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Phytochemical discovery of antifeedant, antimicrobial and antimalarial principles

by

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In Biology

TABLE OF CONTENTS

List of Figures

List of Tables

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Abstract

This thesis examines the phytochemical discovery process from temperate and tropical trees. Thirty extracts of wood and bark of hardwood trees from Eastern North America were examined for insect control and antimicrobial activities. Nine of the bark extracts and four of the wood extracts showed significant growth reducing effects against *Ostrinia nubilalis* at 0.5%, whereas only two bark extracts and one wood extract showed significant antifeedant effect against *Sitophilus oryzae* at the same concentration. Slower growing tree species were more biologically active than fast growing ones. Isolation of the bioactive compounds in one of the active species, *Prunus serotina,* showed that naringenin, its derivative 4'methoxynaringenin, and eriodictyol were responsible for the antifeedant effects

Antimicrobial activity of the hardwood trees was also tested against eight strains of bacteria and six strains of fungi. Eighty-six percent of the bark extracts were active against methicillin-sensitive *Staphylococcus aureus*; 71 % against *Bacillus subtilus* and 79% against *Mycobacterium phlei.* The bark extract of *Juglans cinerea* was active against *Pseudomonas aeruginosa* 187, *Salmonella typhiumurium*, and *Klebsiella pneumoniae*. The wood extracts were less active: 72% were active against *S. aureus* (methicillin-sensitive), 36% against *B. subtilus and* 43% against *M. phlei.* Results from antifungal tests indicated that 36% of the

extracts were active against at least one fungal strain and that bark extracts were more active than wood extracts. The bark extract from *Juglans cinerea* had the broadest spectrum of activities against *Candida albicans, Saccharomyces cerevisiae, Cryptococcus neoformans, Trichophyton mentagrophytes, Microsporum gypseum,* and *Aspergillus fumigatus.* In general, the extracts were more active against gram-positive bacteria than gram-negative bacteria and against filamentous fungi than yeast-like fungi. The study also demonstrated a correlation between frequency of traditional medicinal use by the First Nations people and antimicrobial activity of extracts indicating that the traditional knowledge encompasses an understanding of aspects of chemical ecology.

Tropical trees have been used as a source of traditional remedies for malaria. Bioassay-guided isolation of active principles of the traditionally used antimalarial plant, *Lansium domesticum* from Borneo (Indonesia) was undertaken. Six novel triterpenoids were identified and three derivatives were prepared. All nine compounds were tested for *in vitro* antimalarial activities against chloroquine sensitive (D6) and chloroquine resistant (W2) *Plasmodium falciparum* clones. Four of these compounds exhibited high activity comparable to quinine. *In vivo* studies were conducted using mice infected with *Plasmodium bergheii* in a 4-day suppression test. The results indicated that methyl 15, acetoxylansiolate produced parasitemia clearance level of 50%. It was concluded that the

bitter triterpenoids are responsible for the antimalarial activity of *Lansium domesticum*.

Gedunin from *Cedrela odorata* (Meliaceae), a potent *in vitro* antimalarial agent, was also investigated for its *in vivo* efficacy. When orally administered at 50 mg/kg/day, gedunin was only able to suppress the parasitemia level by 44%. However, at a combination treatment of gedunin and dillapiol (cytochrome P450 inhibitor), the parasitemia clearance increased to 79%. Furthermore, 7-methoxygedunin, a semiderivative stable to degradation by esterases tested at a daily dose of 50 mg/ kg body weight, suppressed the parasitemia level by 67%. When administered in combination with dillapiol, the parasitemia level decreased by 80% similar to the results obtained with gedunin. These results indicated that standardized phytomedicines could potentially be developed using these materials.

Résumé

Le but de cette thèse a été démontrer comment procèder à la découverte de composés phytochimiques provenant d'arbres de régions tempérées et de régions tropicales. Trente extraits de bois et d'écorce d'arbres à bois franc provenant de l'Amérique de l'est et du nord ont été analysés afin de déterminer leur efficacité pour le contrôle des insectes et des micro-organismes. Lors de bioessais avec la pyrale de maïs, *Ostrinia nubilalis* et le charançon de riz, *Sitophilus oryzae*, neuf extraits d'écorce et quatre extraits de bois ont donné une réduction significative de croissance à 0.5%, tandis que seulement deux extraits d'écorce et un extrait de bois ont démontré des effets d'anti-appétence à la même concentration. Les espèces d'abres à croissance lente démontraient une plus grande activité biologique que les espèces à croissance rapide. L'isolation des composés bioactifs d'une espèce, *Prunus serotina*, a démontré que la naringenine, son dérivé, la methoxynaringenine, et l'eriodtyol étaient responsables de l'effet d'anti-appétence observé.

Les arbres à bois franc sont aussi utilisés dans la médecine traditionnelle des Premières Nations. L'activité antimicrobienne sur huit souches de bacteries et six souches de champignons a ete testeé. Quatre-vingt-six pour cent des exraits d'écorce ont eu un effet contre la *Staphylococcus* sensible à la méthiciline; 71% contre *Bacillus subtilus* et 9% contre le *Mycobacterum phlei*. L'extrait de l'écorce de *Juglans cinerea*

a démontré une activité contre *Pseudomonas aeruginosa* 187, *Salmonella typhiumurium,* et *Klebsiella pneumoniae.* Les extraits de bois étaient moins actif: 72% ont eu une activité contre *S. aureus* (sensible à la methicilline), 36% contre *B. subtilus* et 43% contre *M. phlei.* Les résultats obtenus des tests anti-fongiques indiquent que 36% des extraits sont actifs contre au moins une souche de champignons et que les extraits d'écorces ont une plus forte activité que les extraits de bois. Les extraits d'écorce provenant de *Juglans cinerea* ont le spectre d'action le plus étendu contre *Candida albicans, Saccharomyces cervisiae, Cryptococus neoformans, Trichophyton mentagraphytes, Microsporum gypseum,* et *Aspergillus fumigatus.* La tendance générale indique que les extraits ont eu plus d'activité contre les bactéries gram-positives que les bactéries gram-négatives et aussi contre les champignons filamenteux que les champignons ressemblant aux saccharomycètes. Cette étude a démontré également une corrélation entre la fréquence d'utilisation dans la médecine traditionnelle des Premières Nations et l'activité anti-microbienne des extraits analysés. Ce résultat indique que les connaissances traditionnelles des groupes des Premières Nations incluent une compréhension de certains aspects de l'écologie chimique.

Les arbres tropicaux sont utilisés comme source de remède traditionnel contre le paludisme. Nous avons fait une isolation guidée par bioessai des composantes actives de la plante utilisées dans la médecine

traditionnelle contre le paludisme, *Lansium domesticum*, provenant de Borneo (Indonésie). Six nouveaux triterpenoïdes ont été identifiés et trois dérivés ont été synthétisés. Ces neufs composés ont été testés *in vitro* afin de déterminer leur activité anti-paludéenne contre des clones de *Plasmodium falciparum* sensibles à la chloroquine (D6) et résistants à la chloroquine (W2). Quatre de ces composés ont démontré une forte activité anti-paludéenne, comparable à la quinine. Des analyses de suppression en 4-jours ont été effectuées *in vivo* sur des souris infectées par *Plasmodium bergheii.* Les résultats indiquent que le méthyl 15, acétoxylansiolate a reduit le niveau de parasitose de 50%. Les triterpénoides amers sont donc à la source de l'activité anti-paludéenne de *L.domesticum.*

L'efficacité *in vivo* de la gedunin, un agent anti-paludéen *in vitro* puissant provenant de *Cedrela odorata* (Meliaceae), a aussi été étudié. Ce dernier composé a réussi à supprimer le niveau de parasitose de seulement 44% lorsqu'une dose de 50mg/kg de masse corporellea été introduite par voie buccale. Par contre, lors-qu'il est le combiné avec le dillapiol, un inhibiteur de cytochrome P450, l'élimination du parasite parvient à 79%. De plus, le 7-methoxygedunin, un semi-dérivé qui résiste à la dégradation par estérases, a supprimé la parasitose de 67% lorsque'une dose journalière de 50 mg/kg de masse corporelle a été utilisé. Tel qu'observé avec la gedunin, lorsque la 7-methoxygedunin a été

administrée en combinaison avec le dillapiol, il y a eu une réduction de 80% du niveau de parasitose. Ces résultats indiquent qu'il existe un potentiel de développement de phytomedicaments standardisés à partir de plantes qui ont fait le sujet de ce travail.

Chapter 1. General Introduction

1.1 Background

The plant kingdom, with over 250,000 described species, is well defended against insects by secondary compounds (Schoonhoven, 1982). There are expected to be about 500,000 secondary compounds but only 30,000 have been investigated (Bernays and Chapman, 1994). Phytochemicals have received renewed interest in agriculture because of the growth of organic growing practices and the recent registration of new antifeedants from botanical sources in the United States (Isman, 1997; Schumetterer, 1990). In medicine, the discovery of important drugs such as the anticancer drug, taxol, from plants and the increasing desire by the public for alternative medicine has added impetus for research in this field (Beutler et al. 1995; Phillipson and Wright, 1991; Klayman, 1985).

The insecticidal properties of several bitter principles have been investigated in our laboratory in order to seek new leads for the development of biopesticides of plant origin. The group has been successful in isolating a number of new antifeedant limonoids from *Trichilia martiana*, new steroids from *Trichilia hirta* (Arnason et al., 1985; Jimenez et al., 1997) and over 14 unusual C, D spiro triterpenoids from *Ruptiliocarpon caracolito* (MacKinnon et al., 1997) as well as a number of neolignans with insecticidal synergistic properties from the Piperaceae family (Bernard, 1995). Efficacy of these biopesticides in field trials

showed that several of these compounds were effective at low concentration (Assabgui et al., 1997). A review of botanical insecticides indicates that the most promising compounds for use in the future are bitter principles from the Meliaceae and Rutaceae (Jacobson, 1989). Particularly, limonoids from Meliaceae have given the best results (Arnason et al., 1987; Kubo and Klocke, 1981; Champagne et al., 1992).

Plants as sources of medicinal compounds have been known for many centuries. The World Health Organization (WHO) estimates that about 80% of the population in developing nations relys on plant-based traditional medicine for primary health care (Farnsworth et al., 1985). Plants offer an alternative treatment for indigenous people who cannot afford the cost of commercially available synthetic drugs. When the therapeutic effects of these plants are verified, they will also gain wide acceptance by the scientific community. Natural compounds could also provide the pharmaceutical industry with the foundation for development of more effective synthetic compounds. Currently, it is estimated that at least 121 prescription drugs come directly from plants (Soejarto and Farnsworth, 1989).

Traditional healers have used plant preparations for many diseases. Malaria is the most important disease treated with plant preparations in Africa, Latin America and Southeast Asia.

1.2 Malaria: distribution, cause, life cycle, clinical manifestation

Malaria affects an estimated 500 million people worldwide and each year between one to three million deaths are reported in over 90 countries (WHO, 1995). Malaria kills more people today than ever before. The reasons for this spread include resistance of the vector and parasite to the available insecticides and drugs, increased migration, immigration, and global warming (WHO, 1995).

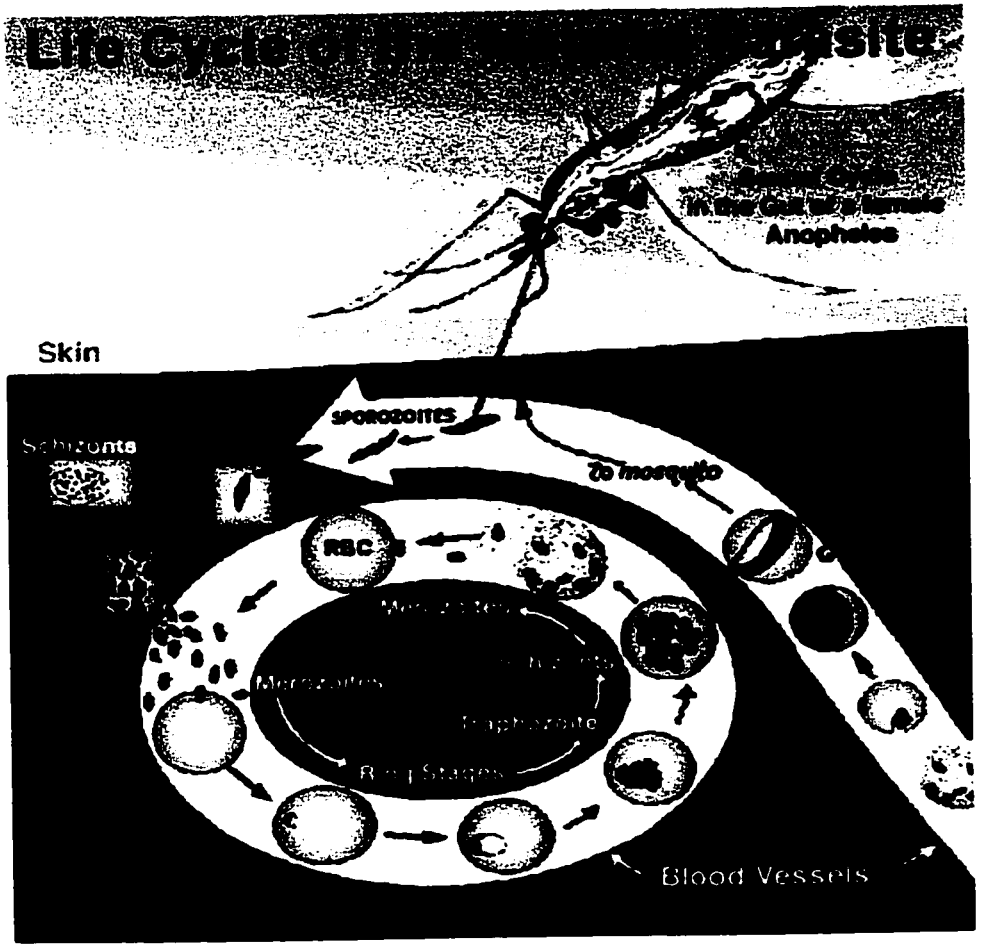
Malaria is caused by the *Plasmodium* parasite that requires two hosts to complete its life cycle. The vertebrate hosts include birds, reptiles, rodents, primates and humans, while the invertebrate host is normally the anopheles mosquito. Human malaria may also be transmitted by blood transfusion, and contaminated syringes (Phillips, 1983). There are four species of *Plasmodium* known to infect human: *P. falciparum*, *P. vivax*, *P. malariae and P. ovale*. The most prevalent of these are *P. falciparum* and *P. vivax*.

1.2.1 *Plasmodium* life cycle

The *Plasmodium* life cycle requires both the mosquito and human host (Figure 1.1). When the female mosquito takes her blood meal, she injects an anticoagulant with the saliva to ensure an even-flowing meal. Sporozoites from the salivary gland are then injected into the capillary

bed of the skin and enter the bloodstream. Sporozoites move to the liver within about half an hour and enter the hepatocytes. Once in the hepatocyte, *P. falciparum* and *P. malariae* sporozoites immediately enter schizogony whereas *P. ovale* and *P. vivax* sporozoites either enter schizogony or develop into dormant hypnozoites (Phillips, 1983). Preerythrocytic schizogony takes between 5 to 15 days depending on the species. The product of this stage is the production of merozoites. Merozoites leave the liver and enter the bloodstream, and within minutes invade red blood cells, where they grow and divide. In the red blood cell, the parasite requires nutrients for growth. Hemoglobin is degraded by a cysteine protease (Salas et al., 1995) and aspartyl protease (Oaks et al., 1991) resulting in amino acids which the parasite uses to synthesize proteins for the developing merozoites. Within the red blood cell, merozoites dedifferentiate into simple trophozoites. These trophozoites produce and insert proteins into the erythrocyte membrane responsible for cytoadherence (Oaks et al., 1991). Every 48-72 hours the erythrocytes rupture, releasing parasites along with waste products and toxins into the blood stream. At this stage, clinical symptoms such as fever and chills arise. The patient feels weak and shows signs of fatigue and episodes of high fever and shivering.

Figure 1.1 Life cycle of malarial parasite (Reproduced from Malaria Foundation with permission)


Anopheles
Skin
SPOROZOITES
To mosquito
RBC
Blood Vessels

Trophozoites that mature into schizonts release merozoites which then invade other erythrocytes. This asexual cycle could be repeated several times, raising the parasitemia level (Phillips, 1983). Some trophozoites develop into the sexual forms, macrogametocytes and microgametocytes. These are the forms that are ingested by the mosquito when she takes the blood meal. The gametocytes once in the midgut of the mosquito, lose the erythrocyte membrane and undergo gametogenesis (Sinden, 1984). The microgametocyte undergoes three nuclear divisions producing eight microgametes but the macrogametocyte only forms one macrogamete. Once mature, fertilization occurs forming a zygote, and after 18 to 24 hours the zygote develops into a motile ookinete. The ookinete elongates and penetrates the midgut wall to lie under the membrane and above the basal lamina. There it differentiates into an oocyst and grows over the next 7-12 days. The hemolymph provides the growing oocyst with nutrients. Sporogony occurs within the oocyst, producing many sporozoites. When the oocyst ruptures, the sporozoites migrate to the salivary gland where they generally remain viable for the life of the mosquito (Beier, 1998), ready to move on to another victim when the mosquito takes a blood meal.

Cytoadherence occurs when parasitized erythrocytes become adhesive, sticking to the endothelial lining of capillaries and sinusoids of the internal organ (Phillips, 1983). Cerebral malaria results in seizures,

coma, and even death with 24 hours (Rowe et al., 1995). Rosetting is another phenomenon which occurs when several parasitized erythrocytes strongly adhere to a non-parasitized one. Whether rosetting itself plays a direct role in the pathogenesis of severe malaria is as yet unknown (Rowe et al., 1995).

1.2.2 Malarial treatment

The bark of the cinchona tree was used by native people of South America for the treatment of malarial related fever. Pelletier and Coventou subsequently isolated quinine in 1820 and it became the first known treatment for malaria (Gentillini et al., 1991). Despite its toxicity, quinine remains an effective drug against many malarial infections. In the 1940's, a synthetic antimalarial, chloroquine, was developed and was available in many parts of the world (WHO, 1995). However, twenty years later, resistance of the *Plasmodium* to this drug appeared and quickly spread around the world. The resistance problem necessitated the development of new drugs. Sulfadoxone / pyrimethamine was developed in the 1960's and twently years later, mefloquine became the first line of prophylactic treatment. Primaquine, halofantrine, proguanil, doxycycline, malarone and artemisinin are also used as antimalarial drugs (Rang et al., 1999).

1.2.3 Plants as sources of antimalarial compounds

The Natural Products Alert database (NAPRALERT) lists about 152 plant genera with a folkloric reputation as malarial remedy throughout *Africa*, Asia and the Americas (Phillipson and Wright, 1991b). Brandao et al. (1992) carried out a survey of antimalarial plants used by natives in the Amazon. The authors were able to obtain more hits using the ethnopharmacological approach (18%), than the random screening approach (0.7%). Their findings provide evidence that the knowledge of traditional healers is invaluable and should be preserved.

In the following literature survey, the plants that had undergone extensive antimalarial activity testing in various laboratories will be briefly summarized. Structures of some of the *in vitro* active compounds are shown in Figure 1.2.

1.2.3.1 Artemisinin

Apart from quinine, the most important antimalarial phytochemical discovered so far is artemisinin (qinghaosu). The Chinese have been using the herb, *Artemisia annua*, for many centuries (Klayman, 1985). This plant is also found in Europe, North America, and Asia (Hien and White, 1993). The active compound was isolated from the leaves of this plant and identified as a sesquiterpene lactone (Klayman, 1985). Structure-activity studies showed that the endoperoxide group confers the antimalarial activity (Hien and White, 1993). Iron-mediated cleavage of the peroxide

group activates the compound by generating an unstable organic free radical. Once the radical is formed, it is believed to oxidize lipids; oxidize and alkylate proteins selectively destroying the membranes of the parasite (Hien and White, 1993). Artemisinin is very effective at preventing cytoadherence and rosetting, and also acts as blood schizontocidal. Several studies have demonstrated its efficacy; because of it's short half-life, recrudescent rates are as high as 49% and it is now given as part of other drug regimens in the treatment of malaria (Lee and Hufford, 1990).

1.2.3.2 Quassinoids from Simaroubaceae

A large-scale study involving the screening of approximately 600 plants revealed that numerous plant extracts from the Simaroubaceae family showed antimalarial activity (Spencer et al., 1949). Quassinoids found in these plant extracts are biodegraded triterpenoids that are bitter principles. Their bitterness is believed to have prompted their use as antimalarials, since quinine is extremely bitter. *In vitro* antimalarial activities against two chloroquine–resistant strains of *P. falciparium*, FCR-1 and FCR-3 were conducted using four quassinoids (Trager and Polonsky, 1981). The most active compound was simalikalactone D (IC_{50}= 1 ng/mL); glaucarubinone, and soularubinone were also active (IC_{50}= 6 ng/mL), however, chaparrinone was ineffective. Ekong et al. (1990) postulated that the steroid-like structure of the quassinoids may facilitate

their uptake by cells. The mechanism of action of these compounds is the inhibition of protein synthesis (Allen et al., 1993). Structural features attributed to the activity are the presence of C-8 methylene-oxy bridge to C-11 or C-13 and the substituents at C-15 as well as the state of oxidation and substitution of the A ring (Allen et al., 1993). So far, there are no reports on *in vivo* studies using these compounds. Given the fairly high cytotoxicity of quassinoids observed, Allen et al., (1993) investigated the selectivity index (cytotoxicity to KB cells / antimalarial activity) of several synthetic derivatives of brutasol. Brutasol was found to be twice as toxic as the other derivatives implying that the esterification at C-3 significantly reduced cytotoxicity. The authors concluded that the esterification may somehow preclude binding of the compound to host ribosomes during protein synthesis. Bray et al. (1987) investigated several quassinoids and found ailanthinone to be the most active (IC_{50} < 10 ng/ mL). Unfortunately, ailanthinone exhibited high toxicity in mice.

1.2.3.3 Napthyllisoquinoline alkaloids

Napthylisoquinoline alkaloids have been used traditionally as antimalarials. Guido et al. (1994) obtained crude extracts and pure compounds from the Ivory Coast. The alkaloids dionocophylline A and B, and dioncopeltine A were obtained from *Triphyophyllum peltantum*, ancistrobrevine D and ancistrocladine were isolated from *Ancistrocladus*

abbreviatus and N-methyldioncophylline was isolated from both *Ancistrocladus abbreviatus* and *Ancistrocladus barteri*. Dioncophylline B and dioncopeltine A were found to be the most active when tested *in vitro* against chloroquine-sensitive *P. falciparum*. Structure-activity relationship of these compounds suggests the presence of a hydroxy group at C-5 is required for activity (Guido et al., 1994). It appears that very minor differences in chemical structures caused large differences in antimalarial activity.

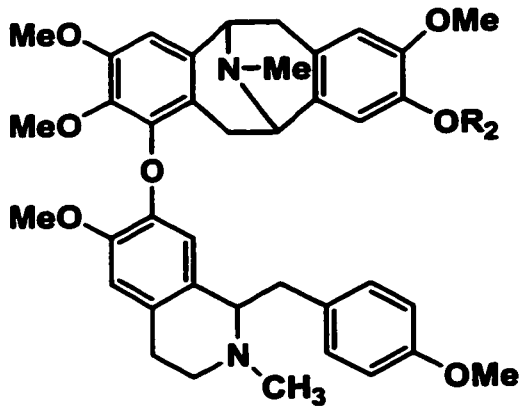
Crytolepine, an indoloquinolide from the roots of a West African shrub, *Cryptolepis sanguinolenta*, is widely used in Ghana (Kirby et al., 1995). It exhibited potent *in vitro* activity and was subjected to clinical trials. This study was conducted where malaria is endemic. Early results showed that it was very successful in reducing parasitemia levels. However, the authors concluded that the patients were likely to have some degree of immunity to the parasite that would affect the results of the trials. (Kirby et al., 1995).

Ratsimamanga-Urveg et al. (1994) isolated isoquinoline alkaloid dimers, Herveline A, B, and C from *Hernandia voyronii* Jumelle, and conducted *in vitro* studies with chloroquine-resistant *P. falciparum* strain FCM 29. Herveline B and C potentiated the effect of chloroquine in a dose-dependent manner, virtually reversing chloroquine resistance. Stuctural examination of these hervelines revealed a benzyl-

tetrahydroisoquinoline (BTIQ) moiety similar to verapamil, a known calcium channel blocker and P-glycoprotein pump inhibitor. Previous studies had shown that calcium channel blockers restore chloroquine sensitivity and BTIQ moeity may be responsible for the potentiation effect. The significance of these results demonstrate that plant materials can be used in conjunction with chloroquine as effective antimalarial treatment.

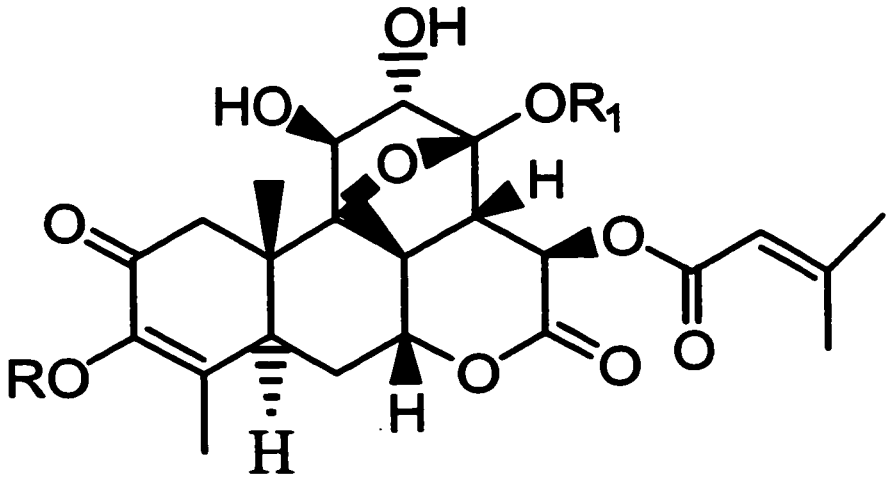
Several other plants are used traditionally in many parts of the world. In Tanzania alone, Gessler et al. (1994) reported the use of 43 species of plants and tested 58 plant samples. Crude extracts from *Maytenus senegalensis* (Celastraceae) and *Zanthoxylum chalybeum* (Rutaceae) exhibited high *in vitro* activities (IC_{50} < 0.7 μg/ mL), but were found to be toxic to KB and HT29 human caricinoma cells. However, the authors noted that this demonstrated toxicity does not preclude the use of these plants since no toxicity were reported by traditional healers in Tanzania and Senegal where they are used for the treatment of malaria. In a subsequent study, Gessler et al., (1995) surveyed 25 traditional healers regarding malarial treatment. Most of the healers mentioned the neem tree, *Azadirachta indica* (Meliaceae) as a widely used antimalarial plant.

