The Bioactivity of Tiger Milk Mushroom: Malaysia's Prized Medicinal Mushroom

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Abstract

The tiger milk mushroom has long been extolled for its medicinal properties and has been used for the treatment of asthma, cough, fever, cancer, liver-related illnesses, and joint pains and as a tonic. The history of usage for tiger milk mushroom dated back to almost 400 years ago, but there were no records of scientific studies done due to unavailability of sufficient samples. Even when there were samples collected from the wild, the supply and quality were inconsistent. With the advent of cultivation success of one of the most utilized species of tiger milk mushroom (*Lignosus rhinocerotis*) in 2009, scientific investigation was done to validate its traditional use and to investigate its safety for consumption and biochemical and biopharmacological properties. Among the properties that have been investigated to date are antiproliferative, anti-inflammatory, antioxidative, nutritional, immunomodulatory, and neuritogenesis activities of the *Lignosus rhinocerotis*. The scientific findings have so far verified some of its traditional applications and revealed interesting data which shows potential for it to be further developed into possible nutraceutical. More scientific investigations are much needed to validate the medicinal properties of tiger milk mushroom across its species and to unveil potential biomolecules that may form a valuable foundation in pharmaceutical and industrial applications.

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Keywords
Biomedical properties • *Lignosus* • Medicinal mushroom • Tiger milk mushroom • Sclerotia

Contents

Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AGE	Advanced glycation end
AKT	Protein kinase B
CAT	Catalase
CBM	Carbohydrate-binding module
CWE	Cold water extract
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ERK	Extracellular signal-regulated kinases
FIP	Fungal immunomodulatory protein
FRAP	Ferric reducing ability of plasma
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GST	Glutathione transferase
HMW	High molecular weight
HWE	Hot water extract
IL-6	Interleukin 6

iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
LMW	Low molecular weight
MALDI-MS	Matrix-assisted laser desorption/ionization coupled with mass spectrometry
LC-MS	Liquid chromatography coupled with mass spectrometry
MCHC	Mean corpuscular hemoglobin concentration
MCPs	Matricellular proteins
MCV	Mean corpuscular volume
ME	Methanol extract
MMW	Medium molecular weight
Mn-SOD	Manganese-superoxide dismutase
NF-κB	Nuclear factor-κB
MIP-1α	Macrophage inflammatory protein- 1α
NOAEL	No-observed-adverse-effect level
PCV	Packed cell volume
RBC	Red blood cell
ROS	Reactive oxygen species
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor alpha

5.1 Introduction

5.1.1 Historical Background

The tiger milk mushroom (also known by a variety of names: *cendawan susu harimau* (in Indonesian/Malay) (Burkill & Haniff 1930, Burkill et al. 1966); *betes kismas* (Haji Taha 2006); *hurulingzhi* (Huang 1999a, b); *hijiritake* (Yokota 2011)) has been documented by Jesuit in 1664 in the "The Diary of John Evelyn" (Evelyn 1664) where it was referred to as *Lac tygridis* (*tigridis*) (tiger's milk) and was recorded to be used by the local communities to treat diseases that "Western druggist and physicians were not able to figure out." Cooke (1879) pioneered the scientific documentation of this fungus and named it as *Polyporus rhinocerus* using a specimen obtained from Penang Island, Malaysia. The subsequent records in Southeast Asia were by H.N. Ridley (Ridley 1890, 1900; Ridley and Curtis 1902) where the mushroom (then referred to as *Polystictus rhinocerotis*) was mentioned to have an important economic value. In "A Dictionary of the Economic Products of the Malay Peninsula," Burkill et al. (1966) listed *Polysticus sacer* as one of the mushrooms that the Malays called *susu rimau*. According to Malaysian folklore, it was believed that the tiger milk mushroom grows where the mother tiger might have disgorged its milk during lactation. There were huge intervals between the historical

mentions, presumably due to the quandary of locating the mushroom for use. The scarcity of the mushroom may be owing to ill-suited weather and growth environment. The decline of this mushroom which is known as an imperative "health guard" to the local communities could also be attributed to its increasing cost due to high demand, overharvesting, deforestation for modern development, pollution (Vikineswary and Chang 2013), and the availability of modern medicine.

5.2 The Morphology and Taxonomy of Three Known Tiger Milk Mushroom Species in Malaysia: The *Lignosus rhinocerotis*, *Lignosus tigris*, and *Lignosus cameronensis*

Lignosus Lloyd ex Torrend, a genus comprising eight species of polyporales macrofungi, namely, *L. dimiticus* Ryvarden, *L. ekombitii* Douanla-Meli, *L. goetzii* (Henn.) Ryvarden, *L. rhinocerotis* (Cooke) Ryvarden, *L. sacer* (Afzel. ex Fr.) Ryvarden, and *L. hainanensis* B. K. Cui, *L. tigris* C.S. Tan, and *L. cameronensis* C.S. Tan (Ryvarden and Johansen 1980; Douanla-Meli and Langer 2003; Cui et al. 2011; Tan et al. 2013). Species in *Lignosus* genus generally have similar gross morphologies. Variations in size and dimensions of either sclerotia or basidiocarps are not characteristically unique to the species. There is also little variation between the hyphal systems and sclereids on a microscopic level. Hence, the sizes of the pores and basidiospores are the two reliable characters for species identification. In Malaysia, there are three main recognized species, namely, the *Lignosus rhinocerotis* (Cooke 1879; Tan et al. 2010), *Lignosus tigris*, and *Lignosus cameronensis* (Tan et al. 2013; Yap et al. 2014a). The pore sizes of the three species are summarized in Table 5.1 and Fig. 5.1a–d.

The molecular taxonomy of *Lignosus* species is shown in Fig. 5.2 where one can note the intra- and interspecific differences among the species. The phylogenetic tree in Fig. 5.2 shows that *L. rhinocerotis* is distinct compared to other members of the genus. *L. cameronensis*, *L. tigris*, *L. sacer* and *L. ekombitii*, and *L. hainanensis* are within the same clade with *L. ekombitii* appearing to be quite closely related to *L. sacer*, while *L. cameronensis* and *L. tigris* remain distinct species with a genetic distance of up to 7.1 % (Tan et al. 2010, 2013). *L. dimiticus* Ryvarden and *L. goetzii* (Henn.) Ryvarden which are also part of the *Lignosus* genus were not included in the taxonomy study done by Tan et al. (2013) due to unavailability of samples.

