ISSN. 0972 - 8406

The NEHU Journal, Vol XV, No. 1, January-June 2017, pp.65-78

Pigskin Poison Earthball Mushroom of Meghalaya: An Identification Paradox

MADHUSMITA BORTHAKUR[1] & SANTA RAM JOSHI[2]

ABSTRACT

Earthball mushrooms or 'Puffball' mushroom from subtropical forest of lower belt of the Himalayan region of India, regularly consumed in eastern India, was identified based on fungal ITS molecular barcode. The phylogenetic analysis revealed that it clades under the genus Scleroderma of order Boletales which was verified by Neighbor Joining (NJ) tool. The detail analysis of the gill section and the spore morphotype revealed lack of gills with abundant veins like connections. Spore morphotype in the scanning electron microscope (SEM) revealed a globular structure with an articulate covering. The morphology, microscopy and molecular data revealed the puffball structure to be of Sclerodermataceae family under the Agaricomycetes group. This study reports the characterization of irregular potato shaped earthball mushroom, Scleroderma from northeast India.

Keywords: Puffball, Wild Mushroom, Molecular Barcode, SEM

Introduction

Over the years, wild mushrooms are known to have ethnic relevance, some as expensive delicacies while others as potent medicine or as supplementary income. Wild mushrooms are becoming global commodities and regional resources are susceptible to worldwide demands and exploitation. Such changes are expected to increase problems for the management of wild mushrooms, with potentially far-reaching implications for the survival of such resources and for the livelihood of the indigenous

---

[1]*Ms. Madhusmita Borthakur is a research scholar at the Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong 793 022, India*

[2]*Dr. S.R. Joshi teaches at the Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong 793 022, India*

[2]*Corresponding author: Dr. S.R. Joshi (srjoshi2006@yahoo.co.in)*

people who partly depend on these resources for their livelihood. Though each society shows diverse preferences, strong relationships between human and wild mushrooms are found across all racial, ethnic and geographic populations. The mushrooms are the most fascinating and striking inhabitants of the natural world with their distinctive flavors used in cooking or as potent medicines.

Meghalaya is an ethnomycophlic society where mushrooms have formed an integral component of tribal dietary habits. The mushrooms are picked every year by the locals and have formed an integral part of their diet during the onset of the monsoon. In view of the intimate association of the tribals with nature, the knowledge of mushroom species is limited to the old-aged villagers and the region still remains to be scientifically explored for its mushroom flora. In spite of immense popularity among the tribes of Northeast India, data on mushrooms identity and toxicity are scarce. More than 95% of the mushroom poisoning occurs as a result of misidentification by the hunters (Horowitz and Hendrickson, 2015). With regard to toxicity, there is a history of relevant practical observations relating to the poisonous nature of mushrooms. With 14 different mushroom poisoning types, the most frequent form is caused in gastrointestinal tract. Presently mushroom poisoning has become an important concern in different parts of India. In Meghalaya, large number of people die every year because of mushroom poisoning. Three tragic incidents of mushroom poisoning were reported in 2016, i.e., on April 6, 8 and 13 where the death toll rose to more than 10 after consumption of wild mushrooms in Mawsawa village of Mawsynram and Nongpriang of Sohra (Anonymous, 2016). On April 22, 2016, the death toll of six people from mushroom poisoning in Rongdong village of Siju in Garo Hill district of Meghalaya was reported (Anonymous, 2016b).

The basidiomycete fungus *Scleroderma citrinum* is also known as ‘Poison Puffball’ mushroom or ‘LeopardEarth-ball’ or ‘Scaly Earth-ball’. Commonly known to be as dead man’s hand, these are prevalent in the climatic zone of Asia, Europe and America. They appear superficially identical to potato with tough warted skin. When cut into a half section, the black soil like “gleba” bearing spores appear. The feature and the morphotypes are alike to that of the common edible puffball mushrooms or Truffles and are often misidentified and consumed by the locals. Researchers have confirmed the presence of cryptic species in fungi. The use of molecular markers like ITS fungal DNA barcode and its phylogenetic studies to substantiate the void between inter and intraspecific taxa are significant for

the identification of these cryptic species. Therefore, it is significant to identify the toxic group of macrofungi that could contribute to proper identification of this cryptic mushroom and provide distinguishing features of edible varieties.

