ANALYSIS OF THE MYCOCHEMICALS COMPONENTS OF THE INDIGENOUS NAMIBIAN *GANODERMA* MUSHROOMS

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

OF

UNIVERSITY OF NAMIBIA

By

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April 2012

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Abstract

Ganoderma lucidum (Fr.) Karst. (Ganodermataceae) is a natural scarce basidiomycetous polypore fungus that has a variety of uses such as providing nutrition and as a medical remedy. It has been used in the Asian countries for many years to treat a wide range of ailments with polysaccharides, triterpenes, sterols, lectins and proteins as major active constituents. However, in Namibia, the traditional usage of the *Ganoderma* mushrooms have been used to relieve stress, calm nerves, heal cold and flu symptoms, treat skin infections and heal wounds. It is also used in the treatment of animal diseases such as lung sickness in cattle, coughing animals and skin rushes in goats. It is for this reason that there is a need for scientific validation of the traditional usage of *Ganoderma* mushroom as medicine in Namibia. Thus, it will be more desirable to know the mycochemical compounds profile of the indigenous *Ganoderma* mushrooms and to validate the antibacterial properties of certain Gram + and Gram – bacteria used in the treatment of skin infection and wounds. The fruit bodies of the indigenous Namibian *Ganoderma* mushrooms were collected from decaying tree stumps, barks, and decomposing roots from the four regions in the north and north-eastern Namibia. The indigenous mushrooms' mycochemical compounds where extracted with aqueous and a series of organic solvents. The classes of compounds present in the crude extracts of the indigenous *Ganoderma* mushrooms were determined through colour reaction detection of the universal spraying reagents on the TLC. The TLC chemical tests of the crude extracts showed that they contained compounds which stained blue–violet, and blue or green when sprayed with anisaldehyde–sulphuric acid, Vanillin sulphur acid reagent and Dragendorff, respectively indicating the presence of triterpenoids. Other classes of compounds detected were the alkaloids, phenols, anthracenes, lipids, flavonoids and polysaccharides making up the Namibian *Ganoderma* mycochemical class of compounds. The major groups of the extracts components detected where the polysaccharides and triterpenes group of compounds. There were some different colour reactions from some of the universal detection reagents suggesting new class of compounds in the Namibian *Ganoderma* which were not reported before in the Literature. The profile of mycochemical compounds in the extracts against the tested and sprayed TLC plates showed variation among the extracting solvents. The antibacterial properties of the crude organic extracts and aqueous extracts were tested against some clinically disease causing microorganisms. The crude extracts of the indigenous *Ganoderma* mushroom exhibited various degree of inhibition against some tested organisms. The widest inhibitory zones (19.0 mm) were obtained with the crude benzene extract of *G. lucidum* against *E. coli and N. meningitidis.* The lowest zone of inhibition (6.0 mm) was demonstrated with the aqueous extract against *E. coli.*

Key Word: Indigenous *Ganoderma*, Antibacterial properties, organic & aqueous extracts mycochemical classes of compounds

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Acronyms

MetOH	Methanol
EtOH	Ethanol
EA	Ethyl Acetate
HWE	Hot Water Extraction
TLC	Thin Layer Chromatography
ZERI	Zero Emission Research Initiative
GC	Gas Chromatography
^{1}H-NMR	proton-Nuclear Magnetic Resonance
^{13}C-NMR	Carbon 13-Nuclear Magnetic Resonance
HPLC	High Pressure Liquid Chromatography
UV	Ultraviolet
IR	Infra Red
SDOC	Spectral Database of Organic Compound
SADC	Southern Africa Developing Countries
NB	Nutrient broth
MHA	Mueller-Hinton Agar
TOCSY	Total Correlated Spectroscopy
HSQC	Hetero-Nuclear Single-Quantum Coherence
COSY	Correlated Spectroscopy
NOEs	Nuclear Overhauser Effect Spectroscopy
2D	Two-Dimension
Gram +	Gram positive (bacteria)
Gram -	Gram negative (bacteria)

Acknowledgements

A number of people have been instrumental in providing me with help, direction and support throughout this MSc. Journey. First and foremost, the Almighty God, my source of my strength, hope, courage, faith and trust; thanks and glory are to Him, for He endures forever. Secondly, my main supervisor, Dr. P M Chimwamurombe, thank you for giving me the opportunity to discover the amazing world of medicinal fungi; Dr. H R Lotfy, thank you for helping me and introducing me to the fabulous world of Analytical techniques in natural products; Dr. M Kandawa-Schulz, thank you for welcoming me to the world of Biochemistry of Natural products. To Mrs. P N Kadhila-Muandingi, thank you for being very courageous, fantastic mentor, being a mother and not just a friend. Thank you for touring with me to the homeland of our native medicinal mushroom, the *Ganoderma (Omapakululu*) mushroom. To Mr. Jean Damascencen Uzabakiriho, thank you for helping me out in the microbiology laboratory and for giving me a helping hand and lot of constructing ideas. To my fellow *Ganoderma* team and my friends, Emmanuel Nepolo, Lempy Ekandjo, Secilia Ilonga, Bertha Nambahu, Helena Naunyango, Rauna, Marius Hedimbi & Mutsa Takundwa; Science Journal Club team; ZERI staff; Chemistry and Biochemistry department staff and Biological Sciences Department staff. I would like to thank the UNDP-SG and the University of Namibia for sponsoring this research project.

I send my love (*Agape, Philia*) and many thanks to you all my wonderful brothers and sisters in Christ from the Hosianna and Emmanuel Youth-Bible Studies. Thank you for your prayers, love (*Philia*) and words of inspiration. Finally, I thank my dear family; Mum, late Dad, sisters, brothers, cousins, aunties and uncles. Thank you for the encouragement and for being so proud of my efforts. Your love (*Storge*), prayers and excitement has helped me believe in God for help.

"Many are the plans in a man's heart, but it is the Lord's purpose that prevails."

Proverbs 19:21

Declaration

I, Laimy T. Shikongo, declare hereby that this study is a true reflection of my own research, and that this work or part thereof has not been submitted for a degree in any other institution of higher education.

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Laimy T. Shikongo

Chapter 1: Introduction

1.1 General introduction

Traditionally, a mushroom has been defined as a fleshy, aerial umbrella-shaped fruiting body of a macrofungi (Miles and Chang, 1997, p. 6; Wasser, 1999, p. 31). In many African countries including Namibia, wild mushrooms have been collected and used for hundreds of years as edible and medicinal resources (Chang and Mshigeni, 2004a, p. 23). In Namibia's rural village communities, mostly *Termitomyces schimperi* (Owowa/Oova), *Terfezia pfeilii* (Kalahari Desert truffles/omatumbula), *Termitomyces sagittiformis* (okahauhwilili/uuhilili), *Panaeolus papilionaceus* (Kakalahambo), and *Termitomyces reticulatus* (Oshaamuya/Oshihamuya) mushrooms are being consumed (Chang and Mshigeni, 2004a, p. 23). During the rainy season such mushrooms are seen sold in local markets and alongside the road as a source of income.

Africa has wide range of climate which brings about the production of enormous quantities of ligno-cellulosic biomass yearly. Unfortunately, African mushrooms, specifically Namibian mushroom biota are poorly researched and documented, yet their biodiversity are known to be extremely high (Chang and Mshigeni, 2004a, p. 16; Mshigeni, 2001, p. 12; Kadhila-Muandingi, 2010). Some mushrooms have been used in Africa in traditional medicinal practices for years, but has never been subjected to comprehensive scientific research (Chang and Mshigeni, 2004a, p. 38). It is increasingly being realized that many species of mushrooms are very effective in boosting the body's immune system (Chen, Su, Kanagarajan, Agrawal and Tsay, 2009, p. 290). In the Namibian ecosystem there are various mushrooms that are of medicinal use which include the *Ganoderma* species. These mushrooms are known to contain mycochemicals which are effective against HIV/AIDS, cancer and other leading killer diseases (Chang and Miles, 2004, p. 241).

1.2 Natural products and the pharmacological industry

The natural products industry has become increasingly popular over the past three decades (SADC, 2006, p. 16). Despite rapid growth on a global scale, the natural products industry in the Southern Africa is not well established. In this regard, Phyto Trade Africa was establishment in 2001 (SADC, 2006, p. 16). The common interests of the Phyto Trade African countries were on the production, processing and sale of natural products through the development of a viable and enduring natural products industry in Southern Africa (SADC, 2006, p. 16). The development of natural products has been considered by pharmaceutical companies to produce affordable and cost effective remedies (Bhat, Nagasampagi, and Sivakumar, 2006, p. 52).

Natural products play a major role as active substances, and model molecules for the discovery and validation of new drug targets. Plants and fungi are the main source of natural compounds used for medicine. Thus, medicinal plants and medicinal fungi have attracted considerable interest because of their wide variety of bioactive metabolites (Cragg, Newman, and Snader, 1997, p. 52). Even though most people associate natural products with extracts from roots and leaves, the discovery of natural products is certainly not limited to plant species.

Many bioactive compounds, especially antibiotics, have been isolated from microbiological sources. Two of the most well-known and often prescribed antibiotic drugs are penicillin and tetracycline. The accidental discovery of penicillin by Alexander Fleming in 1928 is still one of the most important developments in the history of pharmaceutical chemistry. As an inhibitor of the growth of Gram-positive bacteria, it became the first natural product to demonstrate that microorganisms, specifically fungi, are a source of medically useful secondary metabolites (Roberts, 2004, p. 12). According to Roberts (2004, p. 121), the consideration of using natural substances for investigation of medicinal properties must be as follows: the evidence consideration of traditional usage of substances by indigenous populations, the abundance of the species in nature, and the sustainable utilization of the species.

Ganoderma species are one of the most widely researched fungi because of their reported potent bioactive properties. It is evident that the majority of medicinal research within the Ganodermataceae has been performed on *G. lucidum* and it has been reported that this species exist in Namibia (Mshigeni, 2001, p. 12; Kadhila-Muandingi, 2010, p. 45). It was found that *Ganoderma* species in Namibia comprising *G. lucidum, Ganoderma tsugae, Ganoderma neojaponicum* and *Ganoderma* *applanatum* (Kadhila-Muandingi, 2010, p. 45). *Ganoderma* species contains several triterpenoids and polysaccharides, which have been extensively investigated in relation to their physiological effects. Some major constituents which have been isolated from *Ganoderma* species are sterols, lectins and proteins (Sakai and Chihara, 1995, p. 76). Those components can be used to cure various human diseases. The investigation of the local species may yield mycochemicals with novel medicinal as starting materials for the development of therapeutic agents in cancer and for other ailments. It is by the virtue of their unique natural products, which are increasingly being shown to be effective in promoting the body's immunoresponse systems (Chang and Mshigeni, 2004a, p. 21; Mshigeni, 2001, p. 12) that mushrooms are being investigated in this regard. *Ganoderma* is diverse in its mycochemical components, therefore it should be promoted as an effective food supplements for health maintenance. The Namibian industries interested in new products such as pharmaceuticals, cosmetics, flavours and dietary supplements are expected to benefit from this study medicinal finding.

1.3 The Southern Africa *Ganoderma*

In Southern Africa, *Ganoderma lucidum* occurs in the South-Western (Namibia), southern and Eastern Cape Province, Kwazule Natal, Gaunteng regions of South Africa, Mozambique and Zimbabwe. It occurs in all these areas during summer and autumn (Van der Westhuizen and Eicker, 1994, p. 134). The shiny, varnished upper surface is the most distinctive feature of the *Ganoderma* fruit-bodies, although a number of similar species share this feature in these regions. These fungi are important pathogens of broad-leafed trees. Some develop on the trunks of living trees, and stipitate forms attached to roots appear to grow in the soil (Figure 1a) (Van der Westhuizen and Eicker, 1994, p. 134).

The scientific explanation of a *Ganoderma* fruiting body shape can be either environment, genetic or both (van der Westhuizen and Eicker, 1994, p. 135). Therefore, in the Southern Africa, the fruit-bodies grow in single (Figure 1a, 1b) or as compound (Figure1c, 1d) on or near trunks of broad-leafed trees; sessile or stipitate in a wide range of sizes. The fruit-bodies are thick, circular to bracket-shaped, often grown together laterally or arranged in overlapping tiers, corky when fresh, drying to woody, margin thin, entire to somewhat wavy (Figure 1c, 1d). Upper surface flat, occasionally grooved, covered with thin, shiny, lacquer-like crust, yellow to red to brown chestnut-brown, usually extending downwards and covering the stipe as well. Fruiting bodies appears from summer to autumn, usually after a good rains (van der Westhuizen and Eicker, 1994, p. 135).

The woody fruit-bodies are inedible although some of the indigenous locals slice and dry it and traditionally prepare a tea-like decoction which is highly valued as a health drink. *Ganoderma* (*Omapakululu* in Oshiwambo language in Namibia) is prepared as a hot water extract as follows: Thinly sliced or pulverized *Ganoderma* (either fresh or dried) is added to a pot of boiling water, the water is then brought to simmer with a pot covered. The resulting liquid is a fairly bitter in taste, with the redder colour is the tea; the more effective is the extracts. It is believed to possess anti-carcinogenic properties.

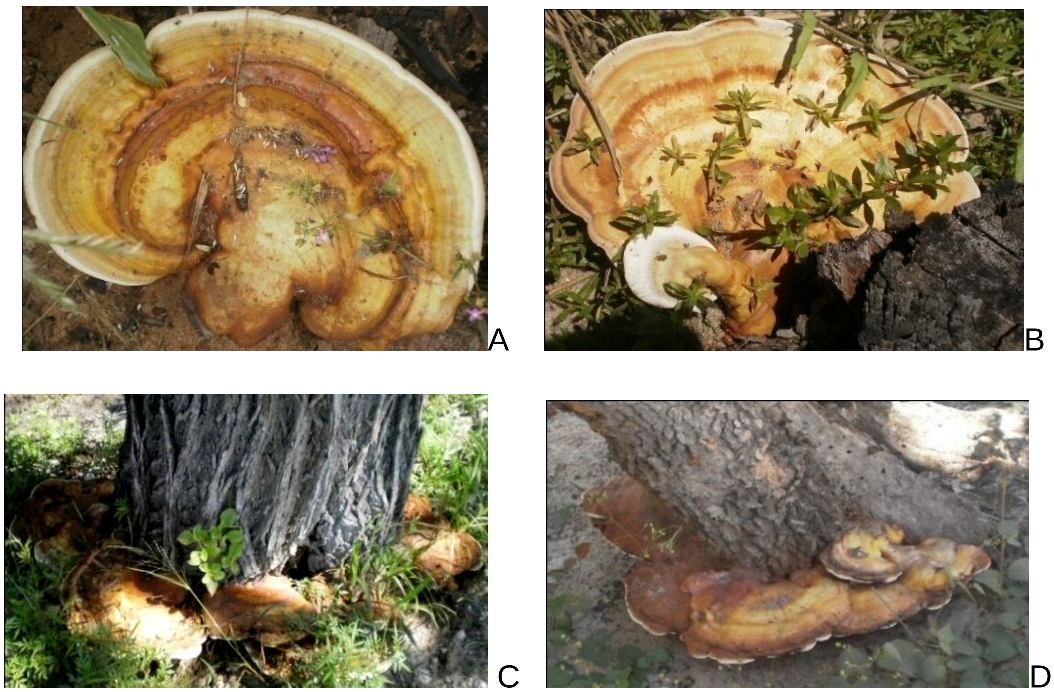

Figure 1 *Ganoderma* fruiting bodies. **(A)**; a single fruiting body attached to a root appear to grow in the soil, **(B)**; a single fruiting body growing from a dead stamp, **(C)** and **(D)**; fruiting bodies growing together laterally as compound,.

The *Ganoderma* mushroom natural product components has been investigated well in other parts of the world where it grows (Paterson, 2004, p. 1888). In other parts of the world, its hosts are certain deciduous trees species that are not found in Namibia. Such tree species are such as Oil palm tree, Rubber tree, Palm tree, Tea tree, Cocoa, Coconut, and Betal palm (Paterson, 2004, p. 1889), Acer, Betul, Castanea, Fagus, Fraxinus, Populus, Quercus, Tilia and very seldom on woods of coniferous trees (Boh, Hodzar, Dolnicar, Berovic and Pohleven, 2000, p. 12). However none of these trees are hosts of the Namibian *Ganoderma,* pointing to the possibility of a novel set of biologically active compounds that are yet to be discovered.

