Studies on the Chemical Constituents of *Pleurotus* Species and *Buxus hyrcana*; Microbial Transformations of Sclareol

By

ZAHEER UDDIN BABAR

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Chemistry
University of Manitoba
Winnipeg, Manitoba, Canada
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This thesis is dedicated to my family

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Abstract

This thesis describes chemical studies on the methanolic-ethyl acetate extract of *Pleurotus* species and *Buxus hyrcana,* and the microbial transformation of sclareol. Chemical studies on *Pleurotus* sp. resulted in the isolation of a steroid known as ergosterol peroxide (**53**). This compound (**53**) exhibited weak antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Corynebacterium xerosis.* Phytochemical investigations on the methanolic extract of the *Buxus hyrcana* have resulted in the isolation of a novel triterpenoidal alkaloid, O^6-buxadiene (**64**). This compound (**64**) showed weak anticancer activity against prostate cancer cell lines (LNCaP). The second part of this thesis describes the microbial transformation of sclareol (**78**), a plant natural product. Two fungi *Curvularia lunata* (ATCC 12017) and *Mucor plumbeus* (ATCC 4740) were capable of oxidizing sclareol into 3β-hydroxysclareol (**79**) and 18α-hydroxysclareol (**80**) by standard two stage fermentation method. Antibacterial assay showed that the oxidation products (**79**) and (**80**) were less bioactive than sclareol (**78**). This indicated that antibacterial activity was lost due to the presence of a hydroxyl group at 3β and 18α in (**79**) and (**80**), respectively. Their structures were established mainly on the basis of mass, IR, UV, 1D and 2D NMR spectroscopic studies.

Acknowledgments

This thesis would not have been possible without the guidance of Dr. Athar Ata. I extend my sincere gratitude to him for allowing me the opportunity to pursue graduate research under his supervision, and to benefit from his knowledge of chemistry. I would like to thank Mr. Ed Segestro and Mr.Terry Wolowiec for their help with the GC/MS and NMR, respectively. I owe my sincere gratitude to Dr. Paul Holloway for his help with the antibacterial studies. I wish to thank Dr. M. H. Meshkatalsadat, Department of Chemistry University of Lorestan, Iran, for supplying us with the crude extract of *Buxus hyrcana*. I would also like to thank the members of the Department of Chemistry at the University of Manitoba, and The University of Winnipeg, especially the academic, technical and administrative staff, for their support. I do greatly appreciate to all those who participated in funding the research on which this thesis is based. Finally, I am forever indebted to my mother and my family for their endless patience and encouragement when these were most required.

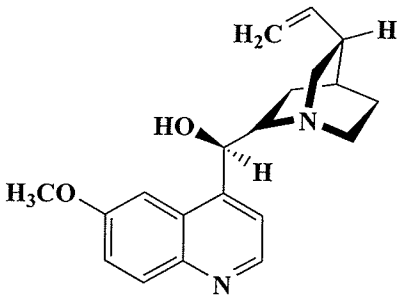
CHAPTER 1

NATURAL PRODUCTS

Natural products are organic compounds derived from marine organisms, plants, microorganisms, and insects. These naturally occurring organic compounds are used as flavoring agents, spices, cosmetics, pharmaceutical and biological agents. It is reported in the literature that about 80% of the world's population rely on traditional medicines for primary health.[1] Citations for the use of natural substances as medicines can be found as far as 78 A.D., when Dioscorides wrote "De Materia Medica", describing the use of thousands of plants as ingredients in crude drug preparations. He further added that these plants serve as the sources of pure chemicals which might be responsible for pharmaceutical activity.[2]

There have been several waves of interest in pharmacognosy or natural products chemistry. The research activity in this area is stronger now than perhaps at any other time. This could be attributed to several factors, including unmet therapeutic needs; the remarkable diversity of both chemical structures and biological activities of secondary metabolites; the utility of novel bioactive natural products as biochemical probes; the development of novel and sensitive techniques to detect biologically active natural products; improved techniques to isolate, purify, and characterize the active constituents; and advances in solving the demand for supply of complex natural products.

Natural products have served as a valuable source of molecular diversity in many drug discovery programs, and several important drugs have been isolated. The medicinal agents such as quinine (**1**), theophylline (**2**), morphine (**3**), cyclosporine A (**4**), and vitamin A (**5**) are the foundation of modern pharmaceutical care.[3]



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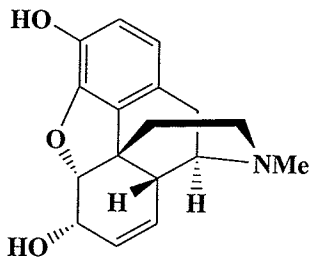

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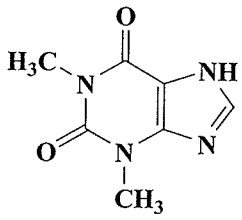

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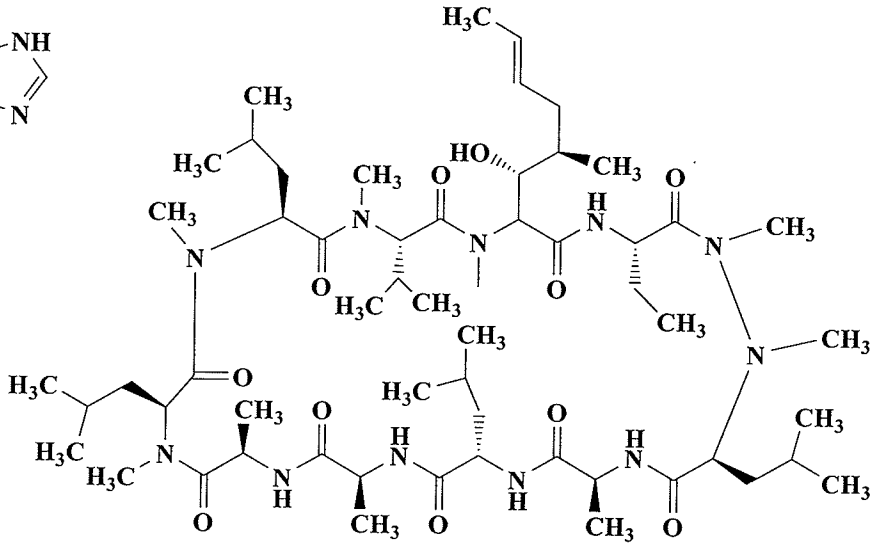

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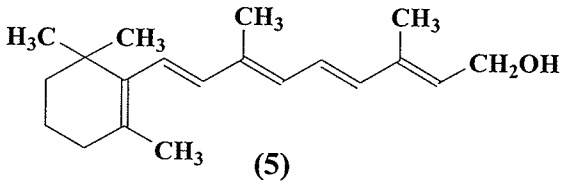

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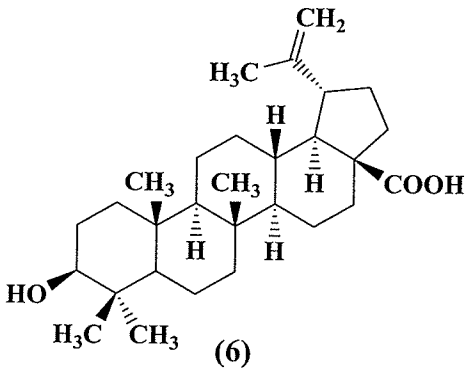
In drug discovery program, bioassay-guided isolation technique plays an important role in the isolation of the new bioactive compound(s). Some of these compounds have been reported to have novel carbon skeletons. Known natural products are also targeted

because of their pharmaceutical activity. For instance, betulinic acid (**6**), one of the most potent anti-HIV agents known, was isolated from *Syzigium claviflorum*.[4]



(6)

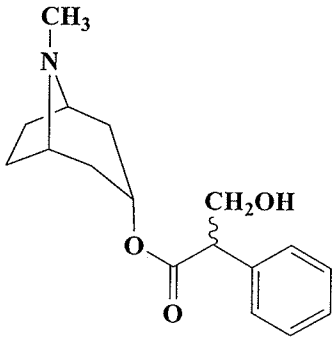
Over the past 25 years, it has been well documented that most of the drugs available in the market are of natural product origin. For instance 50% of the pharmacy based prescription products are natural product-derived entities and half of them are derived from plants.[5]

The approved and regulated chemical armamentarium available today is failing to provide effective health care for even one-third of the human population. Additionally, known therapeutic agents are reported to exhibit resistance to several diseases including cancer, malaria, tuberculosis and other infectious diseases.[6] These days discovery efforts aim at the identification of new therapeutic regimens, but not at the problem to overcome the development of resistance to the already existing therapeutic agents.

1.1 IMPORTANT NATURAL PRODUCT-DERIVED PHARMACEUTICALS

The use of plant and animal products to control disease has been well documented for centuries, the biochemical basis for observed efficacies did not come

under careful scientific scrutiny until the 18th and 19th centuries, particularly in the early to mid 1800's when a number of important, pharmacologically active natural products such as the cardiac glycosides and a variety of bioactive alkaloids, e.g., atropine (**7**), strychnine (**8**) and colchicine (**9**) were discovered.



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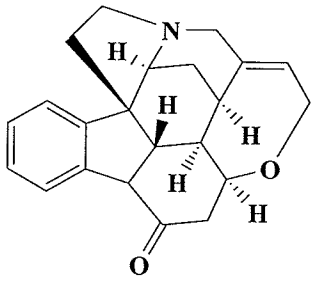

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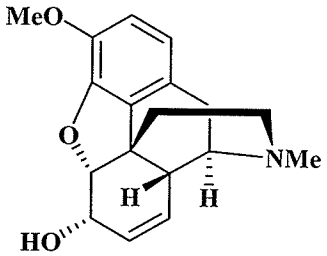

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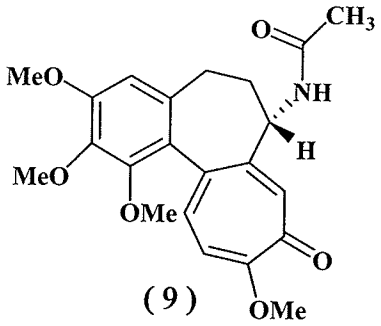

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Many of these biologically active natural products became important not only for their use directly as therapeutic agents or as prototype lead compounds for the development of new drugs, but also as biochemical probes to unravel the principles of human pharmacology, a role for natural products which continues today. Based on the need of

health care, pharmaceuticals are classified into the following classes:

1.1.1 Cardiovascular Drugs

1.1.2 Central Nervous System Drugs

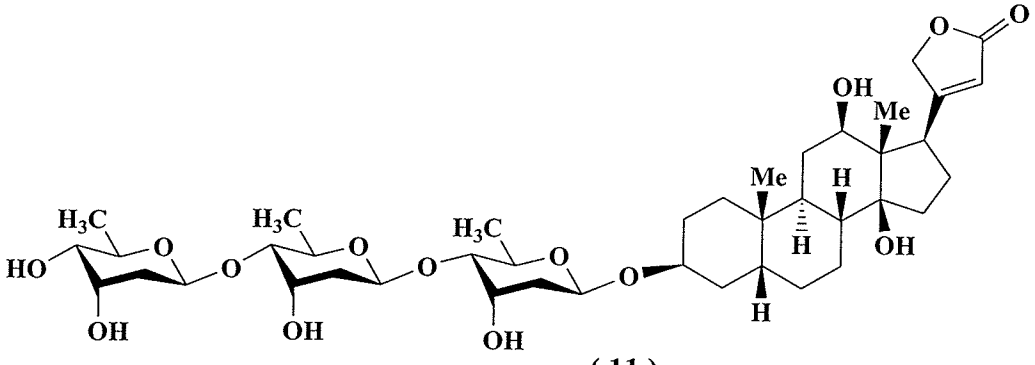
1.1.3 Anti-infectives

1.1.4 Anticancer Drugs

1.1.5 Immunomodulators

1.1.1 Cardiovascular Drugs

Increasing the force of contraction of the heart (positive inotropic activity) is very important for most heart failure patients. Cardiac glycosides including digoxin (**11**),



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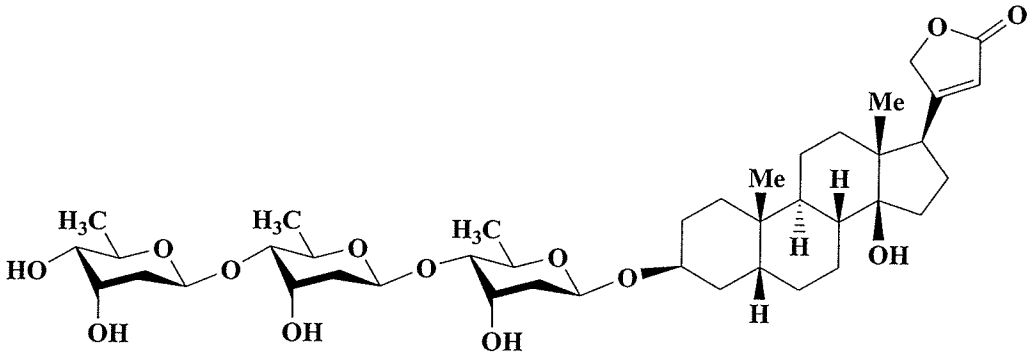
digitoxin (**12**), and deslanoside (**13**), exert a powerful and selective positive inotropic action on the cardiac muscle.



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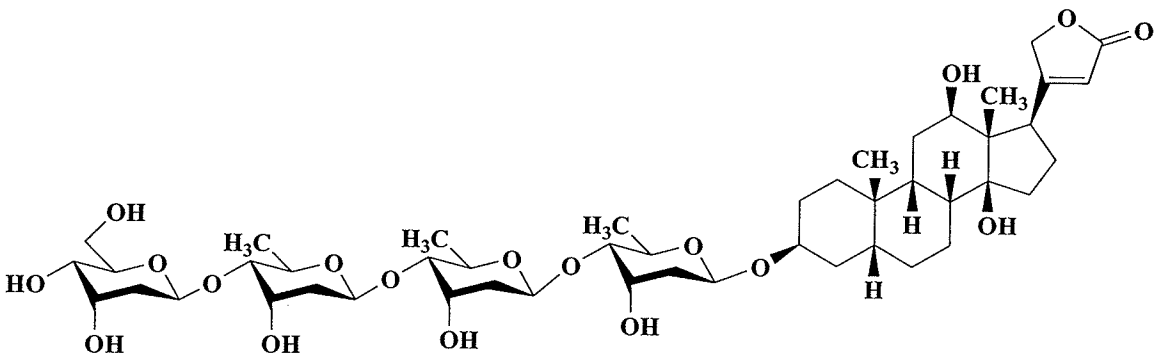

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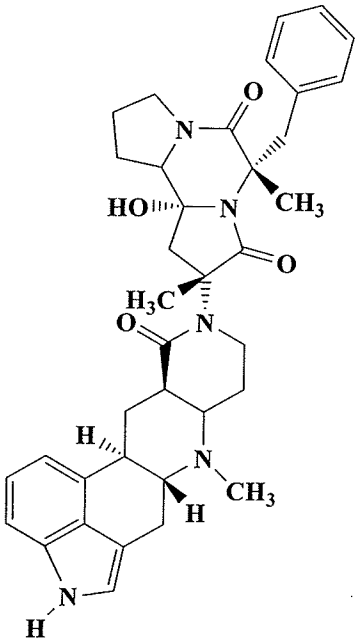
A number of naturally occurring alkaloidal drugs are also being used in the control of various cardiovascular conditions. For instance ergotamine (**14**), an ergot alkaloid obtained from a fungus that infects rye grass, is an important central vasoconstrictor and is used therapeutically to treat migraine headaches.



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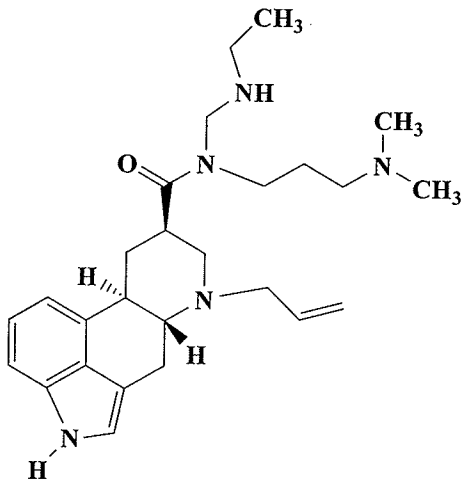
The new long-acting ergoline derivative cabergoline (**15**), launched as an antiprolactin, one of the dopamine D_2 receptor agonists, is also being evaluated as a potential therapy for Parkinson's disease and as an anticancer agent for the treatment of breast cancer.[7]



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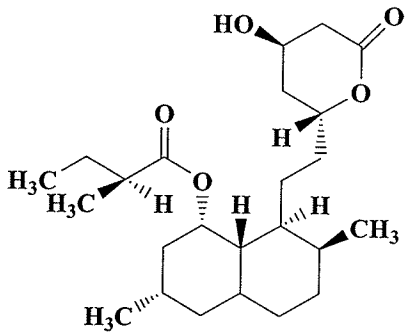
Most of the cholesterol lowering drugs are derived from fungi. They act by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), an enzyme critical in the biosynthesis of cholesterol. For example, Lovastatin (**16**), isolated from the fungus *Aspergillus terreus* inhibits 3-hydroxy-3-methylglutaryl coenzyme A.[8]



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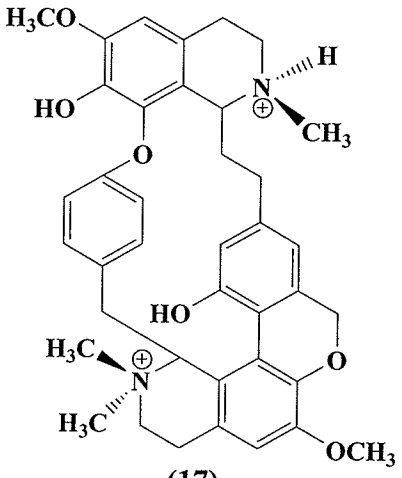
1.1.2 Central Nervous System Drugs

A number of natural products have been reported to exhibit central nervous system (CNS) activity. Examples include, *d*-tubocurarine (**17**), a neuromuscular blocker which was isolated from the South American plant, *Curare*.[3] The opium alkaloids, codeine and morphine (**3**), served as models for the synthesis of naloxone (**18**), an important analog used to treat and diagnose opiate addicts.[9]



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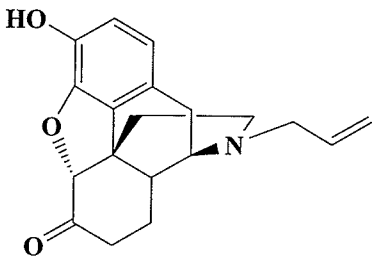

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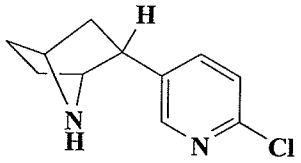
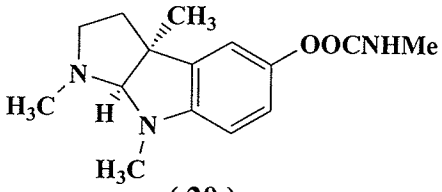
(+)-Epibatidine (**19**), isolated from the skin of the poisonous frog, exhibits exceptional analgesic activity and is a very potent nicotinic acetylcholine receptor (nAChR) agonist. When it binds to the specific receptor, it blocks any neural transmission, thus no pain is felt. Further synthetic studies, have revealed that both enantiomers are potent analgesic agents.[10]



(19)

Physostigmine (**20**), a naturally occurring alkaloid, and its ester, neostigmine are also important acetycholinesterase inhibitors which are used for the treatment of myasthenia gravis and as antagonists to neuromuscular blockade by nondepolarizing blocking agents.