The present work deals with the identification of a puffball mushroom based on its morphotype, microscopic (optical and electron microscope) and molecular analysis to generate detail structure of the mushroom and it is the first report on this mushroom from northeast India.

Methods and Materials

A. Sampling

Morphotypic traits were recorded at the site of collection for the fresh puffball from forests of the East Khasi Hills of Meghalaya. After clustering the macro-morphological traits, the potato shaped mushroom was brought to the laboratory in a sterile plastic bag. All the morphological data including the size, shape, odour, colour and spores were considered. The mushroom was allowed to release the spore print for next 9-12 h. A part of the mushroom section was kept in 4% formaldehyde and preserved in in-house culture collection center of the North-Eastern Hill University.

B. Microscopic Analysis

Transverse sections of the mushroom cap along with the gills were cut into several sections and were mounted in a glass slide with both Lactophenol cotton blue and Melzer's solution (chloral hydrate, potassium iodide and iodine) (Tsujikawa *et al.*, 2003). Sections were viewed in Leica Microsystems (CH-9435, Heerbrugg) after covering the section with a cover slip.

C. Scanning Electron Microscopy Study

Transverse sections of the tissue were dissected and were washed with phosphate buffer saline (PBS) pH 7.3 for several times to remove any dirt to observe under the scanning electron microscope. Later the sections were fixed in 2.5% glutaradehyde in 0.1 M cacodylatebuffer (pH 7.2) and incubated overnight at 4º C. After the incubation hours, the glutaraldehyde was drained and the sections were placed for three consecutive 1 h wash in 0.1 M cacodylate buffer which were later dehydrated twice in a series of acetone (30%-100%) after every 15 min. The washed sections were dried by immersing in TMS for 10 min with two changes at a temperature of 4° C. Finally, it was mounted on an aluminium stubs using double sided adhesive

tape and was layered with gold by sputtering in a vacuum sputter (JFC-1100, JEOL, JAPAN) at various magnification to obtain surface adornment. SEM analysis of the sample was carried out at 20 kV of accelerating voltage (Borthakur and Joshi, 2016).

D. Molecular Analysis

The fungal tissue was considered for extraction of genomic nuclearDNA using HiPurA fungal DNA isolation kit (Himedia, India) and was amplified by twoprimersITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Amplification was carried out in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, USA) under the following parameters: initial denaturation at 95° C for 5 min; 35 cycles of denaturation at 9° C for 1min, annealing at 52° C for 30 s, extension at 72° C for 1 min; and a final extension at 72° C for 10 min. The amplified PCR product was electrophoretically analyzed on 1.5% agarose gel along with 100 bp marker ladder. Amplified ITS ribotypes were purified and sequenced (Xcelris Lab, India). The sequences of ITS gene were searched for its appropriate homologous hits using BLASTN in NCBI database. The analogous sequences which showed close proximity to the query sequence were considered as a hit. The nucleotide sequence coding for rRNA gene were computed for their alignment using ClustalW software. Electropherograms was manually edited to omit the gap using Chromaslite and deposited to NCBI to obtain the accession number. Neighbour joining (NJ) was used to analyze the evolutionary distance matrix between the query sequence and its homologous hits. NJ tree was computed using MEGA 6 software (Tamura *et al.*, 2013). Robustness of NJ was calculated by analyzing the bootstrap of 1000 replicates (Kimura-2 parameter).

E. Extraction of Crude Metabolite

The sample was cleaned and gleba was removed, oven-dried and powdered using mortar pestle. The puffball powder was extracted in methanolic solvent as described earlier (Borthakur and Joshi, 2016). The dried crude was resuspended in aqua methanol solvent at a concentration of 500 mg/mL for its bioactivity analysis.

F. Determination of Antioxidant Assay

a. 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The free radical scavenging activity of the methanolic extract of the mushroom was done with the protocol as described by Devi *et al.* (2008).