Figure 1.2 Structures of compounds with *in vitro* antimalarial activities

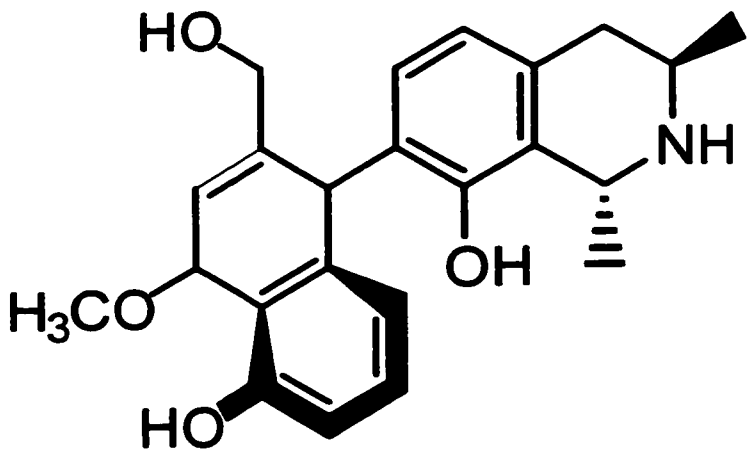


Hervelines A: R_1=H; R_2=CH_3 B: R_1=CH_3; R_2=H;

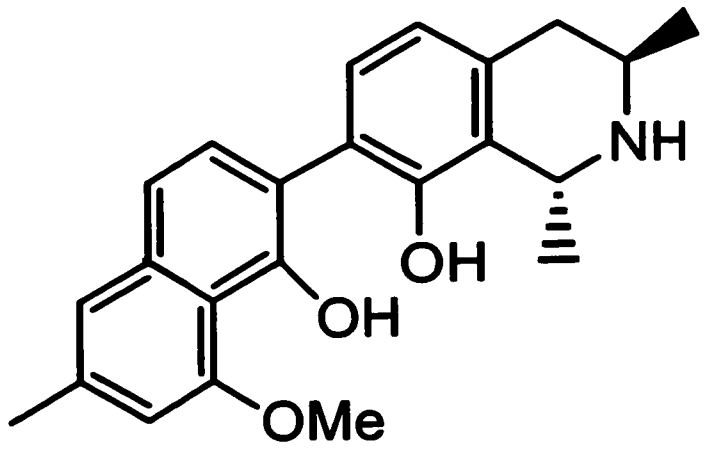
C: R_1=R_2=CH_3



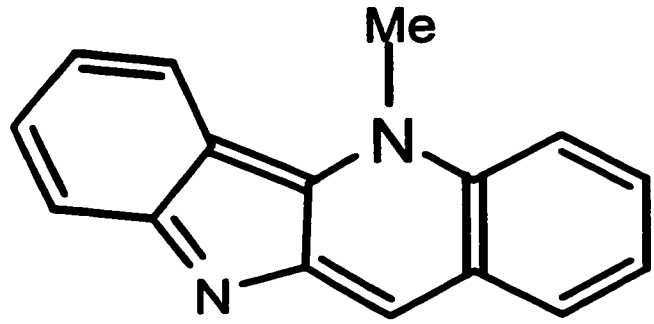
Brutasol



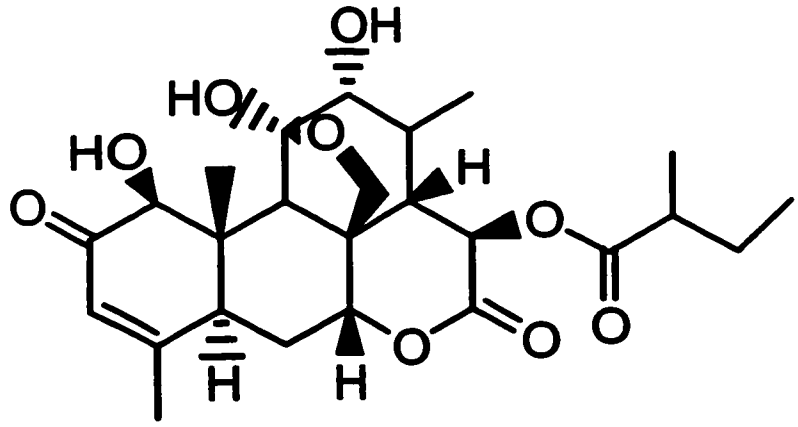
Dioncopeltine A



Dioncopeltine B



Cryptoleptine



Ailanthinone

1.2.3.4 Limonoids

The leaves of *Azadirachta indica* have been used traditionally in Southeast Asia and Africa for the treatment of fever caused by malaria (Rochanakij et al., 1985; Obih and Makinde, 1985). Animal trials conducted with the leaf extracts showed that the treatment suppressed the effect in early malarial infection but was not effective against an established infection (Obih and Makinde, 1985).

Several limonoid compounds have been isolated from this extract including azadirachtin, nimbolides and gedunin. Jones et al. (1994) reported that azadirachtin was found to block the development of the motile microgametocyte *in vitro* and suggested that it may be useful as transmission-blocking agent. Based on *in vitro* studies, Rochanakij et al. (1985) suggested that nimbolide may be the active ingredient. Other *in vitro* studies showed that gedunin as the most active compound (Khalid et al., 1986; Bray et al., 1990; MacKinnon et al., 1997).

1.3 Biological Activity

A successful method for the investigation of phytochemicals of potential biological activities is the screening of plant preparation followed by bioassay-guided fractionation leading to the isolation of pure active principles (Hostettmann and Wolfender, 1997; Vietnick et al., 1995). A number of bioassays are available to test isolated compounds. To be

practical, bioassays should be complemented by *in vivo* trials, as many compounds that exhibited *in vitro* activity may not show any activity *in vivo*.

1.3.1 Insect bioassay

Bioassays for insect control agents are performed using model insects that can easily be reared under laboratory setting. Our laboratory has done extensive work for the identification of insect growth regulating, antifeedant and mosquito larvicidal compounds. The European corn borer, *Ostrinia nubilalis* Hubner, the rice weevil, *Sitophilus oryzae* L. and the mosquito, *Aedes atropalpus* L. have been useful tools for testing minute quantity of crude extracts or pure compounds (Arnason et al., 1985; Xie et al., 1996; Belzile et al., 2000). The life cycles of *O. nubilalis* and *S. oryzae* will be briefly introduced below. Specific details on the bioassays are given in Chapter 2.

1.3.1.1 *Ostrinia nubilalis* life cycle

The European corn borer, *Ostrinia nubilalis* (Lepidoptera:Pyralidae) is a serious pest particularly by borning into the stalks and causing breakage and ear drop of the corn. It has a complete metamorphosis including five stages of instar followed by pupation and

adult formation. The approximate life cycle of *O. nubilalis* is about 28 days.

1.3.1.2 *Sitophilus oryzae* life cycle

The rice weevil, *Sitophilus oryzae* L., (Coleoptera: Curculionidae), is one of the most destructive pests of stored grains; infesting many cereals but favouring wheat and rice (Mills, 1990). The female weevil bites a tiny hole on the grain surface and lays many eggs within the grain. For most of its life, the larva feeds inside the grain excavating a tunnel and passes through four instars in about 25 days. Pupation takes place inside the grain and the adult eats its way out of the grain (Mills, 1990).

1.3.2 Antimicrobial bioassay

The paper disk diffusion bioassay was first developed for bacteria and then was modified for filamentous fungi and yeast (Hadacek and Greger, 2000). In this method, the chemical is applied directly on the filter paper disk which then diffuses into the agar and inhibits the germination and growth of the test microorganism. Specific details of the bioassays are given in Chapter 3.

1.3.3 Antimalarial bioassay

The development of a large-capacity screen for novel compounds with specific antimalarial activity requires the establishment of a relatively simple bioassay for primary screening followed by secondary screening bioassay (Angerhofer et al., 1992).

The microdilution radioisotope technique has several advantages. Its unique screening system mimics the biological picture of an actual infection and the drug permeability through the host red cell membrane is thought to be similar to those encountered *in vivo* during disease progression (Geary et al., 1983; Angerhofer et al., 1992). However, the disadvantage lies in that the blood cells are obtained from different human donors and introduce a degree of variability. As is the case in most *in vitro* testing, false positive or false negative results are also unavoidable. The method is described in detail in Chapter 5.

In vivo trials using murine models are extensively used as part of the secondary assay in the antimalarial drug discovery process. Although the strains of *Plasmodium* that affect rodent and human are different, the similarity in their life cycles makes them a prime target for these studies. The Walter Reed Army Institute of Research (Washington, DC) has made extensive use of these primary and secondary assays. Their discovery of mefloquine and halofantrine attests the success of these bioassays (Angerhofer et al., 1992). This method is described in Chapters 5 and 6.

1.4 Hypotheses

In this thesis, several aspects of the drug discovery process will be examined. At the primary screening level insect bioassays provide a convenient and rapid method of isolation and assessment of mode of action. On the application side, the insecticidal, antimicrobial and antimalarial activities warrant advanced study for the development of promising natural products.

Chapter 2 deals with the process of drug discovery by examining the predictive power of chemical ecology by identifying plant parts suitable for the isolation of bioactive compounds. This chapter was based on a collaborative study with a paper and pulp company, DOMTAR Inc., (Senneville, QC) which was interested in finding value added products in hardwood species in eastern North America.

Hardwood tree extracts from North America also have antimicrobial activity as suggested by ethnobotanical reports (Arnason et al., 1981; Moerman, 1998; Jones, 1999). Chapter 3 examined the ethnobotanical literature to predict antimicrobial activity found in these trees.

Chapter 4 illustrates the isolation of bioactive compounds from the bark of *Lansium domesticum*, a tropical plant used for the treatment of malaria by the Apo Kenyah people (Indonesia). Subsequently, the

antimalarial activity of these compounds was tested *in vitro* and *in vivo* models (Chapter 5).

Chapter 6 examined the antimalarial activities of gedunin and its derivative 7-methoxygedunin and also synergisitic effects with dillapiol. A summary and conclusion are given in Chapter 7.

Hypothesis 1. Extracts of slow growing hardwood trees of North America are predicted to have more insecticidal activity than fast growing iispecies.

Hypothesis 2. Extracts from ethnobotanically used trees will have antimicrobial activity under controlled laboratory conditions.

Hypothesis 3. Bitter principles from *Lansium domesticum* are responsible for the antimalarial activity observed in the crude extracts.

1.5 Objectives

The objectives of this thesis are as follows:

Objective 1: To isolate, identify and biologically characterize hardwood tree extracts from eastern North America for growth regulating and feeding deterrent effects using two insect models.

Objective 2: To investigate the antimicrobial activities of hardwood bark extracts against gram-positive, gram-negative bacteria, filamentous fungi and yeast.

Objective 3: To isolate and undertake structural elucidation of bitter principles from *Lansium domesticum*.

Objective 4: To investigate the *in vitro* and *in vivo* antimalarial activities of crude extracts and pure compounds from *Lansium domesticum*.

Objective 5: To investigate the *in vivo* antimalarial activity of gedunin and 7-methoxygedunin and also to explore the synergistic effect with dillapiol, a cytochrome P450 inhibitor.

Chapter 2. Insect growth-reducing and antifeedant activity in eastern North America hardwood species and isolation of active principles from *Prunus serotina*

2.1 Introduction

The interest in botanical insecticides with antifeedant or growth inhibitory activity has grown because of their natural origin, non-neurotoxic mode of action and low environmental persistence (Arnason et al., 1992; Isman, 1994; Isman, 1997). Complex mixtures of secondary compounds in these plant extracts contribute to synergism which enhances the joint action of active compounds on the insect (Bernard and Philogène, 1993) and reduce the rate of development of insect resistance (Feng and Isman, 1995; Isman et al., 1997). In the past, active phytochemicals from botanicals have been successfully used as a lead to develop new synthetic pesticides that are in worldwide use today (Klocke, 1987).

The vast majority of botanicals developed as commercial pesticides originate from tropical sources. Because of the intensity of plant-insect interactions in the tropics, tropical plants are well defended chemically and have been found to be excellent sources of novel insecticidal substances (MacKinnon et al., 1997). However, there is no lack of insect pressure in temperate forests, as trapping experiments have amply demonstrated the large diversity of wood boring species present at a single forestry site in Eastern Ontario (Chénier and

Philogène, 1989). On this basis long-lasting plant tissues are expected to be well defended. A large number of studies has examined insect deterrents in leaves of trees in Eastern North America (Jacobson, 1990). However, according to our NAPRALERT database analysis (Loub et al., 1985), the insecticidal potential and phytochemistry of temperate wood and bark has not been well investigated.

Depending on the resources availability and the degree of herbivory, the effectiveness of defenses varies widely among plant species (Coley et al., 1985). Thus long-lived tissues and slow growing plants have a greater investment in plant defense mechanisms than fast growing plants. Therefore, slow growing hardwood trees of North America may contain bioactive phytochemicals which could provide a potential lead towards the development of insect controlling substances. In this chapter, the hypothesis that slow growing hardwood trees contain insect growth inhibitory and antifeedant effects is tested using two insect models: the European corn borer, *Ostrinia nubilalis* (Lepidoptera:Pyralidae), and the rice weevil, *Sitophilus oryzae* (Coleoptera:Curculionidae). Since neither species feeds on hardwoods, they may be considered as model lepidopteran and coleopteran species that are not preadapted to defenses in hardwoods. Furthermore, the isolation of active compounds in one of the most active tree species was undertaken and is described.

2.2 Material and method

2.2.1 Plant material

Fifteen samples of trees (n=4) were obtained from Domtar Inc. forestry operation in eastern Ontario. The species collected were *Acer rubrum* L. (Aceraceae, red maple, voucher number UO-18500), *Acer saccharum* L. (Aceraceae, sugar maple, voucher number UO-18501), *Betula allenghaniensis* Britton (Betulacea, yellow birch, voucher number UO-18514), *Betula papyrifera* Britton (Betulaceae, white birch, voucher number UO-18502), *Carya cordiformis* K. (Juglandaceae, bitternut hickory, voucher number UO-18503), *Carya ovata* K. (Juglandaceae, shagbark hickory, voucher number UO-18504), *Fagus grandifolia* Ehrh. (Fagaceae, beech, voucher number UO-18505), *Fraxinus pennsylvanica* Marsh.(Oleaceae, ash, voucher number UO-18506), *Juglans cinerea* L. (Juglandaceae, butternut/walnut, voucher number UO-18507), *Prunus serotina* Ehrh. (Rosaceae, black cherry, voucher number UO-18508), *Populus grandidentata* L. (Salicaceae, hybrid popular, voucher number UO-18509), *Populus sp.* (Salicaceae, voucher number UO-18510), *Quercus rubra* L. (Fagaceae, red oak, voucher number UO-18511), *Tilia americana* L. (Tiliaceae, basswood, voucher number UO-18512), and *Ulmus americana* L. (Ulmaceae, elm, voucher number UO-18513).

Voucher specimens have been retained at the herbarium of the University of Ottawa, Ottawa, Canada.

2.2.2 Extract preparation

The bark and wood from each species were separated and ground into a fine powder (sawdust) by using a Wiley mill. The powdered material was soaked in ethanol (5:1 w/w basis) for 48 hours. The mixture was filtered, and the solvent evaporated to near dryness on a rotary evaporator. The residual water was removed by lyophilization using a freeze-drier.

2.2.3 Compound isolation from *Prunus serotina*

The crude ethanolic extract from the wood of *P. serotina* (3.59 g) was reconstituted with 50 mL of 1:1 water: ethanol and extracted 3 times with 50 mL of hexane. The remaining water: ethanol fraction was evaporated and reconstituted with 50 mL water. The water fraction was extracted three times with 50 mL of ethyl acetate. The combined ethyl acetate fractions were evaporated to yield 2.41 g of yellow gum. An aliquot (2.0 g) was chromatographed on 100 g of silica gel (230-400 mesh) using 7:1 methylene dichloride: ethyl acetate (CH_2Cl_2 :Et_2O) as eluant. Four compounds were separated in order of increasing polarity and identified by comparison with published spectra with literature values

(Mabry et al 1970). NMR spectra were obtained with a 500 MHz Bruker spectrometer. This work was conducted by Matieu Lalonde at the Department of Chemistry, University of Ottawa, Ottawa, ON.

2.2.4 Insect rearing

Initial eggs and pupae of *Ostrinia nubilalis* (European corn borer) were obtained from a wild stock collected at the Central Experimental Farm, Agriculture and Agri-food Canada, Ottawa, ON. Insects were maintained in a growth cabinet at 26°C/19°C day/night temperature regime, 80% humidity and a L/D: 18/6 photoperiod. Adults were moved to a separate growth chamber where eggs were collected on wax paper. Artificial diet was prepared according to Guthrie et al. (1985). The rice weevil, *Sitophilus orzyae*, were obtained from Agriculture and Agri-food Canada, Winnipeg, MB. They were reared on Canada Western Hard Red Spring wheat at 14% moisture content and maintained at 30°C and 85% relative humidity.

2.2.5 *Ostrinia nubilalis* bioassay

Freeze-dried extracts were dissolved in 2 mL of 50% ethanol added to an artificial diet at a final concentration of 0.5% (w/w) basis. The diets were then poured into molds containing 1 cm^3 volume. Second-instar larvae were weighed and placed individually on a diet cube in dram

vials and then stoppered with absorbent cotton. Diet cubes with no extracts were prepared as controls. The larvae were kept in a growth chamber and each larva was weighed before and after the four-day experiment. All experiments were conducted using 30 insects. The relative growth rates (RGR) expressed in mg / mg mean wt. per day were calculated as follows: RGR= ln (final wt / initial wt) / 4 where ln is natural logarithm.

2.2.6 *Sitophilus oryzae* bioassay

Hard red spring wheat flour (200 mg) was added to 1 mL of an aqueous solution containing the test substance at a concentration of 0.5% w/w and mixed using a magnetic stirrer. Aliquots (100μL) of the stirred suspension were placed in a Petri dish aand allowed to dry overnight in air at room temperature. Twenty-five insects were placed in a petri dish with 5 flour disks and kept at 30°C and 85% relative humidity for three days. The weight of the disks before and after flour diet consumption by the insects was compared to the control. Experiments were done in duplicates according to a method developed by Xie et al. (1996).

2.3 Results and Discussion

Many of the wood and bark extracts were significant inhibitors of larval growth of *Ostrinia nubilalis* (Table 2.1). The lyophilized ethanolic bark extracts from *A. rubrum* ($P<0.01$), *A. saccharum* ($P<0.001$), *F. pennsylvanica* ($P<0.001$), *J. cinerea* ($P<0.001$), *P. serotina* ($P<0.001$), *P. grandidentata* ($P<0.01$), *Q. rubra* ($P<0.001$), *U. americana* ($P<0.05$) significantly reduced larval growth of *O. nubilalis* at the concentration of 0.5% in diets. The bark extracts from *B. alleghaniensis, B. papyrifera, C. cordiformis, C. ovata, F. grandifolia, Populus sp. T. americana* did not show any significant inhibitory effect. The wood extracts that caused significant reduction in growth were *A. saccharum* ($P<0.05$), *F. grandifolia* ($P<0.05$), *J. cinerea* ($P<0.0001$), *P. serotina* ($P<0.001$), and *Q. rubra* ($P<0.05$).

A two-way analysis of variance (Table 2.2) with relative growth rate of the *Ostrinia nubilalis* as dependent factor and species, or plant part used to prepare the extracts as independent factor, showed that species effect was significant ($P<0.001$) and plant part was significant ($P<0.001$). The significant interaction ($P< 0.001$) means that plant part effect is bigger from plant to plant. This suggests that defenses vary from species to species as well as the part of the tree. This is attributed to the differences in expression of defenses in different plant parts as well as

the unique defense mechanism of secondary metabolites against insect herbivory employed by each plant species.

These results are in many ways very comparable to growth-reducing effects found previously in similar tests for tropical Rutales extracts (Arnason et al., 1987; Arnason et al., 1992). However, because the present study encompasses several plant families and the tropical collection were mainly from the Meliaceae, a statistical comparison of the tropical and temperate activity is not justified.

It has been proposed that slow growing plants have a higher amount of quantitative defensive secondary compounds than the fast growing trees (Coley et al., 1985). Feeny (1978) proposed that "apparent" (eg. long life cycle, forest dominants) are better defended than "unapparent " plants. In a similar manner to Coley et al. (1985), analyses of several sets of ecological defenses were conducted. The tree species were divided into a group of slower growing species (8 species) and a group of faster growing species (7 species) for comparison of their defensive traits (Table 2.3). This selection was based on available published literature data on relative plant growth rates for a number of species (Walter et al, 1993; Logan et al., 1965; Logan et al., 1967). The slow growing species have a higher number of wood extracts with significant activity ($P<0.001$) in the *Ostrinia nubilalis* growth bioassays compared to the fast growing species (Table 2.4). The mean insect

RGR's for these two groups show similar trends with the slow growing tree group producing a lower insect growth rate than the faster growing tree group. These results are then consistent with the proposal of Coley et al. (1985) explaining a trade-off between growth and defense.

The flour disk consumption test with *Sitophilus oryzae* was found to be reproducible and facilitated the isolation of active compounds because it allowed the bioassay of a large number of extracts or compounds with very small amounts of material which could not have been feasible with the *O. nubilalis* bioassay. Two of the wood extracts (*P. serotina* and *F. grandifolia*) exhibited mean diet consumption levels below the control level (Figure 2.1) and were found to be growth reducers to *O. nubilalis*. Antifeedant effects are not thought to be important in the corn borer growth reduction since previous research has shown that larvae of this species do not respond by reduced consumption when extracts or pure phytochemicals are presented in a meridic diet (Arnason et al., 1985). The antifeedant effects of the *Betula* extracts may be related to triterpenoids from this genus known to reduce feeding (Reinchart et al., 1984) and naphthoquinones (juglone) may be responsible for the feeding deterrence effect of *J. cinerea*. Several of the test diets actually were phagostimulatory to *Sitophilus oryzae* and this may be attributed to the sugars, amino acids, salts and other chemical cues that are known to stimulate insect feeding (Albert, 1990).

Because of its potent activity in both bioassays, *P. serotina* wood was chosen as one of the promising extracts for bioassay guided isolation. Nothing is recorded about the phytochemistry of black cherry wood but the bark is known to contain condensed tannins identified as 5,7,3'4' tetrahydroxyflavin-3,4 diol (Buchalter, 1969).

The *P. serotina* wood extract was separated into hexane, ethyl acetate and ethanol:water fractions and bioassayed for activity. Only the ethyl acetate gave appreciable antifeedant activity and chromatography of this fraction showed relatively few (four) major compounds. These compounds were isolated and identified by comparison of spectral data (Appendix I) with data in the literature (Mabry et al., 1970). The four isolated flavonoids were identified as naringenin, 4'-methoxynaringenin, dihydrokaempferol and eriodictyol (Figure 2.2).

Because of the small amounts available, these materials were only tested in the antifeedant bioassays. As illustrated in Table 2.5, naringenin, naringenin methyl ether and eriodictyol were active, whereas dihydrokaempferol was not active at 0.5%. Naringenin derivatives are well known as bitter principles in grapefruit (Harborne, 1994) and have been extensively investigated by the citrus industry. More recently naringenin has been identified as one of the compounds responsible for increased drug uptake by grapefruit juice (Fuhr, 1998). It is an inhibitor of the cytochrome P450 enzyme (CYP3A4) which is responsible for first

pass metabolism of drugs in the small intestine (Budzinski et al., 2000). The presence of naringenin in black cherry alcohol extract is also medically relevant because of its use as a hydroalcoholic tincture for treatment of coughs in naturopathic medicine (Thomson, 1978). In a chemical ecology context, naringenin in the wood may act as a synergist for other compounds in the defense of black cherry because of its ability to inhibit metabolism. Further investigation of its activity against *O. nubilialis* and insect cytochrome P450 is therefore warranted.

Table 2.1. The effect of Eastern North American wood and bark extracts on larval growth of *O. nubilalis*

Species extract	RGR (mg/mg m.wt/d) bark extract	RGR (mg/mg m.wt/d) wood extract
Control	0.354 ± 0.018a	0.342 ± 0.017a
A. rubrum	0.225 ± 0.012*bde	0.332 ± 0.009a
A. saccharum	0.233 ± 0.013*bde	0.281 ± 0.023*e
B.alleghaniensis	0.284 ± 0.018a	0.337 ± 0.009a
B. papyrifera	0.345 ± 0.007a	0.309 ± 0.014a
C. cordiformis	0.265 ± 0.011*be	0.305 ± 0.013a
C. ovata	0.372 ± 0.011a	0.367 ± 0.009a
F. grandifolia	0.314 ± 0.030a	0.278 ± 0.031*e
F. pennsylvanica	0.108 ± 0.119 *c	0.340 ± 0.012a
J. cinerea	0.170 ± 0.030*de	0.251 ± 0.009*e
P. serotina	0.195 ± 0.037*de	0.249 ± 0.01*e
P. gradidentata	0.237 ± 0.014*de	0.327 ± 0.013a
Populus sp .	0.384 ± 0.009a	0.368 ± 0.025a
Q. rubra	0.227 ± 0.008*de	0.290 ± 0.009e
T. americana	0.263 ± 0.146a	0.338 ± 0.009a
U. americana	0.248 ± 0.011*de	0.315 ± 0.011a

*Significantly different from control at $P<0.05$. The letters a-e denote multiple range tests performed using Tukey's test.

