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New Studies on Hallucinogenic Mushrooms: History, Diversity, and Applications in Psychiatry

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*This paper is based on the lecture presented by the author at the VIII International Medicinal Mushrooms Conference (August 2015, Manizales, Colombia).

ABSTRACT: This paper is a review of the new studies or new explanations of the hallucinogenic mushrooms, regarding their diversity, history, traditions, and problems in their recreational use, new taxonomic studies, and their modern applications in medicine, all of them since the 1970s to the present.

KEY WORDS: medicinal and hallucinogenic mushrooms, neurotropic fungi, advances, knowledge, uses, medicine

ABBREVIATION: LSD, diethylamide of lysergic acid.

I. INTRODUCTION

This work aims to describe new studies on hallucinogenic mushrooms and the psilocybin; there are many, since the 1970s to the present. This article is divided into the following six parts: (1) what are hallucinogenic mushrooms?; (2) who discovered hallucinogenic mushrooms—when and where?; (3) who first used hallucinogenic mushrooms in their traditions?; (4) the importance of taxonomy in the study and application of hallucinogenic mushrooms; (5) the first record of an intoxication of the neurological type with a *Psilocybe*; and (6) the action of hallucinogenic mushrooms in the brain, and how to use them in psychiatry.

II. WHAT ARE HALLUCINOGENIC MUSHROOMS?

Hallucinogenic mushrooms are named in different forms, such as magic, neurotropic, sacred, divines, diviners, psychotropics, psychedelics, hallucinatory, wondrous, and entheogens. Of these, the most appropriate term is *neurotropic*, which means that their action is on the nervous system, a term I used for the first time in 1959.[1] However, I have also used the name *hallucinogen* since 1959,[2] because it is more common in the literature on the hallucinations (and also illusions) that these mushrooms produce. Notwithstanding, the term *entheogen* currently seems to be the most commonly used. This name was proposed by Wasson et al.[3] but as it means to create God inside of us, which I think is not appropriate in scientific and medical writings.

Hallucinogenic mushrooms are a complex of several different fungi,[4] such as the ergot [*Claviceps purpurea* (Fr.) Tul.], a parasitic ascomycete species on rye or wheat tassels (Fig. 1), fly agaric *Amanita muscaria* (L.) Lam. (Figs. 2 and 7c), several species of the genus *Psilocybe* (Figs. 3 and 10–20), some bolets (Fig. 4), and russules (Fig. 5). Of these, the more important are the psilocybes for their great global distribution, traditional uses, and applications. However, the first hallucinogenic substance was obtained from the ergot: LSD (diethylamide of lysergic acid) was discovered and synthesized by Hofmann in 1938.[4,5] The ergot (Fig. 1) seems to be one of the oldest fungi used by humans, based on studies by Wasson et al.,[6] who found that in ancient Greece and in Eleusis, it was used in a potion obtained from the sclerotia of the *C. purpurea*, where LSD is found. This was tested in an experiment by Hofmann.[6]

A. muscaria is another of the oldest mushrooms used by humans, first among some primitive tribes from Siberia in shamanic traditions.[7] However, when

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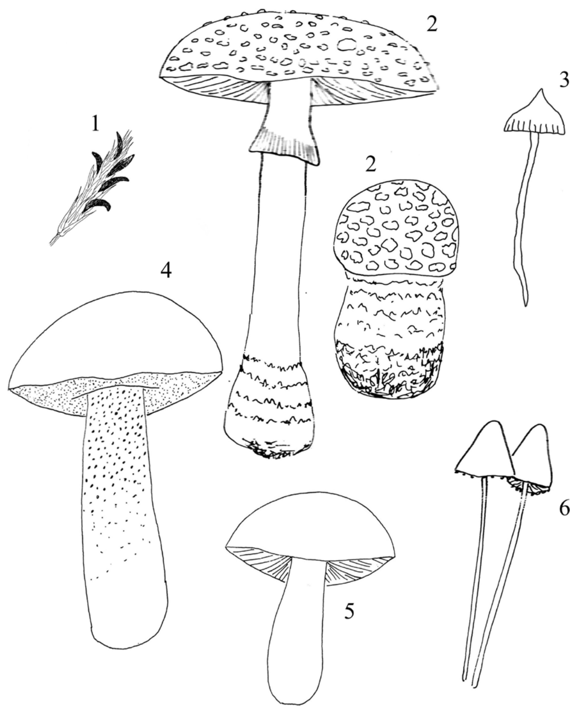
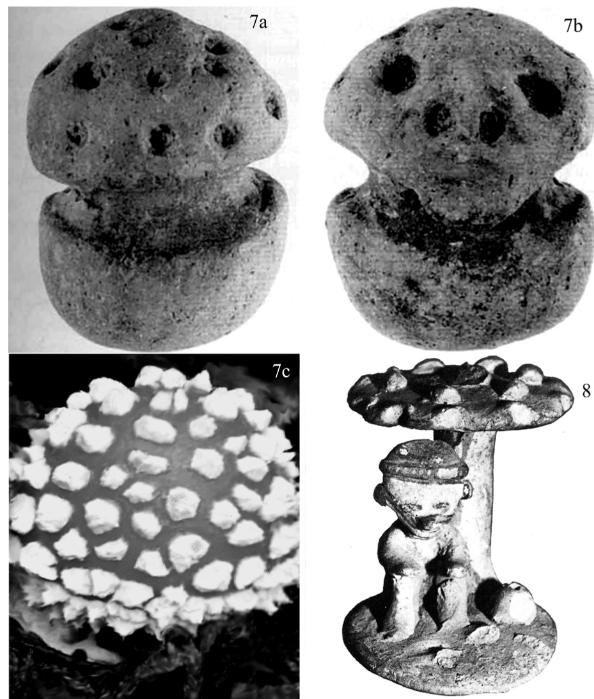


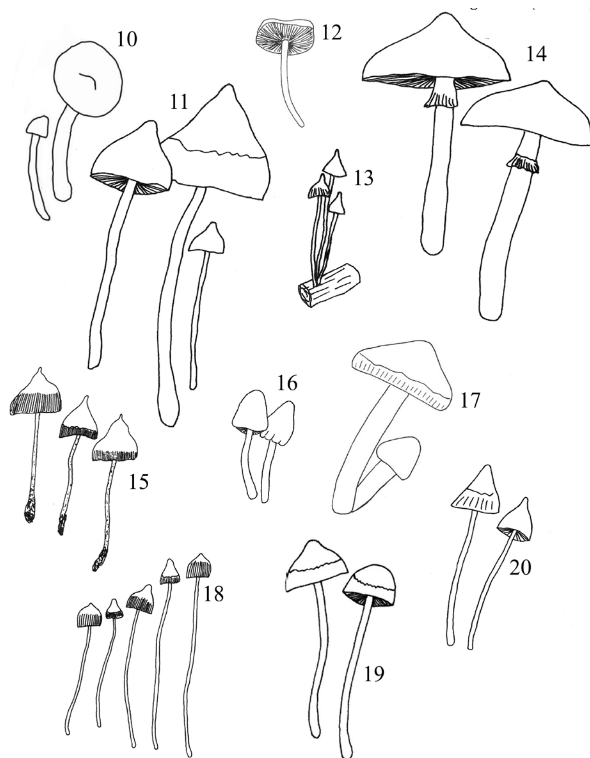
FIGS. 1–6: (**1**) *Claviceps purpurea*. (**2**) *Amanita muscaria*. (**3**) *Psilocybe mexicana* (with pseudorhiza). (**4**) *Boletus* sp. (**5**) *Russula* sp. (**6**) *Panaeolus sphinctrinus* (all by Guzmán).



FIGS. 7–8: (**7**) Piece of *Amanita muscaria*, Purepecha culture (a, b). *A. muscaria* (c). (**8**) Ceramic piece of *A. muscaria*, Capacha culture (all by Guzmán).

FIG. 9: A porcelain Greek amphora depicting two Greeks where they deposited the sacramental potion (from Wasson, pers. commun. to Guzmán).

these tribes needed to move to the southlands for the Glacier Period, through the Bering Strait to reach America, they continued using *A. muscaria*. We can see this in some North American Indians in the Great Lakes region.[8] Nevertheless, these communities continued their emigration to the southern lands, such as Mexico (where several Indian cultures, including the Capachas, Purepechas, and Nahuatls also found *A. muscaria*, as we can see in some anthropological representations[4,9;]; Figs. 2, 7c, and 8) and in Guatemala among the Maya.[10] However, as *A. muscaria* is scarce in Mesoamerica, the Indians needed to look for another mushroom to help them continue their traditions. In this way, they found the psilocybes (Figs. 3 and 10–19), which they began to use, as we can see first in the Capacha and Nahuatl Indian cultures.[9] Notwithstanding, there is a problem with the correct identification of *A. muscaria*, because Singer[11] divided the species taxonomically into subspecies, based on its geographical distribution. Singer claimed that



FIGS. 10–20: (**10**) *P. mairei*. (**11**) *P. zapotecorum*. (**12**) *P. serbica*. (**13**) *P. yungensis*. (**14**) *P. cubensis*. (**15**) *P. hoogshagenii*. (**16**) *P. hispanica*. (**17**) *P. caerulescens*. (**18**) *P. mexicana*. (**19**) *P. aztecorum*. (**20**) *P. semilanceata* (all by Guzmán).

A. muscaria in Mexico is taxonomically different from that found in Siberia.

Concerning the bolets and russules (Figs. 4 and 5), these mushrooms were used in Papua New Guinea some time ago, in some shamanic practices.[12,13] They produced some stages of madness in the people[12–14]; however, we have but little and confusing information on them, even though the first works began in 1947.[14] We also have information on some edible bolets from China,[15,16] where some bolets are sold in the markets, but with the advice from the sellers, that it is necessary to cook the mushrooms well. In some reports, the raw mushrooms produced visions of some little men or soldiers marching on the table.[16] Finally, we have several species of *Panaeolus* that have been reported as hallucinogenic, such as *P. sphinctrinus* (Fig. 6) and others,[1,2,4,5] but there is no group of Indians in Mexico and Guatemala who use them.

III. WHO DISCOVERED HALLUCINOGENIC MUSHROOMS—WHEN AND WHERE?

First, it is necessary to clarify what group of mushrooms will be discussed here because with the above presentation identifying hallucinogenic mushrooms, we have several choices. Certainly *A. muscaria* was an important mushroom among some Siberian tribes that were discovered since the 18th century,[4,7] and ergot, through its LSD, *A. muscaria* was used in ancient Greece as a potion[6] (Fig. 9). Some bolets and russules were used in Papua New Guinea,[4,12–14] and certainly edible bolets producing hallucinations were used in China if they were not well cooked.[15,16] After considering all of these mushrooms, I decided that the hallucinogenic species of *Psilocybe* in Mesoamerica, particularly in Mexico and Europe, is the most important group of hallucinogenic mushrooms (Figs. 3 and 10–20). This is because these mushrooms, although discovered in the 1950s in Mexico, have received worldwide attention from people in general and from medical professionals and mycologists in particular, in order to study them in the field of ethnomycology and to apply them in medicine. Additionally, some psilocybes have also been used in the Sahara Desert in Africa (e.g., *P. mairei* Singer) (Fig. 10) as well as in Europe (e.g., *P. hispanica* Guzmán) (Fig. 16) since prehistoric times. *P. semilanceata* (Fr.) P. Kumm. (Fig. 20) is also used throughout Europe at the present.

Hallucinogenic mushrooms were reported for the first time in Mexico in the 16th century by Sahagún[17] and de Molina,[18] two Spanish friars who were studying the Nahuatl culture (erroneously called Aztec). Sahagún said that the Indians ate some mushrooms named *teonanácatl* (Fig. 30), and de Molina found that those Indians' mushrooms were named *teyuinti*. These mushrooms produced visions and intoxication, according to previous studies.[17,18] For centuries, it was a mystery as to what Sahagún's and de Molina's mushrooms were. Curiously, the name used by Sahagún (its correct orthography is *teotlacuilnanácatl*)[9,24] is more common in the current literature for Mexican hallucinogenic mushrooms, because the name used by de Molina

FIGS. 21–25: (**21**) Reko (from Bol. Soc. Mex. Mic.). (**22**) Weitlaner (from http://deas.inah.gob.mx/sala-roberto-weitlaner/). (**23**) Schultes (courtesy of Schultes). (**24**) Heim. (**25**) Wasson (from *Life*, 1957).

was only reported by Francisco Hernández (a Spanish naturalist sent to Mexico by the king of Spain, Felipe II, to study medicinal plants[7,19]) in 1571–1576.[19] Nevertheless, both names, *teonanácatl* and *teyuinti*, are no longer used by any Indian group in Mexico. Also in the beginning of the last century, Safford in 1915[20] claimed that the *teonanácatl* is "peyote," a cactus [*Lophophora williamsii* (Lem. ex Salm-Dyck) J.M. Coult.] with hallucinogenic properties used by Indians of the southern United States and northern Mexico. This erroneous affirmation by Safford was because he isolated an indolic substance, the mescaline from the "peyote," which produces visions.

Nevertheless, Reko's view[21] (Fig. 21) was opposite to Safford and claimed that *teonanácatl* is a mushroom. Reko's opinion was based on the report that Indians from Oaxaca State (Mexico) eat some rare mushrooms to see visions. He, with the vague description from Sahagún[17] and with the help of Weitlaner (an anthropologist working in Huautla de Jiménez, Oaxaca; Fig. 22), gathered some mushrooms that were sent for identification to different herbaria in the United States. However, because the mushrooms were in poor condition, they only were identified as *Panaeolus* sp. (Fig. 6). Schultes (who was working at the Harvard University Herbarium; Fig. 23), saw Reko's mushrooms and he was interested to identify those mushrooms in the field and learn about their Indian use. He established contact with Reko and both went to Huautla de Jiménez in 1938.[22] There they received two packages of sacred mushrooms from the Indians (Mazatecs), but Reko and Schultes also gathered another type of mushroom in the field, which comprised the third package.[22] Schultes established contact with Miss Pike in Huautla de Jiménez; Pike was a specialist in languages, who had information on the sacred mushrooms from the Mazatecs through an Indian informant. She also sent a letter to Wasson,[7] informing him about the use of such mushrooms. Schultes, with the three packages of fungi obtained, returned to Harvard to make the identifications. There, however, only the the third package (the mushrooms Reko and Schultes had collected) was identified, as *Panaeolus campanulatus* var. *sphinctrinus* (Fr.) Quél. (Fig. 6). With this information, Schultes published his first article on these mushrooms in 1939, but as the *teonanácatl* of the Aztecs.[23] Here started a long line of confusion that has lasted to this day, because the mushrooms gathered by Reko and Schultes in Huautla de Jiménez were from the Mazatecs, not from the Nahuatls (or Aztecs), and the Indians do not use the name "*teonanácatl*" nor do they use any *Panaeolus* in their ceremonies.[24] Moreover, at Harvard, they could not identify the two packages of mushrooms from the Indians, which were the true sacred mushrooms, belonging to *Psilocybe*, as I discuss later in this work.

Singer[25] (Fig. 26) studied the mushrooms of Reko and Schultes at Harvard University in the 1940s. He checked the package on *Panaeolus*, but

FIGS. 26–29: (**26**) Singer. (**27**) Stresser-Péan. (**28**) Heim (by Guzmán). (**29**) Herrera (by Guzmán).

with the name of *P. sphinctrinus* (Fr.) Quél., which is the correct name, and he reported it as a narcotic.[25] He also studied the first package received by Reko and Schultes from the Indians, which he identified as *Psilocybe cubensis* (Earle) Singer[25] (Fig. 14). This was the first time that a *Psilocybe* was considered as a narcotic fungus (!). The problem was that Singer published these two important conclusions in two little paragraphs, one for *Panaeolus* and the other for *Psilocybe*, in an article with more than 800 pages.[25] The result was that noone saw that important information. Later, in the 1986 edition[11] of his paper, Singer provided the information on the *Panaeolus*, because Guzmán stated that no *Panaeolus* (Fig. 6) is used by the Indians,[9,26] information that Singer checked later in his trips to Mexico.[11,25]

The Wassons[7] arrived in Mexico in 1953 to look for information on the *teonanácatl* by Schultes. After several trips to Huautla de Jiménez, they gathered some mushrooms and met María Sabina, the main curandera (medicaster or wise woman) of the town. Late at night, Wasson and his assistant ate the true hallucinogenic mushrooms under the direction of María Sabina; this was the first time that Caucasians ate hallucinogenic mushrooms.[7] Nevertheless, because the Wassons were amateurs, they asked Heim (Figs. 24 and 28) for identification of those rare fungi. Heim found that they belonged to *Psilocybe*[27,28] and arrived in Mexico in 1956 to explore with Wasson (Fig. 25) several regions where he described several new species, including *P. cubensis*, but as *Stropharia cubensis* Earle (Fig. 14). Both Heim and Wasson[29] did not consider the article by Singer[25] where he stated that *P. cubensis* is a narcotic mushroom in Mexico. Later, Singer was in Mexico in 1957 studying the hallucinogenic species of *Psilocybe* and described some new species of *Psilocybe* with Smith.[30] They published the first world monograph of these mushrooms, which they reported were from Mexico, South America, the United States, Canada, and Java.