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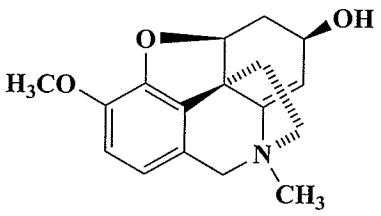

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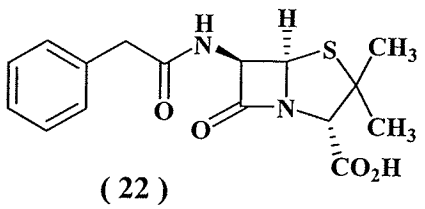
The acetylcholinestrase inhibitor, galanthamine (**21**) is an alkaloid that occurs in the bulbs of daffodils, and is investigated as a possible therapy for cognitive impairment in Alzheimer's disease.[11]

1.1.3 Anti-infectives

Antibiotics are among the most important classes of therapeutic agents and have had enormous impact on both life expectancy and quality of life. With the discovery of the naturally occuring penicillin (**22**), the course of medical history was dramatically changed and the antibiotic era was introduced. Not only these antibiotics serve as important drugs, but also help in the explorations of the mode of action. This type of study helps to explore the utilization of natural products and their analogs as anti-infective agents.



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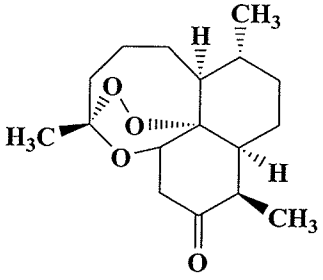
New important anti-infectives are being discovered from microbial, plant, and animal sources. For example, the antimalarial agent, artemisinin (**23**), a peroxy-bridge containing diterpene was isolated from the Chinese medicinal plant, *Artemisia annua.* This plant had been used in China for centuries to cure malaria by the indigenous people. In 1972, the active constituent was isolated and identified as the sesquiterpene endoperoxide, artemisinin (**23**). This compound has shown antimalarial activity against those malaria causing strains of *Plasmodium* spp. that have developed resistance to the currently available drugs. During structure activity relationship studies of compound (**23**), artemether (**24**) has been discovered as one of the most effective antimalarial agents. From extensive studies has emerged artemether (**24**), a derivative that is currently approved for the treatment of malaria in major parts of the world.[12]



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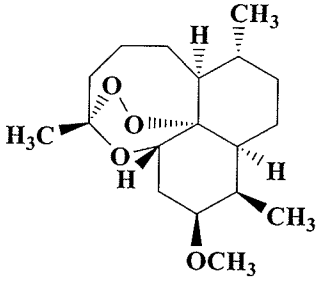

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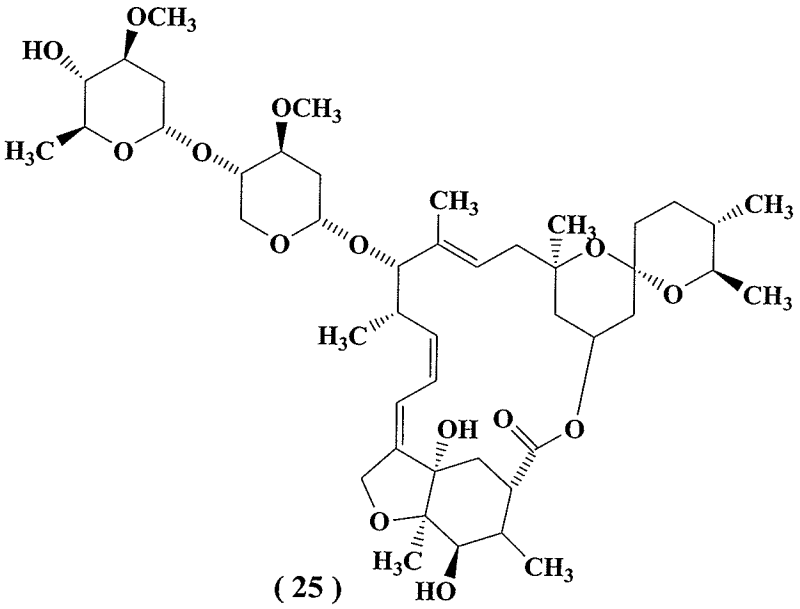
Another important class of anti-infective natural product is the avermectins. This class of compounds has polyketide biosynthetic origin and was isolated from several species of *Streptomyces.*[13]

The major drug of this class, ivermectin (**25**), was originally developed to control nematodes and parasites of livestock.



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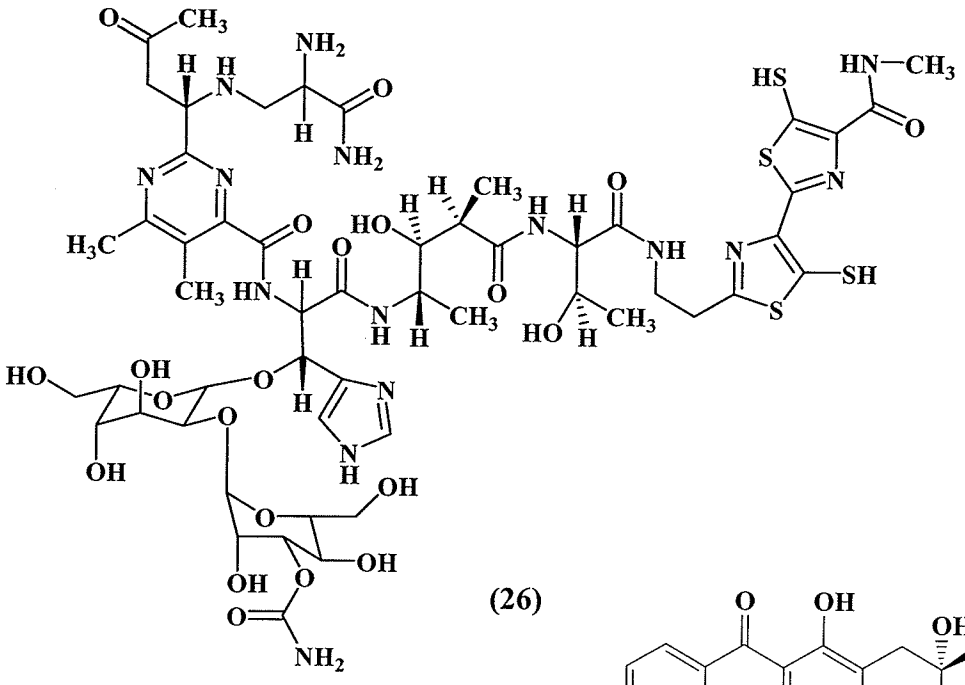
1.1.4 Anticancer Drugs

It is another area of interest for natural product chemists to work on naturally occurring new bioactive chemotherapeutic drugs.[14] The purpose in the search for new anticancer drugs from marine, plant and microorganisms is to discover natural products having specific mode of action. Their target should be the cancerous cells and having little or no harm to healthy cells. A number of natural products or compounds derived from natural products having potential application in the treatment of cancer include, such as bleomycin (**26**), doxorubicin (**27**), daunorubicin (**28**), vincristine (**29**), vinblastine (**30**), mitomycin (**31**), streptozocin (**32**), and paclitaxel (Taxol™) (**33**).



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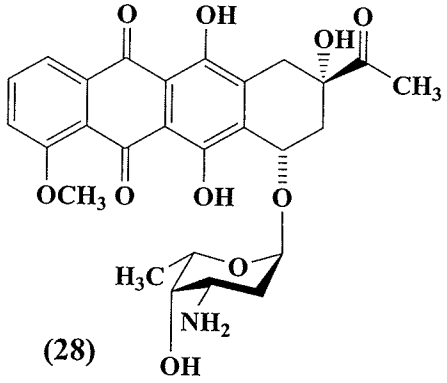

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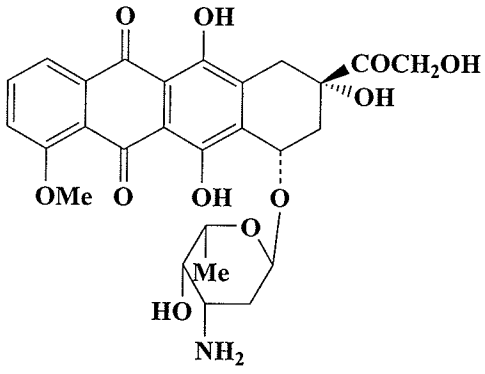

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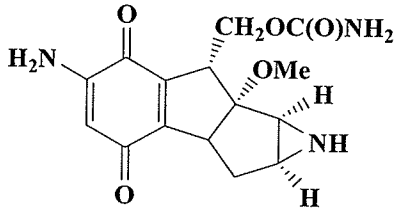

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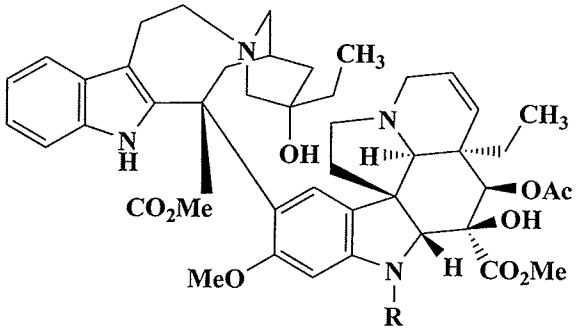
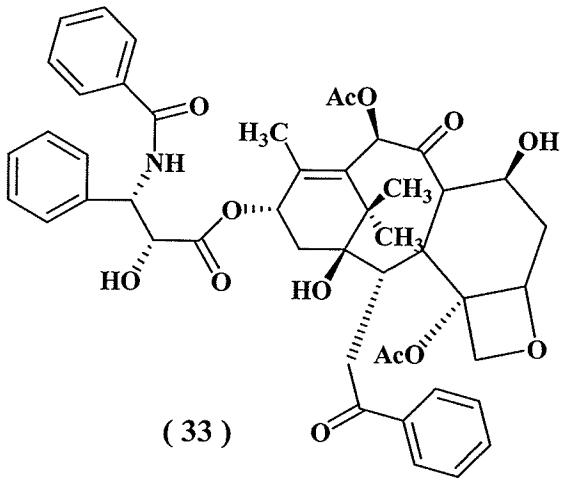


(29)R= CHO,Vincristine
(30)R= Me, Vinblastine



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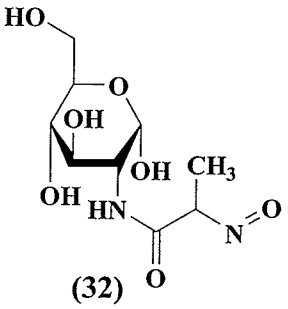

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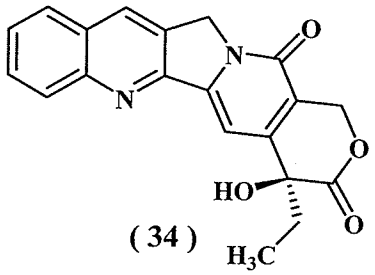
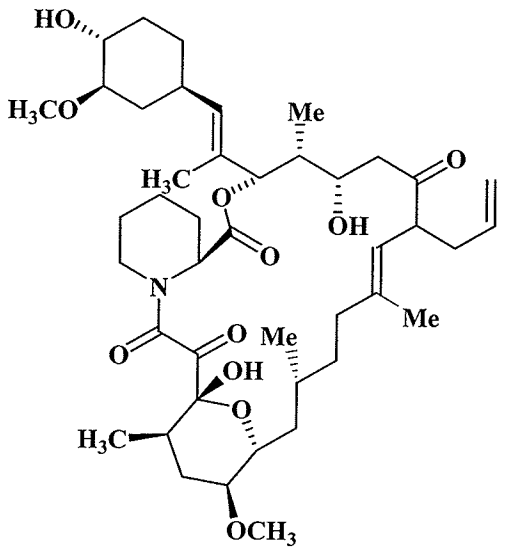


(34)

Studies on the mechanism of anticancer action of paclitaxel revealed that it blocks depolymerization of microtubules.[15] Another important plant-derived anticancer compound is camptothecin (**34**), an alkaloid isolated from the Chinese tree, *Camptotheca acuminate*.[16]

1.1.5 Immunomodulators

A drug used for its effect on the immune system: drugs may be known as immunosuppressants or immunostimulators. Cyclosporin (**35**) is commonly used as an



(35)

Immunomodulator. This compound was isolated from a soil fungus, *Trichoderma polysporum*.[17] Tacrolimus (FK-506) (**35**), a secondary metabolite of *Streptomyces tsukabaensis,* was also use as an immunosuppressant in organ transplantation.[9]

Based on the importance of natural products in drug discovery program, the present project was designed to isolate bioactive compounds from *Pleurotus* sp. and *Buxus hyrcana*. In addition microbial transformation of sclareol was performed to produce a library of compounds to screen them for their antibacterial acitivity.

1.2 References:

1. Farnsworth, N. R.; Akerele, O.; Bingel, A.S; Soejarto, D.D.; Guo, Z. *Bull. WHO,* **1985**, *63*, 965.
2. Soejarto, D. D.; Farnsworth, N. R. *Perspectives Biol. Med.* **1989**, *32*, 244.
3. Tyler, V. E.; Brady, L. R.; Robbers, J. E. *Pharmacognosy*, Lea & Febiger, Philadelphia, **1988**.
4. Fujioka, T.; Kashiwada, Y. *J. Nat. Prod.* **1994**, *57*, 243.
5. Farnsworth, N. R.; Morris, R. W. *Am. J. Pharm.* **1976**, *148*, 46.
6. Henry, C. M. *Chem. Eng. News,* **2000**, *6*, 41.
7. Cheng, X. M. To Market, To Market-1993. In J. A. Bristol, *Annual Reports in Medicinal Chemistry*, Academic Press, **1994**, *29*, 331.
8. Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, C.; Monghan, S. Currie; Stapley, E.; Schonberg, G. A.; Hensens, O.; Hirshfield, J.; Hoogsteen, K.; Liesch, J.; Springer, J. *Proc. Natl. Acad. Sci.* USA. **1980**, *77*, 3957.
9. Hardman, J. G.; Limbird, L. E.; Molinoff, P. B.; Ruddon, R. W.; Gilman A. G., *The pharmacological Basis of Therapeutics*, McGraw-Hill, New York, **1996**.
10. McDonald, I. A.; Cosford, N.; Vernier, J. M. *Nicotinic Acetylcholine Receptors: Molecular Biology, Chemistry and Pharmacology*. In J. A. Bristol (ed.), *Annual Reports in Medicinal Chemistry*, Academic Press, **1995**, *30*,145.
11. Hieble, J. P.; Ruffolo, R. R. Pharmacology of Neuromuscular Transmission. In P. L. Munson, R. A. Mueller, and G. R. Breese (eds.), *Principles of*

Pharmacology: Basic Concepts & Clinical Applications, Chapman & Hall, New York, **1995**, 145.

12. Klayman, D. L.; Lin, A. J.; Acton, N.; Scovill, J. P.; Hoch, J. M.; Milhous, W. K.; Theoharides, A. D.; Dobek, A. S. *J. Nat. Prod.* **1984**, *47*, 715.
13. Davies, H. G.; Green, R. H. *Nat. Prod. Repts.* **1986,** *3,* 87.
14. Loo, I. T. L.; Freireich, E. J. *Cancer Chemotherpeutic Drugs*. In P. L. Munson, R. A. Mueller; G. R. Breese (eds.), *Principles of Pharmacology: Basic Concepts & Clinical Applications*, Chapman & Hall, New York, **1995**, 1475.
15. Schiff, P. B.; Fant, F.; Horwitz, S. B. *Nature,* **1979**, *277*, 665.
16. Wall, M. E.; Wani, M. C.; Cooke, C. E.; Palmer, K. H.; McPhail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3388.
17. Ruegger, A.; Kuhn, M.; Lichti, H.; Loosli, H. R.; Huguenin, R.; Quiqerez, C.; Wartburg, A. V. *Helv. Chim. Acta*. **1976**, *59*, 1075.