Table 2.2. Two-way analysis of variance (ANOVA) of insect RGR by plant species used to prepare extracts and plant part (wood or bark)

Sources of variation	Sum of squares	Degree of freedom	Mean square	F ratio	P value
Plant species	1.464	14	0.105	14.448	<0.001
Plant part	0.618	1	0.618	85.415	<0.001
Species*part	0.960	14	0.069	9.477	<0.001
Error	5.646	780	0.007		

Table 2.3. Classification of relative growth rates of North American species

Slow growing species	Fast growing species
Acer rubrum	*Betula alleghaniensis*
Acer saccharum	*Betula papyrifera*
Carya cordiformis	*Fraxinus pennsylvanica*
Carya ovata	*Populus gradidentata*
Fagus grandifolia	*Populus sp.*
Juglans cinerea	*Tilia Americana*
Prunus serotina	*Ulmus Americana*
Quercus rubra	

Table 2.4. Summary data and comparison between fast and slow growing species for insect growth reducing activity of *Ostrinia nubilalis*

	All species	fast growing species	slow growing species
Frequency of bark extracts active at 0.5% w/w in diet	9/15	3/7	6/8
Frequency of wood extracts active at 0.5% w/w in diet	4/15	0/7	4/8
Mean bark extract (RGR ±SE) mg/mg m.wt./day	0.258 ± 0.019a	0.289 ± 0.017b	0.230 ± 0.028a
Mean wood extract (RGR ±SE) mg/mg m.wt./day	0.312 ± 0.01b	0.328 ± 0.001b	0.298 ± 0.016b

Means followed by the same letter were not significantly different in pairwise comparison :Student's t-test ($P < 0.05$).

Table 2.5. Percentage of flour disks consumed by *Sitophilus oryzae* after treatment by crude extracts and pure compounds isolated from *Prunus serotina* (0.5% w/w)

Compound	disk consumption (% of control ± SE)
Control	100 ± 8.3
Ethanol extract	69 ± 6.5
Hexane fraction	95 ± 7.8
Ethyl acetate fraction	77 ± 4.5
Eriodictyol	25 ± 6.3
Naringenin	47 ± 12.9
4' Methoxynaringenin	62 ± 2.1

Figure 2.1. Percentage of flour disk consumed (± s.d.) by *Sitophilus oryzae* after treatment by North American bark and wood extract (0.5% w/w)
1: A. rubrum; 2: A. saccharum; 3: B. allenghaniensis; 4: B. papyrifera; 5: C. cordiformis; 6: C. ovata; 7: F. grandifolia; 8: F. pennsylvanica; 9: J. cinerea; 10: P. serotina; 11: P. grandidentata; 12: Q. rubra; 13: T. americana; 14: U. americana, 15: Control (ethanol only).

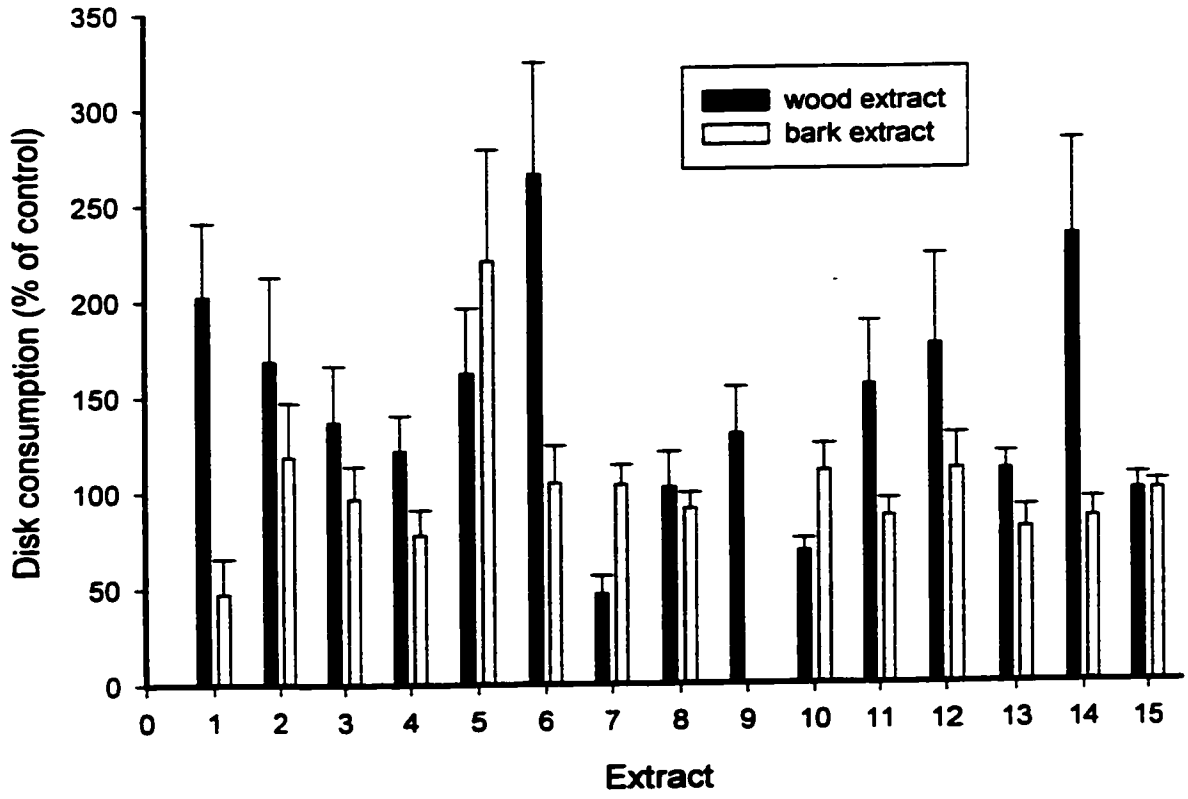
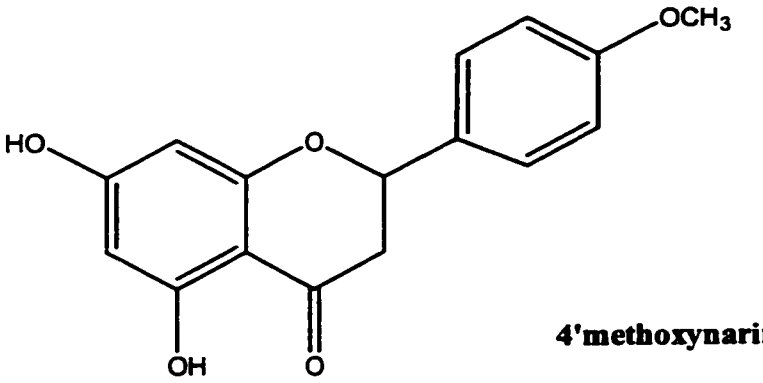
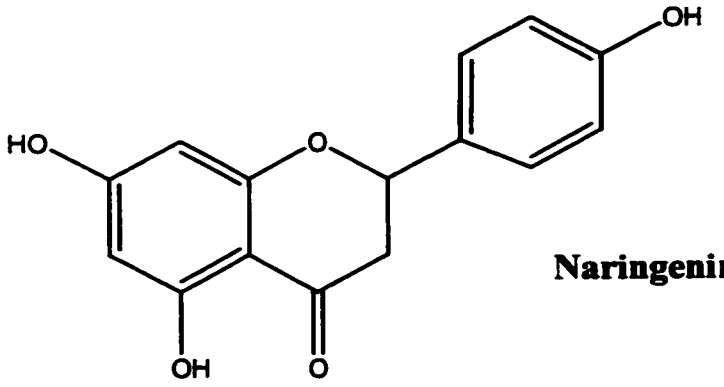

wood extract
bark extract
Disk consumption (% of control)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Extract

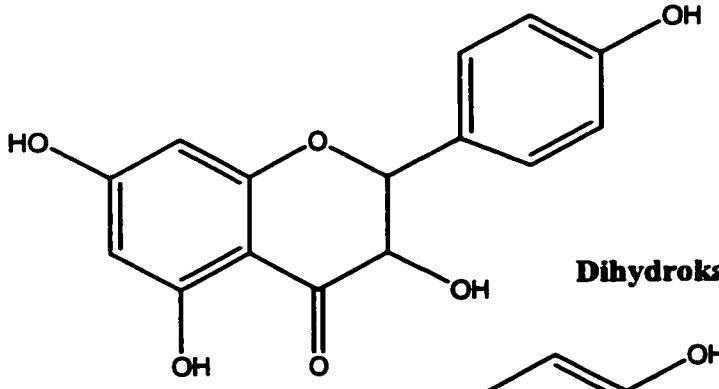
Figure 2.2. Chemical structures of compounds isolated from *P.serotina*



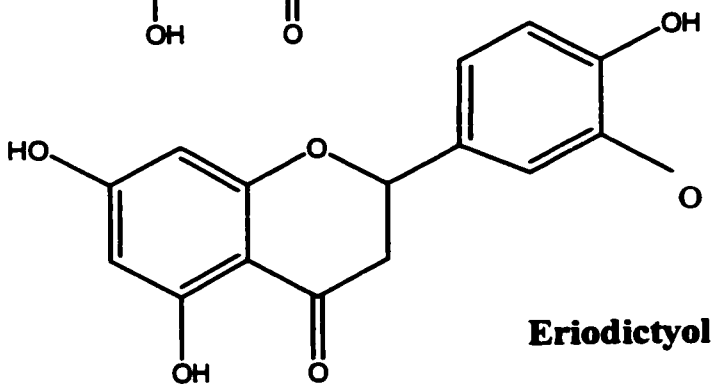
4'methoxynaringenin



Naringenin



Dihydrokaempferol



Eriodictyol

Chapter 3. Screening of antimicrobial activity of extracts of eastern North American hardwood trees and relation to traditional medicine

3.1 Introduction

In a recent compilation of native North American ethnobotany, it was estimated that there are about 4000 plants with recorded use as medicines (Moerman, 1998). The eastern North American flora contains over 400 species that have been traditionally used as medicine by First Nations people against many ailments (Arnason et al., 1981). Many of these plants are associated with infections of microbial origin (Arnason et al., 1981). However, according to the Natural Products Alert database (Loub et al., 1985), it is apparent that the phytochemical and pharmacological properties of the native hardwood trees of eastern North America have not been thoroughly investigated. Based on the prominence of tree extracts as traditional treatments of probable microbial infections, the hardwood tree extracts have antimicrobial activity as suggested by ethnobotanical reports (Arnason et al., 1981; Miguel et al., 1997; Moerman, 1998).

In this chapter, the hypothesis that the hardwood tree extracts will have antimicrobial activity under controlled laboratory conditions was tested. Specifically, the activities of extracts from common hardwood tree extracts against pathogenic bacteria and fungi are reported. Both light

independent and light enhanced activities are reported since many secondary metabolites are shown to be phototoxic in previous research (Arnason et al., 1986). The relationship between traditional medicinal use and antimicrobial activity is also explored.

3.2 Materials and methods

3.2.1 Plant materials

As per described in Chapter 2.2.

3.2.2 Microbial cultures

Bacterial strains tested included the gram-positive strains: *Staphylococcus aureus* (wild) methicillin-sensitive, *Enterococcus faecalis, Mycobacterium phlei, Bacillus subtilus,* and the gram-negative strains: *Escherichia coli* wild strain, *Pseudomonas aeruginosa 187* (wild), *Salmonella typhimurium*, and *Klebsiella pneumoniae.* All cultures were maintained in the laboratory of Dr. G.H.N. Towers, Unviersity of British Columbia, Vancouver, BC. *S. aureus* causes serious food intoxication; *E. faecalis, S. typhimurium, E. coli, B. subtilus, P. aeruginosa 187* (wild), and *Mycobacterium phlei* cause food spoilage and human infection whereas the toxin from *K. pneumoniae* is known for fish poisoning (Frazier and Westhaoff, 1988). The fungal strains used in this study are opportunistic

pathogens of humans except for *Saccharomyces cerevisiae*. *Cryptococcus neoformans* causes generalized mycosis with a predilection for the central nervous system, *Candida albicans* causes oral thrush and systemic infections, *Aspergillus fumigatus* may be associated with respiratory infections. *Microsporum gypseum* and *Trichophyton mentagrophytes* are dermatophytes. The fungal strains were collections maintained in the laboratory of Dr. M.L. Smith, Carleton University, Ottawa, ON.

3.2.3 Antibiotic assay

An inoculum of each of the bacterial strains was suspended in 2 mL of nutrient broth and incubated overnight at 37°C. The culture was then diluted with nutrient broth (1:9). To screen for antibiotic activity, sterile Mueller-Hinton agar plates were used according to the disc diffusion assay (Lennette, 1985). A sterile cotton swab was used for spreading diluted cultures on the plate after which sterile paper discs impregnated with 2 mg of extract in 20 µl of ethanol was placed on the inoculated surface. Plates were incubated at 37°C in the dark, or to test for ultra violet light effects on activity, plates were irradiated with near UV light (10 W / m^2 from four 20 W blacklight, blue tubes, 320-400 nm range) for 2 hours and then incubated at 37°C in the dark. Zones of inhibition were examined after 24

hours except for *Mycobacterium phlei* which was incubated for 48 hours before viewing.

All fungi were grown at 30°C and maintained at 4°C on Sabouraud's dextrose (SD) medium. Inoculum of each yeast strain was prepared from an overnight culture adjusted to an O. D. 600 nm of ~ 2.0 and diluted in SD (1:200). For filamentous fungi, a 1 cm^2 area of actively growing mycelium was removed to 54 mL of sterile, deionized water and fragmented for 1.5 minutes at high speed in a Waring blender. One hundred microliter of fungal cell suspension was spread onto agar plates with a bent glass rod prior to application to paper discs impregnated with 2 mg extract, as described above. All transfers were carried out in a biohazard level 2 laminar flow hood. Dark and UV-treatments with fungal trials were carried out as described above. Zones of inhibition of fungi were measured after 48 hours incubation at 30°C.

For controls, three antimicrobial substances known to be inhibitory were used: Gentamycin (antibacterial agent), the photosensitizing agent 8-methoxy-psoralen (8-MOP) and Berberine (antifungal agent). In addition, dried discs that had been soaked in ethanol served as carrier controls. All trials were performed using three 6.5 mm diameter discs per plate.

3.2.4 Data analysis

The diameter of inhibition zone around each disc was measured and recorded at the end of incubation time. An extract was classified as active when the diameter of the inhibition was equal to or larger than 8 mm. A dashed line (---) denotes no observed activity or an inhibition diameter of less than 8 mm. Mean values between the UV light and dark treatments were statistically tested for significant difference using the Student's t-test ($P<0.05$).

3.2.5 Ethnobotanical data

The primary data set used in this study was compiled and published by Arnason et al., (1981) in an extensive paper entitled, *Use of Plants for Food and Medicine by Native Peoples of eastern Canada*. The report was a special issue of over 100 pages which summarized food, beverage, and medicinal uses of over 400 species presented in 44 ethnobotanical reports. From this review, the medicinal plant usages were compiled into a database facilitating the analysis of data and formation of this report. A 'mention' denotes a record from the ethnobotanical database of medicinal uses of plants by eastern Canada's First Nation peoples as transcribed in the compilation from Arnason et al. (1981). Antimicrobial mention is a record of a treatment of condition of possible microbial origin

such as treatment of sores, skin, respiratory, gastro-intestinal, and urinary tract infections.

3.3 Results and Discussion

The results of testing of the twenty-eight crude ethanolic extracts for antimicrobial activities against 8 bacterial and 6 fungal strains are reported (Tables 3.1-3.3). Table 3.1 shows the effect of bark extracts on bacteria with or without near UV-light exposure. All the bark extracts except those from *F. pennsylvanica* and *P. grandidentata* were active against *S. aureus* (methicillin-sensitive). The extracts from *J. cinerea* tended to have UV-enhanced activities against the gram-positive bacteria. When tested against *B. subtilus*, the bark extracts from *A. rubrum*, *A. saccharum*, *B. papyrifera*, *F. grandifolia*, *J. cinerea*, *P. serotina*, *Populus* sp. and *Q. rubra* were found to be active while the extracts from *C. cordiformis* and *U. americana* were active only when treated in combination with UV-light. Tests against *M. phlei* indicated that all the bark extracts except those from *F. grandifolia*, *F. pennsylvanica* and *P. serotina* were active. Furthermore, UV-light treatment significantly enhanced the activity of *U. americana* extracts against *M. phlei*. Upon testing against the *E. faecalis*, only the extracts from *A. rubrum*, *B. papyrifera* and *U. americana* showed zones of growth inhibition. Further testing against the gram-negative bacteria indicated that only bark extract from *J. cinerea*

was substantially active. UV-induced activity of *J. cinerea* was noted with *E. coli* and *K. pneumoniae*. Likewise, *A. rubrum* was active when exposed to UV light against *E. coli*.

The results of the bark extracts tests against the six fungal species are given in Table 3.2. The extracts from *A. saccharum* and *P. serotina* were found to be active only against *M. gypseum*, with a significantly enhanced effect of UV exposure with *A. saccharum* extract. Extracts from *A. rubrum* and *Q. rubra* were found to be active against both *M. gypseum* and *T. mentagrophytes*. More notably, *J. cinerea* bark extract was active against all fungi except *T. mentagrophytes*.

The activities of the wood extracts against the eight strains of bacteria tested are given in Table 3.3. The extracts from *A. rubrum*, *A. saccharum*, *B. papyrifera*, *C. cordiformis*, *F. grandifolia*, *J. cinerea*, *P. serotina*, *P. grandifolia* and *Q. rubra* were active against *S. aureus* (methicillin- sensitive). When tested against *B. subtilus*, the wood extracts from *A. rubrum*, *F. grandifolia*, *J. cinerea*, *P. serotina*, *Q. rubra* and *T. americana* exhibited activities. Inhibition of *M. phlei* growth was observed by extracts from *A. rubrum*, *B. papyrifera*, *C. ovata*, *J. cinerea* and *P. grandidentata*. None of the extracts inhibited growth of *E. faecalis* nor any of the gram-negative species tested. UV-treatment significantly enhanced the activities of *T. americana* when tested against *S. aureus*, *B. subtilus* and *M. phlei;* and *C. ovata* against *S. aureus* while the extracts from *A.*

rubrum and *Q. rubra* were light activated when tested against *M. phlei*. The effect of wood extracts on fungi tested showed that only *P. serotina* was moderately active against two strains of fungi: *M. gypseum* and *T. mentagrophytes* (inhibition zones of 10 mm in both cases). The rest of the wood extracts did not exhibit any zone of inhibition and UV did not influence the antifungal activities of any extracts.

This study demonstrated that all extracts (either the bark or wood) had inhibitory activity against at least one of the bacterial or fungal strains tested. The extracts were more inhibitory to gram-positive bacteria than the gram-negative in agreement with the general expectation that a much greater number of extracts are active against the gram-positive bacteria (McCutcheon et al., 1992). The extracts were also generally more active against the filamentous fungi than the yeast. Another general observation made was that bark extracts were more inhibitory to both bacteria and fungi strains tested than the wood extracts, inferring that the bark being the outer most protective part of the tree is well defended against microbial attack.

Table 3.4 summarizes the uses of the tree species by First Nations peoples in Eastern Canada. All of the fourteen hardwood species analyzed have four or more medicinal uses. It is noteworthy that six of these nine species (67%) are sought for treatment of conditions associated with microbial infections (antimicrobial mentions) for over 40%

of their uses. Other categories of use include analgesic, blood and heart, general or tonics, liver, kidney, nervous system, reproductive, respiratory and other remedies. The ethnobotanical usage also revealed an interesting trend. Firstly, the bark extracts were categorized into one of the three groups: either "High Usage", "Medium Usage" or "Low Usage". These categories are based on whether the ethnobotanical information shows greater than eight antimicrobial mentions (n=6), from three to eight mentions (n=3) or less than three mentions (n=5).

Figure 3.1 shows the overall antibacterial and antifungal activity calculated by averaging the inhibition zones (in mm) from each of the growth tests in relation to the number of ethnobotanical antimicrobial mentions of the bark for that species. Since only one species, *B. papyrifera*, has an ethnobotanical usage for the wood, a similar analysis of the type done here with bark could not be done with the wood extracts. It is apparent that those bark extracts from the high usage group display more antimicrobial activity than those of the medium and low usage groups. The medium usage group also shows more antimicrobial activity than the low usage group. Also evident is that the highest usage of hardwood bark, that of *P. serotina* does not yield the highest antimicrobial activity. Nor does the second highest, that of *U. americana*. It is the third highest ethnobotanically mentioned hardwood bark that of *J. cinerea* which proved to be the most potent overall inhibitor of the microbes tested.

There may be other factors as to why *J. cinerea* is not the most frequently used for antimicrobial purposes by First Nation's peoples. Interestingly, the two barks least preferred for antimicrobial uses, *F. pennsylvanica* and *P. grandidentata* (with zero antimicrobial usage mentions each) did prove to have the lowest antimicrobial activity.

If the usage groups are combined, a clearer pattern emerges (Figure 3.2). This figure demonstrates that the selection of plants for antimicrobial purposes by First Nations peoples may be correlated with antibacterial and antifungal activity. The traditional medicinal knowledge acquired by these peoples shows an understanding of species and plant parts that can be used for conditions associated with microbial infection. In both the bacteria and fungi tested, the preferred plant parts and preferred species used exhibited greater antimicrobial activities than less preferred sources. These results suggest that indigenous knowledge encompasses some understanding of the chemical ecology of plants.

In recent years there has been an increase in incidence of antibiotic resistance in these pathogenic organisms and the persistence of pathogens in immune-compromised individuals is of great concern (Georgopapadakou and Walsh, 1994). While only a few of these plant extracts may yield pure substances appropriate for the commercial pharmaceutical stream, some relatively unrefined materials may be considered for development as phytomedicines which can be registered in

Canada as traditional medicines. The presence of these materials in trees suggests that there are abundant sources of antifungal and antibacterial agents in forests which can be harvested at modest cost on a very large scale and may be useful in other industrial and agricultural antimicrobial applications.

Table 3.1. Effects of bark extracts on bacteria tested (inhibition zones in mm ± S.E.)
S.a.m.s: Staphylococcus aureus methicillin sensitive, B.s.: Bacillus subtilus, M.p.: Mycobacterium phlei, E.f.: Enterococcus faecalis. E.c.w.: Escherichia coli wild, P.a. 187: Pseudomonas aeruginosa, S.t.: Salmonella typhimurium, K.p.: Klebsiella pneumoniae. Letter a denotes significantly different light enhanced activity ($P < 0.05$, Student's t-test comparison)

Species	*S.a.m.s* -UV	*S.a.m.s* +UV	*B.s.* -UV	*B.s.* +UV	*M.p.* -UV	*M.p.* +UV	*E.f.* -UV	*E.f.* +UV
A. rubrum	10.5 ± 0.7	13.5 ± 0.7	10.0 ± 0.0	8.0 ± 0.0	14.0 ± 2.8	18.5 ± 6.3	8.5 ± 0.7	8.0 ± 0.0
A. saccharum	10.5 ± 0.7	11.5 ± 2.1	9.5 ± 0.7	10.0 ± 0.0	10.5 ± 0.7	12.5 ± 3.5	---	---
B. papyrifera	10.0 ± 0.0	13.5 ± 1.0	10.0 ± 0.0	10.0 ± 0.0	9.5 ± 0.7	11.0 ± 0.0	8.5 ± 0.7	9.0 ± 1.0
C. cordiformis	9.5 ± 0.7	9.5 ± 0.7	---	8.5 ± 0.0	9.0 ± 0.0	10.0 ± 0.0	---	---
C. ovata	9.5 ± 0.7	10.0 ± 1.4	---	---	13.0 ± 2.8	17.5 ± 0.0	---	---
F. grandifolia	9.5 ± 0.7	11.0 ± 1.4	9.0 ± 0.0	9.0 ± 0.0	---	---	---	---
F. pennsylvanica	---	---	---	----	---	---	---	---
J. cinerea	15.6 ± 2.5	22.5 ± 2.1	10.5 ±2.1	11.5 ± 0.0	14.5 ± 0.7	14.0 ± 2.1	---	---
P. serotina	9.0 ± 0.0	10.5 ± 0.7	8.5 ± 0.7	9.0 ± 0.7	---	---	---	---
P. grandidentata	---	---	---	---	9.5 ± 2.1	12.0 ± 5.6	---	---
Populus sp.	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	8.5 ± 0.0	9.0 ± 1.4	11.0 ± 0.0	---	---
Q. rubra	11.5 ± 2.1	15.3 ± 1.5	8.0 ± 0.0	8.0 ± 0.0	11.5 ± 1.4	11.0 ± 0.0	---	---
T. Americana	10.0 ± 0.0	11.0 ± 2.8	---	---	9.5 ± 0.7	10.5 ± 0.7	---	---
U. Americana	10.0 ± 0.0	10.5 ± 0.7	---	9.0 ± 0.0	10.0 ± 0.0	18.0 ± 1.4	---	---
Gentamycin	24.5 ± 0.7	26.0 ± 0.0	23.0 ± 1.2	24.0 ± 0.7	25.0 ± 1.6	29.0 ± 0.7	18.5 ± 1.4	20.0 ± 0.7
Methoxypsolaren	---	11.0 ± 2.0	---	11.0 ± 3.0	---	11.2 ± 2.2	---	12.0 ± 3.1

Species	*E.c.w* -UV	*E.c.w* +UV	*P.a.187* -UV	*P.a.187* +UV	*S.t.* -UV	*S.t..* +UV	*K.p.* -UV	*K.p.* +UV
A. rubrum	---	9.5 ±0.7a	---	---	---	---	---	---
A. saccharum	---	---	---	---	---	---	---	---
B. papyrifera	---	---	---	---	---	---	---	---
C. cordiformis	---	---	---	---	---	---	---	---
C. ovata	---	---	---	---	---	---	---	---
F. grandifolia	---	---	---	---	---	---	---	---
F. pennsylvanica	---	---	---	---	---	---	---	---
J. cinerea	---	9.5 ± 2.2	9.5 ± 2.2	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	---	8.0 ± 0.0
P. serotina	---	---	---	---	---	---	---	---
P. grandidentata	---	---	---	---	---	---	---	---
Populus sp.	---	---	---	---	---	---	---	---
Q. rubra	---	---	---	---	---	---	---	---
T. americana	---	---	---	---	---	---	---	---
U. americana	---	---	---	---	---	---	---	---
Gentamycin	18.5 ± 1.5	20.0 ± 0.0	19.5 ± 0.5	20.0 ± 2.0	21.0 ± 0.5	21.5 ± 0.5	17.0 ± 0.0	20.5 ± 0.5
Methoxypsolaren	---	---	---	---	---	---	---	---

