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Phytochemical & Pharmacological Properties of Northern Prickly Ash, *Zanthoxylum Americanum* Mill. (Rutaceae)

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PHYTOCHEMICAL & PHARMACOLOGICAL PROPERTIES OF NORTHERN PRICKLY ASH, *ZANTHOXYLUM AMERICANUM* MILL. (RUTACEAE)

Nana Fredua A. Bafi-Yeboa

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
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L'Institut de biologie d'Ottawa-Carleton

I trust I have said sufficient to interest our practitioners at least to give the article (*prickly ash berries*) a fair trial in the diseases above named; and should any further discoveries of the value of the remedy be made, I should be pleased to have them published in the columns of this journal.

- Dr. J. KING, *College Journal of Medical Sciences*, 1856.

ABSTRACT

This thesis examines the pharmacological basis for the traditional phytomedicinal uses of *Zanthoxylum americanum* Mill. (Rutaceae). The antifungal activity of six crude extracts was evaluated against 11 clinically relevant pathogenic fungi including *Candida albicans* and *Cryptococcus neoformans*. All extracts demonstrated light-activated antimicrobial action and broad spectrum of activities against at least eight fungal species. A positive correlation was observed between total furanocoumarin content and antifungal activity ($r^2 = 0.88$, $P < 0.001$). Antiviral activity towards *Herpes simplex* virus type-1 was linked to various phytochemical constituents, including three isolated pyranocoumarins suitable for authentication and standardization of commercial raw materials. Anti-HSV activity was significantly enhanced with light treatment (L), for phytochemical fractions rich in polyphenols (+L, MIC = 9.4; -L, MIC > 500) or pyranocoumarins (+L, MIC = 31; -L, MIC > 500), but not in the case of furanocoumarins (+L, MIC = 62; -L, MIC = 94). Phytochemical profiles and average content for phenolic acids and flavonoids contributing to antiviral activity of *Z. americanum* were reported here for the first time.

RÉSUMÉ

Cette thèse a examine les bases pharmacologiques de l'utilisation phytomédicale traditionnelle de *Zanthoxylum americanum* Mill. (Rutaceae). L'activité antifongique de 6 extraits bruts a été évaluée contre 11 pathogènes fongiques d'importance sur le plan clinique, incluant *Candida albicans* et *Cryptococcus neoformans*. Tous les extraits ont démontré une action antimicrobienne activée par la lumière et un large spectre d'activité contre au moins 8 souches fongiques. Une corrélation positive ($r^2 = 0.88$, $P < 0.001$) a été observée entre le contenu total en furanocoumarines et l'activité antifongique. L'activité antivirale contre le virus *Herpes simplex* de type-1 a été reliée à divers constituants phytochimiques dont trois pyranocoumarines qui ont été isolées et qui sont appropriées pour l'établissement de l'authenticité et la standardisation du matériel commercial brut. L'activité anti-VHS a été significativement augmentée lors du traitement à la lumière (L) pour les fractions phytochimiques riches en polyphénols (+L, MIC = 9,4 ; -L, MIC > 500) ou en pyranocoumarines (+L, MIC = 31 ; -L, MIC > 500), mais pas dans le cas des furanocoumarines (+L, MIC =62 ; -L, MIC = 94). Les profiles phytochimiques et les contenus moyens en acides phénoliques et en flavonoïdes contribuant à l'activité antivirale, ont été rapportés ici pour la première fois chez *Z. americanum*.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Plants have long served as the sources of remarkable cures for human ailments. Long before Latin names, chemical formulas or the complex classification systems utilized in medicinal botany were invented, the first healers employed practical botanical cures (Sumner 2000). Early knowledge of plant medicines probably date as far back as our earliest ancestors, since modern great apes are known to use some plant species in behavioural patterns that suggest these plants as being of medicinal rather than nutritional importance (Huffman 2001). As plant gatherers, the first healers crafted an extensive knowledge base of the natural pharmacy of plants with medicinal properties through trial and error. This ethnobotonical tradition is usually imparted orally from healer to healer.

Arguably, the extensive knowledge of practical botany, based on an understanding of which plants could be used safely and reliably to cure specific human ailments was and is deeply rooted in the fabric of indigenous communities. It also serves as a particular socio-cultural identity specific to these indigenous communities. Traditional medicines practiced in the past were based not only on careful observations and experimentation, but also on a community's approach to health and disease. Ultimately, the foundation and future of human medicine is as diverse and pluralistic in nature as there are indigenous communities (Rukangira 2001).

In recent years, there has been a widespread interest and increasing appeal of accessible and affordable traditional medicine (World Health Organization 2001). The scientific evaluation of many ethnomedicinal and ethnoveterinary practices may lead to the discovery of

phytomedicines with promising pharmacological activities useful in treating low intervention conditions. In a recent survey of Canadians, 70% reported having used one or more natural products (World Health Organization 2001). For this reason, it is important that all Canadians have access to safe, effective, high quality phytomedicines and that these products be thoroughly scrutinized before commercialization (Canada Gazette 2001).

Based on the traditional knowledge of plant medicines, the North American botanical market, has capitalized on many native species of medicinal plants such as N. American ginseng, *Echinacea* spp., and goldenseal as lead sources to curb and cure disease. Historically, all of these plants were well-known to Native Americans as part of their medicinal flora.

Zanthoxylum americanum Mill. (Northern prickly ash), has a long extensive history of use for medicinal purposes in Eastern North America. Northern prickly ash, was traditionally used to treat a variety of ailments from ulcers and sores, rheumatism to skin infections and toothaches with considerable success, being widely regarded by our First Nations as one of the most valuable articles of Native American medicine (Erichsen-Brown 1979).

1.1.1 *Zanthoxylum americanum Mill. (Northern prickly ash)*

The Rutaceae (Rue family) constitutes an economically important plant family of trees, shrubs and a few herbs widely distributed in the tropical, subtropical and to a lesser extent in the temperate regions of the world (Harrar and Harrar 1962; Elias 1980). It includes well-known members belonging to the genus *Citrus*, such as the orange, lemon, lime and grapefruit, valued not only for their edible fruits, but also for use as flavourings, spices, incense and in perfumes. The rue family also includes species well-known to indigenous communities and utilized in their ethnomedicinal preparations.

The genus *Zanthoxylum* L. (prickly ash), of the family Rutaceae comprises about 200 species of small aromatic trees and shrubs native to the middle latitudes of North America, South America, Africa, Asia and Australia (Sargent 1961). Based on Linnaeus' system of binominal nomenclature (1753; 1754), the prickly ash genus is known by the name *Zanthoxylum* originating from the Greek words *xanthos* and *xylon* which mean "yellow" and "wood" in reference to the a yellow dye sometimes present in the roots of some species (Hyan and Pankhurst 1995; Quattrocchi 2000). Philip Miller (1691-1771), an English botanist is credited as the authority in the classification of *Zanthoxylum americanum* (Bailey 1949; Hosie 1979; Quattrocchi 2000). The species epithet, *americanum* refers to the plant being endemic to North America (Grimm 1966).

Zanthoxylum americanum is commonly referred to as Northern prickly ash, prickly ash, common prickly ash, yellowwood, toothache tree and angelica tree (Bell *et al.* 1936; Blackburn 1952; Uphof 1968). The last two common names, toothache tree and angelica tree also serve as references to the true Angelica tree, *Aralia spinosa* an unrelated plant or the family Araliacea (ginseng family) belonging to the order Apiales. *A. spinosa* was often confused with *Z. americanum* because of its slightly similar effects and history of use by both early settlers and Native Americans. The prickly ash genus *Zanthoxylum* is classified in the division Magnoliophta, class Magnoliopsida, order Sapindales (Kaertesz 2002). This genus appears incorrectly spelled as *Xanthoxylum* in some literature.

In this thesis, I investigated the biological activity of *Z. americanum* preparations made from various parts of the plant and provided a validated HPLC based method for the analysis of these plant parts. The present work addresses the potential development of *Z. americanum* as a suitable phytomedicine for human and animal health markets worldwide.

1.1.2 *Traditional uses*

The Rutaceae is an extremely important medicinal plant family and well-known throughout the world. Besides, their economic importance in the world market as the sources of all types of citrus fruits, there is growing recognition of the special benefits of rutaceous plants as dietary supplements (Hean 1994). It includes many species with a folkloric use for treating a wide range of ailments. Despite the socio-cultural diversity of traditional medicines, phytomedical uses of *Zanthoxylum* spp. are remarkably similar from one region to another. This may be due in part to the similarity of chemical constituents throughout the genus. For example, indigenous Cameroonian tribes used *Zanthoxylum xanthoxyloides* Waterm. in the treatment of certain infectious disease including skin infections, gonorrhoea, urinary infections and dysentery (Ngane *et al.* 2000). In Chinese traditional medicine, *Zanthoxylum simulans* Hance is used for similar indications as its African relative. The aboriginal peoples of North America utilized Northern prickly ash (*Zanthoxylum americanum* Mill.) for treatment of coughs, fevers, gonorrhoea, rheumatism and external wounds (Erichsen-Brown 1979; Moerman 1998).

In addition to the universal reputed efficacy of *Zanthoxylum americanum* bark as a masticatory for the relief of toothache, lending it the common name "toothache tree", this plant was used in the holistic treatment of many specific conditions (Felter and Lloyd 1983). For example, the Eclectic doctors considered Northern prickly ash as one of the most important medicinal plants employed in indigenous remedies for promoting and maintaing health. A decoction of the bark was used for gonorrhoea as well as inflammations of the throat. The wood was used for toothache and a decoction of boiled roots to increase perspiration. The berries were considered tonic, stimulant, anti-rheumatic and used to make a spray blown on the chest and throat for chest ailments (Erichsen-Brown 1979).

Traditionally, the Chippewa gargled with or drank *Z. americanum* decoctions to treat sore throat, quinsy and swollen or ulcerated throat, as well as a bath to strengthen legs and feet of a weak child, while the Menominee used a decoction of the ripe berries to treat bronchial disease or sores. The Meskwaki used different parts of the plant to make a cough syrup, treat tuberculosis as well as chronic rheumatism and as a stimulant in gastro-intestinal flatulence and diarrhoea (Erichsen-Brown 1979). The extensive uses of *Z. americanum* parts by Native American groups are presented in Table 1.1 and although the berries and bark were most commonly utilized for their medicinal properties, the whole shrub was considered to possess active constituents. The indicated medicinal uses of *Z. americanum* suggest that its physiological effect may be on different systems within the body, such as the nervous system, circulatory system, lymphatic system and mucous membranes.

In spite of the well documented use of *Zanthoxylum americanum* in ethnobotanical literature and identification of a number of the plant's constituents, there are very few scientific articles that have addressed the medicinal properties of this plant and attempted to explain and evaluate them, via biological analysis (Erichsen-Brown 1979; Saqib *et al.* 1990; Moerman 1998).

Table 1.1: Categorical ethnomedicinal uses of *Zanthoxylum americanum* by First Nations groups[1].

Ethnomedicinal use	Group
Abortifacient	Iroquois
Adjuvant	Menominee
Analgesic	Iroquois; Menominee
Anthelmintic	Iroquois
Antiemetic	Iroquois
Antirheumatic (External)	Cherokee; Menominee
Antirheumatic (Internal)	Algonquin
Burn Dressing	Comanche
Cold Remedy	Chippewa; Menominee
Cough Medicine	Meskwaki
Dermatological Aid	Alabama; Menominee
Diuretic	Iroquios
Emertic	Iroquois
Expectorant	Meskwaki
Febrifuge	Comanche
Gastrointestinal Aid	Iroquios
Gynecological Aid	Iroquois
Heart Medicine	Daleware; Daleware, Oklahoma; Mohegan
Hemostat	Meskwaki
Kidney Aid	Iroquois; Meskwaki
Orthopedic Aid	Chippewa; Iroquios
Other	Menominee
Pediatric Aid	Chippewa
Pulmonary Aid	Chippewa; Menominee; Ojibwa
Respiratory Aid	Ojibwa
Sedative	Menominee
Strengthener	Meskwaki
Throat Aid	Chippewa; Comanche; Meskwaki; Ojibwa
Tonic	Delaware, Oklahoma
Toothache Remedy	Alabama; Comanche; Iroquois; Meskwaki
Tuberculosis Remedy	Iroquois; Meskwaki
Venereal Aid	Iroquois; Ojibwa; Potawatomi; Winnebago
Veterinary Aid	Creek; Pawnee

[1]As reported by Erichsen-Brown 1979; Moerman 1998.

1.1.3 *Ecology*

Zanthoxylum americanum is an indigenous deciduous North American shrub occurring in Eastern Canada and throughout the northern portion of the Eastern United States. This species occurs within the temperate deciduous forests of North America, growing beneath such species as beech, red oak, hemlock and black willow (Meilleur *et al.* 1994). This shrub does well in poor soils and in partial sun to full sun, but is intolerant to shade of mature forests. It commonly occurs along riverbanks, moist low-lying grounds as well as in drier well-drained sites such as upland rocky hillsides and open woods (Meilleur *et al.* 1994). The natural distribution of wild *Z. americanum* plants ranges from southern Quebec and Ontario, Canada to Minnesota and south to Louisiana and the peninsula of Florida, both of the USA (Figure 1.1).

1.1.4 *Morphology*

Z. americanum is a dioecious shrub or small tree growing to a height of 1-5 m, with a strong odour reminiscent of the lemon when bruised (Grimm 1966). This aromatic woody plant has a short trunk bearing numerous grey to dark brown branches covered by paired broad based 4 to 8 mm-long nodal prickles at the crescent-shaped leaf scars (Figure 1.2)(Elias 1980). The sharp and strong thorns are not restricted to the trunk and branches, but are found throughout the aerial portion of this plant, including the stout pinnately compound leafstalk (Figure 1.2). The leaf is alternate, with 5 – 11 leaflets, normally 7 dark green ovate leaflets, 2 – 7.5 cm in length, 1 – 3.5 cm wide, glandular-dotted upper surface and paler underside (Grimm 1966; Elias 1980). Staminate and pistillate flowers are borne on separate plants and appear as small clustered greenish-yellow flowers blooming in the spring (April or May) before the leaves emerge.

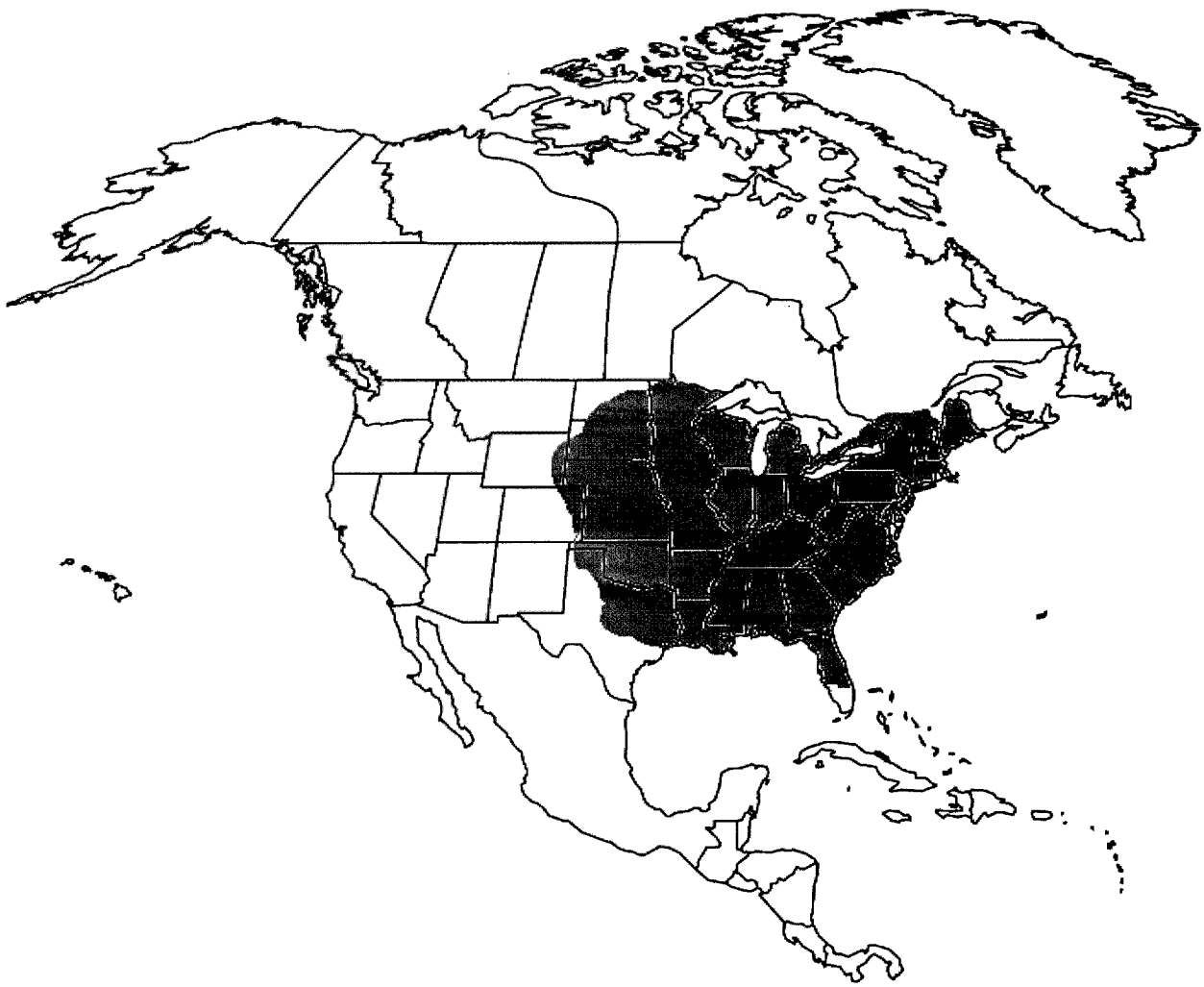


Figure 1.1 Natural distribution of *Z. americanum* Mill. (Northern prickly ash) (USDA 2002).

Figure 1.2 Photographs of *Zanthoxylum americanum* Mill., (Northern prickly ash).
Photos by: **A & D**, Nana Fredua Bafi-Yeboa; **B**, Dr. Donald Farrar and Anna Gardner (with permission) and **C**, John Baker (with permission).

A. Grey to dark brown colouration of *Z. americanum* bark.

B. Pinnately compound leafstalk with berries (insert).

C. Dense clusters of mature *Z. americanum* fruit.

D. Clonal stand of *Z. americanum* shrubs.

Berry-like fruits are produced in dense clusters on previous year's terminal growth as small globose capsules 4 – 5 mm in diameter, housing a single seed. The fruit ripens, turning from green to a reddish-brown in the late summer, with mature capsules opening to discharge a shiny black seed (Grimm 1966; Elias 1980). Fall sowing of untreated seed immediately after collection alleviates the strong dormancy of these seeds and required scarification to germinate (Bonner 1974). *Z. americanum* is easily propagated by wood cuttings because of its underground spreading stems that send up shoots to form monoclonal stands (Figure 1.2)(Elias 1980).

1.2 Phytochemistry

Although numerous chemical and phytochemical studies of the various *Zanthoxylum* species can be found in the literature, there are very few references to previous work on *Z. americanum*. Much of the phytochemical and pharmacological research has focused on *Zanthoxylum* species employed predominately in the Asian and African indigenous system of medicine (Islam *et al.* 2001; Nissanka *et al.* 2001). *Zanthoxylum clava-herculis* (Southern prickly ash) and *Zanthoxylum americanum*, two species native to North American, have shown the occurrence of alkaloids, lignans and coumarins (Fish *et al.* 1975; Duke 1992). Many members within the genus are reported to produce an elaborate variety of biologically active secondary metabolites belonging to the previously mentioned compound classes which demonstrates the chemotaxonomic significance of these compounds within the genus (Gray and Waterman 1978; Ju *et al.* 2001).

1.2.1 *Isoquinoline Alkaloids*

The genus *Zanthoxylum* is a particularly rich source of alkaloids, in particular the benzophenanthridine and aporphine alkaloids in the isoquinoline alkaloid family (Harbonrne and Baxter 1993). Isoquinoline alkaloids represent the largest single class of plant alkaloids derived biosynthetically from phenylalanine and/or tyrosine. Fish *et.al* (1975), in a chemical investigation of the stem and root-bark of *Z. americanum* reported on the occurrence of three benzophenanthridine alkaloids typical of *Zanthoxylum*: nitidine, chelerythrine and tembaterine. The major benzophenanthridine and aporphine alkaloids identified from *Z. americanum* are shown in Figure 1.3. These compounds possess a wide range of biological activities, the most significant being antitumor properties (Krane *et al.* 1984; Tan *et al.* 1991)

Review of phytochemical literature on compounds present in *Z. americanum* as reported in the NAPRALERT database (obtained from the University of Illinois) suggests isoquinoline alkaloids are localized to the root-bark and or stem-bark.

1.2.2 *Coumarins*

Coumarins constitute a major class of O-heterocyclic natural products and belong to the group of compounds known as benzopyrones, all of which consist of a benzene ring joined to a pyrone (Ju *et al.* 2001). Like other phenylpropanoids, the biosynthesis of coumarins normally proceeds from *p*-hydroxycoumaric acid to the hydroxycoumarins, umbelliferone (Harbonrne and Baxter 1993). Isoprenylation of this key intermediate gives rise to the simple prenyl coumarins and derived furanocoumarins and pyranocoumarins. The broad pharmacological profiles of coumarins include anticancer, antiviral, diuretic, carcinogenic and anticoagulent activities (Gray and Waterman 1978; Ju *et al.* 2001).

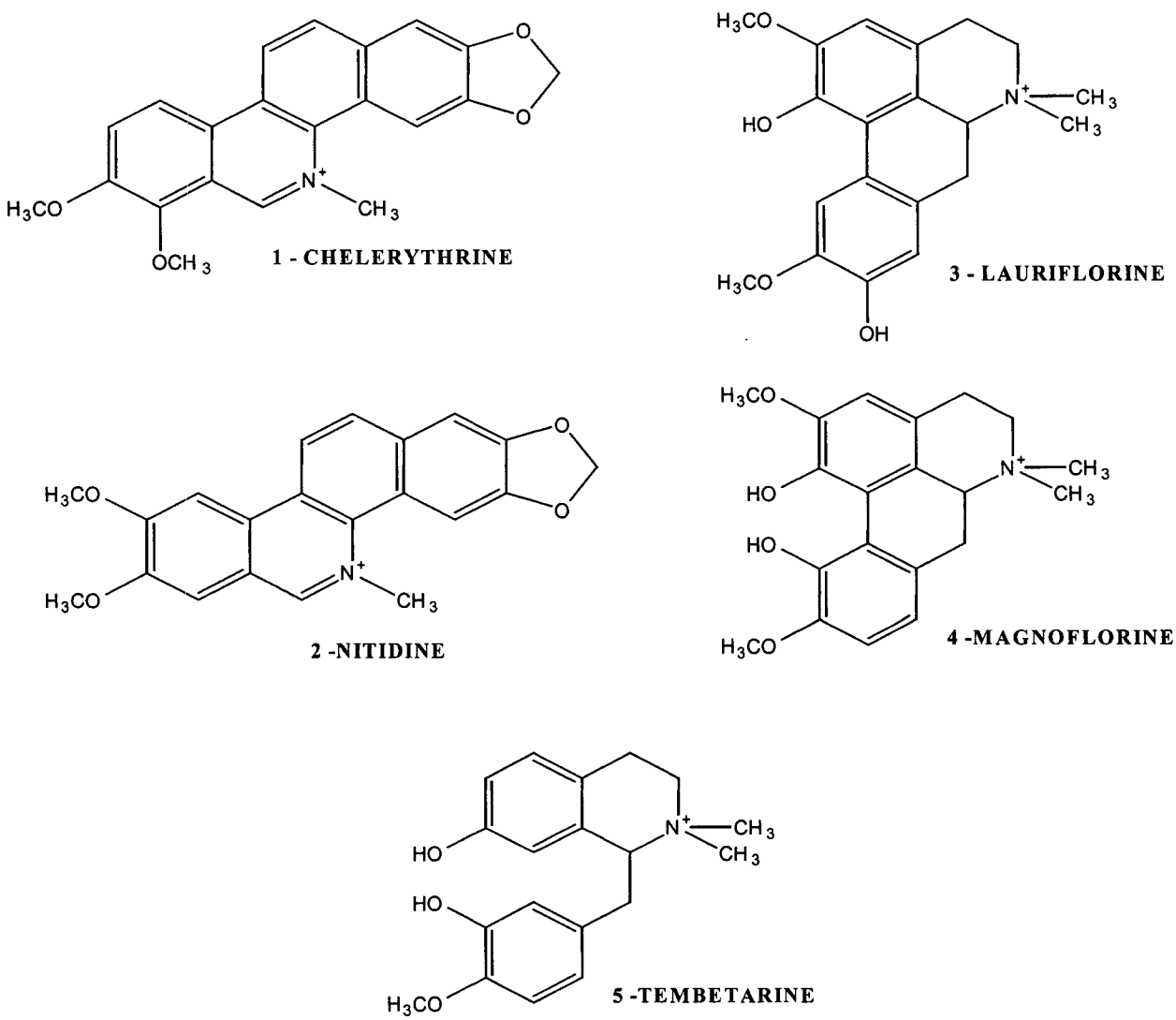


Figure 1.3 Chemical structures of the major isoquinoline alkaloids reported to occur in *Z. americanum* (Stermitz *et al.* 1980; Kane *et al.* 1984; Guinaudeau *et al.* 1988).

Saqib *et.al* (1990) identified several furanocoumarins typically found in the berries of *Z. americanum* whereas work by both Bell *et al.* (1936) and Fish *et al.* (1975) isolated the pyranocoumarins xanthoxyletin, alloxanthoxyletin and xanthyletin as constituents of the bark of *Z. americanum* (Figure 1.4).

1.2.3 *Lignans*

Besides the earlier phytochemical work on the bark and berries resulting in the identification of coumarins and alkaloids, Ju *et al.* (2001) reported on the isolation of two tetrahydrofurofuran lignans, sesamin and asarinin. Lignans of the tetrahydrofurofuran series have been shown to possess some insecticidal as well as medicinal properties including cytotoxic and antimitotic properties (Greger and Hofer 1980). In addition, sesamin, a lignan first characterized in sesame seed oil, is a synergist for pyrethrum insecticides and may synergize many other compounds and also demonstrate similar antitumor activity as noted with asarinin (Ju *et al.* 2001).

1.2.4 *Other constituents*

Z. americanum in addition to containing isoquinoline alkaloids, coumarins and some lignans, contains the furoquinoline alkaloids γ-fagarine and skimmianine, reportedly present in the leaves (Brady and Sun 1985) and the steroid β-sitosterol, a constituent of the root-bark (Fish *et al.* 1975; Duke 1992). This plant may also possess small quantities of other biologically active compounds (terpenes, tannins, saponins etc.) reported in phytochemical characterization of other *Zanthoxylum* species (Ngane *et al.* 2000).

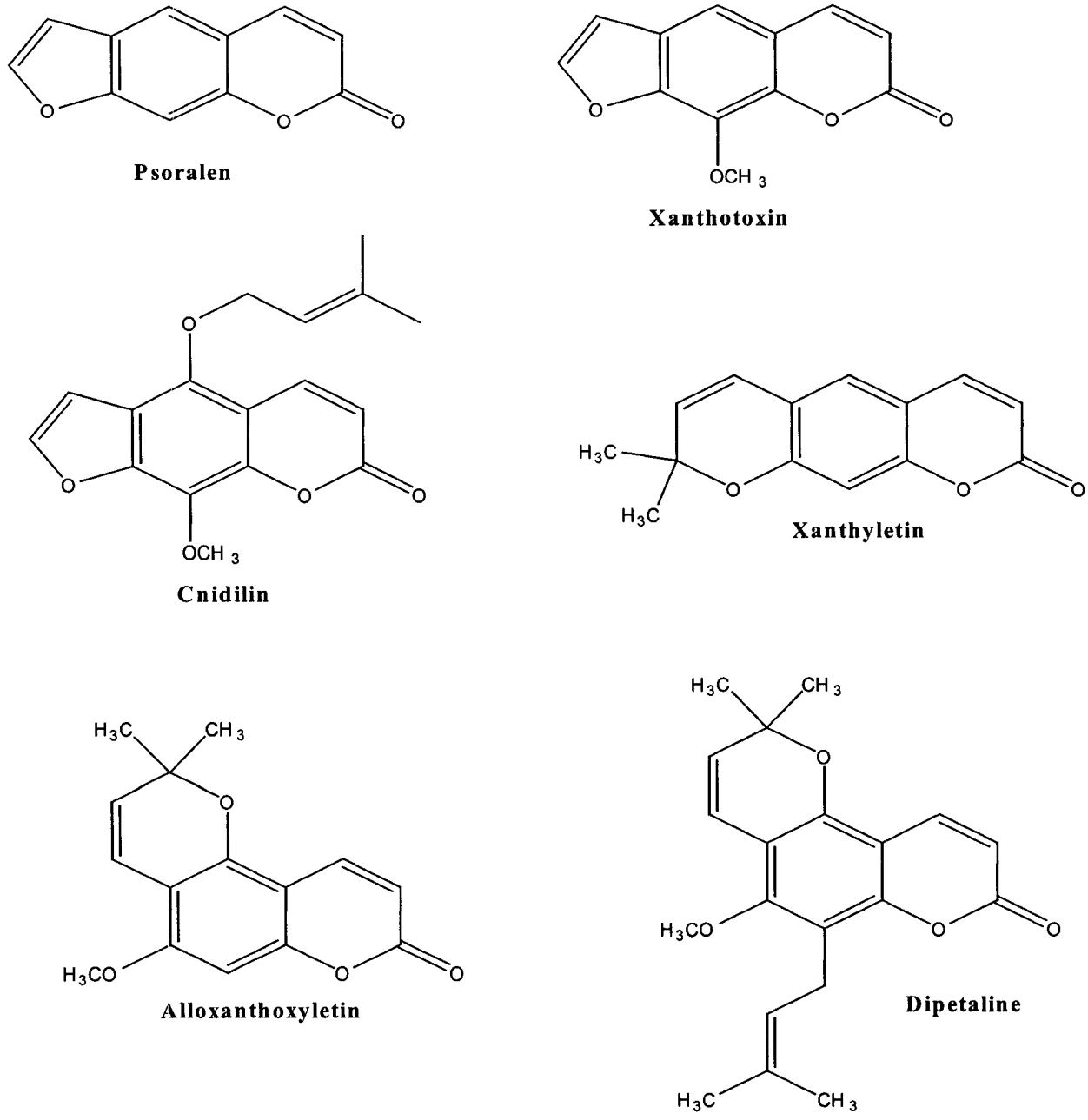


Figure 1.4 Chemical structures of some coumarin compounds identified in *Z. americanum* that have undergone elaborations of the basic structure through isoprenylation.

1.3 Pharmacology of constituents

In spite of the long use of Northern prickly ash by Native Americans and in frontier times as a reputed remedy for many complaints including toothache, rheumatism and external joint pain, few studies have investigated the biological activity of prickly ash. An exception to this was the recent investigations of *Z. americanum* extracts as potential leads for natural antitumor agents (Saqib *et al.* 1990; Ju *et al.* 2001).

