An investigation of the phytochemistry and biological activity of *Asparagus laricinus*

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Doctor Technologiae: Biomedical Technology

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DECLARATION WITH REGARD TO INDEPENDENT WORK

I, SANDILE LAWRENCE FUKU, identity number 820623 5812 083 and student number 211152048, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree DOCTOR TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

Fuku

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18/08/23

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Dedication

I dedicate this thesis to my beloved son Lwazi and my late grandfather Mlulu Fuku . It is the birth of the former and the passing away of the latter that has reaffirmed the unity of opposites- that death stole the one person who rooted my life in necessity, and birth restored the prestige by granting me a life to root in necessity

List of abbreviation

DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EGCG	(-)-epigallocatechin gallate
EC_{50}	50 percent effective concentration
GAE	Gallic acid equivalents
GC	Gas chromatography
GI_{50}	50% growth inhibition
HMG-CoA	Hydroxymethylglutaryl coenzyme A
LC	Liquid chromatography
MAPK	Mitogen-activated protein kinase
MIC	Minimum inhibitory concentration
Min	Minute
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MS	Mass Spectrometry
OD	Optical density
Rf	Retardation factor
RPM	Revolutions per minute
SEM	Standard error of means
SF	Survival fraction
SD	Standard deviation
TGI	Total growth inhibition
TIC	Total ion current chromatogram
TNF	Tumor necrosis factor
VLB	Vinblastine
VCR	Vincristine

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Chapter 1

Literature review

1.1. Introduction

Medicinal plants are part of indigenous people's cultural heritage, thus since ancient times treatment of various diseases using medicinal plants has been part of human culture. The value of medicinal plants to mankind has been very well proven. It is estimated that 70% to 80% of people worldwide rely mainly on traditional health care systems, especially on herbal medicines (Stanley and Luz, 2003).

In many societies the medicinal properties of plants were discovered mostly through trial and error, but use was also influenced by the belief systems of the people involved and often became entangled with religious and mythical practices (Mathias *et al*., 1996). Besides that, medicinal plants are proving to be rich resources of constituents that can be used in drug development and synthesis.

Medicinal plants have been a source of a wide variety of biologically active compounds for many centuries and have been used extensively as crude material or as pure compounds for treating various disease conditions. Between 1% and 10% of plants out of an estimated 250 000 to 500 000 species of plants on earth are used by humans (Boris, 1996).

Plants used for medicinal purposes contribute significantly to the development of major medical drugs that are used today. Most common medicines have compounds extracted from plants as their primary active ingredients and many have provided blueprints for synthetic or partially synthesized drugs (Simpson and Ogorzaly, 2001).

There has been a major resurgence of interest in traditionally used medicinal plants, with a number of international and local initiatives actively exploring the botanical resources of southern Africa with the intention to screen indigenous plants for pharmacologically active compounds (Gurib-Fakim *et al*., 2010; Rybicki *et al*., 2012).

South Africa is considered a "hot spot" for biodiversity and more than 22 000 plant species occur within its boundaries. This represents 10% of the world's species, although the land surface of South Africa is less than 1% of the earth's surface (Coetzee *et al*., 1999).

Plants have also been used by man for various purposes, among others as arrow and dart poisons for hunting, poisons for murder, hallucinogens used for ritualistic purposes, stimulants for endurance and hunger suppression, as well as medicine (Duke *et al*., 2008; Cragg and Newman, 2005).

A derivative of the polyhydroxy diterpenoid ingenol isolated from the sap of *Euphorbia peplus* (known as "*petty spurge*" in England or "*radium weed*" in Australia), which is a potential chemotherapeutic agent for skin cancer, is currently under clinical development by Peplin Biotech for the topical treatment of certain skin cancers (Kedei *et al*., 2004; Ogbourne *et al*., 2004). Combretastatin A-4 phosphate,

a stilbene derivative from the South African bush willow, *Combretum caffrum,* acts as an anti-angiogenic agent causing vascular shutdowns in tumors (Newman *et al*., 2005; Holwell *et al*., 2002).

Further reliance on plants for drug development is demonstrated by the use of galantamine hydrobromide, an alkaloid obtained from the plant *Galanthus nivalis* used traditionally in Turkey and Bulgaria for the treatment of Alzheimer's disease (Howes *et al*., 2003; Heinrich and Teoh, 2004).

The plant chemicals used for the above-mentioned purposes are secondary metabolites, which are derived biosynthetically from plant primary metabolites (e.g. carbohydrates, amino acids and lipids). Secondary metabolites are organic compounds that are exclusively produced by plants and that are not directly involved in the normal growth, development and reproduction of a plant (Firn and Jones, 2003). Yet, they have many functions that are important for the plant's long-term health and appearance.

Plants, being stationary, have to cope with a number of challenges, including engineering their own pollination and seed dispersal, local variation in the supply of the simple nutrients that they require to synthesize their food and the coexistence of herbivores and pathogens in their immediate environment. Plants have therefore evolved secondary biochemical pathways that allow them to synthesize a spectrum of organic molecules, often in response to specific environmental stimuli, such as herbivore-induced damage, pathogen attacks, or nutrient deprivation (Reymond *et al*., 2000; Hermsmeier *et al*., 2001).

The biosynthesis of secondary metabolites is derived from the fundamental processes of photosynthesis, glycolysis and the Krebs cycle to afford biosynthetic intermediates which, ultimately, result in the formation of secondary metabolites also known as natural products (Dewick, 2002).

It is hypothesized that secondary metabolism utilizes amino acids and the acetate and shikimate pathways to produce "*shunt metabolites*" (intermediates) that have adopted an alternate biosynthetic route, leading to the biosynthesis of secondary metabolites (Sarker *et al*., 2006).

Modifications in the biosynthetic pathways that produce secondary metabolites are probably due to natural causes (e.g. viruses or environmental changes) or unnatural causes (e.g. chemical or radiation processes) in an effort to adapt or provide longevity for the plant (Sarker *et al*., 2006). Plants' secondary metabolites can be classified into several groups according to their chemical classes, such alkaloids, terpenoids and phenolics (Harbone, 1984; Wink, 2003).

1.2. Classification of plants' secondary metabolites

The palette of secondary metabolites can be divided into a number of distinct groups on the basis of their chemical structure and synthetic pathways. These groups can, in turn, be broadly differentiated in terms of the nature of their ecological roles and therefore their ultimate effects and comparative toxicity in the consuming animal (Kennedy and Whightman, 2011). The three main groups of secondary metabolites

in plants that are of interest in this study are alkaloids, phenolic compounds and terpenoids (Firn and Jones, 2003; Wink, 2003).

1.2.1. Alkaloids

Alkaloids are complex N-containing heterocyclic organic compounds and are among the most important plant materials for the development and production of drugs (Facchini, 2001). Although no single classification exists, Kennedy and Whightman (2011) argued that alkaloids are often distinguished on the basis of a structural similarity (e.g. indole alkaloids) or a common precursor (e.g. benzylisoquinoline, tropane, pyrrolizidine, or purine alkaloids).

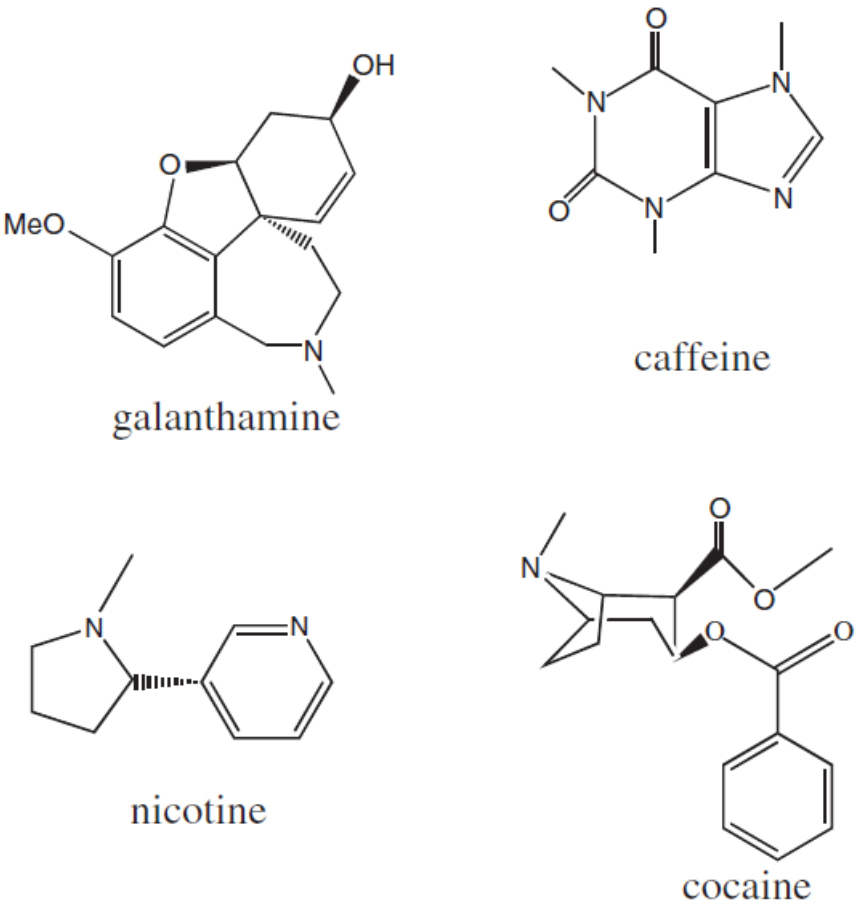

Figure 1.1. Alkaloids (taken from Kennedy and Whightman, 2011).

The recorded use of alkaloids for medicinal purposes stretches back some 5 000 years (Goldman, 2001) and this class of molecules has contributed to the majority of poisons, neurotoxins, traditional psychedelics (e.g. atropine, scopolamine and hyoscyamine, from the plant *Atropa belladonna*) and social drugs consumed by humans, e.g. nicotine, caffeine, methamphetamine (ephedrine), cocaine and opiates, (Zenk and Juenger, 2007).

1.2.2. Phenolic compounds

Phenolic compounds (Harborne and Williams, 2000) are based on phenol (an oxygen linked to a fully saturated C_6 ring), the simplest member of this class of plant substances. Phenolic compounds include, among others, flavonoids and tannins (Harborne and Williams, 2000). Phenols are the compounds containing a hydroxyl group (—OH) directly attached to an aromatic ring.

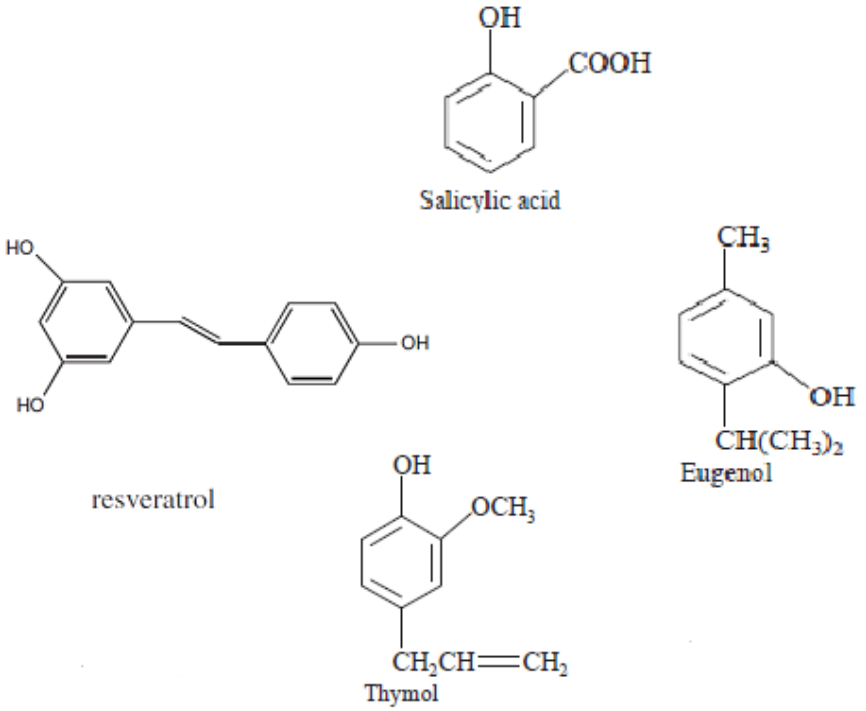

Figure 1.2. Phenols (Aggarwal and Shishodia, 2006; Dewick, 2002)

Phenolic compounds, in particular flavonoids, are generally involved in the protection of plants from attack by microbes and insects (Cushnie and Lamb, 2005; Friedman, 2007).

Special classes of plant phenolics are the tannins, characteristically astringent, bitter plant polyphenols that are toxic to herbivores owing to their capacity to either bind and precipitate, or shrink proteins and other macromolecules (Cushnie and Lamb, 2005). Some phenols are used as chemopreventive agents, e.g. resveratrol (3, 5, 4-trihydroxystilbene) is an oligomeric polyphenol found as dimer, trimer and tetramer. This molecule is implicated in the prevention of cancer and cardiovascular diseases

in vasoprotection and neuroprotection (Ates *et al*., 2007; Delmas *et al*., 2006; Vitrac *et al*., 2004).

Acetylsalicylic acid (aspirin) a time-honored analgesic and antipyretic drug, was derived from salicylic acid, which occurs in willow trees. Some other important salicylic acid derivatives are methyl salicylate, a common ingredient of liniments (Dewick, 2002). Eugenols extracted from cloves are used as an anesthetic and antiseptic in pharmaceutical and dental preparations (Daniel *et al*., 2009).

1.2.3. Terpenoids

Terpenoids are dimers or combinations of isoprene, a common organic compound that is highly volatile because of its low boiling point (Zwenger and Basu, 2008). This class of molecules is diverse and consists of groups of more than 30 000 lipid-soluble compounds. Their structure includes one or more five-carbon isoprene units, which are ubiquitously synthesized by all organisms through two potential pathways, the mevalonate and deoxy-d-xylulose pathways (Rohmer, 1999).

The general chemical structure of terpens is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}) (Arif *et al*., 2011).

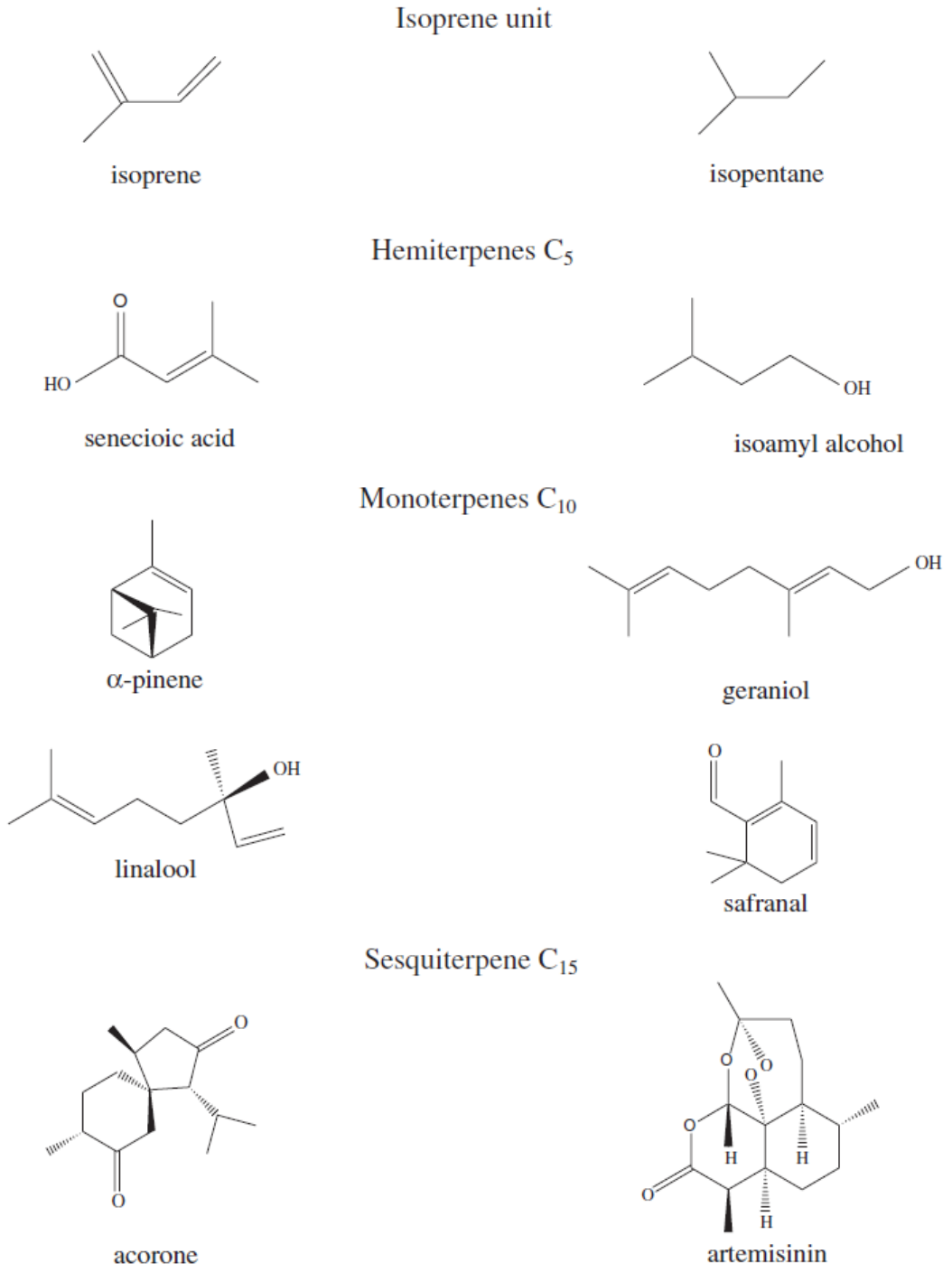

Figure 1.3. Basic building unit and various classes of terpenes (taken from Hao *et al.*, 2013).

Geraniol, an acyclic dietary monoterpene, represents the only monoterpene that has been studied *in vitro* against liver cancer cells. Geraniol was shown to inhibit the growth of HepG2 human hepatic carcinoma cells by decreasing 3-

hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the major rate-limiting enzyme in cholesterol biosynthesis in mammals (Polo and Bravo, 2006).

Ardisiacrispin (A+B), a triterpenoid saponin mixture in the fixed proportion 2:1 of ardisiacrispin A and ardisiacrispin B, is derived from *Ardisia crenata*. This mixture exerted cytotoxic activity against Bel-7402 liver cancer cells through pro-apoptotic, anti-proliferative and microtubule disruptive activities (Li *et al*., 2008).

1.3. Biosynthetic pathways

The synthesis of different classes of secondary metabolites from primary metabolites is presented in schematic form in Fig. 1.4.

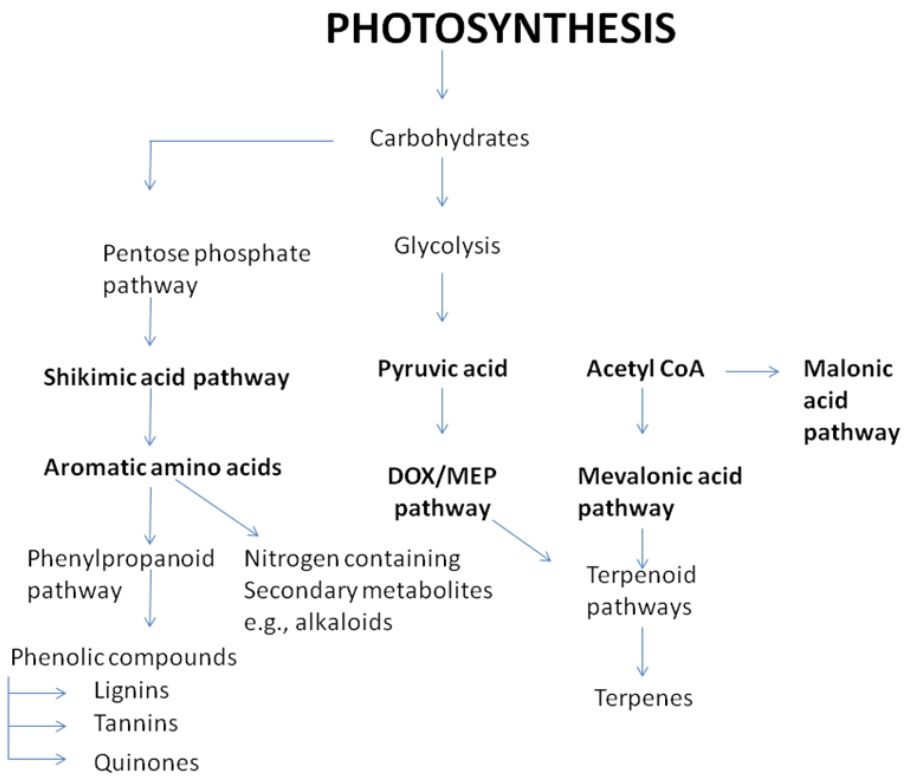

Figure 1.4. Principal biosynthetic pathways leading to synthesis of secondary metabolites. Adapted from Ramawat *et al*. (2009).