Three mL of the mushroom extract with different concentrations (20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL) were mixed with 1 mL of DPPH (0.1 mM) solution in methanol. The mixture was shaken vigorously and left to stand at room temperature in the dark for 30 min. Absorbance was then measured at 517 in UV spectrophotometer. A low absorbance value indicated a high free-radical-scavenging activity. BHT was considered as a positive control for the test. The DPPH scavenging activity of the extract was analyzed using the following equation

$$\text{DPPH scavenging effect (\%)} = 100 \times (1 - A_C/A_D)$$

where A_C is the absorbance of the reaction with test sample and A_D is the absorbance of the DPPH reagent without the test sample.

b. Reducing Power Assay

Reducing power assay was performed using the protocol as described by Oyaizu (1986). To various concentrations of the methanolic extract (2.5 mL) were mixed with sodium phosphate buffer (2.5 mL of 200 mM, pH 6.6) and potassium ferricyanide (1% w/v, 2.5 mL). The mixture was incubated at for 20 min at 50º C. Trichloroacetic acid (10%, 2.5 mL) was added to the above reaction mixture and was centrifuged at 650 rpm for 10 min. The upper layer (5 mL) was mixed with deionised water (5 mL) and 9erric chloride (0.1%, 1 mL), and the absorbance was measured spectrophotometrically at 700 nm. Higher absorbance indicates higher reducing powers. BHT was considered as a standard.

G. Determination of Bioactive Compounds

a. Total Flavonoid

Total flavonoid content of the experimental mushroom was assayed using the protocol of Park *et al.* (2008). The methanolic extract (5 mL) was mixed with 10% of aluminium nitrate (1 mL) and 1 M aqueous potassium acetate (1 mL). The mixture was kept at room temperature for 40 min and the absorbance was noted after the incubation time at 415 nm. Quercitin was used as a standard for the test.

b. Total Phenolic

Folin-Ciocalteau calorimetric method was used to determine the total soluble phenolic matter of the crude extract (Singleton *et al.*, 1991).The methanolic extract (0.5 mL) was mixed with 0.2 mL of *Folin-Ciocalteau* reagent (1 N) and incubated for 15 min at room temperature to which 20% of sodium carbonate (0.6 mL) was added and mixed. The reaction was allowed to stand

at 40° C for 30 min in the dark and the absorbance was recorded at 765 nm after the incubation period. Results were expressed in terms of mg of gallic acid equivalents per gram (mg GAE g^{-1}) of the test mushroom.

Results and Discussion

A. Etymology

The epithet of the test puffball is similar with *Scleroderma citrinum* which grow lonely in moist soil.

B. Taxonomic Analysis

The fruiting body is morphologically similar to a round shaped potato of usually 10 cm in diameter. The texture is rough with scaly irregular warts on the upper surface and is yellowish to brownish in colour. Mostly unpleasant odour result from release of the spores during maturity by rupture of skin, leaving a wide opening. The spore mass inside the thick outermost shell is the "Gleba" which becomes purple brownish on maturity. The round potato shaped mushroom usually grows in acidic soil (Fig. 1).

Fig. 1. Morphology of the common Pigskin Poison Earth-ball mushroom

C. Microscopic Analysis

a. Optical Microscopy

The warty potato shape *Scleroderma cirinum* differs from its related species Boletes (especially *Gyropus*) in its morphological trait. It belongs to the *Scleroderma taceae* family and is often known as a common parasite for *Pseudoboletus parasiticus* and *Hypomyces chrysospermus*. The bumpy spiny pseudoamyloid spores when stained with both lactophenol and melzergive distinct features which differentiates the warty poisonous earthball mushroom from its closely related *Boletus* group (Fig. 2). The spore dimension usually varies from 5 to 12 µ with spiny thick outer cover.

