AN EVALUATION OF THE CHEMOTAXONOMY OF *LIGNOSUS RHINOCERUS*

NORAIDA BINTI MOHAMAD ZAINOOR

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STATEMENT OF SOURCE DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of higher education. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature.

This thesis is being submitted in partial fulfillment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy.

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ABSTRACT

Chemotaxonomy is the classification of plants and microorganisms based on similarities and differences in their natural products and the biochemical pathways involved in their manufacture. The classification and identification of *L. rhinocerus*, a medicinal fungus, for this research was based on secondary metabolites profiles by using chemical analysis that provides more objective and comparable results than traditional descriptions. Furthermore, chemical differentiation products could also be expected to be species-specific which is valuable for assessing the authenticity and quality of THMP containing this fungus.

The aims of this research program were directed towards the characterization of key metabolites of the fungus by evaluating the identified metabolites structure that has significance pharmacological or toxicological impact and chemotaxonomic profiling. This research required to develop appropriate extraction techniques to fractionate biochemical compounds of *L. rhinocerus*, to carry out qualitative and quantitative analyses (TLC, IR, UV, HPLC, LC-MS, GC-MS and NMR) of identified compounds and to establish robust quantitative techniques that may be applied in the routine quality assessment of samples of the fungal material.

The best method of extraction was by using methanol-Et_2O (1:1 v/v) in the presence of NaOH solution. Two identified compounds, ergosterol (0.39 mg/g) and ergone (0.03 mg/g) were isolated from flash chromatographic technique. The robustness of qualitative and quantitative analyses was demonstrated with good data of linearity (r^2, 0.9990) and %RSD value of precision and accuracy were less than 5.0%. Phenolic acid compounds were also detected and its total content (23.15µg/g) was determined by Folin-Ciocalteu method. Both classification of fungal sterols and PACs of *L. rhinocerus* were established using the optimum HPLC (chromatographic fingerprint) that may be applied in the routine quality assessment of samples of the fungal material. The main pharmacological effects of these compounds were anti-oxidant and anti-tumor effects. Consequently, overall aims and objectives of this research have been achieved.

LIST OF ABBREVIATIONS

^{13}C	carbon-13
λ_{max}	maximum absorbance at certain wavelength
%	percentage
°C	degree celsius
µg/g	microgram per gram
µg/mL	microgram per millilitre
µL	microlitre
^{1}H	hydrogen-1
A	absorbance
AC	acidic compound
As	symmetry factor
ATR	attenuated total reflection
b	path length
BC	basic compound
BP	British Pharmacopoeia
BSA	albumin Bovine Serum
BSTFA	bis (trimethylsilyl) triflouroacetamide
c	concentration
C	carbon
CBBG	coomassie brilliant blue G-250 dye
$CDCl_3$	chloroform-deuterium
CE	capillary electrophoresis
cm	centimetre

COX	cyclooxygenase
DAD	diode array detection
DE	diethyl ether
DL	limit of detection
DM	dried material
DNA	deoxyribonucleic acid
DW	dried weight
Ergone	ergosta-4, 6, 8 (14), 22-tetraen-3-one
F	water equivalence factor
FA	formic acid
FC	flash Chromatography
FCA	Folin-Ciocalteu assay
$FeCl_3$	ferric (III) chloride
FF	free form
FTIR	fourier transform infra red
g	gram
GAA	glacial acetic acid
GAE	gallic acid equivalent
GC	gas chromatography
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HSCCC	high-speed counter-current chromatography
ICH	International Conference on Harmonisation
IR	infra red
KF	Karl Fischer
L	litre

LC	liquid chromatography
M	molar
m/z	mass per charge
mg	milligram
mL	millilitre
mL/min	millilitre per minute
mM	millimole
µm	micrometre
mm	millimetre
MS	mass spectroscopy
Na_2CO_3	sodium carbonate
Na_2SO_4	sodium sulfate
$NaHCO_3$	sodium hydrogen carbonate
ND	not detected
nm	nanometre
NMR	nuclear magnetic resonance
NP	normal phase
NPCB	National Pharmaceutical Control Bureau
NPs	non-starch polysaccharides
OH	hydroxyl
P	radiant power
PA	peak area
PACs	phenolic acid compounds
PDA	photodiode array
PFP	pentafluorophenyl propyl
Po	incident radiation of radiant power

ppm	part per million
PS	*Polyporus* sclerotium
Py-GC/MS	pyrolysis with GCMS
QL	limit of quantification
R	resolution
r^2	regression coefficient
R_f	relative movement of compounds to solvent front
RP	reverse phase
RPC	removed phenolic compounds
rpm	revolution per minute
RSD %	relative standard deviation
RT	retention time
sp	species
SPE	solid phase extraction
T	transmittance
TCM	traditional Chinese medicine
TFA	trifluoroacetic acid
THMPs	traditional herbal medicinal products
TIC	total ion count
TLC	thin layer chromatography
TMS	tetramethylsilane
TPC	total phenolic compounds
USP	United States Pharmacopeia
UV/Vis	ultra-violet/visible
V	titre volume
v	volt

ν	frequencies
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight in gram
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION

1.1 Background

The use of alternative medicines, especially traditional herbal medicinal products (THMPs), has increased over the last ten years in Malaysia. Due to this circumstance, the Ministry of Health Malaysia recognised the need for registration of traditional medicine products in 1999. The requirements for registration process involve preliminary screening of the application, followed by testing of products and evaluation of documentary evidence. These requirements are designed to ensure that traditional medicines available in Malaysia are safe and of appropriate quality.

However, problems such as authentication of products (Toth *et al.*, 2006; Xie *et al.*, 2007), product adulterations (Bogusz *et al.*, 2006), misleading claims and counterfeit products are still occurring even though the registration of these products has been enforced. In Malaysia, the most major problem with THMPs is product adulteration. There were 725 of samples received in 2007 for screening and 126 of these samples were found positive for the presence of adulterants (National Pharmaceutical Control Bureau (NPCB) Malaysia Annual Report, 2007) and examples included the presence of sildenafil, whitening and slimming agents.

One of the reasons why this is still happening is due to inadequacy of qualitative and quantitative analysis of products during the registration process. The current regulatory testing of THMPs in Malaysia are based on limits for heavy metals (lead, arsenic and mercury), limits for microbial contamination, absence of steroids as adulterants and limits of disintegration time for products in the tablet or capsule form (NPCB Annual Report, 2006).

In order to minimize these problems, more discerning qualitative and quantitative analysis is required so that the presence and the amount of claimed species or herbal plants in THMPs can be justified. Moreover, these analyses are related to the identification of biochemical compounds that could be plant-specific to the plant producer. In other words, these specific compounds may also assist in the taxonomy of plants which is known as a chemotaxonomy.

1.2 Traditional herbal medicines

According to the World Health Organisation (WHO) definition, the traditional use of herbal medicines covers herbs, herbal materials, herbal preparations and products that contain as active ingredients parts of plants, or other plant materials, or combinations (WHO, 2000). Traditional use of herbal medicines refers to the long historical use of these medicines. Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities. The past decade has seen a significant increase in the use of herbal medicines and there are many guidelines available for their assessment. However, these guidelines have similar aims and objectives, i.e. to ensure the safety and quality of herbal medicines for use in national health care systems.

The WHO guideline for assessing herbal medicines highlights the necessity for a method of identification and, where possible, quantification of the plant material. It also mentions that the identification of an active ingredient (mostly impossible since herbal materials may contain a complex mixture of biochemical compounds with unknown therapeutic effects) should be sufficient to identify a characteristic substance or mixture of substances (for example supplying a "chromatographic fingerprint") to ensure consistent quality in the product.

1.3 Secondary metabolites

Metabolism is the sum of the chemical reactions that occur within living organism and the various compounds formed by these reactions are called metabolites (Isaacs *et al.*, 1996). Metabolism comprises synthesis (anabolism) and degradation (catabolism) processes in which nutrients are changed into structural and functional components of the organism. The primary metabolic pathway synthesizes and utilizes certain essential chemical species such as sugars, amino acids, common fatty acids, nucleotides, and polymers, derived from carbohydrates, proteins, lipids, with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) which are known as primary metabolites. These primary metabolites are essential for the survival and well-being of the organism (Mann, 1987). The secondary metabolic pathway synthesizes and utilizes secondary metabolites (natural products) during particular stages of growth and development or during periods of stress caused by nutritional limitation or microbial attack (Mann, 1987; Griffin, 1994).

The primary and secondary metabolisms are interconnected since the primary metabolism provides a number of small molecules as starting materials for all the crucial secondary metabolic pathways (Mann, 1987; Mann *et al.*, 1994). There are three principal starting materials (building blocks) for secondary metabolism:-

(a) Shikimic acid, the precursor of many aromatic compounds including the aromatic amino acids, cinnamic acids, and certain polyphenols;

(b) Amino acids, leading to alkaloids, and peptide antibiotics including the penicillins and cephalosporins;

(c) Acetate, precursor of polyacetylenes, prostaglandins, macrocyclic antibiotics, polyphenols, and the isoprenoids terpenes, steroids, and carotenoids.

1.4 Chemotaxonomy

The taxon (plural; taxa) is any named group of any rank in the hierarchical classification (taxonomic rank) (Isaacs *et al.*, 1996). The highest rank of hierarchy is called kingdom (such as, bacteria, virus and fungi) and the lowest rank is known as species (such as *Lignosus rhinocerus* and *Ganoderma lucidum*). An example of the hierarchical classification of *L. rhinocerus* is illustrated in Figure 1.1. A classical taxonomy of plants is based on macroscopic and morphological characteristics (Gussem *et al.*, 2007), while chemotaxonomy is mainly based on the comparative characterization of the chemistry of the studied taxa, and the identification of biomarkers, characteristic compounds typical of a given taxonomic rank (Nguyen-Tu *et al.*, 2007). Chemotaxonomy has been an important part of taxonomy of many plants for several decades.

Figure 1.1 *The hierarchical (taxanomic) classification of L. rhinocerus (Index Fungorum Database (IFD), 2009).*

According to the *Concise Science Dictionary*, chemotaxonomy is the classification of plants and microorganisms based on similarities and differences in their natural products and the biochemical pathways involved in their manufacture. Chemotaxonomy has been used extensively in the lichenized fungi and yeasts, and more recently in many other fungal groups (Frisvad *et al.*, 1998). It is normally involved with DNA, RNA, proteins, lipids, carbohydrates, proteins, and cell wall membrane components. However, lately,

chemotaxonomy has turned into a trend of classification and identification of fungi based on secondary metabolites profiles. This methodology has recently been proven to be useful in the study of *Xylariaceous* fungi (Whalley and Edwards, 1995) and during chemotaxonomical studies of *Alternaria* by Andersen *et al.*, (2008). In addition, secondary metabolites of fungi are reported to have medicinal, industrial, or agricultural impact as antibiotics, toxins, anticancer drugs, dyes, growth promoters, hallucinogens and immunosuppressants (Whalley and Edwards, 1997).

The development of analytical and biological methods and advanced instrumentation has resulted in a large number of different approaches to chemotaxonomy. The common approach for classification and identification of secondary metabolites uses a secondary metabolite profile by using a chromatographic technique. In addition, this technique enables the generation of chromatographic fingerprints of samples that have been used for comparison, classification, identification, and have found widespread use in flavour research, forensic investigations, and in the chemotaxonomic characterization of microorganisms and plants (Hansen *et al.*, 2005). The study by Bitzer *et al.*, (2008), has proven that high performance liquid chromatography (HPLC) profiles may be used in conjunction with morphological traits and methods of molecular phylogeny to establish correlations at the suprageneric level (branch points of taxa) in *Xylariaceae* (Stadler and Keller, 2008).

Furthermore, the recent approaches of applying hyphenated technologies such as gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-diode array detection (CE-DAD), HPLC-MS and HPLC-nuclear magnetic resonance (NMR), could provide additional spectral information, which will be helpful for qualitative analysis (Liang *et al.*, 2004) and for the structural elucidation of novel secondary metabolites.

1.5 *L. rhinocerus* fungi

Lignosus as the basidiocarp (spores are borne on specialized cells termed *basidia* underside of the cap) belongs to the tropical *polyporaceae* family and it possess the hymenium (fertile layer) as tubes on the underside of the cap. There are five *Lignosus* species, which are *L. rhinocerus, L. sacer, L. dimiticus, L. goetzii* and *L. ekombitii*, and these, can be found in Australia, Africa, Cameroon and Asia (Ryvarden and Johansen, 1980; Douanla-Meli and Langer, 2003). Among these *Lignosus* species, *L. rhinocerus* (Figure 1.2) has been found in Malaysia particularly at Pulau Pinang, Pahang, Perak and Kelantan.

Figure 1.2 *A picture of air-dried L. rhinocerus.*

The chosen fungus for this study has recently gained the interest of traditional practitioners in Malaysia, especially after it was reported by Leng, (2002) in the press (as attached in Appendix I). *L. rhinocerus* has been used as a traditional herbal medicine for the treatment of coughs, colds, and asthma and has tonic properties (Burkill, 1966). However, this fungus is not cultivated commercially so it has to be collected from the wild, being found in thick forest and hills. Thus, there is uncertainty when THMPs are claimed to contain *L. rhinocerus*. For this reason, the development of qualitative analysis is crucial for assuring the identity, consistency and authenticity of herbal products containing this fungus as well as for building up the chemotaxonomic data of this fungal species.

1.5.1 Source and authentication of material

Air-dried *L. rhinocerus* materials for the usage throughout this research were supplied by Qaisum Enterprise, Kelantan, Malaysia. The material was identified by Dr Chang, Mycologist at the Forest Research Institution of Malaysia, Professor Ryvarden, Polyporologist at the University of Oslo, Norway, Professor Cheung at the Chinese University of Hong Kong and lastly, by the Royal Kew Gardens, London. Two specimens of *L. rhinocerus* have been deposited in the Herbarium of National Museums and Galleries Merseyside (NMGM), Liverpool (certificate of transfer attached in Appendix II).

1.5.2 Nomenclature

L. rhinocerus (Cooke) Ryvarden was formerly known as *Polyporus rhinocerus Cooke* and is still being used by most traditional practitioners in China. Later on, the name was changed to *Polystictus rhinocerus (Cooke) Boedijn* which has been utilized among traditional practitioners in Malaysia for registration purposes. *L. rhinocerus* in the Malay language, is often referred to as" *kulat susu rimau*", meaning tiger's milk fungus (Burkill, 1966; Ismail, 1999; Chang and Lee, 2004).

According to the Index Fungorum Database, there are four other names that are referred to as *L. rhinocerus*. These are *Fomes rhinocerus Cooke, Microporus rhinocerus (Cooke) Imazeki, Polyporus sacer var. rhinocerotis (Cooke) Lloyd* and *Scindalma rhinocerus (Cooke) Kuntze*, respectively.

1.5.3 Description

A labelled drawing picture of *L. rhinocerus* is given below (Figure 1.3 (a)). *L. rhinocerus* is usually easy to identify, being centrally stipitate (a stalk part) and arising from a distinct sclerotium found in the ground (Ryvarden and Johansen, 1980). The genus is microscopically characterised by a trimitic hyphal system (generative hyphae with clamps,

hyaline binding and skeletal hyphae in context), sclerotium (a mass of compacted hyphae) and stipe (stalk). Cystidia (sterile element in the hymenium which is common underside of mushroom cap) and spores (smooth, ellipsoid, hyaline and non-amyloid) are absent (Douanla-Meli and Langer, 2003).

***Figure 1.3** Lignosus species. (a) L. rhinocerus (Ryvarden and Johansen, 1980); (b) L. dimiticus (Ryvarden, 1975).*

Generally, a pileus or cap is of a yellowish brown colour and shows fine, tomentose (hairy) in narrow concentric zones. It has a less circular shape with a diameter of up to 10.0 cm and a thickness of up to 4.0 mm in the centre. The length of stipe or stalk arising from the sclerotium is normally in the range of 5.0 - 7.0 cm while its diameter is in the range of 1.0 - 1.5 cm. The sclerotium is in irregular to elongated shape and has a dimension up to 5.0 cm. It often has a dark and tough outer skin that protects the internal hyphal mass from drying out.

Figure 1.3 (a) shows the dotted line represents ground level and the stipe has grown out from a buried sclerotium (Ryvarden and Johansen, 1980). The sclerotium may produce a new mycelium (a mass of branching (threadlike) of hyphae) when environmental conditions such as humidity and temperature are favorable for it to grow. The rhizomorphs, barely present, have a diameter between 1.0 mm and 3.0 mm while its length can be up to

7.0 cm long (Ryvarden and Johansen, 1980). It usually grows radially out from the stalk and can be found 1.0 cm to 2.0 cm below ground. The pore of *L. rhinocerus* is normally much smaller than *L. sacer*. The round, porous surface is white to light cream in colour and the number of pores is between 7 and 8 pores per 1 mm length while the size of spores is ranging from 1.7 μm to 2.5 μm in diameter (Ryvarden and Johansen, 1980).

1.5.4 Approximate compositions

A fungus normally comprises nucleic acids, proteins, carbohydrates, and lipids. Analyses of the composition of fungi have been made for various purposes and with different degrees of detail and completeness and hence resulted in percentage variations summarised in Table 1.1.

***Table 1.1** Approximate compositions of fungi (Griffin, 1994).*

The beneficial properties attributed to fungi are usually more associated with its sclerotium than its stalk and cap. A study carried out by Wong *et al.*, (2003) suggested the overall compositions of sclerotial air-dried *P. rhinocerus* (other name of *L. rhinocerus*) as given in Figure 1.4. It shows a high amount of carbohydrates and a low amount of protein and lipid in *L. rhinocerus* sclerotia. The sclerotium is also enriched with dietary fibre, the

vegetable residues that withstand digestion by the enzymes of the digestive tract (Bruneton, 1995), and was a novel discovery by Cheung, as with two other mushrooms; *Pleurotus tuber-regium* and *Wolfiporia cocos* (Wong *et al.*, 2003; Wong and Cheung, 2005). According to Wong *et al.* (2003), the main composition of the dietary fibre was mainly based on non-starch polysaccharides (NPs) such as glucosamine and uronic acids. NPs have been reported to possess pharmacological activities such as antitumour, cholesterol-lowering effects and antiproliferative effects on leukemic cells (Cheung and Lee, 2000; Zhang, *et al.*, 2001; Wong *et al.*, 2003; Lai *et al.*, 2008). There are many studies on polysaccharides, metabolites derived from carbohydrate, of *L. rhinocerus*. However, no study on other metabolites derived-carbohydrate of the fungus, such as sterols and phenolic acid compounds (PACs), has been found in the literature. Hence, the evaluation of those metabolites was emphasized in this research.

Figure 1.4 *Approximate compositions of P. rhinocerus in % w/w of dried material (Wong et al., 2003).*

1.5.4.1 Moisture content

Fungi have been found to be organisms that can grow under conditions of low water avaibility. The influence of water availability on longevity has been viewed almost exclusively in the context of sclerotia lying within soil (Ayres and Boddy, 1986) whereby the rate of sclerotium dehydration is relatively slow compared to its ability for the uptake of

water. Figure 1.4 indicates that 15.30% of dried *L. rhinocerus* compositions are derived from moisture content. Due to this reason, the determination of moisture or water content in sclerotial *L. rhinocerus* is important to carry out. Furthermore, moisture is an essential factor in the deterioration of medicinal products especially for products that are susceptible to hydrolysis (Mwesigwa et al., 2005) and could also lead to formulation instability and the risk of microbial growth.

A titrimetric method using Karl Fischer Reagent (KFR) and loss on drying (LOD) are two common methods for determining moisture content in pharmaceutical quality control testings. Generally, the titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer, KFR, that reacts with hydrogen ions (United States Pharmacopeia (USP), 2009). Loss on drying is used in cases where the loss sustained on heating may be not entirely water but also due to volatile matter of any kind that is driven off under the conditions specified (USP, 2009).

1.5.4.2 Total protein content

Protein is an essential macronutrient to the human diet as it supplies nitrogen and amino acids to the body. Amino acids are also the precursors of a large variety of secondary metabolites such as amines, short-chain and heterocyclic acids. There are 300 natural plant amino acids are known, only about 20 are regarded as the normal constituents of proteins (Bruneton, 1995). Many occur in the free state, except in fungi where they are sometimes constituents of small peptides (Bruneton, 1995). The total amount of protein was determined since it may responsible for the tonic activity of *L. rhinocerus*.

There are many assay methods described in publications for determining total protein but the most common method is the Bradford assay due its simplicity, rapid and

inexpensive characteristics. It is based on the binding of Coomassie brilliant blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues (Bradford, 1976). The resulting intensity of CBBG-protein complex (brownish blue colour) was determined at 595 nm using a spectrophotometer.

1.5.5 Chemistry, pharmacological activities, method of analysis and chemotaxonomy

1.5.5.1 Fungal sterols

The secondary metabolites of fungal sterols are mainly derived from carbohydrate and are synthesized from acetate via the melavonic acid pathway. The initial formation of fungal sterols is built up from C_5 isoprene units leading to their characteristic branched chain structure (Hanson, 2008). The number of isoprene units determines the subclasses of terpenoids as tabulated in Table 1.2.

***Table 1.2** The subclasses of terpenoids (Mann et al., 1994).*

The isoprene units are normally linked together in a head-to-tail manner, however, the C_{30} (triterpenoids) and C_{40} (carotenoids) are formed by the dimerization of two C_{15} and

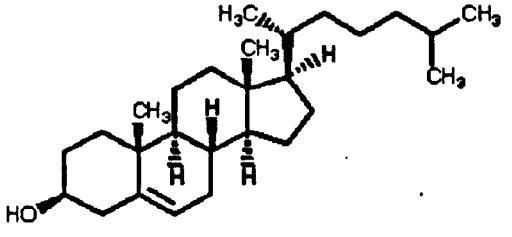
C_{20} units, respectively (Hanson, 2008). Carotenoids are not as significant in fungi as they are in plants because fungi are obviously not photosynthetic (Frisvad, 1998). C_5 isoprene units are condensed to form squalene (triterpenoid) followed by a cyclization process producing steroidal compounds, for example the formation of ergosterol in Figure 1.5.

***Figure 1.5** A simplified biosynthesis of ergosterol (Griffin, 1994).*

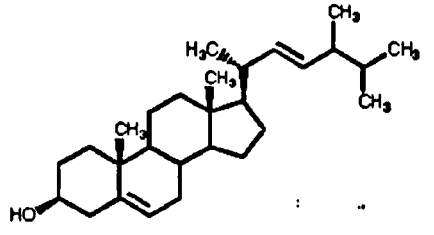
The basic structure of fungal sterols comprises of a steroid ring structure in which a hydroxyl group, usually attached to carbon-3 and hydrocarbon chain can be attached to the rings usually at carbon-17. Fungal sterols differ from major plant sterols in having two double bonds in the steroid structure instead of one (Mattila *et al.*, 2002). They can exist as

free-form sterols or bound-form sterols and the hydroxyl group is often esterified with a fatty acid for example, cholesterol ester. However, most sterol extracts are complex mixtures of C_{27}, C_{28}, and C_{29} sterols of varying degrees of unsaturation which lead to differences in the distribution of steroids among fungi (Griffin, 1994).

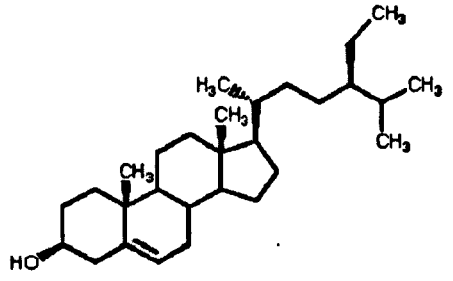
Sitosterol, stigmasterol and campesterol are common in higher plants and occur as free and simple glucosides while ergosterol occurs in lower plants and especially fungi (Harbone, 1973). Ergosterol has the same function as cholesterol in mammals and plays an important role in the membrane structures of fungal cell walls. Other sterol compounds that have been detected in various fungi are desmosterol, 22-dihydroergosterol, ergost-5-enol, ergosta-5, 7-dienol, episterol, fungisterol, fecosterol, fucosterol and 24-methyl cholesterol (Frisvad et al., 1998; Cole and Schweikert, 2003). Figure 1.6 shows some examples of fungal sterols.



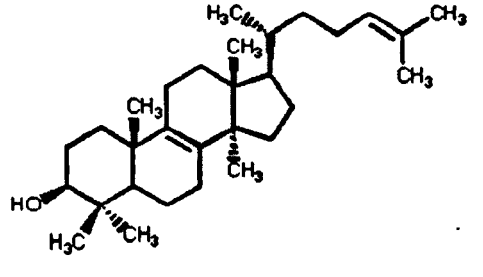
(a) Cholesterol ($C_{27}H_{46}0$; M.W 386.35)



(b) Brassicasterol ($C_{28}H_{46}0$; M.W 398.35)



(c) Sitosterol ($C_{29}H_{50}0$; M.W 414.39)



(d) Lanosterol ($C_{30}H_{50}0$; M.W 426.39)

***Figure 1.6** Fungal sterols.*

1.5.5.1.1 Pharmacological activities

36 TCM preparations of *Polyporus* sclerotium (PS) were used in studies on the marker compounds for the standardization of traditional Chinese medicine (TCM) by Yuan *et al.*, (2003). However, *L. rhinocerus* was not included in these studies even though it belongs to the *Polyporus* family. The study indicated that ergosta-4,6,8(14),22-tetraen-3-one (ergone, Figure 1.7) is the marker compound of PS. Ergone is reported to have an anti-aldosteronic diuretic effect by blocking mineral corticoids (Yuan *et al.*, 2004; Lee *et al.*, 2005).

Figure 1.7 *Chemical structure of ergosta-4, 6, 8 (14), 22-tetraen-3-one (ergone) (Yuan et al., 2003). The number 14 represents carbon (C) that forms a double bond with C-8 instead of forms the double bond between C-8 and C- 9.*

It is commonly stated in the literature that the pharmacological activities of most mushrooms and fungi are mainly due to ergosterol (the principal fungal sterol) and its peroxidation products (compounds that contain the -O-O- group as shown in Figure 1.8) (Kadakal and Artik, 2004; Yuan *et al.*, 2006; Ridder-Duine *et al.*, 2006; Parsi and Gorecki, 2006). A report by Yuan *et al.*, (2006), stated that numerous triterpene derivatives have been isolated from G. lucidum (an established medicinal fungi in TCM) including highly oxygenated lanostane derivatives and common fungal steroids derived from ergosterol. It was also reported that ergosterol and its peroxidation products may contribute to potential health benefits and significant pharmacological activities, including reducing pain related to

inflammation, reducing the incidence of cardiovascular disease, inhibiting cyclooxygenase (COX) enzyme, antioxidant, antimimicrobial, and antitumour activities.

Figure 1.8 *The chemical structure of the ergosterol peroxide (5α, 8α-epidioxy-24(R)-methylcholesta-6, 22-dien-3β-ol as an anti-atherosclerosis agent) isolated from flowers of Erigeron annuus L. (Kim et al., 2005).*

Another medicinal fungus is *Cordyceps sinensis* in which isolated ergosterol and its peroxidation products demonstrated antitumour effects in tumour cell systems and in animal assays (Kahlos *et al.*, 1987; Burczyk *et al.*, 1996; Bok *et al.*, 1999). Research carried out by Nam *et al.*, (2001), also supported that ergosterol peroxide found in *Paecilomyces tenuipes* inhibited human tumour cells growth (gastric tumour cell line, hepatoma cell line, colorectal tumour cell line and murine sarcoma). Besides medicinal fungi, edible mushrooms such as *Grifola frondosa* possessed an anti-inflammatory effect due to the presence of the fungal sterols ergosterol and ergone, which inhibited COX-1 and -2 activities (Zhang *et al.*, 2002; Monthana *et al.*, 2003). *Pleurotus ostreatus* (oyster fungus) was reported to have effects on lowering plasma cholesterol level caused by its isolated compound, ergone (Chobot *et al.*, 1997). Furthermore, dietary ergosterol is absorbed in the alimentary tract, accumulates in the adrenals and other organs, and can be metabolised *in vivo* to generate newer bioactive products, such as 17α, 24-dihydroxyergosterol which has

been found to be able to inhibit the proliferation of skin cells in culture, as demonstrated in human keratinocytes and melanoma cell lines (Slominski *et al.*, 2005).

Ergosterol is also a precursor compound for formation of ergocalciferol (vitamin D_2) when exposed to UV irradiation (Van-Berg, 1997; Mattila *et al.*, 2002). The term vitamin D refers to ergocalciferol (vitamin D_2) or cholecalciferol (vitamin D_3). Ergocalciferol is derived from fungal and plant sources while cholecalciferol is synthesized in the skin from 7-dehydrocholesterol via photochemical reactions with ultraviolet B (UV-B). The fungal sources of vitamin D_2 are mostly reported to arise from the conversion of ergosterol in edible mushrooms (Jasinghe and Perera, 2006; Jasinghe *et al.*, 2007; Teichmann *et al.*, 2007). Mattila *et al.*, (2002), stated that ergosterol was the most abundant sterol found in mushrooms, and its contents were higher in cultivated mushrooms (6.02-6.79 mg/g of dried weight (DW)) than in wild mushrooms (2.96-4.89 mg/g of DW). The study also revealed that vitamin D_2 was almost totally absent in cultivated mushrooms, while some wild mushrooms contained high concentrations of this vitamin (4.70-194.0 µg/g of DW).

Another study on the conversion of ergosterol to vitamin D_2 was done by *Jasinghe and Perera, 2006*, in which fresh Shiitake mushrooms (*Lentinula edodes*), Oyster mushrooms (*Pleurotus ostreatus*), Button mushrooms (*Agaricus bisporus*) and Abalone mushrooms (*Pleurotus cystidus*) were irradiated with UV-B (wavelength 290-315 nm) and the highest vitamin D_2 content (184 ± 5.71 µg/g of DM) was observed in Oyster mushrooms while the lowest vitamin D_2 content (22.9 ± 2.68 µg/g of DM) was observed in Button mushrooms. This means the conversion of ergosterol to vitamin D_2 in opened cap mushroom was about four times higher than the closed cap mushrooms (Figure 1.9). Thus, based on this information, a simple thin layer chromatography (TLC) screening of ergocalciferol in different parts of *L.rhinocerus* was performed. Ergocalciferol has similar

pharmacological activities with vitamin D that is essential to prevent rickets in children and osteomalacia in adults (Jasinghe and Perera, 2005).

***Figure 1.9** A conversion of ergosterol to vitamin D_2 (Endotext, 2009).*

1.5.5.1.2 Method of analysis

A HPLC-DAD, using gradient elution on reversed-phase material, is widely used for the separation and detection of fungal sterols. The use of GC-MS technique is increasingly popular for the analysis of fungal sterols (Mattila *et al.*, 2002; Puglisi *et al.*, 2003; Hajjaj *et al.*, 2005) due to the availability of mass spectral libraries, containing a large number of spectra that can lead to the successful identification of analytes (Rouessac and Rouessac, 2000). Nevertheless, MS instrumentation is still a first choice for some researchers to determine the molecular mass of fungal sterols (Jinming *et al.*, 2001; Lee *et al.*, 2005; San-Martin *et al.*, 2008).

