# PHARMACOGNOSTIC STUDIES AND THE EFFECTS OF *CHLOROPHYLLUM MOLYBDITES* (MEYER:F.) MASSES (*AGARICACEAE*) ON ISOLATED RABBIT JEJUNUM AND GUINEA PIG ILEUM

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**FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA**

**AUGUST, 2012**

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**A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN PHARMACOGNOSY**

**DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT,**

**FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA**

**AUGUST, 2012**

## DECLARATION

I hereby declare that this research work titled "Pharmacognostic Studies and the effects of *Chlorophyllum molybdites* (MEYER:F.) MASSES (*AGARICACEAE*) on isolated rabbit jejunum and guinea pig ileum" was performed by me in the Department of Pharmacognosy and Drug Development under the supervisions of Dr Mujtaba Suleiman Abubakar and Dr. Abdulkarim Agunu. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.


_____ _____ _____

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Department of Pharmacognosy and Drug Development,
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## CERTIFICATION

This thesis titled "Pharmacognostic Studies and the effects of *Chlorophyllum molybdites* (MEYER:F.) MASSES (*AGARICACEAE*) on isolated rabbit jejunum and guinea pig ileum" meets the regulations governing the award of the degree of Masters of Science (Pharmacognosy) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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## DEDICATION

I wish to dedicate this work to late Mallam Abdullahi Nura, whose selfless encouragement and dedication made this work a success. Losing him at a critical moment of this program is the most trying period I faced in this academic endeavour. May Allah's mercy be with Him. Amen.

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## ABSTRACT

Mushroom poisoning by *Chlorophyllum molybdites* /Meyer:Fr (Agaricaceae) is a serious problem that has caused a lot of death due to improper identification. *C. molybdites* found in Zaria Nigeria was investigated to identify the morphological and microscopic features, the secondary metabolites present, the toxicxiy in rats and mice, and also to investigate the effect of the methanolic extract on rabbit and guinea pig jejunum . The external morphology of the pileus showed a whitish domed shaped pileus (brittle texture and the surface bumpy at the centre) with diameter of 5 – 12 cm and a whitish flesh of 1 – 3 cm thick and light brownish patches. Light brownish patches were observed on the scaly pileus with the edges showing radiating lines that follow the lines of the gills. The surface is bumpy and the flesh approximately 1.3 cm thick with a brittle texture. The lamellae was greenish in colour and was at the underside of the cap like pages in a book. It was closely packed on the inside of the cap and had thickness of approximately 1cm from the pileus to the edge the lamellae. The stipe is 1 – 2 cm thick, whitish greyish brown in colour and slightly curved at the base. It has a brittle texture and a smooth surface. The spores on the lamellae were fine greenish powder with greenish colour which changes to brown on storage. The spore print had a diameter of 6.8 – 12.3 cm. The spores of *C. molybdites* was amyloid. The physical constants revealed a moderate total ash value of 4.0% , acid insoluble ash of 0.43%, water soluble and alcohol soluble extractive values were 10.34% and 7.15% respectively. The TLC was done with different solvent system; n-butanol: acetic acid: water in ratio, (4:1:5), Hexane : Ethylacetate in the ration (9:1), Chloroform : Ethylacetate in the ration (2:3) and Chloroform : Ethylacetate : methanol : water (8:15:4:1) chromatograph reveals 5 spots with the solvent system n-butanol: acetic acid: $H_2O$ (4:1:5)

with Ethylacetate : methanol : water (8:15:4:1) and n-butanol: acetic acid: water (4:5:1) being the best solvent system given a total of 5 spots each when sprayed with 10% sulphuric acid and Dragendorff reagent. With Ninhydrine reagent, it showed two spots (n-butanol:acetic acid:Water (4:5:1)). The TLC indicates the presence of highly polar constituents. The methanolic extract of *Cholrophyllym molybdite* is moderately toxic with $LD_{50}$ of 288.53mg/kg and 471mg/kg in rats and mice respectively. The extract also possess smooth muscle contractile effect on the intestinal smooth muscle on both rabbit jujenum and guinea pig ilium mediated via both muscarinic and histaminergic mechanisms. The findings from this investigation are useful in preparation of the monograph of this macrofungi.

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# CHAPTER ONE

## 1.0 Introduction

Mushrooms are macrofungi widely consumed as food throughout the world. Many mushrooms are rich in essential nutrients such as proteins, minerals and vitamins (Lincoff and Mitchel, 1977; Spoerke, 1994). In addition, some mushrooms promote good health and vitality and have been tried with some success in the therapy of cancer, viral diseases, diabetes, hypercholesterolemia, impaired blood platelet aggregation and hypertension (Wang *et. al.*, 1996; Waise and Weis, 1999).

The use of fungi by humans dates back to prehistory; Ötzi the Iceman, a well-preserved mummy of a 5,300 year old Neolithic man found frozen in the Austrian Alps, carried two species of polypore mushrooms that may have been used as tinder (*Fomes fomentarius*), or for medicinal purposes (*Piptoporus betulinus*) (Peintner and Pumpel, 1998). Ancient peoples have used fungi as food sources–often unknowingly–for millennia, in the preparation of leavened bread and fermented juices. Some of the oldest written records contain references to the destruction of crops that were probably caused by pathogenic fungi (Ainsworth, 1976).

In classical botany, fungi are usually regarded as plants, but this is of doubtful validity. If considered superficially, fungi exhibit some of the features of plants; the mushroom, with cap and gills, which catches the eye of the observer, grows on the ground and appears to be rooted like a plant. The same applies to those fungi which grow on living or dead tree trunks; they are rooted in the bark and some of them posses tubes rather than gills. But if these fungi are examined in microscopic detail, one would no longer hold the view that one is dealing with a plant.

Fungi are similar to green plants in bearing fixed and possessing cell walls, but they differ fundamentally in their lack of chlorophyll and therefore cannot photosynthesized their own organic food from carbon dioxide and water. They are therefore forced to live as saprophytes on dead organic matter or as parasites on other living organisms (Miles and Chang, 1997).

The systematic study of fungi is about 250 years old, but the use of this group of organisms in fermentation of wines and baking of bread has been known to man for thousands of years, and it is therefore not surprising to find many references to these uses in the writings of the ancient Greeks and Romans. Yet even today, in the science-concious world, a world in which the surface of the moon has become the playground of the world's scientists, very few people appreciate the importance of fungi (Miles and Chang, 1997).

**Importance of fungi**

Fungi play an important role in our everyday life. They are responsible for the decomposition of organic matter, and as such affect us directly by destroying our food, textiles, timber, plastics, leather, rubber, and even glass. They are the cause of the majority of known plant diseases and many of the disease of animals and men. They are the basis of a number of industrial processes involving fermentation e.g. cocoa bean; they are employed in the commercial production of many organic acids, vitamin preparations and a number of very valuable antibiotic drugs. Fungi are both destructive and beneficial to agriculture. On one hand they damage crops by causing plant diseases, and on the other, they increase the fertility of soil by inducing various changes in it. The fruit bodies of some fungi, such as the mushrooms, truffles, morels, and the sclerotia of *Pleurotus tuber regium* are edible.

Although their nutritive value is not high, the fruit bodies supply vitamins and are valuable as appetizers (Miles and Chang, 1997).

The entire fungal kingdom comprises about 150, 000 species, while many new species are described each year. Although their fruit bodies sow a very wide range in size, shape and morphology, they all serve the same function, i.e. production and dispersal of the spores resulting from the process of sexual reproduction (UN, Training manual on mushroom cultivation technology, 2011).

### 1.1 Statement of the Research Problem

Fungi are regarded as being the second largest group of organisms in the biosphere after the insects. Known fungal species constitute only about 5% of their species in the world. Thus, the large majority of fungi are still unknown. Out of about 70,000 species of fungi identified, around 14,000-15,000 species produce fruiting bodies of sufficient size and suitable structure to be considered as macrofungi (mushrooms). Of these, about 5,000 of the species are considered to possess varying degrees of edibility, and more than 2,000 species from genera are regarded as prime edible mushrooms. But only 100 of them are experimentally grown, 50 economically cultivated, around 30 commercially cultivated, and only about 6 to have reached an industrial scale of production in many countries. Furthermore, about 1,800 are medicinal ones. The number of poisonous mushrooms is relatively small (approximately 10%), of these some 30 species are considered to be lethal (Miles and Chang, 1997).

In Nigeria, proper documentation of mushroom as well as most reports of toxicity and death in humans associated with consumption of mushrooms were often on the pages of

newspaper (Ambali *et. al.*, 2008). Therefore, in view of possible regional differences in the edibility of some wild mushrooms, the need for proper screening becomes pertinent. This is to ascertain the edibility or toxicity of some of the indigenous mushrooms.

In North America, mushroom poisoning has been estimated as accounting for about 17% of outbreaks of food poisoning that have a chemical, rather than a bacterial etiology (Hughes *et. al.*, 1977). In 1986, there were 143 cases of mushroom poisoning reported to the North American Mycological Association and, of these, 75 cases were subsequently linked to the ingestion of a single mushroom type, 11 of the 75 cases were caused by *C. molybdites* (Cochran, 1987). In 1988, *C. molybdites* poisoning accounted for 15 of the 116 symptomatic cases that were reported (Lampe, 1989) and similar proportions were documented for other years. The true incidence of mushroom poisoning is higher as many cases go unreported to the North American Mycological Association. Indeed, in the report of the American Association of Poison Control Centers National Data Collection System for 1989, exposure to mushrooms was reported to Poison Centers 9,388 times (0.59% of total Poison Center referrals covering a population estimated as 73% total US population); and, though most of the exposures were insignificant, 2,294 persons were reported as being treated at a health care facility, moderate and major symptomology developed in 249 persons including 3 persons who died, and minor symptom development was seen in an additional 1,215 cases (Litovitz, 1989). There is the need to study the characteristic features of this fungus so as to make the locals know the distinctive features and prevent it being utilized for gastronomic purposes.

## 1.2 Aims

The aims of this work are to;

i. Evaluate the morphological (macro and microscopic features) of *C. molybdites*

ii. Carry out screening for chemical constituent of *C. molybdites*

iii. *Extraction and evaluation of the toxicity of the extract in mice and rats.*

## 1.3 Hypothesis

The morphological, microscopic features and the secondary metabolites presents in *C. molybdites* can be use to develop the fungus monograph.

## 1.4 Justification of the research

Little is known about the identification and chemical constituents of mushroom found in Nigeria. There is an increase in the interest for both edible and medicinal mushroom. This project/studies will be directed at providing necessary parameter for identifying this species of Nigerian mushroom, the data can serve as basis for monograph of the fungi.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 The diversity of Fungi

A fungus is a member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and moulds as well as the more familiar mushrooms (Moore, 1980). These organisms are classified as a kingdom, Fungi, which are separate from plants, animals, and bacteria. One major difference is that fungal cells have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose. These and other differences showed that the fungi form a single group of related organisms, named the *Eumycota* (*true fungi* or *Eumycetes*), that share a common ancestor (a *monophyletic group*). This fungal group is distinct from the structurally similar mycomycetes (slime molds) and oomycetes (water molds) (Deacon, 2005).

Abundant worldwide, most fungi are inconspicuous because of the small size of their structures, and their cryptic lifestyles in soil, on dead matter, and as symbionts of plants, animals, or other fungi. They may become noticeable when fruiting, either as mushrooms or molds. Fungi perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling and exchange. They have long been used as a direct source of food, such as mushrooms and truffles (Stamets, 2000), as a leavening agent for bread, and in fermentation of various food products, such as wine, beer, and soy sauce (Kulp, 2000). Since the 1940s, fungi have been used for the production of antibiotics, in recent past, various enzymes produced by fungi are used industrially and in detergents (Fincham, 1989). Fungi are also used as biological pesticides to control weeds, plant diseases and insect pests. Many species produce bioactive compounds called mycotoxins,

such as alkaloids and polyketides that are toxic to animals including humans (Schardl *et. al.*, 2006).

The fruiting structures of a few species contain psychotropic compounds and are consumed recreationally or in traditional spiritual ceremonies. Fungi can break down manufactured materials and buildings, and become significant pathogens of humans and other animals. Losses of crops due to fungal diseases (e.g. rice blast disease) or food spoilage can have a large impact on human food supplies and local economies (UN, Training manual on mushroom cultivation technology 2011).

The fungus kingdom encompasses an enormous diversity of taxa with varied ecologies, life cycle strategies, and morphologies ranging from single-celled aquatic chytrids to large mushrooms. Ever since the pioneering 18th and 19th century taxonomical works of Carl Linnaeus, Christian Hendrik Persoon, and Elias Magnus Fries, fungi have been classified according to their morphology (e.g., characteristics such as spore color or microscopic features) or physiology. However, little is known of the true biodiversity of Kingdom Fungi, which has been estimated at around 1.5 million species, with about 5% of these having been formally classified (Alexopoulos *et. al.*, 1996).

Advances in molecular genetics have opened the way for DNA analysis to be incorporated into taxonomy, which has sometimes challenged the historical groupings based on morphology and other traits. Phylogenetic studies published in the last decade have helped reshape the classification of Kingdom Fungi, which is divided into one subkingdom, seven phyla, and ten subphyla (Bruns T. 2006).

### 2.1.1 Characteristics of Fungi

Before the introduction of molecular methods for phylogenetic analysis, taxonomists considered fungi to be members of the Plant Kingdom because of similarities in lifestyle. Both fungi and plants are mainly immobile, and have similarities in general morphology and growth habitat. Like plants, fungi often grow in soil, and in the case of mushrooms form conspicuous fruiting bodies, which sometimes bear resemblance to plants such as mosses. The fungi are now considered a separate kingdom, distinct from both plants and animals, from which they appear to have diverged around one billion years ago. Some morphological, biochemical, and genetic features are shared with other organisms, while others are unique to the fungi, clearly separating them from the other kingdoms (Bruns, 2006; Baldauf, 1993).

### 2.1.2 Relationship of Fungi with other Organisms.

**Relationship of Fungi with Eukaryotes**: Fungal cells contain membrane-bound nuclei with chromosomes that contain DNA with noncoding regions called introns and coding regions called exons. In addition, fungi possess membrane-bound cytoplasmic organelles such as mitochondria, sterol-containing membranes, and ribosomes of the 80s type (Deacon, 2005). They have a characteristic range of soluble carbohydrates and storage compounds, including sugar alcohols (e.g., mannitol), disaccharides and polysaccharides (e.g., glycogen, which is also found in animals) (Deacon, 2005).

**Relationship of Fungi with animals**: Fungi lack chloroplasts and are heterotrophic organisms, requiring preformed organic compounds as energy sources (Alexopoulos *et. al.*, 1996).

**Relationship of Fungi with plants**: Fungi possess a cell wall (Alexopoulos *et. al.*, 1996) and vacuoles (Shoji *et. al*., 2006). They reproduce by both sexual and asexual means, and like basal plant groups (such as ferns and mosses) produce spores. Similar to mosses and algae, fungi typically have haploid nuclei (Deacon, 2005).

**Relationship of Fungi with euglenoids and bacteria**: Higher fungi, euglenoids, and some bacteria produce the amino acid L-lysine in specific biosynthesis steps, called the α-aminoadipate pathway (Zabriskie and Jackson, 2000; Xu *et. al*., 2006).

The cells of most fungi grow as tubular, elongated, and thread-like (filamentous) structures and are called hyphae, which may contain multiple nuclei and extend at their tips. Each tip contains a set of aggregated vesicles—cellular structures consisting of proteins, lipids, and other organic molecules—called Spitzenkörper (Alexopoulos *et. al.*, 1996). Both fungi and oomycetes grow as filamentous hyphal cells (Alexopoulos *et. al.*, 1996). In contrast, similar-looking organisms, such as filamentous green algae, grow by repeated cell division within a chain of cells (Deacon, 2005).

In common with some plant and animal species, more than 60 fungal species display the phenomenon of bioluminescence (Desjardin *et. al.*, 2008).

### 2.1.3 Unique features:

Some species grow as single-celled yeasts that reproduce by budding or binary fission. Dimorphic fungi can switch between a yeast phase and a hyphal phase in response to environmental conditions (plate 2.1) (Alexopoulos *et. al.*, 1996).

The fungal cell wall is composed of glucans and chitin; while the former compounds are also found in plants and the latter in the exoskeleton of arthropods (Alexopoulos *et. al.*,

1996; Bowman and Free, 2006). Fungi are the only organisms that combine these two structural molecules in their cell wall. In contrast to plants and the oomycetes, fungal cell walls do not contain cellulose (Alexopoulos *et. al.*, 1996).



Plate 2.1 *Omphalotus nidiformis*, a bioluminescent macrofungi
Source: Alexopoulos *et. al.*, (1996)

Most fungi lack an efficient system for long-distance transport of water and nutrients, such as the xylem and phloem in many plants. To overcome these limitations, some fungi, such as *Armillaria*, form rhizomorphs (Mihail and Bruhn, 2005), that resemble and perform functions similar to the roots of plants.

Another characteristic shared with plants includes a biosynthetic pathway for producing terpenes that uses mevalonic acid and pyrophosphate as chemical building blocks (Keller *et. al*., 2005). However, plants have an additional terpene pathway in their chloroplasts; structure fungi do not possess (Wu *et. al*., 2007). Fungi produce several secondary

metabolites that are similar or identical in structure to those made by plants (Keller *et. al.*, 2005). Many of the plant and fungal enzymes that make these compounds differ from each other in sequence and other characteristics, which indicate separate origins and evolution of these enzymes in the fungi and plants (Keller *et. al.*, 2005; Tudzynski, 2005).