However the continuing popularity and acceptance of traditional medicines has catalyzed an interest in the study of other *Zanthoxylum* species, their phytochemicals and potential for bioactivity (Kerr et al. 1999; Rukangira 2001). This is especially true for *Zanthoxylum* spp. with applications in the Asian or African traditional medicine system (Nissanka et al. 2001). Such studies have ascribed pharmacological activities to several constituents that are reportedly present in *Z. americanum* as well.

The presence of aporphine and benzophenanthridine alkaloids, mainly magnoflorine, nitidine and cherelythrine in Northern prickly ash could offer some justification for the traditional uses of this plant. This class of quaternary alkaloids in the isoquinoline alkaloid family has been shown to have antitumor activity, antifungal activity, cardiac activity (inotopic effect) and an inhibitory effect on the reverse transcriptase (RT) in RNA tumor virus (Phillips and Castle 1981; Bowen *et al.* 1996). Quaternary alkaloids are also well-known for their effects on neuromascular and ganglionic transmission. Magnoflorine has been reported to have neuromascular blocking activity. Similarly, synthesized laurifoline has been reported to reduce blood pressure through a ganglionic blocking action (Bowen *et al.* 1996).

Furanocoumarins have been successfully used in the treatment of skin disorders such as vitiligo, inhibition of tumour transmitting capacity of tumor cells, inactivation of DNA viruses

and various other applications (Song and Tapley 1979). In addition to their therapeutic application, these compounds have adverse effects in humans. This is because furanocoumarins are photogenotoxic and are therefore biological activity against not only fungi, bacteria or viruses, but also against invertebrates and higher organisms or combinations of these (Towers 1984; Bethea 1999). Although furanocoumarins have been shown to produce singlet oxygen and superoxide radicals in vitro, this does not represent an important mode of action *in vivo* (Towers 1984).

1.4 Rationale for study

The present investigation of *Z. americanum* is aimed at addressing its use by First Nations groups to treat various ailments. In particular I provide biological and scientific evaluation of the traditional medicinal uses of the plant for infectious diseases/conditions and potential pharmacological applications.

1.4.1 *Objectives of the thesis*

The overall objective of this thesis was the phytochemical characterization of *Z. americanum* constituents in relation to biological activity. The specific objectives were as follows:

Objective 1: To measure the light-mediated antifungal properties of *Zanthoxylum americanum* plant extracts against a broad range of clinical pathogenic fungi and to identify active principles.

Hypothesis: Antifungal activity of *Z. americanum* extracts is dependent on furanocoumarin content. A specific prediction tested was that antifungal activity is highly and positively correlated to furanocoumarin content.

Objective 2: To isolate and identify compounds suitable for authentication and standardization of non-photosynthetic parts, bark and wood by HPLC.

Objective 3: To measure the light-mediated antiviral activities of various phytochemical fractions prepared from *Z. americanum* extracts and to characterize their polyphenolic compositions.

Hypothesis: Antiviral activity of *Z. americanum* extracts is dependent on polyphenolic content and composition. In particular, antiviral activity of extracts should increase with increases in the content of polyphenolic constituents (i.e. phenolic acids, tannins and flavonoid).

CHAPTER 2

EVALUATION OF *Z. AMERICANUM* PLANT PARTS FOR ANTIFUNGAL ACTIVITY

2.1 Introduction

Zanthoxylum americanum Mill. (Rutaceae), also known as Northern prickly ash, is a well-known member of the Rue family and a prominent plant in the Native American pharmacopoeia. Native to Eastern North America, this woody shrub is common in rocky woods, thickets and along streams ranging in the south from Virginia to Mississippi and northward to Ontario and Quebec (Erichsen-Brown 1979; Felter and Lloyd 1983). Traditional ethnomedicinal preparations of Northern prickly ash utilized all parts of the plant with the roots, wood, bark and berries, being used predominately to treat rheumatic conditions, toothaches, sore throats, burns and as a tonic for various ailments (Moerman 1998). In addition, *Z. americanum* preparations are also commonly indicated by naturopaths and herbalists for the treatment of infectious and topical conditions such as upper respiratory infections, skin and urogenital infections caused by fungi including candidiasis (Erichsen-Brown 1979; Moerman 1998). A summary of traditional uses by First Nations groups that likely relate to treatment of mycosis is given in Table 2.1.

However, despite its extensive use by Native Americans and its ranking among the most important phytomedicines utilized by the traditional healers, there have been few attempts at exploring the pharmacological properties of prickly ash which may be related to the various ethnomedicinal uses of *Z. americanum*.

Table 2.1 Traditional ethnomedicinal preparations from *Z. americanum* plant parts by different First Nations groups to remedy afflictions originating from, or potentially involving, a fungal origin. (Data compiled from Erichsen-Brown 1979; Moerman 1998).

Condition	Preparation & part used	Form of administration[1]	Group
Bronchial disease/congestion	Infusion of ripe berries	E: sprayed on chest/throat	Menominee; Ojibwa
Burns	Pulverized root	E: root powder applied to area	Comanche
Cough	Decoction of bark & berries	I: taken as a syrup	Meskwaki
	Infusion of bark	I: drank as tea	Chippewa
Itchy skin	Decoction of bark	E: wash for itchy area	Alabama
	Infusion of inner bark	E: rubbed on itchy area	Alabama
Pulmonary trouble	Infusion of the bark	I: taken orally	Chippewa
Ulcerated throat	Decoction of root	I: gargle/drank	Chippewa
	Infusion of berries	I: drank as tea	Ojibwa
	Inner bark	I: placed in throat	Comanche
Wounds/Skin sores	Infusion of ripe berries	E: sprayed onto sore	Menominee

[1]E, external use; I, Internal use

Only limited pharmacological and phytochemical investigations have been undertaken on prickly ash. Saqib *et al.* (1990) demonstrated that extracts from the aromatic fresh berries of *Z. americanum* showed significant toxicity to brine shrimp larvae and were cytotoxic to human tumor cells. Using bioassay-guided isolation, 5 furanocoumarins were identified in the berries. These were isoimperatorin, cnidilin, imperatorin, psoralen and xanthotoxin. The cytotoxicity of other prickly ash plant parts has also been demonstrated. Recently, Ju *et al* (2001) reported on 4 pyranocoumarins and 2 lignans from the stem and root bark that exhibited an inhibitory effect on DNA synthesis in human leukemia cells (HL-60). Nonetheless, the study of antifungal biological activity from *Z. americanum* extracts has yet to be investigated.

Research on the antifungal activity of plants traditionally used for the treatment of infectious and topical conditions has shown that many of these plants have significant activity on a wide range of microorganisms and contain useful anti-parasitic, antifungal, antibacterial and/or antihistamine compounds (Jones *et al.* 2000; Omar *et al.* 2000; Islam *et al.* 2001; Ficker *et al.* 2003). In light of these previous studies and on the basis of the long and extensive use in treating infectious conditions, *Z. americanum* could potentially be an effective antifungal.

In this study, we undertook the evaluation of the antifungal properties of *Zanthoxylum americanum* plant extracts against a broad range of clinical pathogenic filamentous and yeast-like fungi chosen to reflect the causative agents for a variety of infections. This includes opportunistic human skin and systemic pathogens causing diseases such as candidiasis, cryptococcosis and skin infections. Because rutaceous plants often produce prooxidant or phototoxic phytochemicals (Asthana *et al.* 1993), including furanocoumarins against pathogens and herbivore attacks, the contribution of phototoxicity was examined. The hypothesis that

antifungal activity of *Z. americanum* extracts is dependent on furanocoumarin content was tested.

2.2 Materials and Methods

2.2.1 *Plant material and extract preparation*

Plant samples of *Z. americanum* were harvested during the summers of 2002 and 2003 from several wild shrubs growing in Sault Ste. Marie (Northern Ontario), North Gower and Belleville both of Eastern Ontario, Canada and voucher specimens retained at the University of Ottawa Herbarium, Ontario, Canada. Fresh leaves and fruit were separated and ground in a blender with 95% ethanol. Wood, bark, root, husk and seed were dried at ambient temperature and milled to a fine powder (1 mm sieve size) using a Thomas-Wiley laboratory mill (model 4, Arthur H. Thomas Co., PA, USA), prior to extraction with 95% ethanol (biomass to solvent ratio was approximately 1 g to 9 mL). Following vacuum filtration of the suspension (Buchner funnel, Whatman filter paper #1), and rotoevaporation at 50 °C, the crude alcohol extract was stored at 7 °C until use in disk diffusion bioassay. In addition to the crude ethanolic extracts, fractionated extracts for selected plant parts were also tested. Using solvents of increasing polarity (hexane, ethyl acetate, methanol and water), these respective parts were successively extracted (approximately 9 ml/g dry plant material) by sonication and vacuum filtered with resulting fractionated concentrated by rotoevaporation to near dryness.

2.2.2 *Fungal cultures*

The fungal strains used in this study are all opportunistic pathogens of humans. The strains were selected to represent diverse fungal groups and growth forms (Table 2.2). Included in this group were the pathogenic yeast-like fungus, *Candida albicans*, the causative organism in genital, oral and invasive or deep candidiasis and *Cryptococcus neoformans*, which causes cryptococcal meningitis. The filamentous fungi used included species associated with superficial and systemic infections. These fungi were cultured on Sabouraud's dextrose agar medium (SDA, DIFCO 10 g/L neopeptone + 20 g/L dextrose and 15 g/L agar) at 30°C. All manipulations of fungal cultures were carried out in a biohazard level II laminar flow hood (BioKlone 2, Microzone, Ottawa, ON, Canada).

2.2.3 *Validation of antifungal assay*

Two different procedures involving the disk susceptibility method were used to evaluate the antifungal activity of *Z. americanum* extracts. The implemented procedure was dictated by whether the plant extract was used to challenge a yeast-like or filamentous fungus.

The assay used to evaluate antifungal activity of *Z. americanum* extracts against yeast-like fungal species was validated using *Saccharomyces cerevisiae* (procedure A). In this procedure, the inoculum, measured as the absorbance or optical density of the fungal cell suspension read at 600 nm, was varied from about 0.1 to 0.6 and the amount of extract applied tested at 500 and 600 µg/disk.

Table 2.2 Fungal pathogens used to evaluate the biological activity of *Z. americanum* extracts.
(Source: Laskin and Lechevalier 1973; Singleton and Sainsbury 1987; Ficker 2001).

Fungal species	Culture number[1]	Infection types[2]
Filamentous		
Alternaria alternata	OMH-FR9884	Sinusitis, cutaneous and subcutaneous infections
Aspergillus fumigatus	OMH-FR2837	Opportunistic, mucosal, respiratory and subcutaneous infections
Fusarium oxysporum	OMH-FR6448	Opportunistic, keratomycosis, subcutaneous infection
Microsporum gypseum	OMH-FR2385	Cutaneous, Tinea pedis (athletes foot), tinea corporis (smooth skin)
Pseudallescheria boydii	OMH-FR2625	Sinusitis, CNS colonizer, infections of the respiratory tract
Rhizopus sp.	OMH-FR2874	Opportunistic, invasion of tissue and surrounding vessles
Trichophyton mentagrophytes	OMH-T2379	Tinea pedis (athletes foot), tinea corporis (trunk, arms, legs)
Yeast-like		
Candida albicans	OMH-FR2853	Opportunistic, cutaneous, mucocutaneous or systemic infections
Cryptococcus neoformans	OMH-FR2704	Opportunistic, systemic
Saccharomyces cerevisiae	ATCC 48252	Systemic infections in immuno-compromised hosts
Wangellia dermatitides	OMH-FR2236	Can act as a dermotropic and neurotropic pathogen

[1] ATCC, American Type Culture Collection

[2] Classification on basis of tissue levels initially colonized.
Superficial – limited to outer layers of skin and hair
Cutaneous – deeper in the epidermis, hair and nails
Subcutaneous – involve the dermis, subcutaneous tissues and muscles
Systemic – generally originating in the lungs
Opportunistic – involve a variety of body sites

Alternaria alternata and *Trichophyton mentagrophytes* where used to evaluate the disk diffusion assay involving filamentous fungi (procedure B). In this procedure, the appropriate inoculum size was determined by varying the volume (10ml, 30 ml and 40ml) of deionized water and the diameter (7 mm, 10 mm and 12 mm) of the circular plug of mycelium.

2.2.4 *Assay for antifungal activity*

In the case of assays involving a yeast-like fungus (procedure A), a swab was taken from an actively growing culture and transferred into a Sabouraud's dextrose broth (DIFCO 10 g/L neopeptone and 20 g/L dextrose) in 10 x 100 mm culture tubes and allowed to grow overnight. The overnight culture was adjusted to an optical density (OD) of about 0.5 read at 600 nm and used in inoculation of agar plates. For filamentous fungi (procedure B), inoculum was prepared by excision of a circular plug (7 mm diameter) containing actively growing mycelium which was transferred to 10 ml of sterile, deionized water and fragmented for three 30 second cycles in a water chilled Waring blender. A volume of 100 μL of each fungal cell suspension was pipetted onto Sabouraud's dextrose agar plates (15 x 100 mm) and uniformly allotted with a sterile bent glass rod. One hundred and twenty milligrams of the dried extract was dissolved in 10 ml of 95% ethanol for a final concentration of 120 μg/10 μL and a total volume of 50 μL applied to 7 mm diameter sterile filter disk (Whatman filter paper #1) using 10 μL aliquots. Impregnated disks (600 μg of extract per disk) were allowed to air-dry, evaporating the organic solvent, prior to being placed on the inoculated petri plates (n = 4 disks per plate). All experiments were conducted in duplicate to facilitate assessment of antifungal activity in the presence and absence of ultraviolet (UV) light. Parafilm sealed petri dishes were wrapped in tin foil and incubated at 30° C in the case of plates assigned to the dark treatment that required no UV exposure. To

determine ultraviolet light effects on activity, plates were irradiated with near-UV light (10 W/m^2 from four 20 W black light blue tubes, 320-400 nm range) for 2 hours, wrapped in tin foil and then incubated at 30° C in the dark before examination.

Following an incubation period of 48 hours, plates were removed from the incubator and the antifungal activity of that extract evaluated according to the method of Binns *et al.* (1999) by quantifying zones of inhibition of fungal growth. These clear zones were measured in millimeters as the shortest distance from the outer edge of the filter disk to the edge of visible fungal growth.

As a positive control, four filter disks were each impregnated with 600 µg of the photosensitizing agent 8-methoxypsoralen (Sigma-Aldrich, St. Louis, MO, USA) and used in both light treatments (with and without UV exposure). Similarly, a negative (vehicle) control was also carried out by applying 50 µL of 95% ethanol to disks and allowing evaporation of solvent prior to placement on inoculated plates.

2.2.5 *High Performance Liquid Chromatography*

In order to fully test the hypothesis that antifungal activity of *Z. americanum* extracts was related to furanocoumarin content, HPLC analyses of extracts used in antifungal assay were conducted in duplicate. The respective extracts (3–7 mg) were reconstituted into 1 ml of 80% ethanol, filtered (Chromospec 0.2 µm PTFE), 700 µL of the filtrate removed and added to 700 µL of 80% ethanol. Determination of furanocoumarins in extracts was accomplished by injecting 5 µL samples on a reverse phase C-18 column (LiChrospher 125 x 4 mm internal diameter, 5 µm particle size) fitted with a guard column (4 x 4 mm i.d., 5 µm particle size). Separation was achieved using a solvent system of H_2O, sodium dihydrogen ortho-phosphate monobasic buffer

($NaH_2PO_4 \cdot H_2O$) and acetonitrile (MeCN), at a flow rate of 1.0 mL/min. A linear gradient of 20-50% MeCN over 20 minutes, 50-55% for the following 10 minutes with buffer being held constant at 10% throughout the 30 minute run time. Compounds were monitored simultaneously at 200-400 nm, 310 nm, 335 nm and identified by comparison with reference standards at 225 nm. The furanocoumarins used as standards in this study were obtained from the following sources: psoralen and 8-methoxypsoralen (Sigma-Aldrich, St. Louis, MO, USA), angelicin, isopimpinellin and imperatorin (Indofine Chemical Company, Inc., NJ, USA).

2.2.6 *Statistical Design and Analyses*

The zone of inhibition around each disk was measured and recorded for each extract of *Z. americanum* tested against the respective fungal organisms. Mean zones of inhibition ($n = 4$) along with standard deviation were calculated accordingly for each fungal organism and extract combination. Where possible, a comparison between treatment means (with and without UV exposure), for a given extract was statistically tested for significant difference using pairwise *t*-tests. Statistical tests for differences among extracts as to their antifungal efficacy were limited to comparisons within the same trial and for the same treatment. In the event where ANOVA results showed significant differences among extracts tested against the same fungal organism, a matrix of pairwise comparison probabilities based on the Bonferroni Adjustment method was used to determine where differences existed. The relationship between total furanocoumarin content in plant extracts and toxicity to fungi was examined using a linear regression. The zone of clearance (measured as the distance from the outer edge of the filter disk to the edge of visible fungal growth) is more accurately a circumference of inhibition, calculated as $2\pi r$, where r is the zone of inhibition plus the radius of the filter disk (3.5 mm). Recognizing this fact, the log-

transformed circumference of inhibition was regressed on the log transformed total furanocoumarin content. All statistical analyses were performed using SYSTAT 10.1 (SPSS Corp., Chicago, IL, USA).

2.3 Results and Discussion

2.3.1 *Validation of antifungal assay*

Although the disk susceptibility test is regarded as a rapid and reproducible screening method for antifungal activity and accounts for the majority of applied antifungal assays that have been published standardization is still of utmost importance (Hadacek and Greger 2000). In particular, establishing the appropriate inoculum size is important in reducing within trial variability and improving inter-laboratory comparisons, both of which are advantageous in clinical screenings and natural products research of antifungal agents.

The results of preliminary testing of at least three crude ethanolic extracts for antifungal activity against *Saccharomyces cerevisiae* in the presence of UV are shown in Figures 2.1 and 2.2. All extracts were active only when treated in combination with UV-light. Figure 2.1 demonstrated a number of relevant issues with respect to standardization of the disk diffusion assay, mainly the effect of inoculum density and organic solvent concentration on the inhibition zone. With comparisons being limited to within treatments (extracts tested against the same fungal species) only, the variability observed between zones of inhibition is attributable to inoculum density as evidenced by the magnitude of the corresponding mean inhibition zones and associated standard deviation.

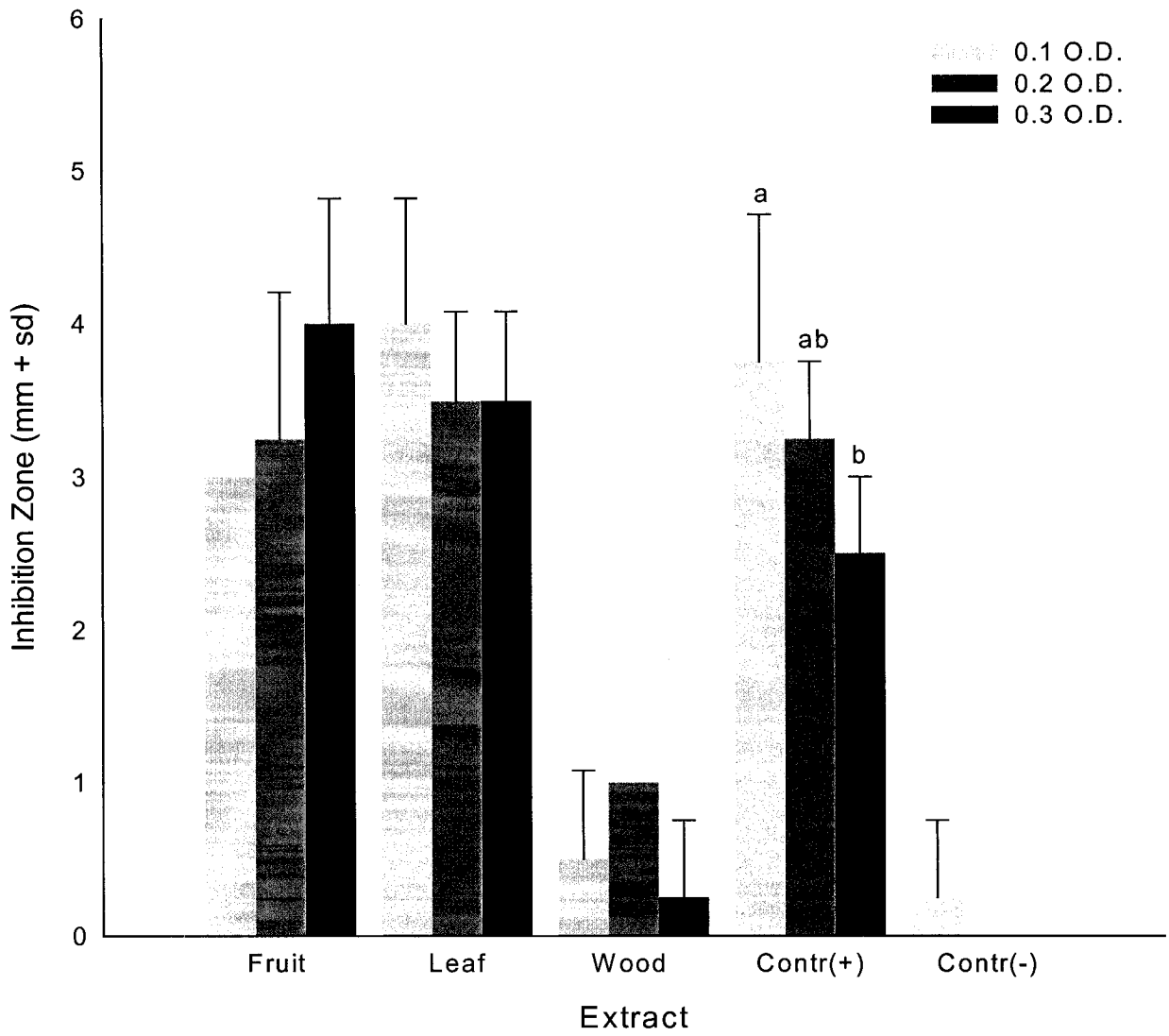


Figure 2.1 Inhibition of *S. cerevisiae* growth (after 48 hours) by *Z. americanum* ethanolic extracts using the disk diffusion method (0.6 mg/disk, $n = 4$). ANOVA and Bonferroni (P=0.05) was used to statistically test the effect of inoculum density (O.D.), measured as the absorbance of the fungal cell suspension read at an optical density of 600 nm, within treatments. Means with the same letter are not statistically different. Experiments conducted in the presence of UV light.

With the exception of the positive control treatment (ANOVA, p=0.036), the effect of the three tested inoculum densities (*S. cerevisiae* cell suspension adjusted to an optical density of about 0.1, 0.2 and 0.3 at 600 nm) on a test compound's ability to inhibit was not statistically different. In the negative control, a slight solvent effect was detected on plates inoculated with an overnight culture adjusted to an optical density of about 0.1, but was not statistically different from plates inoculated with 2-3x that amount.

In Figure 2.2, the actual biological activity of the test compound or extract is illustrated with increases in inoculum density (absorbance) having no impact on inhibition zones within treatments. Therefore, as the general standard methodology for conducting all future antifungal testing involving yeast-like fungi in this study, we adjusted inocula to an optical density at 600 nm to between 0.5-0.6.

Similar to the work carried out with *S. cerevisiae,* efforts were made to standardize test procedures involving filamentous fungi. Our attempt at a standardized quantitative plating method was limited to varying the size of the mycelial plug and the volume of liquid used in its fragmentation in order to derive the stock fungal cell suspension. Results in Table 2.3 indicate that by reducing the volume of deionized water from 30 ml to 10 ml and decreasing the diameter of the mycelial plug from 12 mm to 7 mm respectively, the antifungal efficacy of extracts tested against *Alternaria alternata* increases. This effect was rather unexpected since increases in inoculum size are more likely to lead to smaller inhibition zones as a result of increasing numbers of fungal targets potentially exceeding the given amount of an extract to inhibit growth (Hadacek and Greger 2000). However, this effect was not a response unique to *Alternaria alternata.* Comparable results were also noted with *Trichophyton mentagrophytes* where zones of inhibition increased with a reduction in inoculum size (Table 2.3).

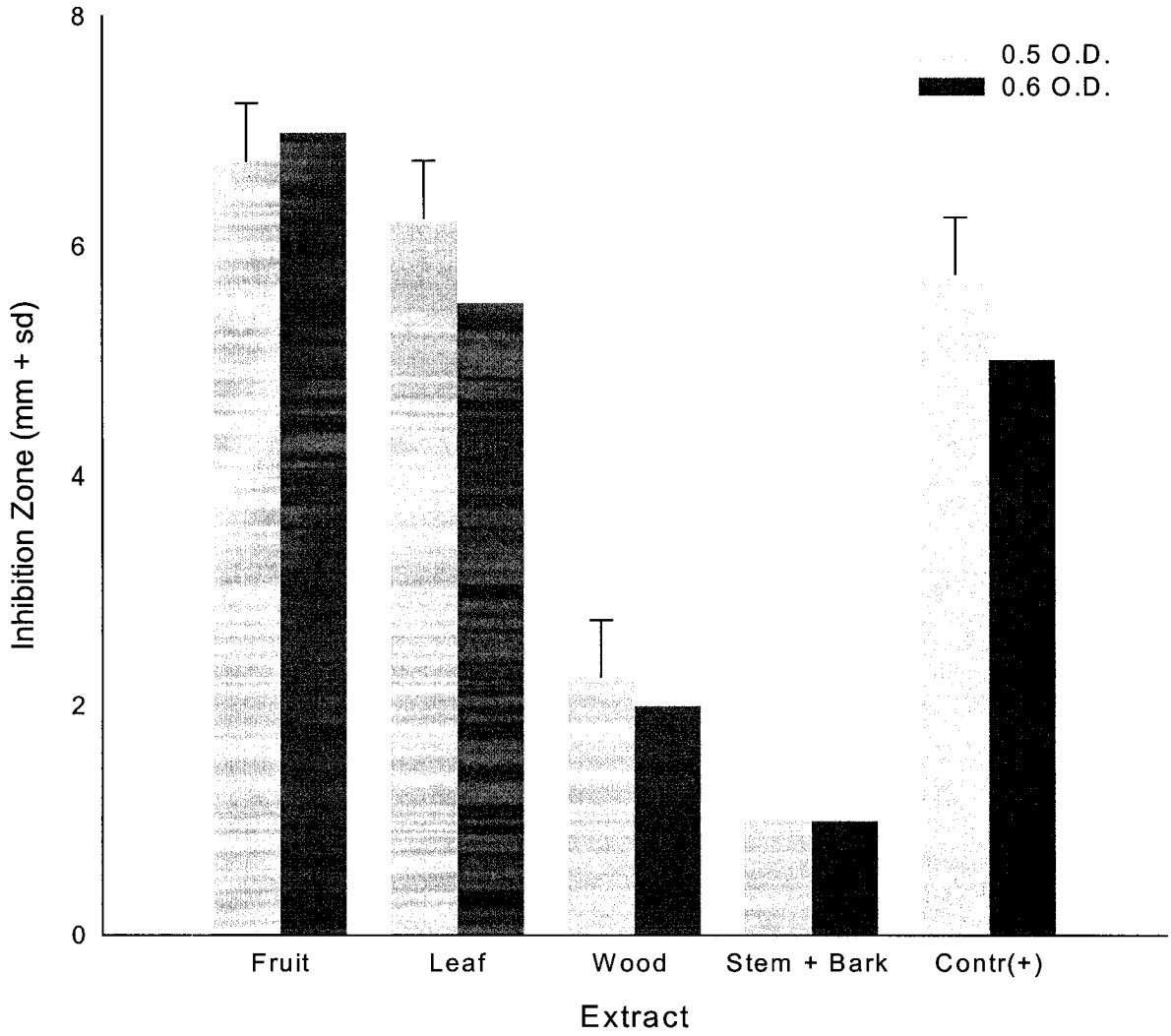


Figure 2.2 *S. cerevisiae* growth inhibition by *Z. americanum* ethanolic extracts after 48 hours using the disk diffusion method (0.6 mg/disk, $n = 4$). Within treatments, the *t*-test was used to determine the effect of inocula density (O.D.) on inhibition. Inhibition of fungal growth was not statistically significant different (P=0.05) within treatments. Experiments conducted in the presence of UV light.

Table 2.3 Effect of inoculum size[a] on antifungal activity of *Z. americanum* extracts[b] tested against the filamentous fungi *Alternaria alternata (A.a.)* and *Trichophyton mentagrophytes (T.m.)*. Inhibition zones (mm ± sd) observed after 48 hours incubation.

Extract	*A.a.*[1]		*A.a.*[3]	
	+UV	-UV	+UV	-UV
Fruit	1.5 ± 0.6	-	6.3 ± 1.0	-
Leaf	1.5 ± 0.6	-	4.5 ± 0.6	-
Stem + Bark	-	-	2.8 ± 0.5	-
Xanthotoxin[c]	2.3 ± 0.5	-	5.8 ± 0.5	-
Extract	*T.m.*[2]		*T.m.*[3]	
	+UV	-UV	+UV	-UV
Fruit	5.0 ± 0.8	0.5 ± 0.6	8.0 ± 0.8	-
Leaf	3.5 ± 0.6	-	7.0 ± 0.0	-
Stem + Bark	1.8 ± 0.5	-	4.8 ± 0.6	-
Xanthotoxin[c]	4.0 ± 0.8	-	7.8 ± 1.0	-

[a] Inoculum sizes based on diameter of mycelia plug and volume of deionized water used for fragmentation from which 100 µL inocula were drawn.
[1] 12 mm diameter mycelia plug fragmented in 30 ml; [2] 10 mm diameter mycelia plug fragmented in 40 ml; [3] 7 mm diameter mycelia plug fragmented in 10 ml.
[b] All extracts showed significantly different light enhanced activity ($p<0.05$)
[c] Control

In this case altering the volume of deionized water from 40 ml to 10 ml and decreasing the mycelial plug diameter to 7 mm from 12 mm respectively modified the inoculum density. Our results concerning *Alternaria alternata* and *Trichophyton mentagrophytes,* suggest that a standardized quantitative plating method based on using a mycelial plug 7 mm in diameter and fragmenting it into 10 ml, not only increases the inoculum density, but also results in higher antifungal efficacies being noted for tested extracts. Such a standardization method is advantageous because it increases reproducibility of the antifungal assay and is applicable in the testing of antifungal agents against other filamentous fungi.

The standardization procedures carried out for yeast-like fungi and filamentous fungi, were based on using as high as possible inoculum density to make certain of homogeneity in the fungal cell suspension. In doing so, we could ensure the formation of a uniform mycelium layer, with negligible effects of organic solvent concentration and inoculum size, since it is only then that reported inhibition zones will closely reflect the actual activity of an extract (Hadacek and Greger 2000).

