CASE-REPORT

DOI: 10.31736/jist/v14.i1.2018.45-54

FORENSIC ANALYSIS OF FUNGAL EVIDENCE: A SYSTEMATIC APPROACH

Journal Homepage :www.jist.org.in; Email: article@jist.org.com

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ARTICLE INFO

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How to Cite this article: Chandel RS, Sharma S, Sharma S. Forensic Analysis of Fungal Evidence: A Systematic Approach. Journal of Indian Society of Toxicology 2018;14(1):1-5. DOI: 10.31736/jist/v14.i1.2018.33-41.

Keywords:
Mushroom poisoning;
Toxicity;
Forensic Science;
Toxicology;
Mycotoxins;
Analysis.

Conflicts of Interest and Fundings: Declared none

Received on 24th April 2018
Accpeted on 30th May 2018
Published on 30th July 2018

Published at JIPMER, Pondicherry, 605006, INDIA Subscription & payment related queries at: toxicology@aims.amrita.edu and rest all types of queries related to the journal to be done at drambika_editor@jist.org.in

ABSTRACT

Forensic mycology is an emerging tool of forensic science. To solve various crime cases, it contributes to many forensic investigations like use of fungi such as trace evidence in linking suspect with the place or object, accidental poisoning cases due to consumption of misidentified mushrooms, illegal trade of narcotic and psychotropic species and to estimate time since death. The evidentiary clue material found at the crime scene in forensic casework is often compromised morphologically and samples of fungi are generally recovered in the form of powder, cooked, dried, on human cadavers, in gut contents and vomit. To know the cause and time since death in such cases is often a challenging task for investigating agencies. So, from forensic toxicological point of view it becomes necessary to correctly identify the species and toxins involved. In the present study, the importance of mycology and a systematic approach for the forensic analysis of fungal evidence when encountered in criminal investigations has been reviewed

INTRODUCTION

The study of fungi of all types (blights, molds, lichens, mildews, plants and human pathogens, rust and smuts, and yeasts) is known as mycology which also includes 'mushrooms' that are a group of fleshy, spore bearing large fruiting bodies. The use of mycological evidence in criminal investigations and its testing in the courts is known as 'forensic mycology'.[1,2] As, forensic investigations generally deal with suicidal, accidental and homicidal cases, mycology can contribute to a variety of forensic investigations, including the determination of postmortem interval from mold growth on corpses, toxicity caused by poisonous and psychoactive substances, and as trace evidence by linking people and objects with places. Fungal spores and other remains may be picked up by any object contacting them, thus can be used as trace evidence in solving many crime cases.[1] In addition, there could be health hazards like respiratory problem from mold growth in buildings. If there is any case in which individual living in a moldy building is facing illness or death has occurred, the fungi could have been the cause.[2] Fungal biodiversity is very well distributed across the world and it provides a treasure trove for ecoclimatic zones. In a recent study, the actual range of the total number of fungi has been properly estimated to be 2.2 to 3.8 million.[3 with figures mostly posited from around half a million to 10 million, and in one extreme case even

 DOI: 10.31736/jist/v14.i1.2018.45-54

Table 1: Showing the importance of various fungal species to correlate the death interval.[15],[17-20]

S.No.	Fungal Species	Observations made in various cases	Advantages	Disadvantages
1.	*Candida* sp. *Penicillium* sp.	Soil fungi might be involved on the body surface but after the major phase of decomposition, molds begin to appear	Fungi can provide a useful means of estimating PMI as information on growth rates of many molds are available.	No information available on the role of particular fungi in decomposition of human corpses, reliability of any estimates depends upon the accuracy of the fungal identification.
2.	*Cladosporium* sp. *Fusarium* sp. *Geotrichum candidum* *Hormodendron* sp. *Mortierella* sp. *Penicillium chysogenum*	In a case of murder, fungal growth on an eyelid and on inguinal skin was observed. They estimated that the death occurred 18 days earlier.		
3.	*Aspergillus terreus* *Penicillium* sp.	White growths dotted on the surface of male corpse found in a kneeling position and partly in water were observed. They estimated that the deceased had been dead for 10 days.		
4.	*Dichotomomyces cejpii*, *Talaromyces trachyspermus*, *Talaromyces flavus*, *Talaromyces* sp.	The mycobiota found in this study clearly differs to mycobiota identified in control sample.		

