

TOXICOLOGICAL EVALUATION OF *INOCYBE VIROSA*

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ABSTRACT

Western Ghats of the Indian sub-continent harbours great fungal diversity with a wealth of mushroom flora, a few genera of which have been found to be poisonous. A new endemic species, *Inocybe virosa*, reported in the Kerala state, is an addition to the group of poisonous mushrooms and is the focus of the present study. HPLC technique was employed for the qualitative analysis and the quantitative estimation of muscarine in *Inocybe virosa*. The HPLC data revealed a concentration of 0.3 mg/g of the hydro-ethanol extract. Further, the toxicological evaluation was carried out *in vitro* by subjecting the mushroom extract to different digestive enzymes and pH variations, simulating the *in vivo* digestion conditions. The toxin was present in the digested fraction and was identified using the chromatographic technique. The digestate obtained on *in vitro* digestion was studied for its cytotoxicity on intestinal Caco-2 cell line and its *in vivo* toxic potential was verified in mice. On oral administration, characteristic symptoms of toxicity viz., perspiration, lacrimation and salivation were observed. As commonly found in the species of Inocybaceae, the basidiomata of this new endemic *Inocybe virosa* too contains muscarine in a concentration which causes undesirable effects and hence, is not recommended for consumption.

Keywords: *Inocybe virosa*, muscarine, Caco-2 cell line, cytotoxicity, Inocybaceae

INTRODUCTION

Mushrooms have been a coveted food item. But mycophagy or mushroom eating is inextricably linked to mushroom poisoning [1]. Mushroom foraging involves a high risk of confusion, wherein the poisonous mushrooms are mistaken for their edible look-alikes. Macroscopic features cannot be the basis to differentiate poisonous mushrooms as it is the chemical nature of the mushroom which determines its edibility or toxicity.

The Indian sub-continent harbours great fungal diversity and in particular, the Western Ghats, recognised as a biodiversity hotspot, has a wealth of mushroom flora. Studies undertaken to explore the diversity of the macro-fungi in the Western Ghats have indicated the occurrence of a few genera of poisonous mushrooms [2-4]. A survey on the agaric flora of the Kerala state has reported a new endemic species, *Inocybe virosa*, previously undescribed in the literature [5].

Among the seven types of mushroom poisoning syndromes [6], muscarine syndrome is mainly due to consumption of *Inocybe* and *Clitocybe* species containing the toxin muscarine which cannot be made non-toxic by cooking, freezing or any other means of processing [7]. The manifestations of the syndrome occur within 15-30 minutes after consumption of the mushrooms. The peripheral parasympathetic nervous system gets activated by muscarine as it mimics the action of acetylcholine at the muscarinic acetylcholine receptors. The toxin stimulates the exocrine glands and causes the characteristic symptoms viz., perspiration, salivation and lacrimation. The poisoning also results in constriction of pupils, blurred vision, muscle spasms, nausea, vomiting, diarrhoea, abdominal pain, slow heart-beat and a drop in blood pressure [8]. Muscarine syndrome can also be fatal in the case of ingesting large quantities of the causative species as profound activation of the peripheral parasympathetic nervous system may end in convulsions and finally death [7].

The presence of the toxin muscarine has been identified in different matrices such as mushroom carpophores and food samples as well as in biological samples like urine using various chromatographic techniques. Brown *et al.* [9] detected muscarine in a few *Inocybe* species by paper chromatography using Thies and Reuther's reagents. Gas chromatography was used to detect muscarine and its isomers in a few selected species of *Inocybe* [10]. The identification of muscarine in *Inocybe napipes* by TLC/MS and LC/SIMS was described by Unger *et al.* [11]. Hydrophilic interaction liquid chromatography (HILIC) in combination with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) on a TSK-Gel Amide 80 column was developed by Chung *et al.* [7] to analyse muscarine along with the cyclopeptide mushroom

toxins in food samples. A Liquid Chromatography/Mass Spectrometry method was developed for rapid and specific quantification of muscarine in human urine by Barбора *et al.* [8]. The HILIC-ESI-MS-MS method developed for simultaneous identification of several mushroom toxins, was modified by Naoki *et al.* [12] to LC-TOF-MS using a PFP (Penta fluoro phenylpropyl) column.