CHAPTER 2

CHEMICAL STUDIES ON *PLEUROTUS* SPECIES

2.0 Introduction

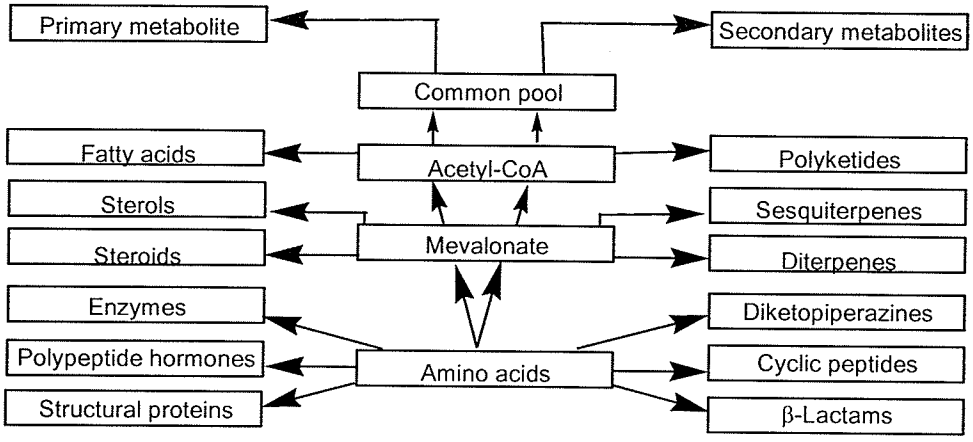
Mushrooms have been found in fossilized wood 300 million years old.[1] Almost categorically ancient people used mushrooms as a food. The great early civilizations of the Greeks, Egyptians, Romans, Chinese and Mexicans valued mushrooms as a delicacy, appreciated their curative nature and, in some cases, used them in religious rites. Literature references indicate[2] that *Auricularia auricula* was cultivated on wood logs in China as early as 600 A.D. Other wood-rotting mushrooms such as *Flammulina velutipes* and *Lentinula edodes* were later grown in similar manner, but the biggest advance in mushroom cultivation came in France around 1600, when *Agaricus bisporus* was cultivated upon a composted substrate. In the Western world *A. bisporus* (champignon or button mushroom) has remained the mushroom that is produced in the greatest amounts. Mushroom cultivation provides both nutritious protein rich food and medicinal products. Cultivated mushrooms have now become popular all over the world. In 1994, the total world production of edible and medicinal mushrooms was estimated to be over five million tonnes, with a value of over 14 billion US dollars.[3] Mushrooms, like all other fungi, lack chlorophyll. They are unable to convert solar energy to organic matter like green plants, but they can convert the huge amount of agricultural and forest waste materials into human food. The byproducts, spent substrates, can be used as animal feed and crop fertilizers. Therefore, sustainable development of mushroom cultivation can be called the "non-green lignocellulosic revolution", because mushroom cultivation can generate equitable economic growth and protect and regenerate the environment.

2.0.1 Classification:

Mushrooms can be divided into four categories: (a) those that are edible and fleshy fall into the edible mushroom category, e.g. *Agaricus bisporus*; (b) those that are considered to have medicinal applications are referred to as medicinal mushrooms, e.g., *Ganoderma lucidum*; (c) those that are proven to be, or suspected of being, poisonous are called poisonous mushrooms or toadstools, e.g., *Amanita phalloides*; (d) a miscellaneous category that may tentatively be grouped together as 'other mushrooms'. This way of classifying mushrooms is by no means absolute since many kinds of mushrooms are not only edible but also possess tonic and medicinal properties. It has been suggested that over 10000 species produce fruiting bodies of sufficient size and suitable texture to be considered as 'macrofungi'.[4]

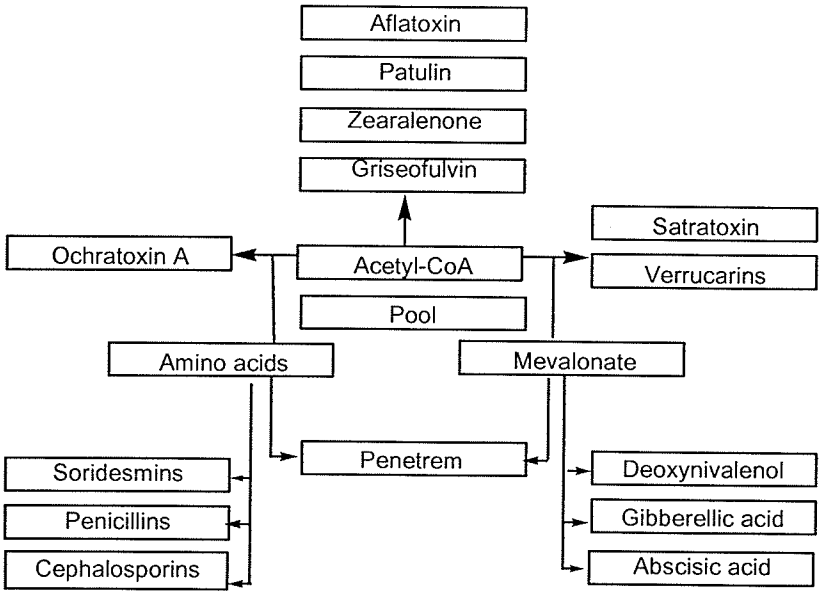
2.0.2 Mushroom Metabolites

Primary metabolism is directly associated with the energy and material requirements of growth and is characterized by the synthesis of proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), polysaccharides and lipids, from a relatively small pool of precursors common to all forms of life on this earth. By contrast, secondary metabolism is not directly associated with growth and involves the formation of a wide diversity of complex, low-molecular weight compounds by the manipulation and transformation of the same pool of metabolites used in primary metabolism (Scheme 2.1).[5]



Scheme 2.1 Primary and secondary metabolism from the same pool of intermediates acetyl-CoA

The biosynthesis of a particular secondary metabolite is often strain-specific, and is usually sensitive to environmental and nutritional conditions. For instance, the diversity of secondary metabolites which are derived from acetyl-CoA, mevalonate and amino acids or combination of them can be visualized in (Scheme 2.2).[5]

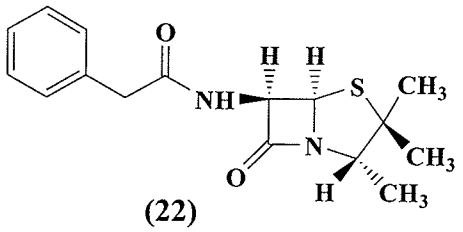


Scheme 2.2 Examples of secondary metabolites derived from the pool of intermediates. acetyl-CoA.

2.0.3 Medicinal Uses of Fungal Metabolites

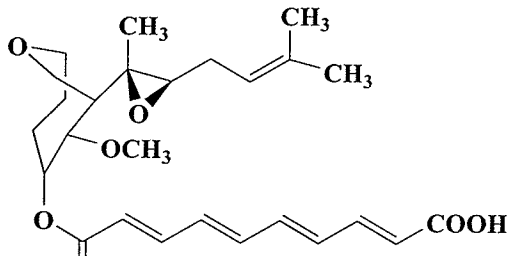
2.0.3.1 Antibiotics

The discovery of the potential of fungi to produce antibiotics is usually credited to Sir Alexander Fleming. In 1928 he observed that a zone clear of colonies of the bacterium *Staphylococcus* surrounded a site of chance contamination of a culture by the mould *Penicillium chrysogenum*. Strains of this same species are still used for the industrial production of benzylpenicillin (**22**), which is a raw material for the synthesis of the large family of modern penicillins. The original natural penicillins had a rather narrow spectrum of activity and bacteria soon acquired resistance to them. Both of these problems were overcome by synthesizing the unnatural analogs of naturally occurring penicillin.[6]



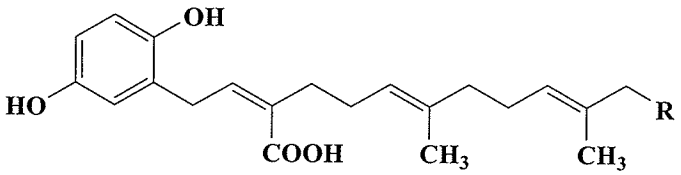
(22)

Fumagillin (**36**), another antibiotic produced by *Aspergillus fumigatus*, is effective against diseases caused by protozoa and and amoebae.

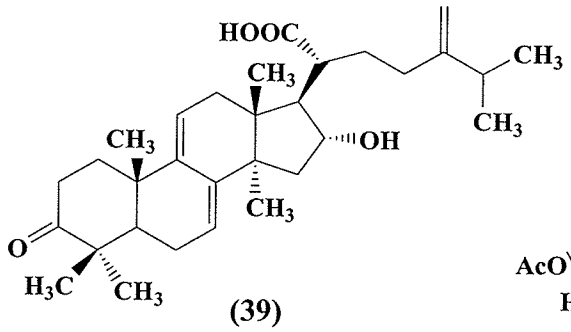


(36)

Two secondary metabolites, ganomycin A (**37**) and ganomycin B (**38**), isolated from *G. pfeifferi* showed moderate antibacterial activity against several bacterial strains.[7] Lanostanoid derivatives, polyporenic acid C (**39**), 3R-acetyloxylanosta-8,24-dien-21-oic acid (**40**), isolated from the polypore *Fomitopsis pinicola* have shown antimicrobial activity against *Bacillus subtilis,* with minimum inhibitory concentration (MIC) values of 0.4-10 μg/mL.[8]



(37) R=OH, (38) R=H



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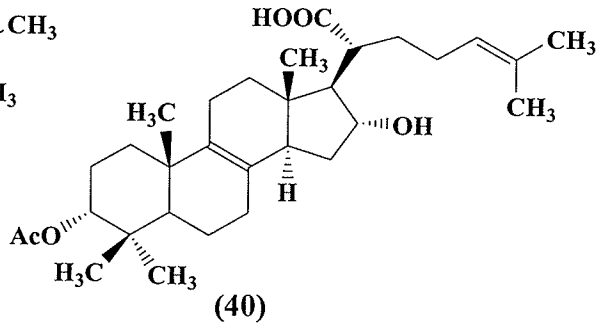

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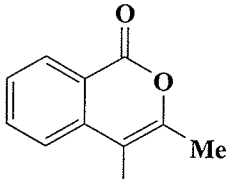
2.0.3.2 Antifungal Natural Products

The antifungal isocoumarin, oospolactone **(41),** was identified as a secondary metabolite of *Gleophyllum sepiarium.*[9] This compound is most active against strains of the asexual ascomycete *Alternaria*, showing MIC values of 12.5-25 µg/ml.



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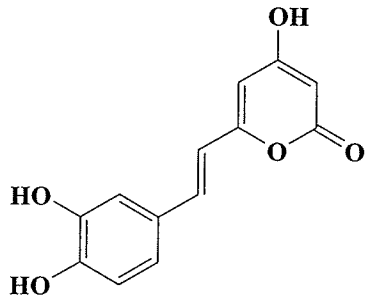
2.0.3.3 Antiviral Metabolites

Brandt and Piraino divided the antiviral compounds from fungi into two major classes: (a) those that act indirectly as biological response modifiers (usually from polysaccharide fractions), (b) Those that act directly as viral inhibitors.[10]

Two phenolic compounds, hispolon (**42**) and hispidin (**43**) isolated from the basidiocarps of *Inonotus hispidus* showed considerable antiviral activity against influenza viruses types A and B in the concentration of 10 mg/ml and 40 mg/ ml respectively.[11]



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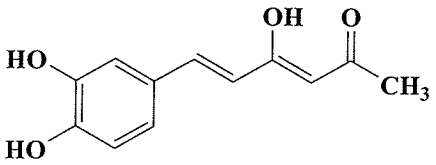
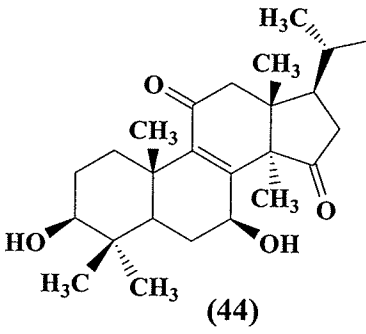


(43)

The fruiting bodies of *Ganoderma lucidum* are the source of antiviral triterpenoids. For instance, Ganoderic acid β (**44**), isolated from the spores of *G. lucidum* showed significant anti HIV-1 protease activity; with an IC_{50} value of 20 μM.[12] The same species also produced ganoderiol F (**45**), which inhibited the enzyme activity to a similar extent (IC_{50}= 0.18 ± 0.32 μM).[13]



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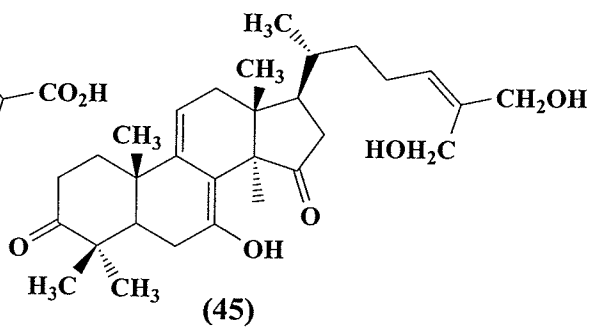

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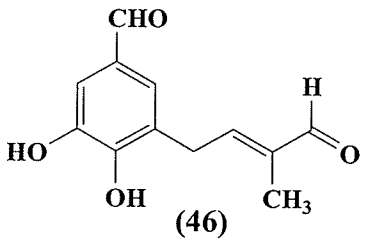
2.0.3.4 Cytotoxic Naturally Occurring Compounds

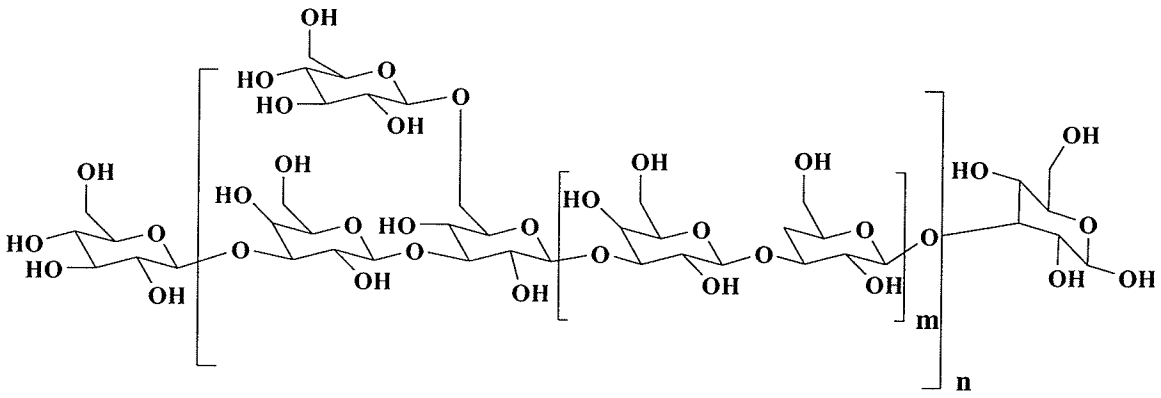
The cytotoxic activities of polypore extracts are mostly associated with the presence of polysaccharides, although numerous smaller molecular weight cytotoxic polypore metabolites also are known.[14]

A rare example of a cytotoxic monoterpene is montadial A **(46)** isolated from the polypore *Bondarzewia montana*[15]. Montadial A is cytotoxic against lymphocytic leukemia L1210 cells in mice at a concentration of 10 μg/mL as well as against promyelocytic human leukemia HL60 cells at 5 μg/mL.



(46)

The vast majority of antitumor β-D-glucans isolated from polypores are *β*-(1→3)-D-glucopyranans with 500000-2000000 mean molecular weight and having characteristic β-(1→6)-D-glucosyl branches (**47**). The level of their activity is closely related to their molecular weight, branching, and solubility in water. Among these preparations, higher antitumor activity is correlated with the higher molecular weight, lower level of branching, and greater water solubility of β-glucans.[16]



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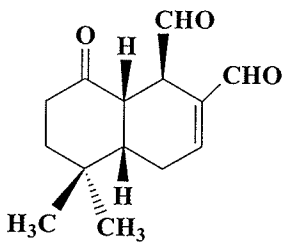
2.0.3.5 Compounds with Miscellaneous Biological Activities

An Australian polypore species of *Panus* biosynthesizes the drimane sesquiterpene panudial (**48**). This compound, with an A/B ring *cis* junction, is isomeric with kuehneromycin B (**49**), isolated from other fungi in addition to polypores. Panudial (**48**) is a potent inhibitor of bovine and human ADP-stimulated platelet aggregation, with IC_{30} values of 2.5 and 6 μg/mL, respectively.[17]



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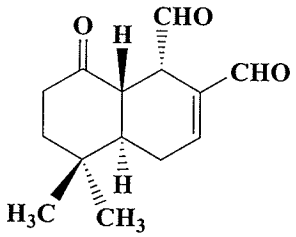

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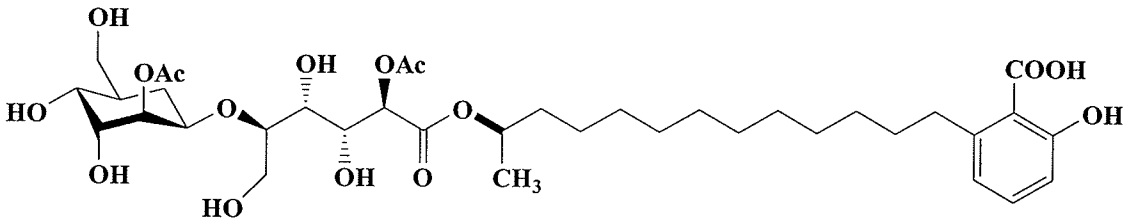
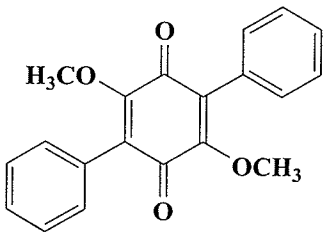
Some secondary metabolites from polypores are also known to exhibit enzyme inhibition. Caloporoside (**50**) isolated from *Caloporus dichrous* inhibits pig's brain phospholipase.[18]



(50)

Two lipid peroxidation inhibitors, betulinans A (**51**) and B (**52**), were isolated from the methanol extract of *Lenzites betulina*.[19] Peroxidative damage of cells and organellar membranes by free radicals has been implicated in pathogenesis of various diseases such as atherosclerosis, arthritis, myocardial ischemia, and cancer. Betulinan A (**51**) was about 4 times more active as a radical scavenger than vitamin E.



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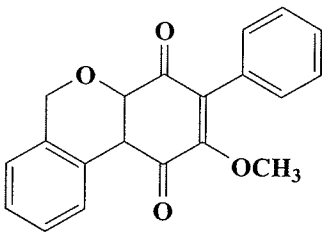


(52)

2.0.3.6 Importance of Mushroom in Agriculture and Forestry

The ability of fungi to kill insect pests and weeds offers an environment-friendly alternative to pesticides. The use of one natural organism to attack another is called biological control. Fungi have proved effective against Gypsy Moth, Colorado Potato Beetle, aphids, whitefly, and mosquito larvae, to name a few. Fungal agents have also controlled weeds including Vetch, Dodder, Bindweed, and Thistle. Some fungi can even control other troublesome fungi.

