2004

Bioactive alkaloids from medicinal plants of Bhutan

Phurpa Wangchuk
University of Wollongong

Bioactive Alkaloids from Medicinal Plants of Bhutan

A Thesis Submitted in Fulfillment of the Requirements for the Award of the Degree

Master of Science (Research)

From

The University of Wollongong

By

Phurpa Wangchuk, B.Sc.(Gen.), A.M.C.

Supervisor: Prof. John B. Bremner

Department of Chemistry

University of Wollongong, Australia

June 2004

Declaration

The ideas and the materials of this thesis, submitted in fulfillment of the requirements for the award of the Master of Science by Research (Medicinal Chemistry) from the University of Wollongong, are entirely of my own work and creation unless otherwise referenced or acknowledged.

Phurpa Wangchuk

18 June 2004

Publications

1. Bioactive alkaloids from the Bhutanese medicinal plant *Aconitum orochryseum* Stapf. Three new C_{20} diterpenoid alkaloids (*Journal of Natural Products*, in preparation).
2. Isoquinoline alkaloids, as novel antimalarial leads from the Bhutanese medicinal plant, *Corydalis gerdae* Fedde (*Planta Medica*, in Preparation).

Acknowledgements

I am highly obliged to my government for giving this M.Sc. scholarship. My unfathomable gratitude owes to Prof. John B. Bremner for being my supervisor without whom this work would have been a far distant dream. I am grateful to Dr. Paul Keller for initially coordinating my studies; Dr. John Korth and Larry Hick for training me to use the mass spectrometer and running my samples for GCMS and HRMS; Dr. Wilford Lee and Dr. Sandra Chapman for giving me NMR training and running experiments on the 500 MHz NMR; Dr. David Perkins for helping me with crystal growing and computer modeling of the structures for my alkaloids; Dr. Chris Bourke, Orange Agriculture Institute, Orange, NSW; Dr. Sumalee Kamchonwongpaisan and Ms. Roonglawan Rattanajak, Protein-Ligand Engineering and Antimalarial Screening Laboratories, National Science and Technology Development Agency, Bangkok, for conducting the antimalarial testing for my samples and Amrad, Melbourne for carrying out the antibacterial testing for my samples.

My sincere thanks goes to Mr. Bill Hawkins for being a good office mate and helping a friend in need especially in initializing my first experiment; Mrs. Siritron for providing me her research skills and encouragement; Ms. Waya for helping on the preparation of preparative plates; Mr. Joseph Ambrus for proofreading my thesis; Mr. James for his productive discussion on structure elucidation; Miss Jane and Mr. Johana for their company and to all my fantastic and helpful friends in the Bremner group. I wouldn't have learnt so much had these people not been there for me.

My utmost thanks also goes to the people working in the Institute for Traditional Medicines Services, especially Dungtshos, Mr. Dorji Thinlay and the medicinal plant collection team from the Pharmaceutical and Research Unit of Bhutan for their belief, faith and earnest

support rendered during the time of my studies in the University of Wollongong. Having said this, it won't be justified if Mr. Samten of Pharmaceutical and Research Unit is not brought into the limelight of this success story. He duly did the entire methanol extractions and supplied me the necessary ethno-medical information to which I owe him my heartfelt gratitude.

Lastly, I thank my wife Mrs. Sonam Choden for her evergreen love, care and constant support through out the study and the Bhutanese friends in the University of Wollongong especially Mr. Tshewang Dorji, Mr. Ugyen Dorji, Mr. Khampa Tshering and Mr. Sonam Dukda for their genuine friendship.

List of Abbreviations

AMRAD	Australian Medical Research And Development
bs	Broad singlet
^{13}C-NMR	Carbon Nuclear Magnetic Resonance
CNS	Central Nervous System
COSY	Correlation Spectroscopy
d	Doublet
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DNA	Deoxynucleic Acid
FDA	Fluorescein Diacetate Assay
GCMS	Gas Chromatography Mass Spectrometer
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HRCIMS	High Resolution Chemical Ionisation Mass Spectrometry
HREIMS	High Resolution Electron Impact Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
HTS	High Throughput Screening
Hz	Hertz
IC_{50}	Inhibitory Concentration with 50% inhibition
IR	Infrared
LRCIMS	Low Resolution Chemical Ionization Mass Spectrometry
LREIMS	Low Resolution Electron Impact Mass Spectrometry

M	Multiplet
MeOH	Methanol
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometer
NITM	National Institute of Traditional Medicine
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
ppm	parts per million
PTLC	Preparatory Thin Layer Chromatography
q	Quartet
Q	Quaternary
s	singlet
STD	Sexually Transmitted Disease
TLC	Thin Layer Chromatography
TOCSY	Total Correlation Spectroscopy
US	United States
UV	Ultraviolet
VRE	Vancomycin Resistant Enterococcus
WHO	World Health Organization

Abstract

Natural products had been indispensably used by many cultures and traditions in folklore medicines for thousands of years. These traditional medicines cater to about 85% of the world population for their primary health care needs. Natural products have been intensively explored also for their bioactive pharmacophores by modern pharmaceutical companies. In fact they are the skeletal framework of about 60% of the modern drugs that are available today. Of these, about 80% of antibacterial drugs and 90% of antimalarial drugs are natural derivatives. One of the interesting secondary metabolite groups which provides these potent drug leads are the alkaloids. Owing to their broad range of bioactivities, bioprospecting for these natural products are undertaken in many countries. In this study, on the molecular basis of traditional medicine of Bhutan, particularly those with potential antimicrobial components, four alkaloid positive medicinal plants, i.e. *Aconitum orochryseum* Stapf, *Corydalis gerdae* Fedde, *Rununculus brotherusi* Freyn and an Australian naturalized plant *Tribulus terrestris* Linn, but also used in Bhutan, were selected for phytochemical analysis.

Bhutanese traditional medicines use *Aconitum orochryseum* (whole parts) for the treatment of bilious fever or high fever related to bile disorders including cold and flu, fever of bile (liver), fever caused by malaria, snake bites and for treating blood infections. *Corydalis gerdae* (whole parts) is used for the treatment of malaria and infections. *Ranunculus brotherusi* (aerial parts) is used for treating wounds, pus, infections and as antipyretics. *Tribulus terrestris* (fruit) is used for the treatment of arthritis, kidney diseases and as a diuretic. Though *Tribulus terrestris* and *Ranunculus brotherusi* failed to give any alkaloids, a number of new as well as known alkaloids were isolated from *Aconitum orochryseum* and *Corydalis gerdae*.

Three new hetisane type diterpenoid alkaloids named orochrine, 2-*O*-acetylorochrine, and lingshinaline, together with two known alkaloids atisinium chloride and virescenine and six other unidentified alkaloids, were isolated from *Aconitum orochryseum*. The structures of the new alkaloids were elucidated by ^{1}H-NMR, gCOSY, gNOESY, TOCSY, ^{13}C-NMR, DEPT, gHSQC and gHMBC spectral data analysis. Atisinium chloride, a major alkaloid of the plant, was identified by single crystal X-ray crystallography.

Four known protopine type and protoberberine type isoquinoline alkaloids (protopine, scoulerine, cheilanthifoline, and stylopine) along with one unidentified alkaloid were isolated from *Corydalis gerdae*. The protopine was the major alkaloid of this plant. The alkaloids were identified by MS and ^{1}H-NMR spectral data comparison.

The antibacterial testing of crude methanol extracts, crude alkaloids and the major alkaloids (atisinium chloride, orochrine and protopine) of these two plants were found inactive against the bacterium *Staphylococcus aureus* (MIC = >125 μg/ml) and Vancomycin resistant *Enterococcus faecium* (MIC = >125 μg/ml). But the *in vitro* antimalarial assay of crude methanol extracts, crude alkaloids, atisinium chloride, protopine, and cheilanthifoline gave very significant activity against *Plasmodium falciparum*, TM4 and K1 strains. Crude methanol extract (AO-ME) of *Aconitum orochryseum* was not that active (IC_{50} of >10 μg/ml for both the strains). Increased activity was observed for its crude alkaloid (AO-CEA) with IC_{50} values of 20.40 μg/ml against TM4 and 19.20 μg/ml against K1 strains. The activity was further increased for atisinium chloride with IC_{50} values of 4.02 μM against TM4/8.2 and 3.59 μM against K1CB1 strains.

The antimalarial activity was better for the extracts and alkaloids of *Corydalis gerdae*. Its crude methanol extract (CG-ME) had the IC_{50} values of 1.00 μg/ml against TM4 strain and

2.56 μg/ml against K1 strain. The activity of crude alkaloid of this plant was far better with IC_{50} values of 0.33 μg/ml against TM4 strain and 0.63 μg/ml against K1 strain. Protopine had IC_{50} values of 4.25 μM against TM4/8.2 and 4.29 μM against K1CB1 strains. Cheilanthifoline had the IC_{50} values of 3.76 μM against K1 and 2.78 μM against TM4 strains.

These alkaloids have the potential to become candidates for antimalarial leads. Similar activities may be expected from other alkaloids including new ones isolated from these two plants. Therefore, the combination of these two plant extracts would make the best antimalarial herbal mixture for Bhutanese traditional medicines. The results proved that ethno-directed biorational approach, combined with an alkaloid focus, is an efficient strategy for drug lead discovery. The results also proved for the first time at the molecular level that Bhutanese traditional medicines have clinical efficacy. The medicinal plants of Bhutan thus have potential to provide important new drug leads. Bioprospecting them would benefit local people, pharmaceutical industries and the patients at large.

Table of Contents

Chapter 1

Natural Product-Based Drug Discovery and Recent Developments

1.1 General Introduction

With the advancement in science and technology, remarkable progress has been made in the field of medicine including diagnosis, treatments and pharmaceutics. Recent drug discovery techniques based on Structure-Activity Relationships, Computer Modelling, Combinatorial Chemistry, High Throughput Screening and Spectroscopy (MS, NMR, and IR) have triggered and spearheaded the discoveries of many natural and synthetic drugs. In 1999, world sales of pharmaceuticals (excluding veterinary medicines) were valued at *ca* US$ 325 billion[1].

Despite these developments, of the known 30,000 human diseases or disorders, only one-third can be treated symptomatically with drugs (either in part or fully) and that too at a great economic and social cost. This is because of the fact that the drugs available today are still not very effective particularly with respect to the fight against drug resistant pathogens and newly emerging infections. This includes infectious diseases such as AIDS, influenza, tuberculosis and malaria as well as other chronic disorders like cancer, autoimmune disorders and central nervous system disabilities (e.g. Alzheimer's disease). The lead cause of death is HIV-AIDS, followed by tuberculosis and malaria. In 1996, the HIV virus had infected at least 21,000,000 people worldwide[2] and in 2001, UNAIDS estimated that over 14,000 new infections occurred daily, nearly half of them in women[3] and strikingly affecting Africa[4]. While malaria continues to affect 1-3 million lives each year[5], ca. 2 billion people (including at least 15 million Americans) are affected by tuberculosis[6,7]. These infectious diseases (microbial) will continue to be the leading causes of premature death in human beings of both developed and developing nations as their resistance to many conventional drugs is increasing. Therefore, microbial resistance especially by bacteria and protozoa to drugs is of special concern to communities worldwide and to researchers.

1.2 Development of Antibacterial Resistance

The discovery of bacteria in 1683 by van Leuwenhoek helped mankind to understand the infectious pathogens and appropriately develop antiseptic and antibiotic protocol in the following years. By the beginning of 20th century, Paul Ehrlich proposed the principle of chemotherapy and his work including Structure-Activity Relationships

significantly contributed to shaping synthetic protocols and helped in the later discoveries of antibacterial drugs[2]. Antibiotics were designed either to kill bacteria (bactericidal) or to nullify growth (bacteriostatic)[8] and three groups of antibacterial agents, which included bacterial cell wall inhibitors, protein synthesis inhibitors[9] and DNA inhibitors, were developed to fight the bacterial infections. The sulphonamides were the first group of effective antibacterials to be developed following a chance discovery in 1932 by Domagk of antibacterial activity in the synthetic azo dye, prontosil.

Many antibacterial drugs were developed in the late 1940s following the first report of resistance in S*taphylococcus aureus* (1941) and in *Mycobacterium tuberculosis* (1940s)[2], a mycobacterium which also developed resistance later to the drugs isoniazid and rifamycin in the 1950s and 1960s. However, by the 1980s most major infectious diseases in the developed world were almost eradicated and half the major pharmaceutical companies in Japan and the USA stopped their antibacterial drug development programs[10]. As a result drug-resistant pathogens were on the rise worldwide[11]. *Streptococci* that causes nosocomial infections showed innate resistance to drugs including cephalosporins, clindamycin and aminoglycoside[2,10]. The bacterium (S*taphylococcus aureus*) has now developed multi-drug resistant strains and threatens to put an end to successful chemotherapy[12]. Vancomycin resistance among enterococci became noticeable in 1987 resulting in a true 'super bug'. A summary of bacterial resistance to chemotherapy is shown in **Table 1**.

Table 1. Bacteria that have acquired resistance to some drug therapy[2].

Resistant type	Bacteria	Disorder	Date (approx.)
Penicillin	*Pneumococci*	Pneumonia, meningitis	Mid 1970s-present
resistant	*Legionella*	Legionnaire's disease	Mid 1970s-present
	Borrelia burgdorferi	Lyme disease	1980s-present
	Salmonella	Gastrointestinal disorders	1980s-present
	Staphylococci	Toxic shock syndrome	1980s
	E.coli	Gastrointestinal disorders	Mid 1980s-present
Multi-drug resistant	*M.tuberculosis*	Tuberculosis	Late 1980s-present
Vancomycin	*Enterococci*	Wound, blood, enteric infections	Late 1980s-present
resistant	*V.cholerae*	Cholera	
Multi-drug resistant	"super bugs"		2002

1.2.1 Mechanism of Bacterial Resistance

The mode of bacterial resistance is either intrinsic (maintained on the bacterial chromosome) or acquired through chromosomal mutations and plasmid transformation, transposition, transductions and conjugation from other species[2,13]. The fundamental mechanisms of resistance generally observable in bacteria include inactivation or degradation of the antibacterial drug by enzymatic action, decreasing or changing of membrane permeability of the bacterial cell wall to antibiotics, the alteration of the bacterial proteins that are antimicrobial targets, and less often, auxotrophic or metabolic by-pass[2]. For example, the resistance to the penicillins and cephalosporins occurs through antibiotic hydrolysis mediated by the bacterial enzyme beta-lactamase while the resistance to trimethoprim, the sulphonamides, the aminoglycosides, chloramphenicol and the quinoline drugs occurs by the productions of antibiotic-modifying enzymes and the synthesis of antibiotic-insensitive bacterial targets[14].

It was found that the degree of resistance may vary based on the level and the capacity of the pathogenic micro-organisms to manipulate, alter and combine all of these biochemical and genetic processes. Several factors, such as inappropriate prescription of drugs (use of doses that are too low), over prescription in trying to meet patients' demand, extravagant prescription due to excessive choices of available drugs, poor infection control practices, the length of therapy (incomplete course taken by patients) and the duration of bacterial exposure to long-acting agents compared to drugs with short half-lives have been found to influence the rate of selection of resistance and play a role in the worsening situation of global resistance[15]. Other determinants such as the state of immunity and the phenotypes of bacterial pathogens also influence the mode of resistance.

1.3 Development of Antimalarial Resistance

Malaria is another pandemic disease endemic to 102 countries[16]. It is an infectious disease caused by four types of parasitic protozoan in the genus Plasmodium: *Plasmodium Falciparum, Plasmodium malariae, Plasmodium ovale* and *Plasmodium vivax*. Falciparum malaria is the most severe form of malaria and it has already developed resistance to many of the currently available drugs. The parasites are transmitted by the bite of female anopheles mosquitoes. Insecticides such as DDT (dichlorodiphenyltrichloroethane) were used in many countries to control the mosquito vector. Though it has partially helped to bring malaria under control, it has also generated many long-term detrimental effects.

The search for agents to combat malaria became particularly intense in the 1800s as British soldiers in India were infected with malaria and as outbreaks of malarial epidemics took heavy tolls in tropical, subtropical and temperate regions of the world [2]. Population growth, urbanization, refugees, conflicts and global warming[17] have contributed significantly to the outbreak of further epidemics. Quinine **1** was the first antimalarial drug discovered in 1820 by Caventou and Pelletier from the bark of *Cinchona officinalis*[18]. By the early and mid 20th century, its synthetic derivatives such as quinacrine, mefloquine and chloroquine became available. In continuation of searches for antimalarial drugs, the first major research on plant extracts was started in 1947 by the screening of 600 species of plants belonging to 126 families but it was only in the mid 1980s that the testing of these plant extracts were completed[19]. The research found several plants, particularly in two families, the Simaroubaceae and Amaryllidaceae, active against avian malarias[18].

With the discovery of many cheap antimalarial drugs, malaria was eliminated from Europe, North America, Australia, South America and Asia and the search for new antimalarial agents became secondary for these countries. The limited armoury of drugs is now greatly compromised by the spread of drug resistant parasite strains[20]. The chloroquine resistant strains of *Plasmodium falciparum* have now developed resistance to mefloquine, halofantrine and quinine[21,22]. Malaria is again claiming millions of lives, mainly affecting children under the age of five in Sub-Saharan Africa[4] (**Figure 1**). Currently, a combination of synthetic drugs like sulfadoxine and pyrimethamine (Fansidar) is used in treating these resistant parasites. Other combination therapy like pyrimethamine with dapsone, quinine/tetracyclines, and atovaquone/proguanil are also used. Nevertheless, resistance to such combined drug therapy has also been reported in Africa, Thailand, Burma, Laos, Vietnam, Cambodia and China.

Artemisinin **2,** which was discovered in 1972 from the Chinese medicinal plant, *Artemisia annua*[22], is the main hope for saving millions of lives from the multi-drug resistant parasite, *Plasmodium falciparum*. Artemisinin and its derivatives such as artemether, arteether, dihydroartemisinin, artelinic acid and artesunic acid (intravenous and intramuscular administration)[22] are currently used worldwide. Recently, the potent antimalarial agents, raphidecurperoxin **3** and polysyphorin **4**, were isolated from the Vietnamese medicinal plants, *Rhaphidophora decursiva*[23]. These natural products showed strong activity against *Plasmodium falciparum in vitro* without any toxicity and may become potential candidates for antimalarial drugs.

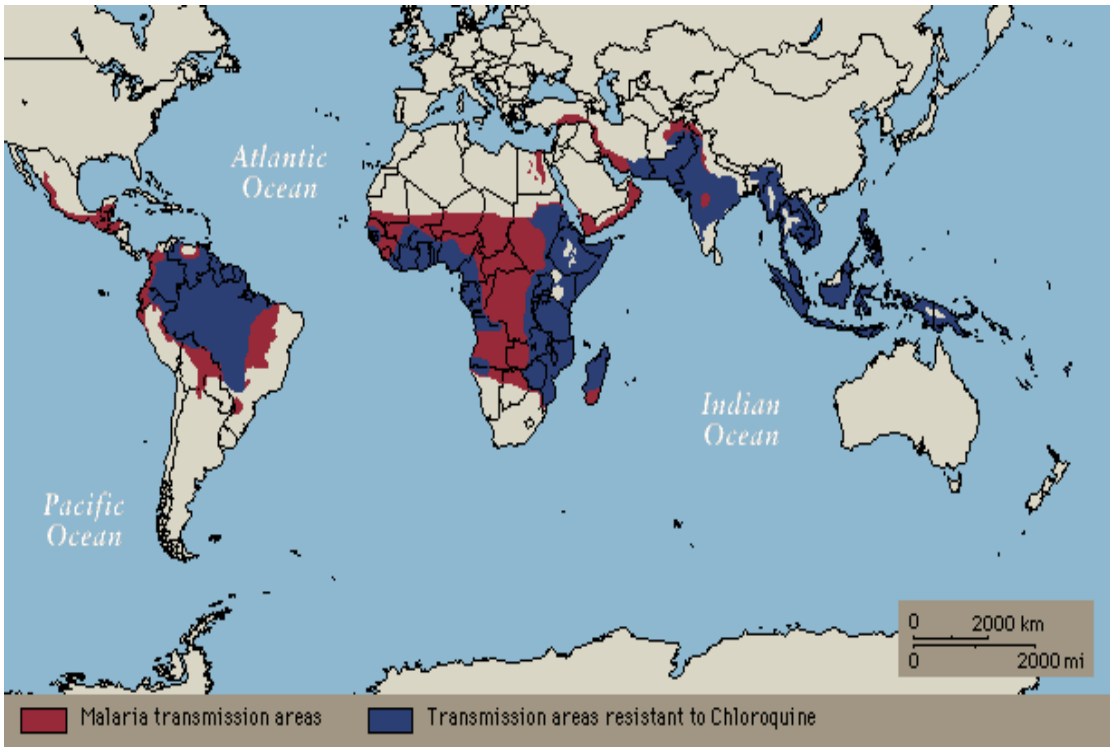
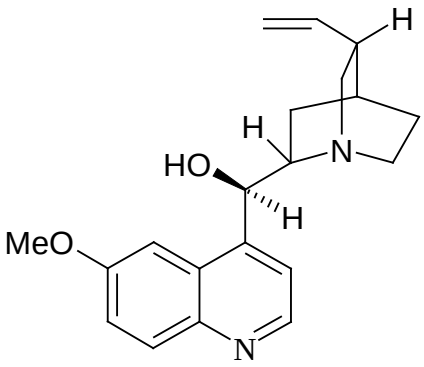
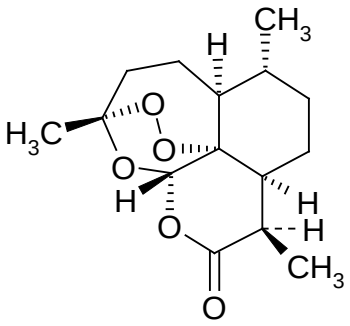


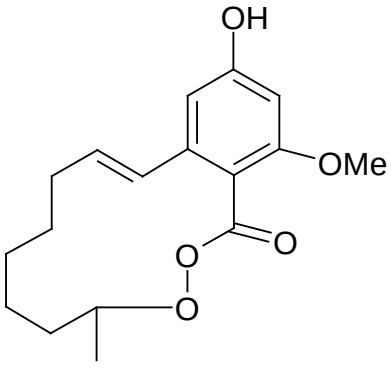
Figure 1. World map showing the occurrence of malaria and chloroquine resistant areas[4].



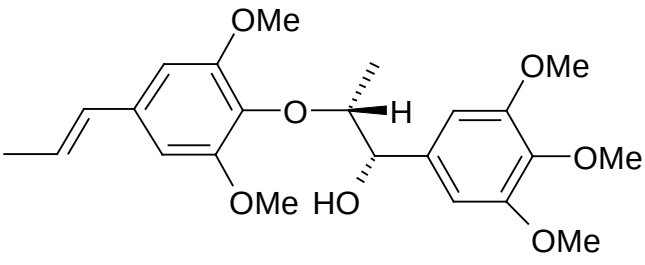
1 Quinine



2 Artemisinin



3 Raphidecurperoxin



4 Polysyphorin

Voacamine (a bisindole alkaloid), isolated from *Voacanga* species (Apocynaceae) has also been found to give promising *in vitro* activity against chloroquine-sensitive and

resistant strains, *Plasmodium falciparum*[24]. Termilignan, thannilignan, 7-hydroxy-3′,4′-(methylenedioxy) flavan, and anolignan B, all isolated from *Terminilla bellerica*, have also shown anti-HIV, antimalarial and antifungal activity *in vitro*[25].

1.3.1 Mechanism of Parasite Resistance

Malarial infections are increasing due to the development of resistance by the parasites to traditional drugs as well as acquisition of resistance to DDT by the vector *Anopheles* mosquitoes. It was suggested that resistance to the inhibitors of dihydro-folate reductase and dihydro-pteroate synthetase enzymes, and the novel antimalarial atovaquone, occurs through genetic point mutations[26]. The mechanism of resistance to quinoline drugs like chloroquine has been attributed to mutations in two genes; *pfmdrl* and *pfCRT* [27]. These mechanisms of drug resistance in parasite are yet to be defined but certainly the mechanism of resistance do seem to vary according to the use and type of drugs, each of which having distinct modes of action on parasites.

1.4 Strategy for Control of Bacterial and Malarial Infections

As the microbial infections continue to plague the nations worldwide due to very few effective drugs that are available today (antibacterial agents make up only 12-15% of the total pharmaceutical business[2]), there is an urgent need to find concrete solutions for combating such epidemics. Prevention of famines, droughts, poverty, floods, wars, political upheaval, economic failures, environmental depletion and pollution would be good solutions to help reduce infections and microbial resistance.

Another measure to prevent microbial resistance (bacterial and malarial) is to use the currently available antimicrobial drugs more judiciously and optimally. In general, resistance is observed higher in areas of high consumption of antibiotics and lower in areas of low consumption[28]. A study conducted on controlling resistance in community-acquired pathogens in Finland in the 1990s, revealed that there was a 50% reduction in the macrolide resistant group-A *Streptococci* when the use of macrolides such as erythromycin were reduced for treating the diseases[29]. Therefore, choosing the correct drug, dose, dose interval and duration of therapy may more efficiently provide clinical benefits while contributing less to resistance than choosing the drug with the narrowest spectrum[30].

Above all, the most important strategy to control, cure and to prevent reinfection is to develop more appropriate treatment regimens including the super drugs. Super drugs could be developed synthetically, but as experience has taught us, natural products are

rich in structurally diverse bioactive molecules like alkaloids, which quite often become potential candidates for the development of drugs.

1.5 Development of Drugs from Bioactive Natural Products

Bioactive natural products are mainly secondary metabolites which are used by the host as defensive and protective mechanisms against their enemies and predators[31]. Generally, screening of these secondary metabolites and development of drugs is a very hard task requiring much effort starting from botanical identification, collection, extraction, isolation, purification and compound identification to pharmacological and clinical testing. However, the enormous chemical diversity and highly unusual structures provided by natural products is greater than that provided by most available combinatorial approaches based on heterocyclic compounds[32,33].

When screening for natural products, three main types of search strategies, namely biorational, chemo-rational and random approaches[34] are followed. The bio-rational approach includes ethno-medically directed screening (as discussed above) and random screening. Random high throughput screening is based on field observation such as pest-plant analysis and plant characteristics. Drugs such as artemisinin, morphine, quinine, and ephedrine were discovered using the ethno-directed bio-biorational approach[35]. Conocurvone[36], an anti-HIV agent was discovered as a result of random high throughput screening. The chemo-rational approach is based on chemo-taxonomical considerations such as alkaloid surveys, and investigation of specific families which are known to contain potential drug leads[37]. Employing these three search strategies as many as 88,000 natural product compounds have been isolated from different sources such as plants, animals, marine organisms, insects and micro-organisms.

It is estimated that ca. 250,000[38] to 500,000 species of plants grow on earth[39,40] and 70% of all the world's biodiversity of vertebrates, higher plants and butterflies are harboured by 12 countries; Mexico, Colombia, Ecuador, Peru, Brazil, Zaire, Madagascar, China, India, Malaysia, Indonesia and Australia[41]. The 18 global biodiversity hotspots support nearly 50,000 endemic plant species[41]. A high rate of endemism is predicted in mountainous areas like Bhutan, oceanic islands like Australia, mutuallistic special pollinators as present in Madagascar and plant symbionts like orchids[42]. These endemic species were found to contain varieties of distinctly new chemical structures. It is reported that only 10-15% of terrestrial biodiversity especially higher plants were explored phytochemically for medicinal applications[40], and the main

categories of plant-derived drugs are terpenes (34%), glycosides (32%), alkaloids (16%) and others (18%)[43]. Around 85% to 90 % of terrestrial resources are still intact and await exploration.

Amongst the animal kingdom, lower groups of animals especially those secreting venoms and toxins such as insects, amphibians, reptiles, wasps, and spiders, produce interesting and novel bioactive compounds. There are 30 million species of insects and very few have been studied for bioactive secondary metabolites[34]. Out of *ca* 100, 000 species of spiders, only *ca* 30 have been studied until date[44]. The marine world represents 70% of earth's surface, but only 5% of the marine organisms have been explored chemically and a huge diversity of marine fauna and flora are yet to be investigated[31]. Marine secondary metabolites are often observed to have anticancer activity. Approximately 300 patents on marine natural products were issued between 1969 and 1999[45] and a number of compounds have progressed to clinical trials.

Micro-organisms have been a frequent source of antibiotics, ever since the discovery of penicillin from the filamentous fungus *Penicillium notatum* by Alexander Fleming in 1928. Cyclosporins and rapamycin (an immunosuppressive agent), ivermectin (an anthelmintics and antiparasitic drugs), streptomycin, chloramphenicol, tetracyclines and cephalosporin (antibiotics) were isolated from *Streptomyces* or *Penicillium* species. Only an estimated 1% of bacterial and 5% of fungal species have been examined to date[38]. Extremophiles such as alkalophiles, halophiles, barophiles, thermophiles and psychrophiles also offer a potentially diverse source of novel bioactive agents[38].

1.5.1 Application of Alkaloids in Medicine

Hesse defined alkaloids as "nitrogen containing organic substances of natural origin with lesser or greater degree of basic character" and include both alkaloids with nitrogen as part of a heterocyclic system as well as the extra cyclic bound nitrogen[44]. However, Roberts and Wink have suggested that a basic character is no longer a pre-requisite for alkaloids[46].

Many workers in the field of alkaloids have attempted classifying alkaloids based on their biogenesis from amino acid derivatives, structural relationships, biological origin, and spectroscopic properties[44]. Since many alkaloids are derived from more than one of the major precursors, there is extreme heterogeneity in alkaloid structures, which makes their orderly classification very difficult. The classification is, thus to some extent, arbitrary and not well established.

Nevertheless, biogenetic groupings of alkaloids are most commonly followed. According to this system[47], four main types of alkaloid groups are distinguishable:

i) Alkaloids of amino acid derivatives, like ornithine, histidine, tryptophan, tyrosine, lysine, anthranilic and nicotinic acids.
ii) Alkaloids of purines like zanthine and caffeine.
iii) Alkaloids of aminated terpenes, like the diterpenoid alkaloid, aconitine and the triterpenoid alkaloid solanine.
iv) Alkaloids of polyketides like coniine and the coccinellines[46].

Alkaloids derived from ornithine are categorized into: simple alkaloids, tropane alkaloids, pyrrolizidine alkaloids, phenanthroindolizidine alkaloids, spermine, spermidine and related alkaloids and the miscellaneous alkaloids. Alkaloids derived from lysine are grouped into: simple piperidines, lobelia alkaloids, lycopodium alkaloids, lythraceae alkaloids and complex and miscellaneous alkaloids. Alkaloids derived from anthranilic acid include the quinoline, furanoquinoline, acridine and quinazoline groups.

Alkaloids derived from tyrosine consist of simple β-phenylethylamine derivatives, Amaryllidaceae alkaloids, and the isoquinoline alkaloids, which are further sub-categorized into many sub-types. Alkaloids derived from tryptophan constitute the largest alkaloid group and include tryptamine derivatives, carbazoles, ergot alkaloids, borreria alkaloids, monoterpenoid and indole alkaloids. Alkaloids derived from aminated terpenes include, monoterpenoid pyridine alkaloids, nuphar alkaloids, celastraceae alkaloids, erythrophleum alkaloids, diterpenoid alkaloids, steroidal alkaloids, and the daphniphyllum alkaloids. Diterpenoid alkaloids are a major and fascinating sub group in this category and they have been the source of many bioactive compounds in the past. It has been found that species in a given genus or related genera yield the same or structurally related alkaloids; for example seven different genera of plants in the Solanaceae contain hyoscyamine[48], while 164 genera of 47 families produce both isoquinoline and indole alkaloids[49].

With alkaloid nomenclature, trivial naming is commonly observed, however about four different types of naming are followed based on[44]:

i) A systematic plant name, e.g Papaverine is derived from *Papaver* species.
ii) The plant discoverer, for example spegazzinine from *Aspidosperma chakensi* SPEGAZZI.
iii) Geographical settings for example tasmanine after Tasmania.
iv) A person's name, for example macrosalhine after the lady Salhiha.

But such naming of alkaloids falls short when several new alkaloids are isolated from a single plant. Judicious naming can be followed based on the chemist's imagination, assigning sufixes like '-ine', '-idine', '- anine', '-aline', and '-inine'[44], since IUPAC naming becomes complex as most of the natural products are very large molecules.

In 1988, the NAPRALERT database listed ca.16000 alkaloids with varying types of nomenclature as described above[40,46]. It is estimated that 10-15% of all vascular plants contain alkaloids, but they are observed particularly in the families: Apocyanaceae, Papaveraceae, Papilionaceae, Ranunculaceae, Rutaceae and Solanaceae[48]. The alkaloids serve plants as a feeding deterrent to protect them from predators and they are also believed to act allelopathically in some plants[44].

Alkaloid distribution in the plant can vary. In many cases higher concentrations are observed in areas where herbivores usually attack, i.e. in the inflorescence, young growing tips and peripheral cell layers of stems and roots[46]. An analysis of 3,589 varieties of plants of various origin in different growth phases and parts of the plant revealed the fact that the maximum amount of total alkaloids is produced by young plants and by green parts of the plant[50]. The alkaloid content and the types of alkaloids also vary from species to species, season to season and region to region. The biosynthesis and the formation of alkaloids in plants involve elaborate chemical reactions that are performed sequentially in a way unique to each species and quite often different from laboratory synthesis[51].

Lipinski and his co-workers[49,52] suggested alkaloids as suitable candidates for orally bio-available drugs. Such drugs should have:

a) Less than five hydrogen bond donor groups (such as –OH and -NH).
b) Less than ten H-bond acceptors in the molecule (such as oxygen and nitrogen atoms).
c) A molecular weight less than 500 Dalton.
d) A Log P value less than five units.

At present, approximately 600 alkaloids have been analysed for their bio-medicinal properties and many of them have become important drug leads or actual drugs in the pharmaceutical industry[46]. For example, the taxol, isolated from *Taxus brevifolia* and the vincristine and vinblastine isolated from *Catharanthus roseus* (Madagascar) are the important anti-cancer drugs derived from plant alkaloids. Very recently, provir, an oral product for the treatment of respiratory viral infections, and virend, a topical antiviral product for the treatment of herpes (both in clinical trials) were developed from plant alkaloids[53]. Hadi *et al*[54] isolated the antimicrobial alkaloids: lombine, cononaridine, and

mataranine A and B from the plant *Vocanga foetida* Rolfe. These alkaloids were reported to have strong activity against *Staphylococcus aureus.* A mixture of mataranine-A and B was found active *in vitro* against *Plasmodium falciparum*. While 25 to 50% of the current pharmaceuticals, including antimalarial drugs, were derived from plants, no ideal antibacterial drugs (antibiotics) have yet been developed from plants[55].

Captopril, an antihypertensive agent[31] was derived from an alkaloid, teprotide (isolated from Snake venom). Epibatidine, which was isolated from the frog *Epipedobates tricolor*[56] was shown to be a novel non-opioid analgesic agent with two hundred times the analgesic potency of morphine. In the mid 1980s, bryostatin-1 was isolated from *Bugula neritina*, a marine bryozoan, and was found to be effective against ovarian carcinoma and non-Hodgkin's' lymphoma[38]. Halobacilin, bisucaberin and homocereulide await clinical applications[57]. Studies on the egg masses of *Dicathais orbita* (a marine mollusc) by Benkendorf *et al*. using an ecologically-directed approach resulted in the isolation of the broad spectrum antibacterial alkaloid derivative, tyriverdin[58].

1.6 Natural Products in Traditional Medicines

Because *Homo sapiens* are herbivores to some degree, they first encountered bioactive agents in vegetal food items[59]. Having lived harmoniously in close association with the environment, they learned to utilise the toxic and medicinal properties of plants and other natural products. Some of those toxic plants were used as poisons for causing death and as arrow poisons for hunting food, warfare, depredating wild animals and for gaining mastery over a hostile environment[46,60]. The most remarkable of all the ancient inventions was the art of utilising these toxic and medicinal natural products for treating various ailments but the ethno-medical knowledge was restricted to a few elites such as priests, medicine men, shamans, magicians and witch doctors[59]. Although some cultures used individual herbs or plants as medicines, many traditions propounded powerful combinations with different ingredients known as poultices, tinctures and mixtures.

It was the Mesopotamians who first used the herbs like oils of cypress, cedar, liquorice and poppy juice for treating different ailments in 2600 B.C[38] followed by the Indian Buddhist system of medicine that dates to 2500 BC[61]. Then came the Egyptian's Ebers Papyrus (1500 BC) that documented some of the 700 drugs including formulas such as gargles, snuffs, poultices, infusions, pills and ointments[38], the Chinese materia medica

Wu-Shi Er-Bing Fang which contains 52 prescriptions dating to 1100 BC[38,62] and the Indian Ayurvedic Medicine that dates to 1000 BC (Susruta and Charaka) documenting the medicinal use of plants like *Datura, Aconitum, Canabis* and *Sarcostemma*[59].

From these ancient cultures, some of the knowledge reached Mediterranean countries through traders and migrations and it was in Hippocrates's (460-377 BC) time that pharmacognosy reached a summit in Greece[59]. Theophrastus (*ca* 300 to 322 BC), who was a philosopher and naturalist, was the first to deal with the history of plants, which later on helped in the classification of plants including herbs. Pedanius Dioscorides, a Greek physician (*ca* 40-90 AD) produced *De materia medica* in 78 AD, which described more than 500 medicinal plants and their uses in detail.

Galen (*ca* 129-199 AD) founded "Galenics" and taught pharmacy and medicine in Rome[63]. Avicenna (980-1037 AD), a Persian pharmacist, physician, philosopher and a poet described 1400 drugs and medicinal plants which greatly contributed to the formation of a codified Graeco-Roman Medicine in the 5th century[38,59]. Paracelsus (1493-1541) administered dosage formulations separating "Arkanum" from non-active ingredients of drugs. Western medicine and pharmacy originated from this medical system.

Tibetan medicine, also called gSo-ba Rig-pa, took shape in the 7th-8th century with the advent of Mahayana Buddhism in the country[61,64]. Bhutanese traditional medicine known as gSo-ba Rig-pa was conceived subsequent to Tibetan medicines in the 8th century and contain more than three hundred herbal formularies and recipes[65,66]. In the USA, homeopathy, that includes hydrotherapy, nutritional therapy, herbal therapy, manual manipulation and midwifery, was founded by German physician Hahnemann (1755-1843) became popular in 1830s.

The discovery of antibiotics and vaccines in the 20th century dramatically changed medical practice worldwide and as a result a separate field of ethno-medicine emerged as an academic specialization focusing on traditional healing systems[67]. The investigation of the principles of drug action of Japanese traditional Sino-medicine at the molecular level have resulted in obtaining many novel compounds and the uncovering of new mechanisms of drug action[68]. Clinical trials of Tibetan medicine, the PADMA Products, also proved successful in the treatment of irritable bowel syndrome[69] and fibrinolysis with stable intermittent claudication[70,71]. This products also furnished new anti-oxidative mechanisms at the molecular level[72].

Thus, traditional medicines were found to be effectively addressing the health needs of millions of people including developed nations by completely different strategies and

well defined approaches, and generally with minimal side effects[67]. Current WHO estimates show that 75% of the French population, 30% of the Vietnamese population, and 40% of Indonesia's population uses traditional medicines[73]. In Germany, 77% of pain clinics provide acupuncture, in Japan 72% of registered western style doctors uses kampo medicine[73] and in Bhutan, traditional medicine caters to 80% of the population. Overall, traditional medicines provide primary health care needs to almost 65-85% of the world's population including developed nations[39,40]. In terms of economic value, traditional therapies contribute to US $ 60 billion a year and the USA alone spends US $ 2.7 billion per year followed by China with US $ 1.8 billion and Australia with Aus $ 1 billion a year[73].

Since almost every traditional medicine regimen uses medicinal plants as the bulk ingredients, they also play significant roles in natural product based drug discoveries. More than 13,000 species of plants are used in traditional medicines and herbal cosmetics[43]. About 8000 of these medicinal plant species are known in South Asia alone[74]. Many pharmaceutical companies have successfully explored these medicinal plants by applying an ethno-directed biorational approach[75]. In fact, among the search strategies, the ethno-directed bio-rational approach has proved to be the shortest and the most effective search strategy for discovering drugs from Nature. For example, the National Cancer Institute, USA, has noted that extracts from medicinal plants in *in vitro* bioassays gave greater rates of bioactivity than those from other plants[75]. In another survey, out of 800 medicinal plant extracts collected from Vietnam and Laos, at least 25 biologically active compounds were isolated; of these 13 were new anti-HIV agents and 3 were antimalarial agents[76]. Similarly, in the USA, out of 119 plant drugs available from 1959 to 1980, 74% of these were discovered as a result of chemical studies directed at isolating the active substances from the plants used in traditional medicines[38].

However, in using this ethno directed search strategy, it is crucial to have intimate understanding of the disease concepts of the culture whose pharmacopeia is under examination. The products used as medicines by local people are usually not those that are tested in the laboratory. Most of the effective brews or formularies are multi-ingredient compounds. Chemical reactions occur within these mixtures or poultices and are most often associated with synergism making them more effective than the single isolated lead compound. When the medicinal plants are subjected to phytochemical screening, researchers often target only one compound, or a few limited compounds, which quite often turn out to be biologically inactive owing to the loss of other active

components during this screening process. Therefore, the ethno-medical indication may not necessarily be productive when screening is directed towards only specific phytochemical isolations.

1.7 Natural Products and Bhutanese Traditional Medicine

Bhutanese Traditional Medicine is locally known as gSo-ba Rig-pa, which means "The Art of Healing Sciences"[65]. It is one of the world's oldest surviving medical traditions, preserved intact, and practised in Bhutan in conjunction with western medicine. Currently it provides primary health care to 80% of the country's population. The history of gSo-ba Rig-pa dates back to the era of Buddha Kashyapa, the predecessor of the historical Sakyamuni Buddha. It was Buddha Kashyapa who transmitted the medical teachings to Brahma and then ultimately propagated them to the human realm as a weapon to fight suffering[66,77]. Buddha Sakyamuni, also sometimes called the Supreme Physician, was then said to have taught a healing system 'Vimalagotra' (Immaculate Lineage) simultaneously with the first turning of the Wheel of Dharma at Sarnath on Four Noble Truths in India, followed by the text gSo-dPyad hBum-pa (One Hundred Thousands Verses of Healing) at Jagoe Phungpoi Ri (Vulture's Peak) and gSer-′od Dampai mdo (Supreme Golden Rays Sutra) that contained a separate chapter on "Nad-thams Cad Zhi-bar bYed-pai rGyud" (The Ways of Completely Curing Diseases) during the third turning of the wheel[78]. Dul-ba rLung (Vinaya Sastra) teaching on moral discipline also contained medical teachings.

He taught his disciples and followers, mostly using disease and healing as metaphors to illustrate his philosophy of the human condition[79]. Though Buddha's teachings spread to many Asian countries, gSo-ba Rig-pa became prominent only in Tibet and Bhutan. It was in 718-785 A.D. that Tibet saw the peak development of astrology, medicine and astronomical science[80]. During that time, Tibet hosted several medical conferences at Samye inviting the great healers from Tibet, China, India, Persia, Byzantium and Himalayan countries like Bhutan and Nepal[79]. It was reported that all the different medical systems were analysed at this conference and the best practices were incorporated into gSo-ba Rig-pa. Pre-Buddhist Shamanistic views and the Bon tradition also influenced the way Tibetan Medicine evolved[81,82].

With the advent of Mahayana (Tantric) Buddhism from Tibet in the 8th century, the gSo-ba Rig-pa (Art of Healing) also took shape in Bhutan as well[65]. The Buddhist philosophy remained as the mainstream of this medical tradition. However, Tibetan Medicine, Indian Ayurvedic Medicine, Unani Medicine, Chinese Traditional Medicine,

Shaman Medicine, Local Ethno-Medicine, Exorcism and the Bon Tradition had a definite influence on the way Bhutanese Traditional Medicine evolved.

Philosophically, gSo-ba Rig-pa is based on the fact that all suffering is caused by ignorance of the nature of reality and the craving, attachment, and grasping that arise from such ignorance and that the path to overcome such suffering is to follow the Noble Eightfold Path of Lord Buddha, which consists of right views, right intention, right speech, right action, right livelihood, right effort, right-mindedness, and right contemplation. Fundamentally, gSo-ba Rigpa states that human beings are composed of three main elements called "Three Humours"; rLung inadequately translates as air, mKhris-pa translates as bile and Bae-dkan, translates as phlegm[64-66]. They are interrelated in all the vital systems and functions of the body such as the nervous system, circulatory system, digestive system, excretory system, reproductive system, respiratory system and musculo-skeletal system. Each of the three humours has different functions in the body (**Table 2).**

Table 2. The Three Humours and Body Function [65,78,79].

Three Humours	Characteristics	Function
RLung (air)	Has the nature of air, rough, light, cold, subtle, hard and mobile	Controls movement and bodily functions such as respiration, excretion, circulation, speech, intellect, impulses, gives clarity to sense organs and sustains life by means of acting as a medium between mind and body.
mKhris-pa (bile)	It has the nature of fire, oily, fluidity, hot, sharp, light, fetid and purgative	Controls digestion, assimilation, and basic metabolism generating heat and energy. Stimulate feelings of hunger and thirst, gives lustre to body complexion and provides courage and determination
Bae-dKan (Phlegm)	Cold in nature, oily, cool, heavy, blunt, smooth, firm, sticky	Firmness of body, stability of mind, induces sleep, connects body joints, generates tolerance, lubricates the body and regulates the balance of energies.

Based on this, body meridians are drawn to help diagnose diseases related to the energy system of the body. Explicit knowledge of body meridians is important in acupressure therapy of gSo-ba Rig-pa, so the Buddhist medical system is more than a study of anatomy, physiopathology and the pharmacopoeia. Physiopathology, as per gSo-ba Rig-pa, is based on the close relationship between the human microcosm and the universal macrocosm[65]. Each of the three humours of the human body is considered a combination of the five basic constituents (proto-elements) of the Universe: Sa (earth),

Chu (water), Mei (fire), rLung (wind/air) and Nam-mKha (space)[78]. They are essential elements for the existence of all life on earth and they coexist in equilibrium. As long as these proto-elements are in their natural state and maintain a proper balance, suffering does not erupt from the environment nor do they affect the three humours. When these three humours are well balanced in an individual, then the person is said to be healthy[65].

Any sign of imbalance or modifications in the three humours are clearly apparent in the pulse, urine and on the surface of the body. Based on this theory, physicians perform pulse readings, physical urinalyses and observe the eyes, tongue and earlobes (in babies) to detect disorders. In modern allopathic medicine, pulse reading is only used to detect anomalies of the heart and the circulatory system, but in gSo-ba Rig-pa, the pulse reading links the network of body meridians and can detect any diseases affecting different parts, organs and systems of the body[66].

The treatment regimen in gSo-ba Rig-pa is holistic in nature and it mainly includes five kinds of healing methods like behavioural modification, herbal physiotherapy, minor surgery and prescription of herbal medicine. Bhutanese traditional medicine texts report the use of as many as 2200 traditional prescription drugs and each of these drugs are multi-ingredient drugs[77,81]. However, in 1969, only 167 formulations, which used 300 different types of ingredients, were selected for the essential drugs list[83]. The essential drugs list was further streamlined based on disease patterns and prevalence in Bhutan and was reduced to 103 after thorough examination and standardization processes[84].

The manufacturing unit in Bhutan produces 8-9 tonnes of traditional medicines annually and these are formulated into different dosage forms which includes pills (39.81%), tablets (30.10%), ointments (3.88%), syrups (1.94%), capsules (12.62%), powders (3.88%), crude extracts (0.97%) and unestablished dosage forms (6.80%)[85]. Some pills like Rinchen Rilbu also uses precious metals such as gold, silver, turquoise, pearls, gems and other rare minerals as ingredients.

There are 46 alkaloid-containing drugs on the Bhutanese essential drugs list. Toxic raw materials are pre-processed and detoxified before formulation following traditional methods. Since 1998, the standardization of traditional drugs has been done using the Total Quality Profiling Method[86,87]. These standardized drugs have the potential to go forward for regulatory approval. Bioprospecting the medicinal plants used in gSo-ba Rig-pa medicine also has an international market value and should play an important role in any new drug discovery programme.

1.8 Research Aims and Objectives

As discussed earlier, microbial (bacteria, fungi, viruses) and parasite (protozoan) resistance to traditional antimicrobial and antimalarial drugs has emerged continuously and has become a great health concern for the communities worldwide. A new arsenal of antiinfective drugs is required for combating these resistant pathogens. Bhutan is rich in its ethno-medical heritage including treatments for infectious diseases (bacterial, fungal and viral infections and also for malaria) and uses more than 300 medicinal plants in different preparations. However, till date, active components of herbal mixtures have not been identified at the molecular level. Based on this ethno-botanical knowledge, analysing Bhutanese medicinal plants for anti-infective agents, with a focus on alkaloidal compounds, were the twin themes of the research described in this thesis.

Therefore, the main objectives of this research project were:

a) To investigate the alkaloidal components of selected medicinal plants of Bhutan where their use suggested antibacterial and antimalarial activities.
b) To determine structures of the new alkaloids isolated from the selected medicinal plants.
c) To assess their antibacterial and antimalarial properties and identify potential new lead compounds.
d) To provide validation for gSo-ba Rig-pa, in an effort to help preserve the gSo-ba Rigpa and to help to develop it as a sustainable traditional medicine.

The medicinal plants of Bhutan selected for the study are discussed in **Chapter 2**. **Chapter 3** presents the isolation of alkaloids from the medicinal plant, *Aconitum orochryseum* Stapf. In **Chapter 4**, the isolation of alkaloids from the plant *Corydalis gerdae* Fedde is discussed. **Chapter 5** and **Chapter 6** present an investigation of the alkaloids from *Tribulus terrestris* Linn and *Ranunculus brotherusi* Freyn respectively. **Chapter 7** discusses the antimicrobial testing conducted on some isolated pure compounds and the crude extracts of the plants. Conclusions and future directions are presented in **Chapter 8**, while **Chapter 9** covers the experimental section of the study.