So far, muscarine has been identified using the liquid chromatography technique in combination with mass spectrometric analysis. Thus, an attempt has been made in the present work to detect muscarine using HPLC independent of mass spectrometry. The method was used for the identification of muscarine in an *Inocybe* species. On establishing the presence of the toxin muscarine in the new species, *Inocybe virosa*, a preliminary toxicological evaluation, *in vitro* and *in vivo* of the mushroom sample was carried out.

MATERIALS AND METHODS

Identification of muscarine in *Inocybe virosa*

Sample preparation: The 50% hydro-ethanol extract of the dried sample of *Inocybe virosa* (Tropical Botanical Garden Trivandrum Herbarium, Accession no. 14191) was prepared. It was purified using the Solid Phase Extraction (SPE) cartridge (Oasis HLB, 1cc) preconditioned with methanol [12]. The extract was dissolved in Milli-Q water and eluted through the cartridge. About 1ml of methanol was then eluted on the same cartridge and the elute was used for the HPLC analysis.

HPLC analysis: Muscarine was determined by HPLC using the JASCO HPLC system fitted with a UV/VIS multi-wavelength detector. Separation was carried out on a HiQ SiL C18 column (250 mm × 4.6 mm, particle size 5µm). The absorbance was monitored at 235 nm. The mobile phase comprised of three solvents: A- buffer containing ammonium formate (2 mM) and formic acid (5 mM) at pH 3.5, B-Acetonitrile and C-Methanol at a flow rate of 0.2 ml/min. The gradient program used is as follows: 0 to 8 min- 4% A and 96% B from; 9-30 min- 10% A, 78% B and 12% C; this ratio of solvents was maintained for the next 20 min; 10% A, 70% B and 20% C for the last 10 min [7]. The standard, (+)-Muscarine (iodide salt) was purchased from Cayman Chemical (Purity ≥95%).

In vitro digestion model

The *in vitro* digestion model applied to mushroom samples by Cristina *et al.* [13] is the method adopted for the hydro-alcohol extract of *Inocybe virosa* with a few changes. 100 g of the sample was mixed with sufficient volume of water and heated to boiling. The sample was then cooled and mixed with 10 ml of volunteer's saliva and 10 ml of phosphate buffer (0.08 M pH-6.7) and ground for 5 min on a mortar with a pestle. The pH was then set to 2 with 6 M HCl and pepsin (5 mg per gram homogenate) was added. The mixture obtained was incubated for 2 h at 37 °C with slow stirring at 60 rpm in a rotary shaker. The sample was subjected to further digestion by adding 20 ml of pancreatic solution (0.08 g pancreatin and 0.5 g bile salts in 20 ml of 0.1 M NaHCO₃) and the pH was adjusted to 7.5 with 0.5 M NaOH. To the digested samples, 1 g of NaHCO₃ was added for maintaining the pH. The mixture was incubated again for 2 h at 37 °C with slow rotary stirring at 60 rpm. On complete digestion of the sample, a mixture of a liquid supernatant (LS) and a solid precipitate was obtained and separated.

Cytotoxicity of the digestate

Cell lines: Caco2, a human colorectal adenocarcinoma cell line obtained from NCCS, Pune, was used to assess the cytotoxicity of the digestate obtained on *in vitro* digestion. The cells were grown and maintained using Minimum Essential Medium (MEM) (Sigma-Aldrich) supplemented with 10% foetal bovine serum (HiMedia), 1% penicillin-streptomycin (Sigma-Aldrich), 1.5 g/l Sodium bicarbonate and 110 mg/l Sodium pyruvate (Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO₂.