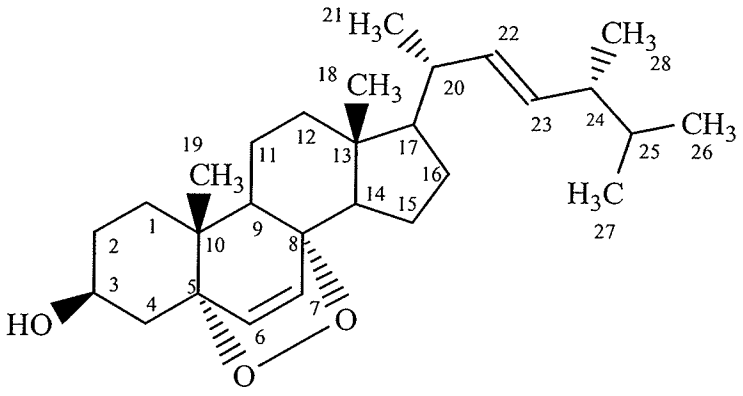
Thus historically, medicinal mushrooms (basidiomycetes) have been shown to have profound health promoting benefits, as we had discussed above, which are now confirming their medical efficacy and identifying many of the bioactive molecules. These facts support the need to further explore the utility of mushroom which is mainly focused on its medicinal aspects having least side effects while interacting in the body to combat the disease.

Based on the importance of mushroom, the present study was conducted to do the chemical investigations on *Pleurotus* sp. and to isolate antibacterial compounds.

2.1 RESULTS AND DISCUSSION

Isolation of Ergosterol Peroxide from *Pleurotus* species

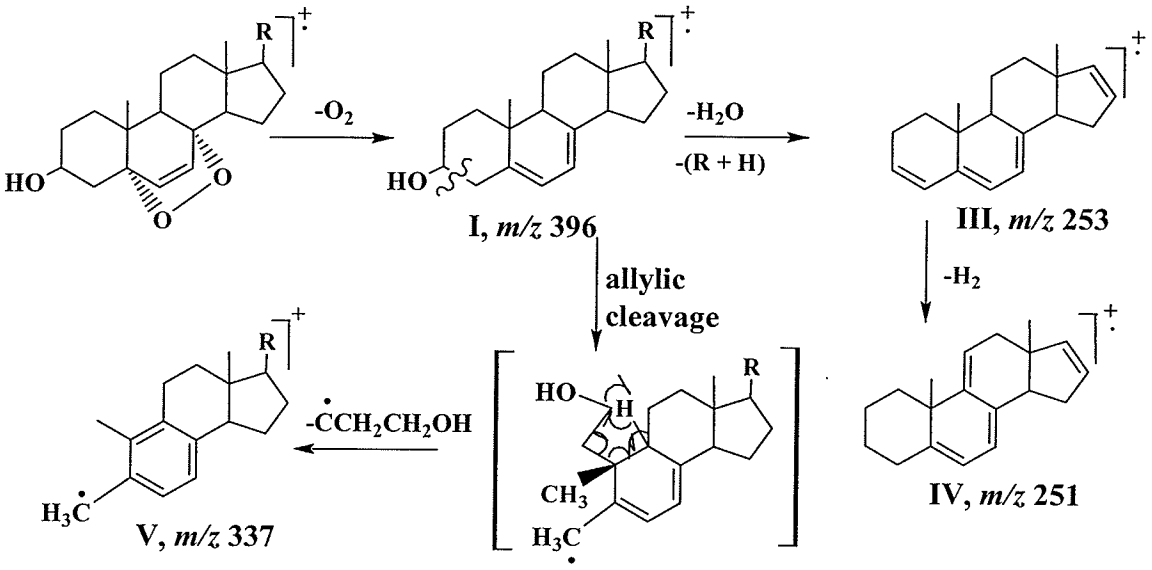
The methanolic and ethyl acetate extract of the *Pleurotus* sp. was subjected to silica gel chromatography with *n*-hexane-ethyl acetate as eluent to give four fractions F_a-F_d. The fraction F_a (See **2.2** experimental) was subjected to preparative TLC on silica plates with *n*-hexane-ethyl acetate (4:6) as eluent to afford compound (**53**) as amorphous white solid.



Ergosterol Peroxide (53)

The IR spectrum of (**53**) showed intense absorption bands at 3386 (OH) and 1180 (C-O) cm^{-1}. Its UV spectrum displayed terminal absorption indicating the lack of a conjugated π-system in the molecule. The electron-impact mass spectrum (EIMS) showed the molecular ion peak at *m/z* 428 and chemical ionization (CI) mass spectrum showed quasi molecular ion $[M+1]^+$ peak at *m/z* 429. The EIMS showed the base peak at *m/z* 410 due to loss of a water molecule. These mass spectra in combination with NMR data provided molecular formula $C_{28}H_{44}O_3$. This molecular formula indicated the presence of seven degrees of unsaturation in the molecule (**53**), six of them were accounted for the steroidal skeleton having two double bonds at C-6/C-7 and C-22/C-23. The seventh degree of

unsaturation was due to the endoperoxide ring at C-5 and C-8. Additionally an intense ion at *m/z* 396 was due to the loss of O_2 from the molecular ion a characteristic feature of epidioxide.[20] Further peaks at *m/z* 253 and 251 indicated the presence of a steroidal skeleton. The most of mass fragments of this compound were found to be similar to those having epidioxy functionality at C-5 and C-8. [21-23] The detailed mass fragmentation of compound **(53)** is shown in scheme (Scheme 2.3).



Scheme 2.3 Mass Fragmentation of 53

The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of **53** revealed the presence of two 3H singlets δ 0.83 and δ 0.88 were assigned to the tertiary C-18 and C19 methyl group protons. Four doublets, integrating for 3H each, at δ 0.84 (J = 6.6 Hz), 0.81 (J = 7.1 Hz), δ 0.91 (J = 6.6 Hz) and δ 1.02 (J = 6.6 Hz) were due to C-26, C-27, C-28 and C-21 secondary methyl protons, respectively. The olefinic C-22 and C-23 methine protons resonated at δ 5.22 and 5.15 respectively. Two signals at δ 6.25 (1H, *d*, J= 8.4Hz) and

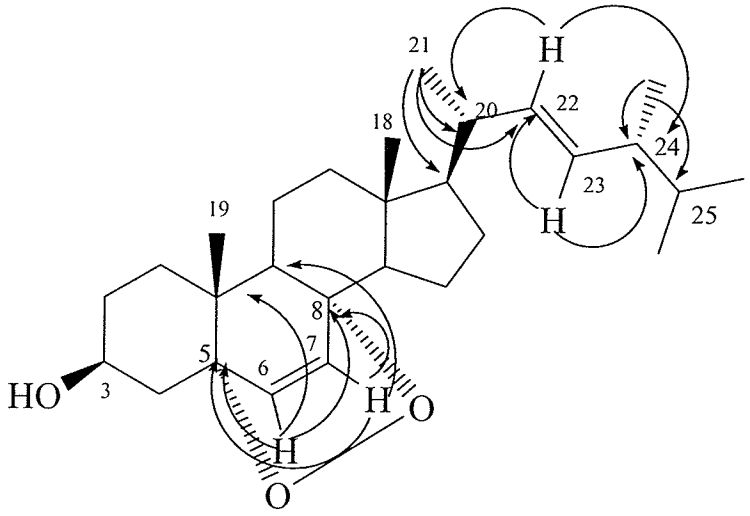
6.50 (1H, *d*, *J*= 8.4 Hz) were assigned to the olefinic protons C-6 and C-7. The multiplicity of these signals indicated that C-5 and C-8 were quaternary carbons. This quaternary nature of these carbons was presumably due to the presence of endoperoxide at these two carbon atoms.

The broad band decoupled ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of **(53)** showed the resonances of all twenty eight carbon atoms. The DEPT spectra indicated that there were six methyl, seven methylene and eleven methine carbon atoms in compound **(53)**. The subtraction of the DEPT spectrum from the broad band decoupled ^{13}C-NMR spectrum showed the presence of four quaternary carbon atoms. Two aliphatic downfield signals at δ 79.3 and 82.1 were assigned to the quaternary carbon atoms C-5 and C-8, respectively. Their downfield values indicated that both C-5 and C-8 were substituted with oxygen atom in the form of endoperoxide. Four downfield olefinic signals at δ 135.1, 130.7, 135.3 and 132.2 were assigned to C-6, C-7, C-22 and C-23, respectively. Another downfield signal at δ 66.3 was assigned to C-3. Complete ^{13}C-NMR chemical shift assignments of compound **(53)** are shown in Table 1.

One-bond ^{1}H/^{13}C one-bond correlations were established by heteronuclear single quantum coherence (HSQC) NMR experiment. The HSQC spectrum showed one-bond correlations of H-3 (δ 3.96), H-6 (δ 6.25) and H-7 (δ 6.50) with C-3 (δ 66.3), C-6 (135.1) and C-7 (δ 130.7) respectively. The chemical shifts and various proton-carbon connectivities are given in Table 1.

The long-range ^{1}H/^{13}C connectivities were determined from HMBC spectrum and this spectrum was very useful in determining the ^{13}C-NMR chemical shift assignments of quaternary carbon atoms. Two protons resonating at δ 6.25 (H-6) and δ 6.50 (H-7)

showed cross peaks with the quaternary carbons C-5(δ 79.3) and C-8 (δ 82.1) confirming that C-6 and C-7 were connected to C-5 and C-8. These HMBC observations suggested that peroxide was present at C-5 and C-8. The C-22 methine proton (δ 135.3) showed long–range heteronuclear couplings with the C-20 (δ 39.6) and C-24 (δ 42.7). Important HMBC interactions of compound **(53)** are shown around structure **(53a)**.



The HMBC correlation for (**53a**)

Based on these spectroscopic studies, compound **(53)** was characterized as an ergosterol peroxide (3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene). The ^{1}H and ^{13}C-NMR data of **53** were distinctly similar to those of 3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene, reported in the literature.[24-25]

Previously, this was isolated from the fungi, *Daedalea quercina* L,[26] *Aspergillus fumigatus*,[27] *Trichophyton schonleini*,[28] *Rhizoctonia repens*,[29] *Cantharellus cibarius*,[30] *Lampteromyces japonicus*,[31] *Penicillium rubrum*[32] and *Basidiomycete* mushroom, *Scleroderma polyrhizum*.[33]

Table 1.^{1}H NMR and ^{13}C NMR spectral data of 53 in ($CDCl_3$)

Carbon No.	^{13}C-NMR Chemical Shifts (δc)	^{1}H-NMR Chemical Shifts $\delta_{H,}$ *J* (Hz)
C-1	34.6	
C-2	30.0	
C-3	66.3	3.96 (*m*)*
C-4	36.8	
C-5	79.3	
C-6	135.1	6.25 (1H, *d*, *J*= 8.4)
C-7	130.7	6.50 (1H, *d*, *J*= 8.4)
C-8	82.1	
C-9	51.0	
C-10	39.3	
C-11	20.6	
C-12	39.3	
C-13	44.5	
C-14	51.6	
C-15	23.4	
C-16	28.6	
C-17	56.1	
C-18	12.8	0.83(*s*)
C-19	18.1	0.88(*s*)
C-20	39.6	
C21	20.8	0.99 (3H, *d*, *J*= 6.6)
C-22	135.3	5.22 (1H, *dd*, *J*= 7.74)
C-23	132.2	5.15 (1H, *dd*, *J*= 8.29)
C-24	42.7	
C-25	33.0	
C-26	19.6	0.84 (3H, *d*, *J*= 6.4)
C-27	19.9	0.81 (3H, *d*, *J*= 7.1)
C-28	17.5	0.91 (3H, *d*, *J*= 6.6)

* (*s*)=singlet; (*d*)=doublet; (*dd*)=doublet of doublet; (*m*)=multiplet

2.1.1 Antibacterial Activity

The compound **(53)** was evaluated for antibacterial activity against *Staphylococcus aureus* var. gold, *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Corynebacterium xerosis,* using the disc diffusion Kirby-Bauer method.[34] The ergosterol peroxide **(53)** exhibited weak antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Corynebacterium xerosis.* The antibacterial data of **(53)** is shown in Table 2.

Table 2 Antibacterial Activities of Ergosterol Peroxide (53) measured in mm after incubating 24 h at 37 °C

Bacterial Strain	Concentrations	
	125 μg	250 μg
S. aureus	6.5	7.0
E. coli	-	-
P. aeruginosa	-	-
B. subtilis	0.0	7.0
C. xerosis	0.0	6.9

2.2 EXPERIMENTAL

2.2.1 General Experimental Conditions

UV spectra were recorded on a Shimadzu UV-240 spectrophotometer. Mass spectra were obtained on a Hewlett Packard 5970 Series (mass selective detector) mass spectrometer. The ^{1}H- and ^{13}C-NMR, spectra were recorded on Bruker 500 MHz spectrometer in $CDCl_3$, at 500 MHz and 125 MHz, respectively. Chemical shifts were recorded in (δ). IR spectra were recorded on a Hartmann and Braun, Bomem, MB-series spectrophotometer The column chromatography was carried out on silica gel 200-400 mesh 60 °A. The purity of the samples was checked on precoated silica gel TLC plates (Merck Kieselgel 600 A° F_{254}). The UV light (λ 254 nm) and 10% H_2SO_4 solution spray reagent were used to visualize these TLC plates.

2.2.2 Fungal Material

Fresh mushrooms, *Pleurotus species* were collected from Antigonish, Nova Scotia during Fall 2003 and these were identified at the Department of Biology, University of Winnipeg.

2.2.3 Extraction and Isolation

The fresh mushroom (100 g) was diced up into small pieces. These pieces were extracted with methanol (2 x 250 ml) followed by extraction with ethyl acetate (250 mL) at room temperature. The extracts were filtered and the filtrate was concentrated under reduced pressure to yield a gum like residue.

2.2.4 Chromatographic Techniques

The ethyl acetate and methanol extract (15 g) was loaded onto a silica gel column. The column was eluted with mixtures of *n*-hexane-ethyl acetate (0-100 %) and then with ethyl acetate-methanol (0-100 %) to get 126 fractions. Then all of the fractions were concentrated under reduced pressure. The analytical TLC systems were used to pool fractions showing spots of same R_f values. This yielded four fractions F_a, F_b, F_c and F_d. Fraction F_a showed the presence of one major compound and (F_b to F_d) minor compounds on analytical TLC. Other fractions showed the presence of polysaccharides. The fraction F_a was subjected to preparative TLC using *n*-hexane-ethyl acetate (4:6) as eluant to afford 7.5 mg of ergosterol peroxide **(53),** as colorless amorphous powder. After spectroscopic studies this compound was identified as ergosterol peroxide **(53)**.

3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene (53): amorphous white powder; IR(KBr) ν_{max} 3386, 2956, 2931, 2871, 1458, 1378, 1044, 967 cm $^{-1}$; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: = See Table 2; ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: = See Table -2

EI-Mass *m/z*: 428(M^+, $C_{28}H_{44}O_3$), 396($C_{28}H_{44}O$, M^+-O_2), 253($C_{19}H_{25}$, M^+-O_2-H_2O-C_9H_{17}), 251($C_{19}H_{23}$, M^+-O_2-H_2O- C_9H_{17}-H_2).

2.2.5 Antibacterial Activity

Ergosterol peroxide **53** was evaluated for antibacterial activity against *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633) and *Corynebacterium xerosis, Escherichia coli* (ATCC 25933) by Kirby-Bauer disc method.[34] These bacterial strains were obtained from the Department of Biology, the University of Winnipeg. Blank paper discs, 6 mm diameter (BBL™) were

impregnated with 125 μg and 250 μg of ethyl acetate solution of ergosterol peroxide **(53)**. The ethyl acetate was evaporated. Two discs having ergosterol peroxide in two different concentrations (125 μg, 250 μg) were placed on the Mueller Hinton agar (preparation of media is described below) inoculated with specific bacterial strain. This was repeated for all five different bacterial strains. These plates were then incubated for 24 hours at 37 °C. The antibacterial activity was determined by measuring the diameters of the inhibitory zone around each disc.

2.2.5.1 Preparation of Media

The Mueller Hinton Agar (19 gm) was dissolved in 500 ml of distilled water. The medium was then sterilized in an autoclave at a temperature of 121 °C and pressure 15 psi for 20 minute. After cooling to about 55 °C, the media was poured into plates. These plates were then stored in refrigerator for antibacterial bioassay.

For the preparation of broth, Muller Hinton (2.625 gm) was dissolved in 125ml water. After sterilization, the medium was allowed to cool before addition of bacteria.

2.3 References:

1. Editorial, The magic of mushroom, *Nature,* **1997**, *388*, 340.
2. Miles, P. G.; Chang, S. T. *Mushroom Biology: Concise Basic and Current Developments*, Singapore: World Scientific, **1997**, p. 193.
3. Chang, S.T. Mushrooms and Mushroom Cultivation. In: *Nature Encyclopedia of Life Sciences*. John Wiley & Sons, Ltd: Chichester, **1998**.
4. Kendrick, B. *The Fifth Kingdom*, Waterloo, Canada: Mycologue Publications, **1985**, p. 364.
5. Maurice, O. M., *Nature Encyclopedia of Life Science,* Macmillan Publishers Ltd, Nature Publishing Group, **2002**.
6. Hare, R. New light on the history of penicillin. *Medical History,* **1982,** *26*, 1.
7. Mothana, R. A.A.; Jansen, R.; Juelich, W.D.; Lindequist, U. *J. Nat. Prod.* **2000**, *63*, 416.
8. Keller, A.C.; Maillard, M. P.; Hostettmann, K. *Phytochem.* **1996**, *41*, 1041.
9. Nakajima, S.; Kawai, K.; Yamada, S.; Sawai, Y. *Agric. Biol. Chem.* **1976**, *40*, 811.
10. Brandt, C. R.; Piraino, F. *Rec. Res. Dev. Antimicrob. Agents Chemother.* **2000,** *4*, 11.
11. Awadh Ali, N. A.; Mothana, R. A. A.; Lesnau, A.; Pilgrim, H.; Lindequist, U. *Fitoterapia,* **2003**, *74*, 483.
12. Min, B. S.; Nakamura, N.; Miyashiro, H.; Bae, K. W.; Hattori, M. *Chem. Pharm. Bull.* **1998**, *46*, 1607.