a sizable portion of the spectacular number of 1 trillion. Here we examine new evidence from various sources to derive an updated estimate of global fungal diversity. The rates and patterns in the description of new species from the 1750s show no sign of approaching an asymptote and even accelerated in the 2010s after the advent of molecular approaches to species delimitation. Species recognition studies of (semi-] They usually grow in rainy seasons as the temperature and other environmental conditions are optimum for their growth.[4,5] Since ages, mushrooms are being consumed as a source of diet as seasonal delicacy which provides nutrition and has traditionally known health benefits.[6,7] Sometimes, inedible (toxic) mushrooms are consumed mistakenly by people in place of edible ones because they lack basic knowledge on morphological features of inedible mushrooms which is a cause of frequent poisoning, that may not only affect individuals, but also can be troublesome for the families.[8–10] The toxic compounds which are extracted or obtained from such mushroom species are classified as mycotoxins. People from various parts of the world which often includes villagers and ethnic tribes consume wild mushrooms as a source of diet, especially during monsoon season.[8,11] Mushroom

 ISSN: 0973-3558, e-ISSN: 0973-3566

poisoning due to accidental and those resulting from crime and suicide attempts are a frequently encountered type of case.[1,10,12] Fungi are also been reported to be used as hallucinogens, neurotropic or psychoactive drugs that has its roots in the old world. Hallucinogenic mushrooms (macrofungi) have been used in spiritual ceremonies by Mexican Indians and Siberian shamans for thousands of years. Soon after 'magic mushrooms' became a drug of abuse. In many parts of the world, the knowing possession of mushrooms that are known as 'magic mushrooms', is illegal.[6] So, cases in which fungi is present at the crime scene in different forms should be examined properly. Care should be taken when it comes to sample collection and data should be carefully interpreted that would help in establishing the cause of death. In criminal investigations, analysis of fungi can be of outmost importance. Mycology provides the basic tool for a wide range of scientific studies including the use of fungi as trace evidence, estimation of time since death, species identification and determination of toxins causing poisoning.

Fungi as Trace Evidence

Locard's Exchange Principle states that every time a person has contact with someone else, a place, or a thing, it results in an exchange of physical material which means 'every contact leaves a trace'. Plant and fungal palynomorphs are readily transferred to textiles (particularly woven, synthetic ones), any article of clothing and footwear, digging implements, vehicles, hair, fur and even hard surfaces. If a suspect denies having visited a place, their statement can be tested by comparing trace evidence profiles from personal belongings with those from samples of nearby vegetation and any other location suspect might have visited. Wiltshire et al.[13] have reported a rape case of young woman in which both palynological and mycological evidences have been used for the first time for identification. In this case, the suspect denied the claim of victim and said that they had consensual sexual relations in a local park, away from the alleged crime scene. Investigators collected samples from each place, clothing, footwear from each party and all the places that were considered to be relevant to the case were visited, and flora was recorded. It was observed that the palynological and mycological profiles yielded by the footwear and clothing of both parties closely resembled to that of the wooded area (claimed by women) and the profile of the wooded site reflected closely its own vegetation. When fungal evidence was proved in the court of law, suspect confessed the crime. It has also been reported that when there is any case of individual's illness or death living in a place having molds, the fungi could be viewed to have been the cause. Another case has been reported by Wiltshire et al.[15] in which a woman was found murdered near the city (Dundee) center in February 2010. Identification was done on the basis of palynological (botanical and mycological) profile obtained from the vegetation (exotic trees, shrubs and climbers) at the crime scene. Suspect's footwear were compared with not only the ground but also with victim's clothing and footwear. Identification and counting was done for each palynomorph and fungal spores. Rare forensic markers were assembled which brought uniqueness to that location and helped in linking crime with the suspect. Thus, mycological evidence has proved to be helpful in providing a separate class of evidence from forensic samples.[1,14] Even fragments of lichens, or fragments of moldy objects, can become detached and caught up in items that are involved in criminal investigations.[2]

Estimation of Time Since Death

Recently, fungi have been reported to be helpful in establishing time since death in criminal cases[1]. Fungal spores can act as an indicator of the environment from which it was derived. If such indicators are identified and quantified, it is possible to reconstruct the nature of the past events embedded in various matrices such as soil, organic and inorganic sediments over time. There are reports of fragments of basidiomes or lichens providing evidence, as well as the fungal colonies on human remains providing information and probative evidence in the estimation of postmortem interval (time since death)[13,14]. Fungal colonies on or associated with human cadavers can give indications of time since death as there is information on growth rates of many molds[15]. Sometimes, bodies are buried at remote locations to hide the evidence of crime and in such cases clandestine graves can be located with the help of fungal growth which comprises a useful tool in investigative process [15,161995]. A case has been reported in which various fungi such as *Hormodendron* sp., *Mortierella* sp., *Penicillium chysogenum* have been identified which were present under decomposed cadaver in the soil. Such investigations can be helpful for locating, recovering and analyzing clandestine graves. These identifications are generally based on the fact that the mycobiota found at