2.3.2 *Results of antifungal assay*

Table 2.4 is a summary of antifungal activity of extracts from *Z. americanum* plant parts harvested from several wild shrub populations growing in Eastern Ontario and Northern Ontario, Canada. The preliminary testing of these extracts against *Saccharomyces cerevisiae* was designed to provide a comparative analysis of variability among extracts and to select extracts for further testing against a panel of opportunistic pathogenic fungi. Conventional (UV-independent) antifungal activity was not observed with any extracts. However, all extracts showed light-mediated (UV-dependent) activity that was superior or equal to growth inhibition observed with the positive control, except for wood (North Gower) and stem + bark (Sault Ste. Marie) extracts, which were less inhibitory than xanthotoxin. The wood (North Gower) and leaf (Belleville) extracts were excluded from further testing.

For the remaining *Z. americanum* plant extracts selected for further testing, antifungal tests were conducted against 11 fungal species (*C. albicans*, *C. neoformans*, *S. cerevisiae*, *A. alternata*, *Rhizopus sp.*, *A. fumigatus*, *M. gypseum*, *F. oxysporum*, *P. boydii*, *T. mentagrophytes* and *W. dermatitides*) representing a wide range of pathogenicities and susceptibilities. The results of the assay with the disk diffusion method are presented in Table 2.5. Against each of the fungal species, there was significant variation in the antifungal activity of the various extracts tested (ANOVA, $P<0.001$). Therefore, antifungal efficacy against the respective fungi was compared among extracts as well as to 8-methoxypsoralen (xanthotoxin) inhibition values, using Bonferroni's multiple comparison test ($P=0.05$). Generally, the antifungal efficacy of tested extracts (for the same fungal species) could be classified into one of three possible groups: that being superior, equal or inferior to xanthotoxin.

Table 2.4 Activity of various *Z. americanum* extracts against *Saccharomyces cerevisiae*. Plant parts were harvested from several wild shrubs growing in Eastern Ontario and Northern Ontario, both of Canada. Statistical differences between means ($n = 4$) are indicated by different letters using Bonferroni's multiple comparison test ($p<0.05$).

		Zone of inhibition (mm ± sd)	
Extract	Collection site	+UV	-UV
Fruit	North Gower	7.3 ± 0.5^{a}	0
Leaf	North Gower	7.0 ± 0.0^{a}	0
Wood	North Gower	2.0 ± 0.0^{c}	0
Stem + Bark	Sault Ste. Marie	1.3 ± 0.5^{c}	0
Leaf	Belleville	5.0 ± 0.5^{b}	0
Seed	Belleville	5.3 ± 0.5^{b}	0
Husk	Belleville	7.3 ± 0.5^{a}	0
Wood	Belleville	4.5 ± 0.6^{b}	0
Xanthotoxin[1]	N/A	4.8 ± 0.5^{b}	0

[1]Positive control

Table 2.5 Growth inhibition zones (mean in mm ± sd) for fungi tested with and without UV treatment. Means (n = 4) followed by the same letter for extracts in the +UV group are not statistically different in Bonferroni's test ($p<0.05$) for respective fungal species.

Species[1]	*C.n.*		*S.c.*		*C.a.*	
	+UV	-UV	+UV	-UV	+UV	-UV
Fruit	4.0 ± 0.8^{a}	0	2.5 ± 0.6^{bc}	0	2.5 ± 0.6^{b}	0
Leaf	2.3 ± 0.5^{b}	0	5.5 ± 0.6^{a}	0	2.3 ± 0.5^{b}	0
Wood	0.5 ± 0.6^{c}	0	1.8 ± 1.3^{c}	0	0.8 ± 1.0^{c}	0
Stem + bark	0.8 ± 0.5^{c}	0	0^{d}	0	0^{c}	0
Seed	0^{c}	0	3.8 ± 0.5^{ab}	0	2.3 ± 0.5^{b}	0
Husk	3.8 ± 0.5^{a}	0	2.8 ± 0.5^{bc}	0	4.5 ± 0.6^{a}	0
8-MOP	2.3 ± 0.5^{b}	0	4.5 ± 0.6^{a}	0	2.5 ± 0.6^{b}	0
Species[1]	*P.b.*		*W.d.*		*A.f.*	
	+UV	-UV	+UV	-UV	+UV	-UV
Fruit	6.5 ± 0.6^{ab}	0	5.5 ± 0.6^{a}	0	12.5 ± 0.6^{a}	0
Leaf	5.3 ± 0.5^{bc}	0	4.3 ± 0.5^{b}	0	9.3 ± 1.0^{b}	0
Wood	3.8 ± 0.5^{d}	0	2.5 ± 0.5^{c}	0	0^{d}	0
Stem + bark	3.8 ± 0.5^{d}	0	3.5 ± 0.6^{bc}	0	0^{d}	0
Seed	4.5 ± 0.6^{cd}	0	3.3 ± 0.5^{bc}	0	5.8 ± 0.5^{c}	0
Husk	7.5 ± 0.6^{a}	0	5.8 ± 0.5^{a}	0	13.5 ± 1.3^{a}	0
8-MOP	5.5 ± 0.6^{bc}	0	3.0 ± 0.0^{c}	0	10.3 ± 0.5^{b}	0

Table 2.5 Continued

Species[1]	*F.o.*		*R.sp.*[2]		*M.g.*	
	+UV	-UV	+UV	-UV	+UV	-UV
Fruit	4.8 ± 0.5^{b}	0	0.8 ± 0.5^{ab}	0	7.0 ± 1.4^{ab}	0
Leaf	3.0 ± 0.8^{c}	0	1.0 ± 0.8^{ab}	0	4.8 ± 0.5^{cd}	0
Wood	2.8 ± 0.5^{c}	0	0.3 ± 0.5^{b}	0	3.5 ± 0.6^{d}	0
Stem + bark	0.5 ± 0.6^{d}	0	0.3 ± 0.5^{b}	0	4.8 ± 0.5^{cd}	0
Seed	3.5 ± 0.6^{bc}	0	1.0 ± 0.8^{ab}	0	4.8 ± 0.5^{cd}	0
Husk	6.5 ± 1.0^{a}	0	2.0 ± 0.8^{a}	0	9.0 ± 1.2^{a}	0
8-MOP	4.8 ± 0.5^{b}	0	1.0 ± 0.8^{ab}	0	6.3 ± 1.0^{bc}	0

Species[1]	*T.m.*		*A.a.*	
	+UV	-UV	+UV	-UV
Fruit	8.0 ± 0.8^{a}	0	6.3 ± 1.0^{ab}	0
Leaf	7.0 ± 0.5^{bc}	0	4.5 ± 0.6^{bc}	0
Wood	5.8 ± 0.6^{cd}	$2.0 \pm 0.0^{*}$	3.3 ± 0.5^{c}	0
Stem + bark	4.8 ± 0.5^{d}	0	2.8 ± 0.5^{c}	0
Seed	6.8 ± 0.5^{ab}	0	4.8 ± 0.5^{bc}	0
Husk	9.0 ± 1.2^{a}	$3.3 \pm 0.5^{*}$	6.8 ± 0.5^{a}	0
8-MOP	7.8 ± 1.0^{ab}	0	5.8 ± 0.5^{ab}	0

[1] *C.n.*: *Cryptococcus neoformans*; *S.c.*: *Saccharomyces cerevisae*; *C.a.*: *Candida albicans*; *P.b.*: *Pseudallescheria boydii*; *W.d.*: *Wangellia dermatitides*; *A.f.*: *Aspergillus fumigatus*; *F.o.*: *Fusarium oxysporum*; *R.sp.*: *Rhizopus sp.*; *M.g.*: *Microsporum gypseum*; *T.m.*: *Trichophyton mentagrophytes*; *A.a.*: *Alternaria alternata*. [2] Inhibition after 24 hours, obliterated after 48 hrs. * Significantly different from +UV treatment (Bonferroni *t*-test, $P<0.05$).

For a given extract, no statistical comparison of antifungal activity was performed across fungal species, since variations in efficacy with respect to the different fungi represents species-dependent susceptibilities evident by the relative activities of the positive control xanthotoxin to these fungi.

All six evaluated extracts demonstrated antifungal activities with the inhibition of at least 7 pathogenic fungi following the 48-hour incubation period. At the concentration tested (600 μg/disk), fruit and husk extracts were the most potent inhibitors of fungal growth, consistently resulting in large zones of clearance superior or equal to that noted with the positive control. In addition, fruit and husk extracts demonstrated the broadest spectrum of antifungal activity inhibiting 10 out of the 11 pathogenic fungi used in this study. Overall, *Z. americanum* extracts did not appear limited in their scope of biological activity and effectively impeded the growth of both yeast-like and filamentous. This was not limited to opportunistic pathogens such as *Candida albicans* and *Cryptococcus neoformans*, but also to *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Wangellia dermatitides*, which are all causative agents in skin infections.

Although all extracts initially (after 24-hour incubation) inhibited *Rhizopus* sp., zones of inhibition were completely obliterated after 48-hour incubation establishing this fungal pathogen as the most resilient to the antifungal properties of *Z. americanum* extracts. *Rhizopus* spp. are well known for their extremely fast growing colonies and capability to colonize many substrates, including a wide variety of soils, decaying vegetation, foodstuffs and animal and bird dung. These traits among others may allow *Rhizopus* physiological compensation for initial growth inhibition and account for the rapid recovery and proliferation of *Rhizopus* sp. surpassing the density threshold required for effective inhibition by tested extracts. In contrast, the fungus *A.*

fumigatus, which causes a number of diseases in man including pneumonia and brain abscesses, aspergilloma (colonization of pulmonary cavities), sinus and postoperative infections in immunocompetent patients, was the most susceptible to inhibition (Singleton and Sainsbury 1987).

In all trials, successful inhibition of fungal pathogens occurred in the UV treated group. The only exception was with wood and husk extracts that showed conventional antifungal activity (without UV irradiation) against *T. mentagrophytes*, but under near UV irradiation, antifungal efficacy was still significantly enhanced (Bonferroni's *t*-test, $p = 0.000$) against *T. mentagrophytes.* These results suggest that the constituents responsible for the biological activity of tested extracts are light mediated and are consistent with the tested hypothesis, since furanocoumarins are generally dependent on photoactivation and considered to have greater activity in the presence of UV-light (Reitz and Trumble 1997).

In addition to evaluating the antifungal activity of crude ethanolic extracts from *Z. americanum* plant parts, fractionated extracts were also assessed. By successively extracting husk, leaf, wood and seed parts collected from Belleville in organic solvents of increasing polarities, it was possible to selectively partition different classes of compounds into specific solvent fractions. The reason for doing so was to determine which fraction or fractions exhibited the greatest antifungal efficacy and the class or classes of compounds potentially present within corresponding fractions. Extracts were fractionated into hexane, ethyl acetate, methanol and water, as well as the crude ethanolic extracts for husk, leaf, wood and seed parts assayed against *Saccharomyces cerevisiae.* The crude ethanolic extracts were included as a positive control to which fractionated extracts could be compared. The choice of *S. cerevisiae* as bioaasay organism was based on its low-virulence and generally moderate sensitivity to previously tested

plant extracts. Although *S. cerevisiae* is often considered of low-virulence, most patients with some form of underlining disease can be subject to associated infections including endocarditis and pneumonia (Anaissie *et al.* 1989). Furthermore, in the context of this study, extracts successfully inhibiting *S. cerevisiae*, also displayed biological activity against at least nine opportunistic human skin or systemic pathogens, suggestive of its potential use for the general screening of antifungal agents.

Since furanocoumarins are non-polar compounds and their solubility increases in aprotic solvents, it was expected that hexane and ethyl acetate fractions would be most active of the fractionated extracts. This was tested using stock solutions of xanthotoxin in the various organic solvents. Contrary to the expected outcome, resulting zones of inhibition for filter disks impregnated with stock solutions of xanthotoxin in hexane, ethyl acetate or methanol were not statistically different (Figure 2.3). However, inhibition of *S. cerevisiae* growth was statistically different when the dissolving solvent was either ethanol or water. Similarly, growth inhibition of *S. cerevisiae* when subjected to fractionated extracts of *Z. americanum* was most pronounced in hexane, ethyl acetate and or methanol fractions, irrespective of the plant part considered (Figure 2.4A - Figure 2.4D), with all fractionated extracts demonstrating light-mediated antifungal activity only.

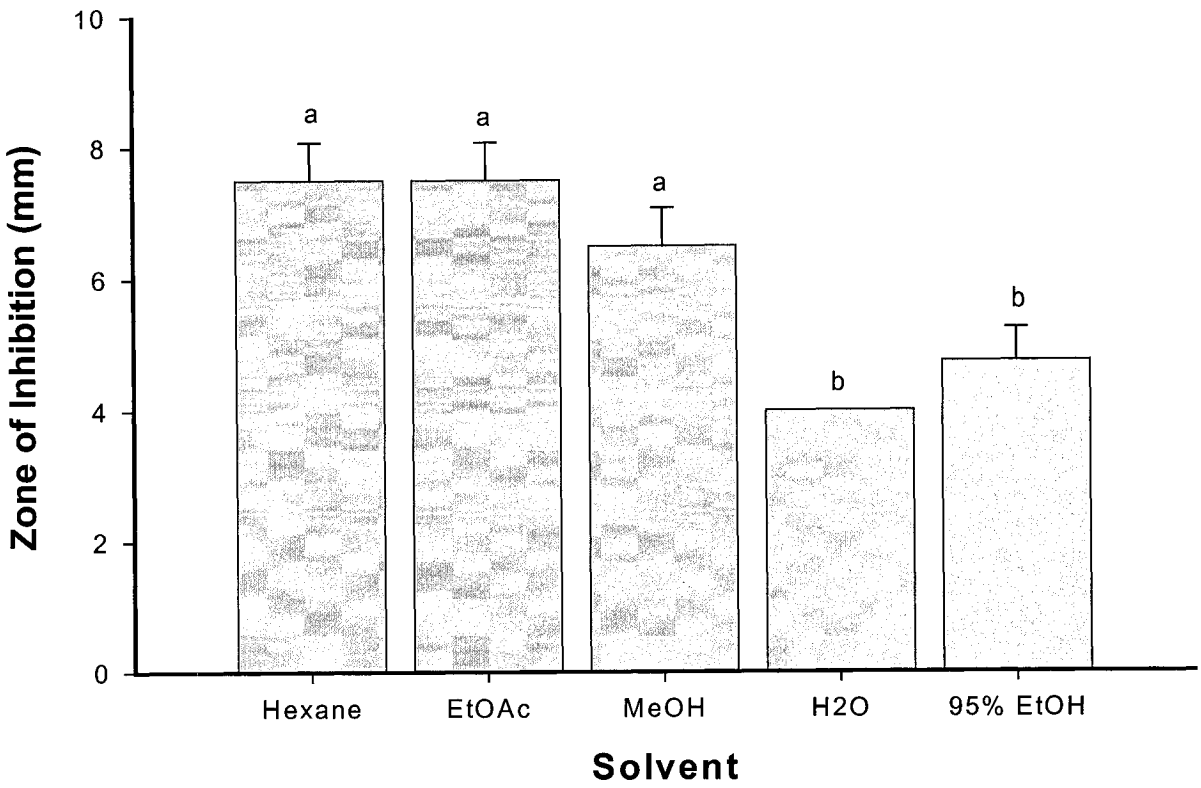


Figure 2.3 Inhibition of *Saccharomyces cerevisiae* in the disk diffusion assay. Growth inhibition based on four filter disks impregnated (600 µg/disk) with a stock solution of xanthotoxin (12mg/ml) in their respective solvents, irradiated with UV light for 2 hours. Means with the same letter are not statistically different (Bonferroni's multiple comparisons test, $p<0.05$), bars above means denote standard deviation.

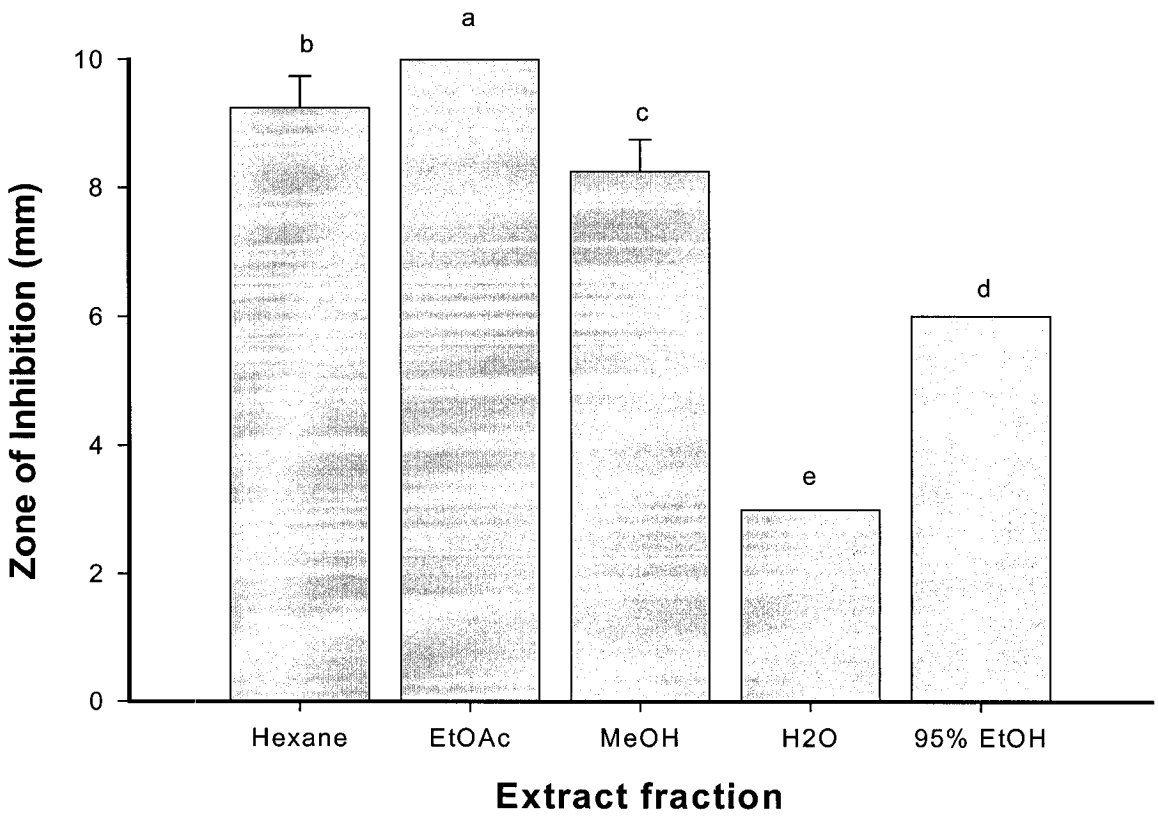


Figure 2.4A Inhibition of *S. cerevisiae* growth (in the presence of UV light) by fractionated husk extracts using the disk diffusion method (600 µg/disk, $n = 4$). Crude ethanolic extract is included as a positive control. Significant differences between means are represented by different letters (Bonferroni's multiple comparisons test, $p<0.05$), bars above means denote standard deviation.

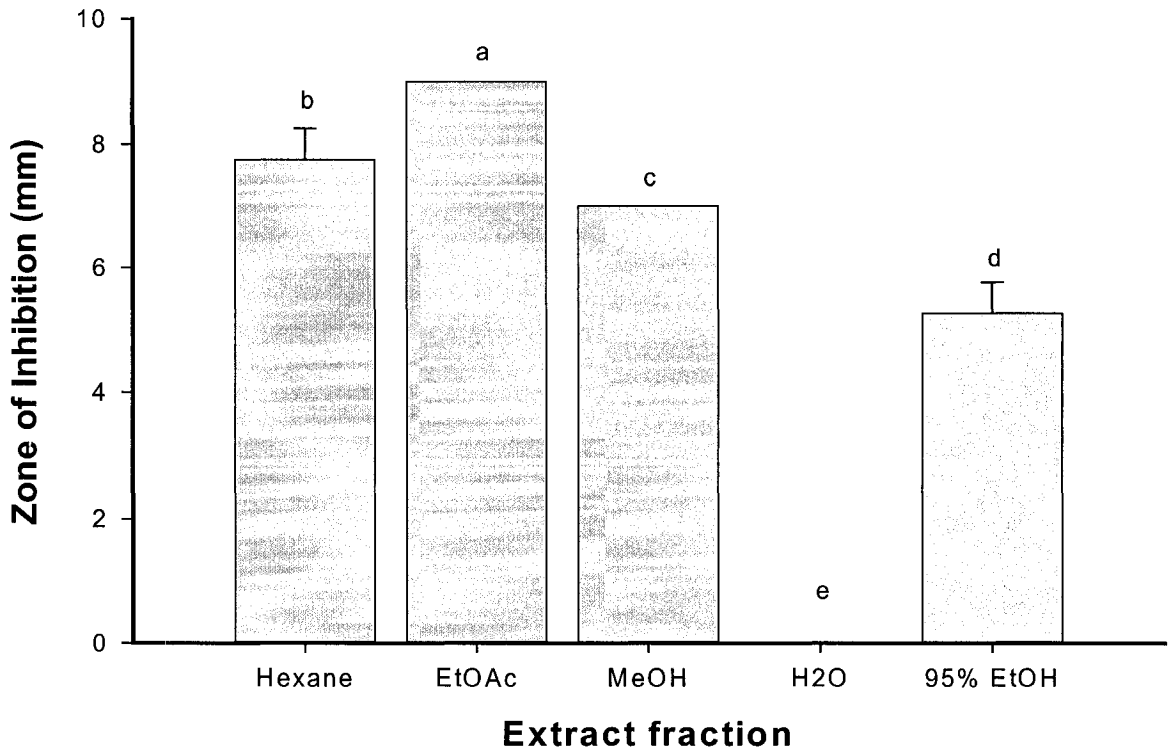


Figure 2.4B Inhibition of *S. cerevisiae* growth (in the presence of UV-light) by fractionated leaf extracts using the disk diffusion method (600 µg/disk, $n = 4$). Crude ethanolic extract is included as a positive control. Significant differences between means are represented by different letters (Bonferroni's multiple comparisons test, $p<0.05$), bars above means denote standard deviation.

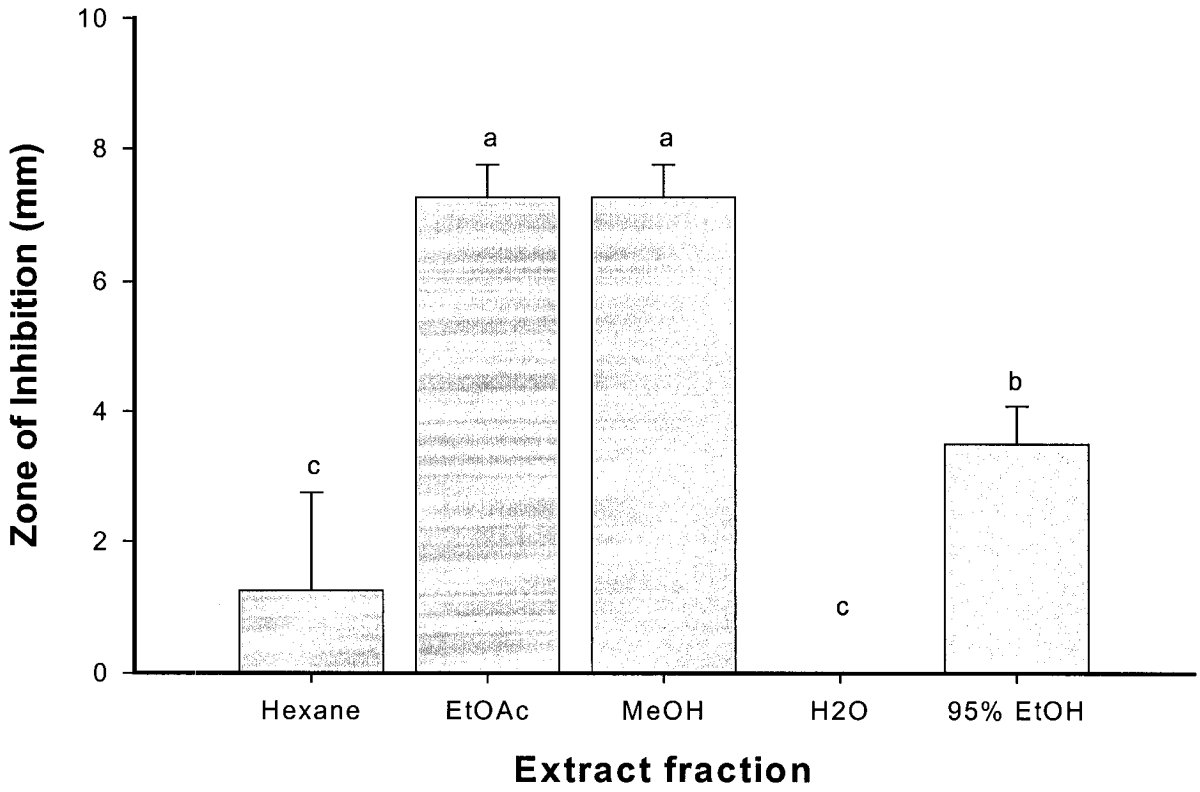


Figure 2.4C Inhibition of *S. cerevisiae* growth (in the presence of UV-light) by fractionated seed extracts using the disk diffusion method (600 μg/disk, $n = 4$). Crude ethanolic extract is included as a positive control. Means with the same letter are not statistically different (Bonferroni's multiple comparisons test, $P < 0.05$), bars above means denote standard deviation.

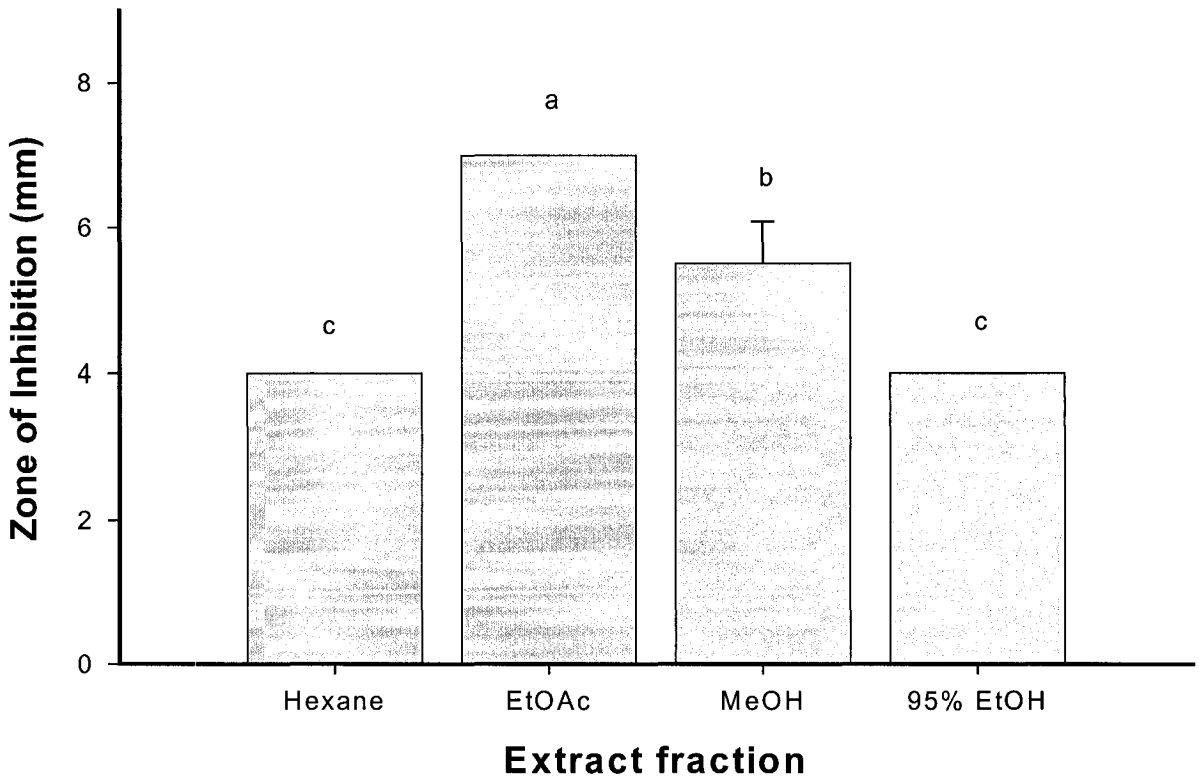
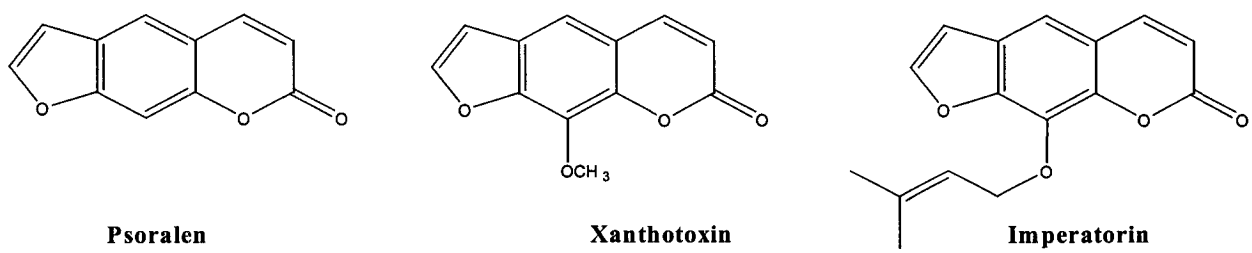


Figure 2.4D Inhibition of *S. cerevisiae* growth (in the presence of UV-light) by fractionated wood extracts using the disk diffusion method (600 μg/disk, $n = 4$). Crude ethanolic extract is included as a positive control. Means with the same letter are not statistically different (Bonferroni's multiple comparisons test, $P < 0.05$), bars above means denote standard deviation.

These results lead to a number of import considerations with respect to the biological activity of fractionated extracts. First, the fact that antifungal activity of extracts was highest when fractionated into hexane, ethyl acetate or methanol compared to little activity in water is consistent with the presence of furanocoumarins. At the same time, the significant amount of variability observed in antifungal activity of fractionated extracts, especially hexane, ethyl acetate and methanol fractions from the same plant part, may reflect the variation in the furanocoumarin content and composition of extracts studied.

Evidence of the variability in the content of the three linear furanocoumarins (Figure 2.5) and their composition in fractionated extracts is provided by phytochemical analysis of fractionated extracts by HPLC (Table 2.6) and may account for the variability observed in antifungal activity. Namely, the variations in total furanocoumarin content closely reflect reported variability in *S. cerevisiae* growth inhibition by fractionated extract and are substantiated by the high degree of conformity between the two variables. Concurrence in this case is based solely on fractionated extracts that are assigned the same letter irrespective of whether biological activity or total furanocoumarin content is considered based on Bonferroni's multiple comparison test. Therefore, the finding that total furanocoumarin content was highest in ethyl acetate fractions and lowest in water fractions for a given extract provides support for the noted trend of ethyl acetate fractionated extracts registering the largest zones of inhibition and water fractionated extracts displaying the smallest zones of inhibition.



Psoralen **Xanthotoxin** **Imperatorin**

Figure 2.5 Structures of 3 linear furanocoumarins present in *Z. americanum* fractionated extracts.