### 2.1.4 Morphology

#### 2.1.4.1 Microscopic structures of macrofungi

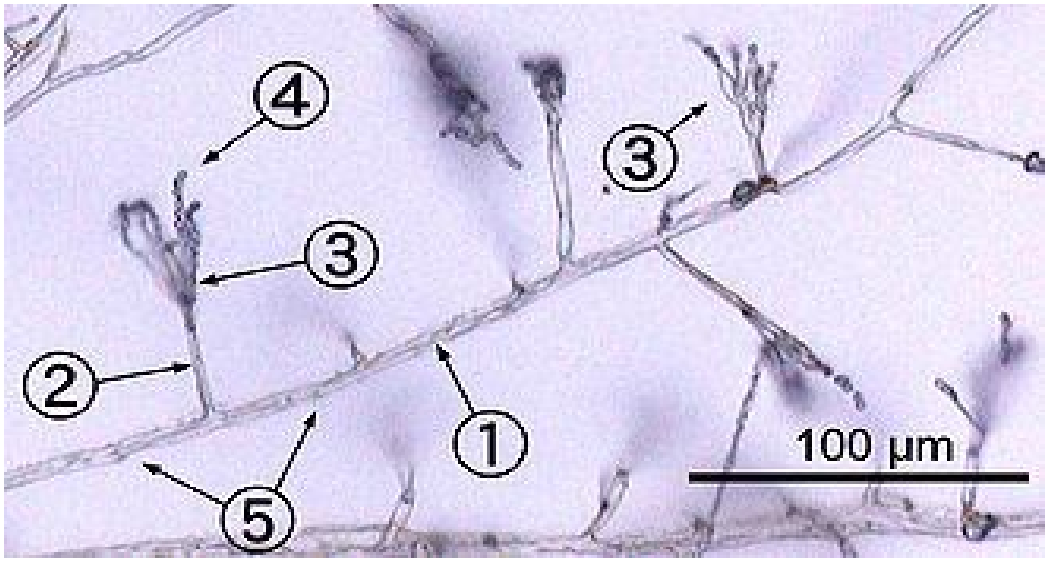



Plate 2.2. An environmental isolate of *Penicillium*; 1. hypha 2. conidiophore 3. phialide 4. conidia 5. septa (Alexopoulos *et. al.*, 1996)

Most fungi grow as hyphae, which are cylindrical, thread-like structures 2–10 µm in diameter and up to several centimeters in length (plate 2.2). Hyphae grow at their tips (apices); new hyphae are typically formed by emergence of new tips along existing hyphae by a process called *branching*, or occasionally growing hyphal tips bifurcate (fork) giving rise to two parallel-growing hyphae (Harris, 2008). The combination of apical growth and branching/forking leads to the development of a mycelium, an interconnected network of hyphae (Alexopoulos *et. al.*, 1996). Hyphae can be either septate or coenocytic: septate

hyphae are divided into compartments separated by cross walls (internal cell walls, called septa, that are formed at right angles to the cell wall giving the hypha its shape), with each compartment containing one or more nuclei; coenocytic hyphae are not compartmentalized (Deacon, 2005).

Septa have pores that allow cytoplasm, organelles, and sometimes nuclei to pass through; an example is the dolipore septum in the fungi of the phylum Basidiomycota (Deacon, 2005). Coenocytic hyphae are essentially multinucleate supercells (Chang and Miles, 2004).

Many species have developed specialized hyphal structures for nutrient uptake from living hosts; examples include haustoria in plant-parasitic species of most fungal phyla, and arbuscules of several mycorrhizal fungi, which penetrate into the host cells to consume nutrients (Parniske, 2008).

Although fungi are opisthokonts—a grouping of evolutionarily related organisms broadly characterized by a single posterior flagellum—all phyla except for the chytrids have lost their posterior flagella (Steenkamp *et. al.*, 2006).

#### 2.1.4.2 Macroscopic Structures of Macrofungi

Fungal mycelia can become visible to the naked eye, for example, on various surfaces and substrates, such as damp walls and on spoiled food, where they are commonly called molds. Mycelia grown on solid agar media in laboratory petri dishes are usually referred to as colonies. These colonies can exhibit growth shapes and colors (due to spores or pigmentation) that can be used as diagnostic features in the identification of species or groups (Hanson, 2008). Some individual fungal colonies can reach extraordinary

dimensions and ages as in the case of a clonal colony of *Armillaria solidipes*, which extends over an area of more than 900 ha (3.5 square miles), with an estimated age of nearly 9,000 years (Ferguson *et. al.*, 2003).

The apothecium—a specialized structure important in sexual reproduction in the ascomycetes—is a cup-shaped fruiting body that holds the hymenium, a layer of tissue containing the spore-bearing cells (Alexopoulos *et. al.*, 1996). The fruiting bodies of the basidiomycetes (basidiocarps) and some ascomycetes can sometimes grow very large, and many are well-known as mushrooms.

### 2.1.5 Taxonomy of Macrofungi

Although commonly included in botany curricula and textbooks, fungi are more closely related to animals than to plants and are placed with the animals in the monophyletic group of opisthokonts (Shalchian-Tabrizi *et. al*., 2008). Analyses using molecular phylogenetics support a monophyletic origin of the Fungi (Hibbett *et. al.* 2007). The taxonomy of the Fungi is in a state of constant flux, especially due to recent research based on DNA comparisons.

There is no unique generally accepted system at the higher taxonomic levels and there are frequent name changes at every level, from species upwards. Efforts among researchers are now underway to establish and encourage usage of a unified and more consistent nomenclature (Celio *et. al.*, 2006; Hibbett *et. al.* 2007). Fungal species can also have multiple scientific names depending on their life cycle and mode (sexual or asexual) of reproduction. Web sites such as Index Fungorum and ITIS list current names of fungal species (with cross-references to older synonyms).

The 2007 classification of Kingdom Fungi is the result of a large-scale collaborative research effort involving dozens of mycologists and other scientists working on fungal taxonomy (Hibbett *et. al.* 2007). It recognizes seven phyla, two of which; the Ascomycota and the Basidiomycota are contained within a branch representing subkingdom Dikarya.

#### 2.1.5.1 Taxonomic Groups of Macrofungi

The major phyla (sometimes called divisions) of fungi have been classified mainly on the basis of characteristics of their sexual reproductive structures. Currently, seven phyla are proposed: Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Ascomycota, and Basidiomycota (Hibbett *et. al.* 2007).

**Microsporidia**: Phylogenetic analysis has demonstrated that the Microsporidia, unicellular parasites of animals and protists, are fairly recent and highly derived endobiotic fungi (living within the tissue of another species) (James *et. al.* 2006; Gill and Fast, 2006). Liu *et. al.* (2006) concludes that the Microsporidia are a sister group to the true fungi, that is, they are each other's closest evolutionary relative. Hibbett and colleagues (2007) suggest that this analysis does not clash with their classification of the Fungi, and although the Microsporidia are elevated to phylum status, it is acknowledged that further analysis is required to clarify evolutionary relationships within this group (Hibbett *et. al.* 2007).

**Chytridiomycota**: The Chytridiomycota are commonly known as chytrids. These fungi are distributed worldwide. Chytrids produce zoospores that are capable of active movement through aqueous phases with a single flagellum, leading early taxonomists to classify them as protists. Molecular phylogenies, inferred from rRNA sequences in ribosomes, suggest that the Chytrids are a basal group divergent from the other fungal phyla, consisting of four

major clades with suggestive evidence for paraphyly or possibly polyphyly (James *et. al.* 2006).

**Blastocladiomycota**: The Blastocladiomycota were previously considered a taxonomic clade within the Chytridiomycota. Recent molecular data and ultrastructural characteristics, however, place the Blastocladiomycota as a sister clade to the Zygomycota, Glomeromycota, and Dikarya (Ascomycota and Basidiomycota). The blastocladiomycetes are saprotrophs, feeding on decomposing organic matter, and they are parasites of all eukaryotic groups. Unlike their close relatives, the chytrids, which mostly exhibit zygotic meiosis, the blastocladiomycetes undergo sporic meiosis (James *et. al.* 2006).

**Neocallimastigomycota**: The Neocallimastigomycota were earlier placed in the phylum Chytridomycota. Members of this small phylum are anaerobic organisms, living in the digestive system of larger herbivorous mammals and possibly in other terrestrial and aquatic environments. They lack mitochondria but contain hydrogenosomes of mitochondrial origin. As the related chrytrids, neocallimastigomycetes form zoospores that are posteriorly uniflagellate or polyflagellate (James *et. al.* 2006).

**Glomeromycota**: Members of the Glomeromycota form arbuscular mycorrhizae, a form of symbiosis where fungal hyphae invade plant root cells and both species benefit from the resulting increased supply of nutrients. All known Glomeromycota species reproduce asexually (Redecker and Raab, 2006). The symbiotic association between the Glomeromycota and plants is ancient, with evidence dating to 400 million years ago (Remy *et. al.*, 1994). Formerly part of the Zygomycota (commonly known as 'sugar' and 'pin' molds), the Glomeromycota were elevated to phylum status in 2001 and now replace the

older phylum Zygomycota (Schüssler *et. al.*, 2001). Fungi that were placed in the Zygomycota are now being reassigned to the Glomeromycota, or the subphyla incertae sedis Mucoromycotina, Kickxellomycotina, the Zoopagomycotina and the Entomophthoromycotina (Hibbett *et. al.*, 2007). Some well-known examples of fungi formerly in the Zygomycota include black bread mold (*Rhizopus stolonifer*), and *Pilobolus* species, capable of ejecting spores several meters through the air (Alexopoulus *et. al.*, 1996).

**Ascomycota**: The Ascomycota, commonly known as sac fungi or ascomycetes, constitute the largest taxonomic group within the Eumycota (Kirk *et. al., 2008*). These fungi form meiotic spores called ascospores, which are enclosed in a special sac-like structure called an ascus. This phylum includes morels, a few macrofungi and truffles, single-celled yeasts (e.g., of the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, and *Candida*), and many filamentous fungi living as saprotrophs, parasites, and mutualistic symbionts. Prominent and important genera of filamentous ascomycetes include *Aspergillus*, *Penicillium*, *Fusarium*, and *Claviceps*. Many ascomycete species have only been observed undergoing asexual reproduction (called anamorphic species), but analysis of molecular data has often been able to identify their closest teleomorphs in the Ascomycota (Samuels, 2006). Because the products of meiosis are retained within the sac-like ascus, ascomycetes have been used for elucidating principles of genetics and heredity (e.g. *Neurospora crassa*) (Radford and Parish 1997).

**Basidiomycota**: Members of the Basidiomycota, commonly known as the club fungi or basidiomycetes, produce meiospores called basidiospores on club-like stalks called basidia. Most common macrofungi belong to this group, as well as rust and smut fungi, which are

major pathogens of grains. Other important basidiomycetes include the maize pathogen *Ustilago maydis* (Valverde *et. al.*, 1995), human commensal species of the genus *Malassezia* (Zisova, 2009), and the opportunistic human pathogen, *Cryptococcus neoformans* (Perfect, 2006).

### 2.1.6 Human use of fungi

The human use of fungi for food preparation or preservation and other purposes is extensive and has a long history. Mushroom farming and mushroom gathering are large industries in many countries. The study of the historical uses and sociological impact of fungi is known as ethnomycology. Because of the capacity of this group to produce an enormous range of natural products with antimicrobial or other biological activities, many species have long been used or are being developed for industrial production of antibiotics, vitamins, and anti-cancer and cholesterol-lowering drugs. More recently, methods have been developed for genetic engineering of fungi (Fincham, 1989), enabling metabolic engineering of fungal species. For example, genetic modification of yeast species (Hawkins and Smolke, 2008) which are easy to grow at fast rates in large fermentation vessels—has opened up ways of pharmaceutical production that are potentially more efficient than production by the original source organisms (Huang *et. al*., 2008).

#### 2.1.6.1 Medicinal value of fungi

Many species produce metabolites that are major sources of pharmacologically active drugs. Particularly important are the antibiotics, including the penicillins, a structurally related group of β-lactam antibiotics that are synthesized from small peptides. Although naturally occurring penicillins such as penicillin G (produced by *Penicillium chrysogenum*)

have a relatively narrow spectrum of biological activity, a wide range of other penicillins can be produced by chemical modification of the natural penicillins. Modern penicillins are semisynthetic compounds, obtained initially from fermentation cultures, but then structurally altered for specific desirable properties (Brakhage *et. al.*, 2004). Other antibiotics produced by fungi include: ciclosporin, commonly used as an immunosuppressant during transplant surgery; and fusidic acid, used to help control infection from methicillin-resistant *Staphylococcus aureus* bacteria (Pan *et. al.*, 2008). Widespread use of these antibiotics for the treatment of bacterial diseases, such as tuberculosis, syphilis, leprosy, and many others began in the early 20th century and continues to play a major part in anti-bacterial chemotherapy. In nature, antibiotics of fungal or bacterial origin appear to play a dual role: at high concentrations they act as chemical defense against competition with other microorganisms in species-rich environments, such as the rhizosphere, and at low concentrations as quorum-sensing molecules for intra- or interspecies signaling (Fajardo and Martínez, 2008).

Other drugs produced by fungi include griseofulvin isolated from *Penicillium griseofulvum*, used to treat fungal infections (Loo, 2006), and statins (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors), used to inhibit cholesterol synthesis. Examples of statins found in fungi include mevastatin from *Penicillium citrinum* and lovastatin from *Aspergillus terreus* and the oyster mushroom (Manzoni and Rollini 2002).

#### 2.1.6.2 Cultured foods made from fungi

Baker's yeast or *Saccharomyces cerevisiae*, a single-celled fungus, is used to make bread and other wheat-based products, such as pizza dough and dumplings (Kulp, 2000). Yeast species of the genus *Saccharomyces* are also used to produce alcoholic beverages through

fermentation (Piskur *et. al.*, 2006). Shoyu koji mold (*Aspergillus oryzae*) is an essential ingredient in brewing Shoyu (soy sauce) and sake, and the preparation of miso (Abe *et. al.*, 2006), while *Rhizopus* species are used for making tempeh (Hachmeister and Fung, 1993). Several of these fungi are domesticated species that were bred or selected according to their capacity to ferment foodwithout producing harmful mycotoxins which are produced by very closely related *Aspergilli* (Jørgensen, 2007). Quorn, a meat substitute, is made from *Fusarium venenatum* (O'Donnell *et. al.*, 1998).

#### 2.1.6.3 Medicinal use of fungi

Certain macrofungi enjoy usage as therapeutics in folk medicines, such as Traditional Chinese medicine. Notable medicinal macrofungi with a well-documented history of use include *Agaricus subrufescens* (Hetland *et. al.*, 2008; Firenzuoli *et. al.*, 2008), *Ganoderma lucidum* (Paterson, 2006), and *Cordyceps sinensis* as shown in plate 2.4 (Paterson, 2008). Research has identified compounds produced by these and other fungi that have inhibitory biological effects against viruses (el-Mekkawy *et. al.*, 1998; El Dine *et. al.*, 2008) and cancer cells (Yuen and Gohel, 2005; Hetland *et. al.*, 2008).

Specific metabolites, such as polysaccharide-K, ergotamine, and β-lactam antibiotics, are routinely used in clinical medicine. The shiitake mushroom is a source of lentinan, a clinical drug approved for use in cancer treatments in several countries, including Japan (Halpern and Miller, 2002; Sullivan, 2006). In Europe and Japan, polysaccharide-K (brand name Krestin), a chemical derived from *Trametes versicolor*, is an approved adjuvant for cancer therapy (Fisher and Yang, 2002).





Plate 2.4 The medicinal fungi *Ganoderma lucidum* (left) and *Cordyceps sinensis* (right) (Paterson, 2008).

### 2.1.6.4 Edible and poisonous species of macrofungi

Edible macrofungi are well-known examples of fungi. Many are commercially raised, but others must be harvested from the wild. *Agaricus bisporus* (plate 2.5), sold as button macrofungi when small or Portobello macrofungi when larger, is a commonly eaten species, used in salads, soups, and many other dishes. Many Asian fungi are commercially grown and have increased in popularity in the West. They are often available fresh in grocery stores and markets, including straw macrofungi (*Volvariella volvacea*), oyster macrofungi (*Pleurotus ostreatus*), shiitakes (*Lentinula edodes*), and enokitake (*Flammulina* spp.) (Stamets, 2000).

Many mushroom species are poisonous to humans, with toxicities ranging from slight digestive problems or allergic reactions as well as hallucinations to severe organ failures and death. Genera with macrofungi containing deadly toxins include *Conocybe*, *Galerina*, *Lepiota*, and most infamously, *Amanita* (Orr and Orr, 1979). The latter genus includes the destroying angel *(A. virosa)* and the death cap *(A. phalloides)*, the most common cause of deadly mushroom poisoning (Vetter, 1998). The false morel (*Gyromitra esculenta*) is occasionally considered a delicacy when cooked, yet can be highly toxic when eaten raw

(Leathem and Dorran, 2007). *Tricholoma equestre* was considered edible until being implicated in serious poisonings causing rhabdomyolysis (Karlson-Stiber and Persson, 2003). Fly agaric macrofungi (*Amanita muscaria*) also cause occasional non-fatal poisonings, mostly as a result of ingestion for use as a recreational drug for its hallucinogenic properties. Historically, fly agaric was used by different peoples in Europe and Asia and its present usage for religious or shamanic purposes is reported from some ethnic groups such as the Koryak people of north-eastern Siberia (Michelot and Melendez-Howel, 2003).