Guzmán, who was Singer's assistant in Mexico in 1957, continued studying alone the hallucinogenic fungi in Mexico.[1,2] In 1983, he published a world monograph of the genus *Psilocybe*.[26] It is important to show that the concept of the genus *Psilocybe* from that time changed recently with the paper of Redhead et al.,[31] in which *Psilocybe* sensu lato was split into two genera according to its properties: bluing and hallucinogenic species as *Psilocybe* sensu stricto, and those not bluing species, that are also not hallucinogenic, as the genus *Deconica*. All of the hallucinogenic species considered in this article belong to *Psilocybe* s. str.

In summary, we consider that hallucinogenic mushrooms belong to *Psilocybe* and were discovered in Mexico through a long chain of specialists beggining with Reko, followed by Weitlaner, Schultes, Pike, Singer, Wasson, and Heim; several

FIGS. 30–34: (**30**) An Indian eating the *teonanácatl* (from Codex Magliabecchiano) (by Schultes). (**31**) Depicted mural in the Tassili of the Sahara Desert (by permission from Samorini G. Fungi allucinogeni. Studi etnomicologici. Dozza [Italy]: Telesterion; 2001).(**32**) Depicted mural in the Tassili of the Sahara Desert (by permission from Samorini G. Fungi allucinogeni. Studi etnomicologici. Dozza [Italy]: Telesterion; 2001). (**33**) Ceramic pieces that show the effect of the sensation of dwarfism of Psilocybe (by Guzmán). (**34**) Ceramic pieces that show the effect of the sensation of dwarfism of *Psilocybe* (by Guzmán).

anthropologists such as Stresser-Péan (Fig. 27) and Herrera (Fig. 29) helped Wasson to find these mushrooms. This conclusion is different from others[4] that stated that Wasson founded the studies of these mushrooms and the ethnomycology.

IV. WHO FIRST USED HALLUCINOGENIC MUSHROOMS IN THEIR TRADITIONS?

These mushrooms have been used since prehistoric times, as we can see on some murals in caverns as shown by Dikov,[32] Samorini,[33] and Akers et al.[34] Of these works, that by Dikov described some anthropoid petroglyphs in Siberia, which seem to be representations of the use of *A. muscaria* by the Siberians. However, Guzmán studied copies of those petroglyphs shown by Samorini[33] and observed some isolated fungoid figures that are similar to some bolets. If this is true, we have an interesting connection with those bolets used in Papua New Guinea and China,[15,16] which needs more study. On the other hand, in Great Britain, there are also some petroglyphs with some approximate representations of both bolets and *A. muscaria*; Samorini[33] reported on the *A. muscaria* but not on the bolets. Samorini studied important murals from Africa, in the Tassili of the Sahara Desert (Figs. 31 and 32). They represent the use of some little mushrooms in the hand of several men running in a line, apparently to deposit them on a primitive altar. In other cases in the murals, there is a shaman covered by mushrooms (Fig. 31). For Guzmán,[9,35,36] the mushrooms represented in these murals are *Psilocybe mairei* (Fig. 10), a hallucinogenic species described from Algeria and Tunisia, that is close to *P. serbica* Moser & Horak (Fig. 12) described from the former Czechoslovakia, belonging to the same Mediterranean region as *P. mairei*.[9,26,36]

Another case of a prehistoric mural with mushrooms was discussed by Akers et al.[34] (Fig. 35) in Spain, where there is a line of 13 mushrooms, in a great scene of a hunt of bulls and deer by primitive men. Guzmán, in Akers' team,[34] supposed from their form and the situation of the mural that the mushrooms are *Psilocybe hispanica* (Fig. 16), which he described[36,37] from the Pyrenees, not too far from the place of the mural. This mushroom grows on cows, and the bull and deer in the mural are indicative of the relationships of this coprophilous habitat. The mushroom is also used recreationally by young people in the Pyrenees locality. Independent of the prehistoric uses of the psilocybes, there are many references to the use of sacred mushrooms among several Indian cultures in Mexico and the Maya in Guatemala, as discussed by Furst,[38] Lowy,[10] and Guzmán,[9,24,26,36,37,39,40] which reported the use of both *A. muscaria* and several psilocybes, respectively.

FIG. 35: Prehistoric mural in Spain (by Piper).

FIG. 36: Sensation of dwarfism for *Amanita muscaria* (taken by postal card).

A. muscaria produces the sensation of dwarfism (Figs. 8 and 36), which also happens in all of the psilocybes (Figs. 33 and 34).

V. THE IMPORTANCE OF TAXONOMY IN THE STUDY AND APPLICATIONS OF HALLUCINOGENIC MUSHROOMS

The first pass in the study of any mushroom and its applications needs to state based on a previous taxonomic research, or at least from a good identification, the correct name following the nomenclature rules. There was a discussion earlier of the great problems that occurred in the identification of the *teonanácatl* by Sahagún,[17] which first was identified as *Panaeolus campanulatus* var. *sphinctrinus* and then as *P. sphinctrinus*.[11,25] However, the name *teonanácatl* was restricted to those mushrooms used by the Nahuatl Indians for several psilocybes, such as *Psilocybe hoogshagenii* R. Heim (Fig. 15), *P. mexicana* R. Heim (Figs. 3 and 18; see the pseudorhiza in Fig. 3, which was not described in the original, Fig. 18), *P. cubensis* (Fig. 14), *P. zapotecorum* R. Heim (Fig. 11), *P. caerulescens* Murrill (Fig. 17), and many others. It is also necessary to know well the synonyms, in order to avoid using inappropriate names. This was the case of *P. cubensis*, which was reported as *Stropharia cubensis*.[29] Ramírez-Cruz et al.[41] reviewed the types of many species of *Psilocybe*, as *P. acutipilea* (Speg.) Guzmán from Brazil, and *P. subacutipilea* Guzmán et al. from Colombia, which are synonyms of *P. mexicana*. In addition, they found that *P. subyungensis* Guzmán from Venezuela is a synonym of *P. yungensis* Singer & A.H. Sm. described from Bolivia[30] (Fig. 13) and reported from Mexico.[26,29] Recently, Guzmán[37] found that *P. zapotecorum* has eight synonyms for problems with the original description of the type.[29] *Psilocybe aggericola* Singer & A.H. Sm. from Argentina, *P. candidipes* Singer & A.H. Sm., *P. bolivarii* Guzmán from Mexico, and *P. barrerae* Guzmán & Cifuentes are all from Mexico[37]; all of them are synonyms of *P. zapotecorum*. Guzmán et al.[42] also found that *P. subcaerulipes* Hongo from Japan has synonyms *P. argentipes* K. Yokoy. also from Japan, *P. taiwanensis* Zhu L. Yang & Guzmán from Taiwan, and *P. thaizapoteca* Guzmán, Karunarathna & Ram.-Guill. from Thailand; all of which are synonyms of *P. zapotecorum*. We said above that with division of *Psilocybe* into *Deconica* and *Psilocybe* s. str. in this way, numerous papers on new combinations on *Deconica* are being published, including works by Noordeloos[43] and Ramírez-Cruz et al.[44]

VI. THE FIRST RECORD OF AN INTOXICATION OF THE NEUROLOGICAL TYPE WITH A PSILOCYBE

Brande in 1799[45] reported a case of mushroom poisoning in an English family, from mushrooms that were gathered in a park in London. The cooked mushrooms were eaten at breakfast, resulting in neurological problems, visions, and laughing. This interesting case was discussed by Sowerby[46] and the

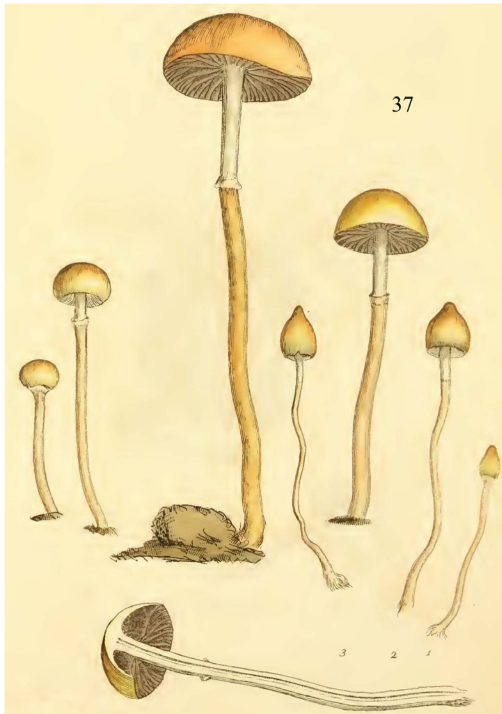


FIG. 37: Plate 248 by Sowerby shows *A. semiglobatus* (without numbers) and *A. glutinosus* (with numbers 1–3 on the plate), the latter is a lectotype of *Psilocybe semilanceata*.

mushrooms are presented in his plate 248 (Fig. 37), below the name *Agaricus semiglobatus* Batsch. This case reported by Brande is the first documented case of hallucinations caused by a mushroom. However, plate 248 has two species of fungi, as discussed by Heim.[47] One of these is *A. semiglobatus* (without numbers), and the other is *A. glutinosus* Schaeff. (with the numbers 1–3 in that plate), following Curtis,[46] but Singer, who sent the Sowerby paper to Wasson, who sent it to Heim, supposed it was *Psilocybe semilanceata* (Heim[47]). Nevertheless Heim[47] did not accept Singer's identification and followed Curtis's[46] determination. Heim proposed that the two mushrooms were *Stropharia semiglobata* (Batsch) Quél. (those with numbers) and *S. semiglobata* var. *glutinosa* R. Heim (the others).

Redhead et al.,[31] studying plate 248 by Sowerby,[46] stated that mushrooms 1–3 are *Psilocybe semilanceatus*, of which they proposed the number 3 as the lectotype of the species. Guzmán (in press) suggested that the lectotypes of *P. semilanceatus* are the three figures, because they belong to one mushroom in different stages of development. However, Singer[11] did not consider *P. semilanceata* as bluing mushrooms; nevertheless, Cooke[48] described *P. semilanceatus* var. *coerulescens* Cooke[48,49] for those bluing specimens. Singer[11] identified Cooke's mushroom as *P. cookei* Singer.[50] Certainly the bluing feature is sometimes difficult to find in some species, as in *P. mexicana* R. Heim, an important hallucinogenic mushroom. Moreover, all of the specimens of *P. semilanceata* gathered by Guzmán in the northwest of the United States in the 1970s, were bluing and little bluing, depending on the state of development. In summary, the report of Brande in 1799[45] was the first case of an intoxication by a *Psilocybe* of the neurological type with laughing, although we need to take into consideration that Sahagún and de Molina in the 16th century made the first reports on hallucinogenic mushrooms (in that case, among the Mexican [Nahuatl] Indians).

VII. ACTION OF HALLUCINOGENIC MUSHROOMS IN THE BRAIN AND HOW TO USE THEM IN PSYCHIATRY

The problems of decreased interest in the study and application of both hallucinogenic mushrooms and psilocybin started in the 1960s, as stated by Guzmán[9] and Kupferschmidt in 2014.[51] This was in part due to both the recreational abuse by young people and the prohibitions by governments, because the latter considered all of these mushrooms as drugs. These criteria stopped many research programs. Recently, the case of D. Nutt, a professor of neuropsychopharmacology at the College at London, was presented. Nutt studies the benefits of psilocybin in depression. He wanted to start a project to produce psilocybin; for this, he received support in the amount of EUR 650.00 from the Medical Research Council, but he needed to pay for a license with a cost of EUR 118 000(!) and unfortunately he stopped his project.[52]

New scientific studies are currently being conducted, such as those by Kupferschmidt et al.,[51] who rediscovered the good properties of psilocybin in their study of some problems related with the brain in persons individuals who are predisposed to illness

(e.g., cancer). Kupferschmidt[51] presented several cases of psilocybin treatments or even with LSD action in some medical experiments. He discussed how the US military and intelligence agencies (e.g., the Central Intelligence Agency) used these drugs in exciting ways of fighting enemies. He also commented on how Hofmann isolated LSD and experimented on himself. "A demon had invaded me, had taken possession of my body, mind and soul," Hofmann said, but as he woke up with a clear head, feeling euphoric, he studied the applications of LSD. Later, in 1958, when Hofmann et al.[53] isolated psilocybin, they continued their studies with several Mexican psilocybes used in ceremonies for centuries. Hofmann and his team[53] continued with these experiments with psilocybin and Sandox produced high quantities of it. This was applied as a beneficial treatment for depression, anxiety, obsessive-compulsive behavior, and psychiatric disorders.[4,29]

Psilocybin is currently used experimentally in several laboratories and hospitals to treat psychiatric disorders, as shown in research by Griffiths et al.,[54] Studerus et al.,[55] Kraehenmann et al.,[56] and Wilkox.[57] Finally, Tyls et al.[58] reported a summary of knowledge and new perspectives about psilocybin and confirmed that this substance has strong therapeutic potential, due to its pharmacodynamics and pharmacokinetics, beneficial safety profile, and zero potential to cause addiction. Recent human studies also suggest its potential therapeutic use in the treatment of several psychiatric and neurological disorders.

In a general summary, I reviewed all of the new discoveries in the hallucinogenic mushrooms, which include ergot, *A. muscaria*, some bolets and russules, and mainly several species of *Psilocybe*, including LSD (from ergot) and psilocybin. This article also reviewed the history of the *teonanácatl* (the devil mushroom from the Nahuatl culture in Mexico) and described the long line of specialists that discovered the complex *teonanácatl* in the 1950s, who all founded the new science of ethnomycology. I also presented the first case of the hallucinogenic mushroom in ancient Greece, through a potion from ergot, as well as the prehistoric origins of the use of hallucinogenic mushrooms (mainly psilocybes and *A. muscaria*). In the new taxonomic studies, the first case of intoxication with a *Psilocybe* was described in Europe in 1799 (this may also be the first such case in the world). I then showed how psilocybin is used in applications in psychiatry. Finally, I presented the bronze doors from the Middle Ages on the Cathedral of Hildesheim in Germany (Fig. 38), where there is a representation of the Tree of the Eden in the form of a large *Psilocybe semilanceata* with three basidiomata, with Adam and Eve, both covering their sex, and God asking them who ate a part (a mushroom) of the tree. Adam points out Eve, and Eve points out a terrible animal. Here we can see another case of the action of the sensation of dwarfism (as in Figs. 8 and 36).

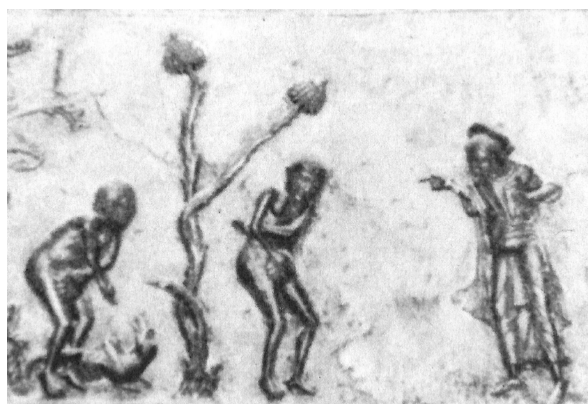


FIG. 38: Door of the Cathedral of Hildesheim at Germany (by Samorini).

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Consequences of Misnomer or Mistakes in Identification of Fungal Species

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ABSTRACT: The author, as a reviewer of many international journals, describes his long-standing experiences with incorrect identification of mushroom and fungal species and the resultant incorrect naming of those species that served as experimental models. From his own praxis, he selected several characteristic examples that sometimes ended in a curious situation. Some recommendations to authors of publications and persons responsible for the proper naming of mushrooms under study are summarized.

KEY WORDS: medicinal mushrooms, taxonomy, nomenclature, mushroom names, valid and invalid names, culture collection contaminations

I. INTRODUCTION

In many manuscripts I have reviewed, some mistakes were repeated frequently, indicating insufficient knowledge in mycology, especially taxonomy and nomenclature. The authors of the manuscripts were partly to blame because they only innocently passed on inaccurate information from the supplier/identifier of the mushrooms and fungal material. However, their mistake is that they did not diligently examine and reconfirm the identification of the material with which they planned to work and therefore submitted their research for publication using incorrect information. Usually there are two main reasons this occurs— an error in the name of the respective mushroom or a wrongly identified organism.