Table 5.1 Pore size of three *Lignosus* species

Species	Pore size (mm)
Lignosus rhinocerotis	5–8 pores per mm
Lignosus tigris	1–2 pores per mm
Lignosus cameronensis	2–4 pores per mm

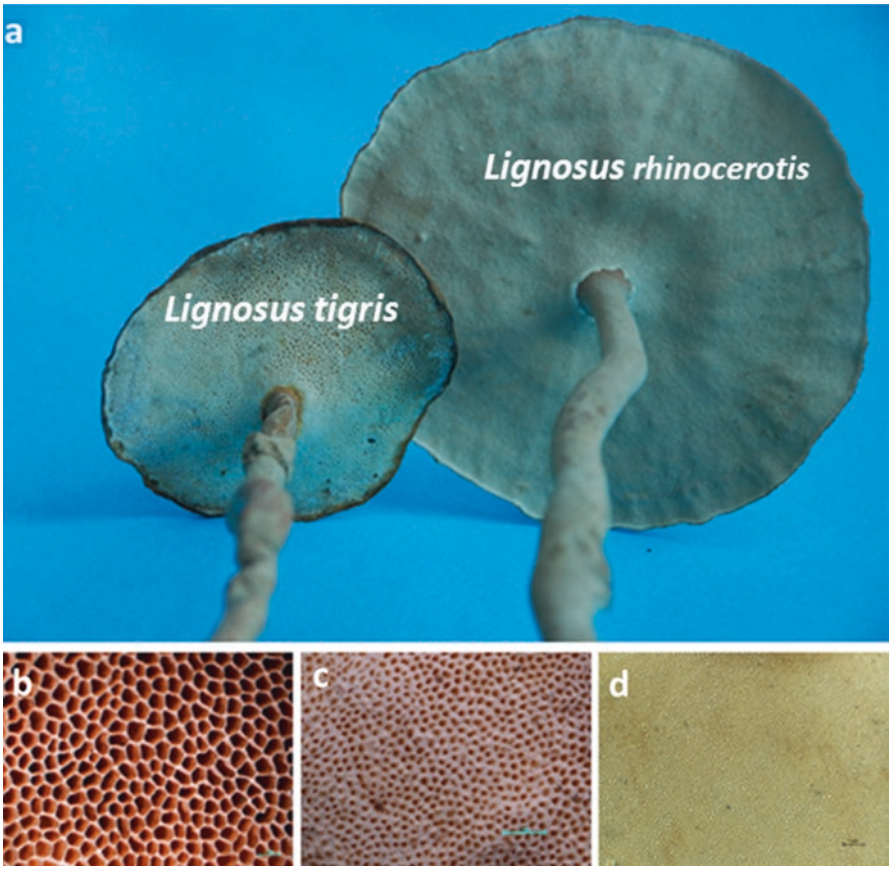

Fig. 5.1 Pores of *Lignosus* species below the pileus and its differences in sizes. (**a**) The structure of *L. tigris* and *L. rhinocerotis.* (**b**) Pore size of *L. tigris.* (**c**) Pore size of *L. cameronensis.* (**d**) Pore size of *L. rhinocerotis*

5.3 Medicinal Usage of Tiger Milk Mushroom: Then and Now

Tiger milk mushroom (particularly referring to the *L. rhinocerotis* species) has been listed as one of the most important medicinal mushrooms used by local communities of Malaysia by a local Malay from Pahang, Tuan Haji Mat Yusop (Corner 1989). Back at the end of the 1890s, Sir H.N. Ridley mentioned that the tiger milk mushroom "has a great reputation for consumption and colds" (Ridley 1897). A technique mimicking cold water extraction has been described by Chan (1953) where the sclerotium is grated on a hard surface such as granite plate along with some water. The resulting mixture is then further diluted with water prior to consumption.

