PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITIES OF SELECTED COLOMBIAN MEDICINAL PLANTS

by

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ABSTRACT

This thesis deals with the search for antibiotic compounds in medicinal plants traditionally used in the treatment of skin morbid conditions by First Nations People in Colombia. The plant collection was carried out in four culturally and geographically different regions of Colombia. Twenty-four chemical plant extracts were screened against different Gram positive and Gram negative bacteria, yeast and viruses. The plant extract from *Piper lanceaefolium* exhibited good activity against the yeast *Candida albicans* and the one from *Iryanthera megistophylla* displayed very good activity against herpes simplex virus and *Staphylococcus aureus* MS (methicillin-sensitive). Four compounds, not described in the literature, were isolated and further characterised from *Piper lanceaefolium*. Phytochemical analyses of *Iryanthera megistophylla* carried out by Dr. Dong Sheng Ming led to the isolation of two new compounds and seven known compounds. Amongst the known compounds a flavolignan, iryantherin K, presented potent activity against *Staphylococcus aureus* MS (methicillin-sensitive) and more importantly against *Staphylococcus aureus* MR (methicillin-resistant). Iryantherin K and its stereoisomer iryantherin L also exhibited good inhibition of the *Candida albicans* secreted aspartic protease, which is a virulent factor in *Candida* infections. The therapeutic potential that is offered by iryantherin K justifies a further toxicological and pharmacological assessment in animal models.
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Chapter 1

Introduction

1.1. Thesis Overview

The search for chemical substances produced by plants, which are biocidal to viruses, yeast, and bacteria is the main focus of this research. A consideration of the traditional uses of a plant would seem to be a sensible approach towards this aim. This manuscript has been divided into six sections: the first section briefly introduces the historical context of natural medicines and presents information on the indigenous cultures I worked with.

The second chapter deals with the field collection of selected Colombian medicinal plants traditionally used in the treatment of skin morbid conditions.

The third chapter deals with the screening of plant chemical extracts against a selected battery of relevant strains of bacteria, fungi and viruses in order to determine biologically active extracts that merited further phyto-chemical analysis. Plant extracts of *Piper lanceaefolium* and *Iryanthera megistophylla* were found to present inhibitory activities against the yeast *Candida albicans* and the herpes simplex virus respectively.

The fourth chapter describes the separation, isolation, purification and structural elucidation of seven compounds, from an acetone extract of *P. lanceaefolium*, of which four are new to the literature and two were biologically active against *Candida albicans*.

The fifth chapter includes a collaborative effort with the post-doctoral fellow Dong Sheng Ming. In this chapter the biological testing of isolated
compounds from *Iryanthera megistophylla* against herpes simplex virus and *Staphylococcus aureus* is described along with an enzymatic assay in order to assess the inhibitory activity of aspartic proteases which are considered a virulence factor in *Candida* infections. The final chapter encompasses a general discussion of the entire research and suggests future possible directions in the field.

1.2. Objectives

1.2.1. General Objective

Search and identification of chemical compounds with biocidal and/or antiviral activities present in traditional medicinal plants from Colombia.

1.2.2. Specific Objectives

➢ Collect and identify traditional medicinal plants from different cultural and geographical areas of Colombia.

➢ Assess biological activities against relevant gram-positive and gram-negative bacteria, fungi and viruses.

➢ Isolate and identify the chemical compounds that individually, or in conjunction with others, are responsible for such biocidal and/or antiviral activity.

1.3. Natural Products and Medicine: a Historical Perspective.

Speaking of the origin of plants as medicinal agents I have always wondered about the number of unfortunate people who had to die or had a miserable time in their efforts to find a suitable cure for a given ailment. Keen
observation of animals’ food consumption behaviour probably provided interesting clues in this regard. A discussion about the origins of medicine and its relationship with natural products is well beyond the scope of this introduction therefore, I will only mention selected historical landmarks of written records regarding the use of plants as medicines.

Chemical ecologists have suggested that the human use of medicinal plants is related to the need for ingestion of plant secondary compounds as a defence mechanism against various parasitic microorganisms. Janzen (1978) suggested that mammals deliberately ingest plants to get rid of intestinal parasites. Antiparasiticility appears to be the primary non-nutrient function that has evolved in animal interactions with plant chemicals. It has been argued that control of parasitic and infectious diseases through the use of plants may have played a role in human plant selection (Johns, 1990).

The way humans have articulated plant medicinal uses in a system of beliefs about nature is an issue that likely will never be resolved. However, I would think that the use of hallucinogenic plant preparations has likely played an important role in this regard. I will divide this short historical account in two parts. I will use Bayer’s semi-synthesis of aspirin as a division because I consider this event a cultural landmark in the sense that it divests the plant or mixture of plants from their intrinsic value to be simply converted to a reservoir of single chemical entities. The notion of plants as resources of different kinds is at the very base of today’s struggle over land use in developing and developed countries alike.
1.3.1. Before Bayer

The first written records of traditional medicine systems are from Mesopotamia, written on hundreds of clay tablets in cuneiform, and date from about 2600 BC. However, it is important to mention they did not place importance on herbs. The herbal remedies of Ancient Egypt are described in exceptional detail by the best known Egyptian pharmaceutical record: the "Ebers Papyrus" dating from 1550 BC. Over 800 remedies are described in it, involving the use of plant extracts, animal organs and minerals. The Assyrians, who ruled in Mesopotamia in the first half of the first millennium BC, greatly consolidated ancient knowledge about herbal remedies. They left a legacy of 1500 years of herbal medicine (c 1900 to 400 BC) that extolled the virtues of around 1000 medicinal plants and remedies including enemas and poultices for exorcism of spirits (Mann, 2000).

The Chinese Materia Medica has been extensively documented over the centuries with the first record dating from about 1100 BC. However, the first herbalist that concentrated on cataloguing and describing therapeutically effective substances appears to have been compiled in China by Shennong Bencao Jing (100-200 AD) and covered 365 herbal drugs at the same time as the materia medica of Dioscorides (Tang, 1992, Unschuld, 1988).

In Chinese herbal medicine formulas are chosen on the basis of patterns of illness or imbalance, not just symptoms. This pharmacopoeia contains almost 6000 herbs usually formulated in mixtures of up to 20 herbs (Simpson, 2001).
Ayurvedic medicine is an old tradition from India and because it literally means "knowledge of life" encompasses more than just medicinal aspects, as it includes psychological, cultural, religious and philosophical concepts. Much of the knowledge is included in the Vedas books of knowledge and in Sambitas which contain the science of Ayurveda. This system classifies individuals according to three determined body types or doshas where the medicinal approach is to restore the balance of the whole body rather than suppress symptoms. Documentation of the Indian Ayurvedic system dates from about 1000 BC and provided the basis for the primary text of Tibetan medicine (Newman et al., 2000). A number of Ayurvedic herbs have become more widely known in the West including Justicia adhatoda (Acanthaceae) which is effective in respiratory conditions and Phyllanthus amarus (Euphorbiaceae) with its beneficial effects in liver disease (Sandberg and Corrigan, 2001).

The concept of hot and cold medicines forms a cornerstone of the Humoral theory that originated in antiquity and developed out of the writings of Hippocrates and Aristotle. Hippocrates maintained that a person's health depended upon a delicate balance of four body humours, blood, phlegm, black bile, and yellow bile, and that an imbalance of these produced illness. This theory of disease, later refined by Galen, was the dominant medical ideology throughout the Renaissance (Furst, 1995). Galen believed that epidemics were caused by "miasmas" or poisonous vapours produced whenever the conjunction of the planets was unfavourable (Mann, 2000).
After the decline of the Greek empire with the death of Alexander the Great (323 BC), several contributions were accomplished and it is worth mentioning the eight volumes of *De Medicina* by Celsus that includes over 250 plant-derived remedies. Probably the most significant contribution was made by Dioscorides, a Greek physician (100 AD), during his travels with Roman armies. He recorded the collection, storage, and use of medicinal herbs. His legacy was a five-volume work entitled *De Materia Medica* written in the first century A. D.; it described 600 plants and plant products. Although poorly organised it became the model for future pharmacopoeias (Simpson, 2001).

Through the so-called “Dark Ages” in Europe, herbal knowledge was kept alive initially through the work of scribes in Constantinople and then in the libraries of the rapidly expanding Arab Empire. The Arabs were responsible for the preservation of much of the Greco-Roman expertise and for expanding it to include the use of their own resources together with Chinese and Indian herbs. The Mansurian Book of Medicine written by Rhazes (c. 865-925 A D) represents a major contribution to pharmacy. Rhazes demonstrated the toxicity of many of the popular remedies based on heavy metal salts, particularly those involving mercury (Mann, 2000). The contribution of Avicenna through works such as *Canon Medicinae* is regarded as “the final codification of all Greco-Roman medicine”. This was subsequently superseded by the comprehensive compilation known as *Corpus of Simples* by Ibn al-Baytar (1197-1248) who practised in Malaga during the Moorish occupation of Spain. This treatise describes many items including 800 plant-derived drugs, 145 from minerals, and 130 from
animals. Also from the fifth to the twelfth centuries, monks in monasteries in Europe preserved the remnants of this Greco-Roman tradition.

Paracelsus (1493-1541) believed that God placed plants on the Earth for human use. Consequently, God had provided signs embodied in the plants to indicate their potential uses. For instance if a plant had red sap, that was a sign that the plant was intended for the treatment of blood disorders. This idea was called the doctrine of signatures and is believed to have directed healers to the potential therapeutic benefits of the vegetation. He was a key figure in the Renaissance and also a pioneer chemist. With his emphasis on the medicinal use of substances such as antimony, arsenic, iron, sulfur and mercury, he laid the foundation of chemical pharmacology (Johns, 1990).

Regarding the American continent, the oldest known medical text is the Badianus manuscript composed by Aztec scholars. It depicts many of the most important plants employed in Aztec medicine and it is the earliest pharmacopoeia we have from the Americas (Furst, 1995).

The number of species employed by Native Americans is staggering. Moerman's two-volume compendium, *Medicinal Plants of Native America* (1998) has 17,000 entries. Unfortunately there is no comparable list for Middle and South America but considering the cultural and natural biodiversity particularly in Central America and in the Amazonian rain forest, the number may well be substantially higher (Furst, 1995). Indigenous people of Mesoamerica had, and still have, extensive knowledge of healing plants.
Conquest and colonisation of the Americas by Europeans were accompanied by an unprecedented blending of Old and New World diseases, ethnomedical systems, and plant-based pharmacopoeias (Voeks, 1993).

The demographics of the slave trade most clearly delimited where and to what degree African magic-medical systems diffused into the New World. Also it has been argued that the religious division between Protestantism in British North America and Roman Catholicism in Latin America played a major role to the degree of cultural retention. Roman Catholic liturgy had some structural similarities with African religions, polytheism amongst them. According to Ribeiro cited in Voeks (1993) the conversion to Catholicism was best performed not by destroying their icons but rather by slowly replacing them with Roman Catholic symbols and rituals.

Although it is not possible to generalise an African ethnomedical system, the idea of illness as a reaction to forces outside the realm of secular comprehension is a common feature. Shaman healers act as intermediates between the material and spiritual universes, and they seek out the otherworld sources of physical and emotional distress.

In The Skeptical Chymist published in 1661 Robert Boyle laid the foundations for an understanding of the chemistry of the drugs. The discovery of the effectiveness of Digitalis by William Withering (1741-1799) for the treatment of dropsy is considered by many as the beginning of modern pharmacology. The old ideas of Aristotle concerning the four elements (earth, air, fire and water)
were swept away and a chemical element was defined as a substance that could not be broken down into simpler substances (Mann, 2000).

1.3.2. After Bayer

In the early 1800's the isolation of the active principles of commonly used plants and herbs such as strychnine from seeds of *Nux vomica* (1817), morphine (1816), atropine (1819), quinine (1820) and colchicine (1820) were achieved. The first semi-synthetic pure drug based on a natural product, acetylsalicylic acid (aspirin), by Bayer in 1899 then followed these isolations. In the first half of the twentieth century many substances with current medicinal use were obtained from traditional plant-derived extracts (Mann, 2000).

Despite the success of single compound drugs in the Western world, it is estimated that over 80% of the world's population still relies on traditional medicines including herbal medicine, for primary health care (Farnsworth, 1985). Regardless of the successful entries of organic synthesis in the post-world war II years, the need for structurally novel therapeutic categories continues and so do bio-prospecting projects world wide on behalf of major pharmaceutical companies.

Ancient medical systems such as Chinese Traditional Medicine and the Ayurvedic system are alive despite the efforts of the pharmaceutical companies to dismiss them claiming health concerns. As a matter of fact, traditional knowledge along with its associated pharmacopoeias has played a major role in drug discovery and it is a matter of political debate between native people and
pharmaceutical companies. It is estimated that at least 88 drugs are derived from plants traditionally used. Compounds such as vincristine, vinblastine, taxol and the family of antimalarials derived from quinine and artemisinin are of great importance and responsible for saving lives worldwide (Farnsworth 1990).

A good illustration of the interplay between traditional medicine and drug discovery is the case of a member in the Apocynaceae family, *Rauvolfia serpentina*. Hindus applied the name *chandra* (moon) to this plant since it was used to treat “moon disease” or lunacy. Indian sadhus have chewed on snakeroots of *Rauvolfia serpentina* for centuries because of its calming effects and as a coadjuvant in reaching “spiritual enlightenment”. It is also used in India and particularly in the Bihar province to treat insanity, epilepsy, and insomnia hence the local name *pagal-ka-dawa* “insanity cure”. In 1949 Emil Schlittler, a chemist at CIBA pharmaceuticals in Basel, Switzerland, extracted from *Rauvolfia* roots an alkaloid and verified its dramatic properties to lower the blood pressure in agreement with previous reports. This compound was named reserpine and became the first major drug to treat one of the most serious illnesses of the Western world: hypertension (Balick and Cox, 1996).

The realisation that “primitive societies” still had something to offer to the western world prompted adventurers, travellers, and scientists from different parts of the world to seek out healers and shamans in an attempt to uncover their knowledge about plants and their curative properties. For a fascinating account of these enterprises in Colombia, the reader is referred to Wade Davis’ book “One River”.

10
Today isolated natural products have become successful pharmaceuticals in the Western world. In a study done in the United States, from January through September of 1993, the top 150 proprietary drugs prescribed were analyzed and their origin determined. Amongst them, 18% of the top 150 most prescribed drugs have their origin in the kingdom Plantae (Grifo et al., 1997).

Numerous scholars have praised the wealth of knowledge of traditional societies and a note of urgency has been sent to rescue and document this body of knowledge. Indeed, Holmstedt and Bruhn (1995) declare, in regard to the “discipline” of ethno-pharmacology: “the objectives of ethno-pharmacology are to rescue and document an important cultural heritage before it is lost, and to investigate and evaluate the agents employed.”

Traditional systems are considered as a collection of items to be investigated with no agency whatsoever. This statement implies the incapacity of non-western cultures in evolving and re-creating themselves in different cultural settings. It would appear that the purpose of the discipline is to obtain information out of a dying culture before it is too late.

1.4. Search for Antibiotics in Medicinal Plants

Henceforth I will use the term “antibiotic” in a pharmacological sense exclusively and I will refrain from implying that such activity plays a role in nature.

Historically, the search for clinically useful antibiotics in plants has been a failure. Until now, there is not a single plant natural product in the pharmaceutical antibiotic armamentarium. However, scientific literature is rich in information
about plant extracts and/or phyto-chemicals with biocidal properties against microbes (e.g. viruses, bacteria and fungi) that often times possess undesirable toxic or pharmacological properties preventing them from becoming useful. Cowan (1999) has recently reviewed antimicrobials of plant origin and Hudson (1990) has compiled a book of antiviral compounds from plants.

Most of the natural products clinically relevant as antibiotics are based on metabolites found in microorganisms (for a review see Newman et al., 2000). A surprisingly limited group of organisms are sources of many antibiotics in use today, more than half of the antibiotics currently used are produced by species *Streptomyces*, filamentous bacteria that commonly inhabit soil (Tortora, 2002).

Two compounds isolated from marine sponges: spongouridine and spongothymidine, became the prototype for a series of a vast number of derivatives (i.e. acyclovir) that were tested extensively as antiviral and anti-tumor agents and are now the current antiviral therapy.

From an evolutionary perspective fascinating proposals have been put forward in regards to the role of antibiotics. Davies (1990) has suggested that low-molecular weight secondary metabolites have played a unique biochemical role in evolution before proteins were available, as many biosynthetic reactions were "catalysed" by low molecular-weight molecules present in "primordial soup reactions".

Whether antibiotic activities found in the laboratory correlate to the traditional use is a matter of debate, and one must exert caution in advancing
hypotheses that sometimes reflect scientist's cultural biases (e.g. euro-centrism) rather than scientific attributes.

The principle that should be accepted is that the possession of any type of biological activity should not in itself be sufficient reason to argue that the substance has been selected by evolution because it possesses that type of biological activity (Finn, 2000).

However the vast majority of small-molecule plant antimicrobials are agents with weak or narrow-spectrum activities, which has led to suggest that “antimicrobials” actually have other functions in the plant and their low-level activity is accidental and largely irrelevant. Others have pointed out that at present there is a lack of a solid rationale for making a functional assignment for the vast majority of plant compounds that have been classified as antimicrobials (Tegos et al., 2002).

Microorganisms can become resistant to antibiotics and chemotherapeutic agents by various mechanisms. One of these mechanisms is by preventing the access of these agents to their target. The active pumping out (efflux) of drugs has been suggested as a major mechanism of tetracycline resistance in bacteria (Nikaido, 1998).

It has been proposed that efflux mechanisms by multi-drug resistance (MDR) provides a reasonable explanation for the apparent ineffectiveness of many antimicrobials in vitro (Tegos et al., 2002).

It is essential to mention the importance of the role of light in mediating natural products' antibiotic activities. Many compounds, when excited by light,
display toxicity towards living cells or organisms. Light mediated biological activities of natural products from plants and fungi have been reviewed by Towers and co-workers (1997). For instance, thiarubrine A, a known constituent of a number of species in the Asteraceae, displays higher activity when exposed to light against *C. albicans* (Towers *et al.*, 1985). Furthermore thiarubrines showed antiviral activity only against membrane containing viruses suggesting that membrane components were the biological targets (Towers *et al.*, 1997).