1.5.5.1.3 Chemotaxonomy

It is widely reported in the literature reported that ergosterol and cholesterol are two major sources of secondary metabolites, the distribution of which varies among fungal species. For example, cholesterol, brassicasterol, ergosterol, fecosterol, campesterol, episterol and sitosterol have been detected, although campesterol and sitosterol were not found in *Trichophyton terrestre*, and campesterol was absent in *Microsporum andouinii* and *M. ferrugineum* (Frisvad, 1998). It is also not unusual that different fungal species possess one

or more secondary metabolites in common (Frisvad *et al.*, 2008) especially ergosterol and fungisterol. Ergosterol is the most common metabolite and in a high concentration can generate fungisterol in most fungal species (Frisvad, 1998).

There are still too few studies on sterol composition available to provide adequate conclusions about their distribution within fungal taxa as stated by Wassef, (1977). Furthermore, most sterol literature is concerned with an emphasis on novel drug discovery and identification of compounds rather than focusing on the utility and importance of fungal secondary metabolites and their genes in polyphasic taxonomy (Stadler and Keller, 2008). Hence, an evaluation of fungal sterols as a chemotaxonomic significance in *L. rhinocerus* is required.

1.5.5.2 Phenolic acid compounds (PACs)

There are over 8000 compounds of known phenolic acid where the flavonoids such as quercetin form the largest group (Mann *et al.*, 1994). The type of phenolic compounds may vary considerably throughout the plant kingdom and are rare in bacteria, algae and fungi. The term *phenolic acid* applies to all organic compounds that comprise at least one phenolic hydroxyl group and one carboxyl group. There are three main types of PACs which are simple phenols, derivatives of benzoic acid and derivatives of cinnamic acids. They are conveniently classified according to the number of carbon atoms in the basic skeleton (Table 1.3) and the number and position of hydroxylation and methoxylation of aromatic ring (Rice-Evans *et al.*, 1997) (Table 1.4).

The types of PACs present in fungal kingdom are simple phenols, phenylpropanoids such as hydroxycinnamic acids, and quinones. They are synthesized via the shikimic acid pathway and may occur in the bound or free form (Ozturk *et al.*, 2007). The production of phenolic polymer causes formation of brown to black pigments in fungi (Figure 1.10) (Griffin, 1994) and this is known as melanin. Melanin helps to prevent ultraviolet radiation

damage, and acts to make the cell wall of fungi even tougher. PACs have major contributions to the taste, flavour and colour of plants and has an important role in avoiding plants from being eaten by herbivores.

Table 1.3 *The major classes of phenolic compounds found in plants (Mann et al., 1994).*

***Table 1.4** Chemical structures of phenolic compounds in different subclasses.*

Figure 1.10 *Simple catechol melanin biosynthetic pathways (Griffin, 1994).*

1.5.5.2.1 Pharmacological activities

PACs have been considered in recent years as potentially protective compounds against cancer and heart disease because of their potent antioxidative properties (Pietta, 2000; 2004; Wen *et al.*, 2005; Barros *et al.*, 2008). Additionally, they exhibit anti-viral, anti-bacterial and anti-inflammatory effects (Lamaison *et al.*, 1991; Proestos *et al.*, 2006), as well as causing reduction in platelet aggregation (Asami *et al.*, 2003). The antioxidative properties of PACs may also prevent other diseases related to aging (Dvorakova *et al.*, 2008) and photosensitivity reactions (Haslam, 1998). PACs are not only widely used in phytotherapy but also are treated as functional food ingredients or as food supplements (Proestos *et al.*, 2006).

1.5.5.2.2 Method of analysis

PACs have widely diversified chemical structures of over 8000 compounds of known PACs as mentioned earlier in part 1.5.5.2. Thus, the total content of PACs, instead of

individual PACs, is usually determined. The most common method in publications is by using the Folin-Ciocalteu assay (FCA). The FCA method is based on the reduction of a phosphotungstate-phosphomolybdate complex by reductants (or antioxidants) to a blue chromogen (Stratil *et al.*, 2006) which is measured using UV/Vis spectrophotometry at a wavelength between 725 nm and 765 nm. The total content of PACs is normally expressed as gallic acid equivalent (GAE) and some reports use catechin as the PACs equivalent (Katalinic *et al.*, 2006). The alternative methods that have recently been developed for total content of PACs are based on spectrophotometric-enzymatic methods (Ma and Cheung, 2007) and flow injection analysis with inhibited chemiluminescent detection (Nalewajko *et al.*, 2008).

Several methods have also been employed for separating and identifying PACs. The majority of reviewed literature used a reverse phase HPLC with DAD detection at 280 nm and operated with a gradient system. Generally, a C_{18} column has been utilized in most of the HPLC methods, followed by C_8 and phenyl column (Hajnos, 1998). Other methods that are used for separating PACs include capillary electrophoresis (Cartoni *et al.*, 1995), LC-MS (Perez-Magarino *et al.*, 1999), GC-MS (Fiamegos *et al.*, 2004) and HPLC-DAD-Electro-spray Ionization Mass Spectrometry (Fang *et al.*, 2007).

1.5.5.2.3 Chemotaxonomy

Among the different PACs, flavonoids have been broadly used in chemotaxonomy of plants as they are widely distributed among plants (almost exclusively in higher plants), and are characteristic constituents of green plants (Robards and Antolovich, 1997) and also responsible for the colour of flowers (Mann, 1987). Flavonoid compounds are chemically stable, structurally variable and relatively easy to establish an analytical method especially using HPLC can be established easily. A recent discovery of flavonoids as

chemotaxonomic marker was luteolin 7-*O*-glucoside, found in the *Hieracium rohacsense* group (Svehlikova *et al.*, 2002).

Besides flavonoids, two caffeic acid esters, (Z,E)-[2-(3,5-dihydroxyphenyl)ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate and (Z,E)-[2-(3,4-dihydroxyphenyl)ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate, were found to be chemotaxonomically significant in *Lamiaceae* (*Grayer et al., 2003*) while (*R*)-3'-*O*-β-*D*-glucopyranosylrosmarinic acid, derived from cinnamic acid, was accepted to be of chemotaxonomic significance in two South African medicinal plants namely *Arctopus* and *Alepidea* (Apiaceae subfamily Saniculoideae) (Olivier *et al.*, 2008).

A literature survey revealed no evidence of PAC contribution to the chemotaxonomic study of *L. rhinocerus*. Therefore, screening of PACs constitutes a step towards upgrading both of the fungal phytochemical and chemotaxonomic data.

1.6 Analytical methods

1.6.1 Extraction methods

Sample preparation is an essential step and often requires many steps before analysis on instrumentation. It normally begins with the extraction process, to separate desired compounds from a mixture by selective solubility (Isaacs *et al.*, 1996). For example, PACs, are usually weakly acidic and water soluble, can be extracted from other fungal constituents with polar organic solvents under slightly acidic conditions. A number of methods, such as maceration, boiling and soxhlet extraction, using organic or aqueous solvents are employed in the extraction of natural products. The current research utilized two extraction techniques; solvent extraction and heating at reflux.

A liquid-liquid extraction, also known as solvent extraction, is the most common extraction technique. It usually allows for the separation of compounds due to their unequal solubilities in two immiscible solvents. This technique is highly effective as the first step

of a fairly large-scale separation of compounds from crude natural products (Sarker *et al.*, 2006). Refluxing is another common technique of extraction. It involves heating and continuous extraction in which an apparatus is arranged so that organic solvent vapour continually circulates through the aqueous solution extracting a small amount of material each time, before being recycled (Harwood *et al.*, 1999).

1.6.2 Isolation techniques

Isolation can be referred as a process to separate a compound or more from a mixture solution. There are several techniques for isolating compounds. The classical ways are by scraping off spots of TLC plate and preparative column chromatography such as flash chromatography. Examples of the modern techniques available for these processes are a solid phase extraction (SPE) and an automatic fraction collector that can be attached to analytical instrument, commonly with HPLC. Three types of isolation techniques were utilized in the present study.

1.6.2.1 Fractionation technique

Fractionation is a separation technique, to isolate individual compounds based on the polarity or solubility. One example is an HPLC system combined with fraction collection. The fraction collector allows specified compounds to be taken from a column effluent and stored in individual vessels that are placed in a rotating disk or in a moving belt which is controlled by a microprocessor. Sample fractions can be collected at equal time intervals and also specific peaks can be collected at a specific time.

1.6.2.2 Thin layer chromatography (TLC)

TLC can be looked upon as being an "open column" system which is not only one of the most widely used techniques for the separation and identification of compounds, but also

useful for isolation of compounds. The method is carried out by scraping off the marked spot of the target compound from TLC plate and the compound is extracted using a suitable solvent. This has been proven to be effective in many reported studies which often dealt with the isolation of lipid compounds (Ruelland *et al.*, 2002; Mishra and Sangwan, 2008).

1.6.2.3 Flash chromatography technique

Flash chromatography (FC) is a rapid form of preparative column chromatography based on a packed column and normally using compressed air to push the solvent through the column at a high flow rate. The common stationary phase for FC is silica gel that is a comparatively fine powder with a relatively narrow particle size ranges. This silica gives better surface contact, and therefore more effective adsorption than traditional long gravity column (Tatchell *et al.*, 1991). Furthermore, the pressure required to drive the solvent through increases the resolution by cutting down band dispersion and considerably reduces the time required or running the column (Tatchell *et al.*, 1991).

Due to some practical difficulties with the original published method by Still *et al.*, (1978), for running flash columns, modifications have therefore been launched over the years. New FC systems can be found as compact systems, comprising pump, gradient mixer, injection valve, column mount, detector, fraction collector, computer and software. This system may be scaled up for separations from a few milligrams to hundreds of grams.

In recent years, FC has been successfully applied to isolate various natural products including fungi (Abraham and Spassov, 1991; Ma *et al.*, 2002; Lee *et al.*, 2005; Davis *et al.*, 2005; Singh *et al.*, 2009; Ho *et al.*, 2009). It has cut down the time taken for not only purification of natural products but also for synthetic drugs and a very powerful and rapid technique for separation of organic compounds.

1.6.3 Qualitative and quantitative analysis

Qualitative and quantitative analysis are two disciplines of analytical chemistry and not only imperative requirements for the quality control of any registered medicine but also important in agricultural, clinical, environmental, forensic, manufacturing, metallurgical, and pharmaceutical aspects. Qualitative analysis can be referred as analyzing the nature of a pure unknown compound or the compounds present in a mixture (Isaacs et al., 1996). It involves the identification of elements, ions, or compounds present in a sample. Analyses may be performed by selective chemical reactions or with the use of instrumentation such as TLC, infra red (IR) spectroscopy, and liquid (LC) and gas chromatography (GC).

Generally, qualitative analysis is used in conjunction with quantitative analysis. The qualitative analysis is functioned to separate desired constituents from a sample while the quantitative analysis is utilized to measure the amounts present. This type of analysis relies mainly on the reproducibility of the separation and on the linear relationship that exists between the injected mass of the compound and the area of the detected peak in the chromatogram (Rouessac and Rouessac, 2000).

A universal method of quantitative analysis is based on LC combined with UV or DAD detection. The UV detector responses to a large number of organic compounds, is inexpensive and shows sensitivity to approximately 0.01 ppm. The DAD provides a powerful qualitative tool which has the ability to resolve overlapping spectra when a chromatographic peak consists of two or more analytes (Christian, 1994). In the last decade, the use of MS as an on-line HPLC detector has significantly increased due to the development of LC-MS interfacing systems. LC-MS is the most sensitive, selective and provides additional information on the mass-to-charge ratio of a compound. However, it is still one of most expensive detectors.

Traditional medicines usually contain a range of pharmacologically active compounds and in many cases it is not known which of these constituents produces the

therapeutic effect. Testing for efficacy in this situation is obviously more complex than with synthetic drugs (Bogusz *et al.*, 2006). Nevertheless, chromatographic techniques have been strongly recommended for the purpose of quality control of traditional herbal medicines (Liang *et al.*, 2004). The reliability and reproducibility of chromatographic system are monitored through a range of tests that are known as system suitability tests. These tests form an integral part of both gas and liquid chromatographic methods that should be run routinely to ensure the best performance of the chromatographic system. Whenever there is a significant change in equipment or critical reagent, suitability testing should be performed before the injection of samples (USP, 2009). Sample analyses obtained while the system fails the requirements of system suitability are unacceptable. There are several tests and formulae to demonstrate system suitability tests, but the most common tests and formulae used in pharmaceutical or quality control analysis are described in the USP (2009) and BP (2009) and are summarised in Table 1.5 below.

***Table 1.5** Common tests and requirements for monitoring the HPLC system suitability.*

Parameter	Definition	Requirement
Resolution (R)	A separation of two peaks.	R greater than 1.5 corresponds to baseline separation (USP, 2009).
Symmetry factor (As) (or tailing factor)	A measure of peak symmetry.	As between 0.8 and 1.5, and 1.0 signifies complete (ideal) symmetry (BP, 2009).
Precision	Replicate injections of standard solution.	Five replicate injections, the percentage of relative standard deviation (% RSD), value is 2.0% or less (USP, 2009).

1.6.4 Validation of analytical method

Analytical validation includes all of the procedures required to demonstrate that a method to quantify the concentration of analyte in a particular solution is reliable for the intended application (International Conference on Harmonisation (ICH), 2005). Currently, for Malaysian regulatory bodies, the pharmaceutical analysis is validated according to Q2 (R1) of the ICH guidelines. According to this guideline, four characteristics are necessary to validate quantify or assay method, namely, specificity, linearity, accuracy and precision. Quantification limit (QL) and detection limit (DL) are preferably used for impurities testing while robustness is necessary for method development.

1.6.4.1 Specificity

Specificity is the ability to assess unequivocally the compound or analyte in the presence of other compounds such as excipient and impurities (ICH, 2005). It is also to ensure that the solvent blank or diluents do not have the same retention time as the analyte. The analyte should have no interference from other extraneous components and should be well resolved from them.

1.6.4.2 Linearity

Linearity is the ability of the analytical method to obtain results that are directly proportional to the concentration (amount) of analyte within a given range (ICH, 2005). The range suggested in ICH is ± 20% of nominal content, and minimum of five concentrations are required. The linearity is normally subjected to the calibration curve and is reported in terms of the regression coefficient (r^2) and linear equation. The best linearity corresponds to the r^2 value equal to 1, and the intercept value should be 2% less than the nominal content.

1.6.4.3 Accuracy

The accuracy of an analytical method is the closeness of agreement (test result) between the value found and the true value of the analyte (Bliesner, 2006). A minimum of nine determinations over a minimum of three levels covering the specified range is required to assess accuracy. It can be reported as the percentage of recovery by the assay of known added amounts of analyte (Bliesner, 2006) or percentage of relative error or coefficient of variation (Miller and Miller, 2005).

1.6.4.4 Precision

The precision is defined as the degree of agreement between replicate measurements of the same quantity (Christian, 1994). Similar to accuracy, a minimum of nine determinations over a minimum of three levels covering the specified range is needed to assess precision of an analytical method. It can be reported as percentage of relative standard deviation as well. According to ICH (2005) and Bliesner (2006), precision may be considered at three levels as described below.

1.6.4.4.1 Repeatability

The use of the analytical procedures within a laboratory by a single analyst, on a single instrument, under the same operation conditions, over a short time interval. Repeatability is also known as method precision.

1.6.4.4.2 Intermediate precision

Intermediate precision is sometimes referred to as ruggedness that is variations within the laboratory such as different days, analysts and equipment.

1.6.4.4.3 Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial, the use of the analytical method in different laboratories. It is normally addressed during the transfer of the method to different sites.

1.6.4.5 Detection limit (DL)

The DL of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (ICH, 2005). There are numerous ways to define the detection but the common way is derived from a signal equal to three times the standard deviation of the background (corresponding to a signal-to-noise (S/N) ratio of 3) and from a visual evaluation.

1.6.4.6 Quantification limit (QL)

The QL of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (ICH, 2005). The limit of quantitation of the peak is equal to ten times the standard deviation of the background (corresponding to an S/N ratio of 10).

1.6.4.7 Robustness

The robustness of an analytical procedure is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability or the analysis of sample (Bliesner, 2006). One consequence of the evaluation of robustness should be that a series of system suitability parameters (section 1.6.3, Table 1.5) is established to ensure that the validity of the analytical procedure is maintained whenever used (ICH, 2005). Examples of typical variations are: stability of analytical solutions; influence of variations of pH; composition of a mobile phase; column type; temperature; and flow rate.

1.7 Aims and objectives

This research programme was directed towards the characterization of key metabolites of the air-dried fungus *L. rhinocerus* by identifying and rationalizing its medicinal value. The research involved qualitative and quantitative analysis of the metabolites, the determination of their concentration in various parts of the fungus and the evaluation of identified metabolites structure which was significant in having important pharmacological or toxicological impact, *in vivo* and also significant for chemotaxonomic profile. The evaluation did not involve a clinical assessment but was based on a literature review of the known properties of any identified metabolites. Thus, the analytical milestones supporting this research programme were:-

1. To develop appropriate extraction techniques required to fractionate the various biochemical compounds.
2. To identify and, if necessary, develop appropriate quantitative and qualitative analytical techniques.
3. To identify qualitatively key metabolites within the fungus and to evaluate its pharmacological activities and its possibility as chemotaxonomic significance.
4. To develop robust quantitative techniques that may be applied in the routine quality assessment of samples of the fungal material.

No publication or report has been found in the literature to the date this thesis was written that related to the chemotaxonomy of *Lignosus* species. A classical taxonomy of *Lignosus* species was based on microscopic morphological characteristics as mentioned earlier in section 1.5.3. The current research into the chemotaxonomy of *L. rhinocerus* is considered to be the first attempt and discovery for this species. However, due to a very limited source of raw materials of *Lignosus* species, comparison within the species was not possible. The comparisons were done with commercials products (as attached in Appendix

IV) which had similar physical properties with *Lignosus* species, such as open caps, mass compact of sclerotia, and belong to *Polyporaceae* family. Because of limited sources, the chemotaxonomic evaluation was mostly carried out by comparing chromatographic fingerprints of *L. rhinocerus* with commercially available products.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

A list of solvents, chemicals and reagents, reference materials, commercial products, filters, TLC plates, columns and analytical instrumentations used throughout the research is provided below.

2.1.1 Solvents, chemicals and reagents

Solvents, chemicals, reagents and other materials used throughout analyses are listed in Table 2.1.

Table 2.1 Solvents, chemicals, reagents and other materials used.

Materials	Grade	Suppliers
Acetone	Analar	Fisher Scientific Ltd., UK.
Acetonitrile	HPLC	VWR International Ltd., U.K.
Acetyl acetate	Analar	Fisher Scientific Ltd., UK.
Ammonium sulfate	Analar	BDH Laboratory Supplies Ltd., U.K.
Benzene	Analar	Merck KGaA., Germany.
Benzoic acid	Analar	BDH Laboratory Supplies Ltd., U.K.
Bis(trimethylsilyl)triflouroacetamide	Chemical	Sigma-Aldrich Company, U.K.
Bradford reagent	Reagent	Sigma-Aldrich Company, U.K.
Chloroform	HPLC	VWR International Ltd., U.K.
Chloroform-deuterium with 0.05% trimethylsilane	NMR	Sigma-Aldrich Company, U.K.

Continued .....

Materials	Grade	Suppliers
Dichloromethane	HPLC	Fisher Scientific Ltd., UK.
Diethyl ether	HPLC	VWR International Ltd., U.K.
Ethanol	HPLC	VWR International Ltd., U.K.
Ferric chloride	Analar	BDH Laboratory Supplies Ltd. U.K.
Folin-Ciocalteu	Reagent	VWR International Ltd., U.K.
Formic acid	Analar	Fisher Scientific Ltd., UK.
Glacial Acetic Acid	Analar	BDH Laboratory Supplies Ltd., U.K.
Hexane	HPLC	VWR International Ltd., U.K.
Hydranal solvent 34800	Reagent	Riedel de Haen, Germany.
Hydranal composite 5K 34816	Reagent	Riedel de Haen, Germany.
Hydrochloric acid	Analar	VWR International Ltd., U.K.
Methanol	HPLC	Fisher Scientific Ltd., UK.
Phenol	Analar	Fision Scientific Company, UK.
Phosphoric acid	Analar	BDH Laboratory Supplies Ltd., U.K.
Potassium bromide	Chemical	Fisher Scientific Ltd., UK.
di-Potassium hydrogen orthophosphate	Analar	BDH Laboratory Supplies Ltd., U.K.
Potassium dihydrogen phosphate	Analar	BDH Laboratory Supplies Ltd., U.K.
Propanol	Analar	Fisher Scientific Ltd., UK.
Pyridine	GC	Sigma-Aldrich Company, U.K.
Sand	Material	VWR International Ltd., U.K.
Salicylic acid	Analar	BDH Laboratory Supplies Ltd., U.K.
Silica powder, 60A, 35-70 micron	Material	BDH Laboratory Supplies Ltd., U.K.
Sodium carbonate anhydrous	Analar	BDH Laboratory Supplies Ltd., U.K.
Sodium Chloride	Analar	BDH Laboratory Supplies Ltd., U.K.

Continued .....

Materials	Grade	Suppliers
Sodium hydrogen carbonate	Analar	BDH Laboratory Supplies Ltd., U.K.
Sodium hydroxide pellets	Analar	BDH Laboratory Supplies Ltd., U.K.
Sodium sulfate anhydrous	Analar	Fisher Scientific Ltd., UK.
Sulfuric acid	Analar	BDH Laboratory Supplies Ltd., U.K.
2,6-*di*-tert-butyl-*p*-cresol	Analar	BDH Laboratory Supplies Ltd., U.K.
Toulene	HPLC	Sigma-Aldrich Company, U.K.
Trifluoroacetic acid	Analar	VWR International Ltd., U.K.
Tris(hydroxymethyl)methylamine	Analar	BDH Laboratory Supplies Ltd., U.K.
Water	HPLC	Fisher Scientific Ltd., UK.

2.1.2 Commercial products

Commercial products used to compare and generate chromatographic fingerprints of *L. rhinocerus* are listed in Table 2.2.

***Table 2.2** Commercial products used.*

Reference material	Batch Number	Suppliers
Coriolus (*Coriolus versicolor*)	07512A	G. Baldwin & Company, U.K
Felyoung (*Poria cocos*)	UNI0025	Long Heh Enterprise, Malaysia
Long Heh (*Cordyceps sinensis*)	JDJCJC	Unifar Sdn, Bhd., Malaysia
Maitake (*Grifola frondosa*)	061104A	G. Baldwin & Company, U.K
Reishi (*Ganoderma lucidum*)	079260	G. Baldwin & Company, U.K

2.1.3 References materials

References (standard) materials used to determine the total protein and for qualitative and quantitative analyses of fungal sterols and phenolic acid compound (PACs) are listed in Table 2.3.

Table 2.3 Reference materials.

Reference material	Batch Number	Suppliers
Apegin	037K1450	Sigma-Aldrich Company, U.K.
Bovine Serum Albumine	BAH62-630	BDH Laboratory Supplies Ltd., U.K
Caffeic acid	047K1609	Sigma-Aldrich Company, U.K.
Chlorogenic acid	127K1640	Sigma-Aldrich Company, U.K.
Cinnamic acid	C80857	Sigma-Aldrich Company, U.K.
p-coumaric acid	078K3736	Sigma-Aldrich Company, U.K.
Ergocalciferol	LB41795	Sigma-Aldrich Company, U.K.
Ergosterol	1302697	Sigma-Aldrich Company, U.K.
Gallic acid	028K0113	Sigma-Aldrich Company, U.K.
Tyrosol	07715PH	Sigma-Aldrich Company, U.K.
Vanillic acid	1306724	Sigma-Aldrich Company, U.K.

2.1.4 Filters, TLC plates, columns and analytical instrumentation

Disposable filters (Whatman PVDF 0.45 μm) and filter papers (Whatman no. 1) were purchased from VWR International Ltd., (U.K). TLC plates (Silica gel plate, F_{254}, 20 cm x 20 cm) were purchased from Sigma-Aldrich, (U.K) and columns used throughout analyses were Alltima, C_{18} (5.0 μm, 250 x 4.6 mm), DB-5 fused silica capillary (0.5 μm, 0.32 mm x 30 m), Kromasil C_8 (5.0 μm, 150 x 4.6 mm), pentafluorophenyl propyl (PFP) (3 μm, 150 x

4.6 mm), Spherisorb S5W (5.0 μm, 250 x 4.0 mm), Waters Symmetry shield, C_8 (5.0 μm, 250 x 4.6 mm), Waters, C_{18} (5.0 μm, 150 x 4.6 mm) and Zorbax C_8 (5.0 μm, 150 x 4.6 mm). A list of analytical instrumentation used throughout the research is provided in Table 2.4.

***Table 2.4** Analytical instrumentation.*

	Instrumentation
1	Alliance Waters 2695 Chromatograph with Waters 486 Tunable Absorbance Detector and LCT Micromass Mass Spectrometer.
2	Bruker Avance NMR Spectroscopy.
3	B & T UV scanner with 254 nm and 365 nm wavelength.
4	Genlab oven.
5	Hewlett Packard 1050 Chromatograph with PDA detector.
6	Hewlett Packard 1090 Chromatograph with PDA detector.
7	701 KFT Titrino.
8	Magnum Finnigan MAT Gas Chromatograph Mass Spectrometer.
9	Mettler Delta 350 pH-Meter.
10	Nicolet Evolution 300 UV/Vis Spectroscopy.
11	Perkin Elmer FT-IR- ATR Spectroscopy.
12	Perkin Elmer Spectrum RX1 FT-IR Spectrometer.
13	Rodleys Discovery Technologies hot plate (magnetic stirrer and temperature controller).
14	Sartorius analytical balance.
15	703 Ti Stand Metrohm.

2.2 Methods

The preliminary works in this research involved the analysis of water and protein contents. The water content (section 2.2.1) was analysed using two methods and were based on; loss on drying and titration using Karl Fisher. The total protein content (section 2.2.2) was determined by spectroscopic analysis using the Bradford assay. An acid-base extraction method (section 2.2.3) was used to screen for the presence of extractable biochemical compounds of *L. rhinocerus*. Further investigations were carried out with different extraction solvents, TLC and HPLC mobile phases and columns as described in the method development section (section 2.2.4). Optimal separation conditions were then employed to isolate and fractionate (section 2.2.5) the biochemical compounds. The isolated compounds were subsequently characterized and subjected to various analytical procedures (section 2.2.6). A validated HPLC method (section 2.2.7) was used to quantify the identified compounds in different parts of *L. rhinocerus* fungal material (section 2.2.8). Section 2.2.9 described a HPLC method used for the generation of chromatographic fingerprints derived from organic extracts while section 2.2.10 illustrated a screening method for the detection of ergocalciferol. Finally, investigations were carried out on water-soluble phenolic acid compounds (PACs) (section 2.2.10 – 2.2.13).

2.2.1 Moisture contents

2.2.1.1 Loss on drying (LOD)

A grated sample of sclerotia (2.0 g) was transferred to a watch glass and dried in the oven at approximately 105 °C until a constant weight of sample was obtained. The sample was removed from the oven and placed in dessicator where it was allowed to cool to room temperature before weighing. The loss of moisture was determined by subtracting the weight of sample before drying with the weight of sample after drying and was then expressed as a percentage of weight (% w/w) (BP, 2009).

2.2.1.2 Titration using Karl Fischer (KF) reagent

The titration vessel was filled with hydranal solvent in order to sufficiently cover the electrode. A syringe was used to transfer 10 µL of distilled water, with known weight, into the vessel and the mixture was stirred and titrated to the amperometric end-point using KF reagent (hydranal composite 5K). The water equivalence factor (F) was obtained in mg/mL and the procedure was repeated with a known weight of grated sclerotial sample. The titer volume (V) of water of the sample was determined and the water content was calculated by multiplying the F with the V value and expressed as percentage of weight of water (% w/w) present in the sample.

2.2.2 Total protein content

2.2.2.1 Preparation of 50 mM tris-hydrochloric acid (HCl) solution (pH 8.0)

Tris base (30.0 g) was transferred into a 1 L volumetric flask and dissolved with distilled water (800 mL). The solution was adjusted to pH 8.0 with dilute HCl and made to volume using the same solvent.

2.2.2.2 Preparation of bovine serum albumin (BSA) stock solution (1000.0 µg/mL)

BSA (50.0 mg) was transferred into a 50 mL volumetric flask and dissolved with 50 mM tris-HCl solution and made to volume with the same solvent.

2.2.2.3 Preparation of calibration standard solutions

Five dilutions of the BSA stock solution (1000.0 µg/mL) in 50 mM tris-HCl were prepared to yield standards covering the concentration ranges 100.0 - 230.0 µg/mL.

2.2.2.4 Preparation of sample solution

A grated sample of sclerotia (5.0 g) was transferred into a 200 mL beaker and distilled water (50 mL) was added. The solution was left to settle for four hours before being filtered. The filtrate was adjusted to pH 4.5 with a few drops of diluted HCl. The solution was saturated with ammonium sulfate (15.0 g). The cloudy upper layer (15 mL) was transferred into a 20.0 mL volumetric flask and was made up to volume with 50 mM tris-HCl solution.

2.2.2.5 UV/Vis spectroscopy

Each of calibration standard solutions (section 2.2.2.3), sample solution (section 2.2.2.4), and the blank solution (0.1 mL distilled water) were mixed with Bradford reagent (3 mL) in a 4 mL plastic cuvette. The absorbance of sample and standard solutions were measured using UV/Vis spectroscopy at a wavelength of 595.0 nm. The amount of crude protein was obtained from calibration standards curve.

2.2.3 Screening method of simple extractable compounds

A flow diagram of extraction processes involved in the sample preparations are summarised in Figure 2.1.

2.2.3.1 Preparation of sample solutions

2.2.3.1.1 Water soluble (WS) compounds

A grated sample of sclerotia (5.0 g) was transferred into a 200 mL beaker containing distilled water (50 mL) and the solution was boiled for 30.0 minutes (Extraction 1). The mixture solution was cooled to room temperature and filtered through a filter paper.

2.2.3.1.2 Free form (FF) compounds

The filtrate (30 mL) from section 2.2.3.1.1 was transferred into a separating funnel. Dichloromethane (30 mL) was added and the flask was shaken. It was allowed to settle and the lower Dichloromethane layer was transferred into evaporating dish. The extraction (Extraction 2) was repeated twice with the same solvent and the dichloromethane layers were combined in the evaporating dish and were evaporated to dryness. The residue was dissolved in methanol (2 mL).