Secondary metabolites are predominantly synthesized via two principal biosynthetic pathways. The first one, the shikimic acid pathway, produces a pool of aromatic amino acids, which in turn are converted into diverse compounds such as phenolics (lignins, tannins, quinones) and alkaloids (Mustafa and Verpoorte, 2007). The second one is the acetyl-CoenzymeA mevalonic acid pathway, which produces a vast array of terpenoids (Eisenreich *et al*., 2004).

1.4. Drug development

Though enormous progress has been made in medicinal chemistry, the development of a novel drug has become more and more difficult. The reasons are manifold, including the fact that good drugs are available for major diseases, and developing a better drug that is active on the same target without being more expensive becomes increasingly difficult in view of the sophistication in industrial production methods (Wess *et al*., 2001).

Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products, particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities (Newman *et al*., 2000; Newman *et al* 2003; Butler, 2004).

Interestingly, of the 877 novel medicines that were developed in the period 1981-2002, 6% were natural products, 27% were derivatives of natural products and 16% were synthetics developed on the model of a natural product (Newman *et al*., 2003).

This demonstrates that nature is an important source for developing novel leads for medicines. Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads. Since the sequencing of the human genome, thousands of new molecular targets have been identified as important in various diseases (Kramer and Cohen, 2004). The history of drug discovery and even drug chemistry that is inexorably bound to the plant kingdom confirms that the process of deriving drugs from plant sources is certainly not a new phenomenon.

Several known compounds isolated from traditionally used medicinal plants have already been shown to act on newly validated molecular targets, as exemplified by indirubin, which selectively inhibits cyclin-dependent kinases (Hoessel *et al*., 1999; Eisenbrand *et al.*, 2004) and kamebakaurin, which has been shown to inhibit NF- $_{k}$B (Hwang *et al*., 2001; Lee *et al*., 2002).

1.4.1. Anti-microbial activity

It is estimated that plant materials are present in or have provided the models for 50% of Western drugs developed today (Robbers *et al*., 1996). Many commercial drugs used in modern medicine today were initially used in crude form in traditional or folk healing practices, or for other purposes that confirmed their potential useful biological activity. Avancini *et al*. (2000) demonstrated the antimicrobial actions of “carqueja” (*Baccharis trimera* Less.) extracts on Gram-positive (*Staphylococcus aureus* and *Streptococcus uberis*) and Gram-negative (*Salmonella gallinarum* and *Escherichia coli*) bacterial strains.

High antimicrobial activity of *Thymus, Origanum* and *Eugenia caryophillus* species has been attributed to their phenolic components such as thymol and carvacrol (Lambert *et al*., 2001; Hazzit *et al*., 2009). Similarly, the antimicrobial activity of *Cinnamomum zeylanicum* has been related to its cinnamaldehyde content (Juliani *et al*., 2009).

1.4.2. Anti-cancer activity

The search for anti-cancer agents from plant sources started in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins (Cragg and Newman, 2005). The first agents to advance into clinical use were the so-called vinca alkaloids, vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthusroseus* (Gueritte and Fahy, 2005). These agents are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemia, lymphoma, advanced testicular cancer, breast and lung cancer and Kaposi's sarcoma.

Combretastatins were isolated from an indigenous South African plant, *Combretum caffrum* (Pinney *et al*., 2005). These molecules have served as a model for the treatment of tumors and as a result many synthetic analogues of Combretastatin-4 have been created in order to improve its cytoxicity and inhibition of tubulin polymerization (Nam, 2003; Ohsumi *et al*., 1998). That has provided an impressive display of the power of a relatively simple natural product structure that can be

altered to produce superior products through medicinal and combinatorial chemistry (Li and Sham, 2002).

1.4.3. Anti-diabetic activity

The aqueous leaf extract of *A. squamosa* has been reported to ameliorate hyperthyroidism (Sunanda and Anand, 2003), which is considered a causative factor for diabetes mellitus. However, the mechanism involved in the extract's inhibition of hyperthyroidism is not known. More than 1000 plant species are being used for the treatment of Type II diabetes mellitus worldwide (Trojan-Rodrigues *et al*., 2011). Similar to the *A. squamosa* extract, very little is known about the mechanism of action of these anti-diabetic plants, thus limiting their use in standard diabetes care. Many plants have shown anti-diabetic action through the release of insulin and some extra pancreatic mechanisms (Jung *et al*., 2006). However, more investigations must be carried out to evaluate the exact mechanism of action of medicinal plants with anti-diabetic and insulin-mimetic activity (Patel *et al*., 2012).

Phytoconstituents such as alkaloids inhibit alpha-glucosidase and decrease glucose transport through the intestinal epithelium and imidazoline compounds stimulate insulin secretion in a glucose-dependent manner (Patel *et al*., 2012). This shows most researchers' preference for pure or semi-pure molecules in evaluating mechanisms of action. This leads to poor validation of the health benefits and biosafety of many plants that are traditionally used.

1.4.4. Anti-oxidant activity

Antioxidant-based drugs/formulations for the prevention and treatment of complex diseases such as atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have emerged in the last three decades (Devasagayam *et al*., 2004). This coincides with the upsurge of interest in the therapeutic potentials of plants as antioxidants in reducing free radical induced tissue injury. Although many synthetic anti-oxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available, their toxicity is a growing concern. Both carcinogenic and anticarcinogenic properties have been reported for the synthetic antioxidants BHA and BHT (Botterweek *et al*., 2000).

In dealing with the toxicity of synthetic anti-oxidants, a worldwide trend towards the use of natural phytochemicals present in berries, tea, herbs, oilseeds, beans, fruit and vegetables has increased (Lee and Shibamoto, 2000; Wang and Jiao, 2000).

Natural antioxidants, especially phenolics and flavonoids from tea, wine, vegetables and spices, are already exploited commercially either as antioxidant additives or nutritional supplements (Schuler, 1990; Chu *et al*., 2000).

Many other plant species have also been investigated in the search for novel antioxidants, but there is generally still a demand to find more information concerning the antioxidant potential of plant species, as they are safe and bioactive.

1.5. *Asparagus laricinus*

Knowledge about the medicinal value of many plants that form part of the rich biodiversity in South Africa is largely contained in the oral traditions of various ethnic groups that constitute the indigenous people of South Africa (van der Merwe *et al*., 2001). Loss of indigenous culture, in favor of western European-derived culture, is an accelerating process among indigenous people around the world (Prance, 1994). Consequently, the traditional knowledge that forms the basis of the use of medicinal plants is in danger of being lost and warrants rigorous scientific investigation.

The current study was prompted by case reports describing unexpected improvement in patients who had been terminally ill with advanced prostate cancer. Clinicians had no explanation for the improvement. When questioned by the clinicians, these patients reported that they had been treated with an extract from the root of a medicinal plant. This plant material was offered to researchers for initial analysis for anti-cancer activity. After characterization by a botanist, the plant was found to be *Asparagus laricinus* (A. laricinus), belonging to a monogeneric family called *Asparagaceae*, under the subfamily *Asparagoidiae* (Brummit, 1992). This plant is commonly known as *lesitwane* among the Batswana clans in South Africa. Traditionally tubers of this plant are used to treat sores, redwater and uterine infection. However, informants in the study attributed the action of this medicinal plant to a physical mechanism that they could not explain owing to lack of knowledge (van der Merwe *et al*., 2001). These claims, the efficacy of the use of the *A. laricinus* plant and its therapeutic potential have not been investigated scientifically.

Objectives of the study: To prepare a crude plant extract of *A. laricinus*, broadly establish the bioactivity and describe the phytochemical properties of *A. laricinus* water extract.

Specific aims:

- To conduct *in vitro* anti-cancer screening of *A. laricinus* extract
- To evaluate the mutagenic and cytotoxic effect of the plant
- To evaluate the radical scavenger and anti-oxidative stress activities
- To evaluate the anti-microbial activity of *A. laricinus* aqueous extract
- To examine the chemical composition of both aqueous and methanol extracts

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Chapter 2

In vitro anti-cancer screening of *Asparagus laricinus* extracts

2.1. Introduction

Cancer is one of the most prominent diseases in humans and currently there is considerable scientific and commercial interest in the continuing discovery of new anti-cancer agents from natural product sources (Kinghorn *et al*., 2003). Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Plants, especially those with ethnopharmacological uses, have been the primary sources of medicines for early drug discovery. Current drug discovery from terrestrial plants has relied mainly on bioactivity-guided isolation methods, which, for example, have led to discoveries of important anti-cancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminate* (Kinghorn, 1994).

The search for natural products as potential anti-cancer agents dates back at least to the Ebers papyrus in 1550 BC. However, the current study begins with investigations similar to those of Hartwell and co-workers in the late 1960s on the application of podophyllotoxin and its derivatives as anti-cancer agents (Hartwell, 1967).

Podophyllotoxin, a bioactive lignan, was first isolated by Podwyssotzki in 1880 from the North American plant *Podophyllum peltatum Linnaeus* (American podophyllum).

Two semisynthetic derivatives of podophyllotoxin, etoposide and teniposide, are currently used in frontline cancer chemotherapy against various cancers (O'Dweyer *et al*., 1985). Since the publication of Hartwell's findings on posophyllotoxin (Hartwell, 1967), natural products and their derived components as therapeutic agents, especially those from plant sources, have increased as a component of modern westernised medicine. Similarly, there has been an increase in publications related to natural products, their chemistry, biological activities and uses.

Currently, over 85 or 48.6% of drugs used in clinical trials for anti-cancer activity are actually derived from natural products or are natural products (Newman and Cragg, 2012). This demonstrates the rationale for the search for novel drug molecules in medicinal plants, especially when literature shows that plant-derived compounds have provided attractive possibilities for treatment strategies (Jain and Jain, 2011).

The aim of the present study was to identify the anti-cancer activity of *A. laricinus* against three human cell lines, namely breast MCF-7, renal TK-10 and melanoma UACC-62. These cell lines were selected because of their high sensitivity to detect anti-cancer activity. The researcher demonstrated here that these extracts exhibit anti-cancer activity against the three human cell lines.

2.2. Materials and methods

2.2.1. Plant material

The plant material (*A. laricinus*) was authenticated by scientists at the National Botanical Gardens in Pretoria, South Africa. The collected root materials were dried at room temperature, pulverised by a Macsalab mill (Model 200, LAB) and weighed. The powder was then stored at room temperature until analysis. Plant material (10 g of the dried roots) was soaked in a volume of 500 ml of ethanol or purified water for 72 hours under shaking conditions (120 rpms). The supernatant was filtered passively through a Whatman® filter paper, 11 cm in diameter. The solvent (ethanol) was removed completely under vacuum by using a speed evaporator (Univapo 100H) at 50°C. The aqueous sample was lyophilised for 72 hours in the VIRTIS 5 L freeze drier (VIRTIS New York, USA) to obtain a dried powdered plant extract. The dried samples were then reconstituted in either water or ethanol.

2.2.2. *In vitro* anti-cancer screening

The human cell lines TK-10, UACC-62 and MCF-7 were obtained from the National Cancer Institute (NCI) in the framework of a collaborative research program between the Council for Scientific and Industrial Research (CSIR) and the NCI. The extracts and compounds were assayed in the three-cell line panel consisting of TK-10 (renal), MCF-7 (breast), and UACC-62 (melanoma) cells. Cell lines were routinely maintained as monolayer cell cultures at 37°C, 5% CO_2 and 100% relative humidity

in Roswell Park Memorial Institute (RPMI) media containing 5% fetal bovine serum, 2 mM L-glutamate and 50 µg/ml gentamicin. The primary anti-cancer assay was performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI (Leteurtre *et al*., 1994; Kuo *et al*., 1993; Monks *et al*., 1991). The extracts or compounds were tested at a single concentration (100 ppm) and the culture was incubated for 48 hours. End-point determinations were made with a protein-binding dye, Sulforhodamine B (SRB).

The growth percentage of the treated cells was evaluated spectrophotometrically versus controls not treated with test agents. All the extracts that reduced the growth of two of the cell lines by 75% or more were tested further at 1/2 log serial dilutions of concentrations ranging from 6.25-100 ppm. The blanks contained complete medium without cells and etoposide was used as a standard. Results of five dose screenings were reported as total growth inhibition (TGI). The biological activities were separated into four categories: inactive (TGI >50 ppm), weak activity (15 ppm< TGI <50 ppm), moderate activity (6.25 ppm< TGI <15 ppm) and potent activity (TGI <6.25 ppm), according to NCI guidelines.

2.2.3. Analysis of results

For each tested extract, one additional parameter was calculated: GI_{50} (50% growth inhibition, as opposed to TGI, which indicates 100% growth inhibition, indicates the cytostatic activity of the test agent).

2.3. Results

The results obtained show significant growth inhibition of three human cancer cell lines by both ethanol and aqueous extracts (Figure 2.1 and 2.2). However, the ethanol extract exhibited more potent anti-cancer activity, where 6.25 µg/ml of the extract inhibits 40% of both TK-10 and MCF-7. In contrast, it required 25 µg/ml of the aqueous extract to achieve the same results. The UACC-62 cell line was the most susceptible to inhibition by both extracts of *A. laricinus*.

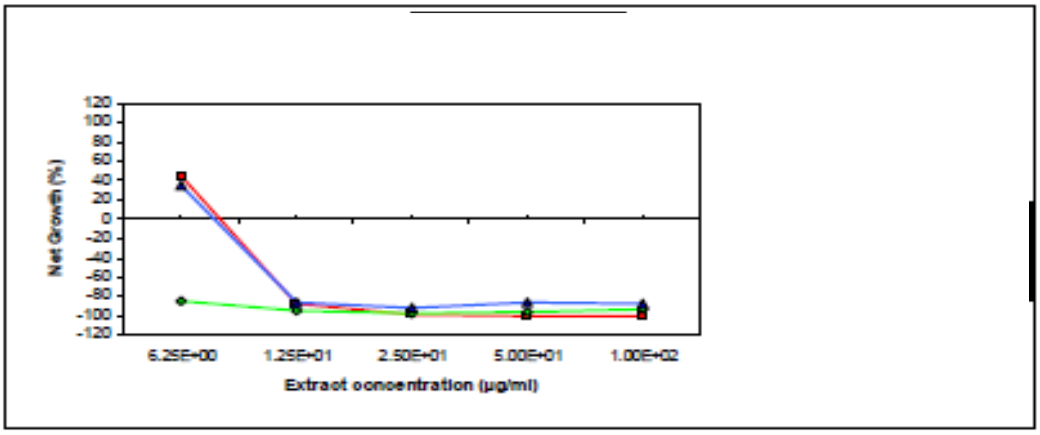

Figure 2.1. Growth inhibitory effect of ethanol extracts of *A. laricinus* on three human cancer cell lines: MCF-7 (▲), TK-10 (▄) and UACC-62 (□).

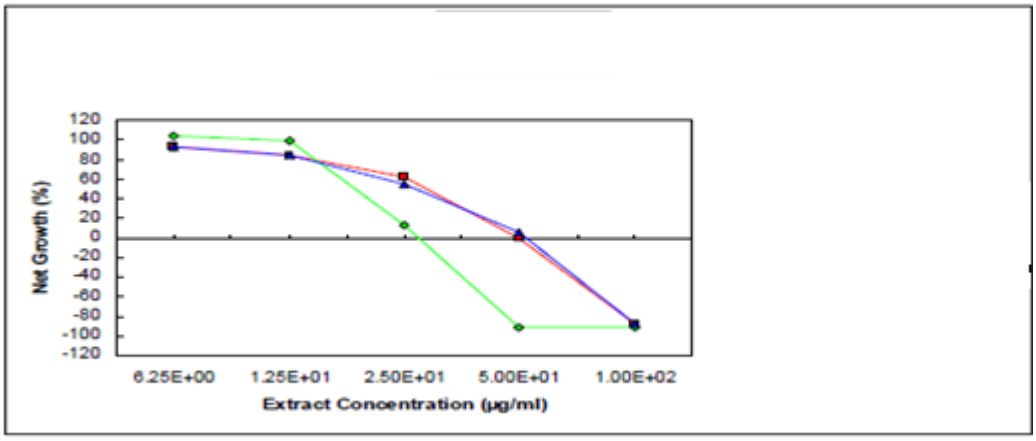

Figure 2.2. Growth inhibitory effect of aqueous extracts of *A. laricinus* on three human cancer cell lines: MCF-7(▲), TK-10(▄) and UACC-62 (□)

The ethanol extract exhibited superior activity to etoposide and aqueous extract on inhibition of the UACC-62. The aqueous extract showed the lowest activity compared to both ethanol extract and etoposide; on all cell lines the activity status was moderate (Table 2.1). The breast cancer cell line (MCF-7) was found to be resistant to etoposide, yet susceptible to both aqueous and ethanol extracts. Interestingly, the ethanol extract showed potent activity against the UACC-62 cell line, while maintaining moderate activity against TK-10 and MCF-7.

Table 2.1. Cytotoxic activity (GI_{50} values) of plant extracts and etoposide that are used in the treatment of cancer cell lines

Treatment	**MCF7 cell line**			**TK-10 cell line**			**UACC-62 cell line**		
	GI50	**TGI**	**Status**	**GI50**	**TGI**	**Status**	**GI50**	**TGI**	**Status**
Ethanol extract	<6.25	8.04	Moderate activity	<6.25	8.34	Moderate activity	<6.25	<6.25	Potent activity
Aqueous extract	27.25	52.83	Weak activity	29.70	49.98	Weak activity	19.59	27.99	Weak activity
Etoposide	<6.25	>100	Inactive	9.72	25.19	Weak activity	<6.25	38.54	Weak activity

2.4. Discussion

The observed anti-cancer activity of water and ethanol extracts of A. *laricinus* at 50 µg/ml concentrations is meaningful and profiles this plant as a potential source of therapeutic compounds. The first report of the anti-cancer activity of *A. laricinus* was published by our research group (Mashele and Kalishnikov, 2010). It is of interest

that the extracts of the plant showed cytotoxicity against the cancer cell line, and if this also occurs *in vivo*, the use of this plant by traditional healers for the treatment of cancer patients would have some scientific basis.

The aqueous extract exhibits low anti-cancer activity compared to the ethanol extract. The latter showed moderate activity while that of the former was weak. Organic solvents have the disadvantage of dissolving molecules that have poor bioavailability, the aqueous extract will be chosen for further experiments due to its suitability in mimic the extraction conditions of most traditional users of the medicinal plants. Fabricant and Farnsworth (2001) argued that proper action needs to be taken to assure that potentially active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples, especially when the plant is selected on the basis of traditional usage.

Pytochemicals isolated from medicinal plants are found to act as potent antioxidants and free radical scavengers. These natural products are supposed to minimize deoxyribonucleic acid (DNA) damage by reacting with free radicals and in this way they could prevent cancer (Rao *et al*., 2008). However, this does not suffice in this study, where the plant extract was found to be cytotoxic to cancer cell lines. Thus, the next chapter will evaluate the mutagenic and cytotoxic effects of *A. laricinus* aqueous extract.

A more plausible proposal of the anti-cancer mechanism of plant extracts is that the anti-cancer action of plants occurs via direct cytotoxic effects and/or indirectly

through immunological modulatory action (Kitakgishi *et al*., 2012). However, this explanation offers no clarity in the cascade of reactions that could be involved.

2.5. Conclusion

It has been proven that both aqueous and ethanolic extracts of *A. laricinus* have anti-cancer activity against TK-10 (renal), MCF-7 (breast), and UACC-62 (melanoma) cell lines. Maximum activity was found in the ethanol extract of the plant. However, the aqueous was chosen for further studies in order to be consistent with the traditional usage of the plant. The aqueous extract of this plant has the potential to be used in dealing with the side effects of cancer.

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Chapter 3

Evaluation of the mutagenicity and cytotoxicity effect of *Asparagus laricinus*

3.1. Introduction

One of the most prominent diseases in humans today is cancer. It is projected that deaths from cancer are continuing to escalate, with an estimated increase from nine million deaths from cancer in 2015 to 11.4 million deaths per year by 2030 (WHO report, 2005). Generally, cancer begins after a mutational episode in a single cell and then it progressively transforms to malignancy in multiple stages through sequential acquisition of additional mutations (Khan and Pelengaris, 2006).

Because mutation is an important factor in carcinogenesis, the incidence of cancer may be reduced by decreasing the rate of mutation. The best way for humans to decrease the rate of mutation is to avoid exposure to or ingestion of mutagens and carcinogens (Kim *et al*., 2006). Currently, there is marked scientific and commercial interest in the continuing discovery of new anti-cancer agents from natural product sources (Kinghorn *et al*., 2003). More than 50% of drugs used in clinical trials for anti-cancer activity were isolated from natural sources or are related to them (Cragg and Newman, 2005; Newman and Cragg, 2007). Hence, the search for natural products to be used in cancer therapy represents an area of great interest in which the plant kingdom is the most important source, providing many anti-tumor agents with novel structures and unique mechanisms of action (Chang *et al*., 1999).

