Bioactivities and Chemical Characterization of the Wild Mushrooms of Nepal

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Bioactivities and Chemical Characterization of the Wild Mushrooms of Nepal

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Thesis Declaration

I, Sonam Tamrakar, hereby declare that all the data described in this thesis are results of my work unless otherwise acknowledged or referenced. This thesis is submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Agriculture at Kyushu University.

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List of abbreviations

NARC: Nepal Agricultural Research Council

TPC: Total phenolic content

GAE: Gallic acid equivalent

ORAC: Oxygen radical absorbance capacity

AAPH: 2,2'- azobis(2-amidinopropane) dihydrochloride

TE: Trolox equivalent

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

RP: Reducing power

EC: Effective concentration

IC: Inhibitory concentration

HPLC: High performance liquid chromatography

LC-MS: Liquid chromatography – mass spectrometry

IT-TOF: Ion trap – time of flight

ESI: Electron spray ionization

UV-Vis: Ultra violet – visible

BPC: Base peak chromatogram

MIC: Minimum inhibitory concentration

MBC: Minimum bactericidal concentration

NMR: Nuclear magnetic resonance

HMBC: Heteronuclear multiple bond correlation

HSQC: Heteronuclear single quantum correlation

MC: Melanin content

CV: Cell viability

MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

BHR: β-hexosaminidase release

Abstract

Mushrooms have been considered a part of a healthy human diet due to its high nutritional benefits and potential medicinal properties. Nepal, is a country rich in biodiversity with abundant natural medicinal resources, including various medicinal mushrooms. However, there is a great lack of scientific research into the bioactivities and chemical characterization of these mushrooms. The present study aims to fill this gap by investigating bioactivities of several wild mushroom species, collected from the forests in different parts of Nepal. Ninety-two samples of wild mushrooms, belonging to 40 different genera were collected from forests in different parts of Nepal (altitudes ranging from 1300 m to 3800m). The samples were dried and sent to Systematic Forest and Forest Product Sciences, Kyushu University, for analysis. The samples were firstly identified based on the morphological characteristics, followed by genetic identification.

The dried samples were ground to a fine powder, and extracted in ethanol and water separately at room temperature for 24 hours. The assays such as total phenol content (TPC), ORAC, DPPH, ABTS, and reducing power were used to test the antioxidant activity. Other tested bioactivities include antibacterial activity against *Staphylococcus aureus* and *Propionibacterium acnes*, inhibitory and stimulatory activity towards melanin synthesis in B16 melanoma cells, anti-allergy activity in RBL-2H3 cells, and anti-cancer activity using the cancer cell lines MCF-7, Hela, HCT-116, HepG2, CCD-841, and NHDF.

Although several species were identified as bearing strong bioactivities, the mushrooms belonging to the Hymenochaetaceae family were some of the most prominent samples. Ethanol extract of *Inonotus clemensiae* showed extraordinarily high values of antioxidant activity (ORAC value 31,966.9 μM trolox equivalent/ g extract), which is one of the highest reported antioxidant activity among food products. Further chemical characterization of this extract by LC-MS and NMR analysis revealed the presence of a highly bioactive compound, “hispidin”. However, the presence of the compound as a single major compound, encompassing around 70% of the extract is a very unique phenomenon. Some other interesting samples with strong bioactive potential were *Cyclomyces setiporus*, *Phellinus conchatus*, and several *Ganoderma* sp. Compounds such as protocatechualdehyde, protocatechuic acid, homovanillic acid, and vanillin were identified from *Cyclomyces setiporus*.

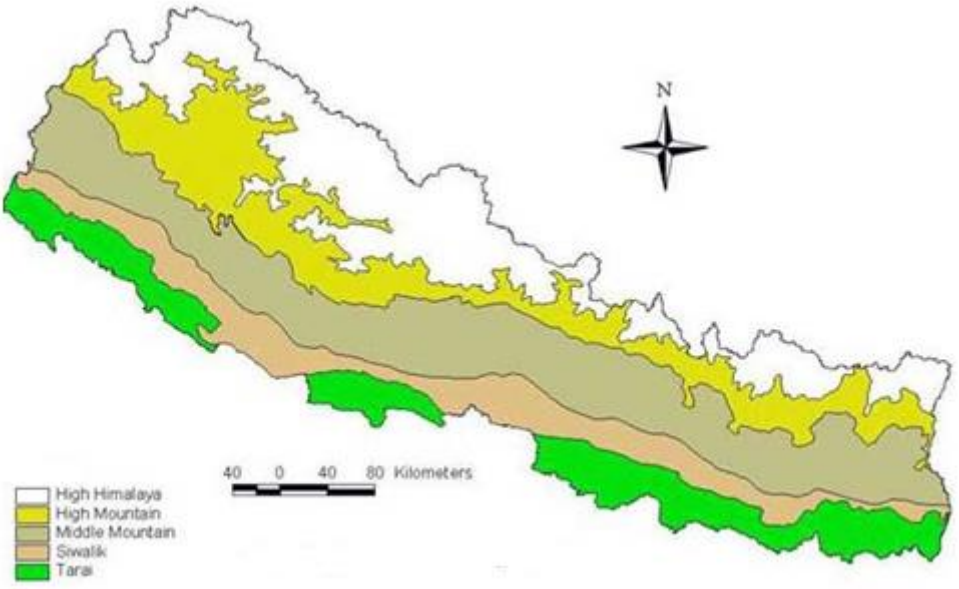
The next step was to focus on the *in vitro* digestion of the bioactive compound hispidin, to confirm the maintenance of bioactivity upon consumption. A two-step process involving the enzymatic and fecal microbial digestion was performed to analyze the effect on the compound. Further experiments are being conducted to clarify this effect and explore the possibilities of biotransformation. Overall, the present research is expected to highlight the bioactive potential of the wild mushrooms of Nepal, opening possibilities to create economic benefits for the local farmers.

Chapter 1

Introduction

1.1 Nepal

Nepal, is a landlocked country nestled between two huge countries India and China. With an area of 147,181 km^2[1], Nepal occupies less than 0.1% of the world land mass. It is located between latitudes 26°22’ and 30°27’ N and longitudes 80°40’ and 88°12’ E[2]. However, the relatively small area of the country must not be mistaken for lack of geographic and biological diversity. The altitudinal variation ranges from 67 meters above sea level to 8848 meters above sea level (Mt. Everest), which also is the highest point in the world. The southern part of the country is made up of tropical alluvial plains, whereas the northern part is occupied by permafrost Himalayas. The entire country is divided into 5 physiographic zones from north to south: the high Himalayas, high mountains, middle mountains, Siwalik hills, and the Tarai plains[3]. With the huge variations in altitudes and the physiographic regions, the climatic conditions also varies from the alpine cold climate in the northern Himalayan region to the tropical climate in the southern Tarai region.



Physiographic regions of Nepal (Source: Soil Science Division, NARC)

1.2 Biodiversity of Nepal

Due to the sharp variations in altitudes and climatic conditions, Nepal is gifted with one of the richest biodiversity in the world. Another important contributing factor is that the country lies in the unique transitional area between two major biogeographic regions, the Indo-Malayan to the south, and the Palearctic to the north. Also, the changes in precipitation, humidity, temperature, and slope effect are some others parameters affecting the diversity within a particular habitat. As a result, Nepal boasts a total of 118 ecosystems, which includes 112 forest ecosystems, 4 cultivation ecosystems, 1 water body ecosystem, and 1 glacier/snow/rock ecosystem[3]. Some researchers argue that due to the utterly complex physiographic and climatic zones, the clear categorization of the vegetation types in Nepal remains extremely difficult; and therefore is still a matter of debate and careful consideration[4]. Nonetheless, broadly the southern lowlands are covered with *Shorea robusta* forests and tall grasslands; the eastern part of Nepal is covered with several species of oak and rhododendron; the western region is dominated by pine forests; and the northern Himalayan region consists of the broadleaved trees and conifers, depending on the altitudes.

Species diversity is an important asset of the country. Although, Nepal occupies only 0.1% of the total land mass in the world, it is home to 2% of the flowering plants, 3% of the pteridophytes, and 6% of the bryophytes of the world flora; and 3.9% mammals, 8.9% birds, and 3.75 of butterflies of the world fauna[4]. There are several flora and fauna that are endemic to Nepal, which includes 284 flowering plants, and 160 animal species. The genetic diversity of the flora and fauna of Nepal has not been explored extensively. The wide-ranging diversity in geographic conditions and species richness all indicate a potentially rich genetic diversity. Some initiatives have been made to study and conserve this diversity, such as the establishment of the gene bank in 2010 at Nepal Agricultural Research Council (NARC).

Despite the prevalence of an extremely rich biodiversity, including several rare, endangered, and endemic species, the biodiversity of Nepal is under serious threat. The 5th National Report to Convention on Biological Diversity of Nepal, has outlined the major risks to the biodiversity as: i) ignorance of the value of biodiversity in government, ii) poverty and lack of other livelihood opportunities, iii) population growth and migration, iv) weak forest governance, v) inadequate awareness, and vi) climate change to name a few[3].

Several conservation efforts are also being made by various governmental, non-governmental, and international organizations. Currently, 23.23% of the total area of the country has been designated as protected areas, including national parks, wildlife reserves, hunting reserves, conservation areas, and buffer zones. Community forestry is another major initiative [5] to protect the biodiversity and increase sustainability of forest resources. Since 2010, 133,579 hectares of forest areas have been designated as protected forest, to enhance biodiversity as well as the livelihoods of the local people[3]. Also, several awareness, education, and research programs are conducted to conserve and strengthen the biodiversity of the country. Nepal has also committed to "Aichi Targets", which is a set of 20 targets for biodiversity conservation, that were developed in the 10th meeting of Conference of Parties' (COP-10) in Aichi, Nagoya, Japan[6].

1.2 Mushrooms of Nepal

The richness of the fungal diversity of Nepal is a mere reflection of its opulent biodiversity. The mycobiota of Nepal has been studied and recorded by researchers from Nepal as well as several other parts of the world [7]. Major part of the research conducted so far has focused on taxonomy, ethnomycology, ecological distribution patterns, mycorrhizal asscociation, and toxicity. Some 608 genera and 2025 species of Nepalese mycobiota have been reported. Among them, 270 genera and 1150 species (157 species in Ascomycota and 993 species in Basidiomycota) have been recorded as mushrooms. Thirty two species of these mushrooms are known to be endemic to Nepal. The mushroom species have been further classified into around 147 edible species, nearly 100 poisonous species, and 73 medicinal species. Furthermore, some 20 species have also been documented for aesthetic or decorative purposes[7]. However, it must be noted that extensive exploration of mushrooms all over Nepal is still an ongoing process. Most of the research so far seems to be concentrated on the central region of Nepal. So, further investigations shall elucidate the species diversity even more elaborately.

The macrofungal population is immensely affected in terms composition and diversity by the surrounding environment such as nutrients, moisture, forest type, climatic conditions, and season. Baral et al. [8] studied the diversity of macrofungi in the *Shorea robusta* forests in the mid-hill region of central Nepal. Within the study area, they found Polyporaceae to be the most abundant family, followed by Clavariaceae. *Coltricia cinnamomea*, *Cantharellus leucocomus*, *Laccaria laccata*, and *Russula aurora* were some of the most common species. Also, some researchers have identified 69 species of wild mushrooms from Sagarmatha National Park, in the northeastern region of Nepal[9]. Among them, the most abundant number of species belonged to Boletaceae family, followed by Russulaceae family.

The collection, consumption and trade of mushrooms has deep rooted ties with several ethnic communities in Nepal. In some communities it is an important resource for livelihood[10], whereas in some communities it is denounced and a subject of taboo [11]. Few reports can be found on the ethnic use of mushrooms in various parts of Nepal. Devkota [12] identified 44 species of mushrooms, including 5 medicinal species such as *Morchella conica*, *Morchella esculenta*, *Morchella elata*, *Lycoperdon pyriforme*, and *Cordyceps sinensis* from the mid-western highlands of Dolpa district in Nepal. Some other ethno-medicinal uses of mushrooms in Nepal have been reported by Adhikari et al. [11], which includes the use of *Grifola frondosa* and *Ramaria botrytis* to relieve muscle pain; and *Coriolus hirsutus*, *Lycoperdon pyriforme* and other species to treat wounds. Christensen et al. [13] documented the use of 228 species of wild edible mushrooms in Nepalese households, across different ethnic groups. They have identified *Boletus edulis, Cantharellus cibarius, Craterellus cornucopioides*, and *Lactarius deliciosus* as species with international trade potential.

Apart from the consumption and use of the mushrooms available in the wild, some species are also cultivated and are a good source of income for local farmers. Some of the most widely cultivated and marketed species include *Agaricus bisporus*, *Pleurotus ostreatus*, and *Lentinula edodes*[14–16]. Since 2004, NARC has also conducted several research related to the cultivation of *Ganoderma lucidum*[17].

Cultivation of *Ganoderma lucidum* at NARC

1.3 Nepal Mushroom Project

Mushrooms have been a part of the human diet since a very long time. Recently, they have attracted a lot of attention as a reservoir of bioactive compounds. The therapeutic benefits of mushrooms have been realized and documented since ancient times in several parts of Asia, mainly in China, Japan, and Korea[18, 19]. However, with increasing research showing promising potential, there has been a global surge in the cultivation and mass production of a specialized group of mushrooms, popularly termed as “medicinal mushrooms”.

Nepal’s unique geographic location, and climatic conditions has contributed to its very rich biodiversity, including some rare natural medicinal resources. Despite, the availability of huge amount of genetic resource, the lack of research and awareness poses a threat to their sustainability; and subsequently to the recognition of their bioactive potential. Nepal is home to more than a 1000 documented species of mushrooms[7], many of them growing in the extreme environmental conditions in the Himalayas. However, the bioactive potential of these mushrooms remain largely unknown.

From an agricultural point of view, the market for mushrooms in Nepal is still in its infancy. Apart from a handful of species including *Agaricus bisporus* and *Pleurotus ostreatus* that are used for consumption, there is a severe lack of variety in the Nepalese mushroom market. Therefore, an in depth research into the therapeutic potential of the mushrooms can contribute to highlight the extremely rich genetic resources in the country, as well as make way for the artificial cultivation of those mushrooms, to enrich the agricultural resources of the local farmers. Consequently, expanded usage of Nepalese mushrooms can be explored, not only for consumption, but also as a source of functional food or functional food ingredients.

Considering the above mentioned situation, the “Nepal Mushroom Project” was planned. All necessary procedures required to collect and analyze Nepalese genetic resources were

implemented, before the initiation of the project. Kyushu University and the Ministry of Science and Technology of the Government of Nepal signed a Memorandum of Understanding (MoU) as prior informed consent (PIC) in 2010. Subsequently, Kyushu University signed a joint research agreement with Nepal Agricultural Research Council (NARC) consisting of mutually agreed terms (MAT) according to the spirit of the international treaty "Convention on Biological Diversity" in July 2011. The project was then started with the aim to search for new usage of Nepalese mushrooms, including development of functional foods, leading to economic benefit in Nepal.[20].

Until now, Nepal has been famous as one of the exclusive habitat for the most sought after mushroom *Ophiocordyceps sinensis*. This has led to the over harvesting of the species threatening its sustainability in the long run [21]. However, the results of the current project could be useful in highlighting the fact that Nepal possesses several other equally if not more therapeutically potent mushroom species. The research papers that have been published so far from the results of this project have shown that Nepalese wild mushrooms have very promising antioxidant and antibacterial properties[22–24]. Moreover, strong effects on melanin biosynthesis, anti-allergy activity, and anti-cancer activity has been observed for various samples. The recognition of the therapeutic as well as the economic potential of the Nepalese wild mushrooms as functional food resources has just begun. We hope that our research will continue to shed more light into the unparalleled capabilities of these mushrooms.

1.4 Bioactivities and chemical components of mushrooms

Mushroom is defined as “a macro fungus with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand”[25]. Taxonomically, mushrooms mainly belong to the class basidiomycetes, with only a few species belonging to ascomycetes. An estimated 140,000 mushroom species exist in world, with a mere 22,000 known species[26]. The history of mushroom consumption, and its usage as traditional medicinal products can be traced back to ancient times. Fruiting bodies of around 200 species are known to be consumed worldwide. The nutritional composition of mushrooms generally comprises of 200-250 g/kg dry matter of proteins, 20-30 g/kg dry matter of lipids, and the remaining dry matter is composed of carbohydrates, minerals, and other constituents[27]. Apart from the basic nutrient contents, higher fungi such as mushrooms are known to produce a wide array of secondary metabolites, as a self-defense and survival tool. Bioactive compounds which are produced as secondary metabolites in mushrooms have proven to be important sources of therapeutic agents[28].

A number of different bioactivities have been attributed to various mushrooms over the years[26, 29–33], with more beneficial effects being discovered as research progresses. Antioxidant, antimicrobial, anti-viral, anti-cancer, anti-hypertensive, anti-diabetic activities are some of the health promoting effects described for mushrooms[34, 35]. This has led to the recognition of a special class of mushrooms, popularly known as “medicinal mushrooms”[36]. Roupas et al.[37] have summarized the studies on edible mushrooms and their components claiming health benefits, with a special focus on human trials. They concluded that the health benefits of mushrooms are mostly related to stimulation or modulation of natural cellular immunity; and many of these immunomodulatory effects arise from the polysaccharide content of mushrooms. Quang et al. have described a wide range of bioactivities including antioxidant,

antimicrobial, nematicidal, anti-human immunodeficiency virus from the bioactive metabolites originating from 22 species of inedible mushrooms[38].

Beta glucans, such as those isolated from *Lentinus edodes* and *Grifola frondosa*; and polysaccharide protein complexes from *Trametes versicolor* were found to inhibit the proliferation of cancer cells[39], and have also been used as adjuvants in cancer therapy[40]. Phenolic compounds are the main source of antioxidants in mushrooms[27]. Moreover, the phenolic compounds along with terpenes, organic acids, and various proteins are also known to contribute for bioactivities like antibacterial activities[41]. Antibacterial activity of mushroom extracts, fractions, and isolated compounds could be detected against several bacteria causing nosocomial infections such as *Pseudomonas aeruginosa*, *Staphylococcus aureus, Klebsiella pneumonia*; as well as multi-drug resistant bacteria[41]. The triterpenoids and related compounds isolated from mushrooms like *Ganoderma lucidum* (Reishi in Japanese), have shown cytotoxic activity against various cancer cells. The fruiting bodies, mycelia, and spores of this species alone has been a source of more than 130 highly oxygenated and pharmacologically active lanostane-type triterpenoids[28]. Apart from the several therapeutic benefits, the commercial potential of mushrooms as cosmetic agents has also been explored by several researchers[42–44].

Therefore, the enormous therapeutic and commercial potential of mushrooms, and the stark lack of research of the bioactivities of mushrooms from Nepal, a country touted as an important biodiversity hotspots, necessitates an in depth research.

Chapter 2

Mushroom Samples

2.1 Introduction

Mushrooms which developed basidiomata were collected from the forests in different parts of Nepal. In this study 92 samples were investigated. The botanical origin, location and habitat of the samples are provided in Table 2.1 (a – d). The pictures of the dried samples are also provided as supplementary data in Appendix Fig A2.1.

2.2 Materials and methods

2.2.1 Identification

The samples were identified on the basis of morphological features and/or genetic analysis. The dried fungal material were mounted in 3% (w/v) KOH or Melzer's reagent and measurements of spore, basidia, cystidia, and other tissue features were made for each specimen using Nikon Eclipse E600 stereomicroscope. For genetic analysis, the DNA was extracted following the procedure described by Hosaka and Castellano [45], with slight modifications. The internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using the primers ITS1 and ITS4B primers [46, 47]. Amplifications were performed with KOD-Plus-Neo polymerase (Toyobo Co. Ltd., Japan). The PCR products were purified using Illustra Exo ProStar kit (GE Healthcare, UK). Both PCR reactions and purification steps were performed in accordance with the manufacturer's protocol. The purified samples were sequenced by the Hokkaido System Science Co, Ltd. The sequences were subjected to a Basic Local Alignment Search Tool (BLAST) search via the National Center for Biotechnology Information. The ITS sequence of the samples that were successfully sequenced, were deposited in the International Nucleotide Sequence Database Collaboration (INSDC) or

DNA Data Bank of Japan (DDBJ). The scientific names and their taxonomic positions used in the present study were described in accordance with the descriptions of the Mycobank.

2.2.2 Sample Preparation

The samples were firstly air dried and then dried in an air ventilated oven at 35 °C for 10 hours followed by 45 °C for 1 hour. The dried samples were ground to a fine powder, and extracted in ethanol. The extractions were performed by shake flask method in an orbital shaker at 200 rpm, for 24 hours at room temperature, and then filtered. Water extracts were freeze-dried and the ethanol extracts were rotary evaporated at 45 °C, and reduced pressure. The extract yield was calculated as the percentage of dried extract obtained from the dry weight of ground mushroom used for extraction. The dried extracts were used for all the analyses.

2.3. Result and discussion

2.3.1 Sample identification

The mushroom samples were identified up to species or genus level on the basis of morphological and/or genetic analyses. The scientific names of the samples, along with the families to which they belong, location, and habitat are listed in Table 2.1 (a - d). The INSDC/ DDBJ accession number of the genetically identified samples are also provided. The 92 samples collected were identified as belonging to 40 different genera. The identity of one of the sample could not be confirmed by both genetic and morphological analyses.

The samples have been grouped into 4 groups depending on the taxonomic order: Hymenochaetales, Polyporales, Agaricales, and the few remaining miscellaneous samples were grouped as “Others”. The highest number of samples belonged to Polyporales with 40 samples, followed by Hymenochaetales with 20 samples, Agaricales with 18 samples, and Others with 14 samples.

The samples were collected from locations ranging from 1300 m to 3800 m from different parts of Nepal. The sample collection sites are shown in Fig. 2.1. The habitat of the samples were mainly wood from living, dead and decayed trees, and also some samples were collected from soil, fallen leaves and branches. The habitat and other environmental conditions of the mushrooms are very important, since it can greatly influence the difference in production of secondary metabolites even within the same strains [48].

2.3.2 Sample preparation

The extract yield of the ethanol extracts have been provided in Table 2.1 (a - d). The extract yields varied from 0.7% to 16.89% for Hymenochaetales; 0.69% to 17.00% for Polyporales; 2.94% to 13.23% for Agaricales; and 2.40 to 14.61% for Others. The wide variations in the extract yield with each group suggests that the amounts of compounds that can be dissolved in ethanol are not dependent on the taxonomic order.

2.4 Conclusion

Mushrooms samples representing the mycoflora of different parts of Nepal were identified and extracted for further investigations. Although the samples consisted of a small fraction of the very rich mushroom diversity of Nepal, this study is the first attempt of its kind to make a concerted study on the mushrooms across Nepal. Further collection and investigation of mushrooms from other parts of the country would be necessary for a deeper understanding of their potential.

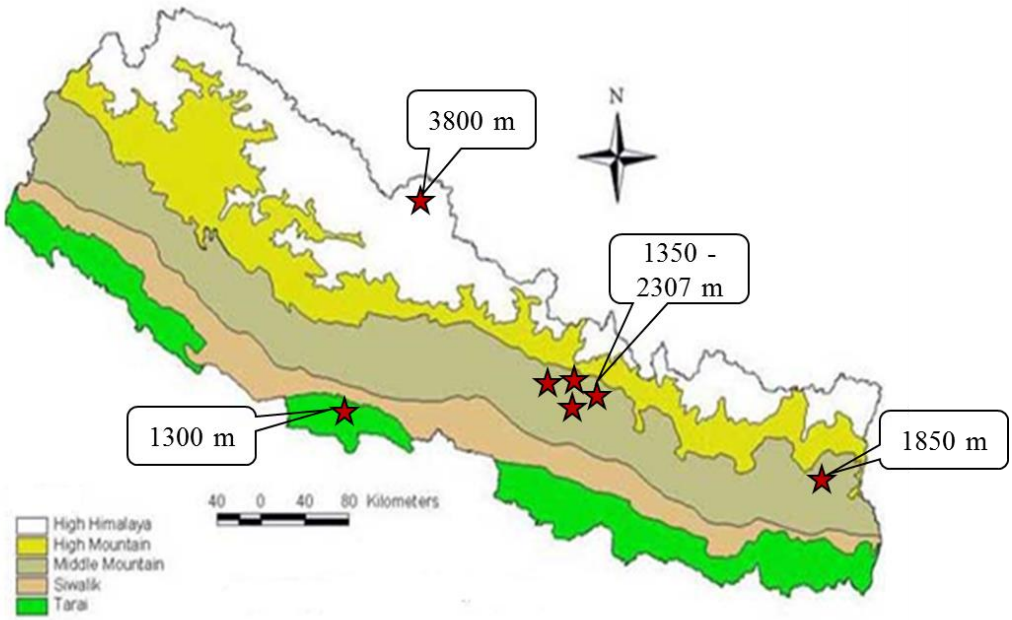


Fig. 2.1 Sample collection sites (Map source: Soil Science Division, NARC)

Table 2.1 (a – d): Botanical origin, location, habitat and yield% of ethanol (EtOH) extracts of 92 wild mushrooms from Nepal

a. Hymenochaetales

S.N.	Scientific name	Family	Location	Altitude	Habitat	INSDC/DDBJ No.	Yield% EtOH
1	*Inonotus andersonii*	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2765 m	Soil	AB811856	3.04
2	*Inonotus clemensiae*	Hymenochaetaceae	Kathmandu,Dawachok	1500 m	stump	-	16.89
3	*Inonotus cuticularis*	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	5.19
4	*Inonotus* sp. 1	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2765 m	Living tree	-	4.73
5	*Inonotus* sp.2	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	-	1.49
6	*Inonotus* sp. 3	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB811865	6.03
7	*Inonotus* sp. 4	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2307 m	oak wood	-	1.73
8	*Inonotus* sp. 5	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2307 m	oak wood	-	1.90
9	*Phellinus gilvus*	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB811862	4.05
10	*Phellinus conchatus* 1	Hymenochaetaceae	Bhaktapur, Nagarkot	2500 m	Decayed wood	AB811863	0.81
11	*Phellinus conchatus* 2	Hymenochaetaceae	Bhaktapur, Nagarkot	2500 m	Decayed wood	AB811864	0.50
12	*Phellinus* sp. 1	Hymenochaetaceae	Makwanpur,Chitlang	2200 m	wood	-	1.25
13	*Phellinus* sp. 2	Hymenochaetaceae	Mustang,Jomsom	3800 m	dead wood	-	2.10
14	*Phellinus adamantinus*	Hymenochaetaceae	Kathmandu,Dawachok	1501 m.	dead wood	LC149611	0.72
15	*Cyclomyces setiporus* 1	Hymenochaetaceae	Makwanpur,Chitlang	2200 m	wood	-	1.91
16	*Cyclomyces setiporus* 2	Hymenochaetaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	1.96
17	*Cyclomyces setiporus* 3	Hymenochaetaceae	Makwanpur,Daman	2320 m	wood	-	0.64
18	*Cyclomyces setiporus* 4	Hymenochaetaceae	Makwanpur,Chitlang	2200 m	dead wood	-	1.11
19	*Cyclomyces setiporus* 5	Hymenochaetaceae	Makwanpur,Chitlang	2200 m	dead wood	-	1.20
20	*Oxyporus* sp.	Schizoporaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	-	6.14

b. Polyporales

S.N.	Scientific name	Family	Location	Altitude	Habitat	DDBJ / INSDC Accession No.	Yield% (EtOH)
21	*Ganoderma australe* 1	Ganodermataceae	Mustang	3150 m	Decayed wood	AB811849	2.52
22	*Ganoderma australe* 2	Ganodermataceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB811850	1.18
23	*Ganoderma australe* 3	Ganodermataceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	-	2.88
24	*Ganoderma australe* 4	Ganodermataceae	Kathmandu, Dawachok	1500 m	Decayed wood	AB811852	1.05
25	*Ganoderma lingzhi* 1	Ganodermataceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	3.43
26	*Ganoderma lingzhi* 2	Ganodermataceae	Makwanpur,Chitlang	2200 m	dead wood	LC149597	4.90
27	*Ganoderma lingzhi* 3	Ganodermataceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB811848	5.02
28	*Ganoderma endochroum*	Ganodermataceae	Makwanpur,Chitlang	2200 m	dead wood	-	3.01
29	*Ganoderma multipileum*	Ganodermataceae	Rupandehi,Lumbini	1300 m	dead wood	LC149613	2.19
30	*Ganoderma carnosum*	Ganodermataceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB764438	5.00
31	*Ganoderma* sp.1	Ganodermataceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	1.39
32	*Ganoderma* sp.2	Ganodermataceae	Bhaktapur,Nagarkot	2,195 m	wood	-	6.89
33	*Amauroderma calcigenum*	Ganodermataceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	2.53
34	*Trichaptum biforme*	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	-	1.23
35	*Trichaptum abietinum*	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	1.22
36	*Trametes versicolor*1	Polyporaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB811855	1.42
37	*Trametes versicolor*2	Polyporaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	AB811857	0.69
38	*Trametes versicolor*3	Polyporaceae	Lalitpur,Mt.Phulchoki	2765 m	Living tree	AB811860	5.00
39	*Trametes versicolor*4	Polyporaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed branch	AB811867	3.85
40	*Trametes versicolor*5	Polyporaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed branch	AB811868	1.34
41	*Microporus xanthopus*1	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC149598	1.11
42	*Microporus xanthopus*2	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC149595	1.08

Polyporales (contd.)