Chapter 2

Selection and Collection of Bhutanese Medicinal Plants

2.1 General Introduction

Bhutan is known to the outside world for its youthful inaccessible mountains, rich biodiversity and unique culture and tradition. It is sandwiched between the two historically, culturally, and politically contrasting giant nations of the world, India and China[88] (**Figure 2**). It has an area of 47000 square kilometres and a population of 600,000.

Geographically, Bhutan is laterally divisible into four zones with a continuous South-North gradation of vegetation. The foothills region ranging from 95 to 2000 metres above sea level receive the heat of the Sahara desert and an annual rainfall of 80-90 inches per annum, and thus supports tropical vegetation such as riverine species, sal evergreen forests and dense savannah grasses.

The interior valleys (ranging from 2000 to 3000 meters) have cool pleasant winters and mild hot summers, which supports temperate vegetation. The annual rainfall ranges between 30-35 inches per annum and most of the flowering plants grow in this region. The alpine zone (starting from 3000 to 6000 metres) experiences intense cold winters and cool summers with scanty rainfall. This region has Tundra vegetation and supports meadows, scrub and shrubs. Most of the rare herbs grow in this zone. The mountaintops are permanently snow clad and don't support any form of vegetation but they are a permanent source of rivers and spring waters in Bhutan. The highest point measures 7800 meters above sea level on the peaks of the major inaccessible mountains[88].

Due to this unique topographical and climatic position[89], the country is blessed with great biological diversity. About 5603 to 7000 species of plants including 60 species of Rhododendron and 600 species of orchids are reported to grow in Bhutan[90,91]. Out of 50,000 endemic plant species reported to thrive in global hotspots of the world[92], about 82 endemic plant species have been discovered in Bhutan[90]. Bhutan has also abundant fungal and parasitic flora. As a result, Bhutan is often described as a "Botanist's Paradise"[93] and, recently, Bhutan was identified as one of the ten global hotspots of biodiversity in the world[94,95]. The conservation and preservation of this rich biodiversity (environment) including the medicinal plants is one of the country's top development strategic priorities. Bhutanese view the environment as an essential component not only for material life but also for medical and spiritual life and thus about 72.5% of the forest is still preserved in its original form[89].

Figure 2. Map showing the strategic location of Bhutan.

The rich Bhutanese biodiversity supports many medicinal plants. In fact, owing to the plentiful medicinal plants thriving in the country, Bhutan was known as “sMan-jong”, the land of medicinal plants[77]. Taking advantage of the availability of such rich medicinal plants and other bioactive natural products applications, the Bhutanese

developed their own medical tradition called gSo-ba Rig-pa. Around 600 species of medicinal plants have been identified to date and 300 of them are in routine use in the day-to-day formulation of gSo-ba Rig-pa medicines.

2.2 Medicinal plants of Bhutan

According to gSo-ba Rig-pa, there are seven categories of medicinal sources: three are of vegetable origin, which comprising of Sgo-sman (High Altitude Medicinal Plants and Herbs above 3000 metres), Khrog-sman (Low Altitude Medicinal Plants below 3000 metres) and Rtsi-sman (resins and extracts); two are of mineral origin comprising of Rin-po-che-sman (precious stones/gems), rdo-sman (base minerals/stone) and Sa-sman (medicinal soils) while one is of animal origin or srog-chags-sman (includes marine organisms). About 2990 species of medicinal plants[73], 35 types of animal parts[96], 18 types of minerals including precious stones and precious metals such as gold, silver, copper and iron are used in traditional medicine in Bhutan[97]. Marine products like crabs, pearls, seashells and other marine products are also used.

Almost 70% of these raw materials, almost all of which are collected in the wild, are available within the country[73]. Only 30% are imported from India. Some rare and expensive animal parts such as rhino's horn, tiger bone, elephant tusk and musk or gLar-tsee (used in 30% of the formulations) are banned from use in the herbal formulations[96]. Low Altitude Medicinal Plants (Khrog-sman) are collected from the temperate and tropical zone (**Figure 3**, Collection site-II) and the High Altitude Medicinal Plants (Ngo-sman) are collected from the alpine region (**Figure 3**, Collection site-I). About 60% of the species are harvested from high altitudes-between 3000 to 6000 metres above sea level and only 40% are collected from low altitudes. The herbs are harvested and collected by the Pharmaceutical and Research Unit in Bhutan once a year employing good harvesting practices and with strict quality control monitoring.

The time of collection is based on gSo-ba Rig-pa guidelines. According to these guidelines, therapeutic properties of the plants are directly affected by the collection season, picking time (morning, afternoon and evening) and the nature of drying conditions that has to be carefully monitored. For example, some medicinal plants need to be picked only in the presence of moonlight. If such plants are picked at other times, it is believed that they lose their efficacy. Plants are collected as whole plants (40 species), fruits (26 species), seeds (20), roots/bulbs (31 species), stems (12), flowers (9 species), leaves (7 species), extracts/resins (7 species) and bark/cambium (3 species)[97].

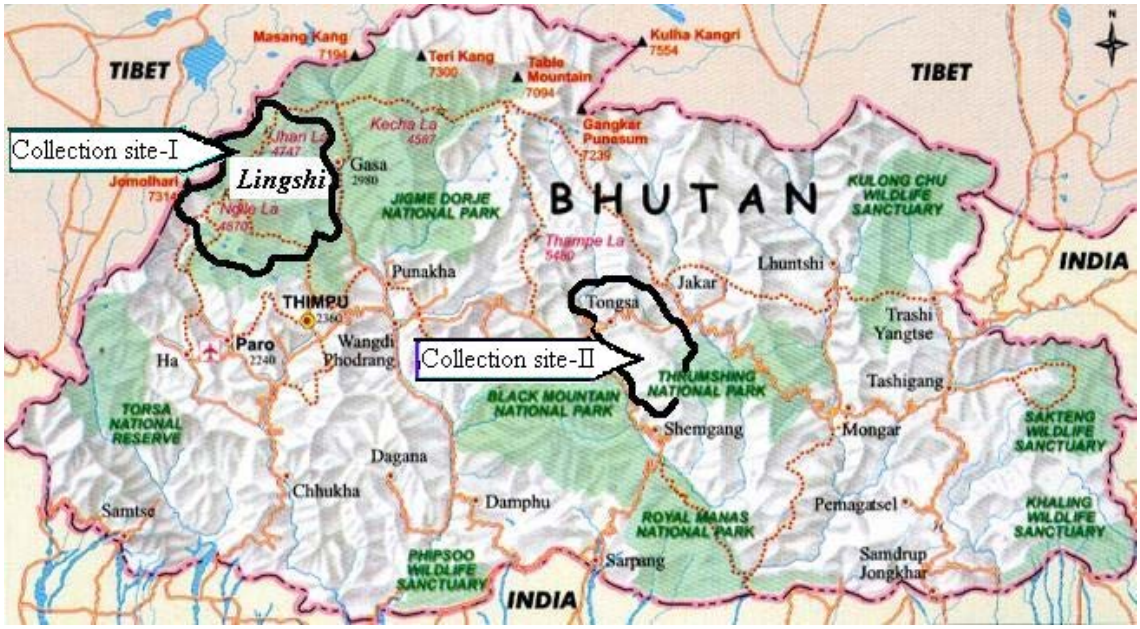


Figure 3. Map of Bhutan showing the collection sites of medicinal plants.

Some medicinal plants that are not used by the Institute have recently been explored by the Aromatic and Phytochemical Section of the Tashi Commercial Corporation of Bhutan and the Japan Himalayan Company which was established in 1990[98].

2.2.1 Sustainability of Medicinal Plants

Bhutanese Traditional Medicine is gaining popularity among people within the country as well as internationally. Many medicinal plants such as *Aquilaria agallocha*, *Rauwolfia serpentine*, *Ephedra gerardina*, *Taxus bacccata*, *Rheum nobile*, *Rheum accuminata*, *Picrorhiza kurroa*, *Nardostachys jatamansi*, *Aconitum* species, *Artemisia species*, *Panax pseudo-ginseng* sub species *himalaicus* and *Cordyceps sinensis* are in high demand for pharmaceuticals and have potential international market value[99]. As a result, the pressures on the alpine medicinal plants have increased undermining their sustainability. In fact, because of the legalisation of the regional trade of highly prized perennial herbs like *Picrorhiza kurrooa*, *Nardostachys grandiflora* and *Cordyceps sinensis* in Asia[100] and highly executed operational networks of illegal traders in the region, these medicinal plants are already at high risk of overexploitation [100,101].

Since Bhutan is an agricultural country, removal of forests for farming has had an adverse impact on the environment, particularly resulting in the loss of the medicinal plant species. Cattle, yak and sheep rearing is another significant economic activity of Bhutan. Overgrazing of the forest by such animals has a detrimental effect on the survival of the grazed plant species. For example, intense browsing by yaks checks the growth of seedlings of the Himalayan yew, *Taxus baccata*[102]. Some plant species like

Senecio and the *Ligularia*, which contain pyrrolizidine alkaloids, were found to be toxic to yaks and cattle[44,103]. Such poisonous plants are cleared by cattle herders in order to protect the cattle. This act significantly contributes to the extinction of many alkaloid-containing plant species.

The threats to medicinal plants also stem from logging activity carried out in Bhutan. This has led to deforestation and the loss of a number of medicinal plants. The natural phenomenon of decaying and dying of Himalayan mountains also contributes to the loss of some plant species[104]. Industrialization poses another significant threat to the medicinal plants of Bhutan.

Although, some medicinal plants may be cultivated, the content of phytochemicals in these plants may not be same as that in the wild species but cultivation is the only proper solution to meet this ever increasing demand. Before all wild plants become extinct or fully domesticated, it is essential to assess them for their chemical content in their original state. In this context, the author has selected some potential medicinal plants used in the traditional medicine of Bhutan for thousands of years and has analysed them for potential antimicrobial and antimalarial lead drug molecules.

2.3 Selection of Medicinal Plants

Based on an ethno-directed biorational approach, the databases of Traditional Medicine of Bhutan[97,105] as well as the inventory carried out by the ethno-botanist[77] and the Forestry Department[99] were analysed and a list of medicinal plants was generated consisting of 156 plant species (**Table 3)**. Every effort has been made to assign individual therapeutic uses based on the text, "Medicinal Flora of Bhutan"[105]. The medicinal plants list was then further analysed and those plants which met all the selection criteria were short listed for collection and phytochemical analysis.

2.3.1 Criteria for Selecting the Medicinal Plants

Because of the large number of possible species for analysis, criteria were established for plant selection as follows:

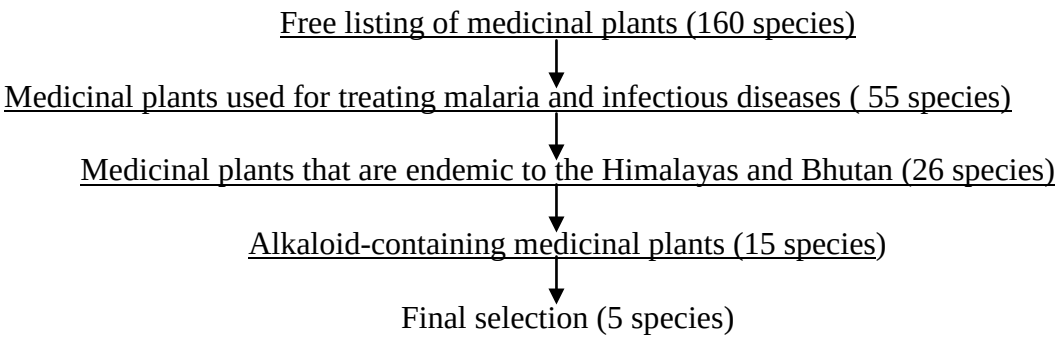
a) Only those medicinal plants that were used for treating malaria or infectious diseases were to be selected.

b) Further selection of medicinal plants was then based on those that were endemic to Bhutan and the Himalayas (high altitude plants, above 3500 metres above sea level).

c) Finally, only alkaloid-containing medicinal plants were to be selected.

Many plants listed in **Table 3** have been used for treating fever arising from different disorders including malaria but only *Corydalis gerdae* was specifically used against malaria. *Aconitum orochryseum* was used for treating infections, snake bite, and bilous fever or fever arising from bile disorders. There were also many medicinal plants used for treating infections including blood infections, skin infections, and infections of internal organs. About fifty five medicinal plants were selected based on the first criterion. The selection was then streamlined by focusing on endemic species growing at high altitudes (*ca* 4000m), since these species were the least studied phytochemically and could contain new molecules that would potentially be new drug leads. About 26 medicinal plants were short listed based on this criterion. Introduced medicinal plant species were not considered here except for *Tribulus terrestris*.

Only those medicinal plants that contained alkaloids were then selected from these 26 species. This is because alkaloids have interesting structural types with a wide range of biological activities and have quite often become potential drug leads. An effort was also made to select medicinal plants representing different families since different families usually contain different classes of alkaloids. So preliminary tests for the presence of alkaloids were conducted on the selected medicinal plants of Bhutan (**Table 4**) at the Pharmaceutical and Research Unit Laboratory in Bhutan using the Culvenor and Fitzgerald[106] test procedure. Only fifteen plants were found to contain alkaloids. The result was reported only as alkaloid positive or alkaloid negative and the degree of alkaloid content was not estimated. Finally, only five alkaloid-containing medicinal plants were selected for chemical analysis (**Table 5**) based on their availability for larger scale collection. The selection methodology is summarised in **Scheme 1.**



Scheme 1. Flow chart for the selection of the medicinal plants based on the four selection criteria.

Table 3. Medicinal plants considered with ethno-medical information.

Botanical name	Family	gSo-ba rigpa name	Alti-tude	Distribution	Part used	Ethno-medical use
Aconitum lacinatum	Ranunculaceae	Bong-nga-nag-po	HA	Himalaya	R/L	Tinging of muscles, meat poisoning and bones
Aconitum orochryseum	Ranunculaceae	Bong-nga-dkar-po	HA	Endemic	A	Flu-fever, infections and antidote for snake bite
Aconitum violaceum	Ranunculaceae	Bong-nga-dmar-po	HA	Himalaya	R	
Aconogonon tortuosum	Polygonaceae	Sna-lo	HA	Himalaya	R	
Acorus calamus	Araceae	Shu-dag-nag-po	LA	Cosmopolitan	R	
Aletris pauciflora	Liliaceae	Dam-bu-ka-ra	LA	Himalaya	W	Lung and liver disorders, pneumonia, cuts and wounds
Allium sativum	Liliaceae	Sgog-skya	LA	Cosmopolitan	B	
Allium wallichi	Liliaceae	Re-sgog	HA	Himalaya	W	Gastritis, tuberculosis, inflammation, and rlung disorders
Amomum subulatum	Zingiberaceae	Ka-ko-la	LA	Himalaya	F	
Androsace strigillosa	Primulaceae	Sga-tig-nag-po	HA	Himalaya	W	Oedema, fever and infections
Anemone rivularis	Ranunculaceae	Srub-ka	HA	Himalaya	S	Antipyretics, blisters, antiseptics, antidote, and to increase body temperature
Aquilaria agallocha	Thymelaceae	A-ga-ru	LA	Bhutan, India and Indonesia	W	
Areca catechu	Palmae	Go-yu	LA	Asia	S	
Arenaria kansuensis	Caryophyllaceae	Rtsva-a-krong	HA	Tibet and Bhutan	Fl	Lund disorders and abscess above chest including head
Aristolochia griffithii	Aristolochiaceae	Ba-le-ka	LA	Himalaya	St	
Asparagus racemosus	Asparagaceae	Nye-shing	LA	Cosmopolitan	R	
Aster flaccidus	Conpositae	Lug-mig	HA	Himalaya	F	Bronchitis, cramps, common cold and to relieves pain
Aster stracheyi	Compositae	Chu-de-ba	HA	Himalaya	A	
Astragalus floridus	Leguminosae	Srad-ser	HA	Himalaya		
Beaumontia grandiflora	Apocynaceae	Dug-mo-nyung	LA	Bhutan and Nepal	S	
Berberis aristata	Berberidaceae	Skyer-pa-dkar-po	HA	Himalaya.	Bk	
Bistorta macrophylla	Polygonaceae	Spang-ram	HA	Himalaya.	R	Diarrhoea and blood dysentery
Bombax ceiba	Bombacaceae	Pad-ma-ge-ser	LA	Tropics	Fl	
Brassica juncea	Brassicaceae	Spong-thogs-pa	HA	Cosmopolitan	A	Meat-poisoning, spasmolytic, lung, heart and blood disorders
Butea buteiformis	Leguminosae	Ma-ru-rtse	LA	Bhutan and India	S	
Capsella bursapastoris	Cruciferae	So-ka-pa	HA	Cosmopolitan	A	
Caragana jubata	Leguminosae	Mdzo-mo-shing	HA	Bhutan and India	St.	
Carum carvi	Umbelliferae	Go-snyod	HA	Cosmopolitan	S/A	Detoxifiers, headache, migraine, nausea and eyes disorders
Cassia fistula	Leguminosae	Dong-ga	LA	Himalaya and Burma		
Cassia tora	Leguminosae	Thal-ka-rdor-rje	LA	South America and Bhutan	S	
Cavea tanguensis	Compositae	Ming-chen-nag-po	HA	Himalaya	A	
Cedrilla toona	Meliaceae	Snying-sho-sha	LA	America and Bhutan	F	
Choenomeles lagenaria	Rosaceae	Bse-yab	LA	Bhutan	F	
Chrysosplenium forrestii	Saxifragaceae	gYaa-kyi-ma	HA	Himalaya	A	Bile diseases
Cinnamomum tamala	Lauraceae	Shing-tsa	LA	Himalaya	R Bk	
Clematis connata	Ranunculaceae	Bbyi-mong-dkar-po	HA	Himalaya.	St.	Sexually transmitted diseases and digestion
Codonopsis bhutanica	Campanulaceae	Klu-bdud-rdor-rje	HA	Endemic	W	Evil affliction, tinging, nephrosis, numbness, gout, leprosy and blood regulator
Codonopsis convulvulaceae	Campanulaceae	Snyi-ba	HA	Himalaya	B	Cough and cold, sore throat, chest pain and decongestant
Cordyceps sinensis	Fungus	Dbyar-rtsva-dgun-bu	HA	Himalaya	W	
Coriandrum sativum	Umbelliferae	hu-su	HA/ LA	Cosmopolitan	S	Bay-kan disorders including digestive system disorders
Corydalis flabellata	Fumariaceae	Re-rdzun	HA	Himalaya	A	Anti-hypertension, blood purifier and nerve pain

Corydalis gerdae	Fumariaceae	Stong-ri-zil-pa	HA	Bhutan and Chumbi valley	W	Antimalarial and infections
Corydalis stracheyi	Fumariaceae	Bya-rgod-sug-pa	HA	Himalaya	A	Blood disorders, liver disorders and analgesic
Cotoneaster microphyllus	Rosaceae	Bya-pho-tsi-tsi	Ha	Himalaya.	F	
Curcuma longa	Zingiberaceae	Yung-ba	LA	Bhutan and India	Rh	
Cynoglossum glochidiatum	Boraginaceae	Nad-ma-gyu-lo	HA	Afghanistan to Bhutan		
Dactylorhiza hatagirea	Orchidaceae	Dbang-lag	HA	Himalaya	R	Suppleness of the body tonics and builds body
Delphinium brunonianum	Ranunculaceae	Bya-rgod-spos	HA	Himalaya	A	Fevers, trembling, bile malfunctioning and as detoxifiers
Delphinium drepanocentrum	Ranunculaceae	Bya-rkang	HA	Endemic to Nepal and Sikkim	A	Dermatitis and dysentery, wounds and abscess
Diospyros spp.	Ebenaceae	Rgun-bdrum-nag-po	LA	Himalaya	F	
Dracocephalum tanguiticum	Labiatae	Pri-yang-ku	HA	Tibet	A	Stomach, lung and the liver disorders and heals wound
Dryopteris fragrans	Polypodiaceae	Re-rel	LA	Cosmopolitan	Rh.	
Elettaria cardamomum	Zingiberaceae	Sug-smel	LA	Sub-tropics	F	
Ephedra gerardiana	Ephedraceae	Mtshe-ldum	HA	Afghanistan to Bhutan	A	Fever, wounds, injuries, bleeding and heals every fever including malaria
Elshoutia eriostachya	Labiatae	Bje-rug	HA		A	Boils, wounds and tuberculosis
Eriophyton wallichiana	Labiatae	Spang-mtshan-spu-ru	HA	W.Nepal to S.W China	W	
Erythrina arborescens	Leguminosae	Mkhal-ma-sho-sha	LA	India, Bhutan, China and Burma	F	
Eugenia fruticosa	Myrtaceae	Sa-bras	LA	Himalaya	F	
Euphorbia griffithi	Euphorbiaceae	Dur-byid	HA	Tropics	R	
Euphorbia spp.	Euphorbiaceae	Thar-nu	HA	Tropics	R	
Euphrasia himalaica	Scrophulariaceae	Zhim-thig-le-dkar-po	HA	Afghanistan to Bhutan	A	
Fragaria indica.	Saxifragaceae	Bri-rta-sa-dzin	HA	Worldwide	W	Anthelmintics, neurological infection, chest infections, and lung inflammation
Fritillaria delavayi	Liliaceae	Dkar-po-chig-thub	HA	Himalaya	B	Detoxifier, blood purifier and as antipyretic
Fritillaria gardneri	Liliaceae	A-bhi-kha	HA	Himalaya	A	Detoxicant and joining bone
Galium aparine	Rubiaceae	Zings-rtsi-dkar-po	HA	Himalaya	W	Bile disorders like jaundice, migraine, and sinusitis
Gentiana algida	Gentianaceae	Spang-rgyan-sngon-po	HA	Kashmir and S.W China	Fl	Anti-dote, inflammation of throat and lung, and pain reliever
Gentiana spp.	Gentianaceae	Spang-rgyan-dkar-po	HA	Himalaya	Fl	Bile related disorders, leprosy and blood disorders
Gentiana urnula	Gentianaceae	Gang-ga-chung	HA	Himalaya	A	Diarrhoea and as Detoxicant
Geranium spp.	Geraniaceae	Gla-sgang	HA	Himalaya	A	Sore throat and bronchitis, intestinal disorders, fevers, tuberculosis and diarrhoea
Geranium tuberaria	Geraniaceae	li-ga-dur	HA	Pakistan, India and Central Asia	R	Common cough and cold and swelling of limbs
Glycyrrhiza glabra	Leguminosae	Shing-mgar	LA	Cultivated	R	
Hemiphragma heterophylla Wall		A-bi-ra	HA	Himalaya	W	Body tonic, for roper blood and menstruation regulation, UTI infection and backache
Heracleum candicans	Umbelliferae	Spru-nag	HA	Himalaya	R	Leprosy, bleeding, headache and neurology
Herpetospermum caudigerum	Cucurbitaceae	Gser-gyi-me-tog	HA	Semi-cultivated	S	Bile disorders and intestinal disorders
Hippophae rhamnoides	Elaegnaceae	Star-bu	HA	Central Asia, India and Pakistan	F	Tuberculosis, anticoagulant and Liver diseases
Hypecoum leptocarpum	Fumariaceae	Par-pa-ta	HA	Himalaya	A	Common cold and detoxifier
Impatiens laxiflora	Balsaminaceae	Byiu-star-ga	HA	Tibet	W	
Inula grandiflora	Compositae	Ming-can-ser-po	HA	Pakistan and Central Nepal	Fl	Abscess/boils, numbness and fever, evil affliction
Inula racemosa	Compositae	Manu	HA	Cultivated	R	
Iris kemaonensis	Iridaceae	Dres-ma	HA	India and Pakistan	F	Anthelmintic and antipyretic

Jaeschkea oligosperma	Gentianaceae	Lcags-tig	HA	Kashmir	A	
Juniperus squamata	Cupressaceae	Shug-pa-tsher-can	HA	Afghanistan, and S.W.China	L/F	
Lancea tibetaca	Scrophulariaceae	Pa-yag	HA	Himalaya	W	Wounds, lung and heart disorders
Lepisorus spp.	Polypodaceae	Brag-spos-pa	HA	Himalaya	W	Bone fracture, burns, wounds and kidney disorders
Luffa aegyptiaca	Cucurbitaceae	Ka-bed	LA	Tropics	S	
Malva sylvestris	Malvaceae	Lcam-pa	HA	Himalaya	A	Revitalising kidney, diuretic, wounds and diarrhoea
Malva verticillate	Malvaceae	So-ma-ra-dza	LA	Pakistan, India, Euro-Asia and Africa	S	
Meconopsis horridula	Papaveraceae	Tsher-ngon	HA	Himalaya	A	Broken bones (skull) and make joints strong
Meconopsis paniculate	Papaveraceae	Ud-pal-ser-po	HA	Utter Pradesh to S.E. Tibet	Fl	
Meconopsis primulina	Papaveraceae	Yi-mo-mdeu-byin	HA	Himalaya	A	
Meconopsis simplicifolia	Papaveraceae	Ud-pel-sngon-po	HA	C. Nepal to S.E Tibet	A	Antipyretics, antimalarial, lungs disorders, liver cirrhosis and blood disorders.
Morina nepalensis	Dipsacaceae	Spyang-tsher-lo-ma-phra-ba	HA	W. Nepal to S.W. China	A	
Myricarea rosea	Tamaricaceae	Chu-shing-om-bu	HA	C.Nepal to S.W China	A	
Myristica fragrans	Myristicaceae	Dza-ti	LA	South East Asia	S	
Nardostachys grandiflora	Valerianaceae	Spang-spos	HA	Utter Pradesh to S.W China	R	Chronic fever, detoxicant, and heart disorders
Onosma hookeri	Boraginaceae	Bri-mog	HA	Himalaya	R	Asthma, hypertension and pneumonia
Oreosolen wattii	Scrophulariaceae	Khron-bu	HA	West Nepal to Bhutan	R	
Oxytropis lapponica	Leguminosae	Srad-dkar	HA	Pakistan and India	Fl	
Oxytropis reniformis	Leguminosae	Sngo--stag-sha	HA	Himalaya	W	Antiseptic, wounds and antidote
Parnassia ovata	Saxifragaceae	Dngul-tig	HA	Himalaya	A	Bile disorders and ganglion blockage.
Pedicularis flagellaris	Scrophulariaceae	Glang-sna	HA	Himalaya	A	Anti-diuretic and wounds
Pedicularis longiflora	Scrophulariaceae	Lug-ro-ser-po	HA	Pakistan to S.W China	A	Dehydration of mouth and tongue
Pedicularis megalantha	Scrophulariaceae	Lug-ru-smug-po	HA	Utter Pradesh to S.E Tibet	A	Antidote, meat poisoning and intestinal disorders
Pedicularis siphonantha	Scrophulariaceae	Lug-ru-dmar-po	HA	Utter Pradesh to S.E Tibet	A	
Phlomis rotate	Labiatae	Rta-lpags	HA	W. Nepal to S.W China	A	
Pholidota recurva	Orchidaceae	Pu-shel-rtse	LA	Himalaya	St.	
Phyllanthus emblica	Euphorbiaceae	Skyu-ru	LA	India and Nepal	F	
Phytolacca esculanta	Phytolaccaceae	Dpah-bo-dkar-po	HA	Cultivated	R	
Picrorhiza kurroa	Scrophulariaceae	Hong-len	HA	Pakistan to Utter Pradesh	R	Blood purifiers
Piper nepalense	Piperaceae	Pi-pi-ling	LA	Himalaya	F	
Plantago erosa	Plantaginaceae	Tha-ram	HA	Cosmopolitan	S	Diarrhoea
Pleurospermum amabile	Umbelliferae	Rtsad	HA	Himalaya	W	Antidote, fever and indigestion
Pleurospermum hookeri	Umbelliferae	Tang-kun-dkar-po	HA	Pakistan to S.W China	R	Heart disorders
Podophyllum hexandrum(emodi)	Podophyllaceae	Hol-mo-se	HA	Afghanistan to S.W China	F	Female gynaecological problems, paralysis, blood disorders and kidney disorders
Polygonatum singalilense	Liliaceae	Lug-mnye	HA	Himalaya	Rh.	
Polygonatum verticillatum	Liliaceae	Ra-mnye	HA	Pakistan to S.E.Tibet	Rh.	Fluid accumulation in joints, anthelmintics, tranquilliser, appetiser and longevity
Potentilla peduncularis	Rosaceae	Gro-ma	HA	Nepal	R	Dysentery
Pterocephalus hookeri	Dipsacaceae	Spang-rtsi-do-bo	HA	W.Nepal to S.W.China	W	Gout, dysentery, fever and hypertension
Punica granatum	Punicaceae	Se-bru	LA	Tropics	F	
Runnunculus brotherusi (nuichillus)	Ranunculaceae	lChe-tsha	HA	Himalaya	A	Antiseptic, wounds and pus and as antipyretic
Ranunculus tricuspis	Ranunculaceae	Chu-rug-pa	HA	Kashmir to S.E.Tibet	W	Relieves nerve pain, hbam-grum and fevers

Rheum australe	Polygonaceae	Chu-rtsa	HA	India and Nepal	R	
Rhodiola himalenses	Crassulaceae	Sro-lo-dmar-pa	HA	Kashmir	R	
Rhododendron anthopogon	Ericaceae	Dvali-dkar-po	HA	Pakistan to S.E Tibet	Fl	
Rhododendron lepidotum	Ericaceae	Dva-li-nag-po	HA	Pakistan to S.W China	L	
Rhus semi-alata	Anacardiaceae	Da-trig	LA	Kashmir, S.W China, Japan, and Burma	F	
Ricinus communis	Euphorbiaceae	Dan-rog	LA	Tropics	S	
Rosa macrophylla	Rosaceae	Se-rgod	HA	Himalaya	F	Constipation, liver and bile disorders, cough and cold, antidote and abscess
Rosa sericea	Rosaceae	Se-bai-me-tog	HA	Himalaya	Fl.	
Roscoea capitata	Zingeberaceae	Sga-skya	LA	Nepal	Rh.	
Rubia cordifolia	Rubiaceae	Brtsod	LA	Pakistan to S.E.Tibet	St.	
Salvia castanea	Labiatae	Jib-rtsi-chen-po	HA	Himalaya	R	
Sambacus adnata	Caprifoliaceae	Yu-gu-shing	HA	W. Nepal to S.W. China	A	
Saussurea gossypiphora	Compositae	Bya-rgod-sug-pa	HA	Kashmir to S.W China	A	
Saussurea spp.	Compositae	Ru=rta	HA	Himalaya	R	
Saxifraga moorcroftiana	Saxifragaceae	Zang-tig	HA	Pakistan to S.W China	W	Antipyretic and bile disorders
Saxifraga parnassiflora	Saxifragaceae	Gser-tig	HA	Utter .Pradesh to Bhutan	W	Wounds, cough cold, and bile disorders including jaundice
Saxifraga umbellulata	Saxifragaceae	Tig-ta	HA	Himalaya	A	Liver and bile disorders, wounds, and fever
Scopolia lurida	Solanaceae	Thang-phrom-nag-po	HA	Cosmopolitan	S	
Selaginella pulvinate	Selaginellaceae	Chu-srin-sder-mo	LA	Himalaya	A	
Shorea robusta	Dipterocarpaceae	Spos-kar	LA	Himalaya	Re.	
Silene satisperma	Caryophyllaceae	Ra-sug	HA	Pakistan to E. Nepal	W	
Soroseris hookeri	Compositae	Srol-gong-ser-po	HA	India to S.E Nepal	W	
Swertia chirata	Gentianaceae	Rgya-tig	LA	Himalaya	W	
Swertia wolfangiana	Gentianaceae	Rgu-drue	HA	Himalaya	A	Wounds and joins nerves
Swertia kingii	Gentianaceae	Kyi-lce-dkar-po	HA	Himalaya	Fl.	
Symplocos ramosissima	Symplocaceae	Byi-tang-ka	LA	India to S.W China	F	
Tanacetum nubigenum	Compositae	Khan-pa-dkar-po	HA	India to Bhutan	W	Abscess, kidney disorders and haemorrhage
Taraxacum officinalis	Compositae	Khur-mong	HA	Europe, Asia and North America	W	Stomach disorders, all fevers and detoxifiers
Terminalia bellerica	Combretaceae	Ba-ru	LA	Tropics	F	
Terminalia chebula	Combretaceae	A-ru	LA	Tropics	F	
Tribulus terrestris	Zygophyllaceae	Gze-ma-ra-go-ma	LA	Tropics	F	Arthritis, diuretics and kidney disorders
Thalictrum reniforme	Ranunculaceae	Sngo-sprin	HA	Nepal to Bhutan	A	Antidote, anti-bacterial, anti-malarial and painkiller
Thermopsis barbata	Leguminosae	Gla-ba-sran-ma	HA	Kashmir to S.W China		
Thlaspi arvense	Cruciferae	Bre-ga	HA	Cosmopolitan	W	Pneumonia and kidney disorders
Tinospora cordifolia	Menispermaceae	Sle-tres	LA	Himalaya.	C	
Verbascum thapsus	Scrophulariaceae	Shing-gi-gser-bye	HA	Euroasia	S	
Veronica himalensis	Scrophulariaceae	Ldom-nag-ldom-mkhri	HA	Himalaya		Ulcers, heals wounds, and haemorrhage
Unidentified	Liliaceae	Byiu-lp-hug	HA	Himalaya	W	
Unidentified		Stab-seng	LA	Himalaya	C	
Unidentified	Moraceae	Seng-ldeng	LA	Himalaya	Wd	

Note: A: aerial, Bk: bark, B: Bulb, C: Cambium, Fl: flower, F: fruit, R: root, Rh: Rhizome, S: seed, St: stem, W: Whole, Wd: Wood, LA: Low altitude, HA: High altitude.

Table 4. Medicinal plants investigated for alkaloids at ITMS using the Culvenor and Fitzgerald procedure[106].

Botanical name	Gso-ba Rig-pa name	Alkaloid Test
Aconitum lacinatum	Btsan-dug	Positive
Aconitum orochryseum	Bong-dkar	Positive
Aletris pauciflora	Dam-bu-kara	Positive
Bistorta macrophylla	Spang-ram	Negative
Chrysosplenium forrestii	gYa-kyi-ma	Positive
Codonopsis bhutanica	Klu-bdud-dorji	Positive
Corydalis flabellata	Re-skon	Positive
Corydalis gerdae	Tong-ri-zil-pa	Positive
Corydalis megacalyx	sTong-zil	Positive
Corydalis stracheyi	Bya-rgod-sug-pa	Positive
Delphinium brunonianum	Bya-rgod-spos	Positive
Fritillaria devalayi	Dkar-po-chig-thub	Negative
Parnassia ovata	Dngul-tig	Positive
Pleurospermum amabile	Rtsad	Positive
Polygonatum verticillatum	Ra-mne	Negative
Ranunculus brotherusi	Che-rtsa	Positive
Rhododendron anthopogon	Da-li	Positive
Rosa macrophylla	Se-rgod	Negative
Thalictrum reniforme	Sgno-sprin	Positive
Thlapsi arvense	Bre-ga	Negative

Table 5. Selected medicinal plants with their indications.

Botanical name	Family	Distribution	Ethno -medical use
Aletris pauciflora	Liliaceae	Himalayas 4100 metres	Pneumonia, liver and lung diseases
Ranunculus brotherusi	Ranunculaceae	Himalayas 4100 metres	Antipyretic, wounds & pus
Codonopsis bhutanica	Campanulaceae	Endemic 4500 metres	Gout, leprosy and infections
Corydalis gerdae	Fumariaceae	Bhutan and Chumbi 4100 metres	Malaria and infections
Aconitum Orochryseum	Ranunculaceae	Endemic 4720 metres	Fever, antidote (snake bite) and infections
Tribulus terrestris	Zygophyllaceae	Australia and Coastal areas	Arthritis, diuretics and kidney diseases

Extracts of two of the selected medicinal plants, *Aletris pauciflora* and *Codonopsis bhutanica*, were damaged on transportation to Wollongong, so as an alternative, the locally available plant, *Tribulus terrestris* L[107] was selected for the study. This plant was known to have caused hepatopathy[108], staggers[109] and locomotor effects[110] in Australian sheep but the specific compound (suspected to be an alkaloid) responsible for neurotoxicity whose symptoms are related to the symptoms of Parkinson's disease in humans, was not known. The plant is also used in the traditional medicine of Bhutan.

It should be noted that the medicinal plants used in Bhutanese traditional medicine were never used as single plant components, but rather as a mixture of two or more active ingredients.

The theory behind using multi-ingredient compounds is that those mixtures of ingredients are believed to act synergistically by neutralising the toxicity of individual ingredient and enhancing the therapeutic values of the mixture. So, quite often the individual therapeutic indication is lost when it is formulated as multi-ingredient products. For example, *Tribulus terrestris* is used for formulating four different multi-ingredient products in combination with other ingredients (**Table 6).** Although the therapeutic properties of *Tribulus terrestris* are retained while formulated, the individual therapeutic properties of other ingredients used in the formulation of 4 products (**Table 6**) could have lost.

Table 6. Traditional medicine products which use *Tribulus terrestris* in their formulation.

Sl. No.	Name of Product	Traditional use
1	Go-yu-28	Diuretic & kidney disorders
2	Seng-lden-25	Paralysis, arthritis, *CNS disorders
3	Ba-sam-lha-rlung	Kidney and renal disorders
4	Bdud-rtsi-nga-lum	Paralysis, gout and arthritis

*CNS: Central Nervous System

Similarly, *Aconitum orochryseum* is used for formulating eighteen different multi-ingredient products (**Table 7**). Only three of these products (Products 8, 9 and 10) have similar therapeutic indications as those of the individual plant and Products 8 and 9 could be possibly good antimalarial compounds since they are used against high fever. The other products (Products 1, 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 15, 16, 17 and 18) have entirely different therapeutic indications to that of the individual therapeutic properties. Two products (Products 5 and 12) are used against poisoning and infections, and to treat liver diseases.

Table 7. *Aconitum orochryseum* Stapf. used in formulating 18 types of multi-ingredient drugs.

Sl. No.	Name of Product	Traditional use
1	Tig-ta-8	Jaundice and Headache
2	Tig-ta-16	Jaundice and yellowing of eyes
3	Gser-mdog-11	Gallstone
4	Hong-len-9	Hypertension and joint pain
5	Thang-chen-25	Poisoning and Infections
6	Gyu-rgyal	Rheumatism
7	Bol-sman-7	Stomach upset
8	Rta-ze-dmar-po	High fever related to cold and flu
9	Spang-rtsi-12	High fever
10	Gtso-bo-8	Cough and Cold
11	Dbang-po-kuen-sel	Sinusitis
12	Gur-gum-13	Liver diseases
13	Man-sil	Poisoning and Bile disorders
14	Ko-la-19	Spleen diseases
15	Dvag-sman-15	Indigestion
16	Brag-zhun-9	Stomach ulcer
17	Mkhris-phyi-7	Dysentery
18	Ded-pon-10	Constipation

Corydalis gerdae and *Codonopsis bhutanica* are used for the formulation of a product called kLu-bdd-8 which is used for treating dermatitis and leprosy.

2.4 Collection of medicinal plants

The collection was done from July to August 2002 by the medicinal plant collection team from the Pharmaceutical and Research Unit of the Institute for Traditional Medicine Services. *Corydalis gerdae* Fedde is endemic to Bhutan and the Chumbi valley in Tibet[111] and was collected from the upper Mochu (Yha-le-La near Lingshi), Chomolhari (4400-4900 m) bases and from nearby mountain screes[77] (**Figure-3,** Collection site-I). *Codonopsis bhutanica*, *Ranunculus brotherusi*, *Aletris pauciflora* were also collected from the Lingshi region (**Figure 3,** Collection site-I). *Aconitum orochryseum* Stapf. has been collected from alpine regions (3950-4720 m)[111] including Thampe la, Zaradingthang and Tolegang[77] in Bhutan. However, for this study, *Aconitum orochryseum* was collected from Chukhalung, Lingshi (**Figure 3**, Collection site-I). Herbarium specimens for each of the plants collected for this study are housed at the Pharmaceutical and Research Unit of the Institute for Traditional Medicine

Services, Thimphu, Bhutan. The herbarium voucher number and the collection codes are noted in **Table 8**.

Tribulus terrestris was collected from near Wellington (Central West, NSW) in February 2002 by Dr. Chris Bourke, Orange Agriculture Institute, Orange, NSW. The air-dried (room temperature) plant material (stems, leaves, flowers and fruits, approx. 8 kg) has been coarse chopped using a petrol driven plant-mulching machine.

Table 8. Site of collection of the selected medicinal plants.

Medicinal Plants	Collection season	Collection sites (Bhutan)	Parts collected	Herbarium No.
Aletris pauciflora	July/August	Zangpothang	Whole	47
Codonopsis bhutanica	July/August	Chewla	Whole	71
Ranunculus brotherusi	July/August	Zangpothang	Whole	18
Corydalis gerdae	July/September	Chukhalung	Whole	07
Tribulus terrestris	February	Wellington, NSW, Australia	Aerial	NA
Aconitum orochryseum	July/August	Jaradingthang	Aerial	83

Except for *Aletris pauciflora* and *Codonopsis bhutanica*, all the plants collected here are rare species[77], and care was taken with respect to the environmental impact. The collected plants were then analysed for their alkaloidal components and the results of the investigations are discussed in the ensuing chapters.

Chapter 3

Alkaloids from *Aconitum orochryseum* Stapf.

Family:	Ranunculaceae
Genus:	*Aconitum*
Species:	*orochryseum*
Common name:	Aconites
gSo-ba Rig-pa name:	Bong-nga-dkar-karpo
Distribution:	Endemic to Himalayas

3.1 Botanical Description and Ethno-medical Use

Aconitum orochryseum is a herbaceous perennial plant and is a close relative of *Delphinium* species[77]. The plant grows to a height of 40-100 cm with pubescent slender stems[77], palmately divided or cleft leaves (3-9 cm diameter) and hairy petalled, 2-10 racemes flowers. There are more than 100 species of *Aconitum* and they are native to temperate regions of the northern hemisphere.

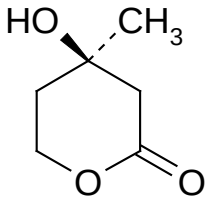
Aconitum and *Delphinium* species both contain biologically active diterpenoid alkaloids. Crude preparations from plants in these two genera have long had a broad range of applications in Asia, Alaska, and Europe[59], ranging from covert human poisons (e.g. *Aconitum napellus* is used in arrowhead dips and reputedly as an agent for euthanasia) to traditional medical uses in neuralgia, gout, hypertension and rheumatism and they have also been included as ingredients in intoxicating liquors[112]. The powdered root of *Aconitum heterophyllum* Wall has been used by Indians (Asia) as a febrifuge and bitter tonic, especially in combating debility after malaria and other fevers[113]. It is also considered efficacious in the treatment of diarrhoea, dysentery, cough, dyspepsia and chronic enteritis[114].

Aconitum leucostomum Vorosch is used in China for the treatment of traumatic injury[115], while *Aconitum hemsleyanum* Pritz has been used as a folk remedy for the treatment of arthritic pain[116]. In Bhutan, three species of aconites: *Aconitum lacinatum, Aconitum violatum* and *Aconitum orochryseum* are used in the formulation of more than 25 traditional medicines[85]. *Aconitum orochryseum,* which is endemic to Bhutan and her bordering areas, has been used for bilious fever, as an antidote against snake bite and also for the treatment of malaria[105].

3.2 Diterpenoid Alkaloids: Biosynthesis and Classification

Diterpenoid alkaloids occur mainly in the families Ranunculaceae (*Aconitum*, *Delphinium*, *Thalictrum* and *Consolida* genera), Cornaceae (*Garr*ya species), Rosaceae (*Spirea japonica*) and Compositae (*Inula royleana*) and possess widely recognised pharmacological and biological activities [117-122]. The majority of the phytochemical studies of these three genera have been carried out with species from Asia, Europe, and North America[123,124]. A series of new and known alkaloids have been isolated and identified from them.

Over two hundred diterpenoid alkaloids are reported to date from 92 different species of *Aconitum* alone[47]. These diterpenoid alkaloids occur commonly either as amino alcohols or as esters of amino alcohols and these permutations and combinations thus give rise to many interesting bases[121]. A number of common biosynthetic pathways have been proposed for the diterpenoid alkaloids which are skeletally closely related. Some authors have suggested, based on the structural similarities, that the diterpenoid alkaloids are derivatives of mevalonate **5**.

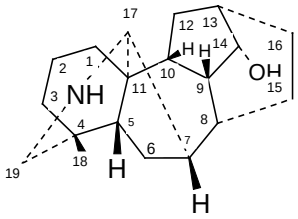


5 Mevalonate

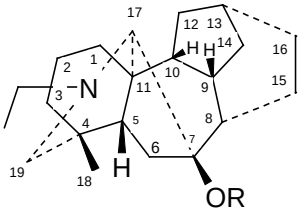
However, the classification of these alkaloids by some authors in this field indicated that the alkaloids arise from cyclization and rearrangement processes. Pelletier and Keith[125], Dalton[121], and Southon and Buckingham[47] have classified these alkaloids mainly into two major structural types, the C_{19} and C_{20} diterpenoid alkaloids. The C_{19} diterpenoid alkaloids have three types of basic skeletal ring systems: the aconitum-type **6**, the lycoctonine-type **7**, and the lactone-type or heteratisane-type **8**.

The aconitane group accounts for the majority of the alkaloids of this diterpenoid class and they differ only in the substitution by hydroxy, methoxy, acetyloxy, benzoyloxy and acyloxy[47] groups. Recently, tuberaconitine and tubermesaconitine, along with the known alkaloids flaconitine, mesconitine, tuberanine and aconitine that belong to the aconitane group have been isolated[126]. Hemsleyatine **9** was the first aconitane-type C_{19} diterpenoid alkaloid bearing an amino group at the C-8 position and it was isolated from the plant *Aconitum hemsleyanum* Pritz[127].

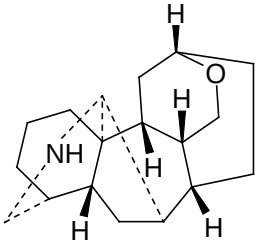
The un-substituted C-14 aconitane ring system with C-7 substitution occurs in the lycoctonine-type alkaloids. Its sub-type, the pyrodelphinine-type **10** of the C_{19} diterpenoid alkaloids, originates as a result of C-8-C-15 double bond formation in the aconitane ring system. The oxidative fission of the C-13-C-14 bond of the aconitane framework forms lactone or heteratisane-type alkaloids as represented by heteratisine[47]. Two new C_{19} diterpenoid alkaloids, jiufengdine and jiufengtine, were also isolated recently from *Delphinium potainii* W.T.Wang varieties *jiufengshanense* W.J.Zhang et G.H.Chen[128].



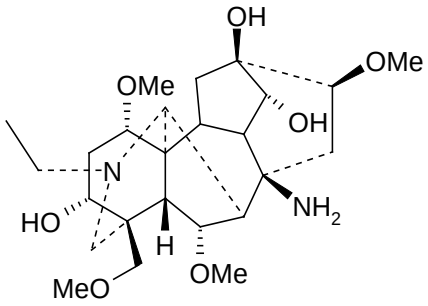
6 Aconitane



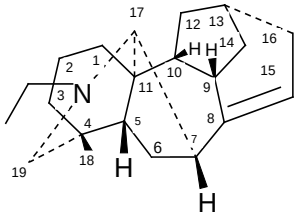
7 Lycoctonine



8 Heteratisane

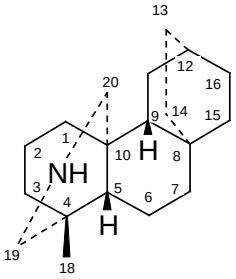


9 Hemsleyatine

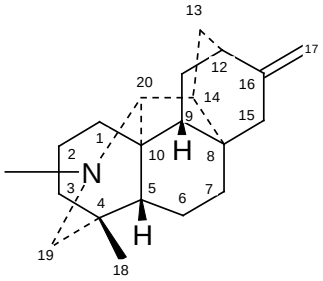


10 Pyrodelphinine

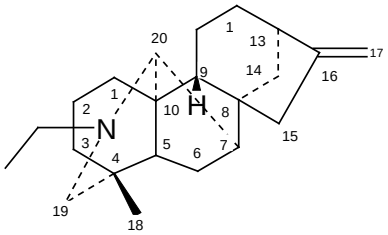
The second major group are the C_{20} diterpenoid alkaloids based on the atidane ring system **11**. Atidine was first isolated from the Himalayan plant *Aconitum heterophyllum* Wall along with the known alkaloids atisine, hetisine, heteratisine, and benzoylheteratisine[129]. The atisane-type alkaloid **12** represents a smaller group with interesting chemical features and complex structures which has been the subject of extensive chemical study[130]. Atisine, the first representative of this type, was isolated as the major alkaloid from the Himalayan plant *Aconitum heterophylloides* Stapf., but it has also been reported from *Aconitum gigas*, *Aconitum anthara* and *Aconitum heterophyllum*[131]. It was suggested[132] that the aconitanes were derived from compounds with the atisine skeleton, through the rearrangement of the C-8-C-9 bond to the C-8-C-15 position and the loss of the C-17 substituent[133]. Hetisane and delnudine together represent the biggest sub-types of atidane-based C_{20} diterpenoid alkaloids and they are discussed separately in section 3.2.1.