Table 3.2. Effect of bark extracts on fungi tested (inhibition zones in mm ± S.E.)
T.m.: Trichophyton mentagrophytes, M.g.: Microsporum gypseum, A.f.: Aspergillus fumigatus, C.a.: Candida albicans, C.n.: Cryptococcus neoformans, S.c.: Saccharomyces cerevisiae
Letter a denotes significantly different light enhanced activity ($P < 0.05$, Student's t-test comparison)

Species	*T.m.* -UV	*T.m.* +UV	*M.g.* -UV	*M.g.* +UV	*A.f.* -UV	*A.f.* +UV
A. rubrum	11.0±0.0	11.0±0.0	11.5 ± 0.0	11.6 ± 1.1	---	
A. saccharum	---	---	--	12.3 ± 2.3a	---	---
B. papyrifera	---	---	---	---	---	---
C. cordiformis	---	---	---	---	---	---
C. ovata	---	---	---	---	---	---
F. grandifolia	---	---	---	---	---	---
F. pennsylvanica	---	---	---	---	---	---
J. cinerea	16.3 ± 1.1	17.0 ± 0.0	22.3 ± 0.0	23.6 ± 0.0	13.0 ± 0.0	11.6 ± 1.1
P. serotina	---	---	11.5 ± 0.0	10.8 ± 1.2---	---	---
P. grandidentata	---	---	---	---	---	---
Populus sp.	---	---	---	---	---	---
Q. rubra	11.5 ± 0.0	9.5 ± 0.0	11.5 ± 0.0	11.5 ± 0.0		---
T. americana	---	---	---	---	---	---
U. americana	---	---	---	---	---	---
Berberine	24.2 ± 5.0	24.8 ± 4.6	18.8 ± 1.2	24.8 ± 1.2	10.2 ± 1.2	10.2 ± 1.2

Species	*C.a.* -UV	*C.a.* +UV	*C.n.* -UV	*C.n.* +UV	*S.c.* -UV	*S.c.* +UV
A. rubrum	---	---	---	---	---	---
A. saccharum	---	---	---	---	---	---
B. papyrifera	---	---	---	---	---	---
C. cordiformis	---	---	---	---	---	---
C. ovata	---	---	---	---	---	---
F. grandifolia	---	---	---	---	---	---
F. pennsylvanica	---	---	---	---	---	---
J. cinerea	---	---	---	---	---	---
P. serotina	---	---	---	---	---	---
P. grandidentata	---	---	---	---	---	---
Populus sp.	---	---	---	---	---	
Q. rubra	---	---	---	---	---	11.5 ± 0.0a
T. americana	---	---	---	---	---	
U. americana	---	---	---	---	---	
Berberine	24.2 ± 5.7	22.0 ± 2.7	25.0 ± 8.7	31.0 ± 1.0	17.5 ± 0.0	25.0 ± 0.0a

Table 3.3. Effects of wood extracts on bacteria tested (inhibition zones in mm ± SE.)
S.a.m.s: Staphylococcus aureus methicillin sensitive, B.s.: Bacillus subtilus, M.p.: Mycobacterium phlei, S.f.: Streptococcus faecalis. Letter a denotes significantly different light enhanced activity ($P < 0.05$, t-test comparison). The extracts were not active on the following species: *Eschericia coli wild, Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae and Enterococcus faecalis*

Species	*S.a.m.s* -UV	*S.a.m.s* +UV	*B.s.* -UV	*B.s.* +UV	*M.p.* -UV	*M.p.* +UV	*E.f.* -UV	*E.f.* +UV
A. rubrum	12.0 ± 0.0	12.0 ± 0.0	8.5 ± 0.7	9.5 ± 0.7	---	15.3 ± 2.2	---	---
A. saccharum	8.8 ± 0.4	8.8 ± 0.4	---	---	---	---	---	---
B. papyrifera	8.5 ± 0.7	10.0 ± 0.0	---	---	---	---	---	---
C. cordiformis	8.5 ± 0.7	---	---	---	---	---	---	---
C. ovata	---	10.0 ± 0.0a	---	---	15.5 ± 0.7	19.0 ± 1.4	---	---
F. grandifolia	10.0 ± 0.0	11.5 ± 0.7	9.0 ± 1.4	9.0 ± 0.0	---	---	---	---
F. pennsylvanica	---	---	---	---	---	---	---	---
J. cinerea	13.5 ± 1.1	18.7 ± 5.5	9.5 ± 0.7	9.5 ± 0.7	16.5 ± 1.4	21.0 ± 1.4	---	---
P. serotina	9.0 ± 0.0	12.0 ± 1.4	9.5 ± 0.7	9.5 ± 0.7	---	---	---	---
P. grandidentata	9.5 ± 0.7	9.0 ± 0.0	---	---	13.5 ± 0.7	15.0 ± 2.1	---	---
Populus sp.	---	---	---	---	---	---	---	---
Q. rubra	15.3 ± 1.5	24.0 ± 5.3a	8.0 ± 0.0	---	---	---	---	---
T. americana	---	11.0 ± 2.0a	---	9.0 ± 0.0a		12.5 ± 3.5a		
U. americana	---	---	---	---	---	---	---	---
Gentamycin	24.5 ± 0.7	26.0 ± 0.0	23.0 ± 1.2	24.0 ± 0.7	25.0 ± 1.6	29.0 ± 0.6	18.5 ± 1.4	20.0 ± 0.7
Methoxypsolaren	---	11.0 ± 2.0a	---	11.0 ± 3.0a	---	11.2 ± 2.2a	---	12.0 ± 3.1a

Table 3.4. Use of hardwood trees for medical purposes by the First Nations People (Arnason et al., 1981)

Species	No.of antimicrobial bark usage mentions	Total no. of bark usage mentions	No.of antimicrobial wood usage mentions	Total no. of wood usage mentions	No. of antimicrobial all parts usage mentions	Total no. of all parts usage mentions
A. rubrum	8	16	0	0	8	17
A. saccharum	2	6	0	0	2	8
B. papyrifera	9	20	3	6	12	28
C. cordiformis	1	3	0	0	1	4
C. ovata	1	5	0	0	1	8
F. grandifolia	3	9	0	0	7	15
F. pennsylvanica	0	3	0	0	0	4
J. cinerea	10	32	0	0	11	34
P. serotina	48	74	0	0	55	90
P. grandidentata	0	4	0	0	0	5
Populus sp.	5	13	0	0	5	14
Q. rubra	4	7	0	0	4	7
T. americana	9	24	0	0	12	30
U. americana	16	28	0	0	16	28

Figure 3.1. The number of antimicrobial usage mentions of hardwood barks and antimicrobial activities (mm inhibition zone) extracts of their bark

High usage: 1: *A. rubrum*; 2: *B. papyrifera*; 3*: J. cinerea*; 4: *P. serotina*; 5*: T. americana*; 6: *U. americana*; Medium usage: 7: *F. grandifolia*; 8: *Populus* sp. 9: *Q. rubra*; Low usage: *10: A. saccharum; 11:C.cordiformis;12:Q.rubra 13: F. pennsylvanica; 14: P. grandidentata*

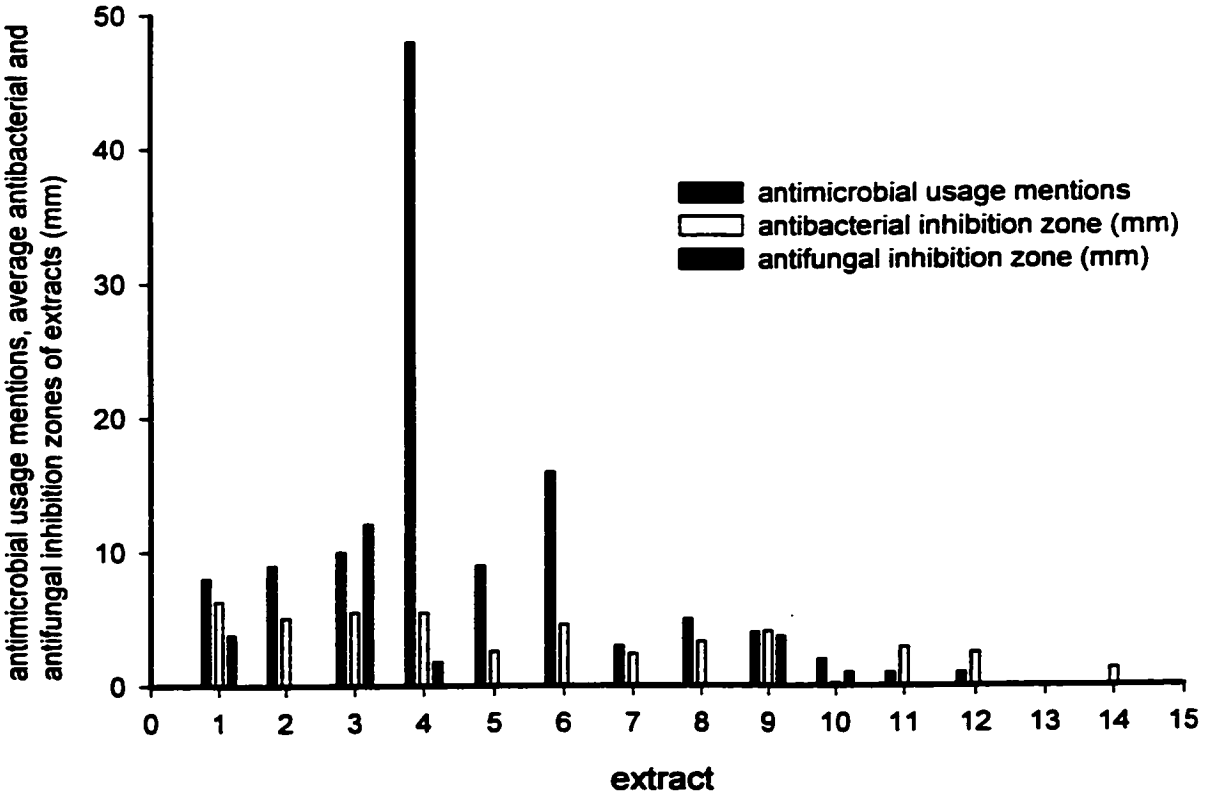
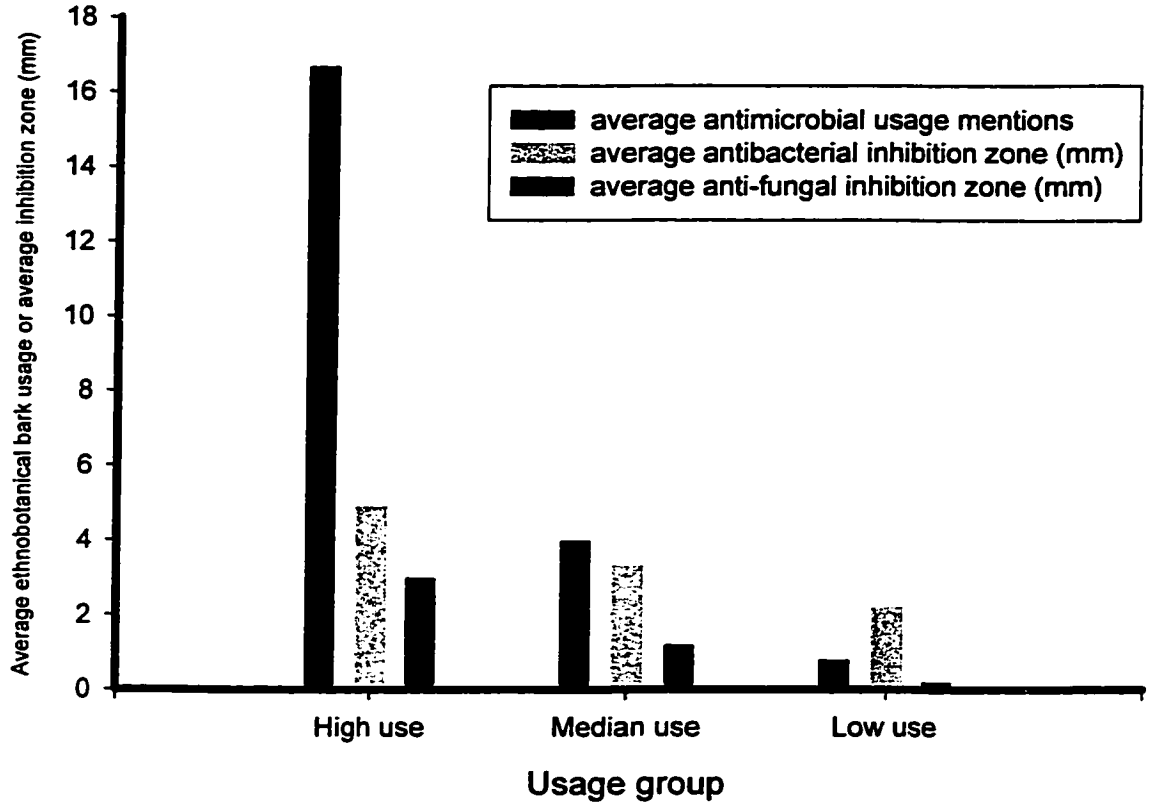

antimicrobial usage mentions, average antibacterial and antifungal inhibition zones of extracts (mm)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
extract
antimicrobial usage mentions
antibacterial inhibition zone (mm)
antifungal inhibition zone (mm)

Figure 3.2. The number of antimicrobial usage mentions of hardwood bark and the average antibacterial and antifungal inhibition zones (mm) of the bark extracts.


Average ethnobotanical bark usage or average inhibition zone (mm)
18
16
14
12
10
8
6
4
2
0
average antimicrobial usage mentions
average antibacterial inhibition zone (mm)
average anti-fungal inhibition zone (mm)
High use
Median use
Low use
Usage group

Chapter 4. Isolation of bitter principles from *Lansium domesticum* and antifeedant activity of terpenoids from tropical plants

4.1 Introduction

Tropical trees of the order Rutales are well known for the production of bioactive terpenoids (Arnason et al., 1993; Isman et al., 1996). In particular the plant family Meliaceae is noted for the production of useful bitter principles which are insect antifeedant and growth reducing substances with low mammalian toxicity (Butterworth and Morgan, 1968; Arnason et al., 1985; Schmutterer, 1995). New bioactive terpenoids isolated from the tropical collection in our laboratory include *Cedrela odorata* and *Ruptilocarpon caracolito* (MacKinnon et al., 1995), and *Swietenia humilis* (Jimenez et al., 1997).

Lansium domesticum Corr. Serr. (Meliaceae) is a tree native to Southeast Asia producing a sweet and aromatic fruit, hence a popular dessert (Wong et al., 1994). The leaves have been used by indigenous people in the Philippines for the control of mosquitoes (Monzon et al., 1994). The peel of this fruit is traditionally known to be toxic to domestic animals. Phytochemical investigations of the peel revealed the presence of triterpene glycosides, and seco-onoceranoids such as lansic acid (Nishizawa et al., 1983). The volatile constitutents of the fruit are sesquiterpene hydrocarbons including germacrene-D (Wong et al., 1994).

The seed and leaf contain tetranortriterpenoids named dukunolides (Nishizawa et al., 1985; Nishizawa et al., 1989). The major triterpenoid compound of the leaf is lansiolic acid and the minor triterpene is characterized as cycloartanoid type carboxylic acid. Some chemical derivatives of these compounds were found to be inhibitors of skin-tumor promoters (Nishizawa et al., 1989).

The bark is used traditionally as an antimalarial remedy by the native people of Borneo (Leaman et al., 1995). Despite the importance of this use, there appears to be no published phytochemical investigation on the bark. In this chapter, isolation of compounds from the bark of *Lansium domesticum* is reported using a *Sitophilus oryzae* L. antifeedant bioassay. Furthermore, the antifeedant activities of related triterpenes isolated recently including humilinolides, C-D spirocaracolitones, and gedunin are also described.

4.2 Material and Methods

4.2.1 Phytochemical isolation

Lansium domesticum (bark) was collected in the Apo Kayan Region, Indonesia by D. Leaman. Voucher specimens were authenticated and deposited at the Herbarium of the University of Ottawa, Ottawa, Canada. The isolation and identification of the compounds were

conducted by Dr. J. Zhang at Department of Chemistry, University of Ottawa, Ottawa, ON.

The bark was finely ground and the sawdust (300 g) was soaked in 0.6 L of 95 % ethanol for two days. The extract was then filtered and dried under vacuum to yield 40 g. Hexane soluble products were removed by the addition of 2 L of 50% hexane and water. The aqueous fraction was then extracted three times with 500 mL of ethyl acetate. The solvents of the hexane, and ethyl acetate fractions were evaporated under vacuum and the water extract was freeze-dried.

The hexane fraction (8 g) was subjected to column chromatography on silica gel (200 g) using 10% ethyl acetate in hexane as eluent. The first three fractions provided less polar materials, namely: fraction A: 0.20 g; fraction B: 0.80 g; and fraction C: 0.50 g.

The ethyl acetate extract (20 g) was subjected to column chromatography on silica gel (400 g) with 10% ethyl acetate in hexane as the first eluent and fraction D (0.60 g) was eluted. The eluents were increased to higher polarity and the more polar materials were eluted as follows: With 25% ethyl acetate:hexane, fraction E (1.50 g); fraction F (0.70 g); and fraction G (0.50 g) were eluted. Fraction H (2.50 g) was eluted with 50% of ethyl acetate-hexane; and fraction I (2.50 g) with 100% of ethyl acetate. Thin Layer chromatography investigation showed that these fractions were mixtures and were susbsequently re-

chromatographed in order to obtain pure compounds as shown in the subsequent section.

Fraction D (0.60 g) was re-subjected to silica gel column chromatography (20 g, 10% of ethyl acetate-hexane) afforded compound II. Fraction F (0.30 g) was re-fractionated via silica gel column chromatography (20 g, 25% of ethyl acetate-hexane) to obtain compound III. Fraction H (2.5 g) was subjected to silica gel column (80 g, 25 % of ethyl acetate-hexane) and compound IV was obtained. No pure compounds could be isolated from fractions E and G.

Fraction I (2.5 g) was dissolved in 20 mL of anhydrous ether at 0 °C and added to an ethereal solution of diazomethane in small portions until gas evolution ceased and the solution acquired a pale yellow color. The solvent was evaporated and the residues were subjected on a silica gel (100 g, 25% of ethyl acetate in hexane). Two methyl esters namely, compounds V and VII were obtained. Hydrolysis of these esters gave compounds VI and VIII respectively.

Acetylation of compound VII was conducted by dissolving 40 mg of the compound in dichloromethane and added to two equivalents of acetic anhydrate, four equivalents of pyridine and a catalytic amount of DMAP. The mixture was stirred at room temperature for 4 hours and then washed with distilled water. The organic phase was dried over $MgSO_4$. After distilling off the solvent, the residues were subjected to column

chromatography (10 g of silica gel, 25% ethyl acetate in hexane), and a colorless product (compound IX) was obtained. NMR spectra was obtained with 500 MHz Bruker spectrometer and mass spectra using VG 7070E instrument.

Gedunin was isolated from the wood of *Cedrela odorata* L. as previously described (MacKinnon et al., 1997), C-D spirocaracolitones were purified from the seeds of *Ruptiliocarpon caracolito* Hammel Zamora (MacKinnon et al., 1995) and the humilinolides were isolated from *Swietenia humilis* Zuccarini (Jimenez et al., 1997).

4.2.2 *Sitophilus oryzae* bioassay

As per methods described in Chapter 3.2

4.3 Results and discussion

4.3.1 Compound isolation

From the hexane fraction, three pure compounds were isolated. Fraction A (0.20 g) was a colorless oil ($C_{30}H_{48}$: MW: 408). This compound was previously unknown and based on its onoceranoid backbone, it was named as iso-onoceratriene. Fraction B (0.80 g) was identified as naphthalene (MW: 128), fraction C (0.50 g) was identified as 1,2-benzenediboxylic acid, bis (2-ethylhexyl) ester MW: 390. Fractions B and

C were exogenous compounds introduced during the plant preservation and extraction processes respectively.

Fraction D (0.60 g) yielded 300 mg of yellow oil ($C_{30}H_{46}O_2$, MW: 438) as its major constituent. Its spectroscopic properties (^1H, ^{13}C NMR and MS) matched those of onoceradienedione previously isolated from the peel of the fruit by Hayashi et al. (1982). Fraction F was chromatographically pure compound identified as $C_{30}H_{48}O_2$, MW: 440. Fraction H (2.5 g) was isolated as an amorphous solid ($C_{30}H_{46}O_3$, MW: 454). These compounds were shown by NMR spectoroscopy to have ketone function at C-3, hence were named 3-keto-22-hydroxyonoceradiene and 3-ketolansiolic acid respectivelyFraction I (2.5 g) gave two methyl esters: 200 mg methyl lansiolate ($C_{31}H_{50}O_3$, MW: 470) and 100 mg of methy lansiolate A ($C_{31}H_{48}O_4$, MW: 484). Methyl lansiolate was hydrolyzed to give lansiolic acid ($C_{30}H_{48}O_3$, MW: 456) and methyl lansiolate A gave a new compound lansiolic acid A ($C_{30}H_{46}O_4$, MW: 470). Acetylation of methyl lansiolate also gave a new compound, methyl 15-acetoxylansiolate A ($C_{32}H_{50}O_5$: MW: 514). Methyl lansiolate and lansiolic acid were isolated earlier from the peel of the fruit (Nishizawa et al., 1985).

The structures of the triterpenoids are depicted in Figure 4.2. The ^1H and ^{13}C NMR assignments of the six new compounds are tabulated in Appedices II and III respectively.

4.3.2 Bioassay with *Sitophilus oryzae*

The results of feeding deterrence studies against *S. oryzae* by the crude extracts and six pure compounds isolated from *L. domesticum* are summarized in Table 4.1. Flour disks prepared using the crude extracts (ethyl acetate and hexane) exhibited total inhibition of diet consumption at 0.5% (w/w) but the water extract was found to be phagostimulatory. Five compounds isolated from the ethyl acetate fraction namely, iso-onoceratriene, 3-keto-22-hydroxyonoceradiene, onoceradienedione, lansiolic acid and lansiolic acid A exhibited significant antifeedant activities at 0.5% (w/w); however, 3-ketolansiolic acid was not active at this concentration. No clear structure activity relationships were evident in this study. In general, triterpenoids have been known to possess strong feeding deterrent activity (van Beek and de Groot, 1986). It was noted that the crude extracts were more active than the isolated compounds and this may be due to synergistic effects of the compounds (Xie et al., 1996) or the presence of other unidentified active compounds. However, the concentration used to observe antifeedant effects was much higher than for the commercially available products such as Margosan-O, active at a 3.75 ppm azadirachtin level (Xie et al., 1996) or toosendanin at 20 ppm (Champagne et al., 1992). Although the commercial application as a stored-product antifeedant may not be practical, the bioassay employed here was useful in isolating bitter compounds present at low

concentrations. Other secondary compounds isolated from tropical sources (Liche xanthone, Formyl – orcinolcarboxyl, methyl orsellinate, gyrophoric acid) were inactive when tested with this bioassay (data not shown).

The chemical structures of other triterpenes isolated from the Rutales are shown (Figure 4.3). The C-D spirocaracolitones B, D and E isolated from *R. carocolito* caused a total feeding inhibition at 0.5% (w/w) and were significantly active at 0.25 % (w/w) (Table 4.2). Spirocaracolitone B and D also showed significantly different activity at 0.05%. Other studies also revealed that these compounds were very active when tested against European corn borer (MacKinnon, 1995) and showed modest antifungal and antimalarial activities (MacKinnon et al., 1997).

The main active compound in the *C. odorata* wood, gedunin, had previously shown moderate antifeedant activity against many insect species (Arnason et al., 1987; Kubo and Klocke, 1986; Champagne et al., 1992). Humilinolides isolated from *Swietenia humilis* showed significant growth reducing activity including delay in time of pupation and adult emergence against *O. nubilalis* (Jimenez et al., 1997). In this study, gedunin, humilinolide C and D were active but humilinolide B was inactive at 0.5% (w/w) (Figure 4.5). The most active compound was humilinolide C. A similar spectrum of activity was noted with *O. nubilalis;* humilinolide C

exhibiting the highest growth reducing activity (Jimenez et al.; 1997). These studies suggested the mode of action of these compound to be a combination of antifeedant action and post-digestive toxicity as seen in other limonoids (Isman et al., 1995; Xie et al., 1995).

Kubo and Nakanishi (1978) indicated that most antifeedant compounds they had isolated exhibited pharmacological activities as well and suggested that the antifeedant bioassay has unexpectedly provided them with a unique system for screening bioactive compounds. Studies conducted in our laboratory have demonstrated that the extracts from *L. domesticum* and *C. odorata* exhibited *in vitro* antiplasmodial activities (Leaman et al., 1995; MacKinnon et al., 1997). *In vitro* and *in vivo* pharmacological activity studies of some of the triterpenoids described here will be the subject of the next two chapters (5 and 6).