DOI: 10.31736/jist/v14.i1.2018.45-54

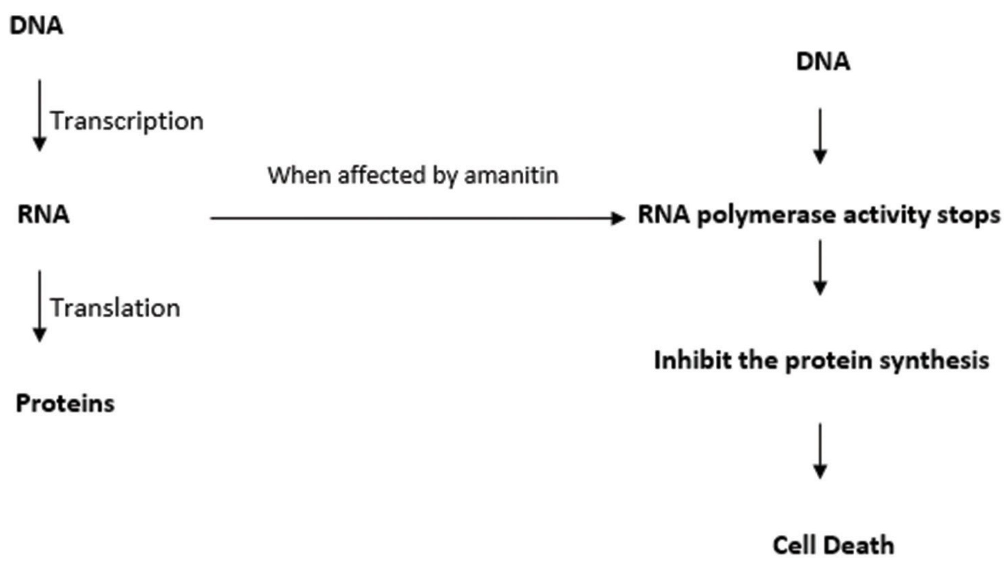
the crime scene in case of decomposed bodies, differs from the control samples. Number of other cases have also been reported in which fungi have been used for the estimation of postmortem interval and time of deposition (Table 1). But the reliability of any estimates will depend on the accuracy of the identification of the fungus, the storage methods for the body, and the availability of data on the temperature and humidity at the site[1].

Mushroom Poisoning

Collecting and consuming wild mushrooms has been a longstanding tradition in various parts of the world. Gonmori and Yoshioka (2006) estimated that in Japan approximately 5000 species of mushrooms are known worldwide and among this 20-25 % have been identified and 3 % causes toxicity. The most common toxic mushroom species belong to genus Amanita, Clitocybe and Inocybe, Cortinarious and Gyromitra.[22] Among mushroom intoxications, the amatoxin syndrome is of primary importance as it accounts for about 90% of fatality.[23–25] Amatoxins are found in genus Amanita that includes approximately 50 members worldwide comprising α-amanitin, β-amanitin and γ-amanitin.[26] The most of the fatalities are often caused by Amanita phalloides (known as death cap), Amanita virosa (known as destroying angel), Amanita verna (known as death angel). The toxicity of Amanita species is related to three distinct groups of toxins: amatoxins, phallotoxins and virotoxins. Amatoxins are heat-stable and resistant to cooking, drying and freezing.[27] As less as 0.1 mg/kg body weight can provide a lethal dose.[28] Amatoxins are bicyclic octapeptides, phallotoxins are bicyclic heptapeptides and virotoxins are monocyclic heptapeptides.[29] Amatoxins (α-amanitin and β-amanitin) are responsible for the toxic effect leading to acute liver failure, renal failure and potential toxicities to pancreas, adrenal glands, and testes whereas phallotoxins are toxic to the cell membrane of enterocytes leading to initial diarrhoea-like illness. Phallotoxins and virotoxins do not actually cause human poisoning as they are not absorbed by the intestine, although reports are present in which fatality occurred of mice and rats within 1-2 h after intra-peritoneal administration. Phallotoxins binds specifically to F-actin, thus stabilising structure of the assembled filaments. Amanitins act via inhibiting eukaryotic RNA polymerase-II and thus interrupting transcription in humans resulting in decreased mRNA and protein synthesis and leading eventually to cell death figure 1. Number of post ingestion fatality occurs due to the misidentification of edible mushrooms from inedible ones. As evidence they are often present in various forms.[12,30,31]