1.5. **Microbial Diseases of the Skin**

The skin supports the growth of certain microbes, which are established as part of the normal microbiota. On superficial skin surfaces, certain aerobic bacteria produce fatty acids from sebum. These acids inhibit many microbes and allow better-adapted bacteria to flourish. Rashes and lesions on the skin do not necessarily indicate an infection of the skin; in fact, many systemic diseases affecting internal organs are manifested in skin lesions (Tortora, 2002). The normal flora of the skin consists primarily of gram-positive bacteria restricted to a few groups. The lack of colonisation of gram-negative bacteria on the skin is probably due to their inability to compete with gram-positive organisms that are better adapted to the dry conditions of the skin.

1.5.1. **Bacteria**

*Staphylococcus* and *Streptococcus* are amongst the most frequent causes of bacterial skin diseases. The genus *Staphylococcus* contains common
pathogens of humans and animals. *S. aureus* a yellow-pigmented form is associated with pathological conditions, including boils, pimples, and impetigo (superficial skin infection) and folliculitis (infection of hair follicle). Because in the hospital environment, patients, hospital staff members, and visitors carry *S. aureus*, the danger of infection is important. It is the most common cause of surgical wound infections and pneumonia, and the second most common cause of blood infections. Many strains are highly virulent and are also resistant to common antibiotics. Extensive use of antibiotics has resulted in the natural selection of resistant strains of *Staphylococcus aureus* such as methicillin resistant strains (Madigan, 2000).

Like other staphylococci *Streptococcus pyogenes* can cause the local infection impetigo. When it infects the dermal layer of the skin, it causes a serious disease, erysipelas, that can eventually lead to local tissue destruction and even enter the bloodstream, causing septicemia. *Pseudomonas aeruginosa* can also cause dermatitis (superficial rash) and otitis externa, however, infection by *P. aeruginosa* is rare in healthy people. These aerobic gram-negative rods are widespread in soil and water and are considered a model of an opportunistic pathogen (Tortora, 2002).

1.5.2. Yeast. *Candida albicans*.

Over the past few years there has been an increase in the incidence of fungal infections in several countries. In the commensal state, *Candida* spp. live as benign members of the microflora of healthy individuals. As a commensal, *C.*
*Candida* albicans causes no disease. *Candida* spp. are “carried” in the oral cavity, gastrointestinal tract, skin, anus and groin of healthy individuals, and also in the vaginal canal and vulva of healthy women (Soll, 2002). However, this yeast can also be considered an opportunistic pathogen. In situations where there is a decrease in host defences, it is able to access different locations in the human body and cause candidosis (Navarro-García *et al.*, 2001).

*Candida* infections of virtually every tissue in the human body have been reported, although by far the most common manifestations of candidosis are superficial lesions, especially infections of the mucous surfaces of the mouth and vagina, and in moist areas of skin. Of the dimorphic fungi, *C. albicans* has the highest incidence of disease. It is a common cause of nosocomial infection; 70% of all women experience *Candida* vaginitis at least once in their lives and 70% of AIDS patients manifest oropharyngeal candidosis (Rooney and Klein, 2002).

*Candida* has a unicellular mode of development and the genus comprises more than 150 species, whose main common feature is the absence of any sexual form (Odds, 1988).

One of the main characteristics of this pathogenic yeast is its ability to switch from a unicellular to a hyphal mode of growth, a property called dimorphism. Dimorphism is triggered in response to changes in certain environmental conditions, such as temperature, pH or serum availability. It is thought that it is this change which allows the organism to invade tissues and, therefore contributes to the virulence of this micro-organism (Navarro- García *et al.*, 2001).
As the host becomes immunocompromised or the competing flora are altered, this opportunist begins displaying cells of the hyphal morphotype (Rooney and Klein, 2002). Dimorphism in C. albicans has caused enormous interest because of the presumption that hypha formation is a process of pathological significance. One rationale is that the hyphal form uniquely expresses molecular virulence factors, which assist in the pathogenesis of candidosis. It is not certain whether yeasts or hyphae consistently play a superior role in the pathogenesis of candidosis. Both morphological forms of the fungus seem to have the ability to initiate and to sustain pathological responses in mammalian hosts, but it is likely that one form may be better adapted than the other to survive in specific ecological microniches in vivo. However, the growth of C. albicans in hyphal form is not invariably associated with tissue invasion in vivo, nor is its growth in the yeast form invariably associated with commensal status. It is believed that all forms are required to maintain an infection. There is a growing body of evidence linking phenotypic switching with pathogenicity of this fungi (Rooney and Klein, 2002). For some authors this dimorphism constitutes a virulence trait per se, but is also co-regulated with other virulence factors, which are associated with cellular morphology (Ernst, 2000).

The three major groups of antifungal agents in clinical use, azoles, polyenes, and allylamine/thiocarbamates, all owe their antifungal activities to inhibition of synthesis of or direct interaction with ergosterol. Ergosterol is the predominant isoprenoid lipid component of the fungal cell membrane. Current available therapies rely mainly on the polyene and azole (synthetic) compounds.
The polyene amphotericin B is the most effective antifungal drug available, but its narrow therapeutic index continues to limit its clinical utility (Ghannoum and Rice, 1999).

Some concepts like antibiotic resistance bacteria leading to hyper-virulent strains have been incorporated into interpretations of fungal pathogenesis. However epidemiological data have not revealed emergence of stable drug-resistant or hyper-virulent strains. Phenotypic switching in response to environmental cues suggests the need to identify the built-in mechanisms these fungi employ for rapidly adapting to challenges (Soll, 2002).

1.5.3. Aspartic Proteases as Virulence Factors

It is very difficult to define virulence for a commensal organism like C. albicans. The concept that virulence is conferred by virulence factors applies best to pathogens that are free-living and able to cause disease in hosts with intact immunity (Casadevall and Pirofski, 2001). This underscores the difficulty in applying classical virulence factors concepts to microbes like C. albicans whose pathogenicity is limited mostly to immunocompromised hosts.

Nonetheless it is possible to point out several factors that seem to contribute to the pathogenicity of C. albicans. Not only factors depending on the micro-organism are involved but also others that are strongly dependent on the host, such as tissue-specific differences in infection susceptibility (Navarro-Garcia et al., 2001). With this note of caution in mind it is important to mention the prominence of secreted aspartic proteases (SAPs) amongst the putative
virulence factors. SAPs constitute a family of isoenzymes encoded by at least nine genes (SAP) that are differentially expressed in vitro and in vivo (De Bernardis et al., 1999).

*C. albicans* strains with disruption of secretory aspartic proteases (SAP 1 to SAP 6) were assessed in a rat vaginitis model. Null sap 1 to sap 3 but not sap 4 to sap 6 mutants lost most of the virulence of their parental strain (De Bernardis et al., 1999).

Inhibition of SAPs with the aspartic protease inhibitor pepstatin A prevented the initial penetration of *C. albicans* through mucosal surfaces but not the dissemination of the fungus once the cells had reached the blood vessels (Fallon et al., 1997). Moreover, pepstatin A prevented *C. albicans* invading and causing tissue damage in oral, vaginal and skin experimental infection models (De Bernardis et al., 1995). HIV aspartic protease inhibitors can inhibit *C. albicans* adherence to epithelial cells (Borg-von Zepelin et al., 1999).

Extracellular matrix and host surface proteins (keratin, collagen, fibronectin) are efficiently degraded by SAP 2. Defence host proteins such as salivary lactoferrin and almost all immunoglobulins, including secretory IgA, which is normally resistant to most bacterial proteases, can also be hydrolysed by SAP 2 (Hube, et al., 2001).

In a mouse gastrointestinal tract model, no experimental support was found for a unique role of individual SAPs. It would seem that SAPs action in colonisation is collective and that absence of one SAP is compensated for by others (Kretschmar et al., 2002). Secreted aspartic proteases by *Candida*
represent a potential target for drug intervention of the disease, and inhibition of SAPs has been proposed as a new approach on the treatment of candidosis (Zhang et al., 2002). Natural products like xanthones and benzophenones present in the Guttiferae family have been reported to have effects against C. albicans secreted aspartic proteases (Zhang et al., 2002) but no powerful plant-derived inhibitor has been commercially developed.

1.5.4. Viruses

In regards to viral disease, herpes simplex virus type 1 is the causative agent of herpes simplex. Frequently, this infection is subclinical, but in many cases causes gingivostoma ("fever blister" or "cold sore") – vesicles around the mouth (Tortora, 2002). Herpes simplex virus (HSV) most frequently produces recurrent painful vesicular eruptions of the skin and mucous membranes. Both HSV-1 and HSV-2 can cause severe protracted and disseminated disease in immunocompromised persons as well as necrosis of infected cells, which is accompanied by a vigorous inflammatory response. Formation of clusters of painful ulceration vesicular lesions on the skin or mucous membranes are the most frequent manifestation of HSV infection (Rubin, 1998). Human poliovirus is a single stranded non-enveloped RNA virus in the family Picornaviridae. This virus will be used to test the different plant extracts because of its resistance to chemical compounds and for comparative purposes since HSV is a DNA virus.
1.6. The Burden of Antibiotic Resistance

Because microbes have the genetic flexibility to develop resistance to any antimicrobial agent it is necessary to accept that the use of antimicrobial therapy is always going to be compromised over time.

The impact of antibiotics in Western society is quite important and nowadays, it is a matter of great concern that no new chemical classes of active antibiotics have been successfully introduced into the clinic for over 30 years (Hancock and Knowles, 1998).

Although there are many instances of spontaneous mutation in the development of resistance to antibiotics (e.g. streptomycin-resistant *Mycobacterium tuberculosis*), mutation is more the exception than the rule. This is because the genetic basis of most antibiotic resistance among clinically significant bacteria is due to the dual process of gene acquisition and gene horizontal transfer (Mazel and Davies, 1999). In the majority of cases, acquisition from exogenous (and still largely unidentified) sources was the primary mechanism by which bacteria obtained genes encoding resistance to antibiotics (Davies, 1994).

The increasing threat of drug-resistant pathogens is causing a renewed interest in the discovery of novel antibiotics. A current trend is to identify essential genes/proteins that are not targets for known antibiotics, and use the purified proteins to search for potent binding ligands from natural sources or combinatorial libraries. A general problem encountered with this approach is the
presence of numerous multidrug pumps found in all bacteria and yeast studied so far (Hsieh et al., 1998). Preventing the access of drugs to the target is one of the various mechanisms microorganisms possess to become resistant to antibiotics. Ubiquitous multidrug resistance (MDRs) pumps, membrane translocases that extrude structurally unrelated toxins from the cell, confer a general and effective defence against antimicrobials' (see Figure 1.1) (Nikaido, 1998).

Figure 1.1.

Gram-positive Bacterial Multidrug Pump: In Gram-positive bacteria, drugs come in unhindered, and are pumped out into the medium (dashed arrows). From Nikaido (1998).
This mechanism involves the active pumping out (efflux) of drugs, a process that has been known since the discovery by Levy and his associates (1992) of active efflux as a major mechanism of tetracycline resistance in bacteria. In recent years, it was found that some of the efflux systems pump out more than one substrate. Nor A is a multidrug pump that belongs to the major facilitator super-family (MFS) of proteins, which use proton motive force as the driving force for efflux (Nikaido, 1998). The NorA MDR pump of *S. aureus* protects the cells from norfloxacin and a number of amphipathic cations, such as the common disinfectants benzalkonium chloride and cetrimide (Hsieh *et al*., 1998).

A mutant of *S. aureus* with a knockout in the norA gene coding for the MDR (multidrug resistance) pump has a substantially increased sensitivity to a large number of antimicrobials, including therapeutically significant compounds (Hsieh *et al*., 1998).

It has been argued that multidrug resistance pumps evolved in response to natural antimicrobial amphipathic cations. For this reason it has been suggested that cells with mutations abolishing the function of their multidrug resistance pumps could be used to increase the sensitivity of screens for new antimicrobial agents (Lewis, 1999). Berberine alkaloids, which are widely spread in the plant world and are prominent in the Ranunculaceae family, appeared to be good substrates for the NorA multidrug resistance pump and for the QacA multidrug resistance pump of *S. aureus*. Consistent with this, mutations knocking out multidrug resistance pumps were found to turn berberine into a very strong
antibiotic. Lewis and co-workers have shown a case (probably the first) of molecular synergy in the medicinal plant *Berberis fremontii* (Berberidaceae) used in Native North-American traditional medicine. This plant produces the weak antibacterial berberine (Figure 1.1) and a potent multidrug resistance pump (MDR) inhibitor identified as 5' - methoxyhydnocarpin (5' -MHC)(Figure 1.2). Berberine used on its own is rendered ineffective due to the presence of the NorA MDR pump. However, when used in conjunction with 5' -MHC, berberine exhibits good activity (MIC=1 μg/mL) (Stermitz et al., 2000).

![Figure 1.2. Structure of Berberine](image-url)
Their experiments showed how two different components of a medicinal plant act in synergy, with one compound disabling a resistance mechanism and potentiating the antimicrobial activity of the antibiotic substance.

Having found natural synergy between berberine and a MDR inhibitor, Tegos and co-workers (2002) tested the general possibility that plant antimicrobials are potentially effective if they are delivered into the pathogen cell while inhibiting the efflux mechanism. A marked increase in the levels of activity of plants antimicrobials was observed, supporting the hypothesis for the apparent ineffectiveness of many antimicrobials in vitro. This is a fascinating illustration that might explain why chemical extracts sometimes present better activity than isolated compounds.

Figure 1.3.
Structure of 5'-methoxyhydnocarpin D (5'–MHC)
1.7. Culture and Environment

Colombia is a country with high cultural and geographical diversity. A census done in 1997 by the National Department of Statistics (DANE) shows a population of 708,860 indigenous people, equivalent to 1.75% of the Colombian population. These populations consist of 80 different ethnic groups, who have territorial rights over 24.5%(279,487 square kilometres) of the national territory (Arango and Sánchez, 1998).

1.7.1. Huitoto

Huitoto people live in the Amazon basin that constitutes a large territory that covers 35% of the Colombian land surface and the 61% of the natural forests. Presently this region is inhabited by 44 different ethnic groups and it is considered the most ethnically diverse in the country (Arango and Sánchez, 1998).

The Huitoto people are also known as murui or muinane. They constitute a typical tribal group of the Amazonian tropical rainforest. They make their living from horticulture, hunting, fishing and fruit collecting and their main transportation means are by foot or canoe. The main crops are yuca (Manihot esculenta), pineapple (Ananas comosus), plantain (Musa paradisiaca), coca (Erythroxylum coca), tobacco (Nicotiana tabacum), corn (Zea mays), sugarcane (Saccharum officinarum) and endemic trees. Each family has two swidden plots: one in production and the other in a growing stage. Both are located within the tropical
rainforest, are distant from one another and several hours away from the main hamlet.

The Huitoto people believe that ailments are caused by supernatural powers. To cure them, they burn aromatic plants around the sick person and use their mouth to extract foreign bodies responsible for the disease.

In the early part of the twentieth century Huitoto were mercilessly exploited during the rubber boom, by the Peruvian Casa Arana –Peruvian Amazon Rubber Company. Violence caused the death of approximately 40,000 Indians (Llanos and Pineda, 1982). Yet the remnants of these once very populous tribes kept their language, culture and knowledge about the properties of plants. Today the Huitotos are the second largest indigenous group in the Colombian Amazon (around 6,245 people) (Arango and Sánchez, 1998).

1.7.2. Kamsá

The Kamsá and Inga indians occupy part of a small highland valley 2,200 m. above sea level in southern Colombia called the Sibundoy Valley. According to the last census, Kamsá people are approximately 4,022 in number in an area of 4,402 ha. (Arango and Sánchez, 1998). The climate of the valley is cool and exceptionally humid. A large non-Indian population also inhabits the valley, and although each group tends to remain apart from the other there is a significant interaction between them, particularly in economic matters. These settlers dominate the local economy and has the best farmland.
The Kamsá and Inga people have become acculturated as a result of schooling, financial transactions and other activities with the white population. All but a few elders are fluent in Spanish, yet they continue to maintain separate identities probably due to the use of traditional language, traditional clothing and separate residences.

Most Kamsá and Inga people have knowledge of herbal remedies and their gardens include several herbs for the treatment of common and minor illnesses. The shamans have received formal training in the use of herbal remedies and the ingestion of the hallucinogen yagé (Bannisteriopsis caapi) is of major importance in diagnostic and therapeutic procedures for illnesses believed to have supernatural causes (Chaves et al., 1995)

Apart from the above, the Kamsá and Inga people have several western medical and para-medical services available in the event of illness yet the healer continues to be regarded more highly than physicians provided by the Government.

1.7.3. Sikuani

The tropical rainforest of Matavén is located north of the Colombian Amazon and surrounded by the Vichada, Orinoco, Guaviare and Chupave Rivers area which is known as the Orinoco region. The Sikuani are approximately 20,544 people inhabiting an area of 2,117,532 ha (Arango and Sánchez, 1998). They are semi nomads and their culture is strongly linked to ecological factors. During dry season the Sikuanis are highly nomadic and rely on hunting, fishing
and fruit collection while in the rainy season they become sedentary and rely upon swidden plots previously established in the higher areas of the basin (Chaves et al., 1995).

As in many other cultures, the shaman cures ailments, provides counseling and teaches prayers that bring good luck, good weather or the opposite. The knowledge associated with the traditional use of medicinal herbs by the Sikuanis is not widespread when compared to other indigenous groups in Colombia (Chaves et al., 1995). Very few ethno-botanical studies have been carried out in the region.

1.7.4. Afro-Colombian Communities

The Pacific littoral is one of the world's rainiest areas, annual precipitation varying between 5,000 and 12,000 mm/year although dry and rainy seasons occur. It is a long (1,300 km) and wide corridor and corresponds to 7% of the national territory (Arango and Sánchez, 1998). The temperature is high with humidity near saturation all of the time.

Afro-Colombian communities inhabit the area since the 16th century and have shared the environment and knowledge with the natives since then. They have profited from plants to treat ailments, obtain food and construction materials since the beginning of the slave trade. Like other communities of African origin inhabiting tropical regions of the world, they share extremely poor living standards despite residing in extremely rich natural and cultural environments.
Until today few ethnobotanical studies have taken place in the region (Caballero, 1995).

As in many communities of African descent, certain features characterize most of their healing traditions: these include theories related to the spiritual realm, the capacity to identify symptoms associated with specific diseases and the ability to prescribe culturally acceptable treatments (Voeks 1993). Cures are most often effected through votive offerings to the ancestors and spirits, observance of taboos, fasting and seclusion, and prescriptions of medicinal plants (Voeks 1993).