II. INVALID NAME OF THE MUSHROOMS

Perhaps the worst example of this is the mushroom named "*Pleurotus florida*." The incorrect naming of this species has been repeated in mycological literature, and in manuscripts sent to this journal, for very many years. In fact, no fungus of this name exists. The fungal culture named "*Pleurotus florida*" that has been distributed in many mycological culture collections is, in fact, *P. ostreatus*. Here is a historical remark to clear up the problem. In 1965, in Germany, Dr. Gerlind Eger published her study on an oyster mushroom that she obtained from Florida in the United States.[1,2] The strain was isolated by a person named Block and sent to Dr. Eger as *P. ostreatus* (Jacq. ex Fr.) Quél. Dr. Eger fructificated the culture and saw that it did not look the same as the *P. ostreatus* fruiting bodies of German origin. She consulted Dr. A. H. Smith (Ann Arbor, MI, USA), who found some similarities with *P. cornucopiae* Paul. ex Fr. Thus, Dr. Eger named the mushroom *Pleurotus florida* **without any description of a new fungal species as is required under nomenclature regulations**. This was a crucial mistake, against all taxonomy and nomenclature rules. Dr. Eger was a recognized expert in the field of mushroom fructification and fruit body regeneration; however, she was probably not experienced in fungal nomenclature. Several fungal taxonomists protested against the name *P. florida* and declared the name invalid (nomen nudum).[3–6] Also, Dr. Eger soon realized her mistake and in the next publication[7] named the fungus "*Pleurotus* **from** Florida." Meanwhile, the cultures of this fungus were distributed into many fungal culture collections and to many mushroom growers because of its good mycelium growth and fruiting body production also at higher temperature, as well as other advantageous features. Current classification of this mushroom is *P. ostreatus* (Jacq.: Fr.) Quél. cv. Florida (cv. means cultivar). Sometimes the cv. is replaced by f. sp. (forma specialis) or the fungus is simply considered as an albino form of *P. ostreatus*. In my opinion, which has been affected by my

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long-time career at the Institute of Microbiology, Czech Academy of Sciences, I could admit naming the fungus "*P. ostreatus* **strain** Florida" since it was distributed not in the form of natural fruiting bodies but as a pure culture that possessed some properties different from *P. ostreatus* cultures standardly used in the mushroom business. Perhaps in the future, modifications of naming this fungus will come about because Hilber[5] and Bresinsky et al.[4] have already stated that strains available under the name "florida" represent two taxa, the majority being *P. ostreatus* and at least one strain being *P. pulmonarius*.

Very similar misunderstandings and inconsistencies as in the case of the so-called "*Pleurotus florida*" also occurred in the naming of one *Agaricus* species. The mushroom was originally found in Brazil and named Royal Sun Agaricus. Because of its very good culinary and medicinal properties, it appeared soon in Japan, where it was cultivated and subjected to an intensive research of its multivarious medicinal properties.[8,9] The mushroom became popular and quickly expanded into other countries. First, it was named *A. blazei* but soon it appeared that two different species named *A. blazei* existed: one *A. blazei* sensu Murrill, and the other *A. blazei* sensu Heinem. With the aim of solving this problem Wasser et al.[10] named this new culinary-medicinal mushroom *A. brasiliensis* S. Wasser et al. However, instead of settling this nomenclature problem, a heated exchange of different views began.[11–13]

Even the Latin name of the well-known cultivated mushroom—white button mushroom—in most parts of the world is *A. bisporus* (J.E. Lange) Imbach, while on the American continent many mycologists insist on the name *A. brunnescens* Peck because it is the oldest and thus correct name for the cultivated mushroom. The name *A. bisporus* has been supported by the fact that it has long been used in the naming of the most common cultivated mushroom. Regardless of the opinion that *A. brunnescens* and *A. bisporus* are the same species, the disputations about the name priority are endless. For example, see Malloch et al.,[14] who insist on the facts that *A. brunnescens* was validly described by Peck in 1900, while *A. bisporus* was described in 1923 by Lange under the name *Psaliota hortensis* forma *bispora*. If it were not for the most common cultivated mushroom, such disputation could be neglected; however, in this case, it introduces confusion in the minds of researchers dealing with cultivated and medicinal mushrooms.

In the Far East, the famous medicinal mushroom used in oriental medicine as lingzhi or reishi, and named *Ganoderma lucidum* (W.Curt.:Fr.) P. Karst. in the scientific literature, has recently been separated into several species on the basis of geographic differences and also using molecular techniques. This difficult and unenviable task was recently explained in detail by Wasser as follows[15]:

> There is now mounting evidence that shows most species previously reported as Lingzhi or Reishi (*Ga. lucidum*) in most pharmacological studies were mistakenly identified. *Ga. lucidum* presents a taxon-linneon or species-complex of which the future subdivision requires caution.[16] Publications, patents, and products are also at risk. Over the years, at least 166 laccate *Ganoderma* species have been described worldwide.[17] Approximately 100 *Ganoderma* species are known from China.[18] It is not known what the taxonomic positions of so-called medicinal Blue Ling Zhi, or Red Ling Zhi, or White Ling Zhi are. For example, Cao et al.[19] published a paper in which they claimed that the so-called *Ga. lucidum* in China is quite different from the *Ga. lucidum* found and described in Europe, and so they introduced a new species, *Ganoderma lingzhi* Sheng H. Wu, Y. Cao et Y. C. Dai, for Chinese mycobiota. This brought more problems and confusion. Who knows which *Ganoderma* species is medicinal? Which species of Lingzhi is the Chinese national mushroom, *Ga. lucidum* or *Ga. lingzhi*? Nobody knows. Young and Feng[20] published a special mini-review dedicated to this problem, called "What is the Chinese 'Lingzhi'?." Molecular phylogenetic analyses based on the ITS (internal transcribed spacer) and 25S ribosomal DNA sequences indicated that most of the collections called "*Ga. lucidum*" in East Asia were not conspecific with *Ga. lucidum* found in Europe.[20] Moreover, in 2012, in the journal *Nature Communications*, a group of scientists published a paper dedicated to genome sequences of the model medicinal mushroom *Ga. lucidum.*[21] The authors reported *Ga. lucidum* 43.3-Mb genome,

encoding 16,113 predicted genes, obtained using next-generation sequencing and optical mapping approaches. However, this very important publication did not solve the problem of the *Ga. lucidum* species complex because the authors studied one dikaryotic strain, CGMCC5.0026, belonging to the *Ga. lucidum* Asian group from China, which was claimed to be, as already mentioned, the new species to science, *Ga. lingzhi*.

It is supposed that *Ga. lucidum* is probably restricted to the western part of Europe, although its distribution range can possibly also include parts of Siberia and western regions of China. Based on molecular phylogenetic evidence, it appears that most collections labeled *Ga. lucidum* in North America do, in fact, best correspond to the taxon labeled *Ga. resinaceum* in Europe, whereas *Ga. tsugae* in North America is genetically very close to the "true" *Ga. lucidum* from Europe.[22] If you check some lists of fungal species, such as http://www.indexfungorum.org/Names/Names.asp, http://www.mycobank.org/quicksearch.aspx, and http://www.speciesfungorum.org/Names/Names.asp, you will see the complicated position of the former simple name *Ga. lucidum.*

The above examples are not a rarity; on the contrary, such incidents happen often for many reasons. Scientific names in use for edible and medicinal mushroom can change as a result of new research on relationships and species limits, and/or issues relating to the international rules regulating publication of scientific names. Other reasons for changing scientific names include the existence of an earlier name which has priority, the replacement of an incorrect name, or the wrong original identification. Implementation of genetics, and biochemical and ultrastructural studies, and the currently still increasing use of methods of molecular biology will surely contribute to the increase in the number of new mushroom Latin names, hopefully resulting in scientific progress.

III. WRONGLY IDENTIFIED SPECIES OF THE MUSHROOM OR THE CULTURE

It is surprising how carefree some researchers are sometimes. If they receive a fruiting body or a culture, they may not obtain complex information about its full name and origin, eventually depositing it in a herbarium or assigning a culture number in a collection, and so forth. When I started isolating pure cultures from mushroom fruiting bodies, I used to go to meetings of the Czechoslovak Scientific Society for Mycology, where freshly collected fruiting bodies were demonstrated as passing regularly through the hands of the best Czech mycology experts. I also brought my own samples, gathered from the forest, to those meetings for verification. Once the mushroom is wrongly identified and then used in experiments, or serves as a source for isolating of pure cultures, the consequences can be formidable; it can bring into the scientific literature a disorder that is not easily reversed, or if a culture of a wrongly indentified fruiting body is distributed among other culture collections, the mistake is sometimes impossible to eliminate.

Mistakes in fruiting body identification, contamination during isolation, or culture reinoculation can happen occasionally; however, it is much worse, when the fungus's Latin name is changed on purpose. Some 53 years ago, when I was a new Ph.D. student in the Laboratory of Experimental Mycology in Prague, a visitor from Bulgaria delivered a lecture on a popular topic at the time—single cell proteins. He named the fungal species he worked with *Polyporus squamosus.* Since my university studies involved work with micromycetes and then my research subjects in my Ph.D. study were cultures of Basidiomycetes, I easily recognized from the slides the Bulgarian lecturer showed that they were not basidiomycetous cultures but rather a micromycete of the genus *Fusarium.* In my adolescent enthusiasm, I told him that the fungus had typical multicellular, crescent-like conidia of a *Fusarium* and that I could identify the species for him with pleasure. My suggestion was strongly refused. A couple of years later, I met Prof. A. S. Buchalo of the Institute of Botany in Kiev, Ukraine, and I retold that story to her. She got excited because the same fellow also visited her laboratory and presented a similar lecture. She also recognized the culture as a *Fusarium*, and she offered him the correct species identification. Because she was more assertive than I had been, she got the culture. Several years after

the story, I received a reprint[23] from Prof. Buchalo, in which she described the *Fusarium* as *F. sambucinum* Fuck. var. *ossicolum* (Berk. et Curt.) Bilai. In the paper, she documented microscope photos of conidia of that micromycete as well as the pictures of how the fungus should look if it were the mushroom culture (including a clamp connection on the mycelium). The reason for that unfair trick was evident. The Bulgarian fellow presented his fungus as a promising organism for food protein production. He declared his fungus as a mushroom because if he admitted that the producing fungus was a *Fusarium*, his chance to succeed in the food protein business would be negligible because many *Fusarium* species are known as producers of dangerous mycotoxins.

The above story is an exception because of its purposely made change of a fungus; however, each fungal culture collection probably has some problem with wrong identification or a contamination, sometimes due to culture exchanges among individual culture collections.

Sometimes the cause of the contamination can be curious. Our Laboratory of Experimental Mycology had a program of cooperation with the Laboratory of Fungal Biochemistry in Saint Petersburg, Russia. Since there was no mycologist at the Petersburg laboratory, during my visits, I had occasionally been asked to check some of their cultures for possible contamination. Once while looking under a microscope I observed the presence of clamp connections on hyphae of fungal species, where clamp connections should not be present. After a long discussion, we came to the conclusion that some time ago someone must have brought a great cluster of oyster mushroom fruiting bodies into the laboratory. Over the weekend, it was left freely on the bench in the laboratory; on Monday, the laboratory staff found every possible surface in the room covered with a layer of basidiospores. Since the same room was used as a place in which manipulation with cultures took place, this was the reason why many test tubes with cultures got contaminated with one fungus—the oyster mushroom characterized with abundant clamp connection formation.

The medicinal mushroom *Oudemansiella mucida* (Schrad.) Höhn., a producer of antifungal antibiotic mucidin discovered in our laboratory, was our research subject for several years. I was involved in studying the effect of the antibiotic on test organisms which included a yeast we received from the Research Institute of Antibiotics and Biotransformation connected to the factory where the antibiotic was manufactured. The culture was believed to be *Candida albicans*, a nonpathogenic strain of that species. During observation of its submerged culture, among vegetative cells I also noticed asci with ascospores. Since it was known that *C. albicans* existed in an anamorphic (imperfect) stage only, there were only two possibilities: either the culture was not *C. albicans*, or I found a telemorph (perfect stage) of *C. albicans.* Thus, I conducted classic fermentation tests and together with microscopic observation they indicated that the yeast was *Saccharomyces fragilis* A. Jörg. with imperfect stage *Candida pseudotropicalis* (Castell.) Basgal based on the current nomenclature *Kluyveromyces marxianus* (E.C. Hansen) Van der Walt. Since yeasts were not the field of my research, I turned to Dr. P. Fragner, who was the top Czech yeast expert at the time. I sent him all the materials, and Dr. Fragner confirmed my identification.

Perhaps the most curious example is the following story. In 1962. H. P. Molitoris published an article[24] in the famous journal *Nature*, in which he described how he found contamination in a prestigious culture collection. The culture collection was the Agriculture Research Service Culture Collection, known under the abbreviation NRRL among microbiologists and mycologists. The collection was established in 1940 and was huge; in 2010, it contained around 9,000 Actinomycetes, 10,000 bacteria, 35,000 filamentous fungi, and 15,000 yeasts, including some 6,000 patented strains. Young H. P. Molitoris was sent by his father to that collection to become familiar with handling fungal cultures. His father had become an owner of a small mushroom farm. Molitoris started studying microscopy cultures of Basidiomycetes, namely, *Agaricus* species. He was surprised when he found conidia and mycelia in all cultures of *A. campestris* that resembled a micromycete rather than a mycelium of a basidiomycete. Therefore, he identified the species and demonstrated

that all three studied strains of "*A. campestris*" were, in fact, micromycetes *Beauveria tenella* (Delacroix) Siem. Some 10 years after that, the whole story was retold to me by Molitoris himself (who meanwhile had become a university professor), at which time he gave me a reprint[25] in which he described the issue in more detail. However, the story did not end here. The purported "*A. campestris*" cultures got into the NRRL from Dr. Humfeld's laboratory, where they served as models for research of submerged cultivation. Humfeld himself explained the unusual look of those cultures and described them as spontaneous mutants. However, Molitoris clearly documented his discovery by comparison with other authentic A*garicus* cultures as well as with real *Beauveria tenella* cultures. It looked like the problem was settled, but it was not. Humfeld, Sugihara, Szuecs, and other coworkers had already published several papers,[26–28] including patents,[29,30] about submerged cultures of "Basidiomycetes," describing growth, role of asexual spores in growth velocity, and so forth, with the aim of demonstrating mushroom submerged cultures as prospective organisms for human food component production. Thus, instead of bringing new scientific information, it brought chaos into the scientific literature. In defense of the above-mentioned authors, it must be noted that at the time, submerged cultivation of mushrooms had just started. Even with standard agar cultures, we were often not sure if the observed spores were a part of the basidiomycete or a contamination. Another important factor was that the above authors declared that the mushrooms under submerged conditions lost their typical "mushroom" smell. This was not true at all; in the room, we grew mushrooms in fermenters under submerged conditions, and we found that the mushroom smell was often like that found in a forest.

IV. WHAT CAN THE POOR AUTHOR WITH LIMITED MYCOLOGY KNOWLEDGE DO?

Each experimentalist must realize that only correctly identified material should be used; if it is not, the resulting publication is of no scientific value. Using incorrectly determined mushrooms is a fundamental mistake. Checking the validity of the fungal name in the above-mentioned fungal lists, or in other mycology literature, or consulting with a mycologist is highly recommended. It is also recommended to require in-depth information about the fungal material, such as the name of the place of the fruiting body collection and the name of the person who identified the fungus; with cultures, it is necessary to know, besides the valid Latin name of the species, the name(s) of the authors, who described the species, as well as the strain number in the respective collection, etc. Verification of the valid name including information about the synonyms in the mycological literature is always very useful.

V. WHAT SHOULD THE AUTHORITIES IN MUSHROOM TAXONOMY DO?

They have already participated in the setting up of the International Code of Nomenclature for Algae, Fungi and Plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress Melbourne, Australia, July 2011. The goal of mycology experts participating in the preparation of the upgraded code version was to maximally explain all possible problems in the nomenclature of fungi. However, there are many other things that could be done. For instance, at every big meeting (conference, seminar, workshop, etc.), the regular insertion of lectures dealing with fungal taxonomy and nomenclature would be useful for scientists who are not mycologists but who use fungi as research subjects. Even in journals in which papers dealing with mushrooms appear only from time to time, reviewer staff should also include mycology experts.

Significant progress has been made in molecular biology. Mycology, as well as other biology branches, urgently needs a reliable instrument for the identification of individual species, distinguishing or confirming the identification of fruiting bodies as well as the cultures, strains, cultivars, etc., originating from different localities or even continents. If the determination would be carried out by molecular biologists as well as in close cooperation with classic mycologists-taxonomists, it could significantly reduce the confusion that has occurred in the past.