S.N.	*Scientific name*	Family	Location	Altitude	Habitat	DDBJ / INSDC Accession No.	Yield% (EtOH)
43	*Polyporus arcularius*	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	LC150016	5.28
44	*Postia stiptica*	Fomitopsidaceae	Kathmandu, Dawachok	1500 m	Decayed wood	AB811853	17.00
45	*Phlebia tremellosa*1	Meruliaceae	Lalitpur,Mt.Phulchoki	2765 m	Soil	AB811854	5.10
46	*Phlebia tremellosa*2	Meruliaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	LC149601	2.23
47	*Lenzites betulina*	Polyporaceae	Lalitpur,Mt.Phulchoki	2765 m	Soil	AB811866	1.04
48	*Rigidoporus* sp.	Meripilaceae	Bhaktapur, Surya Binayak	1400 m	Decayed wood	-	4.70
49	*Laetiporus versisporus* 1	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC150014	4.53
50	*Laetiporus versisporus* 2	Polyporaceae	Makwanpur,Chitlang	2,200 m	dead wood	-	7.36
51	*Laetiporus montanus*	Polyporaceae	Lalitpur,Jawalakhel	1,970 m	dead wood	-	5.68
52	*Mycorrhaphium* sp.	Meruliaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	-	0.79
53	*Grifola frondosa*	Meripilaceae	Lalitpur,Godawari	1515 m.	dead wood	-	4.95
54	*Lentinus* sp.	Polyporaceae	Kathmandu,Dawachok	1500 m.	dead wood	-	4.68
55	*Bjerkandera adusta*	Meruliaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC150020	2.63
56	*Antrodiella zonata* 1	Phanerochaetaceae	Makwanpur,Chitlang	2,200 m	wood	LC149604	1.05
57	*Antrodiella zonata* 2	Phanerochaetaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	LC149607	1.61
58	*Antrodiella zonata* 3	Phanerochaetaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	3.49
59	*Fomes fomentarius*	Polyporaceae	Lalitpur,Mt.Phulchoki	2307 m	oak wood	LC149605	4.26
60	*Abortiporus biennis*	Meruliaceae	Bhaktapur,Nagarkot	2195 m	wood	LC149599	2.93

c. Agaricales

S.N.	Scientific name	Family	Location	Altitude	Habitat	DDBJ / INSDC Accession No.	Yield% (EtOH)
61	*Lentinula edodes*1	Marasmiaceae	Makwanpur,Daman	2320 m	oak wood	LC149603	3.98
62	*Lentinula edodes*2	Marasmiaceae	Lalitpur,Mt.Phulchoki	2307 m	oak wood	LC149606	2.94
63	*Pleurotus ostreatus*1	Pleurotaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	LC150018	3.66
64	*Pleurotus ostreatus*2	Pleurotaceae	Dhankuta,Rajarani	1850 m	wood	-	4.84
65	*Pleurotus ostreatus*3	Pleurotaceae	Dhankuta,Rajarani	1850 m	wood	-	4.94
66	*Pleurotus ostreatus*4	Pleurotaceae	Lalitpur, Khumaltar,	1350 m	straw	LC149608	4.16
67	*Pholiota nameko*1	Strophariaceae	Makwanpur,Daman	2320 m	wood	LC149602	7.75
68	*Pholiota nameko*2	Strophariaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	LC149600	5.02
69	*Marasmius mavium*	Marasmiaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	8.16
70	*Marasmius sp.*	Marasmiaceae	Makwanpur,Chitlang	2200 m	dead wood	-	8.75
71	*Panellus edulis*	Mycenaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	LC150017	13.23
72	*Panellus* sp.	Mycenaceae	Lalitpur,Mt.Phulchoki	2765 m	Decayed wood	-	6.20
73	*Inocybe* sp. 1	Inocybaceae	Lalitpur,Mt.Phulchoki	2765 m	Soil	-	5.35
74	*Inocybe* sp.2	Inocybaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC150015	6.08
75	*Collybia peronata*	Tricholomataceae	Bhaktapur, Nagarkot	2500 m	Fallen leaves	-	3.89
76	*Tricholoma caligatum*	Tricholomataceae	Lalitpur,Mt.Phulchoki	2765 m	Soil	-	12.32
77	*Mucidula mucida*	Physalacriaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC149596	5.19
78	*Gymnopus* sp.	Marasmiaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC149609	4.41

d. Others

S.N.	Scientific name	Family	Location	Altitude	Habitat	DDBJ / INSDC Accession No.	Yield% (EtOH)
79	*Heterobasidion lingzhiense* 1	Bondarzewiaceae	Bhaktapur, Surya Binayak	1400 m	Living tree	AB811859	4.36
80	*Heterobasidion lingzhiense* 2	Bondarzewiaceae	Lalitpur,Mt.Phulchoki	2765 m	Living tree	AB811861	2.40
81	*Lactarius hatsudake*	Russulaceae	Mustang	3150 m	Soil	-	6.04
82	*Lactarius* sp.	Russulaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC150021	3.60
83	*Russula brevipes*	Russulaceae	Bhaktapur,Surya Binayak	1955 m	dead wood	-	7.67
84	*Engleromyces goetzii*	Xylariaceae	Bhaktapur,Surya Binayak	1955 m	dead wood	-	6.54
85	*Xyloborus princeps*1	Stereaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	LC150019	3.67
86	*Xylobolus princeps*2	Stereaceae	Lalitpur,Mt.Phulchoki	2307 m	wood	-	2.65
87	*Xylobolus princeps*3	Stereaceae	Makwanpur,Daman	2320 m	wood	-	2.56
88	*Xylobolus princeps*4	Stereaceae	Lalitpur,Mt.Phulchoki	2307 m	dead wood	-	2.80
89	*Pseudomerulius curtisii*	Tapinellaceae	Kathmandu,Dawachok	1502 m	dead wood	LC149612	14.61
90	*Cantharellus ferruginascens*	Cantharellaceae	Bhaktapur,Surya Binayak	1955 m	dead wood	-	4.08
91	*Neolentinus lepideus*	Gloeophyllaceae	Mustang,Jomsom	3800 m	dead wood	LC149610	5.41
92	*Stereum* sp.*	Stereaceae	Lalitpur,Mt.Phulchoki	2307 m	Dead wood	-	3.00

*identification unconfirmed

Chapter 3

Antioxidant Activity

3.1 Introduction

Free radicals such as the reactive oxygen species (ROS) are formed as a byproduct of normal metabolic processes such as electron transport chain reactions [49]. Under normal conditions a fine balance is maintained between the production of the ROS and its elimination by the anti-oxidative system of the body [50]. However, certain conditions such as excessive exercise [51], chronic inflammation, exposure to pollutants and other xenobiotic substances cause a disturbance in this balance [52]; leading to a condition commonly known as oxidative stress. Oxidative stress can have detrimental effects on cellular lipids, proteins, and DNA; consequently leading to a number of diseases like diabetes, Alzheimer's, cancer, and other cardiovascular and neurological diseases [53].

Dietary antioxidants can play an important role to mitigate the unfavorable effects of oxidative stress [54]. Moreover, due to the ambiguity in the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) for being potentially carcinogenic [55], the prominence of the natural antioxidants from food sources has been heightened even further.

One of the potential sources of dietary antioxidants is mushrooms. Mushrooms have been valued not only for their nutritional properties [27, 56] but also for various medicinal benefits [26, 30, 57]. In recent years a lot of research has been directed towards elucidating the therapeutic capabilities of a wide variety of mushrooms; antioxidant properties being one of the most important among them.

Nepal possesses a rich resource of a wide variety of mushrooms, in different parts of the country [7]. However, apart from consumption of a few species, and some traditional medicinal uses [13], the therapeutic value of Nepalese mushrooms remains largely unexplored. The

present study aims to unveil the antioxidant potential of the wild mushrooms of Nepal; to promote their usage as therapeutic agents or nutraceuticals.

3.2 Materials and method

3.2.1 Total Phenolic Content (TPC)

The total phenolic content was determined by the Folin Ciocalteau method [58], with some minor modifications. In a 1.5 mL microfuge tube, 50 µL of the sample solution or standard or sample solvent (blank) and 100 µL of 10% Folin Ciocalteau reagent was mixed thoroughly. After 2-3 minutes, 400 µL of 7.5% Na_2CO_3 was added, and vortexed for a few seconds. The reaction mixture was incubated at room temperature for 60-90 minutes, and then centrifuged at 6000 rpm for 2 minutes. Two hundred microliters of the supernatant was transferred to the respective wells in a 96-well plate, and the absorbance was read at 765 nm using Molecular Devices FlexStation 3 Microplate Reader. Gallic acid was used as the standard; and the results are expressed as mg gallic acid equivalent (GAE)/g of extract.

3.2.2 Free radical scavenging assays (ORAC, DPPH, and ABTS)

The ORAC assay measures the fluorescence degradation of the fluorescein compound due to the peroxyl radicals generated by the heat treatment of AAPH solution. Antioxidants protect the fluorescein from this oxidative degradation. The method described by Ou et al. was followed with some minor modifications [59]. The extracts were dissolved in 75 mM phosphate buffer (pH 7.4). In a 96 well plate, 20 µL of the sample solution, phosphate buffer (blank), and trolox (standard) solutions were added into the respective wells. Two hundred microliter of fluorescein solution was then added to each well, and the plate was incubated at 37°C for 10 minutes. After incubation, 75 µL of pre-warmed AAPH solution was added, and the fluorescence degradation was measured over a period of 90 minutes at 30 seconds interval using Molecular Devices Flex Station 3 Microplate Reader. The excitation and emission

wavelengths were 485 nm and 535 nm, respectively. After the fluorescence degradation, the values for area under the curve (AUC) were obtained from SoftMax® Pro Data Acquisition & Analysis Software. The AUC value of the blank was reduced from that of the samples as well as the standards. Standard curve was prepared using 0 to 25 µmol trolox solutions. The results are expressed as µmol Trolox Equivalent (TE)/g extract.

The ability of the samples to scavenge the DPPH radicals was determined by following the method described by Miliauskas [60], with some minor modifications. A 1 mL portion of DPPH solution (60 µM), freshly prepared in methanol, was mixed with 33 µL of the methanolic sample solution or methanol (blank). Sample concentration of 2.5 mg/ mL ethanol extract was used. The reaction mixture was then incubated at 37 °C for 20 minutes, in dark condition. The decolorization was monitored by checking the absorbance using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan) at 515 nm. The inhibition percentage was calculated using the following equation

$$\text{Inhibition } \% = [(A_b - A_s)/ A_b] \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where A_b and A_s are the absorbance of the reaction mixtures containing the blank and the samples respectively.

The free radical scavenging ability of the mushroom extracts was also tested using the ABTS assay, following the method described by Zhu et al [61]. The ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting 5 mL of aqueous ABTS solution (7 mM) with 88 µL of potassium persulfate ($K_2S_2O_8$), followed by incubation for 12-16 hours at room temperature in dark condition. The working solution was then prepared by adjusting the absorbance at 0.7 ± 0.02 at 734 nm. A 1 mL portion of the working solution was mixed thoroughly with 10 µL of the sample solution or sample solvent (blank). Sample concentration of 2 mg ethanol extract/ mL

was used. After 4 minutes incubation at 30 °C, the absorbance was read at 734 nm. The percentage of inhibition was calculated by the above formula in equation (1).

3.2.3 Reducing power (RP) assay

The reducing power of the extracts were tested by the method described by Oyaizu [62], with minor modifications. In a 1.5 mL microfuge tube, 100 µL of the sample solution or sample solvent (blank) was mixed with 100 µL of phosphate buffer (200 mM; pH 6.6) and 100 µL of 1% potassium ferricyanide solution. Sample concentration of 1 mg/mL ethanol extract was used. The reaction mixture was placed in a water bath at 50 °C for 20 minutes, followed by rapid cooling in ice bath, and addition of 100 µL of trichloroacetic acid solution (10%). It was then centrifuged at 3000 rpm for 10 minutes. A 100 µL portion of the supernatant was added to the respective wells in the 96 well plate and mixed with 100 µL of ultrapure Milli-Q water, and 20 µL of 0.1% $FeCl_3$ solution. It was then incubated at room temperature in dark condition for 10 minutes, after which the absorbance was measured at 700 nm using the Molecular Devices Flex Station 3 Microplate reader. The reducing power is expressed as the absorbance reading.

3.2.4 Indicators for antioxidant activity

The top 10 samples with the highest TPC were selected for the determination of the EC_{50} values for DPPH, and ABTS assays; and $EC_{0.5}$ for RP assay. The sample concentration resulting in 50% inhibition in DPPH and ABTS assays, and 0.5 absorbance value in RP assay were considered as EC_{50} values and $EC_{0.5}$, respectively [63]. The inhibition percentage for the DPPH and ABTS assays and the absorbance for RP assay were plotted against various sample concentrations, and the equations thus obtained were used to calculate the EC_{50} and $EC_{0.5}$ values.

3.2.5 Statistical analysis

All the assays were conducted at least 3 times, and the results are expressed as mean ± standard deviation. Significant differences between sample groups, grouped on the basis of their taxonomic order, were analyzed by Kruskal Wallis test followed by Dunn-Bonferroni test. Correlation between the total phenolic content and the antioxidant assays were calculated by Spearman's rank correlation. The statistical analyses were performed using SPSS statistics Version 23. The *p*-value less than 0.05 were considered statistically significant.

3.2.6 HPLC analysis

The HPLC analysis was done for the top ten samples with the highest TPC, using the 1220 Infinity LC system, Agilent Technologies, equipped with diode array detector, and fitted with YMC Triart C18 column (250 mm x 4.6mm i.d, 5 µm particle size). The method described by Kim et al. [64] was followed after some modifications. The solvent system comprised of water with 0.15% formic acid as solvent A and acetonitrile with 0.15% formic acid as solvent B. The analysis was carried out at room temperature with a flow rate of 1 mL/min. The gradient flow of the mobile phase was set as: 0 – 12 min, 5 - 15% B; 12 – 18 min, 15 - 17% B; 18 – 20 min, 17 - 20% B; 20 – 35 min, 20 - 25% B; 35 – 40 min, 25 - 40% B; 40 – 60 min, 40 - 42% B; 60 – 68 min, 42 - 90% B; 68 – 70 min, 90 - 100% B; 70 – 72 min, 100% B; 72 – 75 min, 100 – 5% B; and 75 to 85 min, 5% B. The preferred wavelength of detection was 280 nm and the UV-Vis spectra were recorded from 190 to 400 nm. The peaks obtained from the ethanol extracts of mushrooms were compared to the chromatogram of 21 standard phenolic compounds with respect to the retention time and UV-vis spectra. Phenolic compounds present in extracts were quantified by the preparation of standard curves for each standard compound.

3.2.7 LC-MS analysis

LC-MS analysis was done to elucidate the unknown compounds from the HPLC analysis. LC-MS-IT-TOF, Shimadzu, Tokyo was used for the analysis. All the chromatographic conditions

were the same as for HPLC analysis, apart from the flow rate which was reduced to 0.5 mL/min to maintain pressure within permissible limits of the device. For MS, ESI source was used in positive and negative ionization mode with m/z values of 100-2000 for MS and 50-1500 for MS/MS. A probe voltage of ± 4.5 kV, nebulizer gas flow of 1.5 L/min, CDL temperature of 200 °C, and heat block temperature of 200 °C were used.

3.3 Result and discussion

Ethanol extracts of the sixty two samples were tested for their anti-oxidative activity by using four kinds of assays based on the radical scavenging and reducing capabilities. The samples were grouped into four groups, based on their taxonomic order: Hymenochaetales, Polyporales, Agaricales and Others. Since the majority of the samples belonged to the first three groups, the few remaining samples were grouped as "Others". The results for all the assays are listed out in Table 3.1

3.3.1 Total phenolic content

The total phenolic content was measured using the Folin-Ciocalteau method. Samples in the Hymenochaetales group showed a significantly greater phenolic content compared to the other 3 groups. However, *Oxyporus* sp. was a notable exception in this group with the TPC value of only 7.9 mg GAE/g extract. *Oxyporus* sp. is the only sample that does not belong to the Hymenochaetaceae family, within this order. Although there is a lack of detailed research into this mushroom, the major compounds are reported to be sterols and triterpenes in *Oxyporus populinus* [65]. The highest phenolic content was seen for *Inonotus clemensiae* with 643.2 mg GAE/g extract. The TPC of *Inonotus clemensiae* was higher than that reported for the ethanol extract of *Inonotus obliquus* from Thailand with 590.87 mg GAE/g extract [66], which is one of the highest phenolic content reported for mushrooms. The *Ganoderma* species exhibited the highest TPC values within Polyporales group. The lowest phenolic content was seen in Agaricales, with *Mucidula mucida* showing the least TPC value of 0.4 mg GAE/g extract. Some

samples in the Others group, such as *Pseudomerulius curtisii* and *Xylobolus princeps* 4 showed a relatively high phenolic content of 131.6 mg GAE/g extract and 126.6 mg GAE/g extract respectively.

The major antioxidant compounds found in mushrooms belong to the phenolic group [67]. Reducing agents like ascorbic acid are sometimes known to contribute for the seemingly elevated values [68]. However, total phenolics continue to thrive to be good indicators of the anti-oxidative activity.

3.3.2 Free Radical scavenging assay (ORAC, DPPH, and ABTS)

The radical scavenging capacity of the extracts was studied using the ORAC, DPPH and ABTS assays. Again, Hymenochaetales group showed a much higher ORAC activity compared to Polyporales and Agaricales. The highest ORAC value was shown by *Inonotus clemensiae* (31966.9 µM TE/g extract), which outperformed the previously reported highest ORAC value [22] for *Inonotus andersonii* (21015 µM TE/g extract). Significant differences between groups were not seen for Polyporales, Agaricales and Others. In the Others group *Pseudomerulius curtisii* showed a very high ORAC value of 11204.9 µM TE/g extract. The ORAC assay is one of the few assays that monitors the free radical scavenging from the time of sample addition, throughout regular intervals, until the completion of reaction. This enables complete assessment of the reaction providing information about the inhibition time as well as degree of inhibition [69].

The Hymenochaetales group also outperformed the rest of the groups in the DPPH and ABTS assays. However, *Oxyporus* sp. remained as a notable exception within the group, with negligible DPPH and ABTS radical scavenging activity. In the Polyporales group, several *Ganoderma* species showed high DPPH inhibition percentage, with *Ganoderma* sp.1 showing the highest inhibition percentage of 90.9%. Agaricales showed the least DPPH inhibition. In

the Others group, a very high DPPH inhibition percentage was seen in *Pseudomerulius curtisii* (89.0%) and *Xylobolus princeps* (4,2,3,1) (83.3 to 86.3 %).

In the case of ABTS assay, *Inonotus clemensiae* showed the highest inhibition percentage of 92.2%. However, other *Inonotus* sp. could not show comparable activity. Also, considerable variations were seen among the *Ganoderma* sp. of the Polyporales group (6% to 63.2%). In the Others group, high ABTS activity was observed for *Pseudomerulius curtisii* (53.0%) and *Xylobolus princeps* (4,2,3,1) (44.1 to 56.7 %).

The DPPH and ABTS assays are known for its reproducibility, ease of application, and low cost [53]. However, attempts to check the activity at maximum dissolved concentration were hindered due to sample color interference, especially for highly pigmented samples. Although reports for the phenomenon of color interference with regard to mushrooms could not be found, it has been reported for plant extracts [70, 71], especially for DPPH assay. While performing these assays for extracts with unknown composition, it has been recommended to avoid using sample concentrations greatly exceeding the concentrations of DPPH and ABTS solutions [72].

3.3.3 Reducing power (RP)

The ability of the extracts to reduce the ferricyanide complex to its ferrous form was measured by the reducing power or sodium nitroprusside assay. Significant differences were seen between Hymenochaetales, Polyporales and Agaricales groups. In the Hymenochaetales group, *Cyclomyces setiporus* (1,2,3,4,5) along with *Inonotus clemensiae* showed the highest RP values (1.64 to 2.20). Among Polyporales, *Ganoderma* sp.1 exhibited the highest RP value (1.22). Also, in the Others group *Pseudomerulius curtisii* and *Xylobolus princeps* showed relatively high RP values (1.02 to 1.19). A very low variation was seen in the reducing power values among all samples. This could be due to the lower sensitivity of the method compared to other

anti-oxidative assays. The reducing power of mushrooms has been attributed to their ability to donate hydrogen atoms thereby pacifying the free radicals [73].

3.3.4 Correlation between TPC and the antioxidant assays

In order to illustrate the relationship between the anti-oxidative activity and the phenolic content, the anti-oxidative assays were correlated with the TPC values for each group, using the Spearman's rank correlation. Table 3.2 shows the correlation coefficient between TPC and each of the anti-oxidative assays for all four groups. Significant correlations were observed for all the assays in Hymenochaetales, Polyporales, and Others. The highest degree of correlation was seen in Others group, followed by Polyporales and Hymenochaetales. However, in Agaricales only DPPH and TPC could correlate significantly. The major bioactive compounds in Agaricales are known to be ergosterol, lectin, terpenes, and β-glucans [74]. Also, it must be considered that the translation of the phenolic compounds into anti-oxidative activity is dependent on several factors such as the availability of hydroxyl group, and the synergistic, additive or antagonistic activities in the sample matrix [75].

3.3.5 Determination of EC_{50} and $EC_{0.5}$ values

To further clarify the activity of the mushroom samples showing the highest TPC, the EC_{50} values (for DPPH and ABTS assays) and $EC_{0.5}$ values (for reducing power assay) were determined. Table 3.3 shows the EC_{50} and $EC_{0.5}$ values expressed as sample concentration in mg/ml for DPPH and ABTS, and reducing power assays respectively. The lower EC_{50} / $EC_{0.5}$ values indicate stronger anti-oxidative activity. *Inonotus clemensiae* required the least sample concentration to obtain the EC_{50} values for both DPPH and ABTS assays, as well as $EC_{0.5}$ value in reducing power assay; with 0.081 mg/mL for DPPH assay, 0.409 mg/mL for ABTS assay, and 0.031 mg/mL for reducing power assay.

All of the antioxidant assays tested revealed a very high activity for the Hymenochaetales group. Although commonly studied species such as *Inonotus obliquus* have been extensively reported for their antioxidant and other biological activities, the antioxidant activities of *Inonotus clemensiae* and *Cyclomyces setiporus*, have not been reported previously. Within the Polyporales group samples of *Ganoderma* species exhibited a relatively higher activity than other members. The Others group contained a mixture of samples from different orders. However, antioxidant assays and the TPC correlated well in this group. Also, *Pseudomerulius curtisii* and *Xylobolus princeps* (4,2,3,1) consistently showed high activity for almost all the assays tested. Parallels can be observed between the pattern of anti-oxidative activity in the previous study of the antioxidant activities of 29 wild mushroom samples of Nepal [22], where *Inonotus* sp., *Phellinus* sp., and *Ganoderma* sp. had the most predominant anti-oxidative activity. These findings elucidate the presence of high antioxidant activity in previously unreported mushroom species, in Nepal.

3.3.6 HPLC analysis

In order to identify and quantify the phenolic compounds present in the ethanol extracts of top ten samples with the highest TPC values, HPLC analysis was performed. Twenty one phenolic compounds, reported to be commonly found in mushrooms [67], (5-sulfosalicylic acid, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid, chlorogenic acid, (+)-catechin, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, vanillin, rutin, *p*-coumaric acid, ferulic acid, veratric acid, naringin, benzoic acid, abscisic acid, quercetin, *trans*-cinnamic acid, naringenin, and kaempferol) were used as standards. Table 3.4 shows the list of the standard compounds, and the amount present in the respective samples. Also, the HPLC chromatograms of the standards and the samples are provided as supplementary data as appendix in Fig. A3.1 (a to k).

The 10 selected samples showed the presence of 12 out of 21 standard phenolic compounds. Flavonoids such as (+)-catechin, naringin, quercetin, naringenin, kaempferol, along with some other phenolic compounds commonly found in mushrooms such as *p*-hydroxybenzoic acid, benzoic acid, caffeic acid, and ferulic acid were not detected in any of the samples. Also, the presence of some unknown compounds (UC 1 – 4) were found to be in high abundance as indicated by the peak intensity in HPLC chromatograms. The presence of such major unknown compounds in the respective samples are provided in Table 3.4. The compound UC 3 was detected in 4 of the tested extracts, *Inonotus* sp. 4, Cyclomyces setiporus (3,4,5).

3.3.7 LC-MS analysis

The major compounds present in some of the samples did not match with the standards used in the HPLC analysis. Therefore, LC-MS analysis was performed to elucidate the unknown compounds (UC 1 – 4). The retention time and molecular ion m/z values are shown in Table 3.5. Although clear fragmentation patterns could not be obtained, the molecular ion peaks provided some hint for the tentative identification of the unknown compounds. High anti-oxidative activity of mushrooms belonging to *Inonotus* sp. have been largely attributed to styrylpyrone-class compounds, such as hispidin, inoscavin, phelligridin and others [76]. Therefore, it can be predicted that UC 1 with m/z value of 247.0477 in positive mode and 245.0369 in negative mode, is hispidin (exact mass 246.0528 g/mol) [77]. The molecular ion m/z values of the rest of the unknown compounds were detected as follows: 491.0598 in positive mode and 489.0869 in negative mode for UC2, 251.0259 in negative mode for UC3, and 303.0443 in positive mode and 301.0024 in negative mode for UC4.

3.4 Conclusion

A wide range of anti-oxidative activities were observed among the various orders of wild mushrooms species of Nepal. Hymenochaetales was the most active group, which included the

exceptionally potent species, *Inonotus clemensiae*. It was found to exceed the anti-oxidative activity of even *Inonotus obliquus* (Chaga), a mushroom highly revered for its strong anti-oxidative activity among food products. *Pseudomerulius curtisii*, *Xylobolus princeps* (4,2,3,1), and *Ganoderma* sp. 1 emerged as other promising candidates with high anti-oxidative activity, from the rest of the groups. The correlation between the phenolic content and the various anti-oxidative assays across the 4 groups suggest that the phenolic compounds are largely responsible for the anti-oxidative activity of the mushrooms. As is evident from the HPLC and LC-MS analyses, the presence of high concentrations of particular phenolic compounds in some species and the absence of several commonly found phenolic compounds indicates the uniqueness of the phenolic profile of Nepalese mushrooms. This could be a result of its exclusive habitat and growing conditions.

Table 3.1. Antioxidant activities of the ethanol extracts of 62 wild mushrooms of Nepal

Order	TPC	ORAC	DPPH[i]	ABTS[ii]	RP[iii]
Scientific Name	mg GAE/g	µM TE/g	Inhibition %	Inhibition %	Absorbance 700 nm
Hymenochaetales	c	bc	df	bc	ce
Inonotus clemensiae	643.2±21.32	31966.9±198.6	83.2±0.06	92.2±0.05	1.73±0.03
Inonotus cuticularis	102.8±1.73	13228.5±125.5	82.3±0.17	39.8±1.25	0.88±0.01
Inonotus sp. 4	155.7±14.55	6015.9±90.2	89.9±0.11	18.7±0.21	1.00±0.01
Inonotus sp. 5	58.1±1.09	1917.9±27.3	60.3±0.25	18.4±0.43	0.58±0.00
Cyclomyces setiporus 1	423.7±19.65	11017.9±401.3	86.1±0.11	83.3±0.79	1.64±0.03
Cyclomyces setiporus 3	245.0±2.73	6570.9±169.7	85.3±0.17	67.3±1.14	1.77±0.11
Cyclomyces setiporus 2	204.5±0.10	2952.3±71.6	88.0±0.07	70.9±0.58	2.01±0.10
Cyclomyces setiporus 4	155.3±8.73	9988.7±664.5	85.1±0.09	75.2±0.65	2.20±0.06
Cyclomyces setiporus 5	145.0±17.07	7548.79±261.17	86.1±0.10	75.5±0.87	1.97±0.14
Phellinus sp. 1	116.8±5.63	4219.9±71.9	87.4±0.38	46.8±0.56	0.75±0.02
Phellinus sp. 2	66.0±1.77	1829.5±56.6	52.1±1.87	8.6±0.18	0.54±0.07
Phellinus adamantinus	91.0±2.24	2470.1±389.3	77.2±0.57	17.6±0.16	0.84±0.01
Oxyporus sp.	7.9±0.48	328.7±14.7	2.3±0.11	-	0.11±0.00
Polyporales	ad	a	be	a	ad
Ganoderma sp. 1	124.2±4.59	3546.6±212.8	90.9±0.47	63.2±0.45	1.22±0.04
Ganoderma sp. 2	61.1±1.05	2640.2±40	64.4±0.89	20.0±0.98	0.81±0.00
Ganoderma lingzhi 2	63.1±1.13	1450.8±50.3	56.7±0.52	20.5±0.41	0.43±0.01
Ganoderma lingzhi 1	41.2±0.36	936.7±7.9	26.0±0.11	12.8±0.23	0.43±0.01
Ganoderma endochroum	50.2±0.06	1730.4±26.54	40.5±0.90	14.2±0.58	0.43±0.01
Ganoderma multipileum	39.5±0.84	2035.7±65.7	35.0±1.19	6.0±0.26	0.32±0.00
Amauroderma calcigenum	61.8±0.07	1795.4±28.3	46.8±0.53	15.4±0.68	0.60±0.01
Microporus xanthopus 2	14.5±0.33	562.9±38.4	7.0±0.39	1.2±0.25	0.15±0.00
Microporus xanthopus 1	13.3±0.30	196.7±11.9	6.8±0.43	1.5±0.45	0.14±0.01
Fomes fomentarius	32.4±2.89	144.6±29.2	50.8±1.16	24.1±0.05	0.59±0.01
Trichaptum abietinum	9.5±0.34	782.5±19.9	65.8±0.58	-	0.10±0.00
Trichaptum biforme	5.7±0.60	221.9±41.0	4.0±0.18	1.6±0.29	0.12±0.00
Bjerkandera adusta	51.6±2.39	2042.0±180.7	48.3±1.03	9.7±0.08	0.33±0.01
Abortiporus biennis	20.7±0.34	698.5±24.6	11.4±0.14	2.8±0.37	0.18±0.00
Phlebia tremellosa 2	19.7±1.17	649.3±38.3	17.6±0.10	6.1±0.37	0.21±0.05
Mycorrhaphium sp.	8.3±0.23	360.6±56.4	2.9±0.16	-	0.15±0.00
Antrodiella zonata 1	7.5±0.12	201.3±27.0	3.3±0.38	-	0.13±0.00
Antrodiella zonata 2	13.8±0.35	441.0±30.6	6.6±0.48	-	0.13±0.00
Antrodiella zonata 3	13.6±0.32	679.9±51.9	5.0±0.07	1.5±0.44	0.18±0.00
Laetiporus versisporus 1	6.6±0.25	413.7±46.6	2.0±0.18	1.8±0.53	0.16±0.00
Laetiporus versisporus 2	8.6±0.50	478±36.8	4.8±0.18	-	0.13±0.00
Laetiporus montanus	8.4±0.21	167.8±14.0	3.8±0.18	-	0.17±0.00
Grifola frondosa	4.1±0.61	193.9±37.606	2.9±0.03	-	0.14±0.00
Polyporus arcularius	4.3±0.26	383.3±31.4	9.2±0.10	1.0±0.08	0.12±0.00
Lentinus sp.	3.2±0.79	119.1±1.9	4.2±0.04	-	0.13±0.00

Table 3.1 *contd.*

Order Scientific Name	TPC mg GAE/g	ORAC µM TE/g	DPPH[i] Inhibition %	ABTS[ii] Inhibition %	RP[iii] Absorbance 700 nm
Agaricales	a	a	a	a	a
Marasmius sp.	23.6±0.99	647.5±11.0	11.5±0.10	5.1±0.10	0.26±0.00
Marasmius mavium	3.3±0.12	309.3±46.7	2.9±0.26	1.6±0.44	0.11±0.00
Pholiota nameko 2	12.5±0.49	1107.8±91.5	4.3±0.13	-	0.10±0.00
Pholiota nameko 1	1.6±0.20	177.7±17.4	1.7±0.15	-	0.14±0.00
Gymnopus sp.	11.8±0.42	182.3±21.0	3.3±0.15	0.3±1.17	0.21±0.01
Pleurotus ostreatus 4	8.9±0.54	945.9±37.4	3.4±0.03	1.2±0.25	0.12±0.00
Pleurotus ostreatus 2	8.2±0.89	386.0±8.7	2.7±0.15	-	0.11±0.00
Pleurotus ostreatus 1	8.1±0.23	694±17.4	2.3±0.09	1.9±0.42	0.13±0.01
Pleurotus ostreatus 3	5.6±0.19	309.9±27.9	2.0±0.81	-	0.12±0.01
Lentinula edodes 1	4.3±0.04	428.8±11.9	2.0±0.38	-	0.14±0.00
Lentinula edodes 3	6.2±0.63	98.8±45.3	3.0±0.10	1.7±0.39	0.13±0.00
Inocybe sp.	6.0±0.26	343.5±19.1	2.5±0.50	1.8±0.44	0.11±0.00
Panellus edulis	6.1±0.52	556.4±44.9	2.4±0.24	0.7±0.26	0.09±0.00
Mucidula mucida	0.4±0.42	943.15±43.6	0.3±0.39	0.9±0.22	0.12±0.00
Others	bd	cef	ac	ac	bde
Pseudomerulius curtisii	131.6±0.48	11204.9±414.9	89.0±0.29	53.0±0.34	1.10±0.07
Xylobolus princeps 4	126.6±6.62	1290.4±16.4	83.3±0.25	47.8±0.62	1.19±0.12
Xylobolus princeps 2	97.7±2.31	2949.3±265.1	85.1±0.01	56.7±0.56	1.02±0.05
Xylobolus princeps 3	94.4±1.32	2349.5±340.53	86.3±0.22	54.3±0.61	1.19±0.01
Xylobolus princeps 1	57.9±5.29	257.65±29.4	86.3±0.08	44.1±0.11	0.50±0.02
Engleromyces goetzii	30.6±0.14	4666.6±81.88	30.5±0.51	7.8±0.38	0.31±0.03
Neolentinus lepideus	22.7±1.57	631.3±38.3	9.7±0.60	2.1±0.05	0.27±0.04
Lactarius sp.	11.7±0.64	209.0±16.6	7.4±0.42	0.2±0.19	0.14±0.00
Russula brevipes	9.9±2.74	41.4±31.5	3.6±0.19	-	0.13±0.01
Cantharellus ferruginascens	6.3±0.09	170.1±30.8	3.1±0.09	-	0.13±0.00

The values are expressed as "mean ± standard deviation" and n = 3.; [i, ii, iii] indicate sample concentrations of 2.5, 2, and 1 mg/ mL were used for the respective assays, - means the activity was not detected at the tested concentration. Difference between letters in each column means that there is statistical difference between groups at significance level $p < 0.05$.