Table 2.6 Furanocoumarin concentrations (μg/mg) of fractionated *Z. americanum* extracts. Compounds identified by on-line diode array UV spectra and retention times of authentic standards. HPLC analysis conducted in duplicate. Means followed by the same letter are not significantly different in Bonferroni's test ($p<0.05$). No comparisons are performed among fractions of different parts. Crude ethanolic extract (EtOH) included as a positive control.

Part	Fraction[1]	Extract composition (%)			Total
		Psoralen	Imperatorin	Xanthotoxin	(mean ± sd)
Husk	C_6H_{14}	52.3	17.6	30.1	$66 \pm 17^{bc*}$
	EtoAc	17.2	2.0	80.8	$492 \pm 60^{a*}$
	MeOH	39.5	1.0	59.5	$93 \pm 3^{bc*}$
	H_2O	33.4	0.0	66.6	9 ± 1^{c}
	EtOH	31.7	4.0	64.4	203 ± 7^{b}
Leaf	C_6H_{14}	10.6	9.8	79.6	$57 \pm 3^{b*}$
	EtoAc	22.0	6.3	71.7	180 ± 6^{a}
	MeOH	28.0	4.4	67.7	$26 \pm 0^{c*}$
	H_2O	0.0	0.0	100.0	$1 \pm 0^{d*}$
	EtOH	28.3	5.0	66.8	$53 \pm 1^{b*}$
Seed	C_6H_{14}	0.0	0.0	100.0	$1 \pm 0^{c*}$
	EtoAc	7.0	4.7	88.4	15 ± 3^{b}
	MeOH	36.4	0.0	63.6	$25 \pm 1^{a*}$
	H_2O	0.0	0.0	100.0	$0 \pm 1^{c*}$
	EtOH	23.3	0.0	76.7	$12 \pm 0^{b*}$
Wood	C_6H_{14}	19.6	15.2	65.1	10 ± 0^{b}
	EtoAc	30.6	5.3	64.1	$32 \pm 1^{a*}$
	MeOH	22.8	6.1	71.0	$9 \pm 5^{b*}$
	EtOH	35.1	8.0	56.9	11 ± 0^{b}

[1]Extract fractionated into hexane (C_6H_{14}), ethyl acetate (EtOAc), methanol (MeOH) and Water (H_2O).
*Concurrence between total furanocoumarin content and antifungal activity.

Although the total furanocoumarin content of fractionated extracts appears to be related to biological activity, variability in antifungal activity may also be influenced by the potential differences in the composition of the three linear furanocoumarins. Similarly, synergistic, additive and antagonistic interactions may not only exist among these furanocoumarins, but also between the different classes of compounds potentially present in fractionated extracts (Figure 2.6).

The use a regression model to test the hypothesis that a functional relationship exists between antifungal efficacy and total furanocoumarin content provides strong evidence in support for the tested hypothesis (Figure 2.7). The observed coefficient of determination (r^2) of 0.861 resulting from a regression of the log transformed circumference of inhibition on the log transformed total furanocoumarin content, represents a significant positive relationship between the two variables. In spite of 86% of the variance in the light-mediated antifungal activity of tested extracts being explained by the variation in total furanocoumarins content of these extracts, a regression performed with psoralen content as the independent variable results in an r^2 value of 0.986. Coefficients of determination values for regressions of fungal inhibition on imperatorin content or xanthotoxin content only were 0.693 and 0.778 respectively. I interpret this result to mean that all three linear furanocoumarins are phototoxic and variability in the coefficient of determination values observed may simply denote the relative toxicity of the three furanocoumarins to *S. cerevisiae*. This would imply that of the three furanocoumarins, psoralen is the most potent inhibitor of *S. cerevisiae* growth followed by xanthotoxin. These results are in agreement with the well-defined structure-activity relationship exhibited among furanocoumarins, with psoralen being the most active followed by its monomethoxygenated derivatives (Brown 1977; Gray and Waterman 1978).

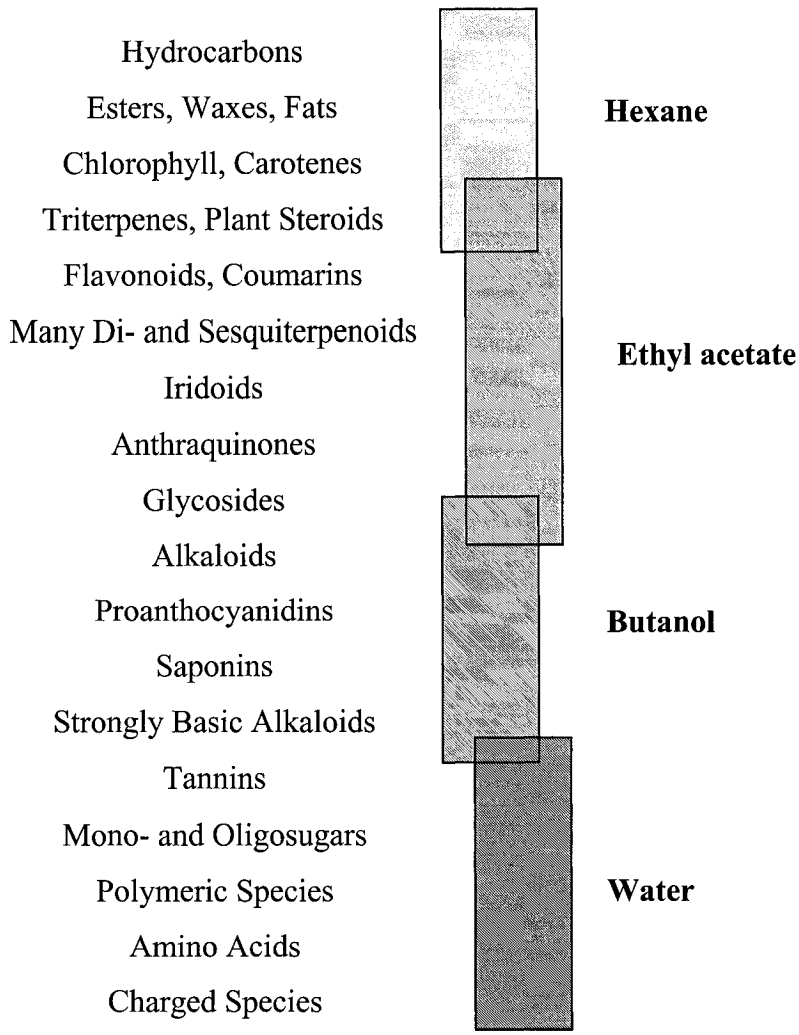


Figure 2.6 Classes of compounds potentially extracted by solvents of varying polarities.

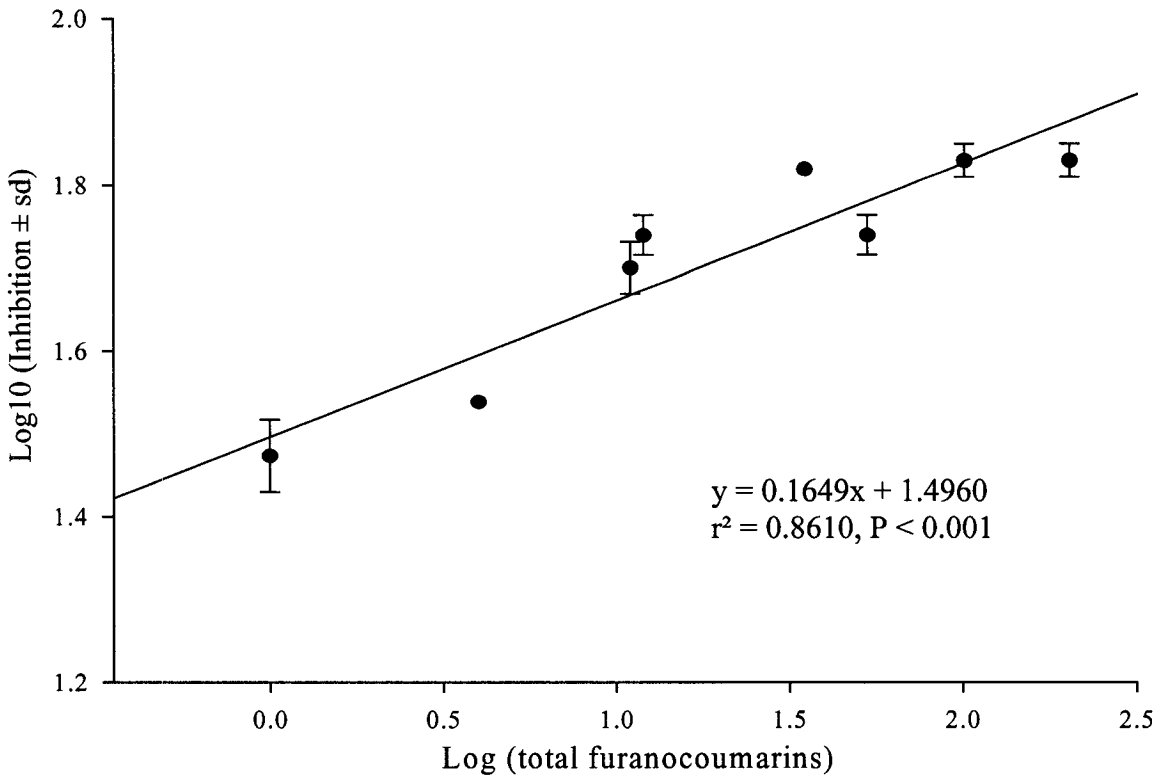


Figure 2.7 Relationship between zone of inhibition of *S. cerevisiae* growth and total furanocoumarin content. Each data point represents mean total furanocoumarin content, based on duplicate HPLC analysis of extracts tested for antifungal activity.

While several mechanisms could account for the phototoxicity of tested extracts, the establishment of furanocoumarins as clearly important in antifungal efficacy provides potential indications as to the associated mode of action. Since the physiological fate of plant secondary compounds is determined by the physical and chemical properties of the compound, in the same way these compounds may be predisposed to certain mechanisms because of their inherent chemical properties (Duffey 1980; Nitao 1990). Consequently, the biological effectiveness of photoactivated furanocoumarins are related to their polarity, solubility and binding constants, allowing these tricyclic, planer compounds to bind and cause damage at the level of DNA (deoxyribonucleic acid)(Towers 1984; Aucoin 1991). The phytochemical addition of furanocoumarins to DNA bases is considered the molecular basis for explaining their photobiological effects and involves electron transfer processes (type I mechanism) leading to the formation of free radicals (Rodighiero and Dall'Acqua 1976). In brief, furanocoumarins act by primarily intercalating with A/T (adenine/thymine) rich regions in DNA and on subsequent irradiation react to form mono- or bi-adducts (Figure 2.8). The impact of this cyclobutane ring formation between the 5, 6 double bond of thymine and the 4', 5' double bond of the furan-side or the 3, 4 double bond of the lactone ring of the furanocoumarin molecule on the cellular properties and function of the cell may encompass a range of effects depending on the capabilities of the organism (Baydoun *et al.* 1989; Bethea *et al.* 1999). Effects may include increased pigmentation on human skin, inactivation or inhibition of DNA and RNA synthesis, as well as lethal or mutagenic chromosomal aberrations (Rodighiero and Dall'Acqua 1976; Song and Tapley Jr. 1979). Alternatively, furanocoumarins may act as photooxidants requiring that the photo-activated phytochemical transfers its energy to molecular oxygen, producing singlet oxygen and superoxide radicals (Aucoin 1991; Binns *et al.* 1999).

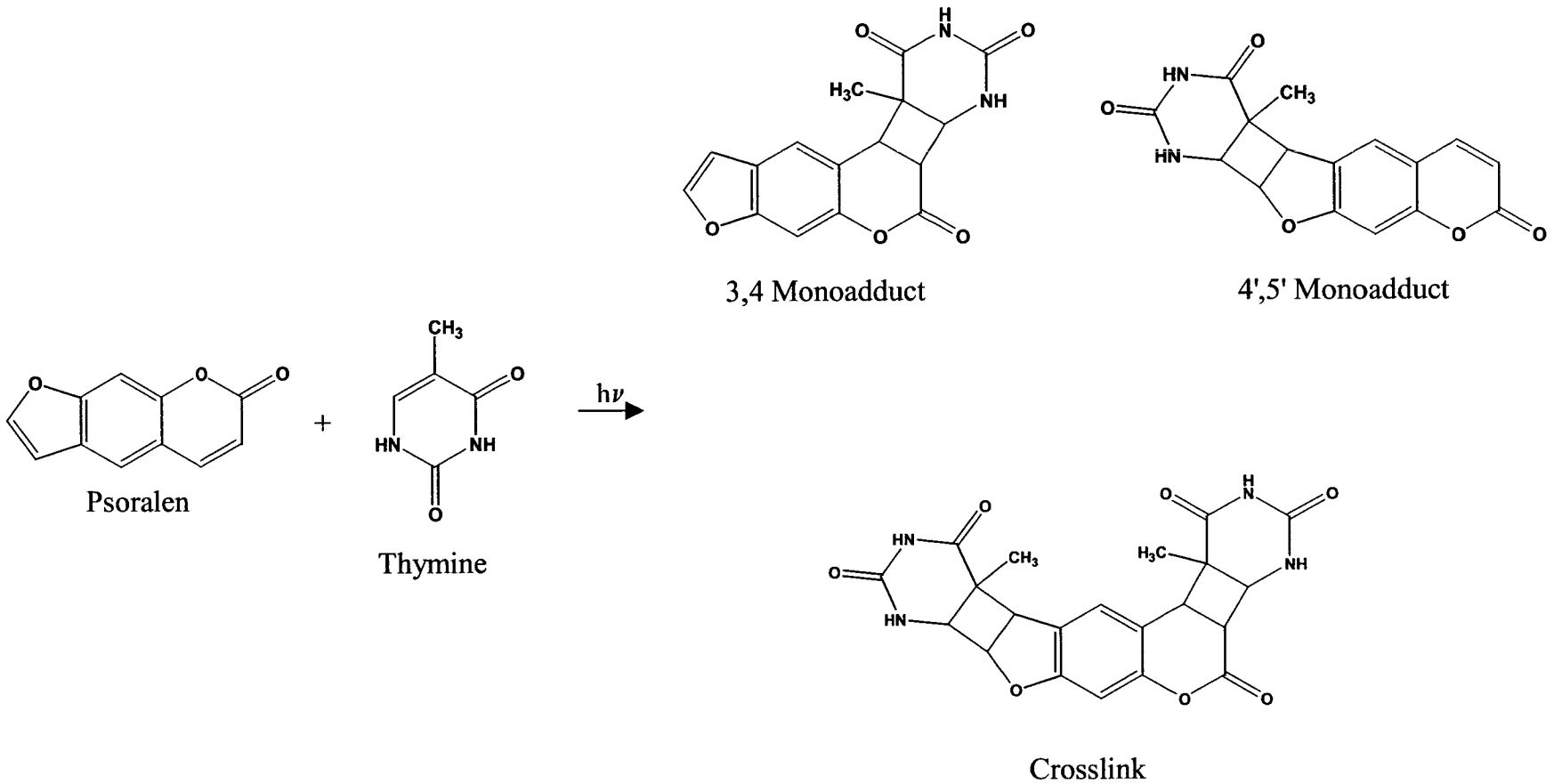


Figure 2.8 Mechanism of light-mediated antifungal activity of UV-treated *Z. americanum* extracts. Process involves three steps: i) non-covalent dark complexing of furanocoumarin within the double helix; ii) absorption of photon resulting in a 3, 4 or 4', 5' covalent monoadduct with thymine and iii) subsequent absorption of a second photon leading to photoaddition with thymine on opposite DNA strand forming an interstrand crosslink (Baydoun *et al.* 1989; Bethea *et al.* 1999).

Elicited by a type II photosensitization process, this oxygen-dependent mechanism of phototoxicity results in lipid peroxidation in the target cell, but is not considered to represent a major mode of action in the phototoxicity of furanocoumarins (Towers 1984).

In view of the fact that furanocoumarins are plant secondary compounds with reported toxicity against a broad spectrum of organisms ranging from bacteria to mammals, furanocoumarins are well regarded as potent plant defense compounds, considered to play an important role in determining plant-herbivore interactions (Diawara *et al.* 1993; Zangerl and Berenbaum 1993). Thus, these compounds are not only important in deterring insect herbivores, but offer protection against organisms including pathogenic fungi. At a broader scale, the furanocoumarin content in *Z. americanum* plants is influenced by the developmental stage of the plant and the environmental conditions under which it was grown, but more interestingly, the within-plant allocation of these compounds has been suggested to occur in accordance with the optimal defense theory (Nitao and Zangerl 1987; Zangerl and Berenbaum 1987).

Because the optimal defense theory predicts that plant organs are defended with respect to their relative reproductive value and susceptibility to attack, a likely outcome is the differential allocations of these compounds among different tissues. Logically, the furanocoumarin content of extracts derived from the different organs of *Z. americanum* plants will reflect the varying degrees to which the respective parts are defended as well as the major sites of accumulation which appear to be present in the aerial portions of the plant such as the leaves and berries (Figure 2.9).

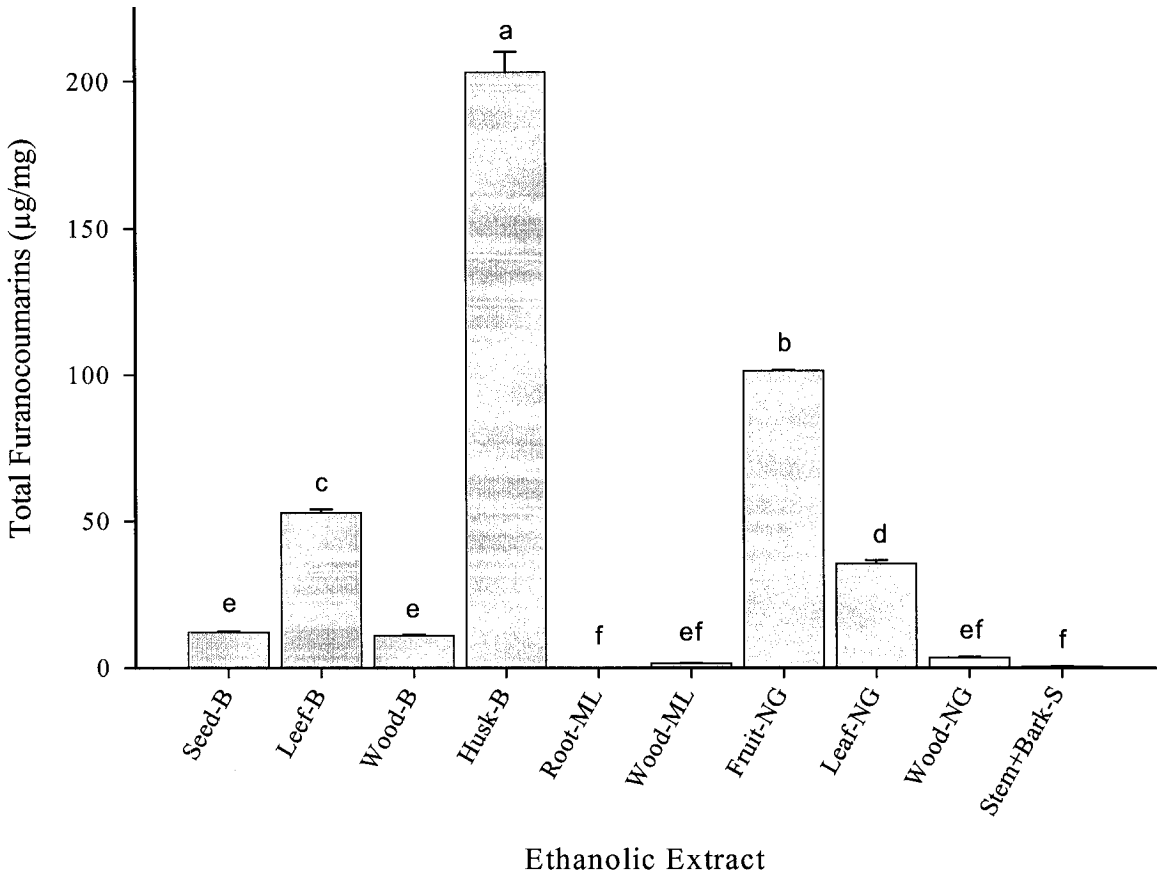


Figure 2.9 Furanocoumarin content (mean ± sd) of various *Z. americanum* ethanolic extracts. Means followed by the same letter are not significantly different in Bonferroni's test ($p<0.05$), total furanocoumarin content based on duplicate HPLC analysis.

This result is rather intriguing because it demonstrates the association of light-mediated compounds with the external regions of the plant, where they are readily exposed to solar radiation and the principal allocation of furanocoumarins to structures of greater reproductive value, specifically the fruit. Such a pattern is to be expected based on the optimal defense theory and has ecological significance as to the proposed mode of action required for furanocoumarin bioactivity activity.

In my investigations of the antifungal properties of *Z. americanum* extracts, all parts of the plant were found to retard or inhibit growth against a majority of the phylogenetically diverse classes of yeast-like and filamentous fungi used in the study. Extracts for the aerial parts of the plant, especially the fruits not only possessed inhibitory activities against the widest spectrum of fungi, but also exhibited the strongest inhibition against pathogens. This finding supports the traditional knowledge that considers the whole shrub to possess medicinal value with the berries being the most active (Erichsen-Brown 1979). Consistent with the tested hypothesis, which was that the antifungal efficacy of *Z. americanum* extracts was dependent on furanocoumarin content, all extracts demonstrated light-dependent toxicities. Therefore the pharmacological basis for the traditional antifungal uses of *Z. americanum* plant parts can be ascribed to the presence of furanocoumarins (psoralen, imperatorin and xanthotoxin). Noteworthy was the fact that these potent plant defense compounds were not only effective against ecologically relevant fungi such as the plant pathogens (*Aspergillus fumigatus* and *Fusarium oxysporum*), but also had similar effects on human pathogenic fungi including *Candida albicans*, *Cryptococuss neoformans* and *Microsporum gypseum*. However, the relative susceptibility or sensitivity of tested fungi to a given extract, is not simply a response to the total furanocoumarin content and composition, but is also indicative of how well the fungal species, put into a cytostatic state where cellular process

may be suspended, can also recover from sublethal damage. The ability of furanocoumarins to act as photogenotoxins makes them capable of killing a wide range of organisms and the presence of these compounds in *Z. americanum* plants offers a potential explanation for the traditional uses of Z. americanum preparations against many infectious diseases.

Since the photobiological effects of furanocoumarins can be ultimately attributed to their reactivity towards nucleic acids, the therapeutic applications of furanocoumarin containing plants, such as *Z. americanum* must be carefully weighed to offset the potential pathological effects on non-target organisms or cells (Song and Tapley Jr. 1979). In our review of the ethnobotanical literature, the use of alcohol or water and prevalent employment of the root and bark in medicinal preparations may imply recognition of this fact by Native American groups. That is to suggest that by preferentially using parts of the plant containing the least amounts of furanocoumarins and mitigating some of its bioactivity through preparation, mild antimicrobial effects could be realized with a relatively low risk of resulting preparations posing a health hazard. Alternatively, it is also logical to assume that the pharmacological basis for the use of plant parts particularly low in furanocoumarins, may be due to other compounds present, especially compounds with light independent activities.

Although there are no published studies on the antifungal properties of *Z. americanum* plants, the findings in this study are consistent with evidence from other Rutaceous shrubs that have demonstrated a correlation between the variation in the phototoxic antimicrobial activity of extracts and the variation in furanocoumarin content (McCloud *et al.* 1992; Asthana *et al.* 1993). Previously, Salib *et al.* (1990) found three furanocoumarins (isoimperatorin, psoralen and xanthotoxin) as the pharmacologically active constituents present in crude extracts of the berries of *Z. americanum*, while demonstrating its therapeutic properties against human tumor cells.

Our results concerning the variability in furanocoumarin content of the different extracts tested are consistent with the interpretations of the optimal defense theory and proposed mode of action, corroborating the results of Asthana *et al.* (1993) and Zangerl and Berenbaum (1990), who showed an association of furanocoumarins with aerial plant parts.

In contrast to these results, Ngane *et al.* (2000) showed that antifungal activity of ethanolic extracts of *Zanthoxylum leprieurii* and *Zanthoxylum xanthoxyloides* against nine pathogenic fungi, five of which were used in the current study, was attributable to nonfuranocoumarin phototoxic constituents, which is a reflection of variability of phytochemistry of the genus.

The use of furanocoumarin-containing plants such as the legume *Psorelea coryfolia* and the Umbeliferous plant *Ammi majus* for medicinal purposes in North African, Hindu and Chinese civilizations dates back to ancient times. Plants containing furanocoumarins are generally well tolerated by humans and include common grocery fruits and vegetables, such as celery, parsnip root, parsley and lime among others (Ataga *et al.* 1993; Diawara *et al.* 2000). In spite of this, furanocoumarins are not devoid of side effects and have been shown to be both mutagenic and carcinogenic (Beier *et al.* 1994). Oral administration of 8-methoxypsoralen to treat psoriasis in human patients has been widely reported to cause basal-cell and squamous-cell carcinomas (Diawara *et al.* 1997). In light of these considerations, development of a commercial phytomedicine to successfully control emerging fungal pathogens must effectively address potential pathological effects resulting from furanocoumarins.

The results of the present study clearly provide pharmacological basis for the traditional use of *Z. americanum* extracts for infectious diseases and topical conditions, especially in phototherapeutic applications. Further phytochemical and pharmacological investigations of the

root, bark or wood extracts should be undertaken, given their diverse and extensive traditional uses, potential therapeutic applications, lower risk to cause health problems and potential presence of other groups of secondary compounds, particularly non-photoactivated constituents.

PREFACE

In the previous (second) chapter of this thesis, phytochemical and pharmacological evidence supporting the traditional antifungal uses of prickly ash plant parts was presented. In our discussion of how total furanocoumarin content may mediate and influence vital cell functions, chapter two succeeded admirably at providing a rationale for the traditional uses of *Z. americanum* to treat various infectious diseases and topical conditions. However, the preferential allocation of furanocoumarins to the aerial portions of the plant (photosynthetic tissues) does not sufficiently explain the pharmacological basis for the uses of *Z. americanum* wood, bark or root.

In the present chapter, I report on the continuing phytochemical examination of *Z. americanum* plant parts by focusing on the development of a fully validated HPLC method to identify and authenticate commercial raw material composed predominantly of plant parts relatively low in furanocoumarins.

CHAPTER 3

IDENTIFICATION AND QUANTITATIVE DETERMINATION OF MARKER CONSTITUENTS BY HPLC/UV FROM WILD *ZANTHOXYLUM AMERICANUM* POPULATIONS

3.1 Introduction

Northern prickly ash, *Zanthoxylum americanum* Mill. (Rutaceae) is a clonally propagated woody North American shrub, widely used in the ethnomedicine of First Nations people. Phytomedicinal preparations based on infusions and decoctions of prickly ash plant parts are employed to treat various ailments. For example, the berries are used for the treatment and cure of pulmonary infections, wounds as well as burns (Erichsen-Brown 1978). Other folkloric applications of prickly ash parts include the use of roots and bark to treat toothache, skin infections and especially to alleviate arthritic and rheumatic conditions (Moerman 1998).

Recently, the exploration of *Z. americanum* extracts as phytopharmaceuticals with relevance to both human and animal health markets has yielded some promising results. Preliminary studies performed with extracts prepared by extracting the terminal parts of the plant using water or ethanol:water mixtures, have been found to be an effective remedy against topical inflammatory conditions, wounds and rashes especially in veterinary applications. In addition, cytotoxic screening of prickly ash plant parts such as the berries and bark, have demonstrated these extracts as strong and selective inhibitors on tumour cells (Saqib *et al.* 1990; Ju *et al.* 2001).

In chapter 2, the pharmacological basis for the antifungal uses of *Z. americanum* plant parts was investigated and the presence of three linear furanocoumarins, namely psoralen, imperatorin and xanthotoxin explained much of the traditional antifungal uses of the aerial parts of this plant on the basis of the biological activity of these secondary metabolites. However, the pharmacological properties of prickly ash root, stem and bark which have distinct phytochemistry from the leaves and fruit is not fully understood. Moreover, the medicinal virtues of non-aerial parts, such as the root and bark cannot be due solely to furanocoumarins, since the allocation of these compounds to non-aerial parts is relatively low to absent.

The potential use of *Z. americanum* extracts as phytopharmaceuticals requires careful control of the active principles and potentially problematic constituents, including furanocoumarins. It is for this reason that the current interest in developing Northern prickly ash as a commercial phytomedicine must not only address the authentication and standardization of raw commercial material, but attempt to relate pharmacological activity with phytochemical constituents. Chemotaxonomic studies conducted on *Zanthoxylum* spp. have established the presence of constituents such as coumarins, lignans and alkaloids with broad pharmacological profiles (Fish *et al.* 1975; Saqib *et al.* 1990; Ju *et al.* 2001), which may be suitable as phytochemical markers for quality assurance.

In continuation of the study on the phytochemistry and pharmacology of Northern prickly ash, an experiment was undertaken aimed at identifying and quantifying phytochemical marker constituents in commercial raw materials by using several chromatographic techniques and spectroscopic methods, including open column, preparative HPLC, HPLC-DAD and HPLC-MS as well as NMR analysis. We report on the presence of four pyranocoumarins isolated from the non-aerial parts of this plant, which are not only suitable as marker constituents, but may also

provide support for some of the biological/pharmacological activities of *Z. americanum* preparations. In addition to this being the first reported rapid and fully validated quantitative method for the determination of amounts of marker constituents, the content of these compounds is assessed in different plant parts as well as in commercial raw materials collected from different geographic locations of Eastern Ontario.

3.2 Materials and Methods

3.2.1 *Plant material*

3.2.1.1 *Isolation of standards*

Stems of *Z. americanum* plants were harvested in July 2001 from several ($n \geq 10$) randomly selected shrubs growing in Mud Lake, Ottawa, Canada and voucher specimens retained at the University of Ottawa Herbarium, Ottawa, Ontario, K1N 6N5, Canada. The fresh stems were chopped into smaller pieces and spread out on a laboratory bench to air dry for 2 weeks prior to use in the extraction procedure.

3.2.1.2 *Determination of the amounts of marker phytochemicals in natural populations*

Authentic plant materials of *Z. americanum* were collected from 27 natural populations in 1998 and seven natural populations in 2001 (Appendix 1.1 and 1.2). In total, Northern prickly ash populations in eight Eastern Ontario counties were sampled throughout the range of this plant. The plant material composed of the terminal portions of the plant (approximately 2-3 feet of growth) potentially included the fresh berries and leaves depending on the time as well as site of collection, but predominantly of stems and twigs were air-dried preceding processing in a

wood mill. All plant materials of *Z. americanum* from the 1998 harvest were provided by Bioniche Botanicals (Belleville, ON, Canada).