There is an excellent 300-year-old quotation of Carl von Linné: "Nomina si nescis, perit et cognito rerum" (If you do not know the species name, you do not understand the core), which I found in one of Prof. Wasser´s papers.[15] I was fascinated at how valid this statement is, even after so many years.

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Isolation and Characterization of a Ubiquitin-Like Ribonuclease from the Cultured Deep Root Mushroom, *Oudemansiella radicata* (Higher Basidiomycetes)

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ABSTRACT: The isolation of a novel 13.5-kDa ribonuclease, displaying a ubiquitin-like inner peptide sequence, from dried fruiting bodies of the cultured mushroom *Oudemansiella radicata* (Relhan: Fr.) Singer (=*Xerula radicata*) is reported. The purification protocol deployed encompassed sequentially, cation/anion exchange chromatography on CM-cellulose, DEAE-cellulose and SP-Sepharose, and FPLC-gel filtration on a Superdex 75 column. The purified enzyme manifested optimum activity at 70°C and pH 4.6, respectively. The activity of the RNase was inhibited by the majority of metal ions tested, especially Al^{3+}, Hg^{2+}, and Cd^{2+} ions, but was promoted by K^{+} ions. It exhibited the highest ribonucleolytic activity toward poly (C), lower activity toward poly (G), and negligible activity toward poly (U) and poly (A). Compared with mushroom ubiquitin-like RNases reported earlier, *O. radicata* RNase possesses a larger molecular mass, distinctive chromatographic behavior on DEAE-cellulose, a lower optimum pH, and a unique polyhomoribonucleotide specificity.

KEY WORDS: medicinal mushrooms, *Oudemansiella radicata*, ribonuclease, bioactivity

ABBREVIATIONS: CM, carboxymethyl; **DEAE,** diethylaminoethyl; **ESI-MS/MS,** electron spray ionization mass spectrum and mass spectrum; **FPLC,** fast protein liquid chromatography; **IC_{50},** half maximal inhibitory concentration; **MALDI-TOF-MS,** matrix assisted laser desorption time of flight mass spectrometry; **NAE,** NEDD8 activating enzyme; **NEDD8,** neural precursor cell-expressed developmentally downregulated 8; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **SP,** sulfopropyl; **UBL,** ubiquitin-like protein.

I. INTRODUCTION

Mushrooms produce a repertoire of molecules with important biological activities encompassing ribonucleases (RNases),[1,2] proteases,[3] laccases,[4,5] antifungal proteins,[6] ribosome inactivating proteins,[6,7] lectins,[8] polysaccharides,[9] and polysaccharopeptides. These mushroom products exhibit a diversity of potentially exploitable activities including antiproliferative,[10] antitumor,[11] immunomodulatory,[12] and HIV-1 reverse transcriptase inhibiting activities.[13,14]

RNases have been isolated and characterized from various mammalian organs comprising the kidneys, liver, and brain, as well as from semen and milk.[15] Mushrooms also produce RNases. A number of mushrooms including *Boletus griseus*,[15] *Lyophyllum shimeiji*,[16] *Pleurotus djamor*,[17] *Russula delica*,[18] *Schizophyllum commune*,[19] and others have been reported to produce RNases. These RNases display a range of molecular masses, a variety of N-terminal sequences, and different pH and temperature maxima.

Deep root mushroom, *Oudemansiella radicata* (Relhan: Fr.) Singer (Physalacriaceae, higher Basidiomycetes), a cultured edible mushroom with tremendous economic value, is distributed in the tropics and subtropics of the Northern Hemisphere. Current cultivation techniques enable production of this mushroom with a high yield. Publications on this mushroom are confined to its antifungal and antibiotic properties,[20] the metabolite oudenone,[21] and the

effects of lead and cadmium on the mushroom.[22,23] To date, there are no reports on the presence of RNase activity or purification of an RNase from this mushroom. We observed that an extract of its fruiting bodies displayed RNase activity. Hence, we set out to isolate the RNase and examine its characteristics.

II. MATERIALS AND METHODS

A. Purification of RNase

Dried fruiting bodies of *O. radicata* (100 g) were collected in Hebei Province, China. Homogenization of the fruiting bodies in 0.15 M NaCl (15 mL/g) was carried out in a Waring blender prior to extraction at 4°C for 8 h. Centrifugation (9000 *g*, 4°C, 10 min) was then performed. The resulting supernatant was dialyzed extensively overnight before ion exchange chromatography on a 5 cm × 20 cm column of CM-cellulose (Sigma, St. Louis, MO, USA) in 10 mM HAc-NaAc buffer (pH 4.6). Unadsorbed proteins (fraction C1) were removed. Adsorbed proteins were desorbed successively with increasing NaCl concentrations, namely, 50 mM NaCl, 150 mM NaCl, and 1 M NaCl in the starting buffer. The adsorbed fraction eluted with 150 mM NaCl (fraction C3) was dialyzed, and then fractionated by ion exchange chromatography on a 5 cm × 10 cm DEAE-cellulose (Sigma) column in 10 mM Tris-HCl buffer (pH 9.0). The column was eluted with 150 mM NaCl, and then with 1 M NaCl in the starting buffer. The fraction (D1) eluted with 150 mM NaCl was dialyzed before applying on a 1 cm × 10 cm SP-Sepharose (GE Healthcare, Uppsala, Sweden) column in 10 mM HAc-NaAc buffer (pH 3.6). Following elution of unadsorbed proteins in fraction SP1, the column was eluted with a linear 0–1 M NaCl gradient in the 10 mM HAc-NaAc buffer (pH 3.6). The adsorbed fraction (SP2) was lyophilized, prior to chromatography on a Superdex 75 HR 10/30 column (GE Healthcare) in 0.15 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare). The first peak (SU1) eluted represented the purified RNase.

B. Determination of Molecular Mass and Amino Acid Sequence

The purified RNase was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) utilizing a 12% resolving gel and a 5% stacking gel for molecular mass determination followed by staining with Coomassie brilliant blue.[24] Gel filtration on an FPLC-Superdex 75 column previously calibrated with molecular mass markers (GE Healthcare) was carried out to ascertain the molecular mass of the RNase. Amino acid sequences of the inner peptides were procured by using matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF-MS) and electron spray ionization mass spectrum and mass spectrum (ESI-MS/MS). The purified protein was subjected to trypsin digestion and MALDI-TOF-MS analysis. Some high-quality peptides were subsequently analyzed by ESI-MS/MS.

C. Assay for Activity of RNase

The ribonucleolytic activity of the purified RNase toward the substrate yeast tRNA (Sigma) was measured by determining the production of acid-soluble, UV-absorbing species.[25] Following incubation of RNase with 200 μg tRNA in 150 μL of 100 mM MES buffer (pH 6.0) at 37°C for 15 min, the enzymatic reaction was terminated by addition of ice-cold 3.5% perchloric acid (350 μL) and leaving the reaction mixture on ice for 15 min. OD260 of the supernatant obtained after centrifugation (15,000 *g*, 15 min) at 4°C, was measured following suitable dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in OD260 of one per minute in the acid-soluble fraction per milliliter of reaction mixture under the specified condition.

D. Effects of pH, Temperature, and Metal Ion on RNase Activity

HAc-NaAc buffer (pH 3.6–5.6, 0.1 M), NaH_2PO_4-Na_2HPO_4 buffer (pH 5.6–7.2, 0.1 M), Tris-HCl buffer (pH 7.2–9.0, 0.1 M), and NH_4HCO_3-$NH_3 \cdot H_2O$

buffer (pH 9.0–9.4, 0.1 M) were used to adjust the pH values of the reaction mixture and the effect of pH on the RNase activity was determined. Incubation of the reaction mixture was carried out at different temperatures from 4°C to 100°C to determine the effect of temperature on RNase activity.

The effect of metal ions on the RNase activity was then determined. Purified RNase solutions were preincubated at 4°C for 1 h with solutions containing different metal ions including K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Al^{3+}, Hg^{2+}, Fe^{2+}, Zn^{2+}, and Pb^{2+} ions at a final concentration of 2.5, 5, and 10 mM. RNase activity was assayed as described above.[17]

E. Activity of RNase toward Polyhomoribonucleotides

Assay of the ribonucleolytic activity of the purified RNase toward various polyhomoribonucleotides was performed with a slightly modified version of the method described by Wang and Ng.[3] The RNase was incubated with 100 μg of a polyhomoribonucleotide, poly (A)/poly (C)/poly (G)/poly (U) in 250 μL 100 mM MES buffer (pH 6.0) at 37°C for 1 h. Ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitrate (250 μL) was then added to bring the reaction to an end. The reaction mixture was centrifuged (15,000 *g* for 15 min at 4°C) after leaving on ice for 15 min. The absorbance of the resulting supernatant, when poly (A), poly (G), and poly (U) were used as substrates, was read at 260 nm following suitable dilution, and measured at 280 nm instead with poly (C) as substrate.

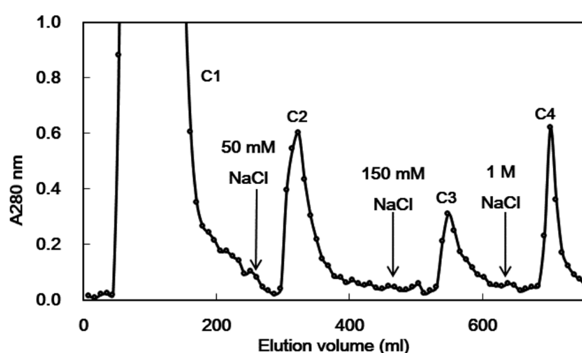

FIG. 1: Cation exchange chromatography of crude extract on a CM-cellulose column. The unadsorbed fraction C1 was eluted with 10 mM HAc-NaAc (pH 4.6) whereas fractions C2, C3, and C4 were desorbed from the column with 50 mM NaCl, 150 mM NaCl, and 1 M NaCl in the same buffer, respectively.

F. Statistical Analysis of Data

Data were analyzed by using analysis of variance.

III. RESULTS

A. Isolation of RNase

Cation exchange chromatography of the *O. radicata* fruiting body extract on CM-cellulose separated it into an unadsorbed fraction (C1) and three adsorbed fractions (C2, C3, and C4) (Fig. 1). Only fraction C3 demonstrated RNase activity. C3 was resolved into two bound fractions (D1 and D2) by anion exchange chromatography on a DEAE-cellulose column. Only fraction D1 manifested RNase activity (Table 1). Cation exchange chromatography of

TABLE 1: Yields and RNase Activities of Various Chromatographic Fractions of *Oudemansiella radicata* RNase toward Yeast tRNA (from 100 g Dry Fruiting Bodies)

Fraction	Yield (mg)	Specific RNase Activity (U/mg)	Total RNase Activity (U)	Recovery of RNase Activity (%)	Purification Fold
Crude extract	4020	1.40	5632	100	1
C3	67.82	21.66	1469	26.08	15.46
C3D1	9.42	83	782	13.88	59.24
C3D1SP2	2.60	220	571	10.14	157
C3D1SP2SU1	0.40	853	343	6.08	609

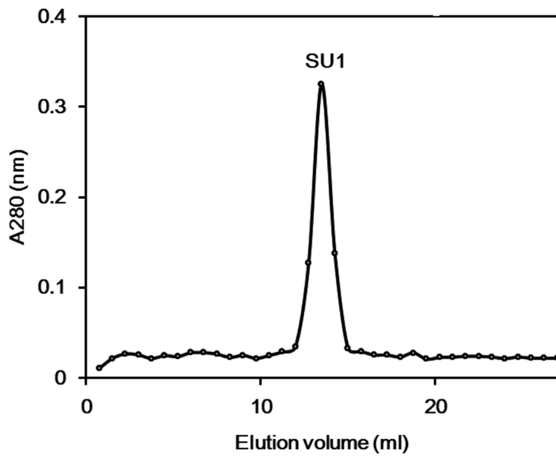

FIG. 2: Gel filtration of fraction SP2 from the SP-Sepharose column on a Superdex 75 HR 10/30 column by FPLC on an AKTA Purifier. Elution buffer: 0.15 M NH_4HCO_3 buffer, pH 8.5. Flow rate: 0.75 mL/min. Fraction SU1 represented purified RNase.

FIG. 3: SDS-PAGE results. Left lane: purified RNase. Right lane: molecular mass marker, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), lactate dehydrogenase (30 kDa), soybean trypsin inhibitor (20 kDa), and alpha-lactalbumin (14.4 kDa).

fraction D1 on SP-Sepharose fractionated it into a broad unadsorbed fraction (SP1) and a sharper adsorbed fraction (SP2). The latter fraction, which possessed RNase activity, appeared as a major peak (MW 13.5 kDa) in FPLC-gel filtration on Superdex 75 (Fig. 2).

B. Characterization of Isolated RNase

The molecular mass of *O. radicata* RNase was below 14.4 kDa as judged by SDS-PAGE (Fig. 3) and 13.5 kDa as estimated by gel filtration on Superdex 75. The amino acid sequences of five inner peptides as determined by ESI-MS/MS were DYNIQKESTLHLVLR, IQDKEGIPPDQQR, RTLSDYNIQKESTLHLVLR, LIFAGKQLEDGR, and MQIFVKTLS, respectively. There was substantial homology to human, *Arabidopsis*, yeast, and the mushroom *Coprinus congregates* (Fig. 4).

The optimum temperature (Fig. 5) and pH (Fig. 6) for the purified RNase were approximately 70°C and pH 4.6, respectively. The activity of the RNase rose steadily from pH 3.6 until it attained its zenith at pH 4.6, and declined from pH 4.6 until it reached residual level at pH 9.4. The activity fell abruptly as the temperature was elevated from 70°C to 90°C. The activity detected at 100°C was analogous to that at 10°C. The RNase activity was strongly suppressed by Cd^{2+}, Hg^{2+}, and Zn^{2+} ions. Among the metal ions tested, only Al^{3+} ions almost completely abrogated the RNase activity. The activity was augmented in the presence of K^+ ions (Table 2).

The RNase displayed high specific ribonucleolytic activity of 12.86±0.17 U/mg and 10.61±0.34 U/mg toward poly (C) and poly (G), respectively, and <1 U/mg toward poly (A) and poly (U).

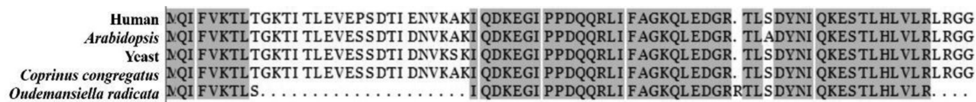

FIG. 4: Alignment of the amino acid sequences of *Oudemansiella radicata* RNase and ubiquitins from humans, *Arabidopsis*, yeast, *and Coprinus congregatus*. The amino acid sequence of *O. radicata* RNase is compared with ubiquitins from humans, *Arabidopsis*, yeast, and *C. congregatus*. Identical residues (shown in gray) are indicated.

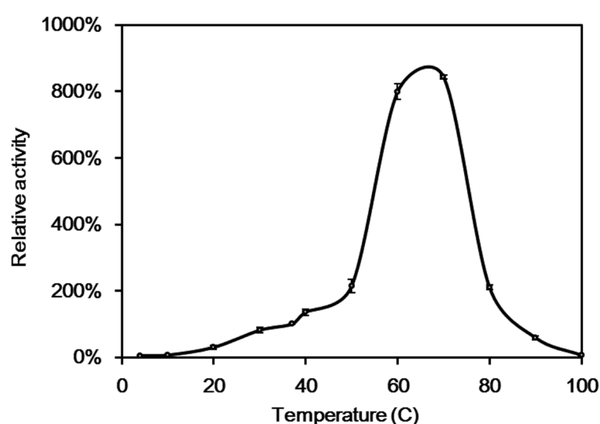

FIG. 5: Effect of temperature on *Oudemansiella radicata* RNase activity. The activity at 37°C was taken as 100%. Results represent means ± SD (n=3). Relative activity at 50°C does not show a statistically significant difference compared with relative activity at 80°C. Each of the other data points is significantly different (p<0.05) from data at other temperatures when analyzed by analysis of variance.