13. El-Mekkawy, S.; Meselhy, M. R.; Nakamura, N.; Tezuka, Y.; Hattori, M.; Kakiuchi, N.; Shimotohno, K.; Kawahata, T.; Otake, T. *Phytochem.*, **1998**, *49*, 1651.

14. Mizuno, T. *Int. J. Med. Mushrooms,* **1999**, *1*, 9.

15. Mizuno, T.; Saito, H.; Nishitoba, T.; Kawagishi, H. *Food Rev. Int.* **1995**, *11*, 23.

16. Sontag, B.; Arnold, N.; Steglich, W.; Anke, T. *J. Nat. Prod.* **1999**, *62*, 1425.

17. Lorenzen, K.; Anke, T.; Anders, U.; Hindermayr, H.; Hansske, F. *Z. Naturforsch. C, J. Biosci.* **1994**, *49*, 132.

18. Atsumi, S.; Umezawa, K.; Iinuma, H.; Naganawa, H.; Nakamura, H.; Iitaka, Y.; Takeuchi, T. *J. Antibiot.,* **1990**, *43*, 49.

19. Lee, I. K.; Yun, B.S.; Cho, S. M.; Kim, W. G.; Kim, J. P.; Ryoo, I. J.; Koshino, H.; Yoo, I. D. *J. Nat. Prod.* **1996,** *59*, 1090.

20. Leslie Gunatilka, A. A.; Gopichand, Y.; Schmitz, F. J.; Djerassi, C. *J. Org. Chem.* **1981**, *46*, 3860.

21. Galli, G.; Maroni, S. *Steroids,* **1967**, *10*, 189.

22. Zurcher, A.; Heusser, H.; Jeger, O.; Geistlich, P. *Helv. Chim. Acta.*, **1954**, *37*, 1562.

23. Bladon, P.; Clayton, R. B.; Greenhalgh, C. W.; Henbest, H. B.; Jones, E. R. H.; Lovell, B. J.; Silverstone, G.; Wood, G. W.; Woods G. G. *J. Chem. Soc.* **1952,** 4883.

24. Serebryakovr, E. P.; Simolin, A. V.; Kucherov, V. F. ; Rosynov, B. V. *Tetrahedron*, **1970**, *26*, 5215.

25. Alder, J. H.; Young, M.; Nes W. R., *Lipids*, **1977**, *12*, 364.

26. Breitmaier, E.; Voelter, W. *Spectroscopy: High Resolution Methods and Applicatioons in Organic Chmistry and Biochemistry*; VCH Verlag: Weinheim, **1987**; p. 342 and 447.

27. Hollinshead, D. M.; Howell, S. C. ; Ley, S. V.; Mahon, M.; Ratcliffe, N. M.; Worthington, P. A. *J. Chem. Soc., Perkin Trans.* I, **1983**, 1579.

28. Torrenegra, R.; Pedrozo, J.; Robles, J.; Waibel, R.; Achenbach, H. *Phytochem.*, **1992**, *31*, 2415.

29. Grandos, A.; Ortiz, A. W. A. *J. Nat. Prod.*, **1990,** *53*, 441.

30. Sholichin, M.; Yamasaki, K.; Miyama, R.; Yahara, S.; Tanaka, O. *Phytochem.*, **1980**, *19,* 326.

31. Hanson, J. R.; Hitchcock, P. B.; Nasir, H.; Truneh, A. *Phytochem.*, **1994**, *36*, 903.

32. Aranda, G.; Kortbi, M. S.; Lallemand, J., Y.; Neuman, A.; Hammoumi, A.; Facon, I., *Tetrahedron* **1991**, *47*, 8339.

33. Betts, R. E.; Walters, D. E.; Rosazza, J. P. N. *J. Med. Chem.* **1974**,*17*,599

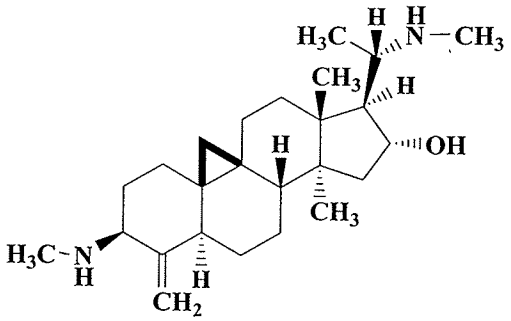
34. Margret, B. *Microbiology Laboratory Exercises*, Wim. C. Brown, Publishers, UK, **1997**, 187.

CHAPTER 3

PYTOCHEMICAL STUDIES ON *BUXUS HYRCANA*

3.0 Introduction

Plants of genus *Buxus* have been used in medicine since ancient times. In the middle Ages, *Buxus sempervirens* L. was reported to be used for many disorders, including skin and venereal diseases.[1] During the nineteenth century, the extract of this plant gained a reputation as a remedy for malaria. The plant was first subjected to chemical investigation in 1830 by Faure´ in France, who was successful in isolating an impure alkaloid, which he designated as "buxine''.[2] Later several authors claimed the isolation of various "buxines" and other alkaloids (parabuxine, buxinidine, parabuxinidine, buxinamine, buxeine) from the leaves or bark of *B. sempervirens,* but none of them obtained in pure form. Keusler and Schlittler for the first time succeeded in isolating seven individual bases (A, B, C, D, L, M, and N) from the leaves of this plant. [3-5] Brown and Kupchan (1962) reported the complete stero structure of cyclobuxine-D, purified from, *Buxus sempervirens*.[6-7] Cyclobuxine-D was shown to be the prototype of a new class of steroidal alkaloids, which contains a cyclopropane ring and has a substitution pattern at C-4 and C-14. This substitution pattern suggested that lanosterol type steroids are the biogenetic precursor of these compounds which is intermediate in the biogenetic scheme between lanosterol and cholesterol type steroids.



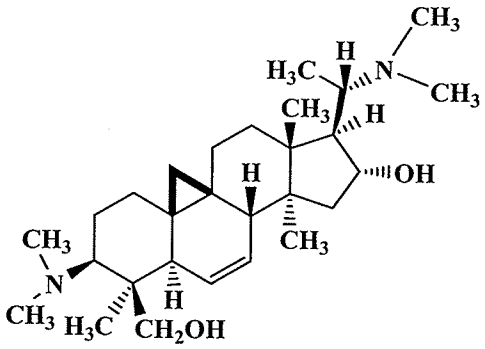
Cyclobuxine-D (54)

All *Buxus* alkaloids are substituted at C-4 by methyl, methylene, hydroxymethylene or alkoxy methyl group and at C-14 by a methyl group, is a characteristic to all (except for irehine) *Buxus* alkaloids. Structurally, *Buxus* alkaloids can be divided into two broad classes:

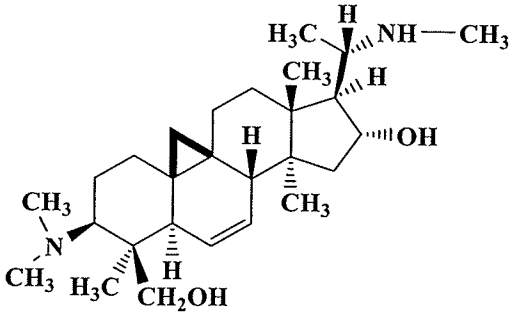
a) Derivatives of 9 β, 19-cyclo-4,4,14-trimethyl-5-pregnane

b) Derivatives of *abeo*-9(10→19)-4,4,14-trimethyl-5-pregnane

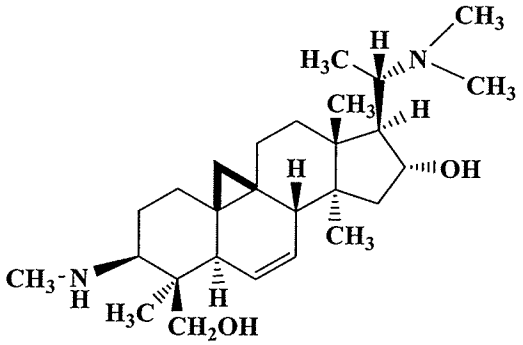
Subsequent study has characterized structurally related alkaloids: cyclomicrophylline-A(**55**), cyclomicrophylline-B(**56**), cyclomicrophylline-C (**57**), cyclobuxamine-H (**59**), cyclovirobuxine-D (**58**), and cycloprotobuxine-C (**60**). Depending on the nature of the skeleton and substitution in positions 4 and 14, the *Buxus* alkaloids containing two atoms of nitrogen can be divided into several groups which are represented by **(55-59)** and designated by the respective trivial names.



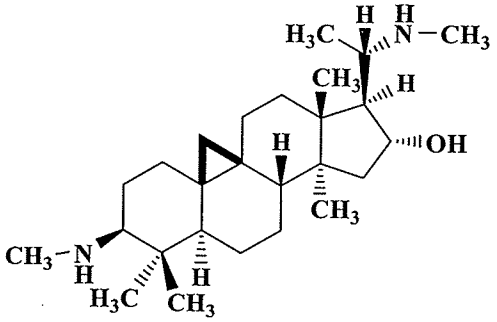
Cyclomicrophylline-A (55)



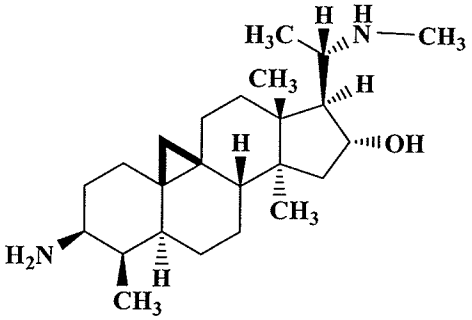
Cyclomicrophylline-B (56)



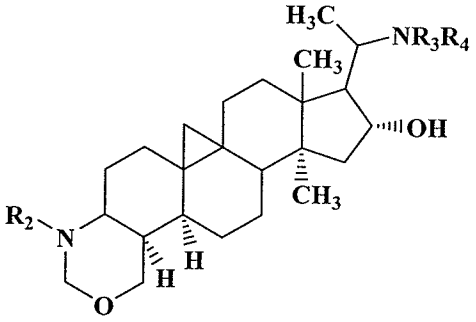
Cyclomicrophylline-C (57)



Cyclovirobuxine-D (58)

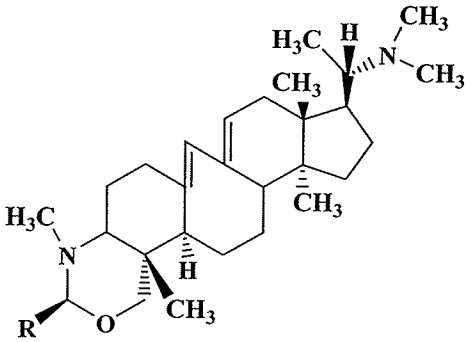


Cyclobuxamine-H (59)



Cyclobuxoxazines (60)

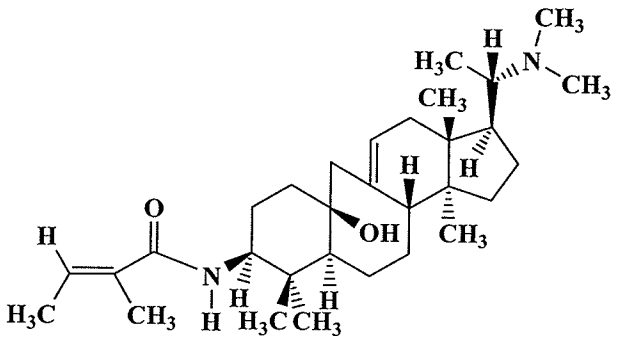
The genus of *Buxus* is a rich source of triterpenoidal alkaloids. Several *Buxus* alkaloids have shown interesting biological activities. For instance cyclovirobuxine D (**58**) has shown active against heart disorders.[8] Two triterpenoidal alkaloids (+)-homomoenjodaramine (**61**), (+)-moenjodaramine (**62**) isolated from *Buxus hyrcana* showed acetylcholinesterase inhibitory activities.[9]



Homomoenjodaramine (61) R= CH_3

Moenjodaramine (62): R=H

The crude ethanolic extract of *B. sempervirens* has been reported to exhibit anti human immunodeficiency virus (HIV) activity.[10] From the leaves of *Buxus hyrcana* one of the alkaloids, (+)-*N*-tigloylbuxahyrcanine **(63)** has shown acetylcholinestrase and butyrylcholinesterase inhibitory activity.[11]



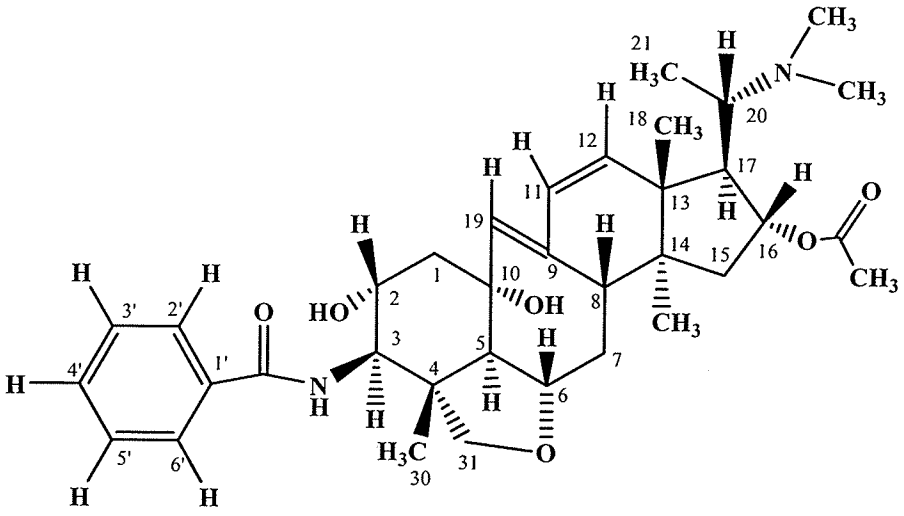
(63)

In this chapter of thesis we report the isolation and characterization of O^6-buxadiene **(64)**.

3.1 RESULTS AND DISCUSSION

The crude methanolic extract of *Buxus hyrcana*, collected from Iran, was loaded onto a silica gel column. This column was eluted with *n*-hexane-ethyl acetate (0-100 %) and ethyl acetate-methanol (0-100 %) to afford several fractions. The fractions F_1 to F_5 obtained with ethyl acetate-methanol (80:20) and F_1, with ethyl acetate-methanol (90:10), were combined having same R_f values. These combined fractions were subjected to preparative TLC using petroleum ether-acetone-diethylamine as mobile phase to afford O^6-buxadiene (**64**) as light yellow colored gum. (See experimental section **3.2.3**)

The UV spectrum of compound (**64**) showed absorption maxima at 239 nm indicating the presence of a 9(10→19) *abeo* diene system. The IR spectrum showed intense absorption bands at 3412 (OH), 1710 (ester carbonyl), 1644 (α,β-unsaturated amide carbonyl), 1602 (C=C) and 1100 (C-O) cm^{-1}.



64

The chemical ionization mass spectrum of (**64**) exhibited the molecular ion peak at *m/z* 592. This mass along with ^{1}H and ^{13}C-NMR data, provided the molecular formula $C_{35}H_{48}N_2O_6$. This indicated the presence of thirteen double bond equivalents in the molecule. The mass fragmentation pattern of compound (**64**) suggested that it has a *Buxus* alkaloid type structure. An ion at *m/z* 105 was consistent with a benzoyl fragment. The base peak at *m/z* 72 indicated the presence of a trimetyliminium ion cleaved from the C-17 of main skeleton.[12] The ion at *m/z* 72 suggested the presence of a *N,N*-dimethyl amino functionality at C-20.[13]

The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of (**64**) showed the resonance of three 3H singlets at δ 0.92, 0.89 and 0.86 due to the tertiary C-18, C-30 and C-32 methyl group protons respectively. A 3H doublet at δ 1.03 (*d, J*= 6.5) was ascribed to the C-21 secondary methyl group. A 6H broad singlet at δ2.27 was a characteristic of the *N,N*-dimethyl protons, substituted at C-20 of the D-ring side chain. Three downfield multiplets integrating for one-proton each at δ 4.08, 3.54 and 5.50 were assigned to the C-2, C-6 and C-16 methine protons respectively. Their downfield chemical shift values were indicative of the presence of geminal oxygen at C-2, C-6 and C-16 respectively. The amidic NH resonated at δ 5.99 as a doublet and 5H multiplets between δ 7.44-7.76 were due to the aromatic protons. The ^{1}H-NMR spectrum also featured a one-proton multiplet at δ 3.75, and was assigned to the C-3 methine proton. Two AB doublets integrating for one-proton each resonating at δ 3.86 and 4.06 were due to the C-31 methylene protons. The downfield multiplet at δ 3.54 was assigned to the C-6 methine proton. The C-11, C-12 and C-19 olefinic protons resonated as doublet at δ 5.71 (*d, J*= 10.7), 5.56 (1H, *d, J*= 10.7) and singlet resonated at δ 5.55 respectively.

The COSY-45° spectrum of **(64)** showed vicinal couplings of C-11 olefinic proton δ 5.55 with the C-12 methine proton δ 5.71 between the olefinic protons H-11/ H-12. The C-2′/C-6′ methine protons exhibited ^{1}H-^{1}H correlations with the C-3′/C-5′ methine protons resonated at δ 7.44.