Table 3.2: Spearman's rank correlation coefficients for TPC and the antioxidant assays ORAC, DPPH, ABTS, and RP

Orders	TPC - ORAC	TPC - DPPH	TPC - ABTS	TPC - RP
Hymenochaetales	0.731**	0.674*	0.852**	0.753**
Polyporales	0.812**	0.821**	0.786**	0.851**
Agaricales	0.327	0.854**	0.165	0.102
Others	0.806**	0.888**	0.900**	0.927**

** $p < 0.01$, * $p < 0.05$

Table 3.3. EC_{50} values of DPPH and ABTS assays, and $EC_{0.5}$ values of reducing power assay for ten samples with the highest TPC values

Scientific Name	**DPPH (EC_{50})**	**ABTS (EC_{50})**	**Reducing Power ($EC_{0.5}$)**
Inonotus clemensiae	0.08±0.004	0.40±0.012	0.03±0.003
Cyclomyces setiporus 1	0.14±0.003	0.71±0.012	0.07±0.030
Cyclomyces setiporus 2	0.20±0.008	0.84±0.016	0.17±0.041
Cyclomyces setiporus 3	0.29±0.041	1.19±0.045	0.13±0.061
Inonotus sp. 4	0.66±0.035	1.24±0.016	0.30±0.256
Cyclomyces setiporus 4	0.15±0.002	0.87±0.003	0.10±0.033
Cyclomyces setiporus 5	0.18±0.013	0.92±0.007	0.11±0.074
Pseudomerulius curtisii	0.73±0.004	2.03±0.024	0.38±0.021
Xyloborus princeps 4	0.57±0.006	2.21±0.041	0.64±0.339
Ganoderma sp. 1	0.47±0.011	1.52±0.015	0.26±0.026

EC_{50} is the sample concentration required for 50% inhibition in DPPH and ABTS assay; and $EC_{0.5}$ is the sample concentration required for 0.5 absorbance unit (700 nm) in reducing power assay. Results are expressed as sample concentration in mg/mL

Table 3.4. Retention time (Rt) and quantification of standard phenolic compounds in top ten samples with the highest TPC value

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	UC1	UC2	UC3	UC4
Inonotus clemensiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	
Cyclomyces setiporus 1	-	-	-	-	54.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	
Cyclomyces setiporus 2	-	1.4	-	11.6	-	-	-	3.2	-	2.1	2.1	-	-	-	-	-	-	-	-	-	-	-	-	+	
Cyclomyces setiporus 3	-	-	-	3.2	24.6	-	-	-	-	-	-	-	-	30.8	-	-	2.6	-	-	-	-	-	-	-	
Inonotus sp. 4	-	2.2	-	2.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Cyclomyces setiporus 4	-	-	-	7.1	-	-	-	-	-	-	-	23.1	-	-	-	-	-	-	-	-	-	-	-	+	
Cyclomyces setiporus 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
Pseudomerulius curtisii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30.9	-	-	-	-	-	
Xyloborus princeps 4	16.1	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Ganoderma sp. 1	-	-	10.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Compound numbers represent: 1. 5-sulfosalicylic acid, 2. gallic acid, 3. pyrogallol, 4. 3,4-dihydroxybenzoic acid, 5. chlorogenic acid, 6. (+)-catechin, 7. *p*-hydroxybenzoic acid, 8. vanillic acid, 9. caffeic acid, 10. vanillin, 11. *p*-coumaric acid, 12. rutin, 13. ferulic acid, 14. veratric acid, 15. naringin, 16. benzoic acid, 17. abscisic acid, 18. quercetin, 19. *trans*-cinnamic acid, 20. naringenin, and 21. kaempferol. UC is unknown compound; + indicates the presence of unknown major compound in the respective sample; - indicates not detected. The values of phenolic compound in each sample is expressed as µg/mg extract.

Table 3.5. Retention time and m/z values of the molecular ions of the unknown compounds

Compound	Rt (min)	$[M+H]^+$	$[M-H]^-$
UC1	44.25	247.0477	245.0369
UC2	48.17	491.0598	489.0869
UC3	26.52	-	251.0259
UC4	38.67	303.0443	301.0024

Rt is retention time, UC is unknown compound, - indicates not detected

Chapter 4

Antibacterial Activity

4.1 Introduction

Staphylococcus aureus and *Propionibacterium acnes* are bacterial species known to have adverse effects on skin health [78]. Colonization of these bacteria on the skin causes the onset and pathogenesis of skin conditions like acne vulgaris, boils, and impetigo [79]. Increasing bacterial resistance and prevalence of side effects from currently available treatments [80], underscores the need for alternative treatment sources. Inhibition of skin problem causing bacteria serves as an important functional property in skin cosmetics too [81].

The dietary and medicinal uses of mushrooms can be traced back to ancient times. Mushrooms have proven to be a vast reservoir of bioactive compounds, exhibiting biological activities such as antioxidant, antibacterial, antiviral, anticancer, anti-obesity, and anti-inflammatory activities [26]. Over the years, mushrooms have transcended from being traditional remedies to bioactive ingredients in medicines and functional foods [31, 82]. The bioactive properties of mushrooms have found important implications in skin health and cosmetics too [42].

Several mushroom species are known to have antibacterial effect on *S. aureus*. However, the antibacterial effect on *P. acnes* is much less studied. *Lentinus edodes*, *Ganoderma lucidum* and *Phellinus linteus* are few examples of mushrooms that have antibacterial effect against *S. aureus* [41]. *Ganoderma pfeifferi* is known to have activity against *P. acnes* and other skin problem causing bacteria [26]. The antimicrobial activity of mushrooms have been attributed to several different compounds, ranging from low molecular weight compounds like phenolic acids, terpenes, steroids, quinolones to high molecular weight compounds like peptides and proteins [41]. Desbois et al. [83] reported the antibacterial activity of polyunsaturated fatty acids against both *S. aureus* and *P. acnes*.

Over a 1000 species of mushrooms have been reported from this country [7]. Despite the diversity and huge potential, the use of mushrooms in Nepal is limited to the consumption, and traditional medicinal uses[13] of a few species. The exploration of the therapeutic potential and the cultivation techniques of the indigenous wild mushrooms could add a new dimension to the Nepalese mushroom market and functional food industry. However, apart from some studies on the antioxidant activities [22, 23], other biological activities of Nepalese mushrooms remain largely unstudied. Therefore the antibacterial property of the ethanol extracts of 90 Nepalese wild mushrooms samples, against *S. aureus* and *P. acnes*. Attempts were also made to investigate the antibacterial activity against the bacteria *Escherichia coli*. However, none of the samples exhibited significant activity against this bacteria. Therefore, the results of the study has been excluded from this chapter.

In depth chemical analysis of selected species with strong antibacterial activity elucidates the nature of the compounds responsible for the antibacterial activity. Therefore, the present study shall elucidate the antibacterial potential and chemical components of different wild mushroom samples of Nepal, for its prospective use as medicinal agents and cosmeceuticals.

4.2 Materials and methods

4.2.1 Antibacterial assay

The antibacterial assay for *S. aureus* was based on the method described by Tanaka et al[84]. A single colony of *S. aureus* (NBRC 1273) was inoculated to 5 mL of nutrient broth, and incubated at 37 °C at 1160 rpm for 18 hours. The bacterial concentration was adjusted to 1×10^5 colony forming units (CFU)/mL. The mushroom extracts were dissolved in dimethyl sulfoxide (DMSO) at the maximum dissolved concentration (final concentration 100 to 400 µg/mL). Sorbic acid (final concentration 400 µg/mL) and DMSO were used as positive and negative controls, respectively. In a 96 well plate, 133.5 µL of the nutrient broth, 15 µL of the

bacterial suspension, and 1.5 µL of the sample suspension/controls were added to each well (*n*=3). Blank wells with 148.5 µL nutrient broth and 1.5 µL sample/ controls were also included. The plate was incubated at 37 °C, 1160 rpm for 18 hours. Bacterial growth was measured using a micro-plate reader (Biotek-ELX800, BioTek) set at 630 nm. The antibacterial activity was calculated as the inhibition percentage with reference to the negative control, DMSO. The samples exhibiting antibacterial activity greater than or equal to the positive control were selected for the determination of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The concentration of the samples were serially decreased, starting from the maximum dissolved concentration, to detect the concentration which inhibited visual growth of the bacteria, i.e. MIC. After obtaining the MIC, the concentration of the samples were serially increased, starting from the MIC, to obtain the minimum concentration in which there was no colony formation upon plating in the nutrient agar plates, i.e. MBC.

In case of *P. acnes*, the method was slightly modified. The stock solution of *P. acnes* (NBRC 107605) was retrieved from the cold storage (-80 °C) and thawed. A 50 µL portion was added to 5 mL Gifu anaerobic medium (GAM) broth. It was then incubated under anaerobic condition using the Anaero Pack jar system at 37 °C for 24 hours. The concentration of the bacterial suspension was adjusted to 1×10^5 CFU/ mL. Benzalkonium chloride (final concentration 200 µg/mL) was used as positive control. The assay was performed in a 96 well plate following the same method as described for *S. aureus*. The plates were then incubated in anaerobic condition at 37 °C for 30 hours. The absorbance was measured at 630 nm. The MIC and MBC values were determined for samples with the inhibition percentage greater than or equal to the positive control.

4.2.2 Statistical analysis.

All the assays were conducted at least 3 times, and the results are expressed as mean ± standard deviation. Significant differences between sample groups were analyzed by Kruskal Wallis test followed by Dunn-Bonferroni test. The statistical analyses were performed using SPSS statistics Version 23. The *p*-value less than 0.05 were considered statistically significant.

4.2.3 Liquid chromatography-mass spectrometry (LC-MS) analysis

High resolution LC-MS analysis was done to elucidate the compounds present in the samples with detectable MIC and MBC. LC-MS-IT-TOF, Shimadzu, Tokyo was fitted with an ODS-3 column (150 mm × 1.5 mm i.d. 5 µm particle size). The solvent system comprised of water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. The analysis was carried out at room temperature with a flow rate of 0.2 mL/min. The gradient flow of the mobile phase was set as: 0 – 10 min, 5 - 30% B; 10 – 15 min, 30 - 40% B; 15 – 30 min, 40% B; 30 – 60 min, 40 - 70% B; 60 – 70 min, 70 – 100% B; 70 – 80 min, 100% B; 80 – 85 min, 100 - 5% B; 85 – 95 min, 5% B. The LC chromatograms were recorded at the wavelengths of 280 and 254 nm.

For MS, electron spray ionization (ESI) source was used in positive and negative ionization mode with *m/z* values of 100-1000 for MS and 50-700 for MS/MS. A probe voltage of ± 4.5 kV, nebulizer gas flow of 1.5 L/min, curved desolvation line (CDL) temperature of 200 °C, and heat block temperature of 200 °C were used. The peaks obtained were partially identified on the basis of retention time, *m/z* values, and comparison with standard compounds.

4.2.4 Nuclear magnetic resonance (NMR) analysis

The structure of some of the compounds in the bioactive samples, were confirmed by NMR analysis. Bruker DRX 600 NMR spectrometer (Bruker Daltonics Inc., MA, USA), was used to obtain the proton (^{1}H) and carbon-13 (^{13}C) NMR spectra. Tetramethylsilane (TMS) was used

as an internal standard for chemical shifts, and the chemical shifts (δ) were expressed in ppm with reference to the TMS resonance.

4.3 Results and Discussion

4.3.1 Percentage inhibition of *S. aureus* and *P. acnes*

The ethanol extracts of 90 mushroom samples were tested for their activity against *S. aureus* and *P. acnes*. The inhibition percentage of the extracts, against both bacterial species are listed in Table 4.1. The samples were grouped into four groups on the basis of their taxonomic order as Hymenochaetales, Polyporales, Agaricales and Others. Since the majority of the sample fell into the first three orders, the few remaining samples were grouped together as "Others". The antibacterial activity was measured using the absorbance obtained at 630 nm, and calculated as the percentage inhibition of the bacterial growth compared to the negative control (DMSO). Therefore, DMSO showed no (0%) inhibition of bacterial growth and the positive controls showed a complete (100%) inhibition.

Overall, *S. aureus* was more susceptible than *P. acnes* towards the antibacterial activity of the extracts. For *S. aureus*, significant differences were seen between the inhibition percentage for the groups Hymenochaetales and Polyporales. Hymenochaetales emerged as the strongest group, with 10 out of 20 samples showing more than 80% inhibition of *S. aureus*. A closer look within the group revealed that *Inonotus andersonii*, *Inonotus clemensiae*, *Inonotus cuticularis*, *Inonotus* sp.2, and *Cyclomyces setiporus* 3 exhibited the highest inhibitory effect. However, interesting contrasts were seen in the samples belonging to the same genera such as *Inonotus* sp.1, 3 and 5, which did not have any inhibitory effect on *S. aureus*. Polyporales showed a medium to low inhibitory effect on *S. aureus*, with the highest inhibitory effect reaching 73% for *Laetiporus montanus*. Almost half (48.7%) of the samples within the group did not show any inhibition on the bacterial growth. Samples belonging to *Ganoderma* sp. showed very low

to undetectable inhibition of *S. aureus*. Varying reports can be found for the antibacterial activity of the ethanol extracts of *Ganoderma* sp. against *S. aureus*. Quereshi et al. [85] reported a much lower inhibitory effect of the ethanol extract of *G. lucidum* compared to methanol and acetone extracts. The inhibitory activity of the Agaricales group also showed some variation. Only 3 samples (*Mucidula mucida, Gymnopus* sp., and *Pleurotus ostreatus*) out of 18 samples showed inhibition percentage greater than 80%. The Others group contained a mixture of low, medium and very high antibacterial samples. *Xylobolus princeps* (2-4), *Pseudomerulius curtisii*, and *Cantharellus ferruginascens* showed the strongest inhibitory effects of more than 90%. Bala et al. [86] also found a strong antibacterial activity of the ethanol and water extracts of *Cantharellus* sp. against *S. aureus*.

Similar to *S. aureus*, *P. acnes* also showed a significant difference between groups for Hymenochaetales and Polyporales. Although Hymenochaetales prevailed as the strongest group; relatively fewer sample showed high inhibition percentage. Only, 3 samples (*I. andersonii, I. clemensiae*, and *I. cuticularis*) out of 20 samples showed inhibition percentage above 80%. For Polyporales, almost half of the samples (48.7%) within the group did not have any inhibitory effect on the bacterial growth. However, complete inhibition of bacterial growth was seen for *Postia stiptica*; and *Ganoderma endochroum* showed 88% inhibition of bacterial growth. Agaricales was limited to an undetectable to medium inhibitory effect, with only one of the samples, *Maramius mavium*, showing inhibition percentage above 80%. In the Others group, the only prominent sample that showed complete inhibition of the bacterial growth was *Pseudomerulius curtisii*.

4.3.2 MIC and MBC

The MIC and MBC were determined for extracts showing inhibition percentage greater or equivalent to the positive control. The MIC and MBC of 11 samples for *S. aureus* and 4 samples

for *P. acnes*, and their respective positive controls are shown in Table 4.2. Around 40% of the samples tested belonged to the Hymenochaetaceae family of the Hymenochaetales group. The MBC could not be detected for 6 out of 12 samples tested for *S. aureus*, whereas for *P. acnes* all 4 samples had detectable MBC values. *I. clemensiae* extract required the least concentration of 100 µg/mL to achieve the MIC and MBC for *S. aureus*; and in the case of *P. acnes*, both *I. clemensiae* and *Postia stiptica* had the least MIC and MBC of 100 µg/mL and 200 µg/mL respectively. Although reports could not be found for *I. clemensiae*, Glamočlija et al. [66] reported the MIC and MBC values of ethanol extracts from *Inonotus obliquus* from Russia as 300 µg/mL and 1500 µg/mL, respectively *for S. aureus*. Apart from *I. clemensiae*, *I. andersonii* and *Pseudomerulius curtisii* were also able to show good inhibitory and bactericidal effects against both *S. aureus* and *P. acnes*. *Postia stiptica* was the only sample that had detectable MIC and MBC values specifically for *P. acnes*. The positive control for *S. aureus*, sorbic acid, showed an MIC value of 350 µg/mL, which was higher than most of the samples tested. However, the positive control for *P. acnes*, benzalkonium chloride, required only 10 µg/mL and 20 µg/mL to achieve the MIC and MBC values respectively.

4.3.3 Partial chemical characterization by LC-MS analysis

LC-MS analysis was performed to elucidate the compounds present in the samples exhibiting detectable MIC and MBC. The retention time and the *m/z* values of the molecular ion and the main fragments are shown in Table 4.3. The LC chromatograms are provided in Appendices in Fig. A4.1 (a – l). Tentative identification of some of the compounds present in the extracts were done on the basis of the *m/z* values of the molecular ions and the major fragments. The major compound that was common to *I. andersonii, I. clemensiae, and I. cuticularis* showed molecular ion peaks with *m/z* values of 247.0467, 247.0437, and 247.0442 in positive mode and 245.0497, 245.0303, and 245.0334 in negative mode respectively. The latter two species also showed an $[2M-H]^-$ ion of 491.0730 and 491.0694 respectively. The compound was

speculated to be hispidin (exact mass 246.0528 g/mol). Furthermore, the retention time and ultra violet-visible (UV-Vis) spectrum were compared with the commercially available standard compound too. However, its presence was not seen in *Inonotus* sp.2. The biosynthesis of hispidin analogs in *Inonotus obliquus* is known to be regulated by exposure to light and presence of fungal elicitors [87]. These factors could be the reason for its absence in *Inonotus* sp. 2.

Cyclomyces setiporus 3 showed the presence of compounds with molecular ion peaks having *m/z* values of 182.9373, 139.0481, 155.0094, and 153.0496 respectively in positive mode; and 181.0792, 137.0034, 153.0040, and 151.1258 respectively in negative mode. Therefore the presence of homovanillic acid (exact mass 182.1733 g/mol), protocatechualdehyde (exact mass 138.1207 g/mol), protocatechuic acid (exact mass 154.1201 g/mol), and vanillin (exact mass 152.0473 g/mol) were predicted. Further comparisons with the respective standard compounds were also done. Although the molecular ion peaks and some main fragments could be determined, the complete identity of the compounds remained elusive for *Postia stiptica*, *Mucidula mucida*, *Gymnopus* sp. *Xylobolus princeps* 2 and 3, *Pseudomerulius curtisii*, and *Cantharellus ferruginascens*.

Phenolic compounds are an important group of compounds responsible for the antibacterial activity of mushrooms [88]. Previous investigations of the total phenolic content of the ethanol extracts of Nepalese wild mushrooms [22, 23] showed very high phenolic content in *I. clemensiae, I. andersonii, I. cuticularis, C. setiporus and Pseudomerulius curtisii*. However, the very low phenolic content in *Postia stiptica*, *Mucidula mucida*, *Gymnopus* sp. and *Cantharellus ferruginascens* indicates the presence of other equally potent antibacterial compounds. The oily nature of the ethanolic extracts of *Mucidula mucida* and *Cantharellus ferruginascens* indicate the presence of unsaturated fatty acids. Unsaturated fatty acids with a length of more than 14 carbons, bearing specific functional groups are known to have strong

bactericidal effect [89]. *Mucidula mucida* is better known for the presence of antifungal compounds like strobilurins and oudemansins [90]. A class of compounds known as *p*-terphenyls exhibiting antifungal and antibacterial activities have been isolated from *Pseudomerulius curtisii*[91, 92]. Kozarski et al. [93] have attributed several bioactive properties, including antibacterial activity of the methanolic extract of *Cantharellus cibarius* to the phenolic content. However, *Cantharellus ferruginascens* used in this study had very low phenolic content [23]. Although reports about the extensive chemical characterization of *Postia stiptica* could not be found, the cultured mycelia are known to produce organic acids like oxalic acid [94]. The bioactive compounds of natural products, including mushrooms, are known to be influenced by several factors including growth stage [95], season, and abiotic stress [96], to name a few. The variations can be observed within population groups of the same species too, as demonstrated by Cirak et al. [97]. In their study, the chemical diversity in the Hypericum populations were attributed to changes in geographic locations, and the phenotypic plasticity of the plant to the varying environmental conditions.

4.3.4 NMR analysis

The structure of the major compounds present in *I. clemensiae* and *C. setiporus* were confirmed to be hispidin and protocatechualdehyde respectively with the help of ^{1}H and ^{13}C NMR analyses, followed by heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum correlation (HSQC) experiments; and also by comparison to previously reported spectral data for hispidin [98, 99] and protocatechualdehyde [100, 101]. The details of the NMR analysis are provided as Appendices in supplementary information A4.1 and A4.2; and Tables A4.1 and A4.2.

4.3.5 MIC and MBC of pure compounds

The compounds identified in the bioactive extracts, were checked again for the MIC and MBC values, to confirm the source of antibacterial activity in the extracts. Table 4.4 shows the MIC and MBC exhibited by the pure compounds. Also, the structure of the compounds investigated for MIC and MBC values are shown in Fig. 4.1. Hispidin showed an MIC and MBC value of 25 µg/mL for *S. aureus*; and an MIC and MBC value of 100 µg/mL for *P. acnes*. However, the extracts of *I. andersonii* and *I. clemensiae* exhibited the same MIC value for both bacterial species. This could be due to the presence of some minor compounds in the extracts which enhanced the antibacterial effect towards *P. acnes*. Protocatechualdehyde exhibited an MIC value of 400 µg/mL for *S. aureus* and the MBC was not detected up to the maximum tested concentration of 400 µg/mL. Also, the MIC and MBC could not be detected for other compounds in *C. setiporus* like homovanillic acid, protocatechuic acid, and vanillin at the maximum tested concentration of 400 µg/mL.

4.4. Conclusion

The present study reports the antibacterial activity of a large number of Nepalese mushrooms against *S. aureus* and *P. acnes* for the first time. Very few studies have reported the inhibitory activity of mushrooms against *P. acnes*. These findings paves the way for potential use of Nepalese mushrooms as functional ingredients, especially for skin health products. Further investigation of the mechanism of the antibacterial activity shall contribute for the expansion of their use in other therapeutic areas too.

Table 4.1 Percentage inhibition exhibited by the ethanol extracts of 90 wild mushroom samples on *Staphylococcus aureus* and *Propionibacterium acnes*

Order Scientific name	*S. aureus*	*P. acnes*	**Order** Scientific name	*S. aureus*	*P. acnes*
Hymenochaetales	**a**	**a**	**Polyporales** *contd.*		
Inonotus andersonii	102.0± 0.7	94.3±4.6	*Ganoderma carnosum*[*]	ND	5.1±1.9
Inonotus clemensiae	100.3±0.1	107.2±8.4	*Ganoderma* sp. 1	38.6±14.1	14.2±11.8
Inonotus cuticularis	98.8±1.3	83.2±3.3	*Ganoderma* sp. 2	16.2±9.8	18.8±5.7
Inonotus sp.1	ND	31.7±3.2	*Amauroderma calcigenum*	12.6±2.6	1.7±2.4
Inonotus sp.2	102.0± 7.9	28.4±4.2	*Trichaptum biforme*	ND	3.3±1.4
Inonotus sp.3	ND	19.2±9.4	*Trichaptum abietinum*	25.0±4.2	ND
Inonotus sp.4	92.2±12.0	27.2±5.2	*Trametes versicolor* 1	1.6 ± 2.1	ND
Inonotus sp. 5	ND	42.4±7.6	*Trametes versicolor* 2	3.5 ± 2.6	16.1±2.1
Phellinus gilvus	25.8 ± 1.8	53.7±7.5	*Trametes versicolor* 3	13.0 ±1.3	35.6±1.7
Phellinus conchatus 1	9.8 ± 1.7	26.9±7.5	*Trametes versicolor* 4	ND	ND
Phellinus conchatus 2	7.9 ± 2.8	38.9±1.3	*Trametes versicolor* 5	ND	ND
Phellinus sp.1	82.8±10.2	60.1±7.6	*Microporus xanthopus1*	ND	5.09±9.7
Phellinus sp. 2	22.0±9.3	ND	*Microporus xanthopus*2	15.4±1.1	ND
Phellinus adamantinus	83.3±1.7	25.4±3.8	*Polyporus arcularius*	19.3±4.0	58.7±6.1
Cyclomyces setiporus 1	93.7±12.6	73.1±10.2	*Postia stiptica*	ND	109.0±1.0
Cyclomyces setiporus 2	28.1±4.3	34.8±5.3	*Phlebia tremellosa* 1	ND	ND
Cyclomyces setiporus 3	103.9±0.1	54.1±4.2	*Phlebia tremellosa* 2	3.3±0.1	57.4±5.1
Cyclomyces setiporus 4	86.3±3.6	ND	*Lenzites betulina*	ND	3.4±3.5
Cyclomyces setiporus 5	59.5±8.4	ND	*Rigidoporus* sp.	ND	ND
Oxyporus sp.	ND	ND	*Laetiporus versisporus*1	11.8±2.0	8.0±5.1
Polyporales	**b**	**b**	*Laetiporus versisporus*2	67.2±1.9	ND
Ganoderma australe 1	ND	11.5±9.7	*Laetiporus montanus*	73.0±12.8	ND
Ganoderma australe 2	ND	ND	*Mycorrhaphium* sp.[**]	ND	ND
Ganoderma australe 3	ND	19.3±7.1	*Grifola frondosa*	67.2±15.4	ND
Ganoderma australe 4	ND	ND	*Lentinus* sp.[**]	49.7±1.7	ND
Ganoderma lingzhi 1	51.8±10.5	ND	*Bjerkandera adusta*	71.9±4.7	ND
Ganoderma lingzhi 2	ND	48.3±9.8	*Antrodiella zonata*1	ND	32.2±7.7
Ganoderma lingzhi 3	ND	6.4±5.5	*Antrodiella zonata*2	10.9±4.9	ND
Ganoderma endochroum	ND	88.0±3.4	*Fomes fomentarius*	ND	ND
Ganoderma multipileum	18.4±5.3	ND	*Abortiporus biennis*	35.2±5.6	54.3±3.4

Table 4.1 *contd.*

Order Scientific name	*S. aureus*	*P. acnes*	**Order** Scientific name	*S. aureus*	*P. acnes*
Agaricales	**ab**	**ab**	**Others**	**ab**	**ab**
Lentinula edodes 1	ND	ND	*Heterobasidion linzhiense*1	ND	40.4±6.3
Lentinula edodes 2	21.3±8.3	2.1±4.2	*Heterobasidion linzhiense*2	ND	59.2±0.6
*Pleurotus ostreatus*1[*]	7.4±0.7	12.4±2.0	*Lactarius hatsutake*	ND	15.7±4.0
Pleurotus ostreatus 2[*]	84.7±14.3	16.3±4.2	*Lactarius* sp.[**]	36.3±7.3	ND
*Pleurotus ostreatus*3[*]	28.2±5.9	13.6±5.8	*Russula brevipes*	28.8±1.3	ND
*Pleurotus ostreatus*4	11.5±0.8	22.0±10.4	*Engleromyces goetzii*	ND	4.6±3.6
Pholiota nameko 1	44.6±8.6	ND	*Neolentinus lepideus*	ND	ND
Pholiota nameko 2	10.2±0.5	11.4±1.8	*Xylobolus princeps* 1	76.5±7.6	15.6±3.9
Marasmius mavium	24.3±6.05	89.4±1.2	*Xylobolus princeps*2	105.7±11.2	27.9±5.7
Marasmius sp.	3.1±0.5	ND	*Xylobolus princeps*3	100.1±8.7	9.4±0.9
Panellus edulis	18.6±8.3	ND	*Xylobolus princeps* 4	96.1±1.7	ND
Panellus sp.	ND	16.4±0.2	*Pseudomerulius curtisii*	102.1±0.9	105.6±1.3
Inocybe sp. 1	ND	23.9±6.4	*Cantharellus ferruginascens*	132.9±2.0	ND
Inocybe sp.2	ND	24.8±1.2			
Collybia peronata	ND	44.5±1.8			
Tricholoma caligatum	ND	26.6±0.9			
Mucidula mucida[*]	113.1±1.8	75.7±2.9			
Gymnopus sp.	113.7±1.2	ND			

S. aureus is *Staphylococcus aureus* and *P. acnes* is *Propionibacterium acnes*. The values are expressed as "average ± standard deviation", and n=3. ND means that there was no detectable inhibition of bacterial growth. Difference in letters in each column means that there is a statistical difference between groups at a significance level of $p < 0.05$. The names of the mushrooms marked with [**] were tested at 100 μg/mL, and those marked with [*] were tested at 200 μg/mL, and the remaining samples were tested at 400 μg/mL.

Table 4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol extracts exhibiting the highest inhibition percentage.

Scientific name	*S. aureus*		*P. acnes*	
	MIC (µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
Inonotus andersonii	200	200	200	200
Inonotus clemensiae	100	100	100	200
Inonotus cuticularis	200	200	-	-
Inonotus sp. 2	400	ND	-	-
Cyclomyces setiporus 3	400	ND	-	-
Postia stiptica	-	-	100	200
Mucidula mucida.	250	400	-	-
Gymnopus sp.	200	ND	-	-
Xylobolus princeps 2	200	ND	-	-
Xylobolus princeps 3	400	ND	-	-
Pseudomerulius curtisii	100	200	200	400
Cantherellus ferruginascens	100	400	-	-
Sorbic acid	350	ND	-	-
Benzalkonium chloride	-	-	10	20

S. aureus is *Staphylococcus aureus* and *P. acnes* is *Propionibacterium acnes*. MIC is minimum inhibitory concentration and MBC is minimum bactericidal concentration. ND means MBC was not detected; - means that the MIC and MBC were not investigated for the particular bacterial species.

Table 4.3 Retention time (Rt) and *m/z* values of the liquid chromatography-mass spectrometry (LC-MS) detectable compounds, in the ethanol extracts of mushrooms showing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against *Staphylococccus aureus* and/or *Propionibacterium acnes*.

Scientific name	Rt (min)	$[M+H]^+$	Main fragments	$[M-H]^-$	Main fragments	Identified compounds
Inonotus andersonii	12.59	247.0467		245.0497		Hispidin (std)
Inonotus clemensiae	12.70	247.0437		245.0303	491.0730 $(2M-H)^-$	Hispidin (NMR)
Inonotus cuticularis	11.97	330.0516	313.1009, 285.0553	328.1289		NI
	12.61	247.0442	229.6337, 212.5372, 163.0358	245.0334	491.0694 $(2M-H)^-$	Hispidin (std)
Inonotus sp. 2	28.80	189.0609				NI
Cyclomyces setiporus 3	6.63	182.9373		181.0792		Homovanillic acid (std)
	8.07	139.0481		137.0034		Protocatechualdehyde (NMR)
	9.28	155.0094		153.0040		Protocatechuic acid (std)
	10.98	153.0496		151.1258		Vanillin (std)
Postia stiptica	14.01	277.2020	253.1551, 239.1103	275.3043		NI
	48.41			631.3535	483.3416	NI
	49.80			629.3742		NI
	71.46	453.3076	435.3409, 284.2561	451.3297		NI
Mucidula mucida	55.75			459.2670	413.2928, 279.2006	NI
	68.71	415.3021	394.2878, 384.2462, 357.3313, 339.2486	413.3745	392.3676, 347.2119, 325.2103, 299.2541	NI
Gymnopus sp.	53.93	295.2167	277.2201	293.1744		NI
	55.363	295.2121		293.2052		NI

Table 4.3 *contd.*

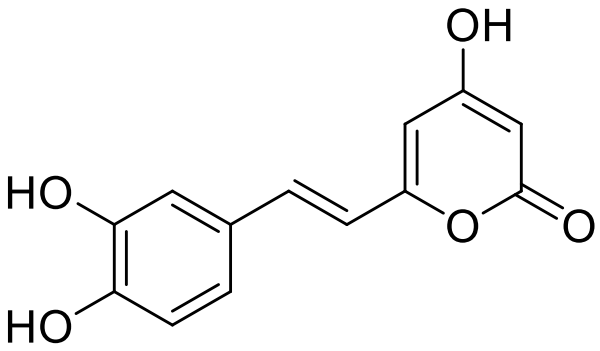
Scientific name	Rt (min)	$[M+H]^+$	Main fragments	$[M-H]^-$	Main fragments	Identified compounds
Xylobolus princeps 2	19.30	318.2628	289.0561			NI
Xylobolus princeps 3	22.77	235.0845	191.0667			NI
	28.51	189.0691				NI
Pseudomerulius curtisii	12.92			497.1496		NI
	13.98			453.0995	429.2301, 324.0877	NI
	15.91	411.0475	363.2784, 266.0553,	409.0760	323.0709	NI
	21.32	545.1939		543.1415		NI
	46.66	591.1802		589.1289	531.2800	NI
Cantharellus ferruginascens	45.86	275.1962	198.1015, 177.6052	273.1675		NI
	52.20	291.1368	273.1826	289.1754		NI
	53.33	295.2618	277.1925	293.1787		NI
	54.26	295.2367	277.2015	293.1874		NI

NI means not identified, standard (std) means that the identity of the compounds were confirmed by comparing with the retention time and ultra violet-visible (UV-Vis) spectra of standard compounds, and nuclear magnetic resonance (NMR) means that the compounds were confirmed by proton (^{1}H) and carbon-13 (^{13}C) NMR analysis, followed by heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum correlation (HSQC) experiments.

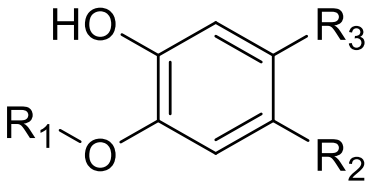
Table 4.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the samples exhibiting highest inhibition percentage.

Compound	*S.aureus*		*P.acnes*	
	MIC (µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
(1) Hispidin	25	25	100	100
(2) Homovanillic acid	>400	>400	-	-
(3) Protocatechualdehyde	400	>400	-	-
(4) Protocatechuic acid	>400	>400	-	-
(5) Vanillin	>400	>400	-	-

S. aureus is *Staphylococcus aureus* and *P. acnes* is *Propionibacterium acnes*. - means that the MIC and MBC values were not investigated for the respective compounds.