11 Atidane type

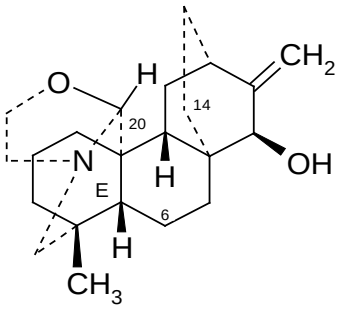


12 Atisane type

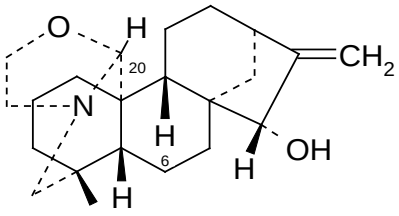


13 Veatchine type

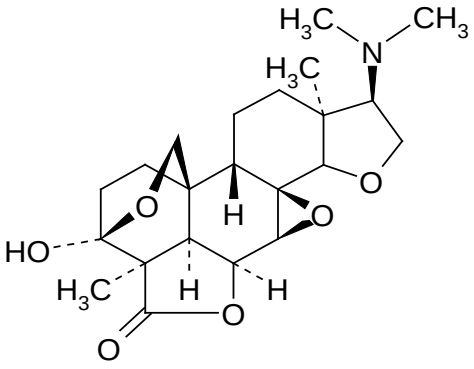
The third major group is represented by the veatchine skeletal type **13** of the C_{20} diterpenoid alkaloids. *Delphinium* species (Ranunculaceae) generally contain veatchine or atisine-type diterpenoid alkaloids[134-136]. A striking similarity is present in the chemistry of atisine **14** isolated from *Aconitum heterophyllum* Wall (Ranunculaceae) and veatchine **15** isolated from *Garrya veatchii* Kellogg (Garryaceae)[137,138] although they belong to different families[139]. A study of the NMR spectra of 44 alkaloids from these two plants confirmed that atisine-type alkaloids which contain an oxazolidine ring, have a boat conformation in ring E **14**[138].



14 Atisine



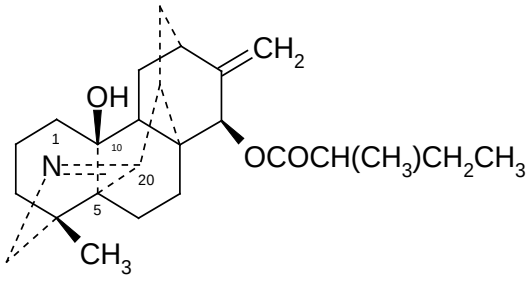
15 Veatchine



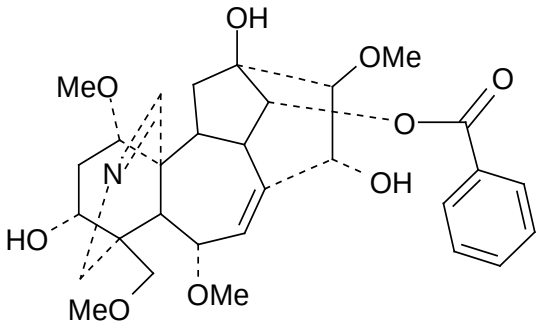
16 Icacine

Varieties of bases, that don't fit into any of the above structural types, could also be formed by either modifications of these skeletal rings or by introduction of a new ring

system (as a result of bond formation) into the main structural skeleton as is the case with the icacine **16** and they are grouped under miscellaneous diterpenoid alkaloids[140]. A new C_{20} diterpenoid alkaloid, arcutinine **17,** that has a C-5-C-20 bond instead of the normal C-11-C-17 and C-10-C-20 bridging bonds, was isolated recently from *Aconitum arcutum* Maxim and is still the only one of its kind[141]. Secokaraconitine **18**, another new diterpenoid alkaloid that has no C-7-C-17 bond, was isolated from the Kyrgystan plant *Aconitum karacolicum* Rapaics[142].



17 Arcutinine



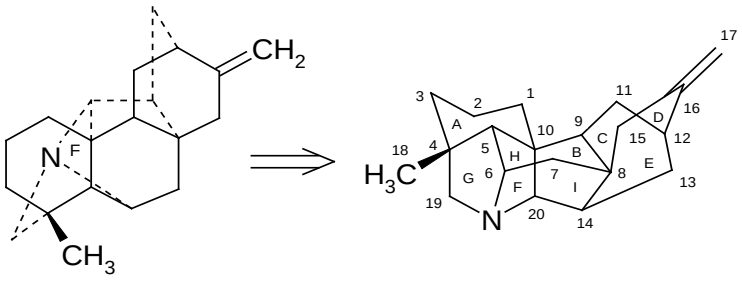
18 Secokaraconitine

3.2.1 Hetisane-type Diterpenoid Alkaloids

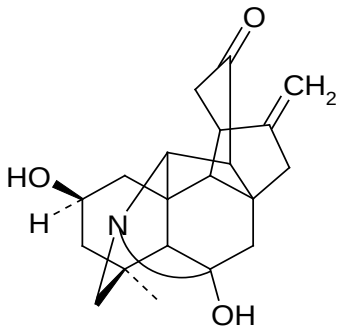
The strong interest in hetisane-type alkaloids is due to their complicated structures, unique chemistry, valuable pharmacological properties and the widespread popularity of plants containing these compounds in folk medicine[122]. The first representatives of this type, paniculatine, hetisine and kobusine, were isolated in the 1930s and 1940's[122]. As of 1998, more than 100 hetisane-type representatives were known[122].

While some sources have suggested that the introduction of additional rings into the atidane ring system by formation of C-14-C-20 and N-C-6 bonds forms the hetisane-type **19** C_{20} diterpenoid alkaloids, a recent review more accurately pointed out that the formation of the bridge bonds C-14-C-20 and N-C-6 bridges in atisine produces the hetisane carbon framework[122,143]. The contraction of ring C in the hetisane forms complex derivatives belonging to the delnudine type of alkaloid as exemplified by delnudine **20**. The 13-O-acetyl derivative of hetisine was isolated from a related plant, *Delphinium nutallianum* Pritz[144].

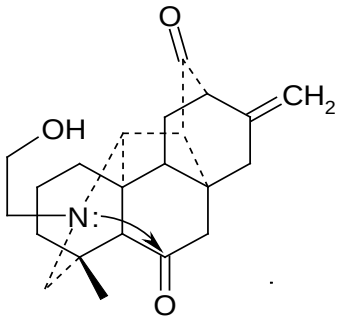
It was proposed that the formation of the N-C-6 bond is based on the structures of the natural alkaloids miyaconitine and miyaconitinone, an analogue of the intermediate **21** [122,143]. Other alternatives for forming the N-C-6 were also studied, proposed and reviewed recently.[118]



19 Hetisane type



20 Delnudine



21 N-C-6 bond intermediate

As illustrated in structure **19**, the skeletal system of the hetisane-type alkaloids is made up of six membered (chair shaped ring-A and ring-B, and a boat shaped ring-C, ring-D, ring-E and ring-F) and five membered (ring-G with a twist-form, ring-H with an envelope form and the ring-I with an equal mixture of the twist and an envelope conformations) rings[145]. For all the hetisane alkaloids the fusion of rings A, B and C is identical. This structural framework and ring conformations of the hetisane type alkaloids significantly helped the author in structural elucidation and the conformational analysis of the compounds isolated from *Aconitum orochryseum* Stapf..

3.3 Pharmacological Investigation of the Diterpenoid Alkaloids from *Aconitum* species.

Aconitum alkaloids have been the subject of extensive chemical and pharmacological investigation owing to their toxicity as well as their potential medicinal values. An aconitane-type diterpenoid alkaloid, such as aconitine, which was isolated as the major component from the poisonous plant, *Aconitum napellus*, was probably the first diterpenoid alkaloid to have been studied pharmacologically[146]. Aconitine-containing liniments like Aconitysat™, Brinpax™, Etermol™ and Pectovox™ have been used in modern medicine for the treatment of rheumatism, neuralgia and sciatica[146]. Another

derivative, deacetylranaconitine, which was isolated from the roots of *Aconitum finetianum*, showed good analgesic activity[47].

Hetisane and atisane type alkaloids are closely related and have been reported to have the lowest cytotoxicity among the different categories of aconitum-diterpenoid alkaloids[114]. About 12 diterpenoid alkaloids were isolated from *Aconitum coreanum* Rapaics collected from the Korean Peninsula and China and have been assessed for structure-biological activity relationships[147]. This study found that atisine chloride, isoatisine and coryphine had the highest toxicity as well as the highest myorelaxant activity among the 12 alkaloids, and hetisine-type alkaloids were found to be less toxic with less myorelaxant activity. This indicated that the therapeutic value is proportional to the toxicological index.

The veatchine-type diterpenoid alkaloids, napelline and its acyl derivatives (isolated from the most toxic plants *Aconitum napellus*, *Aconitum karakolicum* and *Aconitum yesoense*), were studied for their antiarrhythmic activity[148] and it was found that one of the derivatives, 1-O-benzoylnapelline, had maximum activity, even markedly exceeding that of napelline itself and the reference Class I antiarrhythmic drugs novocainamide, quinidine and lidocaine.

Although studies on diterpenoid alkaloids from *Aconitum* species have been extensive and they are still vigorously continued in pursuit of potential new alkaloids, little has been reported on the antimicrobial activity of these diterpenoid alkaloids. In this context, a Bhutanese medicinal plant, *Aconitum orochryseum* Stapf. was analysed for alkaloids and antimicrobial activity of the alkaloids.

3.4 Isolation, Structural Elucidation and Identification of Diterpenoid Alkaloids from *Aconitum orochryseum* Stapf.

The methanol extract of whole parts of air-dried plant material was subjected to a series of extraction, fractionation, separation, purification and crystallization processes (**Chapter 9**). This resulted in the isolation of eleven alkaloids from *Aconitum orochryseum* Stapf.. Comparative literature analysis of data revealed that two of the alkaloids were the known compounds virescenine and atisinium chloride. The structure of virescenine was established by NMR spectral analysis and that of atisinium chloride was confirmed by single crystal X-ray crystallography. Three other alkaloids were identified as new alkaloids and they were named as orochrine, 2-*O*-acetylorochrine and lingshinaline. Orochrine was named after the species *orochryseum*, 2-*O*-acetylorochrine was based on the acetate substituent at the C_2 position of orochrine,

while lingshinaline was named after the place of collection called Lingshi in Bhutan. In the presence of base Na_2CO_3, some of the alkaloids existed in an amino keto form (apply to all the three new alkaloids that were isolated as salts), which would be soluble in chloroform. However, there may have been some left in the aqueous phase.

Among the compounds isolated, atisinium chloride was found to be the major alkaloid from the plant followed by lesser amounts of lingshinaline, orochrine, virescenine, and 2-*O*-acetylorochrine. To identify and establish their structures, physico-chemical and spectral analysis were conducted for each of these three alkaloids and the results are discussed sequentially in the following section. Six other alkaloids were isolated as minor components.

3.4.1 Orochrine

a. Physico-chemical properties

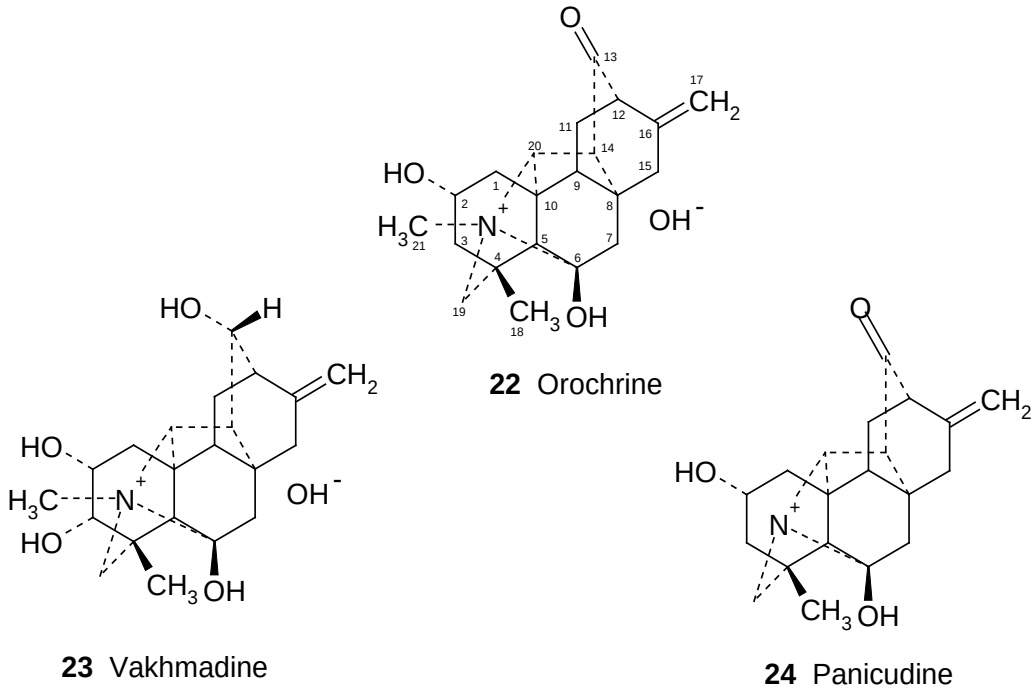
The alkaloid named orochrine **22** was obtained as needle-like crystals (14.9 mg/1 kg dried plant weight) on recrystallization from acetone/diethyl ether. This alkaloid formed a translucent solid in the presence of chloroform. The alkaloid was optically active and had a high melting point.

b. Spectral Analysis of Orochrine.

Low Resolution Chemical Ionisation Mass Spectrometry (LRCIMS) indicated a molecular ion peak at *m/z* 342 (MH^+), which was 18 amu less than that of the alkaloid vakhmadine. High Resolution Chemical Ionisation Mass Spectrometry (HRCIMS) and High Resolution Electron Impact Mass Spectrometry (HREIMS) supported the molecular formula $C_{21}H_{27}NO_3$ for this alkaloid, and hence an index of hydrogen deficiency of 15. In the LREIMS (**Appendix 1**), the molecular ion fragments were observed at *m/z* 341 (M^+), together with major fragment ions at *m/z* 326, 313, 282, 254, 192, 136, 122, 84, 55, and 44 (100%). The number of protons (from integration in the ^{1}H-NMR spectrum) and carbons (from the ^{13}C-NMR) matched with the number of protons and carbons presented by the molecular formula (HRCIMS).

The structure of orochrine **22** was established by the analysis of ^{1}H-NMR (**Appendix 2**), ^{13}C-NMR (**Appendix 3**), DEPT (**Appendix 4**), gCOSY (**Appendix 5**), TOCSY (**Appendix 6**), gNOESY (**Appendix 7**), gHSQC (**Appendix 8**) and gHMBC (**Appendix 9**) spectral data. No signals for aromatic protons were evident in the ^{1}H-NMR spectrum so the index of hydrogen deficiency was structurally indicative of saturated rings or double bonds. Two broad singlets (5.04 ppm, 1H and 4.94 ppm, 1H)

indicated the presence of the H-17 exocyclic methylene group. A strong singlet resonating at 1.48 ppm (3H) was ascribed to the H-18 methyl group. Signals ascribed to the H-20 and H-12 (1H each, bs) protons were observed at 4.27 ppm and 2.98 ppm respectively. The H-19 protons resonated at 3.35 ppm (d, 1H, *J*=11.5 Hz) and 4.30 ppm (d, 1H, *J*=11.5 Hz). Since almost all hetisane type alkaloids contain these four characteristic proton signals[122], it was concluded that the alkaloid (orochrine) belonged to the hetisane type diterpenoid alkaloids (**Section 4.2.1**).



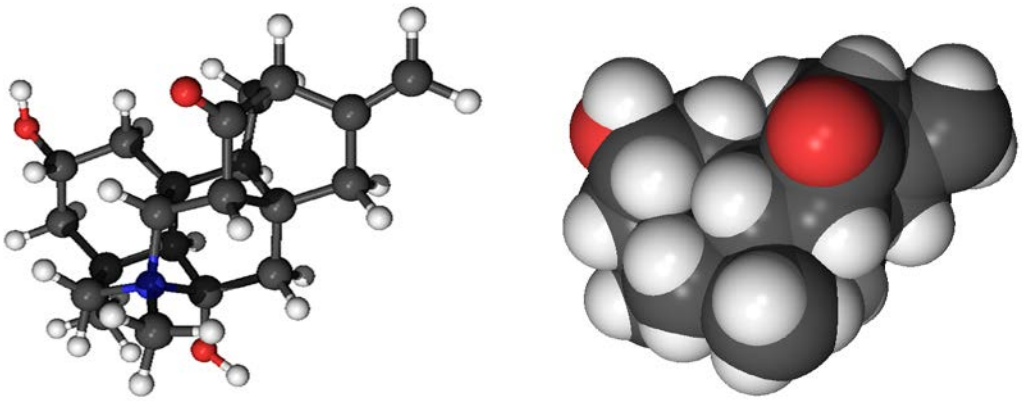


Figure 4. 3D-Model of orochrine (N-blue, O-red, C-black, H-white).

From the gCOSY spectrum, a distinct geminal coupling was observed between the proton signals at 2.69 ppm (d, 1H, *J* = 17 Hz, H-15) and 2.52 ppm (d, 1H, *J*=17.5 Hz, H-15). These geminal protons (2.69 ppm, d, 1H, *J*=17 Hz and 2.52 ppm, d, 1H, *J*=17.5Hz) were coupled to the H-17 exocyclic methylene protons (5.04 ppm, bs,1H

and 4.94 ppm, bs, 1H), clearly indicating that they were close to each other. The gCOSY spectrum also showed strong coupling between the H-19 protons at 4.30 ppm (d, 1H, J = 11.5 Hz), and 3.35 ppm (d, J = 11.5 Hz).

From the ^{13}C-NMR spectrum, the peaks at 142.3 ppm and 112.3 ppm indicated the presence of an alkene group in the structure. A ketone carbonyl group was assigned to the resonance signal at 208.7 ppm. Generally, for the hetisane type C_{20} diterpenoid alkaloids, a doublet for C-2 with chemical shifts of 66.1-67.2 ppm, and its respective protons resonating between 4.02-4.31 ppm a broad singlet, has been assigned to the C-2 α-hydroxyl moiety[149,150]. In the ^{13}C-NMR spectrum of the alkaloid isolated here, the chemical shift ascribed to the C-2 carbon resonated at 65.5 ppm and its associated proton resonated at 4.14 ppm (H-2β) as a broad singlet in the ^{1}H-NMR spectrum. Thus, based on this evidence, a hydroxyl group (C-2αOH) was assigned to the C-2 position of orochrine.

The DEPT spectral analysis showed that there were fifteen protonated carbons. Seven of them were methylene carbon atoms (-CH_2 × 7 including the exocyclic methylene group), six were methine carbon atoms (-CH × 6) and two were methyl carbon atoms (-CH_3 × 2). Based on DEPT, gHSQC, gCOSY and TOCSY spectral analysis, complete carbon-proton connectivity, proton-proton correlations and group assignments were achieved (**Table 9**). The gHSQC correlations found that C-17 had a chemical shift of 112.3 ppm and that of C-18 (methyl carbon) corresponded to the signal resonating at 30.3 ppm.

The structure was then drawn by cross peak analysis of the gHMBC (proton-carbon correlations. of a maximum of 4 bonds away) and gNOESY spectra **(Table 10).** Using the scaffold of the hetisane-type diterpenoid alkaloid **19**, the appropriate carbon peaks of the ring were assigned and a complete structure for orochrine was established as **22**. From the gHMBC correlation (**Table 10**), C-4, C-6, C-8, C-10, C-13 and C-16 were found to be the quaternary carbons. The chemical shifts of C-4 (36.6 ppm) and C-10 (47.4 ppm) matched the range of chemical shifts reported in the literature on hetisane type alkaloids[122].

The gNOESY correlation is also represented diagrammatically in **Figure 5**. Based on this gNOESY correlation, the stereochemistry of orochrine was established. The gNOESY correlation was also consistent with the spatial arrangements of protons and functional groups observed in a computer generated 3D model (**Figure 4**) of orochrine (enantiomeric form, exept at C-2), using the Spartan program. The long range

correlation existing between the *N*-Me and the *C*-14 was clearly seen in this 3D computer model because orochrine has a rigid spatial conformation.

Table 9. The gHSQC, gCOSY and TOCSY NMR data (500 MHz, CD_3OD) of orochrine 22.

Carbon	g HSQC (δC to δH)						gCOSY Correlation	TOCSY Correlation
	δC (ppm)	Assignment	δH (ppm)	Multiplicity	Integration	*J*(Hz)		
C-1	34.5	$-CH_2$	1.59	d	1H	15	H-2β	
			1.75	d	1H	15		
C-2	65.5	-CH	4.14	bs	1H		H-1, H-3	H-1, H-3
C-3	41.4	$-CH_2$	1.60	d	1H	15	H-1	H-1
			1.93	t	1H	15		
C-4	36.6	Q						
C-5	59.1	-CH	2.15	s	1H			H-9
C-6	106.2	Q-OH						
C-7	38.2	$-CH_2$	2.23	d	1H	12.5		
			2.31	d	1H	12.5		
C-8	43.9	Q						
C-9	49.4	-CH	2.20	s	1H		H-11	H-11
C-10	47.4	Q						
C-11	23.3	$-CH_2$	1.86	d	1H	14		H-9
			2.03	d	1H	14.5		
C-12	53.5	-CH	2.97	bs	1H		H-9, C-11	H-11
C-13	208.7	Q,C=O						
C-14	56.3	-CH	2.98	bs	1H			
C-15	32.6	$-CH_2$	2.52	d	1H	17.5	H-17	
			2.69	d	1H	17		
C-16	142.6	Q						
C-17	112.3	$-CH_2$	5.04	bs	1H		H-15	H-15
			4.94	bs	1H			
C-18	30.3	$-CH_3$	1.48	s	3H			
C-19	70.5	$-CH_2$	3.35	d	1H	11.5		
			4.30	d	1H	11.5		
C-20	75.1	-CH	4.27	s	1H			
C-21 (*N*-Me)	37.3	$-CH_3$	2.90	s	3H			H-3, H-7

Note: Q = Quaternary carbon

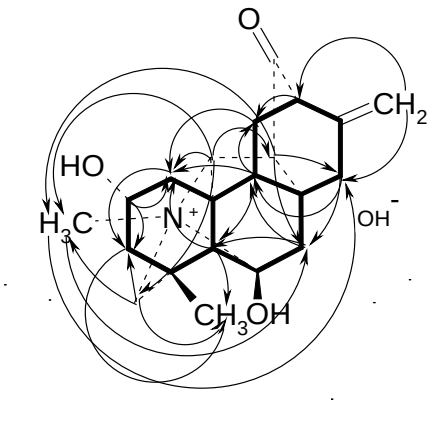


Figure 5. gNOESY Correlation of orochrine.

Table 10. gHMBC and gNOESY NMR correlation (500 MHz, CD_3OD) of orochrine 22.

			gHMBC Correlation		gNOESY
Carbon	**δC (ppm)**	**δH (ppm)**	**δC to δH**	**δH to δC**	**Correlation**
C-1	34.5	1.59, 1.75	H-20	C-2, C-3, C-4, C-5, C-10, C-20	H-3
C-2	65.5	4.14	H-1, H-3	C-20	H-1, H-3
C-3	41.4	1.60, 1.93	H-1, H-18, H-19	C-1, C-2, C-4, C-5, C-10, C-18	H-1, H-18
C-4	36.6		H-3, H-18, H-19		
C-5	59.1	2.15	H-1, H-3, H-7, H-15, H-20	C-9, C-18, C-19, C-20	H-1, H-18
C-6	106.2		H-7, H-9, H-19, H-20, H-21		
C-7	38.2	2.23, 2.31		C-5, C-6, C-8, C-9, C-11, C-14, C-15, C-20	H-5, H-9
C-8	43.9		H-7, H-11, H-15, H-20		
C-9	49.4	2.20	H-5, H-7, H-15, H-20	C-5, C-6, C-8, C-10, C-11, C-12, C-14, C-20	H-1, H-5, H-11
C-10	47.4		H-11, H-12, H-14, H-20 H-1, H-3		
C-11	23.3	1.86, 2.03		C-8, C-9, C-10, C-12, C-13, C-16	H-1
C-12	53.5	2.97	H-9, H-11, H-15, H-17	C-13, C-14, C-15, C-16, C-17	H-11
C-13	208.7		H-11, H-12, H-14, H-20		
C-14	56.3	2.98	H-7, H-12, H-15 (w), H-19, H-20	C-9, C-13, C-15, C-16, C-20	H-7, H-15, H-21 ($N\text{-}CH_3$)
C-15	32.6	2.52, 2.69	H-12, H-14, H-17	C-7, C-8, C-9, C-12, C-14, C-16, C-17	H-7, H-9
C-16	142.6		H-11, H-12, H-14, H-15		
C-17	112.3	5.04, 4.94	H-12, H-15	C-12, C-15, C-16 (w)	H-12, H-15
C-18	30.3	1.48	H-3, H-5, H-19	C-3, C-4, C-5, C-19	
C-19	70.5	3.35, 4.30	H-3, H-5, H-18, H-20, H-21	C-3, C-4 (w), C-5 (w), C-6, C-14, C-18, C-20, C-21	H-3, H-18, H-21 (*N*-Me)
C-20	75.1	4.27	H-1, H-2, H-5, H-9, H-12, H-14, H-19, H-21	C-1, C-4 (w), C-6, C-8, C-9, C-10 (w), C-13, C-14, C-19	H-1, H-14, H-19, H-21
C-21 (*N*-Me)	37.3	2.90	H-19	C-6, C-19, C-20	H-7, H-15

From the gHMBC and gNOESY cross peak assignments, orochrine was assigned to have N-C-6 and C-14-C-20 bonds. The presence of a quaternary nitrogen (attached to C-19, C-20, C-6 and C-18 methyl group) and the quaternary carbon at C-6 (attached to C-5, C-7, N-Me and hydroxyl group) indicated its relation to vakhmadine **23** which was isolated as a quaternary base (i.e. nitrogen bonded to CH_3, C-19, C-20 and C-6) from A*conitum palmatum*[151].

In order to check consistency in the chemical shift assignments of functional groups, the NMR data for orochrine was compared with those for vakhmadine and also with the

range of chemical shifts reported in the literature on C_{20} hetisane type diterpenoid alkaloids[122] (**Table 11**).

Table 11. Comparison of NMR data (500 MHz, CD_3OD) of the orochrine with vakhmadine (300 MHz, D_2O)[151] and the range of chemical shift generally observed for hetisane type alkaloids.

Carbon	Compounds				General range of δ C (ppm) and δH (ppm) for hetisane-type alkaloids*	
	Vakhmadine		Orochrine			
	δC	δH (*J*=Hz)	δC	δH (*J*=Hz)	δC	δH (*J*=Hz)
C-1	30.0		34.5	1.59, 1.75	26.4-39.3	
C-2	69.3	3.97 (bs, 1H, H-2β,)	65.5	4.14 (bs, 1H , H-2β)	66.1-67.2 (d,) with OH	4.02-4.31 (bs)
C-3	73.5	3.33 (d, 1H, *J*= 4.3)	41.4	1.60 (d, 1H, 1.93 (t, 1H)	36.5-43.3 (-OH attached in C-2)	
C-4	40.6		36.6		35.9-38.7	1.29-1.68 (-OH in C-6)
C-5	58.9		59.1	2.15	58.0-62.3	
C-6	105.0		106.2		96.8-107.2	
C-7	40.1		38.2	2.23 2.31	42.5-46.7 (β-effect of -OH at C-6)	
C-8	41.5		43.9		40.5-44.2 (s)	
C-9	45.3		49.4	2.20	48.6-48.9 (due to C=O group on C-13)	
C-10	45.2		47.4		46.0-52.0	
C-11	21.4		23.3	1.86 2.03	22.7-23.4 (with C=O on C-13)	
C-12	41.5		53.5	2.97	53.2-54.0 (with C=O group on C-13)	2.14-2.94 (bs)
C-13	67.8	3.93 (d, 1H,13β, *J*=11.0)	208.7	Ketone group		
C-14	48.1		56.3	2.98	58.8-61.9 (d, with C=O on C-13)	
C-15	31.8		32.6	2.52 (d, 1H, *J*=17.5) 2.69 (d, 1H, *J*=17)	32.3-36.1 (t)	
C-16	148.1		142.6		138.0-147.0 (s)	
C-17	107.2	4.59 (s, 1H) 4.73 (s, 1H)	112.3	4.94 (s, 1H) 5.04 (s, 1H)	104.3-114.4 (t)	4.8-5.52 (bs, 1H each)
C-18	25.3	1.40 (s, 3H)	30.3	1.48 (s, 3H)	28.5-32.0 (q)	
C-19	66.7	4.05 (d, 1H, J=11.7)	70.5	3.35 (d, 1H, *J* = 11.5) 4.30 (d, 1H, *J* =11.5)	62.7-65.0	2.21-3.02 2.61-3.85 (*J* =11.5-14)
C-20	73.2	4.22 (s, 1H)	75.1	4.27 (bs, 1H)	65.0-75.0 (d)	2.04-4.30 (1H, bs)
C-21 (*N*-Me)	36.3	2.58 (s, 3H)	37.3	2.90 (s, 3H)		

Note: The range of chemical shifts were adapted from the literature review by Bessonova et al[122].

From this comparative analysis, the chemical shifts of the carbon and proton signals of orochrine were found to be consistent with those reported for vakhmadine[122,151]. In both cases, the presence of a quaternary nitrogen atom caused a weak deshielding effect on

nearby protons of *N*-Me (2.90 ppm), H-19 (3.35 ppm, d, 1H, *J*= 11.5 Hz and 4.30 ppm, d, 1H, *J*=11.5 Hz) and H-20 (4.27 ppm, bs). Similarly, the hydroxyl group at C-6 caused the protons of C-18 to shift slightly downfield to 1.48 ppm and the *N*-methyl carbon and protons to shift upfield to 37.3 ppm (γ effect)[122] and 2.907 ppm[152] respectively. This C-6 hydroxyl group also affected the chemical shift of C-5 (1H) which resonated at 59.1 ppm.

However, unlike vakhmadine, which has a hydroxyl group at C-13 and an extra hydroxyl group attached at C-3, orochrine **22** does not have a C-3 hydroxyl either. Because of the C-13 carbonyl group, the chemical shifts of C-9 (49.4 ppm), C-11 (23.3 ppm) and C-12 (53.5 ppm) were shifted downfield compared to the same carbon position assignments in vakhmadine **23.**

When orochrine was compared with the structure of panicudine **24** that has a C-13 carbonyl group and no C-3 hydroxyl group, the chemical shifts of C-13 and its neighbouring carbons (C-9, C-12, and C-11) in orochrine were found to be consistent with the corresponding chemical shifts for the carbon and proton signals of panicudine. Panicudine was isolated from *Aconitum paniculatum* Lam[122]. Therefore, the above analysis supported **22** as the structure of orochrine.

The counter ion for the structure of orochrine **22**, 2-O-acetylorochrine **25** and lingshinaline **26** was assigned as a hydroxide ion (OH^-) because only methanol and water had been used for the extraction, separation and purification. Vakhmadine **23** was also isolated as its hydroxide salt.

3.4.2 2-*O*-acetylorochrine

a. Physico-chemical properties

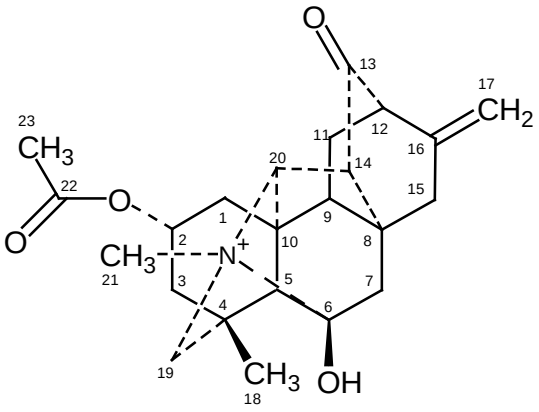
The optically active alkaloid named 2-*O*-acetylorochrine **25** was obtained as a creamy white solid. It dissolved readily in chloroform and methanol.

b. Spectral Analysis of 2-acetylorochrine

LRCIMS gave a peak at *m/z* 384 (MH^+) which was 42 amu more than that of orochrine. HRCIMS and HREIMS supported the molecular formula $C_{23}H_{29}NO_4$ for this alkaloid, and thus an index of hydrogen deficiency of 10. The LREIMS spectra (**Appendix 10**) showed a molecular ion at *m/z* 383 (M^+), plus fragment ions at *m/z* 368, 355, 340, 324, 296, 282, 254, 226, 176, 174, 134, 122, 105, 91, 84, 70, 58, 55, and 43.

Its structure was established by ^{1}H-NMR (**Appendix 11**), ^{13}C-NMR (**Appendix 12),** DEPT (**Appendix 13**), gCOSY (**Appendix 14**), gNOESY (**Appendix 15**), TOCSY

(**Appendix 16**), gHSQC (**Appendix 17**) and gHMBC (**Appendix 18**) spectral data obtained in deuterated methanol (CD_3OD). The structure of this compound differed from orochrine only in the C-2 functional group. This compound had an acetate group rather than a hydroxyl at C-2 position. Such biosynthetically reasonable acetylations are not unusual in these types of alkaloids.



25 2-*O*-acetylorochrine

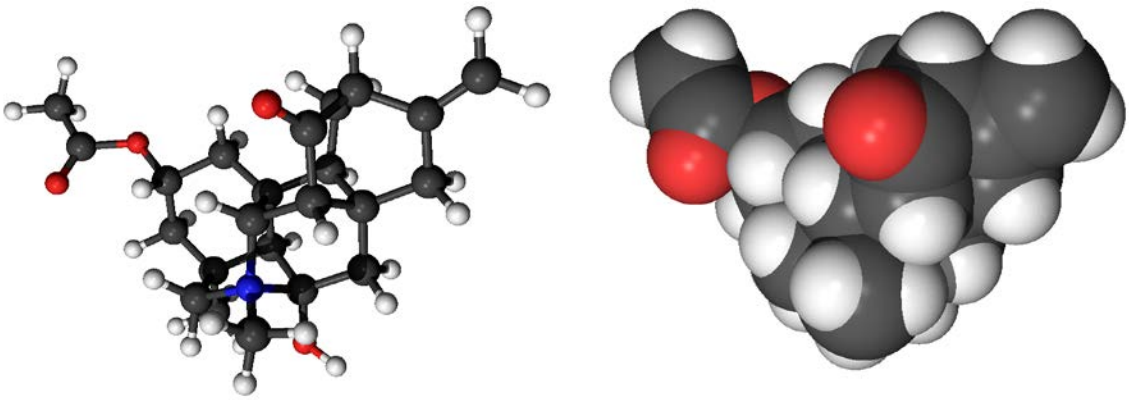


Figure 6. 3D-Model of 2-*O*-acetylorochrine (N-Blue, O-Red, C-Black, H-White).

Like orochrine, no signals for aromatic protons were evident in the ^{1}H-NMR spectrum of **25** and so the index of hydrogen deficiency was structurally indicative of saturated rings or double bonds. However, the presence of an exocyclic methylene group was indicated by two broad singlets (1H each) observed at 4.86 ppm and 4.95 ppm. These protons were attached to the carbon signal resonating at 112.4 ppm in the ^{13}C-NMR spectrum from the gHSQC correlation. The proton signals in this case shifted upfield (+ 0.087 ppm) but the signal for the carbon shifted downfield (+ 0.15 ppm) in comparison to the equivalent signals in orochrine. The signal at 30.1 ppm in the ^{13}C-

NMR was assigned to C-18, while the associated methyl protons resonated at 1.43 ppm (s, 3H).

The N-CH_3 carbon was assigned to the signal at 37.3 ppm, with the methyl protons resonating at 2.90 ppm (s). In addition, another singlet for the acetyl protons was present at 2.06 ppm (s, 3H), which in turn correlated with a carbon signal at 23.3 ppm. A new carbon peak at 171.3 ppm was assigned to the ester carbonyl group. The C-13 carbonyl group resonated at 208.7 ppm. The signal at 106.6 ppm was ascribed to C-6 which was substituted by a hydroxyl group, the assignment being based on the gHMBC spectrum.

From the ^{1}H-NMR and gCOSY spectra, the H-15 geminal protons (2.47 ppm, d, 1H, *J* = 17 Hz, and 2.62 ppm, d, 1H, *J*=17.5 Hz) were coupled to each other and to the H-17 exomethylene protons. The H-19 geminal methylene protons at 3.37 ppm (d, 1H, *J* = 12 Hz), and 3.79 ppm (d, *J* = 12 Hz) were also coupled. Like all hetisane type alkaloids[122], the singlet (1H) at 3.37 ppm for H-20 and the doublet at 2.98 ppm (1H, *J* = 3.5Hz) for H-12 were present. The signals for H-5 (1H) and H-9 (1H) appeared as a partially resolved broad singlet. Unlike orochrine, the signal for H-11 (2H) was a multiplet (half width = 3.5 Hz) and the signal for H-14 was a doublet at 2.97 ppm (1H, *J* = 2Hz). A doublet signal for H-2β was observed slightly downfield at 5.11 ppm (1H, *J* = 3Hz) due to the presence of the adjacent α-acetate group[122]. The chemical shift for the carbon (C-2) to which they were attached was observed at 69.0 ppm.

The DEPT spectra revealed sixteen protonated carbons; seven methylene (-CH_2 × 7 including the exomethylene group), six methine (-CH × 6), and three methyl groups (-CH_3 × 3). The complete carbon-proton correlations for these protons and carbons were established by analysing the gHSQC, gCOSY and TOCSY spectra as presented in **Table 12**. The gHMBC and gNOESY correlations (**Table 13)** provided complete information on the carbon-proton connectivities and their arrangements. The structure was derived by both carbon-proton correlations cross peak analysis and assignments. The gHMBC data analysis generated a structure **25** and the gNOESY spectrum confirmed the relative stereochemistry of this alkaloid. The gNOESY correlation is diagrammatically represented in **Figure 7**.

The gHMBC correlation indicated C-4, C-6, C-8, C-10, C-13 and C-16 were quaternary carbons. The peaks at 36.5 ppm, 47.4 ppm and 58.5 ppm were assigned to C-4, C-10 and C-5 respectively as the chemical shifts were within the normal range noted for these carbon signals in the related alkaloids[122]. A 3D model (**Figure 6**) was generated for 2-*O*-acetylorochrine (enantiomer shown, apart from C-2) using the Spartan

computer program and the spatial arrangements of atoms and functional groups in the structure were analysed. The gNOESY correlations were found to be consistent with the orientations of protons and functional groups in the 3D model of the structure giving added weight to stereochemical assignments.

Table 12. gHSQC, gCOSY and TOCSY NMR data (500 MHz, CD_3OD) of 2-*O*-acetylorochrine 25.

Carbon	gHSQC (δC to δH)						gCOSY Correlation	TOCSY Correlation
	δC (ppm)	Assignment	δH (ppm)	Multiplicity	Integration	*J* (Hz)		
C-1	31.6	$-CH_2$	1.65	d	2H	17.5		
C-2	69.0	-CH	5.11	d	1H	3	H-1, H-3	H-1, H-3
C-3	38.5	$-CH_2$	1.66	d	1H	15.5	H-1	
			1.92	d	1H	16		
C-4	36.5	Q						
C-5	58.5	-CH	2.17	s	1H			
C-6	106.6	Q, -OH						
C-7	38.0	$-CH_2$	2.23	d	1H	14.5		
			2.27	d	1H	14.5		
C-8	44.2	Q						
C-9	49.3	-CH	2.17	s	1H		H-11	H-11
C-10	47.4	Q						
C-11	23.3	$-CH_2$	1.84	m	2H	3.5		
C-12	53.3	-CH	2.89	d	1H	3.5	C-11	H-11
C-13	208.7	Q, C=O						
C-14	56.2	-CH	2.97	d	1H	2		H1, H-7
C-15	32.4	$-CH_2$	2.47	d	1H	17		
			2.62	d	1H	17.5		
C-16	142.3	Q						
C-17	112.4	$-CH_2$	4.86	bs	1H	43.5	H-15	H-15
			4.95	bs	1H			
C-18	30.1	$-CH_3$	1.43	s	3H			
C-19	70.5	$-CH_2$	3.37	d	1H	12		
			3.79	d	1H	12		
C-20	75.1	-CH	3.76	s	1H		H-14	
C-21 (*N*-Me)	37.3	$-CH_3$	2.90	s	3H			H-3, H-7
C-22 (CH_3*COO)	171.3							
C-23 (*CH_3COO)	21.3	$-CH_3$	2.06	s	3H			

*Note: Q = Quaternary carbon, *carbon represented in numbering*

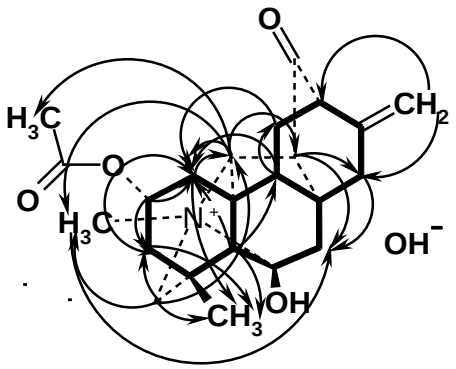


Figure 7. gNOESY correlation of 2-*O*-acetylorochrine 25.

Table 13. gHMBC and gNOESY NMR data (500 MHz, CD_3OD) of 2-*O*-acetylorochrine 25.

Carbon	δC (ppm)	δH (ppm)	gHMBC Correlation: δC to δH	gHMBC Correlation: δH to δC	gNOESY Correlation
C-1	31.6	1.65	H-3, H-11 (w), H-20	C-2, C-3 (w), C-4, C-5, C-10, C-20	
C-2	69.0	5.11	H-1, H-3, H-18 (w)	C-22	H-1, H-3
C-3	38.5	1.66 1.92	H-1, H-19	C-1, C-2, C-4, C-5, C-10, C-18 (w), C-19	H-1, H-18
C-4	36.5		H-1, H-3, H-18, H-19		
C-5	58.5	2.17	H-1, H-3, H-7, H-9, H-18	C-7 (w), C-9 (w), C-14, C-18, C-19, C-20	H-18
C-6	106.6		H-5, H-7, H-19, H-20, H-21		
C-7	38.0	2.23 2.27	H-9 (w), H-15	C-5, C-6, C-8, C-9 (w), C-14, C-15 (w)	
C-8	44.2		H-7, H-9, H-11, H-15		
C-9	49.3	2.17	H-5, H-7, H-11, H-12, H-14, H-15, H-20	C-5 (w), C-7 (w), C-12, C-14, C-20	H-1, H-11
C-10	47.4		H-1, H-3, H-9 (w), H-11, H-14		
C-11	23.3	1.84		C-1 (w), C-8 (w), C-9, C-10, C-12, C-13, C-16	H-1
C-12	53.3	2.89	H-9, H-11, H-15, H-17	C-9, C-13, C-14, C-15	H-11
C-13	208.7		H-11, H-12, H-14, H-20		
C-14	56.2	2.97	H-7, H-9, H-15, H-19 (w), H-20, H-21 (w)	C-9,C-10, C-13, C-20	H-7, H-15
C-15	32.4	2.47 2.62	H-7, H-12, H-17	C-7 (w), C-8, C-9, C-12, C-14, C-16, C-17	H-7
C-16	142.3		H-11, H-15		
C-17	112.4	4.86 4.95	H-12 (w), H-15	C-12, C-15	H-12, H-15
C-18	30.1	1.43	H-1, H-3, H-5, H-19	C-3, C-4, C-5, C-19	
C-19	70.5	3.37 3.79	H-3, H-5, H-18, H-20, H-21	C-4, C-5, C-14 (w), C-18, C-20	H-18, H-21 (*N*-Me)
C-20	75.1	3.76	H-1, H-5, H-14, H-19, H-21	C-1, C-6, C-8, C-9 (w), C-13, C-14 (w), C-19	H-1, H-14, H-20, H-23
C-21 (*N*-Me)	37.3	2.90		C-6, C-19, C-20	H-7
C-22 ($COOCH_3$)	171.3		H-2, H-23		
C-23 (CH_3COO)	21.3	2.06		C-22	

The chemical shifts of protons and carbons of the structure of 2-*O*-acetylorochrine were then compared with the chemical shifts of protons and carbons of orochrine and also with the general range of chemical shifts for the respective carbons and protons reported in the literature for hetisane-type diterpenoid alkaloids. Except for the C-2 acetyl group, all the chemical shifts of protons and carbons in the respective NMR spectra of 2-*O*-acetylorochrine were found to be consistent with those of orochrine and the relevant chemical shifts reported in the literature. Thus, the structure was confirmed

as drawn in **25**. The NMR spectroscopic comparison of 2-*O*-acetylorochrine with the orochrine is tabulated in **Table 14.**

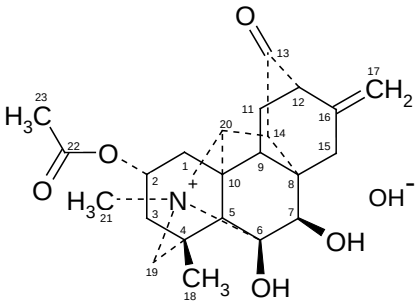
Table 14. Comparison of NMR data of the 2-*O*-acetylorochrine (500 Mhz, CD_3OD) with orochrine (500 Mhz, CD_3OD).

Carbon	Compounds			
	2-*O*-acetylorochrine		Orochrine	
	δC (ppm)	δH (ppm, *J*=Hz)	δC (ppm)	δH (ppm, *J*=Hz)
C-1	31.6	1.65 (d, 2H, *J*=17.5)	34.5	1.59 (d, 1H, *J* =15)
				1.75 (d, 1H, *J* =15
C-2	69.0	5.11 (d, 1H-2β, *J*=3)	65.5	4.14 (bs, 1H-2β)
C-3	38.5	1.66 (d, 1H, *J*=15.5)	41.4	1.60 (d, 1H, *J*=15)
		1.92 (d,1H, *J*=16		1.93 (t, 1H, *J*=15
C-4	36.5		36.6	
C-5	58.5	2.17 (s, 1H)	59.1	2.15, (s, 1H)
C-6	106.6		106.2	
C-7	38.0	2.23 (d, 1H, *J*=14.5)	38.2	2.23, (d, 1H, *J*=12.5)
		2.27 (d, 1H, *J*=14.5)		2.31, (d, 1H, *J*=15
C-8	44.2		43.9	
C-9	49.3	2.17 (s, 1H)	49.4	2.20 (s, 1H)
C-10	47.4		47.4	
C-11	23.3	1.84 (m, 2H, Half width =3.5)	23.3	1.86 (d, 1H, *J*=14)
				2.03 (d, 1H, *J*=14.5
C-12	53.3	2.89 (d, 1H, *J*=3.5)	53.5	2.97 (bs, 1H)
C-13	208.7	ketone group	208.7	ketone group
C-14	56.2	2.97 (d, 1H, *J*=2)	56.3	2.98 (bs, 1H)
C-15	32.4	2.47 (d, 1H, *J*=17)	32.6	2.52, (d, 1H, *J* =17.5)
		2.62 (d, 1H, *J*=17.5)		2.69 (d, 1H, *J*=17)
C-16	142.3		142.6	
C-17	112.4	4.86, (bs, 1H)	112.3	4.94 (bs, 1H)
		4.95 (bs, 1H)		5.04 (bs, 1H)
C-18	30.1	1.43 (s, 3H)	30.3	1.48 (s, 3H)
C-19	70.5	3.37 (d, 1H, *J*=12)	70.5	3.35 (d, 1H, *J*=11.5)
		3.79, (d, 1H, J =12)		4.30 (d, 1H, *J*=11.5)
C-20	75.1	3.76 (s, 1H)	75.1	4.27 (s, 1H)
C-21 (*N*-Me)	37.3	2.90 (s, 3H)	37.3	2.90 (s, 3H)
C-22 ($COOCH_3$)	171.3			
C-23 (CH_3COO)	21.3	2.06 (s, 3H)	Not present	

3.4.3 Lingshinaline

a. Physico-chemical properties

Lingshinaline **26**, was isolated as a pale green solid. The alkaloid was optically active but the $[\alpha]_D$ was smaller than for orochrine or 2-*O*-acetylorochrine. Its melting point was also lower than orochrine or 2-*O*-acetylorochrine.



26 Lingshinaline

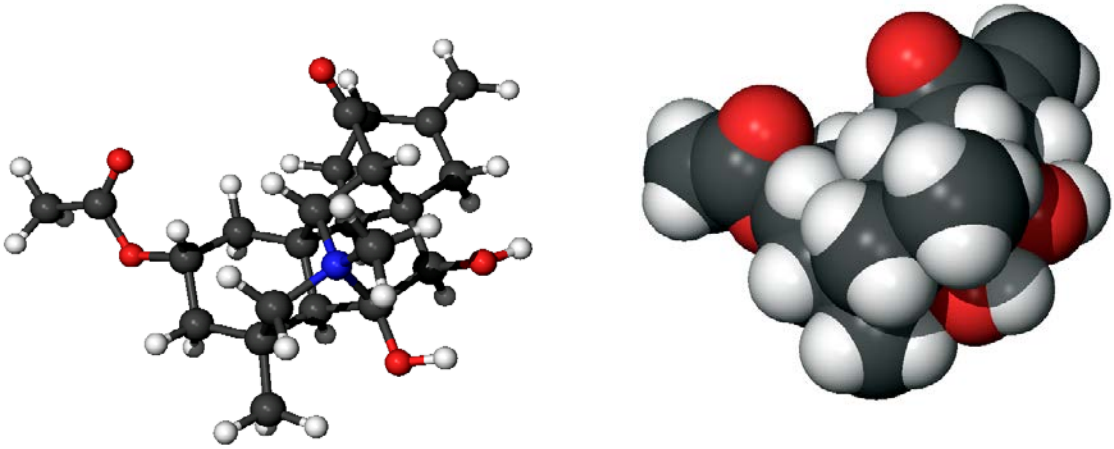


Figure 8. 3D-computer model of lingshinaline (N-blue, O-red, C-black, H-white).

b. Spectral Analysis

HRCIMS indicated the MH^+ peak at *m/z* 400 (calc. 400.2124) and the LREIMS gave the M^+ peak at *m/z* 399 that is 16 amu more than that of 2-*O*-acetylorochrine. HRCIMS and HREIMS supported the molecular formula $C_{23}H_{29}NO_5$ for this alkaloid, and hence an index of hydrogen deficiency of 10. This suggested that lingshinaline had one extra hydroxyl group compared with 2-*O*-acetylorochrine. The LREIMS spectrum (**Appendix 19**) showed ions at *m/z* 399 (M^+), 382, 370, 356, 340, 315, 312, 270, 176, 174, 173, 148, 134, 122, 105, 91, 84, 55, and 43.