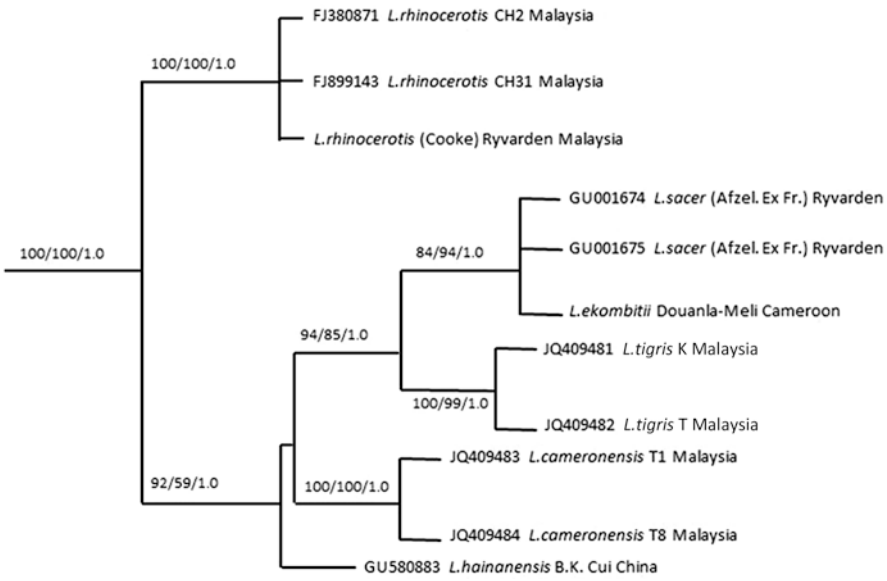

Fig. 5.2 The molecular taxonomy of *Lignosus* species

The Temuans utilized tiger milk mushroom as medicine to treat coughs and asthma and to strengthen weak constitution by consuming the underground sclerotium (tuber-like; the part with medicinal value) in the form of decoction. The Semai aborigines used *Betes kismas* (a common name for tiger milk mushroom; see Sect. 5.1) for the treatment of asthma, cough, fever, cancer, liver-related illnesses, and joint pains. They are also used by men to revitalize their bodies and as medicines for women after childbirth (Chang and Lee 2006). It was documented that in the state of Kelantan, Malaysia (where the mushroom is often given to mothers after childbirth), the sclerotium is pounded with raw rice, infused, and drunk (Burkill et al. 1966; Burkill and Haniff 1930). The latest survey shows that the local Malay and Chinese communities utilized the sclerotium of tiger milk mushroom to treat food poisoning, wounds, stomach cancer, breast cancer, and swellings (LiGNO Biotech Sdn. Bhd. 2012). In Hong Kong and China, traditional Chinese physicians regarded *L. rhinocerotis* sclerotium as an expensive folk medicine to treat liver cancer, chronic hepatitis, and gastric ulcers (Wong and Cheung 2008). Tun Dr. Mahathir bin Mohamad, the fourth Prime Minister of Malaysia and a medical doctor, has mentioned in his opening speech at the International Convention on Biotechnology 2002 that his chronic intractable cough has been cured by tiger milk mushroom (SMPKE Prime Minister's Office 2017).

The Medicinal Mushroom Research Group (MMRG) from the University of Malaya initiated the safety assessment of the cultivated *L. rhinocerotis* sclerotia powder in 2009 after its successful cultivation (Tan 2009b) to ensure that the cultivar is competent for consumption. Results of the safety assessment are discussed in

Sect. 5.6. Following the results of safety assessment, scientific validation of its nutritive composition and bioactive properties such as antiproliferative and anti-inflammatory was investigated alongside the wild type (see Sects. 5.7 and 5.8).

The method used for the cultivation involves using specially formulated culture medium consisting of rice, water, and food-based materials, subsequently incubated in environmentally controlled culture room for up to 6 months to cultivate the sclerotia before harvesting. The proprietary method used for cultivated could greatly affect the quality of the sclerotia and likely to retain more of its medicinal properties.

A quality of life (QoL) survey was conducted recently among the 100 volunteers who had taken the cultivated *L. rhinocerotis* sclerotia powder for various health concerns. The volunteers were given 500 mg of *L. rhinocerotis* sclerotia powder daily for 1–2 weeks consecutively. The volunteers were interviewed or gave written testimonials at the end of the survey period. The volunteers' testimonials were categorized as shown in Table 5.2.

Table 5.2 Usage of tiger milk mushroom based on volunteers' testimonials derived from quality of life (QoL) survey conducted among the 100 volunteers

	Benefit	Summary of comments from volunteers
1.	Relief from respiratory-related illnesses	Ease of breathing
		Rid phlegm with ease (especially noted for volunteers who were smokers)
2.	Relief from asthmatic symptoms	Improves breathing
		Reduce the frequency of inhaler usage
		Shorten the recovery period from an asthmatic attack
		Decreases the recurrence of subsequent asthmatic attacks
3.	Relief from a chronic cough and subsequent recovery	Fewer episodes of a cough
		Recover from a cough
4.	Relief from allergy	Relief from respiratory allergy such as nasal and sinus symptoms
		Relief from skin allergy such as eczema
		Relief from an allergy to food or chemicals (rash subsided within a few days of topical application)
5.	Treatment of joint pains	Effective in the treatment of joint pains (i.e., as a result of dengue fever)
		Relieving joint pains in the elderly, rheumatoid arthritis, and osteoarthritis patients
6.	Improved stamina	Improve alertness and stamina
		Prolongs stamina of athletic volunteers
7.	Anticancer	Reduction in the size of the tumor
		Improved the quality of life and more energetic (volunteers who are cancer patients)

5.4 Sclerotia Versus Mycelia of *Lignosus rhinocerotis*

The morphology of *Lignosus rhinocerotis* as a polypore is unique. The fruiting body (also known as the sporophore) has the characteristic consisting of a centrally stipitate pilei that grow from a subterranean sclerotium (plural sclerotia) in a humid environment.

The different developmental stages of *L. rhinocerotis* are shown in Fig. 5.3a–d. The description of various stages of growth: culture mycelial growth, the formation of sclerotia, mature sclerotia, compacted hyphal mass, mushroom with pileus, stipe, root, sclerotium is depicted. Figure 5.3a shows a 2 week culture of mycelial growth from a spore of *L. rhinocerotis*. Expansion of the mycelium is seen as repeated branching of the germ tube (short, initial hypha) which develops into a circular form (known as the "Tiger's Eyes") (Fig. 5.3b). The color of colony ranges from white to yellow upon maturation; appearance is fluff-/velvet-like. The growing mycelia form crosslink structures among the radiating hyphae to enable nutrient uptake and mobilization (Fig. 5.3c). The mycelia had fully colonized the substrate (1–2 months post-inoculation), and sclerotia had begun to form. Sclerotia forms by the initiation of aggregation of hyphae into small knots within the mycelial mass. As the size of the knots increase, central hyphae accumulate nutrients (reserves) from connected mycelia. Cells of the outer layer are seen to shorten and begin to thicken, resembling barrels as the sclerotium increases in size (Fig. 5.3d). Vigorous mycelial growth promotes the development of mature sclerotia (possible harvesting after between 4 and 6 months) under the soil. The reproduction of *L. rhinocerotis* is likely asexual as it is placed under phylum *Basidiomycota* (Abdul Razak 2009).

The sclerotium is the main source of food storage and medicinal material. It is a compact mass of hardened fungal mycelium and represents one of the stages in the fungal life cycle. This structure is a morphologically variable, nutrient-rich, multihyphal aggregate that serves as a food reserve and can remain dormant until favorable growth conditions arise (Willetts and Bullock 1992). They are long-lived compared to mycelia due to the ability to survive environmental extremes. The sclerotium of *L. rhinocerotis* comes in different shapes and sizes from being spherical to oval or irregular with a diameter of 4–5 cm. (Fig. 5.4) The pale to the grayish-brown outer skin (rind) appears rough and wrinkly to keep the internal compacted hyphal mass from drying out (Fig. 5.4).