Figure 4.1 *Lansium domesticum* tree and fruit (Borneo, Indonesia)

1. *Lansium domesticum* bark

2. *Lansium domesticum* fruit

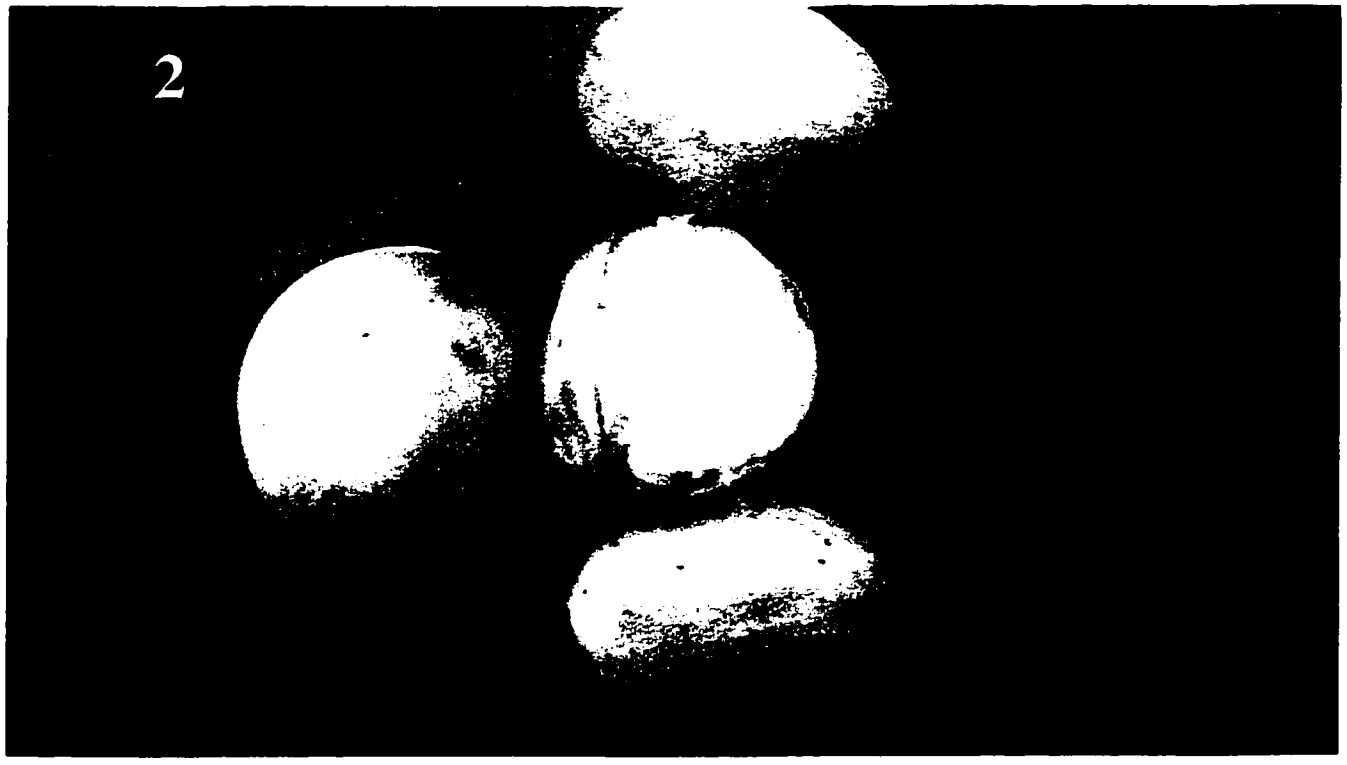


Figure 4.2 Flowchart for the isolation of compounds from *Lansium domesticum*

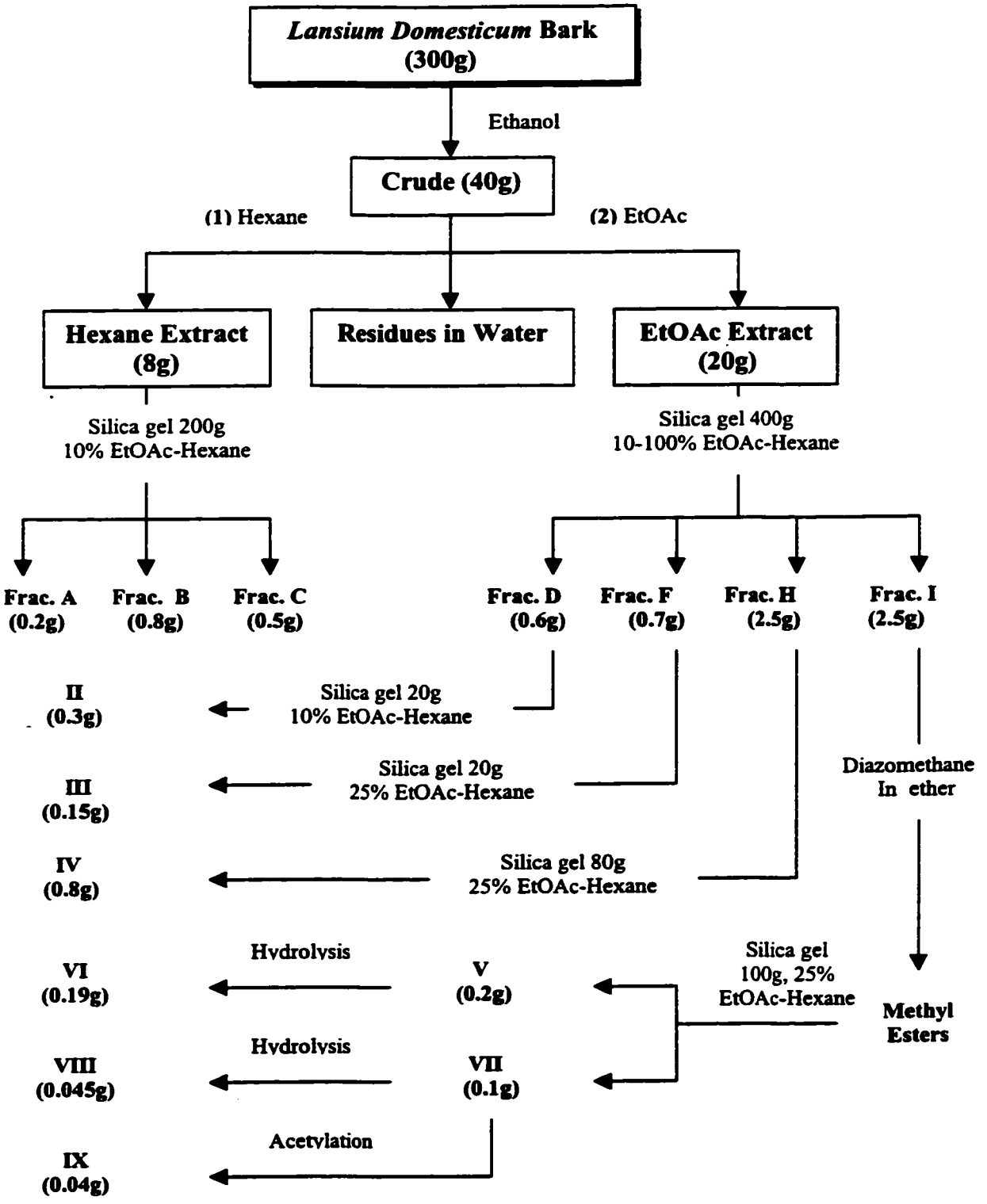
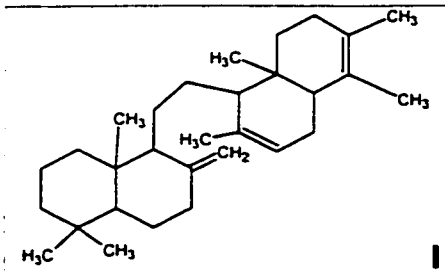
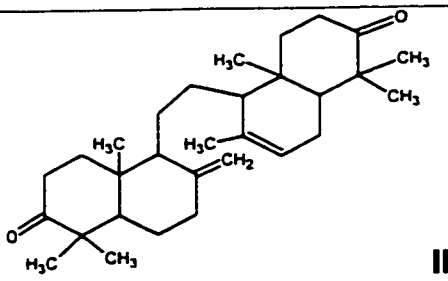

Lansium Domesticum Bark
(300g)
Ethanol
Crude (40g)
(1) Hexane
(2) EtOAc
Hexane Extract
(8g)
Residues in Water
EtOAc Extract
(20g)
Silica gel 200g
10% EtOAc-Hexane
Silica gel 400g
10-100% EtOAc-Hexane
Frac. A
(0.2g)
Frac. B
(0.8g)
Frac. C
(0.5g)
Frac. D
(0.6g)
Frac. F
(0.7g)
Frac. H
(2.5g)
Frac. I
(2.5g)
II
(0.3g)
Silica gel 20g
10% EtOAc-Hexane
III
(0.15g)
Silica gel 20g
25% EtOAc-Hexane
Diazomethane
In ether
IV
(0.8g)
Silica gel 80g
25% EtOAc-Hexane
VI
(0.19g)
Hydrolysis
V
(0.2g)
Silica gel
100g, 25%
EtOAc-Hexane
Methyl
Esters
VIII
(0.045g)
Hydrolysis
VII
(0.1g)
IX
(0.04g)
Acetylation

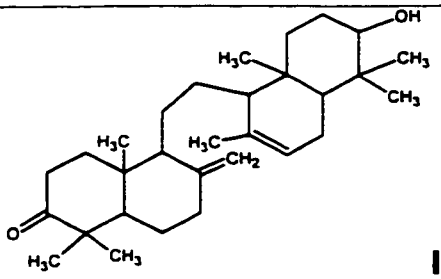
Figure 4.3 Compounds isolated from *Lansium domesticum*
I:onoceratriene, II:Onoceradienedione; III: 3-keto-22-hydroxyonoceradiene; IV: 3-ketolansiolic acid; V: Lansiolic acid; VI: Lansiolic acid A; VII: Methyl lansiolate; VIII: Methyl lansiolate A; IX: Methyl 15-acetoxylansiolate A.



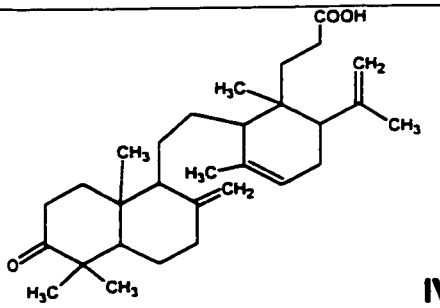
I



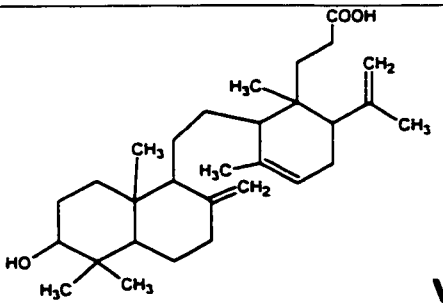
II



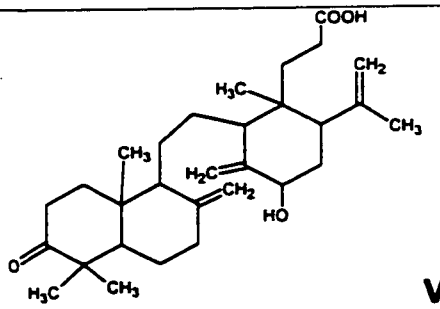
III



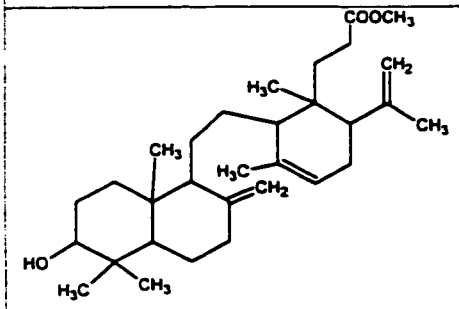
IV



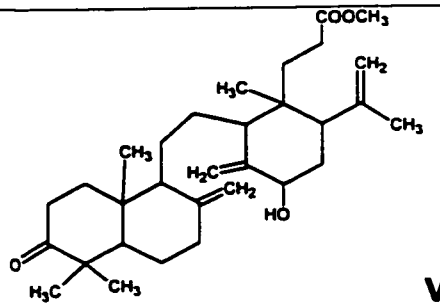
V



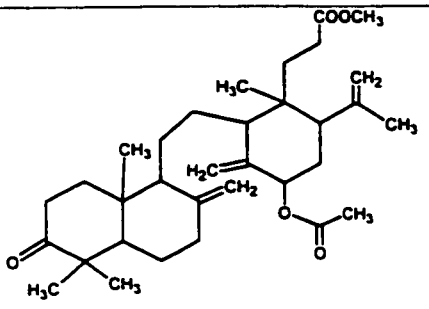
VI



VII



VIII



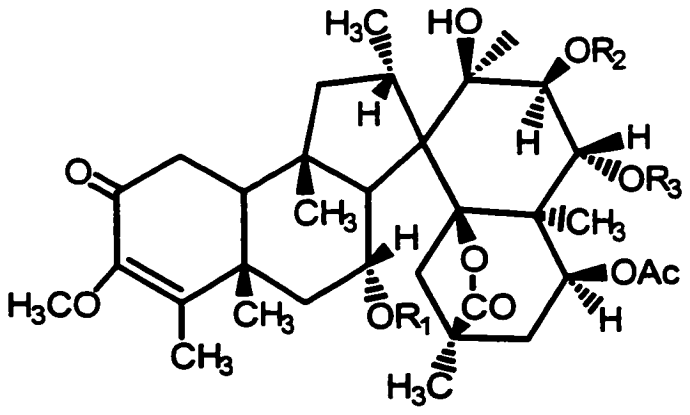
IX

Table 4.1 Antifeedant activities of crude extracts and pure compounds isolated from *Lansium domesticum* against *Sitophilus oryzae* (0.5% w/w)

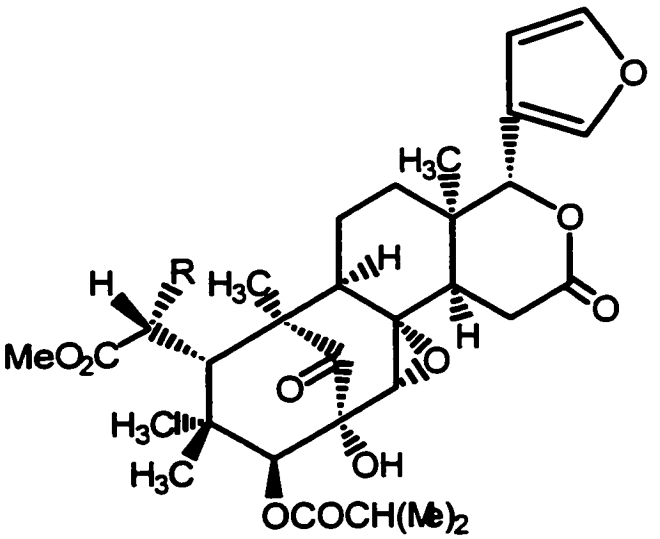
Treatment	Consumption of diet (% control ± SEM)	P value
Control	100.0 ± 10.2a	
Ethyl acetate fraction	0.0 b	P <0.001
Hexane fraction	0.0 b	P < 0.001
Water fraction	184.6 ± 29.1c	P < 0.001
Onoceradienedione	40.1 ± 6.2d	P < 0.001
Iso-onoceratriene	64.7 ± 7.5d	P = 0.03
3-keto-22-hydroxyonoceradiene	53.8 ± 3.4d	P < 0.01
Lansiolic acid	63.2 ± 3.8d	P < 0.01
3-ketolansiolic acid	68.9 ± 17.4a,d	P = 0.09
Lansiolic acid A	56.1 ± 4.5d	P < 0.01

Multiple range test using Tukey's test ($P < 0.05$). Similar letters denote treatments not significantly different from each other.

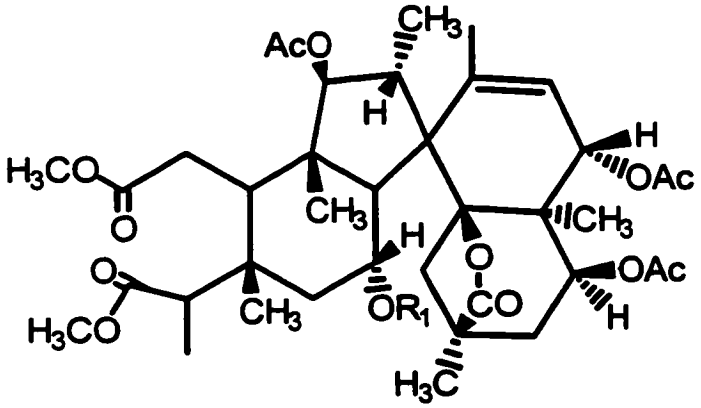
Figure 4.4 Structures of novel triterpenes isolated from tropical sources



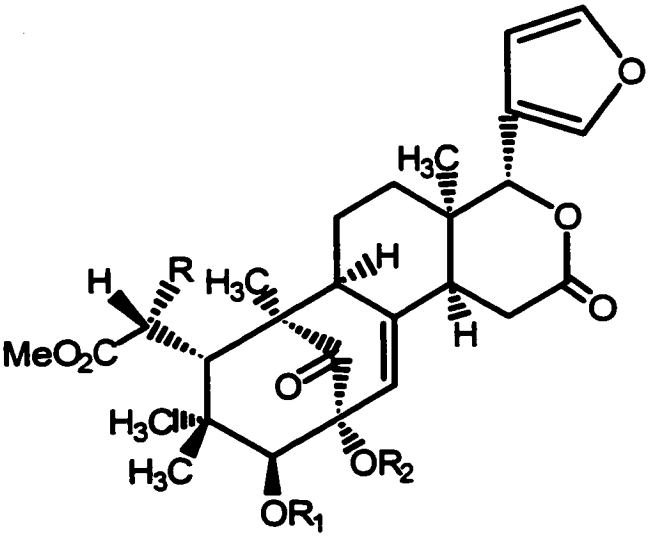
Spirocaracolitone B: $R_1=R_2=R_3=$ Ac



Humilinolide B: R= OH



Spirocaracolitone E: $R_1=$ Ac



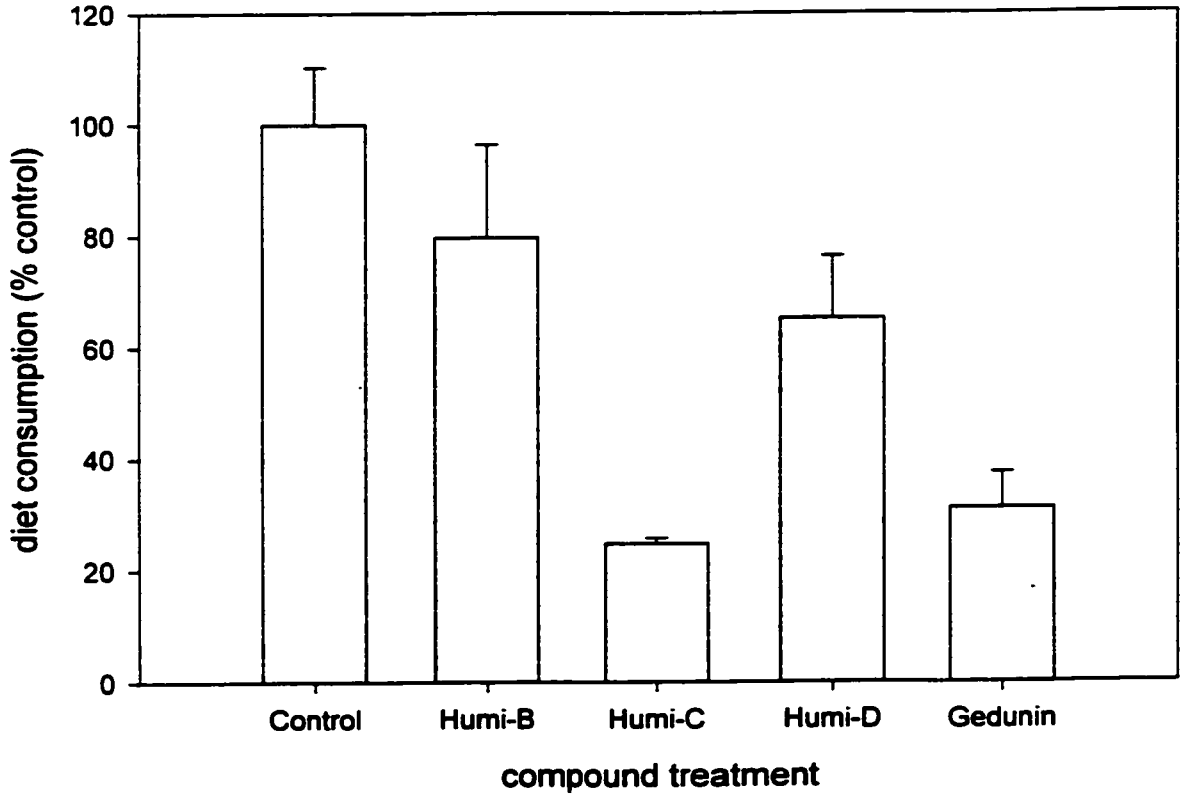
Humilinolide C: R=H, R_1=COC(Me)=CHMe, $R_2=$ Ac
Humilinolide D: R = OAc, R_1=Ac, R_2=H

Table 4.2 Antifeedant activity of Spirocaracolitones against *S. oryzae*

Compound	Concentration (% w/w)	Consumption of diet (% control ± SEM)	P value
Control	0	100.0 ± 8.5a	
Spirocaracolitone B	0.05	67. 1 ± 6.1b	P= 0.03
	0.25	8.8 ± 3.9 c	P < 0.001
	0.50	None	P < 0.001
Spirocaracolitone D	0.05	74.6 ± 6.3 b	P = 0.05
	0.25	2.9 ± 1.3 c	P < 0.001
	0.50	None	P < 0.001
Spirocaracolitone E	0.05	78.3 ± 10.1a,b	P = 0.08
	0.25	19.0 ± 9.9 d	P = 0.001
	0.50	None	P < 0.001

Multiple range test using Tukey's test ($P < 0.05$). Similar letters denote treatments not significantly different from each other.

Figure 4.5 Antifeedant activities of triterpenoids isolated from tropical plants (0.5% (w/w).
Humilinolide C, D and gedunin were significantly active at $P < 0.05$. Humilinolide B was not active.


120
100
80
60
40
20
0
diet consumption (% control)
Control
Humi-B
Humi-C
Humi-D
Gedunin
compound treatment

Chapter 5. Antiplasmodial and cytotoxic activities of crude extracts and pure compounds isolated from *Lansium domesticum*

5.1 Introduction

The discovery of highly effective antimalarial drugs such as quinine and artemisinin from plants has provided the impetus for the investigation of other compounds from natural sources (Phillipson and Wright, 1991a; Angerhofer et al., 1992). In an extensive ethnobotanical survey based on the degree of consensus among traditional healers of the Borneo region (Indonesia), Leaman et al., (1995) reported that the bark of *Lansium domesticum* (Meliaceae) was the most important plant derived antimalarial remedy in the area. Subsequently, crude extracts from this bark and several other traditional phytomedicines were tested for *in vitro* antiplasmodial and cytotoxic activities against chloroquine-sensitive *P. falciparum* clone (D6) and chloroquine-resistant clone (W2). The bark from *Lansium domesticum* was the most active with IC_{50} values below 10 μg/ mL (Leaman et al., 1995). Cytotoxicity was determined by measuring the viability of human epidermal carcinoma KB cells. The results suggested that the survival rate of the cells was high, indicating that the extracts exhibited selective antiplasmodial activity (Leaman et al., 1995).

Phytochemical identification of the compounds from the bark of *Lansium domesticum* revealed the presence of novel triterpenoid

compounds. In this chapter *in vitro* and *in vivo* antiplasmodial activities of crude extracts and nine triterpenoids from the bark *Lansium domesticum* are described.

5.2 Material and Methods

All extracts and pure compounds were prepared as described in Chapter 4.2.

5.2.1 *Plasmodium* Culture System

Cultures of *Plasmodium falciparum* (chloroquine-sensitive clone D6 and chloroquine-resistant clone W2) were maintained in human erythrocytes at Dr. J. Pezzuto's laboratory at the University of Illinois (Chicago) according to an established method (Milhous et al., 1995). Parasites were inoculated into type A+ human erythrocytes at a hematocrit of 6% in RPMI-1640 culture medium supplemented with 32 mM $NaHCO_3$, 25 mmol HEPES and 10% heat-inactivated human plasma type A+. Parasitemia was maintained below 4% under an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in 25 m^2 culture flasks at 37°C.

5.2.2 Antiplasmodium bioassay

The antiplasmodium activity of test compounds was assessed with an *in vitro* radioisotope-incorporation method described in Desjardin et al.,

(1979). A suspension (200 μL) of *P. falciparum*-infected red blood cells (0.5 - 1.0% parasitemia, 1.0% cell hematocrit) was added to each well of a standard 96-well tissue culture plate containing 25 μL of substance to be tested. Each test compound was dissolved in ethanol and assayed over a seven-point concentration range (n=3). In addition, the commonly used antimalarial drugs: quinine, chloroquine, mefloquine, and artemisinin were tested in each experiment over a seven-point concentration range. Microtiter plates were incubated for 24 hours at 37°C in a sealed chamber under an atmosphere of 5% CO_2, 5% O_2 and 90% of N_2. After this incubation period, 0.5 μCi of ^{3}H(G)-hypoxanthine (New England Nuclear Research Products, Boston, MA) was added to each well (25 μL of 20 μCi/ mL). The microtiter plate was returned to the sealed chamber for an additional 18 hours of incubation at 37°C. The assay was terminated by harvesting the contents of each microtiter plate onto a glass fiber filter using a Tomtec Mach III automatic cell harvester. Filters were dried and placed in polyethlene bags with 3.5 mL of biologically safe scintillation cocktail. Radioactivity was determined with a Wallac Microbeta liquid scintillation counter. Concentrations of test compounds and positive controls that inhibited parasite-specific incorporation of ^{3}H hypoxanthine by 50% (IC_{50}) were determined by nonlinear regression analysis. Zero-drug controls conducted equivalent amount of the carrier (ethanol) was defined 100% incorporation.