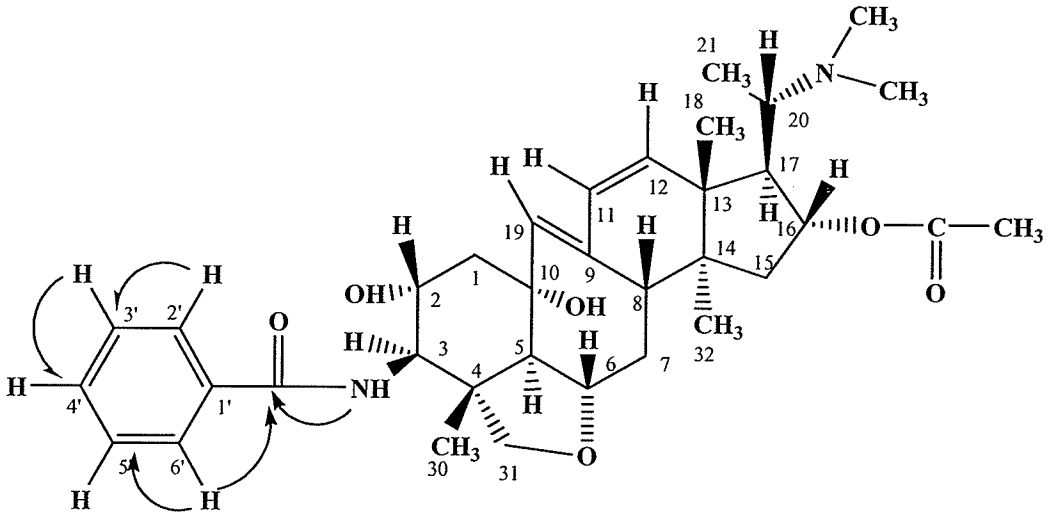
A broad band ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of **(64)** showed the resonance of all 35 carbons. Five downfield resonance signals at δ 76.9, 76.7, 64.8, 62.4 and 76.85 were assigned to C-31, C-6, C-3, C-2 and C-16 carbons atoms, respectively. The downfield value for C-3 is due to the presence of geminal benzamide group. The lowfield resonances for C-2, C-3, C-16 and C-31 suggested the presence of geminal oxygen functionality on these carbon atoms. Complete ^{13}C-NMR chemical shift assignments of **(64)** are shown in Table 1.

The HSQC spectrum of **(64)** was very helpful to determine the $^{1}H/^{13}C$ one-bond shift correlations of all protonated carbon atoms. The $^{1}H/^{13}C$ one-bond correlation of all hydrogen bearing carbons, as deterimined from the HSQC spectrum, are shown in Table-1.

The HMBC spectrum of **(64)** was very informative to assign the ^{13}C-NMR chemical shift of quaternary carbon atoms. H-2′/H-6′ (δ 7.76) showed HMBC interactions with the amidic carbonyl (δ 168). The amidic NH displayed long-range heteronuclear coupling with the amidic carbonyl. These observations indicated the presence of benzoyl group at C-3. Important HMBC interactions are shown around structure **(63b)**. The stereochemistry at various chiral centers was established with the aid of NOESY spectrum. It has been reported in the literature that C-3 methine proton, α-oriented in this class of alkaloids.[14] All of the alkaloids, reported in the literature have α-orientation for

C-2/OH, C-6/O and C-16/acetoxy group.[15-17] This helped us to assume that C-2/OH, C-4 oxygenated methylene, C-16/acetoxy groups might have α-orientation in this compound. It has been reported in the literature that C-31 (C-4 α methyl) undergo preferential oxidation than C-30 (C-4 β methyl), it suggested that C-31 was bonded with C-6 through an ether linkage.[18] The presence of ether linkage was inferred from the IR, mass, ^{1}H and ^{13}C-NMR spectra. The IR spectrum showed intense absorption band at 1100-1200 cm^{-1}. The ^{1}H-NMR spectrum displayed the resonance of C-31 methylene protons as set of two AB doublets at δ 3.86 and 4.06. A one-proton multiplet at δ 3.54 was due to the C-6 methine proton. The ^{13}C-NMR spectrum also exhibited the resonance of downfield signals at δ 76.9 and 76.7 were due to the C-31and C-6 carbon atoms, respectively. The spectroscopic data provided the molecular formula $C_{35}H_{48}N_2O_6$ and this indicated the presence of thirteen degrees of unsaturation in the molecule. Six of these were accounted for steroidal skeleton having two double bonds and six degrees of unsaturation were due the presence benzamide and an ester functionalities. The 13^{th} degree of unsaturation can only be satisfied by the presence of an ether linkage between two carbon C-31 and C-6. The molecular formula showed that six oxygen atoms were present in compound **(64)**. Three of them were due to the presence of C-3 benzamide and C-16 ester functionalities. Two were due to the presence of C-2 and C-10 hydroxyl groups. The remaining oxygen was accounted for by an ether link between C-31 and C-6 atoms.

Based on these spectroscopic studies, structure **(64)** was proposed for this novel compound.



64b

Cytotoxic Activity:

O^6-buxadiene (**64**) was screened for cytotoxicity by using MTT assay and it showed weak anticancer activity against prostate cancer cell lines (LNCaP).

Table 1. ^{13}C NMR spectral data for 64 in ($CDCl_3$)

Carbon No.	^{13}C-NMR Chemical Shifts (δc)	^{1}H- NMR Chemical Shifts δ_H, *J*(Hz)
C-1	30.3	2.60, 1.38
C-2	62.4	4.08 m
C-3	64.8	3.75 m
C-4	44.2	
C-5	51.1	2.00 (1H, *d*, *J*= 12.85)
C-6	76.7	3.54 m
C-7	31.7	1.33, 0.86
C-8	49.8	2.7
C-9	138.0	
C-10	79.1	
C-11	129.0	5.71 (1H, *d*, *J*= 10.73)
C-12	126.2	5.56 (1H, *d*, *J*= 10.76)
C-13	39.2	
C-14	41.3	
C-15	33.1	1.55, 1.35
C-16	76.8	5.50 m
C-17	56.4	2.10
C-18	18.3	0.92 s
C-19	125.1	5.55 s
C-20	52.3	1.99 m
C21	13.9	1.03 (3H, *d*, *J*= 6.5)
C-30	14.7	0.89 s
C-31	76.9	3.86, 4.06 (2H, *d*)
C-32	18.3	0.86 s
Nb-CH3		2.27 br. s
Nb-CH3		2.27 br. s
C-1'	130.0	
C-2'	128.5	7.76 m
C-3'	128.7	7.44 m
C-4'	129.0	7.50 m
C-5'	128.7	7.44 m
C-6'	128.5	7.76 m
Ph-CO-N	168.0	
PhCONH		5.99 (1H, *d*, 9.3)
CH3-CO	171.0	
CH3-CO	20.94	2.16 s

3.2 EXPERIMENTAL

3.2.1 General Experimental Conditions

General experimental conditions of spectroscopic and chromatographic methods were same as those of chapter 2. Page 33.

3.2.2 The Plant Material

The leaves of *Buxux hyrcana,* were collected by Dr. M. H. Meshkatalsadat, Department of Chemistry University of Lorestan, Iran.The plant was identified by Dr. Jahad Sazandegi, Iran.

3.2.3 Extraction of Plant Material

The leaves of *Buxux hyrcana* were dried and extracted with methanol at 25 °C. The solvent was evaporated under reduced pressure to prepare gum. This extract was extracted with chloroform at different pH values (3.5, 7.0 and 9.5).

3.2.2 Isolation of Compound 64

The chloroform extract obtained at pH 7.0 was subjected to column chromatography. The column was eluted with *n*-hexane-ethylacetate (1:0 to 0:1), and then ethylacetate-methanol (1:0 to 0:1) to get several fractions. The fractions F_1 to F_5 obtained with ethyl acetate-methanol (80:20) and F_1, with ethyl acetate-methanol (90:10), were combined having same R_f values and was subjected to preparative TLC using petroleum ether-acetone-diethylamine in different ratios as a mobile phase to afford compound **(64)** as light yellow colored gummy material.

3.3 Reference:

1. Cerny, V.; Sorm, F. Ceskoslov. Akad. Ved, Prague, Czech. *Alkaloids*, Academic Press, **1967**, 9, 305.
2. Faure, M. *J. Pharm.* **1830,** *16*, 428.
3. Schlittler, E.; Heusler, K.; Friedrich, W. *Helv. Chim. Acta,* **1949**, *32*, 2209.
4. Keusler, K.; Schlittler, E. *Helv. Chim. Acta,* **1949**, *32*, 2226.
5. Friedrich, W.; Schlittler, E. *Helv. Chim. Acta,* **1950,** *33*, 873.
6. Brown, K. S. J.; Kupchan, S. M. *J. Am. Chem. Soc.*, **1962**, *84*, 4590.
7. Brown, K. S. J.; Kupchan, S. M. *J. Am. Chem. Soc.,* **1962**, *84*, 4592.
8. Wang, X.; Liang, B. *Faming Zhuanli Shenqing Gongkai Shuomingshu.* **1992**, 6pp.
9. Atta-ur-Rahma; Parveen, S.; Khalid, A.; Farooq, A.; Ayattollahi, S. A. M.; Choudhary, M. I. *Heterocycles*, **1998**, *49*, 481.
10. Durant, J.; Chantre, P.; Gonzalez, G.; Vandermander, J.; Halfon, P.; Rousse, B.; Guedon, D.; Rahelinirina, V.; Chamaret, S.; Montagnier, L.; Dellamonica, P. *Phytomedicine*, **1998**, *5*, 1.
11. Choudhary, M. I.; Shahnaz, S., Parveen, S.; Khalid, A.; Ayatollahi, S. A. M.; Atta-ur-Rahman; Parvez, M. *J. Nat. Prod.* **2003**, *66*, 739.
12. Budzikiewicz, H. Steroids. In: Wailer, G.R. (Ed.), Biochemical Application of Mass Spectrometery. Wiley-Interscience, New York, **1972**, 251.
13. Atta-ur-Rahman, *Handbook of Natural Product Data*: *Diterpenoid and Steroidal Alkaloids*. Elsevier Science, Amsterdam, **1990**, *1*
14. Atta-ur-Rahman, Farhana, N.; Choudhary, M. I.; Zeba, P. *J. Nat. Prod.*, **1997,** 60, 976.

15. Atta-ur-Rahman; Zahida, I.; Rubina, Z.; Choudhary, M. I. *J. Nat. Prod.* **1990,** *53,*319.

16. Atta-ur-Rahman; Ata, A.; Naz, S.; Choudhary, M. I.; Sener, B.; Turkoz, S. *J. Nat. Prod.* **1999,** 665.

17. Guilhem, J. *Tetrahedron Lett.,* **1975**, *34,* 2937.

18. Sangare, M.; Khuong H. L. F.; Herlem, D.; Milliet, A.; Septe, B.; Berenger, G.; Lukacs, G. *Tetrahedron Lett.*, **1975**, *23*, 1791.

CHAPTER 4

MICROBIAL TRANSFORMATION OF SCLAREOL

4.0 Introduction

Microbial reactions have an important role in chemo-enzymatic synthesis of organic compounds. Biocatalysts *i.e.,* pure enzymes or whole microbial cell tender a highly selective microbial transformations which may be applied during multistep organic synthesis. Microorganisms are known for their ability to catalyze a large number of oxidative, reductive, conjugative, and degradative reactions of many classes of natural products.[1,2]

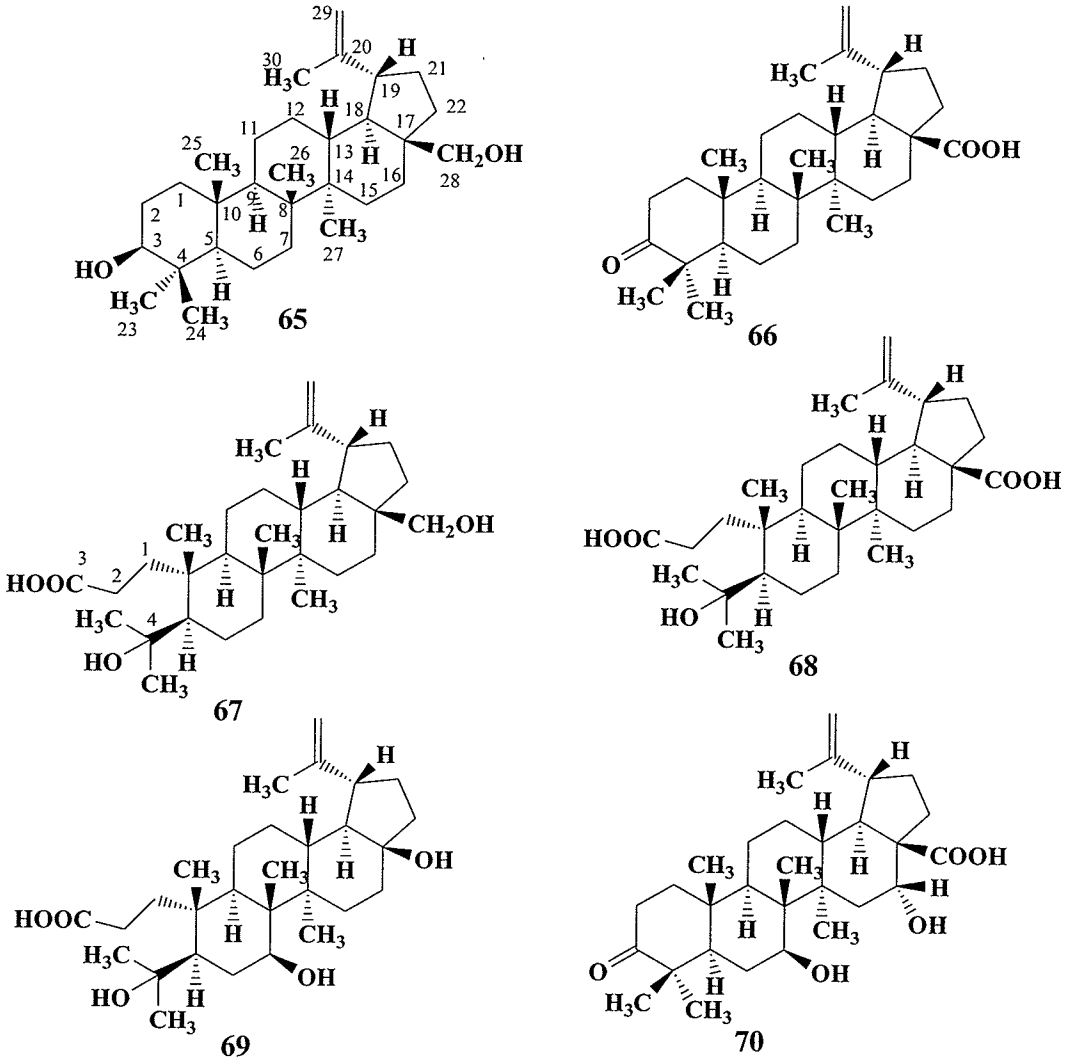
Microbial reactions are of two types:

a) Biosynthetically directed biotransformations: These types of transformations occur when the substrate to be biocatalysed is a biosynthetic intermediate or analogue of secondary metabolites of the particular microorganism performing the transformation.

b) Xenobiotic biotransformations: are those transformations when, the substrate is neither a biosynthetic intermediate nor an analogue of secondary metabolite of the specific microorganism performing the transformation. These types of microbial reaction are responsible for the modification of natural products which are foreign to the microbe. The microbes use natural products as a sole source of carbon or modify the structure as a mean of defense mechanism.[2]

The research conducted for thesis deals with xenobiotic biotransformations. These types of biotransformations are the source to produce lead compounds for drug discovery.

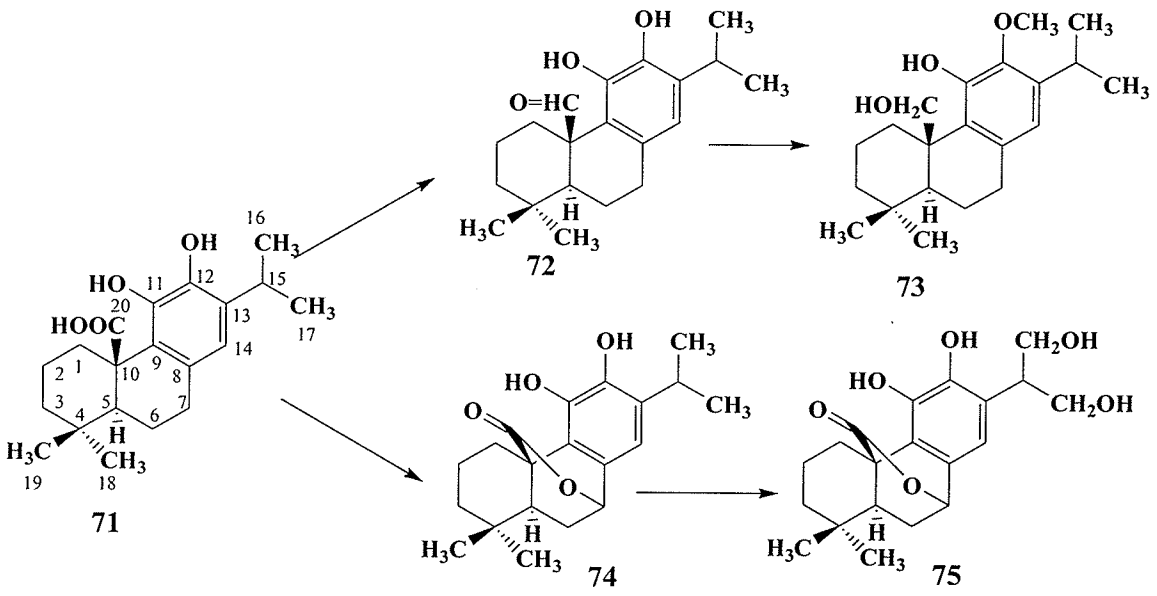
The biocatalytic oxygenation processes are becoming increasingly important since traditional oxygenation methodology is either not feasible or makes use of hypervalent metal oxides, which are ecologically undesirable when used on a large scale. As the use of isolated oxygenases will always be hampered due to their requirement of NAD(P)H-recycling. Thus many useful oxygenation reactions such as mono- and dihydroxylation, epoxidation, sulfoxidation and Baeyer-Villiger reaction will continue to be performed using whole cell systems. Considering the lack of cofactor-dependence, hydrogen-peroxide-dependent peroxidases have become a promising alternative. Microbial reactions are performed on an inaccessible site of the organic compounds. These types of reactions seem to be difficult by chemical methods. Microbial reactions on bioactive natural products may produce metabolite having higher bioactivity and lower toxicity.[3-4] For instance, the betulin (**65**), is a naturally occurring lupane-type triterpene isolated from the bark extract of white birch (*Betula platphylla* Sukatshev var. *japonica*), and its chemical oxidation product, betulonic acid (**66**), were transformed by the fungus *Chaetomium longirostre* to yield the products 4,28-dihydroxy-3,4-*seco*-lup-20(29)-en-3-oic acid (**67**) and 4-hydroxy-3,4-*seco*-lup-20(29)-ene-3,28-dioic acid (**68**) form (**65**) and 4,7β,17-trihydroxy-3,4-*seco*-28-norlup-20(29)-en-3-oic acid (**69**) and 7β,15α-dihydroxy-3-oxolup-20(29)-en-28-oic acid (**70**) from (**66**).[3] The four metabolite (**67-70**) were determined for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells. The transformation products (**67-70**) exhibited more potent effects than parent compounds (**65-66**).[3]



Scheme 4.1 betulin(65) betulonic acid(66) 4,28-dihydroxy-3,4-*seco*-lup-20(29)-en-3-oic acid(67) 4-hydroxy-3,4-*seco*-lup-20(29)-ene-3,28-dioic acid(68) 4,7β,17-trihydroxy-3,4-*seco*-28-norlup-20(29)-en-3-oic acid(69) 7β,15α-dihydroxy-3-oxolup-20(29)-en-28-oic acid(70)

This shows that microbial transformations are valuable in discovering new analogues of bioactive compounds which may be more beneficial than their parent compounds. Since the purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, do survive unaffected. Due to chemo-, regio- and enantioselectivity reactions generally

tend to be cleaner and laborious purification of product from impurities, emerging through side reactions, can largely be avoided. The use of protecting groups can sometimes be eliminated due to this specificity. Another example carnosic acid (**71**), is an abundant abietane diterpene, a powerful antioxidant found in the Lamiaceae herbs rosemary and sage. *Nocardia* sp. (NRRL 5646) was capable of reduction-methylation (**73**), oxidation (**74-75**) of carnosic acid.[5] The biocatalytic reduction of a hindered C-20 carboxylic acid moiety, illustrates the advantages of microbial transformations.