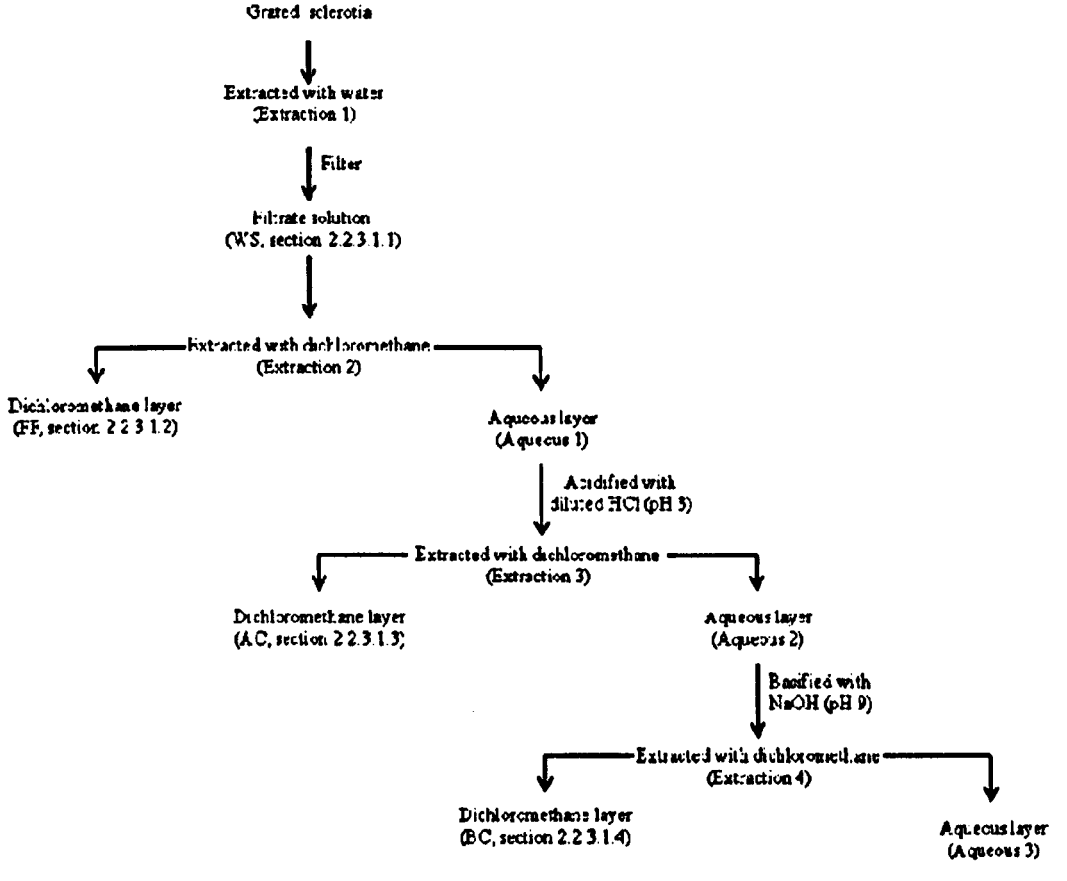


***Figure 2.1** The flow of extraction processes used for screening biochemical compounds of L. rhinocerus. WS: water-soluble; FF: free form; AC: acidic compounds; BC: basic compounds.*

2.2.3.1.3 Acidic compounds (AC)

The remaining aqueous layer (Aqueous 1) from section 2.2.3.1.2 was acidified to pH 3 using 0.05M HCl and was then extracted again with dichloromethane (Extraction 3). The same procedure in section 2.2.3.1.2 was followed.

2.2.3.1.4 Basic compounds (BC)

The remaining aqueous layer (Aqueous 2) from section 2.2.3.1.3 was basified to pH 9 with 1.0 M NaOH solution and extracted again with dichloromethane (Extraction 4). The same procedure in section 2.2.3.1.2 was followed.

2.2.3.2 Qualitative analyses

2.2.3.2.1 TLC analysis

WS, FF, AC and BC solutions (10 µL and 20 µL) (Figure 2.1) were spotted on the TLC plate. The plate was placed in a tank containing 100 mL methanol-chloroform (2:8, v/v). The tank was covered and the chromatogram was allowed to develop for about one hour. The plate was left to dry for a few minutes before visualization under UV irradiation at 254 nm and 365 nm. The observed spots were then marked using a pencil. The plate was sprayed with 50% (v/v) ethanolic sulfuric acid solution and was heated at 105 °C for three minutes.

2.2.3.2.2 UV/Vis spectroscopy

The FF solution and a blank solution (distilled water) were transferred into a separate 4 mL of plastic cuvette. The solutions were subjected to full scan analysis as described in Table 2.5.

***Table 2.5** The UV full scans method.*

HPLC system	Parameter
Start wavelength	200.00 nm
Stop wavelength	500.00 nm
Peak wavelength	Auto
Baseline	Local baseline
Scan speed	500 nm/min
Data interval	Normal
Bandwidth	1.5 nm
Lamp change	Xenon

2.2.3.2.3 HPLC analysis

The HPLC method used in this work was operated into two modes, i.e. reverse phase (RP) and normal phase (NP). The operating conditions used to analyse FF solution involved an isocratic NP-HPLC system (Table 2.6) and a gradient RP-HPLC system (Table 2.7).

***Table 2.6** Isocratic NP-HPLC operating conditions used to analyse FF solution.*

HPLC system	Parameter
Column	Spherisorb, S5W, (5.0 µm, 250 x 4.6 mm)
Flow rate	0.5 mL/min
Wavelength	254 nm
Injection volume	80 µL
Mobile phase	20% (v/v) methanol in chloroform

Table 2.7 *Gradient RP-HPLC operating conditions used to analyse FF solution.*

HPLC system	Parameter	
Column	Alltima, C_{18}, 5.0 µm, 250 x 4.6 mm	
Flow rate	1.0 mL/min	
Wavelength	254 nm , 280 nm and 303 nm	
Injection volume	80 µL	
Mobile phase	Water (%)	Methanol (%)
0-5 minutes	95	5
5-35 minutes	5	95
35-45 minutes	5	95

2.2.4 Method development

2.2.4.1 Preparation 1

A grated sample of sclerotia (2.0 g) was heated at reflux in methanol (20 mL) for one hour at 80 °C. The mixture was filtered through a filter paper (Whatman® 1) and the filter paper was washed with 5% (w/v) aqueous Na_2CO_3 (15 mL). The filtrate was collected and transferred into a separating funnel. Diethyl ether (Et_2O) (20 mL) was added to the filtrate in the separating funnel. It was shaken for about two minutes and then left to stand for the (upper) ether layer to separate. The ether layer was collected into a beaker containing 5.0 g of anhydrous Na_2SO_4. The extraction was repeated and the ether layers were combined. The combined ether solution was transferred into evaporating dish and evaporated to dryness over a period of 24 hours. Glacial acetic acid (GAA) and methanol were used to reconstitute the obtained residue for TLC and HPLC analyses, respectively.

2.2.4.2 Preparation 2

A grated sample of sclerotia (5.0 g) was heated at reflux in methanol (30 mL) and 0.5 M methanolic NaOH (5 mL) for one hour at 80 °C. The mixture was filtered through a filter paper and washed with distilled water (30 mL). The filtrate was extracted with Et_2O (20 mL) and the ether layer was transferred into an evaporating dish. The same procedure described above for Preparation 1 was repeated except that the residue was reconstituted with methanol (2 mL). TLC and HPLC analyses were utilized to analyse the reconstituted solution. The method was repeated using different parts of *L. rhinocerus* (stalks and caps). A number of different solvents, reflux duration, temperatures and NaOH molarities were chosen as listed in Table 2.8 in order to obtain an optimised sample preparation method. A corresponding flow chart was designed and followed throughout the procedure and is summarised in Figure 2.2.

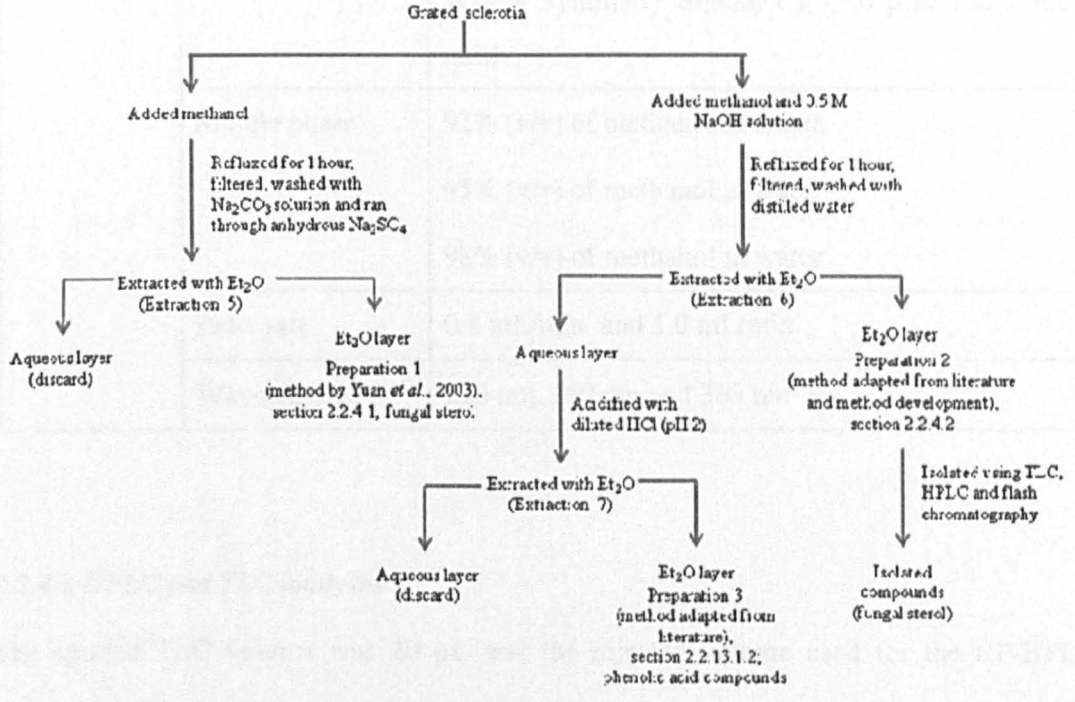


***Figure 2.2** A simplified chart of the main sample preparations involved throughout the research.*

Table 2.8 *A number of different sample preparation conditions, composition of TLC mobile phases and HPLC conditions used to develop an optimised sample preparation method.*

Process	Parameter	
Sample preparation	Solvent	Distilled water, tetrahydrofuran, acetone, chloroform and hexane.
	Reflux duration	30 minutes, 1 hour and 2 hours
	Temperature	50 °C and 80 °C
	NaOH molarities	0.05 M, 0.5 M and 1.0 M
TLC	Mobile phases	Acetone-hexane (1:9, v/v) Acetone -hexane (3:7, v/v) Methanol-chloroform (1:19, v/v) Methanol-chloroform (1:9, v/v)
RP-HPLC	Column	Alltima, C_{18}, (5.0 µm, 250 x 4.6 mm) Waters Symmetry Shield, C_8, (5.0 µm, 150 x 4.6 mm)
	Mobile phase	92% (v/v) of methanol in water 95% (v/v) of methanol in water 98% (v/v) of methanol in water
	Flow rate	0.8 mL/min and 1.0 mL/min
	Wavelengths	220 nm, 280 nm and 365 nm

2.2.4.3 HPLC and TLC analyses

The spotted TLC volume was 20 µL and the injected volume used for the RP-HPLC method was 50 µL, respectively. The remaining operating conditions used for TLC and RP-

HPLC analysis of the resulting sample solutions (Preparation 1 and 2) are listed in Table 2.8.

2.2.5 Fractionation and isolation methods

2.2.5.1 Fractionation by HPLC

2.2.5.1.1 Sample preparation

A grated sample of sclerotia (50.0 g) was heated at reflux in equal volume of methanol and Et_2O (30 mL) and 0.5 M methanolic NaOH (5 mL) for one hour. The mixture was filtered through a filter paper and washed with distilled water (30 mL). The same procedure of extraction in Preparation 2 (section 2.3.4.2) was applied and the obtained residue was reconstituted with methanol (2 mL) for HPLC analysis.

2.2.5.1.2 RP-HPLC analysis

The RP-HPLC operating conditions used to analyse the reconstituted solution are given in Table 2.9.

Table 2.9 *The RP-HPLC operating system used to fractionate biochemical compounds of L. rhinocerus*

HPLC system	Parameter
Column	Alltima, C_8, (5.0 µm, 150 x 4.6 mm)
Flow rate	1.0 mL/min
Column temperature	40 °C
Wavelength	280 nm
Injection volume	50 µL
Mobile phase	95% (v/v) of methanol in water

2.2.5.1.3 Fractionating procedure

Six injections of sample solution were analysed for the repeatability of the selected peak. Fractions were collected at ± 0.5 minute of the RT of the selected peak. The solvent in the combined fractions (about 5 mL) was then left to evaporate until the volume of the fractions was reduced to 1 mL. The resulting solution was analysed again to verify the identity of the selected peak. Fourteen sample injections have been carried out. The combined fractions were then left to evaporate to dryness. The obtained residue was analysed using UV/Vis spectroscopy and FTIR-ATR as described in later sections 2.2.6.1 and 2.2.6.4, respectively.

2.2.5.2 Isolation by TLC

2.2.5.2.1 Sample preparation

The sample residue was obtained from the same procedure as described in Preparation 2 (section 2.2.4.2) except that 10.0 g of grated sclerotia was used.

2.2.5.2.2 Isolating procedure

The residue was dissolved in methanol (5 mL) and spotted (20 µL) on the TLC plate. The plate was placed in a tank containing saturated vapor of mobile phase (35%, v/v, acetone in hexane). The tank was covered and the chromatogram was allowed to develop for about one hour. The plate was left to dry for a few minutes before viewing the chromatograms (spots) under UV irradiation of 254 nm and 365 nm. The observed spots under 254 nm were marked. This procedure was repeated for another seven plates.

The marked spots were scraped off using a spatula and were transferred into a 20 mL volumetric flask and methanol (20 mL) was added. The mixture of silica and methanol was sonicated for 30 minutes and was centrifuged for three minutes at 5000 revolutions per minute (rpm). The supernatant was transferred into an evaporating dish using a pipette. The

solvent of supernatant was then left to evaporate to dryness. The obtained residue was analysed using UV/Vis spectroscopy and FTIR-ATR as described later in sections 2.2.6.1 and 2.2.6.4, respectively.

2.2.5.3 Isolation using flash chromatography

A gradient system, using a solvent composition of hexane-ethyl acetate (3:1) and hexane-acetone-chloroform (6:3:1), was employed to elute biochemical compounds of *L. rhinocerus*.

2.2.5.3.1 Sample preparation

The sample residue was obtained from the same procedure as described in Preparation 2 (section 2.2.4.2) except that 200.0 g of grated sclerotia was used.

2.2.5.3.2 Flash chromatography procedure

Silica gel was measured using the glass column in a fume hood. The silica gel was carefully poured into the column through a plastic funnel to get a volume about 13.0 cm of dried silica gel. The measured silica gel was transferred into a 200 mL beaker and the solvent (14 mL) was added. The mixture was stirred vigorously to remove the air from the silica until fine slurry of the silica gel was formed.

The slurry was carefully and slowly poured back into the column using the funnel (the stopcock was closed at this point). The beaker and the sides of the column were rinsed with the solvent. The sides of the column were tapped gently with the end of a pencil for improving the packing of the silica particles. The stopcock was opened and a compressed air was packed on the top of the column. The silica level shrunk to about half of its original height but the solvent was left just above the top level of the silica. The stopcock was

closed and the sample solution (10 mL) was added slowly using pipette. The stopcock was opened again to let the sample solution just above the top level of silica gel.

The silica surface was then covered with 2 cm layer of sand, to protect the surface from distortion when adding the solvent. The sides of the glass were rinsed with solvent using a pipette and the stopcock was opened to drain the solvent. The column was filled with solvent by using a pipette and when the level of solvent was found to be 2 cm above the surface. The solvent was then added slowly using a beaker until the equivalent of two column volumes was reached.

A 10 mL glass cylinder was placed underneath the column to collect the fraction for every 1 mL. The fractions were monitored by TLC until all compounds were eluted. Fractions containing identical spots were combined in round-bottomed flasks and concentrates for further analyses.

The second solvent composition was added when there were no more compounds eluted and the same procedure was repeated.

2.2.6 Qualitative analyses

2.2.6.1 UV/Vis spectroscopy

A small amount (2.0 mg) of the white and yellow residue isolated from HPLC fractionation (sections 2.2.5.1) and TLC isolation (section 2.2.5.2) was dissolved in methanol and ethanol, respectively. The resulting solutions and a blank solution (methanol and ethanol) were separately transferred into a 10 mm quartz cuvette. The solutions were subjected to UV full scan analysis using the parameters given in Table 2.5.

2.2.6.2 TLC analysis

A small amount (2.0 mg) of each isolated residue was dissolved in methanol and spotted (20 µL) on a silica plate. The chromatograms were developed in mobile phase, acetone-hexane (3:7, v/v). The spots were observed under UV irradiation at 254 nm and 365nm.

2.2.6.3 RP and NP-HPLC analyses

The sample solutions from section 2.2.6.1.2 were filtered and analysed. The operating RP-HPLC conditions used for the analysis were the same as listed in Table 2.9 while the operating NP-HPLC conditions are given in Table 2.10 below.

Table 2.10 *The operating NP-HPLC system used to analyse the isolated compounds.*

HPLC system	Parameter
Column	Spherisorb, S5W, (5.0 µm, 250 x 4.6 mm)
Flow rate	0.5 mL/min
Wavelength	254 nm , 280 nm and 365 nm
Injection volume	80 µL
Mobile phase	10% (v/v) ethanol in hexane

2.2.6.4 FTIR-ATR spectroscopy

The white needles isolated from HPLC fractionation (section 2.2.5.1) were placed onto the diamond ATR plate and the sample was scanned over the range between 4000.0 to 500.0 cm^{-1}. The procedure was repeated with the yellow residue isolated from TLC method (section 2.2.5.2).

2.2.6.5 LC-MS analysis

The isolated yellow residue (Fraction 1) and white needles (Fraction 2), obtained after flash chromatography (section 2.2.5.3), were dissolved in methanol and analysed. The LC and MS operating conditions operating conditions used to analyse the sample solutions are given in Table 2.9 and Table 2.11, respectively.

Table 2.11 *The MS operating system.*

MS system	Parameter
Source temperature	100 °C
Desolvation gas temperature	150 °C
Desolvation gas flow	603 L/hour
MCP (detector) potential	2750 V
Polarity	ES+
Capillary voltage	3000 V
Sample cone	15 V
Extraction cone	1.0 V
RF lens potential	200 V

2.2.6.6 GC-MS analysis

The isolated yellow residue (Fraction 1) and white needles (Fraction 2) were derivatised using a mixture of pyridine and BSTFA (1:3, v/v) (4 mL). The mixture was heated at 100°C for one hour and the resulting solutions were filtered and analysed using the operating conditions as given in Table 2.12.

Table 2.12 *The operating GC-MS system.*

HPLC system	Parameter
Column	DB-5 fused silica capillary (0.5 μm, 0.32 mm x 30 m)
Column temperature	60 °C for 1 min
	30 °C/min to 270 °C
	10 °C/min for 5 min to 320 °C
Gas flow rate	0.7 mL/min
Injection splitless	250 °C
Ion source	280 °C
MS/EI mode	70 eV
Scan range	100-600 m/z
Injection volume	1 μL

2.2.6.7 ^{1}H-NMR spectroscopy analysis

A small amount of isolated yellow residue (Fraction 1) (1.0 mg) and white residue (Fraction 2) (5.0 mg) as placed separately in a NMR tube and of $CDCl_3$ containing TMS (1.5 mL) was added to dissolve the compounds. The ^{1}H-NMR spectra were expressed in parts per million (ppm, δ) relative to TMS at δ = 0 ppm.

2.2.7 HPLC method validation

2.2.7.1 Sample solutions

The sample (1.0 g) was heated at reflux for one hour using a solution containing 0.5 M methanolic NaOH (2 mL), methanol (8 mL) and Et_20 (6 mL). The resulting solution was transferred into a separating funnel and was extracted with equal volume of distilled water and Et_2O (30 mL). The ether layer was transferred to an evaporating dish and the extraction

was repeated twice using the same solvent (distilled water-Et_2O, 30 mL). The combined ether layers were then evaporated to dryness and the obtained residue was reconstituted with methanol (2 mL).

2.2.7.2 Ergosterol reference stock solution

Three weights of ergosterol (100.0 mg) were placed in a separate 100 mL volumetric flask and dissolved in methanol to give a 1000 µg/mL of reference stock solution.

2.2.7.3 System suitability

A standard solution (80.0 µg/mL) was used for the evaluation of system suitability. Six replicate injections were carried out and the precision (repeatability) was expressed as a percentage of relative standard deviation (%RSD) using peak area (PA). The RT variations were measured within ± 1.0 minute of the mean RT value. The peak symmetry factors (As) were also measured for precise integration.

2.2.7.4 Specificity

A diluent (methanol), the sample solution of grated sclerotia, reference solution (80.0 µg/mL) and a spiked sample solution with standard solution (80.0 µg/mL) were used for the determination of the specificity of the method.

2.2.7.5 Linearity

The calibration levels (40.0 µg/mL, 60.0 µg/mL, 80.0 µg/mL, 100.0 µg/mL and 120.0 µg/mL) were prepared using the reference stock solution by serial dilution with methanol.

2.2.7.6 Precision and accuracy

A standard addition method was used for determining precision and accuracy of the method. Known concentrations of standards at three levels (40.0 µg/mL, 80.0 µg/mL and 120.0 µg/mL) were added to a known volume of sclerotial sample solution. Each concentration was done in three replicates. The resulting solution was transferred into a separating funnel and exposed to the same extraction procedure as described in section 2.2.7.1. Precision and accuracy were calculated as a percentage of relative standard deviation and recovery, respectively.

2.2.7.7 Detection limit (DL)

The DL was determined by two methods such as signal-to-noise (S/N) ratio and a standard deviation of the response and the slope.

2.2.7.7.1 S/N ratio method

The S/N ratio was determined by measuring a baseline noise of sample solution and compared to known concentrations of standard. The minimum concentration at which the standard could be reliably detected was established at S/N ratio 3:1 or 2:1 as explained in section 3.7.5.

2.2.7.7.2 Standard deviation of the response and the slope method

The DL may be expressed as:

$$DL = \frac{3.3\sigma}{S} \qquad \text{(Eq.1)}$$

Where, σ = the standard deviation of the response and S = the slope of the calibration curve.

2.2.7.8 Quantification limit (QL)

Both approaches of DL were applied to QL except for the minimum concentration. This was the standard concentration could be reliably quantified at S/N ratio of 10:1. The QL the second approach may be expressed as:

$$QL - \frac{10\sigma}{S} \qquad \text{(Eq.2)}$$

Where, σ = the standard deviation of the response and S = the slope of the calibration curve

2.2.7.9 Stability of analytical solutions

A sclerotial sample solution (section 2.2.7.1) and a standard solution (80.0 µg/mL) were used and analysed over a period of 24 hours in two hour intervals.

2.2.7.10 HPLC analysis

Each of solutions was analysed using the operating conditions as listed in Table 2.13.

***Table 2.13** The HPLC operating conditions used to validate the analytical method.*

HPLC system	Parameter
Column	Waters, Symmetry Shield C_8 (5.0 µm, 150 x 4.6 mm)
Flow rate	1.0 mL/min
Column temperature	40 °C
Wavelength	280 nm
Injection volume	50 µL
Mobile phase	92% (v/v) of methanol in water

2.2.7.11 Robustness

Variations of mobile phase compositions, temperatures and flow rates, different brand columns, HPLC instrumentations and days were carried out to evaluate the robustness of the analytical method and are listed in Table 2.14.

Table 2.14 *Variations of HPLC conditions used.*

Condition	Variation
Column	Kromasil, C_8, (5.0 µm, 150 x 4.6 mm) Waters Symmetry Shield, C_8, (5.0 µm, 150 x 4.6 mm) Zorbax , C_8, (5.0 µm, 150 x 4.6 mm)
Mobile phase	90% (v/v) of methanol in water 92% (v/v) of methanol in water 95% (v/v) of methanol in water
Column temperature	Ambient and 40 °C
Flow rate	0.8 mL/min and 1.0 mL/min
HPLC	Hewlett Packard HPLC 1050 and 1090

2.2.8 Determination of ergosterol in different parts of L. rhinocerus

Sample solutions of sclerotia, stalks and caps of *L. rhinocerus* were prepared in triplicate as described in section 2.2.7.1 and analysed using HPLC conditions as listed in Table 2.13.

2.2.9 Chromatographic fingerprint of fungal sterols of L. rhinocerus

Sample solutions of *L. rhinocerus* (section 2.3.8) and five commercial products solutions (Reishi, Maitake, Coriolus, Long Heh and Felyoung) were prepared as described in section 2.2.7.1 and standard solutions (80.0 µg/mL) were analysed using a HPLC method as shown

in Table 2.15. The obtained chromatograms were compared to generate a suitable chromatographic fingerprint that could be of chemotaxonomic significance to *L. rhinocerus* species.

Table 2.15 *HPLC operating conditions used to produce a chromatographic fingerprint of fungal sterol.*

HPLC system	Parameter
Column	Waters, C_{18} (5.0 µm, 150 x 4.6 mm)
Flow rate	1.0 mL/min
Column temperature	40 °C
Wavelength	280 nm
Injection volume	50 µL
Mobile phase	95% (v/v) of methanol in water containing 0.02% (v/v) trifluoroacetic acid (TFA)

2.2.10 Screening method of ergocalciferol

Sample solutions of sclerotia, stalks and caps (section 2.3.8), ergosterol and ergocalciferol standard solutions (both at concentration of 80.0 µg/mL), and a mixed standard solutions were analysed by TLC. Each solution was spotted (20 µL) on the TLC plate that was placed in a tank containing saturated vapour of 15% (v/v) of acetone in hexane (100 mL) and covered. The plate was allowed to develop for about one hour. The plate was left to dry for a few minutes before inspecting the spots under UV irradiation at 254 nm and 365nm.

2.2.11 Screening method of phenolic acid compounds (PACs)

2.2.11.1 Preparation of solutions

2.2.11.1.1 Phenolic solution

A grated sample of sclerotia (2.0 g) was transferred into a 200 mL beaker and distilled water (50 mL) was added. The mixture was boiled for one hour and was cooled to room temperature before filtered through a filter paper (Whatman® 1) into a separating funnel. $NaHCO_3$ (1 M) solution (10 mL) was added into the filtrate and left for 30 minutes. The solution was then extracted with Et_2O (30 mL) and the ether layer was transferred into evaporating dish. The extraction was repeated for another two times and the ether layers were combined and was evaporated to dryness. The residue was then dissolved in 4 mL of methanol.

2.2.11.1.2 Aromatic carboxylic acid solution

The procedure was similar to (a) except that 1 M of NaOH solution was added into the filtrate

2.2.11.1.3 Reference stock solutions (100.0 µg/mL)

Phenol, benzoic acid and salicylic acid (10.0 mg) were transferred separately into a 100 mL volumetric flask, dissolved and filled to the mark with methanol.

2.2.11.2 Colour test using 1% w/v aqueous iron (III) chloride ($FeCl_3$) solution

The boiled sample solution as prepared in section 2.2.11.1.1, without adding NaOH or $NaHCO_3$ solution and without the extraction was used as a sample solution. Two drops of $FeCl_3$ solution was added into the sample and phenol standard solution (2 mL). The colour intensity of standard and sample solutions was observed.

2.2.11.3 UV/Vis spectroscopic analysis

The sample solutions (section 2.2.11.1.1 and 2), reference solution (2.2.11.1.3) and a blank solution (distilled water) were transferred separately into a 1 mL plastic cuvette. The solutions were subjected to spectroscopic scanning over the range from 200 nm to 400 nm (Table 2.5).

2.2.11.4 HPLC analysis

The HPLC method used to quantify the amount of phenolic and aromatic acid compounds that had been removed from each extraction of sample solution is given in Table 2.16.

Table 2.16 *The HPLC operating conditions used to quantify the amount of phenolic and aromatic compounds removed.*

HPLC system	Parameter
Column	Zorbax, C_8 (5.0 µm, 250 x 4.6 mm)
Flow rate	1.0 mL/min
Wavelength	220 nm, 280 nm and 320 nm
Injection volume	50 µL
Mobile phase	92% (v/v) of methanol in water

2.2.12 Determination of total content of PACs

2.2.12.1 Preparation of solutions

2.2.12.1.1 Free PACs solution

A sample (10.0 g) was transferred into a 200 mL beaker containing a solution of 20% (v/v) of water in methanol (50 mL). The mixture was stirred for one hour, sonicated for 30 minutes and centrifuged at 4500 rpm for 5 minutes. The obtained supernatant (30 mL) was

extracted with Et_2O (30 mL). The ether layer was transferred into an evaporating dish. The extraction was repeated for another two times and the ether layers were combined and was evaporated to dryness. The residue was then dissolved in 3 mL of methanol.

2.2.12.1.2 Bound PACs solution

The remaining aqueous solution from section 2.2.12.1.1 (30 mL) was transferred into a round flask containing 2 M NaOH (20 mL) solution and methanol (10 mL). The solution was then heated at reflux for one hour and the resultant solution was acidified to pH 2 using dilute HCl and then extracted with Et_2O (30 mL). The rest of procedure was the same as described in section 2.2.12.1.1.

2.2.12.1.3 Gallic acid stock solution (1000.0 µg/mL)

Gallic acid (100.0 mg) was transferred accurately into a 100 mL volumetric flask, dissolved and the volume made up with methanol.

2.2.12.1.4 Diluted reference solutions for calibration curve

Five dilutions of the gallic acid stock solution (1000.0 µg/mL) in methanol were prepared to yield reference concentrations of 50.0 µg/mL, 100.0 µg/mL, 150.0 µg/mL, 200.0 µg/mL and 250.0 µg/mL.

2.2.12.2 UV/Vis spectroscopic analysis

Each of the sample solutions (1 mL) and diluted standard solutions (section 2.2.12.1.4) (1 mL) were added separately into a 10 mL cylindrical glass containing 5 mL of Folin-Ciocalteu reagent and 4 mL of 7.5% (w/v) Na_2CO_3 solution. The mixture was agitated and

left to stand for two hours for the reaction to take place and stabilize. The resulting solutions were filtered and analysed at a wavelength of 765 nm using a 10 mm quartz cuvette. The amount of free and bound PACs was determined from calibration standard solutions. The total content of PACs for each part of *L. rhinocerus* was determined by addition of the amount of free and bound PACs.

2.2.13 Chromatographic fingerprint of PACs

2.2.13.1 Preparation of solutions

2.2.13.1.1 Sample solution

The sample solutions were made from three different parts of *L. rhinocerus* (caps, stalks and sclerotia) and the commercial products. The sample went through the same preparation as described in section 2.2.12.1.1.

2.2.13.1.2 Acidic solution

The remaining part of the aqueous solution from section 2.2.4.2 (Preaparation 2, see Figure 2.2) was acidified to pH 2 using dilute HCl and subjected to extraction as described in section 2.2.12.1.1.

2.2.13.1.3 Reference solutions

Phenolic acids standards (gallic acid, vanillic acid, tyrosol, caffeic acid, chlorogenic acid, *p*-coumaric acid, and cinnamic acid) (100.0 μg/mL) were prepared accurately with 50% (v/v) of methanol in water.

2.2.13.2 HPLC analysis

The HPLC conditions used to generate chromatographic fingerprint of PACs is given in Table 2.17.

***Table 2.17** The HPLC conditions used for the chromatographic fingerprint of PACs.*

HPLC system	Parameter
Column	Pentaflluorophenyl propyl (PFP), (3.0 µm, 150 x 4.6 mm)
Flow rate	0.5 mL/min
Wavelength	280 nm and 320 nm
Injection volume	20 µL
Mobile phase	35% (v/v) of methanol in water with 0.2% (v/v) of formic acid

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Moisture contents

The results of moisture content in the sample materials were found to be 13.39% w/w by lose on drying (LOD) method and 10.08% w/w by Karl Fischer titrimetry method and are presented in Table 3.1 and Table 3.2, respectively.