1. Hispidin



2. Homovanillic acid (R_1 = CH_3, R_2 = CH_2COOH, R_3 = H)

3. Protocatechualdehyde (R_1 = H, R_2 = CHO, R_3 = H)

4. Protocatechuic acid (R_1 = H, R_2 = COOH, R_3 = H)

5. Vanillin (R_1 = CH_3, R_2 = CHO, R_3 = H)

Fig. 4.1. Chemical structures of compounds 1 to 5

Chapter 5

Melanin Biosynthesis

5.1 Introduction

Melanin is a pigment responsible for imparting color to skin, hair and other tissues in the body. It is produced in melanosomes located inside melanocyte cells in the basal layer of epidermis[102]. Melanin biosynthesis is initiated by the oxidation of tyrosine by tyrosinase enzyme, followed by the polymerization of the oxidized product[103]. Overproduction of melanin causes hyperpigmentation in skin, leading to conditions such as melasma, solar lentigines, and dark spots[104]; whereas its underproduction makes the skin, hair and eyes more susceptible to the damaging effects of UV radiation[105], potentially causing visual impairment, and skin cancer.

Mushrooms are known to have both inhibitory as well as acceleratory effects on melanin biosynthesis. Yan et al. described the inhibitory and acceleratory effects of *Inonotus obliquus* on tyrosinase activity and melanin formation in B16 melanoma cells[106]. Several mushroom species including *Pleurotus* sp[107], *Ganoderma lucidum*, *Phellinus baumi*, and *Flammulina velutipes* have shown inhibition of melanin biosynthesis, and are therefore being used as natural skin whitening agents[43, 44].

Previous studies on the wild mushrooms of Nepal revealed very strong antioxidative[23], anti-hypertensive[22], and antibacterial activities[24]. Therefore, to obtain a deeper understanding about the bioactive potential of these largely unstudied mushrooms, ethanol extracts of 90 samples were tested for their effect on melanin biosynthesis in B16 melanoma cells. The present study shall reveal the bioactive potential of the wild mushrooms of Nepal, pertinent to medical as well as aesthetic uses.

5.2 Materials and methods

5.2.1 Melanin synthesis assay

The method described by Arung et al[108] was used for the melanin synthesis assay. Briefly, B16 melanoma cells were cultured in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml theophylline. The cells were seeded into a 24 well plate at a density of 1×10^5 cells per well and incubated for 24 hours at 37 °C under 5% CO_2. The medium was then replaced with 998 µL of fresh medium, and 2 µL of the sample dissolved in DMSO/ positive control (arbutin at 100 µg/ mL)/ negative control (DMSO). The samples were tested at various concentrations ranging from 10 µg/mL to 80 µg/mL. After an additional incubation period of 48 hours, the medium was replaced again, along with the addition of sample/ positive control/ negative control. It was then followed by a 24 hour incubation, after which the cells were assayed. All the samples and controls were tested at least 3 times (n=3).

The medium was aspirated from the wells, and the cells were washed with PBS. The adherent cells were dissolved using 1 mL of 1N NaOH per well. The melanin content was then determined by measuring the absorbance at 405 nm using a microplate reader (Bio-Tek, USA). The melanin content was calculated as a percentage value compared with the negative control, DMSO.

5.2.2 Cell viability assay

The cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. To each well 50 µL of MTT dissolved in PBS (5 mg/mL) was added. The plate was incubated for 4 hours at 37 °C and 5% CO_2. The medium was removed and the formazan crystals were dissolved in 1 mL of 0.04 M HCl in isopropanol. The cell viability was estimated based on the absorbance measured at 570 nm, calculated as percentage value compared to the negative control, DMSO.

5.2.3 Tyrosinase assay

The assay was performed as previously described by Arung et al.[108] Briefly, 333 µL of the substrate solution, 2.5 mM L-DOPA/ L-tyrosine, was mixed with 600 µL of phosphate buffer (pH 6.5). Then, 33µL ethanol extract (0.13 to 1.3 mg/mL final concentration), followed by 33µL of mushroom tyrosinase (1380 units/ mL) were added. Immediately after mixing, the linear increase in absorbance, due to formation of dopachrome, was measured at 475 nm. Kojic acid (0.16 mg/mL final concentration) and DMSO were used as positive and negative controls respectively. The tyrosinase activity percentage was calculated as follows:

Tyrosinase activity (%) = $(\Delta A_{\text{test sample}} / \Delta A_{\text{control}}) \times 100$

where ΔA is the unit absorbance per minute (of the initial linear portion of the curve)

5.2.4 LC-MS analysis of melanin biosynthesis inhibiting extracts

High resolution LC-MS analysis was done for the samples showing high melanin biosynthesis inhibition. LC-MS-IT-TOF, Shimadzu, Tokyo was fitted with an ODS-3 column (150 mm ×1.5 mm i.d, 5 µm particle size). The solvent system comprised of water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. The analysis was carried out at room temperature with a flow rate of 0.2 mL/min. The gradient flow of the mobile phase was set as: 0 – 20 min, 3 - 90% B; 20 – 25 min, 90 - 100% B; 25 – 30 min, 100% B; 30 – 32 min, 100 - 3% B; 32 – 40 min, 3% B. For MS, ESI source was used in positive and negative ionization mode with *m/z* values of 100-1000 for MS and 50-700 for MS/MS. A probe voltage of ± 4.5 kV, nebulizer gas flow of 1.5 L/min, CDL temperature of 200 °C, and heat block temperature of 200 °C were used. The peaks obtained were tentatively identified on the basis of retention time, *m/z* values, and comparison with standard compounds.

5.3 Results and Discussion

5.3.1 Melanin content and cell viability

The melanin content (MC) and cell viability (CV) were measured for the ethanol extracts of 90 mushroom samples, using B16 melanoma cells. The ethanol extracts were dissolved in DMSO, and assayed at several concentrations starting from 80 µg/ml to 10 µg/ml. The samples were ranked as A to I according to their MC% and CV%, as shown in Table 5.1. The melanin content and cell viability percentage with individual ranks have been provided as in Table 5.2 (a to d).

Since, cell viability is an important parameter for practical applicability of the samples, CV% of 70% was set as a demarcation point[109]. All samples showing CV% lower than 70% at the maximum tested concentration were further analyzed at lower concentrations. The ranks of particular interest were A and E, representing the samples with CV% greater than 70%; and the lowest (≤ 30), and highest (> 120) MC% respectively. *Ganoderma carnosum* (80 µg/ml), *Pholiota nameko* 1 (80 µg/ml), and *Oxyporus* sp. (20 µg/ml) belonged to rank A. Mycelial extracts of various *Ganoderma* sp. have been reported to be effective tyrosinase inhibitors[110], and have therefore been used as skin whitening agents in facial masks[111]. The positive control arbutin (100 µg/ml), fell into rank B with an MC% of 51% and a CV% of 82%. Arbutin is a commonly used skin whitening agent in cosmetics[112, 113]. However, the present results suggest that several ethanol extracts of Nepalese wild mushrooms can serve as more effective and safer alternatives.

On the contrary, *Fomes fomentarius* extract (80 µg/ml) showed the strongest melanin stimulating activity with an MC% of 144% and a CV% of 103%, becoming the only sample to enter rank E. Melanin glucan complexes are important anti-infective constituents in *F. fomentarius*[114]. So, we can speculate that it consists of some melanin precursors, which could also be responsible for the elevated production of melanin observed in the assay. Also, within rank D, the extracts of *Russula brevipes*, and *Phellinus gilvus* (80 µg/ml); *Ganoderma australe* 4 showed a higher MC% compared to the control (i.e more than 100%). It is worth noting that different samples of *Ganoderma* sp. have shown activity in both extremes of MC

spectrum, with *G. carnosum* (80 µg/ml) showing an MC% of 25% and *G. australe* 4 (40 µg/ml) showing 108%. In a few cases such as *Phellinus sp.* 1, *Cyclomyces setiporus* 1 and *Pleurotus ostreatus* 4, the inhibitory effect on melanin biosynthesis was seen to be increased on lowering the concentration of the extract. Therefore, we can understand that dosage is also a critical parameter for the effectiveness of the extracts.

5.3.2 Tyrosinase assay

The samples belonging to Rank A, *Ganoderma carnosum, Oxyporus* sp., and *Pholiota nameko* 1 were tested for their ability to inhibit tyrosinase enzyme activity. The maximum dissolved concentration in the reaction mixture was chosen for each sample. The inhibitory curve of each sample obtained by monitoring the change in absorbance at 475 nm can be seen in Fig. 5.1 and 5.2. However, almost all of the samples tested were unable to show tyrosinase inhibitory activity, as can be seen in Table 5.3. Only, *Oxyporus* sp. could show a slight inhibitory activity, with 79.2% tyrosinase activity for the substrate L-tyrosine. Inhibition of the tyrosinase enzyme is one of the important mechanism for blocking the melanin biosynthetic pathway[115]. Several mushroom species including *Ganoderma* spp., *Flammulina velupites*, and *Phellinus baummi* have been reported to inhibit melanin biosynthesis through this mechanism[44]. Also, Japanese *Pholiota nameko* have been reported to have very high tyrosinase activity, making it an important source of tyrosinase for purification and characterization[116]. However, the results obtained in this study indicate the presence of alternative mechanisms such as suppression of pigmenting signals like bFGF, ET-1, and α-MSH[109]; prevention of maturation of melanocytes; and presence of antioxidants[112].

5.3.3 LC-MS analysis

LC-MS analysis was performed for the samples belonging to Rank A, *Ganoderma carnosum, Oxyporus* sp., and *Pholiota nameko* 1, to tentatively identify compounds in the extracts

showing strong melanin inhibition. The retention time and the *m/z* values of the molecular ion and the main fragments are provided in Table 5.4. Also, the LC chromatograms obtained at 254 nm are provided in the appendix as Fig A5.1 (a –c).

In *Ganoderma carnosum*, ganoderic acid H, ganodermanontriol, ganoderic acid DM, and ganoderic acid TN were identified; among which the first 3 compounds were compared with standard compounds using the selected reaction monitoring (SRM) mode LC-MS system (data not shown). Ganodermanondiol has been reported to regulate melanogenesis in B16F10 melanoma cells by inhbitng the expression of melanogenesis related proteins such as tyrosinase, TRP-1, TRP-2, and MITF[117].

Ursane type triterpenoids such as tetrahydroxy-urs-12-en-28-oic acid, and 3-oxo-1,19α-dihydroxy-urs-12-en-28-oic acid (annurcoic acid) were tentatively identified in *Oxyporus* sp., by comparing with fragmentation patterns available from previously published report[118]. Although lanostane type triterpenoids are the more common forms of triterpenoids in mushrooms, some ursane type triterpenoids have also been reported[119]. Moreover, preliminary identification of the peak at retention time 17.82 min, suggested it to be an oleanane type triterpenoid of molecular weight 470. However, exact structure of the compound needs to be confirmed.

The available fragmentation patterns obtained from the analysis of *Pholiota nameko* 1, were however insufficient to predict the compounds present in it. Long chain lipids and steroids are known to be potent melanin inhibitors, especially through tyrosinase inhibition. However, the absence of tyrosinase inhibition activity of the extracts suggests the occurrence of alternative mechanisms too.

5.4 Conclusion

The inhibition as well as stimulation of melanin biosynthesis bears great commercial interest, especially in the field of cosmetics. Nepalese wild mushrooms cater to both facets of the concept, with many species inhibiting melanin biosynthesis and some stimulating it too. However, it is equally important to bear in mind that the activity is strongly influenced by the concentration of the extracts used. Several triterpenoid compounds were tentatively identified as the potential bioactive compounds. Pigments such as melanin are important defensive compounds in morphologically and taxonomically higher fungi such as mushrooms, and it is therefore already equipped with the necessary precursors and biosynthetic pathways. So, it is a point of further interest to understand how it can regulate and influence melanin biosynthesis outside its own system too.

Table 5.1 Ranking of samples as A to I on the basis of melanin content (MC) and cell viability (CV)

Rank	Melanin Content (MC)	Cell viability (CV)
A	MC≦30	70≦CV
B	30＜MC≦60	70≦CV
C	60＜MC≦90	70≦CV
D	90＜MC≦120	70≦CV
E	MC>120	70≦CV
F	MC≦30	CV＜70
G	30＜MC≦60	CV＜70
H	60＜MC≦90	CV＜70
I	90＜MC＜120	CV＜70

Table 5.2 The melanin content (MC)% and cell viability (CV)% and their respective ranks of 90 Nepalese wild mushroom ethanol extracts

a. Hymenochaetales

Scientific name	80 µg/ mL			40 µg/ mL			20 µg/ mL			10 µg/ mL		
	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank
Inonotus andersonii	71	95	C	-	-		-	-		-	-	
Inonotus clemensiae	64	89	C	-	-		-	-		-	-	
Inonotus cuticularis	79	123	C	-	-		-	-		-	-	
Inonotus sp. 1	48	64	G	75	78	C	-	-		-	-	
Inonotus sp. 2	58	95	B	-	-		-	-		-	-	
Inonotus sp. 3	47	76	B	-	-		-	-		-	-	
Inonotus sp. 4	20	23	F	82	86	C	-	-		-	-	
Inonotus sp. 5	25	11	F	34	64	G	61	113	C	-	-	
Phellinus gilvus	109	86	D	-	-		-	-		-	-	
Phellinus conchatus 1	24	12	F	39	72	B	-	-		-	-	
Phellinus conchatus 2	21	11	F	44	66	G	84	106	C	-	-	
Phellinus sp. 1	54	36	G	36	69	G	77	101	H	-	-	
Phellinus sp. 2	-	-		70	87	C	-	-		-	-	
Phellinus adamantinus	33	28	G	63	73	C	72	95	C	-	-	
Cyclomyces setiporus 1	44	29	G	33	44	G	32	49	G	61	95	C
Cyclomyces setiporus 2	35	28	G	39	53	G	54	106	B	-	-	
Cyclomyces setiporus 3	43	22	G	42	40	G	47	55	G	63	77	C
Cyclomyces setiporus 4	27	12	F	52	36	G	56	28	G	58	67	G
Cyclomyces setiporus 5	55	43	G	65	93	C	-	-		-	-	
Oxyporus sp.	48	65	G	44	59	G	29	93	A	-	-	

- means that the sample was not tested at the particular concentration

b. Polyporales

Scientific name	80 µg/ mL			40 µg/ mL			20 µg/ mL			10 µg/ mL		
	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank
Ganoderma australe 1	52	66	G	81	83	C	-	-		-	-	
Ganoderma australe 2	54	69	G	87	87	C	-	-		-	-	
Ganoderma australe 3	77	69	H	93	67	I	82	81	C	-	-	
Ganoderma australe 4	63	69	H	108	81	D	-	-		-	-	
Ganoderma lingzhi 1	-	-		69	104	C	-	-		-	-	
Ganoderma lingzhi 2	23	14	F	57	65	G	67	87	C	-	-	
Ganoderma lingzhi 3	41	59	G	65	90	H	-	-		-	-	
Ganoderma endochroum	25	13	F	63	72	C	-	-		-	-	
Ganoderma multipileum	57	75	B	-	-		-	-		-	-	
Ganoderma carnosum	25	74	A	-	-		-	-		-	-	
Ganoderma sp.1	58	99	B									
Ganoderma sp.2	27	19	F	22	56	F	31	93	B	-	-	
Amauroderma calcigenum	92	98	D	-	-		-	-		-	-	
Trichaptum biforme	55	51	G	83	90	C	-	-		-	-	
Trichaptum abietinum	24	13	F	70	42	H	72	80	C	-	-	
Trametes versicolor 1	50	70	B	-	-		-	-		-	-	
Trametes versicolor 2	54	69	G	94	79	D	-	-		-	-	
Trametes versicolor 3	86	85	C	-	-		-	-		-	-	
Trametes versicolor 4	50	82	B	-	-		-	-		-	-	
Trametes versicolor 5	50	83	B	-	-		-	-		-	-	
Microporus xanthopus 1	74	82	C	-	-		-	-		-	-	
Microporus xanthopus 2	67	98	C	-	-		-	-		-	-	
Polyporus arcularius	91	115	D	-	-		-	-		-	-	
Postia stiptica	26	69	F	27	45	F	59	84	B	-	-	
Phlebia tremellosa 1	56	20	G	52	55	G	94	103	D	-	-	

Polyporales *contd.*

Scientific name	80 µg/ mL			40 µg/ mL			20 µg/ mL			10 µg/ mL		
	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank
Phlebia tremellosa 2	25	45	F	40	83	B	-	-		-	-	
Lenzites betulina	45	77	B	-	-		-	-		-	-	
Rigidoporus sp.	55	83	B	-	-		-	-		-	-	
Laetiporus versisporus 1	53	23	G	86	100	C	-	-		-	-	
Laetiporus versisporus 2	30	28	F	35	28	G	38	62	G	45	93	B
Laetiporus montanus	41	46	G	66	75	C	-	-		-	-	
Mycorrhaphium sp.	-	-		80	83	C	-	-		-	-	
Grifola fondosa	81	95	C	-	-		-	-		-	-	
Lentinus sp.	-	-		70	102	C	-	-		-	-	
Bjerkandera adusta	79	89	C	-	-		-	-		-	-	
Antrodiella zonata 1	67	89	C	-	-		-	-		-	-	
Antrodiella zonata 2	34	34	G	37	85	B	-	-		-	-	
Antrodiella zonata 3	58	83	B									
Fomes fomentarius	144	103	E	-	-		-	-		-	-	
Abortiporus biennis	33	63	G	52	88	B	-	-		-	-	

- means that the sample was not tested at the particular concentration

c. Agaricales

Scientific name	80 µg/ mL			40 µg/ mL			20 µg/ mL			10 µg/ mL		
	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank
Lentinula edodes 1	75	109	C	-	-		-	-		-	-	
Lentinula edodes 2	-	-		100	108	D	-	-		-	-	
Pleurotus ostreatus 1	-	-		88	99	C	-	-		-	-	
Pleurotus ostreatus 2	-	-		80	92	C	-	-		-	-	
Pleurotus ostreatus 3	74	73	C	-	-		-	-		-	-	
Pleurotus ostreatus 4	40	58	G	31	74	B	-	-		-	-	
Pholiota nameko 1	30	73	A	-	-		-	-		-	-	
Pholiota nameko 2	68	96	C	-	-		-	-		-	-	
Marasmius mavium	29	51	F	85	91	C	-	-		-	-	
Panellus edulis	70	95	C	-	-		-	-		-	-	
Panellus sp.	58	83	B	-	-		-	-		-	-	
Inocybe sp. 1	50	64	G	59	82	B	-	-		-	-	
Inocybe sp. 2	34	75	B	-	-		-	-		-	-	
Collybia peronata	45	59	G	60	79	B	-	-		-	-	
Tricholoma caligatum	56	81	B	-	-		-	-		-	-	
Mucidula mucida	-	-		99	94	D	-	-		-	-	
Gymnopus sp.	73	80	C	-	-		-	-		-	-	

- means that the sample was not tested at the particular concentration

d. Others

Scientific name	80 µg/ mL			40 µg/ mL			20 µg/ mL			10 µg/ mL		
	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank	MC%	CV%	Rank
Heterobasidion linzhiense 1	75	87	C	-	-		-	-		-	-	
Heterobasidion linzhiense 2	58	83	B	-	-		-	-		-	-	
Lactarius hatsutake	74	88	C	-	-		-	-		-	-	
Lactarius sp.	89	84	C	-	-		-	-		-	-	
Russula brevipes	104	121	D	-	-		-	-		-	-	
Engleromyces goetzii	39	90	B	-	-		-	-		-	-	
Xylobolus princeps 1	45	41	G	74	100	C	-	-		-	-	
Xylobolus princeps 2	65	65	H	72	88	C	-	-		-	-	
Xylobolus princeps 3	35	32	G	39	100	B	-	-		-	-	
Xylobolus princeps 4	68	86	C	-	-		-	-		-	-	
Pseudomerulius curtisii	21	14	F	45	61	G	81	99	C	-	-	
Cantharellus ferruginascens	69	67	H	70	90	C	-	-		-	-	
Neolentinus lepideus	-	-		76	85	C	-	-		-	-	
Arbutin (100 µg/ mL)	51	82	B									

- means that the sample was not tested at the particular concentration

Table 5.3 Tyrosinase activity percentage (with respect to negative control DMSO) of the samples belonging to rank A

Sample (final conc.)	**Tyrosinase activity %**	
	L-Tyrosine	L-DOPA
Oxyporus sp. (0.13 mg/mL)	79.2	100.7
Ganoderma carnosum (0.66 mg/mL)	100.9	102.2
Pholiota nameko 1 (1.3 mg/mL)	102.9	102.3
Kojic acid (0.16 mg/mL)	1.5	2.0

Concentration in brackets represent the maximum dissolved concentation for mushroom samples

Table 5.4 Retention time (Rt) and *m/z* values of the LC-MS detectable compounds, in the ethanol extracts of mushrooms belonging to Rank A

Scientific name	Rt (min)	$[M+H]^+$	Main fragments	$[M-H]^-$	Main fragments	Predicted compounds
Ganoderma carnosum	12.83	487.2821	469.3223, 451.3079		245.0497	-
	13.70		555.3112	571.2801	553.4752, 549.3540, 511.2451, 481.2608	Ganoderic acid H[a]
	14.33	571.23	469.3863, 451.3281, 433.3464	569.2673	553.3875, 549.3209, 531.3420, 445.8326	-
	15.60	489.3208,	471.3505, 413.3070		533.3381	-
	20.22	473.3651	455.3404, 437.3928			Ganodermanontriol[a]
	20.87	469.2450		467.3019		Ganoderic acid DM[a]
	22.90			511.3361	469.4430	Ganoderic acid TN
Oxyporus sp.	10.79	521.3479	503.3225,464.3079	519.3280		-
	12.35	503.3350	318.2955	501.3257	461.2961, 417.2941, 369.0934	-
	13.19		469.2901, 451.3123, 423.3181, 332.3046	503.3378	1007.6489	Tetrahydroxy-urs-12-en-28-oic acid
	13.53	487.2759	469.3700	485.3243	459.2752, 443.2710 (531.3260, 549.3429)	3-oxo-1,19α-dihydroxy-urs-12-en-28-oic acid (annurcoic acid)
	15.61		471.3730, 453.3350, 435.3372	487.3419	(975.6777), 374.2817	-
	16.62	487.2904	469.3005, 451.3216	485.3249	(971.6314), 383.3369	-
	17.82	471.3168	453.3340, 435.3344, 295.2146	469.3293	(941.6886)	-
	25.418		515.3753, 439.3140, 393.2781	455.3466	(911.6994), 502.3392	-
	25.97		573.4735, 427.3506	453.3368	(907.6709)	-
	27.59	631.4848	603.4244, 587.4810, 543.4368			-

Table 5.4 *contd.*

Scientific name	Rt (min)	$[M+H]^+$	Main fragments	$[M-H]^-$	Main fragments	Predicted compounds
Pholiota nameko	12.57		318.2878, 300.2948		329.2300	-
	13.97	536.3227	518.3352, 500.3352		580.3189, 329.2326	-
	16.70	640.3835	606.3472	638.3218	604.3652, 313.2123	-
	19.19	279.2668	(559.4631)		295.2191	-
	19.92	295.2138	277.2444	293.2058		-

- means the compound could not be predicted based on the available fragmentation pattern; [a] means that the presence of the compound was checked by comparing with standard compounds in SRM mode LC-MS system.

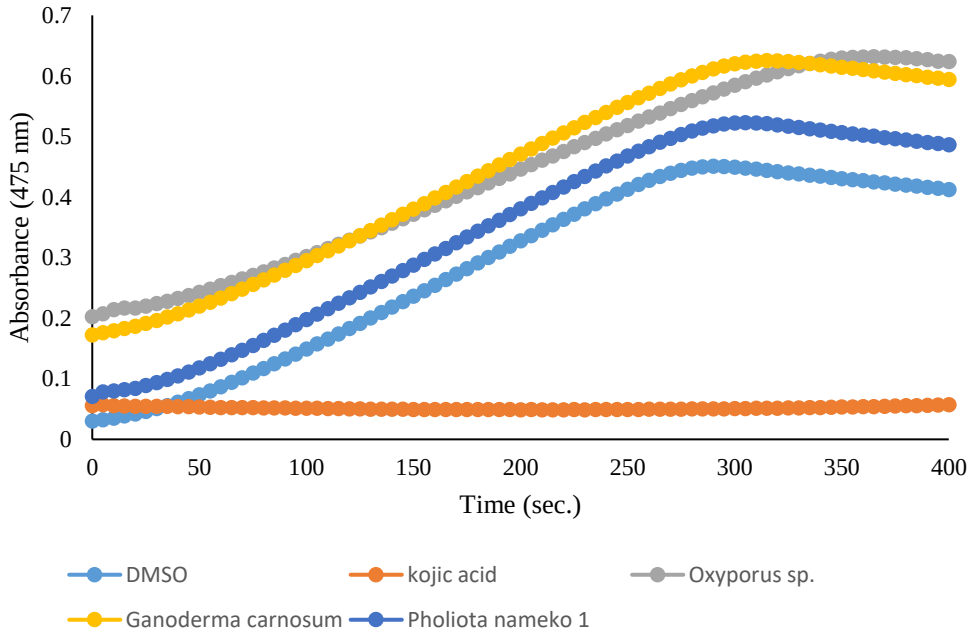


Fig. 5.1 Inhibition curve using L-tyrosine as substrate

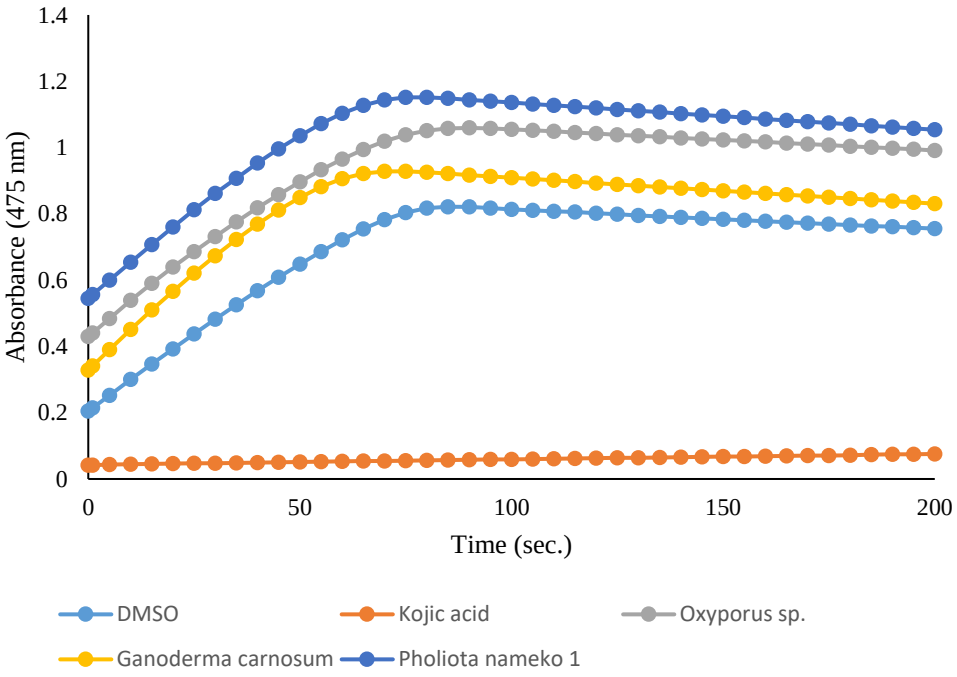


Fig. 5.2 Inhibition curve using L-DOPA as substrate

Chapter 6

Anti-allergy activity

6.1 Introduction

Allergy is defined as the abnormal response of the adaptive immune system against allergens which are non-infectious substances in the environment or non-infectious components of infectious organisms[120]. Allergic reactions like anaphylaxis, hay fever, allergic asthma involve the production of allergen-specific IgE antibody. In the IgE mediated allergic reactions, the IgE antibodies bound to the receptors on surface of mast cells and basophils are cross-linked by the allergen. This results in the degranulation of the cells, allowing the release of a variety of mediators such as histamine, heparin, serine proteases, cytokines, and growth factors. These mediators lead to inflammatory reactions involving bronchoconstriction, vasodilation, increased vascular permeability and mucus production. The incidence of allergy is on an increasing trend worldwide, with allergic rhinitis being the most common form[121]. Depending on the severity of the condition, allergic reactions can not only cause to financial and psychological burden, but also lead to life threatening situations. Currently, the first line of treatment is the non-sedating antihistamines and intranasal corticosteroids[121]. Also, non-steroidal anti-inflammatory drugs are used to reduce inflammation in the body. However, long term usage of these drugs are known to cause severe side effects[122]. Therefore the discovery of a more effective and safer alternative is very important.

The bioactive compounds in mushrooms, such as polysaccharides, terpenes, phenolic compounds, sterols, fatty acids, polysaccharide-protein complexes, and other metabolites are known to have anti-inflammatory activities[122, 123] and anti-allergy activities. The anti-allergic effect of the water extract of *G. lucidum* in animal models were found to prevent experimental asthma and contact dermatitis[124]. *Inonotus obliquus* sclerotia[125] as well as

the mycelium grown on germinated brown rice[126], have been found to suppress the release of proinflammatory mediators. Other mushroom species extensively studied for anti-inflammatory activity includes *Agaricus bisporus, Phellinus linteus, Cordyceps* sp.. *Antrodia camphorate,* and *Pleurotus* sp[123].

The present study of the anti-allergic activity of the wild mushrooms of Nepal is the first of its kind. There are several ways in which the anti-allergic effect can be studied *in vitro* as well as *in vivo*. In this study, the suppression of the degranulation of rat basophil cells, stimulated by the calcium ionophore A23187, were used as the indicator of anti-allergic activity. Calcium influx into the cells is an important step involved in the degranulation of the basophils[127]. So, by using a calcium ionophore to transport calcium directly across cell membrane, the cells can be stimulated for degranulation.

6.2 Materials and method

6.2.1 Anti-allergy activity

The anti-allergy activity was measured using the RBL-2H3 cells, based on the ability of the samples to inhibit the release of β-hexosaminidase, a biomarker of basophil degranulation. Also, the cell viability was measured simultaneously, as described by Kishikawa et al.[128]. Briefly, the 96 well plate was seeded with the cells at a density of 5×10^4 cells per well. The plate was then incubated for 40 hours at 37 °C in a 5% CO_2 incubator. The spent medium was discarded and the cells were washed with Tyrode buffer. Then, 100 µL of the buffer was added to each well, along with 0.5 µL of sample/ positive control (quercetin)/ negative control (DMSO), followed by an incubation period of 1 hour. The buffer was discarded, and replaced with 100 µL of fresh buffer containing 1% v/v of the calcium ionophore A23187 (5 mg/mL); after which the plate was incubated for 1 more hour. Into a new 96-well plate 50 µL of the supernatant from each well was mixed with an equal volume of substrate solution (2mM p-nitrophenyl-N-acetyl-β-glucosamine). The plate was incubated at room temperature in a shaker for 3-4 hours.

Then, 100 µL of stop solution (100mM $NaHCO_3$) was added to each well, and the absorbance was measured at 405 nm.

6.2.2 Cell viability

The cell viability was determined using MTT assay. The cells were cultured in 96-well plates at densities of 5×10^4 cells/well, respectively, in a humid atmosphere of 5% (v/v) CO_2 and 95% (v/v) air at 37 °C. After 48 h, the cells were treated with the extracts dissolved in DMSO at maximum dissolved concentration. Quercetin was used as a positive control. After 1 h, the culture media was discarded, and the cells were washed with 100 µL PBS. Then, 100 µL of the culture medium was added followed by 10 µL of MTT reagent (5 mg/mL). The plate was then incubated for 4 hours at 37 °C in CO_2 incubator. Then the medium was removed and the formazan crystals were dissolved in 100 µL of 0.04 M HCl in isopropanol. The cell viability was estimated based on the absorbance measured at 570 nm, calculated as percentage value compared to the negative control, DMSO.

6.2.3 Determination of IC_{50} values

The top 10 samples exhibiting the least β-hexosaminidase release (BHR) percentage (less than 30%), while maintaining high cell viability (more than 90%) were chosen for further analysis of IC_{50} values. The same method was followed for the anti-allergy activity as described above, with decreasing sample concentrations to obtain 50% β-hexosaminidase release.

6.2.4 Statistical analysis

All the assays were conducted at least 3 times, and the results are expressed as mean ± standard deviation. Significant differences between sample groups were analyzed by Kruskal Wallis test followed by Dunn-Bonferroni test. The statistical analyses were performed using SPSS statistics Version 23. The *p*-value less than 0.05 were considered statistically significant.