The rings on the pileus are formed on each rainy (wet) season; active growth of cells is seen and subsequently ends during the dry season. Another ring is formed in the next wet-dry seasonal cycle (Fig. 5.5). The pileus may be eaten by small animal or rot due to humidity. It is interesting to note that under favorable growth conditions, there is root formation after the nutrient of sclerotia is used for initial sprouting into stipe and pileus. For continued survival, rhizobium-like root structure is formed above the "empty" sclerotium, and the sclerotium is filled with mass and grows larger in size.

Apart from the well-established recognition of the superiority of the sclerotium of *L. rhinocerotis* as a part of the mushroom with high medicinal properties, there has also been a growing emphasis on its mycelium as a source of nutraceuticals

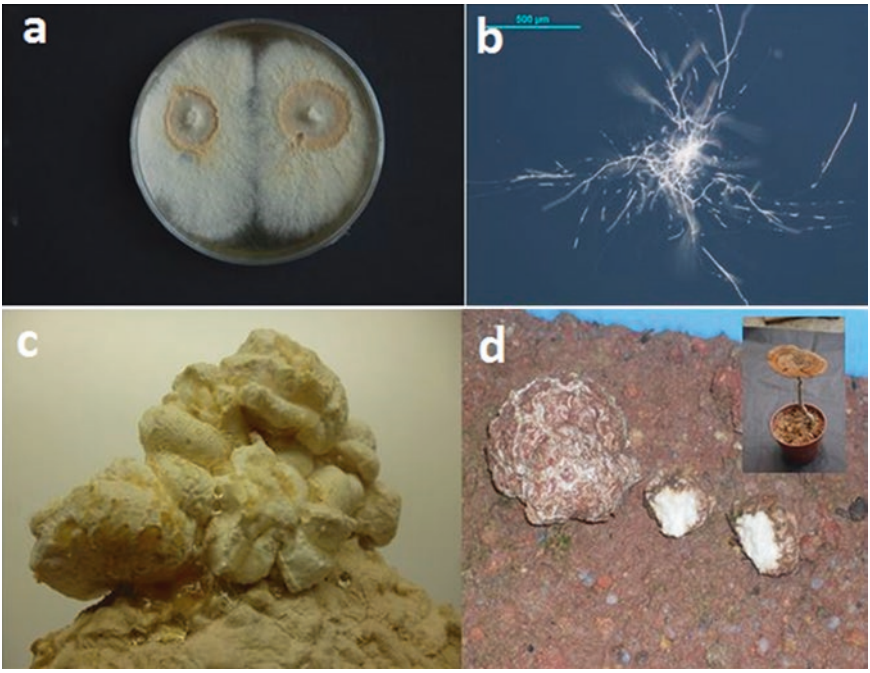

Fig. 5.3 The different developmental stages of *L. rhinocerotis.* (**a**) Mycelia culture; (**b**) mycelia under microscope; (**c**) sclerotium formation from media; (**d**) hardened sclerotia from soil culture

Fig. 5.4 The sclerotium of *L. rhinocerotis*

Fig. 5.5 Rings on the pileus of *L. rhinocerotis*

(John et al. 2013; Lau et al. 2009, 2013, 2014; Phan et al. 2013). Lau et al. (2009, 2013) suggested the use of mycelium as an alternative to sclerotium. They reported the advantages conferred by submerged fermentation as well as the comparable proximate composition and some nutritional attributes of the mycelia. The mycelium was also reported to contain high levels of potassium, phosphorus, magnesium, riboflavin, and niacin and appreciable amounts of essential fatty acids (Lau et al. 2013). They also demonstrated that the mycelium and culture broth of *L. rhinocerotis* exhibited better antioxidant capacity and cytotoxic effect (Lau et al. 2014). However, whether mycelium is a good substitute of sclerotium remains a controversial issue as some researchers are of the opinion that mycelium is not naturally consumed and can only be produced by using artificial chemical-laden liquid culture medium. A simplistic comparison of the bioactivity of mycelium and sclerotia is shown in Fig. 5.6.

5.5 Wild-Type Versus Cultivated *Lignosus rhinocerotis*

The wild-type *L. rhinocerotis* can be located in the forest, by chance. This is due to the fact that the sporophore will only sprout from the underground sclerotium when the environment and conditions are optimum. The sclerotium can remain below the ground for months, years, or even decades without sprouting its stipe; hence it is a mammoth task to locate the spot where the sclerotium lies. The irregularity of supply, coupled with the inconsistency quality and nutritional (medicinal) content of the sclerotium (which is highly dependent on the harvesting conditions) along with