Hallucinogenic Mushrooms

Apart from mushrooms poisoning, in recent years there has also been an increased interest in use and abuse of hallucinogenic mushrooms, also called ‘magic mushrooms’. Now a day, these mushrooms are sold commercially through internet and also in so-called ‘smart shops’. In various countries, the possession and trading of wild mushrooms which contains narcotic substances



is a crime. Mushrooms containing these compounds are Schedule I substances under both federal and state law.[10] Hallucinogenic mushrooms such as ***Psilocybe*** and ***Panaeolus*** are known to synthesize the controlled compounds psilocin (N,N-dimethyltryptamine) and its phosphorylated derivative psilocybin (O-phosphoryl-4-hydroxyN,N-dimethyltyptamine).[32] Psilocin is 1.5 times more active than psilocybin. These substances act on the central nervous system and affect perception, mood and thinking ability. The effects are similar to those caused by LSD and mescaline. In the illegal market, these are found as evidence in processed form like ground to a powder, cut into small pieces, dried, encapsulated for further use but rarely confiscated in a fresh state. Under such circumstances, it may be necessary to find out that a particular preparation contains fungal material from these genera.[33,34food remains, stomach contents, feces, etc] Therefore, precise identification of species of fungi that contain illegal and toxic substances is very important for the purpose of judicial proceedings.

ANALYSIS

The proper identification of the fungal species found as evidence at the scene of crime is very important. In cases where fungal evidence is morphologically compromised, DNA based methods are useful for species identification and is complementary to taxonomic identification (Table 2).[33food remains, stomach contents, feces, etc] Forensic DNA analysis has changed the way in which biological samples can be used as evidence in court of law. DNA barcoding involves sequencing a standard region of DNA as a tool for species identification.[35,36cytochrome oxidase 1, by Canadian mycologists has been fruitful for some fungi, but intron issues and lack of resolution in other taxa prevent its universal application. The momentum established by 15 years of research on the fungal nuclear ribosomal internal transcribed spacer (ITS] The basis of these identifications is variation in nucleotide sequence. Many fungal taxonomic studies have used DNA sequence of the non-coding internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA for resolving relationships at genus and species level.[37–41taxonomic expertise is collapsing. We are convinced that the sole prospect for a sustainable identification capability lies in the construction of systems that employ DNA sequences as taxon 'barcodes'. We establish that the mitochondrial gene cytochrome c oxidase I (COI] The poisonous mushrooms eaten by patients do not retain in their original shape, the molecular genetics play a vital role in

