crude Protein percentage (Factor 4.38 multiplied to Nitrogen content) was done through Elementar Analysensysteme GmbH (CHNS Analyse, Model VarioEL III). The analysis by CHNS Analyser was done according to the protocols prescribed in the manual of the machine in CIF (Central instrumentation facility) Jamia Hamdard, New Delhi.

Procedure

10 mg of dried and homogenized samples of mushroom was taken and packed in tin boats along with the mixture of Tungsten oxide and Wolfram (VI) Oxide mixture (from Merck chemical company). The samples were analyzed along with the blank (Gas-Helium and Oxygen) and standard (Sulfanilic acid procured from Merck chemical company). It was expressed as % / gm powder used.

2.3.3. Estimation of Cadmium (Cd), Arsenic (As), Mercury (Hg), Selenium (Se) and Chromium (Cr).

The status of elements was estimated by the method described by Gupta (1999. The methods are briefly described below:

Analysis of Se, Cr, As, Hg and Cd was done through Atomic absorption spectrophotometer system (AAS system fitted with graphite tube of Wall type, Analytikjena, Germany, model Analytic Xena Zeenit 65) according to the protocols prescribed in the manual of the machine in CIF (Central instrumentation Facility) Jamia Hamdard, New Delhi.

The digested and diluted samples were loaded in the AAS system. The obtained readings were multiplied by the dilution factor. The content of metals in samples were expressed as µg g⁻¹ dw.

2.3.4. Estimation of Copper, Iron, Mn and Zn

Analysis of Cu, Fe, Mn and Zn was performed on Atomic absorption spectrophotometer (Flame based model AAS 4141). The samples were analyzed in triplicates along with sample blanks. The standard curve for each metal analyzed was made utilizing analytical grade standard metal solutions procured from Merck chemical company.
2.3.5. Estimation of Potassium (K) and Sodium (Na)

The status of K and Na was estimated by the method of Lindner (1944) and was analysed in the digested samples using flame photometer. The readings were recorded directly in ppm and converted to actual value of K and Na in samples by multiplying with dilution factor. It was expressed as \( \mu g \, g^{-1} \, dw \) sample.

Procedure

Acid digestion of the samples

0.5 gm of the dried and homogenized sample of mushrooms was taken and was digested in a mixture of concentrated acids (2:1) HNO\(_3\): HClO\(_4\) in the 50 ml digestion tubes over a block digestor. Small funnels were placed over the tubes and the samples were heated to 60\(^\circ\) C for 15 min, further the temperature was increased to 120\(^\circ\) C and the digestion was carried out for 75 min until the samples cleared. Afterwards the samples were cooled down and the volume was made upto 50 ml with milli Q water containing 2\% HNO\(_3\). Suitable aliquots, for determining elements were taken from these acid digested samples. The plastic ware and glassware were cleaned by soaking overnight in a 10\% nitric acid solution and then rinsed with deionised water.

2.4. Statistical analysis

The data obtained for various parameters were statistically analyzed to check the authenticity of the results. The following formulae were put into use:

\[ \text{Mean (} \bar{x} \text{)} \]

The arithmetic mean or the average value was calculated by adding together all the items (values) and by dividing this total by the number of observations involved.
(\bar{x}) = \frac{X_1 + X_2 + X_3 + X_4 + X_5}{N} \text{ Or } (\bar{x}) = \frac{\sum X_n}{N}

Where,
(\bar{x}) = Arithmetic Mean
(X_1 + X_2 + X_3 + X_4 + X_5) = Number of observations
\sum X_n = Sum of all the variables
N = Total number of observations

Standard error

The standard error is used in determination of statistical significance of the values obtained. It is defined as the ratio of standard deviation to the square root of the total number of observations, and expressed as:

$$SE = \frac{\text{Standard deviation (S.D)}}{\sqrt{\text{Number of observation}}}$$