As it is difficult to accurately identify a safe mushroom without proper training and knowledge, it is often advised to assume that a wild mushroom is poisonous and not to consume it (Hall, 2003; Ammirati *et. al.*, 1987).



Plate 2.5 *Amanita phalloides* (Stamets, 2000)

### 2.1.6.4 Pest control using fungi

In agriculture, fungi may be useful if they actively compete for nutrients and space with pathogenic microorganisms such as bacteria or other fungi via the competitive exclusion principle (López-Gómez and Molina-Meyer, 2006), or if they are parasites of these pathogens. For example, certain species may be used to eliminate or suppress the growth of harmful plant pathogens, such as insects (plate 2.6), mites, weeds, nematodes and other fungi that cause diseases of important crop plants (Becker, 1998). Entomopathogenic fungi can be used as biopesticides, as they actively kill insects (Keiller, 2009). Examples that have been used as biological insecticides are *Beauveria bassiana*, *Metarhizium* spp, *Hirsutella* spp, *Paecilomyces* (*Isaria*) spp, and *Lecanicillium lecanii* (Deshpande, 1999; Thomas and Read, 2007).

Endophytic fungi of grasses of the genus *Neotyphodium*, such as *N. coenophialum*, produce alkaloids that are toxic to a range of invertebrate and vertebrate herbivores. These alkaloids protect grass plants from herbivory, but several endophyte alkaloids can poison grazing animals, such as cattle and sheep (Bush, 1997). Infecting cultivars of pasture or forage grasses with *Neotyphodium* endophytes is one approach being used in grass breeding programs; the fungal strains are selected for producing only alkaloids that increase resistance to herbivores such as insects, while being non-toxic to livestock (Bouton *et. al.*, 2002).



Plate 2.6 Grasshoppers killed by *Beauveria bassiana* (Becker, 1998)

**2.1.6.5 Bioremediation using Fungi**

Certain fungi, in particular "white rot" fungi, can degrade insecticides, herbicides, pentachlorophenol, creosote, coal tars, and heavy fuels and turn them into carbon dioxide, water, and basic elements (Christian, 2005). Fungi have been shown to biomineralize uranium oxides, suggesting they may have application in the bioremediation of radioactively polluted sites (Fomina *et. al.*, 2007; BBC, 2008; Fomina *et. al.*, 2008).

Fungi are used extensively to produce industrial chemicals like citric, gluconic, lactic, and malic acids (Schlegel, 1993), and industrial enzymes, such as lipases used in biological detergents (Joseph, 2008), cellulases used in making cellulosic ethanol (Kumar *et. al.*, 2008) and amylases (Olempska-Beer *et. al.*, 2006), invertases, proteases and xylanases (Polizeli *et. al.*, 2005).

### 2.1.7 Mycotoxins from macrofungi

Many fungi produce biologically active compounds, several of which are toxic to animals or plants and are therefore called mycotoxins. Mycotoxins produced by molds causing food spoilage, and poisonous macrofungi are particular relevance to humans. Particularly infamous are the lethal amatoxins in some *Amanita* macrofungi, and ergot alkaloids, which have a long history of causing serious epidemics of ergotism (St Anthony's Fire) in people consuming rye or related cereals contaminated with sclerotia of the ergot fungus, *Claviceps purpurea* (Schardl *et. al.*, 2006). Other notable mycotoxins include; the aflatoxins, which are insidious liver toxins and highly carcinogenic metabolites produced by certain *Aspergillus* species often growing in or on grains and nuts consumed by humans, ochratoxins, patulin, and trichothecenes (e.g., T-2 mycotoxin) and fumonisins, which have significant impact on human food supplies or animal livestock, Ergotamine (figure 2.1), a major mycotoxin produced by *Claviceps* species, which if ingested can cause gangrene, convulsions, and hallucinations . (van Egmond *et. al.*, 2007).

Mycotoxins are secondary metabolites (or natural products), and research has established the existence of biochemical pathways solely for the purpose of producing mycotoxins and other natural products in fungi (Keller *et. al.*, 2005). Mycotoxins may provide fitness benefits in terms of physiological adaptation, competition with other microbes and fungi, and protection from consumption (fungivory) (Demain and Fang, 2000; Rohlfs *et. al.*, 2007).

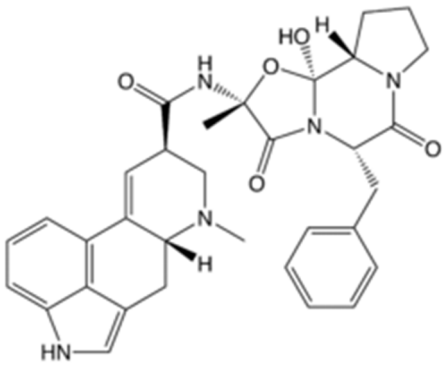



Figure 2.1: Ergotamine, a major mycotoxin produced by *Claviceps* species, which if ingested can cause gangrene, convulsions, and hallucinations.

## 2.2 Macrofungi (Mushroom)

A mushroom is the fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. In a broad sense "Mushroom is a macrofungus with a distinctive fruiting body, which can be either epigeous or hypogeous and large enough to be seen with naked eye and to be picked by hand" (Chang and Miles, 1992). The standard for the name "mushroom" is the cultivated white button mushroom, *Agaricus bisporus*; hence the word "mushroom" is most often applied to those fungi that have a stem (stipe), a cap (pileus), and gills (lamellae, sing. lamella) or pores on the underside of the cap (Dickinson, 1982). Thus, macrofungi need not be basidiomycetes, nor aerial, nor fleshy, nor edible. Macrofungi can be ascomycetes, grow underground, have a non-fleshy texture and need not be edible.

This definition is not a perfect one but can be accepted as a workable term to estimate the number of macrofungi on the earth. The most common type of macrofungi is umbrella shaped with a pileus (cap) and a stipe (stem) i.e. *Lentinula edodes*. Other species additionally have a volva (cup) i.e. *Volvariella volvacea* or an annulus (ring) i.e. *Agarius*

campestris or with both of them i.e. Amanita muscaria. Furthermore, some macrofungi are in the form of pliable cups; others round like golf balls. Some are in the shape of small clubs; some resemble coral; others are yellow or orange jelly-like globs; and some even very much resembles the human ear. In fact, there is a countless variety of forms.

The structure that we call a mushroom is in reality only the fruiting body of the fungus. The vegetative part of the fungus, called the mycelium, comprises a system of branching threads and cord-like strands that branch out through soil, compost, wood log or other lignocellulosic material on which the fungus may be growing. After a period of growth and under favourable conditions, the established (matured) mycelium could produce the fruit structure which we call the mushroom. Accordingly macrofungi can be grouped into four categories: (1) those which are fleshy and edible fall into the edible mushroom category, e.g., *Agaricus bisporus*; (2) macrofungi which are considered to have medicinal applications, are referred to as medicinal macrofungi, e.g., *Ganoderma lucidum*; (3) those which are proven to be, or suspected of being poisonous are named as poisonous macrofungi, e.g., *Amanita phalloides*; and (4) a miscellaneous category which includes a large number of macrofungi whose properties remain less well defined, which may tentatively be grouped together as 'other macrofungi'. Certainly, this approach of classifying of macrofungi is not absolute and not mutually exclusive. Many kinds of macrofungi are not only edible, but also possess tonic and medicinal qualities.

Macrofungi are devoid of leaves, and of chlorophyll-containing tissues. This renders them incapable of photosynthetic food production. Yet, they grow, and they produce new biomass. For their survival, for their growth, and for their metabolism, they rely on organic matter synthesized by the green plants around us, including organic products contained in

agricultural crop residues. The organic materials, on which macrofungi derive their nutrition, are referred to as substrates. Macrofungi are a unique biota which assembles their food by secreting degrading enzymes and decompose the complex food materials present in the biomass where they grow, to generate simpler compounds, which they then absorb, and transform into their own peculiar tissues. These substrate materials are usually by-products from industry, households and agriculture and are usually considered as wastes (UN, 2011).

Macrofungi lack true roots, they are anchored into the substrates by their tightly interwoven thread-like hyphae, which also colonise the substrates, degrade their biochemical components, and siphon away the hydrolysed organic compounds for their own nutrition (UN, 2011).

### 2.2.1 Identification of Macrofungi

Identifying Macrofungi requires a basic understanding of their macroscopic structure. Most are Basidiomycetes and gilled. Their spores, called basidiospores, are produced on the gills and fall in a fine rain of powder from under the caps as a result. At the microscopic level the basidiospores are shot off basidia and then fall between the gills in the dead air space. As a result, for most macrofungi, if the cap is cut off and placed gill-side-down overnight, a powdery impression reflecting the shape of the gills (or pores, or spines, etc.) is formed (when the fruit body is sporulating). The color of the powdery print, called a spore print, is used to help classify macrofungi and can help to identify them. Spore print colors include white (most common), brown, black, purple-brown, pink, yellow, and cream, but almost never blue, green, or red (Dickinson, 1982).

While modern identification of macrofungi is quickly becoming molecular, the standard methods for identification are still used by most and have developed into a fine art harking back to medieval times and the Victorian era, combined with microscopic examination. The presence of juices upon breaking, bruising reactions, odors, tastes, shades of color, habitat, habit, and season are all considered by both amateur and professional mycologists. Tasting and smelling macrofungi carries its hazards because of poisons and allergens. Chemical tests are also used for some genera (Ammirati *et. al.*, 1985).

In general, identification of the genus can often be accomplished in the field using a local mushroom guide. Identification to species, however, requires more effort; one must remember that a mushroom develops from a button stage into a mature structure, and only the latter can provide certain characteristics needed for the identification of the species. However, over-mature specimens lose features and cease producing spores. Many novices have mistaken humid water marks on paper for white spore prints, or discolored paper from oozing liquids on lamella edges for colored spored prints (Stuntz *et. al.*, 1978).

### 2.2.2 Classification of Macrofungi

Typical macrofungi are the fruit bodies of members of the order Agaricales, whose type genus is *Agaricus* and type species is the field mushroom, *Agaricus campestris*. However, in modern molecularly-defined classifications, not all members of the order Agaricales produce mushroom fruit bodies, and many other gilled fungi, collectively called macrofungi, occur in other orders of the class Agaricomycetes. For example, chanterelles are in the Cantharellales, false chanterelles like *Gomphus* are in the Gomphales, milk macrofungi (*Lactarius*) and russulas (*Russula*) as well as *Lentinellus* are in the Russulales, while the tough leathery genera *Lentinus* and *Panus* are among the Polyporales, but

*Neolentinus* is in the Gloeophyllales, and the little pin-mushroom genus, *Rickenella*, along with similar genera, are in the Hymenochaetales (Miles and Chang, 2004).

Within the main body of macrofungi, in the Agaricales, are common fungi like the common fairy-ring mushroom (*Marasmius oreades*), shiitake, enoki, oyster macrofungi, fly agarics, and other amanitas, magic macrofungi like species of *Psilocybe*, paddy straw macrofungi, shaggy manes, etc (Miles and Chang, 2004).

An atypical mushroom is the lobster mushroom, which is a deformed, cooked-lobster-colored parasitized fruitbody of a *Russula* or *Lactarius*, colored and deformed by the mycoparasitic Ascomycete *Hypomyces lactifluorum* (Volk, 2001).

Other macrofungi are not gilled and then the term "mushroom" is loosely used, so it is difficult to give a full account of their classifications. Some have pores underneath (and are usually called boletes), others have spines, such as the hedgehog mushroom and other tooth fungi, and so on. "Mushroom" has been used for polypores, puffballs, jelly fungi, coral fungi, bracket fungi, stinkhorns, and cup fungi. Thus, the term is more one of common application to macroscopic fungal fruiting bodies than one having precise taxonomic meaning. There are approximately 14,000 described species of macrofungi (Miles and Chang, 2004).

### 2.2.3 Morphology of Macrofungi

A mushroom develops from a nodule, or pinhead, less than two millimeters in diameter, called a primordium, which is typically found on or near the surface of the substrate. It is formed within the mycelium, the mass of threadlike hyphae that make up the fungus. The primordium enlarges into a roundish structure of interwoven hyphae roughly resembling an

egg, called a "button". The button has a cottony roll of mycelium, the universal veil, that surrounds the developing fruit body. As the egg expands, the universal veil ruptures and may remain as a cup, or volva, at the base of the stalk, or as warts or volval patches on the cap. Many macrofungi lack a universal veil and therefore do not have either a volva or volval patches. Often there is a second layer of tissue, the partial veil, covering the bladelike gills that bear spores. As the cap expands, the veil breaks, and remnants of the partial veil may remain as a ring, or annulus, around the middle of the stalk or as fragments hanging from the margin of the cap. The ring may be skirt-like as in some species of *Amanita*, collar-like as in many species of *Lepiota*, or merely the faint remnants of a cortina (a partial veil composed of filaments resembling a spiderweb), which is typical of the genus *Cortinarius*. Macrofungi that lack a partial veil do not form an annulus (Stuntz *et. al.*, 1978).

The stalk (also called the stipe, or stem as shown in figure 2.2) may be central and support the cap in the middle, or it may be off-center and/or lateral, as in species of *Pleurotus* and *Panus*. In other macrofungi, a stalk may be absent, as in the polypores that form shelf-like brackets. Puffballs lack a stalk but may have a supporting base. Other macrofungi, like truffles, jellies, earthstars, bird's nests, usually do not have stalks, and a specialized mycological vocabulary exists to describe their parts (Stuntz *et. al.*, 1978).

The way that gills (lamellae as shown in figure 2.2) attach to the top of the stalk is an important feature of mushroom morphology. Macrofungi in the genera *Agaricus*, *Amanita*, *Lepiota* and *Pluteus*, among others, have free gills that do not extend to the top of the stalk. Others have decurrent gills that extend down the stalk, as in the genera *Omphalotus* and *Pleurotus*. There are a great number of variations between the extremes of free and

decurrent, collectively called attached gills. Finer distinctions are often made to distinguish the types of attached gills: adnate gills, which adjoin squarely to the stalk; notched gills, which are notched where they join the top of the stalk; adnexed gills, which curve upward to meet the stalk, and so on. These distinctions between attached gills are sometimes difficult to interpret, since gill attachment may change as the mushroom matures, or with different environmental conditions (Stuntz *et. al.*, 1978).

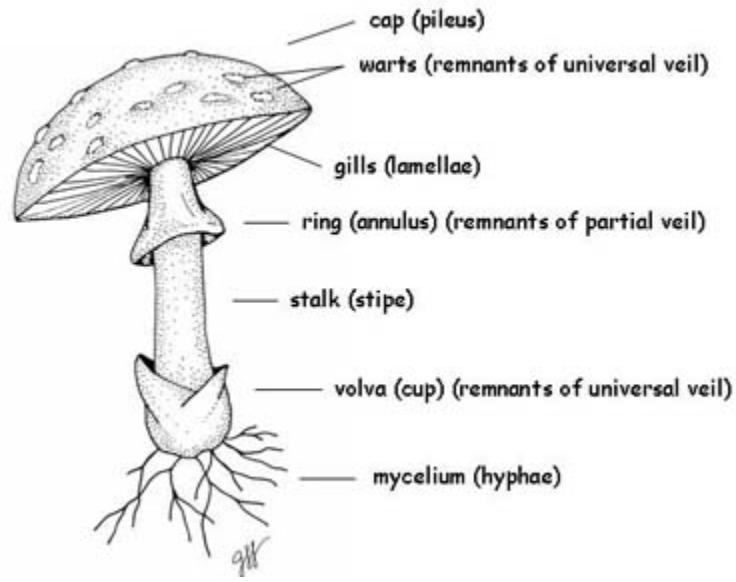



Figure 2.2. Morphological features of a basidiocarp (basidiome or fruiting body)



Plate 2.7. The 8-spored asci of *Morchella elate* viewed with a contrast microscope. Source: Ammirati *et. al.* (1985)

A hymenium is a layer of microscopic spore-bearing cells that covers the surface of gills. In the non-gilled macrofungi, the hymenium lines the inner surfaces of the tubes of boletes and polypores, or covers the teeth of spine fungi and the branches of corals. In the Ascomycota, spores develop within a microscopic elongated, saclike cell called an ascus, which typically contains eight spores (plate 2.7). The Discomycetes—which contains the cup, sponge, brain, and some club-like fungi—develop an exposed layer of asci, as on the inner surface of cup fungi or within the pits of morels. The Pyrenomycetes, tiny dark-colored fungi that live on a wide range of substrates including soil, dung, leaf litter, decaying wood, as well as other fungi, produce minute flask-shaped structures called perithecia, within which the asci develop (Ammirati *et. al.,* 1985).