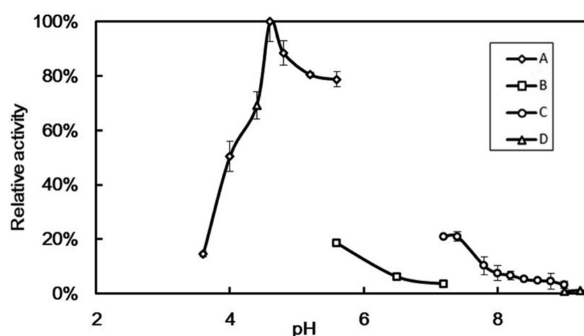

FIG. 6: Effect of pH on *Oudemansiella radicata* RNase activity. (A) HAc-NaAc buffer. (B) Na_2HPO_4-NaH_2PO_4 buffer. (C) Tris-HCl buffer. (D) NH_4HCO_3 buffer. The activity at pH 4.6 was taken as 100%. Results represent means ± SD (n=3). In curve A, data at pH 5 do not show a statistically significant difference compared with data at pH 5.5. In curve C, data at pH 7 do not show a statistically significant difference compared with data at pH 7.25, and data in the pH range 8 to 9 do not show a statistically significant difference from one another. In curve D, all data do not show a statistically significant difference from one another. Otherwise, data on the same curve other than the aforementioned data are significantly different from one another when analyzed by analysis of variance.

IV. DISCUSSION

The present investigation disclosed the production of a RNase, demonstrating inner peptide sequence homology to ubiquitin (Fig. 7), from *O. radicata* fruiting bodies. Ubiquitin from mushrooms displays extensive amino acid sequence resemblance to humans, *Arabidopsis*, and yeast ubiquitins.[26] Three ion exchange steps were utilized in the purification protocol herein, which differed somewhat from the published procedure. A purification protocol comprising sequential anion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, FPLC-cation exchange chromatography on Mono S, and FPLC-gel filtration on Superdex 75 has previously been employed for the isolation of mushroom ubiquitin-like peptides. Ubiquitin-like peptides from the mushrooms *Agrocybe cylindracea*,[27] *Calvatia caelata*,[28] *P. sajor-caju*,[29] *P. ostreatus*,[30] and *Termitomyces globulus*[31] were unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and Mono S. However, *O. radicata* ubiquitin-like peptide resembled only its counterpart from *Cantharellus cibarius*[32] in adsorption on DEAE-cellulose and CM-cellulose.

TABLE 2: Effects of Metal Cations on RNase Activity of *Oudemansiella radicata* RNase

Metal Ions	RNase Activity Remaining (%)		
	2.5 mM	5 mM	10 mM
None	100	100	100
Al^{3+}	0	0	0
Cd^{2+}	41.50	27.91	16.51
Ca^{2+}	94.60	59.31	37.53
Pb^{2+}	93.17	82.95	77.44
Zn^{2+}	58.59	34.24	20.03
Hg^{2+}	38.00	14.06	12.25
Fe^{2+}	109.93	88.04	62.89
Cu^{2+}	79.74	42.44	17.85
Mg^{2+}	91.14	57.10	33.59
Mn^{2+}	81.47	69.16	64.29
K^{+}	165.45	205.92	112.96

Although the molecular mass of *O. radicata* ubiquitin-like RNase resembled those of *P. ostreatus*[30]

```
ORR        1    MQIFVKTLS................................................DYN
                ||||||||.                                                |||
Ubiqutin   1    MQIFVKTLTGKTITLEVEPSDSIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN

ORR        13   IQKESTLHLVLR........................IQDKEGIPPDQQ............
                ||||||||||::                        ||||||||||||
Ubiqutin   61   IQKESTLHLIFVKTLTGKTITLEVEPSDSIENVKAKIQDKEGIPPDQQRLIFAGKQLEDG

ORR        37   RTLSDYNIQKESTLHLVLRL........................................
                ||||||||||||||||||||
Ubiqutin   121  RTLSDYNIQKESTLHLVLRLRGGMQIFVKTLTGKTITLEVEPSDSIENVKAKIQDKEGIP

ORR        57   ......IFAGKQLEDGR.......................
                      |||||||||||
Ubiqutin   181  PDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGN
```

FIG. 7: Alignment of the amino acid sequences of *Oudemansiella radicata* RNase and ubiquitin. The amino acid sequence of *O. radicata* RNase (ORR) is compared with ubiquitin. Identical residues and similar residues are indicated by a vertical bar and a dot, respectively.

and *T. globulus* ubiquitin-like RNases,[31] it appeared to be larger than those of its counterparts from *P. sajor-caju*,[29] *A. cylindracea*,[27] *C. caelata*,[28] and *C. cibarius*.[32]

O. radicata ubiquitin-like RNase shows more pronounced thermostability than *Thelephora ganbajun* RNase.[33] Its activity reached a maximum at approximately 70°C, close to the optimum temperatures for *Lactarius flavidulus*, *Hypsizygus marmoreus*, and *L. shimeiji* RNase.[11] Approximately 60% of its RNase activity remains after exposure to 90°C for 30 min, signifying that it is a thermostable protein. Similar to *O. radicata* ubiquitin-like RNase, *T. globulus* and *C. cibarius* ubiquitin-like peptides required a temperature of 70°C for expression of maximal activity. The RNase activity of *A. cylindracea* ubiquitin-like peptide was stable over the temperature range of 0°C–60°C.

A pH of 4.6 is optimal for the activity of *O. radicata* RNase, *P. djamor* RNase, and *Agaricus bisporus* RNase.[17] The optimal pH is found to be 3.5 for *B. griseus* RNase,[15] 6.5–7.0 for *Clitocybe maxima* RNase, 6.5 for *P. tuber-regium*, and *P. eryngii* RNase[34] and 8.0 for *P. ostreatus*.[35] *T. globulus* ubiquitin-like peptide expressed nearly the same optimal activity over a wide pH range of 5.0–8.0. Maximal activity of *A. cylindracea* and *C. cibarius* ubiquitin-like peptide toward yeast tRNA was observed at a pH of 6 and 7.[32] Thus it appears that *O. radicata* RNase is dissimilar from the majority of mushroom RNases and ubiquitin-like peptides in optimum pH.

The activity of *O. radicata* RNase is strongly inhibited by Al^{3+}, Hg^{2+}, Cd^{2+}, and Zn^{2+} ions. The ribonucleolytic activity of *P. tuber-regium* and *P. djamor* RNases is also inhibited by Hg^{2+} and Zn^{2+}.[17] *P. sajor-caju* RNase[36] resembles *P. djamor* RNase in that KCl has little effect on the RNase activity, but the activity of *O. radicata* RNase was augmented in the presence of K^{+}.

O. radicata RNase exerted ribonucleolytic activity preferentially on poly (C), lesser activity on poly (G), and indiscernible activity on poly (U) and poly (A). Hence, *O. radicata* RNase differed in this aspect from other mushroom RNases including ubiquitin-like peptides. In fact, different RNases including ubiquitin-like peptides may evince distinct polyhomoribonucleotide specificities. *A. cylindracea* ubiquitin-like peptide exhibited ribonucleolytic activity selectively on poly (C), residual activity on poly (U), and virtually undetectable activity on poly (A) and poly (G).[27] *C. cibarius* ubiquitin-like peptide manifested higher ribonucleolytic activity toward poly (A) and poly (C) and lower activity toward poly (G) and poly (U).[32] *T. globulus* ubiquitin-like peptide demonstrated much higher ribonucleolytic activity toward poly (A) and poly (C) than that toward poly (G) and poly (U).[31] RNase from *Russulus virescens*[37] manifested cospecificity for poly (A) and poly (C). *A. bisporus* RNase exhibited ribonucleolytic activity toward poly (A), poly (C), and poly (U).[15]

The changes in expression level of ubiquitin-like proteins in different parts and at different developmental stages of the mushrooms have been reported. A 27.8-kDa ubiquitin-immunoreactive protein, with an isoelectric point of 4.4 and mainly present in the cap of young basidiocarp, was purified from the mushroom *C. cinereus*.[38] During basidiocarp primordium formation, ubiquitin and six ubiquitin-immunoreactive proteins with molecular mass ranging from 15.4 to 30.9 kDa (15.4, 22.5, 26.3, 27.8, 28.6, and 30.9 kDa) increased and during basidiocarp maturation they declined in the cap and upper stipe, but became elevated in level in the lower stipe with the exception of the 27.8-kDa protein and ubiquitin. During sporulation, the levels of ubiquitin and all the ubiquitin-immunoreactive proteins underwent a decline in the cap of the young wild-type basidiocarp. The levels of 15.4- and 30.9- kDa proteins rose temporarily at 6–10 h following the commencement of the last light period, while the ubiquitin level fell notably. No correlation was observed between alterations in levels of the ubiquitin-immunoreactive proteins and the blocked stages in sporulation-deficient mutants.[39] Thus ubiquitin and ubiquitin-immunoreactive proteins play important roles in mushroom development.

Ubiquitin-conjugated proteins are targeted for breakdown by 26S proteasome. The ubiquitin-mediated pathway plays a pivotal regulatory role in endocytosis, receptor downregulation, cell-cycle progression, apoptosis, and development. Defects in ubiquitin-mediated events may be associated with pathological development such as malignant transformation.[40,41] The ubiquitin-like RNases may play similar roles in mushrooms.

The mechanism of ubiquitin-like proteins has recently been disclosed. The bulk of E3 ligases employ a RING domain to activate a thioester-linked E2~ubiquitin-like protein (UBL) intermediate and enhance the transfer of UBL to a distantly bound target protein. Scott et al.[42] reported the structure of a trapped RING E3-E2~UBL-target intermediate representing RBX1-UBC12~NEDD8-CUL1-DCN1, which unravels the mechanism of neural precursor cell-expressed developmentally downregulated 8 (NEDD8) ligation and the way in which a particular UBL and acceptor lysine are matched by a multifunctional RING E3. A diversity of mechanisms specify cullin NEDDylation while inhibiting noncognate ubiquitin ligation. E2-E3-target and RING-E2~UBL modules are not optimized to operate independently, but integration by the UBL and target is needed for maximal reactivity. The UBL and target regulate the catalytic machinery by positioning the RING-E2~UBL catalytic center, licensing the acceptor lysine, and affecting E2 reactivity, hence driving their specific coupling by a multifunctional RING E3.[42]

Among the nine classes of UBLs, NEDD8 has a pivotal role in cell progression and signal transduction. Akin to the ubiquitin pathway, NEDD8 is firstly activated by NEDD8 activating enzyme (NAE) in an ATP-dependent reaction and then transferred to Ubc12, the NEDD8 conjugating enzyme. Eventually, with the coordinated action of Ubc12 and SCCRO (DCN1), the scaffold-type E3 ligase for cullin NEDDylation, NEDD8 is attached to a conserved lysine in the proximity of the C-terminal end of the cullin protein. The NEDDylation of cullin proteins is essential to activate cullin-RING ligases, a subclass of ubiquitin E3 ligases which regulates breakdown and turnover of cancer-related proteins Cdt1 and IκBα. The level of NEDDylation is markedly elevated in cancer cells. Thus targeting the NEDD8 pathway, especially NAE, is highly promising for oncotherapy. Among the small number of available NAE inhibitors, the covalent inhibitors have the highest potency. The most potent compound LZ3 displayed an IC_{50} value of 1.06 ± 0.18 μM.[43]

The viability of breast cancer cells was reduced to half at a *C. caelata* ubiquitin concentration of about 100 nM.[28] *A. cylindracea* ubiquitin-like peptide demonstrated antiproliferative activity on leukemia and hepatoma cell lines, and enhanced nitric oxide production in murine peritoneal macrophages with a potency similar to that of lipopolysaccharide.[27] *P. ostreatus* UBL expressed an inhibitory activity toward human immunodeficiency virus-1 reverse transcriptase, which could be enhanced by succinylation.[30] Hence, it is highly likely that the UBL isolated in the present study exhibits similar health-promoting effects.

V. CONCLUSIONS

An RNase with some novel characteristics, including a distinctive amino acid sequence, a high pH optimum, retention of activity at high temperatures, and susceptibility to inhibition by some metallic chlorides and potentiation by other metallic chlorides, was isolated from *O. radicata* fruiting bodies. This report represents an addition to the existing list of mushroom RNases and ubiquitin-like peptides and broadens the application prospects of *O. radicata*.

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Lion's Mane, *Hericium erinaceus* and Tiger Milk, *Lignosus rhinocerotis* (Higher Basidiomycetes) Medicinal Mushrooms Stimulate Neurite Outgrowth in Dissociated Cells of Brain, Spinal Cord, and Retina: An *In Vitro* Study

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ABSTRACT: Neurodegenerative disease is defined as a deterioration of the nervous system in the intellectual and cognitive capabilities. Statistics show that more than 80–90 million individuals age 65 and above in 2050 may be affected by neurodegenerative conditions like Alzheimer's and Parkinson's disease. Studies have shown that out of 2000 different types of edible and/or medicinal mushrooms, only a few countable mushrooms have been selected until now for neurohealth activity. *Hericium erinaceus* is one of the well-established medicinal mushrooms for neuronal health. It has been documented for its regenerative capability in peripheral nerve. Another mushroom used as traditional medicine is *Lignosus rhinocerotis*, which has been used for various illnesses. It has been documented for its neurite outgrowth potential in PC12 cells. Based on the regenerative capabilities of both the mushrooms, priority was given to select them for our study. The aim of this study was to investigate the potential of *H. erinaceus* and *L. rhinocerotis* to stimulate neurite outgrowth in dissociated cells of brain, spinal cord, and retina from chick embryo when compared to brain derived neurotrophic factor (BDNF). Neurite outgrowth activity was confirmed by the immunofluorescence method in all tissue samples. Treatment with different concentrations of extracts resulted in neuronal differentiation and neuronal elongation. *H. erinaceus* extract at 50 µg/mL triggered neurite outgrowth at 20.47%, 22.47%, and 21.70% in brain, spinal cord, and retinal cells. *L. rhinocerotis* sclerotium extract at 50 µg/mL induced maximum neurite outgrowth of 20.77% and 24.73% in brain and spinal cord, whereas 20.77% of neurite outgrowth was observed in retinal cells at 25µg/mL, respectively.

KEY WORDS: medicinal mushroom, *Lignosus rhinocerotis*, *Hericium erinaceus*, neurite outgrowth activity, neurodegenerative disease

ABBREVIATIONS: BDNF, brain derived neurotrophic factor; **DAPI,** 4′,6-diamidino-2-phenylindole; **DMEM,** Dulbecco's modified Eagle's medium; **FITC,** fluorescein isothiocyanate; **PBS**, phosphate-buffered saline.

I. INTRODUCTION

Aging is an inevitable process and the fraction of world's population over 65 years of age is expected to increase to 80–90 million by the end of 2050.[1] Out of many diseases that threaten aging humans, neurodegenerative diseases such as Alzheimer's disease, dementia, and Parkinson's disease[2] and retinal diseases like diabetic retinopathy, glaucoma, and age-related macular degeneration can be very traumatic.[3] Neurohealth is a major concern as one ages. Retrospective studies have proved that natural products like mushrooms are suitable candidates for neurohealth.[4] Out of 14,000 species of familiar mushrooms,[4] roughly 2000[4] are acknowledged to be fit for human consumption. Researchers have reported that

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many medicinal mushrooms promote neurotrophic properties such as neurite outgrowth stimulation,[5,6] nerve regeneration,[7] neuroprotection,[8] and antioxidation.[9] Mushrooms may have potential in the prevention or treatment of age-related neurodegenerative complaints.[10]

The mushrooms selected for this study were *Hericium erinaceus* (Bull.:Fr.)Pers. (Hericiaceae, higher Basidiomycetes) and *Lignosus rhinocerotis* (Cooke) Rivarden (Polyporaceae, higher Basidiomycetes). *H. erinaceus*, a rare mushroom also known as lion's mane, monkey's head, and Yamabu shitake, is scattered throughout North America, Europe, and Asia.[11,12] It has been used in Chinese and Japanese cuisine and as an herbal remedy to treat various human diseases including gastric ulcers for hundreds of years.[12] A health syrup called "Houtou" is prepared from dried fruit bodies. *H. erinaceus* tablets are used to treat ulcers, inflammation, and tumors of the alimentary canal.[13] It is also evident that *H. erinaceus* has facilitated functional recovery subsequent to peripheral nerve injury.[7] Based on this study, our focus is on regeneration of the central nervous system (CNS) using brain, spinal cord, and retinal explants and cells after dissociation, using chick embryo as a model.

L. rhinocerotis is also known as tiger's milk mushroom or "cendawan susu rimau" in the local language. This species is distributed only in the tropical rainforest in regions of South China, Thailand, Indonesia, Malaysia, Philippines, and Papua New Guinea. *L. rhinocerotis* has been used as a general tonic, antipyretic, and antipruritic; it has also been used to treat fever, cancer, food poisoning, swollen breasts, cough, and asthma and to assist in wound healing, among other uses.[14] Its usage is limited in spite of its medicinal properties due to unavailability. Sclerotium of *L. rhinocerotis* documented enhancement of neurite outgrowth activity in PC12 cells.[15] Based on their potential, in this study, both mushrooms with different concentrations (25–100 μg/mL) were treated with tissue samples and compared with negative and positive controls. Neurite extensions were confirmed by immunofluorescence staining.