Present chemotherapy cancer treatments have proved to be ineffective as a result of their toxicity and cells developing resistance (McWhirter *et al*., 1996; Balducci and Extermann, 2005). Many conventional drugs also induce genetic damage that itself can be carcinogenic. A segment of the research community is thus focusing on identifying novel chemotherapeutic agents in plants that do not induce the destructive effects of conventional cytotoxic therapeutic agents.

Several medicinal plants are traditionally used in the treatment of a variety of ailments, including cancer, in many communities in South Africa and neighboring countries. Since ancient times, herbal medicine has always been one of the main components of the health care system. Plant compounds were initially used as a source of anti-cancer agents by Hartwell in 1967, he used podophyllotoxin and its derivatives as anti-cancer agents (Shams *et al*., 2012).

Many of the plants in phytotherapy have not yet been scientifically assessed for their efficacy or safety to the tissue or organs of recipients. Thus, investigation of traditionally used medicinal plants is valuable as a source of potential chemotherapeutic agents and also to assess the safety of the continuous use of medicinal plants. As a result, many studies are being directed at popular medicine, with the aim of identifying natural products that exhibit therapeutic properties (Hamburger and Hostettmann, 1991; Weisburger, 1996). Phenolic compounds and flavonoids are also widely distributed in plants that have been reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities, as well as anti-inflammatory and anti-carcinogenic effects etc. (Patel *et al*., 2010;

Chu *et al*., 2000). In this regard, this study will determine the phenolic content of *A. laricinus* aqueous extract.

The Ames test (Maron and Ames, 1983; Mortelmans and Zeiger, 2000) was used in this study, as it is widely used in the determination of possible gene mutations caused by extracts. A positive response in any single bacterial strain, either with or without metabolic activation, is sufficient to designate a substance as a mutagen (Zeinger, 2001). The study being reported here evaluated the mutagenic and anti-mutagenic activity of the water extract of *A. laricinus* roots, which are known to be used in the treatment of cancer.

3.2. Materials and methods

3.2.1. Plant material

The plant material (*A. laricinus*) was authenticated by scientists at the National Botanical Gardens in Pretoria, South Africa. The collected root materials were dried at room temperature, pulverised by a mechanical mill and weighed. The powder was then stored at room temperature until analysis. Plant material (10 g of the dried roots) was weighed, pulverised using a Macsalab mill (Model 200, LAB) and soaked in a volume of 500 ml of ethanol or purified water for 72 hours under shaking conditions (120 rpms). The supernatant was filtered passively through a Whatman® filter paper, 11 cm in diameter. The aqueous sample was lyophilised for 72 h in the VIRTIS 5 L freeze drier (VIRTIS New York, USA) to obtain a dried powdered plant extract. The dried samples were then reconstituted in water or ethanol.

3.2.2. The Ames test

The Ames test is a well-known bacterial mutagenicity test (Weisburger, 1996; Mortelmans and Zeiger, 2000). In this test, reverse $His^{-} \rightarrow His^{+}$ mutations are visualized by plating *Salmonella typhimurium* bacteria in a histidine-poor growth medium. In this medium only, His^{+} mutants are able to form visible colonies. Different bacterial strains are available to identify different types of mutations. The researcher used the strains TA97, TA98 TA100 and TA102. The strains TA98 and TA100 are actually those that are most often used, as they detect the great majority

of mutagens. Strain TA98 gives an indication of frame-shift mutations, while a positive response from strain TA100 indicates base-pair substitution. Table 3.1 lists important characteristics of these mutant strains. For a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds should be more than twice the number of colonies produced on the solvent control plates (i.e. a ratio above 2.0). In addition, a dose response should be evident for the various concentrations of the mutagen tested. Toxicity can be checked by investigation of the background layer of bacteria. Anti-mutagenicity was expressed as percentage inhibition of mutagenicity according to the following formula given for the Ames test (Ong *et al*., 1986):

$$\% \text{ inhibition} = [1 - T/M] \times 100$$

where *T* is the number of revertants per plate in the presence of mutagen and the test sample and *M* is the number of revertants per plate in the positive control (mutagen alone). The same formula was also adopted for other tests. An extract was considered to have no or only weak anti-mutagenic properties when the percentage inhibition of mutagenicity (e.g. revertants in the Ames assay) was less than 25%. When the percentage inhibition was between 25% and 40% it was considered that the extract had a moderate anti-mutagenic effect and it was concluded that an extract had a strong anti-mutagenic effect when the percentage inhibition was higher than 40%. Dimethyl sulfoxide was used as a negative control, while daunomycin (10 µl/plate) was used as a positive control. All experiments were performed in triplicate. The metabolic activation (S9) was donated by the MRC Liver Research Centre, Tygerberg, Cape Town.

Table 3.1. List of *Salmonella typhimurium* strains used in Ames test

Strain	Amino Acid Marker		
	Histidine mutation	**Type of mutation**	**Main DNA target**
*Salmonella typhimurium*TA97	*hisD6610*	Frameshift	*rfa*
*Salmonella typhimurium*TA98	*hisD3052*	Frameshift	*rfa*
*Salmonella typhimurium*TA100	*hisG46*	Base pair substitution	*rfa*
*Salmonella typhimurium*TA102	*hisG428*	Base pair substitution	*rfa*

3.2.3. Determination of total phenolic content

The total phenolic content in *A. laricinus* extract was determined employing Folin-Ciocalteu reagent (Singleton *et al.*, 1999), with minor adaptations. Gallic acid was used as a standard phenolic compound. In a test tube, 0.5 ml of plant extract (500 µg/ml) or standard solution was added, followed by 35 ml of H_2O and 2.5 ml of Folin-Ciocalteu reagent. The content was mixed thoroughly. After eight minutes, 7.5 ml $NaCO_3$ (10% w/v) was added. The mixture was allowed to stand in the dark for one hour. Absorbance was measured at 760 nm using a spectrophotometer (Biowave II, WPA). The concentration of total phenolic compounds in plant extracts was determined from the Gallic acid standard curve. Data points were recorded in triplicate and the regression line was drawn with the aid of graphpad prism 5.0.

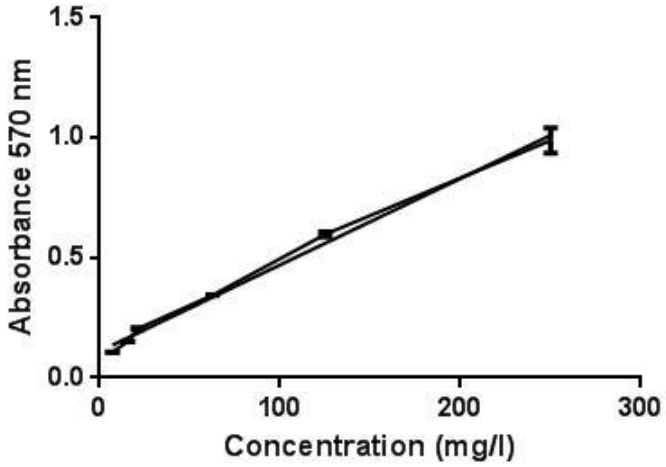

Figure 3.1. Standard curve of absorbance against Gallic acid concentration Y = 0.003604*X + 0.1098, R^2 = 0.9909.

3.2.4. Cytotoxicity

For the cytotoxicity assay, the cells were cultured in 96-well culture plates (Cellstar, Greiner Bio-One, Germany). After incubation for 24 h at 37ºC, cells were exposed to increasing concentrations of the extracts. Assays were carried out in triplicate. Monolayers incubated only with Eagle's Minimum Essential Medium (EMEM) were used as cellular controls. The concentration of the extracts that reduced the viable cell number to 50% (IC50). This colorimetric assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product, which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The IC50 was measured by the MTT method (Mosmann, 1983). Briefly, monolayers treated

with extracts for 48 h at 37ºC were incubated with MTT solution for four hours at 37ºC. Subsequently, the supernatant was removed and acid-isopropanol (0.04N HCl in isopropanol) was added. After gently shaking for 15 min, the absorbance was read on a multiwall spectrophotometer at 570 nm. The optical density (OD) was measured at 560 nm using a microplate ELISA reader (Labsystems Multiskan MS, Finland). The survival fraction percentage (SF %) in the treated cultures was calculated from the OD. In relation to culture controls, that represents 100% viability:

$$SF\ \% = \frac{OD\ \text{treated cells}}{OD\ \text{control}} \times 100$$

3.3. Results

3.3.1. Mutagenicity and anti-mutagenicity

Incubation of *Salmonella typhimurium* standard tester strains TA97, TA98, TA100 and TA102 with three serial dilutions without S9 metabolic activation showed percentage inhibition below 25% for both TA 97 and TA 102; with an increase in dilution the inhibition decreased (Table 3.2). This trend continued even when the tester strains were incubated with both *A. laricinus* and daunomycin. However, this decrease was not concentration-dependent. The results revealed that *A. laricinus* extracts were non-mutagenic towards the *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102 for the assay without metabolic activation.

Table 3.2. Summary of mutagenic/antimutagenic properties of *Asparagus laricinus* aqueous *extract.*

•	**Mutagen/anti-mutagen**				**Mutagen + anti-mutagen**			
Strain	TA 97	TA 98	TA 100	TA 102	TA 97	TA 98	TA 100	TA 102
Dilution	Percentage inhibition				Percentage inhibition			
• 50x	21	49	28.6	22.7	20.2	23.4	31.6	37.5
• 100x	10.4	35.1	12.1	23.5	14.5	19.5	28.4	24
• 200x	9.1	21.9	19	21.5	7.6	17.1	15.5	21

The Ames test without S9 metabolic activation can only detect direct mutagens, while S9 metabolic activation allows the detection of indirect mutagens, often caused by conjugation reactions of metabolic oxidation systems.

The Ames test is carried out by adding the S9 mix to detect an indirect mutagenic effect caused by metabolites of the test *A. laricinus* extract (Table 3.3). The strain TA 102 showed higher colony plate count in *A. laricinus* extract treated cells compared to the negative control. This suggests mutagenic activity from the extract. However, other tester strains (TA97, TA98, and TA100) showed lower counts in cells treated with extract than in the negative controls.

The S9 fraction contains a mixture of xenobiotic metabolizing enzymes, such as the cytochrome P450s and sulfotransferase. *Asparagus laricinus* extract showed an indirect mutagenic effect toward the tester strain TA102 after metabolic activation,

Table 3.3. Mutagenic activities of *Asparagus laricinus* aqueous extract with metabolic activation (+S9).

***S. typhymurium* strain**	**Dilution**	**Plate count (Mean +/- SD)**
TA 97	50X	154 +/-7
TA 97	100x	154+/-10
TA 97	200x	163+/-12
TA 97 control (+)	n/a	351
TA 97 control (-)	n/a	351
\TA 98	50x	40+/-6
TA 98	100x	32+/-8
TA 98	200x	45+/-6
TA 98 control (+)	n/a	830
TA 98 control (-)	n/a	53
TA 100	50x	123+/-4
TA 100	100x	131+/-3
TA 100	200x	132+/-8
TA 100 Control (+)	n/a	1395
TA 100 Control (-)	n/a	183
TA 102	50x	451+/-47
TA 102	100x	406+/-51
TA 102	200x	405+/-50
TA 102 control (+)	n/a	1395
TA 102 control (-)	n/a	391

**SD is standard deviation of three repetitions in the experiment*

3.3.2 Cytotoxicity

The cytotoxic effect of the aqueous extract from *A. laricinus* was tested against *Vero* cells using colorimetric method MTT assay. All the cells were exposed to various concentrations: 0.976, 1.935, 3.906; 7.8125,15.625, 31.25, 62.50, 125.00, 250 and 500 µg/ml (Figure 3. 2).

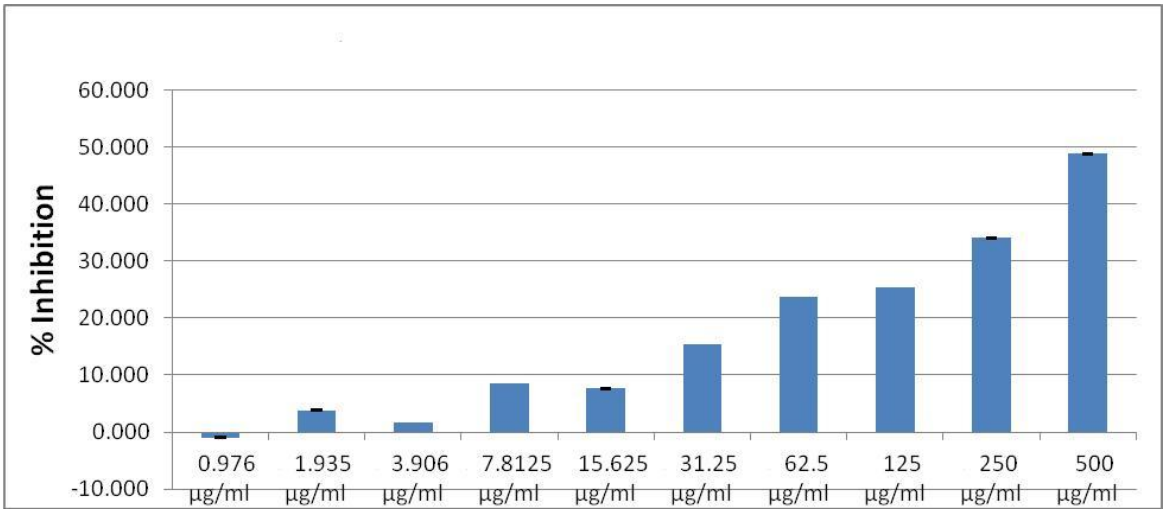

Figure 3. 2. The cytotoxicity of different concentrations of A*. laricinus* aqueous extracts on the growth of *Vero* cells was examined using MTT assay.

The IC50 was determined based on a concentration that induced 50% inhibition on the growth of the treated cells compared to the untreated cells in triplicate; 500 µg/ml was found to induce 50% inhibition of *Vero* cells. The latter observation suggests some cytotoxic effect of the *A. laricinus* extract on the *Vero* cells.

3.4. Discussion

The average His+ revertants observed for all the tester strains caused by the plant extract at all the concentrations without metabolic activation did not satisfy the criteria for mutagenicity. There was no notable dose-dependent increase in the number of revertants and the numbers of revertants were all either not equal to or more than twice that of the negative control (Bulmer *et al*., 2008).

There was also no decrease in the number of revertant colonies to levels far below the negative control (spontaneous reversion), which could also be classified as toxic. The extract had moderate (28%-34%) inhibitory effects using *Salmonella typhimurium* TA100 strain. The upper limit was observed beginning with mutagen and followed by the anti-mutagen (aqueous extract).

The lower limit was observed beginning with an anti-mutagen followed by a mutagen. When both mutagen and anti-mutagen were operative on commencement of a possible disease, 31% inhibition was observed. Similarly, using *Salmonella typhimurium* TA102 strain, the extract showed a low to moderate inhibitory effect (24%-38%) at 500 µg/ml of aqueous extract. The lower limit represented mutagen followed by anti-mutagen and the upper limit was observed when both mutagen and anti-mutagen were operative on commencement of a possible disease. However, using *Salmonella typhimurium* TA97 strain, the extract had a low inhibitory effect (20%-22%).

The degree of inhibition was not affected by the mode of interaction of the mutagen and anti-mutagen. Finally, the inhibitory effect was observed over a wide spectrum using *Salmonella typhimurium* TA98 strain (23%-49%). The upper limit was observed beginning with a mutagen and followed by an anti-mutagen. The lower limit was observed when there was simultaneous interaction between a mutagen and the extract. When both mutagen and anti-mutagen were operative on commencement of a possible disease, 35% inhibition was observed. The significant anti-mutagenic activity of the plant extract against direct-acting mutagen suggests that the extract may directly mitigate events that lead to DNA damage from mutagens (Mussarat *et al.,* 2006; Edenharder and Grunhage, 2003).

The cytotoxicity tests indicated no cytotoxic effect below 500 µg/ml concentration of the *A. laricinus* aqueous extract. This is promising, if the therapeutic benefit would have increased substantially before the 500 µg/ml dose is reached in *in vivo* studies.

The Folin-Ciocalteu method confirmed the total phenolic content in the extracts. These are presented as Gallic acid equivalents (GAE) with reference to the Gallic acid standard curve, which was plotted using the average absorbance values of two independent sets of data against concentrations of Gallic acid in g/l. The *A. laricinus* aqueous extract contained 4.2 g/l GAE. Polyphenols are the most abundant dietary antioxidants and research on their role in the prevention of degenerative diseases and cancer has developed quickly over the last few years (Yang *et al*., 1998). With a transgenic adenocarcinoma of the mouse prostate model of prostate carcinogenesis, it was recently demonstrated that oral consumption of 10 g/l of green tea polyphenols decreased tumor incidence by 65% (Gupta *et al*., 2001). This

polyphenol preparation was enriched with epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%) and caffeine (‘1%). It is worth mentioning that the ratio of individual constituent polyphenols may vary from plant to plant. In a previous chapter the anti-cancer activity of *A. laricinus* aqueous and ethanol extracts on cancer cell lines was demonstrated.

Many researchers have inferred the anti-mutagenic activity of plant extract to the antioxidant activities of the polyphenols. However, there has now been a paradigm shift as a result of a study that showed *Asparagus racemosus* extracts induced excessive production of TNF-alpha compared to controls (Katiyar *et al*., 1997). Naturally, white blood cells produce TNF-alpha protein to stimulate and activate the immune system in response to infection or cancer. Literature suggests that the mode of action of many plant extracts ranges between scavenging for free radicals and modulation of the immune system. The evidence obtained in studies of a tea polyphenol, i.e. (-)-epigallocatechin gallate [(-)-EGCG], indicates that plant polyphenols target many enzymes directly associated with cancer, e.g. proteosome, telomerase, growth factors, MAPKs, etc (Nasaani *et al*., 1998; Hofmann and Sonenshein, 2003; Mukhtar and Ahmad, 1999; Chen *et al*., 2003).

3.5. Conclusion

A. laricinus polyphenol extract exhibited dose-dependent anti-mutagenic activity. The plant extract showed no mutagenic effect on most of the tested strains. However, mutagenic activity was observed with metabolic activation for the TA 102 tester strain only, to the exclusion of TA97, TA98 and TA100. These results indicate

the potential for use of this plant against cancer, despite the moderate cytotoxicity at 500 µg/ml concentration. The results obtained may aid in the development of anti-cancer drugs from *A. laricinus* aqeous extract.

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Chapter 4

Evaluation of the antimicrobial, antiradical and antioxidant activities of *Asparagus laricinus* aqueous extract

4.1. Introduction

Plant-derived phytochemicals with dual functionalities in preventing lipid oxidation and microbial growths have tremendous potential use in phytotheraphy and/or the food industry. Several bioactive flavonoids, such as furocoumarins and furanocoumarins, have been isolated from parsley leaf and are known to exhibit antibacterial activities against both Gram-positive *Listeria* and *Micrococcus* species and Gram-negative *Escherichia* and *Erwinia* species (Manderfield *et al*., 1997; Ulate-Rodriguez *et al*., 1997). Some natural phenolic antioxidants such as caffeic acid, *p*-coumaric acid, chlorogenic acid and ferulic acid exhibit antimicrobial activity (Garrote *et al.*, 2004). Thus, in investigating the biological activities of medicinal plants, it becomes important to determine both the antimicrobial and antioxidant activities.

The validity of the term “antioxidant” depends on the environment of its action, i.e. whether one considers an *in vitro* or *in vivo* action. In this context a precise definition of conditions and processes in which antioxidant action is studied becomes crucial. Outside this context, a statement that some compound is an antioxidant may not yield any biologically meaningful information (Tirzitis and Bartosz, 2010). Thus, this study evaluated anti-oxidative stress, as opposed to antioxidant activity.

Free radicals and reactive oxygen species in general are no longer seen only as destructive factors but also (and perhaps first of all) as messengers involved in intracellular and intercellular signaling (Bartosz, 2005; Halliwell, 2006). The revision of the ideas on the role of free radical reactions in the functioning of cells and organisms has led to a new concept of redox equilibrium.

According to this hypothesis, oxidative stress is a modulation of thiol redox reactions, involved mainly in signaling pathways. Therefore, non-radical oxidants (enzymatically generated hydrogen peroxide, other peroxides, quinones, etc.) play a basic role in the oxidation of thiols for the sake of signaling, without the necessity of formation of free radical intermediates (Ghezzi *et al.,* 2005; Jones, 2006; 2008). Thus, this study will evaluate both the ability to scavenge free radicals (antiradical activity) and the ability to protect the cell from oxidative stress (antioxidant activity**)** as mutually exclusive activities**.**

The aim of this chapter is to evaluate the antimicrobial, antiradical and antioxidant activities of the *A. laricinus* aqueous extract.