6.3 Result and discussion

6.3.1 Anti-allergy activity and cell viability

The anti-allergic activity of the mushroom extracts were calculated in terms of degranulation rate measured by the amount of β-hexosaminidase released. Table 6.1 lists the β-hexosaminidase release (BHR)% and the cell viability (CV)% of the samples at maximum dissolved concentration. Significant difference between groups were seen for Hymenochaetales and Agaricales for the BHR. As for CV, significant differences between groups was seen for Hymenochaetales compared to the rest of the three groups.

Samples showing degranulation rate less than 30%, while maintaining a cell viability greater than 70%, were considered as samples having high anti-allergic potential. All the samples were tested at maximum dissolved concentration, which ranged from 50 to 200 µg/mL. Quercetin at a final concentration of 10 µg/ml was used as a positive control.

The Hymenochaetales group had the largest number of samples having high anti-allergic potential with 6 out of 20 samples showing BHR% less than 30% while maintaining a cell viability of almost 100%. In fact none of the samples were cytotoxic to the cells. *Phellinus adamantinus* showed the least release of BHR with only 19.4% release, followed by *Phellinus* sp.2 with 20.1% release, and *Phellinus conchatus* 1 with 21.6% release. Therefore *Phellinus* sp. was the most prominent genus for this bioactivity, unlike antioxidant and anti-bacterial activity where *Inonotus clemensiae* was most dominant. However, *I. clemensiae* also followed quite closely with a BHR% of only 22.8%. Hispidin was identified as the major bioactive compound in *I. clemensiae*. The anti-inflammatory activity of hispidin has been attributed to the suppression of reactive oxygen species mediated nuclear factor kappa B (NF-κB) pathway lipopolysaccharide induced RAW 264.7 macrophage cells[129]. The mushrooms belonging to *Phellinus* sp. are known to have several bioactive properties including anti-inflammatory and anti-tumor activities. The major bioactive compounds in *Phellinus* sp. are polysaccharide,

flavones, pyranones, furans, terpenes, and steroids[130]. Several researchers have demonstrated the anti-allergic activity of *Phellinus* sp. using a variety of methods especially those involving RAW264.7 macrophages[131], and animal models[132]. The ethyl acetate fraction of *Phellinus linteus* grown in *Panax ginseng* was also found to inhibit β-hexosaminidase release in RBL-2H3 cells[133].

Large variations were seen in the BHR% within the Polyporales group, with only 4 samples falling within the target of less 30% BHR%. *Ganoderma lingzhi* 3 showed the least BHR% of 16.7%, which is also the least value among all the samples tested. It was followed by *Mycorrhaphium* sp. with a BHR% of 20.3%. Ganoderic acids C and D, isolated from the methanolic fraction of *Ganoderma lucidum* have been reported to inhibit the release of histamine from rat mast cells[134]. In the Others group, only *Xylobolus princeps* 4 fell within the target group, with a BHR% of 27.0%.

6.3.2 Determination of IC_{50} values

The top 10 samples showing the least β-hexosaminidase release and high cell viability were chosen for determination of IC_{50} values. The IC_{50} values of the extracts as well as the pure compound is provided in Table 6.2. The samples included 6 samples from Hymenochaetales, and 4 samples from Polyporales. They were as follows: *Inonotus clemensiae*, *Inonotus* sp.2, *Phellinus conchatus* 1 and 2, *Phellinus adamantinus*, *Phellinus* sp.2, *Ganoderma lingzhi*, *Mycorraphium* sp., *Ganoderma endochroum*, and *Ganoderma* sp.1. Moreover, since the bioactive compound in one of the highest activity sample *Inonotus clemensiae* was already identified to be hispidin, the pure compound was also analyzed together with the rest of the samples. The IC_{50} values could be determined for 6 of the samples tested, with *Inonotus clemensiae* and *Phellinus adamantinus* requiring the least concentrations of 51.24 µg/mL and 50.65 µg/mLrespectively for 50% BHR. Interestingly, the major active compound in *I.*

clemensiae, hispidin, required a higher concentration of 82.47 µg/mL to achieve 50% BHR, which indicates that either the compound is more stable and bioactive within the sample matrix, or there is the presence of some minor compounds in the extracts which enhanced the anti-allergic activity.

6.4 Conclusion

Anti-allergic and anti-inflammatory activity bears great value as an indicator of potential therapeutic agents, as chronic allergic reactions can lead to life threatening conditions. Several samples of Nepalese wild mushrooms showed strong inhibition of β-hexosaminidase release in the RBL-2H3 cells, especially those belonging to *Inonotus* sp. and *Phellinus* sp. The bioactive compound in *Inonotus clemensiae*, hispidin, was also able to show strong anti-allergy activity. Further chemical characterization of other highly active samples shall reveal other bioactive compounds too. Moreover, testing the anti-inflammatory activity using other methods based on a different mechanisms shall further deepen the understanding about the anti-allergic activity of these mushrooms.

Table 6.1: Effect of the ethanol extracts of 90 wild mushroom samples on β-hexosaminidase release (BHR) % and cell viability (CV) % of RBL-2H3 cells

Order Scientific name	BHR%	CV%	**Order** Scientific name	BHR%	CV%
Hymenochaetales	a	a	**Polyporales** *contd.*		
Inonotus andersonii	65.5±3.8	104.0±4.9	*Ganoderma carnosum*[*]	44.6±2.0	108.3±8.2
Inonotus clemensiae	22.8±4.7	123.7±2.5	*Ganoderma* sp. 1	30.1±6.8	99.8±1.5
Inonotus cuticularis	62.2±2.8	101.3±1.9	*Ganoderma* sp. 2	35.6±6.1	99.5±2.8
Inonotus sp.1	34.5±1.6	81.9±1.5	*Amauroderma calcigenum*	128.2±20	77.9±3.6
Inonotus sp.2	23.4±2.0	109.3±4.7	*Trichaptum biforme*	123.6±7.6	96.5±15.7
Inonotus sp.3	52.5±7.5	87.7±1.5	*Trichaptum abietinum*	77.7±10.8	94.4±4.3
Inonotus sp.4	45.9±2.0	98.4±3.3	*Trametes versicolor* 1	60.4±6.8	83.4±2.1
Inonotus sp.5	83.6±11.9	103.6±1.2	*Trametes versicolor* 2	53.9±8.3	108.1±3.2
Phellinus gilvus	41.2±3.4	98.8±10.7	*Trametes versicolor* 3	40.4±3.7	64.3±2.4
Phellinus conchatus 1	21.6±2.1	103.3±7.1	*Trametes versicolor* 4	69.4±10.2	97.8±3.0
Phellinus conchatus 2	26.0±0.3	109.4±3.0	*Trametes versicolor* 5	60.0±3.5	97.2±4.3
Phellinus sp. 1	65.6±7.4	97.9±2.4	*Microporus xanthopus*1	75.9±10.2	98.3±1.1
Phellinus sp. 2	20.1±2.3	99.2±1.8	*Microporus xanthopus*2	130.5±5.6	93.5±1.1
Phellinus adamantinus	19.4±5.0	99.5±1.7	*Polyporus arcularius*	160.8±25.5	92.4±6.2
Cyclomyces setiporus 1	57.5±3.1	98.6±1.9	*Postia stiptica*	93.3±11.6	12.6±0.6
Cyclomyces setiporus 2	45.0±3.1	99.5±1.7	*Phlebia tremellosa* 1	89.9±7.1	69.1±1.8
Cyclomyces setiporus 3	65.8±11.8	106.8±3.5	*Phlebia tremellosa* 2	35.2±7.9	93.9±0.9
Cyclomyces setiporus 4	43.8±4.2	109.9±1.6	*Lenzites betulina*	83.5±1.4	91.0±5.2
Cyclomyces setiporus 5	35.5±6.0	107.3±7.4	*Rigidoporus* sp.	127.6±12.0	72.0±6.4
Oxyporus sp.	67.5±5.6	86.3±1.7	*Laetiporus versisporus* 1	122.6±3.7	77.5±0.8
Polyporales	ab	b	*Laetiporus versisporus* 2	53.7±3.4	93.4±0.6
Ganoderma australe 1	31.5±3.4	95.6±1.4	*Laetiporus montanus*	50.0±6.22	81.6±2.3
Ganoderma australe 2	32.0±9.5	97.3±1.4	*Mycorrhaphium sp.* [**]	20.3±1.9	103.9±2.4
Ganoderma australe 3	26.4±2.2	83.6±6.9	*Grifola frondosa*	72.1±1.1	83.9±4.4
Ganoderma australe 4	38.3±6.7	101.4±4.6	*Lentinus* sp. [**]	38.4±5.7	93.0±1.4
Ganoderma lingzhi 1	91.4±30	77.1±4.8	*Bjerkandera adusta*	53.9±1.1	72.5±1.7
Ganoderma lingzhi 2	50.5±4.4	79.5±11.3	*Antrodiella zonata* 1	102.9±5.3	93.1±4.6
Ganoderma lingzhi 3	16.7±3.2	106.1±3.7	*Antrodiella zonata* 2	86.7±11.8	91.6±0.7
Ganoderma endochroum	23.4±2.8	101.0±1.7	*Fomes fomentarius*	95.4±6.4	87.3±1.6
Ganoderma multipileum	32.3±6.5	94.6±0.7	*Abortiporus biennis*	127.1±6.8	102.2±0.4

Table 6.1 *contd.*

Order Scientific name	BHR%	CV%	**Order** Scientific name	BHR%	CV%
Agaricales	b	b	**Others**	ab	b
Lentinula edodes 1	104.5±3.5	91.3±2.9	*Heterobasidion linzhiense* 1	34.0±4.5	68.4±1.6
Lentinula edodes 2	93.0±15.8	94.7±0.6	*Heterobasidion linzhiense* 2	53.3±1.7	71.9±2.5
Pleurotus ostreatus 1[*]	93.3±11.4	98.7±2.0	*Lactarius hatsutake*	122.6±9.6	48.5±5.3
Pleurotus ostreatus 2[*]	108.1±13.2	89.8±3.1	*Lactarius* sp.[**]	64.6±9.4	100.4±3.2
Pleurotus ostreatus 3[*]	71.3±2.7	93.4±2.9	*Russula brevipes*	68.9±6.3	73.7±1.6
Pleurotus ostreatus 4	137.4±15.7	66.5±3.0	*Engleromyces goetzii*	92.0±10.0	112.2±12.8
Pholiota nameko 1	145.6±0.4	98.6±2.2	*Neolentinus lepideus*	51.7±4.2	87.4±0.5
Pholiota nameko 2	62.0±3.2	89.0±1.0	*Xylobolus princeps* 1	60.8±0.8	98.4±1.7
Marasmius mavium	151.1±13.2	57.6±7.2	*Xylobolus princeps* 2	59.0±2.3	88.4±3.8
Marasmius sp.	60.6±2.5	95.9±6.2	*Xylobolus princeps* 3	37.3±5.2	104.3±1.7
Panellus edulis	60.4±1.3	83.4±4.0	*Xylobolus princeps* 4	27.0±1.8	87.1±6.9
Panellus sp.	54.2±4.8	89.3±5.3	*Pseudomerulius curtisii*	15.2±1.3	40.6±4.4
Inocybe sp. 1	116.0±9.2	41.8±8.7	*Cantharellus ferruginascens*	74.5±6.1	64.0±2.9
Inocybe sp. 2	60.2±4.4	92.5±2.6	*Neolentinus lepideus*	51.7±4.2	87.4±0.5
Collybia peronata	56.9±2.7	81.4±0.2			
Tricholoma caligatum	95.5±10.2	80.8±2.0			
Mucidula mucida[*]	110.4±11.0	76.3±19.3			
Gymnopus sp.	70.9±11.1	91.5±1.9			

The values are expressed as "average ± standard deviation", and n=3. Difference in letters in each column means that there is a statistical difference between groups at a significance level of $p < 0.05$. The names of the mushrooms marked with [**] were tested at 50 μg/mL, and those marked with [*] were tested at 100 μg/mL, and the remaining samples were tested at 200 μg/mL.

Table 6.2: IC_{50} values for β-hexosaminidase release (BHR) %of the samples with highest anti-allergy activity

S.N.	Scientific name	IC_{50} value (µg/mL)
1	*Inonotus clemensiae*	51.24±2.5
2	*Inonotus* sp.2	ND
3	*Phellinus conchatus* 1	ND
4	*Phellinus conchatus* 2	ND
5	*Phellinus adamantinus*	50.65±2.0
6	*Phellinus* sp.2	54.00±17.2
7	*Ganoderma lingzhi*	97.65±7.8
8	*Ganoderma endochroum*	ND
9	*Ganoderma* sp.1	ND
10	*Mycorraphium* sp.	72.83±5.2
	Hispidin	82.47±8.8

The values are expressed as as "average ± standard deviation", and n=3. ND means not determined.

Chapter 7

Anticancer activity

7.1 Introduction

Cancer is currently one of the biggest global health problems, and is expected to surge even further in the coming years. The International Agency for Research on Cancer (IARC) reported 14.1 million new cases of cancer, 8.2 million cancer deaths, and 32.6 million people with cancer (within 5 years of diagnosis) globally in 2012[135]. Cancer is the second leading cause of death in the world, and approximately 70% of deaths from cancer occur in low and middle income countries. The most common causes of cancer death are lung cancer, followed by liver, colorectal, stomach, and breast cancers[136]. Apart from being a global health issue, cancer also presents a great economic burden, with US$ 1.16 trillion being spent in the year 2010[136]. Among other factors diet, obesity and physical activity are some of the main parameters having a profound influence on the development and progression of cancer[137]; and it is estimated that between 30 to 50% of cancers can be prevented through healthy lifestyle habits.

The medicinal potential of mushrooms has been recognized in several areas such as antimicrobial, anti-hypertensive, anti-inflammatory activities, and the anti-cancer activity is no exception. Several species of mushrooms are known to have inhibitory effect on various types of cancer[138]. Mushrooms have been found to contain several different kinds of bioactive compounds related to the anti-cancer activity. The bioactive compounds range from low molecular weight compounds like quinones, isoflavones, sesquiterpenes, and others to high molecular weight compounds like polysaccharides, glycoproteins, protein-RNA complex and others[139]. The bioactive polysaccharides and polysaccharide conjugates are eminent anti-cancer agents finding commercial application in anti-cancer therapy. Some of such examples are schizophyllan from *Schizophyllum commune*[140], protein bound polysaccharide K (PSK;

KRESTIN®) from *Coriolus versicolor* [141], lentinan from *Lentinus edodes*, and Befungin from *Inonotus obliquus*[36].

In recent years there has been a growing interest worldwide in the anti-cancer potential of *Ganoderma lucidum*[142, 143]. The most common bioactive compounds in this species are triterpenoids, polysaccharides, proteins, and glycoproteins[144, 145]. These bioactive compounds are found to act upon cancer cells by modulating the host's immune response, exhibiting cytotoxicity to cancer cells, inhibition of tumor-induced angiogenesis and cancer cell proliferation among others[146, 147]. *Inonotus obliquus* is another interesting mushroom possessing several bioactive compounds like lanosterol, inotodiol, and other triterpenes showing selective cytotoxicity towards various cancer cell lines[148]; and in another study various subfractions of the mushroom extract showed inhibition of tumor growth in Balbc/c mice bearing Sarcoma-180 cells[149].

Keeping in mind the abundance of bioresources in Nepal, one can speculate the prospects of several natural products, including mushrooms bearing anticancer activity. However, the severe lack of research dedicated to studying the bioactive properties of Nepalese mushrooms is the major hindrance in the understanding of their bioactive potential. Recently, some researchers have studied the anti-cancer and cytotoxic, and other bioactive properties of some medicinal plants[150] and lichen[151] of Nepal. However, studies related to mushrooms could not be found. Therefore the present study on the anticancer activity of Nepalese mushroom extracts is a first of its kind, and shall definitely be of great value in filling the gap of knowledge that is currently prevalent. By conducting a comparative analysis between the cancer cell lines as well as the normal cell lines, extracts showing selective inhibitory activity towards the cancer cell lines could be selected.

7.2 Materials and methods

Four different cancer cell lines, and 2 normal cell lines were used. Human breast carcinoma (MCF-7), human cervical cancer (HeLa), human colorectal carcinoma (HCT 116), and human hepatocellular carcinoma (HepG2) were used as cancer cell lines. For the normal cell line human colon (CCD 841) and normal human dermal fibroblast (NHDF-Ad) were used. Eagle's minimal essential medium (EMEM), Wako Pure Chemical Industries, Osaka, Japan) was used for culturing MCF 7, HeLa, and CCD 841 cells. Dulbecco's Modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) was used for NHDF-Ad and HepG2 cell lines, and HCT-116 cells were cultured in McCoy's 5A Medium (Wako Pure Chemical Industries, Osaka, Japan). All culture media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin (Gibco BRL, Tokyo, Japan).

7.2.1 Cell viability assay

Cytotoxicity to cancer and normal cell lines was determined using MTT assay[152]. The cells were cultured in 96-well plates at densities of 2×10^5 cells/well, respectively, in a humid atmosphere of 5% (v/v) CO_2 and 95% (v/v) air at 37 °C. After 24 h, the cells were treated with various concentrations of the extracts dissolved in DMSO. 5-Fluorouracil (50 µg/mL) was used as a positive control. After 72 h, cell viability was determined by adding 10 µL of MTT reagent (5 mg/mL), followed by an incubation period for 4 hours. Then the medium was removed and the formazan crystals were dissolved in 100 µL of 0.04 M HCl in isopropanol. The cell viability was estimated based on the absorbance measured at 570 nm, calculated as percentage value compared to the negative control, DMSO.

7.2.2 Statistical analysis

All the assays were conducted at least 3 times, and the results are expressed as mean ± standard deviation. Significant differences between sample groups were analyzed by Kruskal Wallis test

followed by Dunn-Bonferroni test. The statistical analyses were performed using SPSS statistics Version 23. The *p*-value less than 0.05 were considered statistically significant.

7.3 Result and discussion

7.3.1 Cell viability assay

The ethanol extracts of 61 wild mushroom samples from Nepal were tested for *in vitro* anti-cancer activity against 4 cancer cell lines and 2 normal cell lines. The inhibition percentage of the samples against all the cell lines are provided in Table 7.1. In general, most of the samples showed a mixed set of inhibitory effect against the 6 cell lines. No significant difference between groups could be detected for the cancer cell lines HCT 116, and MCF 7; and the normal cell line NHDF-Ad. However, significant difference between groups could be seen for the cancer cell lines HeLa, and HepG2; and the normal cell line CCD 841. Finally, the target was to select the samples that showed strong inhibitory activity against the cancer cell lines, but did not affect the viability of the normal cell lines.

In the Hymenochaetales group, although most of the samples strong cytotoxicity against the cancer cell lines, similar phenomenon was seen for the normal cell lines too. However, some select samples like *Inonotus clemensiae*, *Inonotus cuticularis*, and *Oxyporus* sp. were found to show good selectivity. *I. clemensiae* (400µg/mL) showed an inhibitory effect on the human breast cancer MCF-7 cell line with a CV of 26.4%, while maintaining 63.2% CV for human normal colon CCD 841 cell line. *I. cuticularis* (400µg/mL) had a strong selectivity against human cervical cancer cell, HeLa with a CV of 35.5%, while showing zero inhibitory effect on the normal human dermal fibroblast cells, NHDF-Ad. *Inonotus* sp. are known to possess strong anticancer activities[125, 153, 154]. Subfractions obtained from the methanolic extracts of *Inonotus obliquus* have also been reported to inhibit the MCF 7 cells[149]. Similarly, *Oxyporus*

sp. also had inhibitory effect on HeLa (30.9% CV), and human hepatocellular carcinoma, HepG2 cells (35.3% CV), with no inhibitory effect on NHDF-Ad cell lines. *Phellinus* sp. 1 also had a noteworthy selectivity against HeLa (25.6% CV). With almost no inhibitory effect on NHDF-Ad. Polysachharides have been attributed to the inhibitory activity of *Phellinus baummi* against HeLa and other cancer cells[155].

In the Polyporales group, only 3 out of 24 samples could show selective inhibitory effect against the cancer cell lines. *Bjerkandera adusta* had strong inhibitory effect against HeLa (11.5% CV) and MCF 7 (29.4% CV) cell lines. *Laetiporus versisporus* 2 and *Laetiporus montanus* inhibited the growth of HeLa cells with a CV of 37.9% and 11.0% CV respectively. All three samples had no inhibitory effect on NHDF-Ad cells. However, it must be noted that *B. adusta* and *L. montanus* showed strong cytotoxicity against the other normal cell line CCD 841, with a CV of only 17.5% and 12.6% respectively. *Ganoderma* sp. has attracted much attention in recent years as being potent anti-cancer agents[142, 146]. However, the samples of *Ganoderma* spp. studied in this group could not show strong selectivity, although strong cytotoxic effects against the cancer cells were also seen.

In the Agaricales group, 4 out of 14 samples showed inhibitory effect on cancer cell lines. Surprisingly, the anti-cancer activity of this group was at par with the rest of the groups unlike other bioactivities like antioxidant, antibacterial and anti-allergy. *Pholiota nameko*1 and *Pleurotus ostreatus* 4 showed selective cytotoxicity against HeLa with 14.5% and 11.6% CV respectively. *Lentinula edodes* 2 and *Inocybe* sp. showed strong cytotoxicity against HeLa (CV 23.2% and 20.0% respectively) and MCF 7 (CV 14.4% and 13.4%). All of the samples showed the uninhibited growth of the normal cell line NHDF-Ad only. Although, the anti-cancer activity of species belonging to *Pleurotus* sp. and *Lentinus edodes* have largely been attributed to the polysaccharides[156], evidence of selective cytotoxicity of ethanol extracts of *L. edodes* against murine skin cell carcinoma can also be found[157].

Finally, in the Others group, *Xylobolus princeps* 2 and 1 showed strong cytotoxicity against HeLa (33.6% and 20.0% CV respectively) and MCF 7 (33.4% and 18.3% respectively). Also, *Neolentinus lepideus* and *Lactarius* sp. showed strong inhibition of Hela with CV of 23.0% and 9.5% respectively. Again, no inhibition of normal cell line NHDF-Ad was seen for all the samples. *Neolentinus lepideus* showed a high CV of 82.1% for CCD 841 too.

7.4 Conclusion

Several mushroom species have proven to be important allies in the field of cancer therapy. The chemical constituents in those mushrooms, especially various polysaccharides are known to be biological response modifiers that stimulate the human immune system against the development and proliferation of various types of cancers. However, the strong anti-cancer potential of the ethanol extracts of several Nepalese mushrooms in this study, reveals the presence of other very strong and selective anti-cancer compounds too. Further research into the dose dependency of the extracts and the bioactive compounds in it; as well as the mechanism of anti-cancer activity, will be helpful in establishing these mushrooms as therapeutic agents.

Table 7.1 Cell viability (CV)% of the ethanol extracts of 61 Nepalese wild mushrooms against 4 cancer cell lines (HCT 116, HeLa, HepG2, and MCF 7), and 2 normal cell lines (CCD 841 and NHDF-Ad)

Scientific name	HCT-116	HeLa	HepG2	MCF 7	CCD 841	NHDF-Ad
Hymenochaetales		a	a		a	
Inonotus clemensiae	204.4±16.5	40.2±3.0	95.90±14.6	26.4±8.7	63.2±3.6	37.9±6.7
Inonotus cuticularis	138.6±5.6	35.5±5.6	107.5±10.7	64.8±0.2	58.2±18.4	100.8±13.4
Inonotus sp. 4	106.5±5.4	22.6±1.8	37.2±5.7	17.0±1.0	26.6±5.0	32.5±3.0
Inonotus sp.5	22.4±1.5	32.3±1.6	44.1±10.6	32.6±1.8	43.1±3.8	37.7±1.4
Cyclomyces setiporus 1	11.4±4.1	41.9±2.2	28.9±8.4	43.2±5.3	31.1±6.4	67.1±12.4
*Cyclomyces setiporus*3	58.9±5.3	45.6±4.5	33.7±7.2	13.3±1.3	18.5±3.0	34.4±3.6
*Cyclomyces setiporus*2	64.1±11.8	26.4±2.7	39.8±0.7	37.3±2.7	41.2±0.2	60.7±3.4
Cyclomyces setiporus 4	116.8±3.5	-	88.0±3.7	76.9±5.3	25.1±4.8	49.2±6.5
Cyclomyces setiporus 5	29.9±17.8	37.3±2.5	70.4±5.1	21.3±4.0	41.4±1.5	-
Phellinus sp. 1	74.8±7.0	25.6±3.5	55.2±3.1	54.7±5.8	23.4±0.6	99.3±7.1
Phellinus sp. 2	10.7±0.4	13.3±1.2	8.7±0.7	12.7±3.7	21.0±1.8	30.0±6.8
Phellinus adamantinus	28.4±2.3	19.2±3.5	18.6±5.0	28.7±6.5	21.1±1.1	37.2±2.3
Oxyporus sp.	46.8±5.1	30.9±3.6	35.3±1.2	70.6±8.4	57.1±12.6	138.6±9.7
Polyporales		b	ab		b	
Ganoderma sp. 1	95.1±10.7	21.8±3.2	34.8±6.6	24.7±2.9	25.0±4.7	50.7±4.2
Ganoderma sp. 2	31.6±5.7	29.4±3.7	116.7±17.4	24.5±4.3	15.6±3.8	41.8±10.4
Ganoderma lingzhi 2	14.5±0.3	17.1±0.7	9.2±0.5	21.8±6.7	23.8±1.0	40.4±4.6
Ganoderma lingzhi 1	22.6±7.0	16.1±1.0	14.9±1.0	17.0±4.0	23.9±1.2	36.7±7.0
Ganoderma endochroum	12.0±1.5	14.8±2.2	7.8±0.6	15.4±1.8	21.2±3.5	20.3±0.9
Ganoderma multipileum	9.7±1.7	26.9±2.4	8.8±2.0	18.5±1.8	15.0±2.2	29.6±6.6
Amauroderma calcigenum	110.5±2.8	20.9±6.6	103.6±7.8	15.2±4.5	17.4±7.9	25.2±0.9
Microporus xanthopus 2	103.8±1.7	21.7±1.3	75.7±17.3	27.8±5.5	11.0±0.5	32.3±4.4
Microporus xanthopus 1	10.9±8.7	9.1±0.7	10.0±1.5	14.4±2.1	12.1±0.2	19.2±1.4
Fomes fomentarius	99.1±6.0	87.7±7.0	89.6±5.8	117.5±10.3	58.3±4.1	126.0±15.9
Trichaptum abietinum	18.8±9.6	12.3±1.2	51.4±1.7	11.5±0.2	13.0±1.9	33.0±12.2
Trichaptum biforme	13.6±1.2	12.6±1.3	63.7±2.9	11.8±1.5	15.9±0.3	31.2±0.2
Bjerkandera adusta	49.6±16.7	11.5±1.7	113.9±8.8	29.4±6.8	17.5±5.3	158.2±12.8
Abortiporus biennis	12.2±3.1	11.9±0.9	23.2±2.1	11.6±3.9	13.8±2.1	20.4±3.6
Phlebia tremellosa 2	119.6±2.4	14.1±0.9	12.9±2.4	14.1±2.0	17.7±1.9	28.8±5.2
Mycorrhaphium sp. **	14.0±2.6	16.0±1.9	8.2±0.5	15.7±2.6	10.0±0.5	16.4±1.3
Antrodiella zonata 1	28.8±12.1	11.5±1.4	65.3±1.0	20.4±7.2	12.8±2.1	87.9±14.0
Antrodiella zonata 2	13.2±5.1	10.7±0.4	8.3±0.7	14.4±1.1	37.7±5.4	18.6±1.4
Laetiporus versisporus 1	18.6±4.3	7.7±0.9	108.0±9.0	12.8±1.5	12.3±3.0	15.0±0.5
Laetiporus versisporus 2	81.3±11.8	37.9±4.7	79.3±4.9	70.8±7.3	44.8±6.1	168.5±2.2
Laetiporus montanus	90.4±5.2	11.0±0.3	70.3±6.7	86.4±5.6	12.6±2.0	161.7±7.3
Grifola frondosa	57.5±11.1	27.1±13.5	89.6±3.2	11.8±4.5	10.1±0.1	15.7±0.8
Polyporus arcularius	10.2±2.9	11.5±0.2	75.5±4.1	12.1±1.1	14.2±2.9	16.1±3.2
Lentinus sp. **	71.1±13.3	18.6±3.9	109.2±5.2	9.1±0.2	14.3±0.6	20.2±1.4

Table 7.1 *contd.*

Scientific name	HCT-116	HeLa	HepG2	MCF 7	CCD 841	NHDF-Ad
Agaricales		b	b		b	
Marasmius sp.	8.9±0.5	12.4±1.6	79.3±4.7	14.6±3.6	14.0±2.6	17.6±3.2
Marasmius mavium	10.4±0.9	12.2±3.3	79.0±3.2	26.5±1.8	13.8±0.2	18.0±0.7
Pholiota nameko 2	52.7±6.5	7.0±0.6	100.3±2.7	10.8±1.1	19.5±5.5	20.7±3.6
Pholiota nameko 1	53.1±12.0	14.5±1.5	72.14±5.0	77.7±8.1	12.1±2.0	123.1±4.4
Gymnopus sp.	16.9±11.7	15.6±1.9	101.2±11.3	23.8±3.8	9.48±0.3	17.1±1.5
Pleurotus ostreatus 4	83.2±6.2	11.6±0.7	69.2±4.8	-	15.5±3.4	108.5±8.8
Pleurotus ostreatus 2[*]	82.0±5.7	10.6±1.0	111.3±21.7	-	14.6±2.2	86.1±14.2
Pleurotus ostreatus 1[*]	90.1±4.7	11.3±2.5	86.6±3.9	13.5±1.8	13.8±1.4	52.8±14.7
Pleurotus ostreatus 3[*]	87.0±9.8	13.8±1.4	93.9±3.2	13±0.8	17.4±1.9	55.3±3.1
Lentinula edodes 1	65.1±2.0	86.7±20.8	122.2±6.3	76.0±4.1	80.4±12.9	115.8±9.6
Lentinula edodes 2	116.8±3.5	-	88.0±3.7	76.9±5.3	25.1±4.8	49.2±6.5
Inocybe sp.	58.7±3.7	20.0±2.7	82.5±5.1	13.4±0.1	16.6±0.6	130.7±13.0
Panellus edulis	156.9±18.6	44.1±7.0	82.6±3.4	98.5±3.5	71.2±18.5	99.6±11.9
Mucidula mucida [*]	16.1±1.0	9.1±1.1	78.5±6.5	10.5±0.5	12.4±2.4	16.8±4.4
Others		ab	ab		ab	
Pseudomerulius curtisii	9.7±0.4	49.9±20.5	11.8±3.0	22.9±7.3	21.1±0.1	39.4±1.1
Xylobolus princeps 4	48.4±13.5	37.8±3.0	91.9±6.5	36.2±4.8	32.6±14.3	111.0±1.4
Xylobolus princeps 2	204.4±16.5	33.6±3.7	72.4±4.3	22.3±3.2	33.4±10.9	98.7±16.5
Xylobolus princeps 3	58.9±5.3	25.9±3.2	79.6±6.9	16.5±1.8	16.8±0.9	80.5±7.3
Xylobolus princeps 1	19.8±2.2	20.0±1.0	81.6±3.8	19.0±2.8	18.3±6.7	129.4±17.2
Engleromyces goetzii	108.1±2.0	36.7±3.3	74.18±3.6	74.7±6.4	75.4±6.0	113.±4.6
Neolentinus lepideus	117.2±4.0	23.0±3.9	52.4±8.3	37.1±9.2	82.1±15.2	124.3±12.6
Lactarius sp. [**]	-	9.5±0.5	87.7±6.6	77.3±3.1	37.7±2.1	96.7±3.2
Russula brevipes	11.3±2.1	10.2±0.9	132.4±12.2	14.7±3.0	22.5±16.1	26.2±15.4
Cantharellus ferruginascens	20.1±1.3	9.9±0.2	7.2±0.6	17.4±0.7	12.5±0.9	23.9±4.6

The values are expressed as "average ± standard deviation", and n=3. - means the cell viability against the particular cell line remains undetermined. Difference in letters in each column means that there is a statistical difference between groups at a significance level of $p < 0.05$. The names of the mushrooms marked with [**] were tested at 100 µg/mL, and those marked with [*] were tested at 200 µg/mL, and the remaining samples were tested at 400 µg/mL. – means that the sample could not be tested for the particular cell line.