5.2.3 Cytotoxicity screening

KB cells were cultured in Dulbecco's Modified Eagles's Medium supplemented with 10% fetal bovine serum, and 10 mg/L of each penicillin, streptomycin, fungizone. The cells were kept at 37°C at 100% relative humidity with 5% CO_2 in air. Cells were typically grown to 60%-70% confluence; the medium was then changed and the cells were used for test procedures 1 day later. In each case, 96-well tissue culture plates were used. Test samples were initially dissolved in ethanol, and then diluted ten-fold with water. Serial dilutions were performed using 10% aqueous ethanol as the solvent and 5 X 10^4 cells (in 190 mL of media) were then added to the 96-well plates. Incubation was performed for 72 h at 37°C in a CO_2 incubator with the plates covered by vented plastic lids. After the incubation period, cells were fixed to the plastic substratum by the addition of 50 μL of cold 50% aqueous trichloroacetic acid (TCA). The TCA-fixed cells were then stained by the addition of 0.4% sulforhodamine B (w/v) dissolved in 1% acetic acid (30 minutes) and washed with 1% aqueous acetic acid (4X). The bound dye was solubilized by the addition of 10 mmol unbuffered Tris base, pH 10 (200 μL). The absorption was determined at 515 nm using an Enzyme Linked Immunosorbent assay (ELISA) plate reader. In each case, a zero control was performed by adding an equivalent number of wells of the 96-well plates and incubating at for 10 min at 37 °C. The cells were then fixed with TCA and processed

as described above. The absorbance values obtained with each of the treatment procedures were averaged, and the average value obtained with the zero-day control was subtracted. These values were then expressed as a percentage relative to the solvent-treated control incubation, and ED_{50} values were calculated using percent survival versus concentration.

5.2.4 Dose Preparation

All the crude extracts and the pure triterpenes were insoluble in water, therefore they were co-precipitated with a non-toxic polymer, polyvinyl pyrrolidone (PVP) according to Simonelli et al., (1968). The triterpenes were first dissolved in ethanol (5 mg/mL), sonicated and then added to PVP in ethanol (20 mg/mL). The ethanol was evaporated and the samples were freeze–dried. The co-precipitates were then dissolved in water to give a white, homogenous suspension easy to administer.

5.2.5 *In vivo* antimalarial assay

CD-1 male mice (30 ± 2 g) free of any parasite were obtained from Charles River Laboratories Inc., St. Constant, QC. The animals were housed two per cage at 22°C and 80% relative humidity, and were provided with a standard mouse diet and clean drinking water *ad libitum*.

The mice were first acclimatized for 5 days and then infected intraperitoneally by injecting with 0.2 mL of blood containing approximately 1 X 10^6 intra-erythrocytic parasites of ANKA strain *Plasmodium bergheii* (obtained from Walter Reed Army Hospital, Washington, DC). Daily wellness charts (body weight, dehydration, attitude and demeanor, piloerection, diarrhea, respiratory stress, appetite) were recorded by veterinary technicians at Animal Care Services, Faculty of Medicine, University of Ottawa, Ottawa, ON.

Each mouse was given daily doses of 50 mg/kg/day of the tritepenoids for three consecutive days. Control animals were given equivalent amounts of the vehicle (PVP) dissolved in water. All experiments were done using three animals per treatment. On day four, blood was drawn from the tails and thin films of blood were smeared onto slides and stained with Giemsa stain kit (Jorgesen Laboratories, Loveland, Co). Parasitemia levels were determined by counting the number of infected cells per 5000 erythrocytes (blind-study) under a compound microscope using oil immersion lens (100X). The suppression rate was calculated as follows:

$$\% \text{ suppression} = \frac{\#\text{ of infected cells (control)} - \#\text{ of infected cells (test)}}{\#\text{ of infected cells (control)}} \times 100\%$$

5.3 Results and Discussion

Of the thirteen plant-derived materials tested *in vitro* against the chloroquine sensitive (D6) and chloroquine resistant (W2) clones, one crude extract and five compounds exhibited antiplasmodial activity against both clones (Table 5.1). Methyl lansiolate, methyl lansiolate A and methyl 15-acetoxylansiolate A exhibited potent activity of < 1.0 μg/mL with both clones. When compared with standard drugs, methyl 15-acetoxylansiolate A demonstrated activity as potent as quinine (IC_{50} < 0.3 μg/mL). The crude ethyl acetate fraction, onoceradienedione, and 3-keto-22-hydroxynonoceradiene exhibited weaker activity (IC_{50} 1.66 – 5.61 μg/mL).

To further evaluate the antiplasmodial activity of these compounds, all samples were analyzed for cytotoxicity with human epidermal carcinoma (KB) cells. The choice of KB cells was based on the fact this cell line exhibited an intermediate sensitivity to a large number of cytotoxic agents when compared to a variety of other human tumours (Angerhofer et al., 1999). All thirteen samples tested were found to be non-toxic to these cells at a maximum dose of 20 μg/ mL. Furthermore, only six of the samples showed selective activity against the intra-erythrocytic malarial parasite without any host toxicity (Table 5.1). The selectivity index, defined as the ratio of cytotoxicity over antiplasmodial activity, was calculated in each case and was greater than 100 fold which is indicative of no toxicity.

Since *in vivo* testing of a drug encompasses a much broader spectrum of efficacy and toxicity than *in vitro* trials alone, animal testing was conducted using the compounds that showed potent *in vitro* antiplasmodial activities. When tested against *P. bergheii*-infected mice, onoceradienedion, methyl lansiolate and methyl 15-acetoxylansiolate (all at 50 mg/kg/day) showed a suppression of parasitemia levels of 20%, 25%, and 44% respectively (Figure 5.1). Quinine, tested as a positive control, at the maximum recommended dose of 10 mg/kg showed a parasitemia clearance of only 60%. The crude ethyl acetate fraction, 3-keto-22-hydroxyonoceradiene, methyl lansiolate A had no significant effect of parasite clearance upon these mice. It is presumed that the plant materials that exerted considerable *in vitro* activity but no *in vivo* activity may be explained by the fact that either they lacked bioavailability at the site of infection or underwent biotransformation to yield inactive components (Misra et al., 1991).

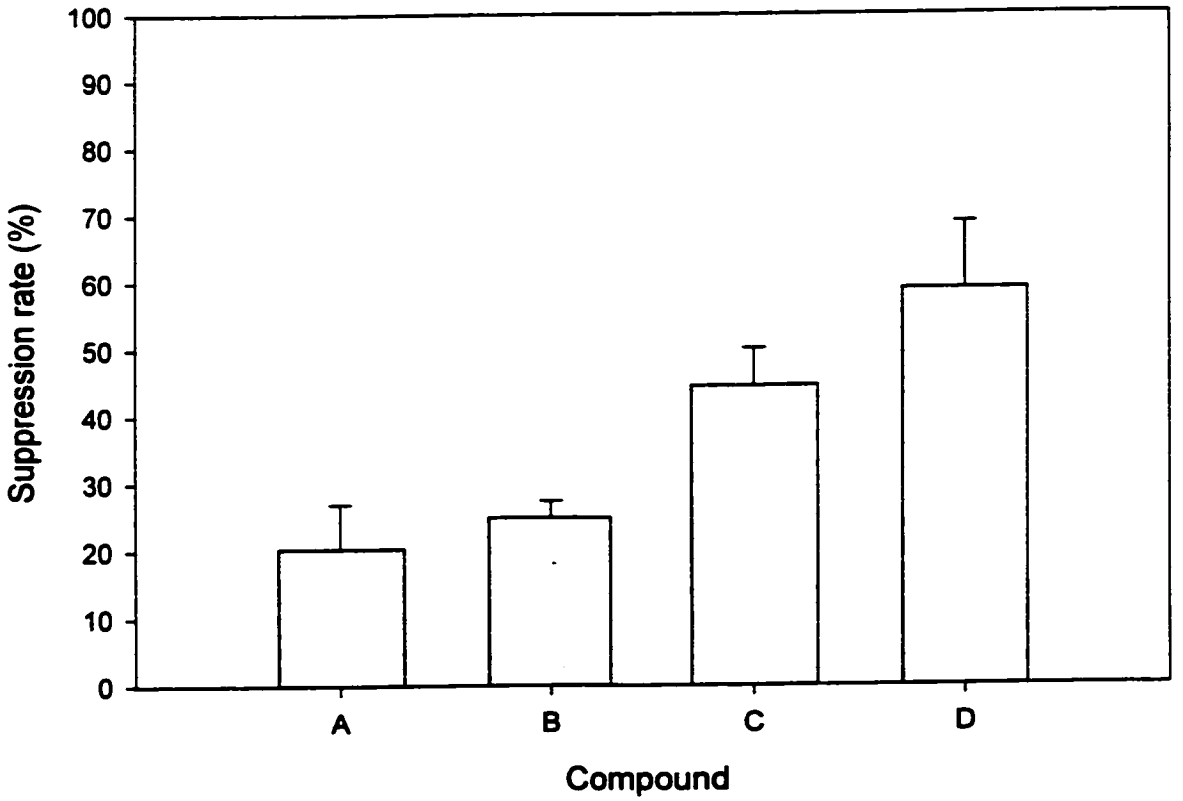
It was interesting to note that the derivatives were more active than the natural compounds. The inclusion of functional groups to the natural compounds enhanced the antiplasmodial activities. Particularly, the presence of an acetoxy group at C-15 position as seen in the methyl 15-acetoxylansiolate considerably enhanced both the *in vivo* and *in vitro* activities.

The suppression levels observed in this *in vivo* study did not show high rate of parasitemia suppression. However, the results obtained suggested that there is some rational basis for the use of the traditional medicine of Borneo from which these compounds were derived. For future work, other derivatives with different substitution of functional groups should be prepared and tested.

Table 5.1 Antiplasmodial and cytotoxicity of crude extracts and pure compounds isolated from the bark of *Lansium domesticum*

Treatment	D6 clone IC_{50} (µg / mL) ± SEM	W2 clone IC_{50} (µg/ mL) ± SEM	KB cells % survival ± SEM
Crude extract (water fraction)	>10	>10	154.3 ± 1.0
Crude extract (ethanol fraction)	>10	>10	149.5 ± 1.9
Crude extract (hexane fraction)	>10	>10	146.1 ± 0.6
Crude extract (ethylacetate fraction)	3.45 ± 0.81	5.61 ± 1.3	140.3 ± 4.2
Iso-onoceratriene	> 10	> 10	108.3 ± 4.1
3-ketolansiolic acid	> 10	> 10	129.1 ± 2.4
Lansiolic acid	> 10	> 10	116.1 ± 0.8
Lansiolic acid A	> 10	> 10	134.5 ± 3.5
3-keto-22-hydroxyonoceradiene	2.41 ± 0.52	> 10	113.9 ± 7.1
Onoceradienedione	1.66 ± 0.11	1.83 ± 0.38	131.5 ± 7.3
Methyl lansiolate	0.65 ± 0.02	0.76 ± 0.24	128.0 ± 2.6
Methyl lansiolate A	0.69 ± 0.05	1.02 ± 0.01	66.7 ± 3.2
Methyl 15-acetoxylansiolate A	0.17 ± 0.06	0.22 ± 0.13	116.0 ± 0.8
Quinine	0.27 ± 0.02	0.19 ± 0.07	Not tested
Artemisinin	0.0015 ± 0.0007	0.0035 ± 0.007	Not tested
Chloroquine	0.0045 ± 0.0021	0.065 ± 0.014	Not tested
Mefloquine	0.0055 ± 0.0007	0.023 ± 0.04	Not tested

Figure 5.1. Antimalarial activity of compounds isolated from *Lansium domesticum* tested against *P. bergheii* (± SEM)
A:Onoceradienedione (50 mg/kg); B: Methyl lansiolate (50 mg/kg); C: Methyl 15-acetoxylansiolate (50 mg/ kg); D: Quinine (10 mg/ kg).


100
90
80
70
60
50
40
30
20
10
0
Suppression rate (%)
A
B
C
D
Compound

Chapter 6. Antimalarial activities of gedunin,7-methoxygedunin and synergistic activity with dillapiol

6.1 INTRODUCTION

Plants from the Meliaceae family are extensively used as traditional remedies against malaria in Africa (Phillipson et al., 1991b; Khalid et al., 1986) and in South America (MacKinnon et al., 1997). Many of these plants produce bitter principles attributed to the presence of limonoid compounds (Champagne et al., 1992). Limonoids are modified triterpenes with a high level of oxidation and have a wide range of biological activities. Recently, MacKinnon et al. (1997) examined sixty extracts from 22 species of Meliaceae by characterizing their antimalarial activity *in vitro* against chloroquine sensitive (W2) and chloroquine resistant (D6) *Plasmodium falciparum* clones Twelve extracts were found to have activity including extracts of *Cedrela odorata* wood (Figure 6.1) and *Azadirachta indica* leaves both containing the limonoid compound, gedunin. Structure-activity studies using gedunin and its nine semisynthetic derivatives indicated gedunin was the most active substance and any modification of its naturally-occuring functional groups reduced antimalarial activity (MacKinnon et al., 1997).

When tested *in vitro* against both chloroquine sensitive (D6) and chloroquine resistant (W2) clones of *P. falciparum* gedunin demonstrated high activity with an IC_{50} of 0.039 and 0.029 μg/mL respectively

(MacKinnon et al., 1997). In fact, gedunin was more active than chloroquine or quinine but less active than artemisinin and mefloquine. Bray et al (1990) conducted an *in vivo* evaluation of gedunin in mice infected with *P. berghei* and concluded that gedunin was non-toxic but ineffective in clearing the parasite at a single dose of 90 mg/kg. It was likely that the parent compound was degraded by esterases or by cytochrome P450 enzymes to inactive metabolites such as 7-deacetylgedunin (MacKinnon et al., 1997; Hantos, 1998).

Two approaches were proposed to address the degradation problem. First, the structural modification of gedunin was undertaken in order to protect the labile acetoxy group at C-7 moiety from possible degradation of esterases. Two derivatives were prepared by substuiting C-7 acetyl group with methoxy and hydroxy groups (Hantos, 1998). *In vitro* of these two derivatives against the *P. falciparum* (D6 and W2 clones) showed potent activities and particularly, the esterase stable 7-methoxygedunin had comparable activity to gedunin (Hantos, 1998). Secondly, the inclusion of a synergist was used to prevent the metabolism of the active compound by cytochrome P450 and facilitate the penetration of the molecule to reach its target (Bernard and Philogène, 1993). Dillapiol is a potent naturally-occuring synergist isolated from the Piperaceae family (MacKinnon et al., 1995). It also acts as an inhibitor of insect cytochrome P450 (Belzile et al., 2000) as well as human cytochrome P450 enzymes

(Budzinski et al., 2000). Dillapiol's low mammalian toxicity (Bernard, 1995) and its potential to act as an inhibitor of drug metabolizing enzymes in human makes it a prime candidate for these studies. In this chapter, the hypothesis that improved formulation of gedunin and 7-methoxygedunin enhances the antimalarial activity was tested. The addition of dillapiol to the formulation was also explored.

6.2 MATERIALS AND METHOD

6.2.1 Sample preparation

Pure samples of gedunin, 7-methoxygedunin and dillapiol (Figure 6.2) were either isolated or synthesized in the laboratory of Dr. T. Durst, University of Ottawa, Ottawa, ON as described in Hantos, (1998); MacKinnon et al., (1997). Gedunin and 7-methoxygedunin were each dissolved in ethanol (5 mg/ mL) and then each separately to 20 mg/mL of PVP in ethanol. Each solution was then evaporated on a rotary evaporator to near dryness and lyophilized. Dillapiol was diluted using standard corn oil.

6.2.2 Pharmacokinetics of orally administered gedunin

The pharmacokinetics of gedunin was investigated to determine the absorption, distribution and excretion of gedunin in mice. Thirty CD-1, male mice, 30 ± 2 g of weight were obtained from Charles River

Laboratories, St. Constant, QC. The animals were housed one per each cage and acclimatized for 5 days. On day 0, twenty-four mice were each orally administered a single dose of gedunin (50 mg/ kg of body weight). As control, 3 mice were administered the vehicle (PVP) exclusively, and 3 mice remained untreated. Feces were collected between days 1-4. Blood samples were taken at different time intervals (3, 6, 9, 12, 24, 48, 72, and 96). The mice were sacrificed by CO_2 asphyxiation each time interval and kidney and liver samples were excised. Urine samples could not be sufficiently collected.

Blood samples (1 mL) were centrifuged for 15 minutes (10,000 rpm) and the serum separated. Serum samples were extracted with 3 aliquots of 1 mL toluene, pooled and evaporated to dryness. Feces (100 mg) were soaked in 10 mL of toluene for 1 hour, filtered using Whatman no. 1 paper and evaporated to dryness. Kidney and liver samples were homogenized at 10% w/v, in toluene, with a Polytron tissue blender. The homogenate was then filtered and evaporated to dryness. Each dried sample was reconstituted in 1 mL volume of acetonitrile, and filtered through a teflon filter (0.45 μm) before injection into HPLC.

6.2.3 HPLC method

Beckman HPLC instrument consisting of a solvent Module (126), a photodiode Array Detector (168), an Autosampler (502), and an Optilex

466/Le computer was used. Separations were achieved using Waters Symmetry C_{18} endcapped column (particle size, 5 μm: column dimensions, 4.6 mm x 150 mm). The mobile phase was an acetonitrile: water mixture with a gradient elution rising from 50% to 90% acetonitrile over a period of 20 minutes. The flow rate of was 1 mL/min. The injection volume was 20 μL. Detection of the analyte was at a wavelength of 230 nm. Comparison of retention time with an authentic standard was used for peak identification.

6.2.4 *In vivo* antimalarial activity of gedunin and 7-methoxygedunin

The antimalarial assay was conducted as described in Chapter 5.2.5. CD-1 male mice (30 ± 2 g wt.) were infected 0.2 mL blood containing approximately 1 X 10^6 *P. bergheii* parasite by intraperitoneal injection. Animals were randomized into five groups (four animals per group and per each treatment) and administered orally different doses of gedunin, 7-methoxygedunin, dillapiol and combinatory treatment of each limonoid with dillapiol for four days. The doses given included: gedunin (25, 50, 100 mg/kg/day), dillapiol (25, 50, 100 mg/kg/day); and a combination of the two compounds: gedunin (25, 50, 100 mg/kg/day) with a fixed dose of dillapiol at 25 mg/kg/day. 7-Methoxygedunin was administered at 12.5 mg/kg/day and 50 mg/kg/day and a combination treatment of 7-methoxygedunin (12.5, 25, 50 mg/kg/day) and dillapiol

(25mg/kg/day) for four days. The control group was given equivalent amount of the vehicle (corn oil and PVP each set at 25 mg/kg/day).

6.2.5 Acute toxicity testing

Five CD-1 male mice (30 ± 2 g) were each given daily dose of 100 mg/kg of gedunin and 25 mg/ kg of dillapiol for five days. Control animals (5 CD-1 male mice of similar weight) were given equivalent doses of corn oil and PVP. Weight changes and wellness were recorded every day for 14 days. The observation included changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system and behavioral pattern. On day 14, blood from three treated animals and three controls were collected via cardiac puncture. Each sample of blood was centrifuged for 20 minutes and the serum was separated. Frozen serum samples were sent for clinical chemistry testing at the Animal Health Laboratory, University of Guelph, Kemptville, ON. The enzymes alkaline phosphatase, aspartate aminotransferase, alaninine aminotransferase were analyzed.

6.3 Results and Discussion

In the formulation studies, the solubility of gedunin was improved with the incorporation of PVP and consequently the oral administration of gedunin was possible. To quantitatively assess the amount of gedunin

reaching the blood, the mice were treated with 50 mg/kg/day and the blood was collected at different time intervals. High-pressure liquid chromatography analysis of the serum of mice gave three major peaks. The online spectrum of gedunin standard was consistent with the online spectrum of the peak observed at a retention time of 10.17 (r= 0.998). Other unidentified peaks presumed to be metabolites were also observed (Figure 6.3). The concentration of the absorbed gedunin was quantifiable only from the serum samples collected at 3 and 6 hours post treatment with the PVP based formulation. The average concentration of gedunin in the blood of each mouse was found by this method to be approximately 6.2 μg/mL. With this formalation, gedunin given at 50 mg/kg/day showed a parasitemia clearance of 44.6% ± 5.6. This suppression rate was insufficient to completely eliminate the infection. However, the improved formulation compares favourably with no suppression observed by Bray et al. (1990) when gedunin was formulated in water without PVP.

It was not possible to detect gedunin in the feces, kidney or liver samples. Gedunin is a reasonably large non-polar molecule and could have passed through the digestive system without adequate absorption and subsequently degraded in the small intestine by the microflora. Although gedunin was found to be stable at acidic conditions (Hantos, 1998), the esterase enzyme in the stomach could easily convert the molecule into inactive derivatives.

A dose-response curve was obtained with the combination treatment of gedunin and dillapiol (Figure 6.4). The parasitemia level was seen to decrease in a dose dependent manner reaching 79.0 % ± 5.3 inhibition. With this treatment, blood levels of gedunin at 3 hours post treatment were also observed to increase to 13.1 μg/mL as compared to 6.2 μg/mL observed earlier without the inclusion of dillapiol. This increase could be due to the inhibition of cytochrome P450 in the small intestine which possibly slowed the degradation of gedunin during first-pass metabolism.

Acute toxicity testing undertaken using the combination treatment of dillapiol and gedunin showed that there was no significant difference in the liver enzyme levels between the treated and the control group (Table 6.1). This suggests that the treatments had no acute mammalian toxicity. However, to assess the long-term effects of these compounds, chronic toxicity studies need to be completed using a large number of animals.

7-methoxygedunin alone when tested at 12.5 mg/kg/day did not show any suppression of parasitemia (Figure 6.5). However, at a dose of 50 mg/kg/day, a suppression rate of 67.5% ± 1.3 was observed. Due to the limited amount of 7-methoxygedunin available for testing, no tests were conducted at high doses (100 mg/kg/day). This study suggests that 7-methoxygedunin was significantly more active than gedunin ($P < 0.05$). Further studies conducted with 7-methoxygedunin and dillapiol showed

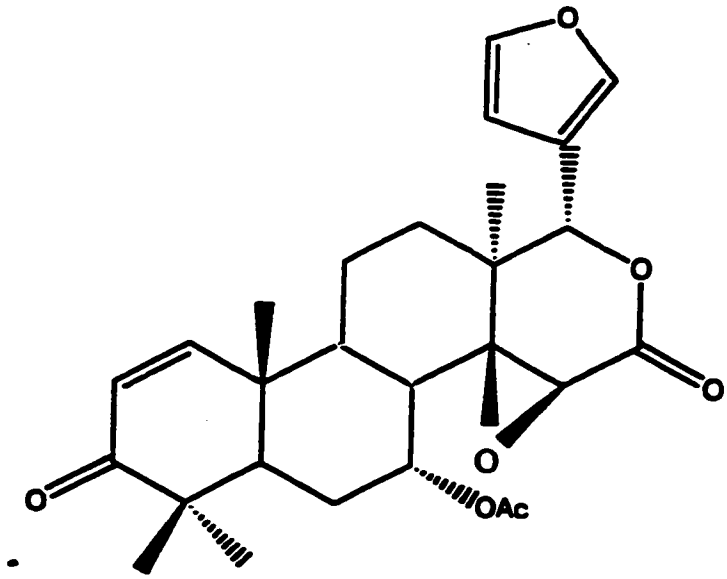
increased suppression in a dose dependent manner. At a maximum dose of 50 mg/kg of 7-methoxygedunin and 25 mg/kg of dillapiol, the clearance level was observed to be 80.7% ± 5.1 comparable to that obtained with gedunin/dillapiol (79.0 % ± 5.3).

From these studies, it can be concluded that the addition of an esterase stable group at C-7 position increased antimalarial activity. It was also evident that the presence of a cytochrome P450 inhibitor such as dillapiol may decrease the degradation of the compound in the stomach and improve bioavailability. This chapter demonstrated the possibility of developing effective phytomedicines based on these traditionally used tropical plants.

