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Amatoxins and phallotoxins in indigenous and introduced South African *Amanita* species

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The production of lethal amatoxins and phallotoxins in species of *Amanita* from South Africa was investigated by HPLC (High Performance Liquid Chomotography) analyses. The indigenous mushrooms *Amanita foetidissima* and *A. pleropus* tested negative for production of these toxins. Several introduced species were analysed; of these, *A. phalloides* var. *phalloides*, *A. phalloides* f. *alba* and *A. reidii* contained amatoxins and phallotoxins. Despite reports of rapid degradation of phallotoxins upon drying, phallotoxins and amatoxins were both readily detectable in dried herbarium specimens up to 17 years old. Previous reports of phallotoxins in *A. rubescens* were not substantiated.

Introduction

Members of the genus *Amanita* Pers., with their characteristic white spores, free gills, and the presence of both a universal and a partial veil, are among the most readily recognised fleshy fungi. This genus has been a subject of intensive study over the past century (Corner and Bas 1962, Bas 1969, Jenkins 1977, Wieland 1986, Reid and Eicker 1991, Yang 1997).

Several species of *Amanita* occur in South Africa (Reid and Eicker 1991, Van der Westhuizen and Eicker 1994). Many species, such as *A. excelsa* (Fr.) Kummer, *A. muscaria* (L.:Fr.) Pers., *A. pantherina* (DC.:Fr.) Krombh., *A. phalloides* (Fr.:Fr.) Link and *A. rubescens* (Pers.:Fr.) Pers., are believed to have been introduced from Europe on trees as mycorrhizal associates (Reid and Eicker 1991). *Amanita reidii* Eicker and Van Greuning was described from a South African specimen but occurs only in association with *Eucalyptus* species and may have been introduced from Australia. *Amanita pleropus* (Kalchbr. and MacOwan) Reid and *A. foetidissima* Reid and Eicker are believed to be indigenous.

Amanita is a large genus, with several hundred species (Hawksworth *et al*. 1995) divided between two subgenera and several sections. Subgenus *Lepidella* is characterised by the blackening of the spores in iodine (amyloid reaction), while subgenus *Amanita* has inamyloid spores. Subgenus *Lepidella* contains sections *Amidella, Lepidella, Phalloideae* and *Validae*. Subgenus *Amanita* contains sections *Amanita* and *Vaginatae*. Sections are *sensu* Corner and Bas (1962), and are further distinguished on the basis of universal and partial veil characters (Corner and Bas 1962, Jenkins 1986).

The genus has been subject to particular scrutiny due to the production of toxins by several species (Wieland 1986). Toxins produced by *Amanita* species include the central nervous system toxin ibotenic acid, produced by certain species of *Amanita* section *Amanita* (e.g. *A. muscaria* and *A. pantherina*) and the hallucinogen bufotenine, produced by *A. citrina* (Schaeff.) Pers and *A. brunnescens* Atk. Most important are the two families of cyclic peptide toxins, amatoxins and phallotoxins, that are produced by several species of *Amanita* section *Phalloideae* (e.g. *A. phalloides, A. virosa* Lamarck, *A. verna* (Bull.:Fr.) Lamarck and others). *Amanita phalloides* is known to produce relatively high quantities of α-, β- and γ-amatoxins, the phallotoxins phalloidin and phallacidin and smaller quantities of related chemicals (Wieland 1986). Amatoxins tend to be localised in the lamellae and annulus, while the area of highest phallotoxin concentration is usually the volva (Enjalbert *et al*. 1989a, b, Enjalbert *et al*. 1993). In species that produce amatoxins and phallotoxins, both types of toxins are detectable in lamellar tissue (Enjalbert *et al*. 1992, Hallen, unpublished results). Phallotoxins have only been reported in the genus *Amanita*. Amatoxins are found in three other genera: *Conocybe* Fayod, *Galerina* Earle and *Lepiota* (Pers.) Gray (Benjamin 1995).