Chapter 8

In vitro digestion

8.1 Introduction

Mushrooms are an important group of food drawing immense attention for its various functional properties and bioactivities. Several mushroom species such as *Ganoderma* sp., *Inonotus* sp., *Phellinus* sp. and others have proven to be a source of various novel and bioactive compounds[18]. The markets are flooded with extracts and other form of functional foods derived from these mushrooms. Therefore, understanding the fate of the bioactive compounds after the various stages of gastrointestinal and colonic digestion becomes equally importance. Various researchers have studied the in vitro metabolism of dietary phenolic compounds[158–160]. Also some studies have been dedicated for the phenolic compounds in mushrooms, using *in vitro* [161] and *in silico* models[162].

The oral administration of drug or bioactive compounds is the most common and convenient mode of administration[163]. After ingestion, these compounds are confronted with a series of metabolic processes which bears important implications in their bioaccessibility [164–166]. Finally, the unabsorbed and undigested materials pass into the colon, which is rich in the strict anaerobic bacterial population such as Bacteroidetes and Lachnospiraceae[167]. The enzymes produced by these bacteria can further metabolize the compounds and facilitate absorption. Several sophisticated *in vitro, ex vivo* and *in vivo* animal models have been designed to simulate the human gut fermentation, as closely as possible[168, 169].

Among the 92 mushroom extracts investigated for various bioactivities, *Inonotus clemensiae* was found to be one of the most bioactive sample. The chemical characterization of this extract revealed hispidin to be the most abundant bioactive compound. Hispidin is one of the styrylpyrone type compound that has been reported from mushroom species such as *Inonotus*

and *Phellinus* spp [76], with several reported bioactivities including anti-oxidant, anti-inflammatory, anti-cancer and others. Therefore, to understand the possible biotransformation, and the maintenance of bioactivity upon the metabolic digestion of the compound, hispidin was subjected to *in vitro* human fecal microbial digestion.

8.2 Materials and Method

8.2.1 Fecal sample preparation

Fecal samples from 14 healthy donors (age 21 to 41) were collected and stored at -80°C until further use. The fecal samples for *in vitro* fermentation were prepared according to the method described by Weickert et al., after slight modification [170]. Briefly, the frozen feacal samples were thawed at room temperature and diluted 10 folds (w/v) using pre-reduced phosphate buffer. To maintain anaerobic conditions, cysteine was used as the reducing agent in the buffer and it was purged with nitrogen gas. The resulting fecal slurry was centrifuged at 300 rpm for 3 min and again at 2000 rpm for 5 min, to remove any non-bacterial particles. Equal volumes of the supernatant of each of the 14 samples was pooled, and the mixture was used as the fecal bacterial stock solution.

8.2.2 Colonic fermentation

The fecal digestion was performed according to previously described method by Ding et al. after slight modifications [171]. Twenty five mL screw cap glass tubes were filled with 20 mL Gifu Anaerobic Medium (GAM) broth and purged for 5 min with filtered nitrogen gas prior to autoclaving. Three groups of tubes were prepared: the first set (positive control) contained 20 mL GAM broth and 5mg hispidin, the second set (test) contained 20 mL GAM broth, 5mg hispidin, and 0.2% v/v fecal bacterial stock solution, and the third set (negative control) contained 20 mL GAM broth and 0.2% v/v fecal bacterial stock solution. The tubes were incubated at 37°C at 250 rpm, and samples were collected from each set at the time points of 0h, 5h, 12h, and 24h. The collected samples were stored at -30°C until further analysis. The

frozen samples were freeze dried, followed by ethyl acetate extraction for at least 3 times. The ethyl acetate extract was then dried using a rotary evaporated. The dried extracts were further used for the analysis of bioactivity and for LC-MS analaysis.

8.2.3 Analysis of Bioactivities

Previous investigations revealed that hispidin is an important bioactive compound responsible for the bioactivities such as antioxidant, antibacterial, and anti-allergy activities [23, 24, 99, 129]. Therefore, to analyze the maintenance of the bioactivity even after the *in vitro* digestion of the bioactive compound, the bioactivities were tested once again for the extracts of the digested product from 3 sample sets as described above. For antioxidant activity, the DPPH radical scavenging activity was tested; for antibacterial activity, the activity against *Staphylococcus aureus* was tested; and for anti-allergy activity, β-hexosaminidase release from RBL-2H3 cells and the cell viability was tested. The same methods were followed as described previously in chapters 3, 4 and 6.

8.2.4 LC-MS analysis

The sample injection volume was 10 µL and flow rate 0.5 mL/min. ODS-3 column with the dimensions 4.6 x 150 mm, 5µm particle size was used. Water with 0.1% formic acid was used as solvent A and methanol with 0.1% formic acid was used as solvent B. A gradient flow from 95% A to 0% A in 40 min was used, followed by 0% A, 40 – 45 min,; and 95% A, 50 – 60 min. The chromatograms were recorded at 254 nm. For MS, electron spray ionization (ESI) source was used in positive and negative ionization mode with *m/z* values of 50-1000 for MS and MS/MS. A probe voltage of ± 4.5 kV, nebulizer gas flow of 1.5 L/min, curved desolvation line (CDL) temperature of 200 °C, and heat block temperature of 200 °C were used.

8.3 Results and discussion

The description of the fecal sample is provide in the appendix in Table A8.1. The samples were collected from 14 healthy individuals between the ages of 21 to 41with no recent history of antibiotic use. The participants included 6 male and 8 female participants. Stock solution of the mixture of fecal samples were prepared and were inoculated together with the pure compound hispidin. The sample plan is shown in Fig 8.1. The samples were divided into 3 groups, with group 1 as positive control, group 2 as test, and group 3 as negative control.

8.3.1 Analysis of bioactivities

The comparative results for the bioactivity of the 3 groups are shown in Fig 8.2 (a - c). The bioactivity of the fecally digested hispidin was comparable with the positive control i.e undigested hispidin, for all of the bioactivities tested. These result show that the antioxidant, antibacterial, and anti-allergy activity of hispidin is maintained even after the digestion process. Also, as expected the bioactivity was absent or negligible for the negative control i.e without hispidin.

A constant DPPH inhibition percentage of above 70% was maintained by both the digested and undigested hispidin until 24h incubation time. As for the antibacterial activity, an almost 100% inhibition was maintained by both undigested and digested hispidin for 12h incubation time. However, the inhibition decreased slightly to 81.02% for the digested hispidin at 24h incubation time. The anti-allergy activity was estimated by the capability of the sample to suppress the release of β-hexosaminidase from RBL-2H3 cells. Therefore lower percentage release indicates higher anti-allergy activity. The β-hexosaminidase release percentage was maintained below 30% by both digested and undigested hispidin until 12h. However, there was a slight increase in the release percentage in the digested sample at 24h to 40.07%. A slight loss of activity was seen for antibacterial and anti-allergy activity upon prolonged incubation. However, an overall maintenance of bioactivity was determined for hispdin even after digestion.

8.3.2 LC-MS analysis

The LC-MS analysis was done to investigate the biotransformation of the compound hispidin after digestion with fecal stock solution. The base peak chromatograms of samples from each group, collected at time points 0h, 5h, 12h, and 24h are provided in Fig 8.3 to 8.6 (a to c). The hispidin peak was detected between 26 to 27 min. As expected, there was not much difference between the chromatograms of the 3 groups, except that group 3 did not show the hispidin peak. Also, the samples collected until the time point 5h did not have much difference.

From the time point 12h, significant differences started to be seen the chromatograms. The most notable difference was the appearance of a prominent peak at the retention time 42.5 min, in set 2. However, this peak was not observed in set 1 and 2. This indicates that the peak is specific to the microbial activity on the hispidin compound. The metabolite in this particular peak, however, did not have any UV –Vis absorbance. The 3D plot of the UV-vis spectrum of samples from each set collected at every time point is provided as appendix in Fig A8.1 to A8.3 (a to d). Even, in the 24h sample of set 2, the particular peak continued to appear.

The analysis of the m/z value of the peak showed a molecular ion peak of m/z value 301.3250 and an $[M+Na]^+$ peak with m/z value of 323.2538 in the positive mode. In the negative mode a molecular ion peak of m/z value 299.2581 was seen. The MS/MS fragmentation in the positive mode showed peaks with m/z values of 247.2500, which is the molecular ion peak for hispidin; and peak with m/z value of 163.2120, which is one of the product ions for hispidin. The MS fragmentations of the peak is provided as supplementary data in Fig A8.4 (a - c). Therefore, from the results obtained it can be speculated that it is the conjugate of hispidin formed in the presence of the fecal bacteria. However, the exact structure of the compound by NMR analysis is necessary for confirmation.

8.4 Conclusion

Hispidin is a well-established bioactive compound having several bioactive properties [172, 173]. So, it is very important to understand the fate of the compound upon digestion to speculate its potential health benfits after consumption as functional food. The results obtained from this study indicate a possible biotransformation of the compound. However, the strong bioactivity of the transformed product is maintained even after digestion of the parent compound. Also, the digested product seemed to contain the conjugate of hispidin. Further studies on the identification of the biotransformed product is needed to confirm the exact structure. The results obtained so far show a promising effect of hispidin even after digestion, which further strengthens the evidence for its use as functional food or ingredient.

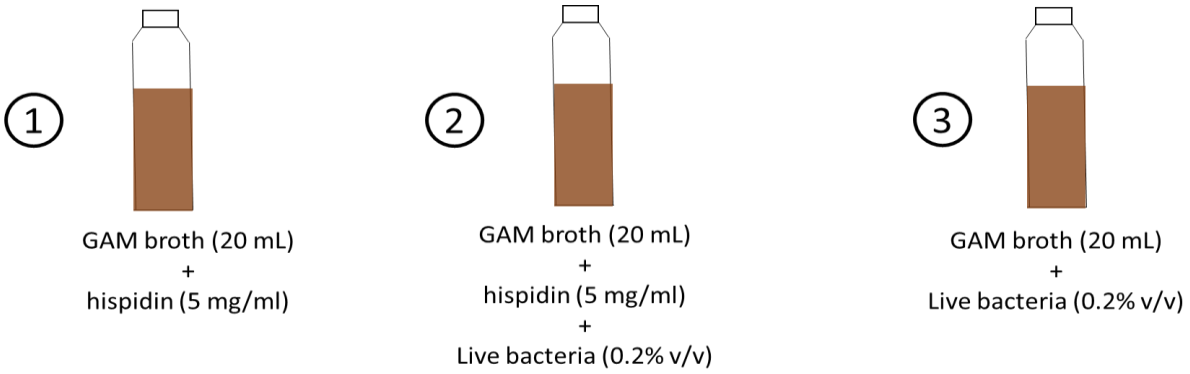
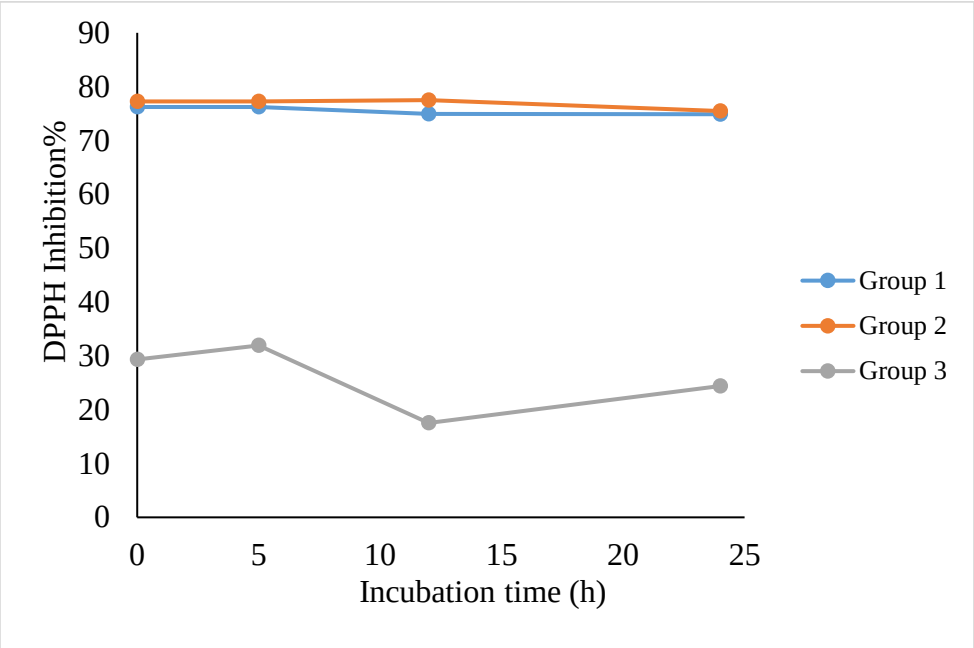


Fig 8.1 Sample plan for the *in vitro* fecal digestion of hispidin

Fig 8.2 Bioactivities of the samples after fecal digestion

(a) DPPH inhibition activity



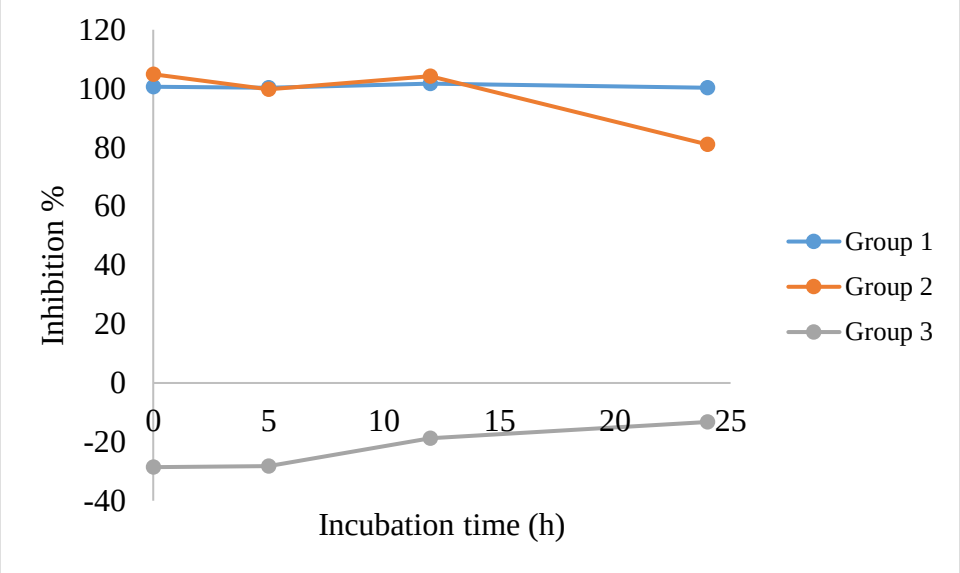
Group 1: no fecal stock + hispidin (positive control)

Group 2: fecal stock + hispidin (test)

Group 3: fecal stock + no hispidin (negative control)

Fig 8.2 Bioactivities of the samples after fecal digestion

(b) Antibacterial activity against *Staphylococcus aureus*



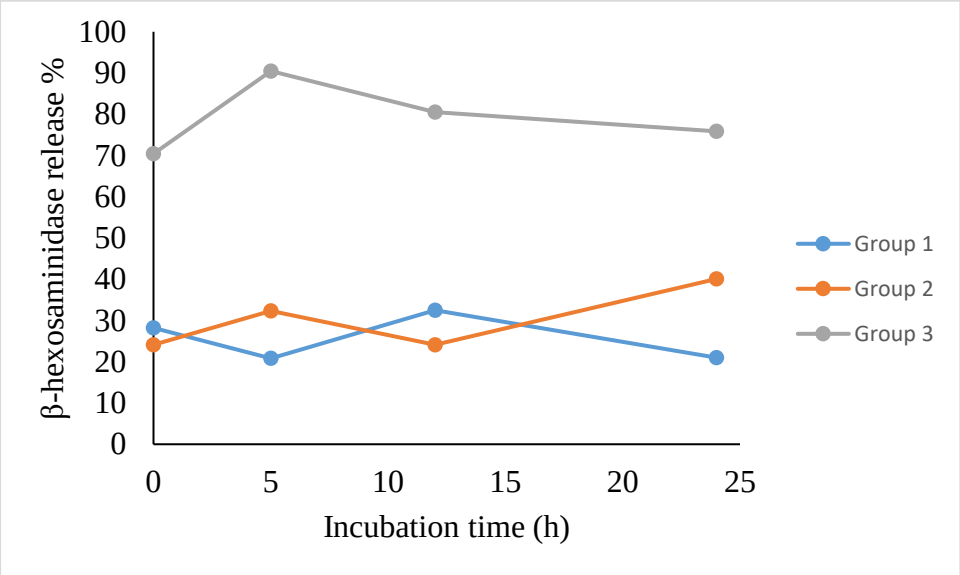
Group 1: no fecal stock + hispidin (positive control)

Group 2: fecal stock + hispidin (test)

Group 3: fecal stock + no hispidin (negative control)

Fig 8.2 Bioactivities of the samples after fecal digestion

(c) Anti-allergy activity (β-hexosaminidase release %)



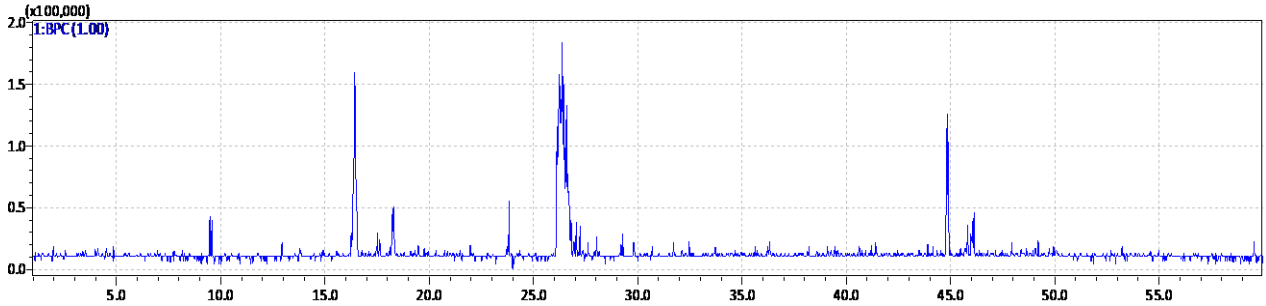
Group 1: no fecal stock + hispidin (positive control)

Group 2: fecal stock + hispidin (test)

Group 3: fecal stock + no hispidin (negative control)

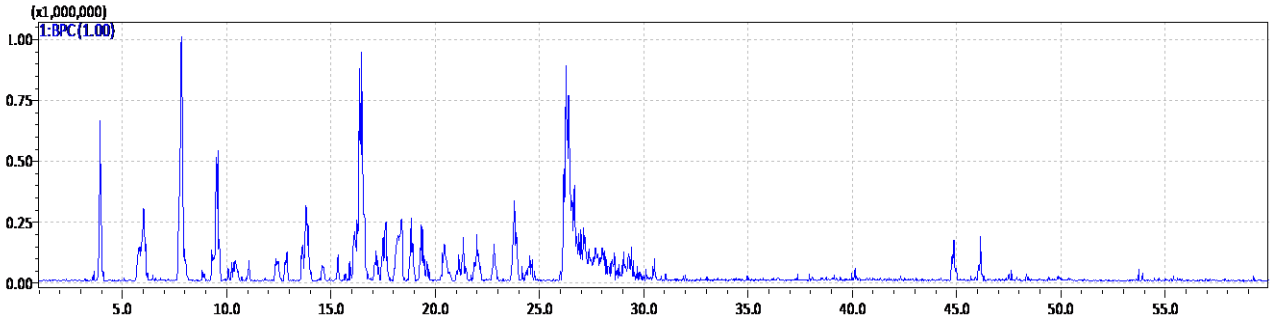
Fig 8.3 Base peak chromatograms (BPC) of the ethyl acetate extracts from 0h samples of set 1, 2, and 3

(a)



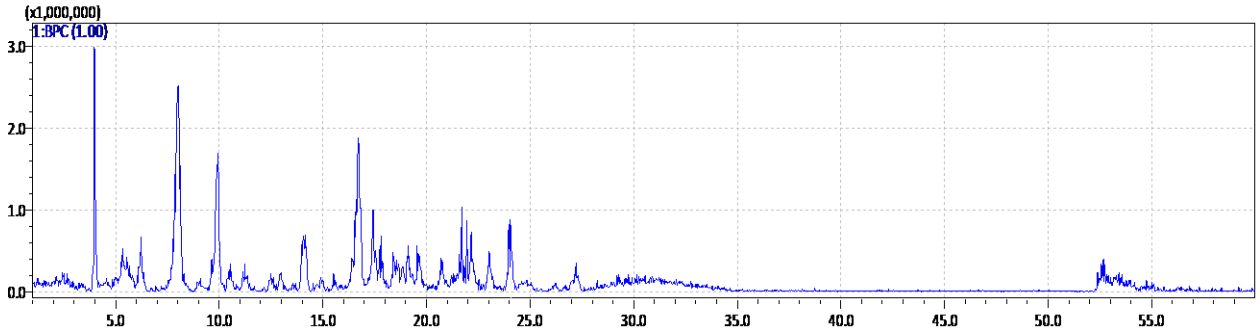
Set 1: GAM broth + hispidin

(b)



Set 2: GAM broth + hispidin + 0.2%v/v fecal stock

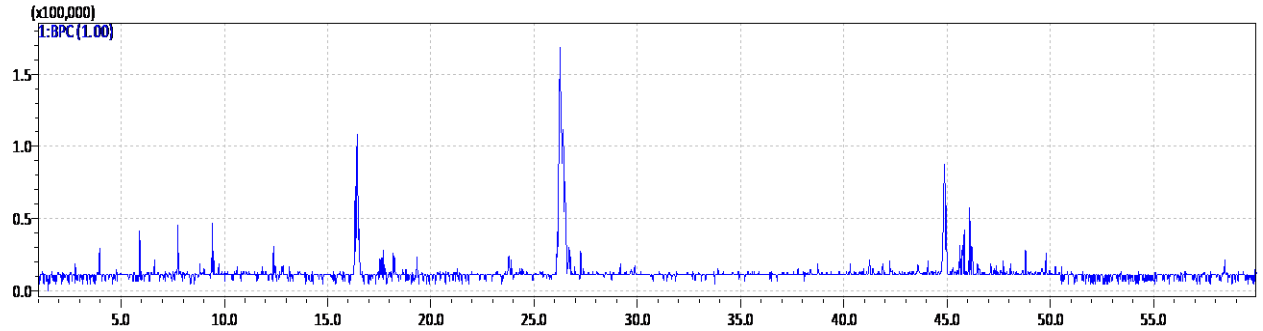
(c)



Set 3: GAM broth + 0.2%v/v fecal stock

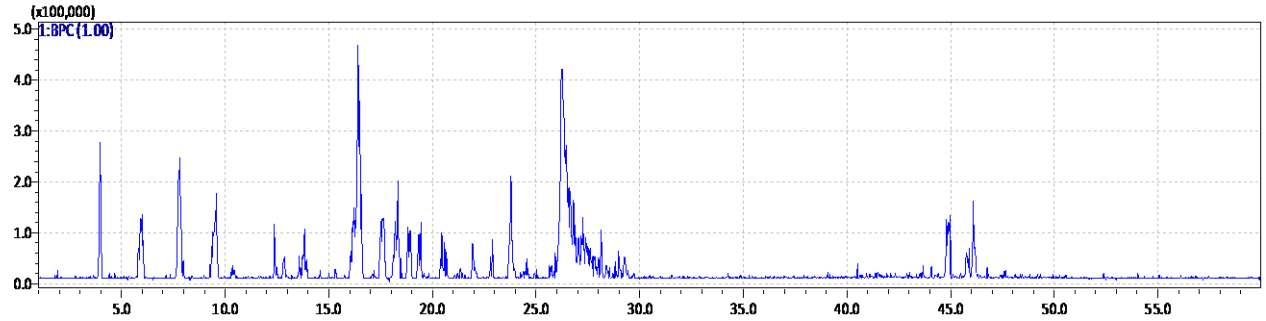
Fig 8.4 Base peak chromatograms (BPC) of the ethyl acetate extracts from 5h samples of set 1, 2, and 3

(a)



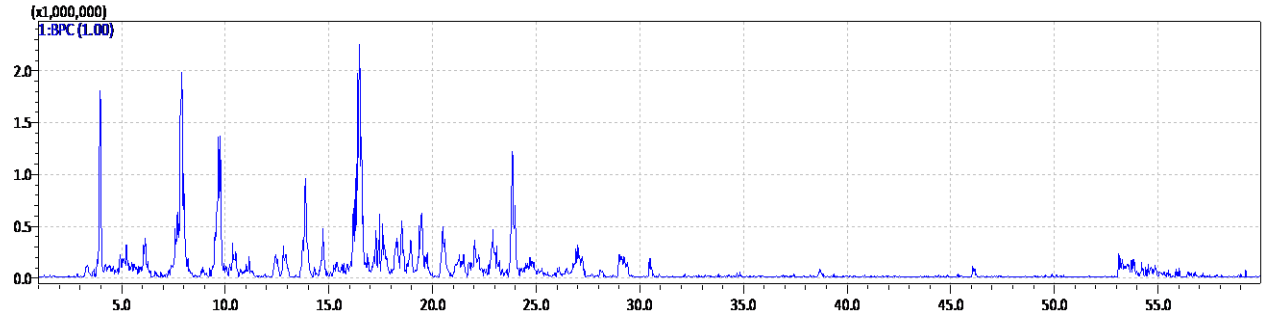
Set 1: GAM broth + hispidin

(b)



Set 2: GAM broth + hispidin + 0.2%v/v fecal stock

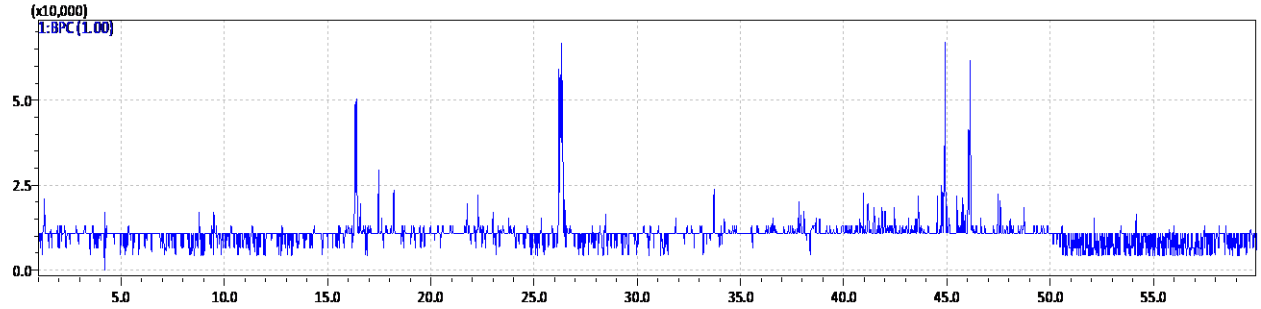
(c)



Set 3: GAM broth + 0.2%v/v bacterial stock

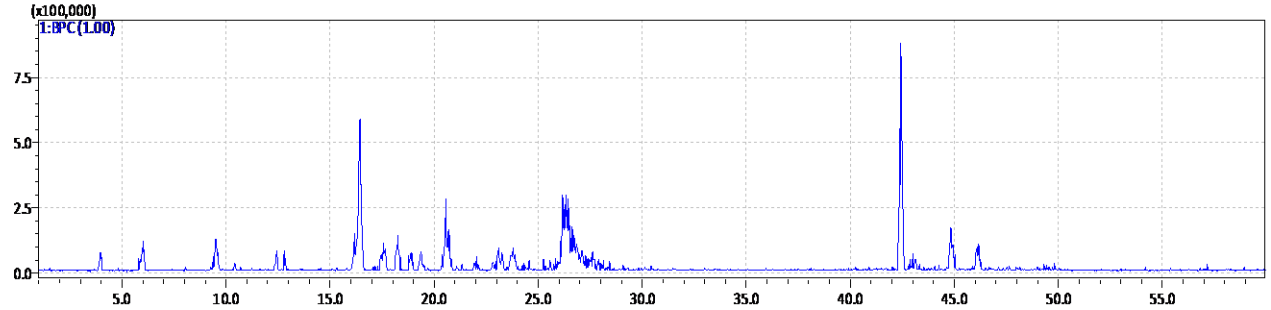
Fig 8.5 Base peak chromatograms (BPC) of the ethyl acetate extracts from 12h samples of set 1, 2, and 3

(a)



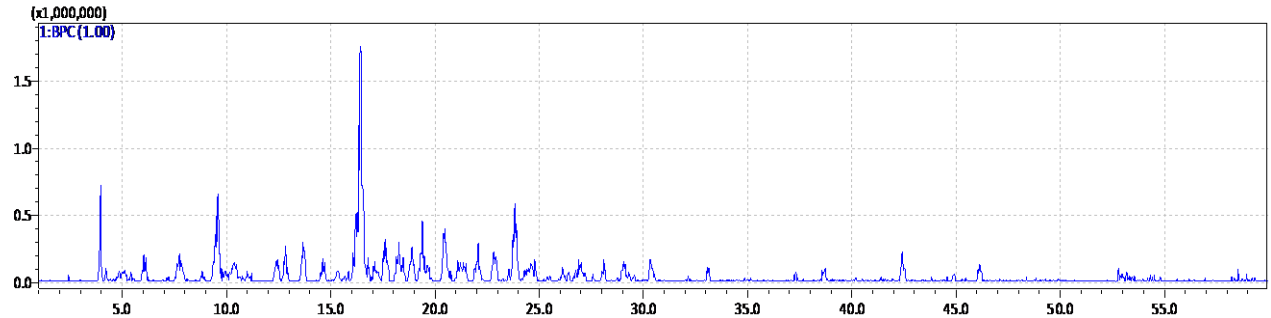
Set 1: GAM broth + hispidin

(b)



Set 2: GAM broth + hispidin + 0.2%v/v fecal stock

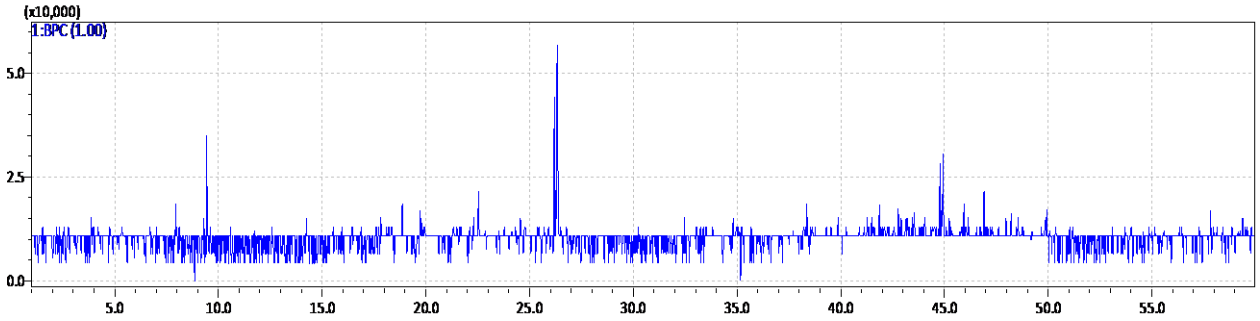
(c)



Set 3: GAM broth + 0.2%v/v fecal stock

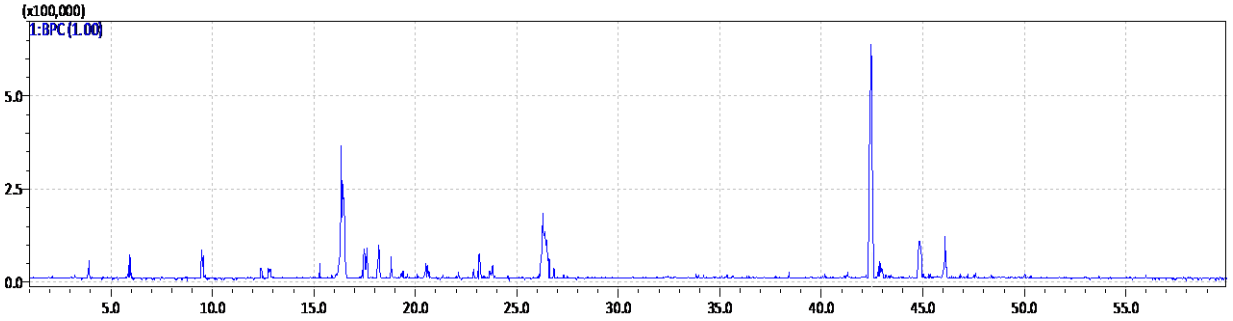
Fig 8.6 Base peak chromatograms (BPC) of the ethyl acetate extracts from 24h samples of set 1, 2, and 3

(a)



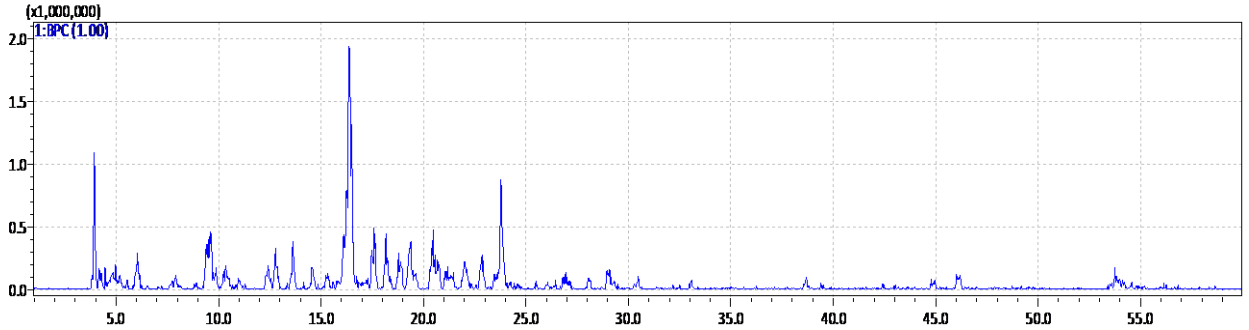
Set 1: GAM broth + hispidin

(b)



Set 2: GAM broth + hispidin + 0.2%v/v fecal stock

(c)



Set 3: GAM broth + 0.2%v/v fecal stock

Chapter 9

Conclusion and Future Perspective

9.1 Conclusion

The popularity of mushrooms as a source of bioactive compounds has seen tremendous increase over the years. Several compounds with high therapeutic potential has been discovered; and the process seems to continue with the escalating pace of research in this field. Consequently, a sharp rise in the mushrooms based functional food is also being observed. In such a scenario, it is but obvious to explore the potential of mushrooms from over the world, especially from largely unexplored places like Nepal. The richness in biodiversity and natural medicinal resources from this country makes it even more lucrative to investigate the bioactive potential in Nepalese mushrooms.