The Namibian *Ganoderma* were found to grow on the following tree species: *Croton gratissimus, Combretum collinum, Colophospermum mopane, Acacia* spp., *Mundulea sericea*, *Terminalia prunioides*, *Terminalia sericea*, *Sclerocarya birrea*, *Acacia sieberana*, *Baikiaea plurijuga*, *Combretum frarans, Grewia retinervis,* and *Combretum zeyheri* (Kadhila-Muandingi, 2010, p. 33). Fungi within the same family can produce a different variety of secondary metabolites as results of stress (Pointing and Hyde, 2001, p. 201) or from exposure to different environmental conditions (Kim, Hwang, Park, Cho, Song, and Yum, 2002, p. 56; Kim, Hwang, Xu, Sung, Choi, and Yum, 2003, p. 123). Namibia is an arid country with a hyper arid zone along the Namib and is in fact considered the driest country south of Sahara. The dry climate and lack of surface water caused a large number of species to evolve adaptations to survive such extreme conditions. Based on that finding, looking at the Namibian extreme environmental conditions, different host species and other factors compared to other parts of the world, the Namibian *Ganoderma* might potentially produce secondary metabolites with different biological activities. Therefore it is important to determine the chemical profile of this indigenous mushroom.

In southern Africa especially Namibia, many health claims by the indigenous group of people have been made on the effect that *Ganoderma* species have on the immune system. According to the information obtained from indigenous people, *Ganoderma* have been used in relieving stress when sniffed as ash mixed with tobacco, calming of nerves when put in water, used as a drink. It is also used in healing of cold and flu symptoms when its smoke is inhaled. Its extracts are applied to infected skin and to treat the wounds on children's heads. This is in support with what Kadhila-Muandingi (2010, p. 34) found out about the traditional uses of *Ganoderma* mushroom in Ohangwena and Oshana regions. The use of crushed *Ganoderma* mixed with ash to be used as ointment in the treatment of skin infections was also confirmed in another study in Cameroon (Yongabi, Agho, and Martinez, 2004, p. 35).

This mushroom is also said to have history in treating animal diseases, especially cattle when suffering from lung diseases and goats when having skin rushes. It is said that the cattle herders crush the fruiting bodies of these mushrooms, add water and mix well before giving the animals to drink. The animals are said to stop coughing after taking the mixture for some days of which the caretaker could not specify as to how many (Kadhila-Muandingi, 2010, p. 57; Yongabi, Agho, and Martinez, 2004, p. 35). Unfortunately, no previous studies were done to confirm this information. The medicinal uses of the mushroom need to be worked out for their biological activities due to a fast increasing number of multidrug resistances in pathogenic microbes like *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *N. meningitides* and *P. vulgaris (*Michael, Madigan, Martink, David, and David, 2011) (also see Appendix). Therefore, it is also important to determine the anti-bacteria properties and effects of the indigenous *Ganoderma* mushroom on specific pathogenic microorganisms which could be inhibited by this medically important mushroom.

1.4 Statement of the problem

There are a number of medicinally and health supplements from mushrooms imported in Namibia. Some of the medicinally and health supplements are from mushrooms which can also be found in Namibia such as the *Ganoderma* mushroom. The main problem is that the Namibian medicinal mushrooms are not researched and as a result, there is lack of scientific validation of the traditional usage of *Ganoderma* mushroom as medicine in Namibia. *Ganoderma* mushrooms are extensively researched elsewhere in the world except in Southern Africa, specifically Namibia. Thus, it will be more desirable if to know the mycochemical compounds profile of the indigenous *Ganoderma* mushrooms is known. Knowing the biochemical make-up of the indigenous *Ganoderma* mushroom will help in the researchers to start cultivating the *Ganoderma* mushrooms and make medicines and health supplements including tea. This will in turn help to generate locally produced nutriceuticals and pharmacological material from indigenous *Ganoderma* mushrooms will be made available.

1.5 The Objectives of the study

This study was aimed at identifying and analysing the mycochemicals present in the indigenous *Ganoderma lucidum* and evaluation of some biological properties. The specific objectives were:

1. To determine the Namibian *Ganoderma* mushroom mycochemicals that make-up the classes of compounds profile with the following points.
    - To determine if the indigenous *Ganoderma* mushroom have some mycochemicals secondary metabolites different from *Ganoderma* mushrooms found elsewhere.
    - To determine if the indigenous *Ganoderma* have some similar types of compounds with *Ganoderma* mushrooms found elsewhere.
2. To evaluate biologically the antibacterial properties of the indigenous *Ganoderma* mushroom against *E. coli, A. faecalis, P. vulgaris, N. meningitidis, B. cereus and S. aureus*.

1.6 Research hypotheses

The study had three research hypotheses, which are:

1. The indigenous Namibian *Ganoderma* mushrooms have produced some secondary metabolites different from those found elsewhere. This is due to the dry climate and lack of surface water can caused a large number of species to evolve adaptations to survive such extreme conditions. However, fungi within

the same family can produce a different variety of secondary metabolites as results of stress or from exposure to different environmental conditions. Thus, the extreme environmental conditions in Namibia, different hosting species and other factors compared to other parts of the world could produce potential mycochemical of different biological activities.

2. The indigenous *Ganoderma* have some similar types of class of compounds with *Ganoderma* mushrooms found elsewhere. The *Ganoderma* mushrooms and its closely related species have been reported to contain some compounds which are similar from even though from different geographical areas.
3. The indigenous *Ganoderma* mushroom extracts have antibacterial properties against *E. coli, A. faecalis, P. vulgaris, N. meningitidis, B. cereus and S. aureus*. This is to validate the traditional usage of *Ganoderma* extracts on wounds and in the treatment of skin infection.

1.7 Significance of the study

The significance of the study is to document information on the mycochemicals profile of the indigenous *Ganoderma* mushrooms as a nutriceuticals and pharmacological material. So far, there is no data in literature on the mycochemical components and antimicrobial properties of the indigenous medicinal *Ganoderma* mushrooms. Therefore, fundamental knowledge of the mycochemical profile of these mushrooms is needed to facilitate effective popularization of the *Ganoderma* mushroom in Namibian. For instance, there is now an increasing evidence that *Ganoderma* mushrooms have a wide range of medicinally important compounds that have anticancer and antiviral activity; offering great hope for the development of new drugs for ailments like HIV/AIDS, Avian influenza and the many cancers that afflict humanity today (Chang and Miles, 2004, p. 241).

This research finding will ensure that the traditional usage of *Ganoderma* mushroom is validated. Therefore, this will lead to the cultivation and commercial productions of these mushrooms in Namibia. This will in turn bring about locally produced health supplements. The locally made *Ganoderma* products can then be marketed and even exported, thus making *Ganoderma* mushrooms a source of income in the country. This study will provide significant insights into the traditional claim of the medicinal usage of *Ganoderma* mushrooms in Namibia through scientific validation.

Chapter 2: Literature Review

2.1 Background information

In a broad sense a "mushroom is a macrofungus with a distinctive fruiting body, which can be either epigeous or hypogeous and large enough to be seen with naked eye and to be picked by hand" (Chang and Miles, 1992, p. 64; Rai, Tidke and Wasswer, 2005, p. 246). Thus, mushrooms need not be Basidiomycetes, nor aerial, nor fleshy, nor edible. Mushrooms can be Ascomycetes, grow underground, have a non-fleshy texture and need not be edible. According to Roberts (2004, p. 11) this definition is not a perfect one but can be accepted as a workable term to estimate the number of mushrooms on the earth. The most common type of mushrooms is umbrella shaped with a pileus (cap) and a stipe (stem) i.e. *Lentinula edodes*. Other species additionally have a volva (cup) i.e. *Volvariella volvacea* or an annulus (ring) i.e. *Agarius campestris* or with both of them i.e. *Amanita muscaria*. Furthermore, some mushrooms are in the form of pliable cups; others round like golf balls. In fact, there is a countless diversity of forms (Roberts, 2004, p. 12).

Accordingly, mushrooms can be grouped into four categories:

1). Mushrooms which are fleshy and edible fall into the edible mushroom category, e.g. *Agaricus bisporus*.

2). Mushrooms which are considered to have medicinal applications, are referred to as medicinal mushrooms, e.g. *Ganoderma lucidum.*

3). Mushrooms which are proven to be, or suspected of being poisonous are named as poisonous mushrooms, e.g. *Amanita phalloides*.

4). A miscellaneous category which includes a large number of mushrooms whose properties remain less well defined, which may tentatively be grouped together as 'other mushrooms' (Chang and Mshigeni, 2004a, p. 18; 2004b, p. 39; Liu, Ooi and Chang, 1995, p. 486). Certainly, this approach of classifying of mushrooms is neither absolute nor mutually exclusive method. Many kinds of mushrooms are not only edible, but also possess tonic and medicinal qualities.

Fungi have been the producers of some of the most useful secondary metabolites and these metabolites have been researched and developed into therapeutic agents (Roberts, 2004, p. 121). The genus most known to have interesting bioactive metabolites is the *Ganoderma*, which belong to the division of Basidiomycota. Fungi from the division Basidiomycota have been of interest recently due to the number of biologically active compounds that have been isolated from them (Eo, Kim, Lee, and Ham, 1999, p. 129). Basidiomycota have a unique criterion for novel bioactive compounds and its potential to produce secondary metabolites (Roberts, 2004, p. 123).

Out of 2,327 useful species of mushrooms catalogued worldwide, barely a hundred can be cultivated and little research has been conducted in developing countries like Namibia on strictly local species i.e. *Ganoderma* species. Tanzania is reported as one of the countries in Africa that is actively exploring *Ganoderma* species of mushroom which are highly prized as dietary supplements (Ogbe, Ditse, Echeeonwu, Ajodoh, Atawodi and Abdu, 2009, p. 1053).

In recent years, more varieties of mushrooms have been isolated and identified, and the number of mushrooms being cultivated for food or medicinal purposes has been increasing rapidly (Mshigeni, 2001, p. 17). There are some prominent and bestselling edible and medicinal mushrooms such as: *Grifola frondosa, Coriolus versicolor, Lentinula edodes, Cordyceps sinensis, Schizophyllum commune, Hericium erinaceus* and medicinal *Ganoderma lucidum* (Chang and Mshigeni, 2004a, p. 14; Smith, Rowan and Sullivan, 2002, p.1839). Among these cultivated mushrooms for food and medicine, *Ganoderma* is cultivated only for medicine. Most traditional knowledge about mushrooms as food and medicinal agents comes from these species. The widespread and regular use of *Ganoderma* and other traditional medicine, in health promotion is based largely on tradition rather than scientific evidence (Wachtel-Galor, Tomlinson and Benzie, 2004, p. 264). Mushrooms from the Ganodermataceae family have been known to be of medicinal interest.

2.2 Ganodermataceae

Ganodermataceae is a widespread family of wood decaying organisms reaching its greatest diversity in the tropics and subtropics where conditions are hot and humid. Wasser and Weis (1999, p. 33) described *G. lucidum* species complex as medicinal fungus species belonging to the Polyporaceae family. The Polyporaceae fungi are classified as such as they have many tiny holes on the underside of the fruiting body, which are pores that contain the reproductive spores. The genus *Ganoderma*, however, was established in the West by a Finnish botanist, P. Karsten, in 1881 (Roberts, 2004, p.14; Bhosle, Ranadive, Bapat, Garad, Deshpande and Vaidya, 2010, p. 249), and more than 120 species have been reported in the world and since then a majority of them have been reported in China (Zhu, Yang, Wang, Zhao, and Chen, 2000, p. 201). There are multiple species of *Ganoderma* scientifically known to be within the *G. lucidum* species complex (Zhu et al., 2000, p. 201). *Ganoderma* species like any other fungi grow wild on living or dead/dying wood log of hardwood and sometimes on dead roots. Typically found at the base of living hardwoods or occasionally on the stumps or roots of a wide range of deciduous hosts (Chang and Mshigeni, 2004b, p. 53; Mshigeni, 2001). The mushroom host range is broad, species within the genus attacking both conifers and hardwoods, some being more generalist while others are host specific. They have a woody or leathery feel and the presence of these pores are obvious characteristics that distinguish polypores from other common types of mushrooms (Chang and Mshigeni, 2004b, p. 53).

There are multiple species of *Ganoderma lucidum*, scientifically known to be within the *Ganoderma lucidum* species complex and mycologists are still researching the differences between species within this complex of species (Moncalvo and Ryvarden, 1998, p. 2). *Ganoderma lucidum* generally occurs in two growth forms, one, found in

North America, is sessile and rather large with only a small or no stalk, while the other is smaller and has a long, narrow stalk or stipe, and is found mainly closer to and in the tropics (Moncalvo and Ryvarden, 1998, p. 3). However, many growth forms exist that are intermediate to the two types, or even exhibit very unusual morphologies, raising the possibility that they are separate species (Huie and Di, 2004, p. 112). Environmental conditions can apparently play a substantial role in the different morphological characteristics. For example, elevated carbon dioxide levels result in stipe elongation in *Ganoderma lucidum*. Other forms show "antlers', without a cap and these may be affected by carbon dioxide levels as well (Paterson, 2006, p. 1986).

2.3 Medicinal uses of *Ganoderma* mushroom

Several *Ganoderma* species are used in traditional oriental medicine (Moncalvo and Ryvarden, 1998, p. 2). *G. lucidum* is called *Ling-zhi* in Chinese, *Reishi* in Japanese and *Young-zhi* in Korean. *Ganoderma* has been used in China, Japan and other Asian countries for more than 2000 years as a medicinal fungus (Liu et al, 2007, p. 1692; Wasser and Weis, 1999, p. 30). The traditional medicinal properties of the fungus have for example been used for the treatment of hepatopathy, nephritis, hypertension, hyperlipemia, arthritis, neurasthenia, insomnia, bronchitis asthma, gastric ulcers, arteriosclerosis, and diabetes (Chang and Mshingeni, 2004b, p. 16; Paterson, 2006, p. 1989). Since the *Ganoderma* species are not classified as edible mushrooms, as the fruiting bodies are thick, corky and, tough and do not have fleshy texture characteristic of true edible mushrooms such as the common white button mushroom, *Agaricus* *bisporus* (Wasser and Weis, 1999, p. 31). Several types of *Ganoderma* products are available in the market although they are not classified as edible, such as capsule, tablets tonic, toothpaste, beauty soaps, tea, and coffee (Jong and Birmingham, 1992, p. 103; Di et al., 2003, p. 88; Bao, Wang, Dong, Fang and Li, 2002, p. 176).

2.3 Bioactive substances in *Ganoderma* species

Mushroom fruit-bodies are complex structures, both morphologically and more physiologically with undoubted variations in chemical composition from batch to batch. The chemical make-up of a mushroom fruit-body will mirror the composition of the basic substrate and supplementary ingredients which can vary considerably since raw materials are derived from lignocellulosic resources (Smith et al., 2002, p. 1842). Also, within any batch of mushrooms, there will be some degree of variation in size and age which will, undoubtedly, influence specific biochemical composition (Gunde-Cimerma, 1999, p. 320; Wasser, 2002, p. 260; Smith et al., 2002, p. 1842). Also, the quality and content of physiologically active substances vary from strain to strain, and also depend on geological location, culture conditions and the growth stage of the fungus (Boh et al., 2000, p. 13).

Many bioactive compounds have been found in *Ganoderma* mushrooms, some of which inhibit the growth of cancer cells *in vitro*, have antiviral activity *in vitro*, which protect the body against free radicals that damage body cells to induce diseases or have other health benefits *in vitro* (Oei, 2003, p. 4; Smith et al., 2002, p. 1842). Recent scientific studies demonstrates that *Ganoderma* mushrooms possess anti-cancer, anti-allergy, anti-oxidation, anti-hypertension, cholesterol reduction, anti-ageing, anti-microbial activities and inhibiting platelet aggregation properties (Chang and Mshigeni, 2004a, p. 21; Boh, Berovic, Zhang and Zhi-Bin, 2007, p. 266). These substances may be useful as starting materials for the development of chemical therapeutic agents in cancer treatment and other ailments (Mizuno, 1995, p. 16). Polysaccharides, triterpenes, protein bound polysaccharides, Germanium, phenolics compound, alkaloids compounds, lectins, amino acids and proteins (Boh et al., 2000, p. 16; Eo et al., 1999, p.130) are some of the major active constituents that have been isolated from *G. lucidum* and its closely related species, with polysaccharides and triterpenes being the most extensively investigated (Chang and Mshigeni, 2001a, p. 26; Chen and Chen, 2003, p. 198).