The structure of lingshinaline was established by ^{1}H-NMR (**Appendix 20**), ^{13}C-NMR (**Appendix 21)**, DEPT (**Appendix 22**), gCOSY (**Appendix 23**), gNOESY (**Appendix 24**), TOCSY (**Appendix 25**), gHSQC (**Appendix 26**) and gHMBC (**Appendix 27**) spectral data analysis obtained in deuterated chloroform ($CDCl_3$).

Like orochrine and 2-*O*-acetylorochrine, the ^{1}H-NMR spectrum of lingshinaline had no signals for aromatic protons and so the index of hydrogen deficiency was structurally indicative of saturated rings or double bonds. However, the exomethylene peaks were clearly present at 4.92 ppm (bs, 1H) and 5.00 ppm (bs, 1H). These protons were

connected to the C-17 carbon, resonating at 112.4 ppm (t) in the ^{13}C-NMR. The C-18 carbon had a chemical shift of 29.7 ppm, and its protons resonated at 1.52 ppm (s, 3H). The *N*-methyl carbon had a chemical shift of 40.4 ppm and its protons resonated at 3.12 ppm (s). A singlet ascribed to acetate ester protons (s, 3H) was present at 2.10 ppm and corresponded to a carbon signal at 21.4 ppm. A carbon peak assigned to the ester carbonyl was present at 169.1 ppm. The C-13 carbonyl group resonated at 206.7 ppm. The signal ascribed to the carbinolamine C-6 substituted by a hydroxyl group resonated at 105.7 ppm. The N-C-6 bond was established using the HMBC spectrum (see **Table 16**).

The ^{1}H-NMR and gCOSY spectra revealed that the H-15 geminal protons (2.48 ppm, d, 1H, *J* = 17.5 Hz, and 2.96 ppm, d, 1H, *J*=17.5 Hz) were coupled to the H-17 exomethylene protons. The H-19 geminal methylene protons at 3.42 ppm (d, 1H, *J* = 11.5 Hz), and 3.61 ppm (d, *J* = 11.5 Hz) were also coupled. Like all hetisane alkaloids[122], the H-20 (3.63 ppm, s, 1H) and H-12 (2.95 ppm, s, 1H) proton signals were also present. The signal for the H-5 proton resonated at 2.12 ppm (s, 1H) and that for the H-9 proton resonated at 2.20 ppm (d, 1H). The signal for H-14 resonated at 3.00 ppm (s, 1H) and that for H-2β (1H) was observed a little downfield as a broad singlet at 5.21 ppm due to the presence of the adjacent α-acetyl group.

The DEPT spectra revealed sixteen protonated carbons: six methylene (-CH_2 × 6, including the exomethylene group), seven methine (-CH × 7), and three methyl groups (CH_3 × 3). In this case the structure has gained one methine and lost one methylene carbon compared to 2-*O*-acetylorochrine. The complete carbon-proton correlations for these protons and carbons were established by analysing the gHSQC, gCOSY and TOCSY (long range couplings) spectra, as tabulated in **Table-15**.

From the gHMBC correlation **(Table 16),** proton-carbon connectivities were established and the cross peaks observed were assigned on the scaffold of the hetisane type alkaloids. Cross peak assignment established C-4, C-6, C-8, C-10, C-13 and C-16 as quaternary carbons. The peaks at 35.9 ppm, 47.7 ppm and 55.8 ppm in the ^{13}C-NMR were assigned to C-4, C-10 and C-5 respectively as the chemical shifts were within the normal range noted for these carbon signals in the related alkaloids[122].

The C-7 (71.7 ppm) carbon was substituted by a hydroxyl group and its proton H-7 was ascribed to the ^{1}H-NMR signal at 4.35 ppm. It was reported in the literature that, as a rule in these alkaloids this C-7 hydroxyl group has the α-orientation and the signal for H-7β should be within the chemical shift range of 3.87-4.50 ppm[122]. Although the chemical shifts for C-7 and its proton (s, 1H, H-7) were within the range noted in the

literature, the gNOESY correlation (**Table 16**) supported a β-orientation of the C-7 hydroxyl group in lingshinaline. From the 3D model (**Figure 8**) of lingshinaline (enantiomeric form shown apart from C-2, C-7 configuration), the C-7 hydroxyl group was found to be protruding to the front and the hydrogen atom going backward thereby correlating with the H-5, H-9 and H-15 protons which also had α-orientations. The gNOESY correlation is diagrammatically represented in **Figure 9**.

Table 15. gHSQC, gCOSY and TOCSY NMR data (500 MHz, $CDCl_3$) of lingshinaline 26.

Carbon	gHSQC (δC to δH)						gCOSY Correlation	TOCSY Correlation
	δC (ppm)	Assignment	δH (ppm)	Multiplicity	Integration	*J* (Hz)		
C-1	31.2	$-CH_2$	1.65	d	1H	16.5		
			1.74	d	1H	15.5		
C-2	66.9	-CH	5.21	s	1H		H-1, H-3	H-3
C-3	37.9	$-CH_2$	1.65	d	1H	17	H-1	
			1.93	d	1H	16.5		
C-4	35.9	Q						
C-5	55.8	-CH	2.12	s	1H			
C-6	105.7	Q						
C-7	71.7	-CH	4.35	s	1H			
C-8	45.3	Q						
C-9	46.7	-CH	2.20	d	1H	7.5	H-11	H-11
C-10	47.7	Q						
C-11	22.1	$-CH_2$	1.86	d	2H	6.5		
C-12	51.6	-CH	2.95	s	1H		H-11	H-9, H-11
C-13	206.7	Q, C=O						
C-14	52.5	-CH	3.00	s	1H		H-9	H-11, H-9
C-15	28.2	$-CH_2$	2.48	d	1H	17.5		
			2.96	d	1H	17.5		
C-16	139.1	Q						
C-17	113.1	$-CH_2$	4.92	bs	1H		H-12, H-15	H-15
			5.00	bs	1H			
C-18	29.7	$-CH_3$	1.52	s	3H			
C-19	70.4	$-CH_2$	3.42	d	1H	11.5		H-14, H-21
			3.61	d	1H	10.0		
C-20	73.5	-CH	3.63	s	1H		H-14	
C-21 (*N*-Me)	40.4	$-CH_3$	3.12	s	3H			H-14
C-22 (CH_3*COO)	169.1							
C-23 (*CH_3COO)	21.4	$-CH_3$	2.01	s	3H			

*Note: Q = quaternary carbon, *carbon represented in numbering.*

Table 16. gHMBC and g NOESY NMR data (500 MHz, $CDCl_3$) of lingshinaline 26.

Carbon	δC (ppm)	δH (ppm)	gHMBC Correlation		gNOESY Correlation
			δC to δH	δH to δC	
C-1	31.2	1.65 and 1.74	H-20, H-3	C-2, C-5, C-8 (w), C-3 (w), C-4	H-3
C-2	66.9	5.21	H-3, H-1, H-18 (w), H-23 (vw)		H-1, H-3
C-3	37.9	1.65 and 1.93	H-1 (w), H-18, H-19	C-2, C-5, C-4 (w), C-1	H-18
C-4	35.9		H-19, H-3, H-18		
C-5	55.8	2.12	H-3, H-1, H-18	C-20, C-7, C-19, C-9, C-18	H-3, H-18
C-6	105.7		H-20, H-21		
C-7	71.7	4.35	H-21 (w), H-5 (w)	C-14, C-10	H-5, H-9
C-8	45.3		H-14, H-11, H-1 (w)		
C-9	46.7	2.20	H-14, H-15, H-5, H-11, H-12		H-5, H-11
C-10	47.7		H-7, H-20		
C-11	22.1	1.86		C-13, C-16, C-8, C-12 (w), C-9 (w)	H-1
C-12	51.6	2.95	H-17, H-15 (w), H-11	C-16,C-17,C-9	H-11
C-13	206.7		H-20, H-14, H-12, H-11		
C-14	52.54	3.00	H-7, H-15	C-13, C-20, C-9, C-8	
C-15	28.2	2.48 and 2.96	H-17, H-7 (w)	C-16, C-17, C-9, C-14	
C-16	139.1		H-15, H-12, H-11		
C-17	113.1	4.92 and 5.00	H-15, H-12	C-12, C-15	H-12, H-15
C-18	29.7	1.52	H-19, H-5	C-19, C-2, C-5, C-3, C-4	
C-19	70.4	3.42 and 3.61	H-20, H-21, H-5, H-18	C-20, C-3, C-4, C-18, C-5, C-21 (w)	H18, H-21
C-20	73.5	3.63	H-19, H-21, H-14, H-5	C-13, C-6, C-10, C-1, C-19 (w)	H-1, H-14, H-21, H-23
C-21 (*N*-Me)	40.4	3.12	H-19 (w)	C-6, C-20, C-7, C-19	H-14
C-22 (CH_3*COO)	169.1		H-23		
C-23 (CH_3COO)	21.4	2.01		C-22, C-2 (w)	

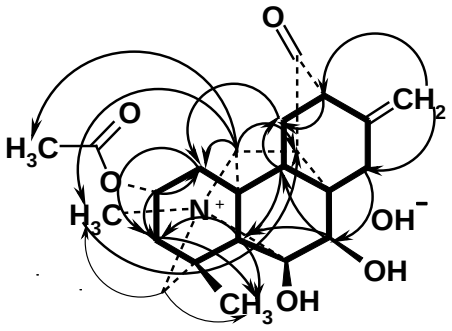


Figure 9. gNOESY correlation of lingshinaline 26.

To confirm the structure, the NMR data for lingshinaline was compared with that of 2-*O*-acetylorochrine **25** (**Table 17**) and the general range of chemical shifts for the

respective carbons and protons reported in the literature on hetisine type C_{20}-diterpenoid alkaloids[122,140]. Except for C-7 (as expected), the ^{13}C-NMR chemical shift values of lingshinaline were found to match closely with those for **25**, and all the chemical shifts were found consistent with the ranges of chemical shifts provided in the literature above.

Slight differences observed were very negligible and could have been caused by either differences in the NMR solvents used or the variations in oxygen containing substituents at C-2 and C-7 positions. Thus the structure of lingshinaline was established and confirmed as **26.**

Table 17. Comparison of NMR data of the 2-*O*-acetylorochrine (500 MHZ, CD_3OD) with NMR data of lingshinaline (500 MHz, $CDCL_3$).

Carbon	Compound			
	2-*O*-acetylorochrine 25		Lingshinaline 26	
	δC (ppm)	δH (ppm, *J*=Hz)	δC (ppm)	δH (ppm, *J*=Hz)
C-1	31.6	1.65 (d, 2H, *J*=17.5)	31.2	1.65 (d, 1H, *J*=16.5)
				1.74 (d, 1H, *J*=15.5)
C-2	69.0	5.11(d, 1H-2β, *J*=3)	66.9	5.21 (s, 1H)
C-3	38.5	1.66 (d, 1H, *J*=15.5)	37.9	1.65 (d, 1H, *J*=17.0)
		1.92 (d,1H, *J*=16		1.93 (d, 1H, *J*=16.5)
C-4	36.5		35.9	
C-5	58.5	2.17 (s, 1H)	55.8	2.12 (s, 1H)
C-6	106.6		105.7	
C-7	38.0	2.23 (d, 1H, *J*=14.5)	71.7	4.35 (s, 1H)
		2.27 (d, 1H, *J*=14.5)		
C-8	44.2		45.3	
C-9	49.3	2.17 (s, 1H)	46.7	2.20 (d, 1H, *J*=7.5)
C-10	47.4		47.7	
C-11	23.3	1.84 (m, 2H, Half width = 3.5)	22.1	1.86 (s, 1H, *J*= 6.5)
				1.87 (s, 1H)
C-12	53.3	2.89 (d, 1H, *J*=3.5)	51.6	2.95 (s, 1H)
C-13	208.7	Ketone group	206.7	Ketone group
C-14	56.2	2.97 (d, 1H, *J*=2)	52.5	3.00 (s, 1H)
C-15	32.4	2.47 (d, 1H, *J*=17)	28.2	2.48 (d, 1H, *J*=17.5)
		2.62 (d, 1H, *J*=17.5)		2.96,(d, 1H, *J*= 17.5)
C-16	142.3		139.1	
C-17	112.4	4.86, (bs, 1H)	113.1	4.92 (bs, 1H)
		4.95 (bs, 1H)		5.00 (bs, 1H)
C-18	30.1	1.43 (s, 3H)	29.7	1.52 (s, 3H)
C-19	70.5	3.37 (d, 1H, *J*=12)	70.4	3.42 (d, 1H, *J*=11.5)
		3.79,(d, 1H, *J*=12)		3.61 (d, 1H, *J*=10.0)
C-20	75.1	3.76 (s, 1H)	73.5	3.63 (s, 1H)
C-21 (*N*-Me)	37.3	2.90 (s, 3H)	40.4	3.12 (s, 3H)
C-22 ($COOCH_3$)	171.3		169.1	
C-23 (CH_3COO)	21.3	2.06 (s, 3h)	21.4	2.01 (s, 3H)

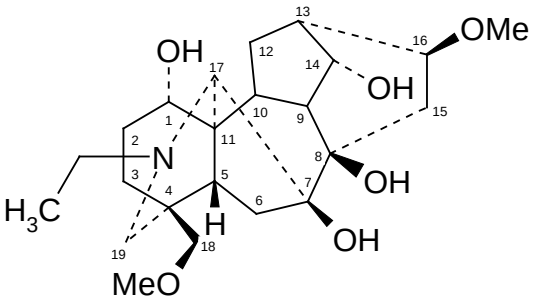
3.4.4 Virescenine

a. Physico-chemical properties

This alkaloid was obtained as an amorphous solid from which a crystalline hydrochloride salt was derived. The alkaloid had a low optical rotation ($[\alpha]_D^{26}$ = + 14.07) and a low melting point (76.7-79.8^oC) compared with the alkaloids **22**, **25**, **26** isolated from the same plant. Unfortunately the crystals of the hydrochloride were not suitable for a single crystal X-ray structure determination.

b. Spectral analysis

The LRCIMS of this alkaloid showed a peak at *m/z* 424 (MH^+ peak). The HREIMS indicated the molecular formula as $C_{23}H_{37}NO_6$ and hence an index of hydrogen deficiency of 20. Its mass was 24 amu more than that of lingshinaline **26**. The LREIMS spectrum (**Appendix 28**) gave molecular and fragment ions at *m/z* 423 (M^+), 408, 405, 406, 390, 374, 365, 352, 336, 178, 164, 148, 122, 108, 98, 85, 71, 58 and 45. The molecular formula of this alkaloid was the same as that of senbusine A[153] and virescenine **27**[154]. Furthermore the optical rotation, melting point, and ion fragmentation pattern were very similar to those for virescenine ($[\alpha]_D^{24}$ = +16.9 in 95% EtOH where *c* =1.0; m.p. = 68-70^oC, Et_2O-C_6H_{14})[47].



27 Virescenine

Virescenine **27** was previously isolated from *Aconitum yesoense* var. *macroyesoense* Tamura[155], *Aconitum delphinifolium* DC[156], *Aconitum nagarum* var.*lasiandrum* W.T.Wang[157], *Delphinium virescens* Nutt[154] and *Aconitum napellus* ssp.*vulgare* (DC.) Rouy et Fouc[158]. So the alkaloid isolated in this study was thought to be virescenine **27**. To substantiate this deduction, the structure of the alkaloid was elucidated by ^{1}H-NMR (**Appendix 29**), ^{13}C-NMR (**Appendix 30**), DEPT (**Appendix 31**), gCOSY (**Appendix 32**), gNOESY (**Appendix 33**), gHSQC (**Appendix 34**), TOCSY (**Appendix 35**) and

gHMBC (**Appendix 36**) spectral analysis. The ^{1}H-NMR showed that the alkaloid had no aromatic protons as well as no H-17 exomethylene and H-18 methyl groups. Instead this alkaloid had two methoxyl signals that resonated at 3.29 ppm (s, 3H) and 3.31 ppm (s, 3H) and an N-ethyl signal whose methyl protons (N-CH_2CH_3) resonated at 1.08 ppm (t, 3H) and the methylene protons (H-18) at 2.95 ppm (q, 1H) and 3.06 ppm (q, 1H). This was the only alkaloid isolated from *Aconitum orochryseum* with an N-ethyl group. The H-19 protons (2H) that were present in orochrine, 2-*O*-acetylorochrine, lingshinaline and atisinium chloride were also present in this alkaloid and their signals resonated at 2.50 ppm (d, 1H, *J*=11 Hz) and 2.76 ppm (d, 1H, *J*=11 Hz).

From the ^{13}C-NMR spectrum, it was found that the alkaloid contained no ester or other carbonyl groups. The DEPT spectrum showed the presence of 19 protonated carbons: eight methylene, eight methine and three methyl groups. Based on the DEPT, gHSQC, gCOSY and TOCSY spectra, the assignment of the groups and carbon-proton connectivities was achieved (**Table 18**) and the complete structure was established using the gHMBC spectrum and gNOESY correlations (**Table 19**).

Table 18. Proton-carbon correlation (from DEPT and gHSQC spectrum) and proton-proton correlation (from gCOSY and long range TOCSY spectrum) of the isolated alkaloid virescenine 27.

Carbon	δC (ppm)	Assignment (From DEPT)	δH (ppm, *J*=Hz) (From gHSQC)	gCOSY	TOCSY
1	72.0	-CH	3.67 (s, H-1β)	H-2, H-3, H-12	H-2, H-18
2	28.7	-CH_2	1.45 (q), 1.57 (d, *J*= 18.5)		
3	26.6	-CH_2	1.63 (dd, *J*=5), 1.82 (dd, *J*=6)	H-2	H-6
4	37.6	Q			
5	41.7	-CH	1.71 (t, *J*=7)		
6	33.3	-CH_2	1.54 (d), 2.26 (d)	H-5, H-10	H-5, H-6
7	85.7	Q			
8	76.0	Q			
9	47.7	-CH	2.15 (t, *J*=5.5)	H-10	H-10
10	43.3	-CH	1.76 (q)		
11	49.3	Q			
12	28.2	-CH_2	1.55 (t), 1.97 (t)	H-10	H-15
13	39.4	-CH	2.27 (m)	H-10, H-12	H-9
14	75.3	-CH	4.17 (t, *J*=4.5, H-14β)	H-9, H-13	H-9, H-13
15	35.8	-CH_2	1.73 (dd, *J*=9.5), 2.89 (m)		H-6
16	81.5	-CH	3.37 (q, H-16α)	H-10, H-15	H-15
17	65.2	-CH	2.83 (s)	H-12	
18	78.4	-CH_2	3.01 (d, *J*=9), 3.16 (d, *J*=9)		H-5
19	55.7	-CH_2	2.49 (d, *J*=11), 2.72 (d, *J*=11)		
20	50.7	-CH_2 (NCH_2CH_3)	2.95 (q), 3.06 (q)	H-21	H-21
21	13.4	-CH_3 (NCH_2CH_3)	1.08 (t, J=7.5)		
22	59.3	-OCH_3 (C-18-OCH_3)	3.28 (s)		
23	56.3	-OCH_3 (C-16-OCH_3)	3.31 (s)		H-17

Note: Q = Quaternary carbon.

Table 19. gHMBC spectrum and the gNOESY correlation of the isolated alkaloid virescenine 27.

Carbon	δC (ppm)	δH (ppm)	gHMBC δC to δH	gHMBC δH to δC	gNOESY Correlation
1	72.0	3.67	H-3, H-5	C-3, C-5	H-2, H-10, H-12, H-15, H-17
2	28.7	1.45, 1.57	H-3 (w)	C-4, C-5, C-11	H-9
3	26.6	1.63, 1.82	H-1, H-18, H-19	C-1, C-4, C-18, C-19	H-2
4	37.6		H-3, H-5, H-6, H-18, H-19		
5	41.7	1.71	H-6, H-10, H-17, H-18, H-19	C-4, C-7, C-10, C-11, C-17, C-18, C-19	H-2, H-12
6	33.3	2.26, 1.54	H-17	C-4, C-5, C-7, C-11, C-17	H-5, H-9
7	85.7		H-5, H-6, H-15		
8	76.0		H-5, H-6, H-9, H-10, H-14, H-15, H-17		
9	47.7	2.15	H-10, H-13, H-15	C-8, C-10, C-12, C-13, C-15	H-10
10	43.3	1.76	H-1, H-2, H-5, H-9, H-13, H-12, H-15, H-17	C-1 (w), C-2, C-8, C-5, C-9, C-11, C-12, C-17	
11	49.3		H-12, H-10, H-17		
12	28.2	1.55, 1.97	H-9, H-10, H-16 (w)	C-5, C-10, C-11, C-13, C-14, C-16	H-15
13	39.4	2.27	H-9, H-12, H-15	C-9, C-10, C-15, C-16	H-12
14	75.3	4.17	H-12, H-16	C-8, C-16	H-6, H-9, H-13,
15	35.8	1.73, 2.89	H-9, H-13	C-6, C-7, C-8, C-9, C-13, C-16	
16	81.5	3.37	H-12, H-14, H-15, H-23	C-12 (w), C-14, C-23	H-2, H-3, H-6, H-12
17	65.2	2.83	H-5, H-6, H-10, H-19, H-20 (w)	C-5, C-6, C-8, C-10 (w), C-11, C-19, C-20	H-6, H-21
18	78.4	3.01, 3.16	H-3, H-5, H-19, H-22	C-3, C-4, C-5, C-19, C-22	H-3, H-5, H-17
19	55.7	2.49, 2.72	H-3, H-5, H-17, H-18, H-20	C-3, C-4, C-5, C-17, C-18, C-20	H-3, H-6, H-21
20	50.7	2.95, 3.06	H-17 (w), H-19, H-21	C-17, C-19, C-21	H-19, H-21
21	13.4	1.08	H-20	C-20	
22	59.3	3.28	H-18	C-18	H-18
23	56.3	3.31	H-16	C-16	H-3, H-6, H-15

Considering the biogenetic relationship in these alkaloids, oxygenation occurs at C-1, C-3, C-4, C-6, C-13, C-16 and C-18. In fact almost all the C_{19} diterpenoid alkaloids bear a hydroxyl or methoxyl group on C-1, C-8, C-14 and C-16[159,160]. Using gCOSY, gNOESY and gHMBC correlations, the three hydroxyl groups of the isolated alkaloid were assigned to the C-1, C-8 and C-14 positions. The hydroxyl groups at C-1 and C-14 had the α-orientation[140,154,159]. The last hydroxyl group was assigned to C-7. The C-7 hydroxyl group is characteristic of the lycoctonine-type **7** C_{19} diterpenoid alkaloids and this hydroxyl group, as well as the hydroxyl group at C-8, has the β-orientation[47,158]. The C-16 methoxyl group was assigned to the β-position while the

adjacent C-16 proton was assigned as α in almost every report of the aconitane-type diterpenoid alkaloids[126].

The NMR spectroscopic data for the isolated alkaloid was then compared with the data for virescenine **27 (Table 20).** The NMR spectra were all obtained in $CDCl_3$; very close chemical shift matches were noted apart from the chemical shifts of C-10 and C-13. The literature on virescenine[140,154] has reported the chemical shift of C-10 as 39.9 ppm and that of C-13 as 43.6 ppm. In contrast, with the alkaloid isolated in this study, the C-10 signal resonated at 43.3 ppm and for C-13 at 39.4 ppm. The assignment of these C-10 and C-13 signals for the alkaloid was supported by the gCOSY and the gHMBC correlations.

From the gCOSY spectrum, the protons of C-6 (1.54 ppm, d and 2.26 ppm, d), C-9 (2.15 ppm, t), C-12 (1.55 ppm and 1.97 ppm, t), C-13 (2.27 ppm, m) and C-16 (3.37 ppm, q) were coupled to the proton (1.76 ppm, q) of the carbon that resonated at 43.3 ppm. This suggested that the carbon at 43.3 ppm was C-10. From the gHMBC correlation, C-10 was connected to the neighbouring carbons C-11 (quaternary), C-9 (-CH) and C-12 ($-CH_2$) rendering it a quartet.

Table 20. Comparison of ^{1}H-NMR and ^{13}C-NMR data of the isolated alkaloid (500 MHz, $CDCl_3$) with that of virescenine[154].

Carbon	Virescenine		Virescenine (Isolated)	
	δC (ppm)	δH (ppm) *J*=Hz	δC (ppm)	δH (ppm) *J*=Hz
1	72.4		72.0	3.67 (s)
2	28.8		28.7	1.45 (q), 1.57 (d, *J*= 18.5)
3	29.3		26.6	1.63 (dd, *J*=5), 1.82 (dd, *J*=6)
4	37.7		37.6	
5	42.0		41.7	1.71 (t, *J*=7)
6	33.6		33.3	2.26 (d), 1.54 (d)
7	86.1		85.7	
8	76.3		76.0	
9	47.9		47.7	2.15 (t, *J*=5.5)
10	39.9		43.3	1.76 (q)
11	49.5		49.3	
12	26.9		28.2	1.55 (t), 1.97 (t)
13	43.6		39.4	2.27 (t)
14	75.5	4.23 (1H, t, *J*=5, H-14β)	75.3	4.17 (t, *J*=4.5, H-14β)
15	35.9		35.8	1.73 (dd, *J*=9.5), 2.89 (m)
16	82.2		81.5	3.37 (q)
17	64.9		65.2	2.83 (s)
18	78.7		78.4	3.01 (d, *J*=9), 3.16 (d, *J*=9)
19	55.9		55.7	2.49 (d, *J*=11), 2.72 (d, *J*=11)
$-CH_2$ (NCH_2CH_3)	50.5		50.7	2.95 (q), 3.06 (q)
$-CH_3$ (NCH_2CH_3)	13.9	1.10 (3H, t, *J*=7.0)	13.4	1.08 (3H, t, *J*=7.5)
$-OCH_3$ (C-18-OCH_3)	59.4	3.33 (3H, s)	59.3	3.28 (3H, s)
$-OCH_3$ (C-16-OCH_3)	56.4	3.35 (3H, s)	56.3	3.31 (3H, s)

The long range gHMBC correlation also found that C-5, C-8, C-11, C-13 and C-17 correlated with the protons (1.76 ppm, q) attached to the carbon that resonated at 43.3 ppm (assigned as C-10, **Table 20**) and this carbon peak (43.3 ppm, δC to δH) correlated with the protons H-1, H-2, H-5, H-9, H-12, H-13, H-15 and H-17. If the carbon peak at 43.3 ppm is assigned to C-13, these correlations would not be possible.

Similarly, from the gHMBC correlation, C-13 (39.4 ppm) for the alkaloid isolated was connected to C-12 (-CH2), C-14 (-CH) and C-16 (-CH). From the ^{1}H-NMR spectrum, the chemical shift of the signal ascribed to the C-13 proton resonated well downfield at 2.27 ppm compared to the C-10 protons that resonated at 1.76 ppm. This was plausible because the C-13 protons were close to the carbons that are substituted with hydroxyl (C-14) and methoxyl (C-16) groups.

There has been little discussion in the literature on the proton-carbon correlations in this alkaloid. It is possible that the original spectra were incorrectly assigned. Since all the ^{13}C-NMR data, other than C-10 and C-13, were in agreement with each other, it was concluded that the alkaloid (**27**) isolated was virescenine.

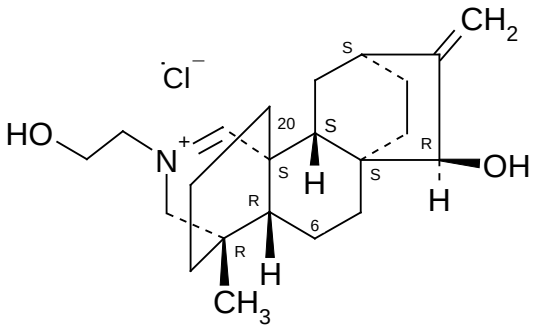
3.4.5 Atisinium chloride

a. Physico-chemical properties

This alkaloid was the major alkaloid of the plant and had been reported before from many *Delphinium* and *Aconitum* species[47,124,161]. The needle-like crystals were obtained from methanol/diethyl ether. The alkaloid had an optical rotation of $[\alpha]_D^{25}$ = + 31.27 (*c*, 0.94 in MeOH) and a melting point of 305.7-308.6^{o}C (decomposition) which was slightly different from the corresponding values noted in the literature {optical rotation varies from $[\alpha]_D^{23}$ = + 22.6 (*c*, 4.9 in 95% EtOH)[162] to $[\alpha]_D^{25}$ = + 28 (*c*,1.10 in H_2O)[47] and the melting point from 311-312^{o}C[47] to 325-326^{o}C[162]}. The alkaloid was the most polar compound of the other alkaloids isolated from the same plant.

b. Spectral analysis

The LRCIMS indicated the MH^+ peak at *m/z* 344. From the HRESMS, the molecular formula was found to be $C_{22}H_{33}NO_2$ (M^+) for this alkaloid. The LREIMS spectrum (**Appendix 37**) had ion peaks at *m/z* 343 (M^+), 342, 328, 314, 300, 284, 257, 241, 186, 171, 159, 143, 131, 105, 91, 79, 72, 55 and 44.



28 Atisinium chloride

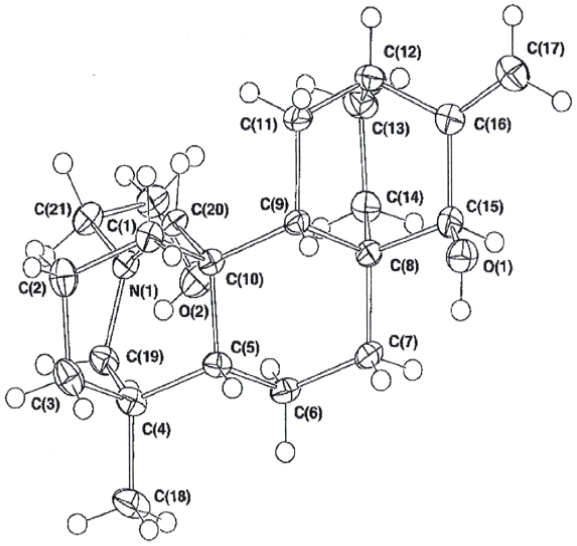


Figure 10. The X-ray structure of Atisinium chloride.

The crystal structure (**Figure 10**) was determined by single crystal X-ray crystallography (by Professor A.H.White, University of Western Australia; **Appendix 38**) and was identified as atisinium chloride **28.** An earlier crystal structure of atisinium chloride (with 4S, 5S, 8R, 12S, and 15S absolute configuration) had been reported in 1976[162].

The torsion angles (deg) (**Appendix 38**), crystal data, data collection parameters, and refinement results (**Table 21**) were compared with the old data reported in the literature[162]. All this data including dihedral angles, fractional atomic position parameters, individual atom parameters, input atom coordinates, bond angles and contact distances were found in agreement to the previous crystal data on atisinium chloride.

Table 21. Crystal data and refinement results of a atisinium chloride.

Description	Atisinium chloride (1976)	Atisinium chloride (2004)
Cell dimensions		
a	14.340	7.670
b	18.180	14.246
c	7.709	17.919
V	2009.7	1958.1
Space group	P212121	P212121
Z	4	4
Mol wt	379.972	379.97
Density, g cm^{-3}		
Observed	1.256	1.289
Calculated	1.256	1.289
$2\theta_{max}$	150.20^{o}	65^{o}
Reflections		
Measured	2377	3372
Observed	2007	3408
Non-observed	370	485
Final agreement		
R	0.038	0.042
R_w	0.037	0.045
Average shift/estimated	0.33	0.64
Max in final difference electron density map, e$Å^{-3}$	0.20	0.36

3.4.6 AO-358

This alkaloid was isolated as a milky white solid. It had a high melting point (122.6-124.2°C) and a large optical rotation ($[\alpha]_D^{27}$ = +86.6). The LRCIMS indicated a peak at *m/z* 358. From the HRCIMS, the molecular formula was found out to be $C_{22}H_{32}NO_3$. In the HREIMS, ion fragments were observed at *m/z* 357 (M^+), 344, 342, 329, 328 (100%), 327, 326, 312, 300, 286, 284, 250, 234, 148, 122, 105, 91, 77, 71, and 60. When the mass spectral library (available on the Department of Chemistry mass spectrometer) was searched for a match, none of the spectra matched the HREIMS spectrum of this alkaloid isolated here.

The ^{1}H-NMR spectrum (nanoprobe) was not clear enough to assign the structure and the sample was insufficient to obtain the ^{13}C-NMR spectrum and 2D spectra. However, the presence of three methoxy groups, a *C*-methyl group and N-ethyl signals were clear in the ^{1}H-NMR spectrum. The three methoxyl protons resonated at 3.23 ppm (s, 3H), 3.38 ppm (s, 3H) and 3.43 ppm (s, 3H). The *C*-methyl protons (probably C-18)

resonated at 1.10 ppm (s, 3H) and the N-CH_2CH_3 protons at 0.90 ppm (t, 3H) with the methylene (-CH_2) protons resonating at 3.12 ppm (q, 2H). Since the alkaloid didn't have signals for an exomethylene group it probably belongs to the C_{19}-diterpenoid type of alkaloid whose structure could be related to virescenine **27**. Nevertheless, the structure was not established due to the lack of spectroscopic data.

Interestingly, another alkaloid fraction was also isolated with the same molecular weight but different molecular formula, and different polarity (on TLC). This compound had a lower Rf value compared to the above compound. Its molecular formula was determined by HRCIMS to be $C_{15}H_5NO_{10}$.

3.4.7 AO-340

This alkaloid was isolated as a milky white solid. The LRCIMS indicated the presence of a peak at *m/z* 340 (MH^+). The HRCIMS supported the molecular formula of $C_{21}H_{26}NO_3$. In the LREIMS, the molecular ion and ion fragments were observed at *m/z* 339 (M^+), 322, 310, 297, 283, 245, 179, 159, 128, 124 (100%), 108, 94, 79, 65, 55, and 42.

3.4.8 AO-330

This alkaloid was isolated as a milky white solid. LRCIMS indicated the MH^+ peak at *m/z* 330. The HRCIMS supported the molecular formula of $C_{20}H_{28}NO_3$. Due to small quantities of sample, it was not possible to obtain adequate NMR data. The mass spectral library was searched for compound matches and none of the spectra in the mass spectrometer library was found to match the mass spectrum of AO-330. Judging by the molecular formula and ion fragmentation pattern, it appears to be a new alkaloid.

3.4.9 AO-372

This alkaloid was isolated as a pale brown solid. The LRCIMS indicated the presence of an MH^+ peak at *m/z* 372. The HRCIMS indicated a molecular formula of $C_{21}H_{26}NO_5$. In the LREIMS, ions were observed at *m/z* 371 (M^+, 100%), 355, 343, 327, 299, 296, 238, 210, 192, 162, 134, 122 and 105.

3.4.10 AO-414

This alkaloid was obtained as a pale green solid. The LRCIMS gave a peak at *m/z* 414 (MH^+) and the HREIMS supported a molecular formula of $C_{23}H_{27}NO_6$. In the LREIMS,

ion fragments were observed at *m/z* 413 (M^+), 385, 369, 354, 342, 310, 280, 252, 220, 212, 148, 134, 122, 105, 91, 79, 67, 57, 55, 44 (100%), 43 and 42.

3.4.11 O-466

This alkaloid was isolated as a pale green solid. The LRCIMS indicated a peak at *m/z* 466 for MH^+. The HRCIMS supported a molecular formula of $C_{25}H_{40}NO_7$. In the LREIMS, ions were observed at *m/z* 465 (M^+), 450, 448, 432, 422, 403, 402 (100%), 390, 374, 358, 342, 329, 300, 284, 194, 164, 148, 122, 98 and 91.

No structures could be assigned for these minor alkaloids (AO-340, 330, 372, 414 or 466) due to the very small quantities available.

Chapter 4

Alkaloids from *Corydalis gerdae* Fedde

Family:	Fumariaceae
Genus:	*Corydalis*
Species:	*gerdae*
gSo-ba rig-pa name:	sTong-ril-zilpa,
Distribution:	Endemic to Himalayas

4.1 Botanical Description and Ethno-medical use

Corydalis gerdae Fedde is an annual flowering herb that grows to about 7-20 cm in height with a long scaly rhizome and stems surrounded by persistent petiole bases of the bipinnatisect (2-4 x 1.5-2 cm) basal leaves that have broadly ovated leaflets[77,111]. The umbellate inflorescence has 3-6 horizontal flowers supported by 10-15 mm pedicels exserting above the bracts (6-12 mm) and the petals are purple or mauve and white with an outer pair broadly crested and lower deflexed lip petal[111].

This species of *Corydalis* is used in Bhutanese traditional medicine in treating malaria[105], coughs and colds, and bile inflammation[77]. Other *Corydalis* species have also been used by many cultures in folklore medicines worldwide[163]. The genus *Corydalis* contains more than 300 species, with a vast variety of isoquinoline alkaloids being isolated from these plants[4,47,163].

4.2 Isoquinoline Alkaloids: Biosynthesis and Classifications

The isoquinoline type alkaloids are the most widely distributed alkaloids and are found in plants of thirty two botanical families[44,164]. Morphine was the first isoquinoline alkaloid to be isolated from *Papaver somniferum* (Papaveraceae) in 1805[165]. To date about 1,200 isoquinoline alkaloids have been isolated from plants belonging to these different types of families mentioned above[44].

The classification of isoquinoline alkaloids is not clearly defined. However, based on their structural types and biogenesis, they are commonly divided into twenty categories, each of which is further branched into different sub-classes[47]. Each group presents striking and enormously varying structural types basically derived from tyrosine, and these types are briefly described below.

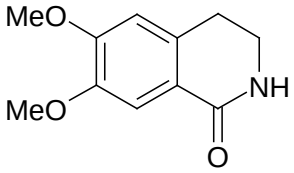
1. Simple isoquinoline derivatives, e.g Corydaldine **29**.
2. Emetine group (derivatives with a monoterpenoid unit) e.g. Emetine **30**.
3. Benzylisoquinolines (derived from two molecules of tyrosine), e.g. Cularine **31**.

4. Proaporphines, e.g. Orientalinone **32**.
5. Aporphines (formed by phenol oxidative coupling of a benzylisoquinoline precursor), e.g. Liriodenine **33**.
6. Protoberberines (derived from benzylisoquinolines by condensation with a one-carbon unit), e.g. Ophiocarpine **34.**
7. Benzophenanthridines (derived from tetrahydro-protoberberine precursors), eg. Chelidonine **35**.
8. Dibenzopyrrocoline alkaloids (derived by oxidation of a benzylisoquinoline precursor), e.g Cryptaustoline **36.**
9. Protopines (formed by oxidative ring fission of protoberberine N-metho salts), e.g Protopine **37**.
10. Rhoeadine alkaloids (derived from two tyrosine units via tetrahydroberberine and protopine intermediates), e.g Rhoeadine **38**.
11. Other categories includes Narceine and Phthalideisoquinoline alkaloids, the Pavine/isopavine group, Spirobenzylisoquinolines[166], the Protostephanine and Hasubanonine groups, Bis-benzylisoquinoline alkaloids, the Morphine group, and the Phenethylisoquinoline group.

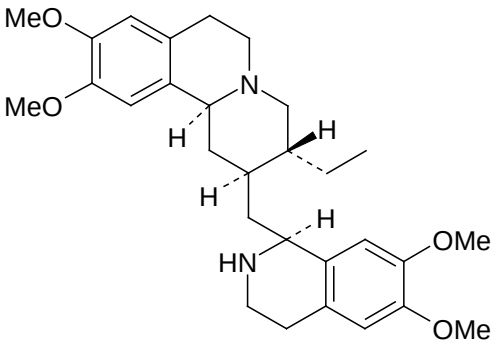
Although these alkaloids are quite often typical and restricted to certain genera of the family as in the case of rhoeadine alkaloids, which are encountered only in the *Papaver* genus, more than one class of alkaloids have been observed commonly even within a genus. The protoberberine alkaloids are distributed among the plant families Alangiaceae, Berberidaceae, Fumariaceae, Hydrastidaceae, Lauraceae, Leguminosae, Menispermaceae, Papaveraceae, Ranunculaceae, Rutaceae and Annonaceae[44]. However, these families also host large reservoirs of other classes of alkaloids. The investigation of the *Annona squamosa* L. of the Annonaceae family resulted in the isolation of ten alkaloids (corydine, annonaine, roemerine, norcorydine, norisocorydine, isocorydine, glaucine, dienone, aporphine and norlaureline) belonging to protoberberine as well as aporphine alkaloids[167].

The protopine group of alkaloids is distributed in a variety of genera in the Fumariaceae (*Corydalis, Dactylicapnos, Dcentra, Fumaria*), Papaveraceae (*Argemone, Bocconia, Chelidonium, Eschscholtzia, Glaucium, Hunnemannia, Hylomecon, Macleaya, Meconella, Meconopsis, Papaver, Roemeria, Romeya, Sanguinaria, Stylomecon*), Hypecoaceae, Berberidaceae (*Berberis*), Sapindaceae (*Pterodophyllum*), Nandinaceae, and the Pteridophyllaceae plant families. Protopine is the principal alkaloid of this group of families. Other groups like protoberberines and

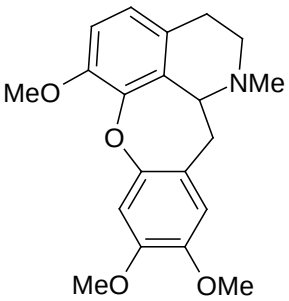
benzophenanthridines were also reported from the same family along with protopine as illustrated by the analysis of *Bocconia cordata* Wild.[168] and *Chelidonium majus* L.(Papaveraceae)[169].



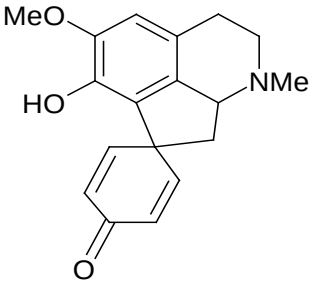
29 Corydaldine



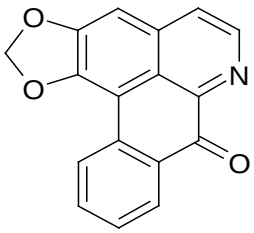
30 Emetine



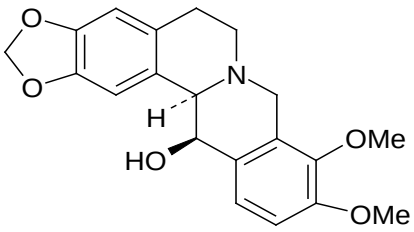
31 Cularine



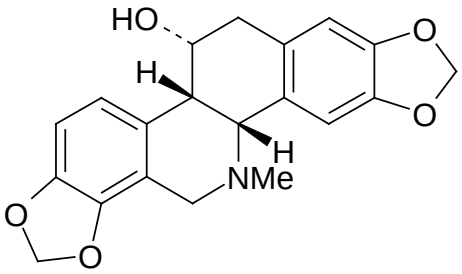
32 Orientalinone



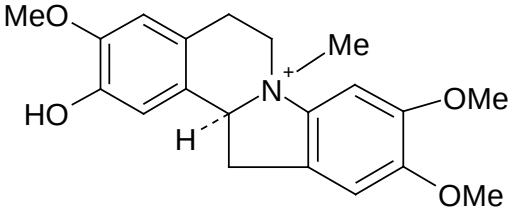
33 Liriodenine



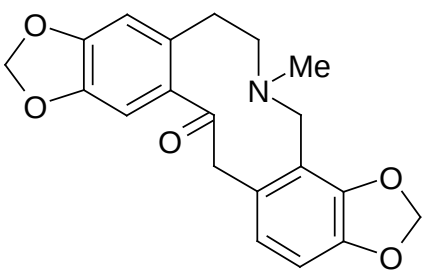
34 (-)-Ophiocarpine



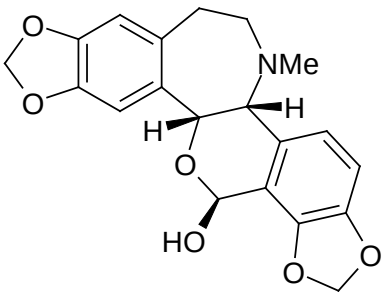
35 Chelidonine



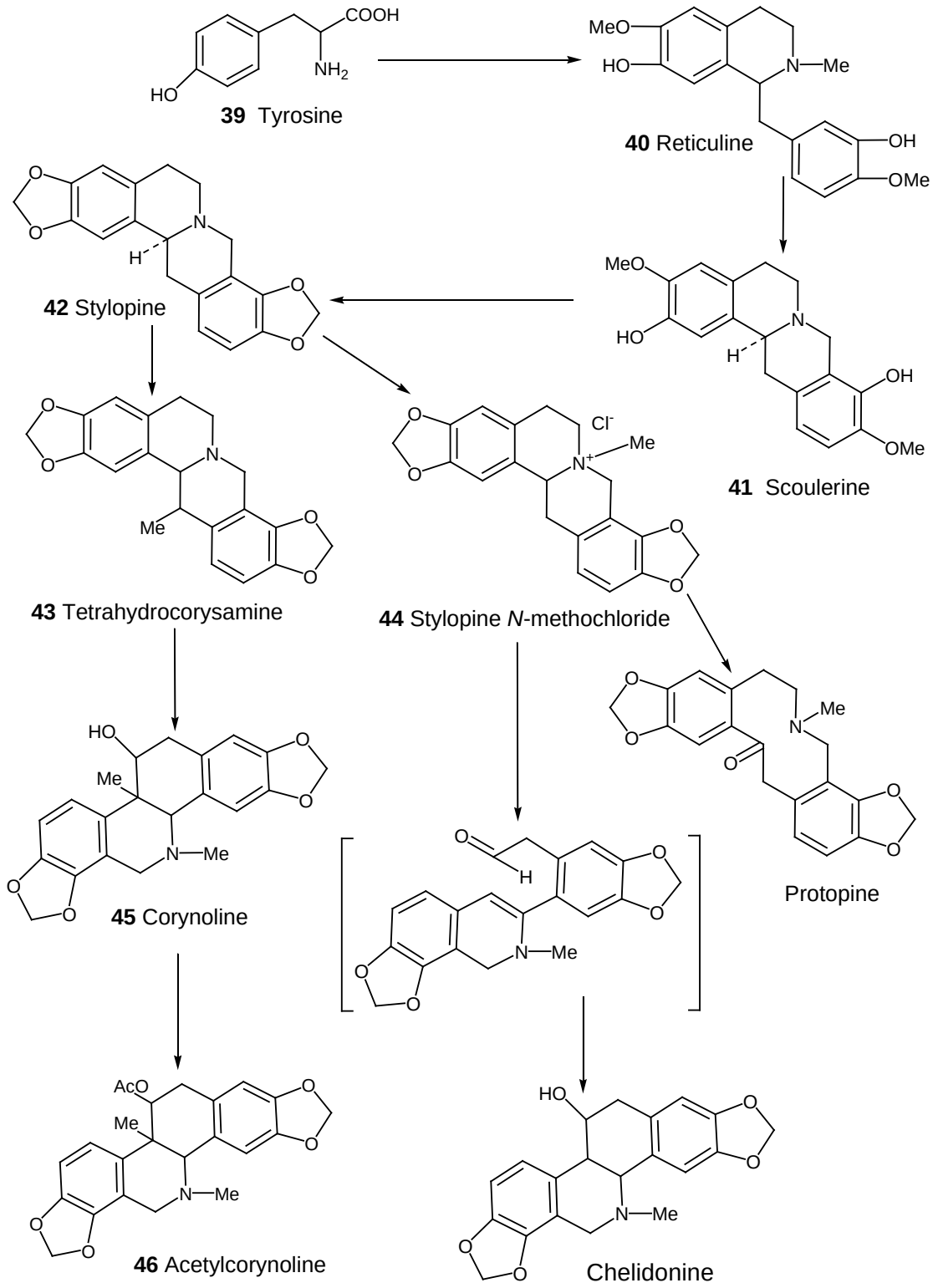
36 (-)-Cryptaustoline



37 Protopine



38 Rhoeadine



Scheme 2 The biosynthetic pathway of the protopine-type and the benzo[c]phenanthridine-type alkaloids.

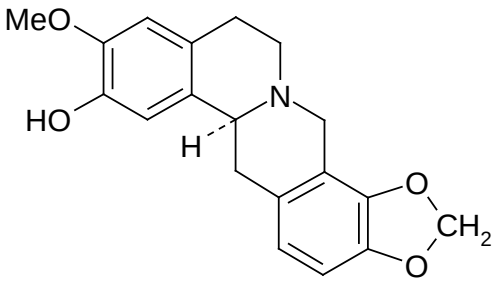
Recently, two new protopine-type alkaloids, argemexicaines-A and B along with thirteen known alkaloids including its related alkaloid, benzo[c]phenanthridine-6-acetonyldihydro-chelerythrine were isolated from the methanol extracts of Formosan *Argemone mexicana* L (Papaveraceae)[170]. Benzo[c]phenanthridine-6-acetonyl-dihydro-

chelerythrine exhibited a significant anti-HIV activity in lymphocytes with EC_{50} and TI (Therapeutic Index) values of 1.77 μg/ml and 14.6 μg/ml respectively[170]. About 20 alkaloids including protopine, cryptopine, stylopine, scoulerine and sinactine[171], and minor alkaloids like fumarofine, fumaricine, fumaritine, and fumariline[172], were isolated and identified from the plant *Fumaria officinalis* (Fumariaceae).

These alkaloids are related biosynthetically and many biosynthetic correlations have been studied for the isoquinolines. The biosynthetic pathways for protopine-type (e.g. protopine) and benzo[c]phenanthridine-type (e.g. chelidonine) alkaloids from *Corydalis incisa* PERS. and *Chelidonium majus* L. are presented. The plants are related to each other and their biosyntheses are relevant to the alkaloids currently isolated from *Corydalis gerdae* Fedde by the author.

It was suggested that the protopine-type and benzo[c]phenanthridine type alkaloids were related biosynthetically as both were derived from the tetrahydroprotoberberine alkaloids. Battersby *et al*.[173] studied the biosynthetic pathway for these alkaloids using *Corydalis incisa* PERS and *Chelidonium majus* L and established (by tracer experiments[174]) the biosynthetic pathway as illustrated in **Scheme 2**. This pathway showed that chelidonine and protopine were biosynthesised in plants from tyrosine **39** via (*S*)-reticuline **40**, (*S*)-scoulerine **41** and (*S*)-stylopine **42**[175].