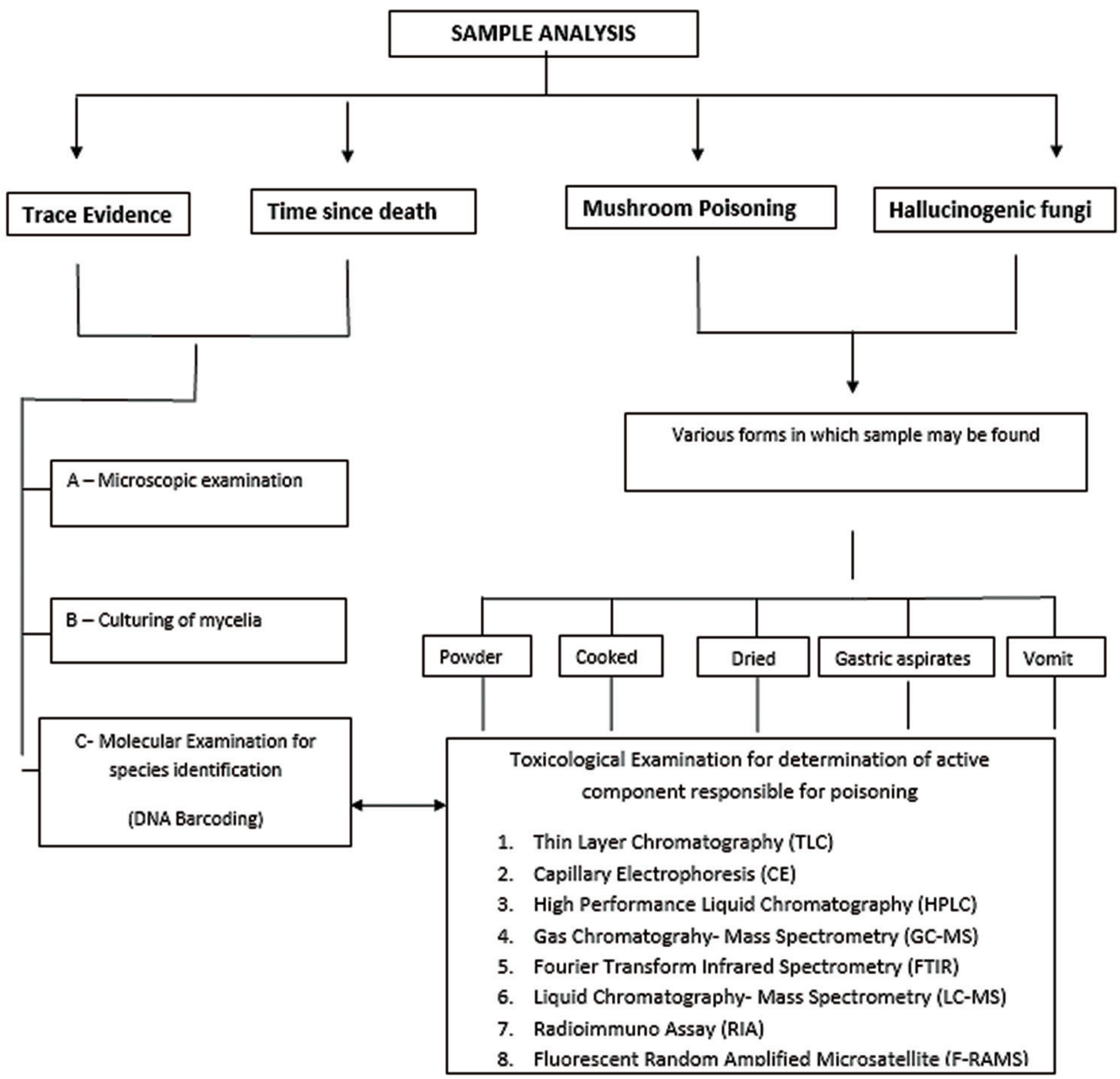
Table 2: DNA analysis technique used for analysis of different types of fungi. [10], [42–44] [45–48], [24]

S.No	Type of fungi	Technique
1.	Hallucinogenic – *Psilocybe cubensis Panaeolus* *Psilocybe semilanceata*	Random Amplification of polymorphic DNA (DNA), Amplified Fragment Length Polymorphism (AFLP), Promega Wizard Genomic DNA purification kit
2.	Poisonous mushrooms- *Cortinarius* sp *Amanita fuliginea* *Amanita oberwinklerana* *Russula subnigricans* *Amanita phalloides* *Anamita verna*	Polymerase Chain Reaction (PCR), Singleplex real-time PCR, Next Generation Sequencing
3.	Others *Candida albicans*	Polymerase Chain Reaction (PCR)

 DOI: 10.31736/jist/v14.i1.2018.45-54

the identification of fungal species. Standard protocol has been designed for rapid PCR-based detection of fungal traces in variety of complex samples. Rapid method which is quick, sensitive and cost-effective of molecular detection for the species-species identification of the major cooked and fresh poisonous mushrooms have been reported using real-time PCR system in various matrices. [12] Various edible and poisonous species of mushrooms have been studied and identification was done using real-time PCR.

Various chromatographic and spectroscopic techniques have been reported for the determination of the active component causing poisoning (Table 3). Reverse Phase High Performance Liquid Chromatography (RP-HPLC) has been reported to measure the toxin levels that can vary among various species and even among various parts of same species. Various species of ***Amanita phalloides var. alba*** and ***Amanita verna*** were collected and taxonomic identifications were made. All parts of dried mushrooms were ground and homogenized in 1g/30 ml extraction medium (methanol-water-0.01 M HCL) using tissue homogenizer. Excellent separation was achieved of amatoxins and phallotoxins with RP-HPLC and DAD detection. It has been found that lowest toxin concentration was detected in spores, volva and



 ISSN: 0973-3558, e-ISSN: 0973-3566

Table 3: Chromatographic and spectroscopic methods used to detect various fungal species from the forensic (mushroom) samples.[34] [53-64] [65–68]