Colorimetric MTT (tetrazolium) assay: The cytotoxic effect was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay according to the method published by Mosmann *et al.* [14]. The cells were grown at approximately 5×10^5 cells per plate in 12 well plate. The digestate was added at 0.5 and 1% in triplicates and incubated over night at 37 °C under 5% CO₂. The medium present in the wells were replaced by 100 µl MMT. The absorbance was read at 570 nm after 2 hours. Per cent cell death was calculated using the formula: Per cent cell death = (sample OD value – digestate standard OD value) / (cells only value – digestate standard OD value) × 100

In vivo* toxicity study of *Inocybe virosa

The *Inocybe virosa* extract was tested for its *in vivo* toxic effects on male albino wistar mice (weighing 25-30 gm). The extract was orally administered at 4 different dosages (25, 50, 100 and 200 mg/kg body weight (bwt)). Each dosage was given to three mice along with three control mice which were administered only water. The behavioural pattern was observed for 4 hours.

RESULTS AND DISCUSSION

Estimation of muscarine in *Inocybe virosa* by HPLC

Muscarine is a characteristic component of the genus *Inocybe* [15] and in fact, the presence of muscarine in Inocybaceae is an ancestral trait [16]. The new *Inocybe* species, *Inocybe virosa*, which by its nomenclature indicates its toxic nature (*virosa*-full of poison), required an analysis for the occurrence of muscarine in its fruiting bodies. Thus an easy and rapid HPLC procedure was employed to test and confirm the presence of muscarine in *Inocybe virosa*, as shown in Fig. 1. The toxin content was quantitatively estimated in the hydro-ethanol extract of dried mushroom sample and it was found to be 0.3 mg/g of the extract.