The same experiment confirmed that (*S*)-cheilanthifoline **47** isolated from *Corydalis incisa* plants was biosynthetically converted to corynoline and protopine and was a probable true intermediate between scoulerine and stylopine[174]. It has also been shown that the protopine-type alkaloids occupy a central position biosynthetically between the tetrahydroprotoberberine-N-metho salts and the benzophenanthridines and between the N-quaternary tetrahydroberberines and the rhoedines[168].



47 (-)-(*S*)-cheilanthifoline

4.3 Phytochemical and Pharmacological Investigation of Isoquinoline Alkaloids from the genus *Corydalis*

Many bioactive alkaloids belonging to different classes of isoquinoline alkaloids (as described earlier in this chapter) have been isolated from *Corydalis* species (Fumariaceae). Sibiricine[176] and ochrobirine[177] were first isolated from *Corydalis siberica* by Manske *et al.* in 1936. The Dictionary of Alkaloids[47] has listed 58 species of *Corydalis* (including unknown species) and 119 different types of alkaloids were reported to have been isolated from these plants. Cheilanthifoline was isolated from *Corydalis cheilanthifolia, Corydalis scouleri, Corydalis siberica* and the *Corydalis gigantea*[178]. Scoulerine and many other alkaloids were reported from *Corydalis tuberosa, Corydalis cava,* and *Corydalis pallida*[179]. Examination of *Corydalis koidzumiana* resulted in the isolation of protopine, α-allocryptopine, (±)-tetrahydropalmatine, (+)-corydaline, (+)-stylopine, (–)-scoulerine, (–)-capaurine, (–)-isocorypalmine, (–)-cheilanthifoline, (+)-corybulbine, sanguinarine, oxysanguinarine, dihydrosanguinarine, (+)-reticuline, sinoacutine and pallidine[180].

Protopine was isolated from *Corydalis ternata* and *Corydalis caseana* A.Gray[181]. Stylopine was also reported from a *Corydalis spp*[47]. Varieties of alkaloids were also isolated from *Corydalis bungeana* Turez[182] and *Corydalis decumbens*[183] and the isolation work is still ongoing.

Amongst different classes of alkaloids isolated from the plants, protoberberine and protopine groups have shown a wide range of pharmacological activities. Berberine was found to be therapeutically useful in the treatment of malaria (*Plasmodium falciparum)*[184]. Seventeen quaternary protoberberine alkaloids related to berberine were tested for antimalarial activity and six of them displayed more potent activity than berberine itself[185].

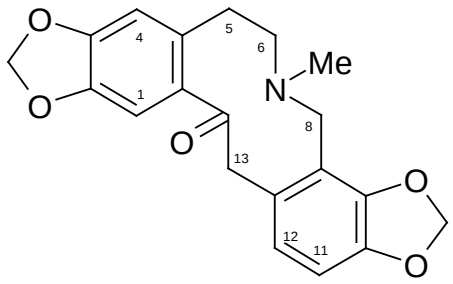
Protopine exhibited both anti-acetylcholinesterase and anti-amnesic properties[186]. Protopine was also found to have phospholipase and thromboxane synthetase inhibitory activity[187], weak spasmolytic, weak anti-tumour activity, smooth muscle stimulant, bactericidal activity and sedative property[47]. When 18 protopine derivatives were tested for antimalarial activity, the derivatives A65, A74, A77 and A20 in which the methylenedioxy moiety was modified by replacing a H atom with Br, NH_2, I and $SbCl_2$ respectively exhibited promising antimalarial activity[188]. Stylopine showed antipsychotic and neuroleptic activity in mice and rats. Dehydrocorydaline, (+)-corydaline and (±)-tetrahydropalmatine isolated from the Chinese *Corydalis tubers* were claimed to be good pain killers and anti-ulcer medicines[189]. With the hope of

finding a novel pharmacophore, we have studied one of the *Corydalis* plant endemic to Himalayas.

4.4 Isolation and Identification of Alkaloids from *Corydalis gerdae* Fedde

The methanol extract of the air dried plant material was subjected to routine procedures of extraction, fractionation, separation and purification (**Chapter 9**). Out of many fractions, only four known alkaloids were isolated and identified by matching their LREIMS spectra with the spectra in the Mass Spectrometer Database. NMR spectra and physico-chemical properties of the isolated compounds were also compared to further confirm their identification. From these analyses, it was confirmed that the alkaloids were protopine, scoulerine, stylopine and cheilanthifoline. Other amide and acid like compounds were also obtained in the process of alkaloid extraction.

4.4.1 Protopine



37 Protopine

a. Physico-chemical properties

This alkaloid, which belongs to the protopine-type isoquinoline alkaloids, was obtained as a white amorphous solid. It was the major alkaloid of the plant and had been isolated before from many plants as described in the earlier part of this chapter. The alkaloid was optically inactive and had a high melting point (203.7-207.8^{o}C, decomposition). An earlier literature has reported the melting point of protopine as 210-211^{o}C[190] and 207^{o} C[47].

b. Spectral Analysis

The LRCIMS indicated an MH^+ peak at *m/z* 354. The HRCIMS and the HREIMS supported the molecular formula as $C_{20}H_{19}NO_5$ and hence a hydrogen deficiency of 12. From the same HREIMS, the molecular ion and ion fragments were observed at *m/z* 353 (M^+), 338, 309, 295, 281, 267, 252, 237, 224, 210, 190, 163, 149, 148 (100%), 134 and 89.

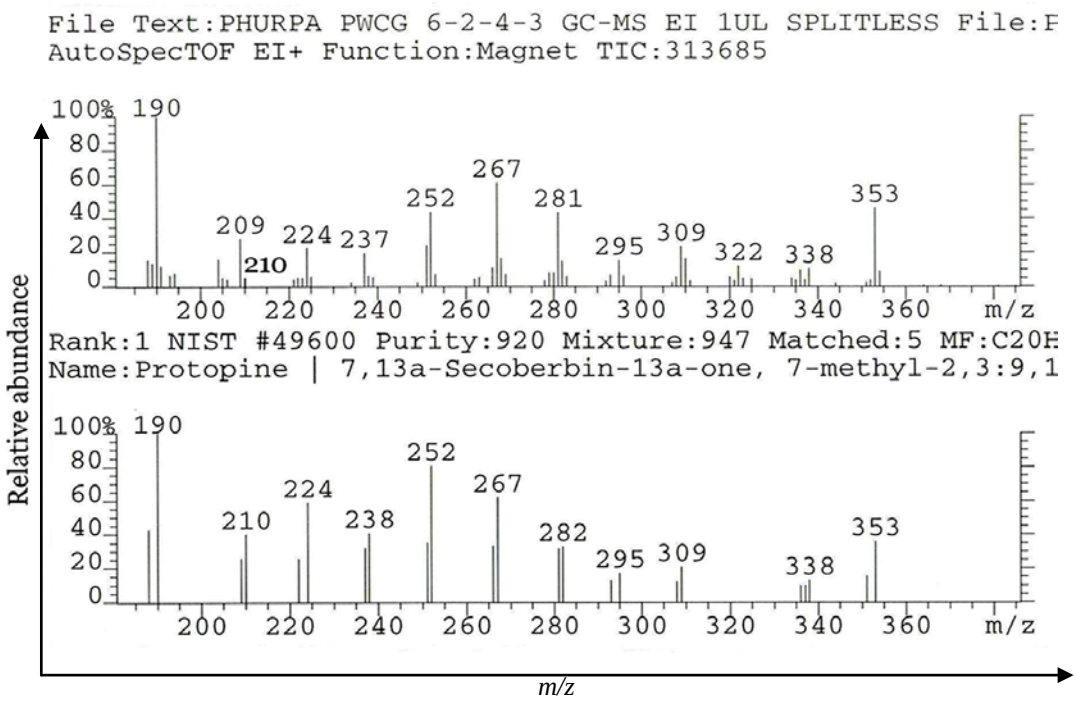


Figure 11. HREI MS library matching of an unknown alkaloid (top) with protopine (below).

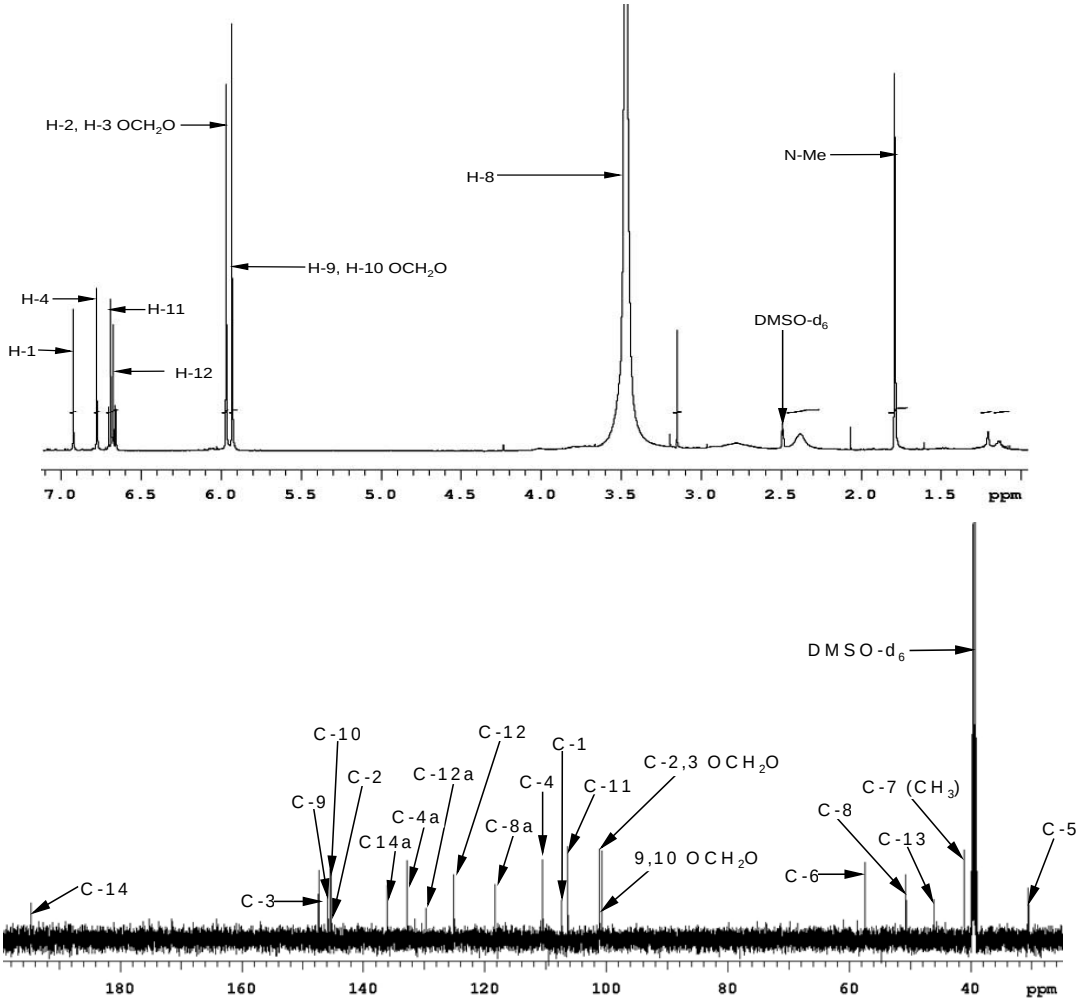


Figure 12. ^{1}H-NMR spectrum (top) and ^{13}C-NMR spectrum (bottom) of the major alkaloid (500 MHz, DMSO-d_6) from *Corydalis gerdae*.

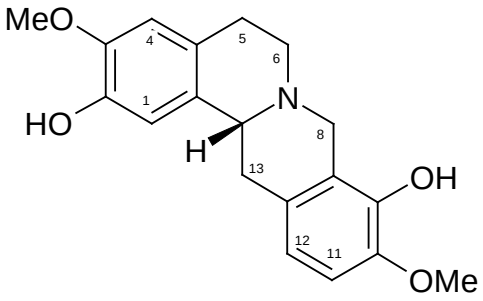
The molecular formula and the ion fragmentation pattern corresponded with that of protopine. From the mass spectrometer library (in the Shimadzu QP-5000 mass spectrometer) matching analogy, the HREIMS spectrum of the isolated alkaloid was found to match that of protopine (**Figure 11**). Thus, this alkaloid was identified as protopine.

The identification was further confirmed by comparing the ^{1}H-NMR and ^{13}C-NMR spectral values of the alkaloid with that of the ^{1}H-NMR and ^{13}C-NMR spectral values (**Figure 12**) reported in an earlier literature for protopine[190]. The ^{1}H-NMR spectrum (300 MHz, $CDCl_3$) of protopine indicated the 2,3-OCH_2O proton at 5.93 ppm (s), the 9,10-OCH_2O at 5.91 ppm (s) and the N-CH_3 protons at 1.93 ppm (s)[190]. These protons were clearly observed in the ^{1}H-NMR spectrum (500 MHz, DMSO-d_6) of the unknown major alkaloid under investigation in which the signal for the 2,3-OCH_2O resonated at 5.96 ppm (s), for the 9,10-OCH_2O at 5.93 ppm (s), and for the N-CH_3 at 1.79 ppm (s). The chemical shifts of the other proton signals as well as the ^{13}C-NMR shift values of the alkaloid corresponded to the chemical shifts of the proton and carbon signals of protopine (**Table 22**) further confirming that the alkaloid was protopine.

Table 22. Comparison of ^{1}H-NMR spectrum (500 MHz, DMSO-d_6) and ^{13}C-NMR spectrum (500 MHz, DMSO-d_6) of the major alkaloid with ^{1}H-NMR spectrum (300 MHz, $CDCl_3$) and ^{13}C-NMR spectrum (75.4 MHz, $CDCl_3$) of protopine[190].

	Protopine (Literature)			Alkaloid (Isolated)		
Carbon	**δC (ppm)**	**δH (ppm)**	***J*** (Hz)	**δC (ppm)**	**δH (ppm)**	***J*** (Hz)
1	108.0	6.89 (s)		107.4	6.92 (s)	
2	145.7			145.3		
3	147.8			147.3		
4	110.3	6.63 (s)		110.4	6.77 (s)	
4a	132.6			132.7		
5	31.7	2.89 (s)	W_H 18	30.5	3.15 (s)	
6	57.7	2.52 (t)	5	57.4	2.38 (bs)	
8	50.6	3.57 (d)	1.5	50.7	3.47 (bs)	
8a	117.7			118.3		
9	146.2			145.8		
10	145.8			145.4		
11	106.6	6.67 (d)	8.5	106.3	6.74 (d)	7.5
12	124.9	6.65 (dd)	8.5, 1.5	125.1	6.66 (d)	8.0
12a	128.8			129.6		
13	46.3	3.79 (s)		46.1		
14	194.8			194.8		
14a	136.0			136.0		
2,3-OCH_2O	101.1	5.93 (s)		101.1	5.96 (s)	
7-CH3	41.3	1.93 (s)		41.1	1.79 (s)	
9,10-OCH_2O	100.7	5.91 (s)		100.7	5.93 (s)	

4.4.2 Scoulerine



41 Scoulerine

a. Physico-chemical properties

This alkaloid was isolated as a dark brown solid compound. The alkaloid was optically active ($[\alpha]_D^{24}$ = –282, *c* = 0.74 in MeOH) and had a high melting point (153-157^o C, decomposition). This compound was isolated previously from many other plants as described earlier in this chapter and had a reported melting point of 155^{o}C and an optical rotation of $[\alpha]_D^{28}$ = –289 (*c*, 0.62 in MeOH)[47]. Thus the optical rotation and melting point of the alkaloid closely resembled that of scoulerine.

b. Spectral analysis of Scoulerine

The LRCIMS gave an MH^+ peak at *m/z* 328 and the HREIMS indicated the molecular formula as $C_{19}H_{21}NO_4$ which suggested an index of hydrogen deficiency of 10. In the HREIMS, the molecular ion and ion fragments were observed at *m/z* 327 (100%, M^+), 326, 312, 310, 207, 179, 178 (100%), 176, 162, 150, 149 and 135. The MS-library was searched for a match and it was found that the alkaloid matched the MS spectrum of scoulerine (**Figure 13**). Major fragments appeared at *m/z* 135, 150, 176, 178 and 326, corresponding to the favoured cleavage of tetrahydroberberines[191,192]. Thus, from this spectral matching and the close proximities of its physico-chemical properties to that of scoulerine, the alkaloid was identified as scoulerine **41.** It is also biosynthetically plausible**.** Slight variations in melting point, optical rotation and peak fragments were observed because of experimental variation and some very minor impurities.

The literature on the ^{1}H-NMR of scoulerine[191] reported the presence of two O-methyl groups (s, τ 6.18), four aromatic protons (τ 3.1-3.5) and the absence of the *N*-methyl group in the spectrum. Another publication on scoulerine[193] reported the presence of signals ascribed to the 2-OH at 5.26 ppm (2H, bs), the 2-OMe group at 3.85 ppm (6H, s), and an AB-quartet at 3.48 ppm and 4.21 ppm with a coupling constant of 16 Hz.

When the [1]H-NMR spectrum of the isolated alkaloid was compared with the above data reported in the literature, they corresponded well.

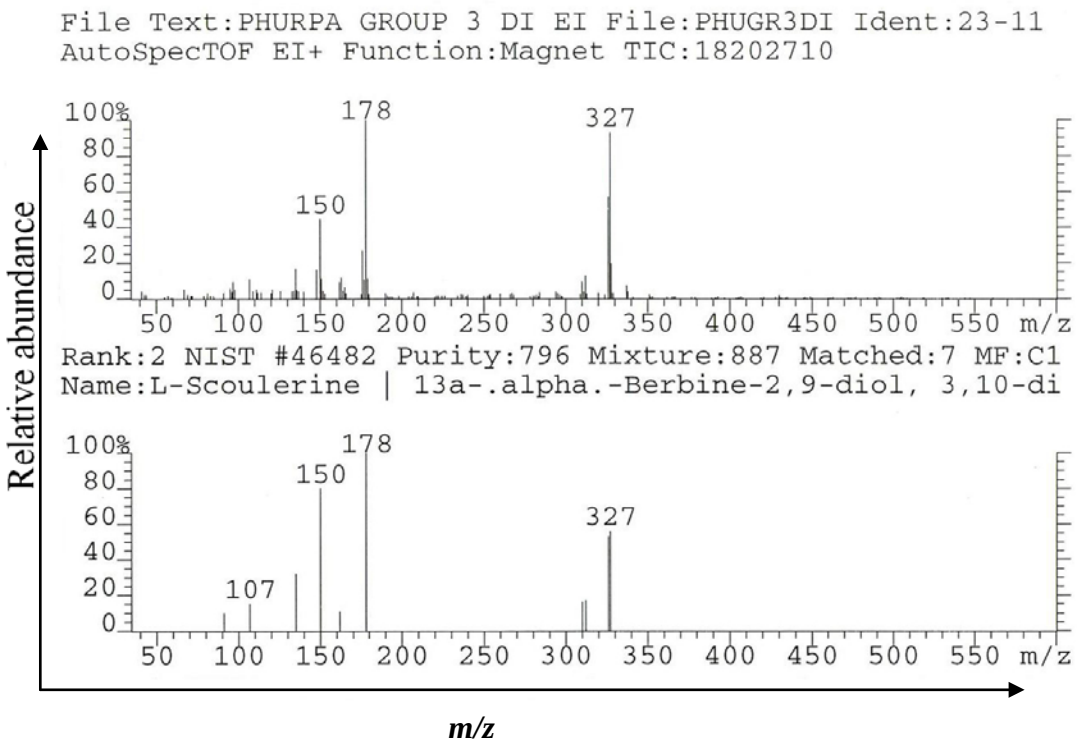


Figure 13. HREIMS spectrum of the alkaloid (top) and that of scoulerine (bottom).

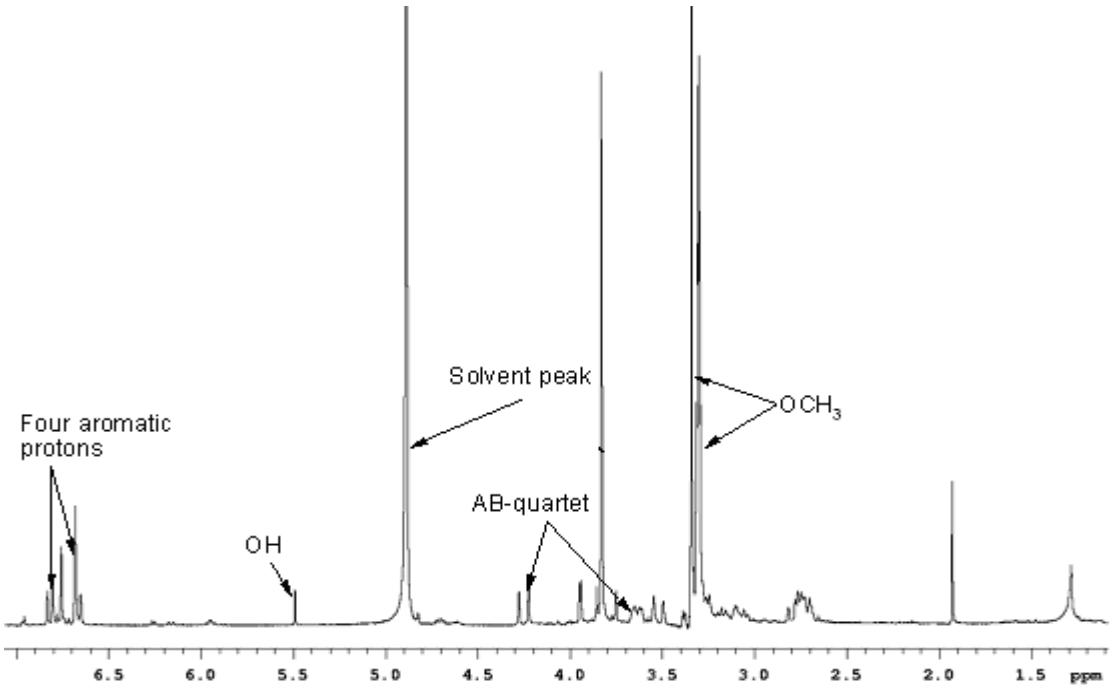
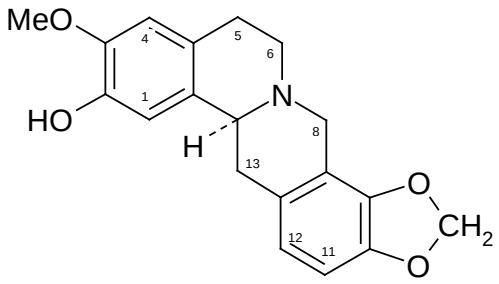


Figure 14. [1]H-NMR spectrum (300 MHz, CD_3OD, TMS) of the alkaloid (identified as scoulerine)

In the ^{1}H-NMR spectrum (**Figure 14**) of the isolated alkaloid, the signal assigned to the 2-OMe group resonated at 3.24 ppm (s) and 3.21 ppm (s), the 2-OH group resonated at 5.38 ppm (s), and the ABX system resonated at 3.52 ppm (q) and 4.14 ppm (d) with a coupling constant of 15.9 Hz. Signals for the four aromatic protons were observed between 6.54-6.72 ppm. The slight differences observed could be due to the different NMR solvents used in the experiments. Therefore, this comparative study further confirmed that the alkaloid was scoulerine.

4.4.3 Cheilanthifoline



47 Cheilanthifoline

a. Physico-chemical properties

This UV active alkaloid was obtained as a pale green solid and was recrystallised from MeOH/diethyl ether. This alkaloid was also a known alkaloid. Its optical rotation ($[\alpha]_D^{20}$= –262 , *c* = 0.60 in $CDCl_3$) and the melting point (168-170.4^{o}C, decomposition) corresponded broadly to that of cheilanthifoline whose melting point was reported as 184^{o}C and its optical rotation as $[\alpha]_D^{20}$ = –311 (MeOH)[47]. In another report, its melting point was reported to be 170^{o} C (decomposition) and the optical rotation of $[\alpha]_D^{20}$ = –315[193]. The difference in optical rotation observed may be due to the different solvent used.

b. Spectral analysis

In the LRCIMS, an MH^+ peak was observed at *m/z* 326 for the alkaloid. The HREIMS indicated its molecular formula as $C_{19}H_{19}NO_4$ and hence an index of hydrogen deficiency of 11. In the LREIMS spectrum of the alkaloid, the main ion fragments were observed at *m/z* 325 (M^+), 177 and 148 (100%) suggesting that the alkaloid was a protoberberine base[194]. When this LREIMS spectrum of the alkaloid was searched in the mass spectrometer library, it was found to correspond with that of

cheilanthifoline[193] (**Figure 15**). Based on this comparative analogy, the alkaloid was identified as cheilanthifoline.

To further confirm the identification, the ^{1}H-NMR spectrum (**Figure 16**) of the alkaloid was obtained and its chemical shifts were compared with the ^{1}H-NMR data of cheilanthifoline reported in the literature[193]. In the ^{1}H-NMR spectrum of the alkaloid isolated here the methoxy group signal resonated at 3.87 ppm (s, 3H, OMe), the methylenedioxy resonated at 5.93 ppm (d, 2H,-CH_2-) and the C-8 methylene protons resonated at 3.53 (q) and 4.09 ppm (d, J=15 Hz).

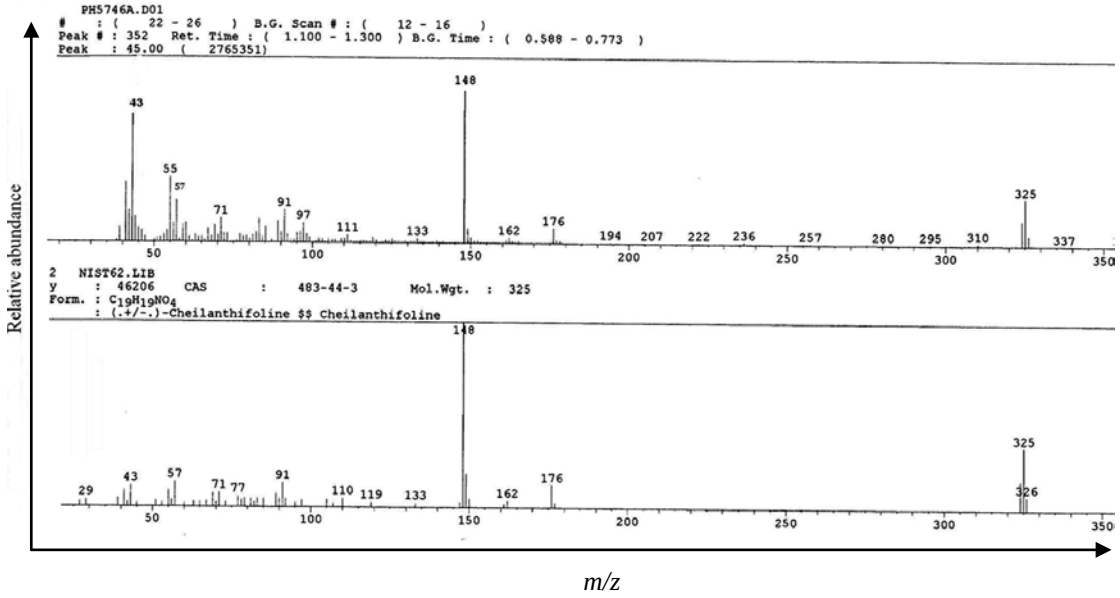


Figure 15. LREIMS of the unknown alkaloid (top) and that of cheilanthifoline (bottom)

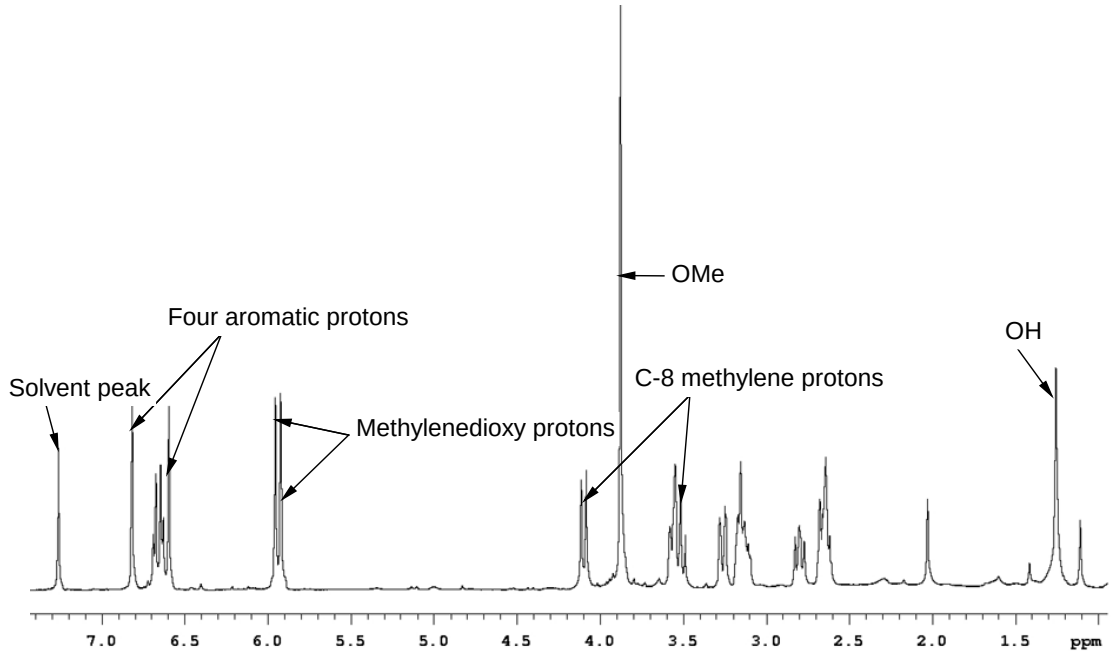
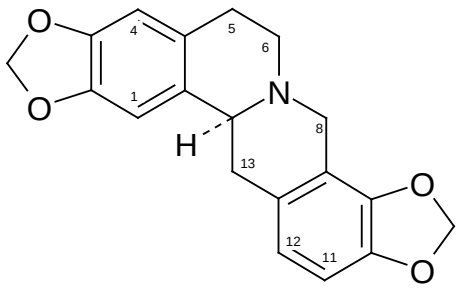


Figure 16. ^{1}H-NMR (500 MHz, $CDCl_3$, TMS) of the alkaloid (cheilanthifoline).

These chemical shifts were in agreement with the ^{1}H-NMR values of cheilanthifoline reported in the literature[193] in which the methoxy group (s, 3H, OMe) signal resonated at 3.85 ppm, the methylenedioxy resonated at 5.90 ppm (d, 2H, -CH_2-) and the C-8 methylene resonated at 3.48 ppm (q) and 4.21 ppm (1H, d, J = 16 Hz). The ^{1}H-NMR spectrum of the alkaloid also indicated the presence of the hydroxy group at 1.25 ppm and the four aromatic protons (m) between 6.59 ppm to 6.81 ppm. The DEPT experiment of the isolated alkaloid, though not clear, distinctly showed the presence of four methylene groups in the structure. This comparative analysis of the data on ^{1}H-NMR further confirmed that the alkaloid was cheilanthifoline **47**.

4.4.4 Stylopine



42 Stylopine

a. Physico-chemical properties

This known alkaloid was isolated as a brown solid. It had a melting point of 163-165^{o} C (decomposition) and was optically active with $[\alpha]_D^{24}$ = –304 (*c*, 0.56 in MeOH). The literature[47] has reported the melting point of stylopine as 204^{0}C and an optical rotation of $[\alpha]_D^{23}$ –310. In other literature on stylopine, its melting point was reported as 220-221°C[175] and 194-195°C[195] and no consistency was observed in the melting points of this alkaloid. The differences in these physicochemical properties could be due to instrumentation error. However, spectral analysis confirmed that the alkaloid was stylopine **42**.

b. Spectral analysis

The LRCIMS gave a MH^+ peak at *m/z* 324. The HREIMS indicated a molecular formula of $C_{19}H_{17}NO_4$ for the alkaloid and hence an index of hydrogen deficiency of 12. In the LREIMS of the alkaloid, the major ion fragment peaks were observed at *m/z* 323 (M^+), 322, 174 and 148 (100%) and this corresponded to the ion fragmentation pattern of stylopine[195]. When the mass spectrometer library was searched for its match, it was confirmed that the alkaloid was stylopine (**Figure 17**).

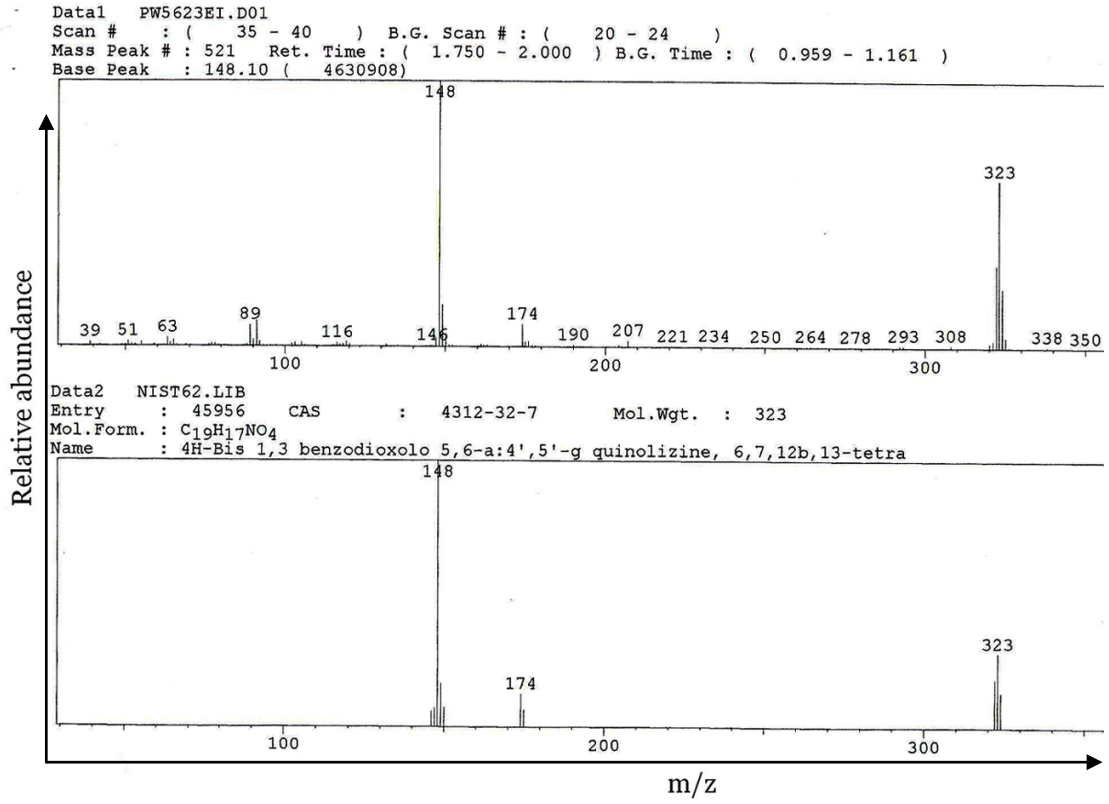
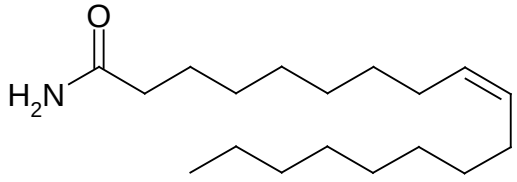


Figure 17. LREIMS of the alkaloid (top) and that of stylopine (bottom).

4.4.5 CG-337

This UV absorbing compound was a minor alkaloid. The HREIMS indicated a molecular formula as $C_{19}H_{15}NO_5$ and hence an index of hydrogen deficiency of 13. Since the amount of the alkaloid was very small, not enough data was obtained for identifying the alkaloid.

4.4.6 9-Octadecenamide



48 9-octadecenamide

This non-alkaloid compound was isolated as white solid. The LRCIMS gave an MH^+ peak at *m/z* 338 and HREIMS confirmed its molecular formula as $C_{22}H_{43}NO$. In the HREIMS, ion fragments were observed at *m/z* 337 (M^+), 319, 277, 136, 122, 97, 83, 72, 69, 59 (100%), 43, and 41.

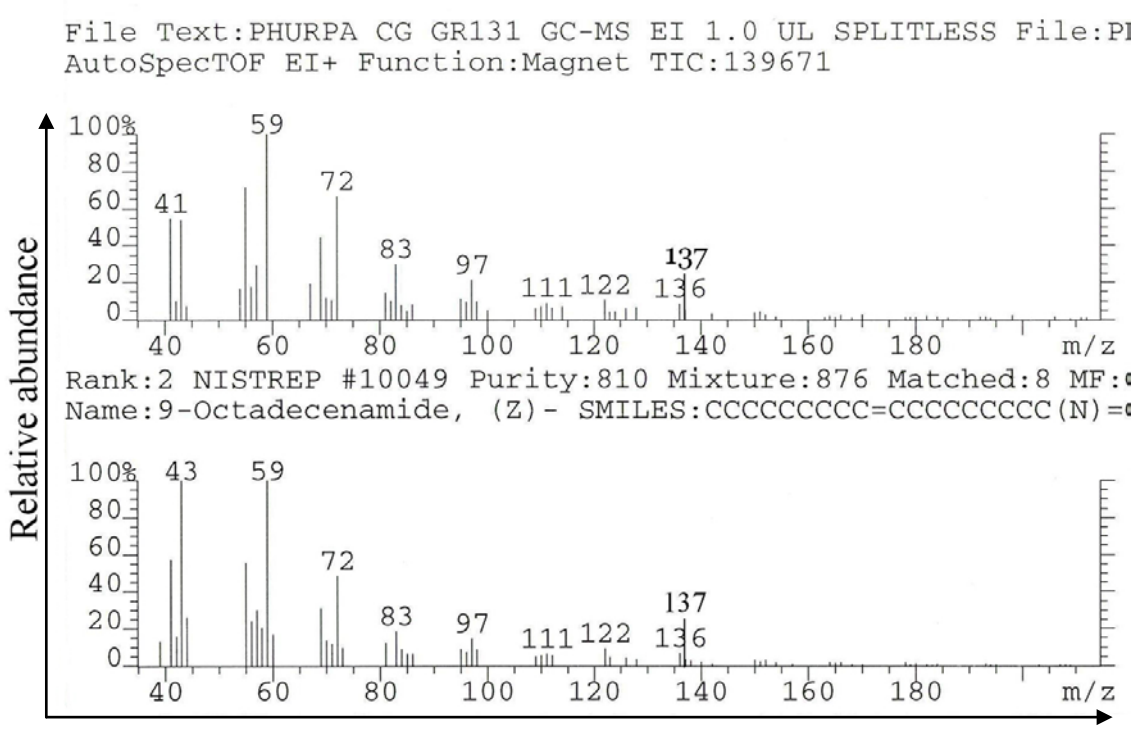


Figure 18. HREIMS of compound (top)and that of 9-octadecenamide (from library, bottom).

The compound was identified as 9-octadecenamide based by the MS library matching (**Figure 18**). This fatty acid amide occurs naturally in plants and has been isolated before from *Vetiveria zizanioides*[196] and *Clausena lansium*[197] (collected in Hainan Island, China). 9-octadecenamide was also isolated from the freshwater green algae *Rhizoclonium hieroglyphicum* growing in the Ural Mountains[198] as its major component (2.26%).

Another two non-alkaloid compounds with molecular mass 337 and 335 were also isolated as white solids (**Chapter 9**).

Chapter 5

Alkaloids from *Tribulus terrestris* Linn.

Family:	Zygophyllaceae
Genus:	*Tribulus*
Species:	*terrestris*
Common name:	Puncture vine or Caltrop fruit
gSo-ba Rig-pa name:	dZe-ma-ra-go-ma
Distribution:	Cosmopolitan

5.1 Botanical Description and Ethno-medical use

Tribulus terrestris is a prostrate annual herb. It is known as goathead in the USA, "Ying-jili" in China and dZe-ma ra-go-ma in Bhutan. The genus *Tribulus* comprises of approximately 20 species which grow as shrubs about 10-60 cm high[199,200]. It is considered a noxious weed and is found abundantly on roadsides and vacant lots with many reddish branches spreading radially from a central point. It has small oppositely arranged pinnate leaves with three to seven branching green oblong leaflets (3-7 cm long)[201]. It gives yellow flowers[202]. The fruits are light brown when mature and measure about a centimeter in diameter. It has sharp spines (2 spines measuring 2-3 cm) accompanied by several spikes around it[201]. The fruits and seeds are of great importance to traditional or natural medicines practiced worldwide (**Table 23**).

Table 23. Traditional medicines using *Tribulus terrestris* Linn

Traditional Medicines	Ethno-medical use
Bhutanese	Kidney and its related disorders, impotence and as diuretics.
Bulgarian	Libido enhancer and treating infertility[203].
Chinese	Calm liver, dizziness, headache, premature ejaculation, cardio-vascular diseases[204], acute conjunctivitis and vertigo[205].
Greeks	As a diuretic, mood enhancer, headache, constipation, sexual dysfunction, hypertension and hypercholesterolemia[206,207].
Indians	As a diuretic, antiseptic and anti-inflammatory[203].
Pakistanis	As a tonic and aphrodisiac.
Turkish	As a diuretic, for colic pain and hypercholesterolemia[207].

However, the plant had been found to have adverse toxic effects on livestock, especially on sheep. The ingestion of its leaf and foliage in large quantities caused the

hepatogenous photosensitization, known as geeldikkop, tribulosis ovis or yellow bighead in sheep and goats[208,209]. The outbreak of this disease has been reported in South Africa[208], Australia[108,109,210], United States[211], Argentina and Iran ever since 1932[209].

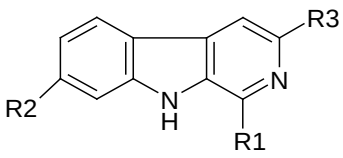
5.2 Chemical and Pharmacological Studies of *Tribulus* species

Owing to ethno-medical claims and also after the tragedy of photosensitization outbreak in sheep, the plant has been extensively investigated by a number of workers in those countries. The presence of steroidal glycosides, steroidal saponins, flavonoids and alkaloids in the plant have been reported[107]. Saponins such as glucopyranosyl, galactopyrans, ruscogenin, hecogenin, gitogenin, titogenin, protodioscin, diosgenin and yamogenin[212,213]; sterols such as sitosterol, and campesterol; flavonoids such as kaemferol, kaempferol-3-glucoside, tribuloside and quercetin[214,215]; and other constituents like fatty acids, polysaccharides, tannins, amino acids and potassium salts have been isolated from this plant.

Alkaloids like norharman **49**, harman **50**, harmol **51**, harmine **52**, 3-hydroxymethyl-norharman **53**, harmalol **54**, harmaline **55**, tetrahydronorharman **56**, tetrahydroharman **57**, 2-ethyltetrahydro-norharman **58**, tribulusterine **59,** and amides such as terrestriamide, terrestribisamide, N-p-coumaroyltryamine and cinnamic amide[110,205,216] were also isolated from the plant. At least five such alkaloids were isolated from the Australian *Tribulus terrestris* and harman and norharman were found out to be the main alkaloids of this plant[107,217]. Recently, the structure of tribulusterine **59** has been revised to that of perlolyrine with a 5-hydroxymethyl substituent in the furan ring[218].

The saponins and the β-carboline alkaloids in *Tribulus terrestris* have been reported to have shown a wide range of pharmacological activity[219]. It was suggested that steroidal saponins, diosgenin and yamogenin caused the geeldikkop diseases[220]. When the synthetic harman and norharman were administered subcutaneously to sheep at a dose rate of 54mg/kg, it was found that both of them caused similar nervous system effects (mainly limb paresis)[110]. This result suggested that the β-carboline alkaloids were responsible for causing staggers in sheep. *Tribulus terrestris* also treats impotence, increases sperm count (50%) and serves as a natural testosterone enhancer that invigorates and boost strength, stamina, muscles growth, fat loss and libido. Protodioscin was reported to be the main active steroidal saponin exhibiting aphrodisiac properties[221,222].

A cream made from an extract of this plant showed very strong antibacterial, anti-inflammation, anti-virus, anti-herpes effects and in some cases was found to block the growth of cancer cells[223]. It was suggested that the activity of the *Tribulus terrestris* extracts could be due to unknown steroidal saponins[224]. Tribulosin and β-sitosterol-D-glucoside from *Tribulus terrestris* showed anthelmintic activity with ED_{50} values of 76.25 and 82.50 μg/ml respectively[225]. Tribusponin was found to be a useful as an antisclerotic agent[226]. Some β-carboline alkaloids, flavonoids, kaemferol and quercetin are known to have antimicrobial effects[227,228].



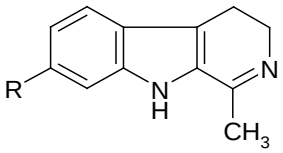
49 Norharman: R1 = H, R2 =H, R3 =H

50 Harman: R1 = CH_3, R2 = H, R3 = H

51 Harmol: R1= CH_3, R2 =OH, R3= H

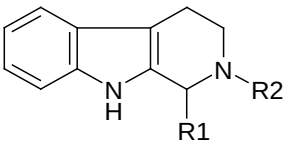
52 Harmine: R1 = CH_3, R2 = OCH_3, R3 = H

53 3-Hydroxymethylnorharman: R1 = H, R2 =H, R3 = CH_2OH



54 Harmalol: R = OH

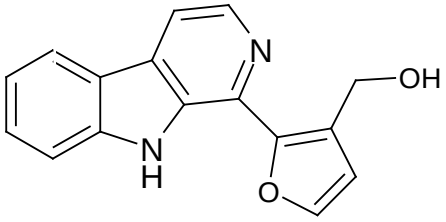
55 Harmaline: R = OCH_3



56 Tetrahydronorharman: R2 = H, R2 = H

57 Tetrahydroharman: R1 = CH_3, R2 = H

58 2-Ethyltetrahydronorharman: R1 = H, R2 = CH_2CH_3



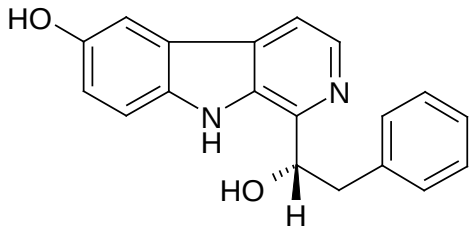
59 Tribulusterine

The search for new such bioactive saponins and β-carboline alkaloids is still proceeding. This study is directed towards the Australian sourced *Tribulus terrestris* species and aimed to isolate new minor alkaloids (if any) present in the plant.

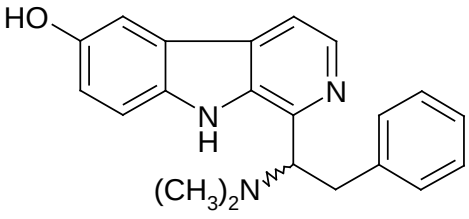
5.3 β-Carboline Alkaloids and Classification

β-Carboline alkaloids are defined as heterocyclic amines with a 9*H*-pyrido[3,4-*b*] indole structure and they are found in various plants including edible plants[229]. These alkaloids are biosynthetically derived from the amino acid L-tryptophan. They were first isolated from the plant *Peganum harmala* L and based on this, the β-carbolines are also known as harmala alkaloids[107]. Since then, they have been reported in a variety of plant families including the Leguminoseae, Malpighiaceae, Rubiaciae, Rutaceae, Graminae, Simaroubaceae and Zygophyllaceae.

The β-carboline derivatives include C-1 unsubstituted β-carbolines (e.g 1, 2, 3, 4-tetrahydro-6-methoxy-2-methyl-β-carboline), the C-1 methyl substituted harman group, the C-1 vinyl, acyl or carboxyl substituted group, and the C-3 substituted group[47]. Many groups of β-carboline alkaloids have also been isolated from marine sources. Only recently two new β-carbolines, eudistomin-W **60** and eudistomin X **61** which exhibited antibiotic activity toward *Bacillus subtilis*, *Staphyloccocus aureus* and *Escherichia coli* and fungicidal activity against *Candida albicans*, were discovered in a Micronesian ascidian *Eudistoma* species[230].



60 Eudistomin W



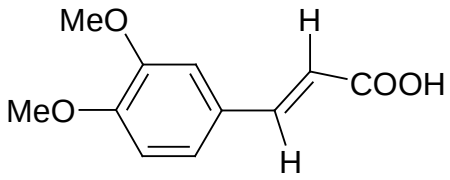
61 Eudistomin X

It was suggested that the pathway for the biosynthesis of these β-carboline alkaloids involves tryptophan, tryptamine and then 1-methyl-1,2,3,4-β-carboline-1-carboxylic acid[107]. Stolle and Gröger proposed that condensation of tryptamine with pyruvic acid formed the β-carboline ring of harman with 1,2,3,4-tetrhydro-β-carboline-1-carboxylic acid as an intermediate product [231].

5.4 Attempted Isolation of β-Carboline Alkaloids from *Tribulus terrestris* Linn.

Aerial parts of *Tribulus terrestris* Linn were collected in New South Wales by Dr. C. Bourke (NSW Agriculture, Orange) and the plant material was dried before processing. The regular process of solvent extraction, fractionation, separation (PTLC) and purification of the plant's crude extract unfortunately gave no alkaloid fractions. The

extraction procedure was kept specific for alkaloids, but the major component isolated gave an MH^+ peak at *m/z* 209 (LRCIMS) and absorbed the UV light but didn't give a positive alkaloid test with Dragendorff's reagent. HRCIMS indicated a molecular formula of $C_{11}H_{13}O_4$. The compound was identified as 3,4-Dimethoxycinnamic acid **62** based on the comparative analysis and the ^{1}H-NMR spectral peaks of the compound with those reported in the literature[47]. Extra peaks observed in the ^{1}H-NMR of the compound were from small impurities.



62 3,4-Dimethoxycinnamic acid

3,4-Dimethoxycinnamic acid occurs naturally and has been isolated before from plants, *Zanthoxylum acutifolium* (leaf)[232] and *Milletia ovalifolia* (seed)[233], and also from beeswax[234].