Lumber operations to secure the availability of large softwoods are quite important to the cash economy of the region and the country. Timber from the interior must be floated down the main rivers to the mangrove delta. Towns emerging along these riverbanks rise and fall in size and importance according to the vicissitudes of external world markets. People inhabiting the wet Pacific littoral have adjusted their lives to such boom-bust economies (Whitten, 1974).
1.8. References


Chapter 2
Field Collection of Colombian Medicinal Plants

“People with pimples and ulcers on the skin, have spoiled blood. It is necessary to clean and purify this blood so they can be cured...”

Domingo Chasoy
Popayán, Colombia

2.1. Introduction

Nothing is further from reality than the idyllic image of the ethnobotanist immersed deep in the forest in search of the shaman who will reveal his/her herbal medicinal secrets to the outsider. This image probably has its origins in earlier accounts from botanists exploring the north-western Amazon. The name of Richard Evans Schultes is the one that comes first to my mind in regard to the search of herbal remedies based on indigenous traditional knowledge. His book entitled “The Healing Forest” (Schultes and Raffauf, 1990) is an impressive treatise of medicinal plants used for medicinal purposes by Huitoto, Muinane and Bora people to name just a few. Schultes, in his introduction, stated the importance of recording this knowledge before it disappeared but assumed that traditional knowledge was not and is not subject to change and consequently can be recorded once and for all.

In his opinion the body of knowledge about therapeutical practices in which plant uses are entwined was static. In other words, the indigenous medical
systems were outside the normal processes of cultural transformation. The names of different tribes were there because of taxonomic compulsion and medical practices had no connection whatsoever to their mythology or religion which might have explained the use of a plant. In short, he saw indigenous populations as passive elements in an idyllic interplay with nature and regarded them as non-historical individuals. Indigenous communities were then transformed into static entities whose knowledge was susceptible to be registered.

Opposing this view, I consider that it is urgent to discuss the use of medicinal plants in an historical context. Indeed when one considers the persecution, displacement and annihilation of the indigenous people along the Caquetá River during the rubber exploitation, one must wonder about the concept of identity and how this concept is reflected in their traditional medical systems. The influence of the Catholic Church in regions like the Sibundoy valley led to a cultural fusion that directly influenced the medical systems of the Kamsá and Inga people. These historical circumstances seem to have been completely disregarded both by botanists and ethnobotanists. The question of how historical processes have transformed traditional medical systems has not been addressed.

It is important to note that the present chapter does not include a historical background of the individuals who helped me during the field collection. Thus I do not consider this field collection to be an ethnobotanical one but rather a collection of plants traditionally used in the treatment of morbid skin conditions
but devoid of a cultural context that would explain their uses. I consider this a drawback whose discussion will be included in chapter six.

I followed the ethnobotanical practice of interviewing, collecting and recording plant uses. The research reported in this chapter may have a limited value from the anthropological point of view, but it gave me the opportunity to compare plant uses in different cultural and geographical communities in Colombia.

2.2. Materials and Methods

2.2.1. Plant Collection

The plants were collected at one site each in four different areas in Colombia (Fig 2.1) guided by local healers and villagers. Information about the therapeutic properties of the plants was gathered by participating in domestic activities and interviewing traditional healers (curanderos) and knowledgeable villagers about those plants used to treat cutaneous conditions of any kind. The information gathered included native and/or common names, part(s) used and recipes.

Plant material was air-dried in the shade and a set of labelled voucher herbarium specimens was made for each collection; these vouchers were filed in the National Herbarium of Colombia (COL) and the Sinchi Institute Herbarium (Santafé de Bogotá, Colombia).
2.2.2. Collection Sites

Distribution of the collection sites were as follows
Figure 2.1
Collection Plants of the Plants Studied

1. Bajo Calima
2. Mataven Forest
3. Sibundoy Valley
4. Middle Caquetá Basin
BAJO CALIMA REGION (Valle Department)

Afro-Colombian community

This area lies in the Chocó biogeographic region south of the Calima River, at 03°57'-04°-10' N and 77°01'-12°W on the Pacific Coast of Colombia near the port of Buenaventura, in the Valle Department. It occupies approximately 60,000 ha, at an elevation between 0 and 300 m and receives 7,000-8,000 mm of rain annually (Motta, 1997). The Bajo Calima site has been inhabited by Afro-Colombian communities for many generations and only few years ago it became a timber concession (1950-1995) to the company Cartón de Colombia. The plant collection was carried out along the Calima River near the coast. Hubert Murillo and his father, Adriano Murillo, who lived in the region, guided the collection of the plants.

MATAVEN FOREST (Vichada Department)

Sikuani community

The Sikuani reserves are mainly located in the Departments of Vichada and Meta. There are 20,544 people in an area of 2,117,532 ha. Mataven forest is north of the Colombian Amazon near Cumaribo in the Vichada Department. The area has an elevation of 166 m and precipitation ranging from 1,500 to 2,000 mm annually (IGAC, 1996). The plant collection carried out in the Cumaribo was guided by Elvira Cariván, a well-respected inhabitant of the region.
SIBUNDOY VALLEY (Putumayo Department)

Kamsá and Inga communities

The valley of Sibundoy lies near the eastern slope of the Andes at an elevation of 2,000 m. The climate of the valley is cool, very humid and with relatively little sunshine. The average annual rainfall is close to 2,136 mm (IGAC, 1996). Kamsá and Inga communities in this valley inhabit an area of 4,402 ha. Although these communities live in the same territory, they belong to different linguistic families, the Kamsá and Quechua respectively.

The plant collection was carried out in the village of Sibundoy and was guided by two traditional healers from the Kamsá community, Don Miguel Chindoy Mutumbajoy and Doña Clarita Buesaquillo.

MIDDLE CAQUETA BASIN (Caquetá Department)

Huitoto community

The middle Caquetá basin is located in the north-western Colombian Amazon and covers 1,300,000 ha, and is dissected by the Caquetá River, which is a tributary of the Amazonas and is one of the basin’s main communication routes. The elevation ranges between 200 and 300 m and the region has an average annual rainfall of 3,000 mm (Galeano, 1992).

Huitoto indigenous communities, along with Miraña, Muinane, and others, inhabit the middle region of the Caquetá River. Plant collection was carried out with two members of a Huitoto family, Oscar and Simon Román. The collection site is located in Aguasal Creek, a small tributary of the Caquetá River.
2.3. Results. Field Ethnomedical Data

A list of the collected plants is enumerated below, with voucher herbarium specimen number indicated in parentheses.

**APIACEAE**

*Hydrocotyle umbellata* L. (Andrés López AL-57)

Collection site: Sibundoy valley

Common name: Chupana

It is said that a mixture with animal fat will “suck” the infections out of the skin.

**ASTERACEAE**

*Ambrosia artemisioides* Willd. (AL-02)

Collection site: Sibundoy valley

Common name: Marco

An infusion of leaves is prepared and used as a bath to “clean” the body.

*Conyza bonariensis* (L.) Cronq. (AL-36)

Collection site: Sibundoy valley

Kamsá name: Tacehsajch

Annual herb in gardens, introduced, common.
It is used to treat dark spots on the face, acne and for the condition known as "carate" (*Tinea alba*). The plant is macerated and applied to the skin. Exposure to sunlight must be avoided.

*Tagetes erecta* L. (AL-59)
Collection site: Sibundoy valley
Common name: Mapán
A decoction of the flowers is used externally in the treatment of skin infections.

*Eupatorium glutinosum* Lam. (AL-01)
Collection site: Sibundoy valley
Common name: Matico
An infusion of the leaves is prepared and used as a bath to "clean" the body.

**CELASTRACEAE**

*Goupia glabra* Aubl. (Oscar Román OR-73)
Collection site: Middle Caqueta Basin
Uitoto name: Jodina (Jodi= to get drunk; -na= tree) The extract of young leaves is used externally to treat blindness caused by cataracts and in the treatment of smallpox. It is used as a cicatricial when applied on the skin.
CLUSIACEAE

_Vismia macrophylla_ Kunth (AL-05)

Collection site: Middle Caquetá Basin

Common name: Lacre

Uitoto name: Yiikoai (Yii= that changes color; -ai= small tree)

The lightly roasted leaves are used as a dressing on snakebites and placed on top of the wounds. The bark is used externally in the treatment of cutaneous infections while the resin is used for the condition known as “carate”. The sap is used in the treatment of problems related with vision.

_Symphonia globulifera_ L. f. (AL-88)

Collection site: Bajo Calima

Common name: Macharé

A decoction of the bark is rubbed on the skin for treatment of cutaneous leishmaniasis.

FABACEAE

_Senna reticulata_ (Willd.) H.S. Irwin et Barneby (AL-33)

Collection site: Bajo Calima

Common name: Galves

A decoction of the leaves is used in the treatment of skin infections particularly in fungal infections that whiten the skin.
JUGLANDACEAE

*Juglans neotropica* Diels (AL-89)

Collection site: Sibundoy Valley

Common name: Cáscara de nogal

An infusion of the bark is prepared and used as a bath to “clean” the body, as an antiseptic and to treat a condition known as “body rottening”. A plant infusion is taken orally as a contraceptive.

LECYTHIDACEAE

*Eschweilera rufifolia* S.A. Mori (OR-71)

Collection site: Middle Caqueta Basin

Common name: Carguero

Uitoto name: Koduiro (kodui= that the bark is easily peeled off)

The inner part of the bark is used externally as an anesthetic and as a cicatrizant in the treatment of wounds. It is also used in the preparation of vegetable salt.

MALPIGHIACEAE

*Byrsonima verbascifolia* (L.) DC. (EM-01)

Collection site: Mataven Forest

Sikuani name: Dorronae

A bath is prepared with a mixture of the leaf and the bark of the root for treatment of cutaneous conditions.
MONIMIACEAE

*Siparuna guianensis* Aubl. (AL-87)

Collection site: Mataven Forest

Common name: Romadizo

Sikuani name: Minisinae

Cuiba name: Bibisine

The bark is macerated in water and used as a bath for skin diseases and headache. This maceration is also good to diminish fever. Also, an infusion from the leaves is used as a bath to "clean" the body.

MYRISTICACEAE

*Iryanthera megistophylla* A.C. Sm. (AL-29)

Collection site: Bajo Calima

Common name: Cabo de indio

The decoction of the bark is used externally in the treatment of cutaneous leishmaniasis.

*Iryanthera tricornis* Ducke (AL-12)

Collection site: Middle Caquetá Basin

Common name: Cabo de hacha, mamita, sangretoro

Uitoto name: Jiikai (Ji= to bend down; kai= refering to an elongated shape and smooth texture). The bark's aqueous extract is used internally in the treatment of measles.
Virola multinervia Ducke (AL-18)
Collection site: Middle Caquetá Basin
Common name: ambil de monte
Uitoto name: Ukukai (uku= star; kai= refering to an elongated shape and smooth texture). The bark is used for the treatment of skin sores and fungal infections by crushing and boiling in water, and applying the decoction externally.

MYRTACEAE

Myrteola nummularia (Poir.) O. Berg (AL-86)
Collection site: Sibundoy Valley
Common name: Guayabilla
Kamsa name: bchichaja
Inga name: paramchichaja
It is a well-known plant commonly sold in market places. A bath of the infusion of the whole plant is claimed to be useful in treatment of skin problems.

PIPERACEAE

Piper lanceaefolium Kunth (AL-75)
Collection site: Sibundoy Valley
Common name: Cueche
The decoction of the leaves is taken as a bath to treat skin infections. Specifically, those that refer to a malignant cutaneous manifestation produced when one gets “the piss of a rainbow”, a term which describes the drizzle that usually accompanies the presence of a rainbow.

POLYGONACEAE

Polygonum punctatum Elliott (AL-35)

Collection site: Sibundoy Valley

Common name: Picantillo

Kamsá name: Fsteshaja

Perennial herb in ditches, native, very abundant.

A decoction of the plant is used externally in the treatment of skin infections. Because of its potency, it should be used with caution. As with Piper lanceaefolium, a bath of the infusion is taken for that purpose. According to Doña Clarita this preparation is used when parts of the body of a living individual are “rotten”. The Kamsá and Inga communities use the word “rotten” when they observe a change in color and swelling in the body tissue, but not necessarily to express the lost of form due to the action of bacteria, fungi etc. Don Miguel noted the benefits when the body is “watery”. It is also used as an insecticide.
PTERIDACEAE

_Adiantum latifolium_ Lam. (AL-26)

Collection site: Bajo Calima

Common name: Montañero; Nena

A decoction of the leaves is used to prevent tissue necrosis produced by the bite of the "bushmaster" snake.

RHAMNACEAE

_Ampelozizyphus amazonicus_ Ducke (AL-13)

Collection site: Middle Caqueta Basin

Muinane name: +minó-gaico

Huitoto name: D+iño; A+foio

( + is a high central vowel; it is pronounced by placing the tongue in the position of _u_ and the lips in the position of _i_; Candre and Echeverri, 1996)

The bark is scraped, added to water and vigorously shaken. The resulting foamy preparation is applied as an antiseptic on wounds.

RUBIACEAE

_Duroia hirsuta_ (Poepp. & Endl.) K. Schum. (OR-74)

Collection site: Middle Caquetá Basin

It is used as a dye by mixing with other plants; the bark is used externally as a cicatrizant.
SIMAROUBACEAE

Picrolemma sprucei Hooker f. (AL-03)
Collection site: Middle Caquétá Basin
Common name: Arbolito de casabe
Uitoto name: Taingorai (taingo= cassava bread; -rai= wooden block)
The leaf extract is applied over an area on the skin affected by an infection.

SOLANACEAE

Solanum sp. (AL-85)
Collection site: Sibundoy Valley
Common name: Chontará
Kamsa name: Beuntajajch or Bebunjaja
It is a well-known vine among the healers of the valley; it serves different purposes. The bark is scraped, toasted and applied over injuries and wounds.
2.4. Discussion

The four collection sites corresponded to different cultural settings and different habitats as well. These differences are reflected in the plant material used for medicinal purposes in these regions.

Eighteen plant families are represented in the collection list. The Asteraceae was represented by four plant species, all of them collected in the Sibundoy Valley and well known in the ethno-medical literature. This probably reflects the interest the healers of the Sibundoy Valley have raised in western researchers. In Uruguay and Argentina, an infusion made of stems, leaves and flowers of *Conyza bonariensis* is orally administered and used as a hepatoprotectant agent. It also has anti-diarrhetic, anti-helmintic, diuretic and antidiuretic effects, and is used in the treatment of rheumatism, gonorrhea and dysentery (Gupta, 1995).

*Eupatorium glutinosum* is also well known in Ecuador because of its reputation for the treatment of peptic ulcer, as a cicatrizant of wounds and ulcers and in the treatment of diarrhea. A decoction of the leaves in water is used and orally administered, or else fresh leaves are crushed and directly applied onto the swollen or ulcerated areas. *Eupatorium* species have been traditionally used in different parts of the world, although some species are known to have a number of adverse interactions in livestock and humans. It is important to bear in mind the fact there is a great variation in the chemistry of the natural products in the different species of *Eupatorium* (Sharma *et al.*, 1998).
A. artemisioides is also a plant with a wide spectrum of medicinal uses. A leaf infusion is used to correct irregularities of the menstrual cycle and in treatment of dysentery, fresh leaves lightly roasted are crushed and rubbed on joints to alleviate the symptoms of rheumatism; an infusion of fresh dried flowers is used as a vermifuge; in Colombia a decoction of the leaves and inflorescences is used as an contraceptive. In cases of hematoma, the leaves are crushed and applied over the area as an anti-inflammatory and analgesic (Gupta, 1995).

Another well known plant is Senna reticulata whose flowered stems are used to decorate the table of the “jaibana” (shaman) during the “chant of the jai” or the “spirits invocation”. An infusion of the leaves is used as a laxative (Caballero, 1995).

In Ecuador an infusion of the leaves of Juglans neotropica is used as an astringent and against diarrhea, particularly in children; it is also used as an antiseptic to wash wounds and ulcers, for vaginal douches in cases of leukorrhea. An infusion of powdered bark of the fruit is used as a purgative (Gupta, 1995).

Fresh latex of Vismia angusta and Vismia ferruginea is used commonly in Colombia and Perú to treat wounds and infected sores (Schultes, 1983). In Nicaragua the leaf of V. macrophylla is used to treat skin infection (Barrett, 1994). Ortiz mentions the use of this plant amongst the Sikuani and Cuiba in the treatment of gonorrhea, without specifying the useful part of the plant (Ortiz, 1985)
Symphonia globulifera is known as “mawinae” in Sikuani and the latex is used as an analgesic to be externally applied. A decoction of the bark is used in Gabon for scabies (Akendengue, and Louis, 1994) and in Panamá the infusion is used to treat bloody vomiting (Joly et al., 1987).

Interestingly, the benefits of Polygonum punctatum when the body is “watery” might be related to the use in traditional Chinese medicine of Polygonum hydropiper ("Water pepper" originary from India) or P. flaccidum which are used to remove dampness and food stagnancy (This terminology corresponds to traditional Chinese Medicine and correlation to western terminology is not evident.) (Huang, 1999).
2.5. References


“Every plant has a spirit of its own, a plant isn’t just something else, it’s a living thing. The spirit of a plant tells us how to cure those who are sick; or shows us more plants. To call on that spirit we take yagé.”

Inga Healer
Mocoa, Colombia

3.1. Introduction

Skin-related morbid conditions are numerous and not necessarily connected to an infection. The rationale is to assume that the plant for which an indigenous use is reported will have some kind of antibiotic and/or antiviral activity, hoping that the extract will be detected as having effect on viruses, bacteria or yeast. This chapter describes the biological screening of chemical extracts of Colombian medicinal plants whose collection has been described in chapter 2.

Because of their current relevance, I became particularly interested in anti-viral (e.g. herpes simplex virus) and anti-Candida activities. *Candida albicans* is an important opportunistic pathogen causing local or systemic infections in predisposed patients who are immunologically compromised or undergoing prolonged antibiotic treatment.
Candidosis has been shown to involve aspartic proteases (SAP) secreted from *Candida albicans* as a major virulence factor in Candida infections (Hoegl *et al.*, 1996; De Bernardis *et al.*, 1999). Inhibition of SAP has been proposed as a new approach in the treatment of this infection. Recently it has been demonstrated that HIV protease inhibitors decrease the adherence of fungal cells to epithelial cells *in vitro* (Bektic *et al.*, 2001).

In view of the inevitable delay between the collection of fresh material and the chemical extraction, plant material collected was air dried in the shade at the respective collection sites in order to avoid microbial contamination. Methanol was the solvent of choice in a preliminary screening because, in comparison to other solvents (ethanol, chloroform, acetone, dichloromethane), the quantity of compounds extracted is greater (Eloff, 1998).