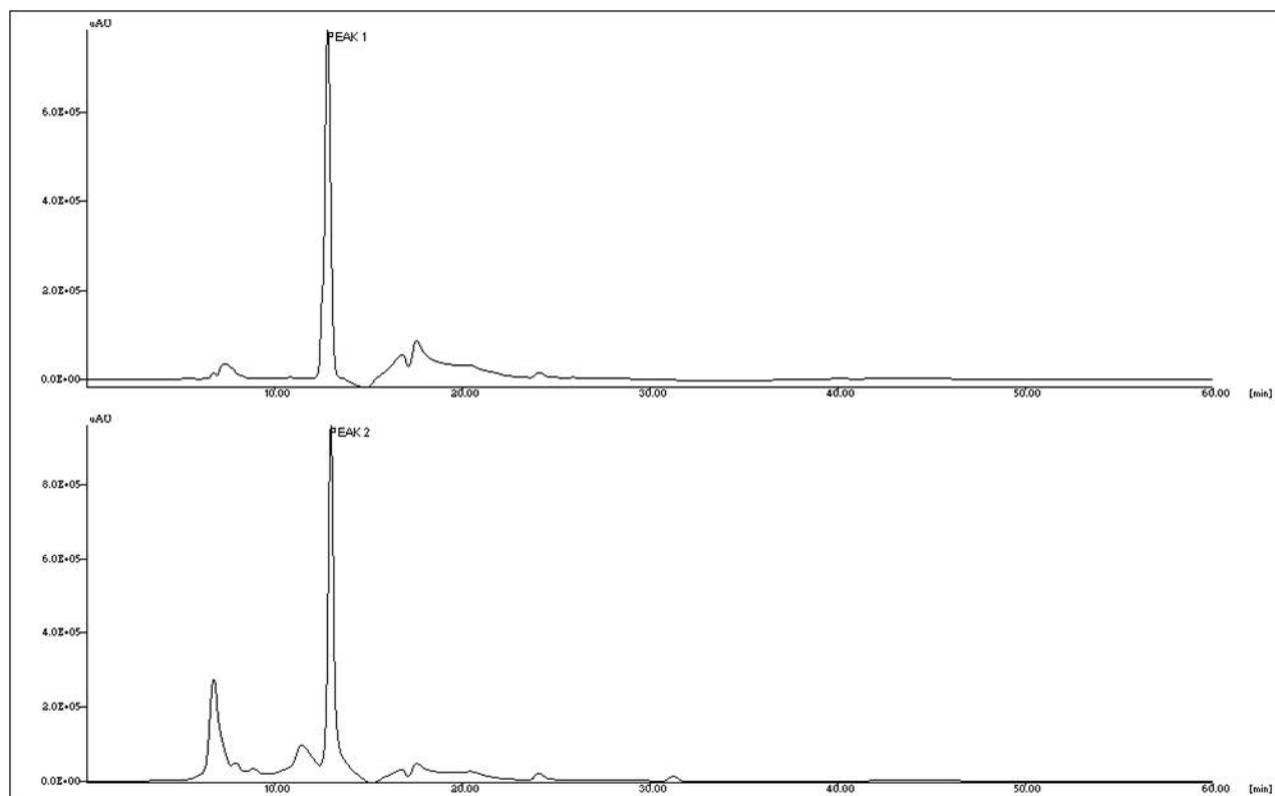


Figure 1. HPLC identification of the presence of Muscarine. PEAK 1: Muscarine Standard; PEAK 2: Muscarine in hydro-ethanol extract of *Inocybe virosa*

Cytotoxic effects of muscarine in the digestate

Since mushrooms are usually consumed along with food, an attempt was made to analyse the effect of digestive enzymes and the extreme pH variations of the digestion process on the compound of interest, muscarine. An *in vitro* digestion model simulating digestive conditions in the mouth, stomach and small intestine was followed. The large intestinal tract was not considered, since during *in vivo* food digestion it is mainly involved in the absorption process [17]. The *in vitro* digestion model for studying the digestibility of the toxin, in combination with Caco-2 cells (representative of the intestinal barrier.), is a useful approach to assess the risk of ingesting the mushroom *Inocybe virosa* containing muscarine.

Muscarine is a fast-acting poison [7] and has a short latency period (symptoms start within 5 hours of consumption) [18]. The initial phase in muscarine poisoning is the gastrointestinal toxicity, reflected in its symptoms like vomiting, diarrhoea and abdominal pain. The liquid supernatant obtained is the bio-accessible fraction which is most likely to be absorbed by the intestinal enterocytes.

Thus, the damaging effects of the bio-accessible fraction was estimated in terms of the cell death caused which is measured by the MTT assay (Fig 2). The extent of damage in case of exposure to 1% of the LS was greater in comparison to 0.5%, evident from the morphological changes observed (Fig 3). A mixture of the digestive enzymes used for the *in vitro* digestion was also applied to the cells to assess their effect on the cells independent of the toxin.

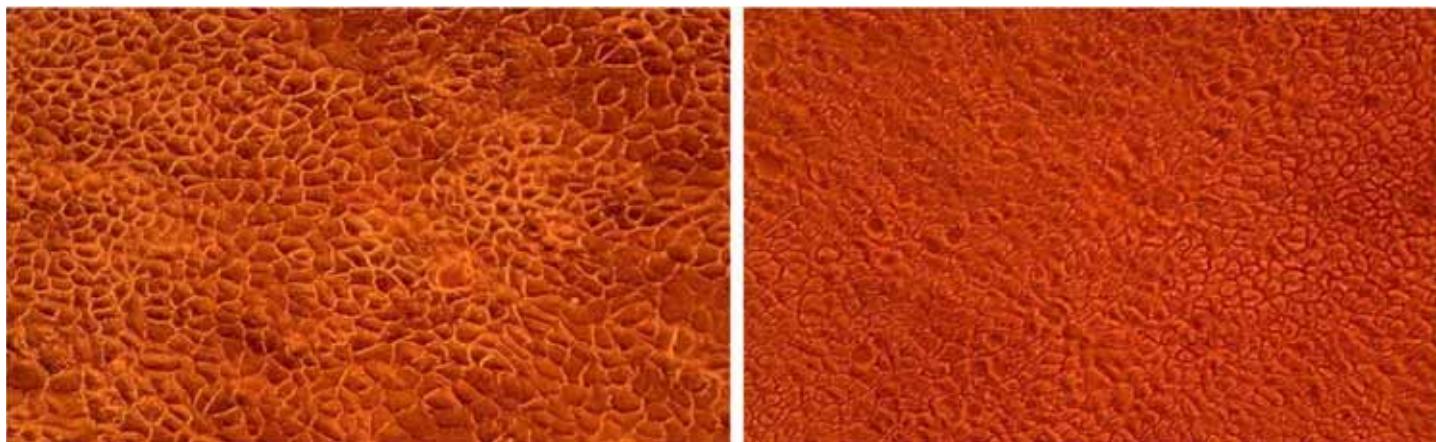


Figure 2. MTT assay to determine the viability of the Caco2 cells upon the exposure to the muscarine digestate. 1-Control, 2-Enzyme treated, 3-0.5% exposure (TEST 1) and 4-1% exposure (TEST 2) of the digestate