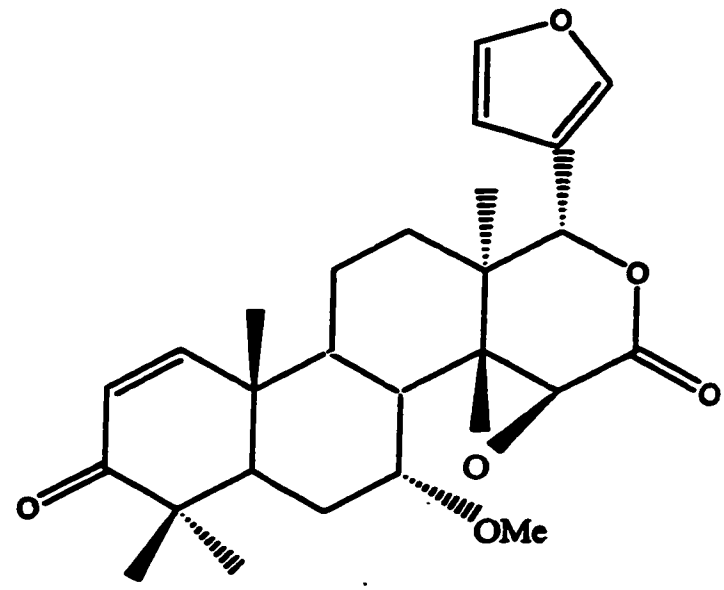
Figure 6.1 *Cedrela odorata* tree (Costa Rica)

Cedrela odorata

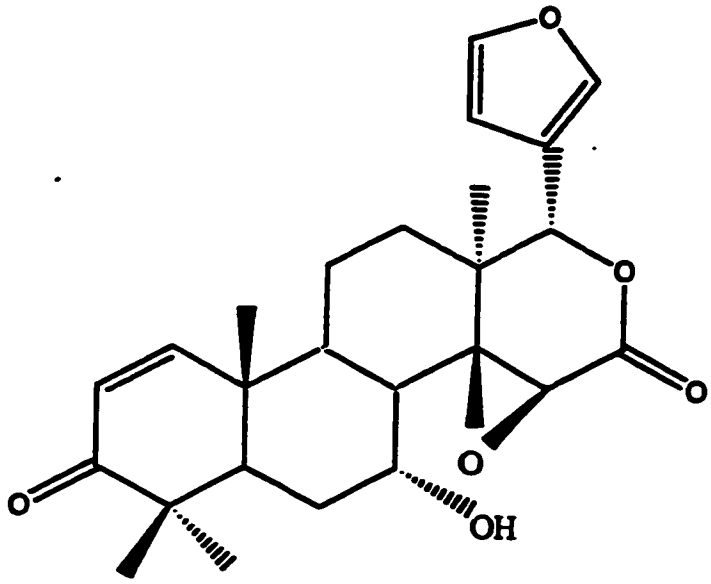
Figure 6.2 Structures of gedunin, 7-deacetylgedunin, 7-methoxygedunin, and dillapiol



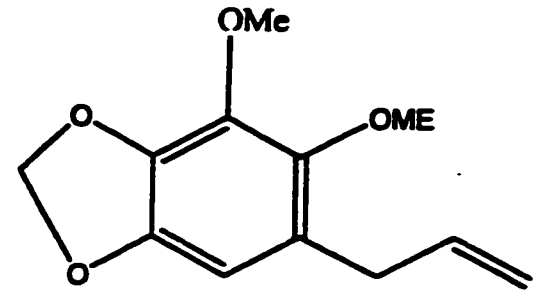
GEDUNIN



7-METHOXY GEDUNIN



7-DEACETYL GEDUNIN



DILLAPIOL

Figure 6.3 Chromatogram showing gedunin and unidentified metabolites from serum obtained from mouse 3 hours post ingestion of gedunin

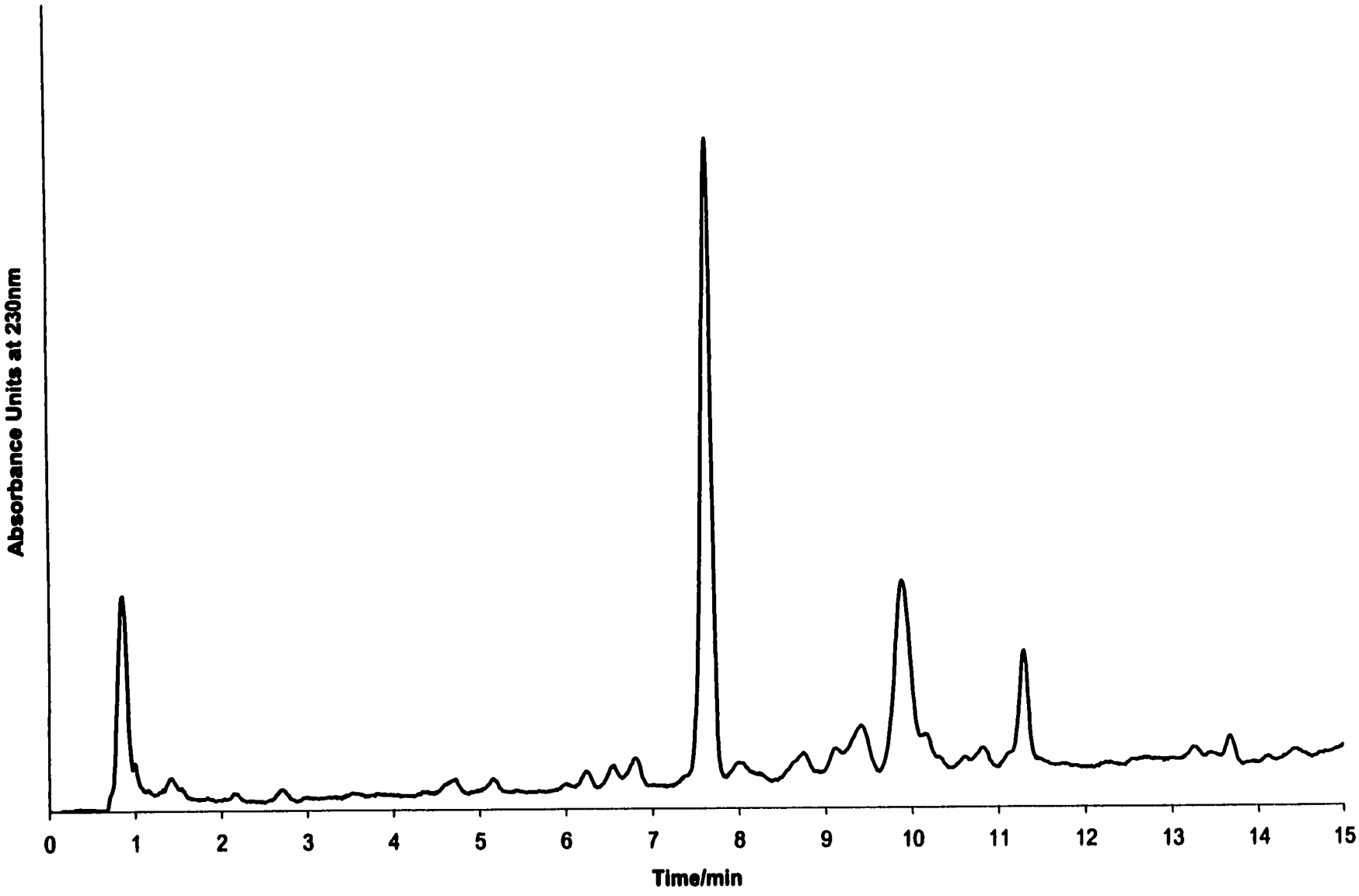

Absorbance Units at 230nm
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Time/min

Figure 6.4 Percentage of parasitemia suppression versus dose of gedunin and dillapiol administered to mice infected with *P. bergheii*

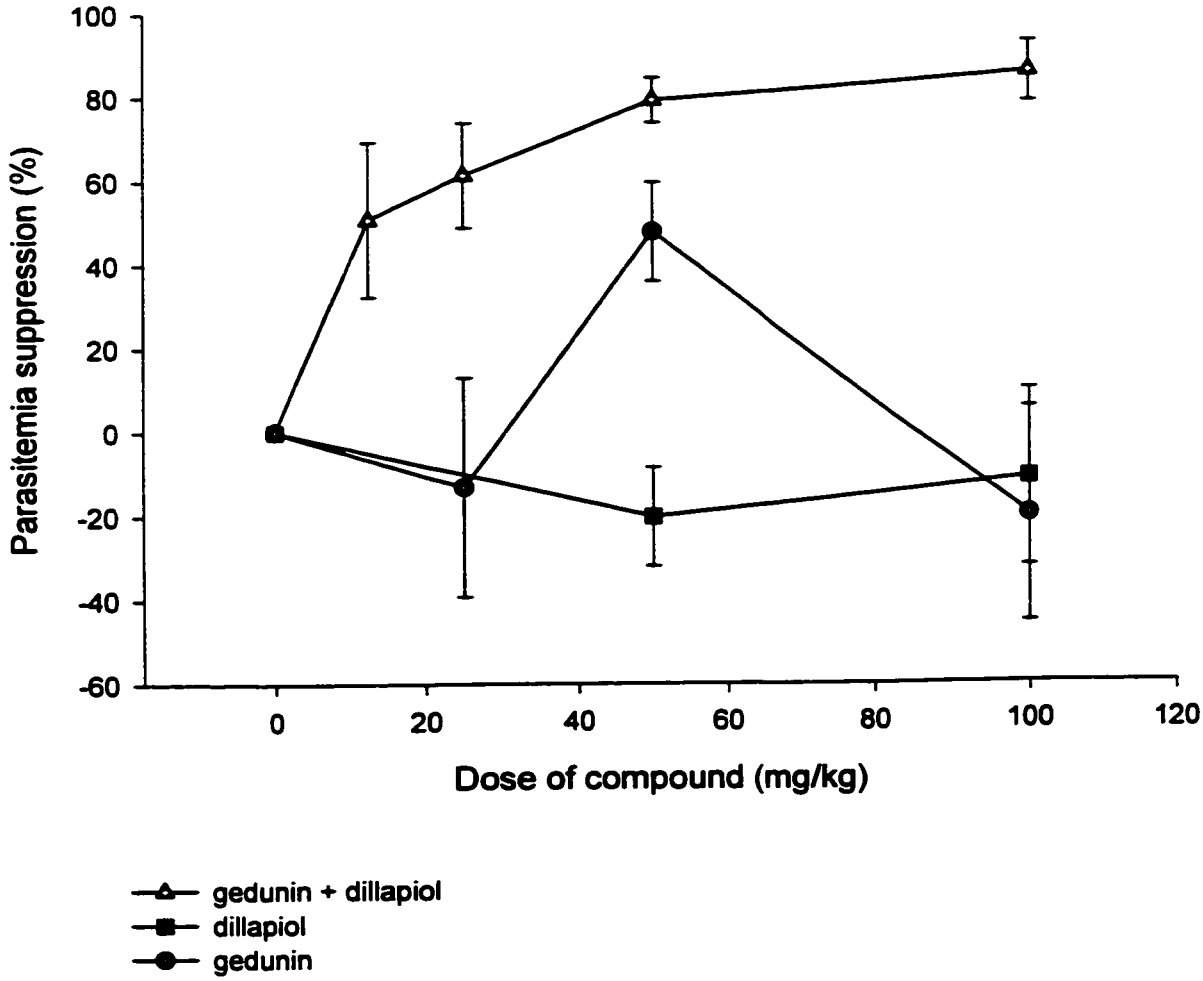

100
80
60
40
20
0
-20
-40
-60
Parasitemia suppression (%)
0
20
40
60
80
100
120
Dose of compound (mg/kg)
gedunin + dillapiol
dillapiol
gedunin

Figure 6.5 Percentage of parasitemia suppression versus doses of 7-methoxygedunin and dillapiol administered to mice infected with *P. bergheii*

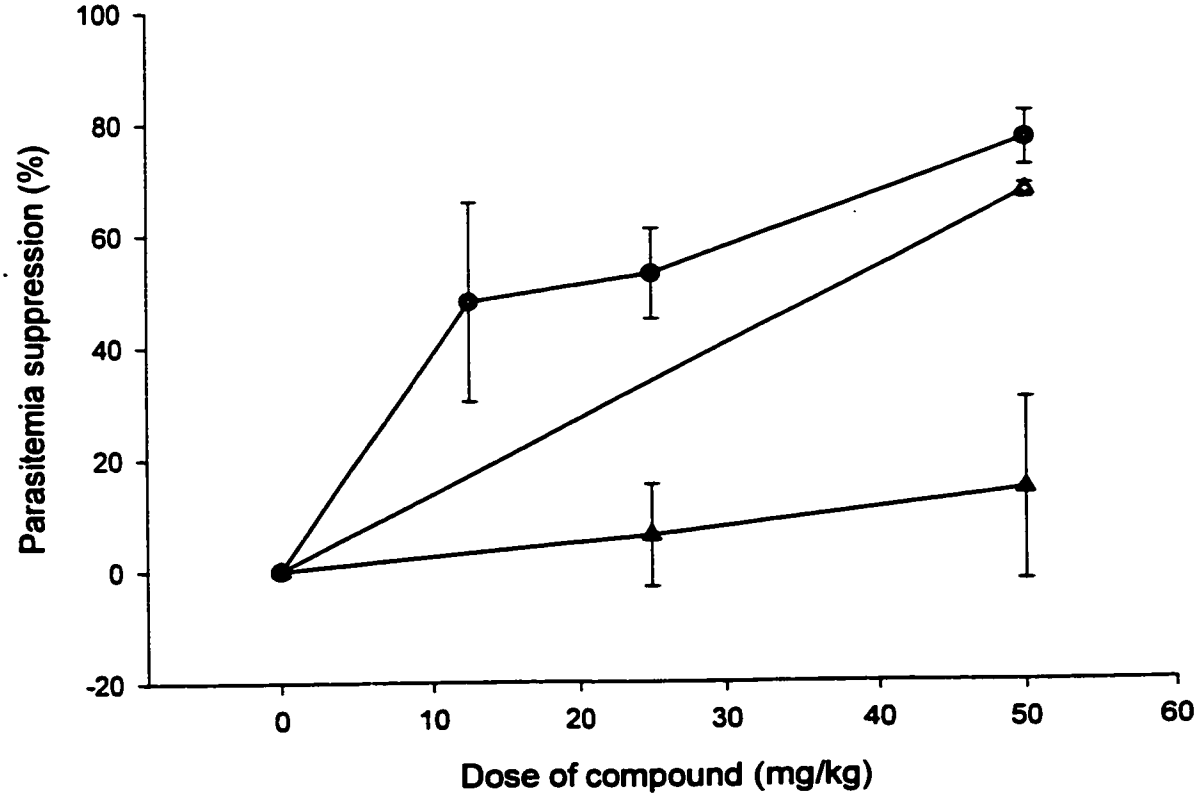

100
80
60
40
20
0
-20
Parasitemia suppression (%)
0
10
20
30
40
50
60
Dose of compound (mg/kg)
methoxygedunin + dillapiol
methoxygedunin
dillapiol

Table 6.1 Serum enzyme levels of mice treated with gedunin and dillapiol

Enzyme tested	Control (U/ L)	Treated (U/L)
Alkaline phosphatase	114 ± 15a	88 ± 7b
Alanine Aminotransferase	93 ± 3c	92 ± 5c
Alanineserine transferase	28 ± 1d	29 ± 2d

Similar letters denote not significantly different (P < 0.05) Student's t-test.

Chapter 7. Summary and conclusions

This thesis has shown that the phytochemical discovery process can be undertaken by using leads from chemical ecology principles or traditional knowledge to increase the identification of bioactive plant parts in ecosystems. When screening for pharmacologically active principles, the crucial factor for success is the selection of the plant species based on ethnomedical information or targetted collection based on chemotaxonomic relationships. Screening programs aimed at insect control agents could also employ field observations and ethnobotanical information.

Chapter 2 examined the insect growth-reducing and antifeedant activity of plants from temperate zones. Coley's hypothesis (1985) which proposes that slow growing plants exhibit a higher amount of quantitative defensive secondary compounds than fast growing trees was tested using hardwood tree extracts against two insects: *Ostrinia nubilalis* (Lepidoptera) and *Sitophilus oryzae* (Coleoptera). The results from these studies indicated that a higher number of insect growth reducing and antifeedant activities were observed in wood extracts from slow growing plants than in the fast growing plants. These results were consistent with the proposal of a trade-off between growth and defense.

Plants produce a wide array of secondary metabolites, many of which are produced as a defense against microorganisms. The earliest

drugs were discovered from plant extracts which led to the isolation of pure products, followed by other natural compounds of known chemical structure and subsequently to many synthetic organic compounds (Farnsworth, 1985).

The efforts to find new drugs from plants have been diminished due to the higher commercial viability of patentable synthetic drugs. One exception is the area of anticancer drugs which are about 65% plant-derived due to publicly funded research. The National Cancer Institute (NCI) has screened more than 35,000 plant species and over 100,000 extracts for their antitumour activities against murine tumours (Cordell et al., 1991). Of these about 7% of these species were found to inhibit various tumours (*in vivo*) and several hundred extracts were found to be cytotoxic (*in vitro*) (Farnsworth, 1985). Eleven of these compounds have been approved for extensive tumour panel testing (Suffness, 1985). Taxol, vinblastine and vincristine are now among the most effective anticancer drugs in the market and new products such as camptothecin are currently being adopted.

It is apparent that the battle against malaria is continuing to lose ground. The WHO conceded that the transmission of malaria is influenced by a complex set of variables including scientific, political, sociological and economic considerations (WHO, 1995). Yet, the greatest problem is the lack of financial aid or incentives for development of new antimalarials. It

is not likely that any simplistic approach to eradicate the disease will succeed. Although several strategies are there to prevent the spread of malaria, drug therapy will remain the most important aspect of a comprehensive program to limit human morbidity and mortality (Angerhofer et al., 1992).

Quantitative methodology in ethnobotany is one of the tools used for assessing the relative value of plants to the people who use them, based on degree of consensus among users (Leaman et., 1995). These techniques have been useful in providing tools for quantitative analysis of ethnobotanical survey data and a means for identifying target plants (Johns et al.; 1990; Leaman et al.,1995; Jones, 1999). Chapter 3 examined the ethnobotanical approach that plants traditionally used as a remedy associated with diseases of microbial origin may have significantly higher antimicrobial activity. In both the bacteria and fungi tests conducted, the plant parts and species preferred by First Nation's peoples exhibited greater antimicrobial activities than the less preferred plants. The results suggested that indigenous knowledge does contribute to the understanding of the chemical ecology of plants.

Plant extracts identified as active in insect bioassays were found to be good sources of new phytochemicals. *Prunus serotina* (black cherry) which was identified as the most active temperate plant in this study yielded four flavonoid compounds: naringenin, 4'methoxynaringenin,

dihydrokaempferol and eriodictyol. Although flavonoids are not noted for their potent insecticidal action, *P. serotina* can be useful Canadian sources of these compounds that could be exploited in the growing botanical or citrus industry.

Chapter 4 described the isolation of compounds from the tropical plant *Lansium domesticum* (bark) identified as an antimalarial by Leaman et al. (1995). Four novel triterpenoid compounds (iso-onoceratriene, 3-ketolansiolic acid, lansiolic acid A, 3-keto-22-hydroxyonoceradiene) and additionally two compounds (onoceradienedione, lansiolic acid) were earlier discovered in the peel of the fruit were isolated.

Triterpenes are synthesized by the acetate-mevalonate pathway. Mevalonic acid produced from 3 acetyl CoA molecules is converted to the 5 carbon compound isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAP). In the biosynthesis of triterpenes, IPP and DMAP condense to form geranyl pyrophosphate followed by the condensation with another IPP to form 15 carbon compound farnesyl pyrophosphate (FPP). When two FPP molecules condense tail to tail, the linear compound squalene is formed which is then converted to the tertacyclic tripterpenes via squalene-2,3-epoxide (Lichtenthaler et al., 1997). Limonoids of the Meliaceae and Rutaceae are derived from tetracyclic triterpenes whose side chain has been converted to a furan, with the loss of 4 carbon atoms (Champagne et al., 1992). Limonoids in

general show a high level of oxidation in comparison to other triterpenes (Das et al.1984).

The study in Chapter 4 also identified potent new insect antifeedants including some that had been isolated previously. Of these, the spirocaracolitones, a novel class of triterpenes and humilinolides (limonoids) exhibited potent activity against the *S. oryzae* although the spirocaracolitone has a better potential for commericialization. The source of the spirocarcolitone compounds was *Ruptilicarpon caracolito*, an endemic rare species found in the humid lowland tropical rainforests in Costa Rica.

The final two chapters (Chapters 5 and 6) examined the possible application of triterpenoid phytochemicals as antimalarials. This study adds limonoids and lansiolides to the known group of compounds found to be effective antimalarials *in vitro* and *in vivo*. Although the compounds isolated from *Lansium domesticum* were not very active *in vivo*, the semi-derivative compounds (methyl lansiolate, methyl lansiolate A, and methyl 15-acetoxylansiolate A) could be modified to give more potent antimalarial compounds. Compounds based on structural modification of these structures could be easily prepared to yield effective antimalarial drugs.

In evaluating traditional medicines, researchers often quickly pass over the preparations from natural sources in laboratory investigations. This study has shown that gedunin previously determined as inactive

antimalarial compound by Bray et al. (1990) can be highly active when properly formulated. Three strategies were developed to investigate this problem; (1) low solubility of gedunin in water was improved by incorporating it to a polymer providing a suspension easy to administer; (2) the possible degradation of gedunin by esterase to its inactive 7-deacetylgedunin was addressed by structural modification to a more stable compound 7-methoxygedunin; and (3) the possible degradation by cytochrome P450 3A4, the major drug metabolizing enzymes was addressed by the addition of dillapiol, a naturally-occuring P450 inhibitor. This study demonstrated that when properly formulated gedunin did show moderate antimalarial activity, however, structural modification to a more stable 7-methoxygedunin demonstrated a more potent antimalarial compound. Furthermore, the addition of dillapiol to either gedunin or 7-methoxygedunin markedly enhanced their activities.

The great diversity of plant resources in the developing world continues to provide health care products at low cost for majority of people in the world. Unfortunately, most of these traditional drugs have not been scientifically tested for their safety and efficacy. In addition, most physicians in these countries who are trained in Western medical schools are hesitant to prescribe such medication to their patients because of the lack of adequate knowledge. However, a survey of attitudes in West Africa has indicated that many patients believe the best treatments are the ones

that consist of both modern and traditional drugs together (Gbeassor et al., 1989). This thesis showed that it is possible to establish cheap, locally produced, standardized phytomedicines based on the tropical plants investigated in this study.

7.1 FUTURE WORK

Plants in eastern North America have been poorly investigated for their potential as insect control agents or sources for phytomedicines. Based on the NAPRALERT search conducted, there is a need to test several of the ehtnobotanical claims in the database using appropriate bioassays followed by isolation and identification of active principles. Tropical trees have contributed to the development of 70% of known plant-based drugs and are better sources of new phytochemicals. Considerably more research is needed to explore the presence of novel compounds from such sources.

An area of study that offers a source of potential malarial drugs is the understanding of biochemical differences of the parasite and human cells. One target is that the malaria parasite has a unique ability to degrade hemoglobin and sequester heme (Phillips, 1983). It is believed the quinoline blood schizontocides (quinine, chloroquine, mefloquine) and the peroxide antimalarials (artemisinin deriviatives) exert some of their effects by interacting with heme or hematin (Ridley, 1997). Other plant

based drugs may have similar properties and their mode of action have to be further investigated.

A second target is the extrachromosomal 35-kb circular DNA that encodes prokaryotic-like transcription and translation in the parasite (Ridley, 1997). Some antibacterial agents such as doxycycline that target prokaryotic translation are found to be effective as antimalarials. This suggest that there are several biochemical pathways associated with the parasite that may be exploited for drug discovery using compounds such as those investigated in this thesis.

Two complementary approaches to drug discovery are chemically-driven and biologically-driven projects (Ridley, 1997). Chemical variation around established classes of antimalarials needs to be further optimized to produce more effective and selective drugs. The understanding of the mechanism of action of these existing compounds is important as it may provide insights into how further modification could be made. The biology-driven projects such as the phytochemical discovery approach taken in this thesis should also be given importance since they could lead to the discovery of novel drugs with new modes of action.

Another aspect of research that has been initiated with the present work is the study of synergism of antimalarial compounds. The possibilities offered by these combinatory treatments could lead to the discovery of new effective treatements. Ultimately, clinical trials,

conducted with the compounds that exhibited high efficacy and low toxicity are the final step to establishing a new, ethical treatment based on plant derived drugs.

REFERENCES

Albert P.J. (1990). A review of some host-plant chemicals affecting the feeding and oviposition behaviors of the Eastern spruce budworm *Choristoneura fumiferana*. Mem. Ent. Soc. Can. 159: 13-18.

Allen D., Toth I., Wright C.W., Kirby G.C. and Phillipson J.D. (1993). *In vitro* antimalarial and cytotoxic activites of semisynthetic derivatives of brusatol. Eur. J. Med. Chem. 28: 265-269.

Angerhofer C. K., Guinaudeau H., Wongpanich V., Pezzuto J.M. and Cordell G.A. (1999), Antiplasmodial and cytotoxic activity of natural bisbenzylisoquinoline alkaloids (1999). J. Nat. Prod. 62: 59-66.

Angerhofer C.K., Konig G.M., Wright A.D., Sticher O., Milhous W.K., Cordell G.A., Farnsworth N.R., and Pezzuto J.M. (1992). Selectivity screening of natural products: a resource for the discovery of novel antimalarial compounds. In Advances in Natural Products Chemistry ed. Attar-Ur Rahman. Hardwood Academic, Chur, Switzerland.

Arnason J.T., Philogéne B.J.R., Berg C, MacEachern A., Kaminski J., Leitch L.C., Morand P., Lam J., (1986). Phototoxicity of naturally occurring and synthetic thiophene and acetylene analogues to mosquito larvae. Phytochem. 25: 7, 1609-1611.

Arnason J.T., Hebda R., Richard J., Johns T., (1981). Use of plants for food and medicine by Native peoples of eastern Canada. Can J. Bot. 59: 2189-2325.

Arnason J.T., MacKinnon S., Durst A., Philogène, B. J. R., Hasbun C., Sanchez P., Poveda L., San Roman L., Isman M.B., Satasook C., Towers G.H.N., Wiriyachitra P., McLaughin (1993). Insecticides in tropical plants with non-neurotoxic modes of action. In Phytochemical Potential of tropical Plants ed. K. R. Downum. Plenum Press, NY.

Arnason J.T., Mackinnon S., Isman M.B., Durst T. (1992). Insecticides in tropical plants with non-neurotoxic modes of action. Rec. Adv. Phytochem. 28: 107-131.

Arnason J.T., Philogène B.J.R., Donskov N., Hudon M., McDougall C., Fortier G., Morand P., Gardner D., Lamber J., Morris C., Nozzolillo C. (1985). Antifeedant and insecticidal properties of azadirachtin to the

European corn borer, *Ostrinia nubilalis* Hubner. Entomol. Exp. Appl. 38:29-34.

Arnason J.T., Philogène B.J.R., Donskov N., Kubo I. (1987). Limonoids from the Meliaceae and Rutaceae reduce feeding, growth and development of *Ostrinia nubilalis*. Entomol. Exp. Appl. 43:221-226.

Assabgui R., Lorenzetti F., Terradot L., Regnault-Roger C., Malo N., Wiriyachitra P. (1997). Efficacy of botanicals from the Meliaceae and Piperaceae. In Phytochemicals for pest control eds Hedin, R.M. Hollingworth E.P. Masler J. Miyamoto D. G. Thompson. ACS Symposium Series 658:38-48.

Beck S.D. and Reese J.C. (1976). Insect plant interactions. Nutrition and Metabolism. Rec. Adv. in Phytochem. 10:41-92.

Belzile A.S., Majerus S.L., Podesznfiski C., Guillet G., Durst T., and Arnason J.T. (2000). Dillapiol derivative as synergists: Structure-activity relationship analysis. Pest. Biochem. Phy. 66: 33-40.

Bentley M.D., Rajab M.S., Alford A.R., Mendle M.J., Hassanali A. (1988). Structure-activity studies of modified citrus limonoids as antifeedant for Colorado potato beetle larvae, *Leptinotarsa decemlineata*. Entomol. exp. appl. 49:189-193.

Bernard C.B. (1995). Biological activities and mode of action of lignans from the piperaceae. PhD thesis. University of Ottawa, Ottawa, Canada.

Bernard C.B. and Philogène B.J.R. (1993). Insecticide synergists: role, importance and perspectives. J. Tox. Env. Health 38: 199-223.

Bernays E.A. and Chapman R.F. (1994). Host plant selection by phytophagous insects. Chapman and Hall, NY.

Beutler J. A., Caredelline J.H., McMahon J.B., Shoemaker R.H., and Boyd M.R. (1995). Phytochemistry of medicinal plants ed. Arnason J. T., Mata R., Romeo J. T. Plenum Press, NY.