4.2. Materials and methods

4.2.1. Antimicrobial activity

Prior to the test, bacterial and fungal cultures were prepared as described below. The bacterial cultures were sub-cultured in nutrient broth (Muller-Hutton) at 37°C for 24 hours. The turbidity of the broth culture was then equilibrated with 0.5 McFarland standard solution.

The minimum inhibitory concentration (MIC) of the aqueous extract was determined by a colorimetric assay. In a 96-well plate, five columns were used, designated “experimental”, and five wells in each column were filled with the same concentration of extract. Three rows in a column were used for negative control (sterile water), another three rows in a column were used for positive control (gentamicin), and two wells were used for sterility control (Figure 4.1).

Bacteria were prepared as described above (approx. 1 × 108 CFU/ml) to provide enough stock to fill all plates for all extracts tested (i.e. all of the tests involving a single bacterium species were performed on the same day). Then, 100 µl of broth culture was placed into each well, except the sterility control wells, and 100 µl of the plant extract at concentrations of 100, 50, 25, 12.5 and 6.3 were added in all wells designated experimental, 100 µl positive control (10 mg/ml of gentamicin) and negative control (sterile water) were also added in their respective wells. The positive control cell (gentimicin) should have no bacterial activity and remain clear, whereas the negative control cell (water) should have bacterial activity and change

to red, providing reference points for the visual analysis of the inhibitory effects of the extract dilutions. The control column only contained 100 µl of the positive control (gentamicin), negative control (water), and extraction dilution solutions (i.e. identical to the experimental column except that no bacteria were present). Plates were prepared in triplicate such that each extract-bacteria combination was replicated across 96-well plates. Plates were covered and incubated at 37°C for 24 hours. After the 24-hour incubation, 40 µl of a 0.2 mg/ml iodonitrotetrazolium violet (INT) indicator solution was simultaneously added to every well and the plate was incubated at 37°C for an additional 30 minutes. The INT indicator solution changes from clear to red in the presence of bacterial activity and the degree of redness is a good measure of inhibitory effects (Eloff, 1998). Following the 30-minute incubation, plates were digitally photographed.

4.2.2. Thin layer chromatography analysis and antioxidant activity of extract constituents

Qualitative screening for antioxidant activity was done using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), according to Tomohiro *et al*. (1994). Thin layer chromatography (TLC) of extracts was developed with the mobile phase that was made of three solvents, namely methanol:chloroform:hexane (70:20:10%). The stationary phase was made of silica gel GF_{254} (Fluka, 20x20 cm^2). The plates were air-dried and sprayed with 0.05% DPPH in methanol. Antioxidant activity was detected on the chromatogram by the appearance of yellow spots produced by

bleaching of DPPH (Bors *et al*., 1992). All detected active antioxidant constituents were noted according to their retention factor (Rf) values. Gallic acid was used as positive control.

4.2.3. Free radical scavenging activity

The free radical scavenging activity of the *A. laricinus* extracts was analyzed by using DPPH assay (Von Gadow *et al*., 1997; Fuhrman *et al*., 2001). Different concentrations (between $10^{-1}x10^{-04}$ mg ml^{-1}) of the extract were prepared. Aliquots (100 µl) of the extract or standard solution (Trolox® and ascorbic acid) were mixed with 2 ml of 0.1 mmol l^{-1} methanolic solution of DPPH radical. The tubes were mixed and allowed to stand for 60 minutes in the dark. Absorbance was read against a blank at 517 nm using a spectrophotometer (Biowave II, WPA). Methanol was used as a blank, while 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox®) and ascorbic acid were used as positive controls. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the extracts was calculated according to the equation used in Turner *et al*. (2011):

$$\text{Inhibition (\%)} = [1- (A_s/A_0)]x100$$

where A_s is the absorbance of the sample (i.e., extracts or standard) and A_0 is the absorbance of the DPPH solution. The EC_{50}, defined as the concentration of the sample leading to 50% of the reduction of the initial DPPH concentration, was

determined at 95% confidence interval, using GraphPad Prism® software. The data on regression graphs represents Mean+/-SEM (standard error of means). The experiment was done in triplicate.

4.2.4. Oxidative stress

Saccharomyces cerevisiae BY4742 strain was grown in YPD (2% glucose, 2% pepetone and 1% yeast extract) agar plate. A single colony was picked and inoculated in 5ml YPD broth and incubated at 30^{o}C under shaking conditions (120 rpms) overnight. This served as the pre-inoculum.

From the overnight culture 100 µl of the pre-inoculum was transferred into a fresh medium. The cells were grown to the exponential phase and harvested when the OD of the culture at 610 nm ($O.D_{610\ nm}$) reached 0.1 absorbance units. At an OD of 0.1 (representing approximately 1.8 x 10^6 viable cells ml^{-1}), cells were harvested by centrifugation at 25 "C (4000 x g for 5 minutes) and resuspended in (5 ml) fresh YPD broth. Three samples were treated with 10 mM concentration of H_2O_2 and 100 µg/ml of aqueous extract; three other samples were treated with H_2O_2 (10 mM) and three untreated samples served as negative control. Aliquots (100 µl) were taken at 30-minute intervals over a period of 1 hour and 30 minutes, diluted in YPD media and plated on YPD plates to obtain viable cell counts. The method was adapted from Izawa *et al*. (1995).

4.3. Results

4.3.1 Minimum inhibitory concentration

The antimicrobial activity of the *A. laricinus* aqueous extract was remarkable against the eight clinical strains of bacteria. The result of the antimicrobial activity, presented in Table 4.1, shows that the lower MIC plant extract was required for the Gram-positive bacteria *Bacillus subtilis* ATCC 269153 (*B. subtilis*), *Streptococcus pneumonia* ATCC 6301 (*S. pneumonia*) and *Staphylococcus aureus* ATCC 29213 (*S. aureus*) .

Table 4.1. Antimicrobial activity of the aqueous extract of *Asparagus laricinus* against selected clinical strains of bacteria .

Microorganism	**Gram-staining property**	**(MIC) µg/ml**
Proteus vulgaris ATCC6386	Negative	100
Proteus mirabilis ATCC12453	Negative	100
Streptococcus pneumonia ATCC 6301	Positive	50
Pseudomonas aeruginosa ATCC 10145	Negative	100
Staphylococcus aureus ATCC 29213	Positive	25
Haemophilus influenza ATCC 1193	Negative	100

Escherichia coli ATCC 133762	Negative	100
Bacillus subtilis ATCC 269153	Positive	50

*American type culture collection (ATCC)
*Gentamicin was used as a positive control

S. aureus was the only tested strain that was inhibited by 25 µg/ml of the plant extract. This was contrary to the 100 µg/ml that was required for all Gram-negative strains (*Pseudomonas aeruginosa* ATCC10145 (*P. aeruginosa*), *Proteus vulgaris* ATCC 6386 (*P. vulgaris*), *Escherichia coli* ATCC 133762 (*E. coli*) and *Proteus vulgaris* ATCC 6386 (*P. vulgaris*)) and the 50 µg/ml in Gram-positive strains such as *Streptococcus pneumonia* (Figure 4.1).

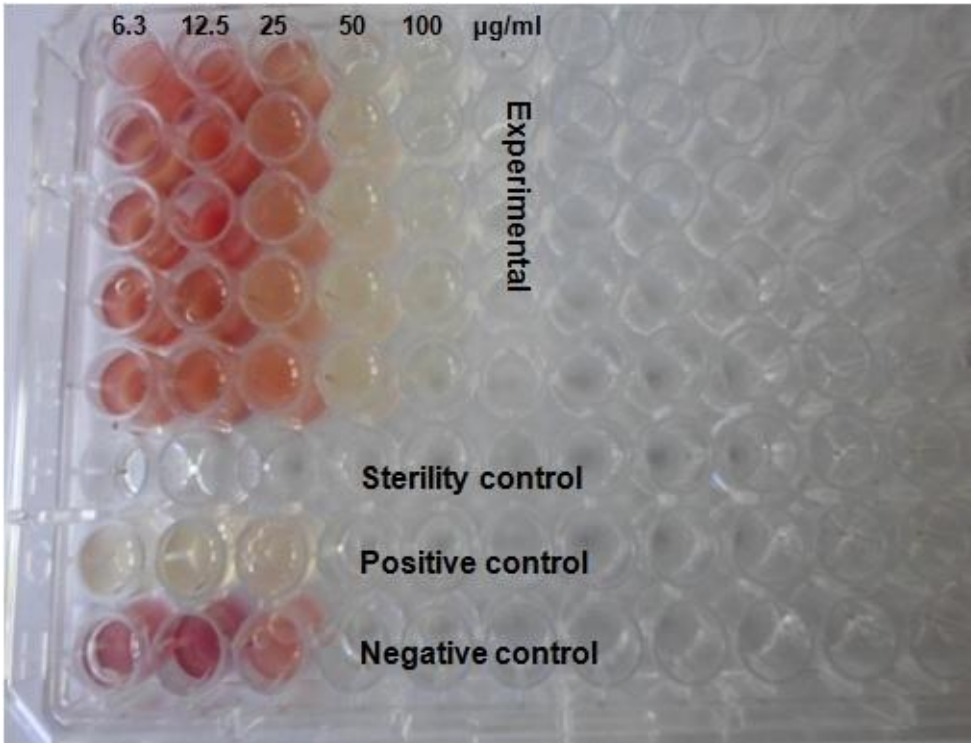

Figure 4.1. A digital photograph of the *Streptococcus pneumonia* ATCC 6301 treated with an increasing concentration of *A. laricinus* aqueous extract.

4.3.2. Thin layer chromatography analysis and antioxidant activity of extract's constituents

Figure 4.2 shows the TLC chromatogram for the qualitative screening for antioxidants. Three spots with Rf of 0.87, 0.38 and 0.35 exhibited antioxidant activity when sprayed with DPPH. This indicated differences in the polarity of the separated antioxidant molecules.

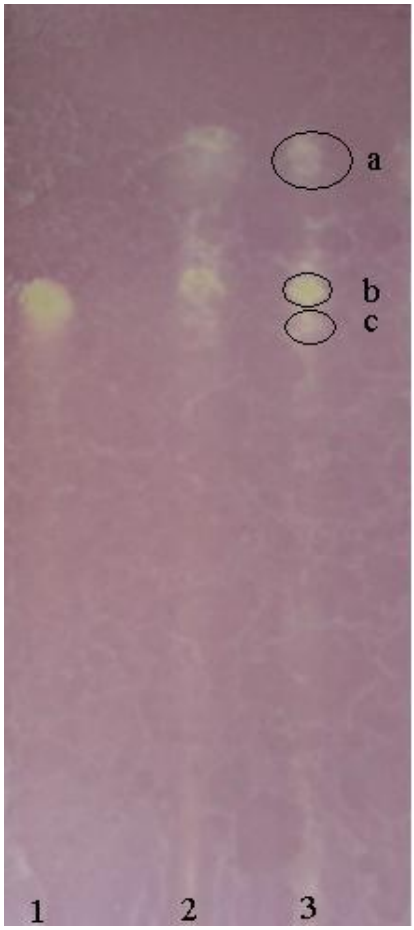

Figure 4.2. Chromatogram of Gallic acid (1) and *A. laricinus* aqueous extract (2 and 3, lane 2 is a twofold dilution of lane 3), separated with methanol:chloroform:hexane (70:20:10%) and sprayed with DPPPH. The yellow spots indicate antioxidant activity (Rf values: a = 0.87; b = 0.38; c = 0.35).

4.3.3. Free radical scavenging activity

The addition of the *A. laricinus* extract to the DPPH solution induced a rapid decrease in absorbance at 517 nm, indicating free radical scavenging activity of the extract (Fig. 4.3). The EC_{50} value of the extract was recorded to be between 0.9205 and 1.188 $mgml^{-1}$ and was higher than that of Trolox® (Figure 4.3d). The radical scavenging activity of the extract was concentration-dependent and similar to that of known antioxidants (Figure. 4.3a, b and c). *A. laricinus* showed concentration-dependent free radical scavenging activity at concentrations above 1 mg ml^{-1}, while ascorbic acid no longer showed increasing activity above the same concentration.

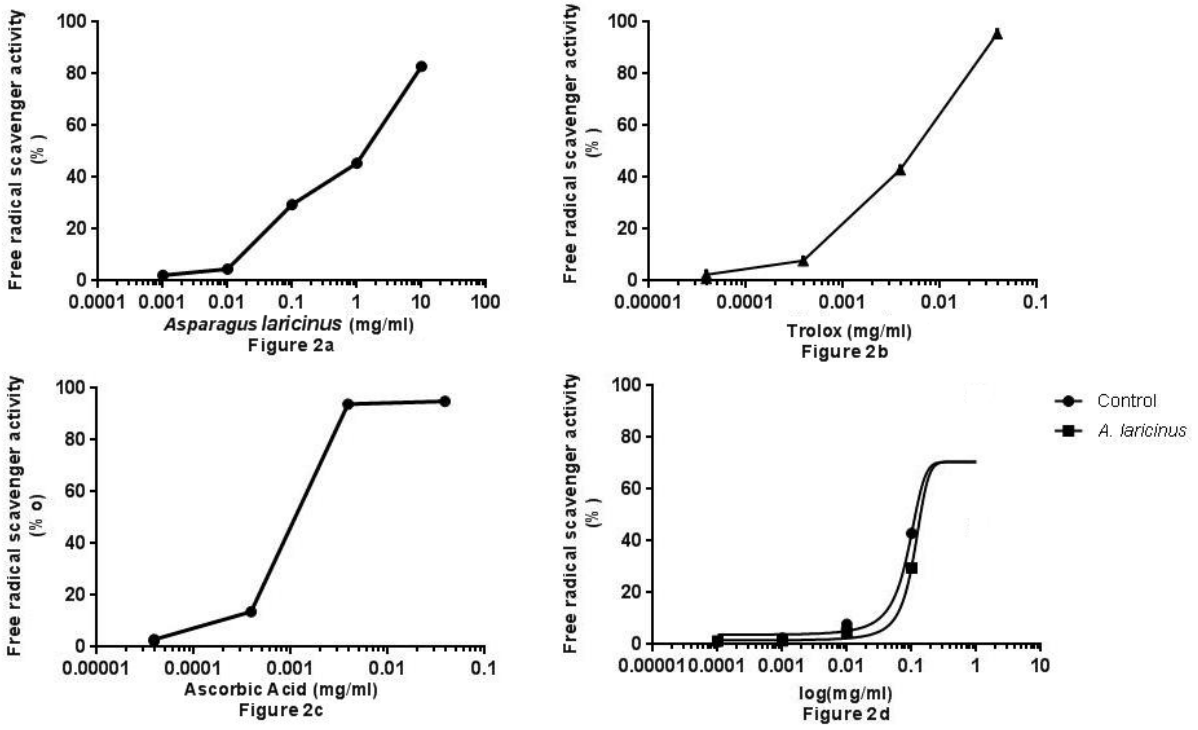

Figure 4.3 (a-d). Scavenging activity of *A. laricinus* aqueous extract on the free radical DPPH. (a) *A. laricinus*, (b) Trolox®, (c) ascorbic acid**,** and (d) EC_{50} shift graph of *A. laricinus* extract with Trolox® as the control.

4.3.3. Oxidative stress

Figure 4.4, illustrates the response of Saccharomyces cerevisiae (BY4742) cells to hydrogen peroxide treatment.

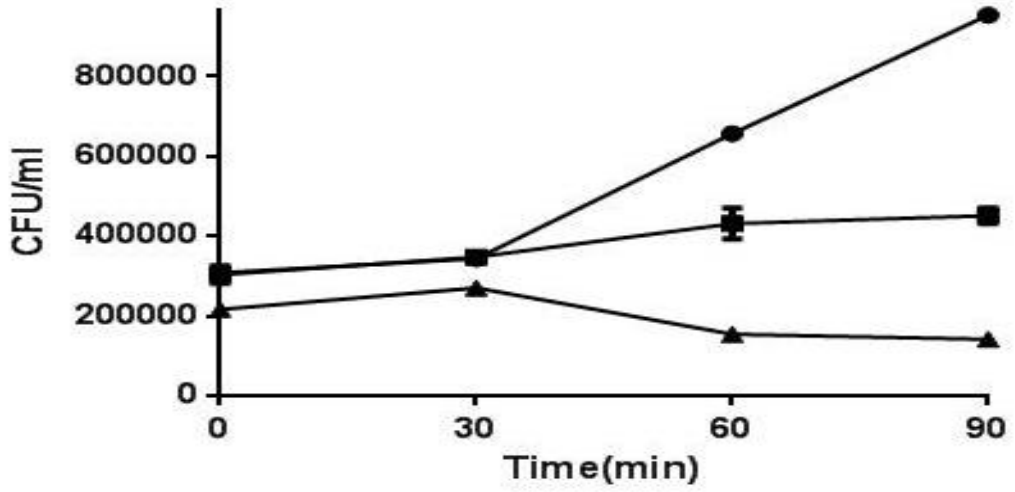

Figure 4.4. Yeast cell (BY4742) sensitivity to hydrogen peroxide. Exponentially growing yeast cells (30^{o}C) were treated with 10 mM (H_2O_2) for 90 minutes. Cells treated with H_2O_2 only (▲), untreated cells (●) and cells treated with H_2O_2 and aqueous extract (■).

Cells that were incubated in hydrogen peroxide only showed a significant decrease in colony-forming units per milliliter (CFU/ml) after 30 minutes. This is contrary to the slight increase in colony-forming units in the cells that were incubated in hydrogen peroxide and aqueous extract. As expected, the untreated cells grew exponentially from the same incubation period.

4.4. Discussion

MICs are considered the gold standard for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all

other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definite answer when a borderline result is obtained by other methods of testing or when diffusion methods are not appropriate (Andrews, 2001).

Inhibition of *Staphylococcus aureus ATCC* 29213 by the *A. laricinus* plant extract demonstrates huge potential for the use of the plant in the treatment of microbial infections, especially in light of the growing antibiotic resistance in microoganisms. The best known example of an antibiotic resistance mechanism is probably the alternative penicillin-binding protein (PBP2a), which is produced in addition to the "normal" penicillin-binding proteins by methicillin-resistant *Staphylococcus aureus* (Hawkey, 1998). The observed antibacterial activity of the extract suggests a possible antibiotic against such resistant strains of *S. aureus*.

The growth inhibition of *Pseudomonas aeruginosa ATCC 10145* and *Escherichia coli ATCC 133762* is important, since it has been reported that patients with neutropenia are found to suffer from infection by the two bacteria mentioned (Schimpff *et al*., 1971; Cometta *et al*., 1996). These results show huge potential in alleviating infection by both Gram-negative and Gram-positive clinical strains.

The antioxidant capacity of crude drugs is widely used as a parameter for evaluating medicinal bioactive components. The present study has demonstrated the antiradical activity of *A. laricinus.* The researcher observed three spots that exhibited antioxidant properties on the TLC chromatograms. The characteristics of the three different antioxidants observed in the *A. laricinus* extracts are yet to be

elucidated. The antioxidant in the plant extract may largely be due to polyphenols (Thabrew *et al*., 1998). Phenolics are the largest group of phytochemicals and most antioxidant activity of plants or plant products is attributed to them. Many studies have shown that natural antioxidants are able to reduce DNA damage, mutagenesis and carcinogenesis. These events are often associated with the termination of free radical propagation in biological systems (Covacci *et al*., 2001; Zhu *et al*., 2002). The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability or radical scavenging ability (Baumann *et al*., 1979). Considering the fact that the *A. laricinus* extract used was crude and the comparison was not on the basis of molar concentration, the above results are promising, despite having a higher EC_{50} value than Trolox®.

H_2O_2 and superoxide anions (O_2^-) are often referred to as reactive oxygen species (ROS) as they can lead to production of more reactive species, particularly in the presence of metal ions. ROS are generated endogenously in many cells as a consequence of metabolic processes. ROS can also be formed by exposure of cells to ionizing radiation, redox-cycling chemicals present in the environment or by exposure to heavy metals (Ames, 1983; Brennan and Schiestl, 1996). Through such mechanisms, all aerobically growing organisms are continuously exposed to reactive oxidants and oxidative stress occurs when the concentration of these oxidants increases beyond the antioxidant buffering capacity of the cell. A side effect of respiration in organisms is the production of ROS that can damage proteins, lipids and DNA (Hererro *et al*., 2008).

The ability to protect *Saccharomyces cerevisiae* (*S. cerevisiae*) from oxidative stress is seen as valuable to understanding the potential effect of the plant extract to humans, since yeasts are eukaryotic cells and are equipped with several elaborate defense mechanisms against different types of stress imposed by the environment, and many of these are similar to mechanisms in higher organisms.

4.5. Conclusion

The aqueous extract of *A. laricinus* showed significant activity as an antioxidant. It showed free radical scavenging activity comparable to Trolox and ascorbic acid. The antimicrobial activity of the aqueous extract indicated potential use of the plant in the treatment of microbial infections. *A. laricinus* aqueous extract has the ability to protect cells from oxidative stress.