Amatoxins and ibotenic acid have both been implicated in fatal human and animal poisonings (Wieland 1986, Benjamin 1995, Naudé and Berry 1997). The isoxazole toxins, ibotenic acid and its metabolite muscimol, will rarely kill an adult; most fatal outcomes are in child or animal poi-

sonings (Benjamin 1995, Naudé and Berry 1997). Amatoxins are frequently lethal, and are responsible for 90% of fatal human mushroom poisonings worldwide (Benjamin 1995). Amatoxins are potent inhibitors of RNA polymerase II (RNA polymerase B), indirectly halting protein synthesis (Wieland 1986). The human LD_{50} is $0.1mgkg^{-1}$ body weight. This is approximately 7mg toxin for an adult male, or approximately $1cm^3$ of tissue from *A. phalloides*. Phallotoxins are structurally similar to amatoxins and are hypothesised to share a common biosynthetic pathway. Phallotoxins have not been implicated in human poisonings because they are not absorbed from the gastrointestinal tract (Benjamin 1995).

In this study, high-performance liquid chromatography (HPLC) has been used to evaluate a number of mushrooms from South Africa for presence of two amatoxins, α- and β-amanitin, and two phallotoxins, phalloidin and phallacidin. We utilised an HPLC protocol that has been proven sensitive enough to detect toxins in nanogram quantities (Enjalbert *et al*. 1992). We further confirmed the presence of the toxins by mass spectrometry. While detailed toxicological studies of many of the northern hemisphere species of *Amanita* have been conducted (Malak 1974, Beutler 1980, Wieland 1986), this is the first report of evaluations of endemic and introduced species collected in South Africa.

Materials and Methods

The specimens evaluated are detailed in Table 1.

Fungi were evaluated for toxins using a modification of the method of Enjalbert *et al*. (1992). Dried specimens were rehydrated in KOH, then rinsed thoroughly with distilled water. Excess water was blotted from the specimens and specimens were then diced and weighed. 8–200mg of the tissues were suspended in 1.5ml extraction medium containing methanol:distilled water:0.01M HCl (5:4:1)g^{-1} tissue. Suspended tissues were incubated at 4°C for 12h. Methanol was HPLC grade (JT Baker, Phillipsburg, New Jersey, USA). Samples were then centrifuged at 1 000 x g and 4°C for 10min, and the supernatant was collected. The pellets were resuspended in 0.6ml extraction medium g^{-1} tissue, incubated at 4°C for an additional 12h and centrifuged. The supernatants from the first and second centrifugation were pooled. Extractions were from lamellar tissue for all samples except *A. rubescens*. Both the lamellae and the volva were used in *A. rubescens* to facilitate testing for phallotoxins which have been reported in this species (Malak 1974).

HPLC analysis of amatoxins and phallotoxins was performed on a Model 114 HPLC apparatus (Beckman Instruments, Inc., Fullerton, California, USA) with detection at 295nm. Amatoxins and phallotoxins were separated using a 0711–0231 C-18 column (Perkin-Elmer Corporation, Norwalk, Connecticut, USA) and a 30min gradient of solution A to solution B. Solution A was 0.2M ammonium acetate, adjusted to pH 5 with glacial acetic acid, and solution B was acetonitrile. Flow rate was $1mlmin^{-1}$. Samples were maintained at a temperature of 4°C until injection. Twenty μl of each sample were injected.

Standards were purified α-amanitin, β-amanitin, phallacidin and phalloidin (Sigma Chemical, St. Louis, Missouri, USA). Each toxin was at a concentration of $100μgml^{-1}$, which is comparable to the concentration of toxins naturally occurring in *A. phalloides* (Enjalbert *et al*. 1992).