2.3.1 Polysaccharides from *Ganoderma*

In recent years, a lot of scientific attention has been focused on *Ganoderma* polysaccharides, which represent a structurally diverse class of biological macromolecules with a wide range of physicochemical properties (Zhang, Cui, Cheung and Wang, 2007, p. 15; Yang and Zhang, 2009, p. 350). Compared with proteins and nuclear acids, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability (Ohno, 2005). Mushroom polysaccharides exist as a structural component of fungal cell wall. The components are composed of two major types of polysaccharides: one is a rigid fibrillar of chitin (or cellulose); the other one is a matrix-like β-glucan, α-glucan and glycoproteins (Zhang et al., 2007, p. 10).

The major polysaccharides isolated from *Ganoderma* species are glucans, β-1-3 and β-1-6 D-glucan. These glucans are linear or branched molecules having a backbone composed of α- or β-linked glucose units, and some of them contain side chains that are attached at different position (Zhang et al., 2007, p. 11; Yang and Zhang, 2009, p. 349). The basic structure is β-1-3 D-glucopyronan with 1 to 15 units of β-1-6 monoglucosyl side chains (Mizuno, 1991, p. 28; Sakai et al., 1995, p. 77). Their 1, 3-linked backbone relatively small side chains and an organized helical structure are beneficial for the immunostimulation (Mizuno, 1991, p. 28). However, the structure of naturally occurring glycans is so diversified that it is difficult to define a universal protocol for their analysis (Zhang et al, 2007, p.11).

Other immunomodulatory polysaccharides have been reported, especially glycopeptides (Zhang et al. 2007, p. 11) and proteoglycans (Boh et al., 2007, p. 268). The anti-tumour polysaccharides differ greatly in their sugar composition and consequently in chemical structure, but one common feature is their relatively high molecular weight (Kim, Cho, Kim, and Choi, 1993, p. 210). Studies have shown that the most active immunomodulatory polysaccharides are water-soluble β-1-3-D and β-1-6-D glucans that can be precipitated by ethanol.

Other anti-tumour polysaccharides of *G. lucidum* are heteropolysaccharides, glycoproteins (polysaccharides connected to proteins), or a group of polysaccharides known as ganoderans A, B and C (Lindequist, 1995, p. 70). It has been reported that polyglucans with a higher molecular weight (10^4 to 10^{-6} Daltons) tend to have greater water solubility and therefore have a more effective anti-tumour activity (Mizuno, 1991, p. 26). The presence of polysaccharides in mushrooms (which are polymers of sugar molecules) suggests they can be useful as natural health-promoters against parasites, bacteria and viruses (Oei, 2003, p. 3).

Selection of an extraction method for polysaccharides depends on the cell wall structure. Hot water extraction method has been a popular approach traditionally. Bioactive water-soluble polysaccharides have been isolated from the fruiting bodies and from the mycelial biomass cultivated in liquid culture of *Ganoderma*. Few have been isolated from the culture medium. Some water insoluble anti-tumor polysaccharides were also identified (Boh et al., 2007, p. 267).

Mizuno (1997, p. 123) developed reliable procedures for successful extraction of polysaccharides from *Ganoderma* fruiting bodies or cultured mycelium. The extraction method can be varied based on the structure and water-solubility of polysaccharides, but the basic rule is to break the cell wall from outer layer to the inner layer with mild to strong extraction conditions (pH and temperature). The natural isolated polysaccharides from mushrooms includes acidic and neutral ones with different types of glycosidic linkages, while some are bound to protein or peptides residues such as polysaccharides protein or peptide complexes (Jong and Birmingham, 1992, p. 103; Mizuno, Wang, Zhuang, Kawagishi, Nishitoba and Li, 1995, p. 154).

Polysaccharides with strong bioactivity action differ greatly in their chemical structures, exhibited by a wide range of glycans extending from homopolymers to highly complex heteropolymers (Ooi and Liu, 1999, p. 200). These glucans are linear or branched molecules having a backbone composed of α- or β-linked glucose units and some of them contain side chains that are attached at different positions. The structure of naturally occurring glycans is so diversified that it is difficult to define a universal protocol for their analysis (Zhang et al., 2007, p. 7; Yang and Zhang, 2009, p. 354). The building blocks (derived from sugar residues) of complex polysaccharides exhibit very similar structures, but their differentiation (diversified linkage style) is more inclusive than that of amino acids. The primary structure of a polysaccharide is defined by monosaccharides composition, configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides, as well as the nature, number and location of appended non-carbohydrate groups (Zhang et al., 2007, p. 8; Yang and Zhang, 2009, p. 354). In addition to the primary structure, a higher structure of polysaccharides such as chain conformation also plays an important role in their bioactivities. Most polysaccharides have remained classified as non specific bioactive substances because their exact mode of action was unknown and chain conformation of the active components was undefined (Wasser, 2002, p. 260).

The analytical methods of determining the anomerity (α or β) of each sugar residue can be done by NMR spectroscopy. The anomeric resonances appear in a clear region in the spectrum and show characteristic doublets with a splitting that is significantly larger for β anomers than for α anomers. NMR spectroscopy is the only method thought to have the potential for full structural characterization of a polysaccharide, with little or no assistance from other methods (Zhang et al., 2007, p. 8; Yang and Zhang, 2009, p. 353). Complete structural elucidation requires the full assignment of both the ^{1}H and ^{13}C NMR spectra using a combination of two-dimensional (2D) NMR techniques consisting of correlated spectroscopy (COSY) (Yang and Zhang, 2009, p. 353) and total correlated spectroscopy (TOCSY) (Yang and Zhang, 2009, p. 354) for ^{1}H, and the hetero-nuclear single-quantum coherence (HSQC) for ^{13}C. Polysaccharides sequencing relies exclusively on 2D 1H NMR spectroscopy, using through-space effects (nuclear overhauser effect, NOEs) as a source of evidence for linking positions and sequence (Yang and Zhang, 2009, p. 354). However the extent to which the primary structure of a polysaccharide has been assessed depends on the available techniques. The contribution from variety of disciplines is required to advance such technologies such as the combination of FTIR, NMR, Raman spectroscopy, GC, GC-MS, and HPLC methods is useful to characterise the chemical structures of bioactive polysaccharides (Zhang et al. 2007, p. 12; Yang and Zhang, 2009, p. 356).

2.3.2 Triterpenes from *Ganoderma*

The fruiting bodies of *G. lucidum* contain some intensely bitter compounds (Lindequist, 1995, p. 72) and this characteristic bitterness of *Ganoderma* is not found in any other mushroom (Mizuno et al., 1995, p. 154). It has been concluded that *G. lucidum* is the only species to contain bitter triterpenoids (Nishitoba et al., 1988, p. 1794; Mizuno, 1995, p. 19; Chen and Chen, 2003, p. 200). It has also been suggested that the bioactivity is related to the bitterness (i.e. the more bitter, the greater the bioactivity), although the relationship is not fully understood (Mizuno, 1997, p. 124). Nishitoba et al. (1988, p. 1793) finds that there is a relationship between the hydrophobic methyl groups of the three functional oxygen atoms that plays a significant role in generating bitterness.

Triterpenes are a class of naturally occurring physiologically active compounds whose carbon skeletons are composed of isoprene C-5 units. The triterpenes molecules are cyclic hydrophobic hydrocarbons with chemical structure based on the ground structure of lanosterol ($C_{30}H_{50}O$) (Lin, Li, Lee and Kam, 2003, p. 2388; Boh et al., 2007, p. 270; Tang, Gu and Zhong, 2006, p. 208), (Figure 2), which is an important intermediate in the biosynthetic pathway for steroids and triterpenes in microorganisms and animals (Chang and Buswell, 1999, p. 141; Chen and Chen, 2003, p. 200). The stereochemical variation of the structure produces a diversity of triterpenes. Their chemical structure contains oxygenated functional groups in a lanosterol skeleton with pairs of a C-3 α/β stereoisomers and C-3/C-15 positioned isomers which is similar to the one of the substances in cholesterol synthesis. Because of their structural similarity and compositional complexity, isolation of oxygenated triterpenoids from fruiting bodies and cultured mycelium for biological study is still difficult. However, over 150 triterpenoids were found in *Ganoderma* spp. (Nishitoba et al., 1988, p. 1793; Chen and Chen, 2003, p. 200) such as ganoderic (major components including about thirty compounds (Chen and Chen, 2003, p. 200)), lucidenic, ganodermic, ganoderenic, lucidic, ganolucidic and applanoxidic acids, lucidones, ganoderals and ganoderols (Boh, et al., 2007, 290; Tang et al, 2006, p. 208; Chen and Chen, 2003, p. 200; Liu et al., 2007, p. 1694; Boh et al., 2000, p.16), though the triterpenoid standards are difficult to be isolated.

Among all other medicinal macrofungi, *G. lucidum* is the only known source of a group of triterpenes, known as ganoderic acids, which have a molecular structure similar to steroid hormones (Shiao, 2003, p. 176; Boh et al., 2007, p. 290). Triterpenes/triterpenoids are bitter components of *Ganoderma* that have received considerable attention owing to their well-known pharmacological activities. Total triterpenes are extracted with a variety of organic solvents and water (Huie and Di, 2004, p. 243). However, the solubility in both polar solvents i.e. hot water, ethanol and methanol imply that it may have hydrophilic functional groups such as hydroxyl-, and carboxyl- groups (Lin et al., 2003, p. 2388).

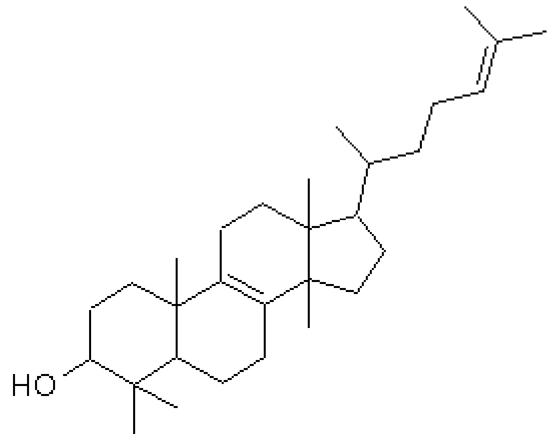

Figure 2: Structure of lanosterol

According to Patterson (2004, p.1985), only M. Hattori et al. 1999 have isolated and quantitated five ganoderic acids, but detailed analytical methods and recoveries have not been described (Chen and Chen, 2003, p. 197). Those triterpenoids were identified using a reverse-phase (RP)-HPLC and semi-preparative HPLC. RP-HPLC is a successful analytical separation tool used for the separation of triterpenoids mixture containing paired stereoisomers and positioned isomers (Shiao, Lin, and Yeh, 1989, p. 874). Nishitoba et al. (1988, p. 1793) investigated the strain specific triterpenoid patterns of *G. lucidum* and found that there was a clear difference in triterpenoid patterns between the strains, while Chen et al. (2009, p. 297) showed that the HPLC analysis of the triterpenoids of different *G. lucidum* strains showed different HPLC patterns, indicating the presence of different triterpenoids, while different *G. tsugae* strains showed similar patterns. They concluded that the spectral patterns between the two mushroom species were different.

The extracted triterpenes profiles was done with the use of a small Sep-pak C_{18} cartridge (especially the ganoderic acids) for a reversed-phase (RP)-HPLC chromatography and a silica gel column chromatography (Huie and Di, 2004, p. 26; Tang et al., 2006, p. 206; Chen and Chen, 2003, p. 200). The total number of hydroxyl and acetoxy groups within the triterpenes plays an important role in governing the polarity of these compounds. In 1989, Shiao et al. (1989, p.874) employed RP-HPLC and a binary solvent system consisting of acetonitrile and water under gradient elution for the separation of 24 oxygenated triterpenoid, including eight pairs of stereoisomers and five pairs of positional isomers, and they were able to correlate the molecular polarities with retention characteristics due to the presence of multiple oxygenated functional groups in these oxygenated triterpenoids (Chen et al., 2009, p. 295).

Triterpenes are qualitatively and semi-quantitatively determined with a silica gel TLC plates. Considering that many triterpenes extracted from *Ganoderma* contained carboxyl groups, solvent systems that consisted of small quantity of acid are preferred for TLC separations, i.e., chemically neutral solvent systems usually lead to band tailing (Paterson, 2006, p. 1989). Also, TLC has been applied for the differentiation of different species of *Ganoderma* by Su, Yang, Ho, Hu & Sheu (2001, p. 96). They found that different species of *Ganoderma* exhibited unique triterpenes patterns on the TLC chromatograms.

2.3.3 Proteins

Bioactive proteins have been isolated from various *Ganoderma* species and characterized by chromatographic and electrophoretic techniques. For instance, a new immunomodulatory protein, known as Ling Zhi-8 (LZ-8) is a polypeptide consisting of 110 amino acid residues with acetylated amino terminus (Roberts, 2004, p. 55). LZ-8 has been shown to have mitogenic activity *in vitro* and immunomodulating activity *in vivo* (Van der Hem, Van der Vliet, Bocken, Kino, Hoistma and Tax, 1995, p. 440). LZ-8 was purified by two chromatographic systems, i.e. gel filtration (Sephadex G-75 column) followed by ion-exchange (DEAE-Sephadex A-25 column) (Van der Hem et al., 1995, p. 440).

Another protein with relative low sugar content which has been extracted from the fruiting body of *Ganoderma* is lectin (Kawagishi, Mitsunaga, Yamawaki, Ido, Shimada, Kinoshita et al., 1997, p. 8; Kino, Yamashita, Yamaoka, Watanabe, Tanaka, Ko, et al., 1989, p. 476). Lectins are carbohydrate-proteins of non-immune origin, which agglutinate cells or precipitate polysaccharides or glycol-conjugates (Goldstein, Hughes, Monsigny, Osawa and Sharon, 1980, p. 66; Kino, Yamashita, Yamaoka, Watanabe, Tanaka, Ko, et al., 1989, p. 474). It was purified by anion exchange chromatography (Superose 12 HR 10/30 column) and affinity chromatography (BSM-Toyopearl affinity column), followed by the use of SDS-PAGE for molecular weight determination. Ye et al. (2002) have found three kinds of bioactive proteins (LZP-1, LZP-2 and LZP-3) from the fruiting body and spores of *Ganoderma* which showed obvious mitogenic activity.

Patterson (2006, p. 1992) demonstrated that the water soluble fraction of *G. lucidum* suppressed platelet aggregation, with the active substance identified as adenosine. Zhang et al. (2007, p. 18) reported the isolation and purification of two kinds of polysaccharides containing selenium in *G. lucidum* using ion-exchange chromatography (DEAE-cellulose column), gel filtration chromatography (Sephadex-100 column) and electrophoresis (PAGE).

2.4 Biomedical applications of *Ganoderma* mushrooms

From a medicinal point of view, the important compounds found in *Ganoderma lucidum* are mainly polysaccharides (β-D-glucans), sterols (particularly ergosterol), triterpenes (ganoderic acids), glycosides, riboflavin, ascorbic acid and amino acids (Wasser, 2010, p. 8; Wasser 2011, p. 308). Furthermore, it possesses dietary fibre (quitin, polysaccharides), i.e. high molecular weight compounds which are not absorbed or transformed into the digestive tract, and hence directly excreted. These compounds exhibit carcinostatic activity, due to their capacity to absorb and excrete carcinogenic substances (Wasser, 2010, p. 8).

There have been a number of reviews published on the biomedical application of mushroom in general (Borcher, Stem, Hackman, Keen, and Gershwin, 1999, p. 130;

Wasser and Weis, 1999, p. 46) and *Ganoderma* species in particular (Su, 1991, p. 10; Paterson, 2006, p. 1999). Over the last decade, there has been an increasing amount of research to investigate *Ganoderma* species for new biomedical applications. Some of the application that the mushroom extracts and constituents have been found to play important roles include: hepatoprotective activity, hypoglycemic activity, anti-inflammatory properties, antihistamine release, anti-platelet aggregation activity, anti-complement activity, antiviral activity, anti-fungal, antibacterial, enzyme inhibition, anti-tumor properties and in the healing of open skin wounds (Chen and Chen, 2003, p. 199; Chang and Mshigeni, 2004b, p. 48).

Recently, *Ganoderma* is best known for its immunostimulating effects in aiding cancer treatment and for its anti-HIV activity (Chang and Mshigeni, 2004a, p. 14; Chang and Mshigeni, 2004b, p. 16: Wasser, 2011, p. 312). *Ganoderma* well investigated bioactive compounds have biomedical applications and the methods of physiological activities are being studied for polysaccharides and triterpenes.