Although some crude fractions also showed CI peaks at *m/z* 213, *m/z* 183 and *m/z* 265 corresponding to those expected for harmine, harman and perlolyrine, none of these could be isolated for structural confirmation. TLC analysis revealed that these compounds had similar retention factors as those of the standards: harmine, harman and perlolyrine. Extractions of the plant material with different solvents were also conducted but no substantial amount of crude alkaloid could be recovered. It should be noted that the dried plant material gave only a very faintly positive Culvenor Fitzgerald test[106] for the presence of alkaloids. This confirmed that the alkaloid content of the plant was extremely low which could have been caused by either inappropriate drying of the plant material or its time of collection. Extreme drying and exposure to light can cause some alkaloids to degrade. As discussed in Chapter 1, alkaloid content may also vary from season to season and with geographical location. Alkaloid yields may be thus improved by collecting the plant at an appropriate time. Further collections of the plant were not conducted due to time constraints.

Chapter 6

Investigation of *Ranunculus brotherusi* Freyn for alkaloids

Family:	Ranunculaceae
Genus:	*Ranunculus*
Species:	*brotherusi*
gSo-ba Rig-pa name:	lche-tsa
Distribution:	Himalayas

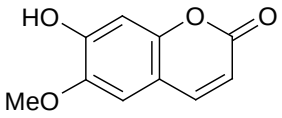
6.1 General Introduction

Ranunculus brotherusi is an erect plant with yellow flowers. This plant is used in the traditional medicine of Bhutan as an antipyretic and for treating infections and wounds. The genus is found in aquatic and terrestrial habitats. Some *Ranunculus* species have been found to cause diarrhoea, drooling, licking air, teeth grinding, excessive chewing, paddling and death in cattle[235]. Other studies have shown that *Ranunculus* species (*Ranunculus acer*) contain a vesicant toxin called ranunculin that is released upon mastication[236,237].

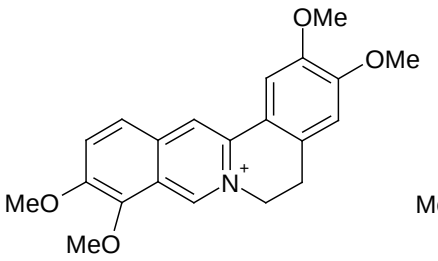
6.2 Phytochemical and Pharmacological Studies of the genus *Ranunculus*

Many saponins, flavonoids and glycosides have been isolated from this plant. Stigmasterol, hexadecanoic acid, eicosanoic acid, nonacosanol, ternatolide A and 5-hydroxy-benzaldehyde were isolated from *Ranunculus ternatus*[238]. Coumarins like aesculetin (6,7-dimethoxycoumarin) and scopoletin **63,** which showed potent antiviral and cytotoxic activity, were isolated from the Egyptian species *Ranunculus asiaticus* L[239]. Four major quercetin triglycosides, namely quercetin 3-sophoroside-7-glucoside, quercetin 3-sambubioside-7-glucoside and quercetin 3-diglucoside were isolated along with other components from an Antarctic *Ranunculus* species[240].

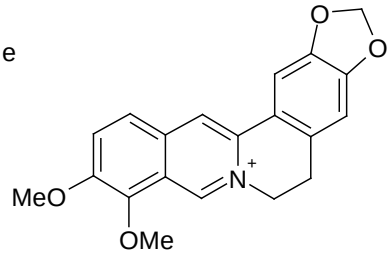
Alkaloids have also been found in *Ranunculus* species. For example, protoberberine-type quaternary alkaloids like palmatine **64**, berberine **65** and columbamine **66,** and the benzylisoquinoline-type magnoflorine **67,** were isolated from the rhizomes of *Ranunculus serbicus*[241]. The leaf extract of *Ranunculus sceleratus* L. showed a rapid fungicidal action, tolerance against heavy fungal inoculum, activity on a broad pH range, broad fungicidal spectrum, and no phytotoxicity with a lethal dose of 1:40 dilution[242]. Phytochemical investigation of this plant species led to the isolation of the glycoside ranunculin, its aglycone protoanemonin **68,** and anemonin **69**[243].



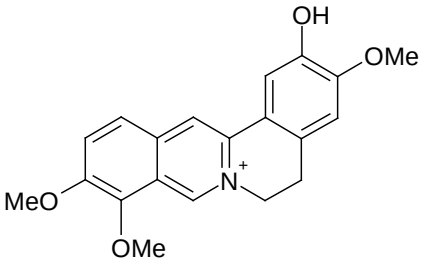
63 Scopoletin



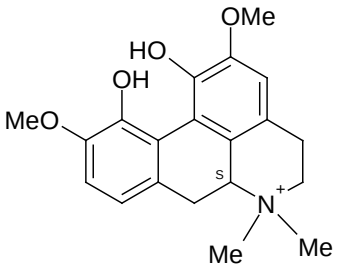
64 Palmatine



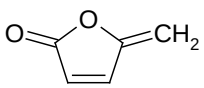
65 Berberine



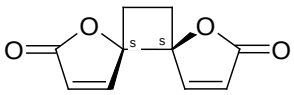
66 Columbamine



67 Magnoflorine



68 Protoanemonin



69 Anemonine

Protoanemonin was also isolated from *Ranunculus oxyspermus* M.B. and *Ranunculus acer*[244]. It was suggested that protoanemonin has hepatotoxic activity[245]. It showed bacteriostatic and bactericidal activity against a large number of Gram-positive and Gram-negative bacteria as well as fungistatic activity against S*accharomyces cerevisiae* and *Candida albicans*[246]. An extract of *Ranunculus japonicus* showed analgesic and anti-inflammatory effects when determined in several animal models (mice and rats)[247].

6.3 Investigation of *Ranunculus brotherusi* Freyn for Alkaloids

The methanol extract of *Ranunculus brotherusi* only afforded a very small quantity of crude alkaloid material after an acid/base separation. This separation gave many UV-active fractions, but none of them gave any positive results when tested for alkaloids with Dragendorff's reagent. The sequential extraction was repeated by increasing the pH from 9 to 11 and then to 13 using aqueous sodium hydroxide. A liquid-liquid extraction was then performed using different solvents like chloroform, dichloromethane and ethyl acetate but still no alkaloids were obtained. The Culvenor-

Fitzgerald test was performed for this plant before the methanol extraction was conducted and it gave a faint positive test. No further work was done on this plant due to time constraints.

Chapter 7

Microbiological Bioassay

7.1 General Introduction

As discussed in Chapter one, medicinal plants offer a vast resource of interesting bioactive lead compounds. Studies of the Structure-Activity Relationships of the leads combined with computer-aided modelling should then result in molecules with optimal activity, better bioavailability, fewer side effects and an acceptable therapeutic index and consequently, good candidates for the development to new drugs[248]. Thus, one of the key steps in the methodology of drug development is the bioassaying or the testing of activities of the plant isolates. To conduct the bioassays, efficient collaborations with pharmacologists, medical doctors, plant pathologists and biologists may sometimes be crucial, and there are relatively few simple laboratory-based *in vitro* methods which could be performed by chemists alone[249].

Bioassays involve quantitation of the response which follows the application of a stimulus to a biological system[250]. The bioassays are performed on crude extracts especially in bioactivity-guided screening or on blindly purified compounds[249]. The bioassays can be best divided into general screening bioassays and the specialized screening bioassays. Antimalarial testing is one example of a specialised screening bioassay. The general screening bioassays can be further classified to broad screening bioassays and primary screening bioassays. Primary screening bioassays include the *in vivo* animal assays (even involving human volunteers) and the *in vitro* microbiological assays[248].

The microbiological bioassays involve the detection of antibacterial and antifungal activities and potencies (Minimum Inhibitory Concentration; MIC) of compounds using different end point methods like plate assays (agar diffusion assay) and tube assays (agar dilution streak assays and broth dilution MIC assays)[250,251]. Due to the requirement of a long incubation time, the agar diffusion assay (plate assay) cannot be used for volatile and unstable compounds and therefore, agar-overlay methods (agar dilution methods or liquid media methods) have normally been followed.

In the agar-overlay methods, the degree of antimicrobial activity and the MIC of a compound are greatly influenced by factors like “compound concentration, carbon dioxide and oxygen concentration, media compatibility with the test compound, solubility and stability of the test compound, pH of the medium, types nutrient of media, temperature, the duration of the exposure to the compound and the types of strains (bacterial, fungal or viral) used in the testing”[54,58]. For these reasons, growth

promoting or inhibiting potencies of test samples have to be standardised and compared with standard preparations of the test compound[250]. Thus, the choice of antimicrobial bioassay method has to be selectively adapted taking the above factors into consideration. Such methods should be sensitive enough to detect active principles, the minimum inhibitory concentrations, and the dose response of the microorganisms (bacteria and fungi). A comparison of the disk diffusion method, the hole-plate diffusion method and the bioautographic TLC assay showed that the nature and amount of substance influence to a great extent the selection of the test method[252].

A number of alkaloids (about 300) have been shown to possess antimicrobial activity[253]. Screening of medicinal plants used in traditional medicines has shown that the incidence of antimicrobial activity found is much higher than for random screening[253]. In conjunction with this, alkaloids isolated from some of the traditional medicinal plants of Bhutan were analysed here for their antibacterial and antimalarial activities.

7.2 Assays for Detecting Antibacterial Activity

To test if a sample can exert antibacterial activity, it should preferably be made up into an aqueous solution by dissolving in water[254]. If it is insoluble in water, ultrasonification should be first tried and if that too fails, water miscible organic solvents like methanol, acetone or the use of emulsifying agents, are other options[254,255]. In such cases, however, care must be taken to avoid false test positives due to lethality of the uncontrolled final concentration of the solvent or emulsifying agents.

The antibacterial activity assay is distinguishable into bacteriostatic activity and bactericidal activity. Bacteriostatic activity of the compounds refers to stopping or inhibiting bacterial growth and multiplication. Bactericidal activity is the property of an antibacterial agent responsible for killing bacteria. Both disk diffusion assay and broth methods can be used to examine bacteriostatic activities but the bactericidal activities can be detected only by microscopic analysis or by the assessment of bacterial cell recovery in fresh media[58] .

Susceptibility of *Mycobacterium tuberculosis* to antimicrobial agents has been tested by the bioluminescent assay (ATP) method, the Mycobacteria Growth Indicator Tube (MGIT) method, the MIC method and the M24-T agar proportion method. It was found that the ATP method was the most rapid and reliable method for the assessment of drug (antibacterial) susceptibility testing[256]. The Fluorescein Diacetate (FDA) Assay, based

on the enzymatic hydrolysis of fluorescein diacetate to fluorescein, is suitable for use with a wide range of different microorganisms and was recommended for determining the MIC of crude extracts and purified compounds[257]. This method is commonly used in antibacterial testing, although problems can arise with fluorescent components in the growth media or fluorescence of the test compound. Kamia *et al*. also has reported that the turbidometric assay was rapid, reproducible and a very useful method for quantitative estimation of antibacterial activity[258].

7.2.1 Results of the Antibacterial Testing

The crude methanol extracts (AO-ME and CG-ME), crude alkaloids (AO-CEA and CG-CEA), atisinium chloride, orochrine and protopine (CG-354) obtained from *Aconitum orochryseum* Stapf. and *Corydalis gerdae* Fedde were sent for antibacterial assay at the Amrad Corporation Laboratories, Melbourne using the antibacterial screening methodology described in **Chapter 9**. All the samples were prepared in DMSO (2.5%) solution. The crude plant extracts and the alkaloids were tested against *Staphylococcus aureus* and all these samples showed very weak activity against this bacterium with MIC values greater than 125 μg/ml. Atisinium chloride and orochrine were tested against vancomycin-resistant and vancomycin sensitive *Enterococcus faecium*. These alkaloids were also found to be essentially inactive against this bacterium with similar MIC values (>125 μg/ml).

Antibacterial tests could not be conducted on the other alkaloids isolated in this project since they were available in very small quantities. However, from the results obtained, antibacterially active components did not appear to be present in *Aconitum orochryseum* or *Corydalis gerdae*, at least for Gram-positive bacteria.

7.3 Assays for Detecting Antimalarial Activity

Different types of antimalarial test methods like biochemical and molecular techniques, colorimetric enzyme assays, flow cytometry, and micro-dilution assays, have been developed over the past years[259]. The colorimetric enzyme assay which was described by Basco *et al*. is based on the measurement (using spectrophotometry)[260] of the activity of the enzyme lactate dehydrogenase in living *Plasmodium falciparum* and *Plasmodium ovale*. A semi-automated micro-dilution assay[261] involves the inhibition of uptake of a nucleic acid precursor, ^{3}[H]-hypoxanthine, in to the malarial protozoa. A modified form of this method was used in this study.

A simple and reproducible method has also been described by Pandey *et al* and modified micro-dilution assay methods has been routinely used in the identification of potential antimalarial compounds[54].

7.3.1 Results of Antimalarial Testing

The pharmacopeia of Bhutanese Traditional Medicine (gSo-ba rig-pa) had indicated *Corydalis gerdae* for the treatment of malaria and *Aconitum orochryseum* for treating infections, snake bites and high fever arising from blood poisoning, cold and flu, bile inflammation and malaria[105]. Based on this ethno-medical information, *in vitro* antimalarial activity testing against *Plasmodium falciparum*, for both an antifolate sensitive strain (TM4) and an antifolate resistant strain (K1) was performed on the crude methanol plant extracts, crude alkaloids and the major alkaloids of these two plants.

The methanol extracts, crude alkaloids and the major alkaloids (protopine and atisinium chloride) of these two plants were found to be active against both strains of *Plasmodium falciparum*. Comparably, *Corydalis gerdae* was found to possess active antimalarial compounds. The methanol extract (CG-ME) of this plant (*Corydalis gerdae*) inhibited the TM4 strain with an IC_{50} value of 1.00 μg/ml and the K1 strain with an IC_{50} value of 2.56 μg/ml. The inhibitory activity of the crude alkaloid (CG-CEA) of the same plant increased significantly with IC_{50} values of 0.33 μg/ml for the TM4 strain and 0.63 μg/ml for the K1 strain. However, decreased antimalarial activities of the alkaloids, protopine and cheilanthifoline, were observed. Protopine inhibited the TM4 strain with an IC_{50} value of 4.25 μM (1.50 μg/ml) and the K1 strain with an IC_{50} value of 4.29 μM (1.51 μg/ml). Cheilanthifoline inhibited the TM4 strain and the K1 strain with IC_{50} values of 2.78 μM (0.90 μg/ml) and 3.76 μM (1.22 μg/ml) respectively. This suggested perhaps that a minor alkaloid was responsible for the high activity exhibited by the crude alkaloid extract. The high activity of this extract could also be due to the synergistic effects of the total range of alkaloids present.

The methanol extract of *Aconitum orochryseum* inhibited the TM4 strain with an IC_{50} value of >10 μg/ml and the K1 strain with an IC_{50} value of >10 μg/ml. The crude alkaloid extract showed increased antimalarial levels and inhibited the TM4 strain with an IC_{50} value of 20.4 μg/ml and the K1 strain with an IC_{50} value of 19.2 μg/ml. The antimalarial activity very significantly increased for the pure alkaloid atisinium chloride (the major alkaloid of the plant). This alkaloid inhibited the K1 Strain with an

IC_{50} value of 3.59 μM (1.35 μg/ml) and the TM4 Strain with an IC_{50} value of 4.02 μM (1.51 μg/ml). Though atisinium chloride was reported previously, no antimalarial testing has been done before on this alkaloid. Thus, from the above antimalarial assay results, it was evident that these two plants, *Aconitum orochryseum* and *Corydalis gerdae* contain bioactive alkaloids which are potential antimalarial drug leads.

The antimalarial assay was not done for the rest of the alkaloids isolated from these two plants since their quantities were too small. It would be worthwhile to synthesise the new alkaloids and test them for malaria along with the known alkaloids isolated from *Corydalis gerdae*. The high activity of the crude alkaloid of *Corydalis gerdae* could be either due to scoulerine or stylopine, which was not tested. It was also possible that some minor alkaloids were responsible for the high activity of this crude alkaloid mixture or all the alkaloids present in the mixture might have acted synergistically to bring in that high activity against K1 and TM4 strains. Alkaloids from *Aconitum orochryseum* could also possibly have antiviral activities since the plant was indicated ethno-medically for treating cold and flu.

Chapter 8

Conclusions and Future Directions

8.1 Conclusions

Infectious diseases like HIV-AIDS, tuberculosis, malaria and other viral and bacterial infections are claiming millions of lives every year. These diseases will continue to be leading causes of death unless researchers find a concrete arsenal of drugs to combat them. One approach is to search for such new drugs from natural sources. Bhutan is one of the countries that is rich in biodiversity as well as being rich in traditional medical knowledge. It thus has the potential to contribute towards discovering natural product-based drugs.

Systematic natural product screening should be based on appropriate search strategies for efficient drug discovery. Since Bhutan has a reservoir of ethno-medical knowledge, the "ethno-directed biorational approach" provided such a strategy in combination with a chemo-directed approach based on alkaloids. The author has successfully engaged this joint strategy for this study in which plant selection was directed by the traditional medicine of Bhutan used for antibacterial or antimalarial treatments (ethno-directed biorational approach) and the presence of alkaloids in these plants (chemo-directed approach).

The alkaloids from six selected plants were investigated. From *Aconitum orochryseum* Stapf., eleven alkaloids were isolated. Three of these were identified as new diterpenoid alkaloids and were named as orochrine, 2-*O*-acetylorochrine and lingshinaline. The crude methanol extract (AO-ME), crude alkaloid mixture (AO-CA), and the alkaloids atisinium chloride (AO-344, major alkaloid) and orochrine (AO-342) were inactive against the Gram-positive human pathogenic bacteria *Staphylococcus aureus* and *Enterococcus faecium*. However, *in vitro* activity was seen against the malarial protozoan *Plasmodium falciparum* (strains TM4 and K1, see **Table 24** for the summarized results). Atisinium chloride gave the highest antimalarial activity among the analytes obtained from this plant. Although this alkaloid had been isolated previously from other species in the genus *Aconitum*, it had not been tested against malaria. Atisinium chloride thus provides a new anti-malarial drug lead. The other alkaloids (orochrine, 2-*O*-acetylorochrine, lingshinaline, virescenine and unidentified alkaloids) isolated from *Aconitum orochryseum* could also have similar activity, but due to the small quantities obtained, it was not possible to conduct an antibacterial assay and antimalarial assay on these alkaloids.

Table 24. The summary of the results of alkaloid investigation of the plants

Plants	Alkaloids	Antibacterial activity (IC_{50} in μM)		Antimalarial activity (IC_{50} in μM or μg/ml)	
		Staph.	VRE	TM4 strain	K1 strain
***Aconitum orochryseum* Stapf.**	Methanol extract (AO-ME)	>125	N/T	>10 μg/ml	>10 μg/ml
	Crude alkaloid (AO-CA)	>125	N/T	20.4 μg/ml	19.2 μg/ml
	Atisinium chloride	>125	>125	4.02 μM	3.59 μM
				1.51 μg/ml	1.35 μg/ml
	Orochrine	>125	>125	N/T	N/T
	2-*O*-acetylorochrine	N/T	N/T	N/T	N/T
	Lingshinaline	N/T	N/T	N/T	N/T
	Virescenine	N/T	N/T	N/T	N/T
	AO-330	N/T	N/T	N/T	N/T
	AO-340	N/T	N/T	N/T	N/T
	AO-358	N/T	N/T	N/T	N/T
	AO-372	N/T	N/T	N/T	N/T
	AO-414	N/T	N/T	N/T	N/T
	AO-465	N/T	N/T	N/T	N/T
***Corydalis gerdae* Fedde.**	Methanol extract (CG-ME)	>125	N/T	1.00 μg/ml	2.56 μg/ml
	Crude alkaloid (CG-CA)	>125	N/T	0.33 μg/ml	0.63 μg/ml
	Protopine	>125	>125	4.25 μM	4.29 μM
				1.50 μg/ml	1.51 μg/ml
	Cheilanthifoline	N/T	N/T	2.78 μM	3.76 μM
				0.90 μg/ml	1.22 μg/ml
	Scoulerine	N/T	N/T	N/T	N/T
	Stylopine	N/T	N/T	N/T	N/T
	CG-337	N/T	N/T	N/T	N/T

Staph. = *Staphylococcus aureus*, VRE = Vancomycin Resistant *Enterococcus faecium*, Red = new alkaloid, Green = known alkaloids, Blue = unidentified, N/T = not tested

From the plant *Corydalis gerdae* Fedde four known alkaloids, namely protopine (major alkaloid), scoulerine, cheilanthifoline and stylopine were obtained, together with one unidentified alkaloid. The crude methanol extract (CG-ME), crude alkaloid mixture (CG-CA) and the alkaloids protopine and cheilanthifoline were analysed for *in vitro* antimalarial activity. These analytes gave interesting and promising antimalarial activity results as summarised in **Table 24**. The inhibitory activity was highest in the case of the crude alkaloid mixture (CG-CA), possibly due to a highly active minor alkaloid. It could be also due to synergistic effects amongst the alkaloids present in the

mixture. The protopine (major alkaloid) and cheilanthifoline also showed significant activity in this assay.

The extracts and isolates of *Corydalis gerdae* showed better antimalarial activity than those of *Aconitum orochryseum*. These results substantiated for the first time at the molecular level the ethno-medical use of these plants in anti-malarial treatment. *Ranunculus brotherusi* failed to yield any alkaloids although a preliminary test on the plant gave a positive test for alkaloids. Similarly, *Tribulus terrestris* also failed to yield any alkaloids. *Ranunculus brotherusi* had not been studied previously. Many β-carboline alkaloids like harman, harmine, norharman and harmaline had been isolated from *Tribulus terrestris* in past work. Thus, it was concluded that these plant materials were either collected at the wrong time or their alkaloids could have been lost on drying.

8.2 Future directions

Since the extracts and the alkaloids of *Aconitum orochryseum* and *Corydalis gerdae* were found potentially active against *Plasmodium falciparum* strains, the immediate follow up work should be focused on doing antimicrobial assays with the other minor alkaloids present in these plants. In doing so, large scale extraction would be needed to get a reasonable amount of the minor alkaloids, especially from *Corydalis gerdae* because the crude alkaloid of this plant showed highest inhibitory activity (**Table 24**). Since the high activity of this crude alkaloid (*Corydalis gerdae*) could also be due to synergistic effects of total alkaloids (protopine, scoulerine, cheilanthifoline and stylopine) present in the crude extract, the antimalarial testing should be done on various mixtures of commercially available alkaloids (protopine, scoulerine, cheilanthifoline and stylopine) so as to find out if the activity of the crude alkaloid was because of synergistic effects.

The six unidentified alkaloids of *Aconitum orochryseum* require further study and further bulk extraction of the plant material will be needed in order to obtain sufficient quantities for their structure elucidation and the evaluation of antimicrobial activities. Modifying the structures of the new alkaloids as well as of the known alkaloids (atisinium chloride, virescenine, protopine, scoulerine, cheilanthifoline and stylopine) which were active against *Plasmodium falciparum* could improve their antimalarial activities. This could lead ultimately to the development of candidates for new antimalarial drugs.

Since *Aconitum orochryseum* is traditionally used against colds, influenza and other microbial infections, it would also be worthwhile undertaking antifungal and antiviral (like SARS and HIV) testing on the alkaloids from this plant. The non-alkaloidal components of the plants studied here may be bioactive or at least could also be acting synergistically with the alkaloids. They should thus also be purified, identified and tested for bioactivity in the future.

The results obtained in this study indicate that the combined ethno-medical-alkaloid search strategy is an efficient and direct one for locating potential new drug leads. The application of the strategy to other medicinal plants in Bhutan should thus be investigated. The chemical and pharmacological investigation of traditional Bhutanese drugs should be pursued in the long term. Such steps would mean a way forward in meeting the WHO standards for such drugs and, most significantly, in providing patients with better herbal medicines.

Chapter 9

Experimental Section

9.1 Equipment and Chemical Specifications

9.1.1. Equipment

Preparative thin layer chromatography plates were made using Merck Kieselgel 60 PF_{254} on glass plates (0.3 mm thickness, 20 × 20 cm). Pre-made silica plates with aluminium backing (0.2 mm thickness) supplied by Merck were used for separating smaller quantities of isolates. Visualization of the separated bands on preparative TLC plates was done using short and long wave UV light. Normal pH paper was used for determining the pH of the extracts and the solutions. Extracting funnels were used for the liquid-liquid separations and sintered glass funnels were used for filtration. A Büchi Rotary Evaporator with a high vacuum pump was used for evaporation of solvents under reduced pressure at 40^{o}C. Nitrogen gas was used for evaporation of smaller quantities of solvents.

Chemical ionisation mass spectra (LRCIMS) were obtained on a Shimadzu QP-5000 (isobutane as the carrier gas) spectrometer by the direct insertion technique. Electron impact mass spectra (at 70 eV) were also obtained on a Shimadzu QP-5000 spectrometer. HRCIMS were run on a Fison/VG Autospec oa-TOF Mass Spectrometer (methane as the carrier gas). Electrospray (ES) mass spectroscopy was performed on a Micro-mass Q-Tof-2^{TM} with acetonitrile as solvent. Gas Chromatographic Mass Spectrometric (GCMS) analysis was conducted using a QP-5000 Shimadzu Spectrometer and VG Autospec (Fisons Instrument) Spectrometer. For the NMR spectra, one dimensional experiments like ^{1}H-NMR, gCOSY, ^{13}C-NMR (at 75.42 MHz), DEPT and NOESY and two dimensional experiments like gHSQC, gHMBC and TOCSY were obtained using a Nanoprobe on a Varian Unity Inova-500 MHz NMR Spectrometer unless otherwise stated.

The melting points were determined using a Reichert hot stage apparatus and were corrected. Melting points were corrected using the 2-benzoylbenzoic acid (m.p.126-129^{o}C) as a standard. The Optical Rotations were measured with a Jasco Dip-370 Digital Polarimeter with a sodium lamp. The specific rotation was calculated by using the equation: $[\alpha]^{t}_{D} = \alpha/l.c.$ where, α = observed rotation at temperature t^{o} Celsius, l = length of polarimeter cell (dm), and *c* = concentration of sample (g/100ml). An average of ten optical rotation readings was taken for the rotation α-value.

9.1.2. Chemicals and solvents

All the solvents were AR (analytical reagent) grade or distilled. DCM, chloroform and methanol were the main solvents used in carrying out the experiments. Deuterated chloroform, methanol, acetone, and acetonitrile were used as NMR solvents. Ethanol, diethyl ether, ethyl acetate, acetone and butanol were used for crystallizations unless otherwise stated. Methanol (10% v/v) in dichloromethane was used as the solvent for dissolving, loading and recovering extracts or compounds from the silica on preparative TLC plates. Sodium sulfate was used for drying traces of water in the separated organic solvents. Sodium carbonate (20% w/v) and potassium hydroxide (10% w/v) were used for the basification of the aqueous plant extracts to a pH of 8-11. Sulfuric acid (0.1 M to 1 M) was used for acidification. To improve separation and overcome tailing of alkaloids on TLC, concentrated aqueous ammonia (28%, 6 drops and 1 ml) was added in the TLC solvent system.

9.2 Detection Methods and Spray Reagent

Dragendorff's reagent and Mayer's reagent were used for the detection of alkaloids in the plant extracts. Mayer's reagent was prepared by dissolving mercuric chloride (1.4 g) and potassium iodide (5 g) in water (100 ml).

Dragendorff's reagent (Munier and Mache-boeuf)[262] was prepared as follows:

a. Bismuth subnitrate (2.5 g) was dissolved in a solution of water (20 ml) and glacial acetic acid (5 ml).

b. Potassium iodide (4 g) was dissolved in water (10 ml).

Solution ***a*** and ***b*** were then mixed together and stored as the stock solution. The spray reagent was prepared by mixing the stock solution (5 ml) with glacial acetic acid (10ml) and water (85 ml). On TLC plates, alkaloids gave an orange-red colouration with this spray reagent.

9.3 Collection of Plant Material

Six medicinal plants: *Aconitum orochryseum* (Ranunculaceae), *Aletris pauciflora* (Liliaceae), *Codonopsis bhutanica* (Campanulaceae), *Corydalis gerdae* (Fumariaceae), *Ranunculus brotherusi* (Ranunculaceae) and *Tribulus terrestris* (Zygophyllaceae) were selected for the study. The first five medicinal plants were collected from Lingshi Dungkhag (Bhutan) in June and August 2002 (see **Chapter 2**, **Table 8**). They were air-dried at room temperature in the drying unit at Lingshi and were then transported to the Pharmaceutical and Research Unit (Bhutan). The herbarium was maintained and stored

in the Research Unit with appropriate labelling including herbarium numbers (see **Chapter 2, Table 8**).

The preliminary test for alkaloids was done in the Pharmaceutical and Research laboratory in Bhutan following the Culvenor and Fitzgerald method[106]. A mortar and pestle was used for grounding plant material (10 g) with acid-washed sand. Dichloromethane (15 ml) and concentrated aqueous ammonia (5 drops) was then added to the ground material and stirred for 5 minutes. The mixture was filtered. The filtrate was acidified with 1 Molar H_2SO_4 (1 ml), shaken thoroughly and then allowed to stand for 30 minutes to effect good phase separation. A portion of the aqueous layer (5 drops) was transferred into a small petri dish. When the Mayer's reagent (K_2HgI_4) was added, the solution turned milky with the alkaloid salt precipitate. The turbidity varied from plant to plant depending on the percentage of alkaloid content in that plant.

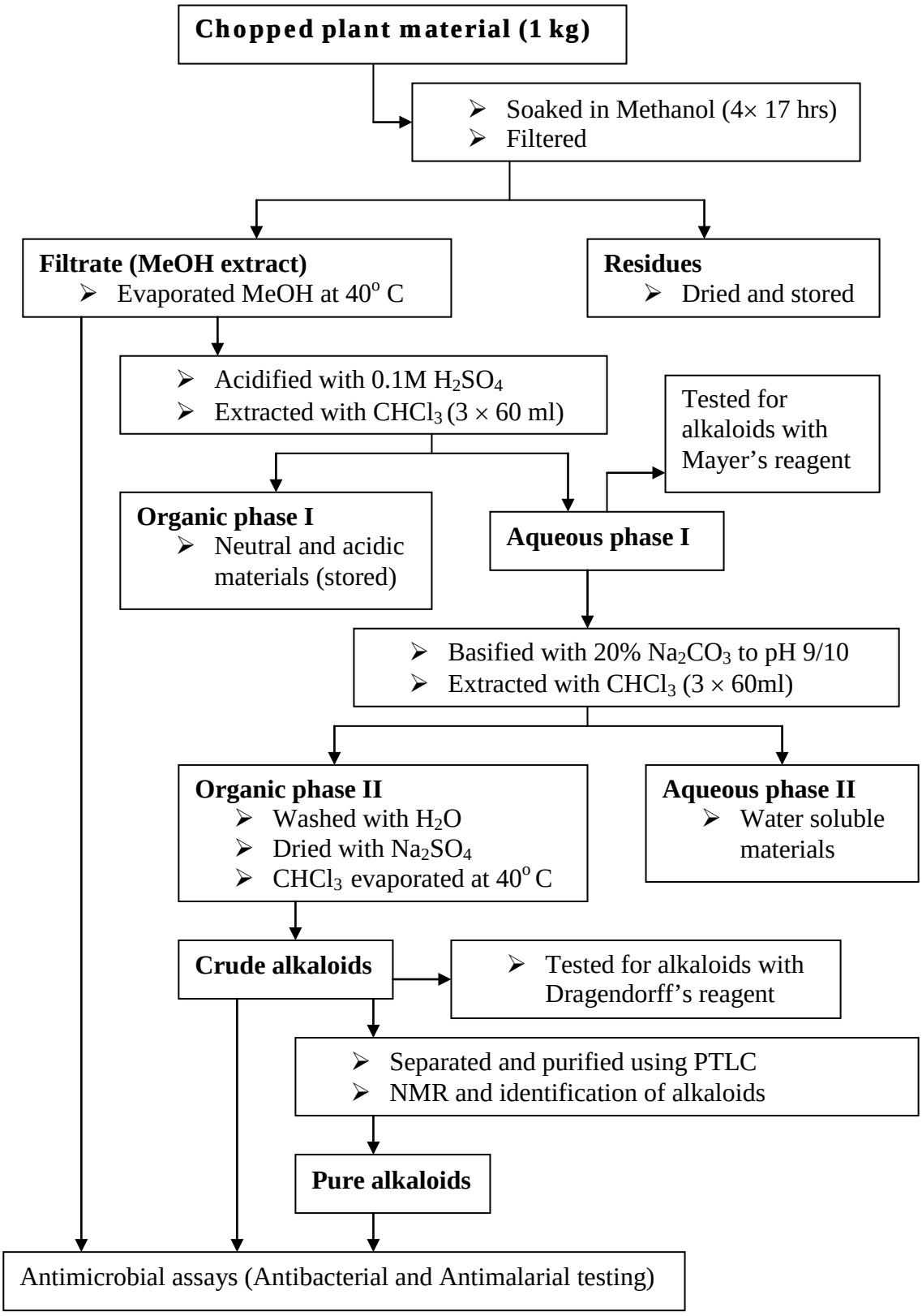
Since two of the plant extracts from *Codonopsis bhutanica* and *Aletris pauciflora* were damaged on transportation to Australia and a *Ranunculus brotherusi* didn't give any alkaloids, a locally available plant *Tribulus terrestris*, which caused neurotoxicity in sheep, was selected for the study. This plant is also used in Bhutanese traditional medicine. The air-dried (room temperature) plant material (stem, leaves, flowers and fruits, approx. 8 kg) which has been coarse chopped following drying using a petrol driven plant mulching machine, was supplied by Dr. C.Bourke, NSW Agriculture, Orange, NSW. It was collected near Wellington (Central West, NSW) in February 2002 (**Chapter 2, Table 8**).

9.4 General Procedure for Extraction and Isolation of Alkaloids.

The alcoholic extraction method was applied to all the selected plants. The plant material (1 kg) was chopped into small pieces. The material was then soaked overnight in methanol (Analytical Grade). The methanol extract was filtered and the process was repeated four times (over 4 days) to ensure no alkaloids were remained in the residues. All the extracts obtained in each set were combined. The methanol was then removed by rotary evaporation. The dried methanol extracts obtained from each plant were then packed in glass bottles (250 ml) and then brought to Australia. The residues from the methanol extracts were air dried and stored with proper labelling for future reference. The extracts were kept refrigerated and away from light (wrapping with aluminium foil) prior to further processing.

Sequential extractions and isolations were done following the schematic procedures outlined in **Scheme 3**. The solvents in the filtrates were evaporated under reduced

pressure and the crude alkaloids were obtained. The crude alkaloids were then separated using Preparative Thin Layer Chromatography on silica gel.



Scheme 3. Outline of the alkaloid separation procedure carried out for the selected medicinal plants of Bhutan and the sources of materials used in the antimicrobial assays.

The alkaloids were recovered from the silica gel as soon as the preparative plate TLC separation was complete to prevent decomposition. The alkaloids were also kept for minimal time in solution in order to avoid reaction and formation of artefacts. The appropriate developing solvent system was determined by manipulating the ratios of the mixture of different solvents, mainly dichloromethane, chloroform and methanol. The TLC separation yielded different fractions of compounds with different polarities. Each fraction was tested for purity using TLC, MS and ^{1}H-NMR analysis. The process of TLC separation and purification was repeated for each fraction till pure compounds were isolated. Concentrated aqueous ammonia solution (28%, 6 drops to 1 ml) was used to prevent tailing in the TLC separation. However, the merging of bands on silica plates occurred when compounds with closer Rf values were present together.

9.5 Purification and Structure Elucidation of Alkaloids from *Aconitum orochryseum* Stapf.

A methanol extract (101.65 g) was obtained from the dried plant material (1 kg) and a reference sample (26.21 g of this extract) was kept refrigerated. A portion of this reference material (20 mg) was used for antimicrobial testing (**Scheme 3**). The remaining crude methanol extract was extracted for alkaloids using the general schematic isolation and extraction procedure (**Scheme 3**). Crude alkaloid (0.561 g) was obtained. Again this crude alkaloid (51 mg) was tested for antimicrobial activity. The LRCIMS of the crude alkaloid revealed ion peaks at *m/z* 344, 342, 384, 400, 358, 424, 440, 406, 314, 448/466 and 298. The ion peak at *m/z* 344 was the major component. Focussing on these individual peaks, preparative plate separations were conducted.

All the crude alkaloid (494.0 mg) was dissolved in MeOH (10%): DCM (90%) solvent and was loaded on PTLC plates (65 g × 8 plates). An appropriate mobile phase was developed using DCM (89%): MeOH (10%): Ammonia solution (28%, 1 ml). Eleven fractions/bands were obtained and each band was monitored using the LRCIMS peaks (**Table 25**). Each band contained a mixture of compounds. All the fractions/bands containing similar mass peaks and with the same Rf values were grouped together as in **Table 25** and were further purified by development in an appropriate solvent system and the process was repeated until pure compounds were obtained.

Group 1 was purified by PTLC on silica gel in DCM (85%): MeOH (14%) : Ammonia solution (28%, 1 ml). This gave two fractions. Fraction one (AO-gr-1-1) contained a LRCIMS peak at *m/z* 344 (MH^+), and gave crystals when it was recovered from the silica. It was recrystallized from methanol/diethyl ether and fine needle-like crystals

(16.9 mg) were obtained. A crystal was sent for X-ray crystallography and was identified as ***atisinium chloride***.

Table 25. Bands separated from the crude alkaloid of *Aconitum orochryseum*

Bands	Net wt. (in mg)	Peaks present (LRMS-CI, *m/z*)	Major peaks in the fractions (*m/z*)	Inferences
1	61.7	326, 344, 342, 424, 358, 400	326, 344, 342	Band 1, 2, 3 and 4 formed group 1
2	24.1	400, 342, 456, 384	400, 342	
3	20.2	384, 342, 400, 440	384, 342	
4	16.3	400, 406, 424, 342, 456, 384	400, 424	
5	21.8	384, 406, 424, 358, 448, 466, 390	384	Band 5 and 6 formed group 2
6	20.3	384, 448, 466, 358	384	
7	31.9	358, 374, 448, 466, 179	358	Band 7, 8, 9, 10 and 11 formed group 3
8	26.7	432, 358, 450, 374	432, 358	
9	38.1	358	358	
10	18.6	358, 303	358	
11	16.9	358, 257, 303	358, 257, 303	

The second fraction (Ao-gr-1-2) still contained a mixture of compounds. Further separation by PTLC on silica gel using DCM (89%): MeOH (10%): Ammonia solution (28%, 1 ml) resulted in the isolation of three compounds: orochrine (LRCIMS peak at *m/z* 342), Ao-372 (LRCIMS peak at *m/z* 372), 2-*O*-acetylorochrine (LRCIMS peak at *m/z* 384) and lingshinaline (LRCIMS peak at *m/z* 400). Recrystallization from several solvents was attempted on each of these fractions. However, only orochrine gave fine small crystals (14.9 mg) from acetone/diethyl ether but the crystals were not big enough to determine the structure by X-ray crystallography. This was then sent for synchrotron X-ray crystallography in the USA. Unfortunately, no results were available at the time of completing this thesis. Crystallization was also attempted on the Group 2 and Group 3 fractions using acetone/diethyl ether but they failed to crystallise.

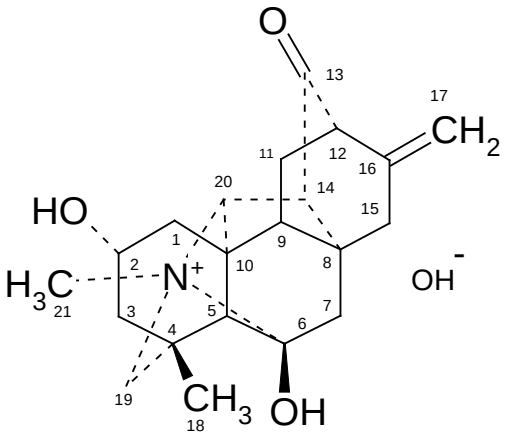
Group 2 was then purified by PTLC on silica gel with MeOH (8%): $CHCl_3$ (91%): Ammonia solution (28%, 1 ml) as the developing solvent. This resulted in the isolation of another alkaloid, virescenine (LRCIMS peak at *m/z* 424).

Group 3 was also subjected to PTLC on silica gel in DCM (95%): MeOH (5%): Ammonia solution (28%, 10 drops). This group yielded the minor alkaloids designated as AO-358, AO-414, AO-372, AO-466, AO-340, and AO-330 (**Table 26**). The final NMR spectra were obtained using a Nanoprobe for all of the five major alkaloids. Due

to very small quantities of sample, it was impossible to obtain useful NMR data for the minor alkaloids like AO-330, AO-358, AO-372, AO-372, AO-414 and AO-466.

Table 26. Alkaloids isolated from each fractions using discrete solvent system.

No.	*Alkaloid*	*MW*	*Molecular formula*	*Wt. (mg)*	*PTLC solvent system*
1	Orochrine	341	$C_{21}H_{27}NO_3$	14.9	10% MeOH:89% DCM:1% NH_3
2	Atisinium chloride	343	$C_{22}H_{33}NO_2$	16.9	85% DCM:14% MeOH:1% NH_3
3	2-*O*-acetylorochrine	383	$C_{23}H_{29}NO_4$	5.2	8% MeOH: 91% $CHCl_3$:1% NH_3
4	Lingshinaline	399	$C_{23}H_{29}NO_5$	8.1	10% MeOH: 89%DCM: 1%NH_3
5	Virescenine	423	$C_{23}H_{37}NO_6$	4.6	6% MeOH: 93% DCM:1% NH_3
6	AO-330	329	$C_{20}H_{27}NO_3$	1.8	10% MeOH:89% DCM:1% NH_3
7	AO-340	339	$C_{21}H_{25}NO_3$	1.9	10% MeOH:89% DCM:1% NH_3
8	AO-358	357	$C_{22}H_{31}NO_3$	2.9	97% DCM:2% MeOH:1% NH_3
9	AO-372	371	$C_{21}H_{25}NO_5$	2.1	10% MeOH:89% DCM:1% NH_3
10	AO-414	413	$C_{23}H_{27}NO_6$	2.2	6% MeOH:93% DCM:1% NH_3
11	AO-466	465	$C_{25}H_{39}NO_7$	3.6	10% MeOH:89% DCM:1% NH_3



22 Orochrine

Orochrine 22: Colourless crystals (needles); m.p. 210.1-213.6^{o}C; $[\alpha]_D^{23} = -65$ (*c*, 0.62 in MeOH); ^{1}**H-NMR** (CD_3OD, 500 MHz, Nanoprobe), δ 2.90 (s, 3H, *N*-Me), δ 5.04 and δ 4.94 (bs, 1H each, H-17 exocyclic methylene), δ 4.14 (bs, 1H, H-2β), δ 1.48 (s, 3H, H-18 methyl), δ 1.59 (d, 1H, H-1, *J* = 15 Hz), δ 1.75 (d, 1H, H-1, *J* =15 Hz), δ 1.60 (d, 1H, H-3, *J* =15 Hz), δ 1.93 (t, 1H, H-3, *J* =15 Hz), δ 2.15 (s, 1H, H-5), δ 2.23 (d, 1H, H-7, *J* =12.5 Hz), δ 2.31 (d, 1H, H-7, *J* =15 Hz), δ 2.20 (s, 1H, H-9), δ 1.86 (d, 1H, H-11, *J* =14 Hz), δ 2.03 (d, 1H, H-11, *J* =14.5 Hz), δ 2.97 (bs, 1H, H-12), δ 2.98 (bs, 1H, H-14), δ 2.52 (d, 1H, H-15, *J* =17.5 Hz), δ 2.69 (d, 1H, H-15, *J* =17 Hz), δ 3.35 (d, 1H, H-19, *J* =11.5 Hz), δ 4.30 (d, 1H, H-19, *J* =11.5 Hz) and δ 4.27 (s, 1H, H-20).

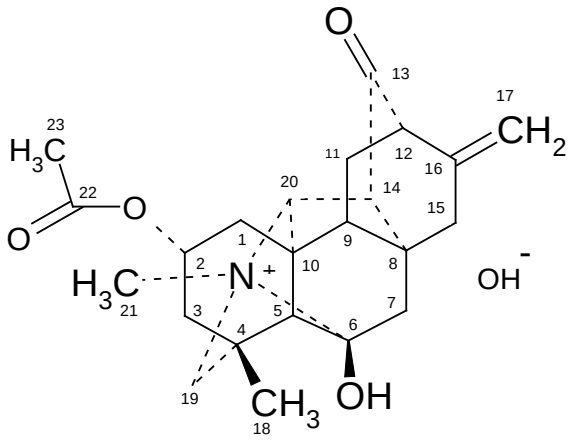
^{13}C-NMR (CD_3OD, 500 MHz, Nanoprobe), δ 34.5 (C-1), δ 65.5 (α-OH, C-2), δ 41.4 (C-3), δ 36.6 (C-4), δ 59.1 (C-5), δ 106.2 (C-6), δ 38.2 (C-7), δ 43.9 (C-8), δ 49.4 (C-9), δ 47.4 (C-10), δ 23.3 (C-11), δ 53.5 (C-12), δ 208.7 (C-13), δ 56.3 (C-14), δ 32.6 (C-15), δ 142.6 (C-16), δ 112.3 (Exocyclic methylene, C-17), δ 30.3 (Me, C-18), δ 70.5 (C-19), δ 75.1 (C-20), δ 37.3 (*N*-Me, C-21).

$_g$HSQC (CD_3OD, 500 MHz, Nanoprobe), δ 1.59 (d) & δ 1.75 (d)/ δ 34.5 (C-1), δ 4.14 (bs, H-2β)/δ 65.5 (C-2), δ 1.60 (d) & δ 1.93 (t)/ δ 41.4 (C-3), δ 2.15 (s)/ δ 59.1 (C-5), δ 2.23 (d) & δ 2.31 (d)/ δ 38.2 (C-7), δ 2.20 (s)/ δ 49.4 (C-9), δ 1.86 (d) & δ 2.03 (d)/ δ 23.3 (C-11), δ 2.97 (bs)/ δ 53.5 (C-12), δ 2.98 (bs)/ δ 56.3 (C-14), δ 2.52 (d) & δ 2.69 (d)/ δ 32.6 (C-15), δ 5.04 (bs) & δ 4.94 (bs)/ δ 112.3 (C-17), δ 1.43 (s)/ δ 30.3 (C-18), δ 3.35 (d) & δ 4.30 (d)/ δ 70.5 (C-19), δ 4.27 (s)/ δ 75.1 (C-20), δ 2.90 (s)/ δ 37.3 (C-21).

$_g$HMBC (CD_3OD, 500MHz, Nanoprobe), **δC to δH, C-1**/H-20; **C-2**/H-1, H-3; **C-3**/H-1, H-18, H19; **C-4**/H-3, H-18, H-19; **C-5**/H-1, H-3, H-7, H-15, H-20; **C-6**/H-7, H-9, H-19, H-20, H-21; **C-8**/H-7, H-11, H-15, H-20; **C-9**/H-5, H-7, H-15, H-20; **C-10**/ H-1, H-3, H-11, H-12, H-14, H-20; **C-12**/H-9, H-11, H-15, H-17; **C-13**/H-11, H-12, H-14, H-20; **C-14**/H-7, H-12, H-15, H-19, H-20; **C-15**/H-12, H-14, H-17; **C-16**/H-11, H-12, H-14, H-15; **C-17**/H-12, H-15; **C-18**/H-3, H-5, H-19; **C-19**/H-3, H-5, H-18, H-20, H-21; **C-20**/H-1, H-2, H-5, H-9, H-12, H-14, H-19, H-21; **C-21** (*N*-Me)/H-19.

δH to δC, H-1/C-2, C-3, C-4, C-5, C-10, C-20; **H-2**/C-20; **H-3**/C-1, C-2, C-4, C-5, C-10, C-18; **H-5**/C-9, C-18, C-19, C-20; **H-7**/C-5, C-6, C-8, C-9, C-11, C-14, C-15, C-20; **H-9**/C-5, C-6, C-8, C-10, C-11, C-12, C-14, C-20; **H-11**/C-8, C-9, C-10, C-12, C-13, C-16; **H-12**/C-13, C-14, C-15, C-16, C-17; **H-14**/C-9, C-13, C-15, C-16, C-20; **H-15**/C-7, C-8, C-9, C-12, C-14, C-16, C-17; **H-17**/C-12, C-15, C-16 (w); **H-18**/C-3, C-4, C-5, C-19; **H-19**/C-3, C-4 (w), C-5 (w), C-6, C-14, C-18, C-20, C-21; **H-20**/C-1, C-4 (w), C-6, C-8, C-9, C-10 (w), C-13, C-14, C-19; **H-21**/C-6, C-19, C-20.

LRCIMS, *m/z* 342 (MH^+); **LREIMS**, *m/z* 341 (M^+), 326, 313, 298, 282, 254, 192, 173, 157, 136, 122, 105, 91, 84, 55 and 44 (100%); **HREIMS,** $C_{21}H_{27}NO_3$ (M^+, measured 341.1974, calc.341.1990).



25 2-*O*-acetylorochrine

2-*O*-Acetylorochrine 25: Amorphous solid; m.p. 148.3-150.1^{o} C; $[\alpha]_{D}^{22}$ = ─82.47 (*c*, 0.82 in $CHCl_3$). ^{1}**H-NMR** (CD_3OD, 500 MHz, Nanoprobe), δ 2.91 (s, 3H, *N*-Me), δ 4.86 and δ 4.95 (bs, 1H each, H-17 exocyclic methylene), δ 5.11 (bs, 1H, H-2β), δ 1.43 (s, 3H, H-18 methyl), δ 1.65 (d, 2H, H-1, *J* =17.5 Hz), δ 1.66 (d, 1H, H-3, *J* =15.5 Hz), δ 1.92 (d, 1H, H-3, *J* =16 Hz), δ 2.17 (s, 1H, H-5), δ 2.23 (d, 1H, H-7, *J* =14.5 Hz), δ 2.27 (d, 1H, H-7, *J* =14.5 Hz), δ 2.17 (s, 1H, H-9), δ 1.84 (m, 2H, H-11, *J* =3.5 Hz), δ 2.89 (d, 1H, H-12, *J* = 3.5 Hz), δ 2.97 (d, 1H, H-14, *J* = 2Hz), δ 2.47 (d, 1H, H-15, *J* =17 Hz), δ 2.62 (d, 1H, H-15, *J* =17.5 Hz), δ 3.37 (d, 1H, H-19, *J* = 12 Hz), δ 3.79 (d, 1H, H-19, *J* =12 Hz), δ 3.76 (s, 1H, H-20), δ 2.06 (s, 3H, H-23/CH_3COO).