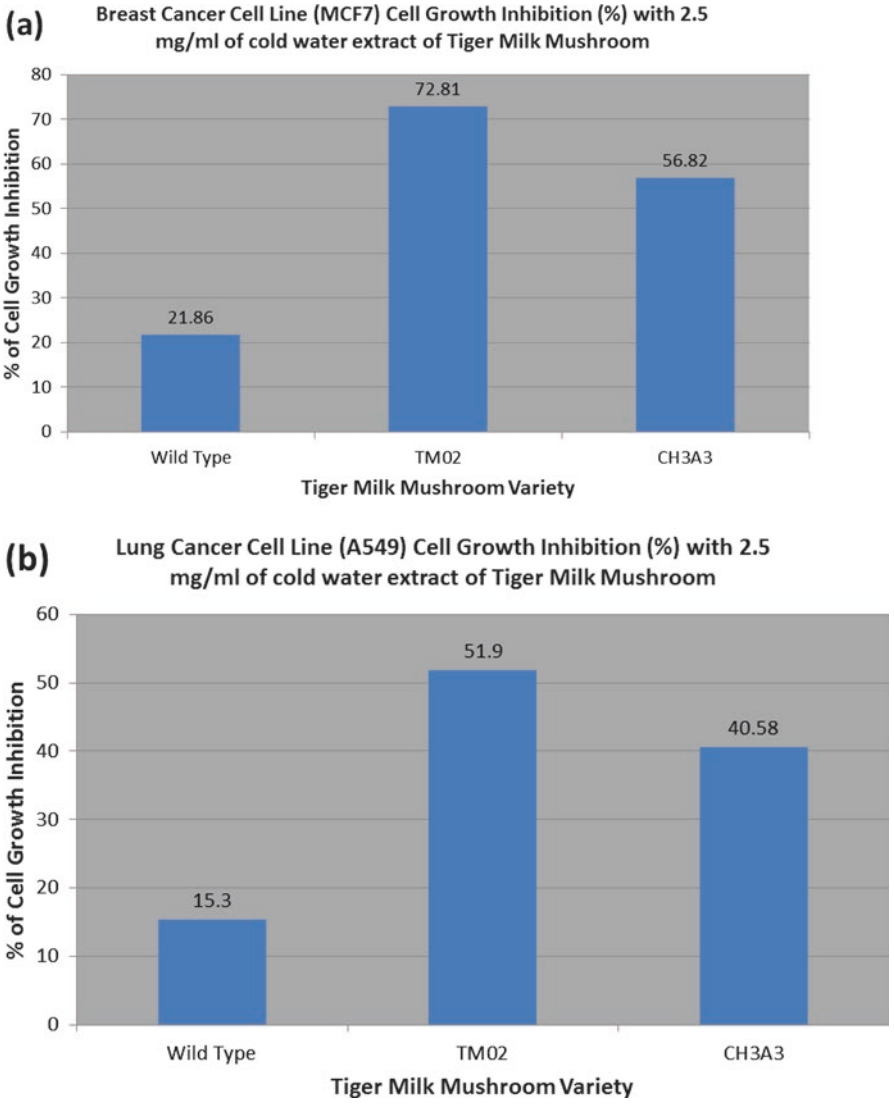

Fig. 5.6 Cell growth inhibition for two cancer cell lines (**a** MCF7, **b** A549) with the treatment of wild type (wild-type tiger milk mushroom),TM02 (sclerotium of *Lignosus rhinocerotis* cultivar), and CH3A3 (mycelium of *Lignosus rhinocerotis*)

the risk of environmental contamination and adulteration of closely related species, has encouraged methods of cultivation for this prized mushroom.

Successful cultivation technique has been reported by Tan (2009a) who cultured mycelium which is placed in a spawn container containing sawdust and buried in soil for the growth of sclerotia. However, the yield was low. Following the spawning container small-scale cultivation success, LiGNO™ Biotech Sdn. Bhd. in joint efforts with a group of scientists further developed a highly efficient method for tiger milk mushroom sclerotium cultivation in rice-based media using specially formulated culture medium consisting of water and other food-based materials. The cultivation is done using standard, sterile, and hygienic protocol, in a controlled environment and harvested in optimum condition. The latter method is highly successful, and LiGNO™ Biotech Sdn. Bhd. was subsequently recognized as the world's first commercial producer of *L. rhinocerotis*, being able to develop an in-house proprietary method for the quick cultivation and mass production of *L. rhinocerotis* (termed TM02). The prevailing successful cultivated method can produce a consistent supply of the *L. rhinocerotis* sclerotia.

As a result of the initiative of the company, tiger milk mushroom is now listed in traditional medicine active ingredient list under the Malaysian National Pharmaceutical Control Bureau in September 2010 and enabling *L. rhinocerotis* to be commercially available (Ligno LiGNO Biotech Sdn. Bhd. 2012). This cultivar has also been filed in 2011 under the New Plant Variety Protection (NPVP) of Malaysia, recognizing the cultivated variety as a distinct in comparison to that of the wild type.

A simplistic comparison of bioactivity between the wild type and LiGNO's cultivar TM02 was made by comparing the antiproliferative activity (more details in Sect. 5.8.1) (Fig. 5.6). The figure shows that the cultivated *L. rhinocerotis* contains at least three times the bioactivity of the wild type.

5.6 Cultivated *Lignosus rhinocerotis* TM02: The Safety Studies (Benefits to Science and Community)

Sclerotia of cultivated *Lignosus rhinocerotis* TM02 are authenticated using a rapid and reliable method. Easily amplified, short, standard genetic markers targeting the internal transcribed spacer (ITS) regions of the ribosomal RNA were developed and used for identification of the cultivated species to ensure that it is identical to the wild type (Tan et al. 2010). Safety studies were conducted (Lee et al. 2011, 2013) to ascertain the innocuous nature of the cultivated species.

5.6.1 Subacute Toxicity

Subacute toxicity was carried out in compliance with the guidelines from the Organization for Economic Cooperation and Development (OECD 1995). This was done using repeated doses of 250, 500, and 1000 mg/kg of cultivated *Lignosus*

rhinocerotis TM02 sclerotial powder. The highest dose used was 1000 mg/kg; this was chosen based on a preliminary 7-day acute toxicity studies where male and female rats ($n = 5$ each) fed with 2000 mg/kg of the sclerotial powder did not reveal any toxicity. There were no significant differences in the hematological parameters of rats fed with cultivated *Lignosus rhinocerotis* TM02 sclerotia throughout the duration of the 28 days of study and that of the control group. Hematological parameters such as red blood cell (RBC) count, hemoglobin, packed cell volume (PCV), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) of *Sprague Dawley* rats in the same group were also found to be normal. Clinical biochemistry revealed that oral consumption of cultivated *Lignosus rhinocerotis* TM02 did not affect the renal functions (urea and creatinine levels were evaluated), hepatic functions (albumin, total protein, SGOT, and SGPT were evaluated), serum electrolytes (calcium, sodium, potassium were evaluated), as well as glucose and total cholesterol levels. Histological examinations supported the clinical biochemistry results. There were no renal or liver damages. Pathological changes in the heart and spleen of the rats were also absent (Lee et al. 2011).

5.6.2 Chronic Toxicity, Genotoxicity, Antifertility, and Teratogenic Effects

The chronic toxicity study was carried out in compliance with the guidelines from the Organization for Economic Cooperation and Development (OECD) (2009). Oral administration of the cultivated *L. rhinocerotis* TM02 sclerotial powder at 250, 500, and 1000 mg/kg did not show any signs of toxicity. There were no significant differences in body weight, urinalysis, hematological examination, and clinical biochemistry, and there was no alteration worthy of note in the microscopic examinations of the organs. In the assessment of fertility and teratogenic effects, the cultivated sclerotial powder did not halt the pregnancy nor alter the number of offspring. It also did not produce any congenital malformation (a birth defect) on any offspring. A biological assay used to assess the mutagenic potential of cultivated *Lignosus rhinocerotis* TM02 sclerotial powder revealed that it did not cause gene mutations (Lee et al. 2013).