For that, it is generally accepted that mushroom polysaccharides have a mode of action that enhances the body's immune response, rather than having direct cytotoxicity towards tumour cells (Patterson, 2006, p. 1988; Gao, Fei, Zhang, Gong, Minami, Nagata et al., 2000, p. 1180. Reports on the pharmacological activity of *Ganoderma* polysaccharides mainly focus on anti-tumor effects, which are linked to immuno-modulation, although other effects, such as regulation and protection of cells, have also been observed (Chang, 1995, p. 48). It has been widely reported that anti-tumour and anti-cancer effects of polysaccharides arise from the enhancement of the host's immune system rather than direct cytocidal effects to the tumour cells, such as via an increase in interleukin, interferon and antibody production, as well as the stimulation of cytotoxic T-lymphocytes (Gao et al., 2000, p. 1181; Wang, Hsu, Hsu, Tzeng, Lee, Shiao, and Ho, 1997, p. 702).

Recently, the mode of action of triterpenes has been of interest to many researchers (Paterson, 2006, p. 1988). The mode of action of triterpenes is different to the mode of action of polysaccharides. Rather than enhancing the immune system, as polysaccharides do, triterpenes have been shown to have direct cytotoxicity against tumour cells (Gonzalez, Leon, Rivera, Padron, Gonzalez-Plata et al., 2002, p. 419). Triterpenes are thought to be potential anti-cancer agents due to the biological activity they have exhibited against actively growing tumours *in vitro* (Lin, Li, Lee, and Kan, 2003, p. 2384).

Phenols are naturally occurring antioxidants identified by Mau et al. (2002) as major constituents in methanolic extracts. *Ganoderma* species were also found to possess higher antioxidant activity, reducing power, and scavenging and chelating abilities than any mushroom studied (Zhu et al., 2000, p. 204). Antibacterial activity has been observed against Gram-positive bacteria from the fruiting body extracts of *G. lucidum* (Kim et al., 1993, p. 209). Yoo et al. (1994) investigated the additive effect on the activity of an aqueous extracts of *G. lucidum* with four known antibiotics and observed that the antibacterial activity increases. Although the isolation process, structural characterization of *Ganoderma* bioactive compounds have been extensively investigated in the past decades, the proper documented and approved method of isolating and structure elucidation of their active components is still not well established (Tang et al., 2006, p. 208).

Chapter 3: Materials and Methods

Overview

The extraction process is an important step in the investigation of biologically active compounds. When extracting compounds from fungi, or any living source, the type of solvent used, the extraction process employed and the age, part of cultivation of living tissue, can all have a marked effect on the type of compound that can be extracted. Aqueous extraction using hot water has been performed traditionally on the fruiting bodies of *Ganoderma* species (and/or any other traditional medicine) and has resulted in the extraction of many proteins, lectins and polysaccharides (Bao et al., 2002, p. 177; Cheong, Jung and Park, 1999, p. 517; Kawagishi et al., 1997, p. 8). When extraction process employs organic solvents such as benzene, chloroform, and/or methanol, which are non-polar and highly polar, respectively, different compounds such as alkaloids, fatty acids, triterpenes, and coumarins are commonly extracted (Cowan, 1999, p. 566; Lin & Shiao, 1988, p. 919; Shiao et al., 1989, p. 874).

3.1 Sample collection and identification

The wild mushrooms fruiting bodies were collected from the natural habitats of the Northern and North Eastern part of Namibia in the months of April-to-June 2010. A number of fresh *Ganoderma* mushrooms were collected from different tree hosting species. The *Ganoderma* mushrooms were found growing on different living and dead trunks of different tree species, and stumps around the villages in four Regions:

Oshana, Ohangwena, Kavango and Caprivi region. The mushroom fruiting bodies were characterized and identified as *Ganoderma lucidum* according to Van der Westhuizen and Eicker (1994, p. 24). The samples were recorded per host per region as follows and were labelled in khaki paper bag as: LRN-X (Laimy, Region, Natural sample number and X-is the local or indigenous name of the hosting species.

Local Name (X)	**Scientific Name**	**Labelled code**	**Region**
Omumbango	*Croton gratissimus*	LC01 LC06 LK08 LK09 LS11 LS01	Caprivi Kavango Oshana
Omusaati	*Colophospermum mopane*	LC07 LC08 LK02 LK04 LH03 LH11	Caprivi Kavango Ohangwena
Omugongo	*Sclerocarya caffra*	LS02 LS07 LS09 LS04	Oshana
Omugolo	*Terminalia sericea*	LC05 LC04 LK01 LK07 LS05 LS12 LH05 LH02	Caprivi Kavango Oshana Ohangwena
Omumbaganyana	*Mundulea sericea*	LC03 LC09 LK03 LK10	Caprivi Kavango
Omuhama	*Terminalia prunioides*	LC10 LC11 LK06 LK11 LH06 LH09	Caprivi Kavango Ohangwena
Eno	*Acacia spp.*	LC02 LC13 LS08 LS10 LH04 LH08	Caprivi Oshana Ohangwena
Omupupwaheke	*Combretum collinum*	LC10 LC12 LS03 LS06 LK05 LK12 LH01 LH07	Caprivi Oshana Kavango Ohangwena

Table 1: The local names and scientific names of the indigenous *Ganoderma* tree hosting species collected in the four regions and few of the samples labelled code given. Scientific names where confirmed in Von Koenen (2001).

3.3 Samples preparation

The freshly collected *Ganoderma* fruiting bodies were air-dried in the shade. Each dried *Ganoderma* sample was broken into pieces placed in a heavy duty commercial blender and crushed to powdered material. Each powdered material was placed in the respective khaki paper bags and stored in the dark and dry place at room temperature till the time of extraction.

3.4 Samples extraction

The bioactive compounds were extracted and isolated from all *Ganoderma* mushroom sample material (Table 1) for each hosting species per region with aqueous and organic solvents, in order to separate the chemical constituents into groups of different polarities. First, a "successive step extraction" was applied to determine the polarity of the active compounds. Different solvents used were known to present different polarity in order to extract successively compounds of different polarities: lipids, sterols, triterpenoids, glycoproteins, glycosides, sugars, amino acids and proteins. The solvents were used in order of increasing polarity (Benzene, Chloroform, Ethyl Acetate (EA), Ethanol (EtOH), Methanol (MetOH) and aqueous extraction as Hot Water (HWE). All reagents were of analytical grade and were used as received.

About 45 grams of each powdered sample was soaked and cold extracted successively with 400 mL of organic solvent at room temperature for 5 days. The organic solvents were used successively with gradient polarity starting with Benzene, Chloroform, (EA), (EtOH), (MetOH) and aqueous extraction HWE. The crude extracts were gravity filtered through a 0.45 μm Whatman No.2 filter paper. The filtrates were concentrated by evaporating excess solvent in a hot water bath and stored in the dark.

3.5 Chromatography

The extracts of *Ganoderma* mushrooms are very complex in composition and the determination of individual or group compounds will require the use of several analytical methods.

3.5.1 Thin Layer Chromatography

The samples were run and developed on several small scaled (i.e. 5 x 10 cm) pre-coated silica-gel TLC plates (silica gel F_{254} nm, bought already made). All the samples filtrates extracted in a series of organic solvents were performed on TLC plates using different development system per solvent extraction i.e. Benzene: Ethyl Acetate, Benzene: Chloroform, Acetic acid: Water, Ethyl acetate: Ethanol, Ethyl acetate: Methanol in varying ratios. The TLC plates were placed in developing chamber glass containers with solvent systems filled for approximate 1 cm from the bottom. The solvents moved to the top of the TLC plates as results of capillary action. Since all compounds in the mixture had unique ways of interacting with the matrix and the solvent, some compounds had moved faster toward the top of the TLC plate than others. The TLC patterns were recorded and observed in the visible light (as some were coloured) and with a UV-light lamp at 254 nm and 365 nm wavelength. The observed band patterns were marked with a pencil and labelled compound ***n*** (where ***n***= 1, 2, 3 …). Suitable separation ratios were successfully obtained for each extraction solvent (Table 2). A number of vague compounds were isolated and separated well by the silica-gel TLC plates.

The plates were viewed under UV light in order to mark compound bands and calculating the compounds R_f values. The R_f value is the ratio of distance of the compound has migrated divided by the distance the solvent has migrated and has by definition a maximum value of 1 (one). The R_f value tends to be constant for a given combination of compound, solvent and matrix so that the comparisons can be made between separations performed at different times. Though R_f value gives some room for uncertainty, two different compounds can have the same R_f-value, therefore further characterization is necessary to establish compound identity with more confidence.

After the proper TLC developing system was obtained, the extracts were separated on a larger scale (20 cm x 10 cm, 20 x 20 cm) TLC plates (Figure 3) in order to scrape the compound band area off the TLC plate where compounds of interest had migrated to, followed by solvent extraction of the matrix. The compounds were collected in labelled 100 mL beakers; each compound in a beaker is in correspondence with the compound number scraped off the TLC plate with a spatula. Each compound was scraped in a different beaker and placed in labelled vials. Each vial had an individual compound per hosting specie per solvent extraction, e.g. Sample Id: **BCG-1** means **B**enzene extracts from host ***C****roton* ***g****ratissimus* isolated compound **one**. The scraped compounds were re-dissolved in their respective solvents, filtered, washed three times with the solvent to ensure maximum removal of the compound and re-developed again on the TLC plate. This was done to find out if the obtained compound bands were composed of a single or more compounds.

3.6 Compound class detection reagents

Spray detection relies on a colour reaction between the compound on the TLC plate and the spray reagent (stain) introduced onto the plate as a fine mist from a spray canister. Universal reagents are used to react with many classes of natural products. The following universal reagents are used to detect classes of compound of the indigenous *Ganoderma* mushroom.

3.6.1 Anisaldehyde- Sulfuric Acid (AS)

AS reagent is used for the unspecific detection of steroids, terpenes, essential oils, bitter principles and saponin drugs. The reagent were prepared by mixing 0.5 mL of anisaldehyde with 10 mL of glacial acetic acid, followed by 85 mL of MetOH and 5 mL of concentrated H_2SO_4. The TLC plates were then sprayed with 10 mL of AS reagent, warmed at 100 $^{\circ}$C for 5 to 10 minutes and evaluated under UV (365 nm) and visible light. The presence of red-violet, brown-red, blue-green and red-grey zones are an indication of steroids, terpenes, essential oils, bitter principles and saponin drug compounds.

3.6.2 Vanillin Sulfuric Acid (VS)

VS reagent is used for the unspecific detection of essential oil, bitter and pungent principles, and saponins drugs. This reagent consisted of two parts. Part one: A 5% (w/v) of 96% ethanolic H_2SO_4 solution, and Part two: A 1% (w/v) of 96% ethanolic vanillin solution. The TLC plates were sprayed with 10 mL of part (one), followed by 5 to 10 mL of part (two), warmed at 100 °C for 5 to 10 minutes and evaluated under visible light. The presence of red and blue colour triterpenes, while red-violet, brown-red, blue-green, blue, grey and red-grey zones are an indication of essential oil, bitter and pungent principles, and saponins drugs.

3.6.3 Liebermann – Burchard Reagent (LBr)

Freshly prepared LBr reagent is used for the detection of triterpenes, steroids (saponins and bitter principles) and sterols (cholesterol and esters). The reagent were prepared by carefully adding 5 mL of acetic anhydride and 5 mL of concentrated H_2SO_4 to 50 mL of absolute ethanol on ice. The TLC plates were sprayed with 5 to 10 mL of LBr reagent, warmed at 100 °C for 5 to 10 minutes and evaluated under visible or UV (365 nm) light. The presence of brown zones in UV_{365nm}, dark brown in visible light, grey zones in UV_{365nm} and weak grey in visible light are the presences of triterpenes, steroids (saponins and bitter principles) and sterols (cholesterol and esters)

3.6.4 Potassium Hydroxide (KOH)

Freshly prepared KOH reagent is used for the detection of anthraquinones, anthrones and coumarins and arbutin drugs. The TLC plates were sprayed with 10 mL of a 10% (w/v) of 96% ethanolic KOH solution, dried and then observed under UV (365 nm) or visible light without warming. The presence of yellow zones are an indication of anthraquinones, anthrones and coumarins and arbutin drug compounds.

3.6.5 Kedde Reagent

Freshly prepared Kedde reagent is used for the detection of cardiac glycoside drugs. The reagent was prepared by mixing 5 mL of a 3% (w/v) of 96% ethanolic 3, 5-dinitrobenzoic acid solution with 5 mL of 2 M NaOH. A volume of 5 to 8 mL of the reagent was sprayed onto each TLC plate and then observed under visible light. The presence of pink zones are an indication of cardiac glycoside drugs compounds.

3.6.6 Berlin Blue Reagent (BB)

Freshly prepared Berlin blue reagent is used for the detection of arbutin drugs, and phenols. The reagent was prepared by adding 10.00g of $FeCl_3$ and 0.50 g of $K_3[Fe(CN)_6]$ in 100mL of distilled H_2O. Each TLC plates were then sprayed with 5 to 8 mL of BB and evaluated in visible light. The presences of blue, purple, green and black zones are an indication of arbutin drugs and phenols compounds.

3.6.7 Iodine

Iodine vapour is used for the detection of compounds with conjugates double bonds system. Each TLC plate was placed into a TLC tank, which had been previously saturated with iodine vapour by the addition of iodine crystals. The TLC plates were removed and evaluated in visible light. The presences of yellow–brown zones are an indication of conjugated double bonds compounds.

3.6.8 Aluminium Chloride

$AlCl_3$ is used for the detection of flavonoids. Each TLC plate was sprayed with 5 to 10 mL of a 1% (w/v) 95% ethanolic $AlCl_3$ solution and evaluated under UV (365 nm) light. The presences of blue, green and purple zones are an indication of flavonoids.

3.6.9 Dragendorff Reagent

Freshly prepared Dragendorff reagent is used for the detection of alkaloids, heterocyclic nitrogen compounds. The reagent was prepared by dissolving 8.00 g of KI in 20 mL of distilled H_2O. This solution was then added to a second solution containing 0.85 g of basic bismuth nitrate in 40 mL of distilled H_2O and 10 mL of glacial acetic acid. Each TLC plate was sprayed with 10 mL and observed under visible light. The presence of orange to red to red colour and yellow zones are an indication of alkaloids, heterocyclic nitrogen compounds.

3.6.10 Natural Products – Polyethykene Glycol (NP-PEG)

NP-PEG is used for the detection of anthracene derivatives, coumarines, arbutin drugs, bitter principles and flavonoids. Ten mL of NP (1% (w/v) methanolic diphenylboryloxyethylamine) followed by 8 mL of PEG (5% (w/v) ethanolic polyethyleneglycol-4000) is sprayed onto the TLC plate. The plate is then observed under UV light at 365 nm.

3.6.10 Fast blue salt (FBS)

Fast blue salt is used for the detection of flavonoids and phenolics compounds. A TLC plate is sprayed with 6 mL to 8 mL of a 0.5 % (w/v) solution of FBS in water, dried and then observed under visible light for the presence of red to brown zones, with or without warming are indications of phenolic compounds.

3.6.12 Fluorescein

Fluorecein is used for the detection of lipids. A TLC plate is lightly sprayed with a 0.01% (w/v) ethanolic solution of fluorescein and dried in warm air. The plate is then lightly sprayed with water and evaluated in visible light. The presences of yellow zones are an indication of lipids compounds.

3.6.13 Folin-Ciocalteu Reagent

Folin-Ciocalteu Reagent is used for the detection of phenolics compounds and is purchased ready made from Merck (Darmstadt, Germany). The TLC plate is sprayed with 5 mL to 10 mL and then evaluated in visible light. The presences of blue zones are an indication of phenolic compounds.

3.7 Biological Evaluation

3.7.1 Antimicrobial properties

The mushrooms extracts from Section 3.2 were used for antimicrobial property screening. In this study the agar disc diffusion method was used to evaluate the antimicrobial properties of the indigenous *Ganoderma* mushroom extracts.

Table 4: Reference bacteria strains used in the susceptibility antibacterial test for the indigenous *G. lucidum* extracts.

Bacteria	Strain	Gram + or -
Escherichia coli	ATCC 25922	-
Pseudomonas vulgaris	ATCC 33420	-
Alcaigenes faecalis	ATCC 8750	-
Neisseria meningitides (Y)	ATCC 35561	-
Staphylococcus aureus	ATCC 25923	+
Bacillus cereus	ATCC 10876	+

3.7.2 Bacterial Media Preparation

All solid and liquid culture media were sterilized. Nutrient broth and Muller-Hinton agar were all prepared according to the manufacturer specifications (Table 5). The mixtures of the NB, MHA were prepared with distilled water and autoclaved at 121 ºC for 15 minutes. After 15 minutes, the broth media was inoculated with the strains of bacteria that were previously lyophilized (Table 4). While the agar media was allowed to cool for a while and poured into the petri dishes. The MHA media was allowed to solidify and incubated for overnight before inoculation with the respective strains of bacteria (Table 4).