^{13}C-NMR (CD_3OD, 500MHz, Nanoprobe), δ 31.6 (C-1), δ 69.0 (α-acetyl, C-2), δ 38.5 (C-3), δ 36.5 (C-4), δ 58.5 (C-5), δ 106.6 (C-6), δ 38.0 (C-7), δ 44.2 (C-8), δ 49.3 (C-9), δ 47.4 (C-10), δ 23.3 (C-11), δ 53.3 (C-12), δ 208.7 (C-13), δ 56.2 (C-14), δ 32.4 (C-15), δ 142.3 (C-16), δ 112.4 (Exocyclic methylene, C-17), δ 30.1 (Me, C-18), δ 70.5 (C-19), δ 75.1 (C-20), δ 37.3 (*N*-Me, C-21), δ 171.3 (C-22, $COOCH_3$), δ 21.3 (C-23, $COOCH_3$).

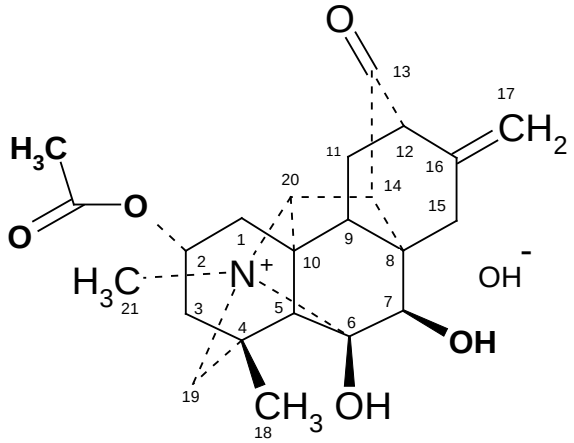
$_{g}$HSQC (CD_3OD, 500 MHz, Nanoprobe), δ 1.65 (d)/ δ 31.6 (C-1), δ 5.11 (bs, H-2β)/δ 69.0 (C-2), δ 1.66 (d) & δ 1.92 (d)/ δ 38.5 (C-3), δ 2.17 (s)/ δ 58.5 (C-5), δ 2.23 (d) & δ 2.27 (d)/ δ 38.0 (C-7), δ 2.17 (s)/ δ 49.3 (C-9), δ 1.84 (m)/ δ 23.3 (C-11), δ 2.89 (d)/ δ 53.3 (C-12), δ 2.97 (d)/ δ 56.2 (C-14), δ 2.47 (d) & δ 2.62 (d)/ δ 32.4 (C-15), δ 4.86 (bs) & δ 4.95 (bs)/ δ 112.4 (C-17), δ 1.43 (s)/ δ 30.1 (C-18), δ 3.37 (d) & δ 3.79 (d)/ δ 70.5 (C-19), δ 3.76(s)/ δ 75.1 (C-20), δ 2.90 (s)/ δ 37.3 (C-21), δ 2.06(s)/ δ 21.3 (C-23, CH_3COO).

$_{g}$HMBC (CD_3OD, 500 MHz, Nanoprobe), **δC to δH, C-1**/H-3, H-11(w), H-20; **C-2**/H-1, H-3, H-18(w); **C-3**/H-1, H-19; **C-4**/H-1, H-3, H-18, H-19; **C-5**/H-1, H-3, H-7, H-9,

H-18; **C-6**/H-5, H-7, H-19, H-20, H-21; **C-7**/H-9(w), H-15; **C-8**/H-7, H-9, H-11, H-15; **C-9**/H-5, H-7, H-11, H-12, H-14, H-15, H-20; **C-10**/H-1, H-3, H-9 (w), H-11, H-14; **C-12**/H-9, H-11, H-15, H-17; **C-13**/H-11, H-12, H-14, H-20; **C-14**/H-7, H-9, H-15, H-19 (w), H-20, H-21(w); **C-15**/H-7, H-12, H-17; **C-16**/H-11, H-15; **C-17**/H-12 (w), H-15; **C-18**/H-1, H-3, H-5, H-19; **C-19**/H-3, H-5, H-18, H-20, H-21; **C-20**/H-1, H-5, H-14, H-19, H-21; **C-22**/H-2, H-23 (CH_3COO).

δH to δC, H-1/C-2, C-3, C-4, C-5, C-10, C-20; **H-2**/C-22; **H-3**/C-1, C-2, C-4, C-5, C-10, C-18, C-19; **H-5**/C-7 (w), C-9 (w), C-14, C-18, C-19, C-20; **H-7**/C-5, C-6, C-8, C-9 (w), C-14, C-15 (w); **H-9**/C-5 (w), C-7 (w), C-12, C-14, C-20; **H-11**/C-1 (w), C-8(w), C-9, C-10, C-12, C-13, C-16; **H-12**/C-9, C-13, C-14, C-15; **H-14**/C-9, C-10, C-13, C-20; **H-15**/C-7 (w), C-8, C-9, C-12, C-14, C-16, C-17; **H-17**/C-12, C-15; **H-18**/C-3, C-4, C-5, C-19; **H-19**/C-4, C-5, C-14(w), C-18, C-20; **H-20**/C-1, C-6, C-8, C-9 (w), C-13, C-14 (w), C-19; **H-21**/C-6, C-19, C-20, **H-23**(CH_3COO)/C-22.

LRCIMS, 384 (MH^+); **LREIMS**, *m/z* 383 (M^+), 368, 355, 340, 324, 296, 282, 268, 254, 226, 176, 174, 173, 148, 134, 122, 105, 91, 84, 70, 55, 44, 43; **HREIMS,** $C_{23}H_{29}NO_4$ (M^+, measured 383.2085, calc.383.2096).



26 Lingshinaline

Lingshinaline 26: Pale green solid; m.p. 110-112^o C; $[\alpha]_D^{25}$ = ─31.76 (*c*, 0.34 in MeOH); ^{1}**H-NMR** (500 MHz, Nanoprobe, CD_3OD), δ 3.12 (s, 3H, *N*-Me), δ 4.92 and δ 5.00 (bs, 1H each, H-17 exocyclic methylene), δ 5.21 (s, 1H, H-2β), δ 1.52 (s, 3H, H-18 methyl), δ 1.65 (d, 1H, H-1, *J* = 16.5 Hz), δ 1.74 (d, 1H, H-1, *J* =15.5 Hz), δ 1.65 (d, 1H, H-3, *J* =17 Hz), δ 1.93 (d, 1H, H-3, *J* =16.5 Hz), δ 2.15 (s, 1H, H-5), δ 4.35 (s, 1H, H-7β), δ 2.20 (d, 1H, H-9, *J* = 7.5 Hz), δ 1.86 (d, 2H, H-11, *J* =6.5 Hz), δ 2.95 (d, 1H, H-12), δ 3.00 (d, 1H, H-14), δ 2.48 (d, 1H, H-15, *J* =17.5 Hz), δ 2.96 (d, 1H, H-15, *J* =17.5 Hz), δ 3.42 (d, 1H, H-19, *J* = 11.5 Hz), δ 3.61 (d, 1H, H-19, *J* =10 Hz), δ 3.63 (s, 1H, H-20), δ 2.01 (s, 3H, H-23, CH_3COO).

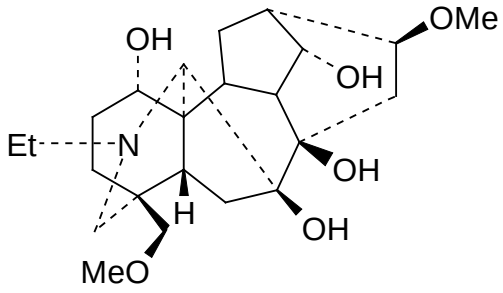
^{13}C-NMR (CD_3OD, 500 MHz, Nanoprobe), δ 31.2 (C-1), δ 66.9 (α-acetyl, C-2), δ 37.9 (C-3), δ 35.9 (C-4), δ 55.8 (C-5), δ 105.7 (C-6), δ 71.7 (C-7), δ 45.3 (C-8), δ 46.7 (C-9), δ 47.7 (C-10), δ 22.1 (C-11), δ 51.6 (C-12), δ 206.7 (C-13), δ 52.5 (C-14), δ 28.2 (C-15), δ 139.1 (C-16), δ 113.1 (exocyclic methylene, C-17), δ 29.7 (Me, C-18), δ 70.4 (C-19), δ 73.5 (C-20), δ 40.4 (N-Me, C-21), δ 169.1 (C-22, $COOCH_3$), δ 21.4 (C-23, CH_3COO).

$_g$HSQC (CD_3OD, 500 MHz, Nanoprobe), δ 1.65 (d) & δ 1.74 (d) /δ 31.6 (C-1), δ 5.21 (s, H-2β)/δ 66.9 (C-2), δ 1.65 (d) & δ 1.93 (d)/ δ 37.9 (C-3), δ 2.12 (s)/ δ 55.8 (C-5), δ 4.35(s)/ δ 71.7 (C-7), δ 2.20 (d)/ δ 46.7 (C-9), δ 1.86 (d)/ δ 22.1 (C-11), δ 2.95 (s)/ δ 51.6 (C-12), δ 3.00 (s)/ δ 52.5 (C-14), δ 2.48 (d) & δ 2.96 (d)/ δ 28.2 (C-15), δ 4.92 (bs) & δ 5 .00(bs)/ δ 113.1 (C-17), δ 1.52(s)/ δ 29.7 (C-18), δ 3.42 (d) & δ 3.61 (d)/ δ 70.4 (C-19), δ 3.63(s)/ δ 73.5 (C-20), δ 3.12 (s)/ δ 40.4 (C-21), δ 2.01(s)/ δ 21.4 (C-23, CH_3COO).

$_g$HMBC (CD_3OD, 500 MHz, Nanoprobe), **δC to δH, C-1**/H-3, H-20; **C-2**/H-1, H-3, H-18 (w), H-23 (-COOCH3, vw); **C-3**/H-1 (w), H-18, H-19; **C-4**/H-3, H-18, H-19; **C-5**/H-1, H-3, H-18; **C-6**/H-20, H-21; **C-7**/H-21 (w), H-5 (w); **C-8**/H-1 (w), H-11, H-14; **C-9**/H-5, H-11, H-12, H-14, H-15; **C-10**/H-7, H-20; **C-12**/ H-11, H-15 (w), H-17; **C-13**/H-11, H-12, H-14, H-20; **C-14**/H-7, H-15; **C-15**/ H-7 (w), H-17; **C-16**/H-11, H-12, H-15; **C-17**/H-12, H-15; **C-18**/H-5, H-19; **C-19**/H-5, H-18, H-20, H-21; **C-20**/H-5, H-14, H-19, H-21, **C-22**($COOCH_3$)/H-23 (CH_3COO).

δH to δC, H-1/C-2, C-3 (w), C-4, C-5, C-8 (w); **H-3**/C-1, C-2, C-4 (w), C-5; **H-5**/C-7, C-9, C-18, C-19, C-20; **H-7**/C-10, C-14; **H-11**/C-8, C-9 (w), C-12 (w), C-13, C-16; **H-12**/C-9, C-16, C-17; **H-14**/C-8, C-9, C-13, C-20; **H-15**/ C-9, C-14, C-16, C-17; **H-17**/C-12, C-15; **H-18**/C-2, C-3, C-4, C-5, C-19 ; **H-19**/C-3, C-4, C-5, C-18, C-20, C-21 (*N*-Me, w); **H-20**/C-1, C-6, C-10, C-13, C-19 (w); **H-21**/C-6, C-7, C-19, C-20, **H-23** (CH_3COO)/C-22 ($COOCH_3$), C-2(w).

LRCIMS, *m/z* 400 (MH^+); **LREIMS**: *m/z* 399 (M^+), 382, 370, 356, 340, 315, 312, 270 176, 174, 173, 148, 134, 122, 105, 91, 84, 55, 44, 43. **HREIMS,** $C_{23}H_{29}NO_5$ (M^+, measured 399.2155, calc.399.2045).



27 Virescenine

Virescenine 27: Colourless crystals (needles); m.p. 76.7-79.8^{o}C; $[\alpha]_D^{26}$ = + 14.07 (*c*, 0.8 in MeOH); **^{1}H-NMR** (500 MHz, Nanoprobe, $CDCl_3$), δ3.67 (s, 1H, H-1β), δ 1.45 and δ1.57 (q, 1H each, H-2), δ1.63 and δ1.82 (dd, 1H each, *J* = 5Hz, 6Hz, H-3), δ1.71 (t, 1H, *J* = 7 Hz, H-5), 1.54 and δ 2.26 (d, 1H each, H-6), δ2.15 (t, 1H, *J*=5.5, H-9), δ1.76 (q, 1H, H-10), δ1.55 and δ1.97 (t, 1H each, H-12), δ 2.27 (m, 1H, H-13),δ4.17 (t, 1H, *J*=4.5 Hz, H-14β), δ1.73 and 2.89 (d, 2H, *J*=9.5Hz, H-15), δ3.37 (q, 1H, H-16), δ2.83 (s, 1H, H-17), δ3.01 and δ3.16 (d, 1H each, *J*=9 Hz, H-18), δ2.49 and δ2.72 (d, 1H each, *J*=11Hz, H-19), δ2.95 and δ3.06 (q, 1H each, N-CH_2-), δ1.08 (t, 3H, *J*=7.5 Hz, N-CH_2CH_3), δ3.28 (s, 3H, C-18OCH_3), δ3.31 (s, 3H, C-16OCH_3).

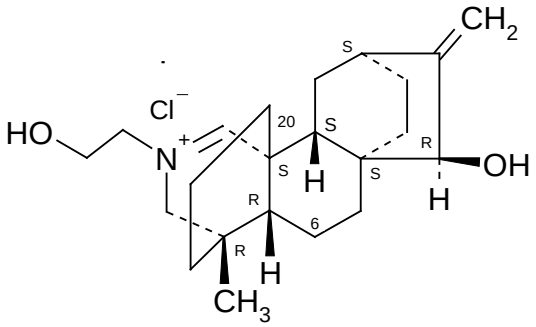
^{13}C-NMR (500 MHz, Nanoprobe, $CDCl_3$), δ72.0 (C-1), δ28.7 (C-2), δ26.6 (C-3), δ37.6 (C-4), δ41.7 (C-5), δ33.3 (C-6), δ85.7 (C-7), δ76.0 (C-8), δ47.7 (C-9), δ43.3 (C-10) , δ49.3 (C-11), δ28.2 (C-12), δ39.4 (C13), δ75.3 (C-14), δ35.8 (C-15), δ81.5 (C-16), δ65.2 (C-17), δ78.4 (C-18), δ55.7 (C-19), δ50.7 (NCH_2-), δ13.4 (NCH_2CH_3), δ59.3 (C-18OCH_3), δ56.3 (C-16OCH_3).

gHSQC (500 MHz, Nanoprobe, $CDCl_3$), δ3.67 (s, 1H, H-1β)/δ72.0 (C-1), δ 1.45 and δ1.57 (q, 1H each, H-2)/ δ28.7 (C-2), δ1.63 and δ1.82 (dd, 1H each, *J* = 5Hz, 6Hz, H-3)/ δ26.6 (C-3), δ1.71 (t, 1H, *J* = 7 Hz, H-5)/ δ41.7 (C-5), δ1.54 and δ 2.26 (d, 1H each, H-6)/ δ33.3 (C-6), δ2.15 (t, 1H, *J*=5.5, H-9)/ δ47.7 (C-9), δ1.76 (q, 1H, H-10)/ δ43.3 (C-10), δ1.55 and δ1.97 (t, 1H each, H-12)/δ28.2 (C-12), δ 2.27 (m, 1H, H-13)/ δ39.4 (C-13), δ4.17 (t, 1H, *J*=4.5 Hz, H-14β)/δ75.3 (C-14), δ1.73 and 2.89 (d, 2H, *J*=9.5Hz, H-15)/ δ35.8 (C-15), δ3.37 (q, 1H, H-16)/ δ81.5 (C-16), δ2.83 (s, 1H, H-17)/ δ65.2 (C-17), δ3.01 and δ3.16 (d, 1H each, *J*=9 Hz, H-18)/ δ78.4 (C-18), δ2.49 and δ2.72 (d, 1H each, *J*=11Hz, H-19)/ δ55.7 (C-19), δ2.95 and δ3.06 (q, 1H each, N-CH_2-)/δ50.7 (C-20), δ1.08 (t, 3H, *J*=7.5 Hz, N-CH_2CH_3)/ δ13.4 (C-21), δ3.28 (s, 3H, C-18OCH_3)/ δ59.3 (C-22), δ3.31 (s, 3H, C-16OCH_3)/ δ56.3 (C-23).

gHMBC (500 MHz, Nanoprobe, $CDCl_3$), **δC to δH**, **C1**/H-3, H-5; C-**2**/H-3 (w); **C-3**/H-1, H-18, H-19; **C-4**/H-3, H-5, H-6, H-18, H-19; **C-5**/H-6, H-10, H-17, H-18, H-19; **C-6**/H-17; **C-7**/H-5, H-6, H-15; **C-8**/H-5, H-6, H-9, H-10, H-14, H-15, H-17; **C-9**/H-10, H-13, H-15; **C-10**/H-1, H-2, H-5, H-9, H-13, H-12, H-15, H-17; **C-11**/H-12, H-10, H-17; **C-12**/H-9, H-10, H-16 (w); **C-13**/H-9, H-12, H-15; **C-14**/H-12, H-16; **C-15**/H-9, H-13; **C-16**/H-12, H-14, H-15, H-23; **C-17**/H-5, H-6, H-10, H-19, H-20 (w); **C-18**/H-3, H-5, H-19, H-22; **C-19**/H-3, H-5, H-17, H-18, H-20; **C-20**/H-17 (w), H-19, H-21; **C-21**/H-20; C-**22**/H-18; **C-23**/H-16.

δH to δC, H-1/C-3, C-5; **H-2**/C-4,C-5,C-11; **H-3**/C-1,C-4, C-18, C-19; **H-5**/C-4, C-7, C-10, C-11, C-17, C-18, C-19; **H-6**/C-4,C-5,C-7,C-11,C-17; **H-9**/C-8,C-10,C-12,C-13,C-15; **H-10**/C-1(w),C-2,C-8,C-5, C-9, C-11, C-12, C-17; **H-12**/C-5, C-10, C-11, C-13, C-14, C-16; **H-13**/C-9, C-10, C-15, C-16; **H-14**/C-8, C-16; **H-15**/C-6, C-7, C-8, C-9, C-13, C-16; **H-16**/C-12 (w), C-14, C-23; **H-17**/C-5, C-6, C-8, C-10, C-11, C-19, C-20; **H-18**/C-3, C-4, C-5, C-19, C-22; **H-19**/C-3, C-4, C-5, C-17, C-18, C-20; **H-20**/C-17, C-19, C-21; **H-21**/C-20; **H-22**/C18; **H-23**/C-16.

LRCIMS, *m/z* 424**; HREIMS,** $C_{23}H_{37}NO_6$ (M^+, measured 423.2619, calc. 423.2620). **LREIMS,** *m/z* 423 (M^+), 408, 407, 406, 390, 374, 365, 352, 336, 98, 85, 71, 58 (100%).



28 Atisinium chloride

Atisinium chloride 28: Crystals (needle shaped); $[\alpha]_D^{25}$ = +31.27 (*c*, 0.94 in MeOH); m.p. 230.7-233.6°C (decomposition); **LREIMS**, *m/z* 343 (M^+), 342, 328, 314, 300, 284, 257, 241, 186, 171, 159, 143, 131, 105, 91, 79, 72, 55, 44. **HRESMS (electro-spray)**, $C_{23}H_{33}NO_2$ (measured 344.2572, calculated 344.2590). The structure was determined and confirmed by single crystal X-ray crystallography (**Figure 10**).

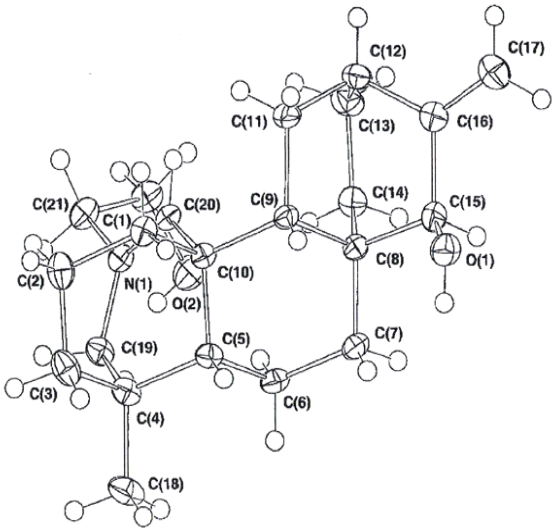


Figure 10. The X-ray structure of Atisinium chloride.

AO- 358: white solid; m.p. 122.6°-124.2°C, $[\alpha]_D^{27}$ = +86.6 (*c*, 0.09 in MeOH); LRCIMS, *m/z* 358 (MH^+); HREIMS**,** *m/z* 357 (M^+), 344, 342, 329, 328 (100%), 327, 326, 312, 300, 286,284,250, 234, 148,122, 105, 91, 77, 71, 60. **HRCIMS,** $C_{22}H_{32}NO_3$ (MH^+, measured 358.2399, calculated 358.2382). Another alkaloid with same LRCIMS peak at *m/z* 358 (MH^+) was obtained. It had a lower RF than the one described above. **HRCIMS**, $C_{15}H_5NO_{10}$ (MH^+, measured 358.9901, calculated 358.9913).

AO-466: pale green solid, **LRCIMS**, *m/z* 466 (MH^+); **LREIMS,** *m/z* 465 (M^+), 450, 448, 432, 422, 403, 402 (100%), 390, 374, 358, 342, 329, 300, 284, 194, 164, 148, 122, 98, 91; **HRCIMS**, $C_{25}H_{40}NO_7$ (MH^+, measured 466.2785, calculated 466.2804).

AO-340: white solid; **LREIMS,** *m/z* 339 (M^+), 322, 310, 297, 283, 245, 179, 159, 128, 124 (100%), 108, 94, 79, 65, 55 and 42. **HRCIMS**, $C_{21}H_{26}NO_3$ (MH^+, measured 340.1912, calculated 340.1912).

AO-372: pale brown solid; **LREIMS**, *m/z* 371 (M^+, 100%), 355, 343, 327, 299, 296, 238, 210, 192, 162, 134, 122 and 105; **HRCIMS**, $C_{21}H_{26}NO_5$ (MH^+, measured 372.1816, calc. 372.1811).

AO-414: pale green solid; **LREIMS,** *m/z* 413 (M^+), 385, 369, 354, 342, 310, 280, 252, 220, 212, 148, 134, 122, 105, 91, 79, 67, 57, 55, 44 (100%), 43, 42; **HREIMS,** $C_{23}H_{27}NO_6$ (M^+, measured 413.1822, calculated 413.1838).

AO-330: white solid; **HRCIMS,** $C_{20}H_{28}NO_3$ (MH^+, measured 330.2066, calculated 330.2069).

9.6 Purification and Structure Elucidation of Alkaloids from *Corydalis gerdae* Fedde.

A methanol extract (58.22 g) was obtained from dried plant material (1 kg). Part of the methanol extract (15.22 g) was kept refrigerated as reference material. Antimicrobial testing was done on a portion of this reference material. The remaining MeOH extract (43 g) was extracted for alkaloids using the general schematic isolation and extraction procedure (**Scheme 3**). This yielded crude alkaloids (303.8 mg) and antimicrobial testing was conducted on a portion of this alkaloid mixture (40 mg). The mass spectral analyses (CIMS) on the crude alkaloids revealed ion peaks at *m/z* 354, 338, 326, 324, and 224. The ion peak at *m/z* 354 was the major component. Focussing on these ion peaks, the isolation and purification was conducted using preparative TLC.

The crude alkaloids (about 260 mg) was dissolved in MeOH (10%):DCM (90%) solvent and then applied to PTLC silica gel plates (65 g × 4 plates). A mixture of DCM (95%): MeOH (4%): Concentrated aqueous NH_3 solution (28%, 1 ml) was found to be the best solvent system for separating this crude extract. The solvent was allowed to saturate the tank for 1 hour and the PTLC separation was then performed. Ten bands were obtained and each of them contained different compounds with CIMS ion peaks (MH^+) as tabulated below (**Table 27**).

Table 27. Bands separated from the crude alkaloid of *Corydalis gerdae*

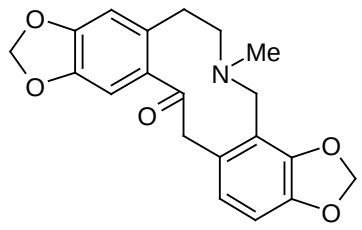
Bands	**Net wt. (mg)**	**Peaks present LRCIMS (*m/z*)**	**Suitable solvent system used for purifying respective bands (PTLC)**	**Compound isolated (*m/z*)**
1+2	24.6	237, 257, 308,354	DCM (83ml):MeOH (17ml):NH_3 (1ml)	354
3+4	21.9	328,354,410	DCM (93ml):MeOH (7ml):NH_3 (1ml)	328
5	28.8	282, 312, 328, 354	DCM(94ml):MeOH (5ml):NH_3 (1ml)	328
6	66.5	354,410	DCM (94ml):MeOH (5ml):NH_3 (1ml)	354
7+8	57.8	257, 312, 326, 354	DCM (98.5 ml) : MeOH (1ml):NH_3 (0.5ml)	326, 338
9	28.0	257, 324	DCM (99ml): MeOH (0.5 ml):NH_3 (0.5 ml)	324
10	12.6	257, 338, 354,371	DCM (99ml): MeOH (0.5 ml):NH_3 (0.5 ml)	282, 338

The bands/fractions were initially not pure and contained mixtures of compounds. They were separated again by PTLC on silica gel by developing in different solvent systems and each of these first bands gave two or more bands with different mass peaks

(LRCIMS). The fractions with the same Rf values and same LRCIMS peaks were combined and were again purified by developing appropriate solvent system and the purification was repeated till pure compounds were obtained (**Table 28**). Crystallization was attempted for all fractions using single solvent and a two different solvents system. In a crystallization which involved two solvent systems (chloroform/ethanol, MeOH/acetone, ethanol/acetronitrile and MeOH/diethyl-ether), the compound was first dissolved in the more powerful solvent, and the second, more volatile and less powerful solvent allowed it to diffuse into it, which facilitated steady growth of large crystals. However, only cheilanthifoline gave small crystals from methanol/diethyl-ether. The crystals were washed with diethyl-ether and was collected and sent for synchrotron X-ray crystallography to USA. This result will further confirm its structure but its result was not available at the time of completion of this thesis. Finally four alkaloids were isolated from this plant.

Table 28. Alkaloids isolated from *Corydalis gerdae*

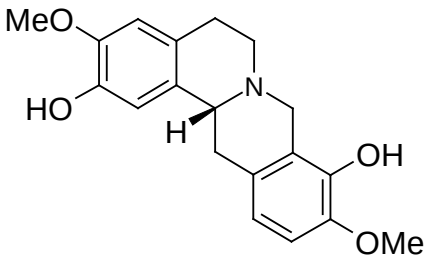
Alkaloids	**Mol.Wt.**	**Molecular formula**	**Wt. obtained**
Protopine	353	$C_{20}H_{19}NO_5$	23.1
Scoulerine	327	$C_{19}H_{21}NO_4$	9.2
Cheilanthifoline	325	$C_{19}H_{19}NO_4$	6.5
Stylopine	323	$C_{19}H_{17}NO_4$	5.9
Unidentified	337	$C_{19}H_{15}NO_5$	1.6



37 Protopine

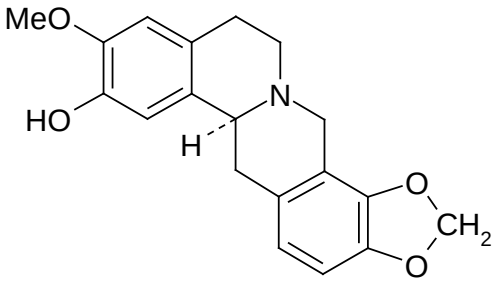
Protopine 37: white amorphous solid; m.p. 203.7-207.8^{o}C (decomposition; optically inactive; **LRCIMS,** *m/z* 354 (MH^+); **HREIMS**, $C_{20}H_{19}NO_5$ (MH^+, measured 354.1342, calc.354.1341); **HREIMS** *m/z* 353 (M^+), 281, 267, 252, 190, 163, 149, 148 (100%) 134 and 89. **^{1}H-NMR** (500 MHz, Nanoprobe, DMSO-d_6), δ6.92 (s, H-1), δ6.77 (s, H-3), δ3.15 (s, H-5), δ2.38 (bs, H-6), δ3.47 (bs, H-8), δ6.74 (d, *J*=7.5 Hz, H-11), δ6.66 (d, *J*=8.0 Hz, H-12), δ5.96 (s, 2,3-OCH_2O), δ5.93 (s, 9,10-OCH_2O), δ1.79 (s, 7-CH_3). **^{13}C-NMR** (500 MHz, Nanoprobe, DMSO-d_6), δ107.4 (C-1), δ145.3 (C-2), δ147.3 (C-3), δ110.4 (C-4), δ132.7 (C-4a), δ30.5 (C-5), δ57.4 (C-6), δ50.7 (C-8), δ118.3 (C-8a),

δ145.8 (C-9), δ145.4 (C-10), δ106.3 (C-11), δ125.1 (C-12), δ129.6 (C-12a), δ46.1 (C-13), δ194.8 (C-14), δ136.0 (C-14a), δ101.1 (2,3-OCH_2O), δ41.1 (7-CH_3), δ100.7 (9,10-OCH_2O).



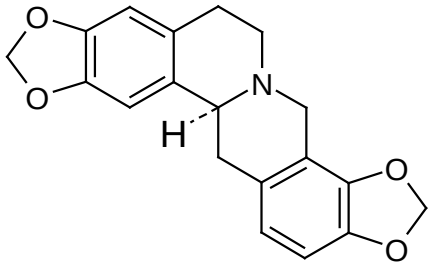
41 Scoulerine

Scoulerine 41: reddish brown solid; m.p. 153-157°C (decomposition); $[\alpha]_D^{24}$ = ─282 (*c*, 0.74 in MeOH); **^{1}H-NMR** (CD_3OD, 300 MHz): δ 3.21 (s, OMe), δ 3.24 (s, OMe), δ 5.38 (s, OH), δ 3.52 and δ 4.14 (AB-quartet, *J* = 15.9 Hz). **HREIMS**, $C_{19}H_{21}NO_4$ (measured 327. 1461, calc.327.1471); **HREIMS:** *m/z* 327 (100%, M^+), 326, 312, 310, 207, 179, 178 (100%), 176, 162, 150, 149 and 135.



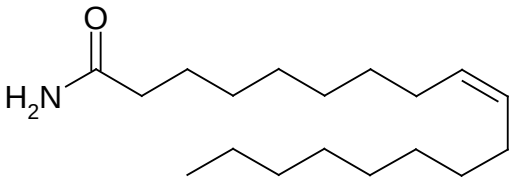
47 (-)-(*S*)-cheilanthifoline

Cheilanthifoline 47: pale green solid**;** m.p. 168-170.4°C (decomposition); $[\alpha]_D^{20}$ = ─262 (*c*, 0.60 in $CDCl_3$); **LRCIMS,** *m/z* 326 (MH^+); **LREIMS,** *m/z* 325 ($M^{+\cdot}$ 100%), 310, 176, 162 and 148 (100%); **HREIMS,** $C_{19}H_{19}NO_4$ (measured 325.1304, calc.325.1314); **^{1}H-NMR** ($CDCl_3$, 500 MHz): δ 3.87 (s, 3H, OMe), δ 5.93 (d, 2H, methylenedioxy), δ 3.53(q) and δ 4.09(d) (C-8 methylene, *J* = 15 Hz), δ 1.25 (bs, -OH), δ 6.59 to δ 6.81 (4 H, aromatic).



42 Stylopine

Stylopine 42: pale green solid; m.p. 163-165^{o} C; $[\alpha]_D^{24}$ = ─304 (*c*, 0.56 in MeOH); **LRCIMS**: *m/z* 324 (MH^+); **LREIMS**: *m/z* 323 (M^+), 207, 174, 148 (100%) and 89. **HREIMS**: $C_{19}H_{17}NO_4$; (measured 323.1145, calc.323.1157).



48 9-octadecenamide

9-Octadecenamide 48: white solid; blue fluorescence under UV; **LRCIMS,** *m/z* 338 (MH^+); **HREIMS,** $C_{22}H_{43}NO$ (measured 337.3382, calc.337.3344); **HREIMS,** *m/z* 337 (M^+), 277, 136, 122, 97, 83, 72, 69, 59 (100%), 43 and 41.

CG-338: white solid; **LRCIMS**, *m/z* 338 (MH^+); **HREIMS**, $C_{19}H_{15}NO_5$ (measured 337.0940, calc.337.0936).

CG-336: white solid; **LRCIMS,** *m/z* 336 (MH^+); **LREIMS,** *m/z* 335 (M^+, 100%), 320, 306, 292, 248, 190, 167, 153, 139, 124, 95, 82, 75 and 63. **HREIMS**, $C_{15}H_{29}NO_7$ (measured 335.1939, calc.335.1944).

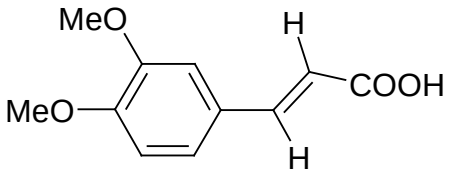
9.7 Attempted Extraction and Isolation of Alkaloids from *Tribulus terrestris* L

9.7.1 Attempt-I.

A methanol extract (127.74 g) was obtained from air dried plant material (1.2 kg). Antimicrobial testing was performed on a part (15 mg) of this extract. The remaining methanol extract was extracted for alkaloids using the general schematic isolation and extraction procedure (**Scheme 3**) and yielded a crude alkaloid fraction (540 mg). However, when the crude fraction was tested with Dragendorff's reagent, it gave a negative test. A portion of this crude extract (25.6 mg) was kept as reference material.

The mass spectral analysis (LRCIMS) on this crude extract revealed ions at *m/z* 144, 163, 175 (major component), 191, 193, 209, 225 and 265.

The crude alkaloid extract was dissolved in MeOH (10%) /DCM (90%) solvent and was loaded on to silica PTLC plates (65 g × 8 plates). After trying different ratios, a mixture of DCM (94%): MeOH (6%): concentrated aqueous NH_3 solution (28%, 3 drops) was selected as the best solvent system for separating this crude extract. The solvent system was left to saturate the tank for 1 hour and the PTLC separation was then performed. Ten bands were separated and each band was tested for alkaloids. None gave a positive test with Dragendorff's reagent. One band yielded 3,4-dimethoxycinnamic acid, as the major component isolated.



62 3,4-Dimethoxycinnamic acid

3,4-Dimethoxycinnamic acid 62: pale green solid; absorbed UV light; **^{1}H-NMR** ($CDCl_3$, 300 MHz), δ 7.70 (d, 1H, *J* = 15.9 Hz), δ 7.07 to δ 7.13 (3H, aromatic protons), δ 6.92 (d, 1 H, *J* = 8.4 Hz), δ 3.80 (s, 3H, OMe) and δ 3.93 (s,3H,OMe); **LRCIMS**, *m/z* 209 (MH^+); **HRCIMS**, $C_{11}H_{13}NO_4$ (MH^+, measured 209.0817, calc.209.0813).

9.7.2 Attempt-II

To recheck the result obtained in the first attempt to isolate alkaloids from *Tribulus terrestris*, the extraction and the isolation of alkaloids from this plant was attempted for a second time using a slightly modified procedure. In this method, the air dried plant material (1.5 kg) was ground using a Grinder (mixer) and was then extracted with analytical grade methanol for 5 days. The separated methanol extract was evaporated and the residue was acidified (to pH 1) with 1 M H_2SO_4 (15 ml). The acidic solution was washed with chloroform and petroleum ether (4 × 60 ml). The aqueous layer was then basified (to pH 10) with Na_2CO_3 solution (20% w/v) and then extracted with chloroform (4 × 60 ml). This chloroform extract was evaporated and the residue tested for alkaloids. The crude extract failed to give any test for alkaloids when tested with the

Dragendorff's reagent and with Mayer's reagent. This confirmed that this batch of *Tribulus terrestris* contained no alkaloids.

9.8 Attempted Isolation of Alkaloid from *Ranunculus brotherusi* Freyn

A methanol extract (85.01 g) was obtained from air dried plant material (1 kg) and a small portion of this extract (15.90 g) was kept as a reference sample. The remaining methanol extract was extracted for alkaloids using the general schematic isolation and extraction procedure (**Scheme 3**) and yielded a crude alkaloid fraction (72.8 mg). This crude extract gave a negative test for alkaloids with Dragendorff's reagent. The **LRCIMS** of the crude alkaloid extract gave ions at *m/z* 225, 209, 207, 197, 195 (major peak), 193, 191, 175, 163, 145, 127 and 86.

All the crude basic extract was separated using PTLC plates (36 g × 2 plates) and the solvent system: DCM (90%):MeOH (10%):Concentrated aqueous NH_3 solutions(28%, 1 ml). The solvent was allowed to saturate the tank for 1 hour and PTLC separation was then performed. Eleven bands were separated and each band contained a mixture of different compounds (on the basis of LRCIMS analyses). None of the fractions gave a positive test with Dragendorff's reagent. No further work was done on this plant extract.

9.9 Antibacterial assay

9.9.1 Sample Preparation

The methanol extract (AO-ME, 10 mg), crude alkaloid (AO-CEA, 10 mg) and atisinium chloride isolated from *Aconitum orochryseum* were packed in small vials. Similarly, the methanol extract (CG-ME, 10 mg), crude alkaloid (CG-CEA, 10 mg) and protopine isolated from *Corydalis gerdae* were packed in small vials. These samples were then sent for antibacterial assays to the Amrad Corporation, Melbourne. All assays used the *Staphylococcus aureus* strain ATCC 6538P, or VRE strains 243, 449, 820 and 987. It should be noted however that two strains, VRE 243 and 987 were in fact sensitive to vancomycin.

9.9.2 Antibacterial Testing Methodology[263]

The Mueller-Hinton Broth (MHB) Medium culture media was prepared with final concentrations of 1 μg/mL $MgCl_2$ and 2 μg/mL $CaCl_2$ and was pre-warmed for 2-3 hour at 37^{o}C before use**.** Mueller-Hinton Agar (MHA) Medium culture media was prepared with final concentrations of 1.5% Agar (Merck Agar 1.01614). *S.aureus* was

streaked onto MHA and the plate was incubated overnight at 37^{o}C. From this plate, 10 cryovial were prepared by looping several colonies into 0.5 mL of 20% glycerol solution and were immediately stored at −140^{o}C. A cryovial was removed from −140^{o}C storage and thawed at room temperature. The MHA plate was streaked with a loopful of bacterial suspension and incubated overnight at 37^{o}C to create a parent plate (P1). The parent plate was stored at 4^{o}C. A daughter plate (D1) was incubated overnight at 37^{o}C and its loop of colony was used to inoculate a 125 mL flask containing 20 mL of MHB containing 25 μg/mL $CaCl_2.2H_2O$) and 12. 5 μg/mL $MgCl_2.6H_2O$. The flask was shaken at 260 rpm for 18 hour at 37^{o}C on an orbital incubator shaker. The parent plates 1 and 2 were each used twice to generate two daughter plates (D1 and D2) before being discarded.

The standardised inocula for assays was prepared as 1/10 dilution of seed cultures by adding 250 μL of the cultures to 2,250 μL of MHB in a disposable cuvette and the required dilution factor was calculated by dividing the observed OD_{650}. Sufficient volumes of the final inoculum cultures were prepared in pre-warmed MHB (37^{o}C) by diluting the standardised cultures to the required final concentration (10^8 dilution).

9.9.3 Assay Procedure for 96-well Microtitre Plates

To each well of the 96-well microtitre plate was added 50 μL of liquid medium and 50 μL of peptoid test solution which was prepared by dissolving in 2.5 % DMSO was added in triplicate to the top of the microtitre plate. A vancomycin control set (triplicate) and a compound negative control set (triplicate) were also set up on each plate. The inoculated culture medium was incubated at 37^{o}C for 30 min shaking it at 130 rpm and using the multichannel pipette and multistepper pipette the adding, transferring and mixing of the inoculum were performed on the wells of the plates. The plates were incubated at 37^{o}C for 18 hrs shaking at 100 rpm in an environment with 90% relative humidity and the results were recorded as the highest dilution of test compound that prevented bacterial growth (MIC). The MIC was also determined for DMSO (2.5%) as a control measure.

9.9.4 Results of Antibacterial Testing

When the methanol extracts, crude alkaloids and the major alkaloids (protopine and atisinium chloride) of the plants were tested, the following results (**Table 29**) were obtained.

Table 29. Antibacterial activities of the plant extracts and alkaloids on *Staphylococcus aureus* and Vancomycin resistant *Enterococcus faecium*

Compound	Culture	MIC (μg/ml)
AO-ME	Staph	>125
AO-CEA	Staph	>125
Orochrine	Staph	>125
	VRE # 243, VRE # 449, VRE # 820 and VRE # 987	>125
Atisinium chloride	Staph	>125
	VRE # 243, VRE # 449, VRE # 820 and VRE # 987	>125
CG-ME	Staph	>125
CG-CEA	Staph	>125
Protopine	Staph	>125
Vancomycin (Standard)	Staph	2.5
	VRE # 243, VRE # 449, VRE # 820 and VRE # 987	1.95 (< 0.98), 62.5, 125 and 1.95 respectively

Note: VRE = Vancomycin resistant Enterococcus; Staph = *Staphylococcus aureus*; Vanc = Vancomycin, MIC = Minimum Inhibitory Concentration.

9.10 Antimalarial assay

9.10.1 Sample Preparation

The methanol extract (AO-ME, 20 mg), crude alkaloid (AO-CEA, 20 mg) and atisinium chloride isolated from *Aconitum orochryseum* were packed in small vials. Similarly, the methanol extract (CG-ME, 20 mg), crude alkaloid (CG-CEA, 20 mg), protopine and cheilanthifoline, isolated from *Corydalis gerdae*, were packed in small vials. These samples were then sent for antimalarial testing at the Protein-Ligand Engineering and Antimalarial Screening Laboratories, National Centre for Genetic Engineering and Biotechnology, National Science and Technology Development Agency in Bangkok, Thailand. The tests were done by Dr. Sumalee Kamchonwongpaisan and by Miss Roonglawan Rattanajak using a Microdilution Radioisotopes Technique.

9.10.2 Antimalarial Assay Method

Samples were made up in DMSO solution. Using the Microdilution Radioisotope Technique, the *in vitro* antimalarial activity of the alkaloids was tested against *Plasmodium falciparum*, TM4 and K1 Strains. The first strain is an anti-folate sensitive one while the second is an antifolate resistant strain.

The sample (25 μl, in the culture medium) was placed in triplicate in a 96-well plate. Red blood cells (200 μl) infected with *Plasmodium falciparum* with a cell suspension (1.5%) of parasitemia (0.5-1%) were added to the wells. The range of the final concentrations of the samples varied from 1 x 10^{-5} to 1 x 10^{-8} g/ml with 0.1% of the organic solvent. The plates were cultured under standard conditions for 24 hours and the ^{3}H-hyphoxanthine (25 μl, 0.5 mCi) was added. The culture was incubated for 18-20 hours. The parasites' DNA was then harvested from the culture onto glass fibre filters. A radiation counter determined the amount of ^{3}H-hypoxanthine. The inhibitory concentration of the sample was determined from its dose-response curves or by calculation.

The Trager and Jensen method[264] was used to culture *Plasmodium falciparum* K1 strain. The parasites were maintained in human red blood cells in a culture medium. RPMI 1640 was supplemented with 25 mM HEPES, 0.2% sodium bicarbonate, and 8% human serum, at 37^{o}C in a CO_2 incubator[54].

9.10.3 Results of the Antimalarial Assay

The following *in vitro* antimalarial test results (**Table 30**) were obtained for the methanol extracts (AO-ME and CG-ME), crude alkaloid mixtures (AO-CEA and CG-CEA), atisinium chloride, protopine and cheilanthifoline.

Table 30. Antimalarial activities of extracts and alkaloids isolated from *Aconitum orochryseum* and *Corydalis gerdae* (IC_{50} value against *Plasmodium falciparum*; TM4 and K1 strains).

		IC_{50}	
Plants	**Sample**	**TM4**	**K1**
Aconitum orochryseum	**AO-ME**	>10 μg/ml	>10 μg/ml
	AO-CEA	20.4 ± 1.74 μg/ml	19.2 ± 3.84 μg/ml
	Atisinium chloride	4.02 ± 0.69 μ M	3.59 ± 1.24 μ M
		(1.51 μg/ml)	(1.35 μg/ml)
Corydalis gerdae	**CG-ME**	1.00 ± 0.34 μg/ml	2.56± 0.46 μg/ml
	CG-CEA	0.33 ± 0.03 μg/ml	0.63 ± 0.14 μg/ml
	Protopine	4.25 ± 0.69 μ M	4.29 ± 1.24 μ M
		(1.50 μg/ml)	(1.51 μg/ml)
	Cheilanthifoline	2.78 ± 0.39 μ M	3.76 ± 1.00 μ M
		(0.90 μg/ml)	(1.22 μg/ml)

An antibacterial and antimalarial testing were not done for *Ranunculus brotherusi* and *Tribulus terrestris* extracts.

References

(1) Hulse, H. J. Ethical issues in biotechnologies and international trade. *J. Chem. Technol. and Biotechnol.* **2002**, *77*, 607-615.

(2) Dax, S. L. *Antibacterial chemotherapeutic agents*; First ed.; Blackie Academic & Professional, Chapman & Hall: London, UK, 1997.

(3) Miller, C. J.; Shattock, R. J. Target cells in vaginal HIV transmission. *Microbes Infect.* **2003**, *5*, 59-67.

(4) *Microsoft Encarta Premium Suite*; 12.0.0.0.0602 ed.; Microsoft Corporation; Website:http://www.microsoft.com/mswish.: Raymond, USA.

(5) A Global Strategy for Malaria Control; World Health Organization: Geneva, 1993.

(6) Rouhi, A. M. Tuberculosis: A tough adversary. In *Chem. Eng. News*, **1999**; pp 52-70.

(7) Rodriguez, A. D.; Ramirez, C.; Rodriguez, I. I.; Gonzalez, E. Novel antimycobacterial benzoxazole alkaloids, from the West Indian Sea Whip *Pseudopterogorgia elisabethae*. *Org. Lett.* **1999**, *1*, 527-530.

(8) Walsh, W. Molecular mechanisms that confer antibacterial drug resistance. *Nature* **2000**, *406*, 775-781.

(9) Hanno, H. Types, characteristics, and break points of antibacterial drugs. *Kagaku Ryohono Ryoiki* **1997**, *13*, 1365-1372.

(10) Williams, R. A. D.; Lambert, P. A.; Singleton, P. *Antimicrobial drug action*; Bios Scientific Publishers Ltd,: UK,, 1996.

(11) Levy, S. B. The challenge of antibiotic resistance. *Sci. Am.* **1998**, *278*, 46-53.

(12) Mitscher, L. A.; Pillai, S. P.; Gentry, E. J.; Shankel, D. M. Multiple drug resistance. *Med. Res. Rev.* **1999**, *19*, 477-496.

(13) Lipsitch, M. The rise and fall of antimicrobial resistance. *Trends in Microbiol. Review* **2001**, *9*, 438-444.

(14) Dever, L. A.; Dermody, T. S. Mechanisms of bacterial resistance to antibiotics. *Arch. Int. Med.* **1991**, *151*, 886-895.

(15) Claude, C.; Raul, I. Narrow versus broad spectrum antibacterials: factors in the selection of pneumococcal resistance to beta-lactams. *Drugs* **2002**, *62*, 1289-1294.

(16) Pradipsinh, K. R.; McErlean, T.; Lee, P. C. Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci, USA* **1997**, *94*, 9389-9393.

(17) Hardwicke, C. J. The World Health Organization and the Pharmaceutical Industry. Common areas of interest and differing views. *Adverse Drug React. and Toxicol. Rev.* **2002**, *21*, 51-99.

(18) Phillipson, J. D. Phytochemistry and medicinal plants. *Phytochemistry* **2001**, *56*, 237-243.

(19) Phillipson, J. D. Radioligand-receptor binding assays in the search for bioactive principles from plants. *J. Pharm. Pharmacol.* **1999b**, *51*, 493-503.

(20) Hyde, J. E. Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes Infect.* **2002**, *4*, 165-174.

(21) Kondrashin, A. V.; Rooney, W. Overview: Epidemiology of malaria and its control in countries of the WHO South-East Asia region. *South East Asian J. Trop. Med. Pub. Health* **1992**, *23*, 13-22.

(22) Van Agtmael, M. A.; Eggelte, T. A.; Van Boxtel, C. J. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *TiPs* **1999**, *20*, 199-206.

(23) Zhang, H. J.; Tamez, P. A.; Hoang, V. D.; Tan, G. T.; van Hung, N. et al. Antimalarial compounds from *Rhaphidophora decursiva*. *J. Nat. Prod.* **2001**, *64*, 772-777.

(24) Federici, E.; Palazzino, G.; Galeffi, C.; Nicoletti, M. Antiplasmodial activity of the alkaloids of *Peschiera fuchsiaefolia*. *Planta Med.* **2000**, *66*, 93-95.

(25) Valsaraj, R.; Pushpangadan, P.; Smitt, U. W.; Adesersen, A.; Christensen, B. S. et al. New anti-HIV-1, antimalarial, and antifungal compounds from *Terminalia bellerica*. *J. Nat. Prod.* **1997**, *60*, 739-742.