Brandao, M.G.L., Grandi T.S.M., Rocha, E.M.M, Sawyer, D. R. and Kretti, A.U.(1992). Survey of medicinal plants used as antimalarial in the Amazon. J. Ethnopharmacology 36: 175-182.

Bray D.H., Boardman P., O'Neill M.J., Chan K.L., Phillipson J.D., Warhust D.C. (1985). Plants of the family Simarobaceae as potential antimalarial agent. R. Soc. Trop. Med. and Hyg. 79: 426.

Bray D.H., Boardman, P., O'Neill, M.J., Chan, K.L., Phillipson, J.D.,Warhust D.C. (1987). Plants as source of antimalarial durgs. Phytother. Res. 1: 22-24.

Bray D.H., Warhust D.C., Connolly J.D., O'Neill M.J. and Phillipson J.D.(1990). Plants as sources of antimalarial durgs. Part 7. Activity of some species of Meliaceae plants and their constituent limonoids. Phytother. Res. 4:1, 29-35.

Buchalter L. (1969). Identification of monomeric and polymeric 5,7,3',4' tetrahydroxylflavan-3-4-diol from tannin extract of wild cherry bark, *Prunus serotina*. J. Pharm. Sci. 58: 10,1272-1273.

Budzinski J.W., Foster B.C, Vandenhoek S., Arnason J.T. (2000). An in vitro evaluation of human cytochrome P450 3A4 inhibition by selected commercial extracts and tinctures. Phytomedicine. 7: 1-10.

Butterworth J. H., and Morgan E.D. (1968). Isolation of a substance that suppresses feeding in locusts. Entomologia exp. appl. 38: 29-34.

Champagne D., Koul O., Isman M., Scudder G.G.E. and Towers G.H.N. (1992). Biological activity of limonoids from rutales. Phytochem. 31:2, 377-394.

Chénier R. and Philogène, B.J.R. (1989). Field responses of certain forest Coleoptera to monoterpenes and ethanol. J. Chem. Ecol. 13: 1729-1745.

Coley P.D., Bryant J.P., Chapin F.S. (1985). Resource availability and plant antiherbivore defense. Sci. 230: 895-898.

Das M.F., Silva G.F.D., Gottlieb O.R., Dreyer D.L. (1984). Evolution of limonoids in rutales, Biochem. System. Ecol. 12: 3, 299-310 .

Desjardin R.E., Canfield C.J., Haynes C.J., Chulay J.D (1979). Quantitative assessment of antimalarial activity *in vitro* by semi-automated microdilution technique. Antimicrob. Agents Chemother. 16: 710-718.

Ekong R.M., Kirby G.C, Patel G., Phillipson J.D. and Warhurst D.C. (1990). Comparison of the *in vitro* activities of quassinoids with activity

against *Plasmodium falciparum*, anisomycin and some other inhibitors of eukaryotic protein synthesis. Biochem. Pharm. 40: 297-301.

Farnsworth N.R., Akerele O., Bingel A.S., Soejarrto D.D., Guo Z. (1985). Medicinal plants in therapy. Bul. World Health Org. 63: 6, 965-981.

Feeny R. (1978). Allyglucoscinolates and herbivorous caterpillars. Sci. 200: 1296-1298.

Feng R. and Isman M.B. (1995). Selection for resistance to azadirachtin in green peach aphid. Experientia 51: 831-833.

Frazier W.C. and Westhoff D.C.,1988. Food Microbiology, 4th ed., McGraw-Hill book company, NY.

Fuhr U. (1998). Drug interaction with grapefruit juice. Extent, probable mechanism and clinical relevance. Drug safety 18: 251-272.

Gbeassor M., Kossou Y., Amegbo K., de Souza C., Koumaglo K., and Denke A. (1989). Antimalarial effects of eight African medicinal plants. J. Ethnopharmacology 25: 115-118.

Gentillini M., Nozais J.P. (1991). Histoire du paludisme. In Davis M. and Machet ed. Paludisme. Ellipses. Paris.

Georgopapadakou N. and Walsh T.J. (1994). Human Mycoses: drugs and target for emerging pathogens. Sci. 264: 371-374.

Gessler M.C., Nkuya M.H.H., Mwasumbi L., B.,Heinrich M. and Tanner M. (1994). Screening Tanzanian medicinal plants for antmalarial acitivity. Acta Tropica 56: 65-77.

Gessler M.C., Tanner M., Chollet J., Nkunya M.H.H., Heinrich M. (1995). Tanzanian medicinal plants used in traditionally treatment of malaria: in vivo antimalarial an *in vitro* cytotoxic activities. Phytother. Res. 9: 504-508.

Guido F., Bringmann G., Phillipson J.D., Assi L.A., Dochez C., Rubenacker M., Schneider C., Wery M., Warhurst D.C. and Kirby C. (1994). Activity of extracts and napthylisoquinoline alkaloids from *Triphyophyllum peltatum*, *Ancistrocladus abbreviatus* and *A. barteri* against *Plasmodium falciparum in vitro*. Phytochem. 35: 1461-1464.

Guthrie W.D., Robbins J.C. and Jarvis J.L. (1985). *Ostrinia nubilalis*. In Handbook of insect rearing eds. Singh P. and Moore R. Elsevier, NY.

Hadacek F., Greger H. (2000). Testing of antifundal natural products: methodologies, comparability of results and assay choice. Phytochem.anal. 11: 137-147.

Harborne J. (1990). The flavonoids, Chapman and Hall, NY.

Hantos S. (1998). The syntheses of trichiliasterone A and B isolated from *Trichilia hirta* and the preparation of derivatives of antimalarial agent gedunin. Master Thesis, University of Ottawa.

Hayashi Y., Nishizawa M., Shiro M., Sastrapraja (1989). New doubly rearranged limonoids from seeds of "duku" (*Lansium domesticum*) as bitter principle. Proceedings of the 6th Asian symp. on med. plants. Bandung, Indonesia.

Hien T.T. and White, N. J. (1993). Qinghaosu. Lancet 341: 603-608.

Hostettmann K., Wolfender J. L. (1997). The search for biologically active secondary metabolites. Pestic. Sci. 51: 471-482.

Isman M.B. (1997). Neem insecticides. Pesticide Outlook October, 32-38.

Isman M.B., Matsuura H., MaKinnon S., Durst, T., Tower, G.H.N., Arnason, J.T. (1997). Phytochemistry of the Meliaceae. Rec. Adv, Phytochem. 30: 155-178.

Isman M.B. (1994). Botanical insecticides. Pesticides Outlook, 5: 26-30.

Jacobson M. (1990). Glossary of plant-derived insect detterents.CRC press Inc. , Boca Raton, FL.

Jacobson M. (1989). Botanical pesticides- past, present and future. In insecticides of plant orgin ed. Arnason, JT, Philogène BJR and Morand P. pp 1-10. Am. Chem.Soc.Symp. Ser. 378, Washington, D.C.

Jimenez A., Mata R., Pereda-Miranda R., Calderon J., Isman M.B., Nicol R. and Arnason J.T. (1997). Insecticidal limonoids from *Swietenia humilis* and *Cedrela salvadorensis*. J. Chem. Eco., 23: 5, 1225-1234.

Johns T., Kokwaro J.O., Kimanini E.K. (1990). Herbal remedies of the Luo of Siaya district Kenya: Establishing quantitative criteria for consensus. Econ. Bot. 44: 369-381.

Jones N. (1999). Quantitative ethnobotany of eastern North America. Master Thesis, University of Ottawa, Ottawa, ON.

Khalid S.A., Farouk A., Geary T.G. and Jensen J.B. (1986). Potential antimalarial candidates from african plants: an *in vitro* approach using *Plasmodium falicparum*. J. Ethnopharmacology 15: 201-209.

Kirby G.C.,Paine A., Warhust D.C., Noamese B.K. and Phillipson J.D. (1995). *In vitro* and *in vivo* antimalarial actitivity of Cryptoleptine, a plant-derived indoquinoline. Phytother. Res. 9: 359-363.

Klayman D. (1985). Qinghaosu (Artemisinin) an antimalarial from China. Sci. 228: 1048-1055.

Klocke J.A. (1987). Natural plant compounds useful in insect control. In Allelochemicals in forestry and agriculture. ACS, Wash. D.C.

Kubo I., and Klocke J. A. (1986). Some terpenoid insect antifeedants from tropical sources. Adv. Pesticide Sci. 2: 284-291.

Leaman D. J., Arnason J. T., Yusuf R., Sangat-Roemantyo H., Soedjito H., Angerhofer C.K., Pezzuto J.M. (1995). Malarial remeides of the Kenyah of the Apo Kayan, East Kalimantan, Indonesian Borneo: a quantitative assessment of local consensus as an indicator of biological efficacy. J. Ethnopharmacology 49: 1-16.

Lee I., and Hufford C.D. (1990). Metabolism of sesquiterpene lactones. Pharmac. Ther. 48, 345-355.

Lennette E.H. (1985). Manual of Clinical microbiology (4th edn). American Association of for microbiology, Washington, DC.

Lichenthanler H.K., Schwender J., Disch A., and Rohmer M, (1997). Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett. 400: 271-274.

Logan K.T. (1965). Growth of tree seedlings as affected by light intensity. Forestry branch departmental publication No. 1121.Environment Canada pp 1-15.

Logan K.T. (1967). Growth of tree seedlings as affected by light intensity. Forestry branch departmental publication No. 1176.Environment Canada pp 1-15.

Loub W.D., Farnsworth N.R., Soejarto D. and Quinn M.L. (1985). NAPRALERT: Computer handling of natural products research data. Chem.Info. Comp. Sci. 25: 99-103.

Mabry T.J., Markham R.K., Thomas M.B. (1970). The systemic identification of flavonoids. Springer Verlag, NY pp 354.

MacKinnon S., Durst, T. and Arnason J.T. Angerhofer C. Pezzuto J.M., Sachez-Vidas P E., Poveda L.J. and Gbeassor F.M. (1997). Antimalarial activity of tropical meliaceae extracts and gedunin derivatives. J. Nat. Prod., 60: 336-341.

Malaria Foundation (1997). Global networking against malarial. Available on line: http://www.malaria.org/FACTPACK. html

McCutcheon A.R., Ellis R.M., Hancock R.E.W. and Towers, G.H.N., (1992). Antibiotic screening of medicinal plants of the British Columbian native peoples. J. Ethnopharmacology 37: 213-223.

Miguel A. Zavala S., Salud Perez G., Rosa M. Perez G. (1997). Antimicrobial screening of some medicinal plants. Phytother. Res. 11: 368-371.

Milhous W.K., Weatherly N. F., Bowdre J.H., Desjardin R.E. (1985). *In vitro* activities mechanisms of resistance to antifol malarial drugs. Antimicrob. Agents Chemother. 27:525-530.

Mills J. T. (1990). Protection of farm-stored grains and oilseeds from insects, mites and molds. Agriculture Canada Publication 1851/E. Winninpeg, MB.

Misra P., Pal N.L., Guru P. Y., Katiyar J.C., and Tandon J. S. (1991). Antimalarial acitivity against erythrocytic stages of *Plasmodium bergheii.* Int. J. Pharmacognosy 29: 1, 19-23.

Moerman D.E., 1998. Native American Ethnobotany. Tiber Press, Portland, OR.

Monzon R.B., Alvior J.P., Luczon L.L.C. Morales A.S., Mutuc R.E.S. (1994). Larvicical potential of five phillipines plants against *Aedes Aegypti* (Linnaeus) *and Culex quinquefasciatus* (Say). Southeast Asian J. Trop. Med. Public Health 25: 4, 755-759.

Moore P.A. (1981). Preparation of whole blood for liquid scintillation counting. Clin. Chem. 27: 4, 609-611.

Morrison B.J, and Franklin R.A. (1978). A rapid, hygienic method for the preparation of fecal samples for liquid scintillation counting. Anal. Biochem. 85: 79-85.

Nakatani M., James J.C. and Nakanishi (1981). Isolation and structure of trichilins, antifeedants against the southern armyworm. J. Am. Chem.Soc. 103:1228-1230.

Nishizawa M., Emura M., Yamada H., Shiro M., Hayashi Y., Tokuda H. (1989). Isolation of a new cycloartanoid triterpene from leaves of *Lansium domesticum* novel skin-tumor promotion inhibitor. Tetrahedaron Lett.30: 41, 5615-5618.

Nishizawa M., Nademoto Y., Sastrapradja S., Shiro M., Hayashi Y. (1985). Structure of dukunolides, bitter principles of *Lansium domesticum*. J. Org. Chem. 50: 5487-5490.

Nishizawa M., Nishide H., Kosela S., Hayashi Y. (1983). Structure of Lansiosides: Biologically active new triterepene glycosides from *Lansium domesticum*. J. Org. Chem. 48: 4462-4466.

Oaks S. C. Jr., Mitchell, V.S. Pearson, G. W. and Carpenter, C.C.J. (1991). Parasite Biology. In Malaria Obstacles and opportunities. National Academic Press, Washington. Pp. 90-129.

Obih P.O. and Makinde J.M. (1985). Effects of azadirachta indica in *P. bergheii* in mice. Afr. J. Med. Sci. 14: 51-54.

Phillips R.S. (1983). Malaria. Studies in Biology no. 152. Edward Arnold, London 58pp.

Phillipson J. D. and Wright C. W. (1991a). Can ethnopharmacology contribute to the development of antimalarial agents. J. Ethnopharmacology 32: 155-165.

Phillipson J.D. and Wright C.W. (1991b). Antiprotozoal agents from plant sources. Planta Medica 57, 1, 53-59.

Pinter R.M. (1973). Malaria: The design, use and mode of action of chemotherapeutic agent. Dorchester, Scientechnica 316pp.

Rang H.P., Dale M.M. and Ritter J.M. (1999). Pharmacology. Fourth edition. Churchill Livingston, London.

Ratsimamaga-Urverg S., Rasoanaivo P., Rafatro H., Robijaona B., and Rakoto-Rastimamanga, A. (1994). *In vitro* antiplasmodial activity and chloroquine-potentiation action of three new isoquinoline alkaloid dimers isolated from *Hernandia voyronii* Jumelle. Ann. Trop. Med. and Parasit. 88: 271-277.

Reinchart P.B., Bryant J.P., Clausen T.P., and Wieland G.D. (1984). Defense of winter dormant paper birch against snow shoe hare. Oecologia 65: 58-69.

Ridely R. (1997), Plasmodium: drug discovery and development- an Industrial perspective. Exp. Parasitology 87: 293-304.

Rochanakij S., Thebtaranonth Y., Yenjai C., and Yuthanvong Y. (1985). Nimbolide, a constituent of *Azadirachta indica*, inhibits *Plasmodium falciparum* in culture. Asian J. Trop. Med. and Pub. Health 16: 66-72.

Rowe A., Obeiro J., Newbold C.I., Marsh K. (1995). *Plasmodium falicparium:* Rosetting is associated with malaria severity in Kenya. Inf. and imm. 27: 810-815.

Salas F., Fichmann J. Lee, G.K., Scott M.D. and Rosenthal P.J. (1995). Functional expression of Falicpain, a *Plasmodium falciparum* cysteine proteinase supports its role as a malarial hemoglobinase. Inf. and imm. 63: 2120-2125.

Schmutterer H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Ann. Rev. Ent. 35:271-297.

Schoonhoven L.M. (1982). Biological aspects of antifeedants. Entomol. exp. appl. 31: 57-69.

Simonelli A.P., Mehta, S.C., and Higuchi W.I. (1969). Dissolution rates of high energy PVP-sulphathiazol co-precipitates. J. Pharm. Sci. 58: 5, 538-549.

Sinden R. E. (1984), The biology of *Plasmodium* in the mosquito. Experientia 40: 130-1357.

Soejarto D., and Farnsworth N.(1989). Tropical rain forests: potential sources of new drugs? Persp. Bio. and Med. 32:244-256.

Spencer C. F., Koninszy F. R., Rogers E.F., Shavel J., Easton N.R., Kaczka E.AAA., Kuehul F.A., Phillips R.F., Walti A., Folkers K. (1947). Survey of plants for antimalarial activity. Llyodia 10: 145-147.

Trager W. and Polonsky J. (1981). Antimalarial activity of quassinoids agaisnt chloroquine-resistant *Plasmodium falciparum in vitro*. Am. J. Trop. Med. and Hyg. 30:3, 531-537.

Vietnick A.J., Pieters L.A.C., Vander Berge D.A. (1995). Bioassay-guided isolation and structure elucidation of pharmacologically active plant substances. In Phytochemistry of medicinal plants ed. Arnason J.T, Mata R. and Romeo J. Plenum Press, NY.

Walter M.B., Kruger E.L.,Reich P.B.(1993). Relative growth rate in relation to physiological and morphological traits for northern hardwood tree seedings: species, light environment and ontogenetic considerations. Oecologia, 96: 219-231.

Wong K.C., Wong S.W., Siew S.S., Tie D.Y. (1994). Volatile constituents of the fruits of *Lansium domesticum* and *Baccaurea motleyana*. Flav. and Frag. 9: 319-324.

World Health Organization. (1995). WHO study group on vector control for malaria and other vector-borne diseases. Tech. Rep. Ser no. 857.

Xie Y.S., Bodnaryk R.P, and Fields P.G. (1996). A rapid and simple flour-disk bioassay for testing substances active against stored-product insects. The Can. Entom. 128, 865-875.

Xie Y.S., Fields P.G., Isman M.B., Chen W.K. and Zhang X. (1995). Insecticidal activity of *Melia toosendan* extracts and toosendanin against three stored-product insects. J. stored Prod. Res. 31: 3, 259-265.

Appendix I. Spectral data used in identification of compounds in *Prunus serotina* wood.

^{1}H NMR naringenin (500 MHz, acetone-d_6) d (ppm): 12.17 (s, H5), 9.53 (vbr s, H7), 8.53 (vbr s, $H4^1$), 7.38 (td, J=2.76, 9.43 Hz, $H2^1$, $H6^1$), 6.89 (td, J=2.84, 9.44 Hz, $H5^1$, $H3^1$), 5.95 (d, J=2.19 Hz, H8), 5.94 (d, J=2.19 Hz, H6), 5.43 (dd, J=2.98, 12.89 Hz, H2), 3.16 (dd, J=12.93, 17.13 Hz, H3), 2.72 (dd, J=3.06, 17.13 Hz, H3).

^{13}C NMR naringenin (125 MHz, acetone-d_6) d (ppm): 197.2, 167.3, 165.3, 164.3, 158.6, 130.8, 129.0, 116.2, 103.2, 96.8, 95.8, 79.9, 43.4.

^{1}H NMR 4'-methoxynaringenin (500 MHz, acetone-d_6) d (ppm): 12.16 (s, H5), 9.59 (vbr s, H7), 7.48 (d, J=8.60 Hz, $H2^1$, $H6^1$), 6.99 (d, J=8.71 Hz, $H3^1$, $H5^1$), 5.96 (d, J=2.10 Hz, H8), 5.95 (d, J=2.05 Hz, H6), 5.49 (dd, J=2.96, 12.79 Hz, H2), 3.82 (s, $H4^1$), 3.18 (dd, J=12.81, 17.12 Hz, H3), 2.75 (dd, J=3.03, 17.07 Hz, H3).

^{13}C NMR 4'-methoxynaringenin (125 MHz, acetone-d_6) d (ppm): 197.1, 167.3, 165.3, 164.3, 161.0, 131.9, 128.9, 114.8, 103.4, 96.8, 95.9, 79.8, 55.6, 43.5.

^{1}H NMR dihydro kaempferol (500 MHz, acetone-d_6) d (ppm): 11.69 (s, H5), 9.55(vbr s,H7), 8.53 (vbr s,$H4^1$), 7.41 (dd, J=1.86, 6.57 Hz, $H2^1$, $H6^1$), 6.89 (dd, J=2.05, 6.58 Hz, $H3^1$,$H5^1$), 5.99 (d, J=2.16 Hz, H8), 5.94 (d, J=2.12 Hz, H6), 5.08 (d, J=11.61 Hz, H3), 4.64 (d, J=11.62 Hz, H2), 2.87 (br s, H3).

^{13}C NMR dihydro kaempferol (125 Mhz, acetone-d_6) d (ppm): 198.2, 167.8, 165.0, 164.2, 158.8, 130.3, 129.1, 116.0, 101.5, 97.1, 96.0, 84.4, 73.1.

Appendix II ^{1}H NMR Spectral Data (500 $CDCl_3$)MHz of compounds in *Lansium domesticum* bark

Compound	Vinyl Protons	Methyl Protons	Other Protons
3-ketolansiolic acid	5.36 (s, 1H, C_{15}-H) 4.88 (s, 1H, C_{26}-H) 4.61 (s, 1H, C_{26}-H) 4.81 (s, 1H, C_{26}-H) 4.67 (s, 1H, C_{26}-H)	1.73 (s, 3H), 1.69 (s, 3H), 1.04 (s, 3H), 0.97 (s, 3H), 0.80(s, 3H), 0.78 (s, 3H)	other 23 H: 2.58, 2.40, 2.38, 2.02, 1.97, 1.81, 1.65, 1.59, 1.57, 1.43, 1.00-0.83
3-keto-22-hydroxy-onoceradiene	5.38 (s, 1H, C_{15}-H) 4.82 (s, 1H, C_{26}-H) 4.51 (s, 1H, C_{26}-H)	1.70 (s, 3H), 1.06 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H), 0.91(s, 3H), 0.74 (s, 3H), 0.64 (s, 3H)	3.23 (dd, 1H, C_{21}-H) other 23 H: 2.66, 2.61, 2.00, 1.90, 1.70, 1.55, 1.35, 1.23, 110, 1.05-0.80
Iso-onoceratriene	5.28 (s, 1H, C_{15}-H) 4.64 (s, 1H, C_{26}-H) 4.57 (s, 1H, C_{26}-H)	1.67 (s, 3H), 1.65 (s, 3H), 1.24 (s, 3H), 0.90 (s, 3H), 0.89 (s, 3H), 0.78 (s, 3H), 0.76 (s, 3H)	other 20 H: 2.16, 2.10, 2.03, 1.92, 1.73, 1.71, 1.67-1.66, 1.64, 1.55, 1.42-1.39, 1.23, 1.22, 0.92, 0.89, 0.86
Methyl lansiolate A	5.04 (s, 1H), 4.86 (s, 1H), 4.83 (s, 1H), 4.67 (s, 1H), 4.65 (s, 1H), 4.58 (s, 1H)	3.60 (s, 3H, OCH_3), 1.65 (s, 3H), 1.05 (s, 3H), 0.96 (s, 3H), 0.77 (s, 3H), 0.60 (s, 3H)	4.29 (m, 1H, HOC<u>H</u>), other 23 H: 2.65 – 0.74
Methy 15-acetoxy-Lansiolate A	5.21 (s, 1H), 4.88 (s, 1H), 4.85 (s, 1H), 4.79 (s, 1H), 4.65 (s, 1H), 4,59 (s, 1H)	3.63 (s, 3H, OCH_3), 2.00 (s,3H, CH_3CO), 1.67 (s, 3H), 1.06 (s, 3H), 0.98 (s, 3H), 0.75 (s, 3H), 0.58 (s, 3H)	5.34 (m, 1H, AcCH), 2.52 (dd, 1H, C_{17}-H), other 21 H: 2.66 – 0.80
Lansiolic acid A	5.09 (s, 1H), 4.90 (s, 1H), 4.86 (s, 1H), 4.70 (s, 1H), 4.68 (s, 1H), 4.62 (s, 1H)	1.70 (s, 3H), 1.08 (s, 3H), 1.02 (s, 3H), 0.80 (s, 3H), 0.63 (s, 3H)	4.34 (m, 1H, HOC<u>H</u>) other 24 H: 2.70 – 0.82

Appendix III ^{13}C NMR Spectral Data (500 MHz, $CDCl_3$) in compounds in *Lansium domesticum* bark

Comp.	C_3	C_8	C_{14}	C_{15}	C_{21}	C_{22}	C_{26}	C_{27}	C_{29}	Other Carbons
3-ketolansiolic acid	217.0	147.4	135.7	121.7	180.1	147.5	107.5	22.9	113.9	57.4, 54,9, 49.0, 48.2, 47.6, 39.2, 38.6, 37.7, 37.4, 34.6, 32.6, 29.5, 28.9, 27.2, 26.2, 26.0, 25.1, 22.8, 21.6, 16.3, 14.0
3-keto-22-hydroxy-onoceradiene	216.9	140.9	135.6	121.6	78.7		106.8			56.7, 55.2, 54.6, 51.5, 47.4, 39.2, 39.1, 38.1, 38.0, 37.8, 36.4, 34.6, 25.0, 24.0, 23.9, 22.2, 28.2, 27.9, 25.8, 25.5, 22.1, 15.3, 14.6, 13.6
Iso – onoceratriene		136.3	134.4	122.4	154.2	153.3	106.5			46.9, 45.1, 44.7, 44.1, 43.7, 39.7, 36.3, 31.5, 30.8, 29.6, 26.6, 26.5, 26.2, 25.8, 25.7, 25.3, 23.8, 21.6, 21.5, 19.9, 19.6, 15.4, 15.1
Methyl Lansiolate A	214.5	149.3	147.5		173.6	145.7	107.6		114.3	56.1, 55.0, 51.5, 47.6, 43.5, 42.8, 41.4, 39.2, 37.7, 37.4, 34.6, 32.2, 27.8, 26.0, 25.1, 23.4, 22.0, 21.9, 21.4, 21.3, 16.4, 13.9
Methyl 15-acetoxy-lasiolate	216.5	147.0	145.7		174.4	144.4	117.5	112.8	114.3	169.9 (Acetyl carbon) 55.7, 55.5, 51.5, 48.9, 43.5, 41.0, 39.1, 37.9, 37.7, 37.6, 34.6, 34.2, 32.0, 28.9, 26.0, 25.0, 22.9, 21.6, 21.5, 21.3, 20.9, 16.4, 13.9,
Lansiolic acid A	217.1	149.2	147.4	73.7	178.8	146.3	107.7	110.1	114.1	56.1, 55.1, 47.7, 43.6, 42.8, 41.5, 39.3, 37.8, 37.5, 36.5, 34.6, 32.0, 29.6, 26.1, 25.1, 23.4, 22.0, 21.6, 21.0, 16.6, 14.1