Chapter 2 provides the description of the 92 samples collected from different parts of Nepal used during the investigation of various bioactivities, including the botanical origin, location, habitat, and also the yield percentage obtained from the ethanol extraction process. A combination of genetic and morphological identification methods were used to establish the identity of the samples.

Chapter 3 describes the antioxidant activity of the ethanol extracts of 62 samples and the phenolic profile of the highest activity samples. Phenolic compounds were found to be largely responsible for the antioxidant activity as shown by the strong correlation of the antioxidant activity with the total phenolic content. *Inonotus clemensiae* was one of the samples with highest antioxidant activity.

Chapter 4 elucidates the antibacterial activity of the ethanol extracts of 90 samples against 2 bacterial species *Staphylococcus aureus* and *Propionibacterium acnes*. *I. clemensiae* showed very strong antibacterial activity against both species. Also the major bioactive compound in

the *I. clemensiae* extract was confirmed to be hispidin, after isolation, and chemical characterization by LC-MS and NMR analyses.

Chapter 5 elaborates on the effect of various concentrations of the ethanol extracts of 90 wild mushrooms, on the melanin biosynthesis in B16 melanoma cells. Strong melanin synthesis inhibiting activity was shown by samples like *Ganoderma carnosum*, *Oxyporus* sp., and *Pholiota nameko* 1. From the LC-MS analysis, the presence of some triterpenoid compounds could be speculated in the 2 former samples.

Chapter 6 and chapter 7 describes the anti-allergy and anti-cancer activities of 90 and 61 ethanol extracts respectively. The inhibition of degranulation of RBL-2H3 cells, with strong cell viability confirmed the anti-allergy activity; and selective cytotoxicity towards the cancer cell lines compared to normal human cell lines confirmed the anti-cancer activity. *Inonotus clemensiae* and *Phellinus adamantinus* required the least concentrations to obtain the IC_{50} values for anti-allergy activity. Also, the bioactive compound of *I. clemensiae*, hispidin, was able to show strong anti-allergy activity.

Chapter 8 explores the fate of the bioactive compound hispidin, which was present in the ethanol extract of one of the strongest bioactive samples *I. clemensiae*. Fecal bacteria was used to act upon the compound under anaerobic conditions. The bioactivities of the digested product was maintained even after digestion. Results from the LC-MS analysis point towards a possible biotransformation into a derivative of hispidin. However, further identification and investigation of the biotransformed compounds seems to be necessary.

9.2 Future Perspective

The present research is a novel attempt to obtain a comprehensive understanding of the bioactivities and the bioactive compounds in the wild mushrooms of Nepal. Five different bioactivities were investigated, along with the chemical characterization of several of the bioactive samples. The results of this research will play an important role to fill the gap of knowledge on the bioactive potential of Nepalese mushrooms. Also, it shall be of great value for the recognition of Nepalese mushrooms as an important source of functional food and functional food ingredients.

However, it is necessary to understand that the present study is limited to the wild species collected from natural habitats in the forests. In order to establish a strong and sustainable market of mushrooms from Nepal as functional foods, artificial cultivation of these mushrooms is necessary for large scale production. One of the most prominent candidate from the results of this study is *Inonotus clemensiae*, with its strong bioactive compound hispidin.

The understanding of the biosynthetic pathways, and the factors affecting the synthesis of this styrylpyrone compound is very important for development of future strategies for cultivation. Previous studies have demonstrated that the styryl group is derived from phenylalanine via cinnamic acid, *p*-coumaric acid and caffeoyl-CoA pathways, and the pyrone ring comes from acetate [76]. Moreover, external factors such as presence of light [174]; and interaction with other fungal species [175] play important roles in the regulation of key enzymes required for the biosynthesis of compounds such as hispidin. Several methods have already been tested for the cultivation of the mycelia of *Inonotus obliquus* (chaga), a closely related species[176].

The establishment of cultivation methods of indigenous mushrooms, optimized for the enhanced production of bioactive compounds will be of crucial importance to propagate Nepalese mushrooms into the functional food industry. Finally, it is hoped that the results of

this study shall attract more research interest in the Nepalese wild mushrooms and their bioactive potential, paving way for their industrial scale cultivation and use.

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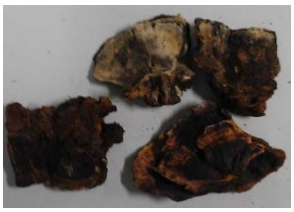
Appendices

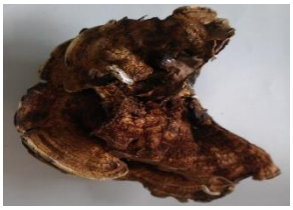
Figure A2.1. Pictures of the dried mushrooms collected from different parts of Nepal



1. *Inonotus andersonii*

2. *Inonotus* sp. 1

3. *Inonotus* sp. 2



4. *Inonotus* sp. 3

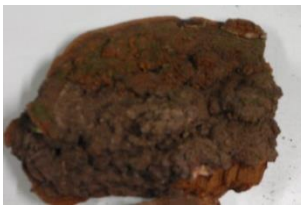
5. *Inonotus clemensiae*

6. *Inonotus cuticularis*

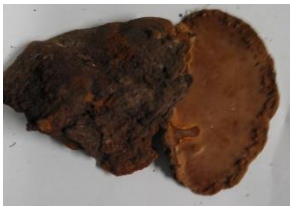
7. *Inonotus* sp. 4

8. *Inonotus* sp. 5

9. *Phellinus gilvus*



10. *Phellinus conchatus*1



11. *Phellinus conchatus* 2

12. *Phellinus* sp.1

13. *Phelinus* sp. 2

14. *Phellinus adamantius*

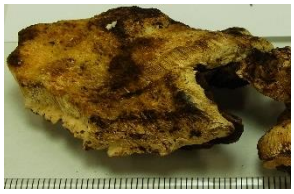
15. *Cyclomyces setiporus1*

16. *Cyclomyces setiporus2*

17. *Cyclomyces setiporus* 3

18. *Cyclomyces setiporus* 4

19. *Cyclomyces setiporus* 5

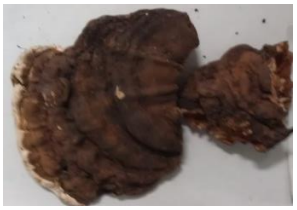


20. *Oxyporus* sp.

21. *Ganoderma australe* 1

22. *Ganoderma australe* 2

23. *Ganoderma australe* 3



24. *Ganoderma australe* 4

25. *Ganoderma lingzhi* 1

26. *Ganoderma lingzhi* 2

27. *Ganoderma lingzhi* 3

28. *Ganoderma endochroum*

29. *Ganoderma multipileum*

30. *Ganoderma carnosum*

31. *Ganoderma* sp.1

32. *Ganoderma* sp.2

33. *Amauroderma calcigenum*

34. *Trichaptum biforme*

35. *Trichaptum abietinum*

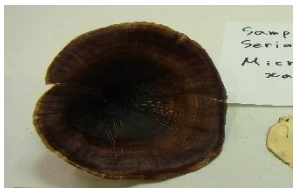
36. *Trametes versicolor* 1

37. *Trametes versicolor* 2

38. *Trametes versicolor* 3

39. *Trametes versicolor* 4

40. *Trametes versicolor* 5



41. *Micropus xanthopus* 1

42. *Micropus xanthopus* 2

43. *Polyporus arcularius*

NO IMAGE

44. *Postia stiptica*

45. *Phlebia tremellosa* 1

46. *Phlebia tremellosa* 2

47. *Lenzites betulina*

48. *Rigidoporus* sp.

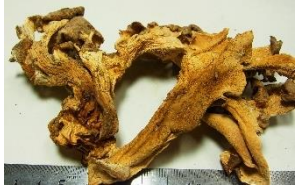
NO IMAGE

49. *Laetiporus versisporus*1

50. *Laetiporus versisporus* 2

51. *Laetiporus montanus*

52. *Mycorrhaphium* sp.



53. *Grifola frondosa*

54. *Lentinus* sp.

55. *Bjerkandera adusta*

56. *Antrodiella zonata* 1

57. *Antrodiella zonata* 2

58. *Antrodiella zonata* 3

59. *Fomes fomentarius*

60. *Abortiporus biennis*

61. *Lentinula edodes* 1

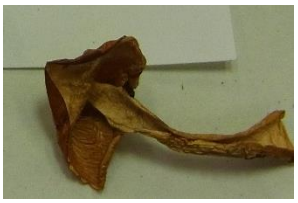
62. *Lentinula edodes* 2

63. *Pleurotus ostreatus* 1

64. *Pleurotus ostreatus* 2

65. *Pleurotus ostreatus* 3

66. *Pleurotus ostreatus* 4



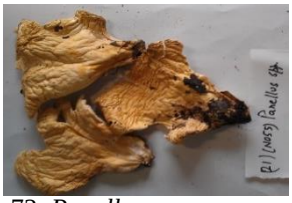
67. *Pholiota nameko* 1

68. *Pholiota nameko* 2

69. *Marasmius mavium*

70. *Marasmius* sp.

71. *Panellus edulis*



72. *Panellus* sp.

73. *Inocybe* sp.1

74. *Inocybe* sp.2

75. *Collybia peronata*

76. *Tricholoma calligatum*

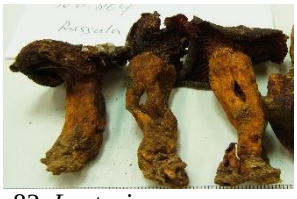
77. *Mucidula mucida*

78. *Gymnopus sp.*

79. *Heterobasidion linzhiense* 1.

80. *Heterobasidion linzhiense* 2

81. *Lactarius hatsutake*



82. *Lactarius* sp.

83. *Russula brevipes*

84. *Engleromyces goetzii*

85. *Xylobolus princeps* 1

86. *Xylobolus princeps* 2

87. *Xylobolus princeps* 3

88. *Xylobolus princeps* 4

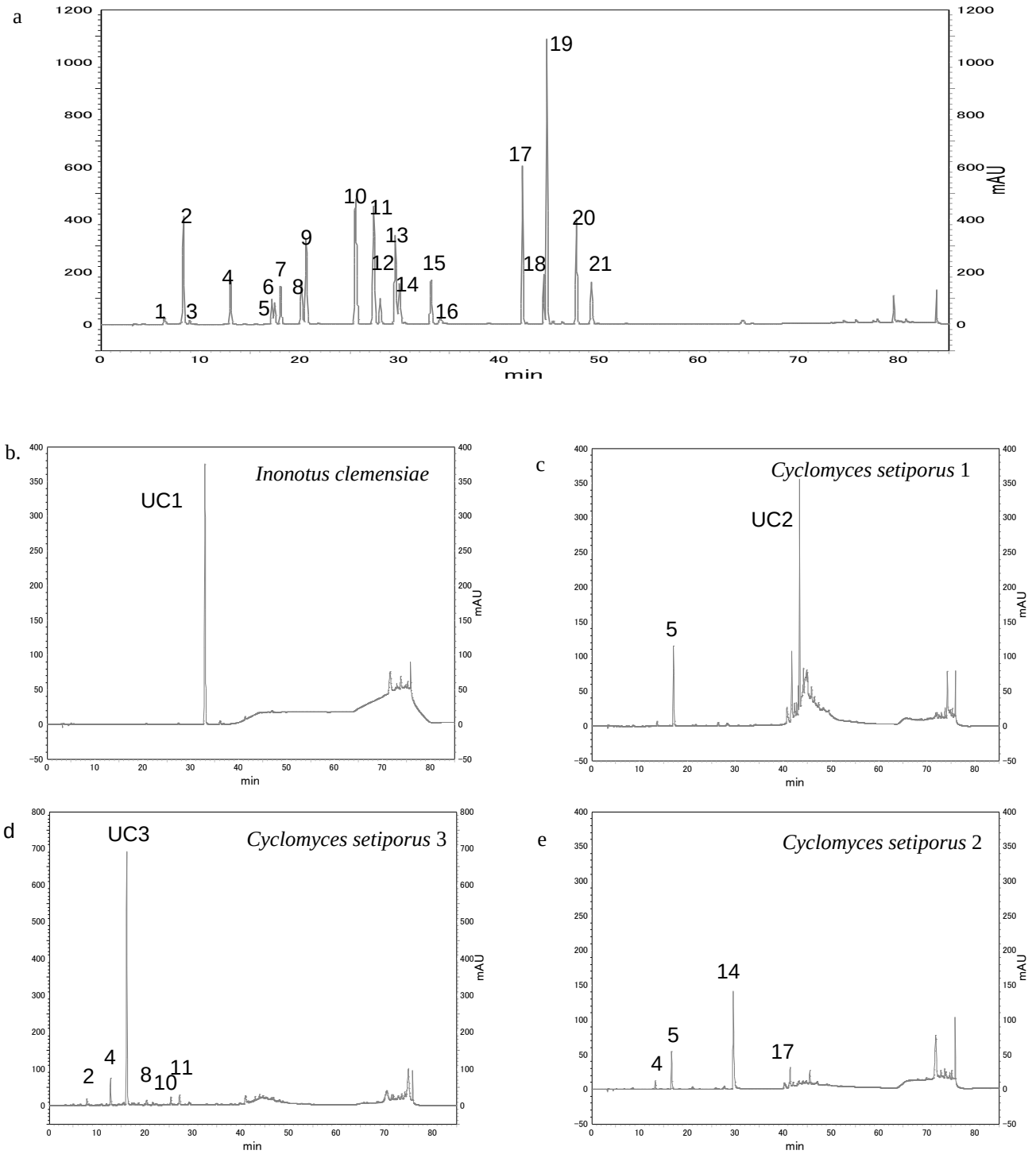
89. *Pseudomerulius curtisii*

90. *Cantharellus ferruginascens*

91. *Neolentinus lepideus*

92. *Stereum* sp.*

* Identifcation unconfirmed by morphological and genetic methods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Cyclomyces setiporus 1
UC2
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UC3
Cyclomyces setiporus 3
e
Cyclomyces setiporus 2

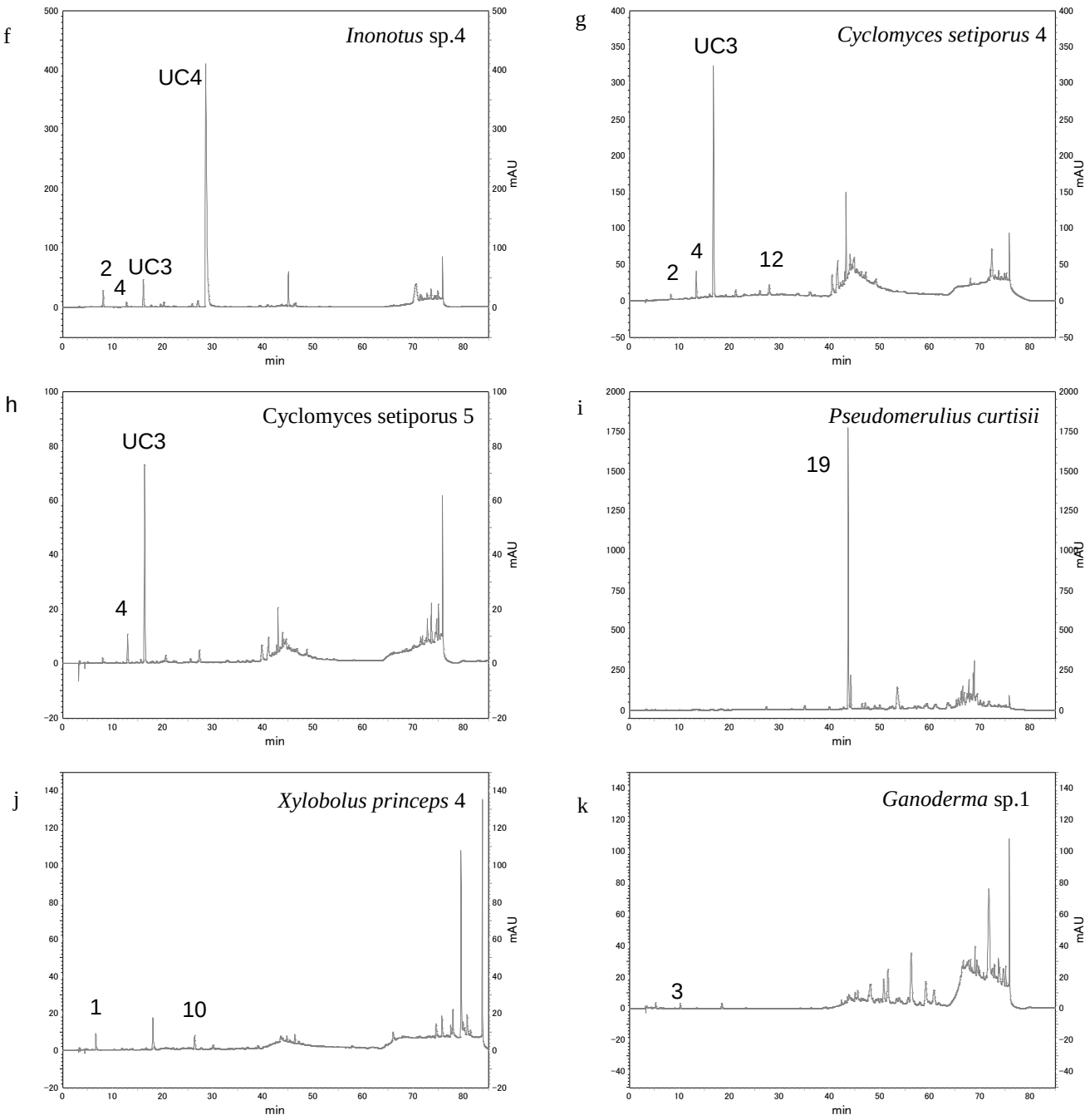
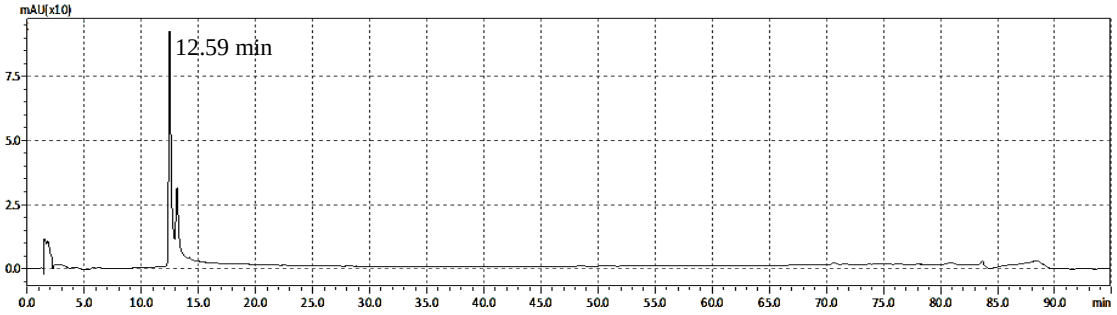
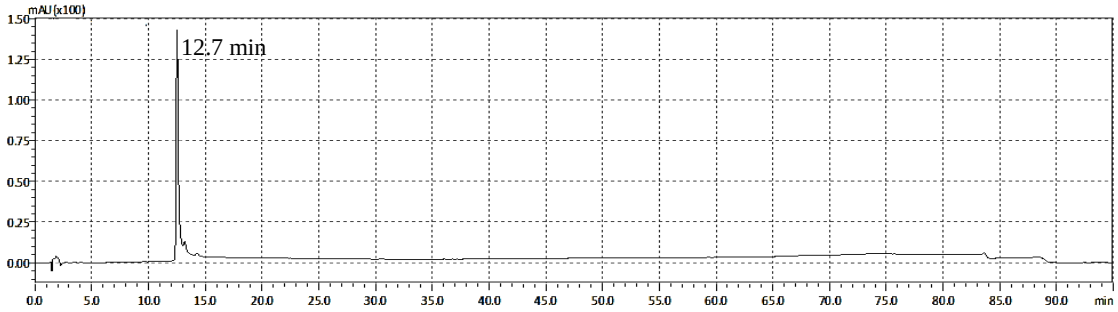


Figure A3.1. HPLC chromatograms at 280 nm of the standard compounds (a) and ethanol extracts of the top ten samples with the highest TPC (b to k). The peaks were identified as 1. 5-sulfosalicylic acid, 2. gallic acid, 3. pyrogallol, 4. 3,4-dihydroxybenzoic acid, 5. chlorogenic acid, 6. (+)-catechin, 7. *p*-hydroxybenzoic acid, 8. vanillic acid, 9. caffeic acid, 10. vanillin, 11. rutin, 12. *p*-coumaric acid, 13. ferulic acid 14. veratric acid, 15. naringin, 16. benzoic acid, 17. abscisic acid, 18. quercetin, 19. *trans*-cinnamic acid, 20. naringenin, 21. kaempferol

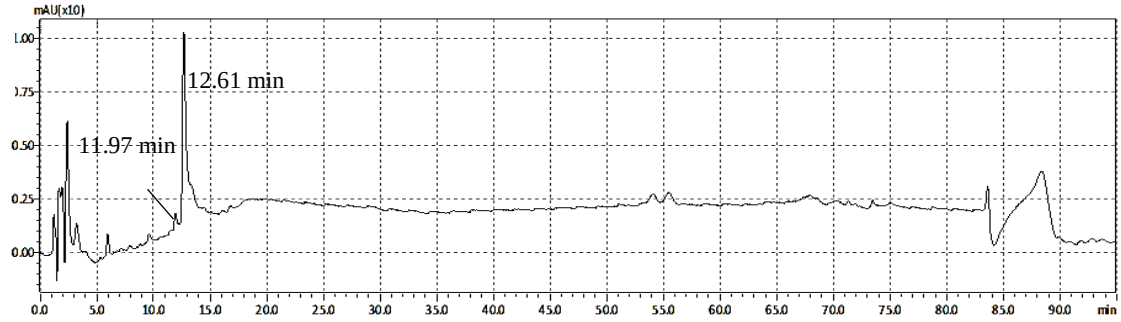
(a)


mAU(x10)
12.59 min
min

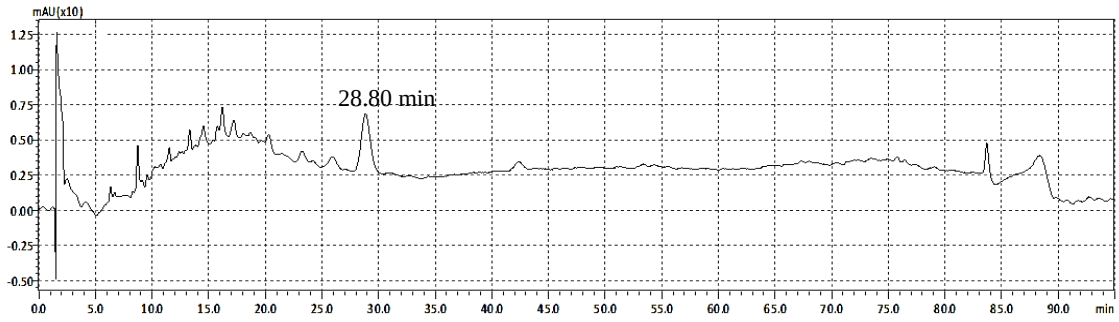
(b)


mAU(x100)
12.7 min
min

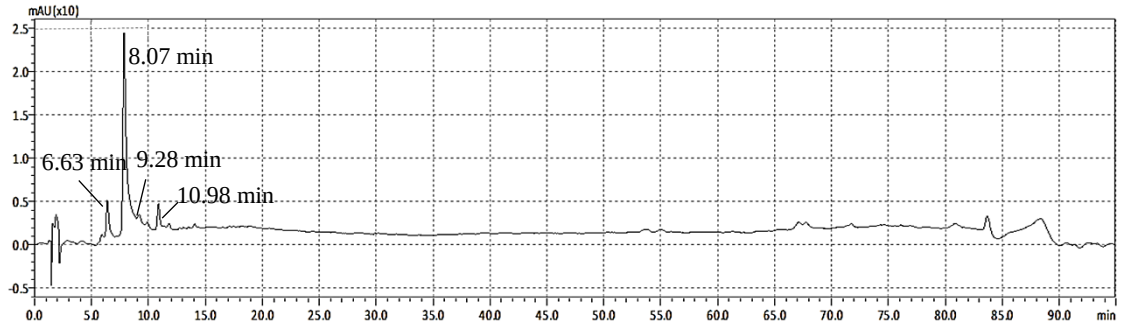
(c)


mAU(x10)
12.61 min
11.97 min
min

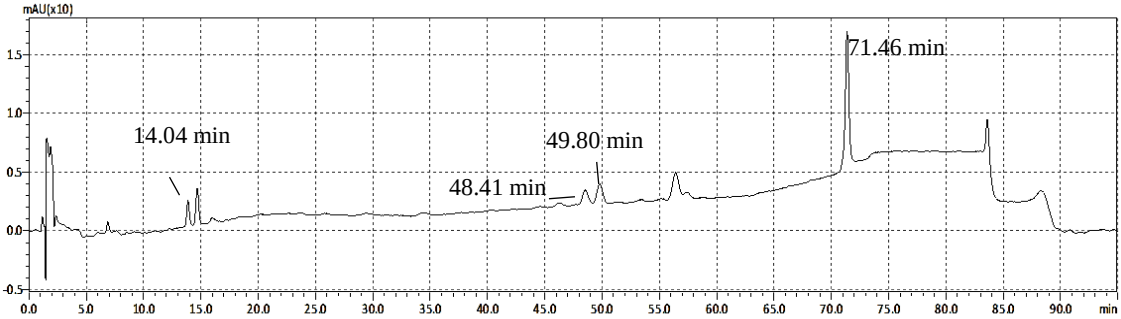
(d)


mAU(x10)
28.80 min
min

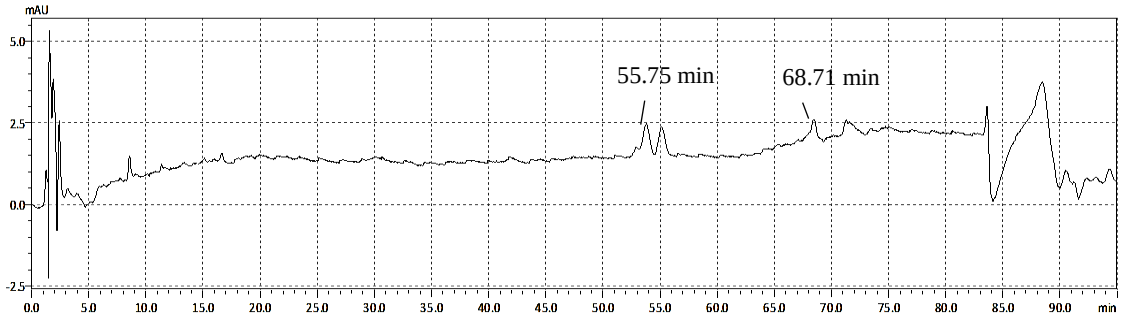
(e)


mAU(x10)
8.07 min
6.63 min
9.28 min
10.98 min
min

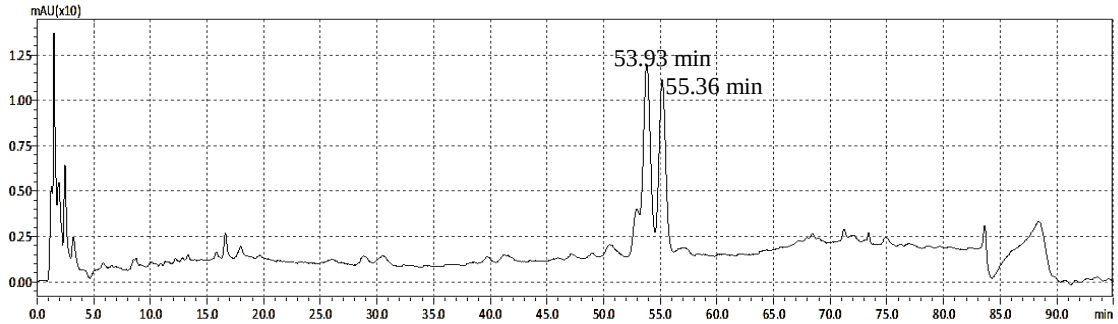
(f)


mAU(x10)
71.46 min
14.04 min
49.80 min
48.41 min
min

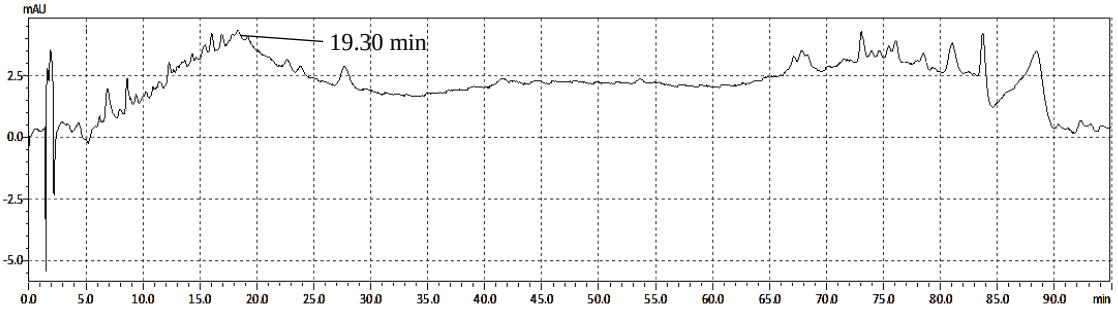
(g)


mAU
55.75 min
68.71 min
min

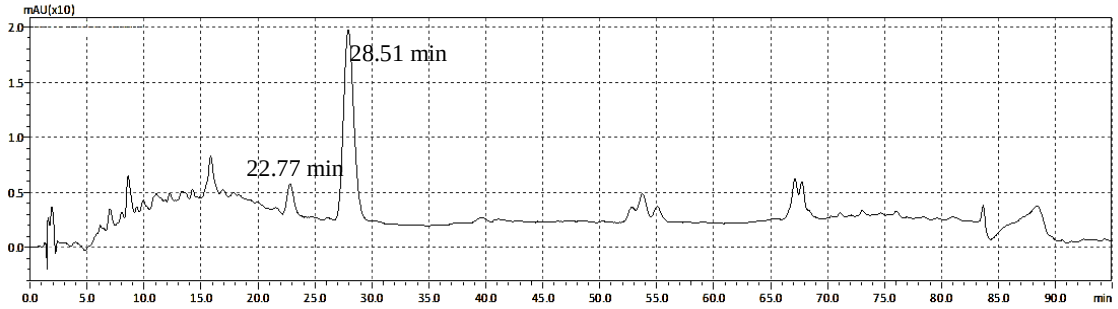
(h)


mAU(x10)
53.93 min
55.36 min
min

(i)


mAU
19.30 min
min

(j)


mAU(x10)
28.51 min
22.77 min
min

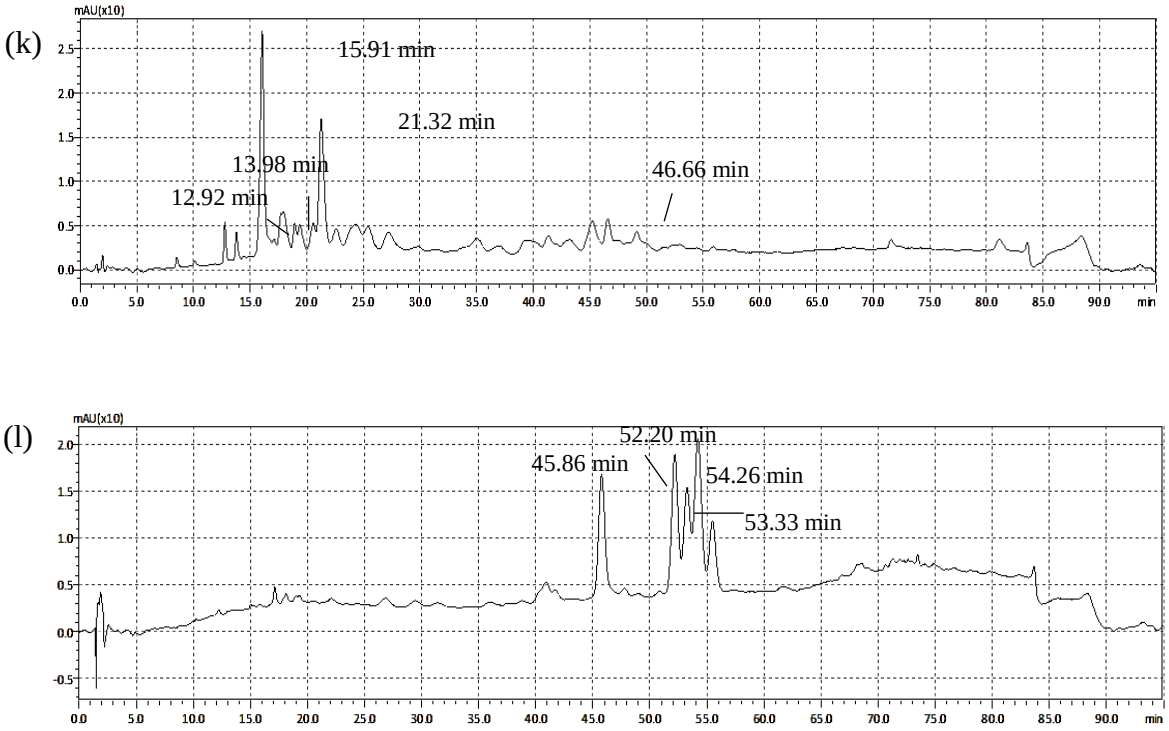


Fig. A4.1. LC chromatograms of the ethanol extracts of mushrooms tested for MIC and MBC values (a) *Inonotus andersonii*. (b) *Inonotus clemensiae*. (c) *Inonotus cuticularis*. (d) *Inonotus* sp. 2. (e) *Cyclomyces setiporus* 3. (f) *Postia stiptica*. (g) *Mucidula mucida*. (h) *Gymnopus* sp.. (i) *Xylobolus princeps* 2. (j) *Xylobolus princeps* 3. (k) *Pseudomerulius curtisii*. (l) *Cantharellus ferruginascens*. All the chromatograms were obtained at 280 nm, except (f) which was obtained at 254 nm.