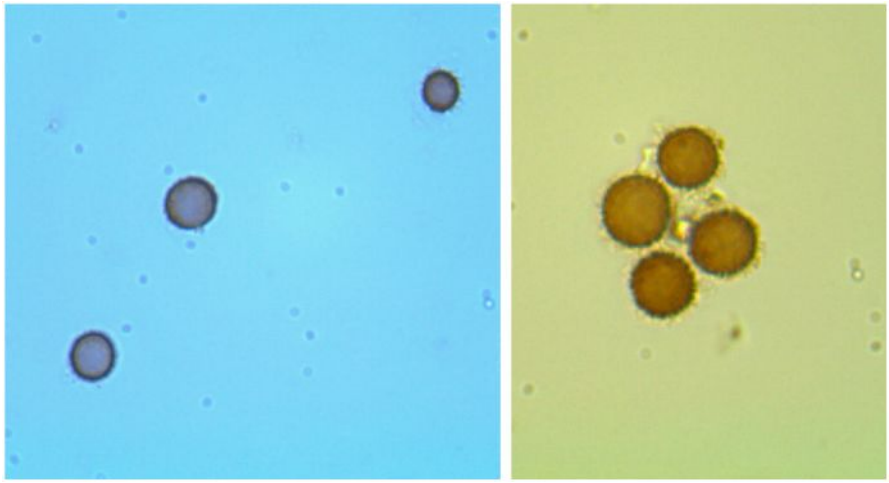

Fig. 2. Spore morphology of the puffball mushroom observed under optical microscope

b. Electron Microscopy

Through SEM, it was possible to observe the surface of the fruiting body. Most of the surface lamellae are filled with thick cylindrical tube shaped hyphaltrama with the presence of spiny basidiospores, the basidioles (Fig. 3). Hyphae are thick walled with apex somewhat cylindrical with wider opening. Certain part of the lamellae appeared damaged, shriveled and twisted due to the loss of water from the tissue during dehydration. Microscopically, it has abundant cylindrical shaped long tube like hyphae, often bulbous to wide at the apex or opened sheath like features. The unique feature of *Scleroderma citrinum* which are the long thick tube like hyphae

with wider opening and the presence of spiny hard coated spores attached to the surface or edge of the lamella has been observed in the section of the gills in optical microscope.

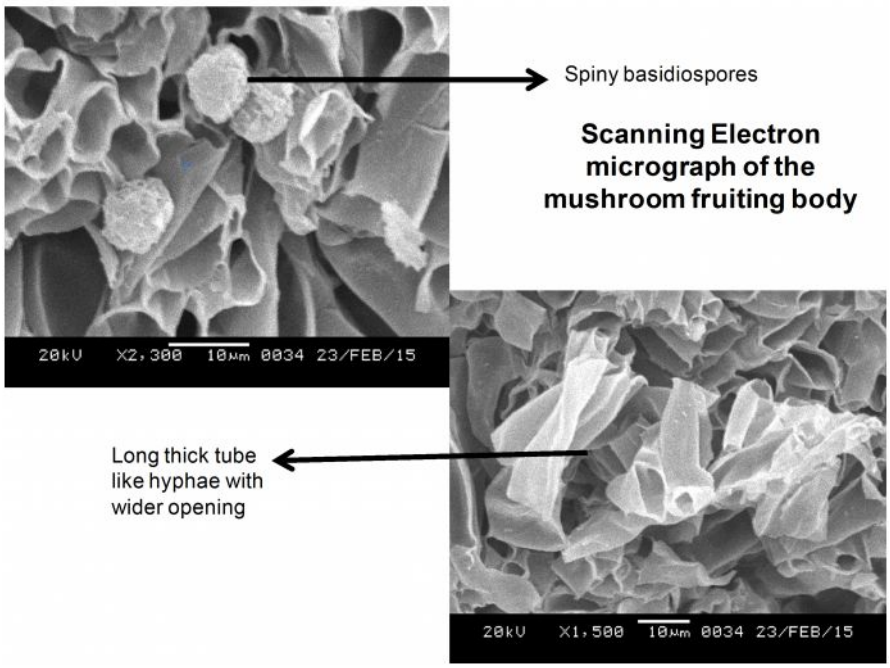

Fig. 3. Scanning Electron micrograph of the mushroom fruiting body

D. Molecular Analysis

Molecular characterization of the conserved rDNA-ITS region of the macrofungi provided confirmatory identification where the sequence data were aligned using BLAST. The ITS sequence for the macrofungi was deposited to NCBI and the accession numbers obtained as (KY883344). The Neighbor Joining (NJ) analysis was performed to check the relationship clade of the sequenced sample. The tree based on the ITS rDNA showed the sequence to clade with *Scleroderma citrinum* with 86% sequence homology with a strong supported bootstrap of 1000 replicates (Fig. 4). The species is identified as *Scleroderma* based on its morphological, microscopic including hyphal cell wall and spiny spores authenticated by molecular characterization using ITS as a standard fungal barcode with the sequence length of 693 bp (KY883344).