Electron Spin Resonance Spectroscopy (ESR) is also termed Electron Paramagnetic Resonance (EPR) and it is a type of spectroscopy similar to Nuclear Magnetic Resonance (NMR). ESR allows studying compounds containing one or more *unpaired* electrons. ESR spectra parameters such as g-factors and hyperfine splitting constants (Hfs) provide information about the structure of some free radicals. This technique can be applied to the detection and identification of ortho and para quinols or quinones in plant extracts, without prior isolation. When these compounds are in alkaline solution they convert to their corresponding semiquinone radicals which are subsequently observed by ESR (Pedersen, 2000).
This technique can be used then to identify compounds in crude extracts allowing selecting a smaller sub-population of crude extracts before engaging in a phytochemical analysis. In this chapter, I will illustrate an application of this technique in the identification of a compound (juglone) in a plant extract that exhibited activity against *C. albicans*.

### 3.2. Materials and Methods

#### 3.2.1. Microorganisms

Viruses used in the antiviral assays were the enveloped double-stranded DNA herpes simplex virus (HSV-1) type 1 and the non-enveloped single-stranded RNA poliovirus (type 1 vaccine strain). Laboratory strains of bacteria and fungi were obtained from the University of British Columbia collection. Eight species of bacteria and one species of fungus were used in the screening process. The bacterial strains consisted of Gram- positive *Bacillus subtilis*, *Streptococcus faecalis* and *Staphylococcus aureus* K147 MS (methicillin-sensitive), while Gram-negative strains consisted of *Escherichia coli* DC10, *Klebsiella pneumoniae*, wild type *Pseudomonas aeruginosa* H187 (wild), *Salmonella typhimurium* TA98, and acid fast *Mycobacterium phlei*. The fungus used was the yeast *Candida albicans* (UBC54).

An inoculum of each bacterial strain was suspended in 5 mL Mueller-Hinton broth (BBL) and incubated overnight at 37°C. The overnight cultures were diluted 1/10 with Mueller-Hinton broth (BBL™) (Becton, Dickinson and Company) before use.
The fungal cultures were prepared by swabbing the plate with a cotton swab and then transferring this to a vial containing 5 mL of Saboraud dextrose broth (BBL™) (Becton, Dickinson and company).

3.2.2. Preparation of Extracts

Dried plant material was grounded manually. 20g of each ground plant were soaked in 200 mL methanol A.C.S. grade for 48 hours at room temperature. The extract was filtered through a Buchner funnel with Whatman No 1 filter paper and the residue was washed with methanol. The filtrate was concentrated and the remaining solvent was further removed under reduced pressure and freeze-dried for 24 hours. The yields of prepared extracts are shown in Table 3.1.

3.2.3. Antiviral Assays. Determination of Minimal Inhibitory Concentration (MIC)

The antiviral assays were performed using the enveloped double stranded DNA herpes simplex virus (HSV-1) type 1 and the non-enveloped single strand RNA poliovirus (type 1 vaccine strain).

Vero cells (African green monkey kidney cell line – American Type Culture Collection) were grown in monolayers in a 5% CO₂ and 95% air atmosphere at 37°C, in Dulbecco’s modified Eagle medium (MEM) (GIBCO-Life Sciences, Ontario) and 25 μg/mL gentamycin sulphate (Sigma), in 96-well microtest trays (Falcon) (Anani et al., 2000). When cells formed confluent monolayers, they were used for the assays. A solution of 40 mg/mL of each methanolic extract was
prepared and filtered through a sterile syringe filter of 0.2 μm pore diameter. Each extract was diluted 1:100 in MEM (Hudson et al., 1994) and serial two-fold dilutions of the extract were made (in duplicate) in MEM across a row of cells in an empty 96-well microtest tray (final concentration of methanol < 1%). The diluted extracts were transferred to the aspirated Vero cell monolayers previously prepared.

These cultures were incubated at 37 °C for 60 min and examined microscopically for possible immediate cytotoxic effects. Then, 100 μL of virus corresponding to one hundred plaque-forming units (PFU) was added to each well. The tray was transferred to an environmental chamber (37 °C) and exposed to a combination of visible light plus UVA (long-wave ultraviolet) for 30 min with continuous gentle shaking (100rpm) of the tray in order to ensure homogeneity in the well.

The fluorescent and black light blue lamps (BLB) were arranged to give approximately 5 watts/m² incident radiation of both visible and UVA light. Following the light exposure, the trays were returned to the cell culture incubator. Cultures were inspected periodically for viral cytopathic effects (CPE). In the case of HSV, complete cell destruction (100% viral CPE) required 4 days, while for the poliovirus, 2 days. Absence of CPE was interpreted as positive results for antiviral activity. Partial inhibition was considered to be a negative result. The MIC was recorded as the minimum concentration of extract, which gave complete inactivation of virus infectivity (i.e., absence of viral CPE). A solution of
methanol in MEM was used as a negative control and antiviral tests were repeated twice.

3.2.4. Cytotoxicity Assays

To test for cytotoxicity, Vero cell monolayers were grown in 96 well microtiter plates (Falcon), and exposed to serial dilutions of the extracts, starting at a final concentration of 20 µg/mL of crude plant extract. The treated cells were then incubated at 37°C for 1 hour, exposed to UV-A light and visible light for 30 min and then re-incubated for 24 hours. The cells were examined microscopically for periodic assessment of changes in cell morphology or visible toxic effects (obvious cellular damage or lysis). A solution of methanol in MEM was used as a negative control (final concentration: 1%). Cytotoxicity assays were repeated twice.

3.2.5. Antimicrobial and Antifungal Assays

The disk diffusion assay (Taylor et al., 1995) was used to screen for antimicrobial and antifungal activities. Samples were tested in duplicate; one in the dark and one exposed to UVA. One hundred microlitres of the diluted culture were spread on sterile Muller-Hinton agar (BBL™) (Becton, Dickinson and company) plates (for bacteria) or sterile Saboraud dextrose agar (BBL™) (Becton, Dickinson and company) plates (for fungi).

Sterile paper discs were impregnated with 20 µL of a solution prepared with 100 mg of extract in 1 mL of methanol, and allowed to dry at room
temperature. The impregnated discs were placed on the plates and incubated for 30 min. to allow for diffusion. Gentamycin 10 mg/ml (for bacteria) or nystatin 5mg/ml (for fungi) was used as a positive control. 8-Methoxypsoralen 1mg/ml (8-MOP) was used as a positive control for light-activated extracts. Methanol was used as a negative control.

To test for light activated antimicrobial/antifungal activity, one replicate plate was exposed to ultraviolet UVA light (5 W/m², 320-400 nm from four Sylvania F20T12-BLB lamps) for two hours while the other was kept in the dark (Taylor et al., 1995). The plates were incubated for 18 h (48 h for Mycobacterium phlei) before the resulting zones of inhibition were observed and recorded. Tests were repeated twice.

3.2.6. SAP Inhibition Screening

The method described by Li and co-workers (2001) was modified to be faster and amenable in an automatic microtiterplate reader (Spectrafluor plus, TECAN GmbH, Salzburg Austria).

3.2.6.1. Induction of SAP Production

The procedure of Capobianco and co-workers (1992) was followed. Briefly, Candida albicans was grown overnight (15 hrs) in a shaker at 50 rpm and 35 °C in Sabouraud dextrose media broth. The cells were centrifuged for 10 minutes at 17,300 rcf. The pellet was washed once with 10mM phosphate-buffered saline (PBS), pH = 7.0, centrifuged for 10 minutes at 17,300 rcf and re-
suspended in 15 mL of filter-sterilized inducing medium (0.2% yeast extract, 0.2% bovine albumin, 2.0% dextrose), and incubated for 14 hours at 35 °C. The SAP-rich solution was centrifuged at 23,500 rcf for 3 minutes, the supernatant was collected, sterilized by filtration (0.2 micron filter from Fisher), and 1 mL aliquots from each fraction were stored at -80 °C until needed.

3.2.6.2. Inhibitory Activity against Secreted Aspartic Proteases.

3.2.6.2.1. Determination of the Optimal Substrate and SAP Extract Concentrations

To determine optimum substrate and SAP extract concentrations an experiment, in which SAP extract and substrate concentrations were varied, was performed in a 96 well microplate. Immediately after adding the renin substrate (Molecular Probes R-2931 Eugene OR) the fluorescence at 360 ex/535 em was measured and fluorescence readings were taken automatically every 10 minutes for the next two hours. The increase in fluorescence during the incubation was determined by subtracting the fluorescence reading from the reading of a blank that contained citrate buffer instead of SAP extract. Optimum concentrations were chosen based on dose response curves and selecting a linear segment of the curve. The selected final concentrations were 40 μM renin substrate and 1/40 dilution of the SAP extract.
3.2.6.2.2. SAP Inhibition Assay for Crude Extracts

A microtiter plate reader (Spectrafluor plus, TECAN GmbH, Salzburg Austria) equipped with an excitation filter at 360 nm (35 nm bandwidth) and an emission filter at 535 nm (25 nm bandwidth) using 96 well microtiter plates (Falcon).

Prior to each set of measurements an automatic fluorescence gain adjustment was performed for the range of fluorescence changes expected. The integration time for the fluorescence signals was 40 μs without lag time at 15 flashes per well. The optimum gain setting of the instrument varied between 130-141. For measurements a microtiter plate was loaded with 50 μL of compound solution for a final concentration of 40 μM in sodium citrate buffer (methanol concentration < 2.0 %) followed by 50 μL of SAP extract (1/40 dilution). The mixture was incubated during one hour at 36 °C in a shaker at 271rpm.

After the incubation time 100 μL of renin substrate was quickly added to obtain final concentrations of 40 μM. The final volume per well was 200 μL.

Plant crude methanolic extracts were tested initially at a final concentration of 50 μg/mL in order to identify extracts with inhibition activity greater than 80%. The fluorescence signals of these extracts were recorded at 37 °C during 20 min. for 10 cycles with a kinetic interval of 120 seconds.

In principle, it is not essential to monitor the time course of fluorescence changes for each experiment. However, it should be visualised at least once to estimate the minimum time required for the reactions to be completed or whether or not there are drifts in the fluorescence signal during the time course of the
experiment. Tests were performed three times for each crude extract. Calculations of non linear regression curves were conducted with Excel (Microsoft) which is compatible with TECAN software. A blank was used to correct the ΔRFU values for non enzymatic renin substrate degradation during the time course of the experiment.

3.2.7. Electron Spin Resonance (ESR)

Twenty microliters of an Ethanol-Water (4:1) extract of *Juglans neotropica* was mixed with five microliters of NaOH (0.2M) and the mixture was shaken in the air. Ten microliters of the mixture in a capillary was introduced into the magnetic resonance cavity and ESR spectra was recorded according to the procedure described by Pedersen (2000).

3.3. Results

3.3.1. Antiviral Activity

Thirteen extracts of the twenty-four species tested exhibited activity against HSV but none of them was active against poliovirus (Table 3.1). Extracts prepared from *Byrsonima verbascifolia* (Malpighiaceae), *Iryanthera megistophylla*, *Vismia macrophylla* (Clusiaceae) and *Eschweilera rufifolia* (Lecythidaceae) exhibited particularly good activity against HSV, comparable to potent extracts from previous studies (Anani *et al.*, 2000). The minimal concentration causing cytotoxicity is reported in Table 3.1.
Extracts from *Tagetes erecta* (Asteraceae), *Vismia macrophylla* (Clusiaceae) and *Picrolemna sprucei* (Simaroubaceae) were found to be cytotoxic. Because of the cell damage produced by these extracts, it was not possible to determine antiviral activity. The results of the active extracts are listed in Table 3.1. The minimal inhibitory concentrations ranged from 2.5 to 25 µg/mL.

### 3.3.2. Antimicrobial Activity

Only *Tagetes erecta* (Asteraceae) extract displayed photoactivity against *Pseudomonas aeruginosa* and *Bacillus subtilis*. None of the extracts was active against the Gram-negative bacteria tested, namely *Escherichia coli*, *Salmonella typhimurium* or *Klebsiella pneumoniae*.

The extracts with the broadest spectrum of activity were: *Adiantum latifolium* (Pteridaceae); *Eupatorium glutinosum* (Asteraceae); *Tagetes erecta* (Asteraceae); *Vismia macrophylla* (Clusiaceae); *Juglans neotropica* (Juglandaceae); *Eschweilera rufifolia* (Lecythidaceae); *Senna reticulata* (Fabaceae); *Symphonia globulifera* (Clusiaceae); *Byrsonima verbascifolia* (Malpighiaceae); *Iryanthera megistophylla* (Myristicaceae); *Siparuna guianensis* (Monimiaceae); *Virola multinervia* (Myristicaceae); *Myrteola nummularia* (Myrtaceae); *Piper lanceaefolium* (Piperaceae); *Polygonum punctatum* (Polygonaceae); and *Duroia hirsuta* (Rubiaceae). Twenty-two of the extracts showed activity against bacteria and two showed activity against *Candida albicans* (Table 3.2). Anti-Candida activity was observed with *Piper lanceaefolium* (Piperaceae) and *Juglans neotropica* (Juglandaceae).
3.3.3. Inhibitory Activity against Secreted Aspartic Proteases

Sixteen of the extracts showed complete inhibition in the aspartic protease inhibition assay (Table 3.3). These were from the following species: *Hydrocotyle umbellata* (APIACEAE); *Eupatorium glutinosum* (Asteraceae); *Symphonia globulifera* (Clusiaceae); *Juglans neotropica* (Juglandaceae); *Eschweilera rufifolia* (Lecythidaceae); *Byrsonima verbascifolia* (Malpighiaceae); *Iryanthera megistophylla, Iryanthera tricornis* and *Virola multinervia* (Myristicaceae); *Siparuna guianensis* (Monimiaceae); *Myrteola nummularia* (Myrtaceae); *Piper lanceae folium* (Piperaceae); *Polygonum punctatum* (Polygonaceae); *Duroia hirsuta* (Rubiaceae) and *Solanum sp* (Solanaceae).
Table 3.1
Antiviral Activity of Extracts of Selected Colombian Medicinal Plants

<table>
<thead>
<tr>
<th>Family / Species</th>
<th>Part</th>
<th>Yield</th>
<th>Cytotoxicity</th>
<th>HSV</th>
<th>Poliovirus</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>μg/mL</td>
<td>μg/mL</td>
<td>μg/mL</td>
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<tr>
<td>APIACEAE</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrocotyle umbellata</td>
<td>AE</td>
<td>8.66</td>
<td>- e</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ASTERACEAE</td>
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- **Part extracted**: AE, aerial parts; LF, leaves; BK, bark; RB, root bark; RT, root; RE, resin; FL, flowers
- **Weight (g)**: Weight (g) of crude methanolic extract per 100 g of dried plant material
- **Minimum concentration causing effect**
- **Minimum concentration causing complete inhibition (MIC)** of viral (HSV and Poliovirus) CPE.
- **Non active extract**
- **Non feasible test**
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Family / Species

Antimicrobial Activities of Extracts of Selected Colombian Medicinal Plants

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*a* (-): no zone of inhibition (negative sign); (+): extract active (positive sign)

*b* Part extracted: AE, aerial parts; LF, leaves; BK, bark; RB, root bark; RT, root; RE, resin; FL. flowers


*d* UV/D: ultra-violet/dark
3.3.4. Inhibition of Secreted Aspartic Proteases

Sixteen extracts presented complete inhibition in the enzymatic assay for secreted aspartic proteases. Only two extracts did not exhibit inhibition. Inhibition percentages are presented in Table 3.3.
Table 3.3
Inhibition of Secreted Aspartic Proteases by Colombian Medicinal Plant Extracts

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<td>Symphonia globulifera</td>
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Table 3.3 (Cont.)
Inhibition of Secreted Aspartic Proteases by Colombian Medicinal Plant Extracts

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<td>Solanum sp.</td>
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3.3.5. Electron Spin Resonance Spectroscopy for *Juglans neotropica* extract.

A spectrum of an ethanol-water extract of *Juglans neotropica* is shown in Figure 3.1. The g value and the proton hyperfine splittings (Table 3.4) are indistinguishable from those of an authentic juglone semiquinone spectrum.
Figure 3.1
ESR Semiquinone Spectrum of Juglone in *Juglans neotropica* Alcoholic Extract
<table>
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<th>Hyperfine Splitting Constants (Hfs)</th>
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Table 3.4

Hyperfine Splitting Constants (a values) (in Gauss) and g values of juglone found in *Juglans neotropica* Extract
3.4. Discussion

Some of the extracts tested appear to be more potent than many of the antiviral extracts from plants examined to date (e.g. Taylor et al., 1996; Anani et al., 2000). The leaf extract of *Byrsonima verbascifolia* presented an inhibitory concentration as low as 2.5 μg/mL which is similar to the best activities found in extracts of seaweeds from British Columbia (Hudson et al., 1999). *Byrsonima crassifolia* (L.) H.B.K. is a tropical tree widely distributed in Mexico, Central and South America. It has been medicinally used since pre-hispanic times (Martínez-Vásquez et al., 1999), and antimicrobial and antifungal activity from bark and leaves has been widely reported (Martínez-Vázquez et al., 1999; Cáceres et al., 1993). However, no bioassay-guided chemical fractionation has been carried out.

Although other members of the genus *Vismia* have been studied extensively, little is known about the species reported here. The related species *Vismia cayennensis* exhibited HIV-inhibitory activity due to the presence of vismiophenone D (Fuller et al., 1999). A structurally related group of compounds collectively named guttiferones (prenylated benzophenone derivatives) were found to be responsible for the HIV-inhibitory activity in an extract of *Symphonia globulifera* (Gustafson et al., 1992). However, these compounds were also cytotoxic to the host cells used in the antiviral assay (Fuller et al., 1999; Gustafson et al., 1992). We detected cytotoxicity against Vero cells for the bark of *Vismia macrophylla* but no cytotoxicity was observed for its resin or in the bark of *Symphonia globulifera*. Further, two cytotoxic xanthones with isoprenoid groups have been reported from the root bark of *S. globulifera* (Nkengfack et al.,...
2002). On the other hand it is interesting to note that *Symphonia globulifera* and *Iryanthera megistophylla*, traditionally used to treat cutaneous leishmaniasis, displayed activity against HSV in our study.

The potent anti-HSV activity (complete virus inactivation at 20 µg/mL) found in the methanolic extract of *Polygonum punctatum* contrasts with the results of Kott and coworkers (1999), who reported that the aqueous extract of the leaves of this species exhibited activity at a much higher concentration (EC$_{50}$ = 169.7 µg/mL). The different conditions used in the extraction procedure might explain in part the difference between these two concentration values. Alternatively, the difference may be due to a lower concentration of bioactive compound(s) in the plant material collected by Kott and to the presence of photoactive antivirals.