Peaks eluted at approximately 70–80% acetonitrile (Figure 1). Putative toxin peaks were identified by comparison with the toxin standards, and eluted fractions were manually collected from the HPLC apparatus. Eluted fractions were subjected to fast atom bombardment (FAB) mass spectrometry, at the Mass Spectrometry Facility at Michigan State University, to confirm identity. FAB mass spectra were obtained using a model HX-110 double-focusing mass spectrometer (JEOL USA, Peabody, Massachusetts, USA) operating in the positive ion mode. Ions were produced by bombardment with a beam of xenon atoms (6kV). The accelerating voltage was 10kV and the resolution was set at 1 000. The instrument scanned from *m/z* (mass to charge ratio) 50 to 1 500.

Results and Discussion

The results of the analyses are shown in Table 1. It was found that only species in *Amanita* section *Phalloideae* showed presence of amatoxins or phallotoxins. These species included *A. reidii, A. phalloides* var. *phalloides*, *A. phalloides* f. *alba* Britzelm (= *A. phalloides* var. *alba* Gillet), and '*A. phalloides* f. *umbrina*' (use of quotation marks is explained below). Each of these species showed HPLC peaks that agreed with the standards of α- and β-amanitin, phalloidin and phallacidin. Other amatoxins and phallotoxins, for which standards were not available, may have been present. Peaks identified by HPLC (Figure 1) were confirmed by FAB mass spectroscopy of the eluted fractions in *A. reidii* and '*A. phalloides* f. *umbrina*'.

The duration between collection of the mushrooms and HPLC analysis ranged from less than one month to 17 years. Despite reports of rapid degradation of phallotoxins upon drying (Stijve and Seeger 1979, Klán and Baudišová 1993), both phallotoxins and amatoxins were readily detectable in dried herbarium specimens of '*A. phalloides* f. *umbrina*' up to 17 years old. We have detected both toxins in *Amanita* species up to 21 years old, but there is a diminution in peak strength with increasing sample age (Hallen, unpublished results). Apparently, following a sharp decrease in the concentration of the heat-labile phallotoxins during drying, there is little degradation over time of the remaining phallotoxins.

The distribution of amatoxins in mushrooms has long been a subject of controversy. Faulstich and Cochet-Meilhac (1976) reported the presence of trace quantities of amatoxins in all mushroom species tested, including the common edible species *Agaricus bisporus* (JE Lange) Pilát, using radioimmunoassay (RIA). Preston *et al*. (1982) also detected trace quantities of amatoxins in edible mushrooms, using *in vitro* inhibition of RNA polymerase II activity. Collectively, these findings were taken to indicate that all Basidiomycetes produce amatoxins. This was rapidly promulgated through the literature (e.g. Wieland and Faulstich 1978, Horgen *et al*. 1978) but these findings were later refuted (Enjalbert *et al*. 1993) because of methodological considerations. The levels