Table 5: Nutrient media used according to the manufacturer specification

Medium	Mass (g) per 1L of Water	Natural pH
Muiller-Hinton Agar (MHA)	37.0	7.4 ± 0.2
Nutrient Broth (NB)	13.0	7.4 ± 0.2

3.7.3 Anti-bacterial Assay using Agar Disc Diffusion method

A 0.5 mL of the each stock culture (Table 4) was inoculated onto the MHA media and spread with a sterile inoculating rod. The Whatmann No. 1 filter papers were used to make 5 mm paper discs by using a punch. The blank sterile filter paper discs (5 mm) were immersed with the respective crude indigenous *Ganoderma* extracts and placed gently onto the agar overlay. The plates were incubated at 37 ºC overnight before inspection. The solvent used for each extraction was used as the negative control. The anti-bacterial activity of each extracts was recorded by measuring any zone of growth inhibition diameter around the disc with a millimeter ruler. The experiments were done in triplicate and the average values were tabulated.

Chapter 4: Results

4.1 Mushroom collection and identification

A number of mushrooms samples (Figure 3) were collected from the four regions and were identified as *Ganoderma lucidum*. The *Ganoderma* mushrooms were picked from dead trees trunks, stamps and from decomposing materials in the soil of different tree hosting species. Figure 3 shows some photos taken at the time of collection of the indigenous *Ganoderma* mushroom in the north and north eastern part of Namibia.

The following were identified *Ganoderma* hosting tree species in the four regions: *Croton gratissimus*, *Combretum collinum*, *Colophospermum mopane*, *Acacia* spp., *Mundulea sericea, Terminalia prunioides, Terminalia sericea, Sclerocarya caffra* (Table 1 for local names). The *Ganoderma* mushrooms exhibited hard woody, shiny red-brownish to yellow-brownish ear-shaped fruiting bodies which occurred in wide ranges of sizes. It was observed that most of *Ganoderma* hosting species were common in all the four regions (Table 1).

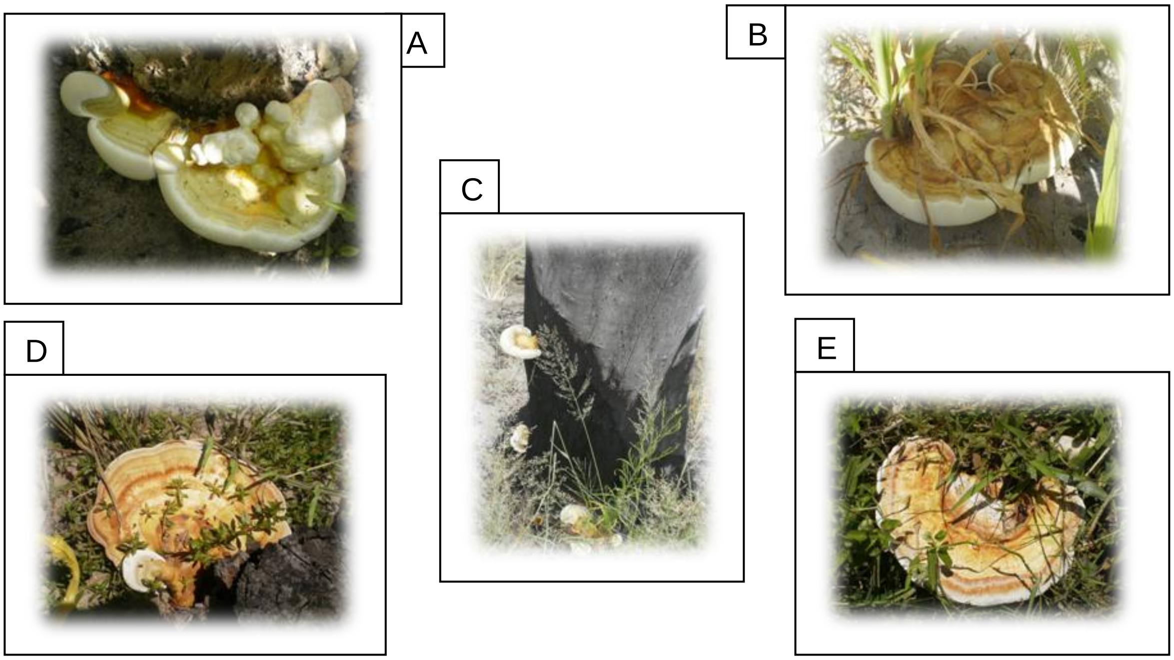

Figure 3: Representative photos of the Namibian indigenous *Ganoderma* species in their natural habitats. (**a**) *Ganoderma* from Ohangwena region, (**b**) *Ganoderma* from Oshana region growing by a Mahangu plant, (**c**) *Ganoderma* from Kavango region growing on a burned tree, (**d**) *Ganoderma* from Caprivi region growing on a stamp, and (**e**) *Ganoderma* from Caprivi region growing from the soil. All *Ganoderma* mushrooms were living at the time of photography and can be seen growing on their substrate which is either wood or organic matter in the soil.

Some fruiting bodies were found to be growing in the open field where there were no stumps or living trees (Figure 3.). However, when examined closely, it was found that there was either a decaying stumps under the soil, decaying the matters in the soil or decaying roots in the soil from which the mushrooms were growing. *Ganoderma* usually found growing on dead matters (i.e. underground roots and barks) and house fencing poles of the hosting species. This is on a contrary with the fact that it is reported that; however, it is not yet a case in Namibia. While in Oshana and Ohangwena region the mushrooms were mostly collected from very old and decomposing dead trees, stamps and underground roots or barks. It was found that most of the mushroom samples collected in the Caprivi and Kavango region were from half burned trees and stamps of which the mushrooms were growing on the burned side of the substrate (Figure 3). It was observed that most *Ganoderma* mushrooms were growing on dead and old decomposing trees stamps.

4.2 Sample extraction

Both aqueous and organic solvents used for the extraction of the indigenous *Ganoderma* fruit body were investigated. Aqueous extraction employed 70 ºC of hot water (HWE). Organic extraction involved cold extraction ranging in increasing polarity at room temperature for 5 days in the dark (i.e. starting with Benzene, Chloroform, EA, EtOH and MetOH extracting solvent). Table 2 and Figure 4 show the appearances and colour of the organic and aqueous after extraction with increasing polarity. Some of the obtained extracts appeared oily with the colour deepening with increased extraction solvent polarity.

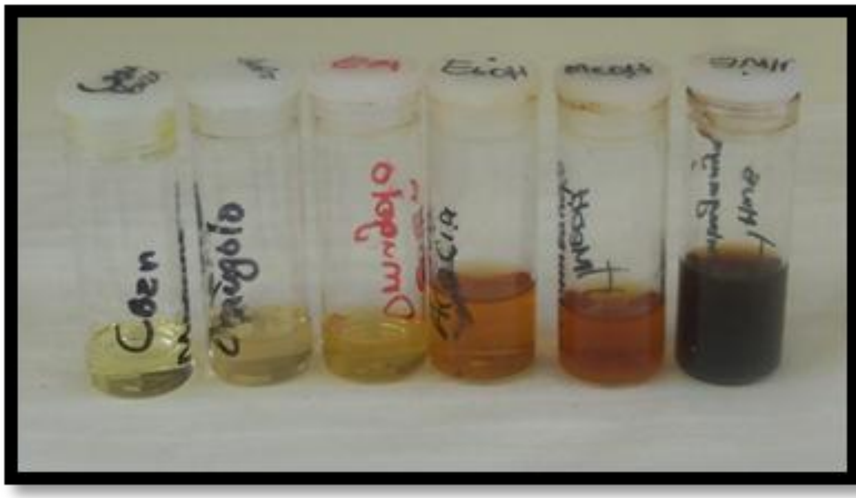

Figure 4: The appearance and consistency of aqueous and organic extracts from the *Ganoderma* mushroom. Vials are in order of increasing polarity; Benzene, Chloroform, EA, EtOH, MetOH, HWE.

The extracts obtained from each *Ganoderma* hosting tree species by the same solvent exhibited similar colours and textures (Figure 3.). All benzene extracts were observed to have a yellow oily consistency. Chloroform extracts was observed to be an orange oily consistency, EA to be dark orange, EtOH to be red, MetOH to be dark red and the HWE to be brown-red (Table 2). These observations were similar to that obtained by Roberts (2004, p. 86), who noted the EA extracts to be a yellow/orange substances and MetOH to be either brown or red. Again, the observation for the crude extracts were similar those obtained by Rosecke and Koning (2000, p. 606), who observed the MetOH extracts to consist of a dark red solid respectively.

Table 3: The appearance and consistency of aqueous and organic extracts from *Ganoderma* mushroom.

Organic and aqueous extracts from *Ganoderma*	
Extraction solvent	**Extracts appearance**
Benzene	Yellow; oily
Chloroform	Orange; oily
EA	Dark orange
EtOH	Red
MetOH	Dark red
HWE	Brown-red

4.3 Chromatography

4.3.1 TLC Chromatography

The initial TLC analysis was performed to determine the number of different components within the crude extract and to identify the components. As the number, the type and the identity of the extraction components were not known, experimentation was required to determine a suitable solvent system. TLC was performed as described in section 3.5.1. A number of TLC plates were spotted with volumes of about 5 to 8 drops of the *Ganoderma* extract. Solvent systems investigated were Benzene: EA (1:1, 1:2, 2:1), EA: EtOH (1:1, 1:2, 2:1), AA: EtOH (1:1, 1:2, 2:1) ratios. The TLC plates were placed into solvent saturated tanks, developed in different solvent systems and then observed for the separation of components under $UV_{254\ nm}$, $UV_{365\ nm}$ or visible light.

Figure 5 show some of the indigenous *Ganoderma* extracted mycochemical compounds on TLC plates under UV lamp ($UV_{254\ nm}$ and $UV_{365\ nm}$). This figure shows a considerable number of components extracted from the indigenous *Ganoderma*. Some components are fluorescing more at $UV_{254\ nm}$ and$UV_{365\ nm}$ .While Figure 6 shows certain compounds which could be of the same structures and minute polarity forming layers.

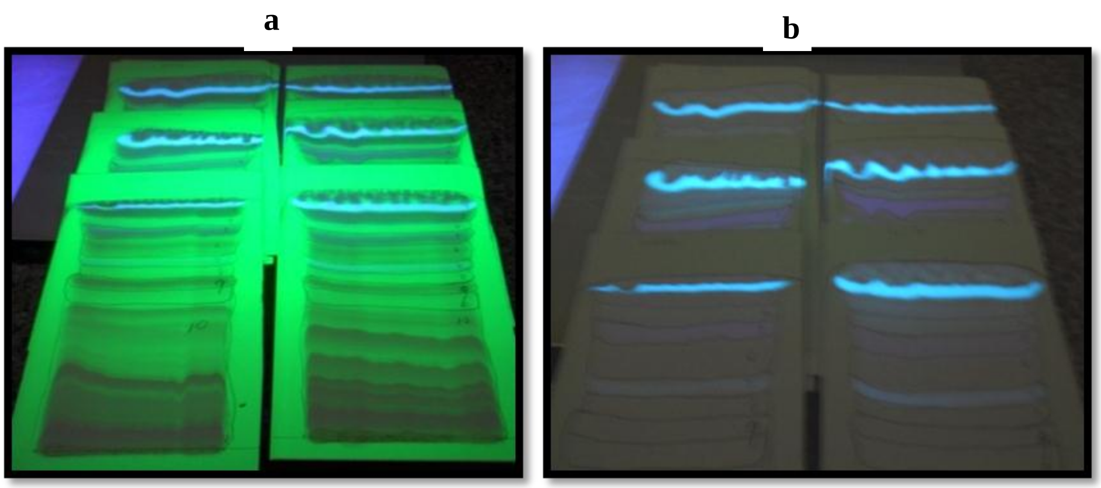

Figure 5: TLC components of Benzene extracts TLC developed in a B:EA (1:1) solvent system under UV lamp with intense colours, appearances, relative concentrations and quenched fluorescence. **(a)** UV at 254 nm, and **(b)** UV at 365 nm.

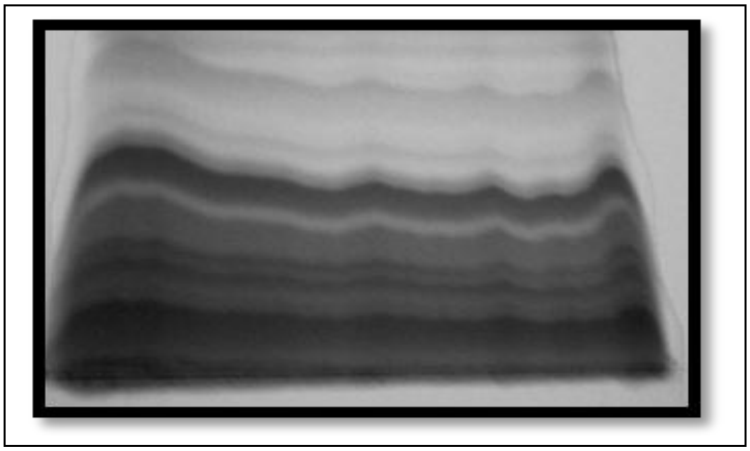

Figure 6: A stratiform of compounds: composed of layers or with a layered appearance/arrangement (these layers were scraped as one as further separation was sought). Compounds that have similar structures tend to migrate close to each other using TLC, making them difficult to separate (Harbourne, 1973, p. 26).

Table 3 shows the estimated number of distinctively clear and visible compounds per solvent extraction on TLC plates and the best developing system sought per solvent extraction. Some compound bands/spots appear to have more than one component when viewed under different UV wavelength. However, for benzene and chloroform extracts there were enormous number of components similar in nature and seemed to be of similar structures which migrated and have moved close together forming a stratiform style.

Table 4: The estimated number of distinctively clear and visible compounds and better sought after developing system per solvent extraction on the TLC plates.

Solvent extracts	**Compounds identified**	**Best developing system (v/v)**
Benzene	$10^{\wedge *}$	B:EA(1:1), Chloroform: EA (1:1)
Chloroform	$10^{\wedge *}$	B: EA (1:1)
EA	$5^{\wedge}$	B: EA (1:1)
EtOH	$8^{\wedge}$	EA: EtOH (2:1)
MetOH	$5^{\wedge}$	EA: EtOH (2:1)
HWE	$1^{\wedge\wedge}$	AA: EtOH (1:1)

Note: * - some compounds are in close layers (stratiform)
^ - compounds more than that value, some not distinctively clear
^^ - compound seemed to be two but not distinctively clear

Table 5 shows the observed colour and fluorescence's components appearing under $UV_{254\ nm}$, $UV_{365\ nm}$ and in the visible light of the indigenous *Ganoderma* benzene extracts. The initial crude extracts of each hosting species per region was performed on different TLC development systems and viewed under UV lamp to separate and determine the number of components in each crude extract. The separation and identification of the components from the same substrate were found to be the same in composition on the TLCs. The extracts were pooled to get a crude extracts of samples Ids: i.e. SCG (O**s**hana, ***C****ombretum* ***g****ratissimus*), HCG (O**h**angwena, ***C****ombretum* ***g****ratissimus*) and CCG (**C**aprivi ***C****ombretum* ***g****ratissimus*): becomes CG (of which C- ***C****ombretum* and G- is ***g****ratissimus*), indicating only the hosting species where the *Ganoderma* sample was hosting.

The Benzene: EA (1:1) (Table 4.3.1) was found to be the best solvent system, yielding good separation of the different components in the *Ganoderma* samples of Benzene extracts, Chloroform extracts and Ethyl Acetate extracts, while MetOH, EtOH extracts were best separated by EA: EtOH (2:1) (Table 4.3.1) and HWE was best separated by Acetic acid: EtOH (1:1) (Table 4.3.1). It was observed that all *Ganoderma* samples components have shown the same migration patterns including the intensity, colour (appearance under UV) and fluorescence intensity of some compounds at 365 nm and 254 nm wavelengths.

The mobile phase, Benzene: EA (1:1) resulted first in the separation of ten components from the benzene crude extract (Table 3.5.1). Four of these components fluoresced at UV_{254nm} (Figure 4.3a) and an additional five components were observed to quench fluorescence at UV_{365nm} (Figure 4.3b). One of these ten components was seen as a creamy-yellow component in the visible light (Figure not shown). Only few compound bands did not fluoresce at both UV (254 nm and 365 nm) wavelengths.

Table 5: TLC of the crude Benzene Extract with colour observation and fluoresces at UV $_{254\ nm}$, UV $_{365\ nm}$ and visible light.