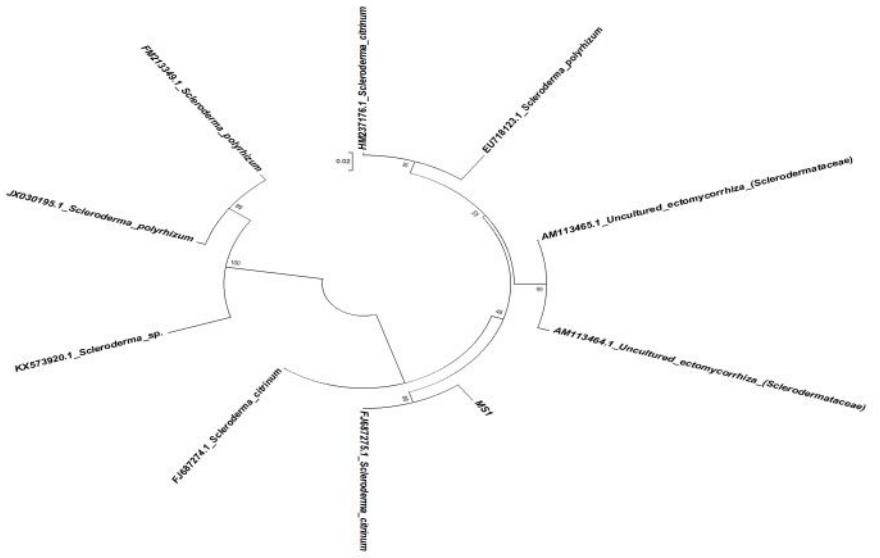

Fig. 4. Evolutionary positions of puffball fungi with its related species based on rRNA-ITS sequence similarity.

E. Phytochemical Screening

The experimental mushroom was analyzed for the presence of phytoconstituents such as alkaloids, flavonoids, terpenes, saponin, cardiac glycosides, phenolics and carbohydrates (Table 1). Among all the phytoconstitutents, the flavonoid content is higher in *Scleroderma citrinum* which corroborated with the findings of Alvarez-Parrilla *et al.* (2007) where the wild mushrooms are known to posses phenols and flavonoid in higher concentration to that of the commercial mushrooms.

Table 1. Major phytochemicals in the crude mushroom extract.

Scleroderma citrinum	Alkaloids	Flavonoids	Terpenes	Saponin	Cardiac Glycosides	Phenolic	Carbohydrates
	+	+++	++	+	++	++	+

+++ indicates intense, ++ indicates moderate and + indicates low quantity

F. Antioxidant Assay

a. DPPH assay

The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is a free radical which when reacted with active antioxidant can donate its hydrogen or electron and gets reduced from dark violet to pale yellowish in colour. The methanolic extracts of the puffball possessed good free radical scavenging activity which increased with concentration when compared to commercially synthesized butylatedhydroxytoluene (BHT) taken as a positive control (Fig. 5a). The observation suggests that the scavenging effect of the methanolic extract of common puffball mushroom was lower than that of commercial BHT. The inhibition was found to be 6.26 to 20.53 from a concentration of 20-100 µg/mL. The IC_{50} value obtained from the regression graph was 227.38 µg/mL.

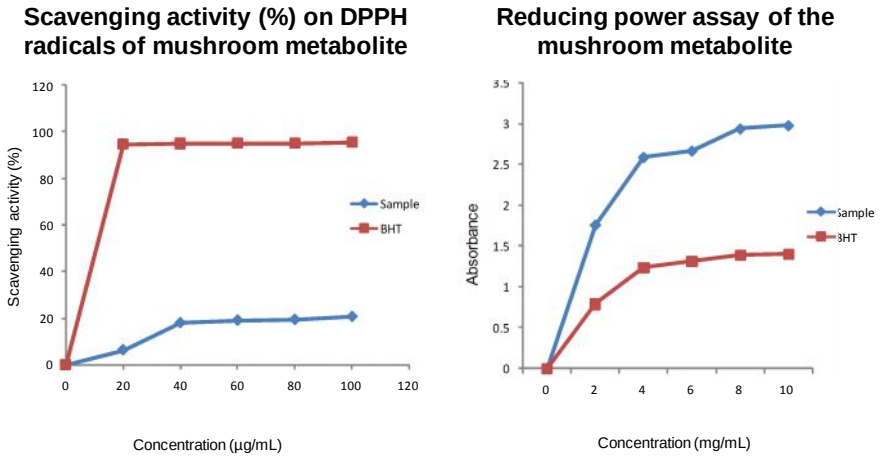

Fig.5. Antioxidant activity of the mushroom metabolite.