In the Basidiomycetes, usually four spores develop on the tips of thin projections called sterigmata, which extend from a club-shaped cell called a basidium. The fertile portion of the Gasteromycetes, called a gleba, may become powdery as in the puffballs or slimy as in the stinkhorns. Interspersed among the asci are threadlike sterile cells called paraphyses. Similar structures called cystidia often occur within the hymenium of the Basidiomycota. Many types of cystidia exist and assessing their presence, shape, and size is often used to verify the identification of a macrofungi (Ammirati *et. al.,* 1985).

The most important microscopic feature for identification of macrofungi is the spores (plate 2.8) themselves. Their color, shape, size, attachment, ornamentation, and reaction to chemical tests often can be the crux of identification. Spores often have a protrusion at one end, called an apiculus, which is the point of attachment to the basidium, termed the apical germ pore, from which the hypha emerges when the spore germinates (Ammirati *et. al.*, 1985).

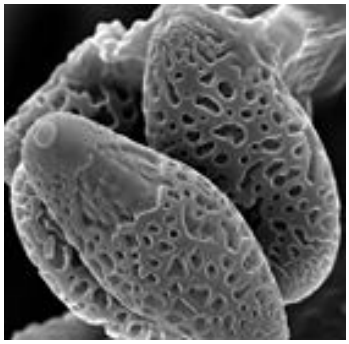


Plate 2.8. *Austroboletus mutabilis* spores viewed using electron microscopy
Source: Ammirati *et. al.* (1985)

### 2.2.4 Biological properties of Macrofungi

Macrofungi with their delicate flavour and texture are recognised as a nutritious food and an important source of biologically active compounds with medicinal values. Generally, macrofungi are low in energy and high in dietary fibre (Emilia *et. al.*, 2006), and an excellent source for antioxidants as they accumulate a variety of secondary metabolites, including phenolic compounds (Jose and Janardhanan, 2000; Cheung *et. al.*, 2003). Antioxidants are substances needed in minute quantity which are capable of counteracting free radicals to prevent oxidative damage. There are about 1200 species of mushroom used in 85 different countries for their gastronomic value and/or medicinal properties (Roman *et. al.*, 2006).

Edible wild macrofungi are often regarded as being nutritionally high and with potential economic value. Many species with medicinal value are widely used in traditional medicine for a broad range of diseases (Roman *et. al.*, 2006). Some species are regarded as therapeutic food for their anti-carcinogenic, anti-cholesterolaemic and anti-viral properties (Emilia *et. al.*, 2006). Oyster macrofungi (Pleurotus ostreatus), shiitake (Lentinus edodes), Jew's ear fungus also known as monkey's ear fungus (Auricularia sp.) and paddy straw

mushroom (Volvariella volvacea) have been utilized for a long time in Malaysia by the Malay, Chinese and Indians for their beneficial effects in addition to being a source of food (Chang and Lee, 2004). A study by Chye *et. al.* (2008), reported that some wild edible macrofungi found in Sabah ( which is located in East Malaysia) were of high nutritional value and exhibited high antioxidant activity.

Medicinal macrofungi are macrofungi or extracts from macrofungi that are used or studied as possible treatments for diseases. Some mushroom materials, including polysaccharides, glycoproteins and proteoglycans, modulate immune system responses and inhibit tumor growth. Some medicinal mushroom isolates that have been identified also show cardiovascular, antiviral, antibacterial, antiparasitic, anti-inflammatory, and antidiabetic properties. Currently, several extracts have widespread use in Japan, Korea and China, as adjuncts to radiation treatments and chemotherapy (Smith *et. al.*, 2002; Borchers *et. al.*, 2008).

Macrofungi have been a subject of modern medical research since the 1960s, where most modern medical studies concern the use of mushroom extracts, rather than whole macrofungi. Only a few specific mushroom extracts have been extensively tested for efficacy (Borchers *et. al.*, 2008). Polysaccharide-K and lentinan are among the mushroom extracts with the firmest evidence. The available results for most other extracts are based on *in vitro* data, effects on isolated cells in a lab dish, animal models like mice, or underpowered clinical human trials (Borchers *et. al.*, 2008). Studies show that glucan-containing mushroom extracts primarily change the function of the innate and adaptive immune systems, functioning as bioresponse modulators, rather than by directly killing bacteria, viruses, or cancer cells as cytocidal agents (Borchers *et. al.*, 2008). In some

countries, extracts like polysaccharide-K, schizophyllan, polysaccharide peptide, and lentinan are government-registered adjuvant cancer therapies (Smith *et. al.*, 2002).

### 2.2.5 Nutritional and Medicinal properties of Macrofungi

Edible macrofungi provide high quality of protein that can be produced with greater biological efficiency than animal protein. They are rich in fibre, minerals and vitamins, and have low crude fat content, with a high proportion of polyunsaturated fatty acids (72 to 85 %) relative to total content of fatty acids. These properties are major contributing factors to the traditional recognition of macrofungi as "healthy" foods. A large number of macrofungi species are not only edible and nutritious but also possess tonic and medicinal qualities. However, some macrofungi are lethally poisonous, and one should eat macrofungi only if one knows their names and their properties with considerable precision.

In the past, the mushroom industry concentrated mainly on the production of fresh, canned and dried macrofungi for food. Thus, the industry had only one leg. In the present era, high-pressure work demands are causing greater stress to the human body, and resulting in the weakening of the human immune system. A variety of proprietary products based on mushroom nutriceuticals and mushroom pharmaceuticals have already been produced and marketed. This trend is expected to increase with wider consumer satisfaction and acceptability. This is the second leg of the industry. These two legs/segments of the mushroom-based industry will not compete but will complement each other (UN, 2011).

#### 2.2.5.1 Nutritional Value of Macrofungi

The greatest difficulty in feeding man is to supply a sufficient quantity of the body-building material -- protein. The other three nutritional categories are: the source of energy food-

carbohydrates and fats; accessory food factors-- vitamins; and inorganic compounds which are indispensable to good health. Of course, water, too, is essential. The moisture content of fresh macrofungi varies within the range of 70 - 95% depending upon the harvest time and environmental conditions, whereas it is about 10 - 13% in dried macrofungus. The protein content of the cultivated species ranges from 1.75 to 5.9 % of their fresh weight. It has been estimated that an average value of 3.5 to 4.0 % would be more representative. This means that the protein content of edible macrofungi in general, is about twice that of onion (1.4%) and cabbage (1.4%), and four times and 12 times those of oranges (1.0 %) and apples (0.3%), respectively. In comparison, the protein content of common meats is as follows: beef, 12-20 %; chicken, 18-20 %; fish, 18 -20 %; and milk, 2.9- 3.3 %. On a dry weight basis, macrofungi normally contain 19 -35 % protein, as compared to 7.3 % in rice, 12.7 % in wheat, 38.1 % in soybean, and 9.4 % in corn. Therefore, in terms of the amount of crude protein, macrofungi rank below animal meats, but well above most other foods, including milk, which is an animal product (Chang and Miles 1989). Furthermore, mushroom protein contains all the nine essential amino acids required by man.

Quantitative data relating to the nutritive value of macrofungi is sparse. In the absence of feeding trials, alternative methods have been used to determine or predict the nutritional value of foods based on their content of essential amino acids (Crisan and Sands 1978).The Essential Amino Acid Index (EA. Index) rates dietary protein in terms of an essential amino acid pattern based on known adult human dietary requirements. The Amino Acid Score (Chemical Score) is the amount of the most limiting amino acid in the food protein expressed as a percentage of the same amino acid present in the reference protein. In an attempt to resolve the difficulties inherent in comparisons between those macrofungi

containing small amounts of high quality protein with those containing larger amounts of a protein of lesser nutritional quality, Crisan and Sands (1978) proposed the use of a Nutritional Index calculated as:

$$\text{Nutritional Index} = \frac{(\text{EAA index} \times \text{percentage protein})}{100}$$

The EAA Indices, the Amino Acid Scores, and Nutritional Indices for various macrofungi and other foods, are reported by Crisan and Sands (1978). EAA Indices and Amino Acid Scores of the most nutritive macrofungi (highest values) rank in potential nutritive value with those of meat and milk, and are significantly higher than those for most legumes and vegetables. The least nutritive macrofungi rank appreciably lower but are still comparable to some of our common vegetables. In addition to their good proteins, macrofungi are a relatively good source of the following individual nutrients: fat, phosphorus, iron, and vitamins including thiamine, riboflavin, ascorbic acid, ergosterine and niacin. They are low in calories, carbohydrates and calcium. It has also been reported that a total lipid content varying between 0.6 and 3.1 % of the dry weight, is found in the commonly cultivated macrofungi. At least 72 % of the total fatty acids are found to be unsaturated in all the four tested macrofungi (Huang, *et. al.*, 1985). It should be noted that unsaturated fatty acids are essential and significant in our diet and to our health.

In recent years, there has been a trend toward discovering ways of treating macrofungi so as to give them added value. For example, Wermer and Beelman (2002) have reported on growing macrofungi enriched in selenium. By adding sodium selenite to compost over a range of 30-300 parts per million, they found that the macrofungi increasingly absorbed

selenium according to the amount in the compost, so that it is possible to grow macrofungi containing a desired concentration. Selenium is an essential micronutrient that has generated much recent interest in nutritional and medical research—and, more recently, within the food industry (Beelman and Royse, 2006). Selenium has numerous physiological functions, but is best known as necessary 30 cofactor for the glutathione peroxidase enzyme system. This system is responsible for removing free radicals from the body, thus reducing oxidative damage.

The desirability of a food product does not necessarily bear any correlation to its nutritional value. However, its appearance, taste, and aroma, sometimes can stimulate one's appetite (preference). In addition to nutritional value, macrofungi have some unique colour, taste, aroma, and texture characteristics, which attract their consumption by humans (UN, 2011).

### 2.2.6 Medicinal Properties of Macrofungi

The second major attribute of macrofungi, their medicinal properties, has long been recognised in China, Korea, and Japan. There has been a great upsurge in activities related to the use of macrofungi products for medicinal purposes in recent years. In 2001, the figure of US$9-10 billion was cited as representing the value of medicinal macrofungi products, including tonics and medicines. The application of modern analytical techniques can be used to establish a scientific basis for the empirical observations that have been made centuries before. According to Chang and Buswell (1996), the term "mushroom nutriceutical" is used for a new class of new compounds extractable from either the mycelium, or the fruiting body of the mushroom.

Mushroom nutriceuticals may possess both nutritional and medicinal properties. Due to present day high pressured work demands resulting in great stress to the human body and

causing a weakening of the human immune system, there are now many new diseases. These have developed as a consequence of lower natural body resistance. There is some evidence that the beneficial treatment of these diseases can be obtained by consumption of macrofungi as a functional food, or through the use of extracted biologically active compounds as a dietary supplement, in order to enhance immune response of the human body, thereby increasing resistance to disease and, in some cases, causing regression of a diseased state. Differing from most pharmaceuticals, these biologically active compounds extracted from medicinal macrofungi have extraordinarily low toxicity, even at high doses. Long viewed as tonics, now it has been known that they can profoundly improve the quality of human health (UN, 2011).

Macrofungi produce several biologically active compounds that are usually associated with the cell wall. Most notably, a group of polysaccharides comprising high molecular weight sugar polymers has been reported to contribute to their immune enhancing and tumour retarding effects. It has been reported that the anti-tumour and anti-cancer effects of the polysaccharides are based on the enhancement of the body's immune systems, including activated macrophages, natural killer cells, cytotoxic T cells, and their secretory products, such as the tumour necrosis factor, reactive nitrogen and oxygen intermediates, and interleukins, rather than direct cytocidal effects (Mizuno *et. al.* 1995; Liu *et. al.* 1996). It should be noted that immune responses are complex reactions involving several types of cells, such as macrophages and lymphocytes. The killing mediated by cytotoxic T lymphocytes and natural killer cells, represents an important mechanism in immune defence against tumours, virus-infected cells, parasites and other foreign invaders. Another group of medicinal compounds found in Ganoderma spp. are triterpenoids, steroid-like

compounds, which contribute cytotoxic, hepatoprotective, and hypolipidmic influence on platelet aggregation, inhibition of angiotensine-converting enzyme, and inhibition of histamine release (Lindequist 1995). Lectins, another group of mushroom bioactive compounds, are proteins or glycoproteins with specific binding sites for sugars, which are not antibodies or enzymes. These have become useful tools in structural studies of the cell surface, oligosaccharides and/or carbohydrate moieties of glycoproteins. Some lectins have been shown to have anti-tumour and immunomodulatory activities (Wang *et. al.* 1996). Other lectins preferentially agglutinate mammalian cells that have been transformed by oncogenic viruses or by chemical carcinogens, as well as spontaneously transformed cells. These and related findings indicate that studies with lectins may lead to a better understanding of cancer. Moreover, some lectins may be used to inhibit the growth of malignant cells. A novel single-chained ibosomeinactivating protein (RIP) was recently isolated from fruit bodies of the edible mushroom, *V. volvacea* (Yao *et. al.* 1998). The mushroom RIP, designated volvarin, exhibited a potent inhibitory action on protein synthesis in the rabbit reticulocyte lysate system. Like most plant RIPs, volvarin acted as an N-glycosidase that depurinated rRNA from rabbit reticulocyte lysate, releasing a characteristic RNA fragment after treatment with aniline. It also exerted a strong abortifacient effect in mice.

Furthermore, the aqueous extracts of Pleurotus sajor-caju (Tam *et. al.* 1986) and Volvariella volvacea (Chiu *et. al.* 1995) have been reported to produce a hypotensive effects in normotensive rats. It has also been reported that dried powder of *Auricularia auricula* and *Tremella fuciformis*, after being fed to the rats, has demonstrated to be effective in lowering both the serum total cholesterol and the low density lipoprotein

(LDL) cholesterol levels (Cheung 1996). Since the macrofungi did not affect the concentration of serum high density lipoprotein (HDL) cholesterol, the reduction of serum total cholesterol by the mushroom diets is believed to be attributable to the fall in LDL cholesterol. It should be noted that LDL is"bad" cholesterol and HDL is "good" cholesterol.

### 2.2.7 Macrofungi (Mushroom) Nutriceuticals

There has been a recent upsurge of interest in macrofungi not only as health vegetables (food) but also as a source of biological active compounds of medicinal value, including use as complementary medicine/dietary supplements for anticancer, antiviral, immunopotentiating, hypocholesterolaemic and hepatoprotective agents. This new class of compounds termed 'mushroom nutriceuticals' (Chang and Buswell 1996), are extractable from either the fungal mycelium or fruiting body and represent an important component of the expanding mushroom biotechnology industry.

Of the 14,000-15,000 species of so-called macrofungi in the world, around 400 have known medicinal properties. However, it has been estimated that there are about 1,800 species of macrofungi with potential medicinal properties. Macrofungi and their root-like structure (called mycelium) produce several medicinal (general immune enhancing) compounds, central of which are the polysaccharides (high molecular weight strings of sugars), triterpenes, and immunomodulatory proteins. Although virtually all macrofungi and many foods have polysaccharides in their cell walls, certain mushroom species have been found to contain polysaccharides which are particularly effective in retarding the progress of various cancers and other diseases, and in alleviating the side effects of chemotherapy and radiation treatment (through cell-level regenerative effects). There are

now many studies in Asia, particularly in China and Japan, documenting life span increases of cancer patients undergoing conventional cancer treatment plus mushroom extract consumption or injection (Mizuno *et. al.*, 1995; Liu 1999). At the same time, due to the enhancement of the immune systems, it can help people reduce the possibility of being infected by other diseases.

Between 80% - 85% of all medicinal mushroom products are derived from the fruiting bodies, which have been either commercially farmed or collected from the wild, e.g., Lentinan, a high molecular weight (1,3)-D-glucan, from *Lentinula edodes* and various products from *Ganoderma lucidum*. Only about 15% of all products are based on extracts from mycelia. The notable examples include PSK-trade name (Krestin) of a polysaccharide peptide, and PSP (polysaccharide-bound peptide) extracted from Coriolus versicolor. A smaller percent of mushroom products are obtained from culture filtrates, e.g., schizophllan, a high-molecular weight (1-3),(1-6)-D glucan, prepared from Schizophyllum commune Fr. and PSPC (a roteinbound polysaccharide complex) from Tricholoma lobayense Hein. However, due to increased quality control and year round production, mycelial products are the wave of the future (UN, 2011).

The market value of medicinal macrofungi and their derivative dietary supplements worldwide was about US$1.2 billion in 1991, and about US$3.6 billion in 1994 (Chang 1996). In 1999, it was estimated to be US$6.0 billion (Wasser *et. al.*, 2000). The market value of Ganoderma-based nutriceuticals alone in 1995 was estimated at US$1.6 billion (Chang and Buswell 1999). The corresponding monetary values were also generated by another famous mushroom, *Lentinula edodes*. Ninety nine percent of all sales of medicinal macrofungi and their derivatives occurred in Asia and Europe with less than 0.1 percent in

North America. The 1999 US market for dietary supplements based mainly on macrofungi was estimated to be US$35 million. However, in recent years, the North American demand is increasing between 20%-40% annually, depending upon species (Wasser *et. al.*, 2000).