Detection of Components in the Benzene Extracts			
Compound band	**UV$_{254\ nm}$**	**UV$_{365\ nm}$**	**Visible light**
1	Dark gray-bluish	Purple* grayish (2)	Cream-yellow
2	Bright lighter blue*	Bright lighter blue*	-
3	Dark blue	Dark blue	-
4	Dull light blue-grayish*	Dull lighter blue-grayish*	-
5	Purple-bluish*	Purple-bluish*	-
6	Dark navy blue	Dark navy blue	-
7	Lighter blue-grayish*	Gray-bluish*	-
8	Plain gray	Plain gray	-
9	Dark purple	Dark purple	-
10	Layers of dark blue, purple, blackish	Layers of dark blue, purple, blackish	-

Note: *- Fluoresced components at UV$_{365\ nm}$

Table 6: Namibian *Ganoderma* HWE and organic solvents crude extracts colour reactions zones on TLC plates after spraying with universal detection reagents. With * fluoresce compounds under UV_{365nm}

Reagent	**EtOH**	**EA**	**Chloroform**	**MetOH**	**Benzene**	**HWE**
EtOH-KOH	fainted yellow	Yellow layers	Intense yellow	yellow	Brighter yellow	-
AS reagent	Yellow, violet, purple, black	Purple, blue, green, red*, violet	Red*, purple, blue, violet, grey, blue-green, black	Purple, yellow, black	Green, yellow, violet, brown, red*-grey, black	Purple
BB	Blue	blue	Blue	Blue	Plenty of Blue	-
Kedde reagent	Pink, blue	Pink, blue, yellow	Pink, blue, green	Pink, blue	Pink, blue, yellow green	-
Folin	Blue	Blue	Blue	Blue	Blue	-
Dragerdorrf	Brown, yellow	Brown, orange, yellow	Brown, orange	Orange,	Brown, orange, yellow	-

Table 6 continue...

Reagent	**EtOH**	**EA**	**Chloroform**	**MetOH**	**Benzene**	**HWE**
EtOH-$AlCl_3$	One Blue*	bright green*, dark purples, blue, bright blue	bright green, dark purples, dark blue and bright blue	blue, grey, bright blue, black, purple, bright green	blue, grey, bright blue, black, purple, bright green	Blue*
Iodine	yellow	yellow	Yellow	yellow	yellow	-
VS	Brown*, red, grey	Brown*, red, grey, blue-green, black	Brown*, red, grey, red-violet, green	Brown*, red, grey, red, black	Red-violet, brown*, red, grey, black	-
LBr	Brown, grey, blue,	Brown, grey, orange	Brown, grey, blue	Brown, grey, blue	Brown, grey, orange	-
FBS	Brown	Red, brown	Red, brown	Red	Red, brown	-
Florecein	Yellow	Yellow	Yellow	Yellow	Yellow	-

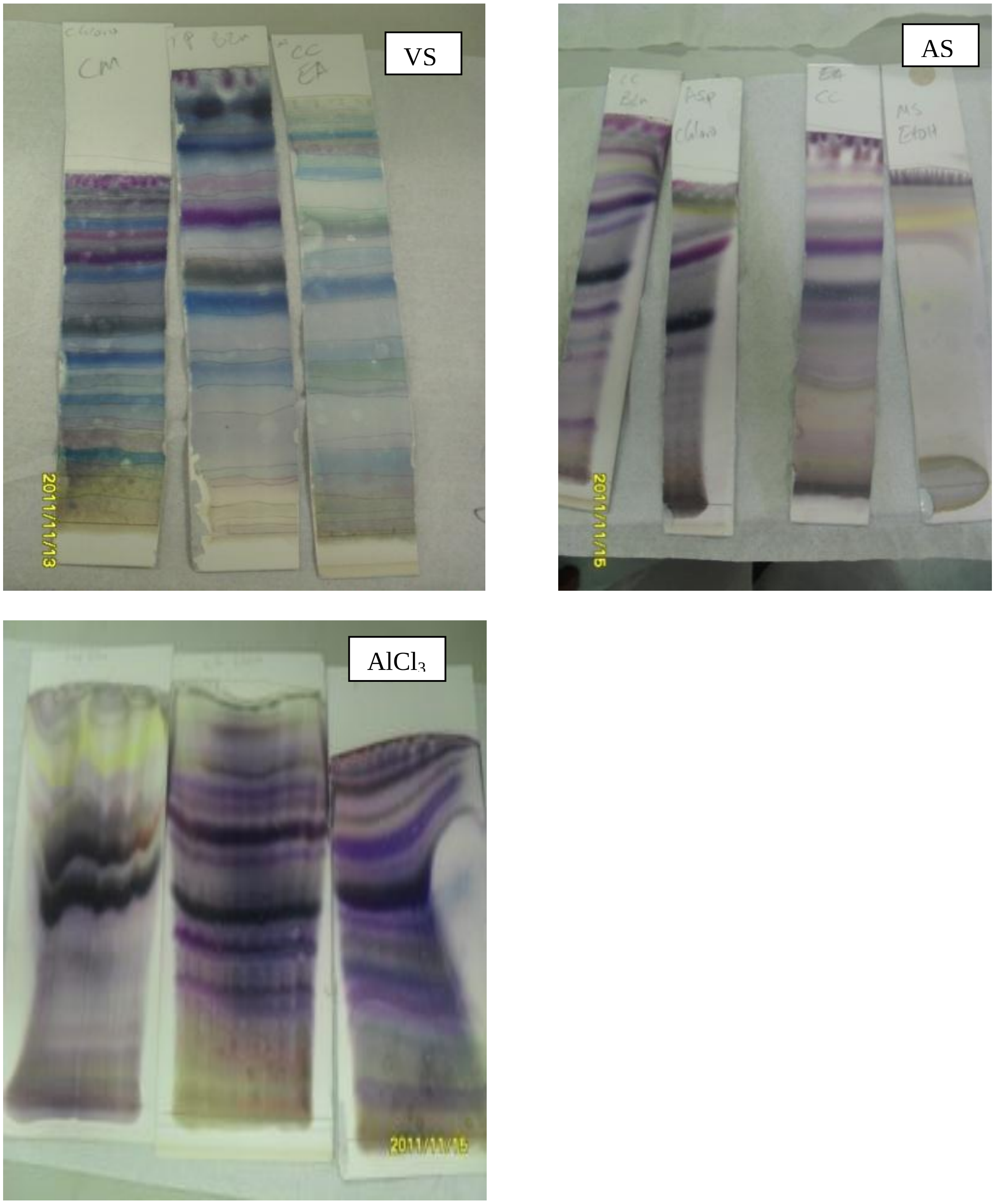

Figure 6: Representative pictures of class of compounds reactions colours to VS, AS and$AlCl_3$ detection spray reagents.

4.5 Antibacterial activity

The inhibitory activities of all crude aqueous and organic extracts were investigated against a range of Gram positive and Gram negative bacteria. Figure 7 gives a representative of inhibition zone disc diffusion assay and measuring the inhibition diameters.

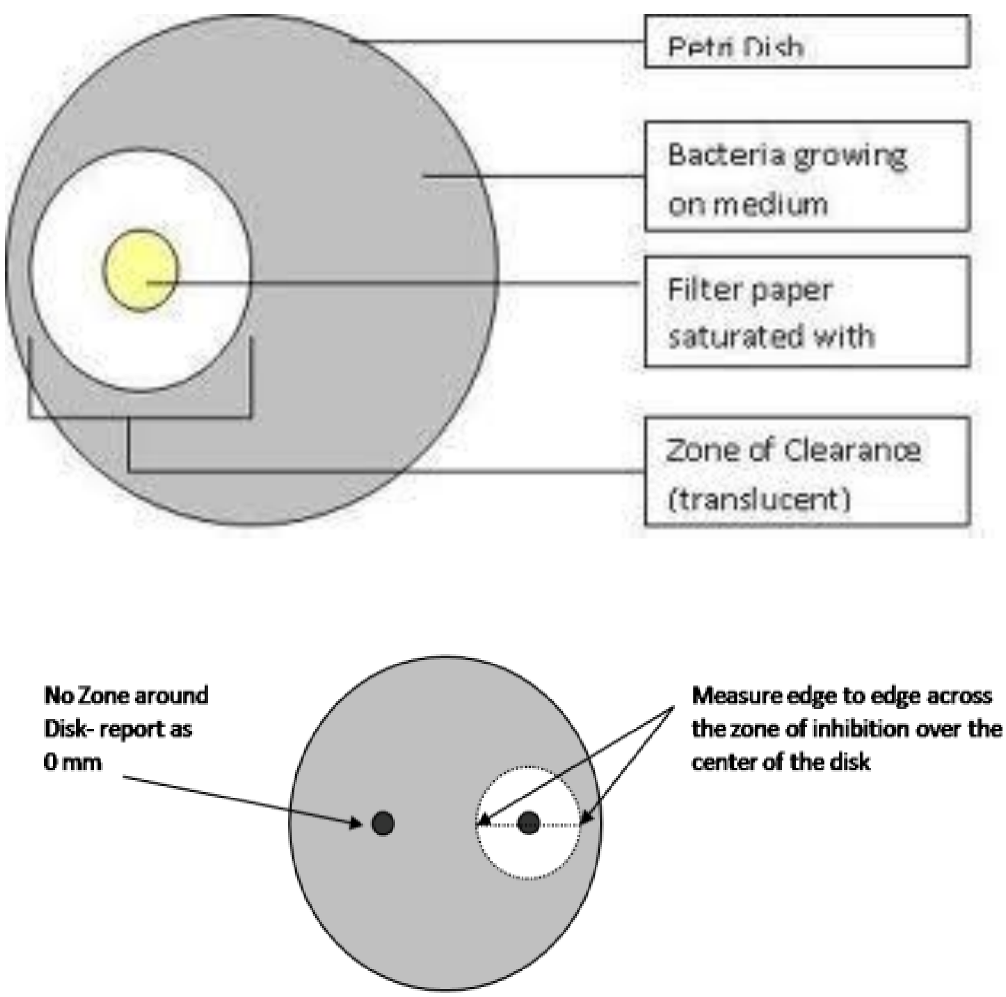

Figure 7: Representative diagram of the inhibition zone disc diffusion assay.

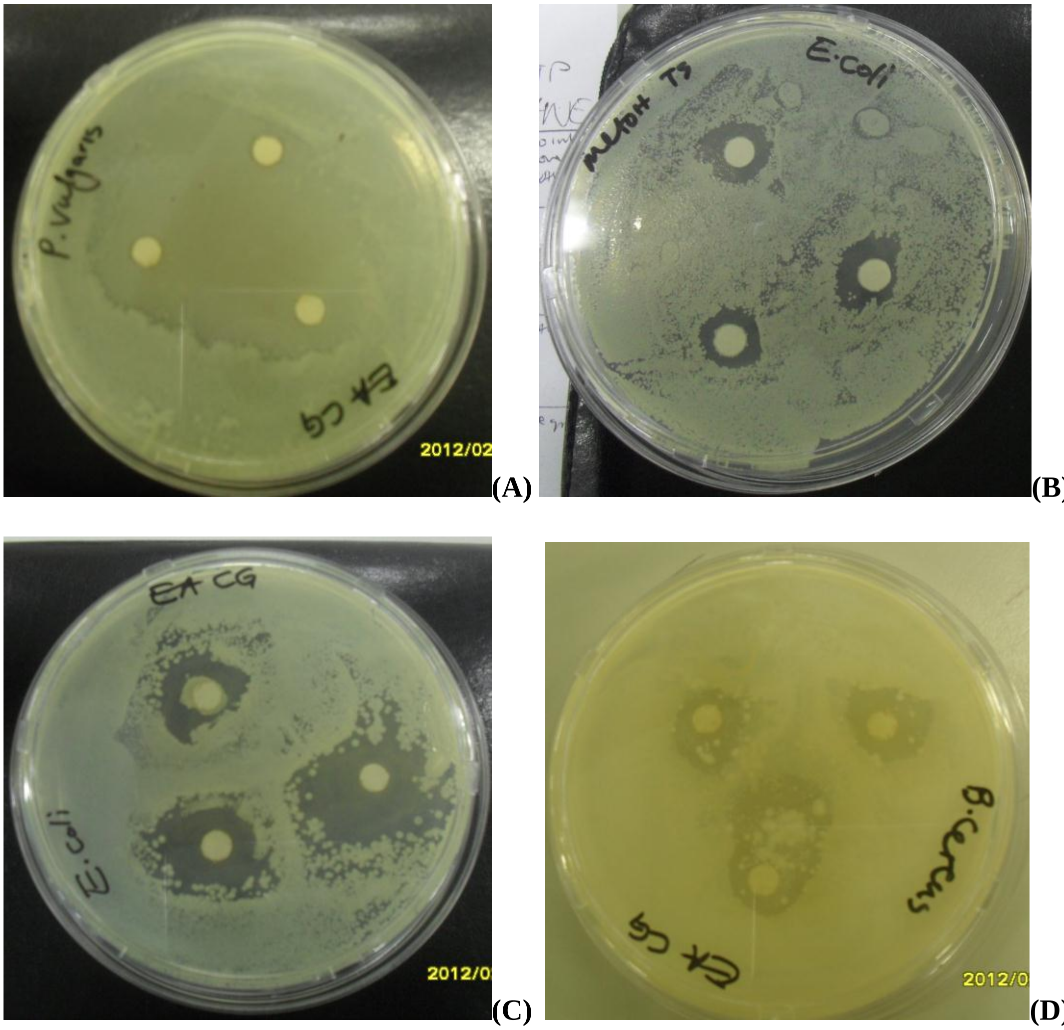

Figure 8: Representative photos of the disc diffusion assay showing (A) and (B) complete inhibition of *P. vulgaris* by the EA *Ganoderma* extracts and *E. coli* by methanol *Ganoderma* extracts; (C) double zone of inhibition of *E. coli* by the EA *Ganoderma* extracts and (D) reduced growth but not complete elimination of growth.

The visual results presented in Figure 8, showed the inhibition zones on the effect of crude *Ganoderma* extracts against (A) *P. vulgaris* by the EA extracts and (B) *E. coli* by MetOH extracts; (C) double zone of inhibition of *E. coli* by the EA extracts and (D) reduced growth but not complete elimination of growth. EA extracts have more antibacterial effects on *P. Vulgaris* than others. But most of the extracts had more effects on the test bacteria.

The results presented in Table 7 and 8 showed the effect of aqueous and organic solvents extracts of *G. lucidum* against the six test bacteria. Antibacterial activity on benzene extracts of *G. lucidum* were the effective zone of inhibition as compared to the other organic solvents against the test bacterial pathogen. The maximum antibacterial activity of aqueous extract of the whole fruit bodies of *G. lucidum* was found 18.0mm against *N. Meningitidis* and minimum 8.0 mm against *E. coli* and *P. vulgaris.*

Whereas the maximum antibacterial activity of organic solvents extracts against the test bacterial pathogen was found 19.0 mm against *E. coli* and *N. Meningitidis* of benzene extracts, and a minimum of 6.0 mm of benzene extracts against *A. feacalis*, 6.0 mm of chloroform extracts against *P. vulgaris* and *A. feacalis*, 6.0 mm of EA extracts against *S. aureus*, and 6.0 mm of MetOH extracts against *A. feacalis*.

Table 7: Antibacterial effects of the aqueous and organic extracts of the indigenous *Ganoderma* mushroom on the clinically important bacteria.

Test Bacteria		Antibacterial activity of crude aqueous and organic extracts					
		Benzene	Chloroform	EA	EtOH	MetOH	HWE
Gram +	*A.feacalis*	+	+	+	--	+	+
	B. cereus	+	+	+	--	+	-
Gram -	*E. coli*	+	+	+	+	+	+
	S. aureus	+	+	+	--	+	+
	P. vulgaris	+	+	+	--	+	+
	N. meningitides	+	+	+	--	+	+

+ Positive inhibition, - No inhibition, -- Not tested

In Table 8, a zone of inhibition >5mm was considered positive. **S** a very slight zone of inhibition observed; **[ ]** incomplete inhibition was observed (i.e. reduced growth, but not complete elimination of growth); **#** a double zone of inhibition was observed. The larger second zone, was a zone of incomplete inhibition, indicated by the parentheses [ ]; **-** no inhibition was observed; **NT** not tested; Negative solvent blanks were also tested. These showed no inhibition (results not shown). Numbers are the average of triplicate discs and triplicate experiments performed on different days.

Table 8: The antibacterial activity of the crude organic extracts from the indigenous *Ganoderma* extracts against a range of bacteria by disc diffusion assays.