b. Reducing power assay

The reducing power assay is based on the ability of metabolite to reduce the yellow ferric iron to the blue ferrous form by donating an electron. The reducing power of the experimental mushroom was found to be significantly different ($p < 0.05$) where BHT was considered as standard. Higher absorbance indicates higher reducing power (Fig. 5b) which might be due to high phenolic or flavonoid content which takes part in electron donating and stabilizing the free radical reaction. A higher absorbance of the mushroom extract indicates better reducing power of the extract (Oyaizu, 1986). Our

study demonstrates higher absorbance which increases with the concentration and is found to be stable at 8 mg/mL. The mean absorbance at 8 mg/mL was found to be 2.940 which established that dried methanolic extract of the common puffball is a good source of antioxidant activity with reducing power potency much higher than the commercial BHT. Our result correlates with the earlier reports as the use of the entire wild mushroom increases the reducing power capacity (Ferreira Isabel *et al.*, 2007). The presence of reductones in the extract is generally responsible for the reducing power properties which donate the hydrogen atom by breaking the radical chain (Ferreira Isabel *et al.*, 2007; Shimada *et al.*, 1992).

G. Determination of Bioactive Compounds

a. Total Flavonoid Content

Flavonoids, commonly occurring phenolic groups in plants are classified as flavanols, flavanones, anthrocyanidins, flavonol, chalcones and isoflavones (Morel *et al.*, 1994). Flavonoids are known to possess strong antioxidant activity to inhibit or scavenge the free radicals. The total flavonoid content in the common puffball was found to be 1.576±0.026 mg QE/g of dry weight in terms of Quercitin (QE) which was considered as the positive control. The high level of flavonoid attributes *S. citrinum* a rich source of antioxidant.

b. Total Phenolic Assay

The total phenolic content of the studied mushroom sample was analyzed spectrophotometrically using Folin-Ciocalteu's reagent which was expressed in terms of gallic acid equivalent (standard curve equation: $y=0.9138x+0.0064$, $R^2=0.9801$). The total phenolic content in the methanolic extracts expressed as gallic acid equivalent (GAE) i.e. mg gallic acid/g dry weight showed a high phenolic content in both the extracts possessing scavenging activity. The value obtained for the concentration of total soluble phenolics was expressed as mg of GAE/g of the dried sample which was estimated to have 0.2 ± 0.02 mg GAE/g in methanolic extract. Soluble phenolic compounds in edible mushroom may prove to be an effective anti-inflammatory, anti-tumor, antibacterial, antioxidant and antiviral agent (Barros *et al.*, 2007). The total phenol in the test species was much less than that of few edible wild mushrooms (Khaund and Joshi, 2015) which could be attributed to certain genetic factors (species to species variations) or certain epigenetic factors which include its storage, variety, climatic, the solvent concentration, solvent to solid ratio, solvent polarity, moisture content of the mushroom (Thomson and Moland, 2000; Abugri and McElhenney, 2013).

A similar study on tea, catechin, and caffeine has also reported much higher total phenol in ethanolic extract than that of methanol or water extract which may be due to solvent inability to permeate in the tissue (Abugri and McElhenney, 2013).

Conclusions

Vast majority of wild mushrooms which are part of dietary supplements of indigenous tribes inhabiting forest catchment areas, still remain untapped and analyzed in term of poisonous nature. Molecular characterization of the fruiting body of the mushroom species showing morphological and microscopic resemblance to *Scleroderma* spp. using the fungal barcode ITS revealed them to be closely related to *Scleroderma citrinum*. Ecologically, *S. citrinum* was first described scientifically in the year 1801 by Christian Hendrik Persoon. There are inconsistent reports on the toxicity with the members of *Scleroderma.* The fruiting bodies of the puffball mushroom are considered to be a worst poison and most prominent mushroom poisoning in UK. Consumption of this species is considered to develop various gastrointestinal disorders along with rhinitis and conjunctivitis from the spores exposures. But, the consumption of this mushroom without any apparent harm was observed among the indigenous tribes of Meghalaya. The loss of intoxication of the analyzed mushroom among the indigenous consumers can be in terms of their cooking practices, the geographical location or the genetic resistance to the toxin or by the presence of bioactive compounds which nullify the toxicity.