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Chapter 5

Chemical composition of both aqueous and methanol extracts of *Asparagus laricinus*

5.1. Introduction

Most medicinal plants are harvested from the wild and the production of medicinal metabolites in plants is affected by plant genotype, cultivation, harvesting, processing and distribution (Crozier, 2006). It has also been noted that the age of the plant, the presence of flowers or pods, different geographical origin, soil and climate play roles in plant adaptation mechanisms (Green, 1997). Because of that, the level of metabolites represents integrative information of the cellular function and hence defines the phenotype plant in response to genetic or environmental changes (Silas *et al.*, 2004).

Since individual plant species have been known to manufacture over 100 000 secondary metabolites (Yuliana et *al*., 2011), robust techniques that are able to analyze these large numbers of metabolites in the shortest time possible are useful in profiling. There is growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity (Prachayasittikul *et al*., 2008; Turker and Usta, 2008; Nogueira *et al*., 2008). Many biologically active compounds isolated from plants lead to the discovery of new medicinal drugs, which play efficient protection and treatment roles against various diseases, including cancer (Sheeja

and Kuttan, 20O7; Mukherjee, 2007). Thus, screening of plant extracts for important biological activities remain crucial in the search for novel molecules with relevant biological activities.

The widespread use of liquid chromatometry – mass spectrometry (LC-MS) for global metabolic profiling is relatively new and the literature indicates that MS-based techniques, particularly in combination with chromatographic technologies, are most popular, as these combine very high analytical precision with equally high detection sensitivity (Lenz and Wilson, 2007; Wilson *et al*., 2005). Electro-spray ionisation (ESl) is one of the most frequently used soft ionisation techniques in MS. ESI eliminates the need for neutral molecule volatilization prior to ionization. In the negative mode, ESI spectra of the phenolics show a predominant molecular ion [M-H] with minimum fragmentation. It is known that the negative ion mode generally provides improved detection limits (Ryan *et al.*, 1999). It is argued that HPLC-MS offers some advantages over the gas chromatography- (GC) MS techniques currently used in metabolomics. In particular, the ability to run samples with minimal sample preparation by HPLC-MS is in contrast to the more extensive sample pre-treatment techniques that are sometimes required to obtain comprehensive metabolite fingerprints by GC-based techniques (Wilson *et al*., 2005).

Based on the literature search, no report is available on the detailed analyses of the *A. laricinus* plant extract. Therefore, the aim of this chapter is to examine the chemical composition of both aqueous and methanol extracts.

5.2. Materials and methods

5.2.1. Phytochemical screening

Chemical tests were carried out on the aqueous and methanol extracts of *A. laricinus*. The samples were first powdered as explained in chapter 2 and subjected to the chemical tests described below (Edoaga *et al*., 2005).

5.2.2. Alkaloid determination using Harborne (1973) method

Five grams (5 g) of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for four hours. This was filtered passively through a Whatman® filterpaper, 11 cm diameter. The extract was concentrated in a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was air-dried and weighed.

5.2.3. Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered through a Whatman® filter, 11 cm diameter. A few drops of

0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

5.2.4. Test for saponin

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath (set at 100^{o}C) and filtered though a. Whatman®, 11 cm diameter. Then 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

5.2.5 Test for steroids

Two milliliters of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2S0_4. The color changed from violet to blue or green in some samples, indicating the presence of steroids.

5.2.6. Test for terpenoids (Salkowski test)

After mixing 5 ml of each extract in 2 ml of chloroform, concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids.

5.3. Gas chromatography-mass spectrometry

GC/MS analysis was carried out on a Shimadzu 2010 QB gas chromatograph with a MSD detector equipped with an HP-5 fused silica capillary column (30 m x 0.25 mm x 25 m film thickness). The aqueous plant extract was injected via an all-glass injector working in split mode with helium as the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was programmed as follows: Injector temperature 200^0C, ion source 200^0C, interphase 200^0C. The column temperature was raised to 45°C (3 minutes hold at 45°C, 4 minutes), then gradually increased to 150°C (3 minutes hold at 150°C, 4 minutes) then raised to 250°C and a 15-minute hold. A split ratio of 1:5 was used (Ajayi *et al*., 2011). Compound identification was accomplished by comparing the retention times with those of authentic compounds or the spectral data obtained from the Wiley Library and National Institute of Standards and Technologies Library, as well as with data published in the literature.

5.4. Liquid chromatography/mass spectometry

The plant extracts were resuspended in 1 ml of a 50% (v/v) mixture of acetonitrile and H_2O containing 0.1% (v/v) formic acid. The suspensions were vortexed for 1 minute then sonicated for 5 minutes, vortexed again for 1 minute prior to centrifugation at 10 000 rpm for 10 minutes. The supernatant (3 µl) was injected into the LC-MS instrument. Metabolites were separated using a gradient of H_2O with

0.1% formic acid (solvent A) and acetonitrile (solvent B), using a Waters UPLC at a flow rate of 0.4 ml min^{-1} on a Waters BEH C18, 2.1x50 mm column. MS was obtained on a Waters SYNAPT™ G2 MS (Manchester, England) using ESI running in positive mode with a cone voltage of 15 V. The injections were repeated once to ensure repeatability.

5.5. Results

5.5.1 Phytochemical screening

Phytochemical screening of the aqueous extract was positive for the presence of alkaloids, saponins, tannins and terpenoids. However, the ethanolic extract only tested positive for tannins and negative for the rest of the tests (Table 5.1).

Table 5. 1. Phytochemical screening of aqueous and ethanol extracts of the roots of *A. laricinus* for different molecular constituents

Constituents	Aqueous	Ethanol
Alkaloids		
Dragendorff's test	+	-
Steroids	+	-
Libarman-Burchard's test	+	-
Terpenes		
Salkowski test	+	-
Tannins		
$FeCl_3$ test	+	+
Gelatin test	-	-
Saponins		
Frothing test	+	-

Key: -: Negative (absent): +: Positive (present)

5.5.2 Gas chromatography-mass spectrometry

The GC-MS chromatogram gave rise to three peaks, suggesting the existence of three secondary metabolites and percentage values of composition of these phytochemicals present in the aqueous root extract of *A. laricinus*. The results suggested the presence of indole-3-carbinol, α-sitosterol and ferulic acid, as shown in Figure 5.1.

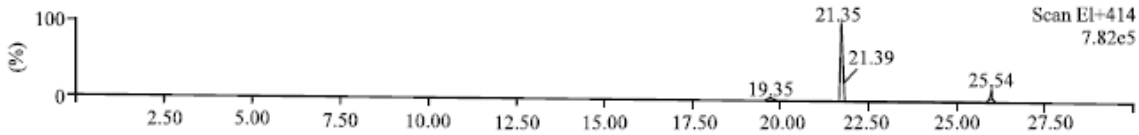


Figure 5.1. GC/MS chromatogram of the aqueous extract of *A. laricinus* roots

5.5.3 Liquid chromatography/mass spectometry

Typical positive and negative ion LC-MS chromatograms for a both aqueous and ethanol extracts of *A. laricinus* (Figures 5.2 and 5.3). The major peaks in each solvent extract differed significantly, thus showing the effluence of solvents on excluding other of molecules during extraction.

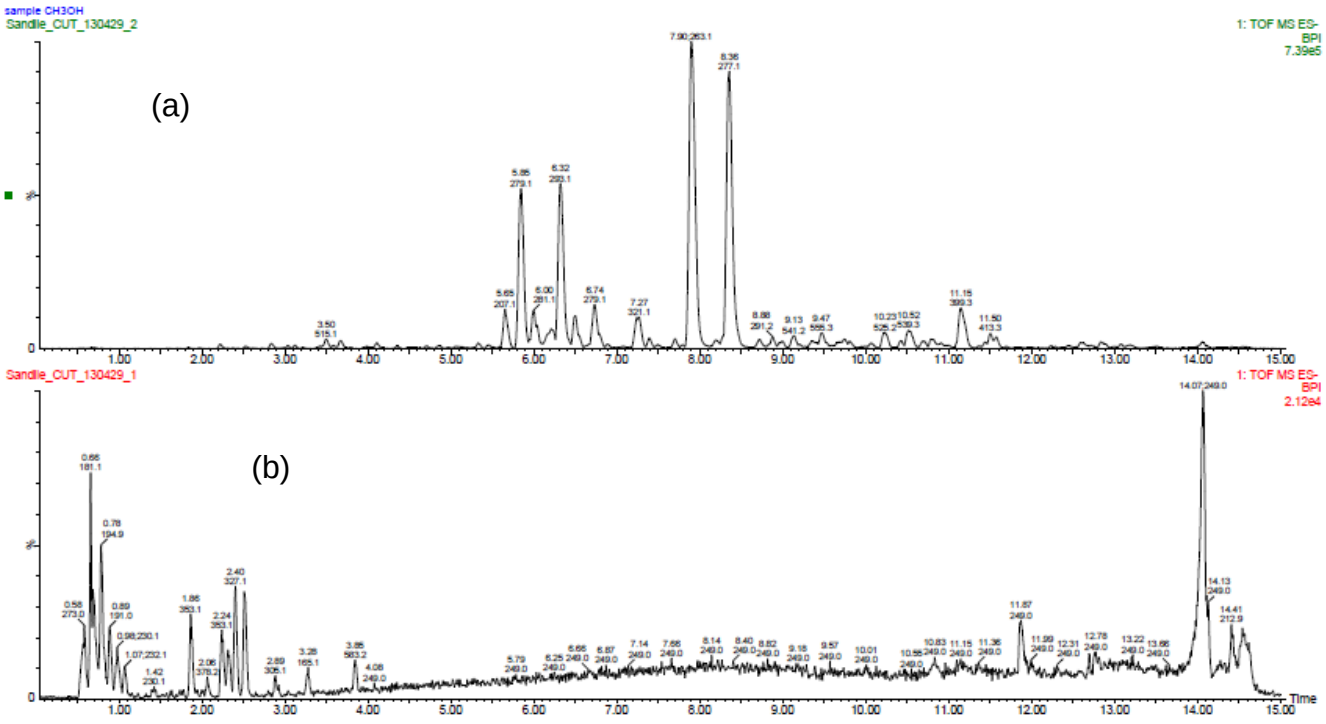


Figure 5.2. Representative LC-MS chromatograms for ethanol extract (a) and aqueous extract (b) in negative ion electrospray.

The aqueous constituents eluted in the predominantly polar regions of the solvent gradient, while ethanol extract constituents appeared in the non-polar region.

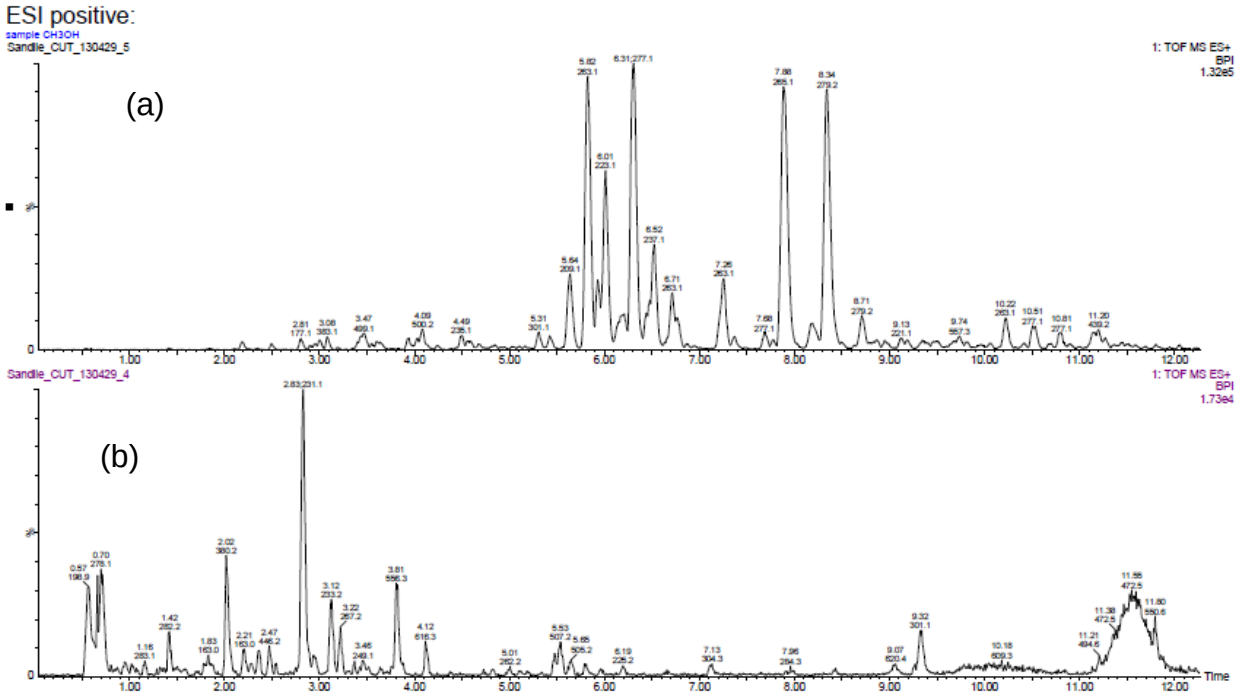
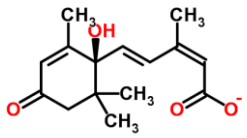
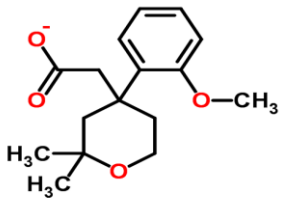


Figure 5.3. Representative LC-MS chromatograms for ethanol extract (a) and aqueous extract (b) in positive ion electrospray.

Various compounds were identified based on their respective molecular formulae (Tables 5.2 and 5.3). The tentative names and putative structures were obtained by searching for structures and names corroborating the molecular formula and the possible double bonds/carbon rings as reflected by the reported (double bond equivalent) DBE.

Table 5.2. Analysis of LC/MS chromatograms of *A. laricinus* ethanol extract

Retention time (min)	Fragmentation ion (M/Z)	Molecular formula	Tentative identification	Putative structure
5.8	263.1275	$C_{15}H_{19}O_4$	5-(1-Hydroxy-2,6,6-triméthyl-4-oxo-2-cyclohexén-1-yl)-3-méthyl-2,4-pentadiénoate http://www.chemspider.com/Chemical-Structure	 http://www.chemspider.com/Chemical-Structure
6.3	277.1435	$C_{16}H_{21}O_4$	4-(2-Methoxyphenyl)-2,2-dimethyltetrahydro-2H-pyran-4-yl]acetate http://www.chemspider.com/Chemical-Structure	 http://www.chemspider.com/Chemical-Structure

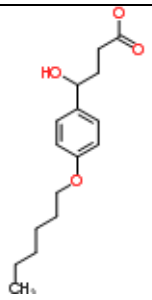
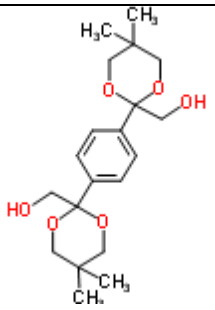
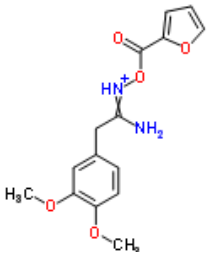
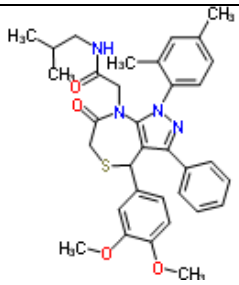
7.8	265.1431	$C_{15}H_{21}O_4$	No inference	No inference
8.4	279. 1594	$C_{16}H_{23}O_4$	4-(4-(Hexyloxy)phenyl]-4-hydroxybutanoat http://www.chemspider.com/Chemical-Structure	 http://www.chemspider.com/Chemical-Structure

Table 5.3. Analysis of LC/MS chromatograms of *A. laricinus aqueous* extract

Retention time (min)	Fragmenta-tion ion (M/Z)	Molecular formula	Tentative identification	Putative structure
2.0	380.2073	$C_{20}H_{30}O_6$	1,4-bis(2-hydroxymethyl-5,5-dimethyl-1,3-dioxan-2-yl)benzene http://www.chemspider.com/Chemical-	 http://www.chemspider.co

			Structure	m/Chemical-Structure
2.8	305.1148	$C_{15}H_{17}N_2O_5$	1-Amino-2-(3,4-dimethoxyphenyl)-N-(2-furoyloxy)ethanimin ium http://www.chemspid er.com/Chemical-Structure	 http://www.chemspider.co m/Chemical-Structure
3.8	556.2529	$C_{34}H_{38}N_4O_4S$	1,4,6,7-tétrahydro-8H-pyrazolo[3,4-e][1,4]thiazépin-8-yl]-N-isobutylacétamide http://www.chemspid er.com/Chemical-Structure	 http://www.chemspider.co m/Chemical-Structure
4.1	616.2509	$C_{39}H_{38}NO_4S$	*5-(3,4,5-Trimethoxyphenyl)-3-(4-dimethylaminophe*	Not assigned

			nyl)-6-(4-phenoxyphenyl)-2-phenyl-3,3a,5,6-tetrahydro-2H-pyrazolo[3,4-d]thiazole (Tugart *et al*., 2007)	

5.6. Discussion

The phyto-chemical test that was done for initial screening of the extract was complemented with detailed results obtained in GC/MS and LC/MS analysis. However, the MS results were more effective, allowing samples to be analysed with the minimum of quantities and analysis and covering as wide a range of classes of metabolite as possible in a short period.

Phytochemical screening of indigenous plants is essential to evaluate their medical value and their potential use in the treatment of various diseases. In the current study of *A. laricinus* extract, three secondary metabolites were found in the aqueous extract. The extract showed the presence of ferulic acid, a phenolic acid derivative that has antioxidant activity and several therapeutic benefits in the treatment of cancer. Ferulic acid is a plant constituent that arises from the metabolism of phenylalanine and tyrosine. It occurs primarily in seeds and leaves, both in its free form and covalently linked to lignin and other biopolymers. Because of its phenolic nucleus and an extended side chain conjugation it readily forms a resonance stabilized phenoxy radical, which accounts for its potent antioxidant effect (Imaida *et al*., 1990; Srinivasan *et al*., 2007). β-Sitosterol has a number of therapeutic and chemo-preventive uses in the medical field (Zak *et al*., 1990; Baskar *et al*., 2010). Indole-3-carbinol is used in prostate cancer (Garikapaty *et al*., 2005). The phytoconstituents detected in the plant materials could be responsible for the cytotoxic activity, though their exact mode of action is poorly understood at present.

The LC-MS chromatograms showed distinct profiles of the extracts. This is important in investigating the health benefit claims in the traditional use of medicinal

plants. It is therefore important to adapt isolation techniques to mimic the extraction methods used in traditional usage of medicinal plants. Consistent with the chemical tests used for initial screening of different classes of metabolites, LC/MS results proved the presence of alkaloids in the aqueous extract, while no alkaloids could be found in the ethanol extract.

It is important to note that the $C_{39}H_{38}NO_4S$ in the aqueous extract was similar to a chemically synthesized derivative of the potential biologically active 4-thiazolidinones (Tugut *et al*., 2007). Predominantly, the aqueous extract had small ring heterocycles containing nitrogen, sulfur and oxygen. Such molecules have been under investigation for a long time because of their important medicinal properties. These types of molecules have been shown to have various important biological activities such as anti-microbial, anti-viral, diuretic, anti-histaminic, anti-cancer, anti-convulsant, anti-inflammatory and analgesic properties (Vigorita *et al*., 2001; Kavitha *et al*., 2006; Kucukguzel *et al*., 2006).

5.7. Conclusion

This analysis of the aqueous and ethanol extracts of *A. laricinus has* helped to predict the compounds present in the respective solvents. The identified molecules supported the evidence of important biological activities that had been observed in *A. laricinus*. The use of chemical formulae in this study limited the identification of the plant extracts' metabolites to a narrow potential search field.