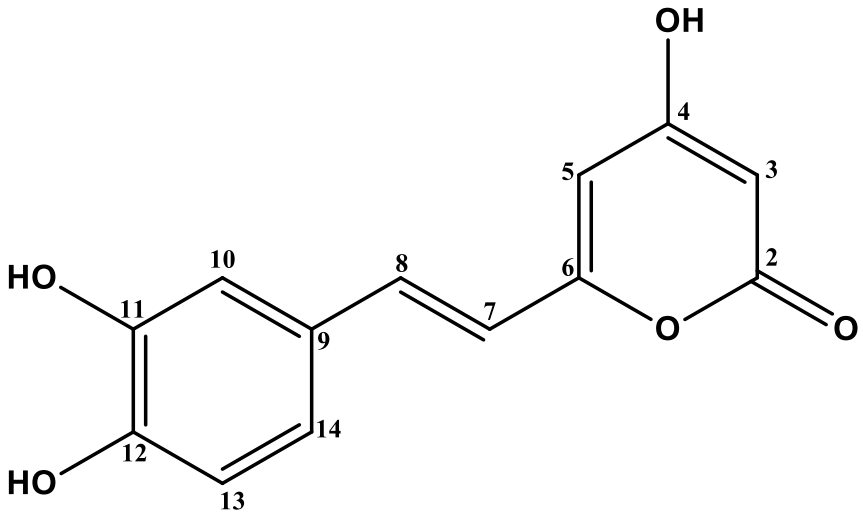
Supplementary information (A 4.1): Nuclear magnetic resonance (NMR) analysis of Compound 1

Compound 1 was obtained as a yellow powder. High resolution-electron spray ionization-mass spectrometry (HR-ESI-MS) showed a protonated molecular ion peak at *m/z* 247.0305 $[M+H]^+$ and a deprotonated molecular ion peak at 245.0451 $[M-H]^-$ in accordance with a molecular formula $C_{13}H_{10}O_5$. The proton (^1H) -NMR spectral data exhibited signals of a 1,3,4-trisubstituted benzene at δ_H 7.01 (d, *J*=1.8 Hz), δ_H 6.91 (dd, *J*=8.4, 1.8 Hz) and δ_H 6.76 (d, *J*=8.4 Hz), two olefinic methines attributed to a *trans*-1,2-disubstituted double bond unit at δ_H 6.53 (d, *J*=15.6 Hz), δ_H 7.24 (d, *J*=15.6 Hz) and two sp^2 singlets at δ_H 5.82 and δ_H 6.03. Guided by the heteronuclear single quantum correlation (HSQC) spectrum, the two olefinic methines at δ_H 6.53 and δ_H 7.24, showed typical resonances of *trans*-olefinic protons, were correlated to δ_C 117.1 and δ_C 136.0, respectively. A partial trisubstituted benzene ring was suggested depending on the previous NMR characteristics. The substitution pattern of the ring was deduced based on the heteronuclear multiple bond correlation (HMBC) correlations observed from δ_H 6.76, 6.91 and 7.01 to two low-field signals at δ_C 146.2 and δ_C 148.3, suggesting oxygenated substituents, probably hydroxyl groups. A long range HMBC correlation was observed from δ_H 7.01 to δ_C 136.0, confirmed the substitution of the benzene ring with a trans-olefinic moiety and a dihydroxystyryl moiety was suggested as a substructure. Based on HMBC spectrum, a six-membered pyrone ring was suggested. A long range HMBC correlation was observed from δ_H 7.24 (δ_C 136.0) with a quaternary carbon at δ_C 160.8 together with correlations of the singlet δ_H 5.82 to the same carbon resonance. In addition, a long range correlation was observed from δ_H 5.82 to δ_C 178.9, assigned for a carbonyl group of the pyrone moiety. Other HMBC correlations are shown in Fig. 2. Consequently, the structure of the compound was identified as 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (hispidin). Further confirmation was done by comparing its spectral data with previously published data of hispidin.

Table A4.1: Nuclear magnetic resonance (NMR) data of compound 1

#	^{1}H-NMR	^{13}C-NMR
2		178.9
3	5.82 (s)	106.3
4		165.1
5	6.03 (s)	105.7
6		160.8
7	6.53 (d, *J*=15.6 Hz)	117.1
8	7.24 (d, *J*=15.6 Hz)	136.0
9		129.3
10	7.01 (d, *J*=1.8 Hz)	114.7
11		146.2
12		148.3
13	6.76 (d, *J*=8.4 Hz)	116.2
14	6.91 (dd, *J*=8.4, 1.8 Hz)	121.7

Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hz. Proton (^{1}H) and carbon-13 (^{13}C) -NMR were measured in CD_3OD at 150 and 600 MHz, respectively.



Hispidin

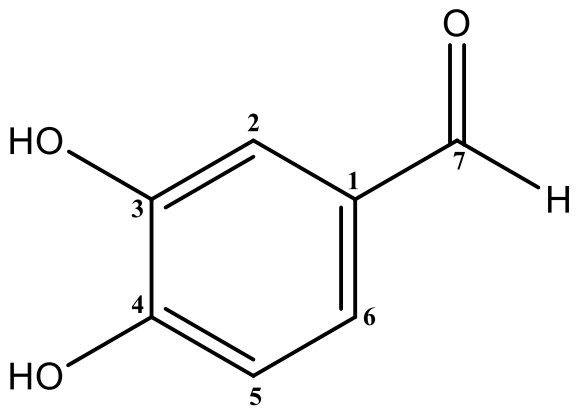
Supplementary information (A4. 2): NMR analysis of Compound 3

Compound 3 was obtained as colorless needles. HR-ESI-MS gave a protonated molecular ion peak at *m/z* 139.1117 $[M+H]^+$, and a deprotonated molecular ion peak at *m/z* 137.0212 $[M-H]^-$, in accordance with the molecular formula $C_7H_6O_3$. It gave a dark blue color with ferric chloride indicating the presence of a phenolic hydroxyl group. ^{1}H-NMR spectral data clearly indicated the presence of three aromatic signals in the range of δ_H 6.91-7.30. This was confirmed by ^{13}C-NMR spectrum which revealed the presence of signals due to a benzene ring resonating between δ_C 115.4 - 153.7 in addition to a signal at δ_C 193.1 assigned to an aldehydic carbonyl carbon. A doublet observed at δ_H 6.91 (J=8.4 Hz) was coupled to another doublet at δ_H 7.30 (J=8.4 Hz) and could be attributed to two *ortho*-coupled protons at C-5 and C-6. The other singlet at δ_H 7.30 which was coincident with H-6 could be assigned to a proton at C-2 and supported by two signals in ^{13}C-NMR spectrum for two oxygenated carbons at δ_C 147.2 and 153.7. On the basis of the above spectral data and by comparison of these values with previously reported data, the identity of compound 3 was confirmed as protocatechualdehyde (3,4-dihydroxybenzaldehyde).

Table A4.2: ^{13}C and ^{1}H-NMR spectral data of compound 3

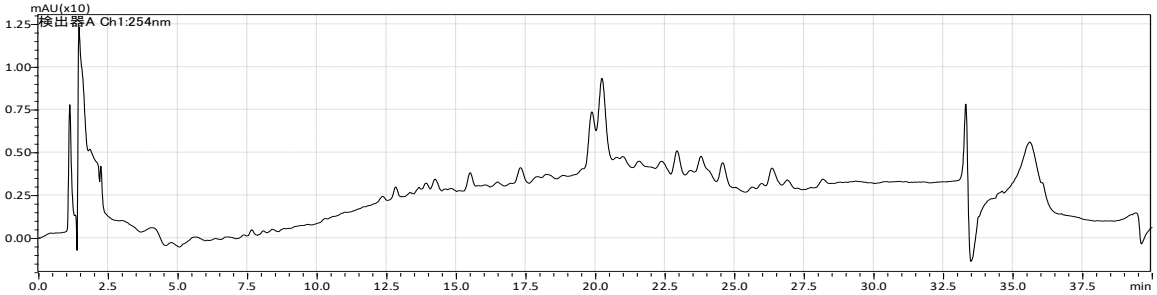
#	^{13}C	^{1}H
1	130.8	
2	116.3	7.30 (s)
3	147.2	
4	153.7	
5	115.4	6.91 (d, *J*= 8.4)
6	126.3	7.30 (d, *J*= 8.4)
7	193.1	9.70 (s)

Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. Proton (^{1}H) and carbon-13 (^{13}C) -NMR were measured in CD_3OD at 150 and 600 MHz, respectively.

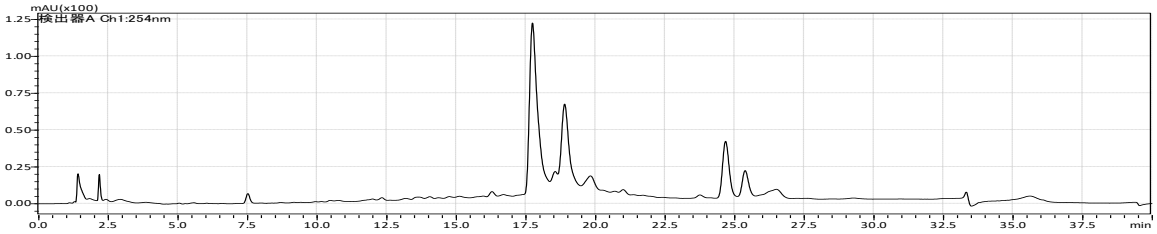


Protocatechualdehyde

(a)



(b)



(c)

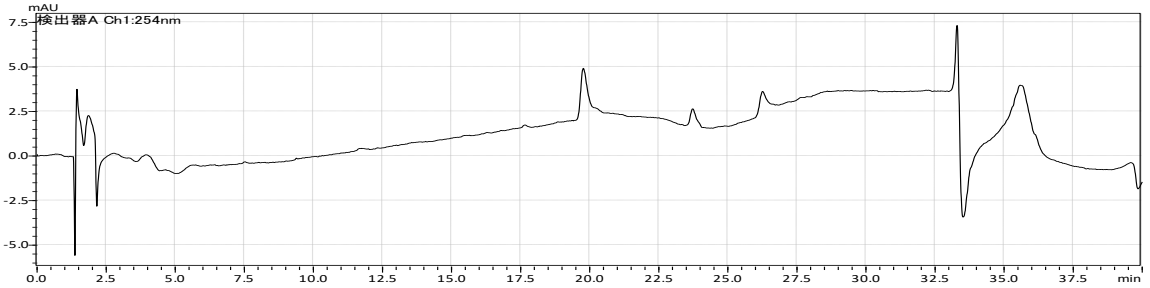


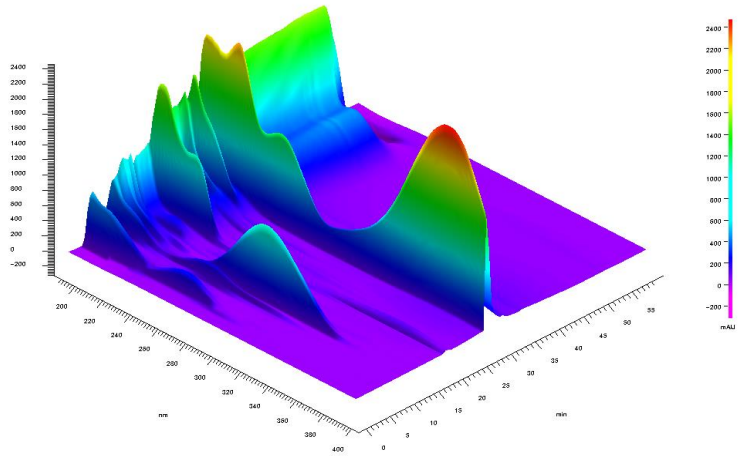
Fig A5.1 LC chromatograms of samples showing melanin synthesis inhibition at 254 nm; (a) *Ganoderma carnosum*, (b) *Oxyporus* sp. (c) *Pholiota nameko* 1

Table A8.1 Description of the fecal samples used for the *in vitro* digestion

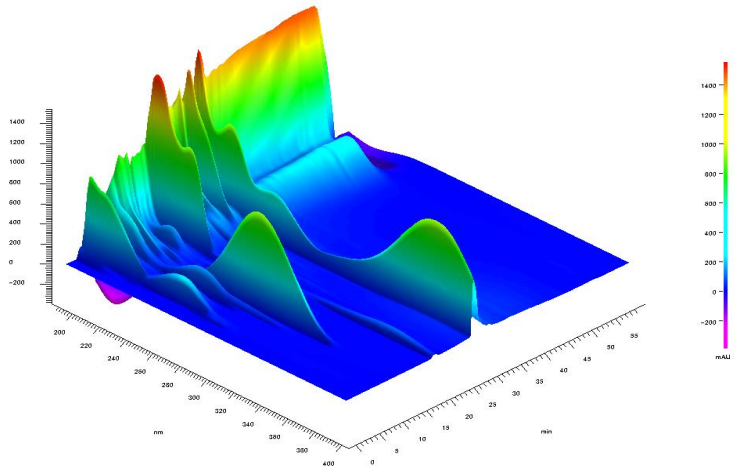
Sample ID	Fecal weight (g)	Age	Gender	Use of antibiotic
JP_A_107_0m	0.259	23	female	NO
JP_A_109_0m	0.223	21	female	NO
JP_A_110_0m	0.538	30	male	NO
JP_A_111_0m	0.954	23	male	NO
JP_A_112_0m	0.896	23	female	NO
JP_A_113_0m	0.774	25	female	NO
JP_A_114_0m	0.195	23	female	NO
JP_A_115_0m	0.524	22	female	NO
JP_A_116_0m	0.743	23	female	NO
JP_A_117_0m	0.471	23	male	NO
JP_A_118_0m	0.543	23	male	NO
JP_A_119_0m	0.341	41	male	NO
JP_A_120_0m	2.237	23	female	NO
JP_A_121_0m	0.227	26	male	NO

Fig A8.1 3D plot of the UV-Vis spectrum of the samples of Set 1 (GAM broth + hispidin), collected at time points 0h, 5h, 12h, and 24h

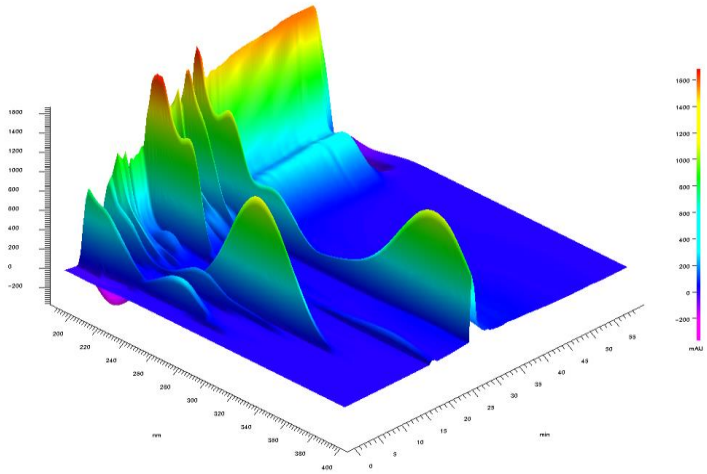
(b) 0 h



(a) 12 h



(c) 5 h



(d) 24 h

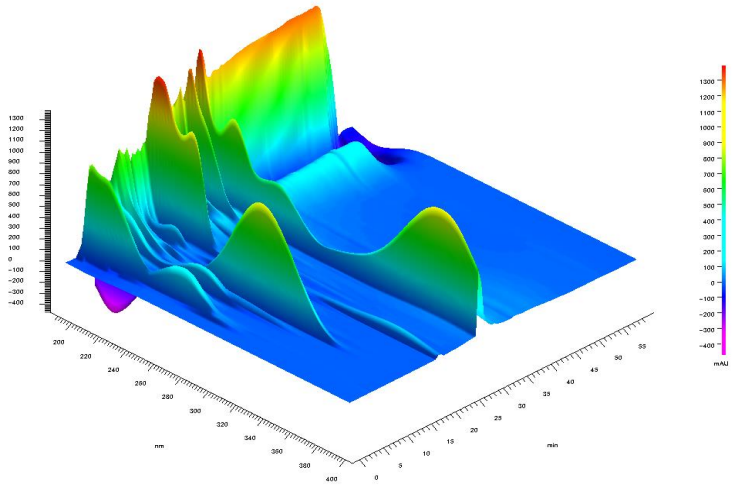
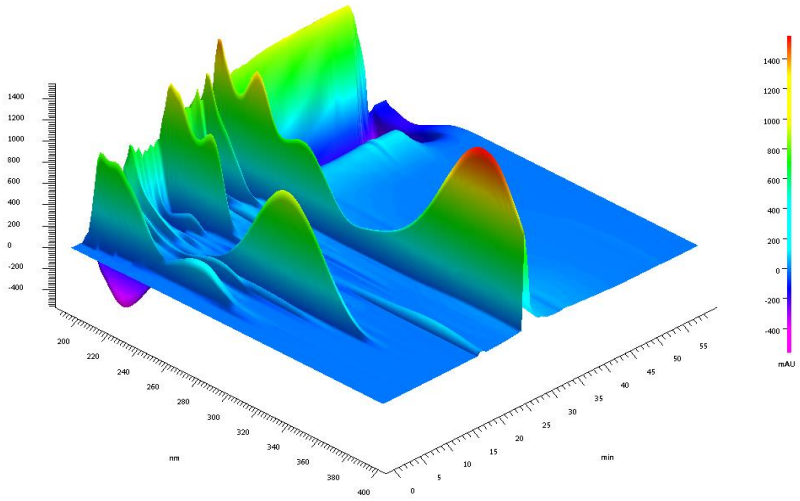
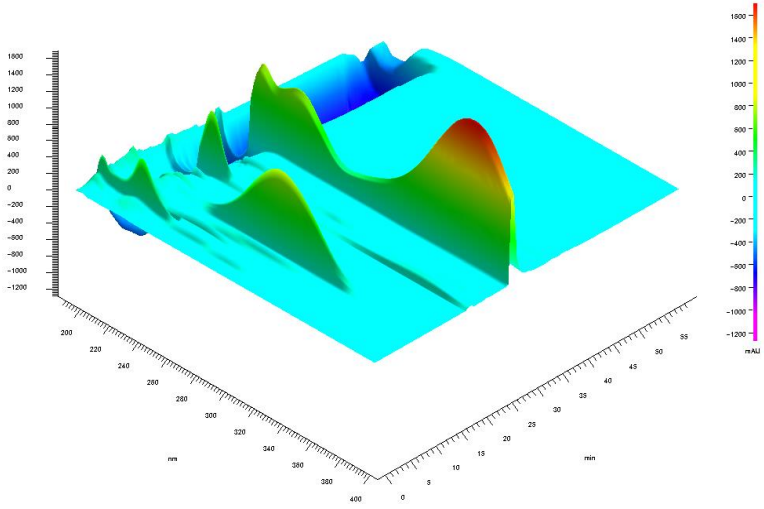


Fig A8.2 3D plot of the UV-Vis spectrum of the samples of Set 2 (GAM broth + hispidin+ 0.2%v/v bacterial stock), collected at time points 0h, 5h, 12h, and 24h

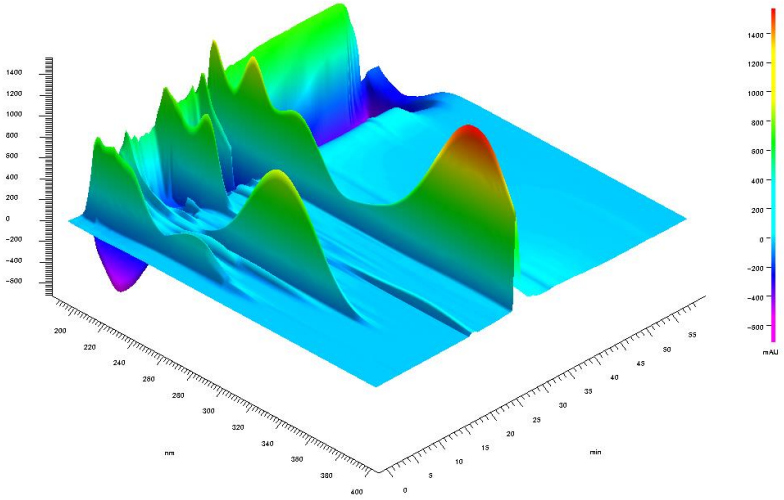
(a) 0 h



(c) 12 h



(b) 5 h



(d) 24 h

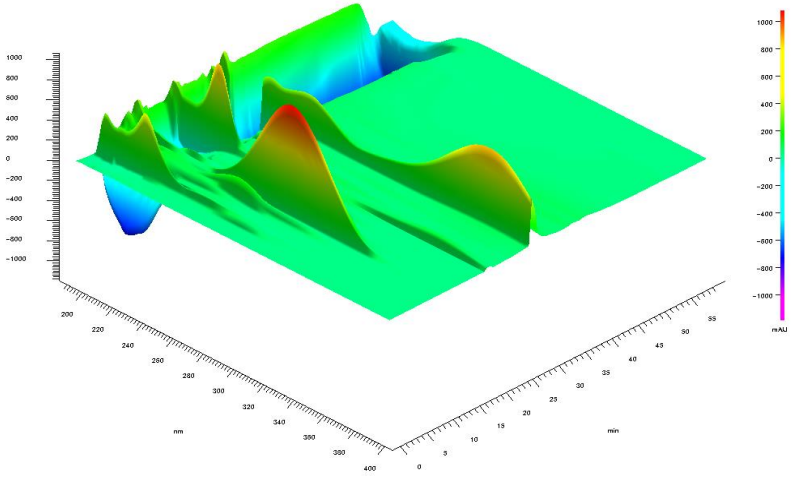
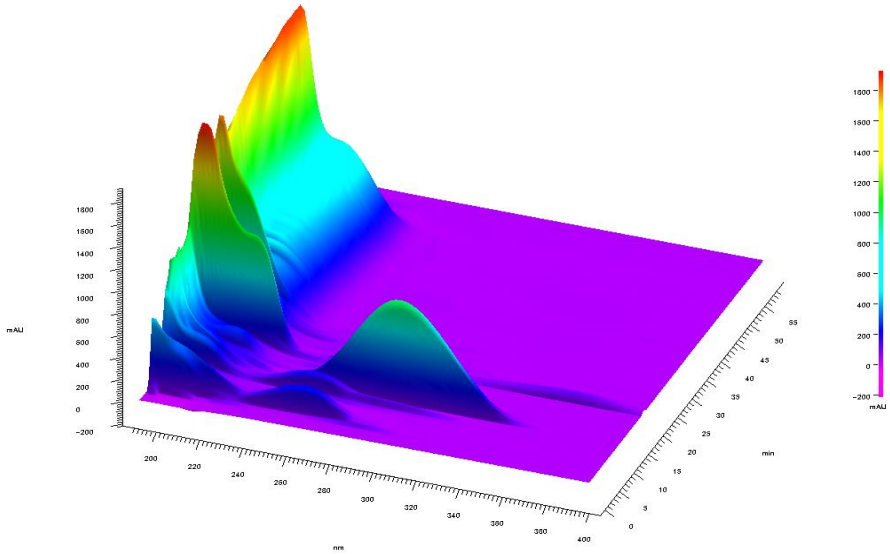
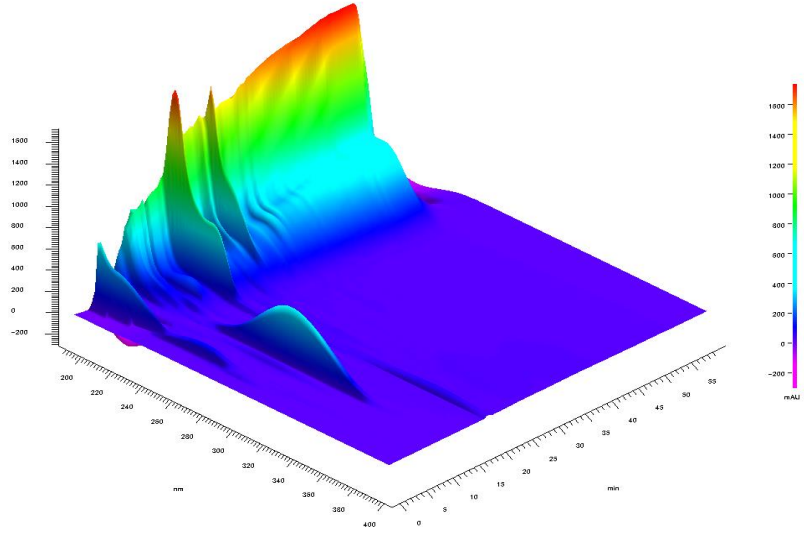


Fig A8.3 3D plot of the UV-Vis spectrum of the samples of Set 3 (GAM broth + 0.2%v/v bacterial stock), collected at time points 0h, 5h, 12h, and 24h

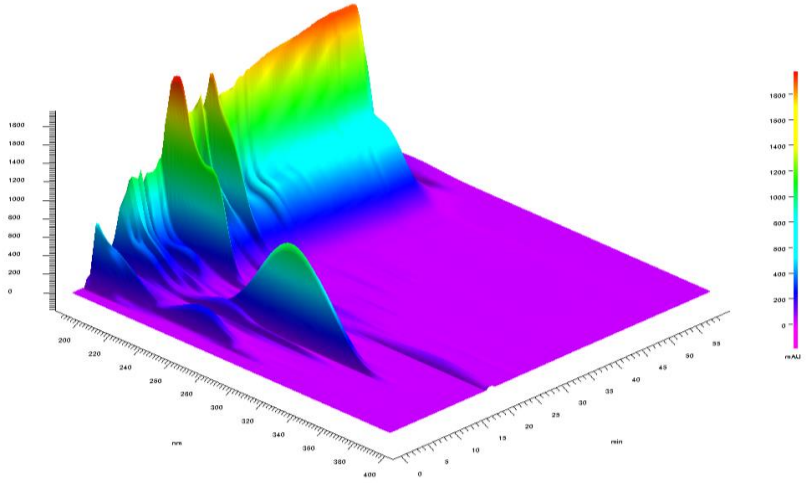
(a) 0 h



(c) 12 h



(b) 5 h



(d) 24 h

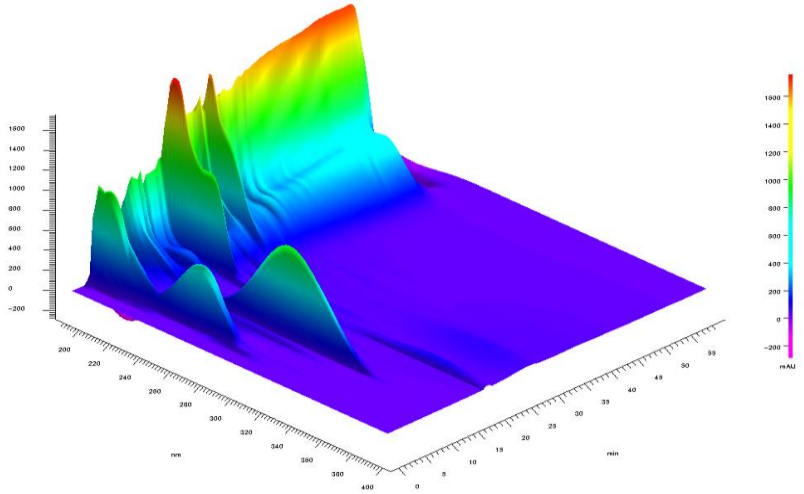
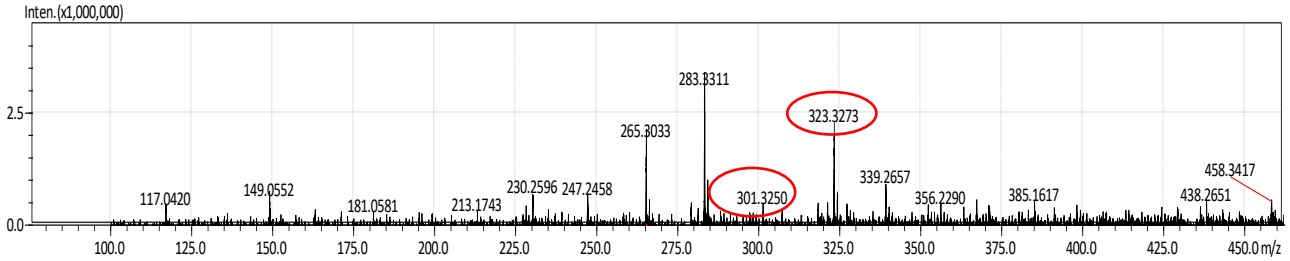
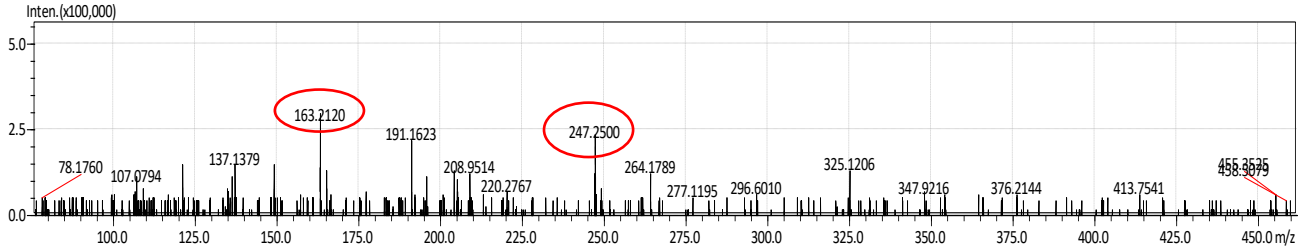


Fig A8.4 Mass spectra of the biotransformed product of hispidin

(a) MS (+)



(b) MS/MS (+)



(c) MS (-)