References

Abugri, D.A. and McElhenney, W.H. 2013. Extraction of total phenolic and flavonoids from edible wild and cultivated medicinal Mushrooms as affected by different solvents. *Journal of Natural Product and Plant Resources* 3(3), 37-42.

Alvarez-Parrilla, E., de la Rosa, L.A., Martínez, N.R. and Aguilar González, G.A. 2007. Total phenols and antioxidant activity of commercial and wild mushrooms from Chihuahua, Mexico. Ciencia Y Tecnologia *Alimentaria* 5(5), 329-334.

Anonymous 2016a. Mushroom poisoning case: Toll rises to five. *The Shillong Times* 13th April, 2016.

Anonymous 2016b. Mushroom tragedy claims 3 more; Toll rises to 6. *The Eastern Today* 22nd April, 2016.

Barros, L., Ferreira, M-J., Queirós, B., Ferreira, I.C.F.R., Baptista, P. 2007. Total phenols, ascorbic acid, â-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. *Food Chemistry* 103, 413-419.

Borthakur, M. and Joshi, S.R. 2016. Micrographical analysis of growth deformities in common pathogens induced by voucher fungi from India. *Journal of Microscopy and Ultrastructure*. 4(4), 203-210.

Devi, K.P., Suganthy, N., Kesika, P., Pandian, S.K. 2008. Bioprotective properties of seaweeds: *In vitro* evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. *BMC Complementary and Alternative Medicine* 8, 38-48.

Ferreira Isabel, C.F.R., Paula, B., Miguel, V.B., Lillian, B. 2007. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. *Food Chemistry* 100, 1511-1516.

Horowitz, B.Z. and Hendrickson, R.G. 2015. Mushroom Toxicity, Medscape: Drugs and Diseases. Department of Emergency Medicine, Oregon Health and Sciences University School of Medicine, Portland, USA: http://emedicine.medscape.com/article/167398-overview, Accessed on 10/01/2016.

Khaund, P. and Joshi, S.R. 2015. Functional nutraceutical profiling of wild edible and medicinal mushrooms consumed by ethnic tribes in India. *International Journal of Medicinal Mushrooms* 17(2), 187-197.

Morel, I., Lescoa, G., Cillard, P., Cillard, J. 1994. Role of flavonoids and iron chelation in antioxidant action. *Methods in Enzymology* 234, 437–444.

Oyaizu, M. 1986. Studies on products of browning reactions: Anti-oxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition* 44, 307–15.

Park, Y.S., Jung, S.T., Kang, S.G., Heo, B.G., Arancibia-Avila, P., Toledo, F., *et al.* 2008. Antioxidants and proteins in ethylene- treated kiwifruits. *Food Chemistry* 107, 640-648.

Shimada, K., Fujikawa, K., Yahara, K., and Nakamura, T. 1992. Antioxidative properties of xanthone on the auto oxidation of soybean in cylcodextrin emulsion. *Journal of Agriculture and Food Chemistry* 40, 945–948.

Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology* 299, 152-178.

Tamura. K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolution genetic analysis version 6.0. *Molecular Biology and Evolution* 30(12), 2725-2729.

Thomson, K.S. and Moland, E.S. 2000. Version 2000: The new beta lactamases of gram-negative bacteria at the dawn of the new millennium. *Microbes and Infection* 2, 1225–1235.

Tsujikawa, K., Kanamori, T., Iwata, Y., Ohmae, Y., Sugita, R., Inoue, H., Kishi, T. 2003. Morphological and chemical analysis of magic mushrooms in Japan. *Forensic Science International* 138, 85-90.

White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A. *et al.* (Ed.), *PCR Protocols, A Guide to Methods and Applications*, Academic Press Publishers, San Diego, pp. 315-332.