Scheme 4.2 carnosic acid (71), metabolite formed by *Nocardia* sp., NRRL 5646, (73-75).

This type of transformations can not be easily achieved by chemical synthetic methods. It requires the use of protecting groups with an overall lengthy and low yielding process. Microorganisms can serve as predictive and reliable models for drug metabolism in mammalian systems.[6-7] Because of the presence of the enzyme cytochrome P-450 monooxygenase, in vitro testing of pharmaceutical agents can be carried out with certain

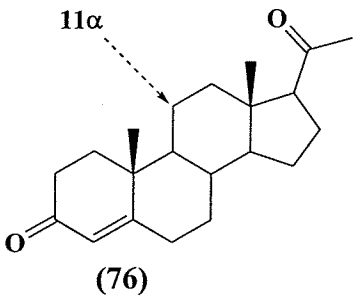
microbes.[8] This may afford significant quantities of metabolites, through large scale fermentation which are easily purifiable and identifiable. These metabolites often parallel those obtained from mammalian biotransformations and can serve as prognostic indicators of pharmaceutical metabolism in human systems.[9,10] This is desirable because metabolites from human trials are often only present in very low and sometimes undetectable quantities which are difficult to purify. There are also other enzymes besides cytochrome P-450 monooxygenase that have been found to be present in certain microbial species which parallel mammalian enzymes. Nitric oxide synthase (NOS), carboxylic acid reductase, aryl aldehyde oxidoreductase, aldehyde reductase, guanylate cyclase, and ornithine transcarbamoylase have all been identified in *Nocardia* sp. (NRRL 5646) and are similar to enzymes found in mammals.[5]

Mechanistic studies on microbial transformations can help to identify and provide information about the enzymes involved in the biotransformations. Identification of the genes governing the expression of these enzymes can allow for their extraction and amplification. Subsequently, vector cloning of these genes into a suitable host will allow for their over expression. Large scale fermentation would make large amounts of a vector cloned enzyme responsible for a biotransformation available to be purified and used outside of the host cell. In this way, microbial transformations can provide an assay to isolate the key enzymes responsible for a biotransformation. This also affords an environmentally friendly "Green Chemistry" a way of carrying out chemical transformations in which harsh reagents and solvents would no longer be needed as it would be the enzymes which perform the desired structural changes.

Studies here are concerned with conducting xenobiotic biotransformations to discover a novel biotransformation on a bioactive natural product substrate and to compare bioactivity studies on the transformed and parent compounds by means of Kirby-Bauer anti-bacterial testing.

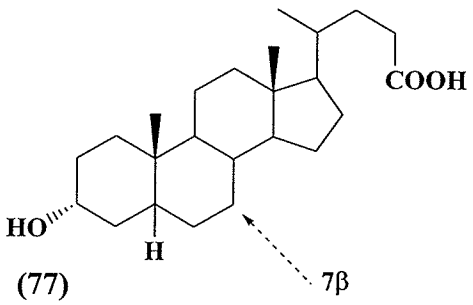
4.1 Hydroxylation of Alkanes

The hydroxylation of nonactivated centers in hydrocarbons is one of the most useful biotransformations due to the fact that this process has only very few counterparts in traditional organic synthesis.[11-12] In general, the relative reactivity of carbon atoms in bio-hydroxylation reactions declines in the order of (secondary> tertiary> primary) which is in contrast to radical reactions (tertiary> secondary> primary).[13-14] There are two main groups of hydrocarbon molecules, which have been thoroughly investigated with respect to microbial hydroxylation, *i.e.*, steroids and terpenoids. Almost any center in a steroid can be selectively hydroxylated by choosing the appropriate microorganism.[15] For example, hydroxylation of progesterone in the 11α-position by *Rhizopus arrhizus*[16] or *Aspergillus niger*[17] made roughly half of the 37 steps of the conventional chemical synthesis unnecessary and made 11α-hydroxyprogesterone available for hormone therapy at a reasonable cost.



(76)

progesterone
Rhizopus arrhizus
or Aspergillus niger



(77)

lithiocholic acid
Fusarium equiseti

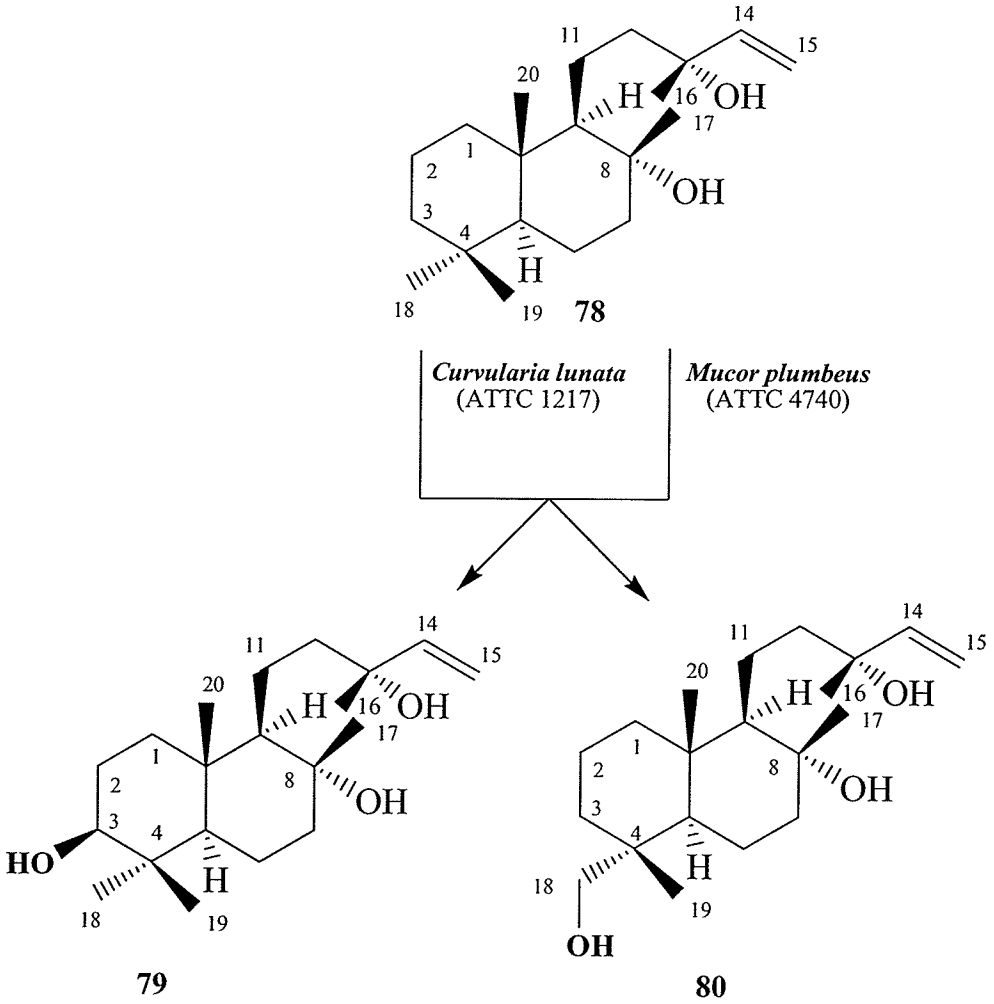
A highly selective hydroxylation of lithiocholic acid in position 7β was achieved by using *Fusarium equiseti*.[18]

The present research is focused on the biocatalytic oxidation, of sclareol, a labdane diterpene, is a widely distributed in nature and an important minor constituent of Clary Sage Oil, the essential oil of *Salvia Sclarea* L. (family Labiatae).[19] The interest in studying labdanes is based on their wide range of biological activities.[20] Sclareol was first isolated from clary sage oil *Sclarea*, Labiatae[21] and it also occurs in many conifers. It was found that sclareol shows activity against the cell cycle and induces apoptosis in leukemic cell lines, while it does not affect normal and PHA stimulated peripheral blood lymphocytes.[22]

In view of the widespread human exposure to sclareol, a prospective approach was undertaken to study the metabolism of sclareol utilizing microorganisms as in vitro models to mimic and predict mammalian biotransformations. Microbial transformations have provided the efficient tool for the stereo- and regioselective transformations of sclareol which are extremely difficult with chemical reactions. In present study, we describe the biotransformation, extraction, isolation, structural elucidation, and antibacterial activity of sclareol.

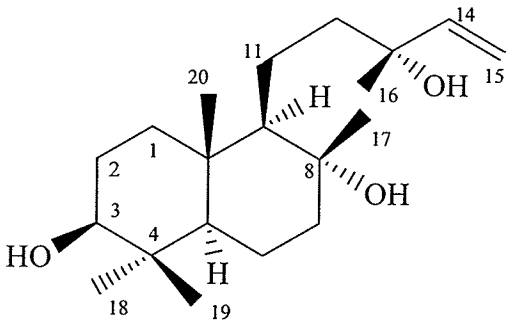
4.2 RESULTS AND DISCUSSION

Two cultures, *Curvularia lunata* (ATCC 12017) and *Mucor plumbeus* (ATCC 4740) were selected to transform sclareol (**78**) at an analytical scale. In our screening experiment, we found on TLC that *Curvularia lunata* (ATCC 12017) and *Mucor plumbeus* (ATTC 4740) were capable of biotransforming the compound (**78**). Incubation of sclareol (**78**) on a preparative scale resulted in formation of (**79**) and (**80**) along with unmetabolized (**78**) (Scheme 4.3). The structures of these metabolites were established with the aid of spectroscopic techniques. The spectral data of (**79**) and (**80**) were in agreement with the previously reported compounds.



Scheme 4.3

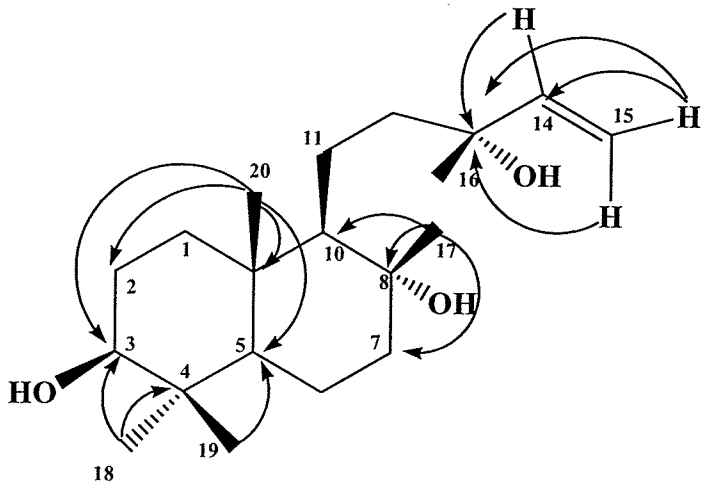
4.2.1 3β-hydroxy sclareol (79)



3β-hydroxy-sclareol 79

The first metabolite, 3β-hydroxy sclareol (**79**) was obtained as an amorphous solid. The IR spectrum revealed absorption at 3388 (OH) and 1133(C-O) cm^{-1}. The EI mass spectrometry (EIMS) of metabolite (**79**), showed a molecular ion at *m/z* 306 (M^+-H_2O). The molecular formula $C_{20}H_{36}O_3$ was deduced from EI mass measurement coupled to NMR data. The ^{1}H-NMR spectrum (CD_3OD, 500 MHz) of metabolite (**79**) was characterized by a new hydrogen signal centered at δ 3.15(IH, *dd*, *J*= 5.0, 5.1 Hz), in agreement with a hydroxylated position at C-1, C-3, C-6 or C-7 adjacent to only one CH_2 group. The NMR data (Table1) of (**79**) were similar to (**78**)[23] except for the proton resonating at δ 3.15 hydrogen geminal to hydroxyl in the molecule. The ^{13}C-NMR spectrum (CD_3OD, 125 MHz) of (**79**) showed the resonance of all twenty carbon atoms in the molecule. It also showed the presence of a new hydroxylated carbon atom at δ 79.6 (C-1 or C-3).[24-25] Analysis of the substituent effect in the ^{13}C-NMR spectrum showed the absence of downfield shift of the C-20 methyl group. The downfield chemical shifts observed for signals of C-2 and C-4 at δ 27.6 and 39.9 for (**79**) These observations had led us to infer that the C-3 position of (**79**) was substituted with hydroxyl group.

Furthermore, the DEPT experiment of metabolite (**79**) showed the presence of a new CH signal at δ 79.6 and the disappearance of a CH_2 signal, which confirmed that metabolite (**79**), was a hydroxylated metabolite of sclareol. The new CH signal at δ 79.6 in the metabolite of (**79**) spectrum was assigned to C-3 based on one-bond and long-range $^1H/^{13}C$ experiments. The HSQC spectrum showed the direct $^1H/^{13}C$ connectivities of H-3 (δ 3.15), H_{cis}-15(δ 5.19) and H_{trans}-15 (δ 5.0) and H-14 (δ 5.91) with their respective carbons *i.e.*, C-3 (δ 79.6), C-15 (δ 111.8) and C-14 (146.8) respectively. Complete ^{13}C-NMR chemical shift assignments and important $^1H/^{13}C$ one-bond shift correlations of hydrogen bearing carbon atoms as determined from HSQC spectrum of compound (**79**) are shown in Table 1. The HMBC data (**79a**) also established the connectivity of new hydroxyl group, C-18 methyl group (δ_H 0.96, δ_C 28.7) showed a cross peak with C-3 (79.6). The stereochemistry at C-8 and C-13 were decided according to its chemical shifts in ^{13}C–NMR and comparison to those recorded for the relevant carbon in sclareol.[26] The stereochemistry at C-3 was confirmed by ^{13}C-NMR spectral data. All the spectral data indicated that metabolite (**79**) is monohydroxylated metabolite of sclareol (**78**). Thus based on all of the evidence, metabolite (**79**) was characterized as 3β–hydroxysclareol.