Leaf extract of *Eupatorium glutinosum* presented activity against *Mycobacterium phlei*, *Bacillus subtilis* and *Staphylococcus aureus*. This activity might be associated with the presence of labdene diterpenes, which have been shown to have weak antimicrobial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. According to El-Seedi and coworkers (2002) this could support the vernacular medicinal use of *E. glutinosum* as an antimicrobial.

Arguably the most important of the extracts which I studied are the root bark and the leaf extracts of *Byrsonima verbascifolia* (see Table 3.1). However, the scarcity of the plant and the difficulty in obtaining adequate amounts made me discard this plant for future work.
Taking the anti-microbial and anti-viral results together, the plant extracts worth further study are: *Eschweilera rufifolia*, *Iryanthera megistophylla*, *Virola multinervia*, *Myrteola nummularia* and *Duroia hirsuta*. These extracts also exhibited complete inhibition in the secreted aspartic proteases inhibition assay.

Based on the anti-Candida assays and the aspartic protease inhibition tests, the most promising extracts are *Piper lanceaefolium* and *Juglans neotropica*. The extract of *Juglans neotropica* was analysed by ESR and although only the semiquinone nucleus is identified by this procedure for some compounds like juglone, this gives an absolute proof of identity.

*Juglans* species, and the compound juglone, have shown very good activity against dermatophytes (Ali-Shtayeh and Suheil, 1999). The presence of juglone detected by ESR may in part explain the wide range of antibiotic activity exhibited by *Juglans neotropica* bark extract.

Based on these results and plant material availability *P. lanceaefolium* and *I. megistophylla* were selected for future phyto-chemical analysis along with associated biological activities. In the next two following chapters I will describe the work accomplished for each of these plant extracts.
3.5. Conclusion

These experiments have revealed some biological activities in Colombian medicinal plants that could account for their traditional uses as herbal medicines. Nonetheless the assumption that pathogenic agents produce the skin diseases might not always be valid. In order to confirm a plant use the scope of the analysis has to be broader than is presented here. This is particularly true when one considers the fact that some traditional uses are disregarded whenever they cannot be apprehended in western terms and instead an assumption about a pathogenic cause is made. In other words there is a tendency to put aside concepts about disease whose epistemology is outside the borders of our cartesian (limited) understanding.

Similarly, I believe that the interplay between secondary metabolites from traditional medicinal plants and the diseased state of the host might be far more complex than merely the result of a direct antibiotic activity exerted by a single chemical entity. Although I have provided evidence of antibiotic and antiviral activity in these plant extracts, it is of great relevance to keep reminding ourselves that plant chemicals have pleiotropic effects and thus the fact of finding interesting antibiotic activities does not confirm, justify or explain the traditional use of a plant.
3.6. REFERENCES


Chapter 4
Antifungal Activity of Benzoic Acid Derivatives from *Piper lanceaefolium* Kunth

"The day after the full, it is bad to bathe:
If you do, the rainbow will urinate on you,
And leave you covered with warts.
For that, the children are to bathe indoors."

Popular Kamså saying cited in Mc Dowell (1989)

4.1. Introduction

Based on the anti-Candida activity results and the inhibition of secreted aspartic proteases, a methanolic extract of *P. lanceaefolium* was chosen for further phyto-chemical analysis.

Locally named in the Sibundoy Valley (southern Colombia) (see Fig. 4.1) as "cueche", a decoction of the leaves of *Piper lanceaefolium* Kunth (Piperaceae) is taken as a bath to treat morbid skin conditions. According to the information provided by Doña Clarita Buesaquillo and Don Miguel Chindoy Mutumbajoy these are malignant cutaneous manifestations whose occurrence is correlated with the drizzle, which usually accompanies the occurrence of a rainbow. This drizzle is thought to be the urine of the rainbow, otherwise known as "cueche". According to the literature, taking a bath outdoors after the full moon, would be the cause of getting the "rainbow's urine" (Mc Dowell, 1989).
Belief in the moon's influence on the human body is by no means exclusive to traditional societies. The apparent relationship between madness and moon is deeply rooted in the popular opinion that the word "lunatic" comes from the Latin word for moon, "Luna" (Zanchin, 2001).

The moon's influence is believed to cause the rainbow to "urinate", source in turn of the referred morbid condition. In my study I assumed that it is caused by a pathogen (e.g. virus, fungus or bacterium). This rationale corresponds to the western pragmatic approach where the body is conceived as a machine and an infection as an extraneous agent that potentially affects the proper functioning of the body and consequently should be removed.

*P. lanceaefolium* extract was selected because of its activity against *C. albicans* and complete inhibition of the secreted aspartic proteases enzymatic assay. This chapter reports the isolation and structural identification of the compounds present in an acetone extract of this plant, the minimal inhibitory concentrations for the active compounds and an assay to determine synergistic effects amongst such compounds. Since resistance against methicillin is such a problem in a clinical setting, the isolated compounds were tested against methicillin resistant *Staphylococcus aureus*.

Species belonging to the genus *Piper* are used in medical systems worldwide. A number of species are used medicinally in Latin American folklore particularly in the western Amazon (Warrier *et al.*, 1995; Schultes and Raffauf, 1992). The genus *Piper* (Piperaceae) has over 700 species distributed in both
hemispheres and its phytochemistry has been the subject of extensive review (Parmar et al., 1997).

Except for a publication about *P. lanceaefolium* essential oils (Mundina et al., 2001) phytochemistry of this species has not been examined.

4.2. Materials and Methods

4.2.1. General Experimental Procedures

Melting points (uncorrected) were recorded using a Gallenkamp melting point apparatus; optical rotations were measured on a JASCO P-1010 polarimeter; IR spectra were obtained using KBr disks on a BOMEM MB-100 spectrophotometer; UV spectra were obtained with a Shimadzu UV 160U spectrophotometer; the NMR spectra were recorded on a Bruker AV-400 at 400 MHz (\(^1\)H) and 100 MHz (\(^{13}\)C); multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC) were obtained using a Bruker AV-400 NMR spectrometer; high-resolution MS spectra were obtained on a Kratos MS 50 mass spectrometer; and TLC analysis was carried out on silica gel F\(_{254}\) plates (Merck). Preparative TLC was performed using silica gel 60 F\(_{254}\) (Merck) 250 micrometers thickness. The isolated compounds were visualized under UV at 254 nm, followed by development with ferric chloride spray reagent.
4.2.2. Plant Material

Leaves of *P. lanceaefolium* were collected in Sibundoy (01° 11' 00" N, 076° 55' 00" W) (Department of Putumayo, Colombia) (See Fig. 4.1) and identified by Dr. R. Callejas, Antioquia University Herbarium (Medellin, Colombia). A voucher specimen (López 56) was deposited in the herbarium of the Sinchi Institute (Santa Fé de Bogotá, Colombia).

4.2.3. Extraction and Bioassay

Fresh leaves were air-dried in the shade and ground. The powder (544 g) was extracted with 300 mL of acetone on a shaker for 25 min and the resulting extract was concentrated to dryness to give 37.8 g of dried material. This was dissolved in methanol and passed through a glass column containing Celite 545 (diatomaceous earth) (Fisher). The resulting extract was dissolved in methanol-water (3:1) and fractionated by liquid-liquid partition with hexane, chloroform, ethyl ether, ethyl acetate, butanol, and water. The different fractions were tested against *Candida albicans* using a disk test assay (Taylor *et al.*, 1995). The antifungal activity was localised in the chloroform and ethyl ether fractions. The water soluble fraction did not present activity against *C. albicans*. In an attempt to follow the traditional preparation method, 20 g of dried leaves were extracted with 500 mL of boiling water, filtered (Whatman No 1) and the resulting extract was freeze-dried. This extract was further tested against the dermatophyte *Trichophyton mentagrophytes* using a disk test assay (See Section 3.2.5) with an
incubation period of 72 h at 30°C. Amphotericin B (Sigma Chemical co.) was used as a positive control and methanol as the negative one.

Figure 4.1
Map of the Sibundoy Valley (Colombia)
4.2.4. Isolation of Compounds

The liquid-liquid partition fractions (chloroform and ethyl ether) (22.3 g) were subjected to column chromatography over silica gel (346 g, silica gel 230-400 mesh, Merck) using a gradient of ethyl acetate in dichloromethane. A total of 68 fractions (230 mL each) were collected and combined into seven pools (I-VII) on the basis of similar thin layer chromatography (TLC) profiles. Pools I and IV, which were eluted with \( \text{CH}_2\text{Cl}_2 \) and \( \text{CH}_2\text{Cl}_2/\text{EtOAc} \) (9:1) respectively, showed activity against *Candida albicans*.

Crystallization of the different pools (except pool IV) produced compounds 1 (1.93 g), 2 (196 mg), 3 (120 mg), 4 (126 mg), 5 (3.06 g) and 6 (6 mg). Fractions in pool IV were combined and applied to a column of silica gel (70-230 mesh) and eluted using continuous gradients of ethylacetate in hexane (0-100%). The fraction that displayed activity against *C. albicans* was subjected to preparative TLC to yield 7 (53 mg).

4.2.5. Direct Bioautographic Assay.

A bioautographic agar overlay assay was used to detect active fractions obtained during the chemical separation. Fractions of 700 µg each were spotted on chromatographic silica gel 60 TLC plates (Merck) and developed using \( \text{CH}_2\text{Cl}_2/\text{EtOAc} \) (50:50) as solvent system. Nystatin (100 µg) was used as a positive control. After thorough drying for complete removal of the solvent, an inoculum of *C. albicans* (ca 10^7 cells/mL) in molten agar (Saboraud broth medium agar, phenol red) was distributed over the chromatograms. The medium solidified
as a thin layer (ca 1 mm layer thickness), and the TLC plates were incubated overnight at 37 °C in a moist and chambered environment. The inhibition zones were visualized by spraying with an aqueous solution of methylthiazolytetrazolium chloride (MTT) (0.1 mg/mL). Active compounds were detected as clear yellow spots against a purple background. Two additional bioautography TLC plates were developed and sprayed with ferric chloride and vanillin/sulfuric acid to visualize the chromatographic profile of phenolic compounds (Rahalison et al., 1994; Saxena et al., 1995).

4.2.6. Minimum Inhibitory Concentration (MIC) Determination.

Minimal inhibitory concentrations values were determined by the broth dilution method using a concentration of 6.0 x 10⁴ colony forming units/mL of Candida albicans grown in Sabouraud broth medium and 5.0 x 10⁴ colony forming units/mL of Staphylococcus aureus MS in Muller-Hinton broth medium. The appearance of turbidity after 24 h. determined the MIC, defined as the lowest concentration of substance that prevented growth. Solutions of test compounds were prepared in methanol, and diluted with Saboraud medium and Muller-Hinton medium to give final dilutions ranging from 400 to 0.4 μg/mL. The final concentration of methanol in the assay did not exceed 2%. The assay was carried out in 96-well microtiter plates. Incubation was at 37 °C for 24 h. Amphotericin B was used as a positive control for yeast and gentamycin for bacteria with a MIC value of 0.055 and 0.1 μg/mL, respectively (Rahalison et al., 1994).
4.2.7. Synergy Effects

In order to study possible synergistic inhibitory activities against *C. albicans*, a microtiter assay was used (see Table 4.1). Different combinations of methanolic solutions were used and tested for activity against *Candida albicans* grown in Sabouraud broth medium (8.0 x 10⁴ colony forming units/mL). The appearance of turbidity after 24 h. determined the inhibitory activity.

Solutions of test compounds were prepared in methanol, and diluted with Saboraud medium to give final concentrations of 100 μg/mL in wells for single compounds (1-7 shown in bold) and 50 μg/mL for wells with combinations of two compounds. The final concentration of methanol in the assay was 2.5%. The assay was carried out in 96-well microtiter plates incubated at 37°C for 24h. Amphotericin B was used as a positive control for yeast and Sabouraud broth medium with methanol (2.5%) as a negative control. Two replicates were carried out.

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Table 4.1

Microtiter Plate Assay to Test for Synergy Effects (numbers inside the grid correspond to the compound identification number)
4.2.8. Secreted Aspartic Proteases Inhibition Assay for Isolated Compounds

Isolated compounds were dissolved in sodium citrate buffer and tested at a final concentration of 40 μM. Reaction rates were determined as described in 3.2.6.2.1. The negative control was secreted aspartic proteases in the presence of renin substrate for a non inhibitory effect. These proteases in the presence of the inhibitor pepstatin (0.006 μg/mL) were used as a positive control (100% inhibition). Only compounds with activity equal or greater than 50% of the positive control were considered for further investigation.

4.3. Results

4.3.1. Plant Description

The botanical description of *Piper lanceofolium* Kunth is as follows: Arborescent; branches nodose, silky; leaves lanceolate, 3-5 wide x 15-27 cm. Long, apex and more or less cuadately acuminate, base inequilaterally cordulate, with the lobes often overlapping, pinnately nerved from below the middle, the impressed nerves 8 or 9 on each side, sharply ascending, more or less finely rugose-bullate and silky-hairy above, at least along the nerves, lacunose and densely villous or subtomentose benneath, drying rather coriaceous, subopaque; petiole nearly suppressed, silky; spikes strongly curved, mostly 3 mm. thick x 8-12cm. Long, often mucronate; peduncle 10-20 mm. long, silky; bracts rounded- or triangular-subpeltate, marginally fringed; fruit suboblong-trigonous, glabrous; stigmas linear, 3 or 4, sessile (Trelease, 1950).
4.3.2. Chemical Isolation and Characterisation

Column chromatography, followed by preparative TLC (see Experimental Section) of the chloroform and ethyl ether fractions, resulted in the isolation of four previously unknown prenylated benzoic acid derivatives (1-4) (Fig. 4.3; 4.4; 4.5; 4.7).

Also known compounds such as taboganic acid (5) (See Figure 4.8) (Terreaux et al., 1998), pinocembrin (6) (See Figure 4.9) (Fukui et al., 1988), and pinocembrin chalcone (7) (See Figure 4.10) (Bremner and Meyer, 1998) were isolated for the first time in this plant, and identified by comparison of their physical and spectral data with those previously reported.

Cyclolanceaefolic acid methyl ester (1): Obtained as an amorphous powder, mp 146-147 °C; [α]D = 5.7° (c 0.5, MeOH); IR (KBr) νmax 3253, 2984, 1713, 1664, 1608, 1587, 1456, 1432, 1394, 1373, 1286, 1229, 1176, 1097, 1008, 947, 929, 903, 771, 751 cm⁻¹; UV (MeOH) λmax (ε) 240 (21 007), 334 (2 195), 203 (6 600); ¹H and ¹³C NMR data, see Table 4.3; EIMS m/z 250 [M]+ (30), 235 (80), 219 (12), 194 (100), 163 (60), 135 (40), 108 (15), 91 (5), 79 (60), 77 (10), 51 (35); HREIMS m/z 250.08334 (calcd for C₁₃H₁₄O₅ 250.08412)

Cyclolanceaefolic acid (2): Isolated as white crystals, mp 244-246 °C; [α]D = -4.0° (c 0.6, MeOH); IR (KBr) νmax 3233, 1683, 1675, 1607, 1587, 1488, 1373, 1292, 1175, 1092, 996, 927, 905, 880, 771, cm⁻¹; UV (MeOH) λmax (ε) 238 (19 920), 338 (2 714), 203 (7 241); ¹H and ¹³C NMR data, see Table 4.3; EIMS
m/z: 236 [M]$^+$ (40), 221 (75), 219 (12), 180 (100), 152 (20), 135 (15), 79 (8), 51 (5); HREIMS m/z 236.06836 (calcd for C$_{12}$H$_{12}$O$_5$ 236.06847).

**Lanceaefolic acid methyl ester (3).** Obtained as yellowish needles, mp 109-110 °C, [α]$^D_{24} = -8.6^\circ$ (c 0.1 MeOH); IR (KBr) $\nu_{\text{max}}$ 3459, 1723, 1713, 1638, 1588, 1482, 1438, 1317, 1231, 1186, 1142, 1093, 1003, 910, 857, 771, 751 cm$^{-1}$; UV (MeOH) $\lambda_{\text{max}}$ (ε) 240 (21 086), 338 (2 525), 203 (4 634); $^1$H and $^{13}$C- NMR data see Table 4.3; EIMS m/z: 250 [M]$^+$ (25), 235 (100), 219 (10), 194 (40), 173 (60), 135 (10), 108 (10), 83 (20), 55 (25); HREIMS m/z 250.08436 (calcd for C$_{13}$H$_{14}$O$_5$ 250.08412).

**Lanceaefolic acid (4):** Obtained as orange-yellow needles (acetone): mp 253-255 °C; [α]$^D_{24} = -14.3^\circ$ (c 0.1, MeOH); IR (KBr) $\nu_{\text{max}}$ 3477, 1713, 1688, 1638, 1584, 1482, 1422, 1313, 1283, 1134, 1096, 1057, 970, 884, 770, 695 cm$^{-1}$; UV (MeOH) $\lambda_{\text{max}}$ (ε) 236 (19 562), 338 (2 898), 205 (8 326); $^1$H and $^{13}$C NMR data see Table 2; EIMS m/z 236[M]$^+$ (30), 221 (100), 180 (55), 152 (10), 125 (10), 83 (15), 55 (15); HREIMS m/z 236.06832 (calcd for C$_{12}$H$_{12}$O$_5$, 236.06847).

**Taboganic acid (5):** Obtained as yellow crystals, mp 170-172 °C EIMS m/z 220[M]$^+$ (6), 205 (100), 165 (18). $^1$H-NMR (400 MHz, acetone-$d_6$): δ 8.58 (1H, d, $J = 2.12$ Hz H-2), 8.13 (1H $dd$, $J = 2.12$, 8.74 Hz, H-6), 7.10 (1H d $J = 1.98$ Hz H-2'), 7.05 (1H $d$, $J = 8.74$ Hz H-5), 2.25 (3H, s, H-4'), 2.07 (3H, s, H-5').
Pinocembrin (6): Obtained as pale yellow needles, mp 198-199 °C EIMS m/z 256[M]+ (100), 238 (12), 179 (84), 152 (85), 124 (88), 105 (43), 104 (33), 103 (35), 77 (64), 69 (56). ¹H-NMR (400 MHz, acetone-d₆ ,): δ : 2.80 (1H, dd, J = 17.1, 3.15 Hz, H-3a), 3.16 (1H, dd, J = 17.1, 12.7 Hz, H-3b), 5.56 (1H dd, J = 3.13, 12.7 Hz, H-2), 5.95 (1H d, J = 2.2 Hz H-6), 5.97 (1H d , J = 2.2 Hz H-8), 7.38-7.60 (5H, m, H of ring B).