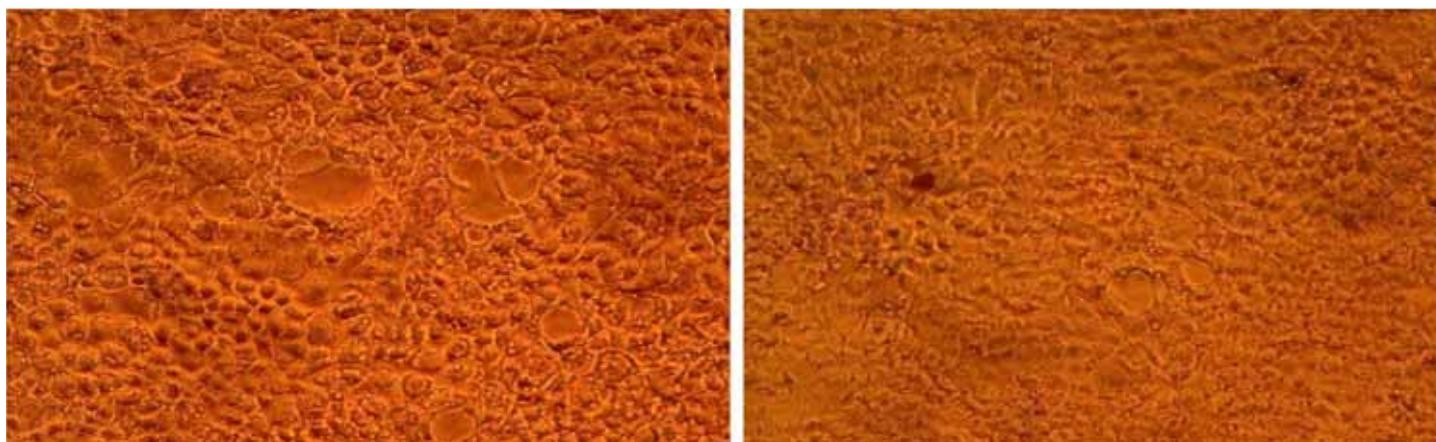


Figure 3. Effect of the digestate on the morphological structure of the cells

***In vivo* toxicity study**

The toxic nature of the mushrooms containing muscarine is well reported [19, 20] and validated by cases of poisoning [21, 22]. A dose dependent study was conducted on mice to assess the effect of consumption of *Inocybe virosa*. The effect of different dosages of the extract is summarized in the table below:

Table 1. Dose-dependent study of *Inocybe virosa* extract

Dosage	Onset of symptoms	Reaction
25mg/Kg bwt	Within 10 minutes	Slight salivation and lacrimation not very evident
50 mg/Kg bwt	Within 10 minutes	Moderate salivation; evident lacrimation
100 mg/Kg bwt	Within 5 minutes	Profuse salivation and pronounced lacrimation
200 mg/Kg bwt	Within 5 minutes	Profuse salivation and pronounced lacrimation
Control	Nil	Nil

All the characteristic symptoms of muscarine poisoning were evident in the study. With an increase in the concentration of the extract administered, the severity of the symptoms was also found to be increasing correspondingly.

CONCLUSION

As commonly found in the species of Inocybaceae, the fruiting body of *Inocybe virosa*, a new and endemic to the Western Ghats of India, also contains muscarine toxin. The muscarine content in the mushroom has been estimated and its toxic effects have been evaluated by this preliminary study. To date, there are no reports available on the toxicity of this mushroom in humans or animals since the mushroom is new. However, to avoid food poisoning due to this mushroom, further studies are warranted in these lines.