### 2.2.8 Non Medicinal Uses of Macrofungi

Macrofungi can be used for dyeing wool and other natural fibers. The chromophores of mushroom dyes are organic compounds and produce strong and vivid colors, and all colors of the spectrum can be achieved with mushroom dyes. Before the invention of synthetic dyes macrofungi were the source of many textile dyes (Mussak and Bechtold, 2009). Some fungi, types of polypores loosely called macrofungi, have been used as fire starters (known as tinder fungi). Macrofungi are currently being employed by companies (e.g. Ecovative Design PLC) to make biodegradable packaging, a direc t replacement for the petroleum based styrofoam. Macrofungi and other fungi play a role in the development of new biological remediation techniques (e.g., using mycorrhizae to spur plant growth) and filtration technologies (e.g. using fungi to lower bacteria levels in contaminated water). The US Patent and Trademark Office can be searched for patents related to the latest developments in mycoremediation and mycofiltration.

## 2.3 Basidiomycota

Basidiomycota is one of two large phyla that, together with the Ascomycota, comprise the subkingdom Dikarya (often referred to as the "higher fungi") within the kingdom Fungi. The Basidiomycota contains about 30,000 described species, which is 37% of the described species of true Fungi (Kirk *et. al.* 2001). The most conspicuous and familiar Basidiomycota are those that produce mushrooms, which are sexual reproductive structures. The

Basidiomycota also includes yeasts (Fell *et. al.* 2001) and asexual species. More specifically the Basidiomycota include these groups: mushrooms, puffballs, stinkhorns, bracket fungi, other polypores, jelly fungi, boletes, chanterelles, earth stars, smuts, bunts, rusts, mirror yeasts, and the human pathogenic yeast Cryptococcus.

Basically, Basidiomycota are filamentous fungi composed of hyphae (except for yeasts), and reproducing sexually via the formation of specialized club-shaped end cells called basidia that normally bear external meiospores (usually four). These specialized spores are called basidiospores. However, some Basidiomycota reproduce asexually in addition or exclusively. Basidiomycota that reproduce asexually can be recognized as members of this phylum by gross similarity to others, by the formation of a distinctive anatomical feature, cell wall components, and definitively by phylogenetic molecular analysis of DNA sequence data(Moore, 1980) . Basidiomycota are found in virtually all terrestrial ecosystems, as well as freshwater and marine habitats (Kohlmeyer and Kohlmeyer, 1979; Hibbett and Thorn, 2001).

The most recent classification (Hibbett, 2007) adopted by a coalition of 67 mycologists recognizes three subphyla (Pucciniomycotina, Ustilaginomycotina, Agaricomycotina) and two other class level taxa (Wallemiomycetes, Entorrhizomycetes) outside of these, among the Basidiomycota. As now classified, the subphyla join and also cut across various obsolete taxonomic groups previously commonly used to describe Basidiomycota. According to a 2008 estimate, Basidiomycota comprise three subphyla (including six unassigned classes) 16 classes, 52 orders, 177 families, 1,589 genera, and 31,515 species (Kirk *et. al.*, 2008). The terms basidiomycetes is frequently used loosely to refer to Basidiomycota.

### 2.3.1 Characteristics of Basidiomycota

Basidiomycota are unicellular or multicellular, sexual or asexual, and terrestrial or aquatic. Indeed, Basidiomycota are so variable that it is impossible to identify any morphological characteristics that are both unique to the group and constant in the group. The most diagnostic feature is the production of basidia (sing. basidium), which are the cells on which sexual spores are produced, and from which the group takes its name. A long-lived dikaryon, in which each cell in the thallus contains two haploid nuclei resulting from a mating event, is another characteristic feature. Finally, clamp connections are a kind of hyphal outgrowth that is unique to Basidiomycota, although they are not present in all Basidiomycota. The following description of the characteristics of Basidiomycota traces the life cycle of a "typical" species, beginning at the site of meiosis.

One of the most fascinating characteristics of Basidiomycota is the production of forcibly discharged ballistospores which are propelled into the air from the sterigma. Ballistospores may be sexual or asexual, and may be produced by basidia, hyphae, yeast cells, or even other ballistospores. This type of spore discharge must have evolved very early in the evolutionary history of the Basidiomycota as it is found in members of the earliest diverging lineages within the group. Ballistospory is associated with forms that disperse their spores directly into the air. Most aquatic Basidiomycota and forms that produce spores inside the fruiting body, such as puffballs, have lost ballistospory.

Ballistospory is associated with the production of a liquid filled "hilar droplet" that forms at the base of the spore, just above its attachment to the sterigma. Resolving the mechanism of ballistospory has been a longstanding problem in mycology (McLaughlin *et. al.* 1985; Webster *et. al.* 1984a, b; Yoon and McLaughlin 1986). In a series of studies, reviewed by

Money (1998), it has been shown that spore discharge occurs when the hilar droplet fuses with a film of liquid on the surface of the spore. The rapid coalescence of the liquids causes a sudden shift in the center of mass of the spore and contributes to its release from the sterigma. This mechanism has been termed a "surface tension catapult" and it results in spores being discharged with a force of about 25,000 g (Money 1998, Pringle *et. al.* 2005).

### 2.3.2 Basidiomycota

Three major classes are strongly supported within the Basidiomycota: 1) Pucciniomycotina includes rusts (Pucciniales) and other taxa (Swann *et. al.* 2001, Aime *et. al.* 2006); 2) Ustilaginomycotina includes smuts (Ustilaginales) and others (Bauer *et. al.* 2001, Begerow *et. al.* 2006); and 3) Agaricomycotina includes mushrooms (Agaricomycetes), jelly fungi (Auriculariales, Dacrymycetales, Tremellales) and others (Hibbett and Thorn 2001, Swann and Taylor 1995, Wells and Bandoni 2001, Hibbett 2006). Monophyly of each of these groups has been supported in phylogenetic analyses of rRNA gene sequences and protein-coding genes (Hibbett *et. al.* 2007). Similarities in the ultrastructure of septal pores and spindle pole bodies (McLaughlin *et. al.* 1995) suggest that Ustilaginomycotina and Agaricomycotina could be sister groups, and some molecular phylogenies also support this topology. The placements of the Wallemiomycetes (a group of osmophilic molds) and Entorrhizomycetes (a group of root-inhabiting Fungi, previously classified in the Ustilaginomycotina [Bauer *et. al.* 2001]) are particularly problematical. At present, these are classified as "incertae sedis" within the Basidiomycota (Hibbett *et. al.* 2007), but with the application of genome-scale datasets their placements may be resolved.

*2.3.2.1 Pucciniomycotina*

The Pucciniomycotina includes the rust fungi, the insect parasitic/symbiotic genus Septobasidium, a former group of smut fungi (in the Microbotryomycetes, which includes mirror yeasts), and a mixture of odd, infrequently seen or seldom recognized fungi, often parasitic on plants. The eight classes in the Pucciniomycotina are Agaricostilbomycetes, Atractiellomycetes, Classiculomycetes, Cryptomycocolacomycetes, Cystobasidiomycetes, Microbotryomycetes, Mixiomycetes, and Pucciniomycetes (Kirk *et. al.*, 2008).

Members of the Pucciniomycotina are remarkably diverse, possessing a wide range of structural and ecological characters. One characteristic many of these fungi have in common, however, is their rather miniscule size, making them challenging to locate and study. The apparent lack of unifying structural features of many members of the Pucciniomycotina has presented a challenge to understanding their phylogenetic affiliations, and the relationships of many of these disparate taxa have been illuminated since 1995 through the acquisition of molecular sequence, biochemical and subcellular data.

The group contains approximately 7,400 species, distributed among approximately 215 genera, but will eventually be much larger as many cryptic species are recognized. More than 95% of the species and 75% of the genera in this group are placed in the Pucciniales (Pucciniomycetes), the plant parasitic rust fungi (Frieders *et. al.*, 2008). The next largest orders, Septobasidiales (Pucciniomycetes) and Microbotryales (Microbotryomycetes), collectively constitute approximately 5% of the species and 4% of the genera. Nearly 20% of the rust genera and 60% of the nonrust genera are monotypic (containing only one

species) (Swann *et. al.*, 2001; Fell *et. al.*, 2001). The rust fungi and several of the yeasts have been more extensively studied than have other taxa, and the surprisingly large percentage of monotypic genera may be artificially high due to the limited research on these often obscure fungi (Frieders *et. al.*, 2008).

The rusts are undeniably the most economically important fungi in this clade, as obligate parasites on a wide range of crop plants including cereal grains, legumes, and trees such as coffee, apple, and pine, where they can cause extensive reduction in yield and even host death (Swann *et. al.*, 2001; Fell *et. al.*, 2001). Other phytopathogenic Pucciniomycotina can be of economic importance, but on a much smaller scale than rust fungi. Species of Helicobasidium (Pucciniomycetes: Helicobasidiales) cause root rot diseases of many economically important plants such as asparagus, beet, mulberry, and pear. Microbotryum violaceum(Microbotryomycetes: Microbotryales) causes anther smut of plants in the Caryophyllaceae, while smut on buckwheat is caused by Sphacelotheca fagopyri (Microbotryomycetes: Microbotryales). These smuts are not closely related to the smuts in the Ustilaginomycotina with which they were confused until molecular sequence data became available (Frieders *et. al.*, 2008).

*2.3.2.2 Ustilaginomycotina*

The Ustilaginomycotina are most (but not all) of the former smut fungi and along with the Exobasidiales. The classes of the Ustilaginomycotina are the Exobasidiomycetes, the Entorrhizomycetes, and the Ustilaginomycetes (Kirk *et. al.*, 2008).

The class Ustilaginomycetes comprises more than 1400 species of basidiomycetous plant parasites, which are distributed in approximately 70 genera. They occur throughout the

world, although many species are restricted to tropical, temperate or arctic regions. Some species of Ustilago and Tilletia, e.g. the barley, wheat or maize smut fungi, are well known because they are of economic importance. For example, from 1983 to 1988 the barley smut fungi reduced annual yields by 0.7% to 1.6% in the prairie provinces in central Canada, causing average annual losses of about U.S. $8,000,000 (Thomas 1989). Tilletia contraversa is important in the international wheat trade (Trione 1982), and 2-5% of the plants in a corn field are generally infected by Ustilago maydis, while up to 80% of a field can be infected if conditions are good for the smut fungus. On the other hand, the galls of U. maydis are considered a delicacy in the Mesoamerican tradition. They are known in Mexico as "Huitlacoche" and in the U.S.A. as "maize mushroom", "Mexican truffles" or "caviar azteca" (Valverde *et. al.* 1995).

The smut fungi are traditionally divided into the phragmobasidiate Ustilaginaceae and the holobasidiate Tilletiaceae, which are sometimes treated as separate orders (Kreisel 1969, Oberwinkler 1987). Difficulties in their classification have been discussed, e.g. by Durán (1973) and Vánky (1987), neither of whom listed higher taxa in this group. Based predominantly on host-parasite interactions and septal pore apparatus, a radical change in the systematics of smut fungi has been proposed by Bauer *et. al.* (1997). As a result, on the one hand the Microbotryales were excluded from the Ustilaginomycetes and on the other the Exobasidiales s. l., Graphiolales and Microstromatales were included in this group. Cellular interaction (Bauer *et. al.* 1997) and cell wall carbohydrate composition (Prillinger *et. al.* 1993) indicate that the class Ustilaginomycetes in this composition is monophyletic, and phylogenetic analyses of rDNA sequences (Berres *et. al.* 1995, Begerow *et. al.* 1997) are consistent with this hypothesis.

*2.3.2.3 Agaricomycotina*

The Agaricomycotina include what had previously been called the Hymenomycetes , the Gasteromycetes as well as most of the jelly fungi. The three classes in the Agaricomycotina are the Agaricomycetes, the Dacrymycetes, and the Tremellomycetes (Kirk *et. al.*, 2008).

The Agaricomycotina is one of three major clades of Basidiomycota (the others are the Pucciniomycotina and Ustilaginomycotina). The Agaricomycotina contains roughly 20,000 described species, which is almost 70% of the (known) Basidiomycota. About 98% of the species of the Agaricomycotina are in a clade called the Agaricomycetes, which includes mushrooms, bracket fungi, puffballs, and others. The other major groups are the Tremellomycetes and Dacrymycetes. These latter groups include "jelly fungi" which have gelatinous, often translucent fruiting bodies as well as many yeast-forming species (Hibbett 2007).

Members of the Agaricomycotina display the full range of ecological strategies that characterizes the Basidiomycota as a whole. To obtain carbon nutrition, Agaricomycotina decompose dead organic matter or enter into diverse associations (both antagonistic and benign) with plants, animals, and other fungi. Mycorrhizal associations with plants are present in many lineages of Agaricomycetes, including certain jelly fungi (Selosse *et. al.* 2002). Mycoparasitism (parasitism of a fungus by a fungus) is especially widespread in the Tremellomycetes (Bandoni 1984). Few Agaricomycotina are medically important. An exception is Filobasidiella neoformans (also called Cryptococcus neoformans), which is a serious pathogen of immunocompromised individuals (Mitchell and Perfect 1995).

The phylogenetic hypothesis and classification adopted here are based on multiple independent analyses, summarized by Hibbett *et. al.* (2007). Three major clades are formally named: Tremellomycetes, Dacrymycetes, and Agaricomycetes (which is here taken to include the heterobasidiomycetous Auriculariales and Sebacinales). Several analyses, including a multi-locus study by Matheny *et. al.* (2007), suggest that the Dacrymycetes and Agaricomycetes form a monophyletic group, which Swann and Taylor (1995) called the Hymenomycetidae. Earlier studies using rRNA genes did not support that grouping, however (Begerow *et. al.* 1997; Weiß and Oberwinkler 2001).

#### *2.3.2.4 Chlorophyllum*

Chlorophyllum is a genus of large agarics similar in appearance to the true parasol mushroom. Chlorophyllum was originally coined in 1898, a time when spore color was the deciding factor for differentiating genera. It was termed in order to describe the poisonous green-spored *C. molybdites* which shared many characteristics of the mushrooms within the genus Lepiota but lacked the all important white spores. The name comes from Greek Chloro meaning green and phyll meaning leaves or gills. It remained as a genus of one lonely member until recently when modern DNA analyses concluded that many of the mushrooms contained in the genus Macrolepiota actually had more in common genetically with the *Chlorophyllum molybdites* than with the other members of the Macrolepiota. The genus has a widespread distribution, especially in tropical regions, and contains 16 species which include; *Chlorophyllum abruptibulbum, C. agaricoides, C. alborubescens, C. brunneum, C. molybdites, C. globosum, C. hortense, C. humei, C. mammillatum, C. neomastoideum, C. nothorachodes, C. olivieri, C. rhacodes, C. sphaerosporum, C. subfulvidiscum, C. subrhacodes* (Kirk *et. al.*, 2008). The best known members are the

edible shaggy parasol, a name applied to three very similar species *Chlorophyllum rhacodes, C. olivieri and C. brunneum, and the poisonous C. molybdites,* which is widespread in subtropical regions around the world (Kirk *et. al.*, 2008).

The fruit bodies of this genus are frequently encountered growing on rich soil in most tropical countries. The epicutis consist of a palisade of erect hyphae on the disc; gills usually becoming green, quite free and remote from the stipe; spore—print of various shades of green, or more rarely colonial buff; spores smooth with thick, complex wall which stains with cresyl blue, with a broad germ pore, large; cystidia absent; cheilocystidia present; hyinenophoral trama almost regular; stipe long, bulbous at the base, but without a volva, the upper part of the stipe has a movable annulus which is tightly attached to the stem in young and fresh specimens but becomes free on drying; context becomes reddish on bruising. containing a poison; hyphae non.-amyloid, without clamp connections (Sundburg, 1971).

Distribution: mostly tropics. Common in tropical America, Oceania, Asia, North America, South America and Africa.

Practical importance: most of the species are poisonous. Some are reported to be edible, but this indication must he regarded with caution.

*2.3.2.5 Chlorophyllum molybdites*

**Scientific classification**

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Subclass: Homobasidiomycetidae

Order: Agaricales

Family: Agaricaceae

Genus: *Chlorophyllum*

Species: *molybdites*

*Chlorophyllum molybdites* is a fairly common and a very large mushroom with a cap that can measure from 20 to 25 cm in diameter; convex to conical when young, becoming convex to broadly convex or nearly flat in age; dry; smooth at first, but soon becoming scaly with brown to pinkish brown scales that are uplifted or flat, and concentrated near the center in age; whitish to tan or yellowish white. Though it may grow singly or in a small group, commonly it forms fairy rings on lawns or in fields in late summer and early autumn. The stem is 5-25 cm long; 1.5-2.5 cm. thick; more or less equal, sometimes slightly enlarged toward base; smooth; firm; white, sometimes discoloring slightly brownish; with a persisting, double-edged ring. The stem has a characteristically smooth surface, elongates early during the formation of the mushroom. The cap, which often retains fragments of its original covering, expands, opens and breaks away from a fibrous ring that remains on the stem. Only when the cap is fully open do the gills take on a greenish tinge as the spores mature. At earlier stages the gills remain white making it

difficult to distinguish the mushroom from edible look alikes (plate 2.9). An extensive, technical description of the fungus is available (Sundberg, 1971).

The geographic distribution of the fungus shows that it is absent from the most northerly parts of the USA, it is distributed widely in North America, Central America and the Caribbean, South America, Central Africa, Oceania, Australia, tropical and subtropical Asia (Pegler, 1983; Zoberi, 1972).



Plate 2.9: *Chlorophyllum molybdites*

There are many reports of *C. molybdites* has been reported to contain protein and essential amino acids (Goyal, 1987).