II. MATERIALS AND METHODS

A. Preparation of Mushroom Aqueous Extracts

Mushrooms require the correct combination of humidity, temperature, substrate (growth medium), and inoculum (spawn) to grow. In Malaysia, *H. erinaceus* is cultivated on a substrate containing rubberwood sawdust, rice bran, and calcium carbonate at a ratio of 100:5:1. After 2 months of spawn run at 27±2 to 32±2°C at a mushroom farm (Ganofarm Ltd., Tanjung Sepat, Selangor, Malaysia), approximately 300 g of fresh fruit body per 800 g of substrate was harvested.[16] Fresh fruiting bodies of *H. erinaceus* were purchased from the mushroom farm. Fresh fruit bodies were sliced, frozen, and freeze-dried. The freeze-dried fruit bodies were then blended in a Waring commercial blender and stored in airtight containers at 4°C prior to assay.[16] *L. rhinocerotis*, a rare species, is found in the forests of Malaysia. In this study, the freeze-dried powder of sclerotia of cultivated *L. rhinocerotis*[10,17] was purchased from Ligno Biotek Sdn Bhd (batch no. TM02). The freeze-dried powders of both mushrooms were then soaked separately in distilled water (1:20, w/v) and were agitated at 150 rpm for 24 h.[15] The mixture was then double boiled in a water bath at 100°C for 30 min, cooled, and filtered by Whatman filter paper No. 4. The aqueous extract was freeze-dried and kept at –20°C prior to use.[6,18]

B. Preparation of Explants Culture and Trypsinization

Fertilized chicken eggs were collected from Charoen Pokphand Jaya Farm (M) Sdn Bhd (Negeri Sembilan, Malaysia) and then were incubated at 39°C in a humidified incubator. The brain (day 4), spinal cord (day 6), and retina (day 9) were dissected on their respective days.[19] Based on a modified method of Gibco Life Technologies (Selangor, Malaysia), the tissue samples (brain, spinal cord, and retina) were finely chopped, washed with phosphate-buffered saline (PBS) twice, and centrifuged at 3000 rpm for 3 min after adding trypsin. The supernatant

was discarded and the cell pellet was resuspended twice with 2–5 mL of prewarmed (37°C) complete media. The samples were centrifuged at 3000 rpm for 3–5 min. The supernatant was discarded and the cell pellet was added to fresh complete media and incubated at 37±2°C in a 5% CO_2 humidified incubator for 24 h.

C. Neurite Outgrowth Assay

Two-day-old cultured cells were seeded into 12-well plates at a cell density of $5×10^4$ cells per well. The mushroom aqueous extracts in Dulbecco's modified Eagle's medium (DMEM) at concentrations of 25, 50, 75, and 100 μg/mL (w/v), and brain derived neurotrophic factor (BDNF) at 10 ng/mL (w/v) were tested for neurite outgrowth stimulation activity. Cells in complete DMEM without treatment served as the negative control. Plates were incubated at 37±2°C in a 5% CO_2 incubator for 2 days.[15]

D. Scoring of Neurites

Neurite extensions were scored under an inverted microscope (Nikon Eclipse TS100) with the aid of a handheld counter. A cell was scored positive for bearing neurites if it had at least one thin extension longer than the diameter of its cell body.[20] In a well, 10 fields with an average of randomly chosen 250–300 cells per well were examined and photographed using a Nikon DS-Fi1 camera and were processed with Nikon's NIS-Elements D imaging software.[6]

E. Neurofilament Staining

A neurofilament was used as an indicator for neurite outgrowth and immunofluorescence staining was used for confirming neuronal extension, which is an increase in axonal length. Based on the standard method,[21] primary neuronal cells were seeded in 12-well plates and exposed to treatment for 2 days. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After two washes with PBS, the cells were incubated with primary antibody, antineurofilament 200 antibody produced in rabbits (Sigma, St. Louis, MO, USA) (1:80 dilution in blocking buffer) for 1 h. The cells were washed and then incubated with secondary antibody, anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibody produced in sheep (Sigma, St. Louis, MO, USA) (1:80 dilution in blocking buffer) for 1 h in the dark at room temperature. The cells were then washed thrice. The coverslips were then mounted with 4′,6-diamidino-2-phenylindole (DAPI), which stained the nucleus. Images were observed and captured with a fluorescent microscope (Nikon Eclipse 80i microscope using FITC and DAPI filters).

F. Statistical Analysis

All experiments were carried out in three replicates. Results were expressed as the means ± SD. All data were subjected to analysis of variance using GraphPad Prism Statistical Software version 7 (GraphPad Software Inc., La Jolla, CA, USA). The differences among samples were evaluated using Duncan's multiple range test, where $p<0.05$ was considered significant.

III. RESULTS AND DISCUSSION

Neurons in the mature CNS are unable to regenerate injured axons and the neurons that remain uninjured are unable to form novel connections that might compensate for ones that have been lost.[22] Subsequently, due to a break in the communication between healthy neurons, a cascade of events takes place that leads to neuronal degeneration and cell death. The factors responsible for failure of regeneration are several and include poor regenerative ability of CNS neurons, inhibitory properties of astrocytes,[23] and inhibitory molecules produced by oligodendrocytes and myelin.[24] Overpowering these issues will facilitate the nerve regeneration for restoration of function following damage, through accident, injury, or neurodegenerative disease.

Natural products have been traditionally accepted as remedies due to the popular belief that they present minor side effects.[25] In traditional Chinese medicine, mushrooms have always been prepared for medicinal use by hot water extraction. The number of mushrooms, however, studied for

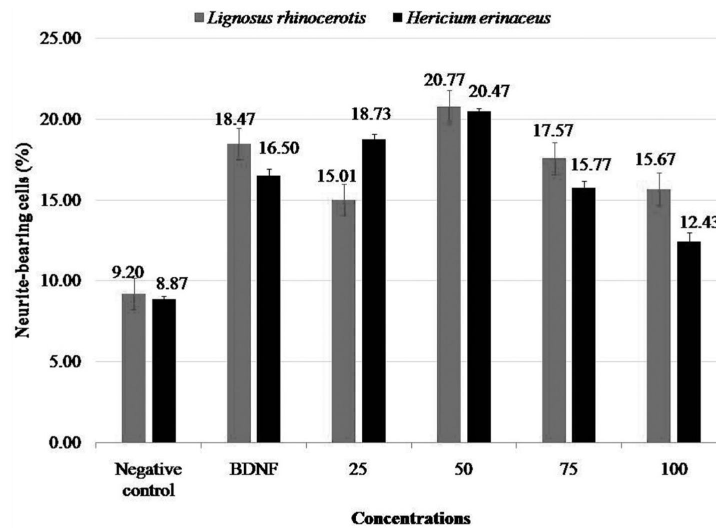

FIG. 1: Effect of varying concentrations of *H. erinaceus* and *Lignosus rhinocerotis* aqueous extracts on neurite outgrowth stimulation on chick embryo's brain cells.

neurohealth activity are few and *H. erinaceus*[10] is one of them. Its chemical composition was documented by Kawagishi et al.[5] Further study on aqueous extracts proved that polysaccharides could induce neuronal differentiation and promote neuronal survival.[26] Ongoing research in our laboratory shows that *H. erinaceus*,[6] *L. rhinocerotis*,[15,27] and *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde (morning glory mushroom, cow's stomach mushroom)[28] exhibit neurite outgrowth stimulatory effects in NG108-15 and PC12 cell lines. Inspired by this, researchers focused their goal on peripheral nerve regeneration following crush injury to the rat peroneal nerve by using aqueous extract of *H. erinaceus*. This study proved that daily administration of aqueous extract of *H. erinaceus* has beneficial effects on recovery of the injured rat peroneal nerve in early stages of regeneration.[7]

Another medicinal mushroom is *L. rhinocerotis.* Retrospective studies have shown that an aqueous extract of *L. rhinocerotis* sclerotium induced neurite outgrowths of 24.4% and 42.1% at 20 μg/mL (w/v) of aqueous extract alone and a combination of 20 μg/mL (w/v) aqueous extract and 30 ng/mL (w/v) of NGF, respectively, in rat pheochromocytoma cells (PC12 cells).[15] Similarly, the present study showed neurite outgrowth of 20.77% and 24.73% at 50 μg/mL in brain and spinal cord cells and 20.77% at 25 μg/mL in retinal cells. Consistent with our previous study, the sclerotia of *L. rhinocerotis* reported neurite outgrowth in N2a.[29] It was recently shown that sclerotial extract performs better than mycelial extract.[29] Advanced study shows maximum neurite extension for *L. rhinocerotis* and curcumin at 21.1% at 20 μg/mL and 29.47% at 10 μg/mL on PC-12 cells.[30] Combining 20 μg/mL of *L. rhinocerotis* with 1 μg/mL curcumin gave 27.2% neurite extension in PC12 cells.[30] Taken as a whole, these medicinal mushrooms have shown neurological properties such as neuronal survival and neurite outgrowth activities including improvement in recovery and function in both *in vivo* and *in vitro* mammalian nervous systems.[31]

The aqueous extracts of *H. erinaceus* and *L. rhinocerotis* showed a gradual dose-dependent twofold increase in neurite outgrowth stimulation at 25 and 50 μg/mL concentration compared to the negative control. Figure 1 shows the neurite outgrowth stimulation on brain cells after 48 h incubation decreased the extension in a dose-dependent manner observed at 75 and 100 μg/mL concentrations. The maximal stimulated outgrowth on brain cells treated with aqueous extracts of *H. erinaceus* and *L. rhinocerotis* was 20.47% and 20.77%, respectively, at 50 μg/mL, comparable to that of the BDNF-treated cells (positive control), whereas *H. erinaceus* showed significant ($p<0.05$) neurite outgrowth of 18.73%

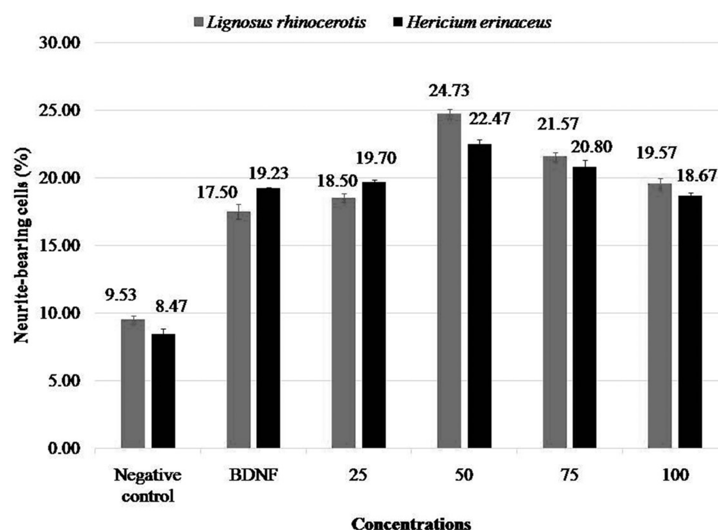

FIG. 2: Effects of varying concentrations of *H. erinaceus* and *Lingnosus rhinocerotis* aqueous extract on *in vitro* neurite outgrowth stimulation on chick embryo's spinal cells.

at a concentration of 25 µg/mL. *H. erinaceus* and *L. rhinocerotis* aqueous extracts showed significant ($p<0.05$) neurite outgrowth of 22.47% and 24.73%, respectively, at 50 µg/mL on the spinal cord (Fig. 2). The aqueous extract of *L. rhinocerotis* showed significant ($p<0.05$) neurite outgrowth (20.77%) at 25 µg/mL on retinal cells; in contrast, the aqueous extract of *H. erinaceus* (21.70%) exerted its maximum neurite growth at 50 µg/mL (Fig. 3). Comparing both of the aqueous extracts, *H. erinaceus* exerted a significantly potent neurite outgrowth on retinal cells at a lower concentration compared to *L. rhinocerotis* aqueous extract. Retinal cell stimulation was significant ($p<0.05$) at 50 µg/mL by *H. erinaceus*. Neurite outgrowth is confirmed by neurofilament staining as shown in Fig. 4. Figures 4A, D, and G show BDNF at the concentration of 10 ng/mL used as a positive control in the brain, spinal cord, and retina. Figures 4B, E, and H show the negative control in all three

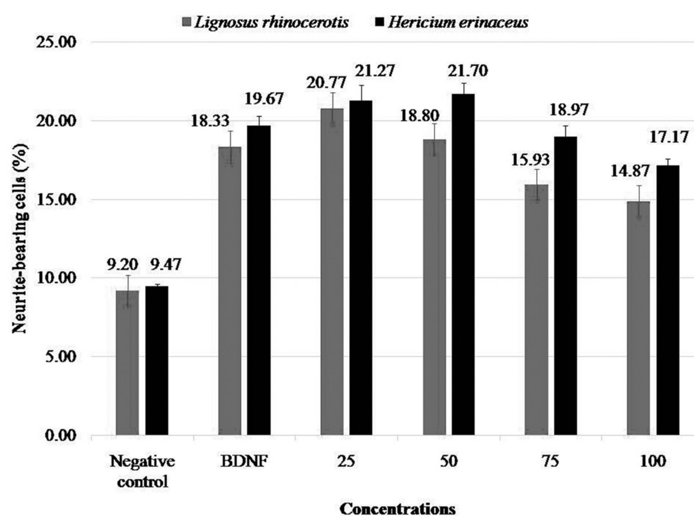

FIG. 3: Effects of varying concentrations of *H. erinaceus* and *Lignosus rhinocerotis* aqueous extract on *in vitro* neurite outgrowth stimulation on chick embryo's retina cells.

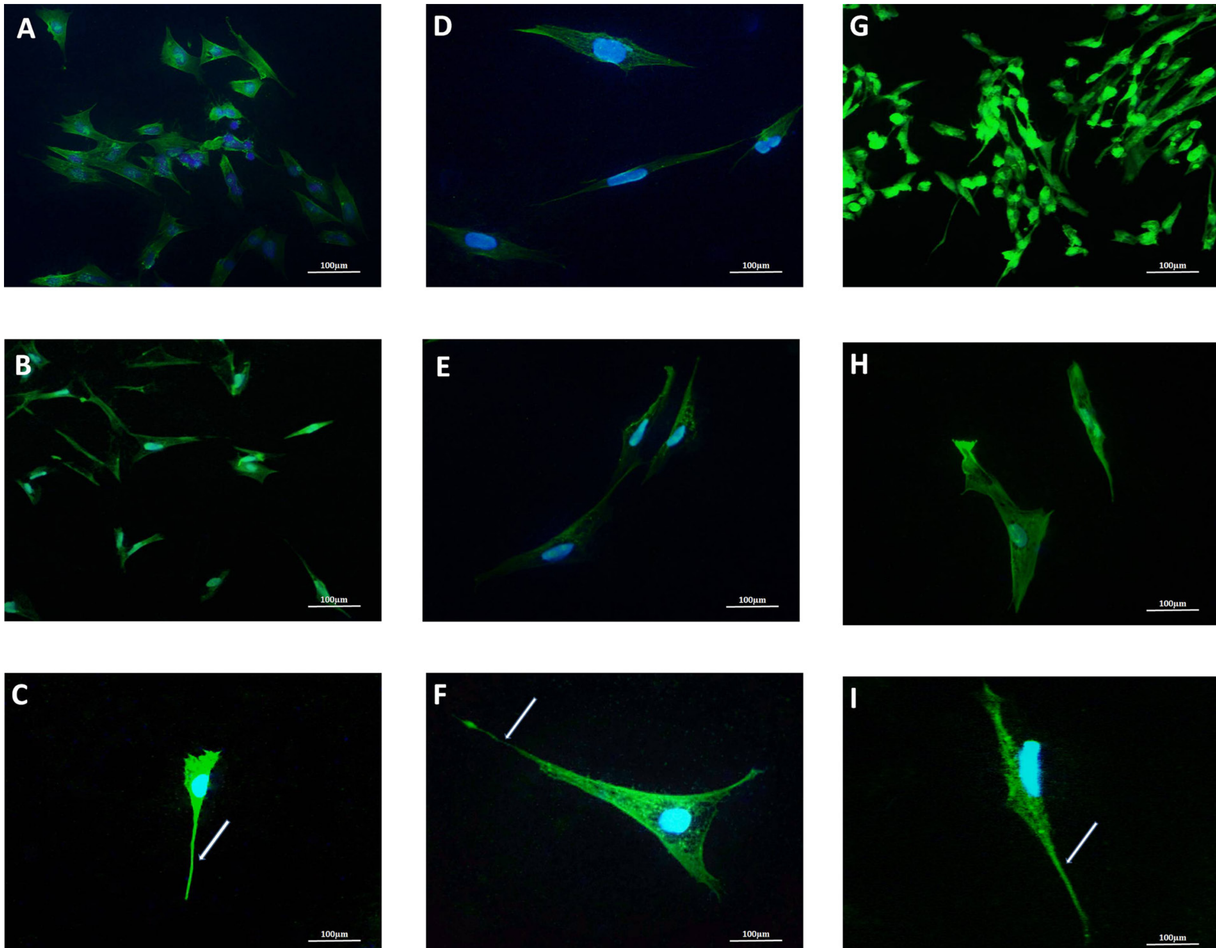

FIG. 4: Fluorescent microscopy image of *in vitro* neurite outgrowth in chicken embryo's brain, spinal cord. and retinal cells by *Lignosus rhinocerotis* aqueous extracts. Brain and spinal cord cells were treated with hot aqueous extract *L. rhinocerotis* at a concentration of 50 and 25 μg/mL for retinal cells after 48 h of incubation at 37±2°C in a 5% CO_2 humidified incubator. (A) Brain positive control: BDNF(10 ng/mL). (B) Negative control of brain cells without extract. (C) Brain cells treated with hot aqueous extract of *L. rhinocerotis* at 50 μg/mL. (D) Spinal cord positive control: BDNF (10 ng/mL). (E) Negative control of spinal cord cells without extract. (F) Spinal cord cells treated with hot extract of *L. rhinocerotis* at 50 μg/mL. (G) Retina positive control: BDNF (10 ng/mL). (H) Negative control of retinal cells without extract. (I) Retinal cells treated with hot aqueous extract of *L. rhinocerotis* at 25 μg/mL. (C), (F), and (I) Cells show an exuberant long neurite outgrowth (arrow) as compared to (A) (D), and (G).

samples, which have either no neurite, or neurite with insufficient length to be scored as positive. Figures 4C, F, and I show neurite outgrowth in the brain, spinal cord, and retinal cells confirmed by neurofilament staining. Neurite extension is marked by an arrow. Recent research suggested that these neurofilaments are closely related to many neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's diseae, and Alzheimer's disease. Using *in vitro* assays, cultures, and transgenic mice, these studies provided new insights into neurofilament function. The function of each subunit, the relationship of neurofilaments with other cytoskeletal elements and their clinical significance are topics of increasing attention.