The recommended daily consumption of the cultivated *Lignosus rhinocerotis* TM02 as nutraceutical is approximately 0.5 g (on an assumption the average body weight is 50 kg). For cancer patients, ten times the amount of sclerotial powder is recommended (up to 100 mg/kg daily). These dosages were taken into consideration for the safety studies. The outcome of the studies shows that the no-observed-adverse-effect level (NOAEL) dose was more than 1000 mg/kg.

The demonstration of the safety profile of cultivated *Lignosus rhinocerotis* TM02 has open doors to more scientific investigations into the application of this medicinal mushroom as a potential nutraceutical, and possible target molecules can be identified for pharmaceutical use.

5.7 Nutritional Value of Wild-Type and Cultivated *Lignosus rhinocerotis* TM02

Wong et al. (2003, 2008) reported that the main components of the dry matter of wild-type *L. rhinocerotis* were mostly made up of carbohydrate (insoluble dietary fiber and non-starch polysaccharides) with low lipid content. In 2013, we reported the comparative nutrition of wild-type and cultivated *Lignosus rhinocerotis* TM02 (Table 5.3) (Yap et al. 2013). The energy value and protein content of cultivated sclerotia are 1.5× and 4.1× higher than the wild type, respectively. The amino acid composition of the protein found in the mushroom sclerotia was also analyzed (Yap et al. 2013). The total essential amino acid content of cultivated sclerotial powder was found to be significantly higher than the wild-type sclerotial powder (6.35 g/100 g dry weight vs. 1.65 g/100 g dry weight).

Carbohydrates are the major constituent of *L. rhinocerotis* sclerotia. Carbohydrate content in cultivated *Lignosus rhinocerotis* TM02 is slightly lower compared to the sclerotia of the wild type. The sclerotial carbohydrate constituent of the wild type is made up of 98.85% insoluble fiber, while the cultivated sclerotia contained 31% insoluble fiber, which is 40.26% of its total carbohydrate content. Further characterization of the sclerotial carbohydrate composition of wild-type and cultivated sclerotial powder using AOAC method shows that there were higher levels of β (1,3/1,6)-glucans in wild-type sclerotial powder, which is 99.78 % of its total glucan content and contains a very low amount of α-glucans. The cultivated sclerotial powder, on the contrary, contains a higher amount of α-glucans (80.44% of its total glucan content) with lower level of β(1,3/1,6)-glucans (14.56% of its total glucan content).

In our most recent study, we investigated the content of β (1,3/1,6)-glucans and α-glucans using Megazyme glucan test kit method. We found that the cultivated sclerotia contain 90.5 % β(1,3/1,6)-glucans and 9.5 % α-glucans. The contradictory results obtained using differing methods of assessment have been discussed by Mccleary and Draga (2016).

Table 5.3 Comparative nutrition values of wild-type and cultivated *Lignosus rhinocerotis* TM02

	Wild-type *L. rhinocerotis*	Cultivated *L. rhinocerotis* TM02
Energy (kcal/100 g dry weight)	201.0	308.0
Proximate composition (g/100 g dry weight)		
Crude protein		
Fat	3.4	14.1
Carbohydrate	0.1	0.8
Dietary fiber	93.3	77.0
	93.3	32.0
Dietary minerals (mg/100 g dry weight)		
Calcium	3.7	19.3
Magnesium	75.8	147.9
Potassium	132.2	203.2
Sodium	8.5	8.8

Table 5.4 Content of adenosine and its derivatives in cultivated *Lignosus rhinocerotis* TM02

Component	Amount in mg/g
Adenosine	0.282
Cordycepin (3′-deoxyadenosine)	0.873
Adenine	0.024
Hydroxyethyl-adenosine	0.268
Ethyl-adenosine	0.399

Among the major elements shown in Table 5.3, potassium is the most abundant mineral in the mushrooms sclerotia followed by magnesium. Calcium, magnesium, and potassium contents of cultivated *Lignosus rhinocerotis* TM02 sclerotia are 5.2×, 2.0×, and 1.5× higher than the wild type, respectively. However, the levels of sodium are comparable. We also determined the content of adenosine and its derivatives according to the method of Furuya et al. (1983) as shown in Table 5.4.

The comparative nutritional studies done thus far indicated that the cultivated *Lignosus rhinocerotis* TM02 sclerotia is superior to the wild-type sclerotia in overall nutritional content by having higher-energy value with more proteins and dietary minerals and is more palatable with the umami ratio higher than the mean ratio of 0.22 (Sun et al. 2012) (umami ratio of cultivated *Lignosus rhinocerotis* TM02 sclerotia was determined to be 0.25, while for wild-type sclerotia, the ratio was 0.20).

5.8 Bioactivities of Wild-Type and Cultivated *Lignosus rhinocerotis* TM02

5.8.1 Antiproliferative Activity

The antiproliferative activity of *Lignosus rhinocerotis* was first reported by Lai et al. (2008). Lai and colleagues reported the growth inhibitory activity of a polysaccharide-protein complex from wild-type *P. rhinocerus* (synonym to *L. rhinocerotis*) sclerotium against a panel of leukemic cell lines mediated by G1 phase cell cycle arrest. We subsequently reported the antiproliferative effect of a sclerotial cold water extract (CWE) from cultivated *L. rhinocerotis* TM02 against breast cancer (MCF7) and lung cancer (A549) cell lines (Fig. 5.6a, b), but not in the two corresponding human non-tumorigenic cell lines. We demonstrated that the antiproliferative activity was due to either the proteins or protein-carbohydrate complex in high-molecular-weight fraction (Lee et al. 2012). In our successive attempt to probe further into the bioactive components responsible for the antiproliferative activity, we characterized the chemical composition of the CWE and found it to contain 77 % carbohydrates and 1.2 % proteins. The extraction at a low temperature of 4 °C likely prevented the excessive degradation of thermolabile constituents including proteins and peptides. Our report was comparable to a cold water extract done reported by Lee et al. (2012) (with 75 % carbohydrates and 1.2 % proteins) and a cold alkaline extract of *P. rhinocerus* sclerotia (with 82 % carbohydrates and 1.3 % proteins) as reported by Lai et al. (2008). Interestingly, Lau et al. (2013) also demonstrated that a cold

aqueous extract preparation from the sclerotium of *L. rhinocerotis* KUM61075 exhibited cytotoxicity against various human cancer cell lines and the cytotoxic component(s) was deduced to be thermolabile, water-soluble protein/peptide(s) (Lau et al. 2013) as cytotoxicity of the cold aqueous extracts diminished when subjected to heat treatment from 60 to 100 °C for 20 min. Further investigations showed that proteins of medium molecular weight could be responsible for the antiproliferative action of *L. rhinocerotis* (unpublished results).