Table 1: Analysis of amatoxins and phallotoxins in *Amanita* species

Taxon	Section	Provenance	Year collected	Accession #[a]	α-AMA[b]	β-AMA[b]	PCD[b]	PHD[b]
Amanita 'capensis'[c,e]	Unknown	Mpumalanga	1992	PRU 3356	–	–	–	–
Amanita excelsa	*Validae*	Belfast, Mpumalanga	1998	MSC 375639	–	–	–	–
Amanita excelsa[d]	*Validae*	Belfast, Mpumalanga	1999	MSC 375640	–	–	–	–
Amanita foetidissima	*Lepidella*	Pretoria	1992	PRU 3505	–	–	–	–
Amanita foetidissima	*Lepidella*	Pretoria	1993	PRU 3498	–	–	–	–
Amanita foetidissima	*Lepidella*	Pretoria	1994	PRU 4168	–	–	–	–
Amanita muscaria	*Amanita*	Pretoria	1998	MSC 377980	–	–	–	–
Amanita nauseosa	*Lepidella*	LC de Villiers sports ground	1989	PRU 2703	–	–	–	–
Amanita pantherina	*Amanita*	Pretoria	1991	PRU 3156	–	–	–	–
Amanita pantherina	*Amanita*	Mpumalanga	1993	PRU 3667	–	–	–	–
Amanita pantherina	*Amanita*	Sabie, Mpumalanga	1998	MSC 375641	–	–	–	–
Amanita pantherina	*Amanita*	Pretoria	1999	MSC 375642	–	–	–	–
Amanita pantherina	*Amanita*	Belfast, Mpumalanga	1999	MSC 375643	–	–	–	–
Amanita phalloides var. *phalloides*	*Phalloidae*	Pretoria	1994	PRU 3959	+	+	+	+
Amanita phalloides var. *phalloides*	*Phalloidae*	unknown	1994	PRU 4258	+	+	+	+
Amanita phalloides var. *phalloides*[d]	*Phalloidae*	Pretoria	1998	MSC 375644	+	+	–	+
Amanita phalloides var. *phalloides*	*Phalloidae*	Saasveld, George, Cape	1983	PRE 47293	+	+	+	+
Amanita phalloides f. *alba*	*Phalloidae*	Bergvliet State Forest, Sabie	1985	PRE 48659	+	+	+	+
Amanita phalloides f. *umbrina*	*Phalloidae*	Bergvliet State Forest, Sabie	1985	PRE 48654	+	+	+	+
Amanita phalloides f. *umbrina*[d,e]	*Phalloidae*	Bergvliet State Forest, Sabie	1984	PRE 48618	+	+	+	+
Amanita pleropus[d]	*Lepidella*	Brummesia National Research Institute Gardens, Pretoria	1984	PRE 47480	–	–	–	–
Amanita reidii[d,e]	*Phalloidae*	Hide-away, Melkrivier, Northern Province	1990	PRU 4306	+	+	+	+
Amanita rubescens	*Validae*	Belfast, Mpumalanga	1998	MSC 375645	–	–	–	–
Amanita rubescens	*Validae*	Belfast, Mpumalanga	1998	MSC 375646	–	–	–	–
Amanita rubescens[d]	*Validae*	Belfast, Mpumalanga	1999	MSC 375647	–	–	–	–
Amanita rubescens	*Validae*	Pretoria	1999	MSC 375930	–	–	–	–
Amanita species[e]	*Lepidella*	Lynnwood Glen Nature Reserve	1993	PRU 3611	–	–	–	–
Amanita species[e]	Unknown	Darow, Cape Province	1996	PRU 4149	–	–	–	–

[a] MSC = Beal-Darlington Herbarium, Michigan State University, East Lansing, MI, USA 48824–1312. PRU = HGWJ Schweickerdt Herbarium, Botany Department, University of Pretoria, Pretoria 0002, Gauteng Province, South Africa. PRE = National Herbarium, National Botanical Institute, Private Bag X101, Pretoria 0001, Gauteng Province, South Africa.
[b] α-AMA = α-amanitin; β-AMA = β-amanitin; PCD = phallacidin; PHD = phalloidin;
– indicates no toxin was detectable; + indicates that toxin was detected.
[c] *Amanita capensis* lacks a type specimen and has never been validly published, so the identification and taxonomic affinities of this taxon are uncertain. Quotation marks are added to indicate its uncertain affinities.
[d] Multiple specimens from this collection were assayed, results were the same for all specimens.
[e] Specimen is being further examined by DNA sequence analysis for phylogenetic analyses and species determination (Hallen, unpublished results).

of toxin detected by Faulstich and Cochet-Meilhac were at the limits of detection for the RIA procedure. These levels could be accounted for by contamination. When Faulstich repeated the assay in a different laboratory using new glassware, no toxins were detected in edible fungi (Wieland 1986). Similarly, Preston and colleagues based their evaluations solely on inhibition of calf thymus RNA polymerase II, without any further assays. The levels of putative toxin detected in nontoxic species, including *Amanita* species such as *A. brunnescens*, were near the limits of detection for this methodology. No toxins have been detected in these species following extensive testing using more sensitive HPLC procedures (Enjalbert *et al.* 1992, 1993, Hallen, unpublished results).