IV. CONCLUSIONS

H. erinaceus and *L. rhinocerotis* aqueous extracts were examined for neurite outgrowth activity in

the brain, spinal cord, and retinal cells of chicken embryo. Four different concentrations (25, 50, 75, and 100 µg/mL) were tested. Among the concentrations, *H. erinaceus* aqueous extract, 50 µg/mL, stimulated neurite outgrowth in brain and spinal cord cells. *L. rhinocerotis* sclerotium extract showed similar neurite outgrowth activity in retinal cells at 25 µg/mL after 48 h of incubation. Immunofluorescence staining confirmed neurite outgrowth.

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Anti-Gastric Ulcer Activity of Polysaccharide Fraction Isolated from Mycelium Culture of Lion's Mane Medicinal Mushroom, *Hericium erinaceus* (Higher Basidiomycetes)

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ABSTRACT: *Hericium erinaceus* is a culinary-medicinal mushroom that is used in traditional medicine, in folk medicine, and as medicinal cuisine in Asian countries such as China, Japan, and Korea. *H. erinaceus* exhibits various pharmacological properties, such as anti-cancer, immunomodulation, anti-dementia, and anti-gastric ulcer effects. The extracts of the fruiting body of *H. erinaceus* demonstrate anti-gastritis activity. However, the active principle in the extract, as well as the mechanism to treat gastric ulcers, remains uncertain. The current study aims to identify the active component, with anti-gastric ulcer function, from the extracts of the *H. erinaceus* mycelium culture. In the experiment, anti-gastric ulcer activity was evaluated using an ethanol-induced ulcer model in mice and with an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay using MC cell lines. The results suggest that the polysaccharide fraction can significantly decrease the ulcerated area compared with the control group and the effect is fairly dose dependent, irrespective of animal or cell experiments. These results indicate that the polysaccharide fraction is the active component of the *H. erinaceus* mycelium culture, which protects against gastric ulcers.

KEY WORDS: medicinal mushrooms, anti-gastric ulcer activity, polysaccharide, *Hericium erinaceus*

ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; **EP,** polysaccharide fraction; **ES,** ethanol supernatant fraction; **GES-1,** human gastric epithelial cell line; **I%,** inhibition percentage; **MC,** GES-1 cells induced by MNNG; **MNNG,** N-methyl-N′ nitro-N-nitrosoguanidine nitroguanidine; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; **OD,** optical density; **UA,** ulcer area; **WP,** water supernatant fraction; **WS,** water precipitate fraction.

I. INTRODUCTION

Lion's mane mushroom, *Hericium erinaceus* (Bull.:Fr.) Pers. (Hericiaceae, higher Basidiomycetes), is a medicinal mushroom that is used in traditional medicine, in folk medicine, and as medicinal cuisine in Asian countries such as China, Japan, and Korea. *H. erinaceus* exhibits various pharmacological properties, such as anti-cancer, immunomodulation, anti-dementia, and anti-gastric ulcer effects.[1] A large number of effects have been attributed to *H. erinaceus*, of which, anti-gastritis is a predominant function and hence, many studies have examined the anti-gastric ulcer effect using the extracts of *H. erinaceus* that include the fruiting body and the mycelium culture.[2,3]

It is known that *H. erinaceus* contains a myriad of components, such as polysaccharides, alkaloids, amino acids, and phenolics,[4] but there has been a paucity of information on the active component that governs the anti-ulcer function. The culturing technology of *H. erinaceus* mycelium is currently well developed, and the mycelium extract is supplied as the target resource for developing drugs and functional foods. "Houtoujun Pian" is one example of a traditional drug in China, made from the mycelium

extract of *H. erinaceus*, which is used to treat gastric ulcers.[5] However, the mycelium extract of *H. erinaceus* has not yet been analyzed for its active component that exhibits the anti-gastric ulcer effect.

Therefore, in the present study, the anti-ulcer activity of cultured *H. erinaceus* mycelium extract was analyzed in different fractions. Activity of the different fractions was analyzed using an ethanol-induced gastric ulcer model in mice. A cell-based 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay of the anti-ulcer activity was also performed using an MC cell line.

II. MATERIALS AND METHODS

A. Materials

The cultured *H. erinaceus* mycelium was obtained from Hangzhou Johncan Mushroom Bio-Technology Co., Ltd. (Zhejiang, China) and complied with the National Drug Standards No. HI4023098. Standard monosaccharides were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Houtoujun Pian, used as a positive control for the anti-gastric ulcer experiment, was gifted by the pharmaceutical company of Jilin University. The other chemicals and reagents were of analytical reagent grade and were obtained from local sources.

B. Extraction and Fractionation of the *H. erinaceus* Mycelium Culture

The cultured *H. erinaceus* mycelium was extracted with 5 volumes of water at 50°C with gentle stirring for 12 h, and the extract was then centrifuged (3000 rpm, 5 min) to obtain the supernatant (WS) and the precipitate (WP) fractions. The precipitate was re-extracted twice with water, as described above, and the supernatants were combined and concentrated under reduced pressure. Then 3 volumes of ethanol were added to 1 volume of WS and maintained for 12 h at 70°C, followed by centrifugation to obtain ethanol-soluble and ethanol-insoluble precipitate fractions. The ethanol-soluble fraction was evaporated to dryness and named as the ES fraction. The ethanol precipitate was washed successively with ethanol and acetone and then freeze-dried to obtain the EP sample. The WS, WP, ES, and EP samples thus obtained were subjected to the animal and cellular anti-ulcer activity evaluations.

C. Mouse Model for Assessing the Anti-Ulcer Effect of *H. erinaceus*

Forty mice (18–22 g, each group included 5 males and 5 females) were obtained from Jilin University College of Pharmacy, with a certificate of conformity for the Chinese ShengChangXuKe (SCXK; 2012-0003). The mice were randomly divided into the following 4 groups: control group, high dosage group, low dosage group, and positive control group. Each group was composed of 10 mice. The low dosage and high dosage *H. erinaceus* groups were administered 1.2 and 2.5 g/kg of *H. erinaceus* mycelium extract, respectively, directly into the stomach by oral gavage, once a day for 5 days. The positive control group was administered 6 g/kg of Wei Lexin by oral gavage. The control mice were administered the same volume of distilled water. On the fifth day of feeding, the mice were deprived of food but allowed free access to bottled tap water for 24 h after the last dose and were then orally treated with 0.1 mL/20 g of anhydrous ethanol to induce gastric ulcers. The animals were sacrificed 1 h later under anesthetized diethyl ether and their stomachs were quickly removed for further studies. The stomach of each experimental animal was opened along the large curvature and rinsed with distilled water to remove the gastric contents. The gastric ulcers appeared as elongated bands on the gastric mucosa with hemorrhagic lesions being parallel to the long axis of the stomach.[6] The mucosa was assessed for damage under a dissecting microscope (1.8×), and a planimeter was used to measure the area of ulceration (hemorrhagic lesions). The length and the width of each lesion were measured, and the sum of the area of all the lesions for each stomach was expressed as the ulcer area (mm^2; UA). The ulcer area (UA) was measured, and the inhibition percentage (I%) was calculated by the following equation[7]:

$$(I\%) = [(UA_{control} - UA_{treated})/UA_{control}] \times 100.$$

D. Cell Model for Evaluating the Anti-Ulcer Activity of *H. erinaceus*

The anti-ulcer effect of *H. erinaceus* was evaluated using a cell model (MC cell lines). The cell line was provided by the Clinical Oncology Hospital and the Molecular Genetics and Molecular Biology Laboratory, Beijing Institute of Oncology, Peking University. The cell lines were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2. Cells in the logarithmic phase were used in the experiments.

The MC cell line is a precancerous stage of the human gastric epithelial cell line GES-1 that is induced by N-methyl-N′ nitro-N-nitrosoguanidine nitroguanidine (MNNG).[8] MNNG is a nitro compound that can induce cancer. Gastric ulcers are commonly considered precancerous lesions. Moreover, MC cell lines are used as a cell model for screening drugs that possess anti-ulcer activity.

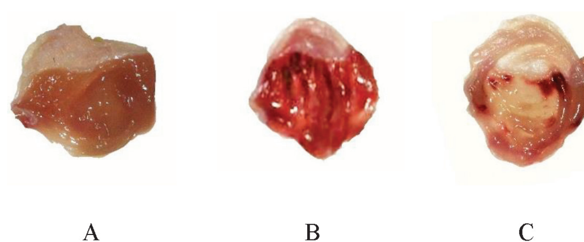
Briefly, the cell toxicity of *H. erinaceus* extracts toward MC cells was assessed using the MTT assay.[9] The MC cells in DMEM (5×10^4 cells) were plated on a well using 96-well microplates and were treated with serum containing increasing amounts of *H. erinaceus* extracts (0.5, 1, 2, and 4 mg/mL). Each experimental group was composed of six wells.

After 48 h of incubation, 10 μL of MTT (5 mg/mL) was added to each well, and the plates were incubated for 4 h in the dark. Following the removal of the medium, 150 μL of dimethylsulphoxide was added to each well to solubilize the formazan crystals formed. The OD value was read at 490 nm using a microplate reader. The cell viability was calculated according to the following formula:

$$\text{Inhibition rate} = (OD_{\text{without drug control group}} - OD_{\text{average of administered group}})/OD_{\text{without drug control group}} \times 100\%.$$

E. Analysis of the Chemical Properties of Different Fractions

The total carbohydrate, uronic acid, and protein contents were quantified by the phenol–sulphuric acid,[10] *m*-hydroxydiphenyl,[11] and Bradford methods,[12] using glucose, glucuronic acid, and bovine serum albumin as standards, respectively.



A B C

FIG. 1: (A) Gastric mucosa of normal mice; (B) gastric mucosa of the normal control group; and (C) gastric mucosa of EP group.

F. Statistical Analysis

The experiments were repeated three times. The values are shown as means ± standard deviation. The Student's *t*-test and one-way analysis of variance were performed for statistical evaluation using the SPSS 16.0 statistical software package (SPSS, IBM, New York, NY, USA). A p value <0.05 indicates statistical significance.

III. RESULTS

A. Anti-Ulcer Activity of *H. erinaceus* Mycelium Extract Evaluated by an Ethanol-Induced Ulcer Model in Mice

When anhydrous EtOH was orally administered in mice for 5 days, typical histological changes due to ulcers occurred in the gastric mucosa, such as injured hyperaemia and oedema, where the tissue became dark red, clear, or with spots, and had linear erosion (Fig. 1B), in contrast with the normal mucosa showing normal epithelial integrity, well-arranged cells in neat rows, and a clean glandular cavity (Fig. 1A). However, these histological changes were significantly prevented in the groups of mice administered with an aqueous extract (WS) of *H. erinaceus* mycelium prior to EtOH administration or with Houtoujun Pian, which was used as a positive control. However, the WP showed no

TABLE 1: Ulcer Area Measurements and the Dose-Dependent Ulcer Inhibition Effects of the WS, WP, ES, and EP Fractions

Groups	*n*	Ulcer Area (mm^2)	Ulcer Inhibition (%)
Normal control group	10	17.24±0.81	
Positive control group	10	12.43±0.74**	27.91
WP(2.5 g/kg)	10	16.62±0.83	3.62
WP (1.2 g/kg)	10	16.57±0.68	3.90
WS(2.5 g/kg)	10	16.38±0.37	4.91
WS (1.2 g/kg)	10	15.18±0.31*	11.90
ES (2.5 g/kg)	10	15.57±1.12	9.70
ES (1.2 g/kg)	10	16.53±0.73	9.93
EP (2.5 g/kg)	10	14.44±0.92**	16.27
EP (1.2 g/kg)	10	15.30±1.00*	11.26

All the results in Table 1 were the typical result of their independent experiments. All of the data are expressed as means ± SD of the mean of 10 mice (performed in triplicate).

*Denoted $p<0.05$, LD group of EP and HD group of WS, respectively, as compared to normal control group using Student's *t*-test.

**Denoted $p<0.01$, HD group of EP and positive control group, respectively, as compared to normal control group using Student's *t*-test.

anti-ulcer effect in the animal model, indicating that the *H. erinaceus* mycelium possessed anti-gastric ulcer activity.

Therefore, the WS was further separated into two fractions, ES and EP, by EtOH precipitation. The precipitate obtained by EtOH is known to concentrate polysaccharides (EP), whereas the EtOH-soluble fraction (ES) contains small organic molecules such as erinacines.[13] The animal model, as mentioned above, evaluated the anti-ulcer activities of the ES and EP fractions. The results obtained are summarized in Table 1. The EP fraction significantly decreased the ulcer area compared with the EtOH-treated control group and the effect was fairly dose dependent (Fig. 1C). In contrast, ES did not inhibit ulcer formation.

B. Anti-Ulcer Activity of *H. erinaceus* Mycelium Extracts in a Cell Line

The anti-ulcer activity assessment was also carried out using the MC cell line. When the cells were treated with the extracts of *H. erinaceus* mycelium, the cell viability was significantly decreased with WS and EP, respectively. However, WP and ES did not give rise to a significant effect on the cell viability. Further, the effects of EP were also dose dependent, as observed in the animal model experiments (Table 2).

C. The Chemical Properties of WS, EP, ES

The chemical and physical properties of WS, ES, and EP are summarized in Table 3. The WP fraction could not be dissolved in water or organic solvent; therefore, the fraction was not studied further. The results demonstrate that EP is mainly composed of carbohydrates. The carbohydrate and protein contents of ES are very high because the mycelium that is mixed with some medium is composed of sugar

TABLE 2: The Effects of WP, ES, and EP Fractions on the MC Cell Viability and the Dose Dependency

Groups	*n*	Inhibition Rate (%)			
		0.5 mg/mL	1 mg/mL	2 mg/mL	4 mg/mL
Normal control group	6	0.12±0.08	0.06±0.05	0.28 ±0.21	0.15±0.09
WP	6	1.30±0.81	2.63±0.65	2.18±0.68	2.16±0.52
ES	6	2.34±0.24	3.11±0.33	4.85±0.32	2.71±0.68
EP	6	13.25±0.12**	15.20±0.63**	17.52±0.48**	21.26±0.57**

All the results in Table 2 were the typical result of their independent experiments. All the data are expressed as means ± SD of the mean of six wells (performed in triplicate).