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REFERENCES

- [1] Kumar M and Kaviyaran V. (1976). Few common poisonous mushrooms of Kolli Hills, South India. *Mycotaxon*. 3(3), 363.
- [2] Farook AV *et al.* (2013). A checklist of agarics (gilled mushrooms) of Kerala State, India. *Mycosphere*. 4(1): 97-131.
- [3] Thiribhuvanamala G *et al.* (2011). Biodiversity, Conservation and Utilisation of Mushroom Flora from the Western Ghats Region of India. In *Mushroom Biology and Mushroom Products. Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products, Arcachon, France, 4-7 October, 2011. Volume 1. Oral presentations.* (pp. 155-164). Institut National de la Recherche Agronomique (INRA).
- [4] Swapna S *et al.* (2008). Diversity of macrofungi in semi-evergreen and moist deciduous forest of Shimoga District, Karnataka, India. *J Mycol Pl Pathol*. 38(1): 21-26.
- [5] Vrinda KB and Pradeep CK. (1996). A new *Inocybe* (Cortinariaceae) from Kerala State, India. *Mycotaxon* (USA).
- [6] <http://www.botany.hawaii.edu/faculty/wong/BOT135/Lect18.htm>
- [7] Chung WC *et al.* (2007). Separation of Polar Mushroom Toxins by Mixed-Mode Hydrophilic and Ionic Interaction Liquid Chromatography-Electrospray Ionization-Mass Spectrometry. *J. Chromatog. Sci.* 45(2): 104-111.
- [8] Merová B *et al.* (2011). Determination of muscarine in human urine by electrospray liquid chromatographic-mass spectrometric. *Journal of Chromatography*. 879(25): 2549-2553.

- [9] Brown JK *et al.* (1962). Paper chromatographic determination of muscarine in *Inocybe* species. *J. Pharmaceu. Sci.* 51(9): 853-856.
- [10] Catalfomo P and Eugster CH. (1970). Muscarine and muscarine isomers in selected *Inocybe* species. *Helvetica Chimica Acta.* 53(4): 848-851.
- [11] Unger SE *et al.* (1981). Identification of quaternary alkaloids in mushroom by chromatography/secondary ion mass spectrometry. *Analytical Chem.* 53(7): 976-981.
- [12] Yoshioka N *et al.* (2014). A simple method for the simultaneous determination of mushroom toxins by liquid chromatography-time-of-flight mass spectrometry. *Forensic Toxicology.* 32(1): 89-96.
- [13] Soler-Rivas C *et al.* (2009). Effect of cooking, in vitro digestion and Caco-2 cells absorption on the radical scavenging activities of edible mushrooms. *Intl. J. Food Sc. Tech.* 44(11): 2189-2197.
- [14] Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65(1): 55-63.
- [15] Catalfomo P and Eugster CH. (1970). Muscarine and muscarine isomers in selected *Inocybe* species. *Helvetica Chimica Acta.* 53(4): 848-851.
- [16] Kosentka P *et al.* (2013). Evolution of the toxins muscarine and psilocybin in a family of mushroom-forming fungi. *PloS one.* 8(5): e64646.
- [17] Versantvoort CH *et al.* (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. *Food Chem. Toxicol.* 43(1): 31-40.
- [18] De Oliveira and Paulo. (2009). Mushroom poisoning. *Medicina Interna.* 16(4): 232-238.
- [19] Malone MH *et al.* (1961). A bioassay for muscarine activity and its detection in certain *Inocybe*. *Lloydia.* 24(4): 204.
- [20] Clark ED and Smith CS. (1913). Toxicological studies on the Mushrooms *Clitocy beilludens* and *Inocybe infida*. *Mycologia.* 5(4): 224-232.
- [21] George P and Hegde N. (2013). Muscarinic toxicity among family members after consumption of mushrooms. *Toxicol. Intl.* 20(1): 113.
- [22] Lurie Y *et al.* (2009). Mushroom poisoning from species of genus *Inocybe* (fiber head mushroom): a case series with exact species identification. *Clinical Toxicol.* 47(6): 562-565.