5.8. References

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Summary

This study reports on the anti-microbial, anti-oxidative stress, phytochemistry and free radical radical scavenging activity of *Asparagus laricinus* Burch (Asparagaceae) in order to explain the anti-cancer properties observed in extracts of this plant. After demonstrating the anti-cancer properties of the *A. laricinus*, the study tested the biosafety of this plant by evaluating the antimutagenic and mutagenic water extract. The root extracts of *Asparagus laricinus* were qualitatively screened for antioxidant properties using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), on silica gel GF_{254} TLC plates. Quantitative Radical scavenging activity using DPPH was also evaluated. The extracts were tested for the presence of alkaloids, saponins, tannins, terpenoids and steroids using standard phytochemical screening methods. Gas chromatography/mass spectrometry (GC/MS) was used to further confirm the phytoconstituents, in addition, LC/MS was used to profile the the phytoconstituents of the plant extract S. Three spots that exhibited antioxidant activity were resolved on the TLC plates. The results on the anti-bacterial activity of *Asparagus laricinus* showed that 50 µg/ml of the plant extract was the (MIC) for the gram-positive bacteria (*Bacillus subtilis*, *Streptococcus pneumonia* and *Staphylococcus aureus*). While 100 µg/ml was required for other gram-negative strains (*Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli* and *Proteus vulgaris*), *Staphylococcus aureus* was inhibited by 25 µg/ml of the plant extract. Evaluation of the anti-oxidative stress activity of *A. laricinus* aqueous extract conclusively demonstrated the ability of the extract to protect *Saccharomyces cerevisiae* (BY4742) cells from oxidative stress when the cells were challenged with 10 Mm (H_2O_2) for 1.5 hours. The results of this

study confirmed the anti-cancer and other biologically significant activities from the aqueous extract of *A. laricinus*. The free radical scavenging activity of *A. laricinus* extract was comparable with that of Trolox and was dependent on the concentration of the extract. This has shown that the effectiveness of the extract can be compared to that of commercial drugs. The aqueous extract of *A. laricinus* contained alkaloids, saponins, tannins and terpenoids. However, the chemical tests on e ethanol extract produced negative results. The phytoconstituents in the aqueous extract were confirmed through the use of GC/MS and indole-3-carbinol, α-sitosterol and ferulic acid were identified. These identified compounds have known biological activities, and that is found to be consistent with the anticancer, anti-microbial and anti oxidative stress activities reported in the current study. The phytochemicals present in the water extracts of *A. laricinus* and the *in vitro* anti-cancer properties indicate the potential use of this plant extract for development of novel drugs. This study has also established a scientific basis for the continued use of *A. laricinus* in traditional medicine for the treatment of cancer and other ailments.

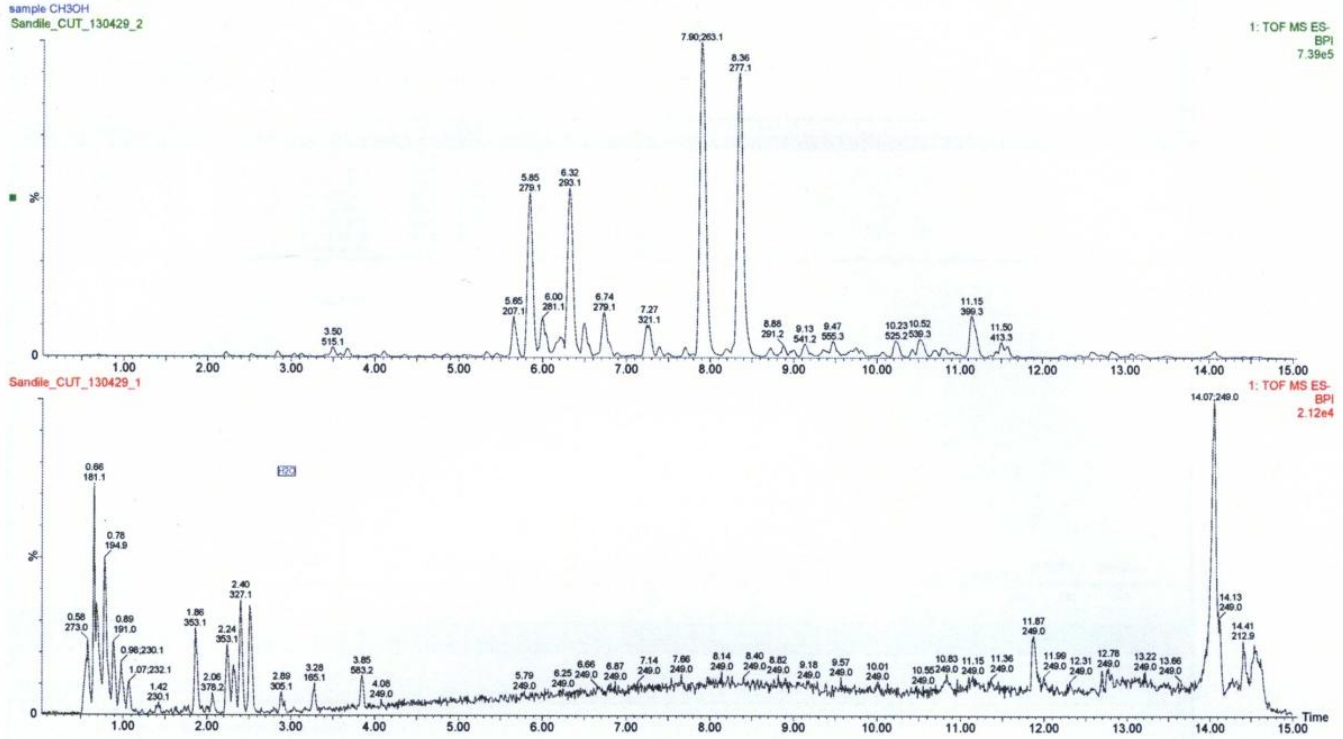
Appendixes:

LC/MS Chromatograms

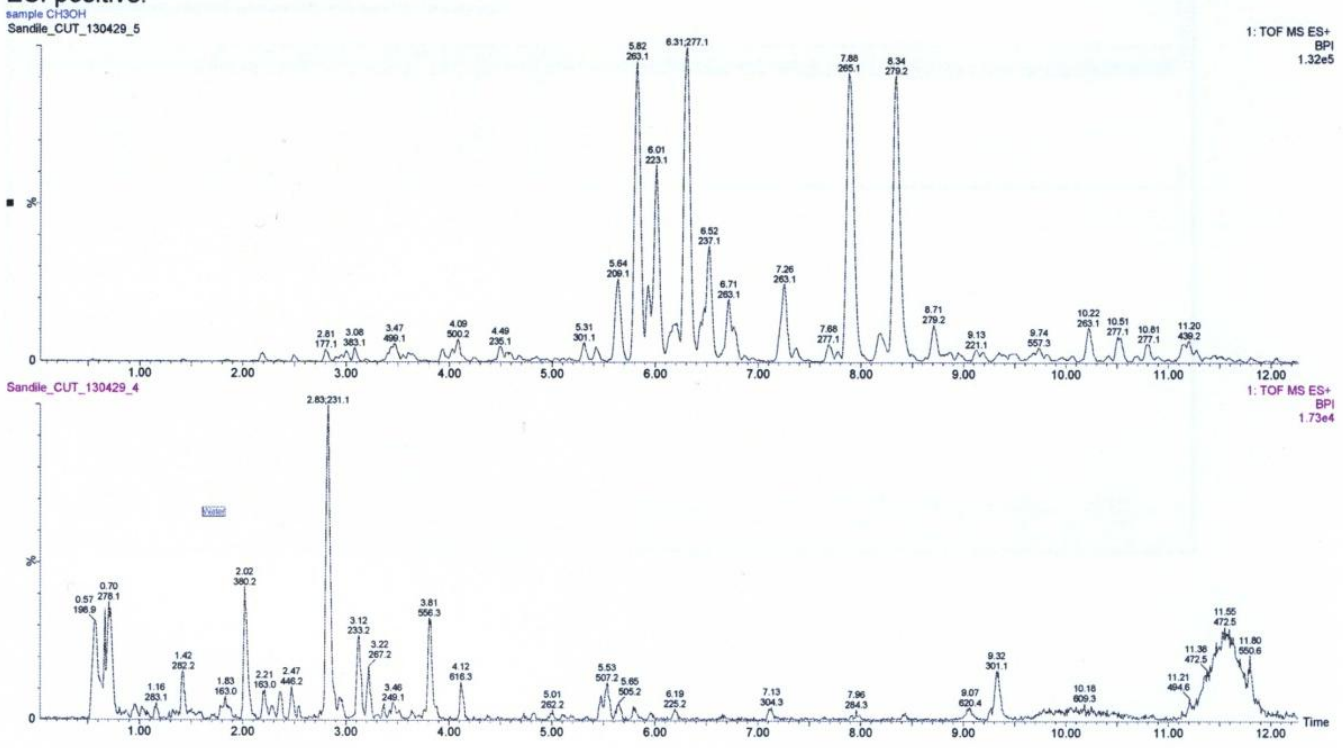
&

Published Articles

Total ion chromatograms:



ESI positive:



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 500.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

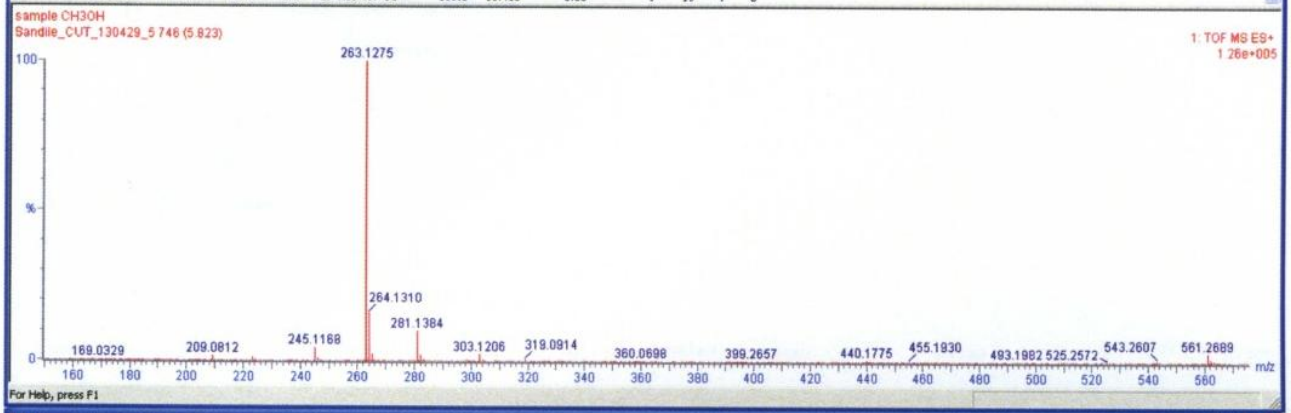
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

557 formula(e) evaluated with 159 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S
263.1275	263.1283	-0.8	-3.0	6.5	C15 H19 O4	188.7	0.806	44.67	15	19		4	
	263.1297	-2.2	-8.4	11.5	C16 H15 N4	195.6	7.698	0.05	16	15	4		
	263.1252	2.3	8.7	1.5	C11 H23 N2 O S2	210.4	22.534	0.00	11	23	2	1	2
	263.1243	3.2	12.2	2.5	C10 H19 N2 O6	198.4	10.548	0.00	10	19	2	6	
	263.1317	-4.2	-16.0	1.5	C12 H23 O4 S	207.6	19.699	0.00	12	23		4	1
	263.1330	-5.5	-20.9	6.5	C13 H19 N4 S	207.1	19.256	0.00	13	19	4		1
	263.1218	5.7	21.7	6.5	C14 H19 N2 O S	207.2	19.340	0.00	14	19	2	1	1
	263.1203	7.2	27.4	-1.5	C5 H19 N4 O8	202.2	14.322	0.00	5	19	4	8	
	263.1355	-8.0	-30.4	2.5	C9 H19 N4 O5	199.3	11.456	0.00	9	19	4	5	



For Help, press F1

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 500.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

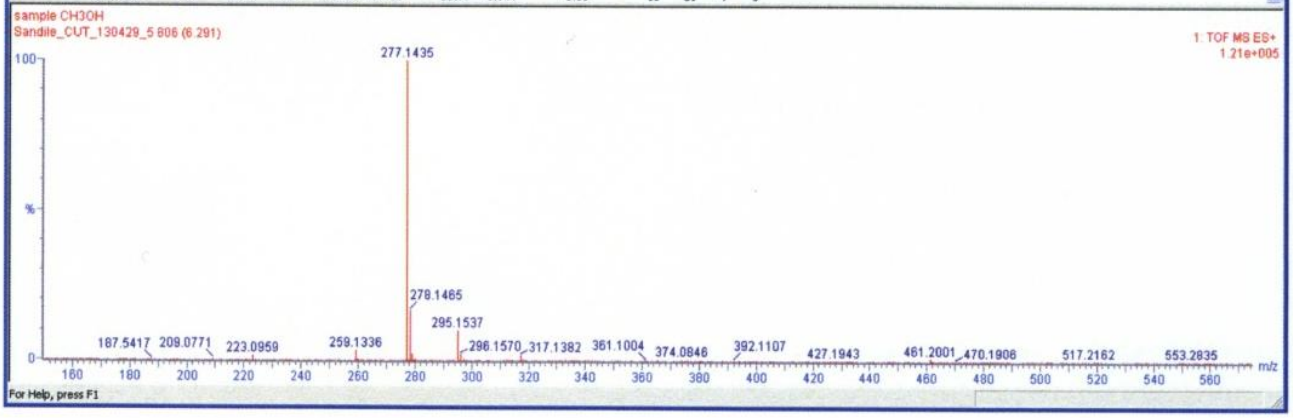
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

617 formula(e) evaluated with 169 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S
277.1435	277.1440	-0.5	-1.8	6.5	C16 H21 O4	147.0	5.511	0.40	16	21		4	
	277.1453	-1.8	-6.5	11.5	C17 H17 N4	152.4	10.909	0.00	17	17	4		
	277.1408	2.7	9.7	1.5	C12 H25 N2 O S2	164.8	23.351	0.00	12	25	2	1	2
	277.1400	3.5	12.6	2.5	C11 H21 N2 O6	150.3	8.782	0.02	11	21	2	6	
	277.1474	-3.9	-14.1	1.5	C13 H25 O4 S	161.6	20.135	0.00	13	25		4	1
	277.1487	-5.2	-18.8	6.5	C14 H21 N4 S	161.3	19.788	0.00	14	21	4		1
	277.1375	6.0	21.6	6.5	C15 H21 N2 O S	161.3	19.795	0.00	15	21	2	1	1
	277.1359	7.6	27.4	-1.5	C6 H21 N4 O8	153.4	11.928	0.00	6	21	4	8	
	277.1512	-7.7	-27.8	2.5	C10 H21 N4 O5	150.4	8.944	0.01	10	21	4	5	



For Help, press F1

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 500.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

623 formula(e) evaluated with 158 results within limits (all results (up to 1000) for each mass)

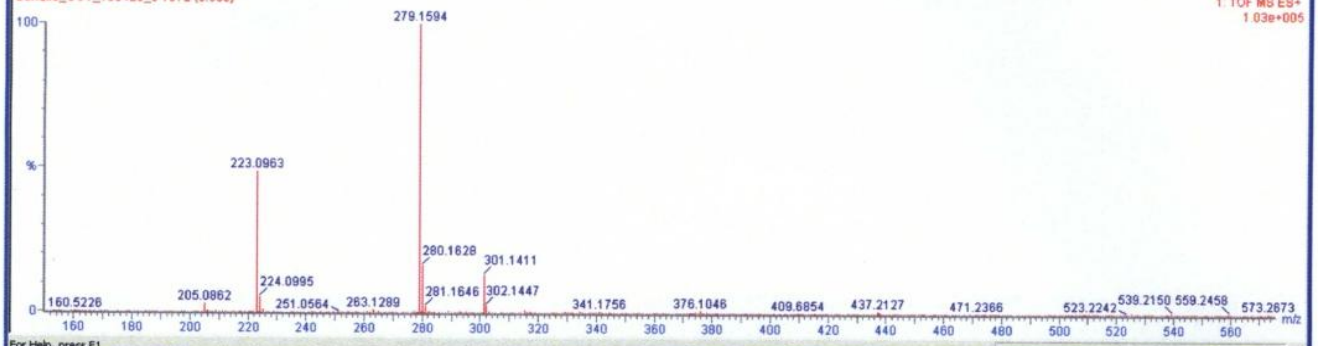
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S
279.1594	279.1596	-0.2	-0.7	5.5	C16 H23 O4	105.8	5.674	0.34	16	23		4	
	279.1610	-1.6	-5.7	10.5	C17 H19 N4	110.9	10.794	0.00	17	19	4		
	279.1565	2.9	10.4	0.5	C12 H27 N2 O S2	125.8	25.652	0.00	12	27	2	1	2
	279.1630	-3.6	-12.9	0.5	C13 H27 O4 S	122.6	22.519	0.00	13	27		4	1
	279.1556	3.8	13.6	1.5	C11 H23 N2 O6	107.5	7.335	0.07	11	23	2	6	
	279.1643	-4.9	-17.6	5.5	C14 H23 N4 S	122.3	22.205	0.00	14	23	4		1
	279.1531	6.3	22.6	5.5	C15 H23 N2 O S	122.4	22.249	0.00	15	23	2	1	1
	279.1668	-7.4	-26.5	1.5	C10 H23 N4 O5	109.9	9.759	0.01	10	23	4	5	
	279.1677	-8.3	-29.7	0.5	C11 H27 N4 S2	125.8	25.652	0.00	11	27	4		2

sample CH3OH

Sandile_CUT_130429_5 1072 (8.358)

1: TOF MS ES+
1.03e+005



For Help, press F1

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 500.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

560 formula(e) evaluated with 150 results within limits (all results (up to 1000) for each mass)

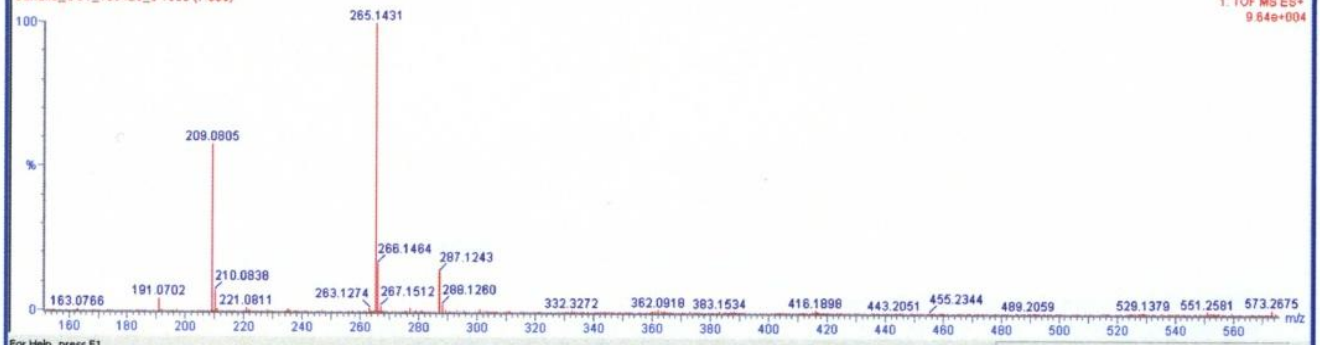
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S
265.1431	265.1440	-0.9	-3.4	5.5	C15 H21 O4	140.3	5.343	0.46	15	21		4	
	265.1453	-2.2	-8.3	10.5	C16 H17 N4	142.9	7.943	0.04	16	17	4		
	265.1408	2.3	8.7	0.5	C11 H25 N2 O S2	158.4	23.378	0.00	11	25	2	1	2
	265.1400	3.1	11.7	1.5	C10 H21 N2 O6	146.8	11.801	0.00	10	21	2	6	
	265.1474	-4.3	-16.2	0.5	C12 H25 O4 S	155.8	20.799	0.00	12	25		4	1
	265.1375	5.6	21.1	5.5	C14 H21 N2 O S	155.5	20.471	0.00	14	21	2	1	1
	265.1487	-5.6	-21.1	5.5	C13 H21 N4 S	155.4	20.399	0.00	13	21	4		1
	265.1512	-8.1	-30.5	1.5	C9 H21 N4 O5	147.4	12.373	0.00	9	21	4	5	
	265.1341	9.0	33.9	10.5	C17 H17 N2 O	143.0	8.004	0.03	17	17	2	1	

sample CH3OH

Sandile_CUT_130429_5 1009 (7.868)

1: TOF MS ES+
9.64e+004



For Help, press F1

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 500.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

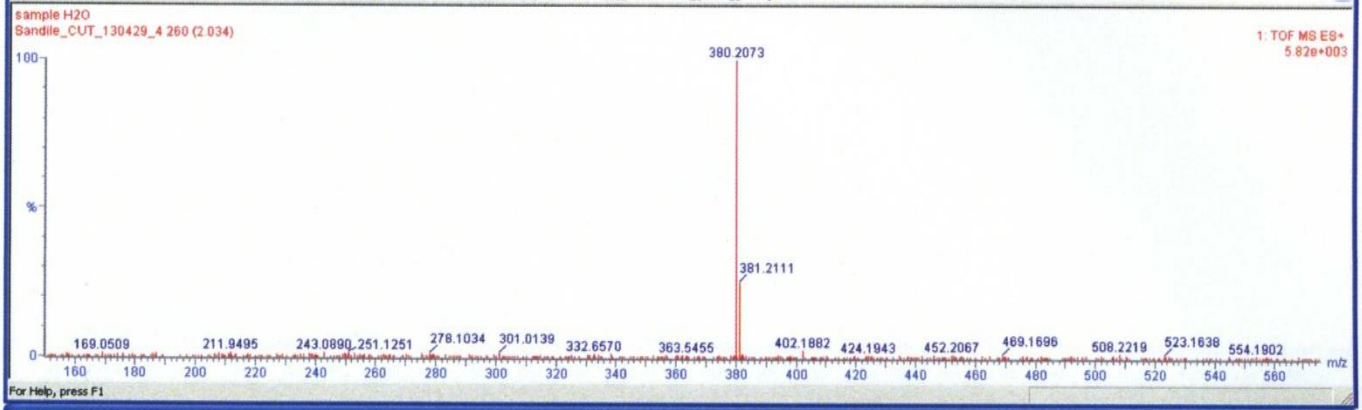
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