3.2.2 *Extraction, purification and isolation of marker constituents*

Air-dried stems of *Z. americanum* (Figure 3.1) were ground to a fine powder (1 mm sieve size) using a Thomas-Wiley laboratory mill (model 4, Arthur H. Thomas Co., PA, USA), and the powdered sample (2.45 Kg) steeped in 95% ethanol (biomass to solvent ratio was approximately 1 g to 9 mL) for 48 hrs and exhaustively extracted by ultrasonication (Branson model 5210R-MT, Danbury, CT 06813, USA) for six 60 min cycles. The extracted plant material was removed by Buchner filtration (Whatman #1 filter paper) and the filtrate concentrated under reduced pressure to yield a dark brown/black slurry. The entire residue was then dissolved in water and successively partitioned with hexane, ethyl acetate and *n*-butanol. The filtrate partitioned into hexane was then placed in a flask and attached to a rotary evaporator, where the hexane was evaporated under reduced pressure until a darkly coloured residue remained. The residue was frozen for several hours at –5 °C and then lyophilized to ensure complete dryness. A portion of the crude extract (2 g) was subjected to repeated open column chromatography over silica gel (33 x 4.5 cm i.d.; 230-400 mesh; 240 g) eluted with hexane: ethyl acetate mixtures in increasing order of polarity as previously reported (Ju *et al.* 2001). Using 500 ml aliquots increased from 9:1 to 1:1, hexane: ethyl acetate following a step gradient, a total of 120 fractions were collected and fractions showing similar TLC patterns pooled. The bulked fractions were further analyzed by preparatory HPLC (Techsphere 5ODS 25cm x 20mm). Three injections of the bulked fractions were performed and 1-minute fractions (5 ml) collected 25 minutes into the run and concluded at the 97th minute for a total of 72 fractions per inject.

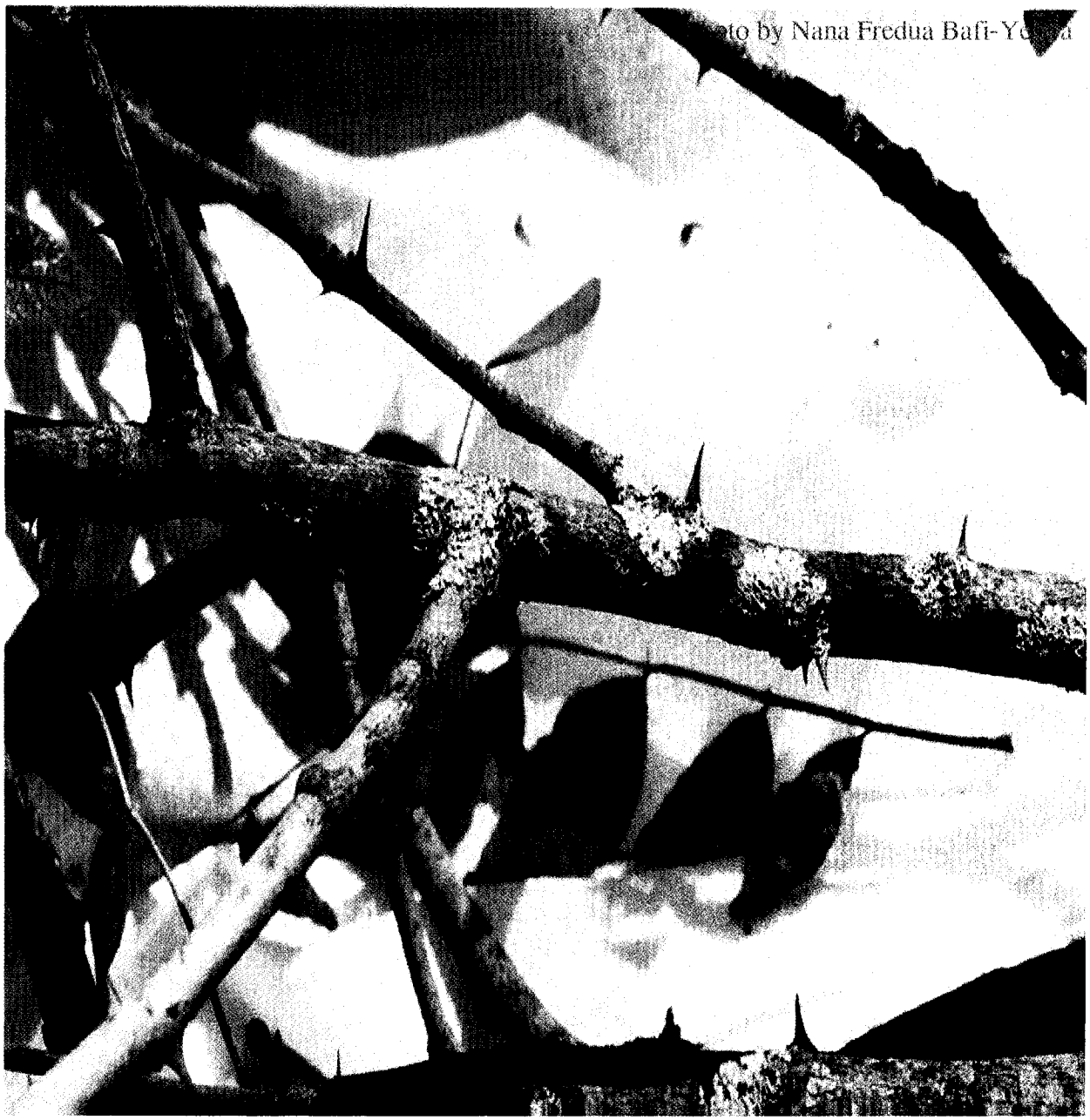


Figure 3.1 Photograph of *Z. americanum* stems being air-dried.

Fractions with the same retention times were combined among injects, concentrated and further analyzed on the basis of the retention times and UV spectra of eluting compounds to yield 15 combined fractions. Six of the combined fractions were further processed since they were comparatively higher in yield (based on the absorbance of the eluting compounds) and less complex in mixture as evidenced by resolution among eluting peaks. Rotary evaporation of these fractions resulted in the isolation of six whitish amorphous solids. Identification and structure elucidation of isolated compounds were facilitated by comparison of melting point, UV spectra, EI-MS and ^{1}H-NMR data (Appendix 2.1-Appendix 2.4) with reported values in the literature (Bell *et al.* 1937; Bell and Robertson 1937; Ju *et al.* 2001). Four of these compounds were definitively characterized as xanthyletin (9 mg), dipetaline (40 mg), allo-xanthoxyletin (7 mg), xanthoxyletin (11 mg) along with the tentative characterization of two coumaric compounds, an unknown pyranocoumarin (2 mg) and 5, 7, 8-trimethoxycoumarin (5 mg).

3.2.3 *Analytical techniques and equipment*

HPLC analyses were performed using a Varian (Mississauga, ON, Canada) Prostar system comprising of a model 230 solvent delivery module, model 410 autosampler and a model 330 photodiode array detector. Separation of coumarin derivatives was carried out on a Merck (BDH, Toronto, Canada) LiChrospher® RP-18 (125 x 4 mm i.d.; 5 μm) column operated at 50 °C and equipped with a Merck LiChrospher® RP-18 (4 x 4 mm i.d.; 5 μm) guard. The mobile phase was (A) water, (B) 25 mM sodium dihydrogen ortho-phosphate ($NaH_2PO_4 \cdot H_20$) adjusted to pH 3.0 using hydrochloric acid and (C) acetonitrile. A two-step linear solvent gradient was used starting from 20% C and increasing to 55% C during a 30 minute period. The amount of C reached 50% between 0 and 20 minutes and finally attained 55% at the end of the run.

A 3-minute equilibration time was used in between runs and the contribution of B was 10% during the entire run and the flow rate maintained 1 mL/min. Eluting peaks were monitored simultaneously at various wavelengths (225, 310 and 335 nm) and online UV spectra collected from 200 to 400 nm for each peak. Detection and quantification of eluting compounds were carried out at 225 nm and Star chromatography workstation software (Version 5.51) used for integration and calibration.

Semi-preparative HPLC analysis was performed using a Varian Prostar model 230 pump equipped with a model 330 diode array detector managed by Star chromatography workstation. The elution profile consisted of a linear gradient of acetonitrile and water rising from 10% to 60% acetonitrile in 40 minutes, after that an increase from 60% to 90% acetonitrile in the next 20 minutes, then held for 30 minutes and followed by a decline from 90% to 10% acetonitrile in 10 minutes. Chromatography using 500 µL manual injects was facilitated on a Techsphere5ODS (250 x 20 mm i.d.; 10 µm; Wellington House, Cheshire, UK) column maintained at ambient temperature. Eluting compounds were monitored at 254 nm and online spectra data was collected from 190-600 nm.

3.2.4 *Assay validation*

3.2.4.1 *Extraction solvent*

Extraction efficiencies were assessed in hexane, ethyl acetate and various concentrations (50, 60, 70, 80, 90 and 100%) of methanol or ethanol in water. Finely ground (0.5 mm) raw plant material (1 g) was extracted for five minutes with 20 ml of solvent using ultrasonic treatments, centrifuged for five minutes and the supernatant collected. The residue was re-extracted as above with 20 ml followed by 10 ml of solvent being used for the successive re-extractions. The

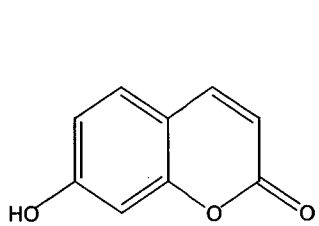
pooled supernatants were adjusted to 50 ml and a portion of the combined supernatant filtered through 0.22 μm PTFE membranes (Chromatographic Specialties, Brockville, Canada) prior to injection of 5 μL into the HPLC column. HPLC analysis of extraction efficiencies were evaluated in duplicate using plant material (site #3) randomly selected out of the natural populations sampled.

3.2.4.2 *Calibration curves*

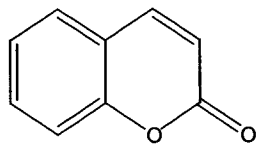
The relationship between peak area and the concentration of reference compounds were determined for xanthyletin (18.3-292.5 μg/ml), xanthoxyletin (13.4-215.0 μg/ml) and allo-xanthoxyletin (8.78-140.5 μg/ml). Calibration curves were also constructed for 5,7,8-trimethoxycoumarin (1.72-27.5 μg/ml), xanthotoxin (13.57-217 μg/ml), psoralen (19.06-305 μg/ml), imperatorin (7.01-112.5 μg/ml), bergapten (10.63-170 μg/ml), isopimpinellin (6.38- 102 μg/ml) and angelicin (15.63-250 μg/ml). Coefficients of determination (r^2) values were established based on a five-point regression curve operating in the noted calibration range for the respective compounds. Although several compounds (Figure 3.2) were detectable with this method and used as external standards, we only evaluated the suitability of xanthyletin, xanthoxyletin and allo-xanthoxyletin as phytochemical markers.

3.2.4.3 *Repeatability of experiments*

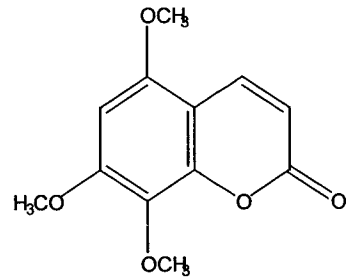
Reproducibility of extraction procedures was evaluated using four different methods. In the first method (A), 1 g quantities of finely powered plant material collected at site #2 were weighed into five plastic centrifuge tubes and each extracted in 20 mL of 80% ethanol with ultrasound treatment for five minutes.



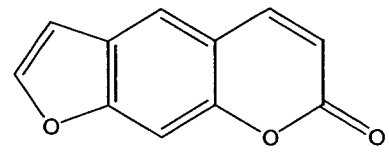
7-hydroxycoumarin (1.521)



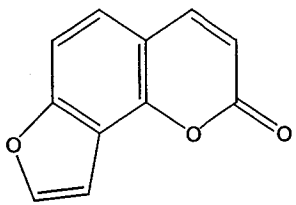
Coumarin (6.464)



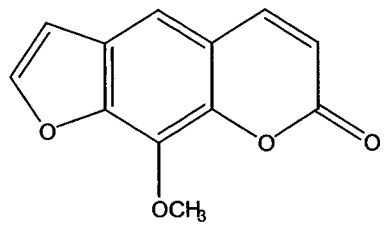
5, 7, 8- trimethoxycoumarin (9.212)



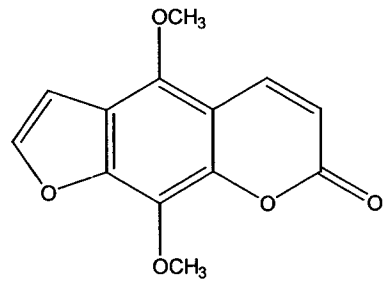
Psoralen (11.872)



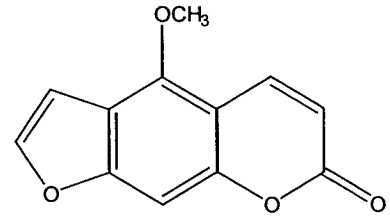
Angelicin (12.282)



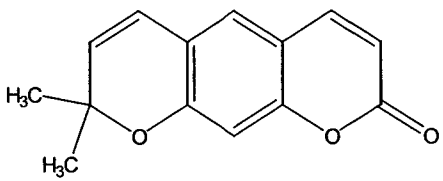
Xanthotoxin (12.515)



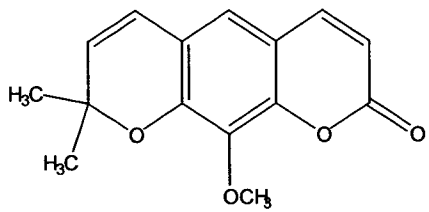
Isopimpinellin (14.625)



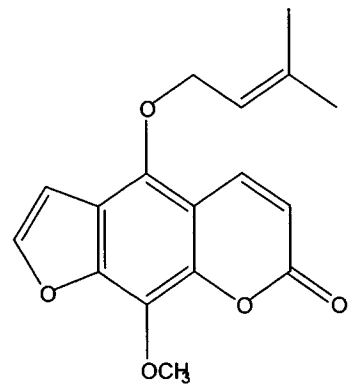
Bergapten (14.880)



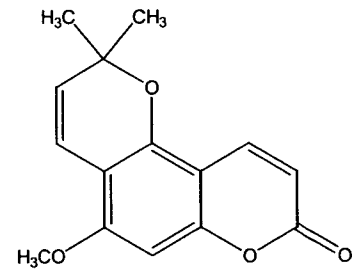
Xanthyletin (18.566)



Xanthoxyletin (20.452)



Imperatorin (21.486)



Alloxanthoxyletin (23.424)

Figure 3.2 Chemical structures and retention times (min) of compounds detectable with HPLC-PAD method.

After ultrasonication, samples were centrifuged for five minutes and the supernatant removed to a clean tube. The entire extraction process was repeated three additional times, but with 20, 20 and 15 mL of 80% ethanol being used in the successive re-extractions. The four resulting supernatants were pooled for each series, evaporated to dryness using a model AES1010 Savant Automatic Environmental SpeedVac System (Holbrook, NY, USA) then re-dissolved into acetonitrile (2 mL). The second method (B) was similar to that above, except pooled supernatants were roto-evaporated to dryness and the residue reconstituted in 10 mL of acetonitrile. A third extraction method (C) was evaluated as to its reproducibility. Using finely powered plant material collected at site #13, 0.5 g quantities were weighed into 10 tubes and extracted three times with 8 mL of 80% ethanol using 5-minute ultrasound treatments for each aliquot. Successive aliquots were pooled and the volume adjusted to 25 mL. In the final extraction method (D), an adaptation of method C, 1 g quantities were weighed into 10 tubes and extracted four times with 11 mL of 80% ethanol using 5-minute ultrasound treatments for each aliquot. Successive aliquots were then pooled and the volume adjusted to 40 mL.

The precision of these methods was expressed by coefficients of variation for each of the standard marker constituents (xanthyletin, xanthoxyletin and allo-xanthoxyletin). For all of the repeatability experiments, supernatants were filtered prior to HPLC analysis.

3.2.4.4 *Effectiveness of successive extractions*

Finely powered plant material (site #2) were placed into five plastic centrifuge tubes (approximately 1 g per tube) and extracted as stated in method B (see 3.2.4.3 *Repeatability experiments*), except for the noted change. Supernatant from successive re-extractions for each

series were not pooled prior to HPLC analysis, but rather analyzed independently to determine the amounts of the marker compounds successively extracted with each ultrasonic treatment.

3.2.4.5 *Recovery experiments*

The efficiency of the HPLC assay procedure was determined first by separately adding volumes (0.5, 1.0 or 2.0 mL) of standard solution for individual marker constituents to finely powdered plant material (n = 2) collected at site #2 (0.5 g). The concentrations of stock solutions of the marker compounds dissolved in acetonitrile were as follows: 0.562 mg/mL for xanthyletin, 0.417 mg/mL for xanthoxyletin and 0.296 mg/mL for allo-xanthoxyletin. Unspiked plants samples received 0.5, 1.0 or 2.0 mL of acetonitrile and were used as controls. Following the addition of the respective marker compounds, samples were placed in a drying oven at 58°C for approximately 24 hours and then extracted using an extraction procedure similar to method D, except pooled supernatants were adjusted to 45 mL.

Recovery experiments were also repeated with the separate addition of various lyophilized crude extracts to 1.0 g equivalents of finely powdered plant samples drawn from site #13. The amounts of the three marker compounds in each of the crude extracts used were determined previously by HPLC (Appendix 3.1). Plant samples were spiked with 50, 100, 200, 300, 400 or 500 mg of the respective crude extracts and subjected to the optimized extraction procedure (method D). The efficiency of the HPLC assay was determined by linear regression of marker compound recovery on amount of exogenous marker compound added.

3.2.4.6 *Minimum detection experiments*

The detection limit for each individual marker constituent in the HPLC assay procedure was also determined. Using serial dilutions of a standard solution containing all 3 marker compounds and serial dilution preparations of a randomly selected plant sample (site #25), detection limits for each of the marker constituents were determined. For plant samples, detection limits were calculated from 4 separate experiments, where as in the case of standard solutions, no replicates were used. The sensitivity of the HPLC assay was determined at a signal to noise ratio of 3 and 5.

3.2.5 *Identification and estimation of marker constituents in the plant*

The determination of the amounts of marker phytochemicals in natural *Z. americanum* populations was performed using samples of finely ground (0.5 mm; 1 g dry weight) plant material that was extracted four times with 11 mL of 80% ethanol using 5-minute ultrasound treatments for each aliquot. Successive aliquots were pooled and the volume brought to 40 mL with 80% ethanol. The pooled supernatants were diluted prior to HPLC analyses, with the removal of 700 μL that was added to an equal volume of 80% ethanol. The diluted 1:1 mix was vortexed and filtered through a Chromospec 0.22 μm PTFE membrane disposable filter and analyzed by RP-HPLC.

To determine the quantity of marker constituents in different plant parts, crude extracts (3-7 mg) from corresponding plant parts were dissolved in 1 mL of 80% ethanol and 700 μL of the filtrate diluted with 700 μL of 80% ethanol.

The determination of each of the marker constituents in *Z. americanum* plants were evaluated in duplicate using 5 μL injects. Identification of eluting compounds were established

using their retention times and both spectroscopic and spectrometric data. The quantities of the marker constituents were determined at a signal to noise ratio of 5 and estimated by using isolated compounds as external standards.

3.2.6 *Statistical analyses*

To determine the extraction efficiency of the various solvents and concentrations, the amount of each marker constituent extracted by the various solvents and concentrations were quantified (peak area/g) and compared using ANOVA and a matrix of pairwise comparison probabilities based on Bonferroni's test used to facilitate multiply comparisons and determine were differences existed. All statistical calculations were performed using SYSTAT 10.1 (SPSS Corp., Chicago, IL, USA).

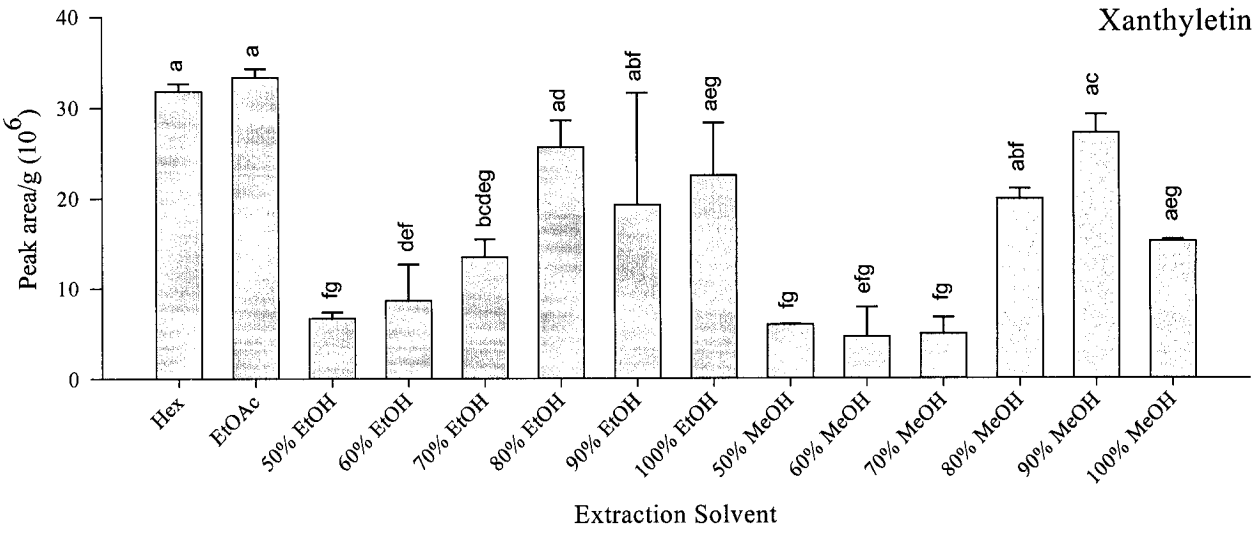
3.3 Results and Discussion

3.3.1 *HPLC assay validation*

3.3.1.1 *Extraction solvents*

In order to develop a rapid and fully validated quantitative method for the determination of amounts of marker constituents from *Z. americanum*, various solvents and solvent concentrations were evaluated to assess their extraction efficiencies with respect to the defined marker constituents, xanthyletin, xanthoxyletin and alloxanthoxyletin (Figure 3.3).

Figure 3.3 Extraction efficiencies of marker constituents (xanthyletin, xanthoxyletin and allo-xanthoxyletin) from the terminal portions of *Z. americanum* plants by various solvents and solvent concentrations. HPLC analyses of extraction efficiencies were evaluated in duplicate using randomly selected plant material from the natural populations (27 in 1998 and 7 in 2001) surveyed. Means followed by the same letter are not significantly different in Bonferroni's test ($p<0.05$). Bars above means represent the standard deviation.


Xanthyletin
Peak area/g (10^6)
40
30
20
10
0
a
a
fg
def
bcdeg
ad
abf
aeg
fg
efg
fg
abf
ac
aeg
Hex
EtOAc
50% EtOH
60% EtOH
70% EtOH
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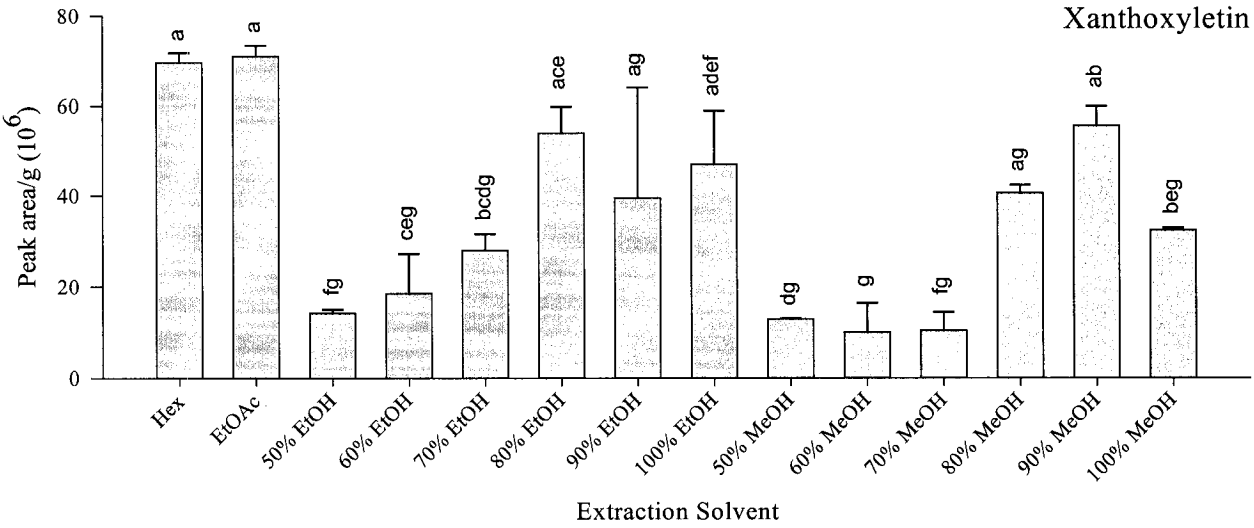
50% MeOH
60% MeOH
70% MeOH
80% MeOH
90% MeOH
100% MeOH
Extraction Solvent


Xanthoxyletin
Peak area/g (10^6)
80
60
40
20
0
a
a
fg
ceg
bcdg
ace
ag
adef
dg
g
fg
ag
ab
beg
Hex
EtOAc
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60% EtOH
70% EtOH
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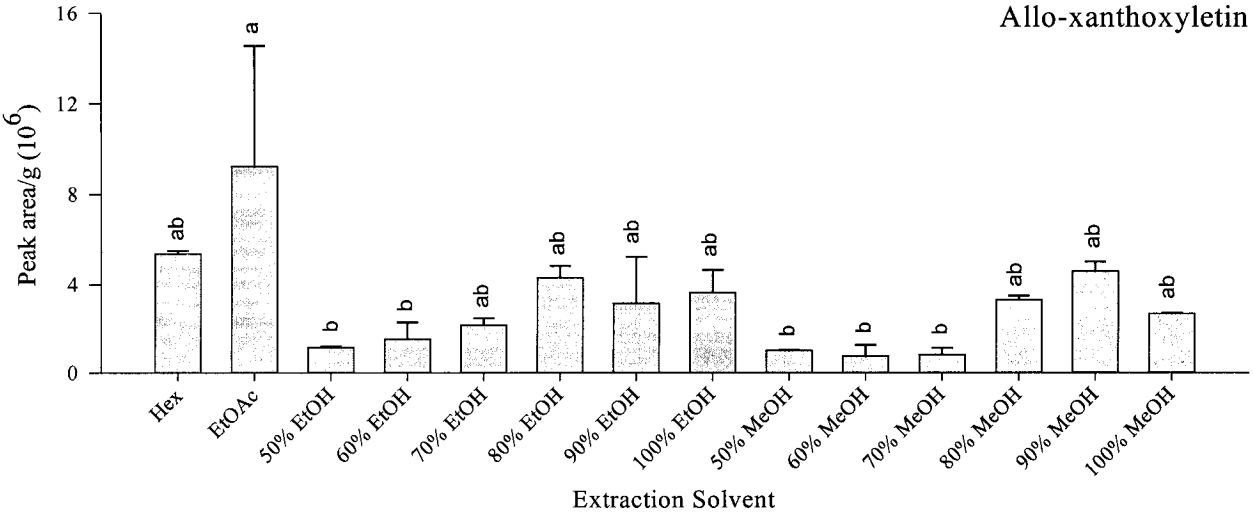
Extraction Solvent


Allo-xanthoxyletin
Peak area/g (10^6)
16
12
8
4
0
ab
a
b
b
ab
ab
ab
ab
b
b
b
ab
ab
ab
Hex
EtOAc
50% EtOH
60% EtOH
70% EtOH
80% EtOH
90% EtOH
100% EtOH
50% MeOH
60% MeOH
70% MeOH
80% MeOH
90% MeOH
100% MeOH
Extraction Solvent

The finding was that all three marker compounds were best extracted with non-polar solvents, in particular hexane or ethyl acetate. This result was not surprising since pyranocoumarins are lipophilic and xanthyletin, xanthoxyletin and alloxanthoxyletin were all isolated from a hexane partitioned extract with increasing concentrations of ethyl acetate in hexane. However, moderately polar solvents, including 80 or 90% ethanol or methanol were equally as efficient as hexane or ethyl acetate at extracting all three phenolic markers (Bonferroni's multiple comparison test, $p<0.05$). Therefore, the choice of 80% ethanol as the extraction solvent was made for a number of reasons, including its lower relative toxicity and lower standard deviation (SD) of mean marker compound extracted based on HPLC-DAD analysis.

3.3.1.2 *Efficiencies of successive extractions*

In addition to determining the best extraction solvent, the extraction of marker compounds with each successive ultrasound treatment was also determined. The reason for doing so was to ensure the exhaustive extractions of marker constituents from raw commercial materials was achieved. Using four successive extractions and determining the mean ($n = 5$) amount of the respective marker compound extracted with each re-extraction, it was found that the first extraction removed between 63.7% (SD = 1.9) and 71.9% (SD = 2.2) of the total amount of a given pyranocoumarin marker present, where as after two successive extractions an estimated 90.0% (SD = 3.0) to 93.2% (SD = 4.1) of the marker present had been removed (Table 3.1). Further, the amount of a given marker constituent extracted with each successive extraction tends to diminish, with the cumulative amount extracted showing an asymptotic relationship with the number of extractions performed.

Table 3.1 Cumulative amounts of marker constituents extracted from *Z. americanum* plant material with successive ultrasound treatments. Samples were re-extracted a total of four times and the aliquots from each extractions analyzed separately.

	Content dry weight, % (mean ± standard deviation)[a]		
Extraction	Xanthyletin	Xanthoxyletin	Alloxanthyletin
1	69.5 ± 1.9	71.9 ± 2.2	63.7 ± 1.9
2	92.7 ± 4.1	93.2 ± 4.1	90.0 ± 3.9
3	98.6 ± 4.4	98.6 ± 4.4	97.8 ± 4.5

[a] Experiments conducted with five sub-samples ($n = 5$).

This is indicated by a small relative difference (of less than 3%) in the cumulative amount extracted for each marker compound after three successive extractions as opposed to four successive extractions. These results indicate that in order to exhaustively extract marker compounds from raw materials of *Z. americanum*, a minimum of 3 successive ultrasound treatments must be used.

3.3.1.3 *Reproducibility of extraction method*

As part of the stringent standards required for the development of a quality control method for raw commercial material, the coefficient of variation of various extraction methods was determined. The comparison of four extraction methods using 80% ethanol is provided for xanthyletin, xanthoxyletin and alloxanthoxyletin (Table 3.2). The coefficient of variation for these three compounds varied in the range of 13.7–14.6%, 9.8–11.9%, 5.6-6.2% and 1.5-1.7% with extraction method A, B, C and D respectively. Because methods A and B both employ additional steps to concentrate and reconstitute the supernatant, these high coefficient of variation values suggest that the reproducibility of the extraction method improves with the elimination of these additional steps. This is particularly advantageous because by limiting the number of steps between sample extraction and analysis, we not only eliminate time-consuming steps (such as that required for the concentration of supernatants), but reduce the inherent error and improve reproducibility by reducing potential degradation of active constituents including marker compounds. With respect to extraction methods C and D, the latter appears to superior and suggests that the use of four successive extractions with 1.0 g equivalents of raw plant material as a better alternative than three successive extractions with 0.5 g equivalents of raw plant material.