Test Bacteria		Antibacterial activity of crude aqueous and organic extracts (mean values)					
		Benzene	Chloroform	EA	EtOH	MetOH	HWE
Gram positive	*A. feacalis*	6.0[s]	6.0[s]	[8.0]	NT	6.0[s]	9.0
	B. cereus	10.0	8.0	7.0	NT	[7.0]	-
Gram negative	*E. coli*	19.0	11.0	[8.0]	11.0	9.0	8.0
	S. aureus	[7.0]	10.0	6.0[s]	NT	10.0	10.0
	P. vulgaris	[8.0]	6.0	[7.0]	NT	12.0[#]	[8.0]
	N. meningitidis	19.0[#]	14.0[#]	[7.0]	NT	11.0	18.0

Chapter 5: Discussion

5.1 Mushroom collection and identification

In this study, the investigations of the *Ganoderma* mushroom samples were collected in the four regions namely: Oshana, Ohangwena, Kavango and Caprivi region. In general *Ganoderma* is very scarce in nature, thus the mushrooms collected were picked at any substrate which the mushrooms were growing. They were picked from old woods of dead stamps and decomposing trees. This is observation is in support with the description of the growing environments and requirements of *Ganoderma* mushrooms in the southern Africa by Van der Westhuizen and Eicker, (1994, p. 182). Namibia has unique hosting species of *Ganoderma* mushrooms from other regions in the world where *Ganoderma* is grows and is researched (Table 1.). It is evident that the isolated *Ganoderma* mushroom in the four regions shows host preferences since the hosting species are common in the four regions (Table 1.) (Kadhila-Muandingi, 2010, p. 34).

The collected mushrooms were identified to be from the Ganodermataceae family because of typical characteristics they had in common with other members of this family. They are regarded as polypores because they possessed tiny pores underside their cap which contained reproductive spores as described by Van der Westhuizen and Eicker, (1994, p. 182) and Bhosle et al. (2010, p. 255). According to Moncalvo and Ryvarden, (1998, p. 38) the reproductive spores are the obvious characteristic that distinguishes polypores from other common types of mushrooms. The colour of the *Ganoderma* mushrooms caps which were collected ranges from shiny brown, to yellow-brownish, with reddish-brown being typical of the polypore (Van der Westhuizen and Eicker, 1994, p. 183). The collected *Ganoderma* mushrooms exhibited a kidney and/ or ear-shaped fruiting bodies in wide ranges of sizes as described by (Van der Westhuizen and Eicker, 1994, p. 183). The macroscopic characteristic of the fruiting bodies of the indigenous *Ganoderma* were found to be thick, woody and tough hard to break with hands as been described previously by several authors (Van der Westhuizen and Eicker, 1994, p. 183; Bhosle et al., 2010, p. 255).

Namibia being a dry and hot country, it flourishes *Ganoderma* mushrooms during the rainy season as the *Ganoderma* species are known to grow under hot and humid conditions in subtropics and tropical regions including Namibia (Van der Westhuizen and Eicker, 1994). It was found that most of the *Ganoderma* mushrooms collected in the four regions were from dead tree stumps. None of the dead tree species were known to be killed and parasitized by *Ganoderma* mushrooms as reported by Fernando (2008, p. 323; Smith et al., 2002, p. 1842). *Ganoderma* is known to parasitize certain oil palm trees (Patterson, 2006, p. 1986) and other certain tree species from other parts of the world. Even, some of the *Ganoderma* mushroom samples that were collected from a live *S. caffra* (Marula) trees, were found growing on old and decomposing barks. According to the indigenous knowledge and available information on tree deaths obtained from local people in both four regions, none of the tree species are known to have been killed by *Ganoderma* before. Smith et al., 2002, p. 1842) reported that *Ganoderma* kills and is a parasite to broad-leafed trees causing a white rot fungus. It is interesting to know that the indigenous *Ganoderma* does not cause a heart rots or white rot on the indigenous hosting tree species but instead decompose already dead wood. This is so because some tree species are known to have very hard wood in comparison to others as described by Patterson (2006, p. 1369). On a contrary, some of the locally known tree species with very hard wood and even though old enough, none where preferred by *Ganoderma*.

5.2 Mushroom Extraction

Solvents of increasing polarity were used in the extraction process to try and extract components of varying polarity into the different solvents. Generally, extraction using a less polar solvent (e.g. benzene) will extract compounds of a similar polarity, and extracting using a more polar compound (e.g. MetOH) will then extract compounds that have greater polarity. Ideally, different compounds will be extracted into the solvents at different phases in the cold soaking extraction process. However, it is rare achieve complete separation of constituents and the same compound may be recovered (in varying proportions) (Harbourne, 1973, p. 20). Nevertheless, cold extraction with non-polar and polar solvent has yielded most of the components of varying polarity. Therefore, using solvents of increasing polarity in a cold soaking extraction is a classical chemical procedure for obtaining chemical constituents of different class from dried mushroom materials.

5.3 Crude extract compositions

The components of all samples crude extracts of *Ganoderma* were separated by TLC and observed by visualization at UV-254 nm and UV-365 nm wavelength. TLC was performed as described in section 3.5.1. and the retention factor was calculated for some compound (results not shown). However, the term retention factor, R_f, is commonly used to described the chromatographic behaviour of sample solutes. The R_f value for a given component is the distance it has travelled from the origin divided by the distance travelled by the solvent front (mobile phase) (Harbourne, 1973, p. 20).

The centre for each spot or band is the point taken for reference. Since silica gel retains the more polar compounds, the non-polar compounds were eluted first (and moved further up the TLC plate). Hence, the more polar the components the lower the R_f value, and the less polar the component the larger the Rf value. The R_f value of a component will not be the same when a different solvent system is used. For this reason, the R_f value can only be compared if the same mobile phase is used and the time used for the development of the plate was the same which was not the case in this regard. Therefore, in this investigation, it was found that the same samples were run on TLCs with the same mobile phase and also placed in the development chamber at the same time yielded compounds that seemed similar with different R_f-values and compounds that seemed different with the same R_f-values. Such samples did not even develop fully at the same time in the chamber system. Though R_f value gives some room for uncertainty, two different compounds can have the same R_f-value, therefore further characterization is necessary to establish compound identity with more confidence.

According to Harbourne (1973, p. 21), the intensity and size of the spot/band on the TLC plate is a measure of the concentration of that component in a mixture. Therefore, relative concentrations of the components can be estimated. It was clear that all the organic solvent extracts and water extracts had components present in varying concentration, as different intensities of the components were observed under UV light (Figure 5). The component that fluoresced with a brighter blue colour at UV 365 nm (component number two of benzene extract from the top), was present in a much greater concentration than any other component of all solvent extracts (Figure 5).

The stratiform layers of compounds component in benzene and chloroform extracts suggested that they were slightly polar with similar structure differing slightly in polarity (Figure 6). Compounds that have similar structures tend to migrate close to each other using TLC, making them difficult to separate (Harbourne, 1973, p. 21). Thus, the identification of stratiform compounds into the same class of compounds may explain why there was difficulty in separating those components on the TLC.

Compound band/spot that appeared to contain more than one component were subjected to different TLC solvent systems in an attempt to separate the active components (Figure 5). This resulted in the active components moving further up the plate yielding larger R_f values. This strongly suggested that some of the active components were very similar in structures (Figure 6) as some compounds are in a stratiform manner. When components are similar in structures they can be very difficult to be identified as the separation systems required becomes very complex (Harbourne, 1973, p. 21). For instance, the triterpenoids are structurally similar and their compositional is complex, and the building blocks (derived from sugar residues) of complex polysaccharides exhibit very similar structures too, thus their components may migrate closely together because of differing slightly in polarity.

It was observed that each organic solvent had different compounds and the number of compounds from the others solvent extraction. All samples from different region hosted by the same tree species have shown the same migration patterns including the concentration, colour (appearance under UV) and intensity of compounds at 365 nm and 254 nm wavelengths. It appears that the indigenous *Ganoderma* has an enormous amount of compounds, extraordinarily compounds of all polarities. It was found that each solvent extract exhibited unique compound patterns on the TLC chromatograms.

However, this investigation was interested in the identification of the classes of compounds present in the indigenous *Ganoderma*.. Further separation, purification and structure elucidation need further studies to take on the step ahead. Extracts subjected to TLC where the classified using universal and specific TLC reagents, UV-254 nm, and UV-365 nm light (Roberts, 2004, p. 66) for the identification of the classes of compound in the indigenous *Ganoderma*.

Although the isolation of pure, pharmacologically active constituents from natural sources remains a long and tedious process, there is no established method of extracting mycochemical components of *Ganoderma* mushrooms (Hostettmann, 1999, www.iupac.org/symposia/proceedings/phuket97/hostettmann.html, IUPAC electronic source. A number of secondary metabolites have been purified and identified for *Ganoderma* species using column chromatography and HPLC/GC techniques (Gonzalez, Leon, Riven, Munoz and Bermejo, 1999, p. 1701; Gonzalez, Leon, Riven, Padron, Gonzalez-Plata, Zuluaga et al., 2002, p. 418; Kleinwachter et al., 2001; Rosecke & Koning, 2000, p. 608). These secondary metabolites have generally been isolated, identified and then tested for bioactivity. Solvents of increasing polarity were used in the extraction process of this study to try and extract components of varying polarity into the different solvents. Generally, extraction using a less polar solvent (e.g. Benzene) will extract compounds of a similar polarity, and extraction using a more polar solvent (e.g. MetOH) will then extract compounds that have a greater polarity. Ideally, different compounds will be extracted into solvents at different phases of extraction process. However, one rarely achieves complete separation of constituents and the same compound may be recovered in varying proportions in several fractions (Harbourne, 1973, p. 28; Roberts, 2004, p. 143). Never-the-less, using solvents of increasing polarity is a classical chemical procedure for obtaining chemical constituents from dried materials.

A review by Cowan (1999, p. 572) reported that the most active components are generally water insoluble, hence, the expectation that low polarity organic solvents would yield more active extracts. This is in contrary with the fact that traditionally, people use water to extract the active component from the mushroom and drink it as tea. This made the tea (water extract) to gives greater activity and effectiveness because water is a more polar solvent. In addition, the well-known major active components of *Ganoderma* mushrooms are the polysaccharides and triterpenoids, which are polar compounds and can be extracted by a polar solvent such as EtOH, MetOH or Water. However, Roberts (2004, p.177), in his investigation found out that the aqueous extracts exhibited less activity than the organic extracts. In addition Robert reported that the extracts he obtained using the less polar solvents, such as hexane, dichloromethane (DCM) and EA, gave greater activity than those obtained using the highly polar solvent MetOH. This implies that the polysaccharides and triterpenoids were extracted by the less polar solvents (i.e. hexane, dichloromethane (DCM) and EA).

Up to now, numerous methods of extraction have been developed with the objective of obtaining extracts with higher yields and lower costs. Such is the case of extraction with organic solvents such as methanol, ethanol, acetone, hexane, Ethyl acetate. So, water has been shown to be capable of extracting different classes of compounds depending on the temperature used, more especially because it is available for the extraction of hydrophobic substances. It was the technique of using none or least polar extracting organic solvent and polar organic solvents with increasing polarity. This technique extracted the majority of the active mycochemical elements in the indigenous *Ganoderma* mushroom including beta glucans and triterpenoids including ganoderic acids and alcohols respectively. Nevertheless, the *Ganoderma* mycochemical components are still poorly characterised, due basically to them being difficult to isolate and identify well, thus there is no established method of extracting *Ganoderma* mycochemical components.

5.4 Classes of compounds

The crude extracts of the organic and aqueous solvent on the TLC plates were used to determine the classes of compound of active components. R_f values of the components and in turn the corresponding colour reaction were determine and compared. A number of TLC plates were spotted with the extracts, developed, dried and then sprayed with different spray reagents to determine the presence of classes of compounds.

Since there appear to be few reports on using spray reagents for the preliminary identification of constituents in basidiomycetous fungi, the spray reagents chosen were those that have been used in the Phytochemical identification of plants constituents (Ahmad and Beg, 200, p. 316; Ebi and Kamalu, 2001, p. 74; Van der Watt & Pretorius, 2001, p. 89). The identification of active constituents in plants using TLC spray reagents has been performed for many years and only recently have the same techniques been used in the identification of constituents in *Ganoderma* species (Aryanha , Adinda and Kusmaningati, 2002, p. 126; Roberts, 2004, 176). In the case where a first spray reagent detected a certain class of compound, a second spray reagent was then used for confirmation.

Separation of the components was attempted using different solvent systems but did not improve successful, which suggested that some of the components were structurally similar with minute polarity. Nevertheless, for the purpose of class of identification, this was not necessary as the extracted components had different R_f values.

For ease of discussion, the components in each extract that exhibited R_f values were numbered n (where n is 1, 2, 3...). The number of components which were labelled as individual components had appeared to show two or more different components with different colour reactions upon spraying with detection reagents. This made it difficult to assign and number the compounds as n (where n= 1, 2, 3...) as was the initial idea. In addition, rather than separately discussing the results of each spray reagent reaction for the different active components, the different classes of compounds have been grouped and discussed. This enabled the identification of the classes of compounds to be performed by taking into account all the results obtained from the detection reagents, as well as from observation at $UV_{365\ nm}$ or $UV_{254\ nm}$.

5.4.1 Alkaloids and lipids

The appearance of yellow/brown spots in the visible light immediately after spraying with Dragendorrf's reagent indicated the presence of alkaloids (Wanger, Bladt and Zgainski, 1984, p. 351). The appearance of yellow zones after spraying with 0.01% (v/v) fluorescein was an indication of the presence of lipids in the extracts (Harbourne, 1973, p. 29). All these colours were observed after spraying with the two reagents (table 6), thus the Namibian *Ganoderma* mushrooms contain alkaloid and lipid classes of compounds. In the benzene extracts on the TLC plate after spraying with Dragendorff's reagent a fluoresced bright blue at UV 365nm was an indicative of alkaloids (Harbourne, 1973; Wagner et al., 1984).

5.4.2 Phenols

A number of detection reagents have been developed to detect phenolics compounds. Such detection gave the appearance of blue spots in visible light after spraying with Folins reagent and the giving the red-brown zones (Table 6) in visible light after sprayed with Fast Blue Salt reagent (FBS) (Wagner et al., 1984), while some appeared with intense green, purple, blue and black colour in visible light (Table 6) after sprayed Berlin Blue (BB) reagent (Harbourne, 1973, p. 29). Some of the colour reactions in the detection reagents where never described in the detection of alkaloids suggesting different compounds (Satyajit, Zahid and Alexander, 2006, p. 89; Ofodile**,** Numa, Kokubun, Grayer, Ogundipe, and Simmonds, 2005, p. 310).

5.4.3 Flavonoids and Coumarins

Coumarins and flavonoids are phenolics structures (Cowan, 1999, p. 568) and have been shown to exhibit antibacterial activity from plants (Ahmad & Beg, 2001, p. 116; Nostro, Germano, D'Angelo, Marino and Cannateli, 2002, p. 382). However, there appear to be no reports of fungal flavonoids or coumarins exhibiting anti-bacterial activity. The indigenous *Ganoderma* extract components reacted with KOH and $AlCl_3$, indicating that there was coumarins and flavonoids type compounds (Table 6). This was confirmed using the spray reagent FBS (results not shown in the table), which reacted with flavonoids to produce blue to blue violet zones in visible light (Wagner et al., 1984, p. 351; Roberts, 2003, p. 178).

5.4.4 Anthracene Derivatives

Ethanolic KOH was also used for the detection of anthracene derivatives. When the TLC plate was sprayed with ethanolic KOH, anthraquinones appeared red in visible light and exhibited red fluorescence in UV-365 nm, while anthrones and anthronol appeared yellow in visible light and exhibited yellow fluorescence in UV-365 nm (Wagner et al., 1984, p. 351). A lot of components yielded these colours when this spray reagent was applied, indicating that they were not derivatives of anthracene (Table 6). In addition, there were yellow and red to brown fluorescence exhibited by the components at UV-365 nm, which also indicated they were anthracene derivative (Wagner et al., 1984, p. 351).

5.4.5 Terpenoids

Non-specific detection of triterpenoids are performed by spraying the plates with vanillin-sulphuric acid reagent (VS), anisaldehyde-sulphuric acid reagent (AS) or Libermann-Burchard reagent (LBr), heating the plate at 100°C for 5 to 10 minutes and the observing for colours in visible light (Wagner et al., 1984, p. 351). All components exhibited distinct colours, ranging from violet/purple to blue, red and green, with these detection reagents, indicating the presence of triterpenoids (Table 6). The colour reactions with these reagents were strong, suggesting that there were triterpenoids type components. Some of the colour reactions in the detection reagents where never described in the detection of triterpenes i.e. black, brown, grey zones suggesting different compounds (Satyajit et al., 2006, p. 88; Ofodile et al., 2005, p. 310).