Pinocembrin chalcone (7): Obtained as yellow powder, EIMS m/z 256[M]+ (100), 238 (10), 179 (78), 152 (59), 124 (30), 105 (28), 104 (22), 103 (24), 77 (33), 69 (23). ¹H-NMR (400 MHz, acetone-d₆ ): δ 7.45 (2H, m, H-3, 5), 7.69 (2H, m, H-2,6), 7.75 (8-H d, J = 15.7), 8.23 (7-H d J = 15.7)

4.3.3. Biological Activity

Growth inhibitory effects against Candida albicans were detected in lanceaefolic acid methyl ester (3) and pinocembrin chalcone (7) using disk diffusion and bioautographic assays (Terreaux et al., 1998) (See Figure 4.11). The aqueous extract of the leaves of P. lanceaefolium did not present activity against C. albicans nor against Trichophyton mentagrophytes. These compounds exhibited a minimal inhibitory concentration of 100 µg/mL for Candida albicans. Pinocembrin and its chalcone resulted also in a minimal inhibitory concentration of 100 µg/mL for Staphylococcus aureus MS and none of the compounds presented at a concentration less than 320 µg/mL for S. aureus MR.
Figure 4.2

Bio-autography with *Candida albicans*

No synergy effects were detected for the combination of compounds used. Only compounds 3 and 7 presented activity against *C. albicans* and only their combination presented inhibitory activity.
4.3.4. Aspartic Proteases Inhibition Assay

None of the compounds caused 50% or more inhibition of aspartic protease at 40 μM. Consequently they were not selected for further characterisation (i.e. inhibition studies). Table 4.2 shows the inhibition percentages for the aspartic proteases assay.

Table 4.2
Inhibition of Secreted Aspartic Proteases by compounds from *P. lanceaefolium*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.9</td>
</tr>
<tr>
<td>2</td>
<td>15.3</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
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<td>11.9</td>
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<td>6</td>
<td>15.6</td>
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<tr>
<td>7</td>
<td>16.1</td>
</tr>
<tr>
<td>Position</td>
<td>$\delta_H$</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>81.8 (s)</td>
</tr>
<tr>
<td>3</td>
<td>2.83 (s)</td>
</tr>
<tr>
<td>4</td>
<td>191.8 (s)</td>
</tr>
<tr>
<td>4a</td>
<td>123.4 (s)</td>
</tr>
<tr>
<td>5</td>
<td>7.96 (d, 2.1)</td>
</tr>
<tr>
<td>6</td>
<td>121.2 (s)</td>
</tr>
<tr>
<td>7</td>
<td>7.62 (d, 2.1)</td>
</tr>
<tr>
<td>8</td>
<td>147.9 (s)</td>
</tr>
<tr>
<td>8a</td>
<td>152.7 (s)</td>
</tr>
<tr>
<td>9</td>
<td>166.5 (s)</td>
</tr>
<tr>
<td>10, 11</td>
<td>1.48 (s)</td>
</tr>
<tr>
<td>CH$_3$O</td>
<td>3.86 (s)</td>
</tr>
</tbody>
</table>

* Spectra were recorded in CD$_3$OCD$_3$; chemical shifts are reported as $\delta$ values (ppm) from TMS at 400MHz for $^1$H and 100MHz for $^{13}$C; signal multiplicity and coupling constants (Hz) are shown in parentheses.
4.4. Discussion

4.4.1. Structural Elucidation

Compound 1 was obtained as a white amorphous powder and had a molecular ion peak at m/z 250.08334 in HREIMS suggesting a molecular formula of C_{13}H_{14}O_{5}. The IR spectrum indicated the presence of two carbonyl groups (1713, 1664 cm\(^{-1}\)) and an aromatic ring (1608, 1587 cm\(^{-1}\)). The assignments of proton and carbons were obtained by analysis of its 2D NMR (HMQC and HMBC). The \(^1\)HNMR spectrum (Table 4.3) exhibited two aromatic proton resonances at \(\delta\) 7.62 (1H, d, \(J = 2.1\) Hz) and 7.96 (1H, d, \(J = 2.1\) Hz). On the basis of chemical shifts and coupling patterns, a 1, 2, 3, 5- tetrasubstituted phenyl ring was suggested to be present in the molecule. In addition, the \(^1\)HNMR spectrum displayed signals for one methylene group at \(\delta\) 2.83, and three methyl groups at \(\delta\) 1.48 (6H, s) and 3.86 (3H, s). The \(^{13}\)C-NMR spectrum (Table 4.3) and DEPT experiments of 1 showed 13 signals: three methyls, one methylene, two methines, and seven quaternary carbons, of which the signals at \(\delta\) 152.7, 123.4, 121.1, 119.2, 121.2, 147.9, 166.5, and 52.3, together with a sharp singlet (3H) at \(\delta\) 3.86 in the \(^1\)H NMR spectrum, implied the presence of a methyl ester of 3,4,5-trisubstituted benzoic acid. The signals at \(\delta\) 191.77, 48.92, 81.75, and 26.47 (x2) together with the methylene resonance (singlet) and two aliphatic methyl groups of magnetic equivalence in the \(^1\)H NMR spectrum, as well as the HMBC (Figure 4.6) correlations between C-2, C-4 and methylene (H-3) revealed the connectivity CO (4)-CH\(_2\)(3)-C(2)-(CH\(_3\))\(_2\). The HMBC spectrum of 1 also showed correlations from the carbonyl group at \(\delta\) 191.77 to H-5, from the carboxyl group to H-5 and
H-7, and from C-8a to H-7 and H-5, which indicated that the ketone carbonyl and ester groups were attached at C-4a and C-6, respectively. Thus, the structure of 1 was determined to be cyclolanceaefolic acid methyl ester (methyl ester of 8-hydroxy-2,2'-dimethyl-6-carboxy-chroman-4-one).

![Chemical Structure](image)

**Figure 4.3**

Cyclolanceaefolic acid methyl ester (1)

Compound 2 was isolated as white crystals. The HREIMS of this compound gave a molecular ion at $m/z$ 236.06836 corresponding to a molecular formula of $\text{C}_{12}\text{H}_{12}\text{O}_{5}$. IR absorptions at 3233, and 1683 cm$^{-1}$ suggested the presence of hydroxyl and carbonyl groups. $^1\text{H}$ and $^{13}\text{C}$-NMR data (Table 4.3) and DEPT experiments revealed 12 signals: two methyls, one methylene, two methines, and seven quaternary carbons. Compounds 1 and 2 were found to have similar structures by comparison of their NMR spectra. The observed difference was the loss of the methoxy signals ($^1\text{H}$-NMR at $\delta$ 3.86 and $^{13}\text{C}$-NMR at $\delta$ 52.27) in the NMR spectrum of 1, which indicated that the methyl ester carbonyl was replaced...
in 2 by a carboxyl group. Thus, the structure of 2 was assigned as cyclolanceaeafolic acid (8-hydroxy-2,2'-dimethyl-6-carboxy-chroman-4-one).

Figure 4.4
Cyclolanceaeafolic acid (2)

Compound 3 was obtained as yellowish needles. The HREIMS indicated a molecular formula of C\textsubscript{13}H\textsubscript{14}O\textsubscript{5}. The IR spectrum indicated two carbonyl groups at 1730 and 1724 cm\textsuperscript{-1}. The \textsuperscript{1}HNMR spectrum contained two aromatic protons, with the chemical shift and splitting pattern typical of H-2 and H-6 of a 1,3,4,5-tetra substituted benzene ring. Furthermore the signals at \(\delta\) 196.88, 120.23 and 161.79 in the \textsuperscript{13}C-NMR spectrum and a signal at \(\delta\) 7.06 in the \textsuperscript{1}H-NMR spectrum implied a conjugated ketone system. The signals in the \textsuperscript{1}HNMR spectrum at \(\delta\) 2.10 (3H, s), 2.24 (3H, s) were assigned to vinyl methyl groups positioned on a trisubstituted double bond. A sharp, 3H singlet at \(\delta\) 3.85, together with the \textsuperscript{13}C NMR resonances at \(\delta\) 166.48, and 52.29 suggested the presence of a methyl
ester. Correlations in the HMBC spectrum from C-7 to H-2 and H-6, from C-8 to H-2, and from C-3 to H-9 suggested that the ester carbonyl and the ketone carbonyl were attached to C-1 and C-3 of the benzene ring, respectively. COSY, HMQC and HMBC NMR spectra permitted the complete assignment of all the protons and carbons as shown in Table 2. The above evidence led to the elucidation of structure 3 as lanceaefolic acid methyl ester [Methyl ester of 4,5-dihydroxy-3-(3-methyl-2-butenoyl) benzoic acid], which was confirmed by comparing the NMR data with the known compounds taboganic acid methyl ester (Roussis et al., 1990) and piperoic acid (Ampofo et al., 1987).

![Figure 4.5]

Lanceaefolic acid methyl ester (3)
Compound 4 was obtained as orange-yellow needles. The HREIMS showed a molecular ion at m/z 236.06832, suggesting a molecular formula ion of C\textsubscript{12}H\textsubscript{12}O\textsubscript{5}. The presence of a 1,3,4,5- tetrasubstituted aromatic ring in the structure became clear from the consideration of resonances attributed to the aromatic rings in the \textsuperscript{1}H and \textsuperscript{13}C NMR spectra. Compounds 3 and 4 showed very similar NMR spectra except that the signals of the methyl group at \(\delta\text{H} 3.85\) and \(\delta\text{C} 52.29\) were only present in the spectra of 3. Thus, the structure of lanceaefolic acid [4,5-dihydroxy-3-(3-methyl-2-butenoyl) benzoic acid] was assigned as 4.
Figure 4.7
Lanceaefolic acid (4)

Figure 4.8
Taboganic acid (5)
3-(1'-Oxo-3'-methyl-2'-butenyl)-4-hydroxy-benzoic acid
Most of the chalcones isolated from the *Piper* species are oxygenated at the C-2', C-4' and C-6' positions and the B ring is generally unsubstituted (Parmar et al., 1997). Pinocembrin chalcone, isolated for the first time in *Piper* species, follows this substitution pattern. On the other hand, 5,7-Dihydroxyflavanone, (pinocembrin) has been isolated in *P. hostmannianum* (Diaz et al., 1987). Taboganic acid was reported for the first time as a natural product in *Piper*
dilatatum L.C. Rich (Terreaux et al., 1998) although its methyl ester had been already isolated from *Piper taboganum* (Roussis et al., 1990).

### 4.4.2 Biological Activity

Determination of the minimal inhibitory concentration of the compounds confirmed that lanceaefolic acid methyl ester (3) and pinocembrin chalcone (7) are at least partly responsible for the antifungal activity of *Piper lanceaefolium*. Also it appears that pinocembrin and its chalcone are at least partially responsible for the antibacterial activity of leaf extracts.

It is interesting to note that only pinocembrin chalcone but not pinocembrin, exhibited activity against *C. albicans*. There is a loss of activity by cyclization to the corresponding flavanone. Similarly only lanceaefolic acid methyl ester (3) but not cyclolanceaefolic acid methyl ester (1) presented activity against *C. albicans*. It would appear that cyclization is correlated with the loss of activity.

![Figure 4.11](image)

Chalcones (1,3-diaryl-2-propen1-ones) general skeleton
Tsuchiya and co-workers (1994) reported anti-fungal activity of different synthetic hydroxy-chalcones against *Candida* species. The authors stated that substitution of the chalcones with hydroxyl groups enhances the anti-*Candida* activity. This would provide both lipophilic and hydrophilic moieties to the chalcones. However, the authors report activity in chalcone A and none in chalcone B that has an additional hydroxyl in 2' position (see Figure 4.12). Interestingly, a synthetic preparation of pinocembrin chalcone displayed activity against *C. glabrata* but not *C. albicans*. The importance of the double bond was also highlighted.

![Figure 4.12](image)

**Figure 4.12**

Effect of 2' Hydroxyl Group Removal in Chalcones Antifungal Activity (Tsuchiya, 1994)
Similarly Sato and co-workers (1994) tested a group of synthetic chalcones against *Candida* species. They found that 2'-hydroxyl group was important for significant antifungal activity however no minimal inhibitory concentrations were reported. It is interesting to note in these two publications the effect of the removal of 2'-hydroxyl as illustrated in Figure 4.11 and 4.12. It would seem that 2' hydroxyl group plays a significant role in decreasing antifungal activity probably due to its withdrawing electronic effect.

![Active Inactive](image)

**Figure 4.13**

*Effect on Antifungal Activity of the Removal of 2'-Hydroxyl (Sato, 1994)*

Pinocembrin has been reported in leaf resin of eastern cottonwood (*Populus deltoides*) (Shain and Miller, 1982), aerial parts of *Teloxys graveolens* (Camacho *et al.*, 1991), in heartwood of *Pseudotsuga menziesii* (Douglas-fir) (Dellus *et al.*, 1997), aerial parts and roots of *Lippia graveolens* (Oregano) (Dominguez, *et al.*, 1989); and along with chrysin in leaves of *Anomianthus dulcis*
(Sinz et al., 1999). Galangin, chrysin and pinocembrin, among others, are characteristic flavonoids from propolis (bee glue) and bee wax. However no studies have been published in regards to ecological role for pinocembrin or its chalcone.

The large amount of taboganic acid extracted from the plant (3.06 g) is noteworthy. Although no activity was shown against C. albicans, this compound presented activity against the plant pathogenic fungus Cladosporium cucumerinum in a bioautographic test on TLC (5 µg), although it did not exhibit activity in a dilution assay (Terreaux et al., 1998). Its methyl ester showed activity in the leaf cutter repellence assay and consequently it might constitute a natural defence against ants (Roussis et al., 1990).

In regards to antifungal activity of chalcones and their structure-activity relationships, S. N. López and co-workers (2001) tested a series of chalcone derivatives (this list does not include pinocembrin chalcone) against known dermatophytes (Mycosporum canis, M. gypseum, Trichopyton rubrum and Epidermophyton floccosum). The authors' conclusions were that planar forms in chalcones have the spatial ordering needed to produce the antifungal response, whereas β- and carbonyl carbons are most susceptible sites for a possible nucleophilic attack of the enzyme or receptor. However none of the compounds tested were active against Candida albicans, Saccharomyces cerevisae or Cryptococcus neoformans nor against the filamentous fungi Aspergillus niger, A. fumigatus or A. flavus.
It has been reported that a slight structural modification of pinocembrin chalcone brought an increase in activity against *S. aureus* MS. Indeed, 2′, 6′, 4′-trihydroxy-4′-methoxydihydrochalcone (asebogenin) exhibited activity against *S. aureus* MS, although the minimal inhibitory concentration is not reported (Joshi *et al.*, 2001).

![Figure 4.14](image)

**Figure 4.14**

Asebogenin (2′, 6′, 4′-trihydroxy-4′-methoxydihydrochalcone) (Joshi, 2001).

Regarding the search for new inhibitors for methicillin-resistant *Staphylococcus aureus* strains, chalcones seem to display higher activity than flavones and flavanones. The carbonylic region along with molecular co-planarity may play an important role in anti-MRSA activity (Alcaráz *et al.*, 2000).
4.5. CONCLUSION

It is crucial to bear in mind that the traditional use of this plant is an aqueous extract used externally to alleviate skin conditions. The two compounds found to be active presented a weak activity against fungi and bacteria. Minimal inhibitory concentrations such as the ones reported here cannot sensibly be regarded as meaningful. Also, no synergy effects were observed for the isolated compounds. On the other hand, the fact that the aqueous extract did not present activity against C. albicans or T. mentagrophytes challenges our hypothesis even further because this is the extract likely to be obtained by the traditional method. I am tempted to affirm that the traditional use of P. lanceaefolium in the Sibundoy valley has nothing to do with counteracting infectious agents such as bacteria, fungi or viruses.

It is possible that the traditional treatment is meant to physically and/or to psychologically alleviate the symptoms of discomfort caused by a still-to-be-identified agent. Association with the moon may signify the cyclic appearance of such an agent.
4.6. References


Find a toad generous in size,
And wash its belly thoroughly.

Hold the toad by its limbs and gently
Rub the stomach on the affected area.

Crucify the toad at dawn and cremate after death.
Repeat the treatment for eight days.
Home remedy for Herpes

5.1. Introduction

Iryanthera is a neotropical genus and some of its species are used in the treatment of different ailments. The Waorani from Ecuador for instance, shred the inner bark of I. juruensis, I. paraensis or I. elliptica and use the "resin" to treat fungal infections of the skin. They apply the resin directly onto infected areas, where it kills the fungus "just like the dart poison" (Davis and Yost, 1983). Likewise the Puinaves from Colombia employ the soft inner bark of Iryanthera species on areas of fungal infection of the skin (Schultes and Raffauf, 1990).

On the other hand I. tessmannii is used in the Iquitos (Perú) region for treatment of diarrhoeas. In Perú the resin of the bark of I. ulei is used in the treatment of "patco", a disease in which a white substance appears in a child's mouth (Schultes and Raffauf, 1990). The reference unfortunately does not provide further information about this ailment. Whereas Iryanthera extracts' are
used as anti-fungal agents in the Amazon Basin, the Afro-Colombian communities of the Pacific Coast use it to treat cutaneous leishmaniasis. The phyto-chemistry of this genus has been studied (Vieira et al., 1983, Garzon et al., 1987, Conserva et al., 1990, Silva et al., 2001). The distribution of flavonoids has been reviewed (Martínez, 2000) and Ming and co-workers (2002) have studied the phyto-chemistry of *Iryanthera megistophylla*.

Based on the results of the anti-viral and anti-bacterial assays described in chapter 3, *Iryanthera megistophylla* appeared to be a good candidate to undertake phytochemical analysis. Indeed not only did the methanolic extract of this plant display good activity against herpes simplex virus but also against *Staphylococcus aureus* methicillin-sensitive MS. This extract presented good inhibition against secreted aspartic proteases in the inhibition test described chapter 3 (Section 3.2.6.2.2). In this chapter I will describe and discuss the anti-viral and anti-microbial activity of four of the seven compounds isolated by Dr. Dong Sheng Ming from *I. megistophylla*. Also I will describe and discuss the inhibitory activity of these compounds against secreted aspartic proteases from *C. albicans*.

Considering the chemical nature of some of the isolated compounds (flavolignans) and their putative role in inhibiting resistance pumps, I will describe and discuss the multidrug resistance test (Guz *et al.*, 2001).

The activity of iryantherin K against a battery of Gram positive, Gram negative and yeast will be presented along with its cytotoxicity in a mammalian
system. The potential of iryantherin K in the treatment of *Staphylococcus aureus* methicillin-resistant MR infections will be discussed.