Table 3.2 Coefficient of variation (%) measurements of marker phytochemicals. Finely ground plant materials comprising of the terminal portions of *Z. americanum* were divided into 0.5 or 1.0 g equivalents and successively extracted by various methods using 80% ethanol.

	Coefficient of variation (%)		
Method[1]	Xanthyletin	Xanthoxyletin	Alloxanthyletin
A	14.5	14.6	13.7
B	10.5	11.9	9.8
C	5.6	5.8	6.2
D	1.5	1.7	1.7

[1] **A**: Four successive extractions with reconstitution of concentrated supernatants by SpeedVac, $n = 5$; **B**: Four successive extractions with reconstitution of concentrated supernatants by RotoVap, $n = 5$; **C**: Three successive extractions with no concentration of pooled supernatants, $n = 10$; **D**: Four successive extractions with no concentration of pooled supernatants, $n = 10$.

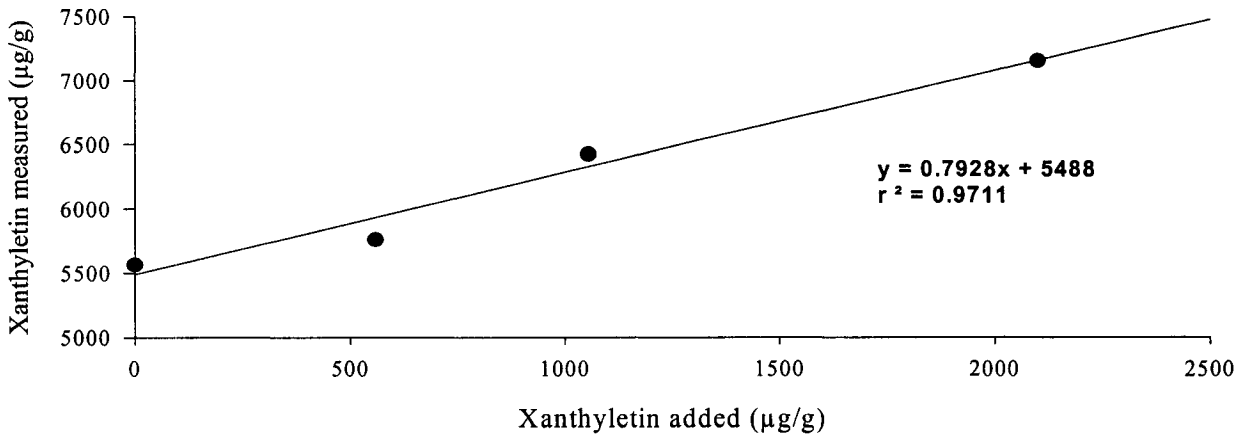
The reason for this may be two-fold. First by performing a fourth extraction, the exhaustive extraction of plant material is maximized and equally important a 1.0 g equivalent of raw plant material may represent a more homogenous sub-sample of the analysed material.

3.3.1.4 *Recovery experiments*

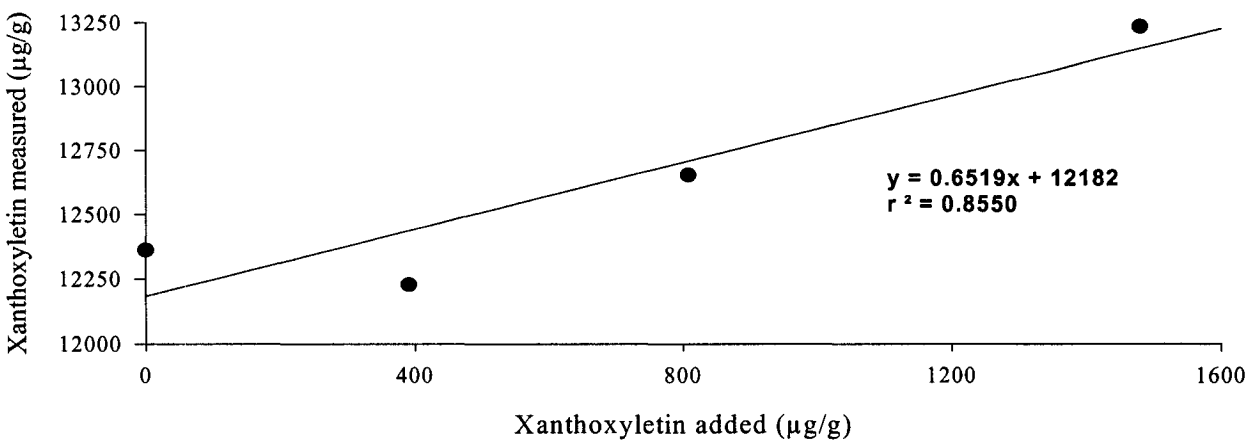
Due to the small amounts of standards present and the difficulties associated with isolation and purification, additions of the pyranocoumarin markers were conducted using standard solutions in contrast with the conventional additions of standards as a solid. Using 0.5, 1.0 or 2.0 ml of each standard solution, samples were spiked with varying amounts of xanthyletin (281, 562 or 1124 mg) xanthoxyletin (208, 417 or 833 mg) and alloxanthoxyletin (148, 296 or 592 mg) prior to extraction. Recovery estimates were then determined over the appropriate range of concentrations by the linear regression of the amount of the marker compound added on the amount recovered (Figure 3.4). The mean recoveries estimated from the slopes ($n = 2$) were 79% for xanthyletin, 65% for xanthoxyletin and 80% for alloxanthoxyletin. However, the reported recoveries for the marker phytochemicals was not indicative of the actual efficiency of the developed HPLC method and at best should be viewed as a lower estimate of recover efficiencies. The reason for this is attributable to the fact that recovery experiments are best performed with the addition of exogenous amounts of standards encompassing about one-half of what would naturally be present in the plant, upwards to about 3-4 times this amount, in order to minimize within sample variability. Therefore, if we consider the maximum amounts of exogenous standard added as a percentage of the total amount naturally present for the respective phenolic markers, we obtain approximately 37%, 12% and 27% for xanthyletin, xanthoxyletin and alloxanthoxyletin.

Figure 3.4 Recovery experiments for *Z. americanum* marker compounds. Finely powdered plant materials (1.0 g) were spiked using 0.5, 1.0 or 2.0 mL stock solutions of xanthyletin (5619 μg/mL), xanthoxyletin (4166 μg/mL) or allo-xanthoxyletin (2958 μg/mL) and the solvent (acetonitrile) allowed to evaporate (24 hrs at 58 °C). As a control, unspiked samples received 0.5, 1.0 or 2.0 mL of acetonitrile. The efficiency of the RP-HPLC assay was determined by the linear regression of amount measured on amount of exogenous marker compound added. All experiments were performed in duplicate.

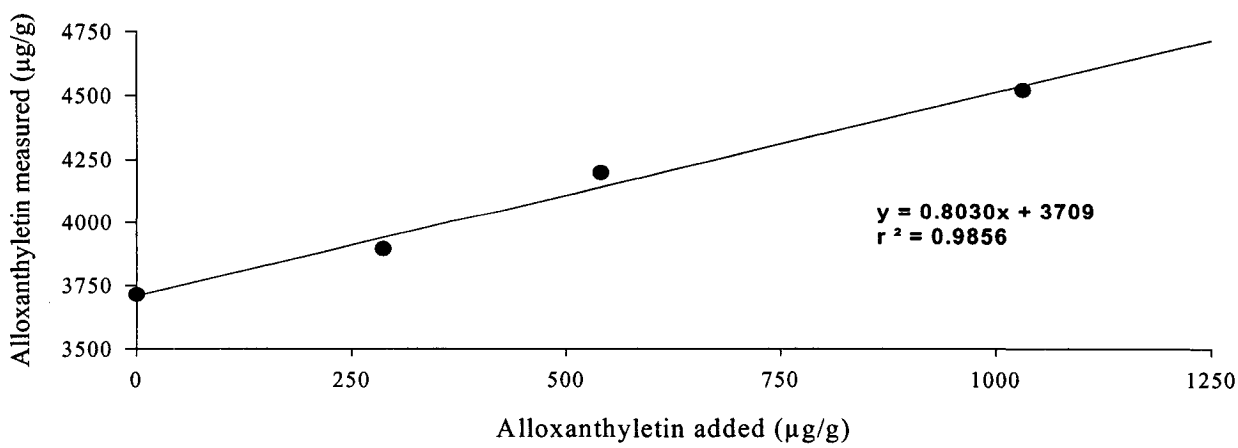
Recovery of xanthyletin from finely ground *Z. americanum* plant material



Recovery of xanthoxyletin from finely ground *Z. americanum* plant material



Recovery of alloxanthyletin from finely ground *Z. americanum* plant material



This measurement undoubtedly correlates with recovery efficiencies and suggest that introduced variability (exogenous standard added) will be obscured unless the amounts of exogenous standard added exceed the natural variability within samples. In doing so, the within sample variability can be minimized and rendered negligible.

To better approximate the recovery efficiencies for the three pyranocoumarin markers, we repeated spiking experiments in which the addition of various crude ethanolic extracts to finely ground plant material was carried out. Despite the fact that all samples were dosed with 50, 100, 200, 300, 400 or 500 mg of the respective crude extracts (24-70% EtOH, 24-90% EtOH, 8-50% MeOH and 8-75% MeOH), the added amounts for each marker compound differed from extract to extract and was determined from the content of crude extracts (for the respective marker compounds) based on previous phytochemical analysis by HPLC-DAD performed in duplicate. For all three marker phytochemicals, reliable recovery efficiencies over a range of concentrations in the order of one-tenth of the normal levels present in samples to four times this amount were obtained. Recoveries between 97-98% for xanthyletin (Figure 3.5), 90-104% for xanthoxyletin (Figure 3.6) and 90- 97% for alloxanthoxyletin (Figure 3.7) are rather remarkable because the use of crude extracts in spiking experiments could potentially pose a problem not encountered when using purified standards. This is because spiking experiments with purified standards essentially determine the efficiency of an extraction method at liberating exogenous addition of standards from raw material. However with the use of crude extracts, recovery efficiencies can be adversely affected by the presence of other compounds along with marker constituents.

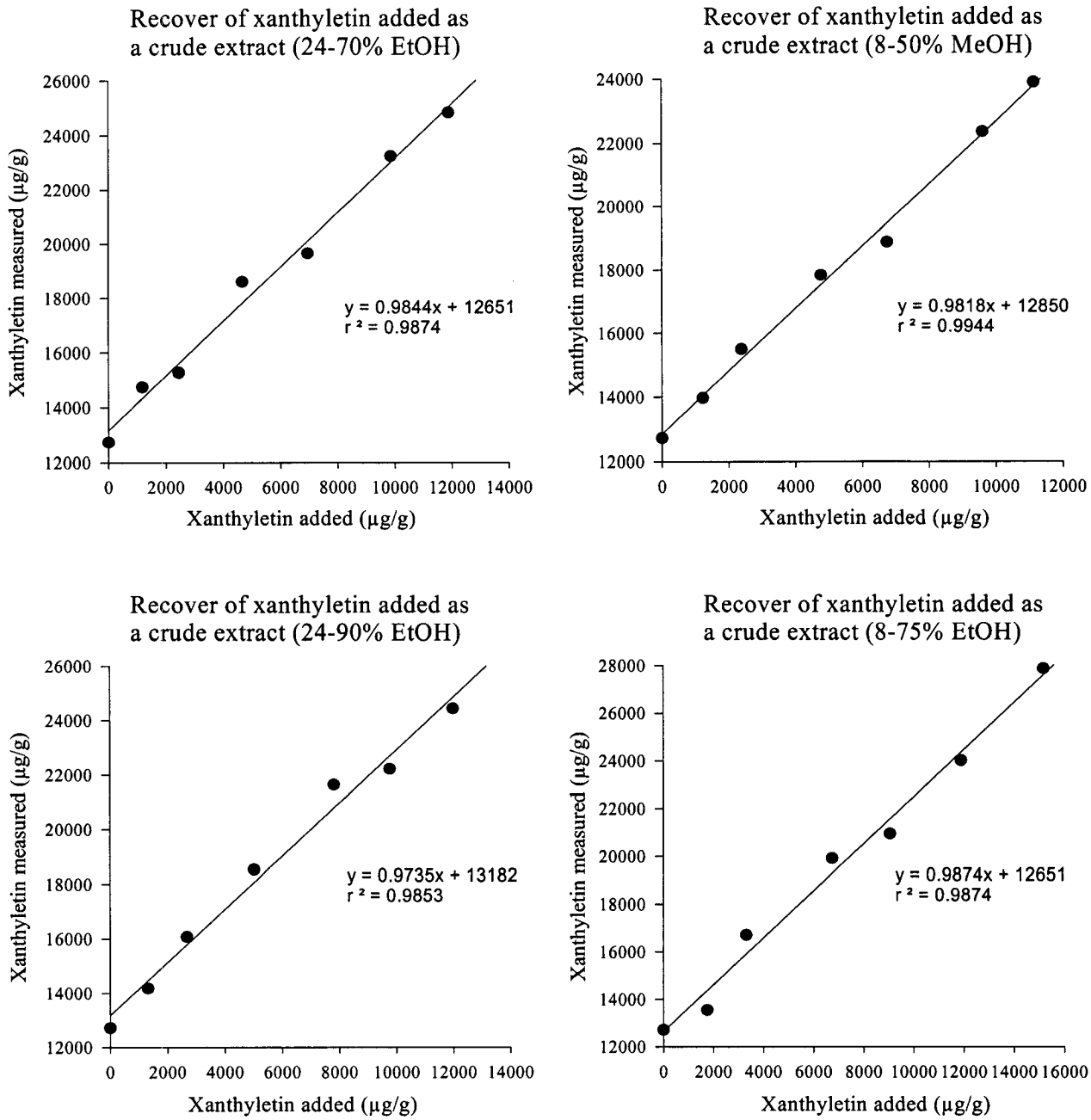


Figure 3.5 Recovery experiments with different amounts of xanthyletin added to 1.0 g powdered plant materials of *Z. americanum*. Samples were spiked by the addition of 50, 100, 200, 300, 400 or 500 mg of various crude *Z. americanum* extracts. A linear regression of amount measured on amount added was used to determine recovery estimates. Amounts of exogenous xanthyletin added were calculated based on content of respective crude extracts (24-70% EtOH: 24.69 µg/mg; 24-90% EtOH: 26.10 µg/mg; 8-50% MeOH: 23.95 µg/mg; 8-75% EtOH: 32.29 µg/mg).

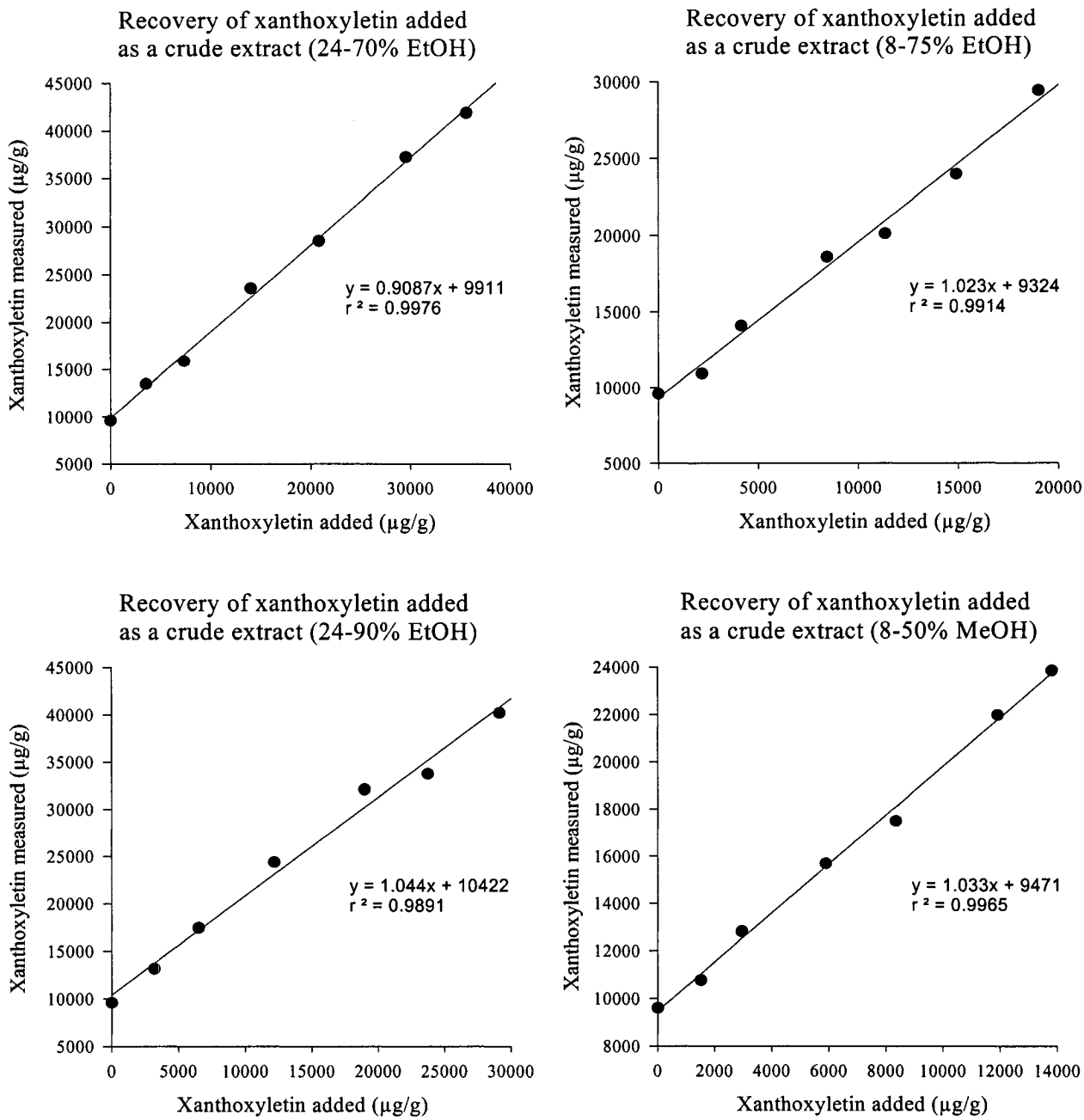


Figure 3.6 Recovery experiments with different amounts of xanthoxyletin added to 1.0 g powdered plant materials of *Z. americanum*. Samples were spiked by the addition of 50, 100, 200, 300, 400 or 500 mg of various crude *Z. americanum* extracts. A linear regression of amount measured on amount added was used to demonstrate the efficiency of the assay. Xanthoxyletin content were previously determined for respective extracts (24-70% EtOH: 73.96 µg/mg; 24-90% EtOH: 63.42 µg/mg; 8-50% MeOH: 29.65 µg/mg; 8-75% EtOH: 40.48 µg/mg).

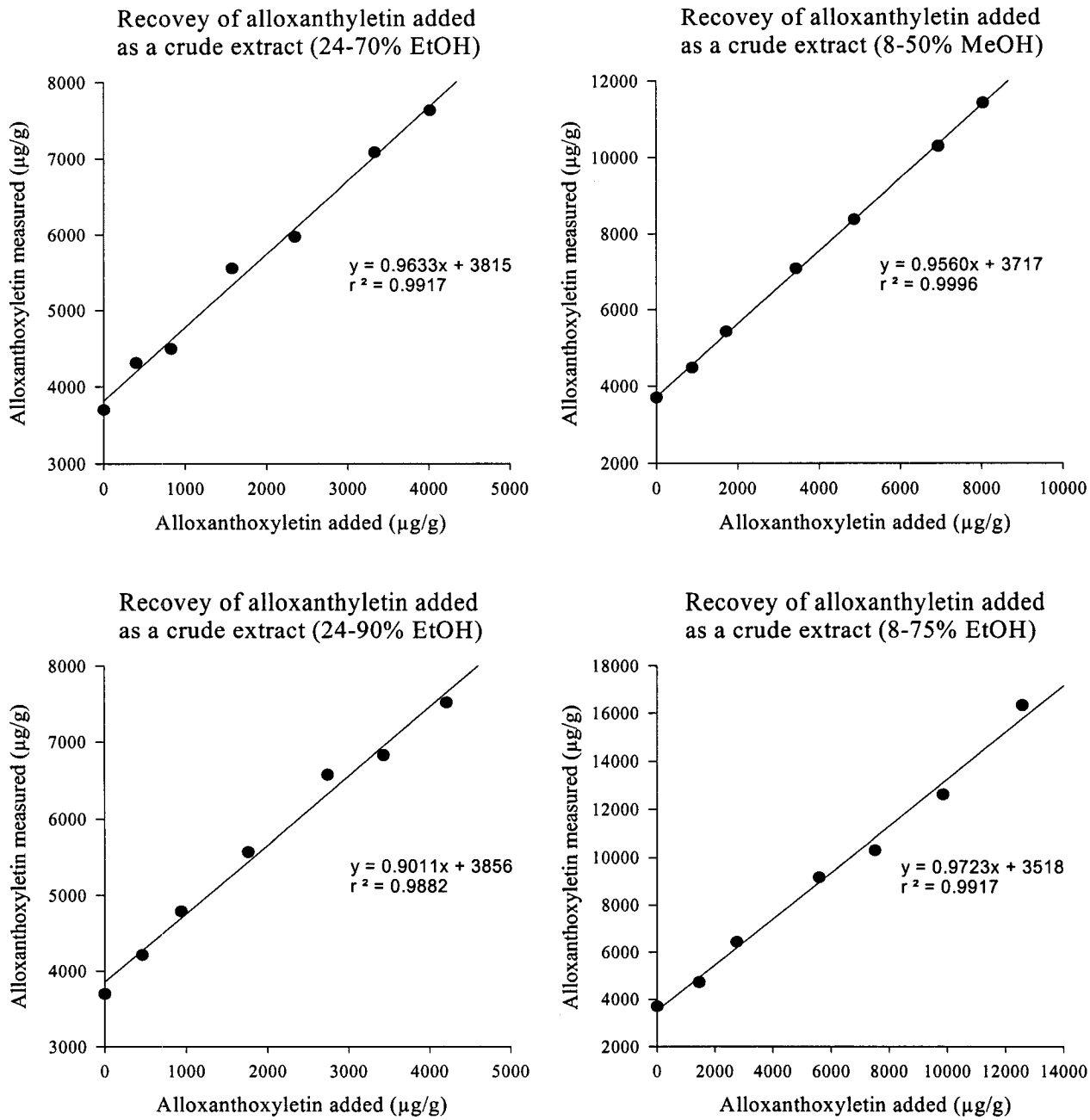


Figure 3.7 Recovery experiments with different amounts of alloxanthoxyletin added to 1.0 g powdered plant materials of *Z. americanum.* Samples were spiked by the addition of 50, 100, 200, 300, 400 or 500 mg of various crude *Z. americanum* extracts. A linear regression of amount measured on amount of exogenous marker compound added was used to illustrate the efficiency of the assay. Alloxanthoxyletin content were previously determined for respective extracts (24-70% EtOH: 8.34 µg/mg; 24-90% EtOH: 9.16 µg/mg; 8-50% MeOH: 26.79 µg/mg; 8-75% EtOH: 17.28 µg/mg).

Consequently, the determination of recovery efficiencies in which crude extracts are used will also reflect how well the extraction method can liberate marker phytochemicals from a complex mixture of potentially binding compounds.

3.3.1.5 *Calibration curves*

The relationship between peak area and the concentration of reference compounds was established using a five-point regression curve for all three pyranocoumarin markers operating in the range 18.3-292.5 μg/ml for xanthyletin, 13.4-215.0 μg/ml for xanthoxyletin and 8.78-140.5 μg/ml for allo-xanthoxyletin. A good linearity of the calibration curves was obtained for all three marker constituents with coefficient of determination (r^2) values > 0.999 (Table 3.3). Quantification was also performed with standard solutions obtained from several furanocoumarins, including xanthotoxin (13.57-217 μg/ml), psoralen (19.06-305 μg/ml) and imperatorin (7.01-112.5 μg/ml), all with r^2 values > 0.999.

3.3.1.6 *Minimum detectable limits*

The sensitivity of the proposed HPLC assay at detecting marker compounds was evaluated using serial dilutions containing all three marker standards, but such experiments although informative do not address or incorporate the complexities of actual samples. Namely, standard solutions provide well-resolved and defined peaks, which simplify the distinction of eluting compounds from baseline noise. Therefore to provide a more realistic estimate as to the minimum detectable limits, serial dilutions with a randomly selected sample were also conducted.

Table 3.3 Calibration factors and retention times of ten coumaric compounds potentially present in raw materials from *Zanthoxylum americanum* plants.

Compound	R_t[a] (min)	Range (μg/ml)	Equation $y = ax + b$	r^2
5,7,8-trimethoxycoumarin	9.21	1.72-27.5	$y = 9.47 \times 10^3x - 14143$	0.9997
Psoralen	11.87	19.06-305.0	$y = 5.77 \times 10^3x + 25825$	0.9999
Angelicin	12.31	15.63-250.0	$y = 8.37 \times 10^3x - 77291$	0.9999
8-methoxypsoralen	12.52	13.57-217.0	$y = 1.18 \times 10^4x + 29695$	0.9999
Isopimpinellin	14.61	6.38-102.0	$y = 1.33 \times 10^4x + 154$	0.9999
5-methoxypsoralen	14.88	10.63-170.0	$y = 2.39 \times 10^4x + 50051$	0.9999
Xanthyletin[b]	20.05	18.28-292.5	$y = 1.19 \times 10^3x - 290826$	0.9996
Xanthoxyletin[b]	21.70	13.44-215.0	$y = 1.11 \times 10^4x - 275658$	0.9995
Imperatorin	22.48	7.01-112.5	$y = 2.28 \times 10^4x - 170829$	0.9999
Allo-xanthoxyletin[b]	24.88	8.78-140.5	$y = 7.57 \times 10^3x - 74932$	0.9998

[a] Retention times measured using analytical HPLC under gradient elution conditions with UV detection at 225 nm.
[b] Evaluated as marker constituents for authentication and standardization of raw commercial *Z. americanum* plant materials.

The minimum detectable limits for marker constituents in both standard solutions ($n = 1$) and samples ($n = 4$) are provided in Table 3.4. The results, as expected, indicate that a signal to noise (S/N) ratio of five as opposed to three is more rigorous with respect to the differentiation of eluting peaks from baseline noise and represents a more stringent standard in the analysis of commercial raw material. Secondly, minimum detectable limits are slightly higher when determined with samples and more relevant. For example, the absence of an eluting peaking for alloxanthoxyletin in raw plant materials indicates 21.79 ± 1.12 ng as what would be the maximum level in the plant for non-detection, as opposed to 13.18 ng.

Table 3.4 Minimum detectable amounts of marker phytochemicals corresponding to a 5 μL inject onto HPLC column. Detectable limits were established based on serial dilution preparations from a randomly selected plant sample ($n = 4$) with results being integrated at a signal to noise ratio of 3(**1**) and 5(**2**). Detection limits of marker compounds in stock a solution (**3**) are also provided for comparison.

	Minimum detectable amounts (ng) (mean ± standard deviation)		
Marker constituent	**1**	**2**	**3***
Xanthyletin	3.29 ± 0.25	8.05 ± 1.18	5.16
Xanthoxyletin	6.66 ± 0.88	6.66 ± 0.88	6.96
Alloxanthoxyletin	9.86 ± 0.84	21.79 ± 1.12	13.18

* No replicates used for stock solution, at a signal noise ratio of 5.

3.3.2 *Estimation of marker constituents in the plant*

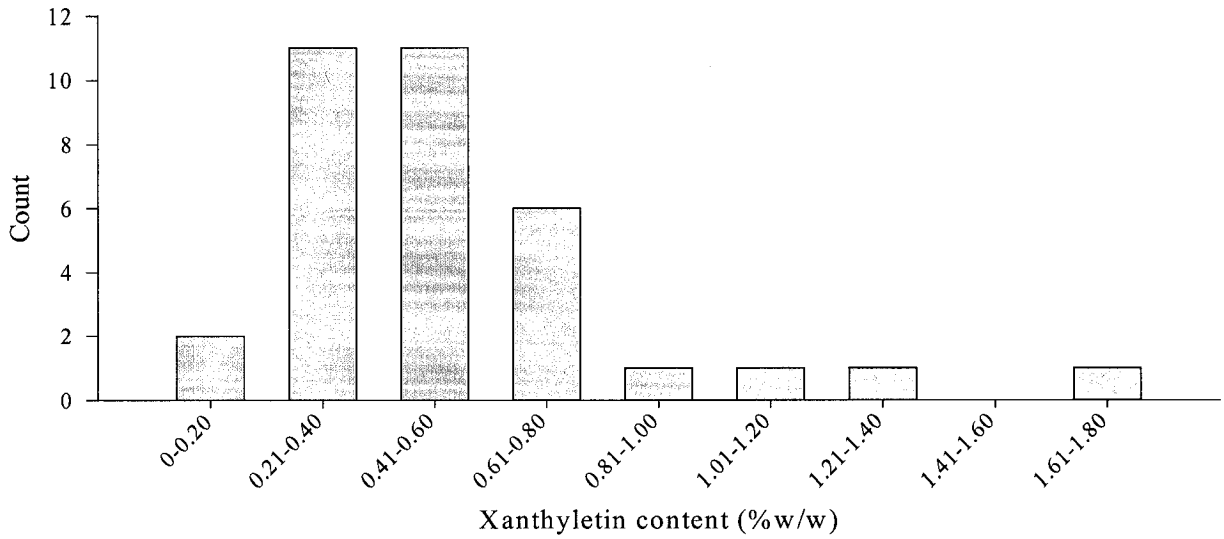
3.3.2.1 *Distribution in raw plant material*

Using the fully validated HPLC assay described here, the distribution of the defined marker constituents were evaluated in commercial raw materials drawn from 34 sites throughout the range of *Z. americanum* in Eastern Ontario, Canada. The content (% w/w) of xanthyletin, xanthoxyletin and alloxanthoxyletin in plant samples varied from 0.1-1.8%, 0.2-1.9% and 0.05-0.68% respectively (Figure 3.8). These results, contrary to our initial expectations do not show the bimodal distribution of marker compounds among populations, but are rather suggestive of a skewed normal distribution. Because prickly ash plants are clonally propogated, it would not be unlikely for distinct genotypes to exist resulting in bimodal (or greater) distributions for observed phytochemicals. Despite the lack of support for this contention, clear differences exist among samples with regard to both the composition and content of marker compounds (Figure 3.9). As well the amounts of these marker compounds do not appear to be correlated.

3.3.2.2 *Distribution within plant*

The distribution of marker constituents in *Z. americanum* plant parts was estimated by determining the sum of xanthyletin, xanthoxyletin and alloxanthoxyletin in various crude ethanolic extracts. Phenolic markers were present almost exclusively in the non-photosynthetic tissues of the plant, namely the wood (including stems and bark) and root and typically followed an allocation pattern opposite to that documented for furanocoumarins (Figure 3.10). These findings are of much interest because they could be suggestive as to the role of xanthyletin, xanthoxyletin and alloxanthoxyletin as defence compounds within these plant parts.