Table 3.1 *The moisture content result by LOD method.*

	Sample A	Sample B
Before drying		
Weight of container (g)	31.98	34.35
Weight of container and sample (g)	33.63	36.22
Weight of sample (g)	1.65	1.87
After Drying (19 hours)		
Weight of container and sample (g)	33.42	36.01
After drying (20 hours)		
Weight of container and sample (g)	33.41	35.97
After drying (21 hours)		
Weight of Container and sample (g)	33.41	35.97
% Loss on drying = weight lost (g) / Weight of sample (g) x 100	0.22 / 1.65 X 100 = 13.33	0.25 / 1.87 X 100 = 13.37
Average % (w/w) of water	13.35	

Table 3.2 *The moisture content result by Karl Fischer titrimetry method. F: water equivalence factor; V: titer volume; W: weight of sample.*

(a) Standardization of KFR	Standard 1	Standard 2
Weight of syringe and water (mg)	7542.89	7543.00
Weight of empty syringe (mg)	7532.86	7533.00
Weight of water (mg)	10.03	10.00
Titer (mg/ml)	4.83	5.06
Average Titer, F (mg/mL)	4.94	
(b) Water content	Sample A	Sample B
Weight of beaker and sample (mg)	6077.75	6094.73
Weight of empty beaker (mg)	5919.26	5918.48
Weight of sample, W (mg)	158.49	176.25
KFR, V (mL)	2.92	3.94
% of Water = (F x V x 100)/W	9.11	11.05
Average % (w/w) of water	10.08	

The result of moisture content was considerably high for an air-dried fungus. These results were expected to be higher since the sclerotial fungi have the ability to rehydrate rapidly in the presence of moisture or water. This was reported by Trevethick and Cooke, (1973) where oven-dried sclerotia incubated above a free water surface attained a water content of 15.0% - 21.0% within 24 hours. The result of LOD was also similar to the previous study by Wong *et al.*, (2003), concerning the moisture content of *P. rhinocerus*. However, some other factors such as the interferences of Karl Fisher reagent with chemical

constituents and volatile compounds of *L. rhinocerus* may have also contributed to high moisture content.

The LOD method was time consuming. A constant weight was obtained after 21 hours, when the difference between two successive weighings corresponded to less than 0.05% (w/w) of sample taken (USP, 2009). Furthermore, the dried sclerotia may have been exposed to atmospheric moisture during the weighing process and this could have contributed to rehydration. The Karl Fischer titrimetry method was found to be fast and simple. The result obtained was also more reliable and exact than the LOD method. A blank determination (standardization) was used to eliminate residual water from the measurement cell. A suitable amount of water was titrated and the water equivalent was determined to achieve the desired accuracy (BP and USP, 2009). Furthermore, the amperometric end-point was clearly observed as a change in colour, i.e. from colourless to yellow. In addition, the apparatus for this titrimetric method was commercially available, generally comprised of a closed system and was kept dry with silica beads to prevent atmospheric moisture from entering the system.

3.2 Total protein content

The total protein content of the dried sclerotial *L. rhinocerus* was found to be 2.84% w/w. The result was obtained from the standard calibration curve as is shown in Figure 3.1 and was in agreement with the result reported by Wong *et al.*, (2003). The total-protein was considered to be a general test, but it was a useful characteristic of a fungus, which could therefore be used for quality control purposes. In order to assess how useful this test was, it would be necessary to consider some other factors such as sample batch, age of the fungi and growing condition.

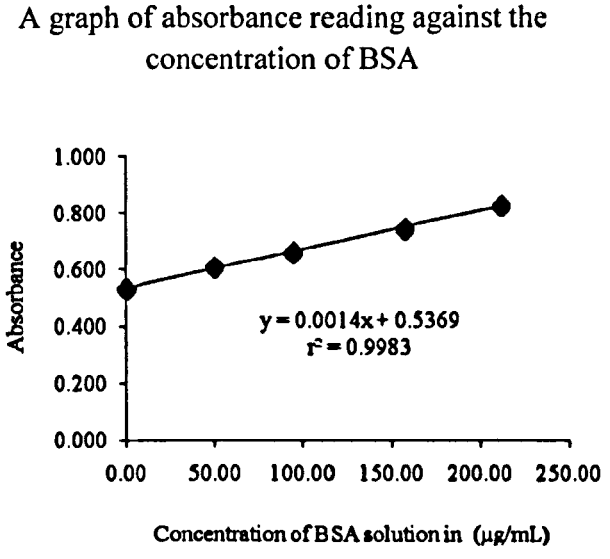


Figure 3.1 The calibration standard curve of BSA concentrations.

3.3 Screening of simple extractable compounds

3.3.1 TLC analysis

A simple acid-base extraction was used to extract biochemical compounds of sclerotial *L. rhinocerus.* The TLC chromatograms obtained after water extraction (WS) and acid-base extractions (FF, AC and BC, refer to Figure 2.1, page 42) are shown in Figure 3.2. One main black spot at R_f 0.92 under 254 nm irradiation and a fluorescent spot under 365 nm irradiation was detected in all solutions except for the WS solution. The WS solution (water extraction) represented a commonly used preparation method used for traditional herbal medicines which is a decoction process. As a result, a solvent was introduced to extract the biochemical compounds of *L. rhinocerus.*

Dichloromethane was selected as the solvent for extraction because it fulfilled all the main requirements: immiscibility with water, different density to water, good solubility and volatility and less toxicity than chloroform (Harwood *et.al.*, 1999). All extracted residues were reconstituted using methanol since it was a good all-purpose solvent for preliminary work as suggested by Harborne, (1973). The detected spots from FF, AC and

BC solutions turned to brown spots after being sprayed with 50% ethanolic sulfuric acid and gently heated. These brown spots indicated the presence of organic compounds while the fluorescent compound showed the possible presence of an aromatic system (Rouessac and Rouessac, 2000).

Figure 3.2 also shows that more spots were obtained from the FF solution when compared to the AC and BC solutions, suggesting that more free base form were readily extracted using dichloromethane than acidic and basic compounds. The FF solution was analysed using the same TLC conditions but using different spotted volumes (10 µL – 50 µL). Four spots were clearly observed using a spotted volume of 30 µL as illustrated in Figure 3.3. The FF solution was then used for UV full scan and HPLC analysis.

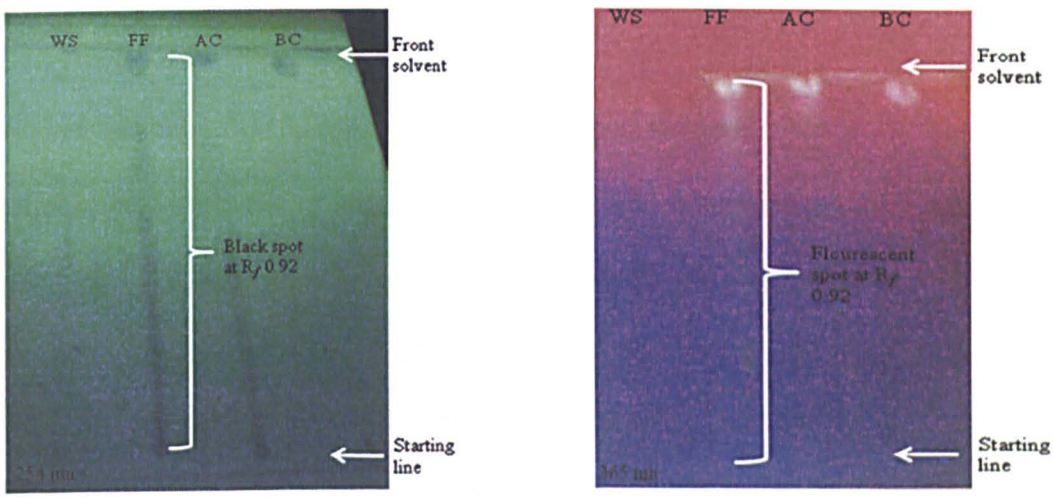
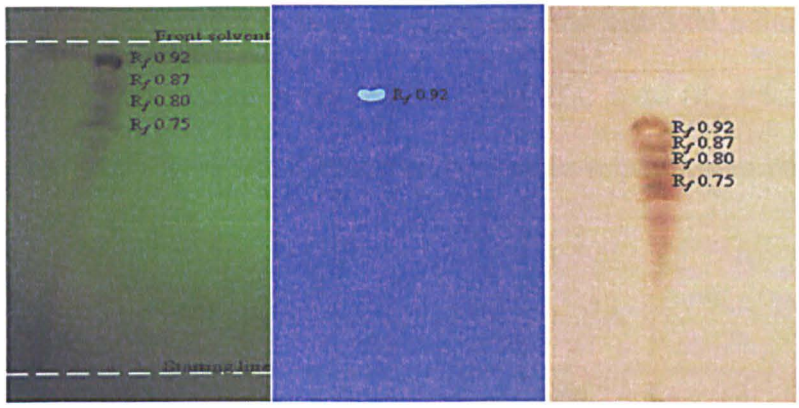


Figure 3.2 *Chromatograms of water-soluble (WS), free-form (FF), acidic (AC) and basic compounds (BC) solutions (extraction method; see Figure 2.1, page 42) after developed in the mobile phase of 20% (v/v) methanol-chloroform.*



(a) *(b)* *(c)*

Figure 3.3 *Chromatograms of FF solution after developed in 20% (v/v) methanol-chloroform as the mobile phase; spots were observed (a) under UV 254 nm (R_f: 0.92, 0.87, 0.80 and 0.75), (b) one blue fluorescent spot under UV 365 nm (R_f: 0.92) and (c) after sprayed with 50% v/v ethanolic sulfuric acid.*

3.3.2 UV/Vis Spectroscopy

A full scan spectrum obtained from the FF solution is shown in Figure 3.4. The spectrum exhibited a maximum absorption (λ_{max}) at 287 nm and a broad absorption band between 300 nm and 400 nm. The λ_{max} at 287 nm may imply the presence of conjugated chromophores; molecules containing the electrons responsible for the absorption such as π-bonded electrons (double bonds). As the number of double bonds in conjugation increases, the wavelength also increases (Williams and Fleming, 1995). Consequently, the broad band between 300 nm and 400 nm may indicate the existence of small amount conjugated chromophores such as polyenes, substituted benzene rings and polycyclic aromatic hydrocarbons in the FF solution.

3.3.3 HPLC analysis

The obtained chromatogram from the isocratic NP-HPLC method of FF solution is given in Figure 3.5. The chromatogram displayed two resolved peaks at 4.28 minutes and 10.16

minutes and unresolved peaks at RTs between 2.50 minutes and 3.60 minutes. These unresolved peaks may correspond to the close position of TLC spots (R_f; 0.92, 0.87, 0.80 and 0.75) as illustrated in Figure 3.3. Consequently, further improvement on separation using NP-HPLC was required.

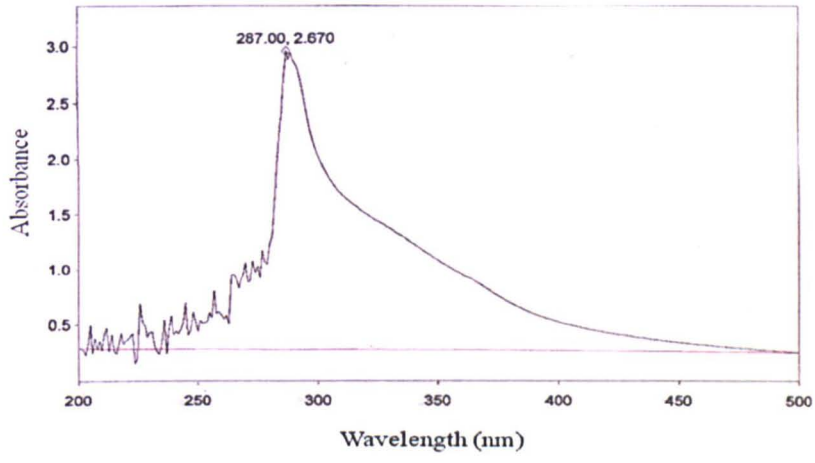


Figure 3.4 *The UV spectrum of FF solution from Extraction 2 (see Figure 2.1, page 42) using the UV scanning method as illustrated in Table 2.5, page 44.*

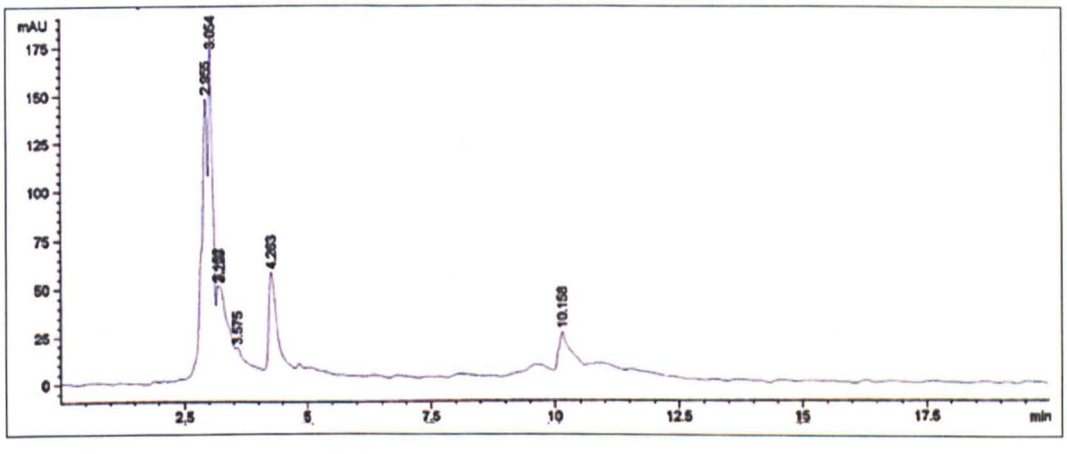


Figure 3.5 *The chromatogram of FF solution (Extraction 2, Figure 2.1) analysed using the isocratic NP- HPLC method as described in Table 2.6.*

Chromatograms of FF solution analysed using the gradient RP-HPLC method are depicted in Figure 3.6. Three wavelengths, 254 nm, 280 nm and 303 nm were chosen based on the UV spectrum (Figure 3.4). In addition, the wavelength at 254 nm is the common wavelength to detect organic compounds and is used to view TLC spots. Some peaks were eluted at the early retention times (RT) between 1.50 minutes and 3.00 minutes using 5% (v/v) methanol-water as the mobile phase composition. No peak appeared as the composition of methanol was increased to 85% (v/v). Some peaks started to resolve at RTs between 28.00 minutes and 35.00 minutes at 90% (v/v) methanol-water. The chromatogram (Figure 3.6) revealed two major characteristics of compounds present in the FF solution. The first characteristic was very polar compounds represented by the early RTs and the second was slightly non polar compounds (eluted at the end of analysis). Hence, extended analyses were carried out to look into the best choice of solvent, columns and mobile phase compositions (see Method Development section 3.4) that were able to extract and separate most of compounds contained in the sclerotia of *L. rhinocerus*.

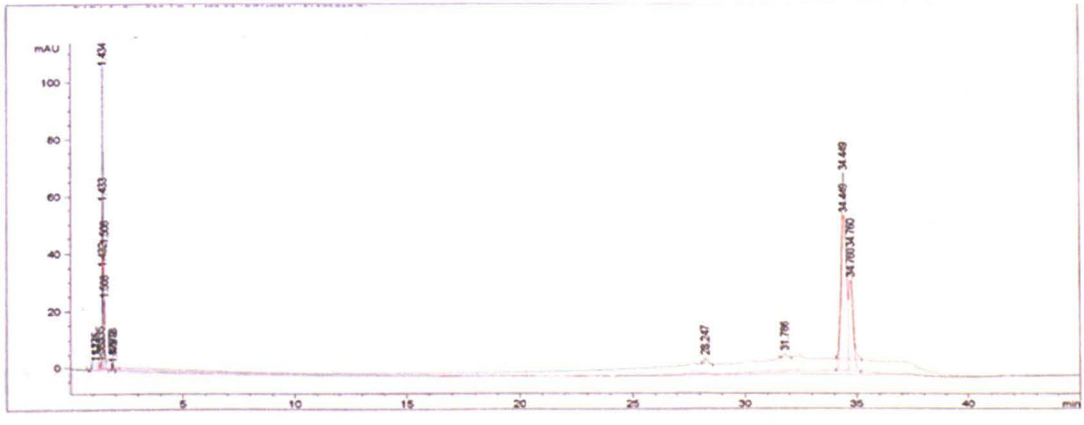


Figure 3.6 *Chromatograms of FF solution (Extraction 2, Figure 2.1) analysed using the gradient RP-HPLC method as described in Table 2.7.*

3.4 Method development

3.4.1 Sample preparations

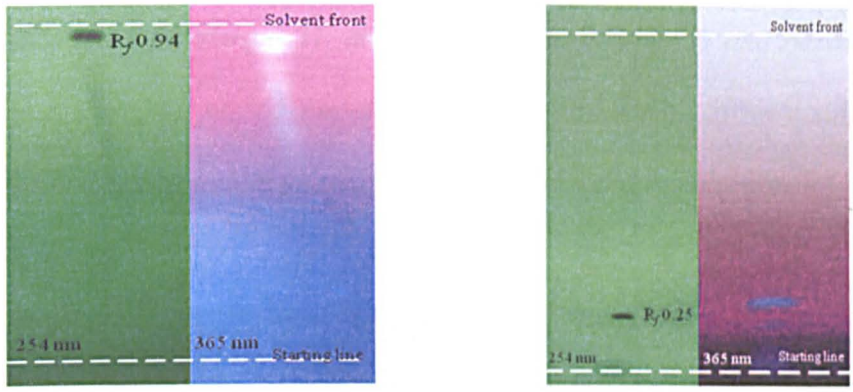
Preparation 1 (section 2.2.4.1 and see Figure 2.2, page 46) was employed by Yuan *et al.*, (2003), while Preparation 2 was a modified method based on reported methods in the literature. A NaOH solution (instead of Na_2CO_3 solution) was used in the second preparation to break free any bound compounds, such as esterified compounds (Weete, 1989). The anhydrous Na_2SO_4 was replaced with distilled water to remove water-soluble compounds which were separately analysed in PACs section (section 2.2.13.1.2). The GAA used to reconstitute the dried organic extract residue was replaced with methanol. This was based on the fact that the solution representing reconstitution with GAA took longer time to dry and produced a large spot at the starting line on the silica plate.

Many organic reactions are run at elevated temperatures and are complete within a convenient time scale (Harwood et al., 1999). As a result, different solvents, refluxing times (30 minutes, 1 hours and 2 hours), temperatures (50 °C and 80 °C) and NaOH molarities (0.05 M, 0.5 M and 1.0 M) were examined to find the best conditions to yield more biochemical compounds of *L. rhinocerus.* Preparation 2 was found to represent optimum conditions; methanol as the solvent extraction, refluxing time for 1 hour at 80 °C using 0.5 M methanolic NaOH. The selected conditions were optimized and supported by TLC and HPLC analysis as shown below.

3.4.2 TLC analysis

The Yuan *et al.*, (2003) method employed Preparation 1 (section 2.2.4.1 and Figure 2.2) and two different mobile phase compositions; polar (5% v/v of methanol in chloroform) and non-polar (10% v/v of acetone in hexane). The TLC chromatograms obtained from this method are represented in Figure 3.7 (a) and (b). According to Moffat (1986) the separation of organic compounds is the movement of compounds across the plate at

different rates depending on their polarities, stationary phases and mobile phase compositions. Polar organic compounds moved faster across silica plate with polar mobile phase compositions and moved slower with non polar mobile phase. One main spot at R_f 0.94 (polar compound) was clearly seen using 5% (v/v) of methanol in chloroform as the mobile phase. However, the previous screening method (Figure 3.3) gave better separation (four spots had been detected) than this method suggested by Yuan *et al.*, (2003). Consequently, this may imply that the Yuan method may not suitable to extract most of organic compounds from sclerotial *L. rhinocerus*. Hence, Preparation 1 was then modified to Preparation 2.

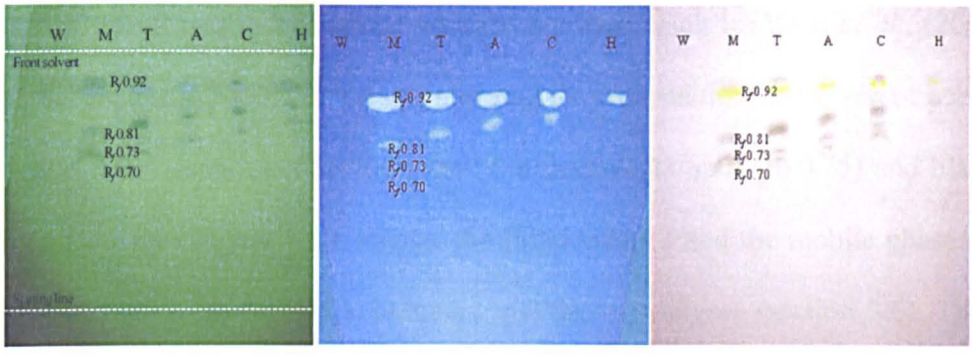


(a) *(b)*

Figure 3.7 *Chromatograms of Preparation 1 using mobile phase of (a) 5% (v/v) of methanol in chloroform and (b) 10% (v/v) of acetone in hexane.*

Six solvents; methanol, water, tetrahydrofuran, acetone, chloroform and hexane, were employed and methanol turned out to be the best solvent for extraction. Four compounds were separated (at R_f 0.92, 0.81, 0.73 and 0.70) using methanol as displayed in Figure 3.8. The Preparation 2 and methanol as the extraction solvent, was once again applied to different parts of *L. rhinocerus* (stalks and caps). Two different mobile phase compositions; polar (5% v/v of methanol in chloroform) and non-polar (30% v/v of acetone

in hexane) were employed and the chromatograms are illustrated in Figure 3.9 and Figure 3.10, respectively.



(a) *(b)* *(c)*

Figure 3.8 *TLC chromatograms of Preparation 2 using six different solvents; methanol (M), water (W), tetrahydrofuran (T), acetone (A), chloroform (C) and hexane (H), were viewed (a) at 254 nm, (b) at 365 nm and (c) after sprayed with 50%v/v sulfuric acid in ethanol and heated, using mobile phase of 5% v/v of methanol in chloroform.*

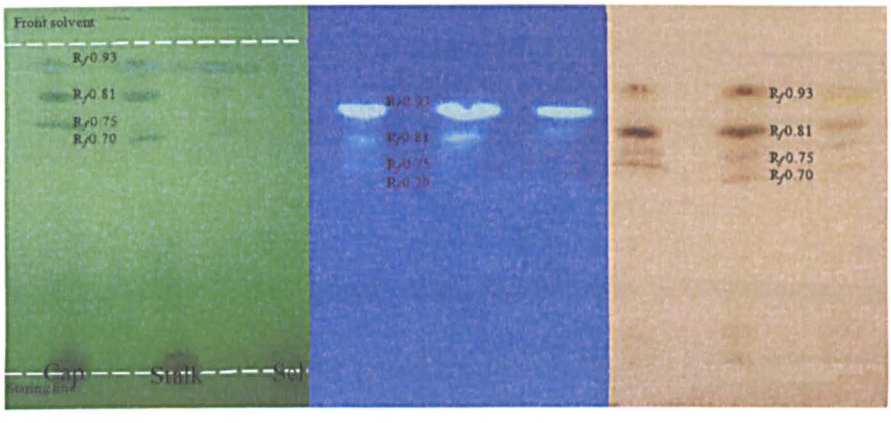


Figure 3.9 *TLC chromatograms of cap, stalk and sclerotia (Scl) solutions prepared using Preparation 2 were viewed under UV 254 nm and 365 nm, and after sprayed with 50% v/v sulfuric acid in ethanol and heated, using the mobile phase of 5% v/v methanol-chloroform.*

A blue fluorescent spot was viewed under 365 nm and turned to yellow after being sprayed with 50% v/v sulfuric acid in ethanol and gently heated. This provided some indication that ergone (R_f0.75, Figure 3.10) may have been present in all parts of the fungi since the TLC result was in agreement with the reported result by Yuan *et al.*, (2003) and Cole and Schweikert, (2003). The non-polar mobile composition (30% v/v of acetone in hexane) gave better separation between the blue fluorescent spot (R_f0.75) and black spot (R_f0.55) as shown in Figure 3.10. Hence, the Preparation 2 and the mobile phase of 30% v/v acetone-hexane were used in subsequent qualitative analyses (section 3.6). This TLC result also verified that apart from sclerotium these biochemical compounds also existed in stalks and caps.

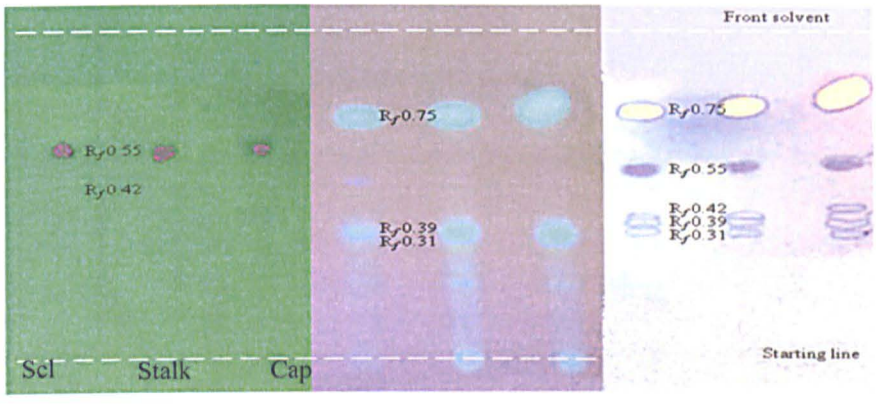


Figure 3.10 *TLC chromatograms of cap, stalk and sclerotia (Scl) solutions prepared using Preparation 2 were viewed under UV 254 nm and 365 nm, and after sprayed with 50% v/v sulfuric acid in ethanol and heated, using the mobile phase of 30% v/v acetone-hexane.*

3.4.3 HPLC analysis

The Preparation 1 (section 2.2.4.1), C_{18} column (5.0 µm, 250 x 4.6 mm), a mobile phase of 98% (v/v) of methanol in water and flow rate 1.0 mL/min were utilized in Yuan *et al.*, (2003). The resulting chromatograms for this method are depicted in Figure 3.11. Three

wavelengths, 254 nm, 280 nm and 365 nm, were employed to find the best wavelength for sensitivity and quantification. There were unresolved peaks detected at early RTs (2.00-6.00 minutes) for all the three wavelengths. Three peaks were well resolved at RTs of 15.36 minutes (Peak 4), 17.64 minutes (Peak 5) and 19.77 minutes (Peak 6) when using a wavelength of 254 nm. Peaks 4 and 5 were detected with high intensity at 365 nm and 280 nm, respectively. The run time of analysis was 40 minutes but no peak was obtained after 20 minutes after the last peak (Peak 6).

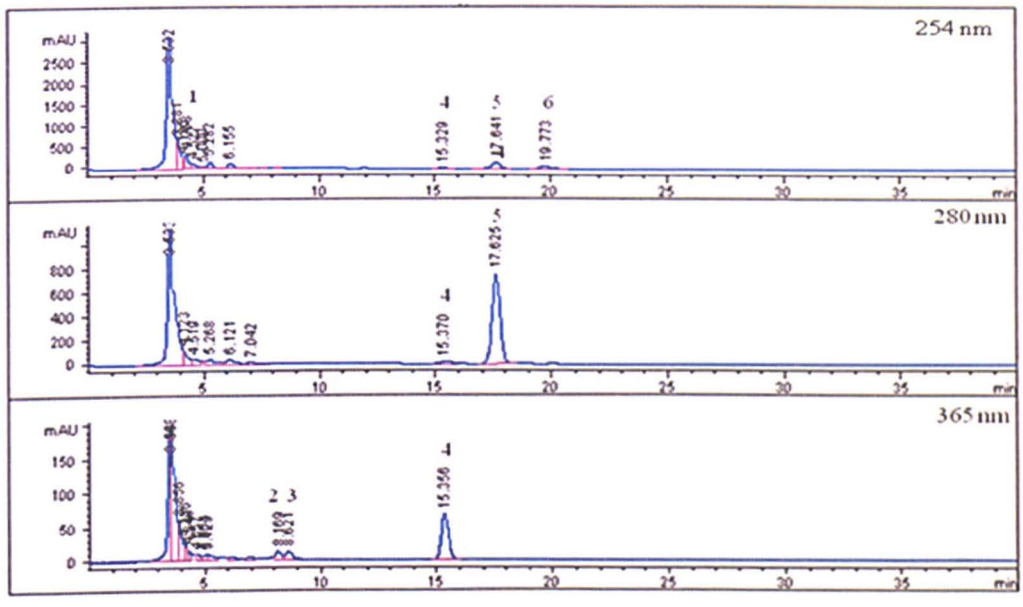


Figure 3.11 *Chromatograms obtained from Preparation 1 using RP-HPLC method; C_{18} column (5.0 µm, 250 x 4.6 mm), 98% (v/v) of methanol in water as mobile phase and at flow rate of 0.8 mL/min.*

The sample solution was also analysed using an isocratic NP-HPLC method as described in Table 2.6. The chromatogram showed unresolved peaks at the early RTs and one small broad peak at 10.23 minutes. Hence, an isocratic RP-HPLC was found to be a suitable method to separate most of compounds when compared to the gradient and NP-HPLC method. Figure 3.12 shows chromatograms generated from Preparation 2 without

treatment with NaOH solution and heating time was 30 minutes at a temperature of 50 °C. There was a reduction of peak height at the early RTs, especially at a detection wavelength of 280 nm. The peak height of Peak 5 was significantly improved at 280 nm and followed by Peak 4 and 6 at 365 nm and 254 nm, respectively. Hence, this result suggested that impurities could be minimised in extractable compounds by washing the sample with distilled water instead of Na_2CO_3 solution. These chromatograms also gave an indication that Peaks 4, 5 and 6 were free-form compounds which did not require NaOH treatment to induce release from the bound-form and could readily be extracted. The analysis was repeated with different conditions of sample preparation and the results are summarized in Table 3.3.

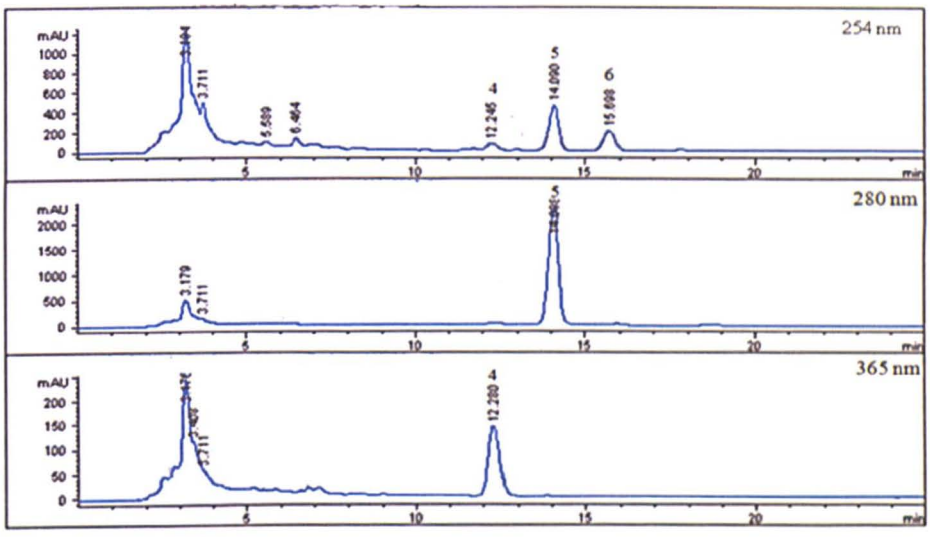


Figure 3.12 *Chromatograms obtained from the sample solution without treatment with NaOH solution, 30 minutes of refluxing time and temperature at 50 °C using the RP-HPLC method; C_{18} column (5.0 µm, 250 x 4.6 mm), mobile phase of 95% (v/v) methanol-water and flow rate at 1.0 mL/min.*

Table 3.3 *Different conditions for sample preparation and its results.*

Refluxing hour	Temperature	Molarity	Result
30 minutes	50 °C and 80 °C	0.05 M, 0.5 M, 1.0 M	Chromatographic profile as described in Figure 3.12.
1 hour	50 °C and 80 °C	0.05 M	Chromatographic profile similar to Figure 3.12.
	50 °C	0.5 M, 1.0 M	Peak 1, Peak 2 and 3 were traced at 254 nm and 365 nm.
	80 °C	0.5 M	Chromatographic profile as described in Figure 3.13.
		1.0 M	Precipitation was formed and emulsion was produced during extraction.
2 hours	50 °C	0.05 M	Chromatographic profile similar to Figure 3.12.
	80 °C	0.5 M 1.0 M	Precipitation was formed and emulsion was produced during extraction.