## 2.4 Microscopy of Macrofungi

Microscopy is an important tool for the identification and taxonomy of macrofungi species. Each macrofungi species has unique microscopic characteristics that distinguish it from other species and genera. To observe these characteristics and classify them accordingly,

one will need to be familiar with using a microscope and preparing microscopic slides. Most commonly, macrofungi spores are use for microscopy. But the tissue culture, mycelium or raw macrofungi can also be useful (Kuo, 2006).

A spore to students of archegoniate plant (Bryophyta and Pteridophyta) is definable as a unicellular asexual reproductive body formed within a sporangium following meiosis .However, the mycologists uses the term in a much wider sense. To him, a spore is any simple reproductive unit often unicellular but frequently multicellular, and usually with some food reserves. He recognizes many different kind of spore: zoospores, sporangiospores, chlmydospores, zygospores, oospores, ascospores, uredospores, basidiospores, teleutespores, brand spores and various types of conidia such as pycnidispores, aleuriospores, phialospores, arthrospores and blastospores. With the possible exception of ascospores and basidiospores none of these can be considered as homologous with the spores of archegoniate plants. However, all these kind of spores agree in being microscopic, normally devoid of vacuoles and, more often than not, unicellular (Ingold, 1953)

Spore shape generally fit into one of the following five categories;

Round or nearly round (globose or sub-globose)

Tear shaped; (lacrimoid)

Elliptical or nearly so, (elliptical, ellipsoid etc)

Football shaped; (fusiform)

Funky; (spores are incredibly diversed, all kind of funky shaped can be found and the range is too broad to cover here) (Largent *et. al.*, 1973; Kuo, 2006).

Observe spore surface characteristics to view spore surface characteristic generally fall under one of the following eight categories:

- Pitted spores
- Dextrinoid spores
- Reticulate spores
- Smooth, inamyloid spores
- Warty spores
- Partially reticulate spores
- Amyloid spiny spores
- Spiny spores (spinose, echinate, echinulate) (Kuo, 2006)

### 2.4.1 Organoleptic Properties of Macrofungi

To study various organs of the macromycetes under studies for the following features: gross anatomy of each plant, shape, size, colour, odour and taste. Some of the organ common to macromycetes may include, Pileus (cap or fruit), Stipe (stem or stalk), annulus (ring around the stipe), veil (whether partial or universal), volva (cup-like structure common to Amanitaceae and important key to their identification), hymenia (spore making reproductive surfaces specific to basidiomycetes), Lamellae (gills; its type and attachment to the pileus or stalk that free or not free gills), Pellis (cortex or cuticle on the surface of the pileus hence names pileipellis or stipitipellis specific to basidiomycetes on the pileus and stipe),

## 2.5 Macroscopy of Macrofungi

Many macrofungi have short gills touching the pileus but not reaching the full length of other gills and not touching the stipe. This is not to be confused with free gills where none of the gills touch the stipe (Spoerke and Rumack, 1994). Looking at one lamella as if it were a knife blade, the download sharp edge will have varying features, depending on the species.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Materials

Materials used were obtained from the department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. Some include; Pocket knife, basket, brush, rackets, stick, hand lens, Digital camera, glass wares, retort stand, sharp razor blades.

### 3.1.1 Collection of mushroom

*Chlorophyllum molybdites* was collected around Demonstration Secondary School, Ahmadu Bello University Zaria, Nigeria in April, 2009. The cap or fruit of the fungus was handled carefully since the cap can be used to differentiate between species especially its shape, size, colour, spots and type of spores observed below it. It was identified by a taxonomist Mal. Umar Galla of the herbarium, Department of Biological Sciences, Ahmadu Bello University Zaria, Nigeria, where a voucher specimen 900167 has been deposited. The Mushroom was dried under shade and its size reduced using mortar and pestle. It was then stored in a tight container until required for further use (Kuo, 2006).

## 3.2 Methods

### 3.2.1 Macroscopy of *Chlorophyllum molybdites*

#### 3.2.1.1 Organoleptic Characters of *C. molybdites*

The features of the various organs of the macromycetes under study were observed for: gross anatomy, shape, size, colour, odour and taste.

The colour and odour of the pileus surfaces including lamellae or pores were observed and recorded. Colorations of the matured spores were observed directly from the spore prints of the species. The procedure followed was as described by Michael (2006).

### 3.2.2 Microscopy of *C. molybdites*

#### 3.2.2.1 Spore print

The cap or pileus of *C. molybdites* was carefully cut from the point of attachment to the stipe and placed on top of a white piece of paper that is previously placed on the table. Cap was allowed on the paper facing down for a day and then carefully removed to view the shape and arrangement of spores deposited on the white sheet of paper. This was photographed with a digital camera and a small portion of the spores was scraped and placed on a glass slide for microscopic analysis (Largent *et. al.*, 1973; Kuo, 2006).

#### 3.2.2.2 Chemo microscopy of *C. molybdites*

*Meltzer's reagent (modified)*

100g of Chloral hydrate, 5g of Potassium iodide and 1.5g of Iodine were weighed. They were then transferred into 100 $cm^3$ of distilled water. The mixture was thoroughly stirred until dissolved before the remaining water and chloral hydrate were added.

The specimen was first wetted in ammonia for a few seconds on a glass slide, after which the ammonia was completely removed using a filter paper and a large excess of the reagent (Meltzer's reagent) added.

Determination of whether spores were amyloid, dextrinoid or inamyloid was done by adding Meltzer's reagent to the sample slide. The iodine contained in Meltzers reagent causes one of the following three color reactions;

Bluish black = amyloid

Reddish brown = dextrinoid

No noticeable change in color = inamyloid

### 3.2.3 Physicochemical Constants of *C. molybdites*

#### 3.2.3.1 Determination of total ash

The dried powdered mushroom (2.0g) was weighed and transferred to a clean and dry metal crucible,it was then heated in a furnace at 450 $^{o}$C until it charred on a Bunsen burner. Heating continued until all organic matter was burnt. It was allowed to cool in a desicator and weighed. The experiment was carried out in triplicate. The total ash value was calculated with reference to the air dried material as follows:-

$$\text{Total ash value} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

#### 3.2.3.2 Moisture content determination of *C. molybdites*

Exactly 2.0g of the mushroom was weighed into a clean, dry and previously weighed metal crucible. The crucible and its content were heated in an oven at 105$^{o}$C for 1 hour. It was removed from the oven, cooled in a desiccator, and weighed. Heating, cooling and weighing were repeated until a constant weight was obtained. Two more samples were treated similar as above. The percentage moisture content was calculated with reference to the original sample from the following formula;

$$\text{Moisture content} = \frac{\text{weight of water loss}}{\text{weight of sample}} \times 100$$

### 3.2.3.3 Alcohol soluble extractive value

From the dried mushroom powder, 10g was weighed and transferred into a conical flask. 100ml of alcohol was measured, added to the flask and stoppered. The flask and its content were placed on a mechanical shaker for six hours and allowed to stand for another eighteen hours. The content was filtered. 20ml of the filtrate was taken into a clean, dried and previously weighed evaporating dish. The filtrate was evaporated to dryness on a water bath, allowed to cool in a dessicator and weighed.This was carried out in triplicate and the percentage of the alcohol soluble extractive value with reference to the air dried material was examined from the formula:-

$$\text{Alcohol soluble extractive value} = \frac{\text{weight of residue}}{\text{weight of sample}} \times 100$$

### 3.2.3.4 Water soluble extractive value

From the dried mushroom powder, 10g was weighed and transferred into a conical flask. 100ml of water was measured, added to the flask and stoppered. The flask and its content were placed on a mechanical shaker for six hours and allowed to stand for another eighteen hours. The content was filtered. 20ml of the filtrate was taken into a clean, dried and previously weighed evaporating dish. The filtrate was evaporated to dryness on a water bath, allowed to cool in a dessicator and weighed. This was carried out in triplicate and the percentage of the alcohol soluble extractive value with reference to the air dried material was examined from the formula (British Pharmacopoeia, 1985):-

$$\text{Alcohol soluble extractive value} = \frac{\text{weight of residue}}{\text{weight of sample}} \times 100$$

### 3.2.3.5 Determination of acid-insoluble ash

To the crucible containing the residues obtained from the determination of ash, 25mls of hydrochloric acid (70g/l) TS, was added and covered with a watch glass and was gently boiled for five minutes. The watch glass was rinsed with 5mls of hot water and the rinsed was added to the crucible. The insoluble matter was collected on previously weighed ashless filter paper and was washed with hot water until the filtrate was neutral to litmus. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant weight (British Pharmacopoeia, 1985).

### Test for Alkaloids

The powdered drug (10g) was weighed in to a beaker. It was moistened with concentrated ammonia solution and allowed to stand for 20mins. 50ml of chloroform was added; the mixture was stirred for 10mins and filtered through a plug of cotton wool. The marc was washed twice with 5ml chloroform and the washings were added to the filtrate. The combined filtrate was evaporated to dryness on a water bath. The residue was cooled and then dissolved in 20ml of chloroform. The chloroform extract was transferred to a separating funnel and shaken with 25ml of 5% sulphuric acid. The two layers were allowed to separate. The chloroform (lower) layer was drained off and discarded. 10ml of chloroform was further added, shaken, drained off and discarded until the acid layer was colourless.

The acid layer was made alkaline with concentrated ammonia solution and extracted with 20ml of chloroform. The extract was retained and evaporated to dryness. The residue was dissolved in 10ml of methanol and the following tests were carried out:-

**General test**: To 1rnl of the methanol extract in separate test tubes, 3 drops of dragendorff"s reagent were added.

### 3.2.5 Thin Layer Chromatography (TLC) of Methanolic Extract of *Chlorophyllum molybdites*

Thin Layer Chromatography (TLC): stationary phase: Silica gel (60 $F_{254}$, Pre-coated plates manufactured by Merck) of 0.25 mm thickness were used. Spotting was done manually using glass capillary tubes. The plates were developed at room temperature using the following solvent systems in a chromatographic tank: n-butanol: acetic acid: water(4:1:5), Hexane : ethylacetate (9:1) and chloroform : ethylacetate (2:3). The chromatograms were then allowed to dry and sprayed with different spraying reagents.

### 3.2.6 Spraying Reagents

Preparation of Coomassie brilliant blue G-250 reagent was improved to fit TLC cyclotides detection sensitively, which are smaller than proteins. The preparation was as follows: 100 mg G-250 was dissolved in 20 mL ethanol, 20 mL phosphoric acid and 160 mL 50% ethanol was added.

A Coomassie G-250 dye is a disulfonated triphenylmethane compound that is commonly used as the basis of stains for detection of proteins in gel electrophoresis and Bradford-type assay reagents for protein quantitation. The G-250 (green-tinted) contains two methyl groups. Typically, in acidic conditions, the dye binds to proteins primarily through basic amino acids (primarily arginine, lysine and histidine), and the number of coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. Protein-binding causes the dye to change from

reddish-brown to bright blue (absorption maximum equals 595nm). The ninhydrin reagent was 0.2% ethanol solution (Xu *et. al.*, 2008).

Dragendorff's reagent was prepared as follows: 0.85 g Bismuth subnitrate dissolved in 10 ml glacial acetic acid and to which 40 ml $H_2O$ was added was taken as solution A; 8 g potassium iodide dissolved in 20 ml $H_2O$ was taken as solution B; 1 ml solution A was mixed with 1 ml solution B, to which was added 2 ml acetic acid and 10 ml $H_2O$ (Xu *et. al.*, 2008).

### 3.2.7 Acute Toxicity Studies ($LD_{50}$) on Methanolic Extract of *Chlorophyllum molybdites* in mice

Oral $LD_{50}$ determination was conducted on the methanolic crude extract of *Chlorophyllum molybdites* using the method of Lorke (1983) in mice. Briefly; the method was divided into two phases. In the initial phase, 3 groups each containing three mice were treated with the methanolic crude drug extract of the macrofungi at doses of 10,100 and 1000 mg/kg body weight orally, and observed for signs of toxicity and death for 24 hours. In the second phase, 4 groups each containing one mouse was administered with four more specific doses (140 mg/kg, 225 mg/kg, 370 mg/kg and 600 mg/kg) of the extract based on the result of the initial phase (Phase I). The LD50 value was determined by calculating the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived (0/1and 1/1), respectively. The same procedure was repeated in rats.

### 3.2.8 Effects of methanolic extract of *Cholorophyllum molybdites* on isolated rabbit jejunum

The rabbit was sacrificed by a blow on the head. Segments of the jejunums, about 3.0 cm long were removed and dissected free of adhering mesentery. The intestinal contents were removed by flushing with Tyrode solution of the following compositions in millimoles (mM): NaCl, 136.8; KCl, 2.7; CaCl, 1.3; NaHCO3, 12.0; MgCl, 0.5; NaPO4, 0.14; glucose, 5.5. The tissue was mounted in a 25 ml organ bath containing Tyrode solution maintained at 35Oc and aerated with air. An initial tension of 0.5 g was applied to the segments and 60 min equilibration period was allowed while the physiological solution was changed every 15 min. At the end of the equilibration period, the effect of acetylcholine ($1.4 \times 10^{-7} – 1.1 \times 10^{-6}$M) and the methanolic extract of *Chlorophyllum molybdites* (0.08-1.6 mg/ml) were investigated non-cumulatively. The contact time for each concentration was 1 min which was followed by washing three times. The tissue was allowed a resting period of 15 min before the next addition. The effect of atropine ($5 \times 10^{-7}$ M) on effect of both acetylcholine and the extract were also evaluated.

### 3.2.9 Effects methanolic extract of *Cholorophyllum molybdites* on isolated guinea pig ileum

The guinea pig was sacrificed by a blow on the head. Segment of the ileum was removed The intestinal contents were removed by flushing with Tyrode solution of the following compositions in millimoles (mM): NaCl, 136.8; KCl, 2.7; CaCl, 1.3; $NaHCO_3$, 12.0; MgCl, 0.5; $NaPO_4$, 0.14; glucose, 5.5. The tissue was mounted in a 25 ml organ bath containing Tyrode solution maintained at 35$^{o}$C and aerated with air. An initial tension of 0.5 g was applied to the segments and 60 min equilibration period was allowed while the physiological solution was changed every 15 min. At the end of the equilibration period,

the effect of hsitamine ($11 \times 10^{-7} - 8.6 \times 10^{-6}$M) and the methanolic extract of *Chlorophyllum molybdites* (0.08-1.6 mg/ml) were investigated non-cumulatively. The contact time for each concentration was 1 min which was followed by washing three times. The tissue was allowed a resting period of 15 min before the next addition. The effect of mepyramine ($5 \times 10^{-7}$ M) on effect of both histamine ($1.4 \times 10^{-7}$ M) and the extract were also evaluated.

# CHAPTER FOUR

# RESULTS

## 4.1 Macroscopy

### 4.1.1 Organoleptic Characters

The organs common to macromycetes include, Pileus (cap or fruit), Stipe (stem or stalk), annulus (ring around the stipe), veil (whether partial or universal), volva (cup-like structure, important key to their identification) and hymenia.

The external morphology of the pileus is as follows;

A whitish domed shaped pileus (appendix A) with diameter of 5 – 12 cm and light brownish patches. It has scales with the edges having radiating lines that follow the lines of the gills. The surface is bumpy and the flesh approximately 1.3 cm thick with a brittle texture. The summary of the physical assessments of the fungus is presented in Table 4.1

Table 4.1: Pileus external morphology

| Parameter | Description |
|---|---|
| Diameter | 5-12cm |
| Colour | Whitish |
| Patches | Light brownish patches |
| Scales | Scaly |
| Edges | Radiating lines that follow the lines of the gills |
| Shape | Dome in shape |
| Texture | Brittle |
| Surface | Bumpy at the centre |
| Flesh | Approximately 1.3cm thick |

#### 4.1.2.1 External Morphology Underside the Pileus

Underside the pileus, the lamellae (appendix A) which was greenish in colour was at the underside of the cap like pages in a book. The greenish colour was uniform on individual lamellae. The lamellae were closely packed on the inside of the cap and had thickness of approximately 1cm from the pileus to the edge the lamellae.

#### 4.1.2.3 External Morphology of the Stipe

The stipe is whitish greyish brown in colour and slightly carved at the base, was found to be 1 – 2 cm thick. It has a brittle texture and a smooth surface (appendix A). The flesh when young is light brown and is fleshy and pith filled when young (Table 4.2).

Table 4.2: External morphology of stipe

| Parameter | Description |
|---|---|
| Thickness | 1-2cm thick |
| Shape | May be slightly curved at base |
| Colour | Whitish greyish to brownish |
| surface | Smooth |
| Texture | Brittle |
| Flesh | Fleshy and pith filled when young |
| Flesh colour when fresh | Light brownish |

#### 4.1.2.4 The Spore Print

The spores on the lamellae (appendix D) were fine greenish powder which changes to brown on storage. The spore print had a diameter of 6.8cm – 12.3 cm (table 4.5).

### 4.1.3 Chemo-microscopy

The Meltzer's reagent when added to the specimen on the glass slide gave a bluish-black colour indicating that the spores of *C. molybdites* are amyloid.