Figure 3.8 Distribution of content (%w/w) of marker constituents (xanthyletin, xanthoxyletin and alloxanthoxyletin) in several wild populations of *Z. americanum* plants. Authentic plant materials were collected throughout the range of this plant from 34 sites (27 in 1998 and 7 in 2001) within eight Eastern Ontario counties.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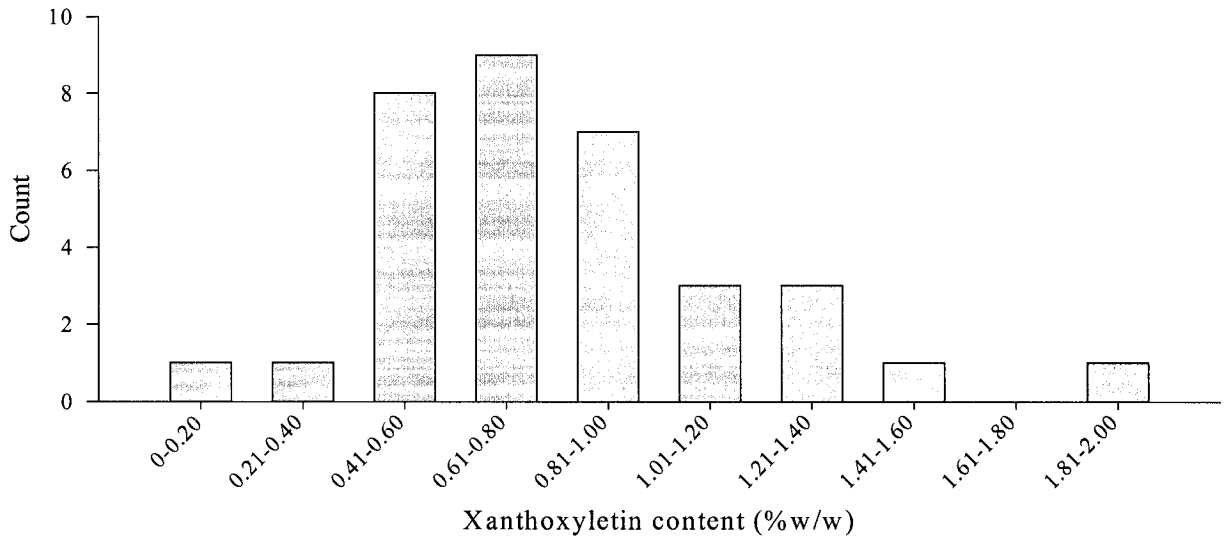
10
8
6
4
2
0
0-0.20
0.21-0.40
0.41-0.60
0.61-0.80
0.81-1.00
1.01-1.20
1.21-1.40
1.41-1.60
1.61-1.80
Xanthyletin content (%w/w)


Count
10
8
6
4
2
0
0-0.20
0.21-0.40
0.41-0.60
0.61-0.80
0.81-1.00
1.01-1.20
1.21-1.40
1.41-1.60
1.61-1.80
1.81-2.00
Xanthoxyletin content (%w/w)

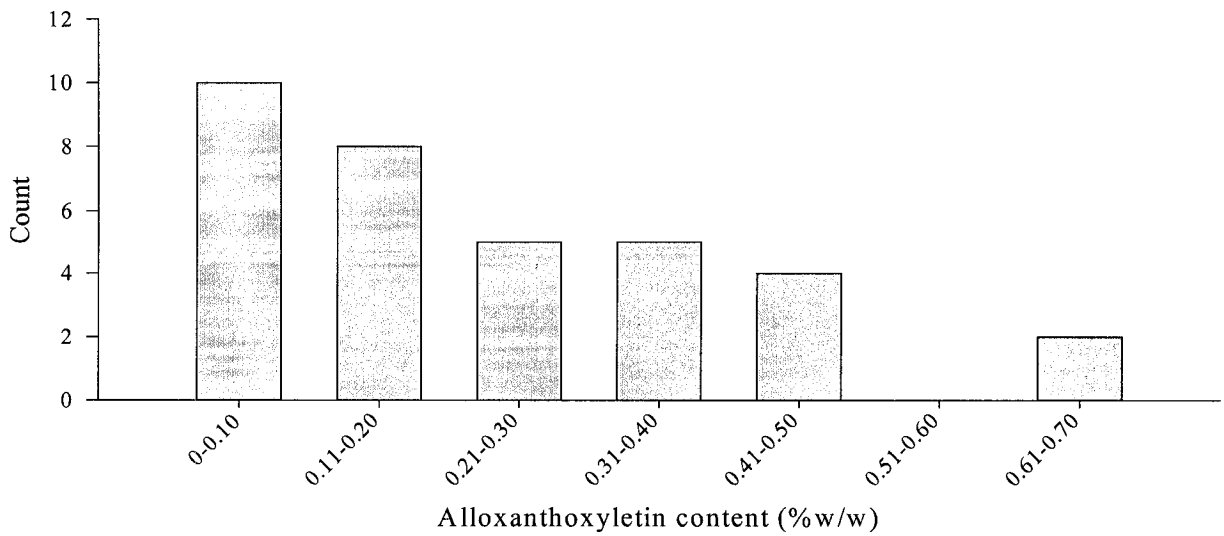
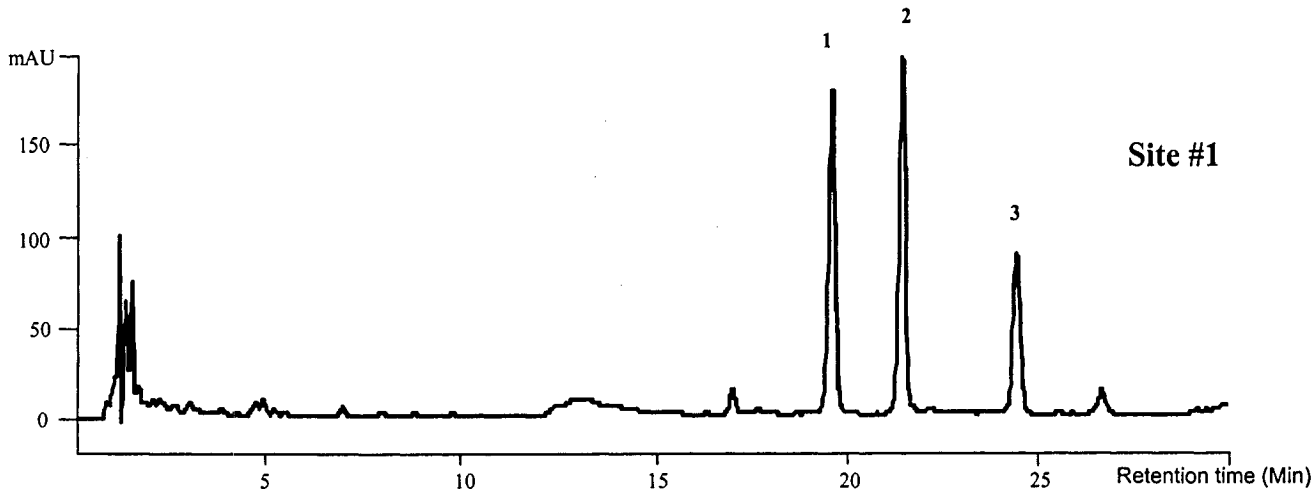

Count
12
10
8
6
4
2
0
0-0.10
0.11-0.20
0.21-0.30
0.31-0.40
0.41-0.50
0.51-0.60
0.61-0.70
Alloxanthoxyletin content (%w/w)

Figure 3.9 HPLC chromatographs of *Z. americanum* samples acquired by HPLC-DAD at 225 nm, showing the separation of marker constituents from 4 collection sites. Key to peak identities: **1**, xanthyletin; **2**, xanthoxyletin and **3**, alloxanthoxyletin. Gradient conditions are described in the Material and Methods section.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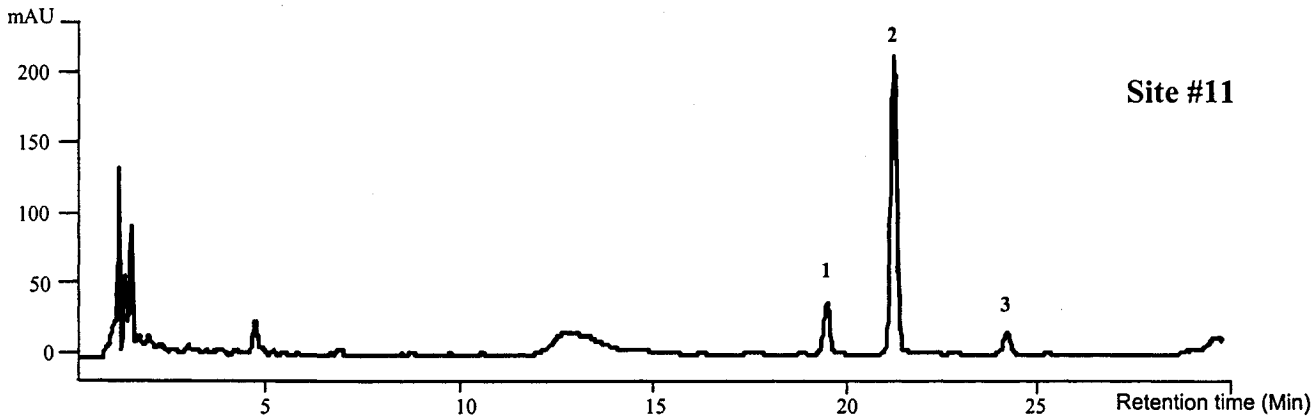
100
50
0
1
2
3
Site #1
5
10
15
20
25
Retention time (Min)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100
50
0
1
2
3
Site #11
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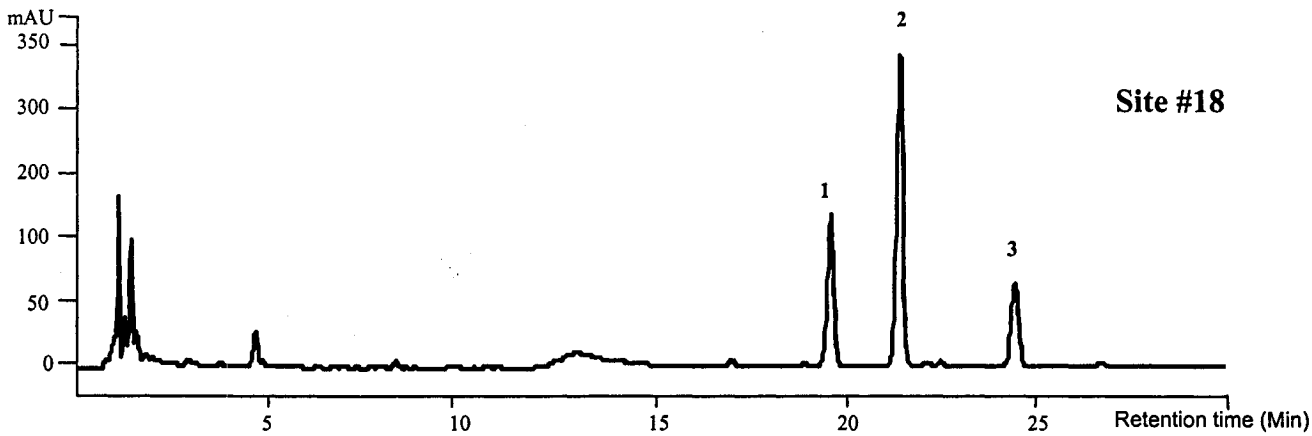
10
15
20
25
Retention time (Min)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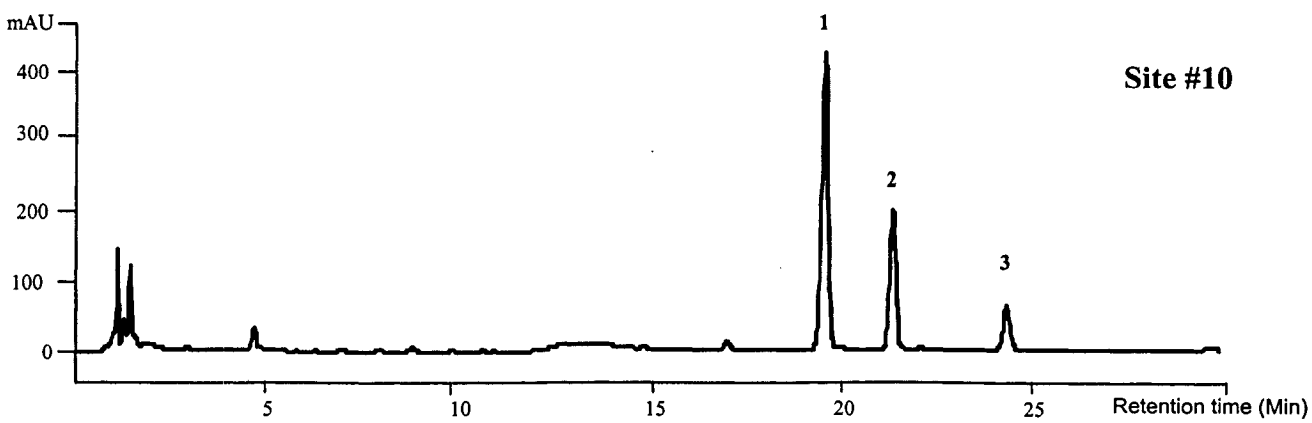
300
200
100
50
0
1
2
3
Site #18
5
10
15
20
25
Retention time (Min)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Site #10
5
10
15
20
25
Retention time (Min)

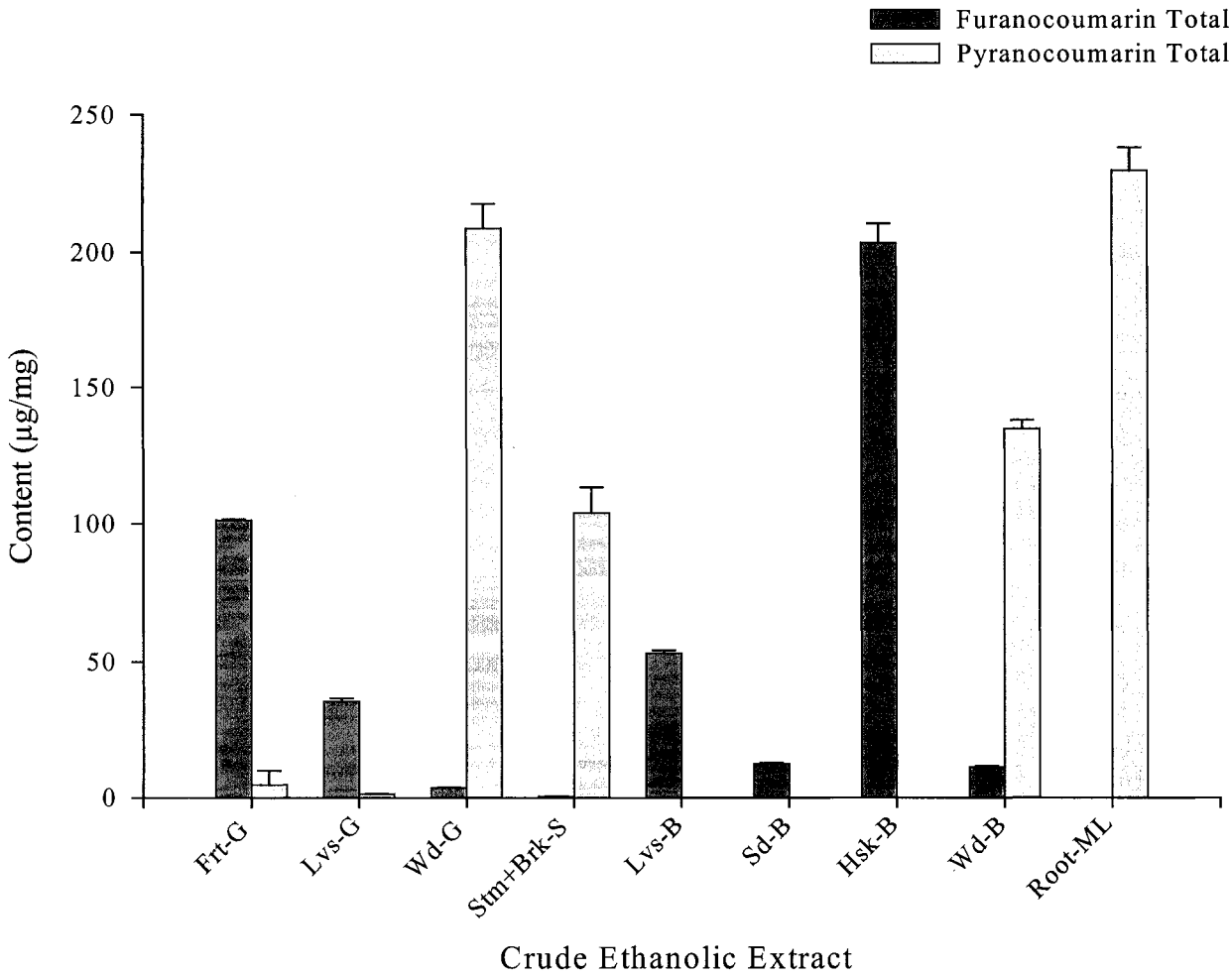


Figure 3.10 Differential allocation of coumaric compounds among *Z. americanum* plant parts. Total furanocoumarin (xanthotoxin, psoralen and imperatorin) and total pyranocoumarin content (xanthyletin, xanthoxyletin and alloxanthoxyletin) are based on duplicate HPLC analyses. Bars above means denote standard deviation. Crude ethanolic extracts were prepared from samples collected at North Gower (**G**), Belleville (**B**), Ottawa (**ML**) and Sault Ste. Marie (**S**).

The observation of marker constituent amounts ascertained from crude extracts of root and wood samples provides some support for this view (Figure 3.11). The results reveal that all three marker compounds are most abundant in the bark and root and largely absent from the vascular tissues of the wood. These results are all consistent with the accumulation of biologically active compounds in the cuticle of plant organs as is the case for many defence compounds (Towers 1984). Furthermore, the presence of pyranocoumarins in the bark and root of this plant may be of relevance to the various ethnomedicinal uses of these parts, including in the treatment of burns and afflictions of the respiratory system (Erichsen-Brown 1979; Moerman 1998).

3.3.2 *Suitability of chosen phytochemical markers*

The development of products of herbal origin for animal and human health markets requires the control of the quality of raw materials (Rukangira 2001). Consistent quality products can only be assured if the starting plant materials are defined in a rigorous and detailed manner (Mehta *et al.* 2001; Rukangira 2001). Consequently, identification and authentication is an absolute necessity.

In the present study, we have tried to define specific compounds, generally referred to as marker constituents, for the authentication and standardization of *Zanthoxylum americanum* Mill. raw plant material. The chosen marker phytochemicals (xanthyletin, xanthoxyletin and alloxanthoxyletin) are all recognized as potential low-toxicity anticancer agents with anti-proliferative activities against several cancer cell lines including human lung carcinoma (A549) and human leukemia (HL-60) cells (Gunatilaka and Kingston 1994; Ju *et al.* 2002; Kawii *et al.* 2001).

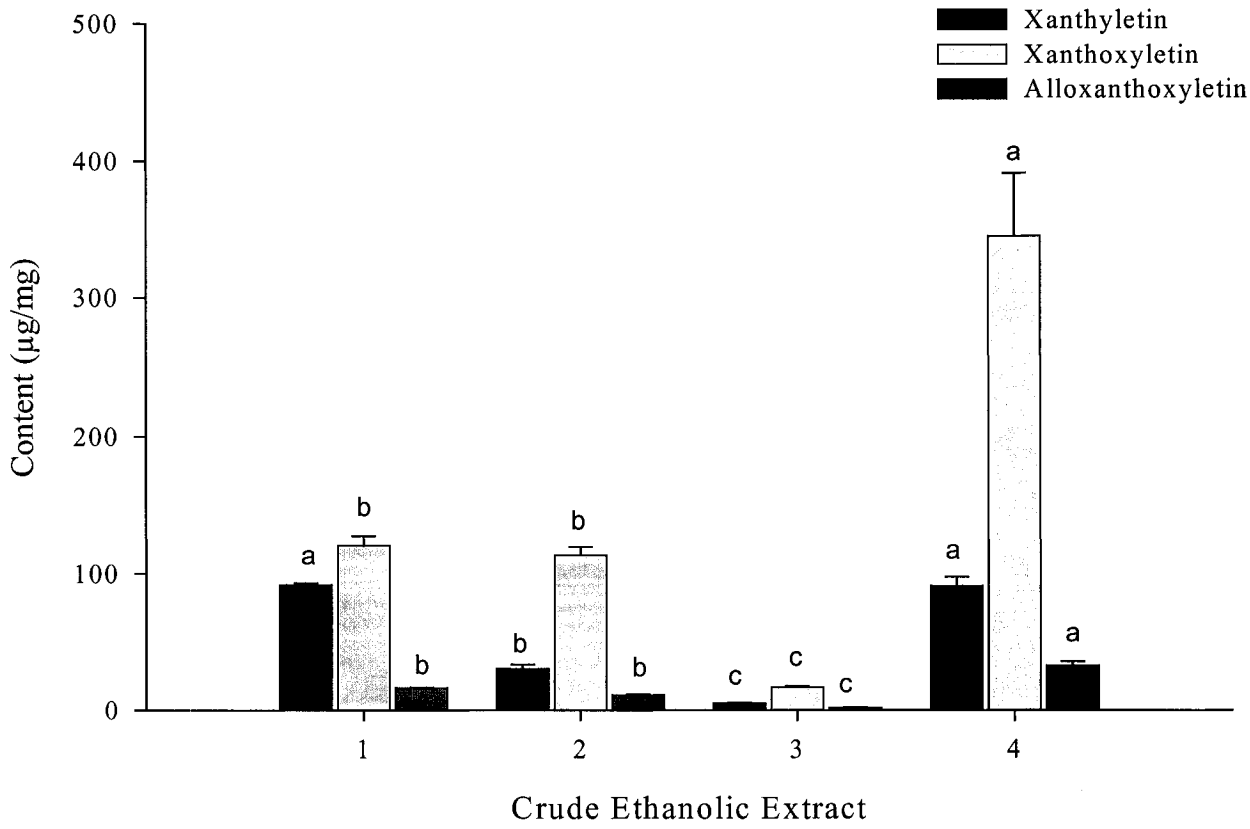
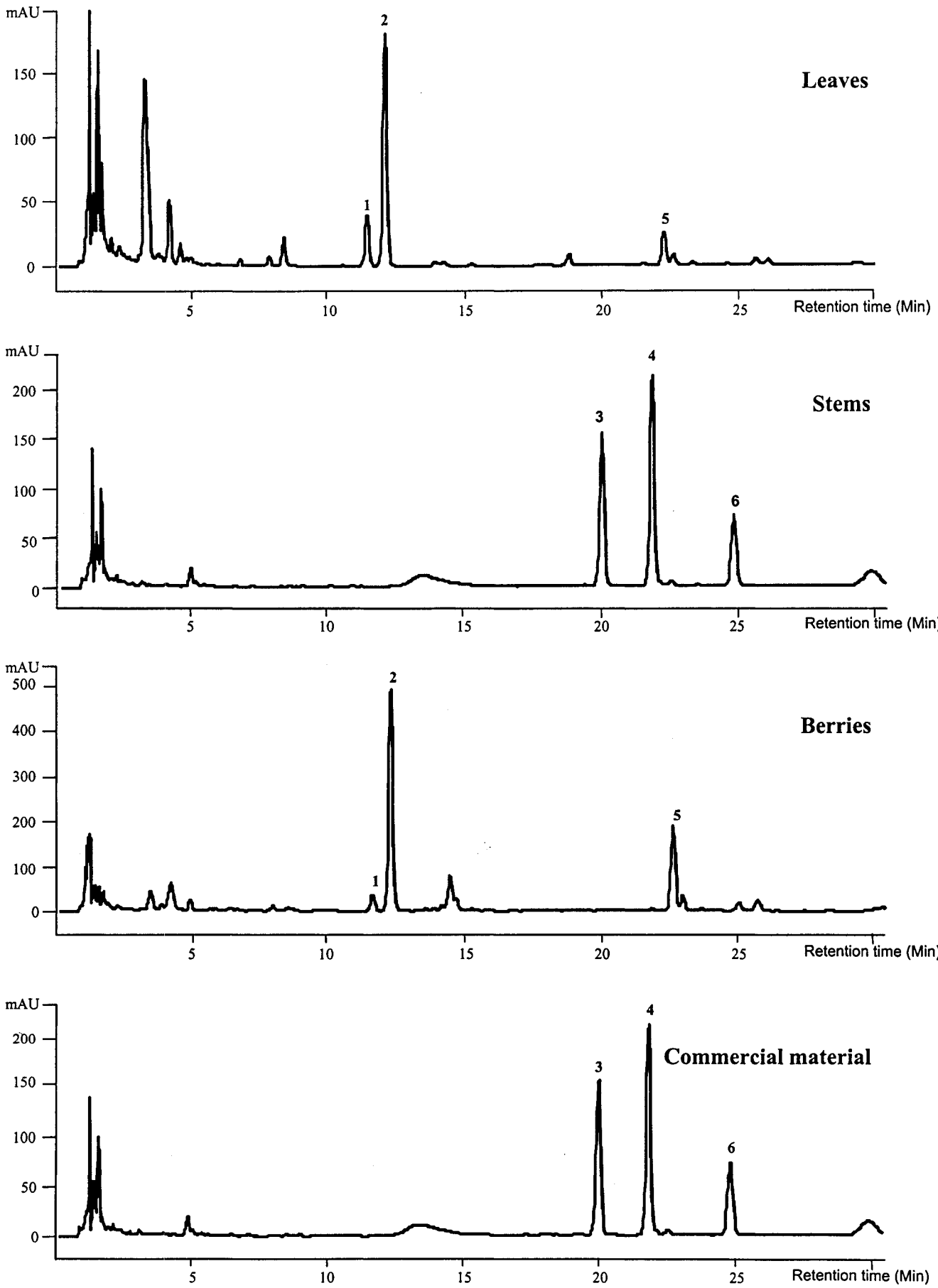


Figure 3.11 Marker constituent compositions in various non-photosynthetic plant parts of *Zanthoxylum americanum.* Letters above means were determined using Bonferroni's matrix of pairwise comparisons, whereby extracts were compared with respect to content of marker compounds. No statistical analysis was performed for markers within extracts. Key to extract identities: **1**, root (*n* =2); **2**, wood (*n* =2); **3**, wood without bark (*n* =2); **4**, bark only (*n* =4). Bars above means denote standard deviation.

In addition, our phytochemical analysis of *Z. americanum* plants allows for the detection of several furanocoumarins. This is because raw materials of prickly ash consist of the terminal portions of the plant (leaves, berries, stems and twigs), therefore requiring a standardization method that included other relevant classes of compounds (Figure 3.12). This not only allows for the control of potentially active principles, but potentially toxic compounds as well.

In evaluating the amounts of marker constituents at 34 randomly selected sites throughout the range of *Z. americanum* in Eastern Ontario, obvious inherent variations were observed. These are most likely a result of genotype and environmental interactions. Undeniably, factors such as temperature, soil, humidity, light or biotic interactions will influence the abundance and distribution of secondary metabolites including marker compounds from *Z. americanum*. It is also conceivable and highly probable that variability among the different geographic locations surveyed do not comprise all possible ranges for the distribution of marker compounds in all Northern prickly ash populations. Because secondary metabolites mediate part of the evolutionary process of plants (Romani *et al.* 2002), continued bioprospecting of wild *Z. americanum* plants is essential in order to understand environment-induced changes in marker compounds. In addition, controlled environment studies should be used to complement this approach and may prove useful in addressing potential genetic differences among *Z. americaum* plants.

Figure 3.12 Chromatographic profiles of crude ethanolic extracts (leaves, stems and berries) and commercial raw material of *Z. americanum* acquired by HPLC-DAD detected at 225 nm. Key to peak identities: **1**, psoralen; **2**, 8-methoxypsoralen; **3**, xanthyletin; **4**, xanthoxyletin; **5**, imperatorin; and **6**, alloxanthoxyletin. A LiChrospher® RP-18 column was used at 50 °C and eluted with water, 25 mM sodium dihydrogen ortho-phosphate at pH 3.0 (with hydrochloric acid and acetonitrile at a flow rate of 1.0 mL/min following the two-step linear gradient system described in the Materials and Methods section.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2
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Leaves
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Retention time (Min)
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3
4
6
Stems
5
10
15
20
25
Retention time (Min)
mAU
500
400
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200
100
0
1
2
5
Berries
5
10
15
20
25
Retention time (Min)
mAU
200
150
100
50
0
3
4
6
Commercial material
5
10
15
20
25
Retention time (Min)

CHAPTER 4

ANTIVIRAL ACTIVITY OF *ZANTHOXYLUM AMERICANUM* EXTRACTS AGAINST *HERPES SIMPLEX* VIRUS *IN VITRO*

4.1 Introduction

Ethnomedicinal preparations from *Zanthoxylum americanum* Mill. (Rutaceae), a North American woody shrub are widely used for the treatment of rheumatism, toothaches and topical conditions including burns and wounds (Moreman 1998). In addition, *Z. americanum* preparations are commonly indicated for the treatment of infectious conditions. Traditionally, the bark or root and or berries are used by First Nations groups, particularly for skin diseases and for the treatment of problems associated with the respiratory apparatus such as pneumonia, bronchitis and coughing (Erichsen-Brown 1979; Moerman 1998). In Table 4.1, a summary of the ethnomedicinal uses of Northern prickly ash by Native Americans to treat infections possibly caused by a variety of microorganisms including *Herpes simplex* virus is provided. To date, there has been no attempt to relate the ethnobotanical use of *Z. americanum* by First Nations groups with the biological activity of secondary metabolites present in the plant.

In common with many members within the principally tropical *Zanthoxylum* genus (Sargent 1961), *Z. americanum* is reported to produce an elaborate variety of secondary metabolites, including several classes of phenolic compounds with wide-ranging pharmacological activities (Gray and Waterman 1978). Equally important, is the fact that members of this genus typically grow and express secondary metabolites under severe stress conditions, such as drought and high light (Mellieur *et al.* 1994).

Table 4.1 Traditional medicinal uses of *Z. americanum* plant parts by different First Nations groups to treat afflictions potentially involving viral origins (Erichsen-Brown 1979; Moerman 1998).

Condition/use	Preparation & part used	Group
Bronchial disease	Infusion of ripe berries	Menominee
	Infusion of bark	Chippewa
Bronchitis	Infusion of ripe berries	Ojibwa
Cough medicine	Decoction of bark & berries	Meskwaki
	Infusion of bark	Chippewa
Skin disease/sores	Decoction of bark	Alabama
	Infusion of inner bark	Alabama
	Infusion of berries	Menominee
Sore throat	Decoction of bark & berries	Ojibwa
	Decoction of root	Chippewa
Tonsillitis	Decoction of bark & berries	Ojibwa; Chippewa

As a result, the presence of polyphenols in these plants may be of ecological importance by preventing plant cells from the detrimental effects of solar radiation and reactive oxygen species (Husain *et al.* 1987; Shirley 1996). In addition to their protective role in plants, polyphenols and phenolic compounds are considered the most important group of natural antioxidants. In addition they posses a wide range of physiological and pharmacological actions, including antiviral activity (Haslam 1996; Islam *et al.* 2002). A summary of some biological activities of natural polyphenols is given in Table 4.2.