Chromatograms (Figure 3.13) were obtained from Preparation 2; treated with 0.5 M methanolic NaOH solution, 1 hour heating at reflux and temperature at 80 °C. Peaks 1 - 3 became visible, indicating that the compounds were present in bound-form and were released by alkaline hydrolysis. As mentioned previously, these conditions (Preparation 2) were found to be optimal for sample preparation and were used throughout the research to analyse fungal sterols of *L. rhinocerus*. In addition, there was one main peak (Peak 5) significantly detectable at 280 nm that could be isolated using HPLC fractionation.

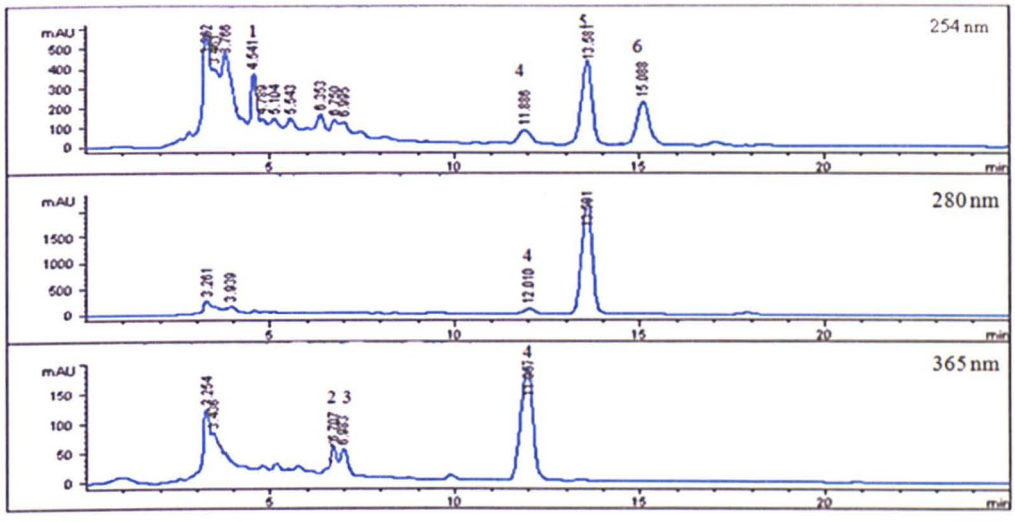


Figure 3.13 *Chromatograms obtained from Preparation 2; treated with 0.5 M NaOH solution, 1 hour of refluxing time and temperature at 80 °C, using RP-HPLC method; C_{18} column (5.0 µm, 250 x 4.6 mm), mobile phase of 98% (v/v) methanol-water and flow rate at 1.0 mL/min.*

3.5 Fractionation and isolation methods

3.5.1 Fractionation using HPLC method

The C_{18} column (5.0 µm, 250 x 4.6 mm) was substituted with C_8 column (5.0 µm, 150 x 4.6 mm) and used to fractionate Peak 5. The run time of analysis using the substituted column was shortened from 14.00 minutes to 6.00 minutes. The Peak 5 eluted at RT of 4.87 minutes (Figure 3.14) and was collected and concentrated. The concentrated solution was injected to evaluate the reproducibility and repeatability of the peak. The peak eluted at 4.84 ± 0.01 minutes indicating that the RT was reproducible. Good repeatability was achieved with %RSD values of 0.77% and 0.18% for peak areas and RT, respectively, as shown in Table 3.4.

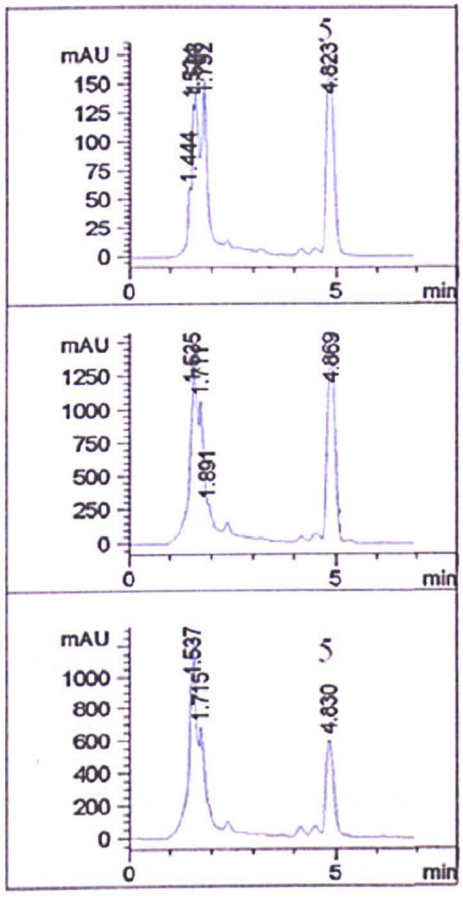
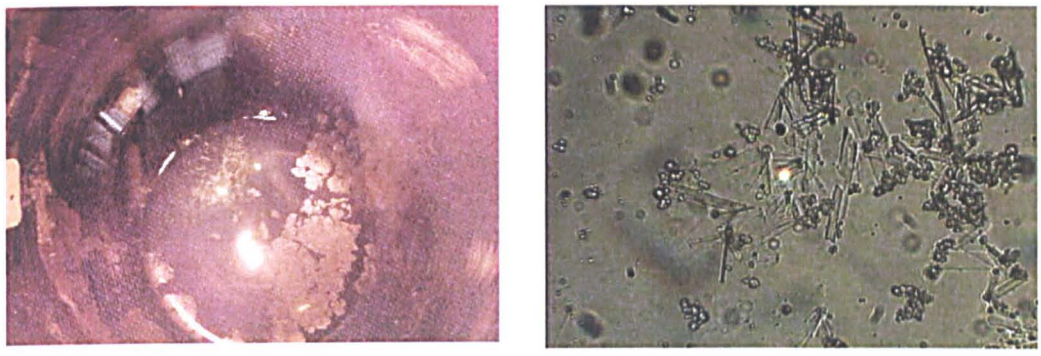


Table 3.4 *The repeatability of peak area and retention times (RTs).*

Injection	Peak area	RTs
1	3786.57	4.85
2	3788.23	4.84
3	3849.83	4.84
4	3841.31	4.83
5	3841.62	4.83
6	3843.74	4.83
Std. Dev.	29.46	0.01
Average	3825.21	4.84
%RSD	0.77	0.18

Figure 3.14 *The reproducible peaks at 280 nm, using Preparation 2 and the isocratic RP-HPLC as described in Table 2.9.*

The peak fraction was collected manually where the outlet tubing from the HPLC detector was directed towards an evaporating dish. The fraction collection was carried out between 4.10 and 5.1 minutes and about 15 mL of combined fractions were collected and were concentrated. Figure 3.15 (a) shows the presence of elongated white needless after slow solvent evaporation. A small amount of crystal formation was obtained and viewed under a polarizing microscope (Figure 3.15 (b)). The obtained weight of the residue was 7.29 mg or 0.15 mg/g of dried sclerotial *L. rhinocerus*.

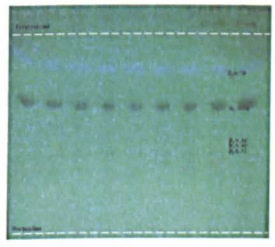


(a) *(b)*

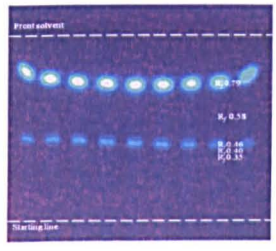
Figure 3.15 *(a) White needles became visible after solvent evaporation; (b) crystal formation under the polarizing microscope.*

3.5.2 Isolation using TLC method

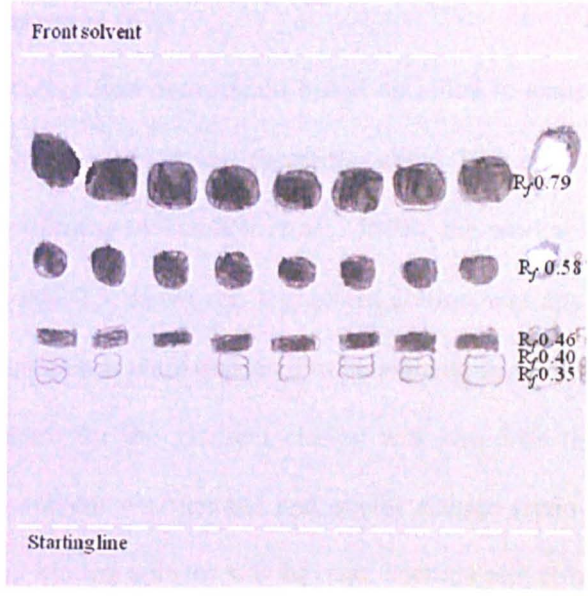
Extra precautions were taken during scraping of the spotted TLC plate since silica materials may cause irritation to skin, eyes, and respiratory tract. This work was done in a fume hood and gloves and mask were worn as protection. Figure 3.16 shows the TLC spots at R_f 0.79, 0.58, 0.46, 0.40 and 0.35 for sample solution which was prepared using Preparation 2. Two spots at R_f 0.79 and 0.58 were scraped while spots at R_f 0.46, 0.40 and 0.35 were too close to each other and hence were difficult to isolate. A yellow residue was generated from the spot at R_f 0.79 while a white residue was obtained from spot R_f 0.58 after evaporating the solvent. The weights for yellow and white residues were about 1.24 mg and 2.30 mg, respectively. These residues were dissolved in 2 mL methanol and analysed using the isocratic RP-HPLC method (Table 2.15). The chromatogram showed that the white and yellow residue corresponded to Peaks 5 and 4, respectively (Figure 3.17).



(a)



(b)



(c)

Figure 3.16 *TLC chromatograms prepared using Preparation 2 were developed in 30% v/v acetone-hexane of mobile phase and viewed under UV (a) 254 nm, (b) 365 nm and (c) the scraped spots at R_f 0.79 and 0.58.*

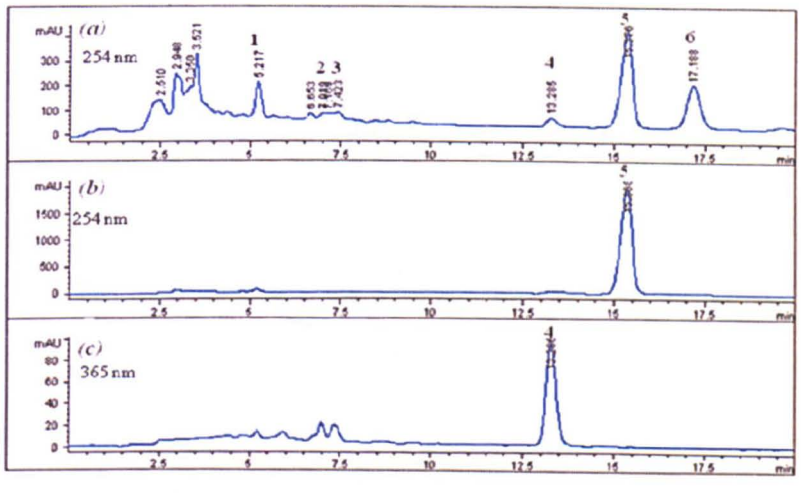


Figure 3.17 *Chromatograms of (a) sample solution (Preparation 2), (b) white needles (Peak 5) and (c) yellow residue (Peak 4) analysed using RP-HPLC method; C_{18} column (5.0 µm, 250 x 4.6 mm), mobile phase of 92% (v/v) methanol-water and flow rate at 1.0 mL/min.*

3.5.3 Isolation using flash chromatography (FC)

The amount of silica used in this process was determined based on silica to sample ratio 30:1 w/w. The yield of crude sample was 0.31 g and therefore, about 10.0 cm^3 of dried silica gel was measured and used. According to Tatchell *et al.*, (1991), the workable range of R_f for FC should be between 0.2 and 0.3. However, a gradient elution was applied for this process since more than three compounds were expected to be isolated from the sample solution. The selection of mobile phase for the gradient elution was based on this TLC result (Table 3.5). The first elution solvent was hexane and acetyl acetate (ratio 3:1) to isolate Spot 1 and 2 while the second elution solvent was hexane, acetone and chloroform (ratio 6:3:1) to isolate Spots 3 - 6.

Five fractions were collected after TLC monitoring. The amount of residue obtained from each fraction was weighed (Figure 3.18). Among these fractions, Fraction 2 had given 78.6 mg of white needles (0.39 mg/g of dried *L. rhinocerus*). The total amount of isolated residue was 143.39 mg which referred to 45% based on crude residue (310.12 mg). Hence, 55% of the crude residue may have represented impurities and/or compounds lost during the flash chromatography process.

The position of isolated compounds was identified using TLC (Figure 3.19), NP-HPLC (Figure 3.20) and RP-HPLC (Figure 3.21) analysis. The TLC chromatograms (Figure 3.19) showed each fraction spots whereby the compounds at R_f 0.79 (Fraction 1) and R_f 0.58 (Fraction 2) were successfully isolated. Fractions 3 - 5 contained the combinations of compounds at R_f 0.46, 0.40 and 0.35 which was also illustrated in the chromatograms of NP and RP-HPLC methods (Figure 3.20 and Figure 3.21, respectively). Hence, these compounds were still difficult to separate when using gradient flash chromatography.

Table 3.5 *TLC results using Preparation 2 and different mobile phase compositions. nd: not detected.*

Composition of mobile phase (% v/v)	R_f and number of spots						
	1	2	3	4	5	6	7
Hexane : ethanol : acetone; 60 : 10 : 30	0.91	0.82	0.65	0.62	0.61	0.55	0.51
Hexane : Ethyl acetate; 3 : 2	0.82	0.70	0.53	0.45	nd	nd	nd
Hexane : acetone ; 70 : 30	0.74	0.61	0.47	0.44	0.42	0.39	0.33
Hexane : Ethyl acetate: Acetic acid; 93 : 7 : 0.5	0.31	0.19	0.15	0.13	nd	nd	nd
Hexane: Ethyl acetate; 3 : 1	0.28	0.15	nd	nd	nd	nd	nd
Methanol : chloroform; 5 : 95	SF	0.90	nd	nd	nd	nd	nd

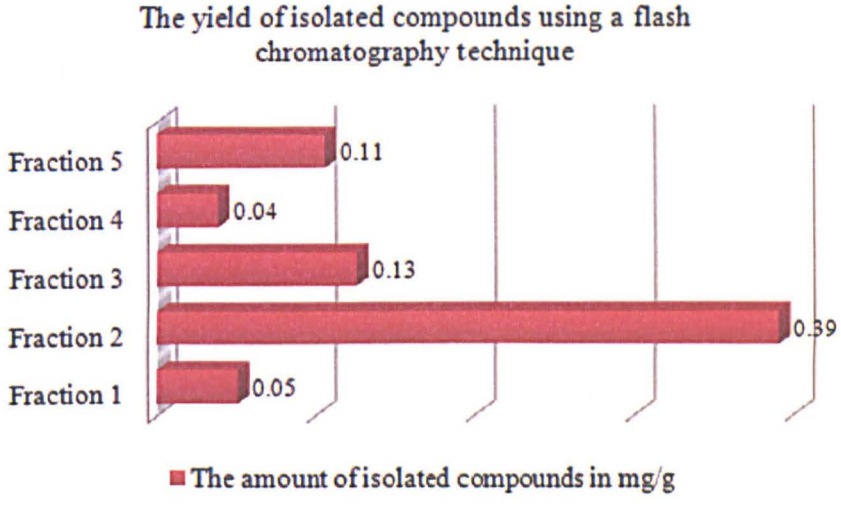


Figure 3.18 *The amount of residue obtained from each fraction.*

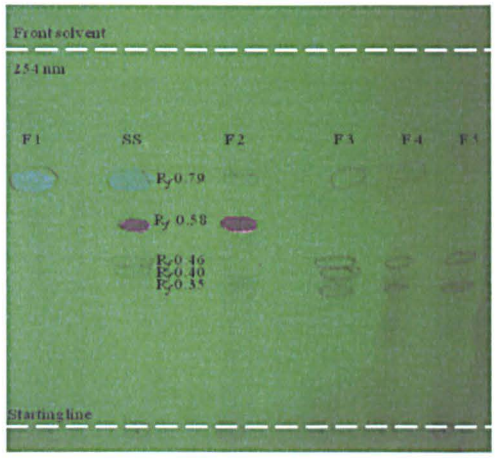
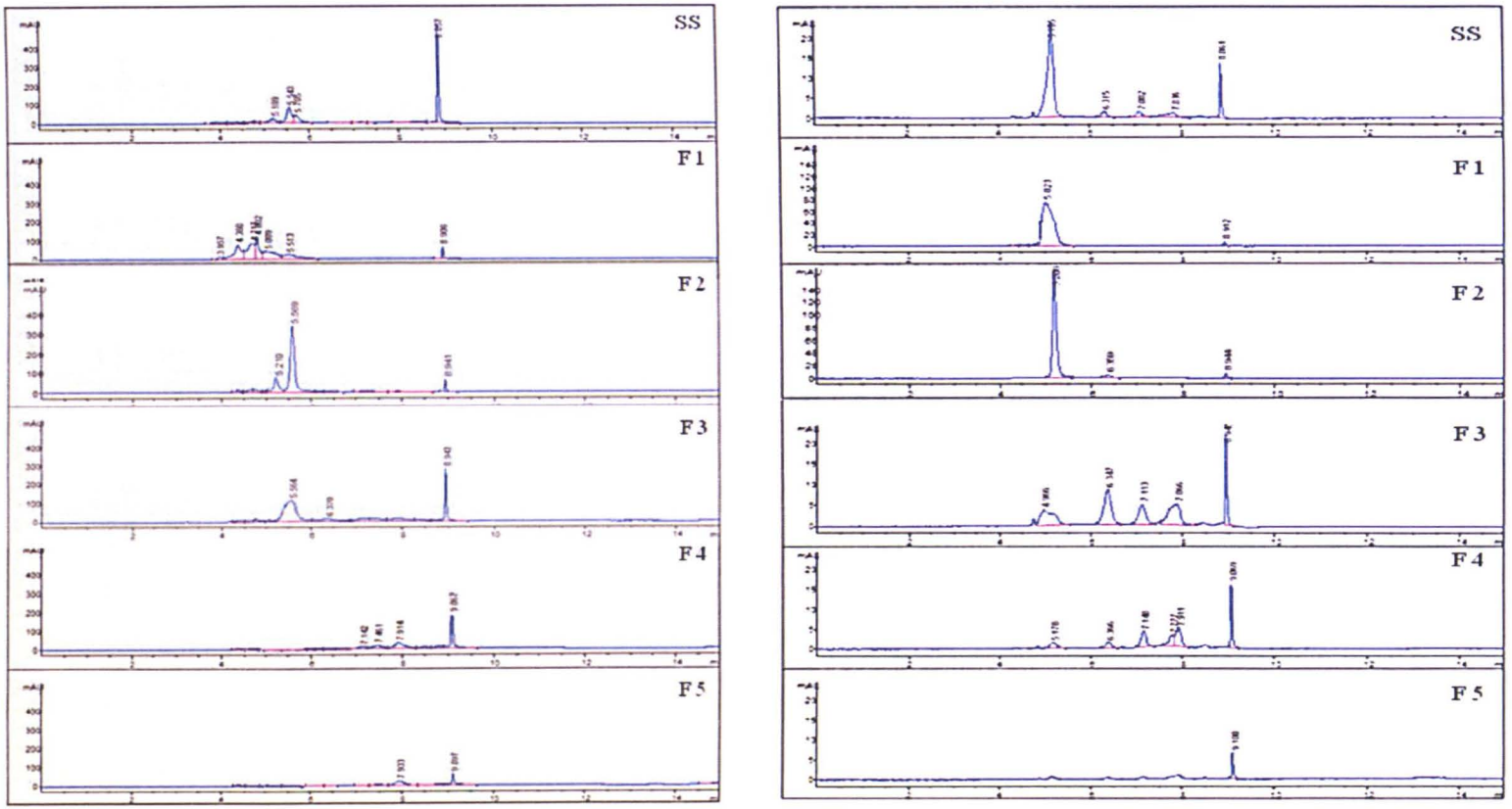


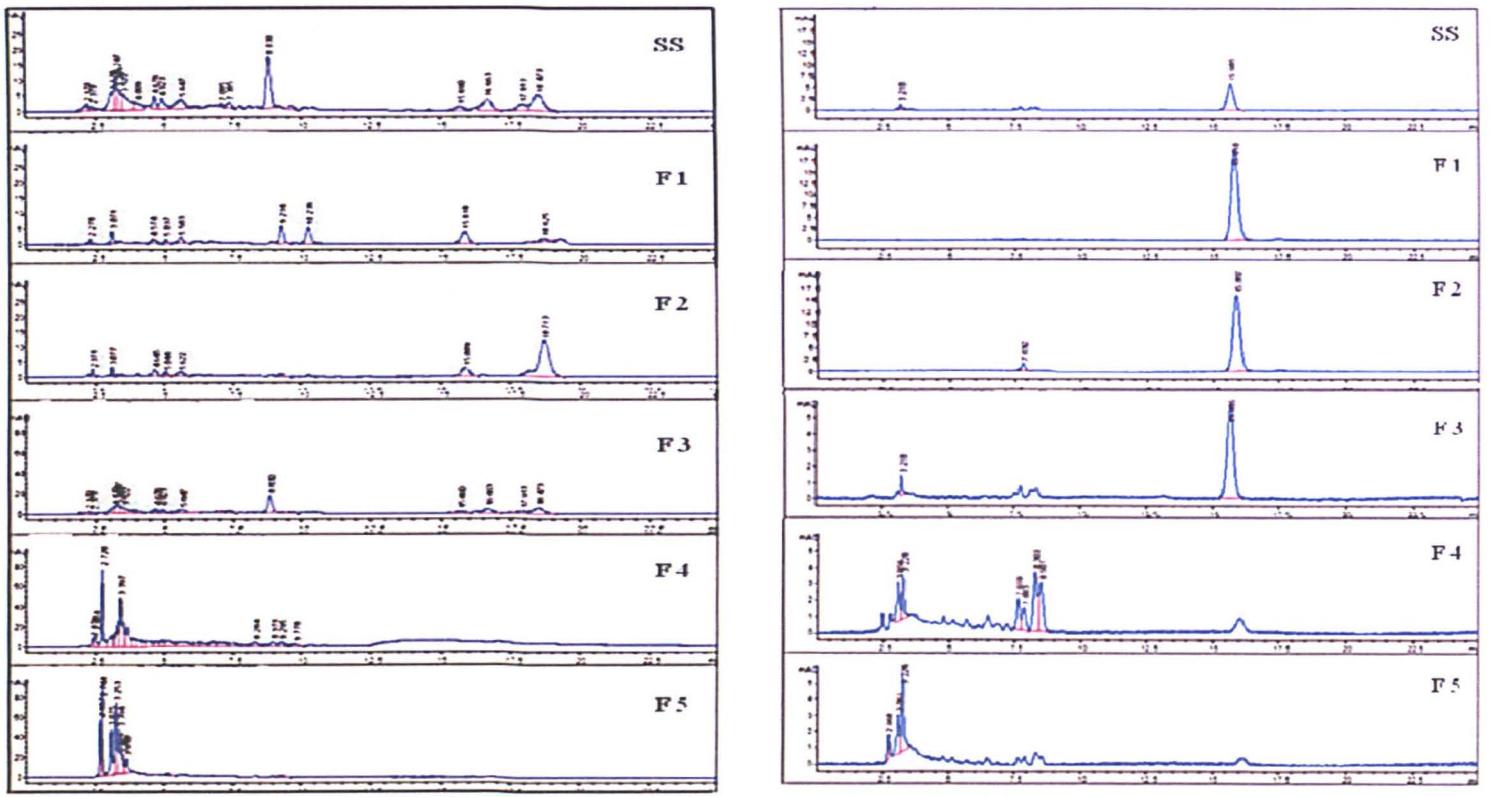
Figure 3.19 *TLC chromatograms of fractions 1-5 (F1, F2, F3, F4 and F5) and sample solution (SS) using a mobile phase of 30% acetone in hexane (v/v).*

The chromatogram scale for Fraction1-2 and Fraction 3-5 (Figure 3.20 and Figure 3.21) were different in order to show that more than one peak were observed in each fraction. This may imply that the compounds present could easily be oxidised by atmospheric oxygen during the isolation process. The investigation of oxidised compounds had been carried out and is covered in section 3.10. Under the conditions used it was found that TLC and flash chromatography methods were not be suitable for the isolation of compounds at R_f 0.46, 0.40 and 0.35. In addition, the amount of these three compounds was very small and required a large quantity of raw materials. However, an automatic fractionation of HPLC coupled with a high sensitivity of detector would be useful in order to achieve this task.



(a) *(b)*

Figure 3.20 *Chromatograms of fractions 1-5 (F1, F2, F3, F4 and F5) and sample solution (SS) analysed at wavelength (a) 254 nm and (b) 365 nm using NP-HPLC method; Silica column, 250 x 4.6 mm, mobile phase; 10% (v/v) ethanol in hexane, flow rate; 0.5mL/min nm.*



(a) *(b)*

Figure 3.21 *Chromatograms of fractions 1-5 (F1, F2, F3, F4 and F5) and sample solution (SS) analysed at wavelength (a) 254 nm and (b) 365 nm using RP-HPLC method; Column; C_8, 250 x 4.6 mm, mobile phase; 95% (v/v) methanol in water, flow rate; 1.0 mL/min .*

3.5.4 HPLC-DAD for peak purity checking

Peak purity checking is an essential tool when dealing with unknown compounds as it verifies the absence or presence of potentially present impurities that may contribute to the peak. Purity of a chromatographic peak can be checked by overlaying normalized spectra from different selections of the peak (Fong and Lam, 1991). A good overlapping spectra shape) and matching RTs indicate the detection of a pure peak. For example there is 50 out of 50 overlaying spectra at particular RTs indicates 100% similarity curve or purity level meaning a pure peak. The confirmation of peak purity however should be performed before quantitative information is obtained from a chromatographic peak. Figure 3.22 demonstrates a 100% purity level of Peaks 1, 4 and 5. The purity level of Peaks 2, 3 and 6 were less than 70% which indicated that the peaks showed co-elution of impurities. Hence, qualitative analyses were emphasized on the compound represented by Peaks 4 and 5.

The DAD scanning for Peak 5 revealed a similar UV profile as ergosterol as reported by Yuan *et al.*, (2003), Slominski *et al.*, (2005) and Yuan *et al.*, (2006). Consequently, the qualitative analyses for Peak 5 (white needles) were compared to ergosterol reference material. The Peak 4 (yellow residue) exhibited a similar UV profile as ergone that was studied by Tanaka *et al.*, (1996) and Yuan *et al.*, (2003 and 2004). There was no reference material commercially available for ergone and therefore, its spectral data were compared with reported spectra in the literature.

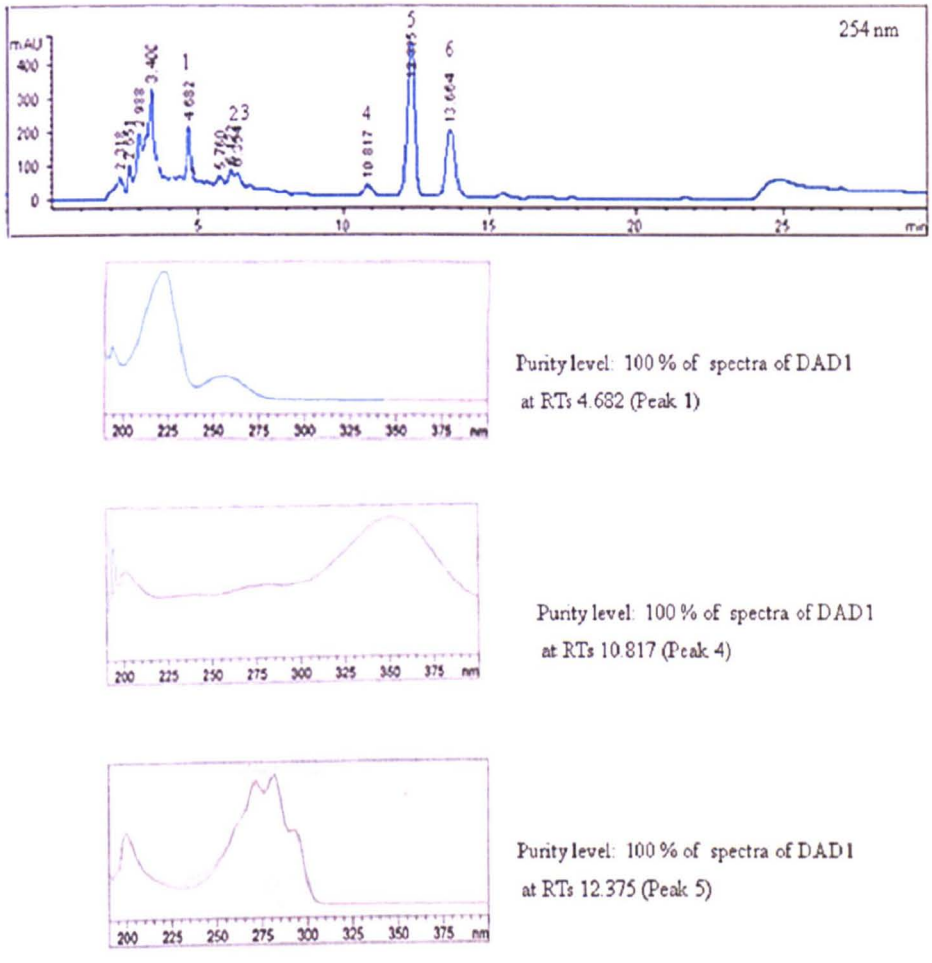


Figure 3.22 *DAD scanning with respective RTs for peak purity levels of Peaks 1, 4 and 5. The chromatograms obtained from Preparation 2 using RP-HPLC method: C_{18} column (5.0 µm, 250 x 4.6 mm), mobile phase of 98% (v/v) methanol-water and flow rate at 1.0 mL/min.*

3.6 Qualitative analyses

3.6.1 UV/Vis spectroscopy

UV determination can be a useful analytical tool for supporting data for qualitative analysis. Both spectra derived from the white needles and ergosterol reference (100 µg/mL) were superimposed and exhibited the same absorbance wavelengths at 262 nm, 271 nm, 282 nm and 293 nm (Figure 3.23). Hence, this provided support for the fact that both

candidates may have shared the same conjugated chromophores groups that are present in ergosterol. A spectrum of yellow residue in ethanol exhibited a λ_{max} at 239 nm, 282 nm and 351 nm (Figure 3.24). The spectrum may suggest the existence of an aromatic compound (239 nm and 282 nm) with the combination of conjugated aliphatic hydrocarbon (351 nm). The spectral data of this yellow residue were in good agreement with reported data on ergone (Cole and Schweikert, 2003).