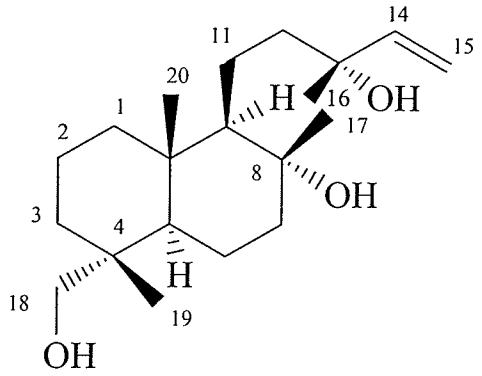


The HMBC correlation for 79a

Table 1. ^{13}C-NMR and ^{1}H-NMR Chemical Shift Assignments for 3βhydroxy sclareol 79 (CD_3OD)

Carbon No.	^{13}C-NMR Shift (δ)	Multiplicity (DEPT)	^{1}H-NMR Chemical Shift (δ)
1	39.4	CH_2	
2	27.6	CH_2	
3	79.6	CH_2	3.15 (1H, *dd J*= 5.0, 5.1)
4	39.9	-C-	
5	56.6	CH	
6	21.2	CH_2	
7	45.0	CH_2	
8	75.0	-C-	
9	62.5	CH	
10	40.2	-C-	
11	20.7	CH_2	
12	46.6	CH_2	
13	74.4	-C-	
14	146.8	CH	5.91 (1H, *dd*, J_{cis}=10.8, J_{trans}=17.4)
15	111.8	CH_2	5.19(1H, *dd*, J_{trans} = 17.4, H_{trans}-15); 5.0 (1H, *dd*, J_{cis}= 10.8, H_{cis}-15)
16	27.4	CH_3	1.23 *s*
17	24.0	CH_3	1.11 *s*
18	28.7	CH_3	0.96 *s*
19	16.0	CH_3	0.82 *s*
20	16.1	CH_3	0.75 *s*

4.2.2 18α-hydroxy-sclareol (80)

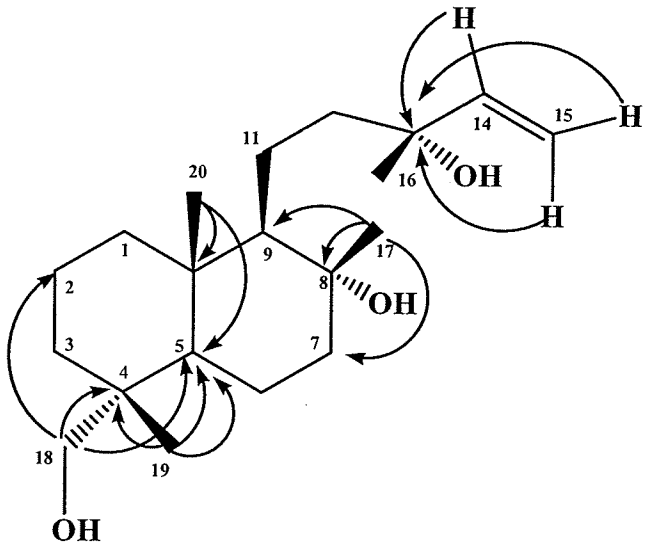


18-hydroxy-sclareol **80**

The second metabolite, 18α-hydroxy sclareol (**80**) was obtained as a colorless amorphous solid. The IR spectrum of (**80**) displayed bands at 3447(OH) and 1049(C-O) cm^{-1}. The mass spectrum indicated the parent peak at *m/z* 306 and the molecular formula, was inferred to be $C_{20}H_{36}O_3$ by using EI mass spectrum and coupled with NMR data. The ^{1}H-NMR (CD_3OD, 500MHz) spectrum of (**80**) (Table 1) showed four methyl singlets instead of five at δ 1.23, 1.11, 0.85 and 0.71 for the C-16, C -17, C-19 and C-20 tertiary methyl groups, respectively. In addition, one oxygenated methylene was resonated at δ 3.0 and 3.34. A broad band decoupled ^{13}C-NMR (CD_3OD, 125 MHz) spectrum of (**80**) showed all twenty carbon atoms resonances. The DEPT experiment indicated there were four CH_3, nine CH_2, three CH and by the difference from broad band decoupled spectrum, four quaternary carbon atoms. Thus DEPT experiment showed the disappearance of a methyl group at δ 28.7 and appearance of CH_2 group at δ 72.0. Additionally on comparison with ^{13}C-NMR spectrum with that of sclareol

revealed that C-4 is deshielded (δ_C 33.2 to 38.6, while C-3 is shielded (δ_C 42.0 to 36.4) requiring hydroxylation at one of the geminal methyl groups. The ^{13}C-NMR spectrum also revealed that (**80**) is exo-alcohol because the resonance of the adjacent methyl group was at δ_C 17.8. A high field shift of δ_C 3.7 is in the range of predicted γ-effect at C-19 of the hydroxyl group. One bond ^{1}H/^{13}C correlations were established by heteronuclear single quantum coherence (HSQC) NMR experiment. The HSQC spectrum of (**80**) showed the direct ^{1}H/^{13}C chemical correlations of H-14 (δ 5.92), H_{trans}–15 (δ 5.19), H_{cis}-15 (δ 5.0), H-16 (1.23), H-17 (δ 1.11), H-18 (δ 3.34, 3.0), H-19 (δ 0.85) and H-20 (δ 0.71) with C-14 (δ 146.7), C-15 (δ 111.7), C-16 (δ 27.3), C-17 (23.9), C-18 (δ 72.0), C-19 (δ 17.8) and C-20 (δ 16.3) respectively. The long range ^{1}H/^{13}C connectivites were determined from an HMBC spectrum. The C-18 methylene protons (δ 3.34, 3.0) showed long range heteronuclear shift correlations with C-4 (δ 38.6), C-2 (δ 18.8) and C-5 (δ 50.2) were established the position of hydroxyl at C-18. Important interactions are shown around structure (Scheme 4.4, **80a**) Furthermore, in comparison with related 18(α)- or 19 (β)-hydroxylated diterpenic homologous structures, the ^{13}C-chemical shifts of the CH_2OH group fits better with a 18 (α)-OH (about δ 72) than a 19 (β)-OH (about δ 65) structure.[27-28]

On the basis of above spectroscopic studies, structure (**80**) was characterized as (18α hydroxy sclareol).



The HMBC correlation for 80a

Table 2. ^{1}H-NMR and ^{13}C-NMR spectral data for 18-α-hydroxy-sclareol (80) (CD_3OD)

Carbon	^{13}C-NMR Chemical Shift (δ)	Multiplicity (DEPT)	^{1}H–NMR Chemical Shift (δ)
1	40.7	CH_2	
2	18.8	CH_2	
3	36.4	CH_2	
4	38.6	-C-	
5	50.2	CH	
6	21.1	CH_2	
7	44.7	CH_2	
8	75.1	-C-	
9	62.8	CH	
10	40.4	-C-	
11	20.7	CH_2	
12	46.6	CH_2	
13	74.4	-C-	
14	146.7	CH	5.92 (1H, *dd*, J_{cis}=10.8, J_{trans}= 17.3, H-14)
15	111.7	CH_2	5.19 (1H, *dd*, J_{trans}= 17.4, H-15$_{trans}$), 5.01 (1H, *dd*, J_{cis}= 10.8, H-15$_{cis}$)
16	27.3	CH_3	1.23 s
17	23.9	CH_3	1.11 s
18	72.0	CH_3	3.34, 3.0
19	17.8	CH_3	0.85 s
20	16.3	CH_3	0.71 s

4.2.3 Antibacterial activities of 3β-hydroxy sclareol 79 & 18α-hydroxy sclareol 80

The antibacterial activity of biotransformed metabolites of sclareol, 3β-hydroxy sclareol (**79**) and 18α-hydroxyl sclareol (**80**) was evaluated by Kirby-Bauer method against *E. coli, P. aeruginosa, C. xerosis, B. subtilis, and S. aureus.* It showed that metabolized products lost activity as compared to sclareol. The compound (**80**) did show weak activity against *B. subtilis* gave a 6.5 mm and 7.0 mm inhibition zone at 250 μg and 500 μg, respectively.

4.3 EXPERIMENTAL

4.3.1 General Experimental Conditions

The general experimental procedures were similar to those reported earlier in Chapter 2.

4.3.2 Microorganisms

Fungi, *Mucor plumbeus* (ATCC 4740) and *Curvularia lunata* (ATCC 12017 were obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA and maintained on Potato Dextrose Agar and stored in a refrigerator at 4 °C.

All five types of bacteria used for Kirby-Bauer anti-bacterial testing were obtained from the Department of Biology, The University of Winnipeg, Winnipeg, Manitoba. The bacteria used were: *Staphylococcus aureus* var. gold, *Escherichia coli*, *Pseudomonas aeruginosa*, *Corynebacterium xerosis* and *Bacillus subtilis.*

4.3.3 Microbial Substrates

Soy broth culture was used for carrying out the biotransformation experiment. Fermentation medium was prepared by mixing 20 g dextrose (EM Science), 5 g yeast (Bacto yeast extract, DIFCO), 5 g NaCl (EM Science), 5g K_2HPO_4 (EM Science), and 5 g soy flour (Vita Health) per liter of distilled H_2O and adjusted to pH 7 before autoclaving. Stock cultures of all fungi were maintained on potato dextrose agar (PDA) (potato infusion 200.0 g/L, bacto dextrose 20.0 g/L, bacto agar 15.0 g/L, pH 5.6 ± 0.2, DIFCO) slants at 4 °C and PDA petri plates at room temperature.

For Kirby-Bauer anti-bacterial testing, *Staphylococcus aureus* var. gold, *Escherichia coli coli*, *Pseudomonas aeruginosa, Corynebacterium xerosis and Bacillus subtilis* were grown on Mueller Hinton Agar (MH) (beef extract powder 2.0 g/L, acid digest casein

17.5 g/L, soluble starch 1.5 g/L, pH 7.3 ± 0.1, DIFCO). All broths and agar medium were sterilized at 121 °C and 15 PSI.

4.3.4 Biotransformation Procedure

A two-stage fermentation protocol was used for screening experiment to isolate metabolite (**79**) and (**80**).[29] Stage I culture broths of *Mucor Plumbeus* (ATCC # 4740) and *Curvularia lunata* (ATCC 12017) were each started in 50 ml of soy broth in 250 ml Erlenmeyer flasks. They were incubated on a rotary shaker at 250 rpm and room temperature for 48 hours. After 48 hours, 5 ml of the stage I culture broths was used as inoculum for the 50 flasks and incubated on a rotary shaker at 250 rpm and at room temperature for their lifecycles, 24 hours after inoculation, sclareol was added to each of the different stage II culture broths to make a final concentration of 0.4 mg/ml. The sclareol was added as solution of (**78**) in ethyl acetate. The extract was then tested by thin layer chromatography (TLC). The whole culture broth was extracted with 3×500 ml of ethyl acetate each after the substrate had been incubating with the fungi for 10 days. These whole extracts were also tested by TLC to monitor the results. Fungal blanks were kept in which the fungi were incubated in the same broth, under the same conditions, and for the same time period, but without the substrate present. Substrate blanks were kept in which the substrate was incubated in the same broth, under the same conditions, and for the same time period, but without the fungi present. This was done to monitor if any suspected transformations were actually fungal metabolites or caused by the broth.

4.3.5 Isolation of biotransformed Products

After 10 days of incubation on the shaker, the suspensions were pooled and extracted three times with ethyl acetate. The ethyl acetate extract of whole culture, was evaporated under reduced pressure to afford a crude brown gummy extract. This extract was subjected to silica gel column chromatography and eluted with stepwise gradient of *n*-hexane-ethyl acetate (0-100%) to give 65 fractions for each fungus. Thin layer chromatography was used to check the purity of biotransfromed products. The solvent system used for TLC was *n*-hexane-ethyl acetate (1:1). The separated compounds were detected by spraying the plate with a solution 10% H_2SO_4 and heating it on the hotplate at 120 °C for 3 minutes. The fractions having same R_f value were pooled together. Removal of solvent resulted in two major biotransformed products (**79**) and (**80**).

4.3.6 Kirby-Bauer Anti-bacterial Testing:

Anti-bacterial testing was performed on biotransformed metabolite (**79**) and (**80**) using Kirby-Bauer method, as previously described in chapter 2.

3β-hydroxy sclareol (**79**)*:* IR(KBr) ν_{max} cm^{-1}: 3388, 2935, 1463, 1387, 1040, 992, 916; EI-MS: *m/z* (rel. int. %), 306(M^+-18, 8), 291(8), 255(4), 175(27), 149(83), 121(105), 95(35),71(45) 81(288), 43(100); ^{1}H-NMR (CD_3OD, 500 MHz): δ 5.91 (1H, *dd,* J_{cis}=10.8, J_{trans}=17.4), 5.19 (1H, *dd,*, J_{trans} = 17.4, H_{trans}-15); 5.0 (1H, *dd,* J_{cis}= 10.8, H_{cis}-15), 3.15 (1H, *dd J*= 5.0, 5.1), 1.23, 1.11, 0.96, 0.82, 0.75 (16-, 17-, 18-, 20- and 19-CH_3, 5 *s*); ^{13}C NMR (CD_3OD, 125 MHz) δ: 146.8(C-14), 111.8(C-15), 79.4(C-3), 75.0 (C-8), 74.4(C-13), 62.5(C-9), 56.6(C-5), 46.6(C-12), 45.0(C-7), 40.2(C-10), 39.9(C-4),

39.4(C-1), 27.6(C-2), 27.4(C-16), 24.0(C-17), 21.2(C-6), 20.7(C-11), 16.1(C-19) and 16.0 C-20).

18 α-hydroxyl sclareol (80): IR(KBr) ν_{max} cm^{-1}: 3347, 2933, 1462, 1389, 1049, 993, 914; EI-MS: *m/z* (rel. int. %): 306(M^+-18, 8), 279(8), 257(16), 177(77), 149(83), 121(105), 95(201), 81(288), 43(29); ^{1}H-NMR (CD_3OD, 500 MHz): δ 5.92 (1H, *dd,* J_{cis}=10.8, J_{trans}= 17.3, H-14), 5.19 (1H, *dd,* J_{trans}= 17.4, H-15_{trans}), 5.01 (1H, *dd,* J_{cis}= 10.8, H-15_{cis}), 3.34, 3.0 (18-H), 1.23 *s*, 1.11 *s*, .85 *s*, 0.71 *s* (16-, 17-,19, and 20-CH3, 4*s*); ^{13}C-NMR (CD_3OD, 125 MHz) δ: 40.7(C-1), 18.8 (C-2), 36.4 (C-3), 38.6 (C-4), 50.2(C-5), 21.1(C-6), 44.7(C-7), 75.1(C-8), 62.8(C-9), 40.4(C-10), 20.7(C-11), 46.6(C-12), 74.4(C-13), 146.7(C-14), 111.7(C-15), 27.3(C-16), 23.9(C-17), 72.0 (C-18),17.8 (C-19) and 16.3(C-20).

4.4 References:

1. Raja, M. S.; Wu, G. S.; Grad, A.; Rosazza, J. P. *J. Nat. Prod.* **1982**, *45*, 231.
2. Ata, A.; Atta-ur-Rahman; Choudhary, M. I. *Recent Discoveries in Natural Products Chemistry*. Eds. Atta-ur-Rahman, M. I. Choudhary, and M. S. Shekhani. Elite Publishers, Karachi, **1995**.
3. Akihisa, T.; Takamine, Y.; Yoshizumi, K.; Tokuda, H.; Kimura, Y.; Ukiya, M.; Nakahara, T.; Yokochi, T.; Ichiishi, E.; Nishino, H. *J. Nat. Prod.,* **2002**, *65,* 278.
4. Fenica, W.; Pawilik, J. R. *Marine Ecology-Progress Series*, **1991**, *71*, 1.
5. Hosny, M.; Johnson, H. A.; Ueltschy, A. K.; Rosazza, J. P. N. *J. Nat. Prod.* **2002**, *65*, 1266.
6. Orabi, K. Y.; Clark, A. M.; Hufford, C. D. *J. Nat. Prod.* **2000**, *63,* 396.
7. Orabi, K. Y.; Li, E.; Clark, A. M.; Hufford, C. D. *J. Nat. Prod.* **1999**, *62,* 988.
8. Schoken, M. J.; Mao, J.; Schabacker, D. J. *J. Agri. Food Chem.* **1997**, *45*, 3647.
9. Abourashed, E. A.; Clark, A. M.; Hufford, C. D. *Curr. Med. Chem.* **1999**, *6*, 359.
10. Davis, P. J. *Antibiotics and Microbial Transformations* Eds. S. S. Lamba and Walker. CRC Press, Boca Raton, FL. **1987,** 47.
11. Alexander, L. S.; Goff, H. M. *J. Chem. Educ.* **1982,** *59*, 179.
12. Kieslich, K. *In: Biotechnology*, Rehm H-J, Reed G(eds), Verlag Chemie, Weinheim, **1984,** *6a*, p. 1.
13. Holland, H. L. *Curr. Opinion Chem. Biol.* **1999,** *3*, 22.
14. Kim, K. H.; Kwon, D. Y.; Rhee, J. S. *Lipids,* **1984,** *19*,975.
15. Valivety, R. H.; Brown, L.; Halling, P. J.; Johnston, G. A.; Suckling, C. J. *Opportunities in biotransformations,* Elsevier, London, **1990,** p.81.

16. Peterson, D. H.; Murray, H. C.; Eppstein, S. H.; Reineke, L. M.; Weintraub, A.; Meister, P. D.; Leigh, H. M. *J. Am. Chem. Soc.* **1952,** *74*, 5933.

17. Fried, J.; Thoma, R. W.; Gerke, J. R.; Herz, J. E., Donin, M. N.; Perlman, D. *J. Am. Chem. Soc.* **1952,** *74*, 3692.

18. Sawada, S.; Kulprecha, S.; Nilubol, N.; Yoshida, T.; Kinoshita, S.; Taguchi, H. *Appl. Eviron. Microbio.* **1982,** *44*, 1249.

19. Lawrence, B. M., *Progress in Essential Oils, Perfumer and Flarorist*, **1986,** *11*, 111.

20. Singh, M.; Mahesh, P.; Sharma, R. P. *Planta Med.* **1999,** *65*, 2.

21. Decorzant, R.; Vial, C. A. *Tetrahedron,* **1987,** *43*, 1871.

22. Dimas, K.; Demetzos, C.; Vaos, V.; Ioannidis, P.; Trangas, T. *Leuk. Res.* **2001,** *25*, 449.

23. Barton, D. H. R.; Beloeil, J. C.; Billion, A.; Boivin, J.; Lallemand, J. Y.; Lelandais, P.; Mergui, S. *Helv. Chim. Acta* **1987**, *70*, 2187.

24. Breitmaier, E.; Voelter, W. *Spectroscopy: High Resolution Methods and Applications in Organic Chemistry and Biochemistry*; VCH Verlag: Weinheim, **1987**, p. 342 & 447.

25. Hollinshead, D. M.; Howell, S. C. ; Ley, S. V.; Mahon, M.; Ratcliffe, N. M.; Worthington, P. A. *J. Chem. Soc., Perkin Trans.* I, **1983**, 1579.

26. Torrenegra, R.; Pedrozo, J.; Robles, J.; Waibel, R.; Achenbach, H. *Phytochem.,* **1992**, *31*, 2415.

27. Grandos, A.; Ortiz, A. W. A. *J. Nat. Prod.*, **1990,** *53*, 441.

28. Sholichin, M.; Yamasaki, K.; Miyama, R.; Yahara, S.; Tanaka, O. *Phytochem.,* **1980**, *19,* 326.

29. Betts, R. E.; Walters, D. E.; Rosazza, J. P. N. *J. Med. Chem.*, **1974**, *17*,599.

CONCLUSIONS

During this research project, we were successful in isolating bioactive natural products:

An ergosterol peroxide (3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene) was isolated from crude extract of methanolic-ethyl acetate of *Pleurotus* sp., which showed weak antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Corynebacterium xerosis* but it was remained inactive against *Escherichia coli, Pseudomonas aeruginosa.* The purification of the crude methanolic extract of *Buxus hyrcana*, resulted in another bioactive triterpenoidal alkaloid, O^6-buxadiene. And it showed weak anticancer activity against prostate cancer cell lines.

Additionally, we also did the microbial transformation of sclareol, a plant natural product, by using standard two stage fermentation method. Two fungi *Curvularia lunata* (ATCC 12017) and *Mucor plumbeus* (ATCC 4740) were capable of oxidizing sclareol into 3β-hydroxy sclareol and 18α-hydroxy sclareol .The antibacterial activity of biotransfromed metabolites of sclareol was evaluated, against *E. coli, P. aeruginosa, S. aureus*, *B. subtilis* and *C. xerosis.* The 18α-hydroxy sclareol showed weak antibacterial activity against *B. subtilis.* It showed that metabolized products lost activity as compared to sclareol.