(26) Nosten, F.; Brasseur, P. Combination therapy for Malaria, the way forward? *Drugs* **2002**, *62*, 1315-1329.

(27) Foote, S. J.; Kyle, D. E.; Martin, R. K., et al. Several alleles of the multidrug resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* **1990**, *345*, 255-258.

(28) Austin, D. J.; Kristinsson, K. G.; Anderson, R. M. The relationship between the volume of antimicrobial consumption in human community and the frequency of resistance. *Proc. Natl. Acad. Sci, USA* **1999**, *96*, 1152-1156.

(29) Sepala, H. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. *New Engl. J. Med.* **1997**, *337*, 441-446.

(30) Guillemot, D.; Carbon, C.; Balkau, B. Low dosage and long treatment duration of β–lactam.Risk factors for carriage of penicillin-resistant *Streptococcus pneumoniae*. *JAMA* **1998**, *279*, 365-370.

(31) Patrick, G. *Medicinal Chemistry-Instant Notes*; First ed.; Bios Scientific Publishers Limited, UK: Leeds, UK, 2001; 77-83.

(32) Gyllenhaal, C. Intellectual property rights, naturally derived bioactive compounds, and resource conservation. Meeting report. *J. Nat. Prod.* **1996**, *59*, 334-337.

(33) Harvey, A. L. Medicines from nature: are natural products still relevant to drug discovery? In *TiPS*, 1999; pp 196-198.

(34) Verpoorte, R. Chemodiversity and the biological role of secondary metabolites, some thoughts for selecting plant material for drug development. *Bioassay Methods in Natural Product Research and Drug Development*; Kluwer Academic Publishers: The Netherlands, 1999; pp 11-23.

(35) Bremner, J. B. *Lecture/Tutorial course outline-natural products in drug discovery*; Department of Chemistry, University of Wollongong: Wollongong, 2002.

(36) James, A. A.; Robert, W. B.; Wayne, M. B.; Lindsay, T. B.; Jack, R. C.; Steven, M. C.; Anthony, R. G.; Neville, G. M.; Neil, R.; Melvyn, V. S.; Colette, G. S.; Zdzislaw, E. S.; Robert, D. T. Constituents of *Conospermum brachyphyllum*. Improved methods for the isolation and synthesis of (+)-Conocurvone and the structure of (+)-Brachyphyllone. *Aust. J. Chem.* **1999**, *52*, 57-62.

(37) Hostettmann, K.; Marston, A.; Wolfender, J. L. Strategy in the search for new biologically active plant constituents. *Phytochemistry of Plants Used in Traditional Medicine*; Oxford University Press Inc. New York: Laussane,Switzerland, 1993; pp 17-45.

(38) Cragg, G.; Newman, D. Chemists' toolkit, Nature's bounty. *Chem. Brit.* **2001**, 22-26.

(39) Merrilyn, A. K. *Complementary Therapies for Health Care Providers*; Lippincott williams and Wilkins: Baltimore, 1999.

(40) Kinghorn, A. D.; Balandrin, M. F. *Human Medicinal agents from plants*; ACS Symposium Series 534, Washington, DC., 1993.

(41) Elmqvist, T. Plant biodiversity-evolutionary and ecological perspectives. *Bioassay Methods in Natural Product Research and drug Development*; Kluwer Academic Publishers: Biomedical Centres of Uppsala University, Sweden, 1997; pp 1-9.

(42) Heywood, V. H. Global biodiversity assessment,; UNEP, Cambridge University press, 1995; pp 174-185.

(43) Wilkinson, J. A.; Wahlqvist, M. L.; Clark, J. New food and pharmaceutical products from agriculture . Rural Industries Research and Development Corporation: Australia, 2002; pp 1-30.

(44) Hesse, M. *Alkaloids, nature's curse or blessing?*; Verlag Helvetica Chimica Acta and Wiley-VCH: Zurich, 2002; 293.

(45) Fusetani, N. Introduction. *Drugs from the Sea*; Karger: Tokyo, Japan, 2000; pp 1-5.

(46) Roberts, M. F.; Wink., M. Introduction. *Alkaloids: Biochemistry, Ecology, and Medicinal Application*; Plenum press: New York, 1998; pp 1-7.

(47) Southon, I. W.; Buckingham, J. *Dictionary of Alkaloids*; Chapman and Hall: London, 1989.

(48) Pelletier, S. W. Introduction. *Chemistry of the alkaloids*; Van Nostrand Reinhold Company: New York, 1970; pp 1-10.

(49) Cordell, G. A.; Quinn-Beattie, M. L.; Farnsworth, N. R. The potential of alkaloids in drug discovery. *Phytother. R.* **2001**, *15*, 183-205.

(50) Yunusov, S. Y. Dynamics of accumulation, formation of alkaloids, and their role in plants. *Akad. Nauk Uz. SSR* **1966**, *2*, 104-106.

(51) Polonovski, M. The method of formation and the role of alkaloids in plants. *Bull. Soc. Chim.* **1926**, *35*, 1365-1398.

(52) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **2001**, *46*, 3-26.

(53) King, S. R.; Tempesta, M. S. From shaman to human clinical trials: the role of industry in ethnobotany, conservation and community reciprocity. *CIBA Foundation Symposium*; Shaman Pharmaceutical Inc.: South San Francisco, 1994; pp 197-213.

(54) Hadi, S. Bioactive alkaloids from medicinal plants of Lombok (PhD Thesis). PhD Thesis; University of Wollongong: Wollongong, 2002

(55) Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564-582.

(56) Daly, J. W.; Garaffo, H. M.; Spande, T. F.; Decker, M. W.; Sullivan, J. P. et al. Alkaloids from frog skin: the discovery of epibatidine and the potential for developing novel non-opiod analgesics. *Nat. Prod. Rep.* **2000**, *17*, 131-135.

(57) Jensen, P. R.; Fenical, W. Marine microorganisms and drug discovery: Current status and future potential. *Drugs from the Sea*; Karger: Tokyo, 2000.

(58) Benkendorf, K. Bioactive molluscan resources and their conservation: biological and chemical studies on egg masses of marine molluscs (PhD Thesis). PhD; University of Wollongong: Wollongong, 1999

(59) Wink, M. A short history of alkaloids. *Alkaloids-Biochemistry, Ecology, and Medicinal Applications*; Plenum Press: New York, 1998; pp 11-44.

(60) Neuwinger, H. D. Alkaloids in Arrow Poisons. *Alkaloids; Biochemistry, Ecology and Medicinal Applications*; Plenum Press: New York, 1998; pp 45-84.

(61) Smanla, A. T. A brief introduction to traditional Tibetan medicine in Ladakh (online); Website:http://home.t-online.de/home/520097278994-0001/yuthog/engl/med.html; Neugebauer, A., 1998.

(62) Wangchuk, P. Natural product-based drug discovery: Recent development (literature review); University of Wollongong., 2002.

(63) Mann, J. *Murder, Magic and Medicine*; Oxford University Press: London, 1992.

(64) Tokar, E. Tibetan medicine (online); Website: http://www.tibetanmedicine.com; Eliot Tokar, 1998.

(65) Dompnier, R. The Art of Healing. In *Tashi Delek, Druk air's in-flight magazine*, 1998.

(66) Dorji, P.; Morisco, P. *An Introduction to Traditional Medicine in Bhutan*; National Institutes for Traditional Medicines, Ministry of Health and Education: Thimphu, 1989.

(67) Bright, M. A. Paradigm shifts. *Holistic Health and Healing*; F.A. Davis Company: Philadelphia, USA, 2002; pp 3-30.

(68) Kimura, M. The molecular patho-pharmacological studies for novel drug design by a principle of drug action in Japanese traditional Sino-medicine system. *Yakugaku Zasshi* **1997**, *117*, 133-154.

(69) Ligumsky, M.; Sallon, S.; Shapiro, H.; Ben-Ari, E.; Davidson, R. et al. Treatment of irritable bowel syndromme (IBS) with Tibetan herbal multicompound, PADMA-179: A controlled, double-blind study. *Gastroenterology* **1999**, *116*, *G4473*.

(70) Drabaek, H.; Mehlsen, J.; Himmelstrup, H.; Winther, K. A botanical compound, PADMA-28, increases walking distance in stable intermittent claudation. *Angiology-The Journal of Vascular Diseases* **1993**, *44,11*, 863-867.

(71) Winther, K.; Kharazmi, A.; Himmelstrup, H.; Drabaek, H.; Mehlsen, J. PADMA-28, A botanical compound, decreases the oxidative burst response of monocytes and improves fibrinolysis in patients with stable intermittent claudation. *Fibrinolysis* **1994**, *8, Suppl.2*, 47-49.

(72) Suter, M.; Richter, C. On the effect of PADMA-28: Antioxidative mechanisms at a molecular Level; Institut fur Biochemie, Eidgenossische Technische Hoschschule ETH Zurich: Switzerland, 2000; pp 17-22.

(73) Bagozzi, D. Traditional and Alternative medicine, Fact Sheet No 297; World Health organization; Website: http://www.who.int/, 2002.

(74) Karki, M. Medicinal and Aromatic Plants Program in Asia (MPPA); IDRC /CRDI, 2001.

(75) Prance, G. T.; Chadwick, D. J.; Marsh, J. Ethnobotany and the search for new drug discovery. *Ethnobotany and the search for new drugs*; John Wiley and Sons: England, 1994.

(76) Soejarto, D. D.; Pezzuto, J. M.; Fong, H. H. S.; Tan, G. T.; Zhang, H. J. et al. An international collaborative program to discover new drugs from tropical biodiversuty of Vietnam and Laos. *Nat. Prod. Sci.* **2002**, *8*, 1-15.

(77) Ugyen. *Sustainability: An assessment of medicinal plants in Bhutan*; University of Kent at Canterbury, Durrel Institute of Conservation, The Royal Botanic Gardens: Kew, UK, 1999.

(78) Men-tsee-khang (Homepage); Tibetan Medical and astrologyl Institute, Men-tsee-khang, India (online); Website:http://www.mentseekhang.org, 2002.

(79) Tibetan medicine-an alternative and complementary medicine resource guide; Alternative medicine foundation, Inc., Bethesda, Maryland; Website: http://www.amfoundation.org/tibetanmedicine.htm, 2000.

(80) Dagthon, J. G. An introduction to Tibetan astronomical science; The Office of Tibet, London; Website: http://www.tibet.com/Med_Astro/astro.html, 2003.

(81) Meyer, F. The world of Tibetan Medicine. *Oriental medicine-an illustrated guide to Asian arts of healing*; Serindia Publications: London, 1995; pp 107-143.

(82) Angele, E. Notes on history of Tibetan medicine. *Tibetan medicine*; Library of Tibetan Works and Archives: New Delhi, 1984; pp 1-14.

(83) Report of research laboratory; National Institute of Traditional Medicine: Thimphu, 1995.

(84) Norbu, K. *Fundamentals of Tibetan medical practice*; D.L.Tashigangpa: Ladakh, 1974.

(85) *NITM Database: National Essential Traditional Medicine List*; Institute for Traditional Medicine Services: Thimphu, 2001; 1-8.

(86) *Bhutan to unveil herbal secrets (online); London, Europe world, Website:http://www.europaworld.org/issue15/bhutantounveilherbalsecrets291200.htm*, 2003.

(87) Kingdom of Bhutan poised to offer west their ancient medicinal heritage thanks to funding by the European Commission (online); Oxford Natural Products, UK.; Website:http://www.oxfordnaturalproducts.com/site/onp/oooo6, 2000.

(88) Karan, P. P.; Jenkins, W. M. J. *Bhutan: A physical and cultural geography*; University of Kentucky Press: Lexington, 1967.

(89) The biodiversity of Bhutan; Bhutan Trust Fund for Environmental Conservation (online); Website:http://www.bhutantrustfund.org/biodiversity.htm, 2003.

(90) Grierson, A. J. C.; Long, D. G. *Flora of Bhutan: Including a record of plants from Sikkim*; Royal Botanic Garden: Edinburgh, 1994.

(91) Introduction to Bhutan; Lhayul Tours and Treks (online); Website:http://www.lhayul.com/bhutanintro.htm, 2002.

(92) Bohlin, L.; Bruhn, J. G. Bioassay methods in natural product research and drug development. *Proceedings of the Phytochemical Society of Europe*; Kluwer academic publishers, The Netherlands,. 1999.

(93) Bhutan (online); Indianvisit tours, 2002. Website:http://www.indianvisit.com/ivnew/destinationguides/neighboursofindia/bhutan.htm.

(94) Giri, S. *Country Programme Strategy-Second Operational Phase (1999-2001)* **1999**, 1-17.

(95) Introduction to Bhutan; Lhayul Tours and Treks. Website:http://www.lhayul.com/bhutanintro.htm.

(96) Mainka, S. A. The use of animal ingredients in Bhutanese traditional medicine; RGoB and Commision of European Communities: Thimphu, 1996; pp 4-53.

(97) NITM Database*:* List of Ingredients of Traditional Medicine; NITM: Thimphu, 2001; pp 1-10.

(98) Chhetri, B. B. Vegetable oil and ornamental plants. *Non-wood forest products of Bhutan*; RAP Publications-FAO United Nations: Bangkok, Thailand, 1996; pp 1-16.

(99) Ngawang, R. Medicinal plants. *Non-wood forest products of Bhutan*; RAP Publications-FAO United Nations: Bangkok, Thailand, 1996.

(100) Mulliken, T. Implementing CITES for Himalayan Medicinal Plants *Nardostachys grandiflora* and *Picrorhiza kurrooa*. In *Traffic Bulletin*, 2000; pp 1-16.

(101) Emmett, S. Mountain medicines; New agriculturist; Website: http://www.new-agri.co.uk/03-1/focuson/focuson5.html, 2003.

(102) Norbu, P. W. Basic information on Bhutan's Himalayan yew (*Taxus baccata*). *Non-wood Forest Products of Bhutan*; RAP Publications/FAO, UN.: Bangkok, Thailand, 1996; pp 1-3.

(103) Winter, H.; Seawright, A. A.; Noltie, H. J.; Mattocks, A. R.; Jukes, R. et al. Pyrrolizidine alkaloid poisoning of yaks: identification of the plants involved. *Vet. Rec.* **1994**, *134*, 135-139.

(104) Klatzel, F.; Kirby, K. E. S.; Wangdi, K. *The Natural World of Bhutan*; Royal Society for Protection of Nature-World Wildlife Fund: Thimphu, 1999; 4-24.

(105) *Medicinal Flora of Bhutan*; NITM, KMT Printing Works: Thimphu, Bhutan, 1999.

(106) Culvenor, C. C. J.; Fitzgerald, J. S. A. A field method for alkaloid screening of plants. *J. Pharm. Sci.* **1963**, *52*, 303-304.

(107) Sengpracha, W. Synthesis of tribulusterine, a potent toxic alkaloid from *Tribulus terrestris*, M.Sc. Thesis;University of Wollongong, Wollongong, 2001; pp 78

(108) Bourke, C. A. Hepatopathy in sheep associated with *Tribulus terrestris. Aust. Vet. J.* **1983**, *60*, 189.

(109) Bourke, C. A. Staggers in sheep associated with the ingestion of *Tribulus terrestris*. *Aust. Vet. J.* **1984**, *61*, 360-363.

(110) Bourke, C. A.; Stevens, G. R.; Carrigan, M. J. Locomotor effects in sheep of alkaloids identified in Australian *Tribulus terrestris. Aust. Vet. J.* **1992**, *69*, 163-165.

(111) Grierson, A. J. C.; Long, D. G. *Flora of Bhutan*; Royal Botanic Garden: Edinburg, 1984.

(112) Baillie, L. C.; Batsanov, A.; Bearder, J. R.; Whiting, D. A. Synthesis of the A/E/F sections of conaconitine, napelline and related diterpenoid alakloids of the aconitine group. *J. Chem. Soc. Perkin Trans.1* **1998**, 3471-3478.

(113) Chopra, R. N.; Chopra, I. C.; Handa, K. L.; Kapur, L. D. *Chopra's indigenous drugs of India.*; U.N.Dhur and sons Private Ltd.: Calcutta, 1958; 54-57.

(114) Pelletier, S. W.; Aneja, R.; Gopinath, K. W. The alkaloids of *Aconitum heterophyllum* Wall.: Isolation and characterization. *Phytochem.* **1968**, *7*, 625-635.

(115) Yue, J.; Xu, J.; Zhao, Q.; Sun, H. Diterpenoid alkaloids from *Aconitum leucostomum*. *J. Nat. Prod.* **1996**, *59*, 277-279.

(116) *Flora Reipublicae Popularis Sinicae*; Institute of Botany, Chinese Academy of Sciences & Institute of Materia Medica, Chinese Academy of Medical Sciences.Science Press: Beijing, 1979; 235-236.

(117) Mesia, L. R.; Madinaveitia, A.; Reina, M.; Rodriguez, M. L.; Fuente, G. d. l. et al. Four new alkaloids from *Consolida glandulosa*. *J. Nat. Prod.* **2002**, *65*, 496-499.

(118) Pelletier, S. W.; Joshi, B. S. *Alkaloids: Chemical and Biological Perspectives*; Springer: New York, 1991; pp 297-565.

(119) Benn, M. H.; Jacyno, J. M. *Alkaloids: Chemical and biological perspectives*; John Wiley & Sons.: New York, 1983; 153-210.

(120) Grandez, M.; Madinaveitia, A.; Gavin, J. A.; Alva, A.; Fuente, G. d. l. Alkaloids from *Consolida oliveriana*. *J. Nat. Prod.* **2002**, *65*, 513-516.

(121) Dalton, D. R. *The Alkaloids: The fundamental chemistry. A biogenetic approach*; Marcel Dekker, Inc.: New York, 1979; 1-789.

(122) Bessonova, I. A.; Saidkhodzhaeva, S. A. Hetisane-type diterpenoid alkaloids. *Chem. Nat. Compd.* **2000**, *36*, 419-477.

(123) Pelletier, S. W.; Mody, N. V.; Joshi, B. S.; Schramm, L. C. *Alkaloids: Chemical and Biological Perspectivess*; Wiley-Interscience: New York, 1984; pp 205.

(124) Diaz, J. G.; Ruiz, J. G.; Fuente, G. d. l. Alkaloids from *Delphinium staphisagria*. *J. Nat. Prod.* **2000**, *63*, 1136-1139.

(125) Pelletier, S. W.; Keith, L. H. The diterpene alkaloids. *Chemistry of the Alkaloids*; van Nostrand Reinhold Company: New York, 1970; pp 503-590.

(126) Boronova, Z. S.; Sultankhodzhaev, M. N. Alkaloids of *Aconitum tuberosum*. Structure of tuberaconitine and tubermesaconitine. *Chem. Nat. Compd.* **2001**, *37*, 269-271.

(127) Zhou, X. L.; Chen, Q. H.; Chen, D. L.; Wang, F. P. Hemsleyatine, a novel C_{19}-diterpenoid alkaloids with 8-amino group from *Aconitum hemsleyanum*. *Chem. Pharm. Bull.* **2003**, *51*, 592-594.

(128) Shen, X. L.; Zhou, X. L.; Chen, Q. H.; Chen, D. L.; Wang, F. P. New C_{19}-diterpenoid alkaloids from the roots of *Delphinium potaninii* var.*jiufengshanense*. *Chem. Pharm. Bull.* **2002**, *50*, 1265-1267.

(129) Pelletier, S. W. The diterpene alkaloids:The structure of Atidine. *J. Am. Chem. Soc.* **1965**, *87*, 799-802.

(130) Pelletier, S. W.; Mody, N. V. The conformational analysis of the E and F rings of Atisine, Veatchine, and related alkaloids. The existence of C-20 Epimers. *J. Am. Chem. Soc.* **1977**, *99*, 284-286.

(131) Pelletier, S. W.; Mody, N. V.; Moore, J. F.; Desai, H. K. The structure and absolute configuration of heterophylloidine. *Tetrahedron Lett.* **1981**, *22*, 313-314.

(132) Cookson, R. C.; Trevett.M.E. Aconitum and *Delphinium* alkaloids. Part II. Interrelation of the functional groups of delpheline. *J. Chem. Soc.* **1956**, 3121.

(133) Hanson, J. R. *The tetracyclic diterpenes*; First ed.; Pergamon Press Ltd.: London, 1968; 1-127.

(134) Rahman, A. *Handbook of natural products data, diterpenoid and steroidal alkaloids*; Elsevier Science Publishers: Amsterdam, 1990.

(135) Rahman, A.-u.; Nasreen, A.; Akhtar, F.; Shekhani, M. S.; Clardy, J. et al. Antifungal diterpenoid alkaloids from *Delphinium denudatum*. *J. Nat. Prod.* **1997**, *60*, 472-474.

(136) Grina, J. A.; Schroeder, D. R.; Wydallis, E. T.; Stermitz, F. R. Alkaloids from *Delphenium geyeri*. Three new C_{20}-diterpenoid alkaloids. *J. Org. Chem.* **1986**, *51*, 390-394.

(137) Pelletier, S. W.; Parthasarathy, P. C. The diterpene alkaloids. Further studies of atisine chemistry. *J. Am. Chem. Soc.* **1965**, *87*, 777-798.

(138) Pelletier, S. W.; Oeltmann, T. N. NMR studies of diterpene alkaloids. *Tetrahedron* **1968**, *24*, 2019-2038.

(139) Pelletier, S. W.; Locke, D. M. The diterpene alkaloids.Correlation of the atisine and Garrya series of alkaloids. *J. Am. Chem. Soc.* **1965**, *87*, 761-776.

(140) Rahman, A. U. *Handbook of natural products data: Diterpenoid and Steroidal alkaloids.*; Elsevier Science Publishers B.V., 1990; 1-962.

(141) Saidkhodzhaeva, S. A.; Bessonova, I. A.; Abdullaev, N. D. Arcutinine, a new alkaloid from *Aconitum arcuatum*. *Chem. Nat. Compd.* **2001**, *37*, 466-469.

(142) Sultankhodzhaev, M. N.; Tashkhodzhaev, B.; Averkiev, B. B.; Antipin, M. Y. Secokaraconitine, a new diterpenoid alkaloid from *Aconitum karacolicum*. *Chem. Nat. Compd.* **2002**, *38*, 78-82.

(143) Ichinohe, Y.; Yamaguchi, M.; Katsui, N.; Kakimoto, S. On the structure of miyaconitine. *Tetrahedron Lett.* **1970**, 2323.

(144) Benn, M.; Richardson, F. Hetisine 13-O-acetate, a new diterpenoid alkaloid from *Delphinium nutallianum* Pritz. *Heterocycles* **1986**, *24*, 1605-1607.

(145) Takayama, H.; Okazaki, T.; Yamaguchi, K.; Aimi, N.; Haginiwa, J. et al. Structure of two new diterpene alkaloids, 3-epi-ignavinol and 2,3-dehydrodelcosine. *Chem. Pharm. Bull.* **1988**, *36*, 3210.

(146) Schemeller, T.; Wink, M. Utilization of alkaloids in modern medicine. *Alkaloids: Biochemistry, Ecology and Medicinal Applications*; Plenum Press: New York, 1998; pp 435-459.

(147) Dzhakhangirov, F. N.; Bessonova, I. A. Alkaloids of *Aconitum coreanum*. X. Curare-like activity-structure relationship. *Chem. Nat. Compd.* **2002**, *38*, 74-77.

(148) Shakhidoyatova, N. K.; Dzhakhangirov, F. N.; Sultankhodzhaev, M. N. Medicinal plants: Antiarrhythmic acitivity of diterpenoid alkaloids of the napelline-type and their acylated derivatives. *Pharm. Chem. J.* **2001**, *35*, 266-267.

(149) Venkateswarlu, V.; Srivastava, S. K.; Joshi, B. S.; Desai, H. K.; Pelletier, S. W. 13-*O*-Acetylvakhmatine, a new diterpenoid alkaloid from the seeds of *Consolida ambigua*. *J. Nat. Prod.* **1995**, *58*, 1527.

(150) Sakai, S.; Yamaguchi, Y.; Takayama, H.; Yamamoto, I.; Okamoto, T. The structure of a new diterpene alkaloid: Sanyonamine. *Chem. Pharm. Bull.* **1982**, *30*, 4576.

(151) Jiang, Q.; Pelletier, S. W. Two new diterpenoid alkaloids from *Aconitum palmatum*. *J. Nat. Prod.* **1991**, *54*, 525-531.

(152) Bai, Y.; Sun, F.; Benn, M.; Majak, W. Diterpenoid and norditerpenoid alkaloids from *Delphinium nuttallianum*. *Phytochemistry* **1994**, *37*, 1717.

(153) Konno, C.; Shirasaka, M.; Hikino, H. Structure of senbusine A, B and C, diterpenic alkaloids of *Aconitum carmichaeli* roots from China. *J. Nat. Prod.* **1982**, *45*, 128-133.

(154) Pelletier, S. W.; Mody, N. V.; Venkov, A. P.; Jones, J. S. B. Alkaloids of *Delphinium virescens* Nutt. Virescenine and 14-acetylvirescenine. *Heterocycles* **1979**, *12*, 779.

(155) Bando, H.; Wada, K.; Amyiya, T.; Kobayashi, K.; Fujimoto, Y. et al. Studies on the Aconitum species. V. Constituents of *Aconitum yesoense* var. Macroyesoense (Nakai) Tamura. *Heterocycles* **1987**, *26*, 2623.

(156) Aiyar, V. N.; Kulanthaivel, P.; Benn, M. The C_{19}-diterpenoid alkaloids of *Aconitum delphinifolium*. *Phytochemistry* **1986**, *25*, 973.

(157) Chen, S. Y.; Li, S. H.; Hao, H. J. The diterpenoid alkaloids of *Aconitum nagarum* var. Lasiandrum W.T.Wang and its chemotaxonomic significance. *Acta Botanica Sinica.* **1986**, *28*, 86.

(158) Chen, Y.; Koelliker, S.; Oehme, M.; Katz, A. Isolation of diterpenoid alkaloids from herb and flowers of *Aconitum napellus* ssp.*vulgare* and electrospray ion trap multiple MS study of these alkaloids. *J. Nat. Prod.* **1999**, *62*, 701-704.

(159) Joshi, B. S.; Glinski, J. A.; Chokshi, H. P.; Chen, S.; Srivastava, S. K. et al. Deltasine, a new C_{19}-Diterpenoid Alkaloid from *Delphinium tatsienense* Franch. *Heterocycles* **1984**, *22*, 2037-2042.

(160) Joshi, B. S.; Puar, M. S.; Bai, Y.; Panu, A. M.; Pelletier, S. W. The structure of andersonbine, a new diterpenoid alkaloid from *Delphinium andersonii* Gray. *Tetrahedron* **1994**, *50*, 12283.

(161) Han, Y.; Liu, J.; Wang, M. Chemical constituents of Korean monkshood (*Aconitum coreanum*). *Zhongcaoyao* **1994**, *25*, 619-621, 624.

(162) Pelletier, S. W.; De Camp, W. H.; Mody, N. V. Epimerization and isomerization in C_{20}-diterpenoid alkaloids. Crystal and molecular structures of Atisinium chloride, Dihydroatisine, and Veatchine. *J. Am. Chem. Soc.* **1978**, *100*, 7976-7987.

(163) Caius, J. F. *The medicinal and poisonous plants of India*; Scientific Publishers: Jodhpur, India, 1986.

(164) Vennerstrom, L. J.; Klayman, D. L. Protoberberine alkaloids as antimalarials. *J. Med. Chem.* **1988**, *31*, 1084-1087.

(165) Kametani, T. *The chemistry of the isoquinoline alkaloids*; Hirokawa Publishing Company, Inc.: Tokyo, 1969; 266.

(166) Hughes, D. W.; Nalliah, B. C.; Holland, H. L.; MacLean, D. B. ^{13}C nuclear magnetic resonance spectra of the benzylisoquinoline alkaloids and related model compounds. *Can. J. Chem.* **1977**, *55*.

(167) Bhakuni, D. S.; Tewari, S.; Dhar, M. M. Aporphine alkaloids of *Annona squamosa*. *Phytochemistry* **1972**, *11*, 1819-1822.

(168) Iwasa, K.; Okada, M.; Takao, N. Protopine-N-oxide, an alkaloid from *Bocconia cordata*. *Phytochemistry* **1983**, *22*, 627-628.

(169) Han, L.-F.; Nowicky, W.; BGutmann, V. Reversed-phase high-performance liquid chromatographic separation of tertiary and quaternary alkaloids from *Chelidonium majus* L. *J. Chromat.* **1991**, *543*, 123-128.

(170) Yuh-chwen, C.; Pei-Wen, H.; Fang-Rong, C.; Ru-rong, W.; Chih-Chuang, L. et al. Two new protopines argemexicaines A and B and the anti-HIV alkaloid, 6-acetonyldihydrochelerythrine from formosan *Argemone mexicana*. *Planta Med.* **2003**, *69*, 148-152.

(171) Yu, C. K.; Sauders, J. K.; MacLean, D. B. The structure of fumarofine. *Can. J. Chem.* **1971**, *49*.

(172) MacLean, D. B.; Bell, R. A.; Sauders, J. K.; Chen, C. Y. Structures of three monor alkaloids of *Fumaria officinalis* L. *Can. J. Chem.* **1969**, *47*.

(173) Battersby, A. R.; Staunton, J.; Wiltshire, H. R.; Francis, R. J.; Southgate, R. Biosynthesis. Part XXII.1,2. The origin of chelidonine and of other alkaloids

derived from the tetrahydroprotobererine skeleton. *J. Chem. Soc. Perkin I.* **1975**, 1147-1156.

(174) Takao, N.; Iwasa, K.; Kamigauchi, M.; Sugiura, M. Studies on the alkaloids of papaveraceous plants. XXV. Biosynthesis of alkaloids of *Corydalis incisa* Pers and *Chelidonium majus* L. Incorporations of tetrahydroprotoberberines, N-methosalts of tetrahydroprotoberberines, and protopine. *Chem. Pharm. Bull.* **1976**, *24*, 2859-2868.

(175) Battersby, A. R.; Francis, R. J.; Hirst, M.; Ruveda, E. A.; Staunton, J. Biosynthesis.Part XXI.1,2 Investigations on the biosynthesis of stylopine in *Chelidonium majus*. *J. Chem. Soc. Perkin I* **1975**, 1140-1147.

(176) Manske, R. H. F.; Rodrigo, R.; MacLean, D. B.; Gracey, D. E. F.; Sauders, J. K. Structure of sibiricine, an alkaloid of *Corydalis sibirica*. *Can. J. Chem.* **1969**, *47*.

(177) Manske, R. H. F.; Rodrigo, R.; MacLean, D. B.; Gracey, D. E. F.; Sauders, J. K. The structure of ochrobirine. *Can. J. Chem.* **1969**, *47*.

(178) Margvelashvili, N. N.; Prisyazhnyuk, N. P.; Kislov, L. D.; Tolkachev, O. N. Alkaloids from *Corydalis gigantea*. *Khimiya Prirodnykh Soedinenii* **1976**, 832.

(179) Kaneko, H.; Naruto, S. Constituents of Corydalis species.VII.Constituents of *Corydalis pallida* var.tenuis. *Yakugaku Zasshi*. **1971**, *91*, 101-108.

(180) Tani, C.; Nagakura, N.; Hattori, S.; kao, M. T. Alkaloids of papaveraceae plants.XIX. Alkaloids of *Corydalis koidzumiana*. *Yakugaku Zasshi*. **1976**, *94*, 844-851.

(181) Manske, R. H. F.; Miller, M. R. The alkaloids of fumaraiceous plants. XVII.*Corydalis caseana* A.Gray. *Can. J. Res.* **1938**, *16*, 153-157.

(182) Xu, X. Y.; Li, B. M.; He, L. Y.; Sagara, K.; Ohshima, T. et al. Determination of isoquinoline alkaloids in *Corydalis bungeana* Turez by reversed-phase ion-pair high-performance liquid chromatography. *Yaoxue Xuebao.* **1994**, *29*, 785-789.

(183) Wu, C. Z.; Kadota, S.; Li, J. X.; Basnet, P.; Namba, T. A comparative study of alkaloidal constituents in C*orydalis decumbens* tubers by high-performance liquid chromatography. *Shoyakugaku Zasshi* **1993**, *47*, 440-445.

(184) Vennerstrom, J. L.; Klayman, D. L. Protoberberine Alkaloids as Antimalarials. *J. Med. Chem.* **1988**, *31*, 1084-1087.

(185) Iwasa, K.; Nishiyama, Y.; Ichimaru, M.; Moriyasu, M.; Kim, H.-S. et al. Structure-activity relationships of quaternary protoberberine alakloids having an antimalarial activity. *Eur. J. Med. Chem.* **1999**, *34*, 1077-1083.

(186) Kim, S. R.; Hwang, S. Y.; Jang, Y. P.; Park, M. J.; Markelonis, G. J. et al. Protopine from *Corydalis ternata* has anticholinesterase and antiamnesic activities. *Planta Med.* **1999**, *65*, 218-221.

(187) Shiomoto, H.; Matsuda, H.; Kubo, M. Effects of protopine on blood platelet aggregation.III. Effect of protopine on the metabolic system of arachidonic acid in platelets. *Chem. Pharm. Bull.* **1991**, *39*, 474-477.

(188) Zhao, Y.; Zheng, J.; Huang, S.; Li, X.; Lin, Q. et al. Experimental studies on the antimalarial effect of protopine derivatives. *Yaoxue Tongbao* **1981**, *16*, 7-10.

(189) Nagai, Y.; Matsukura, H.; Naruta, S.; Nanba, K. Analysis of alkaloidal components in *Corydalis tubers. Pharm. Tech. Japan* **1988**, *4*, 37-43.

(190) Takahashi, H.; Iguchi, M.; Onda, M. Utilization of protopine and related alkaloids.XVII. Spectroscopic studies on the ten-membered ring conformations of protopine and α-allocryptopine. *Chem. Pharm. Bull.* **1985**, *33*, 4775-4782.

(191) Brochmann-Hanssen, E.; Nielsen, B. The Isolations of (-)-Scoulerine from Opium. *Tetrahedron Lett.* **1966**, *20*, 2261-2263.

(192) Budzikiewicz, H.; Djerassi, C.; Williams, D. H. Structure elucidation of natural products by Mass Spectrometry. *Alkaloids*; Holden-Day Inc.: San Francisco, 1964; pp 181.

(193) Furuya, T.; Nakano, M.; Yoshikawa, T. Biotransformation of (RS)-reticuline and morphinan alkaloids by cell cultures *Papaver somniferum*. *Phytochem.* **1978**, *17*, 891-893.

(194) Ohashi, M.; Wilson, J. M.; Budzikiewicz, H.; Shamma, M.; Slusarchyk, W. A. et al. Mass Spectrometry in structural and stereochemical problems. XXXI. Aporphines and related alkaloids. *J. Am. Chem. Soc.* **1963**, *85*, 2807.

(195) Narasimhan, N. S.; Mali, R. S.; Kulkarni, B. K. Synthetic application of lithiation reactions-XVI. Syntheses of (+/−)stylopine and (+/−) sinactine. *Tetrahedron* **1983**, *39*, 1975-1982.

(196) Huang, J.; Li, H.; Yang, J.; Chen, Y.; Liu, Y. et al. Chemical components of *Vetiveria zizanoides* volatiles. *Yingyong Shengtai Xuebao* **2004**, *15*, 170-172.

(197) Zhao, J.; Nan, P.; Zhong, Y. Chemical composition of the essential oils of *Clausena lansium* from Hainan Island. *J. Biosci.* **2004**, *59*, 153-156.

(198) Dembitsky, V. M.; shkrob, I.; Rozentsvet, O. A. Fatty acid amides from freshwater green alga *Rhizoclonium hieroglyphicum*. *Phytochemistry* **2000**, *54*, 965-967.

(199) Bedir, E.; Khan, I. K. New steroidal glycosides from the fruits of *Tribulus terrestris*. *J. Nat. Prod.* **2000**, *63*, 1699-1701.

(200) Yan, W.; Ohtani, K.; Kassai, R.; Yamasaki, K. Steroidal saponins from fruits of *Tribulus terrestris*. *Phytochemistry* **1996**, *42*, 1417-1422.

(201) Terra, E. A Simpler Way: *Tribulus terrestris*; A Simpler Way; Website: http://www.asimplerway.com/archives/000008.html, 2002.

(202) Ganzera, M.; Bedir, E.; Khan, I. A. Determination of steroidal saponins in *Tribulus terrestris* by reversed-phased high-performance liquid chromatography and evaporative light scattering detection. *J. Pharm. Sci.* **2001**, *90*, 1752-1758.

(203) Higher power *Tribulus terrestris* (online); Website: http://www.bodybuilding.com/store/trib.htmlBody; Body Building.Com, 2003.

(204) Tribulus terrestris facts, information, and research (online); Website:http://www.bodybuildingforyou.com/pro-hormones/tribulus-terrestris-facts.h; Body Building for You.Com, 2003.

(205) Wu, T. S.; Shi, L. S.; Kuo, S. C. Alkaloids and other constituents from *Tribulus terrestris*. *Phytochemistry* **1999**, *50*, 1411-1415.

(206) Sahelian, R. *Tribulus terrestris*: underrated roadside sex weed that works; Ray Sahelian; Website: http://www.raysahelian.com/tribulus.html, 2003.

(207) Arcasoy, H. B.; Erenmemisoglu, A.; Tekol, Y.; Kurucu, S.; Kartal, M. *Effect of Tribulus terrestris L.saponin mixture on some smooth muscle preparations: a preliminary study.* **1998**, *2003*.

(208) Henning, M. W. Virus and deficiency diseases, plant poisonings. *Animal diseases in South Africa*: South Africa, 1932; pp 855-867.

(209) Aslani, M. R.; Movassaghi, A. R.; Mohri, M.; Pedram, M.; Abavisani, A. Experimental *Tribulus terrestris* poisoning in sheep: Clinical, laboratory and pathological findings. *Vet. Res. Commun.*; Kluwer Academic Publishers: Netherland, 2003; pp 53-62.

(210) Glastonbury, J. R.; Doughty, F. R.; Whitaker, S. J.; Sergeant, E. A syndrome of hepatogenous photosensitisation, resembling geeldikkop, in sheep grazing *Tribulus terrestris*. *Aust. Vet. J.* **1984**, *61*, 314-316.

(211) McDonough, S. P.; Woodbury, A. H.; Galey, F. D.; Wilson, D. W.; East, N. et al. Hepatogenous photosensitization of sheep in California associated with ingestion of *Tribulus terrestris* (puncture vine). *J. Vet. Diagn. Invest.* **1994**, *6*, 392-395.

(212) Xu, Y.; Xie, S.; Zhao, H.; Han, D.; Xu, T. et al. Studies on chemical constituents of *Tribulus terrestris*. *Yaoxue Xuebao* **2001**, *36*, 750-753.

(213) Xu, Y.; Chen, H. S.; Liang, H. Q.; Gu, Z. B.; Liu, W. Y. et al. Three new saponins from *Tribulus terrestris*. *Planta Med.* **2000**, *66*, 545-550.

(214) Zafar, R.; Nasa, A. K. Quercetin and kaempferol from the fruits and stem of *Tribulus terresris. Indian J. Nat. Prod.* **1987**, *3*, 17-18.

(215) Bhutani, S. P.; Chibber, S. S.; Seshadri, T. R. Flavonoids of the fruits and leaves of *Tribulus terrestris*: Constitution of tribuloside. *Phytochem.* **1969**, *8*, 299-303.

(216) Wu.;, T.-S.; Shi.;, L.-S.; Kuo., S.-c. Alkaloids and other constituents from *Tribulus terrestris*. *Phytochemistry* **1999**, *50*, 1411-1415.

(217) Zafar, R.; Nasa, A. K. Quercetin and kaempferol from the fruits and stem of Tribulus terrestris Linn. *Indian J. Nat. Prod.* **1987**, *3*, 17-18.

(218) Bremner, J. B.; Sengpracha, W.; Southwell, I.; Bourke, C.; Slkelton, B. W.; White, A. H. A revised structure for the alkaloid, tribulusterine, from *Tribulus terrestris* L. *Aust. J. Chem.* **2004**, *57*, 273-276.

(219) Reinehart, K. L. J.; Kobayashi, J.; Harbour, G. C.; Gilmore, J.; Mascal, M. et al. Eudistomins A-Q, β–carbolines from the antiviral Caribbean Tunicate *Eudistoma olivaceum*. *J. Am. Chem. Soc.* **1987**, *109*, 3378-3387.

(220) Miles, C. O.; Wilkins, A. L.; Erasmus, G. L.; Kellerman, T. S. Photosensitivity in South Africa.VIII.Ovine metabolism of *Tribulus terrestris* saponins during experimentally induced geeldikkop. *Onderstepoort J. Vet. Res.* **1994**, *61*, 351-359.

(221) Gauthaman, K.; Adaikan, P. G.; Prasad, R. N. V. Aphrodisiac properties of *Tribulus terrestris* extract (protodioscin) in normal and castrated rats. *Life Sci.* **2002**, *71*, 1385-1396.

(222) Adimoeja, A. Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunctions. *Int. J. Androl.* **2000**, *23*, 82-84.

(223) Alexis, B. Natural antibacterial, anti-inflamation,anti-virus, anti-herpes cream containing *Tribulus* extract. *PCT Int. Appl.* **2001**, 21.

(224) *Tribulus terrestris* (online); Chinese Herbs &Co.; Website: http://www.chinese-herbs.org/tribulus-terrestris/, 2002.

(225) Deepak, M.; Dipankar, G.; Prashanti, D.; Asha, M. K.; Amit, A. et al. Tribulosin and β-sitosterol-D-glucoside, the anthelmintic principles of *Tribulus terrestris*. *Phytomedicine* **2002**, *9*, 753-756.

(226) Kemertelidze, E. P.; Pkheidze, T. A.; Kachukhashvili, T. N.; Turova, A. D.; Sokolova, L. N. et al. Tribusponin-an antisclerotic agent; All-Union Scientific-Research Institute of Medicinal and Aromatic Plants: U.S.S.R, 1977.

(227) El Gammal, A. A.; Mansour, R. M. A. Antimicrobial activities of some flavonoid compounds. *Zentralblatt fuer Mikrobiologie* **1986**, *141*, 561-565.

(228) Ahmad, A.; Khan, K. A.; Sultana, S.; Siddiqui, B. S.; Begum, S. et al. Study of the invitro antimicrobial activity of harmine, harmaline and their derivatives. *J. Ethnopharmacol.* **1992**, *35*, 289-294.

(229) Allen, J. R. F.; Holmstedt, B. R. The simple β-carboline alkaloids. *Phytochemistry* **1980**, *19*, 1573-1582.

(230) Schupp, P.; Poehner, T.; Edrada, R.; Ebel, R.; Berg, A. et al. Eudistomins W and X, Two new β-carbolines from the Micronesian tunicate *Eudistoma* sp. *J. Nat. Prod.* **2003**, *66*, 272-275.

(231) Stolle, K.; Gröger, D. Studies on the biosynthesis of harmine. *Arch. pharm.* **1968**, *301*, 561.

(232) Arruda, M. S. P.; Fernandes, J. B.; Da Silva, M. F. d. G. F.; Vieira, P. C.; Pirani, J. R. Quinolone alkaloids from *Zanthoxylum acutifolium*. *Phytochemistry* **1992**, *31*, 3617-3619.

(233) Krishnamurti, M.; Islam, A. Isolation of isolonchocarpin, 3,4-dimethoxycinnamic acid, and heptacosanol from *Milletia ovalifolia* seeds. *J. Bangladesh Acad. Sci.* **1987**, *11*, 133-135.

(234) He, Z.; Pan, w.; Lu, Y.; Zhang, C.; Yuan, M. Study on organic acids in beeswax of Guizhou. *Zhongcaoyao* **2002**, *33*, 405-406.

(235) Davis, U. C. Lab notes; CVDLS, 1999.

(236) Murphy, J. E.; Beckmen, K., B.; Johnson, J. K.; Cope, R. B.; Lawmaster, T. et al. Toxic and feeding deterrent effects of native aquatic Macrophytes on exotic grass carp(*Ctenopharyngodon idella*). *Ecotoxicology* **2002**, *11*, 243-254.

(237) Schumacher, E.; Hulftegger, E. The action of *Ranunulus acer* on the PH and fermentation in the rumen and on the surviving rumen wall of cattle. *Schweiz. Arch. Tierheilk.* **1958**, *100*, 415-427.

(238) Li, H.; Du, S.; Hou, F.; Wang, D.; Liu, G. et al. Study of chemical constituents of *Ranunculus ternatus* Thumb. *Shenyang Yaoke Daxue Xuebao* **1999**, *16*, 24-27.

(239) Addel-Kader, M. S.; EI-Lakany, A. M.; EI-Fiky, F. K. Coumarins with antiviral and cytotoxic activity from Egyptian *Ranunculus asiaticus* L. *J.Pharm.Sci.* **1996**, *10*, 5-7.

(240) Gluchoff-Fiasson, K.; Fiasson, J. L.; Favre-Bonvin, J. Quercetin glycosides from antarctic *Ranunculus* species. *Phytochemistry* **1994**, *37*, 1629-1633.

(241) Bonora, A.; Tosi, B.; Dall'Olio, G.; Bruni, A. Quaternary alkaloids in rhizomes of *Ranunculus serbicus. Phytocheistry* **1990**, *29*, 2389-2390.

(242) Misra, S. B.; Dixit, S. N. Antifungal properties of leaf extract of *Ranunculus sceleratus* L. *Experientia* **1978**, *34*, 1442-1443.

(243) Saber, A. H.; Mahran, G. H.; EI-Alfy, T. Phytochemical investigation of *Ranunculus sceleratus L. Planta Med.* **1968**, *16*, 231-238.

(244) Zechner, L.; Wolhlmuth, H. Anemonin and protoanemonin.I. New method of isolation of anemonin from *Ranunculus acer*. *Scientia Pharm.* **1954**, *22*, 74-89.

(245) Kelch, W. J.; Kerr, L.; Adair, H. S.; Boyd, G. D. Suspected buttercup (*Ranunculus bulbosus*) toxicosis with secondary photosensitization in a *Charolais heifer*. *Vet. Hum. Toxicol.* **1992**, *34*, 238-239.

(246) Tocan, V.; Baron, O. Antibiotic activity of protoanemonine from *Ranunculus oxyspermus* M.B. *Boll. Chim. Farm.* **1969**, *108*, 789-791.

(247) Cao, B. J.; Meng, Q. Y.; Ji, N. Analgesic and anti-inflamatory effects of *Ranunculus japonicus* extract. *Planta Med.* **1992**, *58*, 496-498.

(248) Vlietinck, A. J. Screeening methods for detection and evaluation of biological activities of plant preparations. *Bioassay Methods in Natural Product Research and Drug Development*; Kluwer Academic Publishers: The Netherlands, 1999; pp 37-52.

(249) Marston, A.; Hostettmann, K. Biological and chemical evaluation of plant extracts and subsequent isolation strategy. *Bioassay Methods in Natural Product Research and Drug Development*; Kluwer Academic Publishers: The Netherlands, 1999; pp 67-80.

(250) Hawcroft, D.; Hector, T.; Rowell, F. *Quantitative bioassay, analytical chemistry by open learning*; John Wiley& Sons: London, 1987; 1-300.

(251) Venturini, M. E.; Blanco, D.; Oria, R. In vitro antifungal activity of several antimicrobial compounds against *Penicillium expansum*. *J. Food Protect.* **2002**, *65*, 834-839.

(252) Brantner, A.; Pfeiffer, K. P.; Brantner, H. Applicability of diffusion methods required by the pharmacopoeas for testing antibacterial activity of natural compounds. *Pharmazie* **1994**, *49*, 512-516.

(253) Verpoorte, R. Antimicrobially active alkaloids. *Alkaloids: Biochemistry, Ecology, and Medicinal Applications*; Plenum Press: New York, 1998; pp 397-434.

(254) Spooner, D. F.; Skyes, G. Laboratory assesment of antimicrobial activity. *Methods in Microbiology 7B*; Academic Press Inc.: London, 1972.

(255) Janssen, A. M.; Scheffer, J. J. C.; Baerheim, S. A. Antimicrobial activity of essential oils: A 1976-1986 literature review. Aspects of the test methods. *Planta Med.* **1987**, 395-398.

(256) Toshio, Y.; Naoki, S.; Kenya, Y.; Yutaka, O.; Hajime, A. et al. Reliability of bioluminescent assay (ATP method) for testing antimicrobial susceptibility of

Mycobacterium tuberculosis. *Rinsho Byori (Japanese Journal of Clinical Pathology)* **2003**, *51*, 194-200.

(257) Chand, S.; Lusunzi, I.; Veal, D. A.; Williams, L. R.; Karuso, P. Rapid screening of the antimicrobial activity of extracts and natural products. *J. Antibiot.* **1994**, *47*, 1295-1304.

(258) Kamiya, H.; Muramoto, K.; Ogata, K. Antibacterial activity in the egg mass of a sea hare. *Experientia* **1984**, *40*, 947.

(259) Croft, S. L.; Weiss, C. R. Natural products with antiprotozoal activity. *Bioassay Methods in Natural Product Research and Drug Development*; Kluwer Academic Publishers: Dordrecht, 1999; pp 81-100.

(260) Basco, L. K.; Marquet, F.; Makler, M. M.; Le Bras, J. *Plasmodium falciparum* and *Plasmodium vivax*-lactate dehydrogenase activity and its application for *in vitro* drug susceptibility assay. *Exp. Parasitol.* **1995**, *80*.

(261) Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. Quantitative assessment of antimalarial activity in vitro by a semiautimated Microdilution technique. *Antimicrob. Agents Chemother.*, 1979; pp 710-718.

(262) Svendsen, A. B.; Verpoorte, R. Chromatography of alkaloids. *Journal of Chromatography Library*, 1983; pp 502.

(263) Witchard, H. The development of novel carbazole-based peptoid antibacterials to challenge the deadly superbugs (PhD Thesis, Appendix). PhD; University of Wollongong: Wollongong, 2002; pp 367

(264) Trager, W.; Jensen, J. B. Human malaria parasites in continuous culture. *Science* **1976**, *20*, 673-675.