The edible species *A. rubescens* did not contain detectable toxins in our studies. Neither phallotoxins nor amatoxins were detected in either lamellar or volval preparations of *A. rubescens*. This contradicts the report of the detection of phallotoxins in *A. rubescens* using thin layer chromatography (Malak 1974). The edible *A. excelsa,* and the poisonous *A. pantherina* and *A. muscaria,* contained no detectable amatoxins or phallotoxins using our analytical

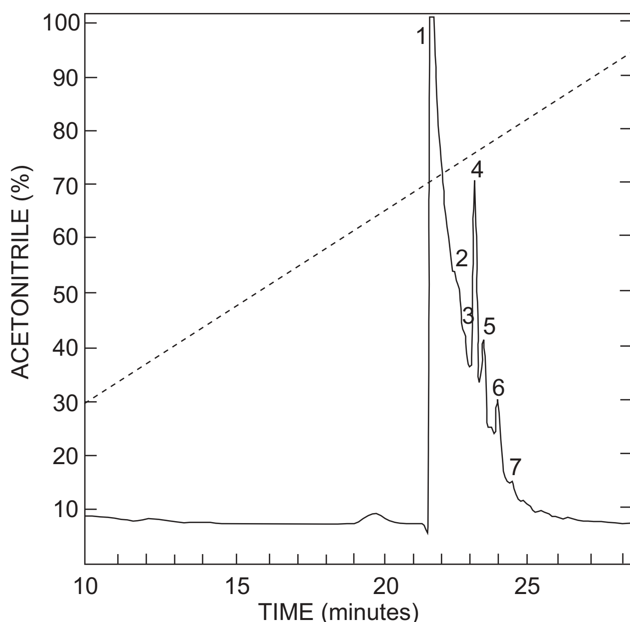

Figure 1: HPLC results for *Amanita phalloides* f. *umbrina* (= *A. reidii)* PREM 48654. The dashed line indicates the percent acetonitrile. Solid line peaks show absorbance and do not correspond to units of measure. Peak #1 represents β-amanitin, 3 α-amanitin, 5 phallacidin and 6 phalloidin. Peak 4 is likely γ-amanitin; this could not be confirmed due to the lack of a γ-amanitin standard

techniques. However, our methods do not detect other toxins, notably ibotenic acid or muscimol. Neither amatoxins nor phallotoxins were detected in the indigenous species *A. foetidissima, A. pleropus*, the species of uncertain identity *A. 'capensis'*, or the unidentified species PRU 3611 and PRU 4149. Tests for other fungal toxins need to be performed, and more specimens need to be examined, before these species are considered safe to eat.

Despite the fact that *A. phalloides* f. *umbrina* was originally used to describe aged specimens of *A. phalloides* (Ferry 1911), the name came into colloquial use in South Africa in referring to the streaked, gray-brown mushroom now known as *A. reidii* (Eicker *et al.* 1993). Thus the colloquial usage (which we have denoted by quotation marks: '*A. phalloides* f. *umbrina')* may be considered synonymous with *A. reidii,* as in Van der Westhuizen and Eicker (1994), while the original sense is not taxonomically valid (Eicker *et al*. 1993). *Amanita reidii* may be synonymous with the Australian *A. marmorata* ssp. *marmorata* Cleland and Gilbert and the Hawaiian *A. marmorata* ssp. *myrtacearum* OK Miller, D Hemmes and G Wong (R Tulloss, personal communication). These taxa are all mycorrhizal on *Eucalyptus*, and appear to have accompanied their host plant from Australia. *Amanita reidii* was placed in section *Phalloideae* based on morphological characters, and has been presumed toxic due to its affinities. This study is the first direct analysis of amatoxins and phallotoxins in this taxon, and confirms the presence of the toxins.

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