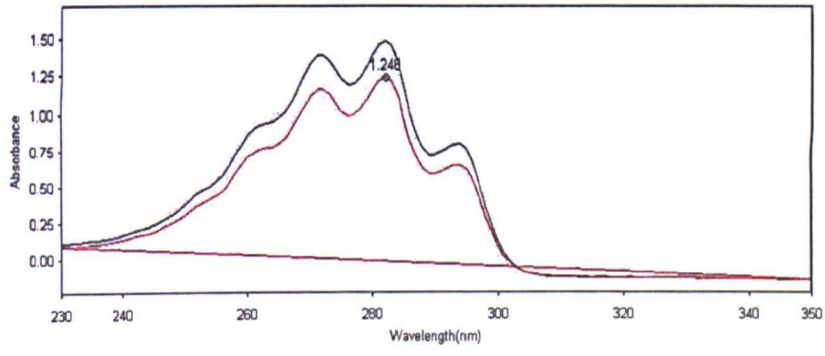


Figure 3.23 *The UV spectrum of white residue (black) overlaid with the spectrum of ergosterol reference material (red).*

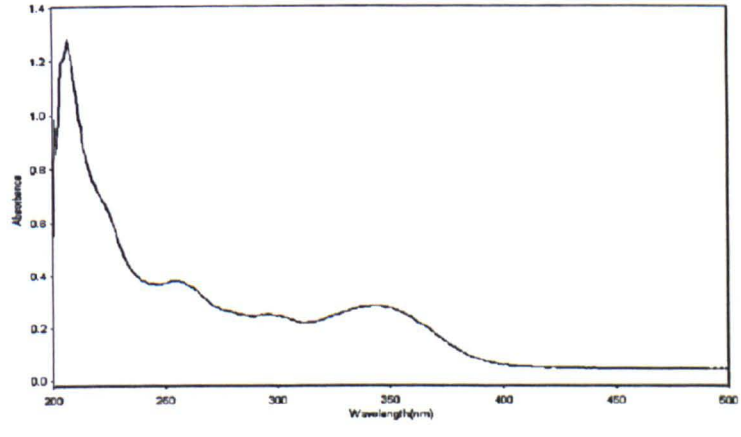


Figure 3.24 *The UV spectrum of yellow residue.*

3.6.2 FTIR -ATR analysis

Each molecule will have a complete absorption spectrum unique to that molecule, and so a 'fingerprint' of the molecule is obtained (Christian, 1994). FTIR analysis provided information on fingerprint region (1200 to 700 cm^{-1}) and functional groups (4000 to 1200 $cm^{-1)}$ present in the molecule (William and Fleming, 1995; Hill *et al.*, 2005). An unknown molecule can be identified by a comparison of its unique absorption in the fingerprint region with known spectra.

Two identical spectra were obtained from the white needles and ergosterol reference material as illustrated in Figure 3.25. A typical broad peak at 3363 cm^{-1} indicated the presence of a hydroxyl group. Both spectra exhibited the same profile in the fingerprint region, supporting the hypothesis that the white needles were of ergosterol.

Most of reported IR data (frequencies, ν) of ergone were obtained from FTIR using potassium bromide (KBr) disc while the ν data produced in this analysis was from FTIR-ATR. The application of FTIR-ATR is increasing due to its ease of use and the time-saving sample preparation. Normally, no sample preparation is required for pure samples, and this technique is ideal for rapid qualitative and quantitative analyses. A spectrum of the yellow residue is shown in Figure 3.26 and the ν were 2916, 2849, 1710, 1650, 1471, 1378, 1264, and 1095 cm^{-1}, comparable to IR data reported by Jinming *et al.*, (2001); Cole and Schweikert (2003), Lee *et al.*, (2005) and San-Martin *et al*, (2008). The ν of 2916 and 2849 cm^{-1} represented general alkyl groups while 1710 and 1655 cm^{-1} indicated the present of a ketone group in the sample and 1458 cm^{-1} represented benzene carbon-hydrogen bending motions. There was no absorbance band at ν of 3363 cm^{-1} indicated the absence of hydroxyl group. Hence, this spectrum suggested that the yellow residue may have displayed similar structural features represented by ergone.

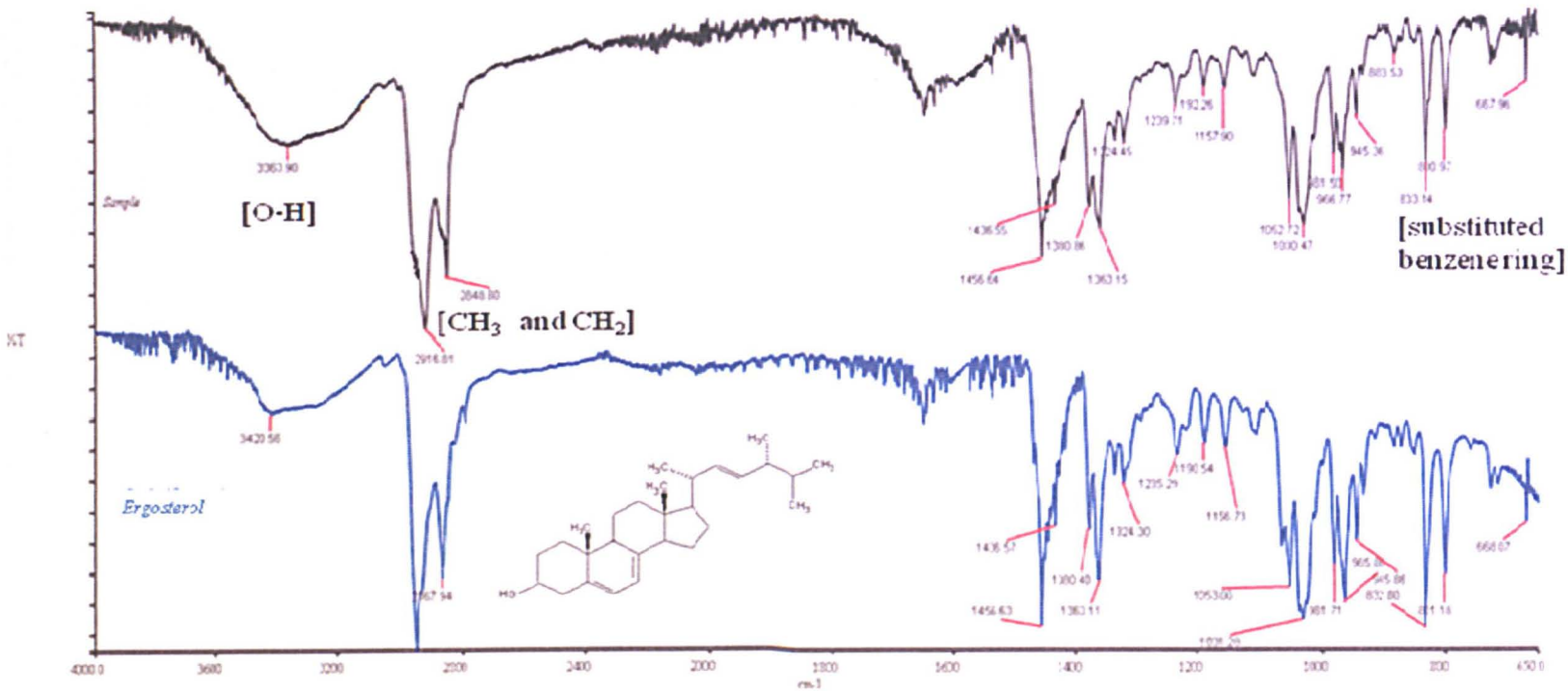


Figure 3.25 *The FTIR spectrum of white residue (black) overlaid with the spectrum of ergosterol reference material (blue).*

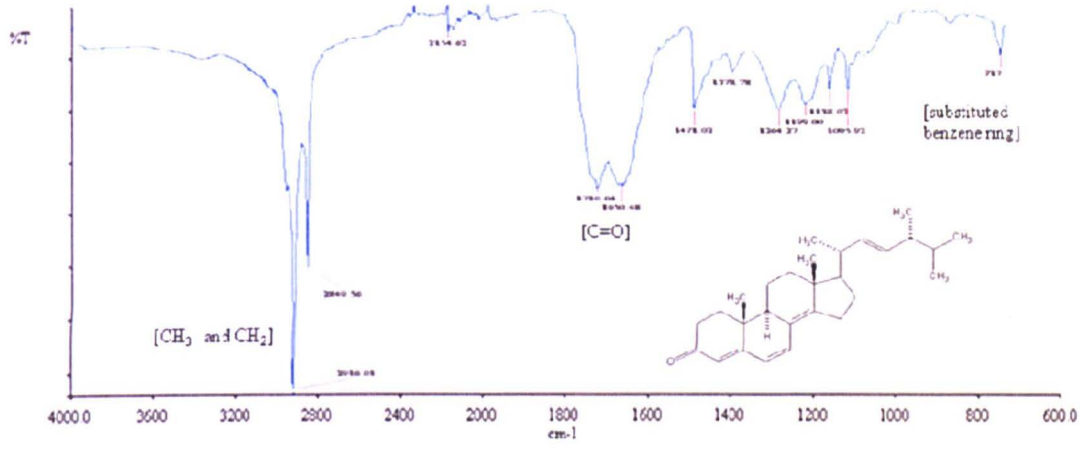


Figure 3.26 *The FTIR-ATR spectrum of yellow residue.*

3.6.3 TLC and HPLC analyses

The ergosterol reference solution (100.0 µg/mL) and white needles solution were spotted on a TLC plate and analysed by TLC (section 2.2.6.2). The spot of ergosterol reference solution was at the same position and size when compared with the sample spot at R_f 0.58 (demonstrated in Figure 3.19 page 86). The reference and sample solutions were also analysed using the RP-HPLC method (section 2.2.6.3) and the RTs of ergosterol was similar to Peak 5 of sample solution (refer to Figure 3.17 page 83). Hence, these results provided supporting evidence for the presence of ergosterol.

3.6.4 LC-MS and GC-MS analyses

Several attempts, including modifications to ESI-MS conditions and direct infusion of examined solutions, were made to obtain a mass spectrum of ergosterol from the sample solution, ergosterol reference solution and a mass spectrum of ergone from the yellow residue solution but the attempts were not successful. This may indicate that the compounds were not fully ionized under electro-spray conditions. This result was reported by Heimark *et al.*, (2002), who stated that sterol compounds such as ergosterol responded poorly to electro-spray and atmospheric pressure chemical ionization conditions. Hence, the

analysis was repeated using GCMS and electron ionization based on an adapted and modified method reported by Mattila *et al.*, (2002) and Hajjaj *et al.*, (2005).

The white residue was derivatized with TMS and the corresponding mass spectrum and its mass molecular ions (M) are shown in Figure 3.27. The M of ergosterol-TMS ether (m/z 468) was not detected in this spectrum. However, the mass spectrum exhibited the same base peak of M (m/z 363; without TMS and two methyl groups in the hydrocarbon chain) as the ergosterol reference material mass spectrum. The mass peak at m/z 143 is characteristic for sterols with two double bonds in the ring structure (Mattila *et al.*, 2002).

Signals in the mass spectrum of the yellow residue were not well resolved and hence unuseful for any interpretation. This could be due to the amount of sample used being too small and insufficient for derivatisation, or the adapted derivatisation procedure was not suitable for the yellow residue. Mass spectral data has been reported for ergone (Jinming *et al.*, 2001; Cole and Schweikert, 2003; Lee *et al.*, 2005; San-Martin *et al*, 2008). Fragments included the presence of m/z 377 ($C_{27}H_{37}O$, without one CH_3 group), 349 ($C_{25}H_{33}O$, without three CH_3 groups), 268 ($C_{19}H_{24}O$, base peak without the hydrocarbon chain), 253 ($C_{18}H_{21}O$), 240 ($C_{17}H_{20}O$), 214 ($C_{15}H_{18}O$), and 173 ($C_{12}H_{14}O$). This result may indicate the possibility of ergone (M.W = 392, $C_{28}H_{40}O$) existence in the yellow residue.

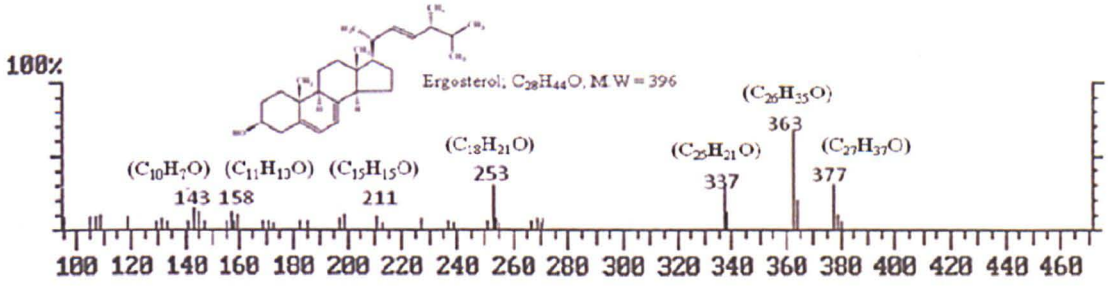


Figure 3.27 *The white needles mass spectrum and its mass molecular ions.*

3.6.5 ^{1}H-NMR spectroscopic analysis

NMR signals are frequently split into groups of related peaks, called doublets, triplets, multiplets; this splitting is due to the presence of permanent magnetic fields emanating from adjacent nuclei, particularly from other protons within two to five valence bonds (BP, 2009). The ^{1}H-NMR spectra for both white needles and yellow residue were too complex to interpret, especially where there were complex signals at regions δ 1.2-2.0. Most of the splitting groups were not clearly seen in the spectra due to excessive peak overlaps. However, multiplets signals at δ 5.18-5.21 were detected in both the residue spectra and ergosterol reference material spectrum. These signals may be assigned to methine protons (C-22 and C-23) of double bond in the hydrocarbon chain (Figure 3.28) (Slominski *et al.*, 2005). Signals at δ 3.60, δ 5.34 and δ 5.56 were seen in both of the white needles and ergosterol reference spectra, indicating that one methine proton was attached on an oxygenated carbon (Wang *et al.*, 2008) and methine protons of double bonds in the ring structure (C-6, and C-7) (Slominski *et al.*, 2005). These signals were not seen in the yellow residue spectrum. Overall, a conclusive chemical structure for white needles and yellow were not fully achievable. Nevertheless, all supportive data from UV, TLC and HPLC especially IR analyses suggested the presence of ergosterol (white needles) and ergone (yellow residue) in sclerotial *L. rhinocerus*.

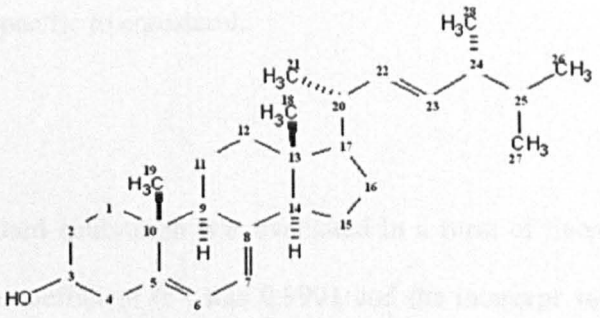


***Figure 3.28** The numbered carbons of ergosterol structure.*

3.7 HPLC method validation

Ergosterol has been reported in numerous publications concerning its beneficial effects and pharmacological activities as mentioned in section 1.5.5. Hence, a validated method was required to be established in order to quantify the amount of ergosterol present in *L. rhinocerus*. The quantification was performed only on ergosterol due to the accessibility of its reference material and it was considered to be an important marker molecule since it is the most commonly found metabolite in fungal species. The isocratic RP-HPLC method at wavelength 280 nm was used to validate the method due to high sensitivity of ergosterol detected at this wavelength. This validated method could be then applied to the routine quality assessment of fungal material samples.

3.7.1 Specificity

The %RSD of peak area and RTs and the symmetrical factor (As) of ergosterol reference material are shown in Table 3.6. The %RSD value was determined after six replicate injections and was found to be below 2.0% while the RTs were within ± 1.0% of the average RT. The As value was found to be between 1.08-1.13 which provided support for the reliability of the method. The ergosterol peak clearly appeared at RT 4.88 min for both sample and spiked sample solutions (Figure 3.29). There was no peak detected in the diluent of sample solution that has the same RTs to ergosterol. Hence, these data indicated that the method was specific to ergosterol.

3.7.2 Linearity

The linearity of standard calibration was evaluated in a form of linear regression (Figure 3.30). The regression coefficient (r^2) was 0.9991 and the intercept value (52.77) was less than 2.0% of the peak area (4175.71) at the working concentration (80.0 µg/mL).

Consequently, the results demonstrated that the linearity within the selected range was satisfactory.

Table 3.6 *System suitability tests of ergosterol reference solution.*

Injection	Peak area	Retention time	Symmetry factor
1	4177.41	4.85	1.15
2	4200.64	4.84	1.13
3	4206.87	4.84	1.08
4	4183.80	4.83	1.09
5	4190.17	4.83	1.08
6	4185.69	4.83	1.15
Average	4190.76 ± 11.59	4.88 ± 0.01	1.10 ± 0.03
% RSD	0.26	0.12	0.27

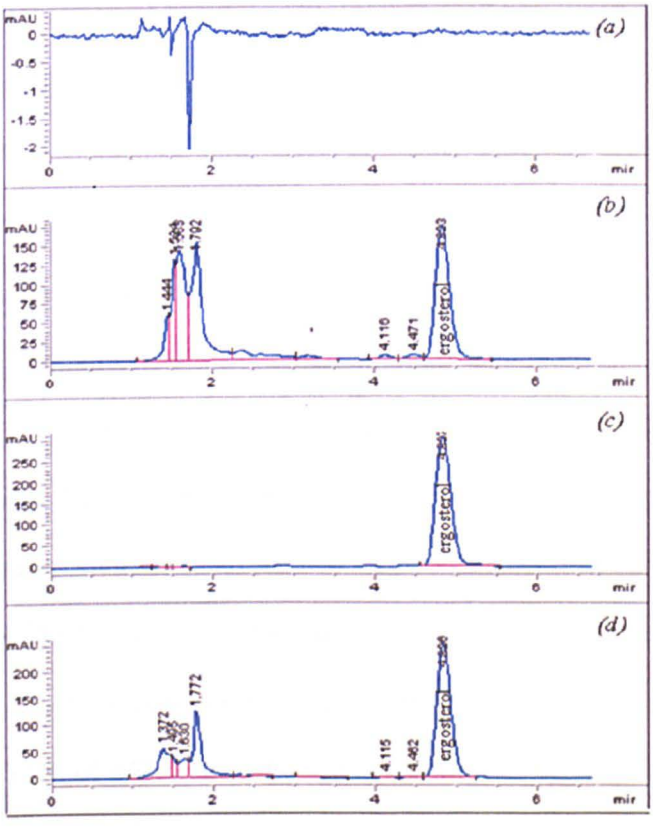


Figure 3.29 *Chromatograms of (a) diluents, (b) sample solution (Preparation 2), (c) ergosterol reference solution and (d) spiked sample solution analysed using RP-HPLC method (Table 2.13) to assess the specificity of method.*

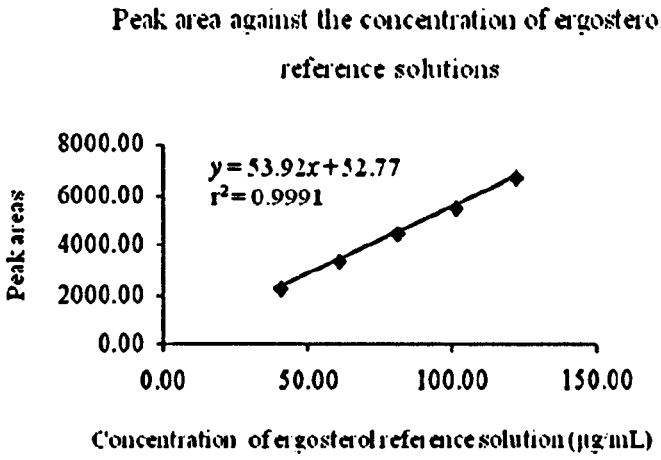


Figure 3.30 Standard calibration curve for assessing the linearity of the method.

3.7.3 Precision and accuracy

The recovery of the HPLC method was assessed using nine determinations of sample solutions spiked with a known amount of ergosterol reference material. The reference working concentration used at the 100% level was 80.0 µg/mL. This working concentration was selected based on the similarity of ergosterol peak area and height in the reference solution to ergosterol peak in sample solution (Preparation 2). According to Q2 (R1) of the ICH guidelines, three levels of sample solutions were required to assess the recovery. The sample preparation involved an extraction procedure, hence different levels of the reference solution were added to the sample solution to evaluate recovery by the extraction procedure.

All recovery results were obtained from a calibration standard curve (Figure 3.30) and the percentage recoveries of extracted ergosterol are summarised in Table 3.7. The mean value of recovery was 99.10% and the true value was within a 95% confidence limit of 99.10 ± 2.99 %. These data also represented the repeatability of the method. All % RSD values of three replicates from each level were between 3.83% and 4.35%, i.e. less than 5.0% of RSD. The intermediate precision (ruggedness) was determined with two different

HPLC instruments on three different days and the obtained % RSD values were less than 5.0% (Table 3.8). These results indicated that the HPLC method was accurate and precise for determining the ergosterol content in *L. rhinocerus*.

***Table 3.7** Summaries of recoveries and precision results.*

No	Sample Levels	Standard added (µg/mL)	Results (µg/mL)	% Recovery
1	50.00%	40.00	38.16	95.40
2	50.00%	40.00	39.60	99.50
3	50.00%	40.00	41.64	104.10
4	100.00%	80.00	83.68	104.60
5	100.00%	80.00	80.40	100.50
6	100.00%	80.00	77.20	96.50
7	150.00%	120.00	111.12	92.60
8	150.00%	120.00	119.76	99.80
9	150.00%	120.00	118.20	98.50
			Mean	99.10 ± 2.99 %.
			%RSD	3.90

3.7.4 Robustness

The robustness of the HPLC method was assessed for variations of different column brands, different composition of mobile phase, flow rate, temperature and the stability of analytical solutions. The percentage of recoveries and RSD values for the robustness of the method are summarised in Table 3.8. Variation of mobile phase composition by 6.5% and column use did not significantly affect the precision of the HPLC system. The variation of

flow rates and temperatures showed only minor impact on RT and symmetry of the ergosterol peak. The HPLC system of Day 1 was selected for routine analysis and was based on the relatively low column back pressure. Furthermore, ergosterol was stable at 40.0°C as demonstrated by the stability of sample and standard solutions within 24 hours at 40.0°C (Figure 3.31). The variations of peak areas were within ±1.0% of the mean peak area and the $RSD_{\%}$ of RT were less than 0.5%, indicating that the solutions were stable under these conditions.

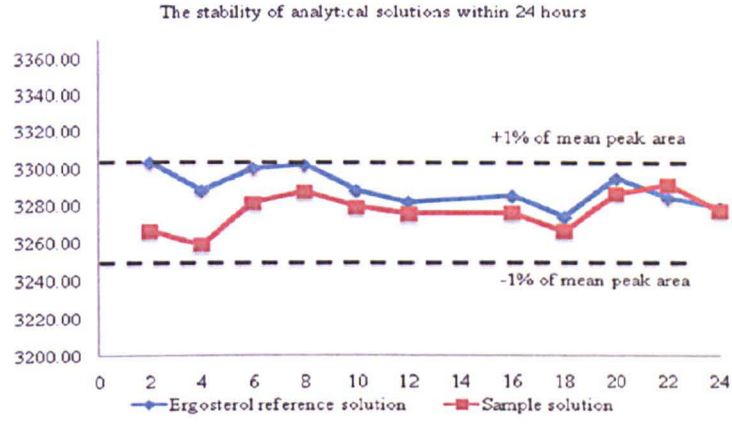


Figure 3.31 *The stability of sample (Preparation 2) and standard solutions within 24 hours at 40.0°C.*

3.7.5 Quantification (QL) and detection limits (DL)

Two approaches were utilised for determining the QL and DL. The first approach was based on S/N since the HPLC instrument can exhibit baseline noise while the second approach was chosen for comparative reasons. The baseline chromatogram of sample solution was amplified and measured (Figure 3.32).

Table 3.8 *The variations of column brands, mobile phase composition, flow rates and temperatures used to evaluate robustness of the method.*

Peak area	Theoretical (µg/mL)	Actual (µg/mL)	% Recovery
1143.41	42.86	41.81	97.54
1065.54	42.86	39.94	93.19
1083.27	42.86	40.61	94.74
2314.97	81.64	82.42	100.95
2268.21	81.64	79.99	97.98
2379.79	81.64	82.68	101.28
3658.54	121.56	128.40	105.63
3532.81	121.56	123.99	102.00
3463.54	121.56	122.35	100.65
		Mean	99.33%
		%RSD	3.88%
		95% of Confidence limit	99.33 ± 2.96%

Day 1

HPLC conditions; HP 1050, Kromasil C_8 (5.0 µm, 150 x 4.6 mm), 92% methanol in water, flow rate 1.0 mL/min, temperature at 40.0°C.

Peak area	Theoretical (µg/mL)	Actual (µg/mL)	% Recovery
1340.74	42.86	41.33	96.45
1390.09	42.86	42.39	98.91
1301.96	42.86	40.14	93.66
2410.53	81.64	81.81	100.21
2392.69	81.64	82.05	99.26
2423.55	81.64	82.08	100.54
3658.26	121.56	127.50	104.89
3631.19	121.56	120.66	99.26
3642.53	121.56	120.94	99.57
		Mean	99.19%
		%RSD	3.06%
		95% of Confidence limit	99.19 ± 2.33%

Day 2

HPLC conditions; HP 1090, Waters C_8 (5.0 µm, 150 x 4.6 mm), 95% methanol in water, flow rate 1.0 mL/min, ambient temperature.

Peak area	Theoretical (µg/mL)	Actual (µg/mL)	% Recovery
1316.66	42.86	40.89	95.40
1343.41	42.86	42.65	99.50
1405.41	42.86	44.62	104.10
2322.13	81.64	85.39	104.60
2310.39	81.64	82.05	100.50
2229.52	81.64	78.78	96.50
3185.07	121.56	112.56	92.60
3439.60	121.56	121.32	99.80
3388.00	121.56	119.74	98.50
		Mean	99.05%
		%RSD	3.95%
		95% of Confidence limit	99.05 ± 3.00%

Day 3

HPLC conditions; HP 1050, Waters C_8 (5.0 µm, 150 x 4.6 mm), 98% methanol in water, flow rate 0.8 mL/min, temperature at 30.0°C.

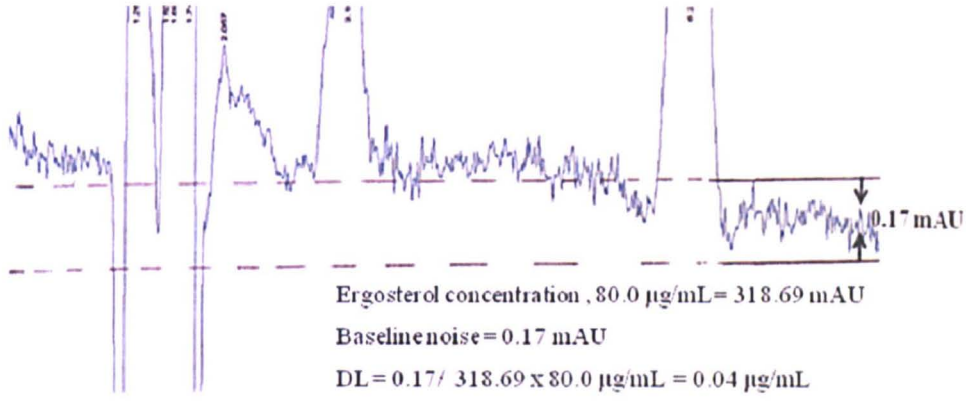
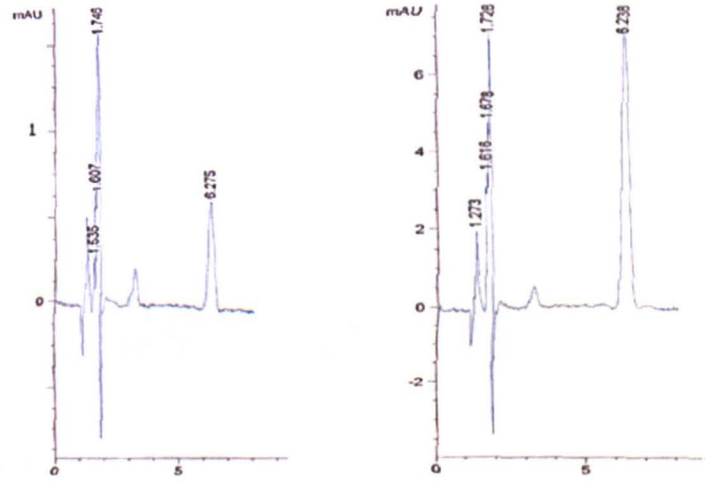


Figure 3.32 *Amplified baseline of chromatogram of sample solution.*

A direct proportion method was selected in which the peak height was directly proportional to analyte concentration in the attempt to determine the lowest concentration of ergosterol. This was found to be 0.04 µg/mL. The ergosterol reference solution was prepared at a concentration of 0.04 µg/mL and was detected at 6.27 minutes (Figure 3.33 (a)) while the quantified concentration (10 x DL) was detected at a concentration of 0.4 µg/mL, respectively (Figure 3.33 (b)).



(a) *(b)*

Figure 3.33 *Chromatograms of (a) detected and (b) quantified concentration.*

The second approach was based on the standard deviation of *y*-intercepts obtained from three regression lines. The DL and QL concentrations were found to be 1.31 µg/mL and 3.98 µg/mL, respectively. These values were larger than those obtained from the former approach which may be due to high value of standard deviation of *y*-intercepts and which can be improved by more regression lines. It was decided to adopt the first S/N approach for DL and QL determinations because it gave rise to improved sensitivity.

3.8 Determination of ergosterol in different parts of *L. rhinocerus*

Overall results of the specificity, linearity, precision, accuracy and robustness showed that the selected HPLC method (Table 2.13) was valid to quantify the amount of ergosterol in caps, stalks, and sclerotium of *L. rhinocerus*. Figure 3.34 illustrates the distribution of ergosterol in different parts of the fungus. The average amount of ergosterol (n = 3) in caps, stalks, sclerotia with and without growth were *0.37 ± 0.06 mg/g, 0.11* ± 0.03 mg/g, 0.13 ± 0.02 mg/g and 0.11 ± 0.02 mg/g of dried material, respectively.