1097 formula(e) evaluated with 300 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S
380.2073	380.2073	0.0	0.0	6.5	C20 H30 N O6	104.0	3.949	1.93	20	30	1	6	
	380.2062	-0.9	-2.4	5.5	C21 H34 N O S2	108.8	8.700	0.02	21	34	1	1	2
	380.2087	-1.4	-3.7	11.5	C21 H26 N5 O2	103.8	3.680	2.52	21	26	5	2	
	380.2048	2.5	6.6	10.5	C24 H30 N O S	108.6	8.553	0.02	24	30	1	1	1
	380.2042	3.1	8.2	1.5	C16 H34 N3 O3 S2	108.7	8.586	0.02	16	34	3	3	2
	380.2107	-3.4	-8.9	1.5	C17 H34 N O6 S	108.1	8.074	0.03	17	34	1	6	1
	380.2033	4.0	10.5	2.5	C15 H30 N3 O8	104.5	4.428	1.19	15	30	3	8	
	380.2120	-4.7	-12.4	6.5	C18 H30 N5 O2 S	107.9	7.877	0.04	18	30	5	2	1
	380.2127	-5.4	-14.2	15.5	C26 H26 N3	104.3	4.194	1.51	26	26	3		



For Help, press F1

Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 500.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

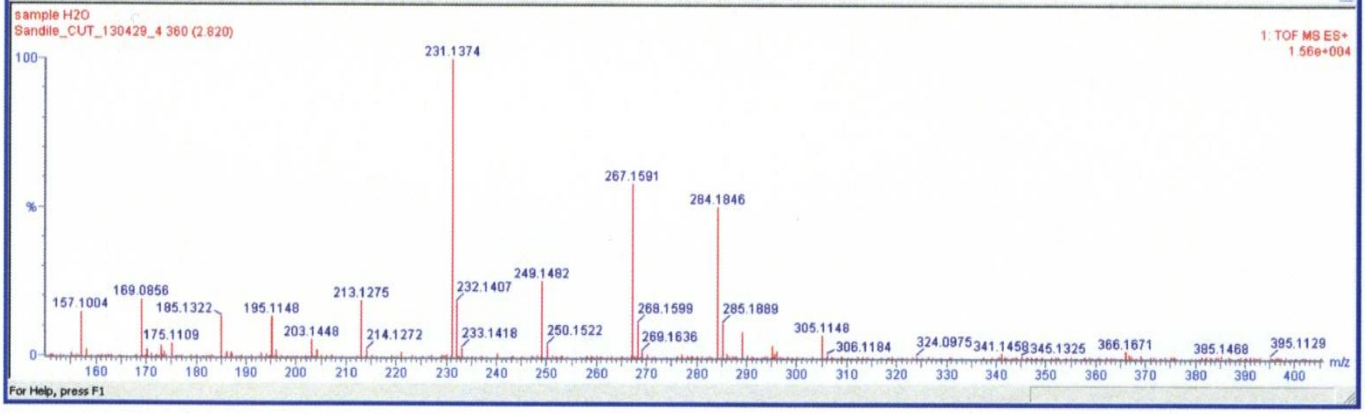
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

746 formula(e) evaluated with 250 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S
305.1148	305.1146	0.2	0.7	7.5	C16 H21 N2 S2	57.0	7.808	0.04	16	21	2		2
	305.1137	1.1	3.6	8.5	C15 H17 N2 O5	53.4	4.268	1.40	15	17	2	5	
	305.1131	1.7	5.6	-0.5	C7 H21 N4 O7 S	55.1	5.964	0.26	7	21	4	7	1
	305.1171	-2.3	-7.5	3.5	C12 H21 N2 O5 S	53.7	4.540	1.07	12	21	2	5	1
	305.1178	-3.0	-9.8	12.5	C20 H17 O3	55.3	6.135	0.22	20	17		3	
	305.1112	3.6	11.8	12.5	C19 H17 N2 S	56.2	7.058	0.09	19	17	2		1
	305.1106	4.2	13.8	3.5	C11 H21 N4 O2 S2	57.1	7.925	0.04	11	21	4	2	2
	305.1196	-4.8	-15.7	-0.5	C8 H21 N2 O10	54.0	4.888	0.75	8	21	2	10	
	305.1097	5.1	16.7	4.5	C10 H17 N4 O7	52.6	3.423	3.26	10	17	4	7	



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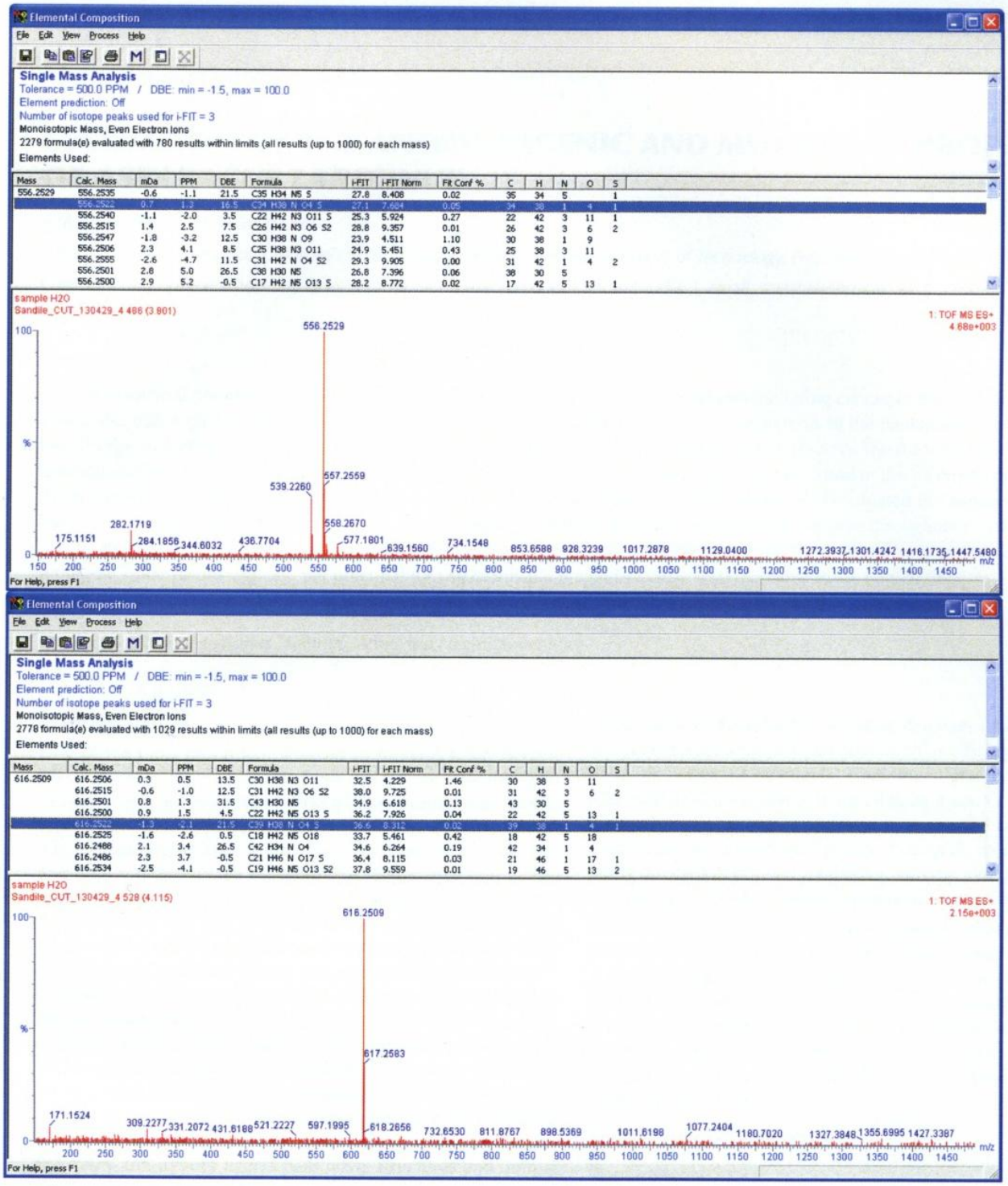

Helpful hints in interpreting MS results from Masslynx data

1. The ionization mode is indicated in the top right hand corner of spectra:

ESMS and LCMS lab (more suitable for non-volatile compounds):

ES+ = Electrospray positive: Typically a M+H or M+Na ion is observed
ES-= Electrospray negative: Only used for molecules that can be negatively charged like phenols and carboxylic acids, typically a M-H or M+Cl is observed

APCI = Atmospheric pressure chemical ionization: A softer technique for non-polar molecules – rarely used

GCMS (more suitable for smaller and volatile compounds):

EI = Electron impact, this is a harsher ionization technique. A fragmentation pattern and M^+ is sometimes observed

Peer reviewed **ORIGINAL ARTICLE**

EVALUATION OF THE ANTIMUTAGENIC AND MUTAGENIC PROPERTIES OF *ASPARAGUS LARICINUS*

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Abstract

Several medicinal plants are traditionally used in the treatment of a variety of ailments including cancer in the rural areas of South Africa and our neighbouring countries where herbal medicine is one of the main components of the healthcare system. Many of these herbs, including *Asparagus laricinus*, have not been scientifically assessed for efficacy or safety. The study on which this article is based evaluated the antimutagenic and mutagenic water extract of *Asparagus laricinus* roots used in the treatment of cancer. The Ames test recommended for mutagenicity screening was employed. The mutagenic activity was evaluated in *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102. The mutagenicity tests of aqueous extracts were negative throughout (1:10, 1:25, 1:50. 1:100, and 1:200). The highest antimutagenic effect in the order of 49% was observed using TA 98 (5µg daunomycin/plate), a strong mutagen. These results indicate the potential for use of this plant against cancer and oxidative stress-related diseases.

Keywords

Medicinal plants, *Asparagus laricinus*, Ames test, antimutagenicity

INTRODUCTION

One of the most prominent diseases in humans today is cancer. It is projected that deaths from cancer are continuing to escalate, with an estimate of nine million deaths from cancer in the year 2015 and 11.4 million deaths by 2030[1]. Generally, cancer begins after a mutational episode in a single cell and then it progressively transforms to malignancy in multiple stages through sequential acquisition of additional mutations[2]. In view of the fact that these initial events are the underlying causes of the whole progression of carcinogenesis, their inhibition would therefore be an efficient preventive measure[3]. Currently, there is marked scientific and commercial interest in the continuing discovery of new anti-cancer agents from natural product sources[4]. More than 50% of drugs used in clinical trials for anti-cancer activity were isolated from natural sources or are related to them[5,6]. Hence, the search for natural products to be used in cancer therapy represents an area of great interest in which the plant kingdom is the most important source, providing many anti-tumour agents with novel structures and unique mechanisms of action[7].

Present chemotherapy cancer treatments have proved to be ineffective as a result of their toxicity and cells developing resistance[8]. Many conventional drugs also induce genetic damage that itself can be carcinogenic. A segment of the research community is thus focusing on identifying novel chemotherapeutic agents in plants that do not induce the destructive effects of conventional cytotoxic therapeutic agents.

Several medicinal plants are traditionally used in the treatment of a variety of ailments, including cancer in many communities in South Africa and neighbouring countries. Since ancient times, herbal medicine has always been one of the main components of the healthcare system. In this study *Asparagus laricinus* belonging to Asparagaceae family was investigated. This is a monogeneric family, which was previously included within the Liliaceae family[9]. The genus Asparagus comprises approximately 100 species and consists of herbs, shrubs and vines. Many of these herbs, including *Asparagus laricinus*, have not yet been scientifically assessed for their efficacy or safety to tissue or organs of recipients. Thus, investigation of traditionally used medicinal plants is valuable as a source of potential chemotherapeutic agents and also to assess the safety for the continuous use of medicinal plants. As a result, most studies are being directed at popular medicine, with the aim of identifying natural products which exhibit therapeutic properties[10,11].

The Ames test[12] was used in this study as it is widely used in the determination of possible gene mutations caused by extracts. A positive response in any single bacterial strain either with or without metabolic activation is sufficient to designate a substance as a mutagen[13]. The study being reported here evaluated the mutagenic and antimutagenic activity of the water extract of *Asparagus laricinus* roots which are known to be used in the treatment of cancer.

MATERIALS AND METHODS

Plant material

The plant material (*Asparagus laricinus*) was authenticated by scientists at the National Botanical Gardens in Pretoria, South Africa. The collected root materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored in a cool place until analysis. Plant material (10 g of the dried roots) was weighed, pulverised and soaked in a volume of 500 ml of purified water for 72 hours with occasional stirring. The extracts were filtered and the solvent was removed completely by employing a rotatory evaporator.

The Ames test

The Ames test is a well-known bacterial mutagenicity test[11,14]. In this test, reverse $His^- \rightarrow His^+$ mutations are visualised by plating *Salmonella typhimurium* bacteria in a histidine-poor growth medium. In this medium only, His^+ mutants are able to form visible colonies. Different bacterial strains are available to identify different types of mutations. We used the strains TA97, TA98,

Table 1: Summary of mutagenic/anti-mutagenic properties of Asparagus laricinus *extract.*

	Mutagen/anti-mutagen				Mutagen + anti-mutagen			
	TA 97	TA 98	TA 100	TA 102	TA 97	TA 98	TA 100	TA 102
Dilutions	Percentage inhibition				Percentage inhibition			
50X	21	49	28.6	22.7	20.2	23.4	31.6	37.5
100X	10.4	35.1	12.1	23.5	14.5	19.5	28.4	24
200X	9.1	21.9	19	21.5	7.6	17.1	15.5	21

TA100 and TA102. The strains TA98 and TA100 are actually those that are most often used as they detect the great majority of mutagens. Strain TA98 gives an indication of frame-shift mutations, while a positive response from strain TA100 indicates base-pair substitution. For a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds should be more than twice the number of colonies produced on the solvent control plates (i.e. a ratio above 2.0). In addition, a dose-response should be evident for the various concentrations of the mutagen tested. Toxicity can be checked by investigation of the background layer of bacteria.

Antimutagenicity was expressed as percentage inhibition of mutagenicity according to the following formula given for the Ames test[15]:

$$\% \text{ inhibition} = [1 - T/M] \times 100$$

where *T* is the number of revertants per plate in the presence of mutagen and the test sample and *M* is the number of revertants per plate in the positive control (mutagen alone). The same formula was also adopted for other tests. An extract was considered to have no or only weak antimutagenic properties when the percentage inhibition of mutagenicity (e.g. revertants in the Ames assay) was less than 25%. When the percentage inhibition was between 25% and 40% it was considered that the extract had a moderate antimutagenic effect and it was concluded that an extract had a strong antimutagenic effect when the percentage inhibition was higher than 40%. Dimethyl sulfoxide (DMSO) was used as a negative control, while daunomycin (10µl/plate) was used as a positive control. All experiments were performed in triplicate. The metabolic activation (S9) was donated by the MRC Liver Research Centre, Tygerberg, Cape Town.

Determination of total phenolic content

Total phenolic content in *Asparagus laricinus* extract was determined employing Folin-Ciocalteu reagent[16], with minor adaptations. Gallic acid was used as a standard phenolic compound. In a test tube, 0.5ml of plant extract or standard solution was added, followed by 35 ml of H_2O and 2.5ml of Folin-Ciocalteu reagent. The content was mixed thoroughly. After 8 minutes, 7.5ml $NaCO_3$ (10% w/v) was added. The mixture was allowed to stand in the dark for 1 hour. Absorbance was measured at 760nm using a spectrophotometer. The concentration of total phenolic compounds in plant extracts was determined from the gallic acid standard curve.

RESULTS AND DISCUSSION

The standard plate incorporation methods for the Ames test using *Salmonella typhimurium* standard tester strains TA97, TA98, TA100 and TA102 exposed to five dilutions with and without S9 metabolic activation of the herbal mixtures were performed. The Ames test without S9 metabolic activation can only detect direct mutagens while with S9 metabolic activation allows the detection of indirect mutagens, often caused by conjugation reactions of metabolic oxidation systems. Table 1 and 2 present the spontaneous reversion response of the *Salmonella typhimurium* tester strain to the different dilutions of the Asparagus laricinus extract. The results revealed that Asparagus laricinus extracts were non-mutagenic towards the *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102 for the assay without metabolic activation. The average His^+ revertants observed for all the tester strains caused by the extracts at all the concentrations without metabolic activation did not satisfy the criteria for mutagenicity. There was no notable dose-dependent increase in the number of revertants and the numbers of revertants were all either not equal to or greater than twice that of the negative control[17]. There was also no decrease in the number of revertant colonies to levels far below the negative control (spontaneous reversion) which could also be classified as toxic. The extract had moderate (28%-34%) inhibitory effects using *Salmonella typhimurium* TA100 strain. The upper limit was observed beginning with mutagen and followed by the anti-mutagen (extract). The lower limit was observed beginning with an anti-mutagen followed by a mutagen. When both mutagen and anti-mutagen were operative on commencement of a possible disease, 31% inhibition was observed. Similarly, using *Salmonella typhimurium* TA102 strain, the extract showed a low to moderate inhibitory effect (24%-38%). The lower limit represented mutagen followed by anti-mutagen and the upper limit was observed when both mutagen and anti-mutagen were operative on commencement of a possible disease. However, using *Salmonella*

Table 2: Mutagenic activities of Asparagus laricinus *extract with metabolic activation (+S9).*

S. typhymurium strain	Dilution	Mean +/- SD
TA 97	50X	154 +/-7
TA 97	100X	154+/-10
TA 97	200X	163+/-12
TA 97 control (+)	n/a	351
TA 97 control (-)	n/a	351
TA 98	50X	40+/-6
TA 98	100X	32+/-8
TA 98	200X	45+/-6
TA 98 control (+)	n/a	830
TA 98 control (-)	n/a	53
TA 100	50X	123+/-4
TA 100	100X	131+/-3
TA 100	200X	132+/-8
TA 100 Control (+)	n/a	1395
TA 100 Control (-)	n/a	183
TA 102	50X	451+/-47
TA 102	100X	406+/-51
TA 102	200X	405+/-50
TA 102 control (+)	n/a	1395
TA 102 control (-)	n/a	391

SD is standard deviation of three repeats in the experiment

typhimurium TA97 strain, the extract had a low inhibitory effect (20%-22%). The degree of inhibition was not affected by the mode of interaction of the mutagen and anti-mutagen. Finally, the inhibitory effect was observed over a wide spectrum using *Salmonella typhimurium* TA98 strain (23%-49%). The upper limit was observed beginning with a mutagen and followed by the anti-mutagen. The lower limit was observed when there was simultaneous interaction between a mutagen and the extract. When both mutagen and anti-mutagen were operative on commencement of a possible disease, 35% inhibition was observed.

The Ames test with metabolic activation was carried out by addition of the S9 mix so as to detect an indirect mutagenic effect caused by metabolites of the test *Asparagus laricinus* extract. The S9 fraction contains a mixture of xenobiotic metabolising enzymes, such as the cytochrome P450s and sulfotransferase. *Asparagus laricinus* extract showed an indirect mutagenic effect toward the tester strain TA102 after metabolic activation but not in the other tester strains (TA97, TA98, and TA100).

The rationale for screening for antimutagenicity was that antimutagenic compounds can possibly also be anticarcinogens. These results have stimulated us to carry out ongoing work to determine the active ingredients since these may prove valuable lead compounds in the light of their antimutagenic ability. No data relative to cytotoxic components of *Asparagus laricinus* had been reported and further phytochemical work on the isolation and identification of active structures of this plant is presently in progress.

As assessed by the Folin-Ciocalteu method, the total phenolic content in the extracts are presented as gallic acid equivalents (GAE) with reference to the gallic acid standard curve which was plotted using the average absorbance values of two independent sets of data against concentrations of gallic acid in g/l. The *Asparagus laricinus* aqueous extract contained 4.2g/l GAE. Polyphenols are the most abundant dietary antioxidants and research on their role in the prevention of degenerative diseases and cancer has developed quickly over these last few years. With a transgenic adenocarcinoma of the mouse prostate model of prostate carcinogenesis (TRAMP), it was recently demonstrated that oral consumption of 10g/l green tea polyphenols decreased tumour incidence by 65%[18], the latter polyphenol preparation was enriched with epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%), and caffeine (‘1%). It is worth mentioning that the ratio of individual constituent polyphenols may vary from plant to plant. Evaluation of the *Asparagus laricinus* aqueous extract on human melanoma cell lines is presently in progress.