5.2. Materials and Methods

5.2.1. Anti-viral assays. Determination of Minimal Inhibitory Concentration (MIC)

Assays were carried out with 100 plaque-forming units (PFU) of HSV-1 in Vero cells grown in 96-well culture trays, as described in chapter 3 section 3.2.3. The MIC is the minimum concentration of compound, which caused complete inactivation of virus infectivity (i.e., absence of viral CPE). A methanolic leaf extract of *Adansonia digitata* was used as a positive control (minimum antiviral concentration in UVA, 5 μg/mL) (Hudson *et al.*, 2000).

5.2.2. Cytotoxicity Assay

5.2.2.1. Cytotoxicity Visible Assessment

To test for cytotoxicity, Vero cell monolayers were grown in 96-well microtiter plates (Falcon) and exposed to serial dilutions of the corresponding compounds starting at 320 μg/mL. The treated cells were then incubated at 37°C for 1 h, exposed to UV-A light and visible light for 30 min and then re-incubated for 24 hrs. The cells were examined periodically through the microscope for assessment of changes in cell morphology or visible toxic effects (obvious cellular damage or lysis).
5.2.2.2. **Cell Proliferation Assay for Iryantherin K.**

Mouse fibroblast cells (NIH3T3) were seeded at 1000 cells/well in 96-well plates, grown overnight, and treated or not treated with iryantherin K in a dilution series (50-0.001 µg/mL) for 24 hours. Iryantherin K was removed, and cells were allowed to grow in fresh medium until those not treated with the compound approached confluence, which was typically 4-6 days. Cell proliferation was measured as follows: 25 µL of a 5 mg/mL solution of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline was added to cells in the presence of 100 µL of cell culture medium. After a 2 hours incubation at 37 °C, 100 µL of 20% sodium dodecyl sulfate dissolved in dimethylformamide/water (1:1), pH 4.7, was added, and the absorbance at 570 nm was measured after overnight incubation (Curman et al., 2001).

5.2.3. **Antibacterial and Antifungal Assays.**

5.2.3.1. **Minimum Inhibitory Concentration (MIC) determination**

As described in 4.2.6

5.2.3.2. **Test for Multidrug Resistance (MDR) Inhibitory Activity**

*S. aureus* ATCC 25923 MS (methicillin-sensitive) was cultured in 3 mL of Mueller-Hinton (MH) broth overnight at 37°C. Cells were then diluted 1:2000 into MH broth (4.1x10⁸ cells/mL) and 0.05 mL were dispensed into each well of microtiter plates. Test substances were serially diluted 2-fold in the wells for a volume of 0.075 mL per well. A methanolic solution of berberine (0.075 mL) was added to each well at a sub-inhibitory concentration (30 µg/mL, 1/8 MIC). Minimal
inhibitory concentration was defined as the minimal concentration that completely inhibited cell growth in presence of 30 μg/mL of berberine. The final volume of the well was 0.2 mL. (Guz et al., 2001).

5.2.4. Secreted Aspartic Proteases Inhibition Assay

Secreted Aspartic Proteases Inhibition Assay for isolated compounds is de in section 4.2.8. IC 50 values were derived from the dose-effect curves calculated according to the regression analysis (least-square method) by plotting the log concentration against the percentage of inhibition.

5.2.5. Pepsin Inhibition Assay

Optimum final pepsin (from Porcine Stomach Mucosa- Sigma) and substrate concentrations were determined to be 15 U/mL (471 Units/mg enzyme) and 40 μM respectively. The assay was performed as for secreted aspartic proteases except pepsin was substituted for the SAP extract and 50 mM sodium citrate buffer (pH= 4.0) was used as diluent.

5.3. Results

5.3.1 Cytotoxicity

None of the compounds (1-9) presented visible cytotoxic effects were at concentrations up to 320 μg/mL. In the cell proliferation assay iryantherin K was found to be cytotoxic at 50 μg/mL but it did not present cytotoxic effects at lower concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 μg/mL).
5.3.2 Antiviral and Antimicrobial activities

Biological activities of isolated compounds are summarised in Tables 5.1; 5.2 and 5.3. Compounds 6 and 7 (See Figures 5.1 and 5.2) were found to be active against HSV-1 at a minimal inhibitory activity of 20 μg/mL.

Figure 5.1
Structure of Cinchonain lb (6)

Figure 5.2
Structure of Cinchonain la (7)
Figure 5.3
Procyanidin B₂ or catechin – (4β - 8) epicatechin (8)

Figure 5.4
Structure of Cinchonain IIa (9)
Table 5.1
Minimal Inhibitory Concentrations of *I. megistophylla* compounds against herpes simplex virus, *C. albicans*, *S. aureus* MS (methicillin-sensitive) and *S. aureus* MR (methicillin-resistant)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HSV-1 (µg/mL)</th>
<th><em>C. albicans</em> (µg/mL)</th>
<th><em>S. aureus</em> MS (µg/mL) ATCC 25923</th>
<th><em>S. aureus</em> MS (µg/mL) ATCC 25923 MDR Test *</th>
<th><em>S. aureus</em> MR (µg/mL) SAP 0017 (clinical isolate)</th>
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<tbody>
<tr>
<td>1</td>
<td>_a</td>
<td>_b</td>
<td>_c</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>2</td>
<td>_a</td>
<td>_b</td>
<td>_c</td>
<td>ND</td>
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<td>9</td>
<td>_a</td>
<td>150</td>
<td>_c</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Multidrug resistance Inhibitory Test

_a* no detectable activity at 320 µg/mL.

_b* no detectable activity at 300 µg/mL.

_c* no detectable activity at 200 µg/mL.

_d* ND: minimal inhibitory concentration was not determined

Amphotericin and gentamycin were used as positive controls for *C. albicans* and *S. aureus* MS respectively.
Table 5.2
Minimal Inhibitory Concentrations (µg/mL) of Iryantherin K against Gram positive Bacteria

<table>
<thead>
<tr>
<th>Compounds</th>
<th>S. aureus SAP0017 MR</th>
<th>S. aureus ATCC 25923 wt</th>
<th>S. epidermidis ATCC 25923 C621 wt</th>
<th>E. faecalis ATCC 25912 wt</th>
<th>B. subtilis ATCC 6633 wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iryantherin K</td>
<td>2.5</td>
<td>1.25</td>
<td>2.5</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Methicillin</td>
<td>16 (64)</td>
<td>2</td>
<td>4 (16)</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>16 (32)</td>
<td>32</td>
<td>16</td>
<td>&gt;64</td>
<td>64</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>32</td>
<td>0.125</td>
<td>32</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

- Results shown are 24 hours, 48 hrs shown in brackets if greater than 2 fold change
- S. aureus methicillin resistant 0017 and S. epidermidis C621 are clinical isolates.

Table 5.3
Minimal Inhibitory Concentrations (µg/mL) of Iryantherin K against Gram negative Bacteria

<table>
<thead>
<tr>
<th>Compounds</th>
<th>P. aeruginosa H187 wt</th>
<th>P. aeruginosa H188 abs</th>
<th>E. coli UB1005 DC2 abs</th>
<th>E. coli DC2 abs</th>
<th>S. typhimurium ATCC 14028 wt</th>
<th>S. typhimurium C610 abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iryantherin K</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Methicillin</td>
<td>128</td>
<td>0.5 (2)</td>
<td>&gt;128</td>
<td>4</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.06</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

- Results shown are 24 hours, 48 hrs shown in brackets if greater than 2 fold change
- P. aeruginosa (H187 and H188), E. coli (UB 1005 and DC2) are Hancock's laboratory strains
- S. typhimurium C610 is a defensin sensitive mutant of S. typhimurium ATCC 14028
Iryantherin K (4) and iryantherin L (5) are active against S. aureus MS (methicillin-sensitive) at 1.25 μg/mL and iryantherin K (4) presented very good activity (2.5 μg/mL) against S. aureus MR (methicillin-resistant). Also it presented very good activity against others Gram positive bacteria (see Table 5.2). There was inadequate material to test other compounds. Compounds 6 and 7 showed a weak activity in the multidrug resistance inhibitory test.

Figure 5.5
Iryantherin K (4)
The MIC for compounds 6,7,8,9 against C. albicans was of 150 μg/mL. The remaining compounds were not active against C. albicans or S. aureus methicillin-sensitive MS at the upper concentration tested in the broth dilution assay (300 and 200 μg/mL, respectively) (Table 5.1). Compounds 1, 2 and 3 were not active in any of the tested systems.
Figure 5.7
Megislignan (1)
[2,3-dimethyl-4-(4-methoxyphenyl)-6-hydroxynaphthalene]

Figure 5.8
Megislactone (2)
[(2R,3R,4R)-3-hydroxy-4-methyl-2-(hexacos-17-enyl) butanolide]
5.3.3. Secreted Aspartic Proteases Inhibition

Only compounds megislignan (1), iryantherin K (4) and iryantherin L (5) were inhibitory at 40 μM in the secreted aspartic protease inhibition assay.

Table 5.4
Secreted Aspartic Proteases Inhibition Activity of compounds from *I. megistophylla*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
</tr>
</tbody>
</table>

Compounds No 2, 3, 6, 7, 8 and 9 did not present activity.
5.3.4. Inhibitory Activity of Iryantherin K (4) and L (5).

Iryantherin K and L were selected for evaluation of effects against *Candida albicans* secreted aspartic proteases. The IC$_{50}$ values were calculated based on the corresponding log-dose curve, (See Table 5.3). Pepstatin, which was used as a positive control, showed an IC$_{50}$ of 0.003 µg/mL. To determine if the inhibitory effects of these compounds were selective for *Candida* secreted aspartic proteases (SAP), their activities against an aspartic protease, pepsin, were determined. Due to limitations in the amount of material the inhibitory activity against pepsin was determined at a concentration close to the compounds’ IC$_{50}$ values. Iryantherin K caused inhibition of 56.2% at 15 µg/mL while Iryantherin L presented a value of 37.6% at 20 µg/mL.
Table 5.5
Inhibitory Activity of Iryantherin K and L against Secreted Aspartic Proteases (SAP's) by *C. albicans*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μg/mL)</th>
<th>IC 50 (μg/mL)</th>
<th>IC 50 (μM)</th>
<th>Pepsin Inhibition (%) At 15 μg/mL</th>
<th>At 20 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iryantherin K</td>
<td>3.4 ±0.46 23 ± 0.51 32 ± 1.10 59 ± 0.98 72.4±0.78 73 ± 1.2</td>
<td>20.95</td>
<td>36.8</td>
<td>56.2</td>
<td>-</td>
</tr>
<tr>
<td>Iryantherin L</td>
<td>3.9 ± 1.70 9.7 ± 0.85 27.1 ± 1.3 38.9±0.92 52.1±0.82 44.5 ±0.58</td>
<td>23.21</td>
<td>40.7</td>
<td>-</td>
<td>37.6</td>
</tr>
</tbody>
</table>
5.4. Discussion

Cinchonain lb (6) and la (7) (See Figure 5.1 and 5.2) showed potent activity against HSV-1 at a minimal concentration of 20 μg/mL (Table 5.1). Compounds 6 and 7 were thus partly responsible for the antiviral activity detected in the crude extract of *I. megistophylla* (López *et al.*, 2001). The fact that the crude extract exhibited a minimal inhibitory concentration of 10 μg/mL (See Table 3.1 in Chapter 3) and 20 μg/mL for each one of the pure compounds, might indicate that a synergistic effect took place which was responsible for the overall observed anti-viral effect.

Cinchonain lb (6) and la (7) were first isolated by Nonaka and co-workers (1982) from the bark of *Cinchona succirubra*, which is the source of quinine and quinidine. They have been isolated as well from *Uncaria rhynchophylla* (Rubiaceae), *Kandelia candel* (Rhizophoraceae), *Polygonum bistorta* (Polygonaceae) and *Raphiolepsis umbellata* (Rosaceae). They exhibited poor activity (150 μg/mL) against *C. albicans* and were inactive against *S. aureus* MS. Aside from the activities reported here, compound 7 has also been shown to be hepatoprotective. It has been suggested that this compound, by scavenging reactive oxygen species (ROI's), interferes with the signal transduction triggered by TNF-α (activator of phagocytic cells) and thus protects the cell from subsequent injury (Fan *et al.*, 1999; Xiong *et al.*, 2000).

One can speculate that because of the polyphenolic nature of compounds 6 and 7, they are likely to bind to the protein coat of the virus or to the host's cell membrane. It is believed that virus adsorption and consequently virus penetration
is thus arrested. For instance, Fukuchi et al. (1989) using radiolabeled virus particles showed the anti-viral activities on HSV-1 and HSV-2 of high molecular weight polyphenols (i.e. tannic acid, coriariin A, rugosin D) on African green monkey kidney cells were due to the inhibition of virus adsorption.

In this regard, procyanidin B-2 (8) (Fig. 5.3) and cinchonain Ila (9) (Fig. 5.4), which are characterised by a large number of phenolic groups, did not exhibit antiviral activity to HSV-1 in this assay. In an extracellular virucidal (cells pre-treated with virus) assay it was found that procyanidin B-2 reduced the number of plaques to 50% at 35 µg/mL (Takechi et al., 1985). Also procyanidin B-2 at 100 µg/mL presented $10^4$ fold reduction of HSV-1 virus titer in Vero cells (De Bruyne, et al., 1999a).

Procyanidin B-2 or catechin – (4β – 8) epicatechin (8) is a well-known proanthocyanidin (previously referred to as “condensed” tannins), which are oligomers and polymers composed of flavan-3-ol nuclei (De Bruyne et al., 1999b). It has been reported to have hydroxyl radical scavenging activity and presented inhibitory activity in a microsomal lipid peroxidation assay ($IC_{50}$= 3.3 µM) (Shahat et al., 2002). Procyanidin B-2 (8) and cinchonain Ila (6) exhibited antileishmanial activity in vitro (Kolodziej et al., 2001), which may eventually explain the traditional use of I. megistophylla in the treatment of leishmaniasis.

Procyanidin B-2 showed weak activity against S. aureus MS (MIC=100 µg/mL) and against C. albicans (MIC=150 µg/mL). These concentrations cannot be regarded as meaningful because of the high concentration of compound that has to be used to inhibit the activity. The concentration of this compound in the
ethanolic extract was very low compared to the amount found for iryantherin K for example (for iryantherin K 0.4% referred to the ethanolic extract).

There is indeed a tendency to allocate biological activities of tannins because of their capacity to bind proteins in a non-specific manner. However, on the one hand, characteristics of both the tannin and the protein as well as the conditions of reaction influence tannin-protein interactions. On the other hand it has been demonstrated that some phenolic compounds (i.e. procyanidin B-2) show specific activities at receptor levels which cannot solely be explained in terms of non specific protein binding (Zhu et al., 1997).

Iryantherin K (4) and L (5) have been isolated previously from bark of I. ulei (Conserva et al., 1990) and the pericarp of I. lancifolia (Silva et al., 1999). These iryantherins belong to a complex group of dihydrochalcones that have been isolated exclusively from Iryanthera species (so far). Iryantherin K and its stereoisomer iryantherin L showed good activity against S. aureus MS (MIC=1.25 μg/mL). Also iryantherin K was the more abundant compound in the ethanolic extract whereas iryantherin L was present only in small amounts. More importantly, iryantherin K presented potent activity against S. aureus MR (methicillin-resistant) and presented no cytotoxic effects at 10 μg/mL in mouse fibroblast cells. This may prove a very important finding from the therapeutic perspective.

Iryantherin K and L had inhibitory effects against C. albicans secreted aspartic proteases. These compounds were also active in the same range of concentrations, against the aspartic protease pepsin. It is not surprising since the
overall architecture of the SAP’s from *Candida* conforms with the classical aspartic protease fold typified by pepsin (Sielecki *et al*., 1990). However, the structures of the SAP’s from *Candida* present unique features in both the amino and carboxy lobes (Abad-Zapatero, 1998). Considering that secreted aspartic proteases are a virulence factor in candidosis it would be interesting to test these compounds in an animal model.

Interestingly, compounds 4 and 5 have been found to have potent antioxidant activities as measured by their ability to inhibit spontaneous lipid peroxidation of brain homogenates (Silva *et al*., 1999). A necessity to maintain the chemical integrity of fruit tissue rich in easily oxidizable fatty acids and triglycerides has been suggested (Silva *et al*., 1999).

In regards to antioxidant activity it has been suggested that flavonoids multiple biological activities may result, at least, in part from their antioxidant and free radical-cavenging abilities. The protective effect of flavonoids against membrane lipoperoxidative damage has been well established, and seems to depend both on their structure and ability to interact with and penetrate the lipid bilayers. During pathological processes reactive oxygen species accumulate and the mitochondria membrane may undergo lipid peroxidation and/or increase in permeability. Agents that inhibit these processes may be of high pharmacological potential. However it has been shown that same flavonoids could behave as both antioxidants and prooxidants, depending on concentration and free radical source (Cao *et al*., 1997).
Although members of the genus *Iryanthera* are traditionally used to treat fungal infections, no significant activity was found against the yeast *C. albicans* (MIC=150 μg/mL). It may be worth testing these compounds against other fungi, such as dermatophytes, that are found in conditions similar to the site of collection. It is also important to recall the fact that it is the resin that is used to treat fungal infections rather than the bark. In general resins are composed mainly of terpenoid-like compounds that have been shown to have a wide range of antibiotic activity. However very few aspartic proteases have been isolated up to now in bacteria, and those described so far seem not to be involved in pathogenesis (Supuran et al., 2002).

Cinchonain Ib (6) and Ia (7) presented weak synergistic activity in the presence of berberine against *S. aureus* MS at minimum inhibitory concentration of 100 μg/mL. In the absence of berberine these compounds do not present antibiotic activity. A number of flavolignans have been found to act as potentiators of otherwise weak antimicrobials and it has been suggested that their action is due to inhibition of the *S. aureus* MDR efflux pump protein NorA (Guz et al., 2001). It would be interesting to compare these *S. aureus* NorA putative inhibitors with some inhibitors of the mammalian MDR P-gp efflux proteins since any inhibitor of a microbial MDR pump developed for therapeutic use should not affect P-gp, which plays a role in xenobiotic efflux of normal tissues.
Although cinchonain lb and la presented synergistic activity in the presence of berberine, they did not potentiate any activity in the presence of iryantherin K (4) nor iryantherin L (5). This indicates that there is no antimicrobial synergistic effect amongst the compounds found in this plant.