5.8.2 Anti-inflammatory Activity

We investigated the in vitro and in vivo anti-inflammatory activity (Lee et al. 2014). In vitro studies with CWE, along with its high- (HMW) and medium-molecular-weight (MMW) fractions, exhibited an inhibitory effect on TNF-alpha production in LPS-induced macrophages. We demonstrated that the cold water extract exhibited anti-acute inflammatory activity by reducing paw edema induced by carrageenan up to 200 mg/kg, in all three phases of edema development. The fashion in which the CWE resulted in anti-inflammatory action was similar to 10 mg/kg of indomethacin (a nonsteroidal anti-inflammatory drug) in all three phases. The CWE (200 mg/kg) showed ~88 % paw edema inhibition (greater than 10 mg/kg indomethacin) during all the phases of edema development. Further investigations showed that the anti-inflammatory activity was mainly contributed by high-molecular-weight fractions (with possible synergistic effect with lower-molecular-weight fractions) of the CWE. 35 mg/kg of high-molecular-weight fraction had comparable activity with 200 mg/kg of CWE. Cotton pellet-induced granuloma test used widely to assess transudative, exudative, and proliferative phase of inflammation (Swingle and Shideman 1972) revealed that 200 mg/kg of CWE did not inhibit the transudative and proliferative phase of chronic inflammation (Lee et al. 2014). In our attempt to elucidate the active principal causing the anti-inflammatory activity, we determined the ratio of protein and carbohydrate content of the most potent fractions (the HMW fraction). The ratio of protein to carbohydrate in the HMW fraction was 1:20. Further isolation of the protein components in the HMW fraction revealed that the isolated proteins contained a large amount of carbohydrate (1 protein to 8 carbohydrates, mostly alpha glucans). The nonprotein components (mainly alpha-glucan and 3.2 % beta-glucan) were devoid of anti-inflammatory activity. There is a possibility that the anti-inflammatory effect could be due to a polysaccharide-protein complex which has yet to be elucidated. Preliminary studies with the CWE on airway relaxation revealed that CWE was able to fully relax both the trachea and bronchus (unpublished results). Its link with anti-inflammatory activity and its possible application to respiratory ailment have yet to be established.

5.8.3 Antioxidative Activity and Presence of AGE Inhibitors

The antioxidant capacity of the various *L. rhinocerotis* sclerotial extracts (hot water (HWE), cold water (CWE), and methanolic (ME)) for both the cultivated and wild type was found to be generally comparable and very low reducing activity when compared to the positive controls (Yap et al. 2013). The inhibition of free radicals by the extracts was found to be dose dependent. The IC_{50} values for DPPH• scavenging activity of the extracts were found to be generally comparable or even lower than most hot water extracts of medicinal mushrooms such as *G. lucidum*, *Lentinula edodes*, and *Pleurotus eryngii*, with IC_{50} values, ranging from 5.28 to 19.09 mg/ml (Abdullah et al. 2012). In comparison with other extraction methods, methanolic extracts (ME) were found to exhibit higher activity in FRAP, DPPH•, and ABTS•+ assays in spite of their lower phenolic content in terms of mg GAE/g extract. This suggests that the extracts may contain other types of antioxidant/reducing compounds which might also contribute to their reducing/electron-donating ability (Mau et al. 2004). Similarly, the methanolic extract of *L. rhinocerotis* sclerotia showed remarkably potent O2•− scavenging activity in contrast with their lower activity in FRAP, DPPH•, and ABTS•+ assays which suggest the presence of other non-phenolic compounds that have the ability to scavenge O2•−.

In a separate study done to investigate the antioxidant and anti-glycation properties of the fractions of CWE, the sum of phenolic compounds of the three fractions (18.27 mg GAE/g) was found to be significantly lower compared to CWE (28.23 ± 0.50 mg GAE/g) and also possess weaker antioxidant activities than CWE, with the exception of O2•− scavenging activity. Degradation and loss of the phenolic compounds might have occurred during fractionation due to external factors such as light and air (Khoddami et al. 2013). The highest terpenoid content was found in low-molecular-weight fraction. This corresponded to the whole-genome analysis of cultivated *L. rhinocerotis* which shows a high amount of terpenoid biosynthesis genes (see Sect. 5.9). The MMW was shown to be a promising O2•− scavenger among the three fractions, with activity comparable to CWE and the positive controls suggesting the presence of compound(s) with SOD-like activity in the fraction. This provides support for the idea that complex polysaccharides and secondary metabolites such as phenolics and terpenoids which are abundantly present in HMW and LMW may not play a significant role in the potent O2•− scavenging activity of *L. rhinocerotis* sclerotium. All these results still did not give any conclusion as to what are the responsible agents for the antioxidant activity and warrant further investigations.

There is a positive correlation between glycation inhibitory activity and antioxidative potency as antioxidants have been shown to protect against glycation-derived free radicals and may possess therapeutic potential (Elosta et al. 2012; Ramkissoon et al. 2013). Our studies revealed that these fractions showed overall weaker glycation inhibitory activities than the CWE itself. The ability of the fractions to reduce glucose-induced AGE-derived fluorescence decreased in an order similar to their secondary metabolites content and antioxidant potential: LMW> MMW > HMW (unpublished results).

Even though studies to date has yet to elucidate the responsible agent as antioxidant, these preliminary results indicate that consumption of the whole *L. rhinocerotis* sclerotial powder is beneficial due to the synergistic effect of bioactive compounds and that long-term multi-antioxidant diet may possibly lead to the prevention of AGE-associated diabetic complications development and progression.