Natural terpenoids have cyclic structures with one or more functional groups (e.g. hydroxyl, carbonyl) (Harbourne, 1973). They have conjugated double bond systems which appeared as yellow zones in visible light when was exposed to iodine vapour (Wagner et al., 1984). Nearly all components in Benzene, Chloroform and EA extracts, and few in MetOH and EtOH extracts displayed this reaction when exposed to Iodine vapour, confirming that they were compounds that contained conjugated double bonds (Table 5). This confirms the positive reactions obtained with the triterpenoids universal detection reagents including VS, AS and LBr reagents (Table 5). Phenolics unit can also be encountered in triterpenoids (as a functional group) (Harbourne, 1973, p. 28) and may explain why there were positive reactions with some of the detection reagents that detected phenolics compounds. In addition, the colour reactions observed for phenolics were less intense than those for triterpenoids, which suggested that they were a substituent attached to the main triterpenoids compound.

Triterpenoids encompass a number of compounds including volatile mono- and sesquiterpenes (C15) (essential oils) through to the less volatile diterpenes (20) to the involatile triterpenoids and sterols (C30) and carotenoids pigments (C40) (Harbourne, 1973, p. 28; Patterson, 2004, p. 1989). Unfortunately, there is no sensitive universal reagent for the detection classes of triterpenoids (Harbourne, 1973, p. 28). Thus, differentiation of the different types of triterpenoids is generally impossible by using TLC spray reagents.

6.4 Antibacterial activity

The inhibitory activities of all crude aqueous and organic extracts were investigated against Gram positive and Gram negative bacteria. The results indicated that both aqueous and organic extracts from *Ganoderma* possessed activity against a range of bacteria (Roberts, 2004, p. 137). Some important clinically important pathogenic species were investigated i.e. *E. coli*, *P. vulgaris*, *A. faecalis*, *B. cereus*, *S. aureus*, and *N. meningitides*. Effective concentrations of the active organic extracts were not investigated in this study. So the Minimum Inhibitory Concentration (MIC) and

Minimum Bactericidal Concentration (MBC) assays were not investigated in this study.

6.4.1 Antibacterial activity screening of aqueous extracts from *Ganoderma*

The antibacterial screening was performed to investigate whether the aqueous and organic extracts possessed any biological activity. The antibacterial screening was performed using a disc diffusion assay. The data in Table 7 show that benzene extracts exhibited a complete inhibition against the Gram negative bacterium, *N. meningitidis*. *Ganoderma* also exhibited activity against *E. coli* according to Roberts (2004, p. 139). There were activities observed for the Benzene extracts against the tested microorganisms of the Gram positive bacteria *B. cereus* as the highest 10.0 mm inhibition diameter zone (Roberts, 2004, p. 139). Yoon et al., (1994), investigated the bioactivity of aqueous extracts which also exhibited inhibitory activity towards the *Bacillus* species. In addition, they found their aqueous extracts to have strong activity against *N. meningitidis*. It has been suggested that there can be variation in the bioactive components during different stages of growth of fungal fruiting bodies, there can be structural changes of the bioactive components (Chen & Miles, 1996, p. 65).

The appearance/consistency of the samples for all *Ganoderma* mushrooms were dark brown powder, however the extracts were dark brown to orange extracts of which Roberts (2004, p. 136) and Yoon et al. (1994, p. 56) also observed similar colours. The indigenous *Ganoderma* organic extracts roughly exhibited reasonable strong zones of inhibition against the tested microorganisms of 6.0 mm to 19.0 mm inhibition zone i.e. *E. coli*, *P. vulgaris*, *A. faecalis*, *B. cereus*, *S. aureus*, and *N. meningitides* (Table 7). Roberts (2004, p. 137) found that organic extracts from *Ganoderma* species displayed inhibitory activity towards these bacteria.

However, there is much debate regarding the use of the diameter of zone of inhibition as a reliable measure of antibacterial activity, as the size of the zone of inhibition is affected by the rate of diffusion of the compound through the agar gel, and different compounds diffuse at different rates (Roberts, 2004, p. 141). The observation of the larger zone highlighted this issue and illustrated that inhibition of each bacterium depends largely on the ability of the extracts to diffuse through the medium. The organic solvents were used as controls without any extracts, of which no inhibition zoning activities were recorded (Table not shown) and observed against the tested microorganism.

Overall, the aqueous extracts from the indigenous *Ganoderma* exhibited a small range of antibacterial activity against the tested Gram positive and Gram negative bacteria. These activities appeared to be much lower than that reported in the literature (Roberts, 2004, p. 137). However, it must be noted that the aqueous extraction process employed in this investigation (boiling water) may inactivate some types of potential antibacterial compounds, such as proteins. All organic extracts exhibited antibacterial activity against all the tested bacteria.

Chapter 6: Conclusion

6.1 Sample collection and identification

The indigenous Namibian mushroom samples collected and analysed in this study were confirmed to be from the Family Ganodermataceae according to a genetic diversity identified by Kadhila-Muandingi (2010) using molecular tools. According to the available information and the knowledge of tree death in the four regions, none is known to be caused by the *Ganoderma* mushrooms. Therefore, the indigenous *Ganoderma* mushroom is not known to parasitize and/or kill any tree in Namibia according to the local people's current knowledge. It does grow mainly on old, decomposing materials and burned wood of the respective tree hosting species. It is evident that the indigenous *Ganoderma* mushrooms have host preferences common in the four (Oshana, Ohangwena, Kavango and Caprivi) regions. However, the macrofungus is very rare in nature rather not sufficient for commercial exploitation. Thus, domestication and the cultivation on solid substrates need to be established to meet the increase demand in the Namibian market and eventually the international market.

6.2 Sample extraction and chromatographically identification

The hot water and cold organic extraction in increasing polarity of the indigenous mushroom sample were investigated. The indigenous *Ganoderma* was found to contain a massive number of metabolites of all polarities from less polar components to more polar components. This was true when a range of less polar and more polar organic extracting solvents used yielded a massive number of uncountable components on TLC plates. As a general rule of thumb: like dissolves likes. The TLC method is one of the easiest method to do but trickier to develop a good development system suitable for all components especially when you have a lot of components of which more are structurally similar and with minute polarity difference. A neutral system ratio of B: EA (1:1) was sought to elute better Benzene, Chloroform and Ethyl Acetate extracts, while for EtOH, MetOH and HWE was EA: MetOH/ EA: EtOH (1:1). The secondary metabolites of these mushrooms were chemically diverse components and unable to separate them completely on the TLC plates.

6.3 Class of compounds identification

The mycochemicals identification of class of compounds of the extracted indigenous *Ganoderma* mushroom was investigated with universal detection reagents (Section 5.). The classes of compounds identification is in general consensus with reports in the literature that polysaccharides and triterpenes (a subclass of terpenoids) are major groups constituents isolated from *Ganoderma* that have been shown to possess biological activity (Kim and Kim, 1999, p. 83; Mizuno et al., 1995, p. 156; Wu et al., 2001, p. 1122; Roberts, 2004, p. 168; Patterson, 2004, p. 2000). Interestingly, the indigenous *Ganoderma* was found to contain certain compounds reactions to universal detection reagents which are not reported in the literature. This has suggested possible compounds produced by the indigenous *Ganoderma* mushroom due to the adaptation of the harsh dry and hot conditions in Namibia. Thus the first research hypothesis confirmed that the indigenous Namibian *Ganoderma* mushrooms have produced some secondary metabolites different from those found elsewhere. This was due to the dry climate and lack of surface water can caused a large number of species to evolve adaptations to survive such extreme conditions. Thus, the extreme environmental conditions in Namibia, different hosting species and other factors compared to other parts of the world could produce potential mycochemical of different biological activities

The following classes of compounds were found to make-up the indigenous *Ganoderma* mycochemicals profile: anthraquinones, anthrones, coumarins, arbutin drug compounds, cardiac glycoside drugs, alkaloids, heterocyclic nitrogen compounds, conjugated double bonds compounds, triterpenes, steroids (saponins and bitter principles) and sterols (cholesterol and esters), essential oil, bitter and pungent principles, saponins drugs, terpenes and essential oils. This is in support with Boh et al., (2000, p. 13), Eo, Kim, Lee and Ham, (1999, p.130) that polysaccharides, triterpenes, protein bound polysaccharides, germanium, phenolics compound, alkaloids compounds, lectins, amino acids and proteins are some of the major active constituents that have been isolated from *G. lucidum*. Therefore, the indigenous *Ganoderma* have variety of compounds classes similar to those researched elsewhere. However, until further structure elucidation is performed, one can only speculate as to what type or class of compounds is present. Thus the second research hypothesis confirmed that the indigenous *Ganoderma* have some similar types of class of compounds with those found elsewhere. The *Ganoderma* mushrooms and its closely related species have been reported to contain some compounds which are similar from even though from different geographical areas. To identify the exact type of compounds, further work is required which would involve complete purification of the components and further analysis using chromatographic and spectroscopic techniques.

Finally, the findings from this investigation are particularly interesting from a commercial aspect. Potential possibilities exist to establish research and development of bioactive metabolites from Namibia indigenous *Ganoderma* species. However, there is a need to bring about the law to comply with the production of such metabolites from the natural environment of the indigenous *Ganoderma* species. This is the first report on the classes of mycochemicals class of compounds from the fruiting body of the indigenous Namibian *Ganoderma* mushroom.

6.4 Antibacterial biological properties

The antibacterial activity of the indigenous *Ganoderma* mushroom was investigated. The extraction processes were employed, hot water and organic extraction (Chapter 3). The results indicated that both the aqueous and organic extracts from the indigenous *Ganoderma* mushrooms possessed activity against a range of tested pathogenic bacteria. Hot water extracts exhibited activity against the Gram negative bacteria *N. meningitides* (Section 4.). However, organic solvents extracts exhibited antibacterial activity against Gram negative and positive bacteria (Section 4.) of which the Benzene, EA extract appeared to have the strongest antibacterial effect. Anti-microbial activities from the *Ganoderma* extracts were reported by Boh et al., (2007, p. 300) and Chang and Mshigeni, (2004a, p. 38). Other reports have also shown organic solvents extracts from different *Ganoderma* species to possess biological activity toward Gram positive bacteria (Roberts, 2004, p. 168; Mothana, Awadh Ali, Jansen, Wagner, Mentel and Lindequist, 2000, p. 178). More antibacterial activity was exhibited against Gram negative bacteria for the most of the organic extracts.

The MHA medium supported the great mycelia growth of the selected clinically important bacteria also yielded great antibacterial activity. The Benzene, Chloroform, EA, EtOH and MetOH extracts from this fungus exhibited antibacterial activity (Section 4.) towards some Gram positive and negative bacteria, *E. coli, N, meningitides, P. vulgaris, B. cereus, S. aureus,* and *S. faecalis.* In particular, the antibacterial activity exhibited against a number of clinically important pathogens, was of great significance, as these bacteria are among those that are reported to have resistance to a number of currently available antibiotics. Thus the third research hypothesis confirmed that the the indigenous *Ganoderma* mushroom extracts have antibacterial properties against *E. coli, A. faecalis, P. vulgaris, N. meningitidis, B. cereus and S. aureus*. This is to validate the traditional usage of *Ganoderma* extracts on wounds and in the treatment of skin infection, wounds treatment. Therefore, the indigenous *Ganoderma* mushroom extracts have antibacterial activities. It is recommended that further study need to be embarked upon the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays.

These mushrooms are not only used in treating human ailments, but are also used in treating animals, for example their use in cattle for lung diseases, goats for skin diseases and chickens (Ogbe, Ditse, Echeonwu, Ajodoh, atawodi and Abdu, 2009, p. 1052; Kadhila-Muandingi, 2010, p. 68). This is the first report on the antibacterial assay of the fruiting body of the indigenous Namibian *Ganoderma* mushroom.

Chapter 7: References

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Appendix

Table 2. Local names and regions where the mushrooms were collected.

Local Name (X)	Scientific Name	Labelled code	Region
Omumbango	*Croton gratissimus*	LC01 LC06 LK08 LK09 LS11 LS01	Caprivi Kavango Oshana
Omusaati	*Colophospermum mopane*	LC07 LC08 LK02 LK04 LH03 LH11	Caprivi Kavango Ohangwena
Omugongo	*Sclerocarya caffra*	LS02 LS07 LS09 LS04	Oshana
Omugolo	*Terminalia sericea*	LC05 LC04 LK01 LK07 LS05 LS12 LH05 LH02	Caprivi Kavango Oshana Ohangwena
Omumbaganyana	*Mundulea sericea*	LC03 LC09 LK03 LK10	Caprivi Kavango
Omuhama	*Terminalia prunioides*	LC10 LC11 LK06 LK11 LH06 LH09	Caprivi Kavango Ohangwena
Eno	*Acacia* spp.	LC02 LC13 LS08 LS10 LH04 LH08	Caprivi Oshana Ohangwena
Omupupwaheke	*Combretum collinum*	LC10 LC12 LS03 LS06 LK05 LK12 LH01 LH07	Caprivi, Oshana Kavango, Ohangwena

Table 6: Namibian *Ganoderma* HWE and organic solvents crude extracts colour reactions zones on TLC plates after spraying with universal detection reagents. With * fluoresce compounds under UV_{365nm}

Reagent	**EtOH**	**EA**	**Chloroform**	**MetOH**	**Benzene**	**HWE**
EtOH-KOH	fainted yellow	Yellow layers	Intense yellow	yellow	Brighter yellow	-
AS reagent	Yellow, violet, purple, black	Purple, blue, green, red*, violet	Red*, purple, blue, violet, grey, blue-green, black	Purple, yellow, black	Green, yellow, violet, brown, red*-grey, black	Purple
BB	Blue	blue	Blue	Blue	Plenty of Blue	-
Kedde reagent	Pink, blue	Pink, blue, yellow	Pink, blue, green	Pink, blue	Pink, blue, yellow green	-
Folin	Blue	Blue	Blue	Blue	Blue	-
Dragerdorrf	Brown, yellow	Brown, orange, yellow	Brown, orange	Orange,	Brown, orange, yellow	-

Table 6 continue...

Reagent	**EtOH**	**EA**	**Chloroform**	**MetOH**	**Benzene**	**HWE**
EtOH-$AlCl_3$	One Blue*	bright green*, dark purples, blue, bright blue	bright green, dark purples, dark blue and bright blue	blue, grey, bright blue, black, purple, bright green	blue, grey, bright blue, black, purple, bright green	Blue*
Iodine	yellow	yellow	Yellow	yellow	yellow	-
VS	Brown*, red, grey	Brown*, red, grey, blue-green, black	Brown*, red, grey, red-violet, green	Brown*, red, grey, red, black	Red-violet, brown*, red, grey, black	-
LBr	Brown, grey, blue,	Brown, grey, orange	Brown, grey, blue	Brown, grey, blue	Brown, grey, orange	-
FBS	Brown	Red, brown	Red, brown	Red	Red, brown	-
Florecein	Yellow	Yellow	Yellow	Yellow	Yellow	-

Description of tested bacteria against the indigenous *Ganoderma* extracts from Michael et al. (2011, Brock Biology of Microorganisms Book).

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Neisseria meningitidis, is a Gram-negative diplococcus bacteria, often referred to as *meningococcus*, is a bacterium that can cause meningitis and other forms of meningococcal disease such as meningococcemia, a life threatening sepsis. *N. meningitidis* is a major cause of morbidity and mortality during childhood and has been responsible for epidemics in Africa and in Asia.

Proteus vulgaris is a rod-shaped, bacterium that inhabits the intestinal tracts of humans and animals. It can be found in soil, water and fecal matter. It is an opportunistic pathogen of humans. It is known to cause urinary tract infections and wound infections.

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. *E. coli* bacteria constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination.

Alcaligenes faecalis is a Gram-negative, rod-shaped, that is commonly found in the environment. When an opportunistic infection does occur, it is usually observed in the form of a urinary tract infection.

Staphylococcus aureus is a Gram-positive coccal bacterium species. It is frequently found as part of the normal skin flora on the skin and nasal passages. *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, to life-threatening diseases such as pneumonia, meningitis and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections.

Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped bacterium. Some strains are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for animals. In food animals such as chickens, rabbits and pigs, some harmless strains of *B. cereus* are used as a probiotic feed additive to reduce *Salmonella* in the intestines and cecum.