5.5. Conclusion

The anti-viral activity of *Iryanthera megistophylla* reported in Chapter 3 might be due to a synergistic effect amongst different compounds. In a bio-assay guided phytochemical separation, cinchonain lb (6) and la (7) were found to have anti-viral activity against herpes simplex virus. It is possible that the polyphenolic nature of these compounds is related with the anti-viral activity but no definitive evidence has been brought forward.

Also it would be worth investigating an eventual correlation between inhibition of aspartic proteases and the anti-oxidant properties reported elsewhere.

Iryantherin K activity against *S. aureus* MR represents an important finding and it might hold promise as a new powerful antibiotic active against this resistant bacteria.
5.6. References


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196.

Phytochemistry 55: 505-511.


6.1. Search for Antibiotics in Medicinal Plants

In the course of this project I have isolated, purified and structurally identified seven compounds from *Piper lanceaefolium*, which is traditionally used in the Sibundoy Valley (Colombia) for the treatment of a skin morbid condition caused by the "urine of the rainbow". These compounds are fairly inactive against micro-organisms such as yeast (*C. albicans*) and bacteria (*S. aureus*). Their lack of potency thus prevents me from considering them as antibiotics. A role for these compounds in nature and their effect in human health has yet to be determined.

In contrast, anti-viral and anti-bacterial activities were found for compounds isolated from *Iryanthera megistophylla* (Ming *et al.*, 2002). Probably the most important result from a pharmacological perspective is the very potent activity of iryantherin K against methicillin-resistant *Staphylococcus aureus*. Iryantherin K and its estereoisomer iryantherin L also presented moderate activity to *C. albicans* secreted aspartic proteases. Iryantherins seem to be a very interesting type of flavolignans whose mechanisms of action remain to be
elucidated but powerful antioxidant properties might be related to their antibiotic and enzymatic inhibition activities. Traditional use of *I. megistophylla* in the treatment of cutaneous leishmaniasis warrants further research to study the effect of these compounds in this parasitic disease.

It is probably time to ask whether the different activities found were the consequence of our premises regarding the uses of traditional plants. It is also time to ask why, every time we inquire about antibiotic activities as I did, the result seems to be affirmative.

In a large screening for antibiotic activities in medicinal plants used by British Columbian native peoples, McCutcheon and co-workers (1992) found that 69 plant extracts out of 73 presented antibiotic activity. Reports of this sort are common in the literature but often these studies are not followed by the corresponding phyto-chemical analyses, which makes it difficult to assess the potency of individual compounds compared to the whole plant extract activity.

I believe that most of these “antibiotic” activities are detected regardless of their traditional use because of a methodological bias. Also I think that these putative “antibiotic” activities do not reflect a plant’s ecological role nor do they explain traditional medicinal uses.

From the methodological point of view, the disk diffusion assay used throughout this study has a drawback particularly dealing with plant extracts. Large concentrations of plant extract are generally used, which are not realistic and certainly greater than the ones expected in a plant. It is also possible to
increase the number of apparently active extracts by selectively using particular bacteria or fungi that are very susceptible to chemical plant extracts.

These two factors together may make the disk diffusion assay a unreliable methodology that affords many false positives and in consequence meaningless results.

The fundamental question is then whether the likelihood of finding antibiotic compounds is increased by using ethno-botanical criteria instead of a random plant collection. In this regard it is interesting to note that plants collected within different medicinal categories (general tonic, non-antibiotic medicine, related medicinal species and unspecified medicine) also presented a good portion of plants with antibiotic activity. Eighteen extracts out of a collection of twenty-three turned out to be active giving a success rate of 78% which seems to be a very high proportion considering that these plants are not traditionally used as “antibiotics”. Furthermore, two out of four plants under the category non-medicinal, had antibiotic activity (McCUTCHEON et al., 1992). Interestingly the authors interpret this data as “indicative of the loss or distortion of knowledge concerning the specific uses of a given plant” (Mc CUTCHEON et al., 1992). Rather than examining the biological meaning of the screening, the authors assumed a disruption in the traditional knowledge.

This is not the first time that these types of concerns have been raised and alternative “experiments” have been proposed. For instance, it has been suggested to perform a “random” collection of plants and analyse their antibiotic activities and compare them with those traditionally used by a given community.
This proposition assumes that nature is a neutral field in the absence of human influence. It does not acknowledge the actual and/or past role of culture in shaping of the landscape. This culture/nature divide is the centre of great deal of academic activity and its implications are profound, but are well beyond the aim of this discussion. Suffice to say I do not hold any hope in finding such a thing as a random collection of plants, as Haraway (1992) pointed out “nature is a semiotic place”. The representation of nature as the untouched wilderness is cherished by most Westerners and its political significance has been discussed elsewhere (Willems-Braun, 1997).

However, whether this is testable or not I do not think is worth putting effort into finding a “random” collection of plants in the so-called “wilderness”. Suffice to say that antibiotic screenings are not serving their purpose as “sieves” or “indicators” of extracts with antibiotic activity.

Regardless of the potency of antibiotic activity found in each of these compounds, I believe that one should continuously bear in mind multiple effects of phenolic compounds in organisms. Closer attention must be paid then to the host-pathogen interaction during an infection event. For instance, quinine (present in Cinchona officinalis) and chloroquine, exert their effects by inhibiting host – encoded functions (Ridley, 1997).

This of course brings about the challenge of dealing with complex systems. The effect of a single compound or group of them at the cellular level in organisms (eukaryote or prokaryote) is complex and difficult to model. The concept of cause and effect is blurred in networks where feedback mechanisms
are the rule. Events occurring simultaneously are more likely to reflect reality better than cascade of events in a timely manner. In teasing apart these interactions, there is no obvious path to be followed and one should be able to overcome the horror that produces the mere idea of what complexity implies for our cause-effect mindset. In other words we need to develop intellectual tools that will allow us to look at the whole system with well-identified players. This implies the capacity to detect relevant cellular events without falling into simplified models that too often reflect our own limitations rather that a "real" phenomenon.

There is a more sensitive issue that deals with the way we approach different cultures. This issue is particularly difficult to tackle for it implies an examination of the scientific community as a culture. This challenging undertaking will compel us to look differently at other's healing practices.

6.2. Understanding other's Medical Practices

Very little attention has been paid (this thesis is an example) to the preparation and the cultural context in which a given plant is used as a therapeutic agent.

We need to do a better job of studying traditional practices and in understanding traditional therapeutical practices. We must keep in mind for instance that "infection" is not a universally accepted category and recognise its cultural relativity as a western concept deeply rooted in the germ theory. It is intriguing to see how traditional knowledge is continuously praised as an
example of keen observation of nature and as a reflection of a profound knowledge of biochemical properties present in plant extracts without considering cultural contexts of illness.

Many important biologically active compounds have been isolated from plants traditionally used in different parts of the world. However it is not uncommon to find bioactive compounds with activities that do not have evident correlation with their use in traditional societies. Lowering blood pressure effects of reserpine have not been studied in relation with Rauvolfia serpentina traditional use as a coadjuvant in reaching spiritual enlightenment.

P. lanceaefolium is a case in point. It is not a minor task to correlate the chemical nature of these compounds with a cryptic traditional use associated with the moon cycle and the appearance of the rainbow.

In this particular case one can observe how the moon cycle has traditionally been associated with crop rotation and in consequence with the whole subsistence in agricultural societies. It is likely these cycles are associated with insect pests that can eventually act as vectors of infectious diseases such as malaria or leishmaniasis. The genus Piper has been found to have important insecticidal chemical principles. The presence of taboganic acid and its action as an insect repellent might be an indication of possible role of these compounds in this regard. Although these hypotheses have not been tested yet I believe these alternatives are more likely than a putative antibiotic activity whose potency has been shown to be very low. Arguments over rationality and science endeavour
are expected. However these undertakings are at the very core of what the role of ethno-pharmacology should be as a discipline.

If one desires to understand the true use of medicinal plants by different cultures it is crucial to shift our mindset and be creative in generating hypotheses that take into account others' beliefs no matter how challenging the task might appear.

Also, and probably more fundamental, is the need for the scientific community to realise that medicines (substances used in the treatment of sickness) have culturally defined meanings as well as bio-chemical properties that endow them with specific qualities and powers (Reynolds and Van Der Geest, 1988). The task of uncovering the meaning of medicines is not a trivial one. We should be able to accomplish this by looking in more detail into the humoral theories of disease in traditional societies as well as assimilating the idea that therapy is about restoring a balance between contrasting symbolic qualities (Etkin, 1988).

In this regard assessing the efficacy of natural remedies is highly problematic because of different understandings. For instance, North-American indigenous people traditionally use plants known to be irritant and have vesicant use, which is not easily understood by westerners. However, the inducement of irritation, resulting in change of skin color and vesication, is the physical evidence that disease entities are encountering the surface to leave the body. Treatment and healing are then processual and only a series of outcomes will fulfill expectations (Etkin, 1988).
Thus efficacy as cultural construct is not necessarily consistent with biomedical criteria so when one cannot make sense of a plant use one should be wary in invoking the Doctrine of Signatures as an alternative exegesis.

Concepts like “validation of traditional knowledge” illustrates western pretensions in accommodating indigenous knowledge only in western terms and advocating the right to dismiss other uses as *irrational* under the realm of superstition.

Similarly we need to revisit the way we are drawing from indigenous knowledge and decide whether we wish to bridge cultures or we are just pragmatically interrogating traditional uses in order to increase efficiency in a long-term molecular farming project.

At the beginning of the twenty-first century, research in medicinal plants used by traditional societies opens up a yet to be established path towards a better understanding of the role of herbalists (yerbatero) in a post-modern society.
6.3. References


APPENDIX 1A
Nuclear Magnetic Resonance Spectrum of Cyclolanceaefolic acid methyl ester (\( ^1H \)) 400 MHz (1)

sample no: 5262  a.lopez  al151x /mta
1h  acetone-d6
APPENDIX 1B

Nuclear Magnetic Resonance Spectrum of Cyclolancefolic acid methyl ester ($^{13}$C) 100 MHz (1)
APPENDIX 1C
Two-Dimensional ($^1$H $^1$H) Nuclear Magnetic Resonance Spectrum (COSY) of Cyclolanceafolic acid methyl ester (400 MHz) (1)

JOB NO: 5308  NADRES LOPEZ  AL 151*
n15308 2 1  cosygr expt
APPENDIX 1E
High Resolution El Mass Spectrum of Cyclolanceaeofolic acid methyl ester (1)

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File Title : AL 151*
Instrument : MS50 EI

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Selected Isotopes:

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H       | auto| 1   |      | Hydrogen-1
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APPENDIX 1F

Infrared Spectrum of Cyclohexenyl acid methyl ester (1)

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2968.19
2984.29
3253.94
3394.81
3744.66
3854.22
3855.18
3870.07
3880.56
3885.96

100 500 1000 2000 3000 4000
APPENDIX 2A
Nuclear Magnetic Resonance Spectrum ($^1$H) of Cyclolanceaefolic acid (2) (400 MHz)
APPENDIX 2B
Nuclear Magnetic Resonance Spectrum (\(^{13}\text{C}\)) of Cycloleucine folic acid (2) (100 MHz)
APPENDIX 2C
Low Resolution EI Mass Spectrum of Cyclolanceaefolic acid (2)

SCAN GRAPH, Haggng=Nom. M/z.
APPENDIX 2D
High Resolution El Mass Spectrum of Cyclolanceaefolic acid (2)

ATOMIC COMPOSITION REPORT

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Sort Field: Hr M/z (descending).
Scan Filter: Minimum Intensity= 10.00%
Mass Range= [220:240]

Selected isotopes:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Min</th>
<th>Max</th>
<th>Vcy</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>30</td>
<td>4</td>
<td>Carbon-12</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>auto</td>
<td>1</td>
<td>Hydrogen-1</td>
</tr>
<tr>
<td>O</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>Oxygen-16</td>
</tr>
<tr>
<td>N</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>Nitrogen-14</td>
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Allowable error = minimum of 10.0 ppm, 30.0 mmu.
Ring/Double Bond limits = [-0.5 : 100.0]

Scan 77#:0:07:04 - 86#:0:07:54
Number of Peaks=31, filtered down to 3.
Base Peak= 221.04559, 100% Intensity=56322.

<table>
<thead>
<tr>
<th>Mass</th>
<th>%age</th>
<th>Calculated</th>
<th>ppm</th>
<th>mmu</th>
<th>R/DB</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>236.06836</td>
<td>25.94</td>
<td>236.06847</td>
<td>0.5</td>
<td>0.1</td>
<td>7.0</td>
<td>C_{12}H_{12}O_{5}</td>
</tr>
<tr>
<td>222.04893</td>
<td>12.19</td>
<td>222.04695</td>
<td>-8.9</td>
<td>-2.0</td>
<td>16.0</td>
<td>C_{18}H_{6}</td>
</tr>
<tr>
<td>221.04559</td>
<td>100.00</td>
<td>221.04500</td>
<td>-2.7</td>
<td>-0.6</td>
<td>7.5</td>
<td>C_{11}H_{6}O_{5}</td>
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<tr>
<td></td>
<td></td>
<td>221.04768</td>
<td>9.5</td>
<td>2.1</td>
<td>12.0</td>
<td>C_{14}H_{7}O_{2}N</td>
</tr>
</tbody>
</table>

***** End of Atomic Composition Report *****
APPENDIX 3A
Nuclear Magnetic Resonance Spectrum (1H) of Lanceaeolic acid methyl ester (3) (400 MHz)
APPENDIX 3B
Nuclear Magnetic Resonance Spectrum (\(^{13}\text{C}\)) of Lanceaefolic acid methyl ester (3) (100 MHz)
APPENDIX 3D
High Resolution El Mass Spectrum of Lanceaeefolic acid methyl ester (3)

ATOMIC COMPOSITION REPORT

File Name: C:\MASPEC\Data\H14580.ms2
File Date/Time: 5/22/01 at 14:04:57
File Type: V/E Data - Cld
File Source: Acquired on MASPEC II system [1132/9949]
File Title: A 1-16R
Instrument: MS50 E1

Sort Field: Hr M/z (descending)
Scan Filter: Minimum Intensity= 10.00%

Mass Range= [233:255]

Selected isotopes:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Min</th>
<th>Max</th>
<th>Vcy</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>Carbon-12</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>auto</td>
<td>1</td>
<td>Hydrogen-1</td>
</tr>
<tr>
<td>O</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>Oxygen-16</td>
</tr>
</tbody>
</table>

Allowable error = minimum of 10.0 ppm, 30.0 mnu.
Ring/Double Bond limits = [-0.5 : 100.0]

Scan 53#0:04:53 - 62#0:05:42

Number of Peaks=52, filtered down to 3.
Base Peak= 235.06052, 100% Intensity=13854.

<table>
<thead>
<tr>
<th>Mass</th>
<th>%age</th>
<th>Calculated</th>
<th>ppm</th>
<th>mmu</th>
<th>R/DB</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>250.08436</td>
<td>22.38</td>
<td>250.08412</td>
<td>-0.9</td>
<td>-0.2</td>
<td>7.0</td>
<td>C_{13}H_{14}O_{5}</td>
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<td>236.06352</td>
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<tr>
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<td>235.06065</td>
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<td>0.1</td>
<td>7.5</td>
<td>C_{12}H_{11}O_{5}</td>
</tr>
</tbody>
</table>

***** End of Atomic Composition Report *****
APPENDIX 4A
Nuclear Magnetic Resonance Spectrum ($^1$H) of Lanceaefolic acid (4) (400 MHz)
APPENDIX 4B
Low Resolution El Mass Spectrum of Lanceaefolic acid (4)
APPENDIX 4C
High Resolution El Mass Spectrum of Lanceaefolic acid (4)

ATOMIC COMPOSITION REPORT

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>File Date/Time</td>
<td>5/22/01 at 13:01:43</td>
</tr>
<tr>
<td>File Type</td>
<td>V/E Data - Ctd</td>
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<tr>
<td>File Source</td>
<td>Acquired on MASPEC II system [II32/9949]</td>
</tr>
<tr>
<td>File Title</td>
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<tr>
<td>Instrument</td>
<td>MS50 EI</td>
</tr>
<tr>
<td>Sort Field</td>
<td>Hr M/z (descending)</td>
</tr>
<tr>
<td>Scan Filter</td>
<td>Minimum Intensity= 10.00%</td>
</tr>
<tr>
<td></td>
<td>Mass Range= [220:240]</td>
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</tbody>
</table>

Selected Isotopes:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Min</th>
<th>Max</th>
<th>Vcy</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>Carbon-12</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>auto</td>
<td>1</td>
<td>Hydrogen-1</td>
</tr>
<tr>
<td>O</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>Oxygen-16</td>
</tr>
</tbody>
</table>

Allowable error = minimum of 10.0 ppm, 30.0 mmu.
Ring/Double Bond limits = [-0.5 : 100.0]
Scan 111#0:10:11 - 120#0:11:01
Number of Peaks=116, filtered down to 3.
Base Peak= 221.04452, 100% Intensity=417050.

<table>
<thead>
<tr>
<th>Mass</th>
<th>%age</th>
<th>Calculated</th>
<th>ppm</th>
<th>mmu</th>
<th>R/DB</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>236.06832</td>
<td>23.31</td>
<td>236.06847</td>
<td>0.7</td>
<td>2.0</td>
<td>7.0</td>
<td>C_{12}H_{12}O_{5}</td>
</tr>
<tr>
<td>222.04762</td>
<td>12.53</td>
<td>222.04695</td>
<td>-3.0</td>
<td>-0.7</td>
<td>16.0</td>
<td>C_{18}H_{16}</td>
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<tr>
<td>221.04452</td>
<td>100.00</td>
<td>221.04500</td>
<td>2.2</td>
<td>0.5</td>
<td>7.5</td>
<td>C_{11}H_{9}O_{5}</td>
</tr>
</tbody>
</table>

***** End of Atomic Composition Report *****
APPENDIX 5A

Nuclear Magnetic Resonance Spectrum of Taboganic acid (5) (H) 400 MHz
APPENDIX 5B
Low Resolution EI Mass Spectrum of Taboganic acid (5)
APPENDIX 6A

Nuclear Magnetic Resonance Spectrum of Pinocembrin (6) (H) 400 MHz
APPENDIX 6B
Nuclear Magnetic Resonance Spectrum of Pinocembrin (6) \(^{13}\text{C}\) 100 MHz
APPENDIX 6C
Low Resolution El Mass Spectrum of Pinocembrin (6)
APPENDIX 7A
Nuclear Magnetic Resonance Spectrum of Pinocembrin chalcone (7) (¹H) 400 MHz
APPENDIX 7B
Low Resolution El Mass Spectrum of Pinocembrin chalcone (7)