## 4.2 Physical constants

The physical constants were found as follows; percentage moisture content was found to be 16.27 ± 0.01%, the percentage ash value was found to be 4.0 ± 0.12%, the acid insoluble ash value was found to be 0.43 ± 0.01%, the alcohol soluble extractive value was found to be 10.34 ± 0.01% and the water soluble extractive value was found to be 7.15 ± 0.02% as shown in Table 4.3.

Table 4.3: Physicochemical constants

| Parameter | % Physical constants ± SEM |
|---|---|
| Moisture content | 16.27 ± 0.01 |
| Ash value | 4.00 ± 0.12 |
| Acid insoluble ash value | 0.43 ± 0.01 |
| Alcohol soluble extractive value | 10.34 ± 0.01 |
| Water soluble extractive value | 7.15 ± 0.02 |

Where SEM = Standard Error of Mean

### 4.3 Phytochemical Screening of *C. molybdites*

#### 4.3.1 Test for Alkaloids

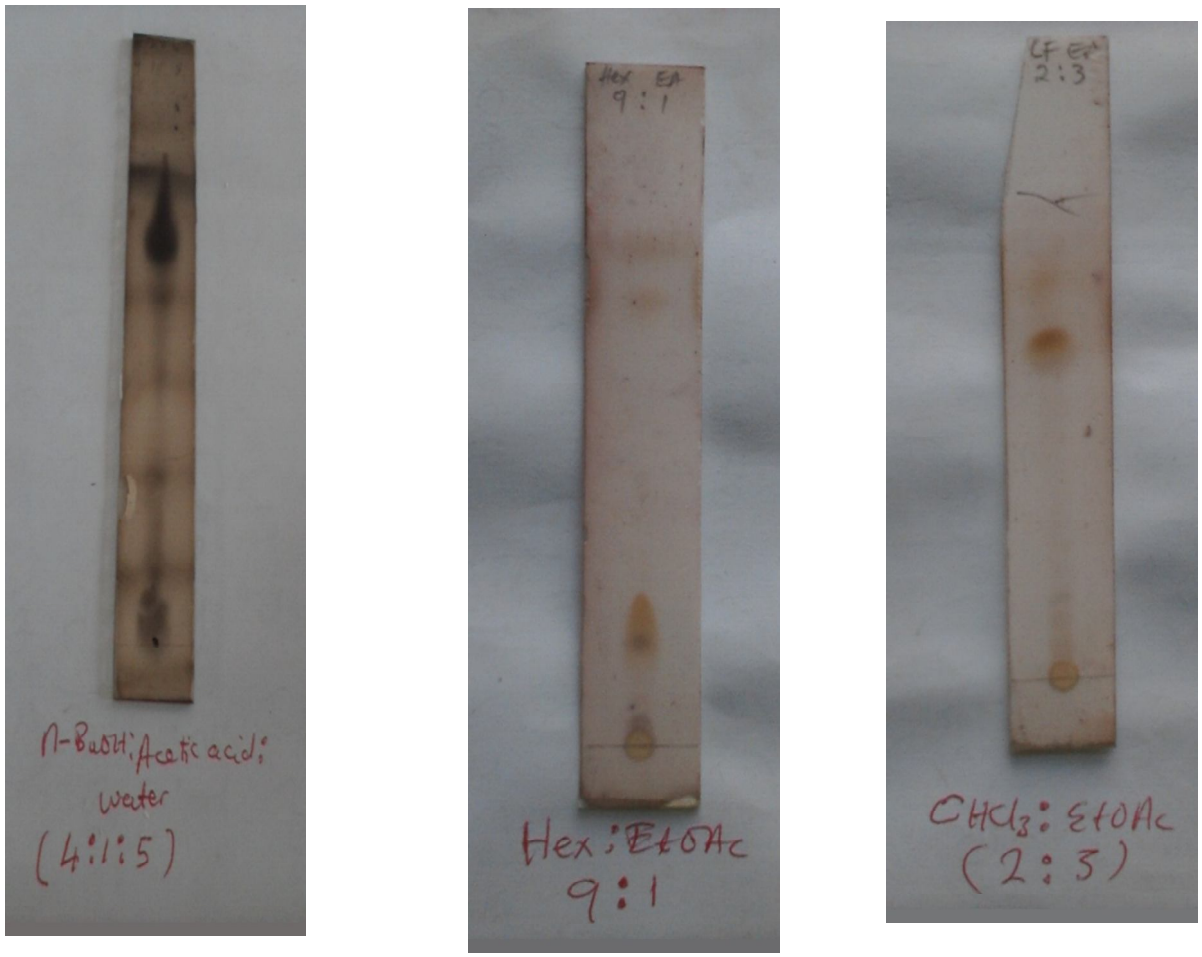
a. **Drangendorff's reagent:** No orange-yellow precipitate was observed when the alkaloidal extract was treated with Drangendorff's reagent, confirming the absence of alkaloids.

### 4.4 Thin Layer Chromatography (TLC)

The TLC of the methanolic extract of *Chlorophyllum molybdites* was carried out using the following solvents systems n-butanol : ethylacetate : acetic acid : water (4:1:5); Hexane : ethylacetate (9:1) and chloroform : ethylacetate (2:3) as shown in table 4.8.

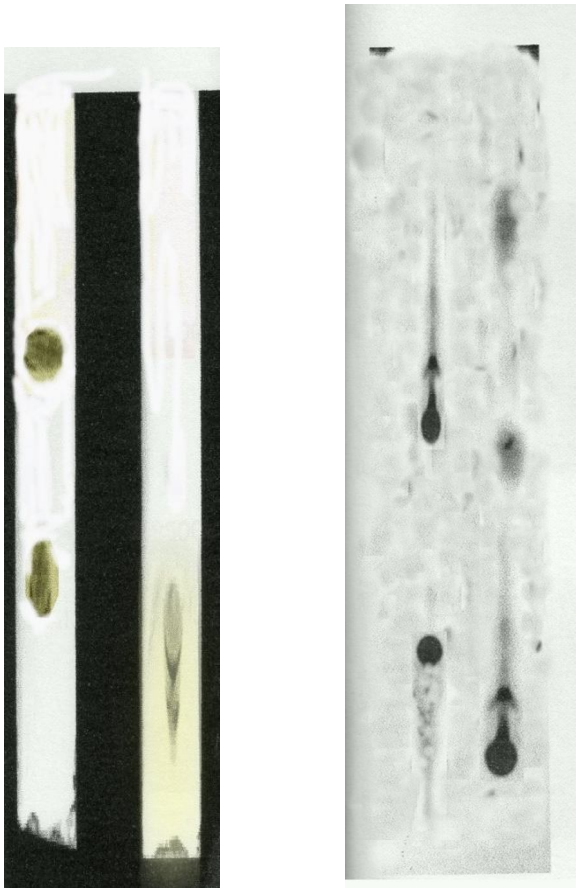
Table 4.4. TLC of the methanolic extract of *Chlorophyllum molybdites* using different solvent systems

| Solvents system | Ratio | Number of spots | Rf value |
|---|---|---|---|
| n-butanol: acetic acid: water | 4:1:5 | 5 | 0.1, 0.3, 0.5, 0.7, 0.8 |
| Hexane : Ethylacetate | 9:1 | 2 | 0.2, 0.9 |
| Chloroform : Ethylacetate | 2:3 | 2 | 0.7, 0.9 |




**a** **b** **c**

**Plate 4.1. TLC of methanolic extract of *Chlorophyllum molybdites* sprayed with 10%$H_2SO_4$ (a = n-butanol: acetic acid: water in ratio, 4:1:5; b = Hexane : Ethylacetate in the ratio 9:1; c = Chloroform : Ethylacetate in the ratio 2:3)**



a b

**Plate 4.2. TLC of methanolic extract of *Chlorophyllum molybdites*.**

a = ( n-butanol: acetic acid: water in ratio, 4:1:5) sprayed with Coomasie brilliant blue.

d = (Chloroform : Ethylacetate : methanol : water 8:15:4:1) with Dragendorf reagent.

### 4.5 Acute Toxicity Studies in Mice and Rats

The Acute Toxicity (**$LD_{50}$**) was determined to be 471mg/kg in mice and 288.53mg/kg in rats respectively.

### 4.6 Effects of methanolic extract of *Cholorophyllum molybdites* on isolated rabbit jejunum

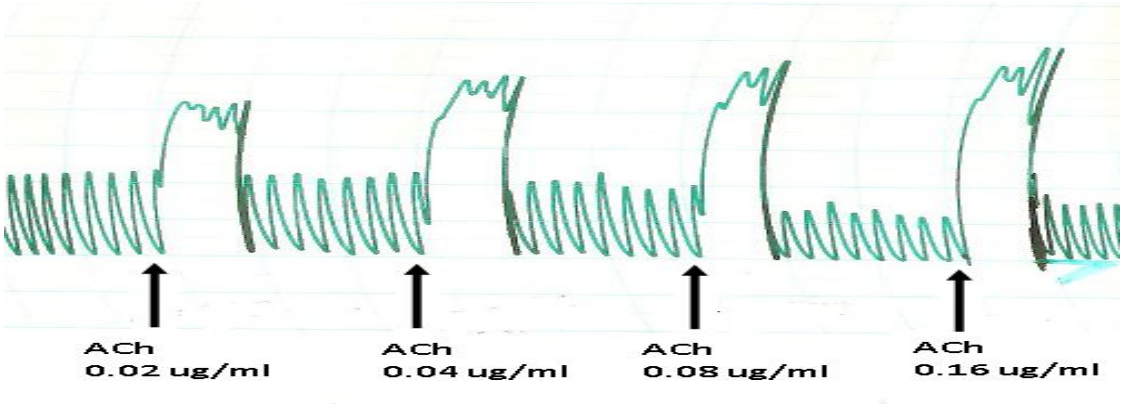



Figure 4.1 Effect of Acetylcholine on spontaneous contraction of isolated rabbit jejunum

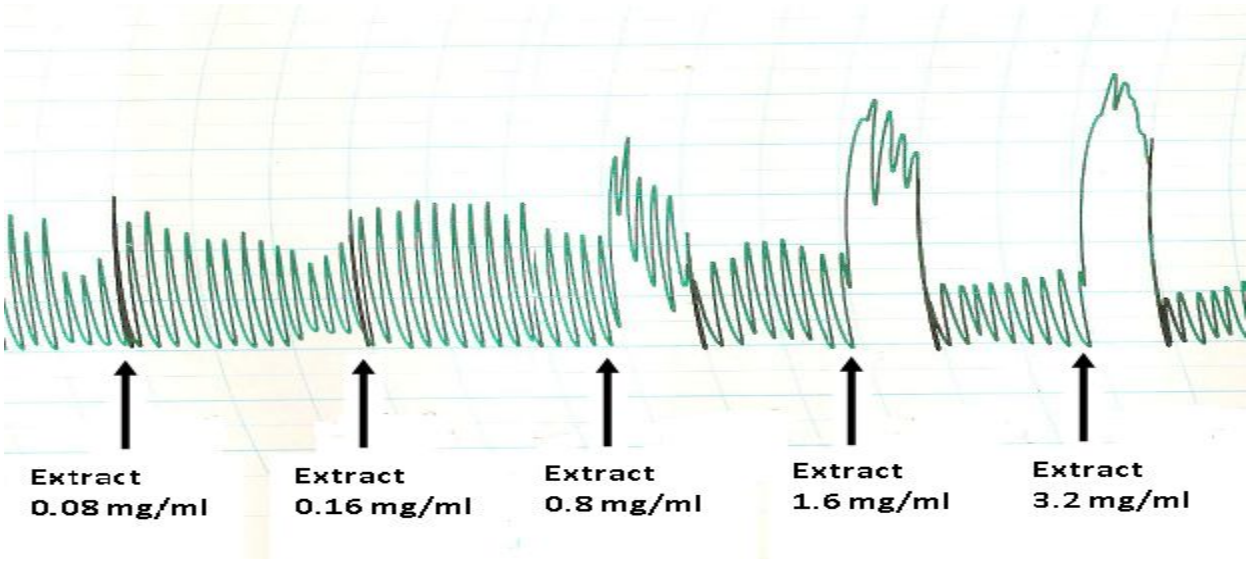



Figure 4.2 Effect of methanolic extract of *Chlorophyllum molybdite* on spontaneous contraction of isolated rabbit jejunum

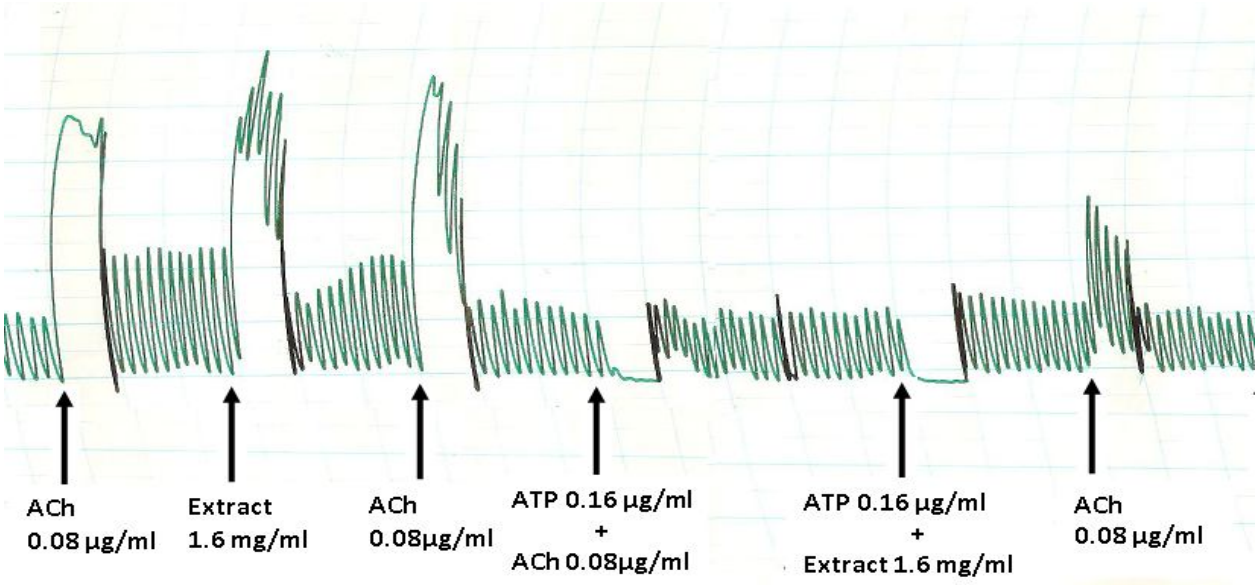



Figure 4.3 Effect of Atropine on smooth muscle contractile effect of Acetylcholine and methanolic extract *Chlorophyllum molybdites* using isolated rabbit jejunum

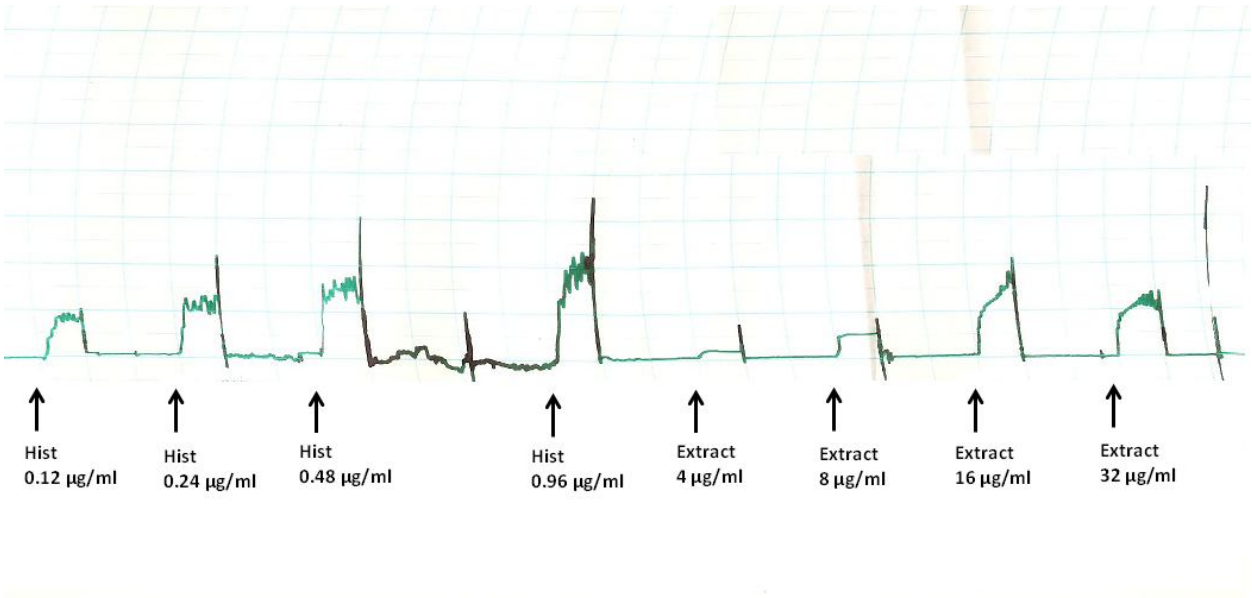



Figure 4.4 Effect of Histamine and methanolic extract of *Chlorophyllum molybdite* on spontaneous contraction of guinea pig ileum