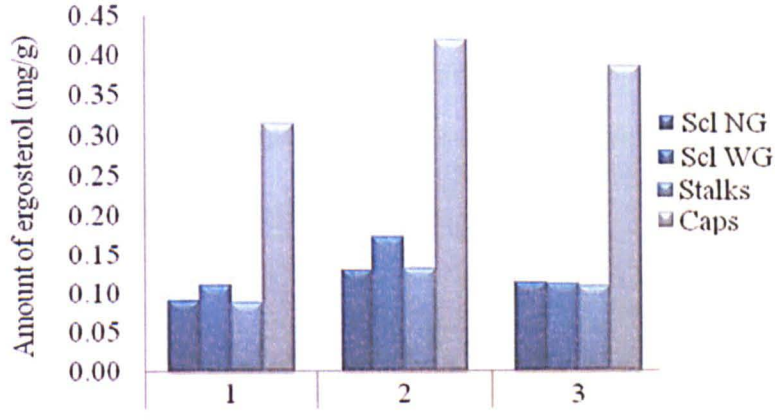


Figure 3.34 *The distribution of ergosterol in different parts of the fungi (n = 3). Scl NG: sclerotia without any growth of caps and stalks; Scl WG: sclerotia with growth of caps and stalks.*

These results revealed that ergosterol was not only present in the sclerotium but also in the stalk and cap. Hence, the potentially useful parts of *L. rhinocerus* were not restricted to sclerotia, as reported in publications and anecdotal information. The result also showed that the distribution of ergosterol tended to be higher at the younger part of fungi (caps) than the older parts (stalks and sclerotium) which was in agreement with a study reported by Jasinghe and Perera (2005). The authors also suggested that the higher amount of ergosterol found in caps were rationalized by its function to stabilize the membrane structure from UV exposure. However, the results may vary due to some other factors such as age of the fungus, growing condition and post-harvest storage.

3.9 Screening of ergosterol in commercial products

Chromatograms of the sample and reference solutions (Figure 3.35) showed that ergosterol was detected in Long Heh (*C. sinensis*) and Coriolus (*C. versicolor*) but not in Reishi (*G. lucidum*), Maitake (*G. frondosa*) and Felyoung (*P. cocos*) products. This indicated that the amounts of ergosterol present in these products were too small to detect. The reported ergosterol amount for each fungus is shown in Figure 3.36. The employed HPLC method showed that it was suitable for the screening and determination of ergosterol in THMPs and that it should provide a useful approach towards routine quality control assessment of traditional medicinal products of fungal materials.

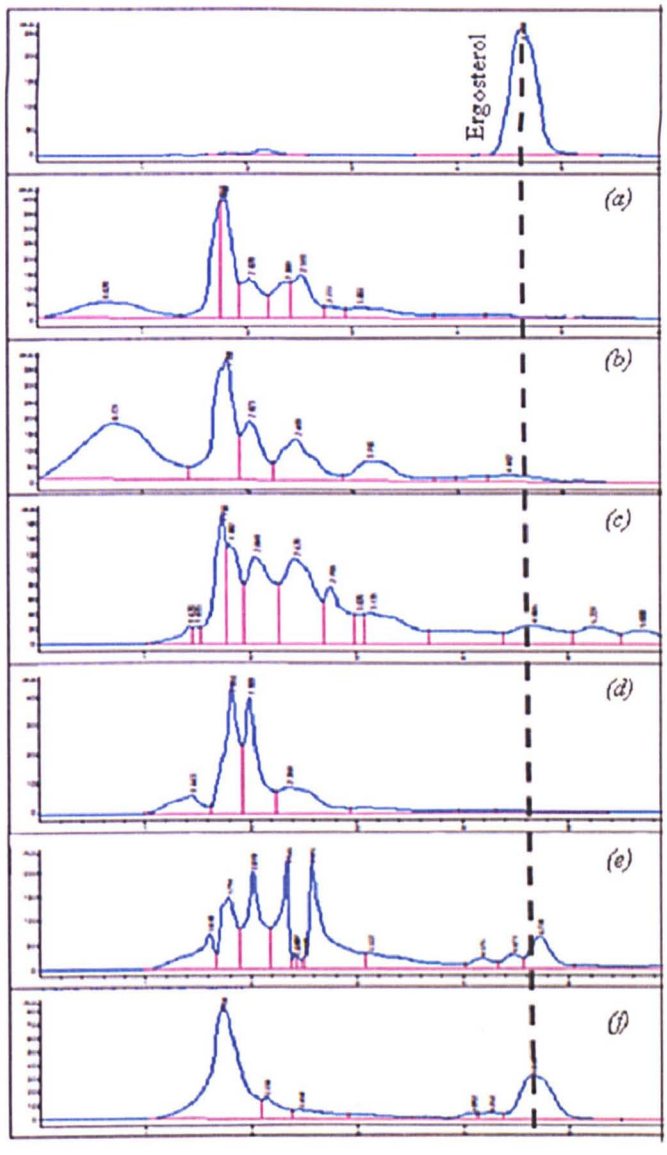


Figure 3.35 *Chromatograms of ergosterol reference, (a) Reishi (G. lucidum), (b) Maitake (G. frondosa), (c) Coriolus (C. versicolor), (d) and Felyoung (P. cocos), (e) Long Heh (C. sinensis) and (f) L. rhinocerus sample solutions (Preparation 2) analysed using RP-HPLC method (Table 2.13).*

Figure 3.36 *Pictures of fungi in the commercial products except for L. rhinocerus and with its respective reported amount of ergosterol.*

3.10 Chromatographic fingerprint of fungal sterols

The sample used for this work consisted of mixed parts (caps, stalks and sclerotia) of *L. rhinocerus* in order to ensure that most fungal sterols were extracted and therefore, could represent a chromatographic fingerprint. Figure 3.37 shows a representative

chromatographic fingerprint after extraction of mixed parts of *L. rhinocerus*. The peak at RT 5.75 minutes corresponded to Peak 1 (Figure 3.22, page 90) as judged from the same UV profile. Peak 1 would therefore be suggested to serve as a chemotaxonomic marker compound since it was only detected in *L. rhinocerus* sample solutions when compared to chromatograms of other commercial products (Figure 3.36).

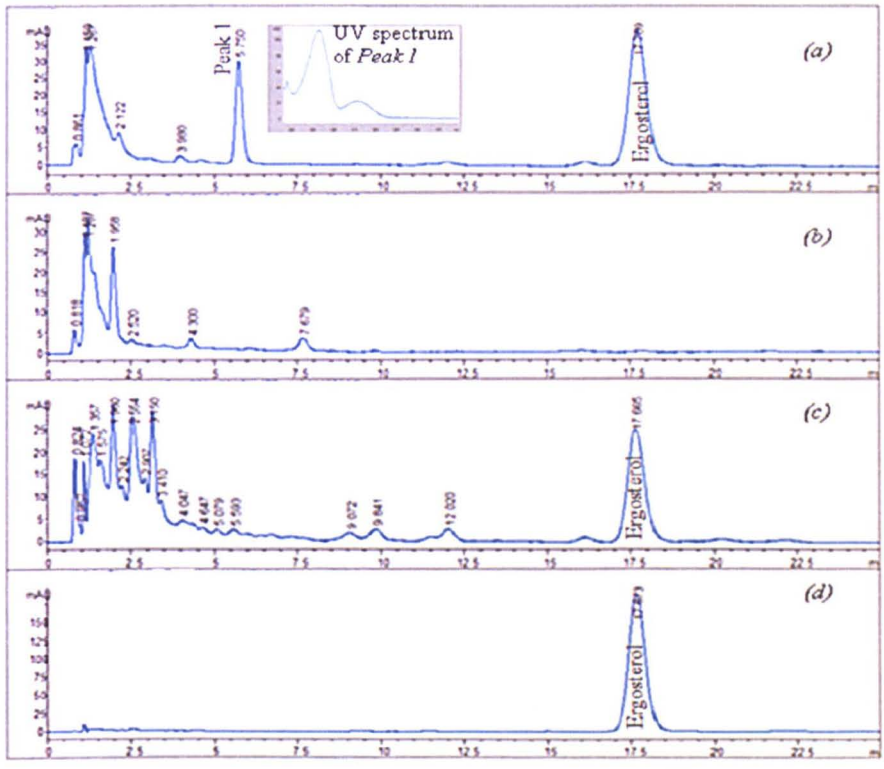
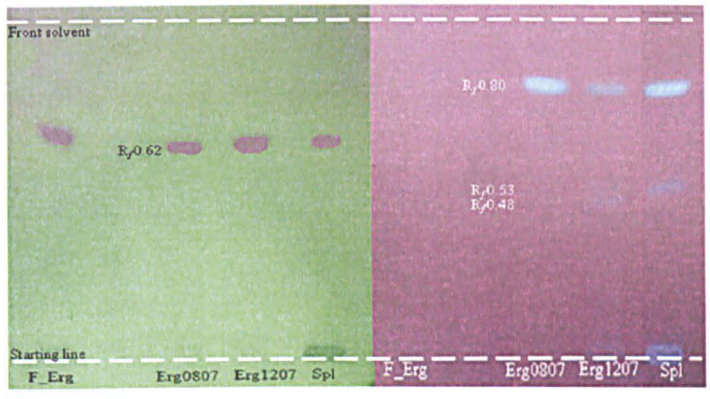


Figure 3.37 *Chromatograms of (a) L. rhinocerus sample, (b) Reishi (G. lucidum), (c) Long Heh (C. sinensis) and (d) ergosterol reference solutions from Preparation 2 and RP-HPLC method; C_{18} column (5.0 µm, 250 x 4.6 mm), mobile phase of 92% (v/v) methanol-water at flow rate 1.0 mL/min and wavelength 254 nm.*

However, Peaks 2 - 4 and 6 (Figure 3.22, page 90) were not detected in this chromatogram of the sample solution (Figure 3.37). Further investigations revealed that these compounds could be oxidised compounds of ergosterol. The investigations were

carried out by monitoring the ergosterol reference solution (80.00 µg/mL) every four months. The solution was kept in a HPLC vial and stored at 4 °C. Figure 3.38 illustrates the comparison between TLC spots obtained from a freshly prepared reference solution, sample solution (Preparation 2) and the monitored reference solution. There was no spot detected under 254 nm conditions, besides the ergosterol spot at R_f 0.62. The blue fluorescent spots at R_f 0.80 and between R_f 0.48-0.53 became visible in the monitored reference solution under UV 365 nm. These results suggested that the TLC spot at R_f 0.80 (blue fluorescence) was an oxidation product of ergosterol since the spots corresponded to ergone (see Figure 3.9, page 75). Further oxidation of ergone may have given rise to other oxidised compounds leading to additional blue fluorescent spots between R_f 0.48-0.53 (Figure 3.38).



254nm *365nm*

Figure 3.38 *TLC spots and HPLC for oxidised ergosterol. F-Erg: freshly prepared ergosterol reference solution; Erg0807 and Erg1207: month and year of the prepared ergosterol solution; Spl: sample solution (Preparation 2) and 30% (v/v) acetone in hexane was used as the mobile phase.*

Support for this finding came from Tanaka et al. (1996) who reported on the photochemical reaction of ergone. The study used an ergone solution (4 mg/mL) that was stirred at room temperature under irradiation with UV light (100 W high-pressure mercury arch) through Pyrex for five hours and the reaction was monitored by HPLC. It was found that ergone reacted easily with two molecules of oxygen to give 6α,7α-epidioxy-14α-hydroperoyergosta-4,7,22-triene-3-one which was transformed successively to 6α,7α:8α,9α-diepoxy-14α-hydroperoyergosta-4,22-diene-3-one and 14α-hydroperoxy-9α-hydroperoyergosta-4,7,22-triene-3,6-dione (Figure 3.39).

Figure 3.39 *Proposed photochemical reaction of ergone (1) to give 6α,7α-epidioxy-14α-hydroperoyergosta-4,7,22-triene-3-one (2), 6α,7α:8α,9α-diepoxy-14α-hydroperoyergosta-4,22-diene-3-one (3) and 14α-hydroperoxy-9α-hydroperoyergosta-4,7,22-triene-3,6-dione (4) (Tanaka et al., 1996).*

Consequently, Peaks 2 - 4 and 6, seen in the chromatograms of sample solution and the monitored reference solution, may have also derived from oxidized ergosterol or ergone (Figure 3.40). Relevant TLC and HPLC findings are summarized in Table 3.9.

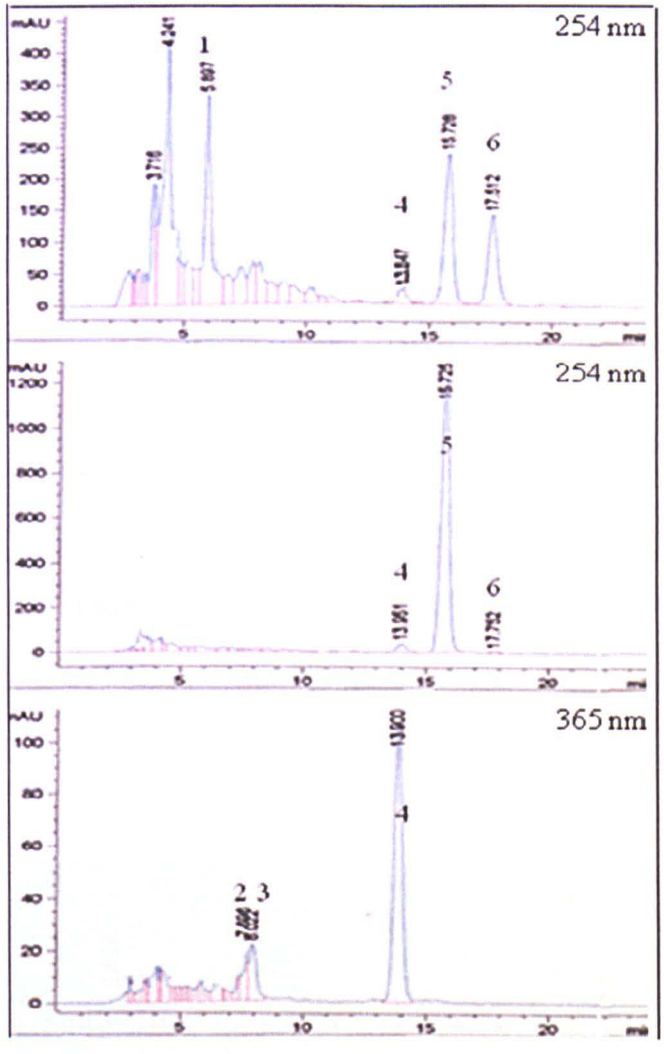


Figure 3.40 *Chromatograms of sample solution (Preparation 2) and the monitored ergosterol reference solution (Erg1207; the month and year). HPLC method as described in Table 2.15.*

Table 3.9 *The TLC and HPLC findings.*

Peak of HPLC (Figure 3.16, page 83)	R_f of TLC (Figure 3.37, page 107)	The possible compound
Peak 1	Not detected	Potential as chemotaxonomic indicator. A bound-form of sterol (Unknown 1)
Peak 2	Between 0.48 and 0.53	Oxygenated form of ergosterol / ergone (Unknown 2)
Peak 3	Between 0.48 and 0.53	Oxygenated form of ergosterol / ergone (Unknown 3)
Peak 4	0.80	Ergone
Peak 5	0.62	Ergosterol
Peak 6	Not detected	Oxygenated form of ergosterol / ergone (Unknown 4)

3.11 Screening of ergocalciferol

The results of TLC spots (Figure 3.41) indicated that vitamin D_2 was not detected in any parts of *L. rhinocerus* in which may be due to very low amount of ergosterol. Furthermore, most of *L. rhinocerus* species were collected from hills and forests which meant that these species were exposed to reduced levels of UV light.

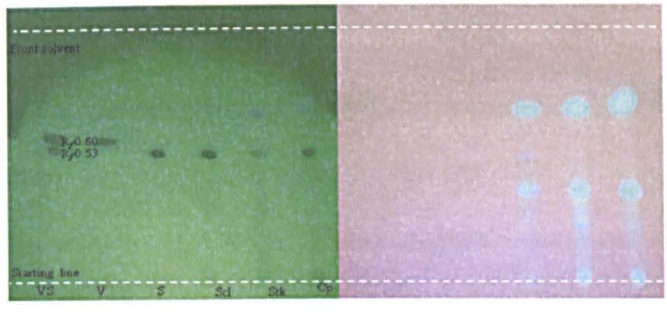


Figure 3.41 *TLC spots of ergosterol (S) at R_f 0.53, vitamin D_2 (V) at R_f 0.60, sclerotia (Scl), stalks (Stk) and caps (Cp) solutions under UV irradiations (254 nm (left) and 365 nm (right)), developed in 15% (v/v) of acetone-hexane as the mobile phase.*

3.12 Phenolic acid compounds (PACs)

3.12.1 Colour test using 1% (w/v) aqueous iron (III) chloride ($FeCl_3$) solution

A violet colour was produced upon addition of the 1% (w/v) $FeCl_3$ solution to the phenolic reference solution (100.0 µg/mL). The colour intensity of this solution was compared to the sample solution. A serial dilution of the standard solution was carried out to get the same colour intensity of sample solution. This observation showed that the colour intensity of the sample solution was comparable with the standard concentration at 10.0 µg/mL. Hence, no more than 10.0 µg/mL of water-soluble phenolic or polyphenol compounds may have been present in the sample solution.

3.12.2 UV/Vis spectroscopy

Both the phenolic and aromatic carboxylic acid solutions gave similar spectra which consisted of absorption bands at 220 nm and a broad band between 250 nm and 300 nm. The sample spectrum was compared to phenol, benzoic acid and salicylic acid reference spectra (Figure 3.42). The sample spectra displayed a UV profile similar to phenol and benzoic acid reference spectra. Hence, UV scanning suggested that phenolic and carboxylic acid compounds may have been present in *L. rhinocerus* sample solutions.

3.12.3 Removal of phenolic and aromatic carboxylic compounds by HPLC analysis

Phenols, as weak acids, are usually insoluble in aqueous $NaHCO_3$ solution but soluble in aqueous NaOH (Harwood et al., 1999). Hence, $NaHCO_3$ solution was used to extract phenols from the sample solution while phenolic and carboxylic compounds will retain in the aqueous layer when extracted using NaOH solution.

Figure 3.42 *Overlaid UV spectra of sample solution (SS) (preparation as described in section 2.2.11.1.1) with (a) phenol, (b) benzoic acid and (c) salicylic acid reference solutions.*

The total peak area between RTs 1.90 minutes and 5.00 minutes obtained at a wavelength of 220 nm (the highest absorbance among other wavelengths at 280 nm and 320 nm) (Figure 3.43) was used to calculate the amount of phenolic compounds which had been removed. The percentage value removed by each extraction (total of three extractions) was estimated using Equation 3 and the collected peak area are represented in Table 3.10.

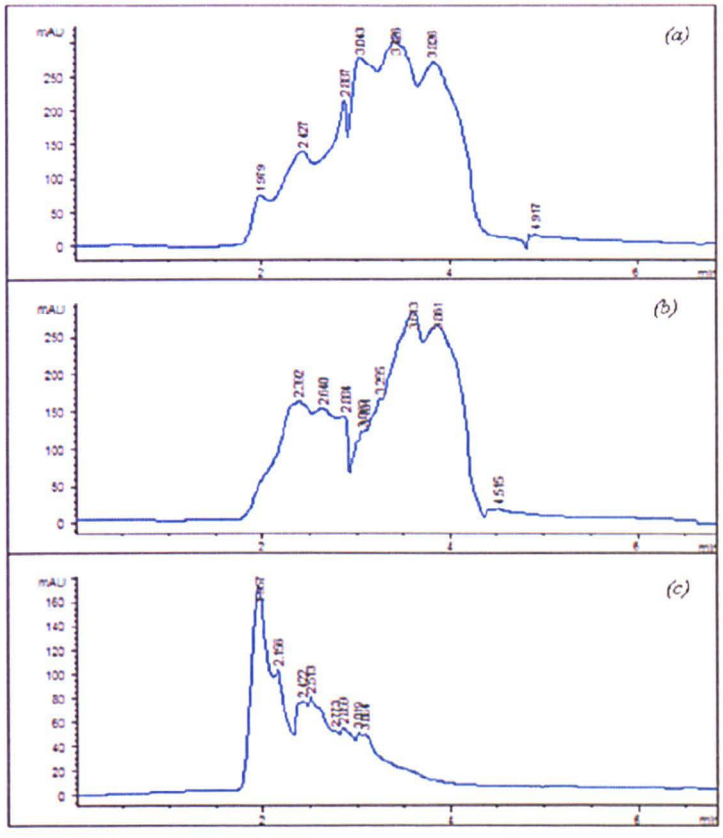


Figure 3.43 *Chromatogram of (a) an aqueous solution before extraction; (b) aqueous solution after extraction; (c) phenolic solution. Preparation as described in section 2.2.11.1.1 and analysed using HPLC method (Table 2.16).*

Table 3.10 *Total peak areas detected between 1.90 minutes and 5.00 minutes for sample solution before (Solution 1) and after extraction (extracts 1-3).*

Peak area			
Solution 1	Extract 1	Extract 2	Extract 3
39944.30	4272.28	16.60	1148.25
8167.18	3050.61	1901.80	223.32
6589.77		1315.50	779.75
16312.60		1108.37	448.79
		137.82	257.82
		1299.77	270.14
		391.24	343.44
			16.25
Total = 71021.59	7328.02	6177.27	3491.63

$$\text{Percentage of phenolic compounds removed} = \frac{\text{Total peak area of extract}}{\text{Total peak area of sample solution before extraction}} \times 100 \qquad \text{(Eq. 3)}$$

The amount of carboxylic compounds present in the sample solution was determined by subtracting the total amount of phenolic and carboxylic compounds removed with the removed phenolic amount only (Figure 3.44). It appeared that more phenolic compounds (average of 19.45%) had been removed from the solution compared to aromatic carboxylic acids (average of 7.98%). These screening tests provided some indication that the majority of water soluble compounds of *L. rhinocerus* may have comprised polyphenolic species or compounds with the combination of hydroxyl and carboxylic group.

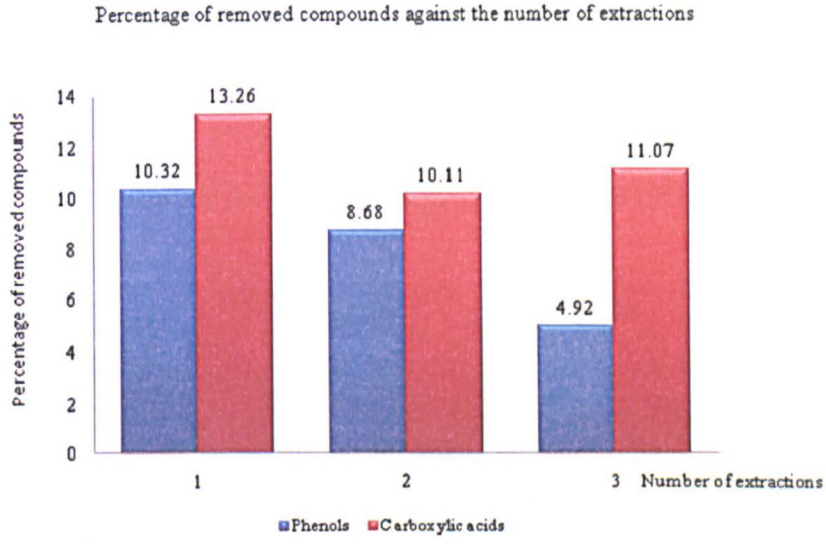


Figure 3.44 *The percentage of removed compounds*

3.13 Determination of total content of PACs

Phenolic acids are hydroxylated derivatives of benzoic acid and commonly found in free form, as well as their ester or glycoside counterparts (Bruneton, 1995). They tend to be

water-soluble due to their combination with sugar, for example, as glycosides. Free phenolic acids are generally extracted with alcohol and water and in this study 20% (v/v) of water in methanol was used.

Phenolic acids that are hydroxylated derivatives of cinnamic acid are often found to be esterified. This property lends itself to release from plant tissue under acidic or alkaline hydrolysis conditions and subsequent extraction into organic solvents such as diethyl ether (Harborne, 1973; Bruneton, 1995). In this study, a NaOH solution was used to release the bound PACs. In order to have a sensible measurement, the combined amounts of free and bound PACs were calculated for the total content of PACs.

As mentioned earlier in section 1.5.5.2.2, the wavelength used in the application of the Folin-Ciocalteu assay (FCA) ranged between 725 nm and 765 nm. The best wavelength that gave a maximum absorbance for this current analysis of total PACs was found to be at 765 nm. All absorbance readings were plotted against its corresponding concentration of gallic acid. The correlation coefficient (r^2) of the linear equation was 0.9970 (Figure 3.45).

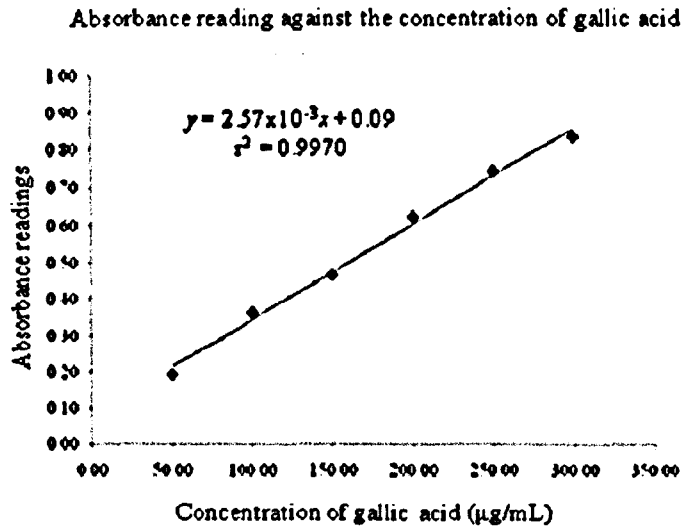


***Figure 3.45** The calibration curve of gallic acid standard solution*

The result of total PACs in *L. rhinocerus* is tabulated in Table 3.11. The majority of PACs originated from the free form and varied in different parts of the fungus. More free PACs were detected in caps than stalks and sclerotia, presumably reflecting open exposure of the cap to the environment. The results showed very low amounts of total PACs that may also be affected by the time of collection and storage conditions of the fungi. Based on these results, further investigation was deemed necessary using a HPLC method.

Table 3.11 *The result of total PACs in L. Rhinocerus. nd: not detected.*

Part of *L. rhi*nocerus	PACs form (μg/g)		Total PACs (μg/g)
	Free	Bound	
Caps	39.95	4.38	44.33
Stalks	21.46	2.35	23.81
Sclerotia	1.17	nd	1.17

3.14 Chromatographic fingerprint of PACs

The HPLC method used to generate a chromatographic fingerprint of PACs was based on adapted literature methods (Table 3.12). Little information appeared to be available concerning the detection of flavonoids in fungi (Matilla *et al.*, 2001). As a consequence, gallic acid, vanillic acid, tyrosol, caffeic acid, chlorogenic acid, *p*-coumaric acid, and cinnamic acid were chosen as representative examples for this study. This decision was also consistent with the fact that these reference materials are commonly reported in the chromatographic literature when dealing with the analysis of PACs (Table 3.12).

Author	Montedoro *et al.*, 1992	Wen *et al.*, 2005	Proestos *et al.*, 2006	Dvorakova *et al.*, 2008	Veberic *et al.*, 2008
Preparation of sample solution	Methanol/water (7:3, v/v) and extracted with petroleum ether 40-70.	Methanol/water/TFA (50:50:0.1, v/v)	62.5% v/v aqueous methanol, BHT (1 g/L) and 6 M HCL.	Methanol/water (7:3, v/v)	Methanol and 1% w/v of BHT
Column	Erbasil, C_{18}, 150mm x 4.6 mm	Agela XBP-C_{18}, 5 um, 4.6 mm x 150 mm	Waters Spherisorb ODS2, 5um, 4.6 mm x 250 mm	LiChroCart RP-18, 125mm x 3 mm, 3 um.	Phenomenex Synergi 4u MAX – RP 80 A
Mobile phase	Gradient; (A) 2% v/v acetic acid in water and (B) methanol	Gradient; (A) 0.02%v/v TFA in water and (B) 0.02%v/v TFA in methanol	Gradient; (A) 1%v/v GAA in water, (B) 6%v/v GAA in water and (C) water/acetonitrile/GAA (65:30:5, v/v)	Gradient; (A) methanol and (B) 0.1%v/v in water	Gradient; (A) 0.01 M phosphoric acid and (B) methanol
Flow rate	1 mL/min	0.5mL/min	0.5mL/min	0.2 mL/min	1 mL/min
Detector and phenolic acid	239 nm and 278nm Gallic acid, vanillic acid, caffeic acid, syringic acid, coumaric acid, cinnamic acid and (3,4-dihydroxyphenyl)ethanol	254nm-anisic, vanillic 275nm-gallic, trans-cinnamic, syringic acid 305nm- salicylic acid 320nm- chlorogenic, gentsic, caffeic, sinapic, p-coumaric, ferulic, rosmarinic acid	280nm-phenolic acids 320nm - Apigenin and luteolin 370nm -quercetin	280 nm -(+)- catechin and (-)- epicatechin and gillic acid 320 nm –caffeic, *p*-coumaric, ferulic and sinapinic acid 250 nm - protocatechuic and vanillic acid	280 nm. 210 and 350 nm gallic acid, chlorogenic acid (5-*O*-caffeoylquinic acid), (-)-epicatechin, (+)-catechin, syringic acid, and rutin (quercetin-3-*O*-rutinoside).

Table 3.12 *Different HPLC methods obtained from literature sources for the analysis of PACs*

Two types of sample preparations were used for this study. The first preparation (section 2.2.13.1.1) was chosen based on the highest PACs results (Table 3.11) for free form compounds that could introduce additional peaks to the chromatogram of sample solutions. The second preparation (section 2.2.13.1.2, Preparation 3, Figure 2.2, page 46) was selected since it produced optimum separation.

A number of columns have been evaluated for the separation of PACs and included C_{18}, C_8, silica gel, phenylhexyl and diol stationary phases. Representative examples of chromatograms obtained from different columns and mobile phase compositions are shown in Figure 3.46 and 3.47, respectively.