5.8.4 Immunomodulatory Activity

Liu et al. (2016) reported a novel water-soluble polysaccharide-protein complex (a mannoglucan-type heteroglycan-protein complex) isolated from the sclerotia of *Polyporus rhinocerus* which could significantly activate murine macrophages RAW264.7 in vitro. The signaling pathway involved was reported to be via the activation of ERK and AKT and iNOS (without NF-κB) together with the secretion of NO, IL-6, TNF-α, G-CSF, GM-CSF, MCPs, and MIP-1α. This is a subsequent report of their previous studies (Wong et al. 2009,2011) which documented the involvement of sclerotial polysaccharides (both water and alkaline soluble) to stimulate human innate immune cells. Guo et al. (2011) also reported hot aqueous extract of *L. rhinocerotis* sclerotial which promoted pinocytosis and increased the level of reactive oxygen species (ROS) and nitric oxide (NO), as well as the production of TNF-α. We have also reported a novel fungal immunomodulatory protein (FIP-Lrh) cDNA which was isolated from *L. rhinocerotis*, with the closest protein sequence identity to *G. lucidum* (64.55 %) and very similar predicted 3-D structure to FIP-fve from *Flammulina velutipes*, GenBank: ADB24832.1 (Pushparajah et al. 2016). Like other FIPs, it is a sugar-binding protein, and the more positively charged putative CBM pocket of FIP-Lrh predicted a stronger interaction with N-acetylgalactosamine and N-acetylglucosamine. A functional recombinant 6xHisFIP-Lrh was successfully produced in *E. coli* cells and was shown to be cytotoxic against several tested cells lines, notably the MCF7 cell line.

Consolidation of present reported results shows that *L. rhinocerotis* inarguably possesses immunomodulation activity for preventive and therapeutic potentials, such as anti-anaphylaxis and antitumor effect. Much remains to be explored in this area for the development and utilization of medicinal proteins and their subsequent connection with polysaccharides in their activity.

5.8.5 Neuritogenesis Activity

Studies have been done to investigate the neuritogenesis activity of *L. rhinocerotis* sclerotia for maintenance and regeneration of the neuronal communications network to subsequently be used as a preventative measure and as therapeutic agents for neurodegenerative disorders. Neuritogenesis was seen in PC-12 cells (pheochromocytoma of the rat adrenal medulla which has a mixture of neuroblastic cells and eosinophilic cells) using hot water extract of cultivated *L. rhinocerotis* sclerotia (Eik et al. 2012; Seow et al. 2015). The neuritogenic activity was comparable to nerve

growth factor (NGF) albeit at a much higher concentration (The hot aqueous extract (25 μ g/ml) stimulated neuritogenic activity that was comparable to NGF (50 ng/ml, 500×) and hence could mimic the neuritogenic activity of NGF and induce neuritogenesis in PC-12 cells via the NGF-responsive pathway. It is postulated that the neuritogenesis activity of hot aqueous extract may be mediated through the phosphorylation of TrkA receptor and ERK1/ERK2 signaling pathway in PC-12 cells.

5.8.6 Antiviral and Anti-microbial Activities

Wild-type *L. rhinocerotis* sclerotium has been screened for antiviral and antimicrobial activities (Mohanarji et al. 2012; Kavithambigai et al. 2013). It was reported that methanol and aqueous extracts (suggesting that the active components are polar) exhibited significant inhibition against several Gram-positive and Gram-negative bacteria at 30 mg/ml. The aqueous extract (at a concentration of 2.5 mg/ml) also showed moderate inhibitory effect (<40 %) against dengue virus type 2. There have been no other studies done thus far to substantiate the screening results reported.

5.9 Future Potentials with the Genome, Transcriptome, and Proteome Data of *Lignosus rhinocerotis*

We reported the genome of *Lignosus rhinocerotis* (Yap et al. 2014b) and revealed that it encodes 10,742 putative genes with 84.30 % of them having detectable sequence similarities to others available in public databases. It has a close phylogenetic relationship with medicinal mushrooms from the polyporoid clade, namely, the *Ganoderma lucidum*, *Dichomitus squalens*, and *Trametes versicolor*. Functional annotation of genes showed that the genome encodes for genes responsible for carbohydrate and glycoconjugate metabolism, cytochrome P450s, putative bioactive proteins (such as lectins, fungal immunomodulatory proteins, and laccases), and secondary metabolite biosynthesis, particularly sesquiterpenoid biosynthesis genes. The revelation of the genome contents has provided valuable insights into the biomolecule discovery and provided the foundation for future research and exploitation of *L. rhinocerotis* in pharmacological and functional food applications. For instance, the putative FIP genes (GME7566_g and GME10641_g) were cloned and expressed and subsequently characterized (structure modeling inclusive of carbohydrate binding site and binding affinity) (as discussed in Sect. 5.8.4). The bioactivity of recombinant FIP-Lrh produced in*E. coli* was also verified. Transcriptomic studies (Yap et al. 2015a) confirm the expression of the large number of transcripts involved in the processing of gene information; genes encoding for carbohydrate metabolism (biosynthesis of glucans), terpene synthases, non-ribosomal peptide synthetases, and polyketide synthases; production of cysteine-rich cerato-platanin, hydrophobins, and sugar-binding lectins; and cytochrome P450 sequences were identified. Systematic profiling and identification of proteins from proteomics studies (Yap

et al. 2015b) revealed only a few identified proteins with public databases using the matrix-assisted laser desorption/ionization coupled with mass spectrometry (MALDI-MS) which confirms that *L. rhinocerotis* proteins are indeed structurally quite different from other known fungal proteins. Using liquid chromatography coupled with mass spectrometry (LC-MS) with *L. rhinocerotis* genome as references database, more proteins were identified. These proteins play roles in nutrient mobilization and defense mechanisms. Putative lectins, immunomodulatory protein, aegerolysin, and antioxidant proteins such as Mn-SOD, CAT, and GST were also identified. These data forms a valuable foundation for future research in the exploitation of the *Lignosus rhinocerotis* in pharmacological and industrial applications.

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