Category	Genus	Species	Active constituent	Technique
Poisonous mushrooms	Amanita	*Amanita phalloides, Amanita virosa, Amanita pantherina, Amanita muscaria, Amanita exitialis, Amanita fuliginea, Amanita pallidorosea, Amanita rimosa, Amanita smithiana, Amanita proxima*	α-amanitin β – amanitin γ - amanitin Phalloidin Phallacidin Virotoxins Ibotenic acid Muscimol	Paper chromatography, A Reversed phase High Performace Chromatography (RP-HPLC), Gas Chromatography- Mass Spectrometry (GC-MS), Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), Liquid chromatography-electrospray ionization-time-of-flight-mass spectrometry (LC-TOF-MS), Capillary Electrophoresis (CE) with diode array detection (DAD), Liquid Chromatography- electrochemical detection (LC-ECD), Nuclear Magnetic Resonance (NMR)
	Cortinarius	*Cortinarius orellanus, Cortinarius rubellus, Cortinarius speciosissimus*	Orellanine	Thin Layer Chromatography (TLC), Electrophoresis, Electron Spin Resonance, Gas Chromatography- Mass Spectrometry (GC-MS), High Performace Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR)
	Gyromitra	*Gyromitra esculenta*	Gyromitrin	High Performace Liquid Chromatography (HPLC)
	Clitocybe and Inocybe	*Clitocybe amoenolens Clitocybe dealbata Clitocybe rivulosa Clitocybe candicans Clitocybe cerrusata Clitocybe phyllophila*	Muscarine	High Performace Liquid Chromatography (HPLC), Gas Chromatography- Mass Spectrometry (GC-MS)
Hallucinogenic mushrooms		*Panaeolus* *Psilocybe cubensis* *Catha edulis FORSK*	Psilocin Psilocybin	High Performace Liquid Chromatography (HPLC), Liquid Chromatography-Ultraviolet detection (LC-UV), Attenuated Total Reflectance (ATR) and Transflection IR Spectroscopy

stipe than other parts of same species. The amount of toxin in ***Amanita verna*** is more than ***Amanita phalloides*** and ***Amanita phalloides var. alba*** In a case report of mushroom poisoning, the identification of cooked mushroom fragments through HPLC-UV detector was done to detect α-amanitin in gastric juice showing that amatoxins containing mushrooms were also included in the prepared meal. Gas Chromatography- Mass Spectrometry (GC-MS) analysis proved the presence of muscimol in urine. [24,34,49–51]

Analysis of mushrooms has been applied to forensic studies in a meaningful way for a long time and in many countries it has reached its most varied and highest level of development and utility. The general approach for the identification of fungal genera is usually based upon the observation of combination of the macroscopic and microscopic characteristics which includes gill structure and spores. It is well known that in forensic casework, the samples are morphologically compromised and often present in trace quantities, so these characteristics become difficult to identify. Cases in which samples are not damaged morphologically systematic analysis of fungi could be done by microscopic examination, but if evidence does not contain the necessary morphological or histological features to identify a species at the genus or species level, DNA based methods are used. In some cases, DNA barcodes may not be available for the species involved, so identification can be done by microscopic examination. After the species have been identified, an attempt can be made to know the active component responsible for poisoning by using various chromatographic and spectroscopic techniques (Figure 2).`

CONCLUSION

Forensic mycology has been known to provide important forensic evidence in a variety of ways and in some cases, it has proved useful in giving convictions. But, it is rarely employed especially in developing countries due to lack of awareness among crime scene investigators especially toxicologists and pathologists. Investigators should work in collaboration with skilled mycologists to gain expertise in this field. In particular, they should know the evidential value of fungi and should not overlook its presence. Fungi can be very helpful in solving cases of poisoning, illegal trade, as trace evidence by linking suspect with crime scene and estimating interval of time since death. So, there is a need to generate proper identification manuals to make critical identification of various species. For estimating time since death, no data is available on the growth rate of fungi on human cadavers. As the application of forensic mycology is at the stage of infancy so comprehensive research work and database related to all types of fungi that are useful for investigations in various types of cases is required for its use in forensic casework. Samples can be examined by using microscopic methods and identifications using molecular sequence data can be made in certain well-researched group of fungi. For the analysis of active component responsible for poisoning, analytical approach could be used. Now, it is a need of time to make an effort and develop more expertise in this particular field. There is also a need to aware general public regarding the after effects of poisonous mushrooms when consumed without proper knowledge of differentiating between edible and inedible ones.

Acknowledgement

The authors are thankful to University Grants Commission (UGC) for providing fellowship.

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 ISSN: 0973-3558, e-ISSN: 0973-3566