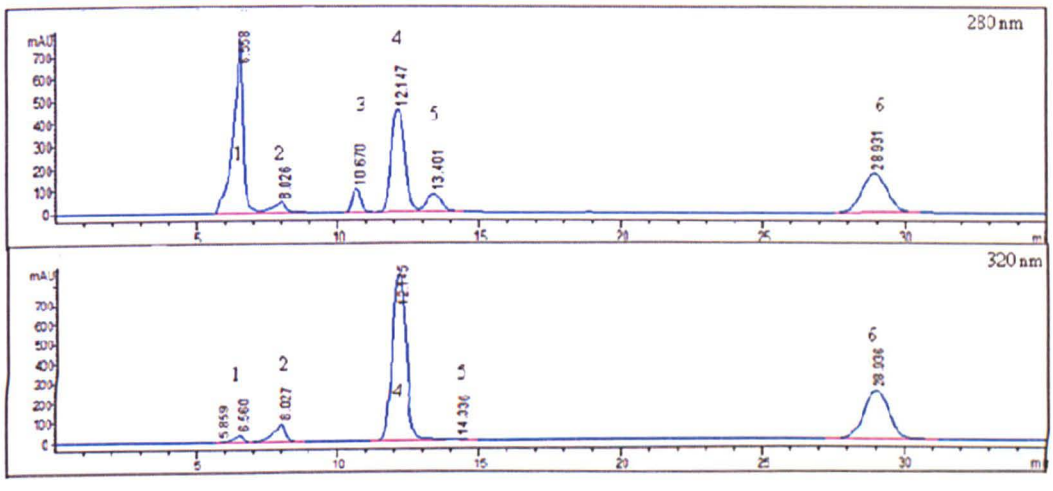


Figure 3.46 *Chromatograms of phenolic acid reference solutions. (1) gallic acid, (2) chlorogenic acid, (3) tyrosol, (4) caffeic acid, (5) p-coumaric acid and (6) cinnamic acid obtained from HPLC analysis. C_8 column (5 µm, 250 x 4.6 mm), 35% (v/v) methanol in water as the mobile phase and flow rate 0.8 mL/min.*

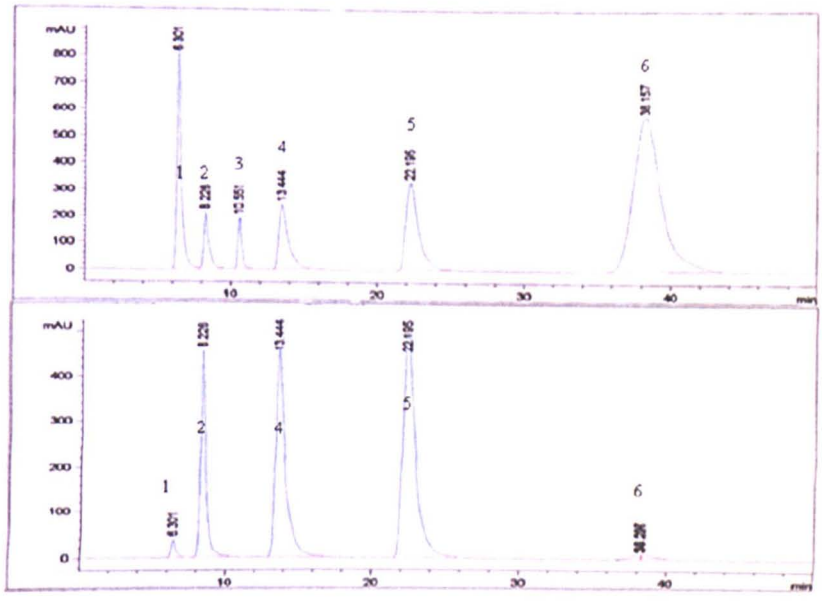


Figure 3.47 *Chromatograms of phenolic acid reference solutions. (1) gallic acid, (2) chlorogenic acid, (3) tyrosol, (4) caffeic acid, (5) p-coumaric acid and (6) cinnamic acid obtained from HPLC analysis. Phenylhexyl column (5 μm, 250 x 4.6 mm), 35% (v/v) methanol in water containing 1% (v/v) glacial acetic acid as the mobile phase and flow rate 0.8 mL/min.*

HPLC analysis of PACs was carried out in the isocratic mode since the current analysis did not involve with flavonoids which normally employed a gradient system for peaks separation. A literature search revealed that a polar-non polar solvent system, containing 2 mL acetic (GAA) or formic acid (FA) per 100 mL or phosphate buffer at low pH, has been employed to separate these acids (Ozturk et al., 2007). Correspondingly, three different acids (TFA, GAA and FA) were used as modifiers. It was found that when the separation of the six phenolic acid reference materials was carried out with the addition of FA in the presence of methanol/water (35:65, v/v) better resolution was observed for gallic and chlorogenic acid. Moreover, higher peak symmetry, as well as precision, was also observed. All adjacent peaks were resolved from each other with resolution above 1.2. In

addition, the analysis with PFP column was completed in about 20 minutes (Figure 3.48) while other columns required between 30 and 40 minutes for complete separation.

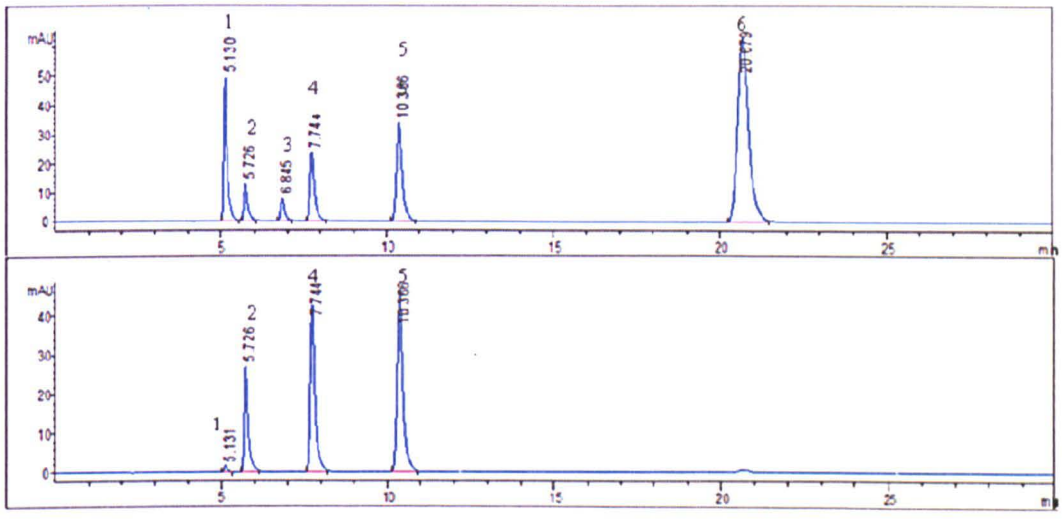


Figure 3.48 *Chromatograms of phenolic acid reference solutions. (1) gallic acid, (2) chlorogenic acid, (3) tyrosol, (4) caffeic acid, (5) p-coumaric acid and (6) cinnamic acid obtained from HPLC analysis. PFP column (3 μm, 150 x 4.6 mm), 35% (v/v) methanol in water containing 0.2% (v/v) formic acid as the mobile phase and flow rate 0.5 mL/min.*

The selection of two wavelengths (280 and 320 nm) was based on optimum sensitivity when compared to wavelengths at 220, 254 and 365 nm. According to Harbone (1973) benzoic acid derivatives of PACs can be detected at lower wavelengths (280 nm) while cinnamic acid derivatives (chlorogenic, caffeic and *p*-coumaric acids) are found to respond better at 320 nm due to the presence of one extra double bond in the compound (Table 1.4, page 21).

Figure 3.49 shows the chromatograms obtained from the analysis of caps, stalks and sclerotia solutions obtained at 280 nm. The retention times of sample solutions were compared with the retention times of the six reference materials but none of the reference

compounds were detected in the sample solutions. In addition, there was a massive unresolved peak at the beginning of the chromatogram which did not allow for the unambiguous detection of gallic, chlorogenic, tyrosol and caffeic acid. The most resolved peaks were obtained from stalks and acidic solutions at 320 nm (Figure 3.50). This indicated that the sample solutions may have contained other phenolic acid derivatives in addition to the selected reference materials. At this stage it was not possible to draw any conclusion about the potential presence of simple PACs in the sample solutions. Consequently, further improvements of the purification procedure were deemed desirable in order to improve information content.

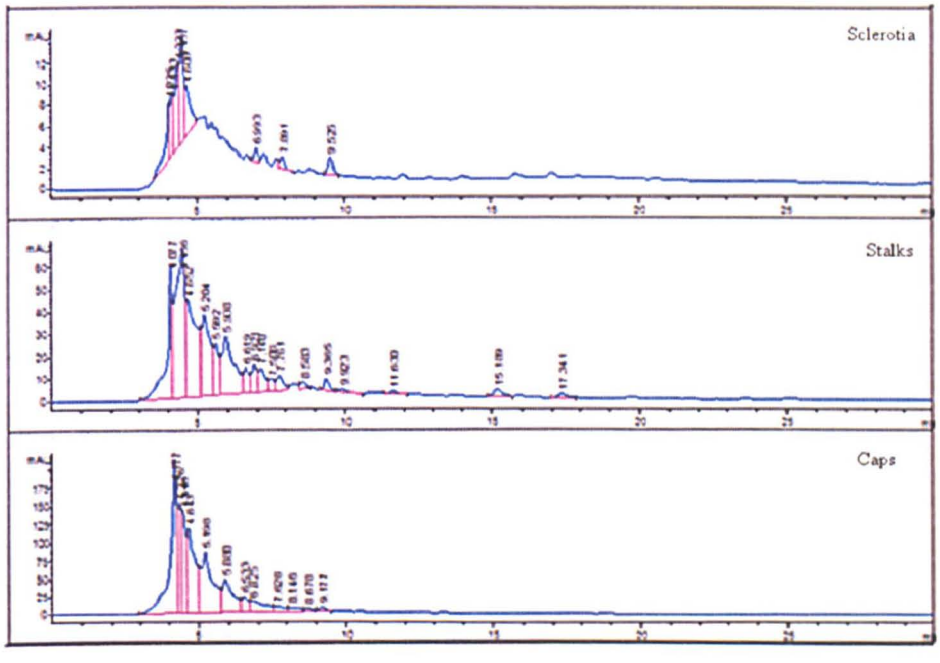


Figure 3.49 *Chromatograms of sclerotia, stalks and caps analysed by HPLC. PFP column (3 μm, 150 x 4.6 mm), 35% (v/v) methanol in water containing 0.2% (v/v) formic acid as the mobile phase, UV 280 nm and flow rate 0.5 mL/min.*

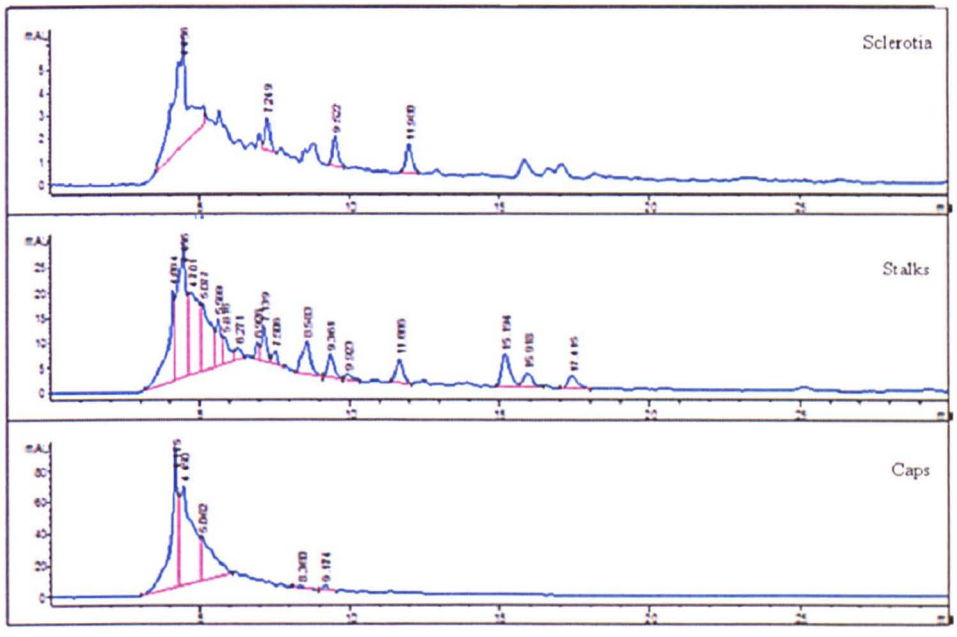


Figure 3.50 *Chromatograms of sclerotia, stalks and caps analysed by HPLC. PFP column (3 μm, 150 x 4.6 mm), 35% (v/v) methanol in water containing 0.2% (v/v) formic acid as the mobile phase, UV 320 nm and flow rate 0.5 mL/min.*

It was found that the use of acidic sample solution provided improved chromatographic fingerprints when applied to the quality control of THMPs that are known to consist of all parts of *L. rhinocerus*. Three chromatograms of commercially available products (Reishi, Coriolus and Maitake) were obtained for comparison using the acidic sample solution (Figure 3.51). One compound (peak X) was detected at 15.387 minutes (Figure 3.51) and the fact that it appeared to be absent in the other traces raised the question whether it could serve as a potential chemotaxonomic marker for *Lignosus* species. However, further investigations are required with other *Lignosus* species for verification.

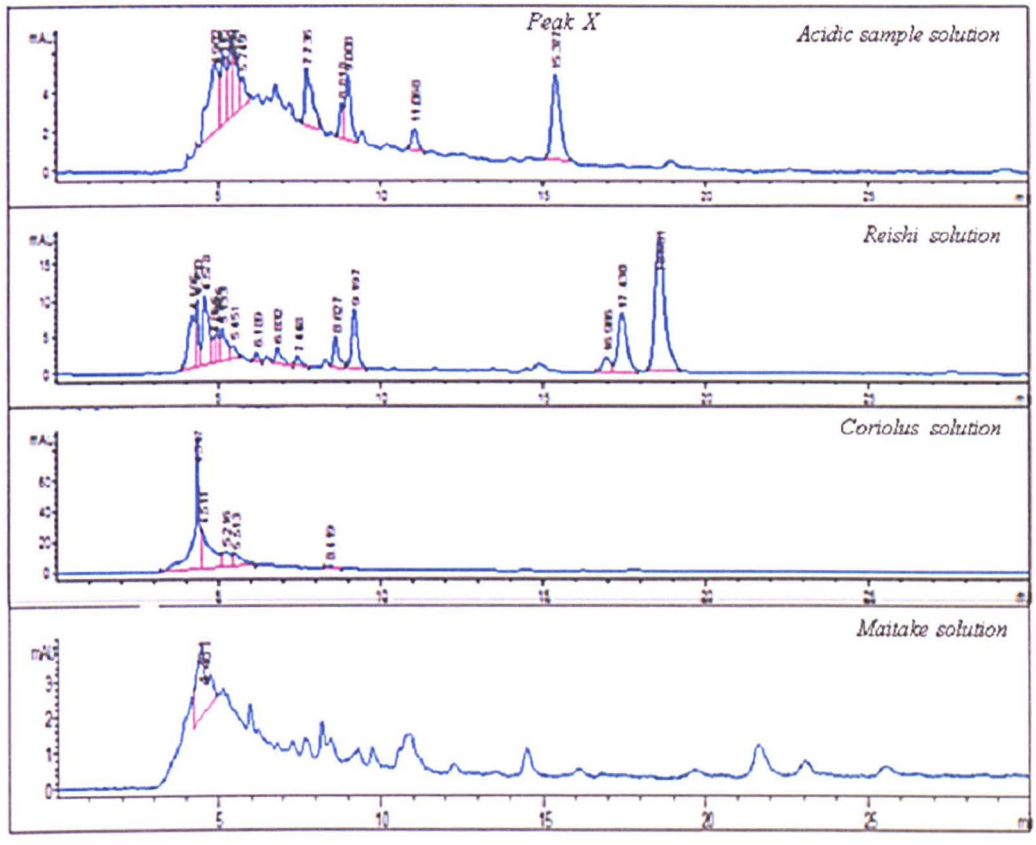


Figure 3.51 *HPLC chromatograms of acidic sample, Reishi, Coriolus and Maitake with one potential chemotaxonomic marker compound (peak X). PFP column (3 μm, 150 x 4.6 mm), 35% (v/v) methanol in water containing 0.2% (v/v) formic acid as the mobile phase, UV 320 nm and flow rate 0.5 mL/min*

CHAPTER 4: CONCLUSION AND SUGGESTIONS FOR FURTHER WORK

4.1 General conclusion

The aim of the research presented here was to characterize the key metabolites present in the *L. rhinocerus* fungus and to develop a validated methodology that could be used for the application of quality control procedures to traditional herbal medicinal products (THMPs). This study provided the first steps towards a chemotaxonomic approach to the classification of *L. rhinocerus* which was based on the detection and fingerprint analysis of biochemical compounds (secondary metabolites). One of the reasons for limited information on the composition of *Lignosus* species was mainly due to the difficulty in getting access to these rare species.

The initial work involved the evaluation of approximate compositions of *L. rhinocerus*. The reported components were identified as carbohydrates (86.91%), water (9.15%), proteins (2.80%) and lipids (0.02%), respectively. Polysaccharides have been reported to show anti-tumor properties. The moisture content was found to be considerably high (10.08-13.39% (w/w) of dried materials (DM)) in sclerotia of *L. rhinocerus* even though it was a dried material. The sclerotia of fungi are known to have relatively high affinity to water which indicated that the presence of water formed a crucial part within the routine quality assessment of *L. rhinocerus* medicinal products, since high water content may have significant effects on the formulation of products. The best method for the determination of moisture was found to be Karl Fischer titrimetry because it was simpler, accurate and fast when compared to the loss on drying method. The protein content of *L.*

rhinocerus is normally related to tonic properties which is claimed to be therapeutically useful. The Bradford assay was used to determine the total content of protein (2.78 % (w/w) of DM by BSA calibration). Total protein analysis is considered to be a general test but was found to be useful information which was decided to be included into the quality control assessment or specifications of this fungus.

Preliminary work on extraction techniques were carried out on the water extract (decoction) and involved the use of dichloromethane-water. There were a significant number of compounds which could be readily extracted when using dichloromethane-water. Several solvents, ranging from polar (methanol) to non-polar (hexane) solvents, were used to establish an appropriate extraction technique and methanol was found to be optimal. In addition, further improvements were achieved when using the extraction. The method required refluxing the sample with a mixture of methanol-Et_2O (1:1, v/v) in the presence of NaOH solution. This method led to the detection of four compounds by TLC analysis. This extraction technique was then utilized throughout the sample preparations.

Three approaches were employed for isolating the biochemical compounds. The first approach was based on fractionation of a principal peak detected under isocratic HPLC conditions, using a short C_8 column and 95% (v/v) methanol in water as the mobile phase. This approach was found to be a convenient method since the principal peak was well resolved from other peaks and as a result, a pure white residue was obtained (7.29 mg, 0.15 mg/g of dried sclerotia). The other two approaches involved the isolation of TLC spots and flash chromatography which was not be suitable to isolate compounds at R_f 0.46, 0.40 and 0.35 since the compounds residues were very little and required a large quantity of raw material for the isolation. These both methods were also not suitable for compounds susceptible to oxidation. In addition, both methods were time consuming and used a lot of solvent and sample material. Hence, from these two approaches, only two compounds were

successfully isolated: a white residue, 78.63 mg or 0.39 mg/g of DM and a yellow residue, 5.76 mg or 0.03 mg/g of DM.

The white residue was identified as ergosterol by comparing its retention time and spectra to ergosterol reference material. Ergosterol is a secondary metabolite ultimately derived from carbohydrate and is synthesized from acetate via the mevalonic acid pathway. The HPLC method was validated for quantifying the amount of ergosterol in different parts of *L. rhinocerus*. The validation showed that the method was selective, precise (RSD 3.90%) and accurate (recovery 92.60 - 104.60%) with good linearity ($r^2 = 0.9990$) while the QL and DL were determined at 0.04 µg/mL and 0.40 µg/mL, respectively. The robustness of the HPLC method was demonstrated by selective variations of analytical conditions in which the RSD values of intermediate precision were below 5.0%. The ergosterol distribution was found to vary in different parts. Caps, stalks, sclerotia with and without growth of the fungi were 0.37 ± 0.06 mg/g, 0.11 ± 0.03 mg/g, 0.13 ± 0.02 mg/g and 0.11 ± 0.02 mg/g of DM respectively. These results indicated that the younger part of *L. rhinocerus* had more ergosterol than the older. This also indicated that in addition to the sclerotia the beneficial material obtained from *L. rhinocerus* included the caps and stalks as well.

The HPLC-UV chromatographic fingerprint of *L. rhinocerus* species revealed the presence of fungal sterols. A Waters C_{18} column (150 x 4.6 mm i.d, 5.0 µm) was employed using 95% (v/v) methanol in water containing 0.02% (v/v) TFA at a flow rate of 1.0 min/mL and a wavelength at 280 nm. Chromatographic analysis showed potential for chemotaxonomic profiling of *L. rhinocerus* since Peak 1 (Figure 3.22, page 90) was detected in the sample solution when compared to chromatograms obtained from other commercially available fungal products where this peak was absent.

Ergosterol, which is the principal fungal sterol and its peroxidation products, has been reported to have numerous pharmacological properties including anti-inflammatory, anti-tumor and anti-oxidant effects. Ergosterol is also a precursor of vitamin D (ergocalciferol) via photochemical reactions using UV-B. However, vitamin D was not detected in *L. rhinocerus* under the conditions used.

The second isolated yellow compound was found to be ergosta-4, 6, 8 (14), 22-tetraen-3-one (ergone) as judged from comparison with spectral data reported in the literature. The pharmacological activity of ergone is known to be related to ergosterol and include anti-aldosteronic diuretic properties.

The UV and HPLC screening tests revealed that the *L. rhinocerus* may contain phenolic acid compounds (PACs). The total PACs content (average 23.15 μg/g) was determined by the Folin-Ciocalteu method. Anti-oxidant effects are related to pharmacological effects of PACs. The chromatographic fingerprint was determined by an optimized HPLC method. One compound was detected at 320 nm that may serve as a potential chemotaxonomic marker molecule within *L. rhinocerus*.

Both qualitative and quantitative analyses and the chemotaxonomic profiling of fungal sterols and PACs by HPLC methodologies were found to be suitable for the classification of *L. rhinocerus* species. It was possible to conclude that the developed procedures should be applicable to routine quality control assessments of traditional medicinal products of fungal materials. Routine analysis and screening for active ingredients should also enable the determination of environmentally-based variations that are naturally observed in herbal and fungal products. Factors include age of the fungus, growth conditions and post-harvest storage.

4.2 Suggestions for further work

The current research used a very limited source of raw materials of *L. rhinocerus* which placed some difficulties on the isolation and unambiguous identification of Peak 1 (fungal sterols, Figure 3.22, page 90) and Peak X (Figure 3.51, page 125) from PACs. This problem may be resolved when using more advanced techniques such as HPLC-NMR. This technique has become the most promising technique for isolation, quantification, and structural elucidation of metabolites from biological fluids such as urine, blood or plasma and natural products in low concentration (Zhou *et al.*, 2007; Weber *et al.*, 2008; Djukovic *et al.*, 2008). Traditionally, NMR measurements required milligram amounts of the analyte in question to obtain sufficient signal-to-noise ratios, whereas the newly developed micro-coil NMR probes currently offer detection limits below 10 ng (Djukovic *et al.*, 2008).

The isolation of biochemical compounds using silica gel plates enabled the separation of ergone only but did not result in separation from other fluorescent compounds. However, these compounds could possibly be isolated using an automated-fractionating HPLC technique with fluorescent detection which is 1000-fold more sensitive than absorbance measurements (Christian, 1994). A high-speed counter-current chromatography method (HSCCC) is another example of potentially efficient separation that could be used in the future. It is a support free liquid-liquid partition chromatographic technique that can eliminate irreversible adsorption of samples on solid support in conventional column chromatography (Wu *et al.*, 2009). HSCCC is a very effective tool for the preparative separation and purification of natural products and Chinese traditional herbs (Zhao and He, 2007).

Purification of fungal sterols and PACs may be improved by using solid phase extraction (SPE) rather than using flash chromatography and HPLC-based fractionation. SPE has drastically changed the classical approaches of solvent extraction and allows not only isolation of analytes but also its preconcentration (Rouessac and Rouessac, 2000). The

technique can minimize the consumption of solvent and packing materials, especially silica powder which is hazardous to health.

Ergosterol is a unique component found in fungi, and hence, a good indicator for fungal contamination. Its quantification is more reliable than chitin (a component of fungal cell walls) which forms the major part of the insect cuticle and therefore, the analysis for fungal chitin could be contaminated with insects (Frisvad *et.al*, 1998). Furthermore, the accuracy of chitin assay techniques (e.g. hydrolysis followed by chromogen development procedures and spectrophotometric determination) depends on the conversion factor relating to the glucosamine content (Nilsson and Bjurman, 1998; Ng *et al.*, 2007) whereas quantification of ergosterol is possible by direct comparison with reference material.

Subsequently, this HPLC method may become an alternative method to the conventional plate count methods used for the determination of microbial contamination assessment as described in the BP and USP. The conventional methods only measure culturable fungi and often require long incubation periods on selective agar media (Ng *et al.*, 2007). Many studies have found a significant correlation between the ergosterol content and fungal dry mass in soil and aquatic systems, rice, tomato products and corn grains (Pasanen *et al.*, 1999; Kadakal and Artik, 2004; Ridder-Duine *et al.*, 2006; Parsi and Gorecki, 2006). There was a study by Ng *et al.*, 2007 who used an ergosterol assay as a rapid tool for the assessment of fungal contamination in grains and feeds. In the assay, *Aspergillus niger* was used, cultured in agar media and the colonies of the fungi were scraped and extracted with methanol. The HPLC result of this study showed a good correlation between the growth of *A. niger* and ergosterol content which indicated that ergosterol was a good indicator for mould growth. The suggested quality limit from this study was less than 3.0 μg of ergosterol per gram of sample and values above 8.0 μg/g of ergosterol were considered to reflect poor quality. Hence, this study supported the idea of employing the HPLC method as an alternative for traditional microbial assessment of

pharmaceutical products in the future. Furthermore, this HPLC method may facilitate the exploration of a wide range of fungal species apart from *A. niger* and *Candida albicans which* are indicators for fungal contamination found in medicinal products (BP and USP, 2009).

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Appendix I: The Press Release of *L. rhinocerus*

The Star Online >Nation Friday, October 04, 2002

Cough cure tiger's milk is a fungus

By FOONG THIM LENG

IPOH: The tiger's milk which cured Prime Minister Datuk Seri Dr Mahathir Mohamad chronic cough is a fungus found in Malaysian jungles, said local Chinese herbalist Liew Shou Lin, 63.

JUNGLE CURE...Liew showing the 'tiger's milk' fungus in Ipoh Thursday.

He said the Malays and the orang asli called it *kulat susu rimau* as according to folklore, the fungus would grow at the spot where milk from a tiger had dropped on. He said the fungus grew well in cool and wet areas in the fringe of the jungle or rubber estates.

Dr Mahathir had during the opening of the International Convention on Bio-technology at the Putra World Trade Centre in Kuala Lumpur on Tuesday, said that the chronic cough he used to suffer from stopped after a friend persuaded him to take Chinese medicine. His friend had said the medicine was tiger's milk but was not willing to divulge its secret. Dr Mahathir said that so far, no attempt had been made to analyse and identify the active ingredient or to test and produce the medicine commercially.

Liew said the *kulat susu rimau* belonged to the same family as the lingzhi (*ganoderma lucidum*), another popular fungus known for its various curative properties in treating ailments like insomnia, hypertension, chronic bronchitis, kidney infection, asthmatic cough and low blood cell count.

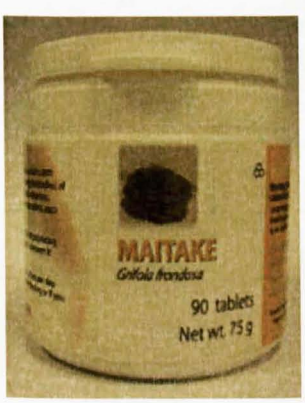
The *kulat susu rimau* fungus is a local herb not found in the Chinese pharmacopoeia but its effectiveness in treating asthmatic cough, gastritis, and indigestion especially among infants, has been known not only to the older generation among the Chinese but also the Malays and the orang asli.

The orang asli used to collect the fungus for Chinese medical halls in towns and villages, he said. He said the effective part of the fungus was its hard, tuber-like body found in the top soil. The part is normally cut into pieces and boiled in water for drinking. It can be ground into powder Form and mixed with Chinese rice wine for external application to treat lumps, boils and sores, said Liew.

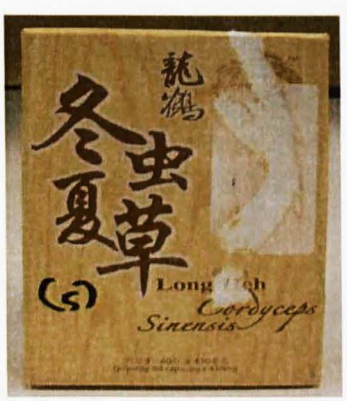
Appendix III: Commercials products

Reishi (*G. lucidum*),

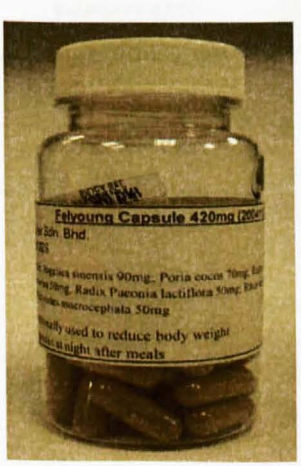
Coriolus (*C. versicolor*),



Maitake (*G. frondosa*),



Long Heh (*C. sinensis*)



Felyoung (*P. cocos*),

Appendix IV: Validation data

(a) Calculation of limit of detection (DL) and quantification (QL) based on the standard deviation (σ) of y-intercepts and slope (S):-

Run 1

No.	Concentartion (μg/mL)	PA
1	40.89	2028.13
2	61.33	3218.25
3	81.78	4222.96
4	102.22	5589.99
5	122.66	6381.41
	S	54.19
	y-intercepts	-143.17
	r^2	0.9951

Run 2

No.	Concentartion (μg/mL)	PA
1	39.5058	1986.65
2	61.3320	3214.26
3	81.7760	4175.71
4	102.2200	5617.48
5	122.6640	6479.00
	S	54.96
	y-intercepts	-184.58
	r^2	0.9955

Run 3

No.	Concentartion (μg/mL)	PA
1	40.90	2007.39
2	60.33	3205.25
3	80.51	4210.96
4	100.52	5333.99
5	120.17	6341.31
	S	54.32
	y-intercepts	-152.42
	r^2	0.9990

Calculation

	y-intercepts	***S***
	143.17	54.19
	184.58	54.96
	152.42	54.32
σ	21.74	0.41
Average	160.06	54.64

$$DL = \frac{3 * \sigma}{S}$$

$$= \frac{3.3 * 21.74}{54.64}$$

$$= 1.31\ \mu g/mL$$

$$QL = \frac{10 * \sigma}{S}$$

$$= \frac{3 * 21.74}{54.64}$$

$$= 3.98\ \mu g/mL$$

(b) Stability of analytical solutions:-

Run 1

No.	Sample		Reference	
	RT	PA	RT	PA
1	6.37	2781.59	6.34	4678.35
2	6.39	2778.73	6.42	4750.71
3	6.40	2766.33	6.39	4725.90
4	6.47	2777.25	6.45	4721.71
5	6.50	2782.85	6.48	4780.50
6	6.49	2764.94	6.48	4766.24
7	6.48	2763.84	6.50	4746.20
8	6.47	2758.20	6.40	4730.01
9	6.39	2766.20	6.41	4712.86
10	6.36	2748.30	6.42	4695.72
11	6.33	2730.40	6.43	4678.57
12	6.39	2712.51	6.43	4661.42
average	6.42	2759.05	6.44	4724.53
sd	0.06	21.35	0.04	37.03
RSD%	0.92	0.77	0.68	0.78

Run 2

No.	Sample		Reference	
	RT	PA	RT	PA
1	4.33	3361.69	4.44	3266.74
2	4.44	3288.33	4.45	3259.30
3	4.45	3300.01	4.45	3281.53
4	4.46	3302.13	4.46	3287.55
5	4.47	3288.34	4.46	3279.43
6	4.46	3281.91	4.45	3275.60
7	4.40	3270.57	4.45	3280.91
8	4.38	3260.46	4.44	3292.94
9	4.37	3250.35	4.44	3296.96
10	4.45	3240.24	4.43	3260.99
11	4.44	3230.13	4.42	3240.01
12	4.45	3220.02	4.42	3241.99
average	4.43	3266.59	4.44	3272.47
sd	0.04	38.57	0.01	18.53
RSD%	0.99	1.18	0.30	0.57