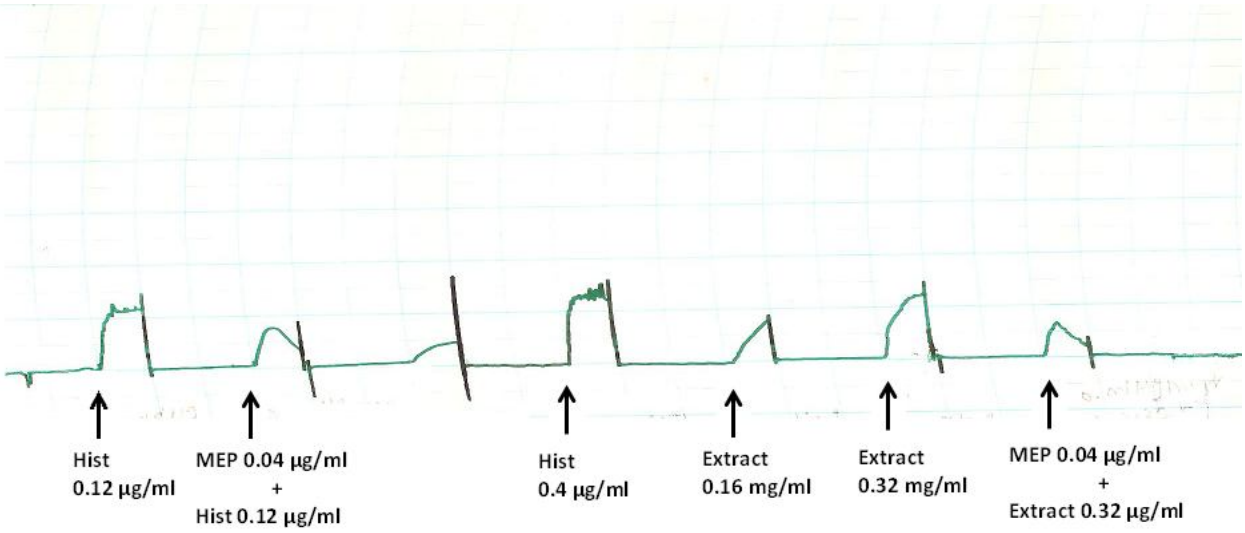



Figure 4.5 Effect of mepyramine on smooth muscle contractile effects of Histamine and methanolic extract of *Chlorophyllum molybdite* using guinea pig ileum

# CHAPTER FIVE

# DISCUSSION

Macrofungi have been known to possess medicinal properties for thousands of years. Higher basidiomycete macrofungi have been used in clinical nutrition because they exhibit anti-tumour, immune modulating, cardiovascular and anti-microbial activities (Wasser and Weis, 1999). The benefits of mushroom nutrition on these clinical conditions has attracted great interest in the scientific community in order to understand the molecular mechanism responsible for their action (Hobbs, 1995). Mushroom biomass contains many complex substances of therapeutic interest such as secondary metabolites (i.e terpenes, alkaloids, tannins, lactones etc) (Ossowski *et. al.*, 1996; Gubareva, 1998).

The morphological features revealed that the pileus with a dome shape had a diameter of 5 – 12 cm and the flesh 1 – 3 cm thick was whitish in colour. Light brownish patches were observed on the scaly pileus with the edges showing radiating lines that follow the lines of the gills. It had a brittle texture and the surface bumpy at the centre.

The stipe, whitish greyish brown in colour and slightly carved at the base, was found to be 1 – 2 cm thick. It has a brittle texture and a smooth surface. The flesh when young is light brown and is fleshy and pith filled when young.

The spores on the lamellae were fine greenish powder with greenish colour which changes to brown on storage. The spore print had a diameter of 6.8 cm – 12.3 cm.

The Meltzer's reagent when added to the specimen on the glass slide gave a bluish-black colour indicating that the spores of *C. molybdites* are amyloid.

The acid insoluble ash of 0.43% is an indication that the crude drug sample contains some small amount of dirt. The water soluble and alcohol soluble extractive values were 10.34% and 7.15% respectively. This result showed that more constituents are soluble in water than alcohol. The alcohol and water soluble extractive values are meant to detect exhausted and already utilized drug which could be fraudulently used as substitutes and adulterants (Elujoba, 1999).

Direct contamination such as by sand or earth is immediately detected by the ash value. The total ash of any material, normally consist of inorganic mixture of metallic salt and silica (Danmalam, 2000). Total ash value is particularly significant in the evaluation of the purity of the drug and depends largely on the absence of foreign organic matter. Total ash usually contains carbonates, phosphates, silicates and silica (Evans, 2001; Wallis, 2002).Thus the total ash value of 4% is relatively high showing that there is presence of foreign organic matter in the mushroom.

The moisture content obtained of 16.27% is high, this indicates higher possibility of the crude drug extract being exposed to microbial attack and hence degradation of the secondary metabolites contained. To circumnavigate this problem, storage of the crude drug has to be done in dry condition or if possible a dessicator may be employed.

The TLC chromatograph reveals 5 spots with the solvent system n-butanol: acetic acid: water (4:1:5). It also showed 5 spots with solvent system Chloroform : Ethylacetate : methanol : water (8:15:4:1). Thus Chloroform : Ethylacetate : methanol : water is the best solvent system for *C. molybdites.* This shows that the macrofungi contains highly polar constituents. Using n-hexane : ethylacetate (9:1) reveals the presence of 2 spots with $R_f$ of

0.2 and 0.9 likewise using chloroform : ethylacetate (2:3) also showed two spots when sprayed with 5% sulphuric acid. Spraying the TLC chromatograph with Coomasie brilliant blue, the presence of brilliant blue colour of the spots as shown in plate 4.2 (a) indicates the presence of proteins in *C. molybdites*.

Based on the calculated values of $LD_{50}$ in both rats and mice, which were 288.53mg/kg and 471mg/kg respectively, which shows that the crude methanolic extract of *C. molybdites* was moderately toxic according to Matsumura (1975). This shows that the crude methanolic extract of *C. molybdites* has the ability to cause toxicity in humans by extrapolating results obtained from the animal studies. This observation is in tandem with the result obtained in the isolated tissue study which showed that *C. molybdites*, by its ability to cause continuous contraction of smooth muscle has tendency to cause diarrhea and hence dehydration in subjects that accidently ingest this specie. The results of the LD50 and the effects of the extract on the two animals' organs could therefore be used to properly catalogue information obtained in this study to form basis of a monograph of *C. molybdites*.

The extract increases the spontaneous muscle contractions of both isolated rabbit jejunum and guinea pig ileum (figure 4.2 and 4.4), similar to the standard agents, acetylcholine (figure 4.1) and histamine (figure 4.4), respectively. The effect of the extract on the rabbit jejunum was blocked by atropine, a muscarinic antagonist. While its effect on the guinea pig ileum was blocked by mepyramine, a histamine H1 antagonist. These findings suggest that the methanolic extract of *C. molybdites* possess smooth muscle contractile effect on the intestinal smooth muscle mediated via both muscarinic and histaminergic mechanisms.

This finding is significant as it indicates possible exploitation of the extract of *Chlorophyllum molybdites* in the preparation of purgative agents at small dose, due to its possible toxicity in cases of constipation. On the other hand, smooth muscles contractile effect can lead to precipitation of diarrhea and hence dehydration on subjects that accidentally ingest this mushroom. Therefore , communities especially those who use mushroom as substitute to meat, should be careful and be enlightened on ways of identifying this type of macrofungus, due to its nature of fruit body in particular and all other macrofungi in general with a view to avoid mushroom poisoning.

# CHAPTER 6

# SUMMARY, CONCLUSION AND RECOMMENDATIONS

## 6.1. SUMMARY

This study has provided some pharmaocognostic standards for the monograph of *Chlorophyllum molybdite*. The pileus of this mushroom has a diameter of 5 – 12 cm with a dome shape and a whitish flesh of 1 – 3 cm thick. Light brownish patches were observed on the scaly pileus with the edges showing radiating lines that follow the lines of the gills. It had a brittle texture and the surface bumpy at the centre. The stipe; 1 – 2 cm thick, whitish greyish brown in colour and slightly carved at the base. It has a brittle texture and a smooth surface. The spores on the lamellae were fine greenish powder with greenish colour which changes to brown on storage. The spore print had a diameter of 6.8cm – 12.3 cm. The spores of *C. molybdites* was amyloid.

The total ash value of 4% is relatively high showing that there is presence of foreign organic matter in the mushroom.

The acid insoluble ash of 0.43% is an indication that the crude drug sample contains some small amount of dirt. The water soluble and alcohol soluble extractive values were 10.34% and 7.15% respectively. This result showed that more constituents are soluble in water than alcohol.

The methanolic extract of *Cholrophyllym molybdite* possess smooth muscle contractile effect on the intestinal smooth muscle mediated via both muscarinic and histaminergic mechanisms.

## 6.2 CONCLUSION

The results of the macroscopic, microscopic, physical constants and chemo-microscopic, TLC and Phytochemical evaluations provide a number of information which can be included in the monograph of the mushroom for proper identification. The methanolic extract of *Cholrophyllym molybdite* also possesses smooth muscle contractile effect on isolated rabbit jejunum and guinea pig ileum.

## 6.3 RECOMMENDATIONS

The following recommendation is proposed for further study;

- Testing the anti-tumour, immune modulating, cardiovascular and anti-microbial activities of this mushroom.
- Isolation and characterization of the secondary metabolites present in it.
- Make the monograph known and readily available so that locals can readily identify this specie of mushroom.
- Determine the nutritive content of this mushroom

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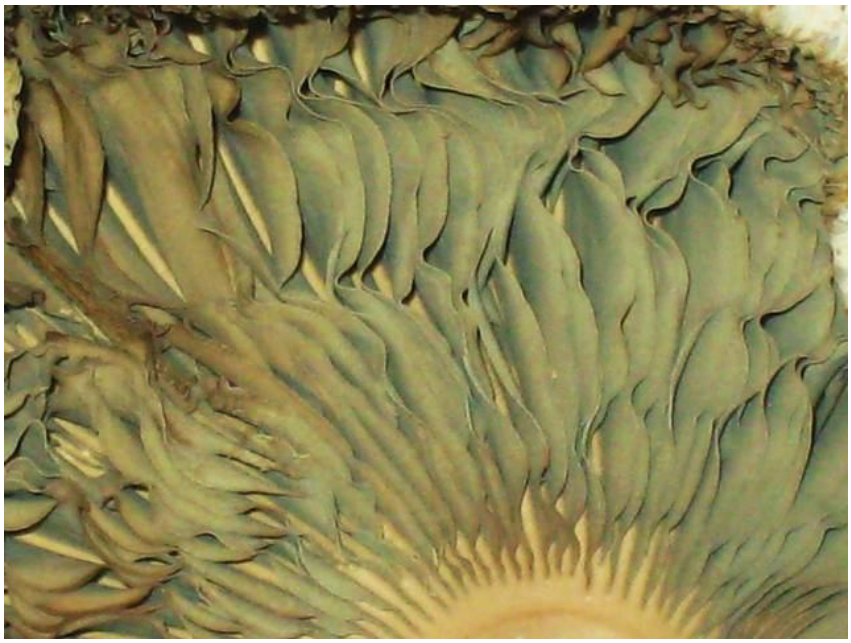
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## APPENDIX A

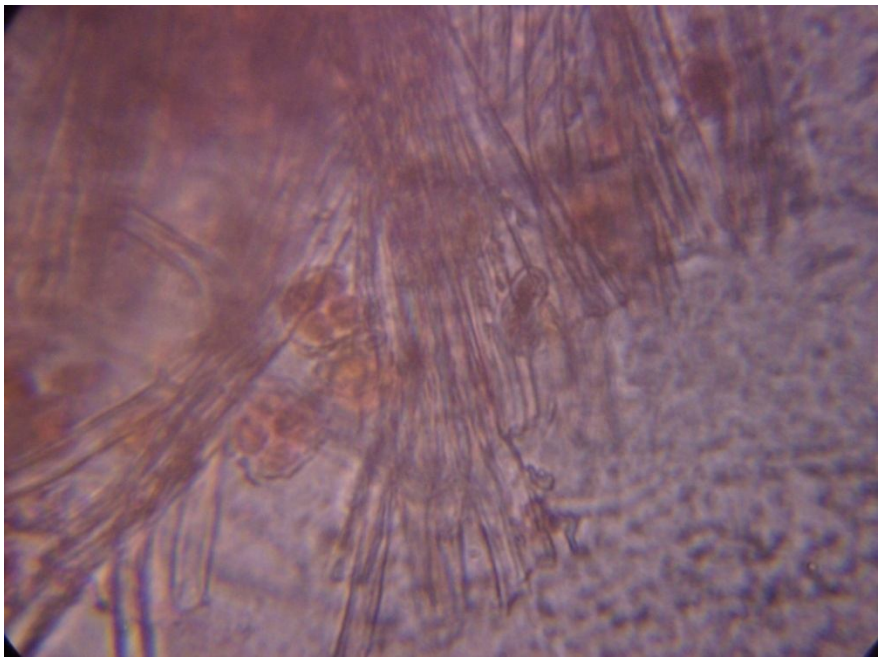


***Chlorophyllum molybdites*** **showing pileus**

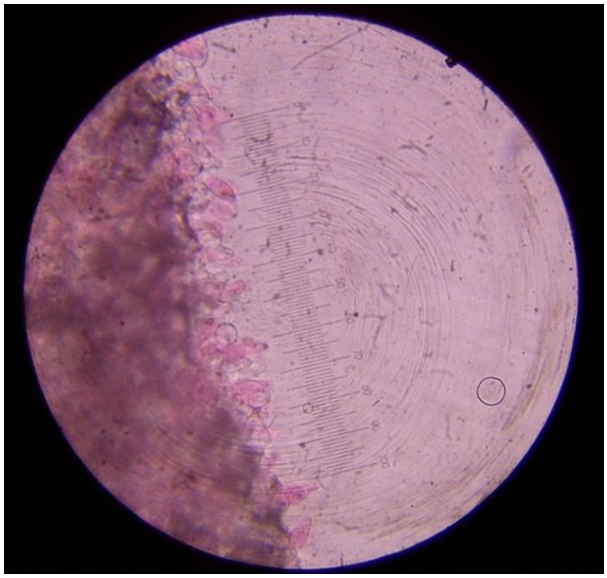


**Lamellae of *Chlorophyllum molybdites***

## APPENDIX B



**Asci of *Chlorophyllum molybdites***

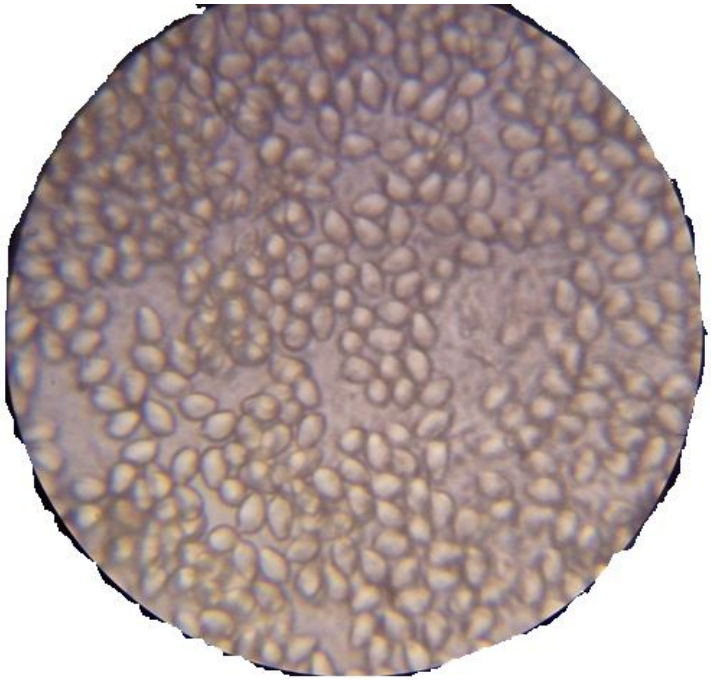


**Basidia of *Chlorophyllum molybdites***

## APPENDIX C



***C. molybdites*** **showing the pileus**



**Spores of *C. molybdites***

## APPENDIX D



Spore print of *C. molybdites*



*C. molybdites*

## APPENDIX E

**Table of LD50 in mice and rats**

**Phase one**

| | *Mice* | | *Rats* |
|---|---|---|---|
| **Dose** | **Animals** | **Remark** | **remarks** |
| **10mg/kg** | **1** | **S** | **S** |
| | **2** | **S** | **S** |
| | **3** | **S** | **S** |
| **100mg/kg** | **1** | **S** | **S** |
| | **2** | **S** | **S** |
| | **3** | **S** | **S** |
| **1000mg/kg** | **1** | **D** | **D** |
| | **2** | **D** | **D** |
| | **3** | **D** | **D** |

**KEY : S = survival, D = died**

**PHASE TWO**

| *Dose* | *Mice* | *Rats* |
|---|---|---|
| **140** | **S** | **S** |
| **225** | **S** | **S** |
| **370** | **S** | **D** |
| **600** | **D** | **D** |

**KEY : S = survival, D = died**

$LD_{50}$ (mice) = $\sqrt{370 \times 600}$ = 471mg/kg

$LD_{50}$ (rat) = $\sqrt{225 \times 370}$ = 288.53mg/kg