Many researchers have inferred the antimutagenic activity of plant extract to the antioxidant activities of the polyphenols. However, there is now a paradigm shift. Owing to a study that showed *Asparagus racemosus* extracts induced excessive production of TNF-alpha as compared to controls[19], similar reports are increasing. Naturally, white blood cells produce TNF-alpha protein to stimulate and activate the immune system in response to infection or cancer. The current state of knowledge suggests that the mode of action of many plant extracts is between scavenging for free radicals and modulation of the immune system. The evidence obtained in studies of a tea polyphenol i.e. (-)-epigallocatechin gallate [(-)-EGCG], indicate that plant polyphenols target many enzymes directly associated with cancer (e.g. proteosome, telomerase, growth factors, MAPKs, etc.)[20,21,22,23]

CONCLUSION

Asparagus laricinus polyphenol extract exhibited a dose dependent antimutagenic ability. The plant extract showed no mutagenic effect on all tested strains. However, mutagenic activity was observed with metabolic activation for the TA 102 tester strain only, to the exclusion of (TA97, TA98, and TA100). These results indicate the potential for use of this plant against cancer and oxidative stress-related diseases. Although these results support the use of *Asparagus laricinus* for treatment of cancer, further investigation is required, for this promising plant.

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Phytochemistry and Free Radical Scavenging Activity of *Asparagus laricinus*

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Abstract: This study reports on the phytochemistry and radical scavenging activity of *Asparagus laricinus* Burch (Asparagaceae) in order to explain the anti-cancer properties it possesses. The root extracts of *Asparagus laricinus* were qualitatively screened for antioxidant properties using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), on silica gel GF_{254} TLC plates. Quantitative Radical scavenging activity using DPPH was also evaluated. The extracts were tested for the presence of alkaloids, saponins, tannins, terpenoids and steroids using standard phytochemical screening methods. Gas chromatography/mass spectrometry (GC/MS) was used to further confirm the phytoconstituents. Three spots that exhibited antioxidant activity were resolved on the TLC plates. The free radical scavenging activity of *A. laricinus* extract was comparable with that of Trolox® and was dependent on the concentration of the extract. The aqueous extract of *A. laricinus* contained alkaloids, saponins, tannins and terpenoids while the ethanol and dichloromethane extracts produced negative results. The phytoconstituents confirmed through the use of GC-MS were indole-3-carbinol, α-sitosterol and ferulic acid. The phytochemicals present and the antioxidant activity demonstrated by extracts of *A. laricinus* explain its *in vitro* anti-cancer properties as well as its continued use in traditional medicine for the treatment of cancer and other ailments.

Key words: *Asparagus laricinus*, antioxidant, free radical scavenging, phytochemical screening

INTRODUCTION

The use of medicinal plants in all cultures is well documented. Plants have been the main component of traditional pharmacopoeias for generations and continue to supply new remedies for the treatment of various maladies. For instance, an estimated 80% of the world's population mainly uses plant medicines for primary health care. Traditional healers in South Africa attend to approximately 60% of South Africans. The bulk of traditional medicine in the country is based on nearly 3000 plant species (Taylor *et al*., 2001; Masoko *et al*., 2010).

Modern pharmaceutics also have benefited from medicinal plants. For example, only 39% of the 877 molecules used in drug development between 1981 and 2002 were truly synthetic in origin. The rest were naturally inspired or derived. Furthermore, more than half of the drugs used in cancer treatment are of natural origin (Gurib-Fakim, 2006; Newman and Cragg, 2007) with the therapeutic alkaloids (vinblastine and vincristine) epipodophyllotoxins, taxanes and camphothecins as examples of plant-derived anti-cancer compounds that are currently in clinical use (Balunas and Kinghorn, 2005). Plants therefore are potent sources of new drugs and drug leads (Balunas and Kinghorn, 2005; Newman and Cragg, 2007; Harvey, 2008; Shyur and Yang, 2008).

The therapeutic properties of plants are attributed to the broad spectrum of secondary metabolites (Pieters and Vlietinck, 2005; Kaur *et al*., 2005) including polyphenols, alkaloids and flavonoids. These phytochemicals are produced to protect the plant against herbivory and microbial attacks. Secondary metabolites are also manufactured by plants to attract pollinators and symbionts, as well as to respond to abiotic stresses (McRae *et al*., 2007). These ecological functions form the basis of the bioactivity exhibited by the compounds and has resulted in antimicrobial medicines (Briskin, 2000), anti cnacer drugs and plant-based antioxidants. The medicinal potency of a plant may result from a single compound or the synergistic or additive action of several constituents in the plant (Shyur and Yang, 2008; Van Vuuren, 2008; Eloff *et al*., 2008). Therefore, the phytochemical screening of plant extracts in the process of drug discovery is very important.

This Study reports on the phytochemical screening of *Asparagus laricinus* Burch (Asparagaceae). *A. laricinus* is part of traditional medicine in many

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communities in South Africa. The leaves and stem are used medicinally in South West Gauteng (Dzerefos and Witkowski, 2001). The use of the roots of *A. laricinus* as a diuretic and to treat tuberculosis is reported in Khoi-San and Cape Dutch medical ethnobotany (Van Wyk, 2008). The roots also have ethnoveterinary use for the treatment of sores, redwater, urine infections, umbilical cord inflammation and general ailments among the Setswana people of the North West Province of South Africa (Van der Merwe *et al*., 2001). *A. laricinus* is also used in the treatment of cancer (Mashele and Kolesnikova, 2010).

Previous studies have indicated that alcoholic and aqueous extracts of *A. laricinus* have active anti-cancer properties in-vitro against three human cancer cell lines (Mashele and Kolesnikova, 2010). The aqueous extract of the plant also showed antimutagenic effects (Mashele and Fuku, 2011) using the Ames test, whilst the mutagenicity tests were negative. Traditional medicinal use of plants in South Africa is strongly related to physiological and pharmacological activity of active plant ingredients. The phytochemical characterization of the plant may validate its use in traditional medicine for the treatment of cancer. Oxidative stress is involved in the development of diseases such as cancer due to the overproduction of free radicals (Adewusi and Afolayan, 2009; Aremu *et al*., 2011). *A. laricinus* was also screened for antioxidant (radical scavenging) properties in an effort to further evaluate its potential as a comprehensive anti-cancer agent.

METHODS

Collection and validation of samples: *Asparagus laricinus* was collected from traditional healers in Pretoria, South Africa in July 2011. The plant was cross-identified by its vernacular names and later validated at the National Botanical Gardens in Pretoria, South Africa (Voucher specimen: Mash 002).

Preparation of extracts: Two hundred and fifty gram roots of *Asparagus laricinus* were cleaned with tap water to eliminate dust and soil, then air dried under shade. The dried material was sliced into small fragments and extracted at room temperature thrice with ethanol, distilled water and dichloromethane for 72 h. Finally, the extracts were concentrated using a rotary-evaporator (R215 Buchi Instrument, Switzerland) at a reduced pressure and at <40°C. The recovered weight of the plant material obtained was 20% of the dried material (Al-Azzawi and Al-Juboori, 2012).

Phytochemical analysis: The presence of phytochemicals in the three extracts, such as alkaloids, saponins, tannins (5% ferric chloride), terpenoids (2, 4-dintrophenyl hydrazine) and steroids (Liebermann-Burchard test) were evaluated according to the methods described by Edeoga *et al*. (2005).

Thin layer chromatography analysis of antioxidant constituents: Qualitative screening for antioxidant activity was done using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) according to Tomohiro *et al*. (1994). Thin layer chromatography (TLC) of extracts was developed with methanol: Chloroform: Hexane (70:20:10%) on silica gel GF_{254} (Fluka, 20×20 cm^2) as the stationary phase. The plates were air dried and sprayed with 0.05% DPPH in methanol. Antioxidant activity was detected on the chromatogram by the appearance of yellow spots produced by bleaching of DPPH (Bors *et al*., 1992). All detected active antioxidant constituents were noted according to their Rf values. Gallic acid was used as positive control.

Free radical scavenging activity: The free radical scavenging activity of the *Asparagus laricinus* extracts was analyzed by using 2,2-diphenyl-2-picrilhydrazyl (DPPH) assay (Von Gadow *et al*., 1997; Fuhrman *et al*., 2001). Different concentrations (between $10^{-1}\times10^{-04}$ mg mL^{-1}) of the extract were prepared. Aliquots (100 μL) of the extract or standard solution (Trolox® and ascorbic acid) were mixed with 2 mL of 0.1 mmol L^{-1} methanolic solution of DPPH radical. The tubes were mixed and allowed to stand for 60 min in the dark. Absorbance was read against a blank at 517 nm using a spectrophotometer. Methanol was used to blank the spectrophotometer, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) and Ascorbic Acid were used as positive controls. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the extracts was calculated according to the equation used in Turner *et al*. (2011):

$$\text{Inhibition (\%)} = [1 - (A_s/A_0)]\times100$$

where, A_s is absorbance of sample (i.e., extracts or standard) and A_0 is the absorbance of the DPPH solution, the EC_{50}, defined as the concentration of the sample leading to 50% of the reduction of the initial DPPH concentration, was determined at 95% confidence interval, using GraphPad Prism® software. The data on regression graphs represents Mean+/-SEM (Standard error of means). The experiment was done in triplicate.

Gas chromatography/mass spectrometry (GC/MS): GC-MS analysis was carried out on an Shimadzu 2010 QB gas chromatograph with a MSD detector equipped with an HP-5 fused silica capillary Column (30m×0.25mm×25m film thickness). The aqueous plant extract was injected via., an all-glass injector working in split mode with Helium as the carrier gas at a flow rate of 1 mL min^{-1}. Temperature program: Injector temperature 200°C, Ion source 200°C, Interphase 200°C. Column temperature was raised to 45°C (3 min hold at 45°C, 4°C min), then gradually increased to 150°C (3 min hold at 150°C, 4°C min) then raised to 250°C and a 15 min hold. A split ratio of 1:5 was used (Ajayi *et al*., 2011). Compound identification was accomplished by comparing the retention times with those of authentic compounds or the spectral data obtained from the Wiley Library and National Institute of Standards and Technologies Library, as well as with data published in the literature.

RESULTS

Thin layer chromatography analysis of antioxidant constituents : Figure 1 shows the TLC chromatogram for the qualitative screening for antioxidants. Three spots with Retention Factors (Rf) of 0.87, 0.38 and 0.35 exhibited antioxidant activity when sprayed with DPPH. This indicated differences in the polarity of the separated antioxidant molecules.

Free radical scavenging activity: The addition of the *A. laricinus* extract to the DPPH solution induced a rapid decrease in absorbance at 517 nm indicating free radical scavenging activity of the extract (Fig. 2). The EC_{50} value of the extract was recorded to be between 0.9205 to 1.188 mg mL^{-1} and was higher than that of Trolox® (Fig. 2d). The radical scavenging activity of the extract was concentration-dependent and similar to that of known antioxidants (Fig 2a, b and c). *A. laricinus* showed a concentration-dependant free radical scavenging activity at concentrations above 1 mg mL^{-1} while ascorbic acid no longer showed increasing activity above the same concentration.

Phytochemical analysis: Phytochemical screening of the aqueous extract was positive for the presence of alkaloids, saponins, tannins and terpenoids. However, the ethanolic and dichloromethane extracts had negative results for all the chemical tests that were carried out, as shown in Table 1.

GC-MS study: The GC-MS chromatogram gave rise to three peaks suggesting the existence of three secondary metabolites and percentage values of composition of these phytochemicals present in the aqueous root extract of *Asparagus laricinus*. The results suggested the presence of indole-3-carbinol, α-sitosterol and ferulic acid, as shown in Fig. 3.

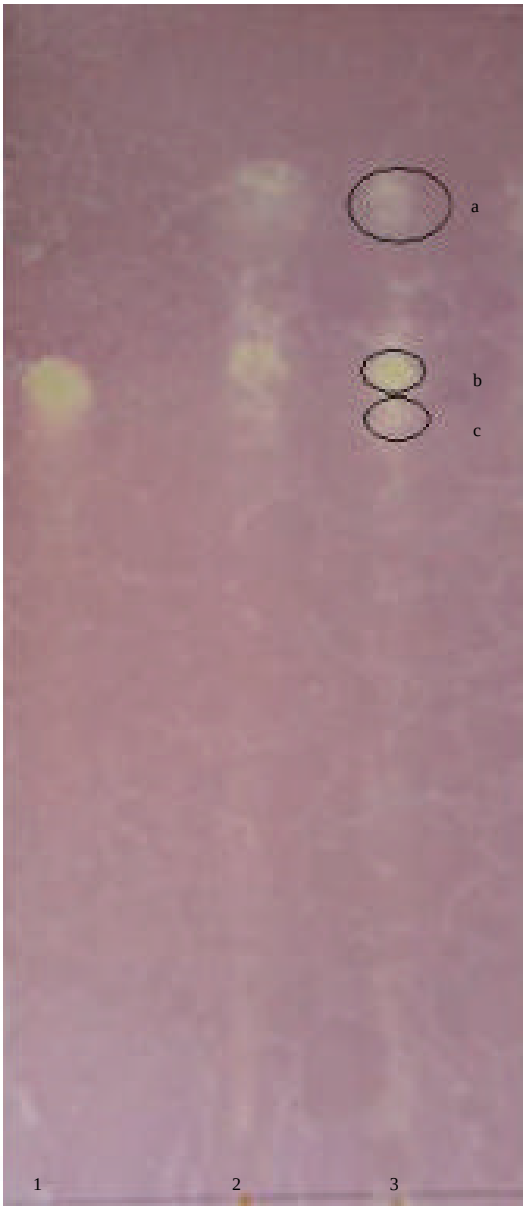

Fig. 1: Chromatogram of gallic acid (1) and *A. laricinus* aqueous extract (2 and 3), separated with methanol:chloroform: hexane (70:20:10%) and sprayed with DPPPH. The yellow spots indicated antioxidant activity (RF values: a = 0.87; b = 0.38; c = 0.35)

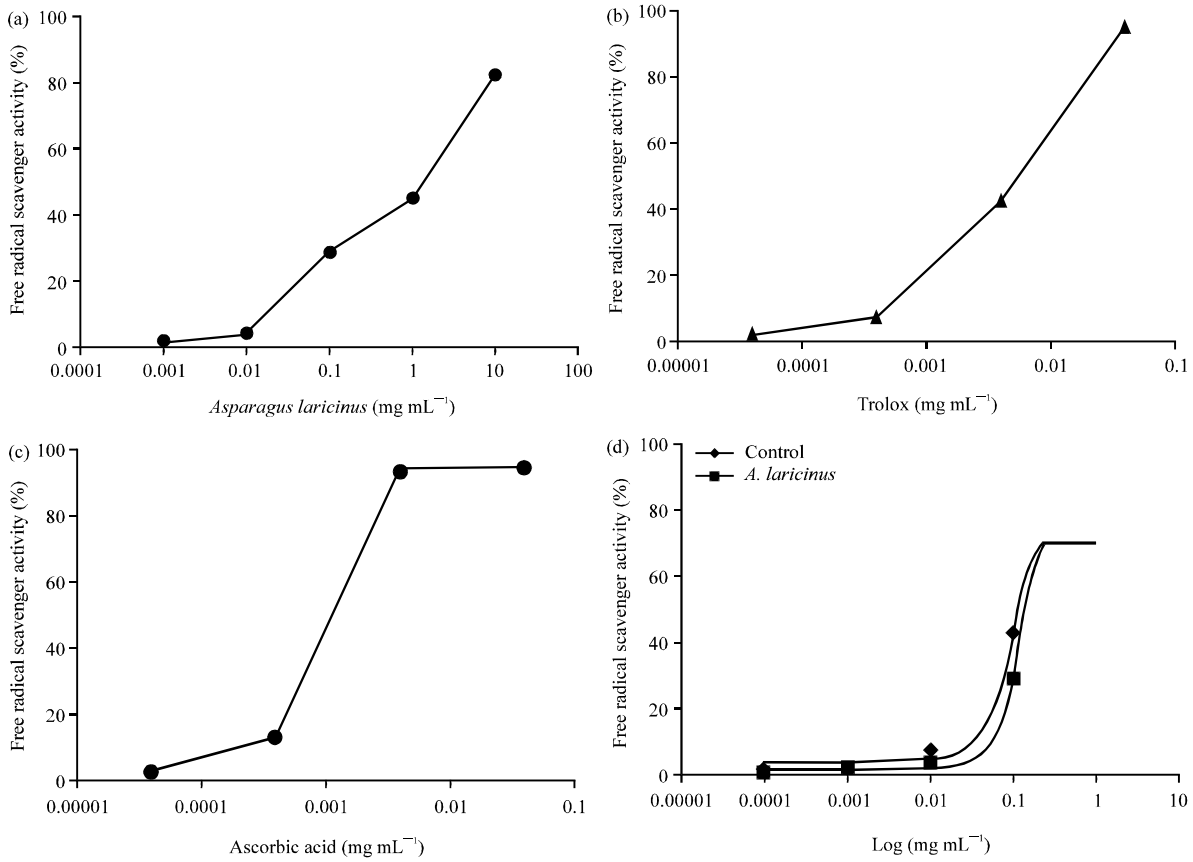

Fig. 2(a-d): Scavenging activity of *A. laricinus* aqueous extract on the free radical DPPH, (2a) *A. laricinus* extract, (2b) Trolox®, (2c) and ascorbic acid, (2d) EC_{50} shift graph of *A. laricinus* extract with Trolox® as the control

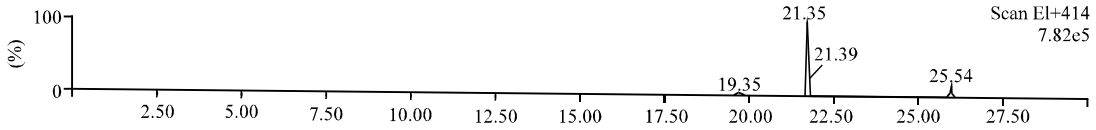

Fig. 3: GC-MS chromatogram of the aqueous extracts of *A. laricinus* roots

Table 1: Phytochemical screening of ethanol, aqueous and dichloromethane extracts of the roots of *A. laricinus*

Constituents	Aqueous	Ethanolic	Dichloromethane
Alkaloids			
Dragendorff's test	+	-	-
Steroids			
Libarman-Burchard's test	+	-	-
Terpenes			
Salkowski test	+	-	-
Tannins			
FeCl3 test	+	+	-
Gelatin test	-	-	-
Saponins			
Frothing test	+	-	-

Key: -: Negative (absent): +: Positive (present)

DISCUSSION

The antioxidant capacity of crude drugs is widely used as a parameter for evaluating medicinal bioactive components. The present study has demonstrated the antiradical activity of *A. laricinus*. We observed three spots that exhibited antioxidant properties on the TLC chromatograms. The characteristics of the three different

antioxidants observed in the *Asparagus laricinus* extracts are yet to be elucidated. The antioxidant in the plant extract may largely be due to polyphenols (Thabrew *et al*., 1998). Phenolics are the largest group of phytochemicals and most of the antioxidant activity of plants or plant products is attributed to them. Many studies have shown that natural antioxidants are able to reduce DNA damage, mutagenesis and carcinogenesis. These events are often associated with the termination of free radical propagation in biological systems (Covacci *et al*., 2001; Zhu *et al*., 2002). The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability or radical scavenging ability (Baumann *et al*., 1979). Considering the fact that the *A. laricinus* extract used was crude and the comparison was not on the basis of molar concentration, the above results are promising, despite having a higher EC50 value than Trolox®.

Phytochemical screening of indigenous plants is essential to evaluate their medical value and their potential use in the treatment of various diseases. In the current study of the *A. laricinus* extract, three secondary metabolites were found in the aqueous extract. The extract showed the presence of ferulic acid, a phenolic acid derivative that has antioxidant activity and several therapeutic benefits in the treatment of cancer. Ferulic acid is a plant constituent that arises from the metabolism of phenylalanine and tyrosine. It occurs primarily in seeds and leaves both in its free form and covalently linked to lignin and other biopolymers. Due to its phenolic nucleus and an extended side chain conjugation it readily forms a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential (Imaida *et al*., 1990; Srinivasan *et al*., 2007). β-Sitosterol has a number of therapeutic and chemo preventive uses in the medical field (Zak *et al*., 1990; Baskar *et al*., 2010). Indole-3-carbinol is used in prostate cancer (Garikapaty *et al*., 2005). The phytoconstituents detected in the plant materials could be responsible for the cytotoxic activity, though their exact mode of action is poorly understood at present.

CONCLUSION

The aqueous extract of *Asparagus laricinus* roots showed significant activity as an antioxidant. It showed free radical scavenging activity comparable to Trolox and Ascorbic acid. The free radical scavenging activity of the extract may be attributed to Ferulic acid, β-Sitosterol and/or indole-3-carbinol. The results of this study further justify the use of the plant in traditional medicine and highlight its potential for use in drug development.

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