**Denoted $p<0.01$, EP as compared to normal control group using Student's *t*-test.

TABLE 3: Properties of the WS, ES, and EP Fractions

Fractions	Carbohydrate Content (%)	Uronic Acid Content (%)	Protein Content (%)
WS	61.72	5.62	33.16
ES	54.21	5.53	42.6
EP	79.24	Not detected	20.12

and amino acids and it has no anti-ulcer activity. The active fraction, EP, is also mainly composed of carbohydrate and protein, but the carbohydrate is different from that of ES since the fraction has larger molecules. The results also reveal that the polysaccharides of the EP mycelium culture are an active component in the anti-ulcer effect.

IV. DISCUSSION

In the present study, mycelium extracts were evaluated for their anti-ulcer activity both by an EtOH-induced gastric ulcer model *in vivo* and by an MC cell model *in vitro*. Ethanol may damage the gastric mucosa. The preventive activities of mycelium extracts against ethanol-induced gastric ulcer were demonstrated and the results were consistent.

Numerous studies indicate that gastric ulcer patients are more likely to develop gastric cancer. Gastric ulcer is considered a direct neoplastic precancerous lesion.[14] It is reasonable to use MC cell lines as the cell models of precancerous lesions of gastric cancer to evaluate anti-ulcer activity. The fraction (EP) displayed the maximum inhibition of the growth of MC cells and thus showed the best anti-ulcer activity.

Polysaccharides are the best known and most potent mushroom-derived antitumor and immunomodulating substances. On the other hand, plant polyphenolic antioxidants are an important group of secondary metabolites because of their contribution to human health and their multiple biological effects, such as antioxidant, anti-mutagenic, and anticarcinogenic activities, as well as anti-inflammatory action. However, there is a dearth of data regarding their content in mushrooms, especially in blended mushroom extracts.

This work determined the content of soluble polysaccharides and their anti-ulcer capacity. It also revealed that the activity is located in the EtOH-precipitated fraction of the mycelium aqueous extract (WS). The EtOH-insoluble fraction containing high molecular weight components, including polysaccharide(s)[15,16] of *H. erinaceus*, was shown to have anti-oxidative stress and anti-inflammatory activities both *in vitro* and *in vivo*.[17] Numerous compounds showed protective effects in ethanol-induced gastric ulcers by decreasing oxidative stress and increasing antioxidant enzyme activity.[18] In addition, several reports on the crude fractions isolated from the fruiting body of *H. erinaceus* indicated polysaccharide(s) to be a possible active ingredient against gastric ulcer.[2] Based on the data of these reports and our present observations, it is reliable to conclude that the polysaccharide(s) is the active component responsible for the anti-ulcer activity of *H. erinaceus*. Indeed, our preliminary study indicates that the EP fraction contained heteroglycans composed of mannose, galactose, fucose, and glucose (not published). Further studies are underway to characterize the chemical properties of the active polysaccharides in the EP fraction and to clarify if the same polysaccharides work as the active components in the mycelium and the fruit body of *H. erinaceus*.

The observed anti-gastric ulcer activity may be due to both the cytoprotective and the antioxidant polysaccharides present in *H. erinaceus*. However, the exact mechanism of anti-gastric ulcer is not known. A further detailed study on various other parameters of mucosal defense factors could shed more light on other mechanisms of action.

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Medicinal Mushroom Cracked-Cap Polypore, *Phellinus rimosus* (Higher Basidiomycetes) Attenuates Acute Ethanol-Induced Lipid Peroxidation in Mice

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ABSTRACT: Alcohol abuse and alcoholism remain one of the major health issues worldwide, especially in developing countries. The protective effect of *Phellinus rimosus* against acute alcohol-induced lipid peroxidation in the liver, kidney, and brain as well as its effect against antioxidant enzyme activity such as superoxide (SOD) and catalase (CAT) in the liver was evaluated in mice. Ethyl acetate extract of *Ph. rimosus* (50 mg/kg body wt, p.o.) 1 h before each administration of alcohol (3 mL/kg, p.o.; total 2 doses at 24-h intervals) protected against lipid peroxidation in all organs and attenuated the decline of SOD and CAT activity in the liver. The fold increase in lipid peroxidation, including conjugated diene and thiobarbituric acid reactive substance (TBARS) levels, was highest in the liver. There were 2.6- and 1.5- fold increases in TBARS levels in the liver of the alcohol alone– and alcohol+*Ph. rimosus*–treated groups, compared with that of the normal group. Activity of SOD and CAT in the liver of alcohol– and alcohol+*Ph. rimosus*– treated animals was 9.05±1.38, 18.76±1.71, and 11.26±1.02, 31.58±3.35 IU/mg protein, respectively. Extract at 1 mg/mL inhibited 50.6% activity of aniline hydroxylase (CYP2E1) in liver homogenate. From these results, we concluded that the extract significantly protected against the lipid peroxidation. Protection in the liver may be due to the inhibitory effect on CYP2E1 as well as the direct radical scavenging effect of *Ph. rimosus*, which warrants further research.

KEY WORDS: medicinal mushrooms, alcohol abuse, antioxidants, lipid peroxidation, oxidative stress, *Phellinus rimosus*

ABBREVIATIONS: CAT, catalase; **CYP2E1,** cytochrome P450 2E1 isozyme; **CYP450,** cytochrome P450; **EtOAc,** ethyl acetate extract; **MDA,** malondialdehyde; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase; **TBARS,** thiobarbituric acid reactive substance; **WHO,** World Health Organization.

I. INTRODUCTION

Many synthetic antioxidant compounds exhibit toxic effects that have directed attention to the naturally occurring antioxidants. A multitude of natural antioxidants have already been isolated from different plant materials. Basidiomycetes represent a major untapped source of powerful new pharmaceutical products. Among the higher Basidiomycetes, medicinal macromycetes and their secondary metabolites have an established history of application in medicine.[1] Extracts and powders of mushrooms in the form of capsules and tablets are being marketed.

Although research was focused on the therapeutic effects of the medicinal mushrooms, scientific evidence on their biological properties is limited. *Phellinus* is a large and widely distributed genus. Some of the species of *Phellinus* are extensively studied in China, especially *Ph. linteus*, which has been considered as a traditional Chinese medicine.[2] Cracked-cap polypore, *Ph. rimosus* (Berk.) Pilát (Hymenochaetales, Hymenochaetaceae, higher Basidiomycetes), is frequently found growing on jackfruit tree trunks in Kerala, India. Investigations on the pharmacological activities of *Ph. rimosus* are fragmentary. Among the activities demonstrated, ethyl acetate extract of *Ph.*

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rimosus showed profound antioxidant, hepatoprotective, and antimutagenic activities.[3,4] Recently, we have reported the ameliorating effect of ethyl acetate extract of *Ph. rimosus* against 7,12 dimethyl benz[a] anthracene–initiated, croton oil–promoted skin papilloma formation in mice.[5] The protective effect of ethyl acetate extract against croton oil–induced lipid peroxidation in mice was also demonstrated.[5] Nevertheless, to our knowledge, a protective effect of *Ph. rimosus* against acute alcohol-induced changes in the liver has not yet been reported.

Alcohol abuse and alcoholism represent one of the major health, social, and economic issues worldwide, especially in developing countries. According to the 2011 World Health Organization (WHO) global status report on alcohol and health, almost 4% of all deaths worldwide are attributed to alcohol.[6] Public health objectives have been initiated by the WHO to reduce the health burden caused by the harmful use of alcohol. Biochemical and molecular studies undertaken in recent years have shown that oxidative stress plays an important role in disease and injuries due to alcohol abuse.[7,8] Oxidative stress due to both acute and chronic alcohol exposure increased lipid peroxidation.[7] The highest degree of oxidative damage occurs in the liver.[9] This study examined the protective effect of *Ph. rimosus* against acute alcohol-induced lipid peroxidation in the liver, kidney, and brain of mice and the results are reported herein.

II. MATERIALS AND METHODS

A. Preparation of Extracts

Sporocarps of *Ph. rimosus* were collected from the outskirts of Thrissur, Kerala, India. The specimen was identified with the available literature. The identification was confirmed by Prof. K. M. Leelavathi (Department of Botany, Calicut University, Calicut, Kerala, India) and a voucher specimen was deposited in the Herbarium of the Centre for Advanced Studies in Botany (University of Madras, Chennai, India; HERB MUBL 3171).

Ethyl acetate extract of *Ph. rimosus* was prepared as described in our previous publication.[3] Briefly, 200 g of the powdered fruiting body of *Ph. rimosus* was defatted with petroleum ether and then extracted with ethyl acetate for 8–10 h using a Soxhlet apparatus. The solvents were completely evaporated at 40°C using a rotary vacuum evaporator. The residue was designated as ethyl acetate extract (2.5%), which was initially dissolved in a trace amount of 95% ethanol and made as a fine suspension in distilled water (net concentration of ethanol kept as 0.01%, v/v) and used for the study.

B. Animals

Male Swiss albino mice (30±2 g) and a male Sprague-Dawley rat (200 g) were used for the study. They were purchased from the Small Animal Breeding Centre (Kerala Agricultural University, Mannuthy, Thrissur, Kerala, India) and were kept for a week under environmentally controlled conditions with access to standard food and water *ad libitum*. The experiment was carried out according to the guidelines of the Government of India Committee for the Purpose of Control and Supervision of Experiments on Animals and approved by the Institutional Animal Ethics Committee, Amala Cancer Research Centre (Amala Nagar, Thrissur, Kerala, India).

C. Determination of the Effect of *Ph. rimosus* against Alcohol-Induced Lipid Peroxidation

Swiss albino mice were divided into three groups of six animals each. Group I was treated with vehicle (10 mL/kg of 0.01% ethanol, v/v) and kept as the normal group. Group II was treated with 95% ethanol (3 mL/kg, p.o.) once daily for 2 days and kept as control. Group III was treated with ethyl acetate extract of *Ph. rimosus* (50 mg/kg body wt, p.o. in 10 mL/kg), 1 h before each ethanol administration. Ethanol and *Ph. rimosus* were administered orally using a gavage needle. Twenty-four hours after the second administration of ethanol, the animals were sacrificed with ether anesthesia.

The liver, brain, and kidney were excised and rinsed thoroughly in ice-cold saline to remove blood. Homogenate (10%) was prepared in 0.05 mol/L phosphate buffer (pH 7) using a polytron

TABLE 1: Effect of Ethyl Acetate Extract of *Phellinus rimosus* (EtOAc) on Alcohol-Induced Conjugated Diene Level in the Liver, Kidney, and Brain in Mice

Groups	Treatments (p.o.)	Liver (mmol/100 g)	Kidney (mmol/100 g)	Brain (mmol/100 g)
Normal	25.0±2.1	25.0±2.1	51.0±4.3	35.7±3.4
Ethanol (control)	3 mL/kg	46.1±3.3*	74.8±5.6*	47.7±2.8*
Ethanol+EtOAc	50 mg/kg	30.3±1.3[a]	55.0±4.0[a]	38.8±3.0[a]

Values are means ± SD, n=6.

*P<0.01 (least significanat difference) significantly different from normal.

[a]P<0.01(Dunnett's t-test) significantly different from control.

homogenizer. A part of this homogenate was used for the determination conjugated diene. The rest of the homogenate was centrifuged at 10,000× g for 20 min to remove the cell debris, unbroken cells, nuclei, erythrocytes, and mitochondria. The supernatant was used for the estimation of biochemical parameters.

Conjugated diene was determined according to the method of John and Steven[10] and was calculated using the molar extension coefficient $2.5210^4\ M^{-1}\cdot cm^{-1}$ and expressed as mmol/100 g tissue. Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) and expressed as nanomoles of malondialdehyde according to the method of Ohkawa et al.[11] Liver homogenate was subjected to estimation of antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT).[12,13] Protein content was determined according to the method of Lowry et al.[14]

D. Effect of *Ph. rimosus* on *In Vitro* Aniline Hydroxylase Activity

The Sprague-Dawley rat was treated with sodium phenobarbitone (80 mg/kg body wt, p.o.) for 5 days.[15] After an overnight fasting, the animal was killed by decapitation and 10% liver homogenate (in 0.1 mol/L phosphate buffer, pH 7.4) was prepared. Aniline hydroxylase was determined in the post-mitochondrial supernatant and in the presence and absence of various concentrations of the *Ph. rimosus* ethyl acetate (0.25, 0.5, and 1 mg) as described by Bourrie et al.[16] The amount of p-amino-phenol formed was calculated using the millimolar extinction coefficient 284 cm^{-1}. The specific activity of aniline hydroxylase was expressed as nanomoles of p-aminophenol formed/mg protein/min. The activity was compared to the reaction mixture devoid of the extract.

E. Statistical Analysis

All data were represented as means ± SD. The mean values were statistically analyzed using one-way analysis of variance (using the GraphPad Instat software package, La Jolla, CA, USA) followed by post-test. The significant differences between the ethanol alone–treated (control) and normal groups was analyzed by least significant difference. The *Ph. rimsus*–treated group with control was analyzed by Dunnett's t-test. A p-value less than 0.05 was considered significant.

III. RESULTS

A. Inhibition of Lipid Peroxidation Induced by Ethanol

The effect of ethyl acetate extract of *Ph. rimosus* on ethanol-induced lipid peroxidation is given in Tables 1 and 2. Ethanol ingestion increased (p<0.01) the TBARS and conjugated diene levels in the kidney, liver, and brain in the control group of animals compared to the normal group. Extract of *Ph. rimosus* significantly inhibited (p<0.01) the ethanol-induced lipid peroxidation in the liver, kidney, and brain. The conjugated diene level in the liver, kidney, and brain was inhibited 75%, 83%, and 74%, respectively, in the extract-treated group of animals. The level of

TABLE 2: Effect of Ethyl Acetate Extract of *Phellinus rimosus* (EtOAc) on Alcohol-Induced Lipid Peroxidation (TBARS) Level in the Liver, Kidney, and Brain of Mice

Groups	Treatments (p.o.)	Liver (nmol/mg Protein)	Kidney (nmol/mg Protein)	Brain (nmol/mg Protein)
Normal	Vehicle (10 mL/kg of 0.01% ethanol)	0.77±0.11	1.36±0.21	0.86±0.11
Ethanol (control)	3 mL/kg	2.03±0.15*	3.38±0.64*	1.64±0.20*
Ethanol+EtOAc	50 mg/kg	1.16±0.15[a]	1.99±0.26[a]	1.09±0.14[a]

Values are means ± SD, n=6.
*P<0.01 (least significant difference) significantly different from normal.
[a]P<0.01(Dunnett's t-test) significantly different from control.

TBARS also decreased significantly in the extract treated group. The level was inhibited 69%, 68.8%, and 70.5% in the liver, kidney, and brain of animals treated with extract, respectively.

B. Effect on Ethanol-Induced Decline of SOD and CAT Activity in the Liver

Effect of ethyl acetate extract of *Ph. rimosus* against alcohol-induced declined activity of SOD and CAT in the liver is depicted in Table 3. Activity of CAT was significantly (p<0.01) decreased in the control group and the decline was attenuated in the presence of the extract. However, we could not observe a greater decline of SOD in the control group.

C. *In Vitro* Aniline Hydroxylase Inhibiting Activity

Ethyl acetate extract of *Ph. rimosus* inhibited the aniline hydroxylase activity *in vitro* (Table 4). Ethyl acetate at 1 mg/mL inhibited the 50.6% activity of aniline hydroxylase.

IV. DISCUSSION

Results of this study revealed that the extract of *Ph. rimosus* is effective in ameliorating ethanol-induced lipid peroxidation in the liver, kidney, and brain. Further, the oxidative stress–induced decline of antioxidant enzyme (i.e., SOD and CAT) activity in hepatocytes was improved by the extract. The single dose of extract selected in this study was based on our previous observations, in which we found that 50 mg/kg is effective in preventing the organ injuries induced by oxidative stress.[17,18] In the previous studies, we did not find any change in the biochemical parameters of liver function and renal function or hematological parameters of animals treated with the vehicle (10 mL/kg of 0.01% ethanol, v/v); hence, this was selected as the vehicle for making a suspension of the ethyl acetate extract.[3,18]

TABLE 3: Effect of Ethyl Acetate Extract of *Phellinus rimosus* (EtOAc) against Alcohol-Induced Declined Activity of Superoxide Dismutase (SOD) and Catalase (CAT) in the Liver

Groups	Treatments (p.o.)	SOD (U/L)	CAT (U/L)
Normal	Vehicle (10 mL/kg of 0.01% ethanol)	12.42±1.21	35.30±4.91
Ethanol (control)	3 mL/kg	9.05±1.38*	18.76±1.71*
Ethanol+EtOAc	50 mg/kg	11.26±1.02[a]	31.58±3.35[a]

Values are means ± SD, n=6.
*P<0.05 (least significanat difference) significantly different from normal.
[a]P<0.01(Dunnett's t-test) significantly different from control.