In this study, the therapeutic potential of *Z. americanum* extracts for viral infections was evaluated based on its biological activity against the *Herpes simplex* virus type 1 (HSV-1), a DNA virus with a membrane that causes skin infections and cold sores (Hudson *et al.* 1999; Anani *et al.* 2000; Binns 2001). Following this preliminary testing of extracts for antiviral activity, selected extracts were tested against Sindbis virus (SINV), a close relative of the Western, Eastern and Venezuelan equine encephalitis viruses and poliovirus type 1 (PV-1), a RNA virus without a membrane (Table 4.3).

The objectives of the present study were to 1) determine the antiviral activities of various phytochemical fractions prepared from *Z. americanum* extracts, 2) to characterize tested *Z. americanum* extracts in relation to their polyphenolic compositions and 3) to provide biological and scientific evaluation of the ethnomedicinal uses of *Z. americanum* preparations to treat infectious conditions. The hypothesis that antiviral activity of *Z. americanum* extracts is dependent on polyphenolic content and composition was tested. To the best of our knowledge, no specific study has been carried out to elucidate the polyphenolic compositions (phenolic acids, flavonoids and tannins) of Northern prickly ash shrubs.

Table 4.2 Some physiological and pharmacological actions of polyphenols determined *in vitro*. Source: Haslam (1996).

	Activity
i	Bactericidal action
ii	Molluscicidal action
iii	Anthelminitic action
iv	Antihepatoxic action
v	Stimulation of phagocytic cell iodination
vi	Inhibition of human immunodeficiency viral replication (HIV)
vii	Inhibition of human simplex virus (HSV)
viii	Inhibition of glucosyl transferases of *Streptococcus mutans* (dental caries)
ix	Inhibition of ascorbate autooxidation (green tea)
x	Inhibition of lipoxygenase dependent peroxidation; "French Paradox"
xi	Host-meditated antitumor activity: cytotoxic effects, inhibition of tumor promotion, inhibition of Ornithine decarboxylase (ODC) response
xii	Inhibition of xanthine oxidase and monoamine oxidase

Table 4.3 Properties of viruses used for the evaluation of the antiviral efficacy of *Z. americanum* plant extracts (Hudson *et al.* 1999).

Virus	Abbreviation	Host	Genome	Membrane
Herpes simplex type 1	HSV-1	Human	DNA	+
Poliovirus type 1	PV-1	Human	RNA	-
Sindbis virus	SINV	Mammals, mosquitoes	RNA	+

4.2 Materials and Methods

4.2.1 *Phytochemical fractions studied for antiviral activities*

In total, four types of phytochemical fractions derived from *Z. americanum* shrubs were evaluated for antiviral activities (Table 4.4). Evaluated extracts could be classified into: 1) the polar fraction containing the alkaloids (mainly magnoflorine, nitidine and chelerythrine), 2) the lipophilic faction containing the furanocoumarins (essentially psoralen, xanthotoxin and imperatorin, 3) a second lipophilic fraction containing the pyranocoumarins (primarily xanthyletin, xanthoxyletin and alloxanthoxyletin) and 4) the polyphenolic fraction containing phenolic acids, flavonoids and possibly tannins.

4.2.2 *Extraction of phytochemical fractions*

To preferentially remove polar constituents from *Z. americanum* shrubs, the terminal portions of the plant were harvested and extracted following a modified procedure previously described for several *Zanthoxylum* species (Stermitz *et al.* 1980). In brief, air-dried stems (approximately 1.6 Kg) were milled to a fine powder (1 mm sieve) and extracetd successively in hexane then metanol (approximately 1 g biomass to 9 mL solvent). The methanol filtrate was concentrated to 400 mL and an equal amount of water added. The soluble portion was evaporated to dryness and the residue redissolved in 50 mL of methanol and precipitated with acetone. The precipitate was added to water, filtered and the solutions concentrated. The resulting residue (A1R) could potentially contain the alkaloids chelerythrine, magnoflorine and nitidine along with other polar constituents if present in *Z. americanum* terminal parts.

Table 4.4 *Z. americanum* plant extracts studied for antiviral activities. Extracts are grouped into four phytochemical fractions based on the different types of compounds targeted during extraction.

Targeted compounds	Plant part used	Extraction solvent
Polar compounds (i.e. alkaloids)	Terminal portions	Acidified methanol
	Terminal portions	50% methanol
	Terminal portions	Hot water
Furanocoumarins	Husk	95% ethanol
	Husk	Ethyl acetate
Pyranocoumarins	Root	95% ethanol
	Bark only	95% ethanol
Tannins and related polyphenols	Terminal portions	70% acetone
	Terminal portions	70% acetone

A summary of the procedure appears in Figure 4.1. In addition to the alkaloid residue, finely ground plant material randomly drawn from commercially available raw materials was extracted using 50% methanol or hot water (approximately 1 g of plant biomass to 9 mL of solvent) and resulting filtrate concentrated to yield corresponding crude extracts (Sample 8-50% methanol and Sample 8-hot water).

Lipophilic phytochemical fractions rich in furanocoumarins (husk-95% ethanol and husk-ethyl acetate) were those previously evaluated for antifungal activities and their extraction procedures are described in chapter 2 (Material and Methods) of this thesis. Extracts rich in pyranocoumarins (bark-95% ethanol, root-95% and isolated marker compounds) were extracted following the procedure outlined in chapter 3 (Material and Methods) of this thesis.

The extraction of tannins and related polyphenols was carried out as previously reported by Hagerman and Butler (1991). Using randomly selected samples drawn from the natural populations of *Z. americanum* shrubs surveyed (chapter 3), air-dried and mill powdered plant materials (terminal portions) were exhaustively extracted with 70% acetone (Me_2CO) using ultrasound treatments. Approximately 9 ml of solvent was used for each gram of dry plant material. Following suction filtration, the acetone filtrate was then evaporated to dryness under vacuum at 50 °C and the residue freeze-dried to obtain a crude extract (D1, pooled collection from 1998 harvest of sites 7, 5 & 27; D2 pooled collection from 1998 harvest of sites 11, 4 and 2001 harvest of sites 3, 4, 6 & 7). Because the aim of this study was to investigate the polyphenolic phytochemical constituents of *Z. americanum* extracts in more detail, extracts of *Acer saccharum* (sugar maple) were include and used only to ensure protocols employed for identification of *Z. americanum* constituents were functioning efficiently.

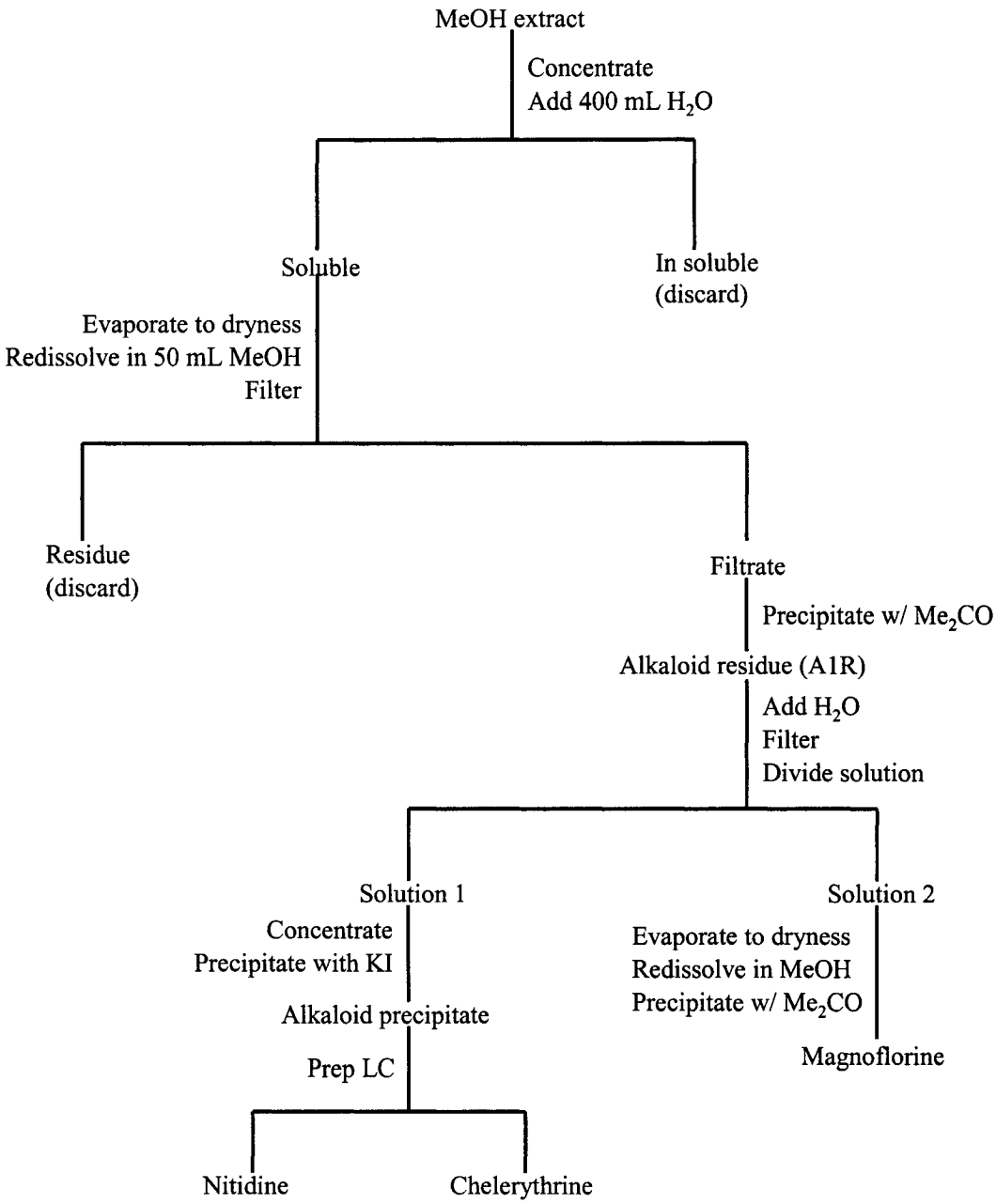

Figure 4.1 Procedure for extracting alkaloid residue sample. Modified from Stermitz *et al.* 1980.

Leaf material of *A. saccharum* was collected in October of 2002 from mature trees (estimated to be between 30-35 years old) located on the University of Ottawa campus, (Ontario K1N 6N5 Canada). Fresh foliage (approximately 30 g) with petioles removed were steeped in 95% ethanol or 70% ethyl acetate for 12 hours, macerated in a commercial food processor and exhaustively extracted using ultrasound treatments. Resulting filtrates (approximately 225 mL) were evaporated under reduced pressure and crude extracts obtained (M1, 95% EtOH; M2, 70% Me_2CO) following methods described for previous extracts.

4.2.3 *Antiviral bioassay*

The technique used and described below is a modified form of the "virucidal" protocol reported in detail previously (Morales 1992; Hudson *et al.* 1999, 2000; Anani *et al.* 2000).

4.2.3.1 *Cells and viruses*

Vero cells (monkey kidney line, American Type Culture Collection) were propagated in a 5% carbon dioxide atmosphere at 37 °C in Dulbecco Modified Eagle Medium (MEM-minimal essential medium) with 5% fetal bovine serum (FBS) (Gibco Life Sciences, Ontario), in 96-well tissue culture trays (Falcon), 0.1 mL per well. Once confluent monolayers were obtained for cultivated cells, they were used for assays. *Herpes simplex* virus type-1 (HSV-1), Sindbis virus (SINV) and poliovirus type-1 (PV-1) were clinical isolates that had undergone several propagations in Vero cells (Hudson *et al.* 1999).

4.2.3.2 *Antiviral test ("virucidal" protocol)*

Each plant extract was prepared as a duplicate series of two-fold dilutions (in 100 µL of extract into 100 µL MEM without serum) to give a range of final concentrations from 500 to 1 µg/mL, across a row of wells in an empty 96-well microtest tray. With the aid of a multipipetting device, 100 µL of the virus in MEM (without FBS) comprising of 100 plaque-forming units (pfu) was added to each well (except cell controls). The virus-extract mixtures were transferred to a shaker platform inside an environmental chamber at 30 °C and exposed to a combination of fluorescent and long-wave UV (covering the range of 320-600 nm). After 30 minutes of exposure, the virus-extract mixtures were transferred to a tray of aspirated Vero cell monolayers in another 96-well tray and returned to the incubator.

Control cultures were subjected to identical light exposure and included two types of control cells: 1) cells without virus and no plant extract, in which case the cells should remain healthy for the duration of the test and 2) infected untreated cells (infected with the virus, but without plant extract added), which should display maximum cytopathic effects (CPE) in the time indicated. All cultures were examined microscopically and assessed for viral characteristic cytopathic effects. In the case of HSV-1, complete cell destruction (100% viral CPE) required 4 days; for SINV, 3 days and 2 days for poliovirus in infected untreated cells (those incubated without *Z. americanum* extracts). In the event where no CPE were evident, the virus (comprising of a 100 pfu) was assumed to be completely inactivated or inhibited. Partial inactivation of the virus, i.e. a 50% decrease or less in CPE compared to untreated virus were also recorded and represented inactivation or inhibition of a fraction of the 100 pfu present in the standard virus dose (Hudson 1999; Anani *et al.* 2000; Binns 2001). The minimum inhibitory concentration

(MIC) reported was that dilution of the extract that gave rise to complete (MIC-100) or partial inactivation of the virus determined in duplicate.

Although a high consistency exists among MICs determined with a given experiment, with two consecutive experiments usually being equally reproducible, potential variability can exist among several experiments. Therefore, *Flaveria* spp. extract was used as a reference antiviral to guard against potential discrepancies. The choice to use this extract was primarily due to the fact that *Flaveria* spp. of the plant family Asteraceae, contain many biological active secondary metabolites including the antiviral tricyclic thiophenes, such as alpha-terthienyl (Marles *et al.* 1992).

4.2.3.3 *Modified Antiviral tests*

In the first modified antiviral test (light versus dark protocol), the implemented test procedure was similar to that previously described above (virucidal protocol), except that all test trays were duplicated and assigned to one of two treatments following the addition of virus to extract. Trays assigned to the dark treatment were wrapped in tin foil to exclude light where as light assigned trays were exposed to light.

A second adaptation of the virucidal protocol was to distinguish between the effects of long-wave ultraviolet (UVA) and visible light (VIS). In this modification, viral-extract mixtures were exposed to UVA (320-400 nm) lamps only, fluorescent (400-600 nm) lamps only or covered with foil as above, within the environmental chamber (Hudson *et al.* 2000).

All antiviral bioassays were performed by Dr. J.B. Hudson at the Department of Pathology, University of British Columbia, Vancouver, Canada.

4.2.4 *Analysis of phytochemical constituents*

4.2.4.1 *Test for the presence of condensed tannins*

The presence of proanthocyanidins (i.e. condensed tannins) in crude extracts was determined by employing an acid treatment, resulting in their conversion to anthocyanidins (Porter *et al.* 1986; Harborne 1998; Tanner *et al.* 2000). Approximately 50 mg of each crude extract was dissolved in 5 mL of 2*N* HCl and placed in a water bath at 100 °C for 20 minutes. After allowing acid treated extracts to cool, samples were span down and the removed supernatant extracted with an equal amount of *n*-butanol. The production of a reddish water insoluble pigment extractable into butyl alcohol signifies the presence of proanthocyanidins (hydrolyzed to give rise to anthocyanidins). The presence of proanthocyanidins in crude extracts was further verified by LC/MS analysis using the method described below.

4.2.4.2 *Test for the presence of hydrolysable tannins*

To determine the presence of hydrolysable tannins, which are esters of gallic acid or ellagic acid and glucose (Tanner *et al.* 2000), a similar procedure to the test for condensed tannins was used except the removed supernatants were not extracted into butyl alcohol, but directly analyzed by LC/MS (method described below).

4.2.4.3 *Measurement of total phenolics*

Determinations of total polyphenols were made according to the Folin-Ciocalteau method with slight modification (Hagerman and Butler 1991; Nicol 1996). Crude extracts (between 1 to 7 mg) were dissolved in 1 mL of 95% ethanol and a 100 μL of the dissolved alcohol extract diluted with 7 mL of distilled water. Phenol reagent (500 μL) was added to the diluted alcohol

extract, thoroughly mixed and allowed to stand. After 3 minutes, 1 mL of saturated sodium carbonate was added and the alcohol extract mixtures transferred to a dark enclosure for a period of an hour after which point their absorbance was determined at 725 nm. Absorbance readings for alcohol extracts were measured in 1 cm glass cuvette using a Beckman DU640 spectrophotometer. As a blank, 100 μL of 95% ethanol with 7 mL of distilled water and 500 μL of phenol reagent was used following the same protocol described above. A standard linear calibration curve was obtained from standard solutions of chlorogenic acid (0.05-1.2 mg/mL). Total phenolic content was calculated as chlorogenic acid equivalents (CAE) based on the calibration curve (Figure 4.2). The results were expressed as μg per mg dry crude extract. All extracts were analyzed in 3 separately prepared replicates, all with an absorbance reading within the range of the standards.

4.2.4.4 *Sample preparation (LC/MS analysis of polyphenols)*

A small volume (700 μL) of the removed supernatants from hydrolyzed alcohol extracts was diluted with an equal amount of methanol, filtered (Chromospec 0.2 μm PTFE), and 1μL of the filtrate injected onto the HPLC column. For non-hydrolyzed samples, the respective crude extracts (3–8 mg) were reconstituted into 1.5 ml of methanol, filtered and 1μL of the filtrate injected onto the HPLC column.

4.2.4.5 *Identification and quantification of polyphenolics by LC/MS*

Separation of polyphenols was carried out on a YMC^{TM} ODS-AM column (120 Å, 2.0 x 100 mm) at a flow rate of 0.3 mL/min, using a Hewlett-Packard 1100 Series chromatograph system (Agilent Technologies, Waldbronn, Germany) equipped with a UV-Vis photodiode

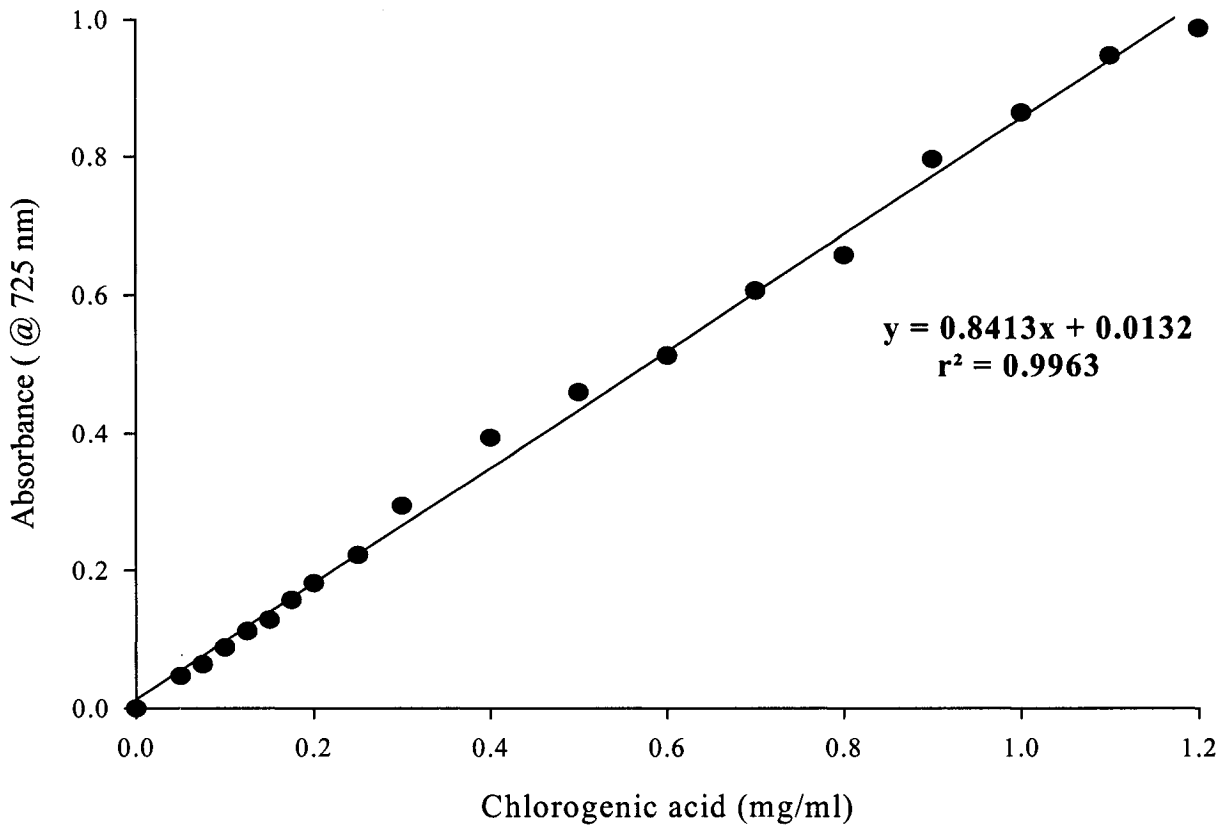

Figure 4.2 Calibration curve for quantitative estimation of total phenolics, using the Folin-Ciocalteau colorimetric assay (Hagerman and Butler 1991). Phenolic content calculated as chlorogenic acid equivalents (CAE).

detector. The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.05% TFA); initial conditions were 8% A followed by a linear gradient to 35% A over 12 minutes, increasing to 100% A over the next 3 minutes, before retuning to initial conditions from 15.5 to 19.5 minutes. Starting conditions were maintained for an additional 4 minutes to facilitate re-equilibration between samples. The column oven temperature was set at 50 °C. Polyphenols were monitored simultaneously at 190-600 nm, 280 nm, 350 nm, 520 nm and identified at 325 nm. To identity the eluting peaks, the retention times, UV-Vis spectra and MS data (were available) were compared with reference standards. Eluting compounds were compared to over 90 authentic polyphenols (Appendix 4.1).

HPLC-MS analysis was performed using the elution program described above. The HPLC-PAD equipment detailed above was interfaced with a Hewlett-Packard 1100 MSD APCI (atmospheric pressure chemical ionization) operating in positive ion mode and scanning from m/z 100 to 1000. HPLC-MS chromatograms were recorded and integrated using Agilent ChemStation for LC/MS systems Revision - A.09.01 12-Dec-2001 (Agilent Technologies, Waldbronn, Germany).

4.2.5 *Statistical Design and Analyses*

The total phenolic content among non-hydrolyzed extracts was tested using ANOVA and significant differences among extracts determined by a matrix of pairwise comparison probabilities based on the Bonferroni Adjustment method. All statistical calculations were performed using SYSTAT 10.1 (SPSS Corp., Chicago, IL, USA).

4.3 Results and Discussion

4.3.1 *Preliminary antiviral assay (HSV)*

In an effort to ensure that all promising antiviral extracts are detected, it has been suggested that antiviral test procedures must be optimized to detect photosensitizers. This is because many phytochemicals have light activated antiviral activity (Anani *et al.* 2000; Hudson *et al.* 2000). For this reason, preliminary testing of *Z. americanum* extracts were conducted with the inclusion of light.

The results of the preliminary antiviral assay conducted with HSV-1 *in vitro* when exposed to a combination of visible and UVA light are summarized in Table 4.5. Nine of the 10 evaluated extracts had activity against HSV-1 and antiviral efficacy varied according to plant extracts, as well as by phytochemical fraction. For the pyranocoumarin markers (MC: xanthyletin, xanthoxyletin and alloxanthoxyletin) as well as extracts classified to polar fractions (A1, A2 and A3) the maximum testable concentrations (6.5-13 μg/mL, MC; 100 μg/mL, A1, A2, A3) were limited, since the residual solvent (DMSO) had to be kept at a minimum. This, in addition to other factors, such as an absence of or lower abundance of bioactive phytochemicals may, be largely responsible for the marginal and, in the case of A3, no detectable activity against HSV-1. The bioactivity ranking of extracts (i.e. with better than marginal activity) according to their minimum antiviral concentration inhibiting HSV-1 growth (from most active to least) was: D1 > D2 > C2 > B1 > B2 > C1. D1-wood 1998 (70% acetone) extract (MIC < 8.5 μg/mL) and D2-wood 1998/2001 (70% acetone) extract (MIC = 19 μg/mL) were the most potent inhibitors of HSV-1, comparing favourably with the *Flaveria* spp. extract (MIC < 6 μg/mL).

Table 4.5 Antiviral activity of *Z. americanum* extracts and isolated compounds against *Herpes simplex* virus type 1 (HSV-1). Infected Vero cells were exposed to a combination of fluorescent and long wave UV covering the range 320-600 nm.

Extract[a]	Code[b]	MIC-100 (μg/ml)*
Alkaloid residue[1]	A1	±
Sample 8 (50% MeOH)[1]	A2	±
Sample 8 (Hot H_2O)[1]	A3	Nd
Husk (95% EtOH)	B1	90
Husk (EtOAc)	B2	100
Root (95% EtOH)	C1	150
Bark (95% EtOH)	C2	55
Wood 1998 (70% Me_2CO)	D1	<8.5
Wood 1998/2001 (70% Me_2CO)	D2	19
Isolated compounds (MC)[2]	MC	±
Flaveria extract (positive control)	CON	<6

[1]Tested at 100 μg/ml; [2] Xanthyletin, xanthoxyletin and alloxanthoxyletin tested individually and in combination (ratio 3.5:9:1) at 6.5-13 μg/ml

[a]Acetone (Me_2CO), ethanol (EtOH), ethyl acetate (EtOAc), methanol (MeOH) or water (H_2O).

[b]Laboratory codes for extracts, preceding letters identifies compounds preferentially extracted; **A**, polar compounds (i.e. alkaloids); **B**, furanocoumarins; **C**, pyranocoumarins; **D**, tannins and related polyphenols; **MC**, marker constituents and **CON**, positive control.

***MIC-100** = minimum concentration required to completely inhibit 100 infectious units of HSV-1

± denotes viral cytopathic effects in < 50% cells (= partial inhibition);

nd, no activity detected.

4.3.2 *Antiviral photosensitizers*

Based on the results from the preliminary study of antiviral activity among different extracts and phytochemical fractions, a subset of the extracts previously evaluated was selected for further testing. In this case, experiments were designed to identify the role of light in resulting antiviral activity.

Table 4.6 shows the minimum antiviral concentrations required to completely inhibit 100 pfu of HSV-1 in the presence of light (UVA and visible light) and in the dark. On the basis of these results, several conclusions can be made. Generally, antiviral activity was increased with the inclusion of light and in certain cases surpassed values within the range of doubling dilutions for end-point MICs, suggesting significantly enhanced activity. Using the same method as the current study, extracts without photosensitizers lead to dark/light (D/L) ratios of approximately 1.0 (± 0.5) for tested phytochemical or extracts (Hudson *et al.* 2000). Consequently, D1, D2 and to a lesser extent B1 extracts all demonstrates photoactivated antiviral activity (since D/L ratios ranging from 2 to 53). Again, the anti-HSV activity of D1 (D/L ratio > 53) was most interesting when compared to that of the *Flaveria* spp. extract (D/L ratio > 83).

It is worth noting that all extracts exhibited "dark" activity, indicating antiviral activity in the absence of photoactivation. However, extracts that had previously demonstrated potent light-mediated anti-HSV activity (namely D1 and D2) faired poorly without light exposure (MIC > 500 μg/mL) and may be due to bioactive constituents that are predominantly photoactive. This view is further substantiated by the fact that the dark antiviral activity of D1 and D2 was comparable to that of the *Flaveria* spp. extract, known to be rich in numerous photoactivated antiviral compounds (Marles *et al.* 1992).

Table 4.6 Effect of light on the antiviral activity of *Z. americanum* extracts against *Herpes simplex* virus type 1 (HSV-1).

Extract	Minimum antiviral concentration (μg/ml)[a]		Ratio D/L[b]
	+ light	- light	
Husk (95% EtOH)	62	125	2.0
Husk (EtOAc)	62	94	1.5
Bark (95% EtOH)	55	78	1.4
Wood 1998 (70% Me_2CO)	9.4*	>500	>53.2
Wood 1998/2001 (70% Me_2CO)	31*	>500	>16.1
Flaveria extract (positive control)	6*	>500	>83.3

[a]Minimum concentration required to completely inhibit 100 infectious units (MIC-100) of HSV-1
[b]Dark/light activity
*Significantly enhanced light dependent activity

In contrast, the lack of an absolute requirement for light noted with B1, B2 and C2, suggests these extracts as having different phytochemical profiles than D1 or D2. In particular, the anti-HSV activity of B1, B2 and C2 may be the result of photoactive and non-photoactive constituents and or the presence of two or more different antiviral compounds.

4.3.2 *Comparison of UVA and visible light*

Given that the most promising antiviral activities of tested extracts against HSV-1 were all light-mediated, it was important to elucidate whether photoactivated compounds were responding to UVA or visible light. Antiviral tests were conducted using three treatments involving the exposure of culture trays to UVA lamps only, fluorescent lamps only or no light at all, with the exclusion of both UVA and visible light. Table 4.7 shows the results for the most promising extracts. Extracts that had failed to demonstrate significantly enhanced light-mediated anti-HSV activity were not amenable to this comparison.

Once again, both D1-wood 1998 (70% acetone) extract and D2-wood 1998/2001 (70% acetone) extract showed substantial light enhancement, but this was equally evident for UVA as well as visible light. The most likely explanation for this result may be due to the fact that a slight overlap occurs between the emission spectra of the UVA and fluorescent lamps. That is to say that in the case where the bioactive compound(s) absorbs around 400 nm, irradiation with UVA or visible light could elicit similar antiviral activities. However, the results of this particular experiment must be interpreted with caution since they are based on a single experiment, with UVA and VIS doses not being adjusted relative to each other or to combinations used in previous experiments. This may also account for part of the variation noted for the reported "dark" activity of extracts when compared with that of the previous

Table 4.7 Antiviral activities of *Z. americanum* extracts towards *Herpes simplex* virus type 1 (HSV-1). Extracts tested under long wave ultraviolet (UVA), visible light (VIS) or without UVA/VIS light.

	MIC-100 (μg/ml)[a]		
Extract	+ VIS light	+ UVA light	- light
Wood 1998 (70% Me_2CO)*	<4	<4	86
Wood 1998/2001 (70% Me_2CO)*	39	55	400
Flaveria extract (positive control)*	1.2	1.8	>40

[a]Minimum concentration required to completely inhibit 100 infectious units (MIC-100) of HSV-1, based on a single experiment. Doses of UVA and VIS not adjusted to each other or to combinations in previous experiments.

*Significantly enhanced light dependent (+VIS/UVA) activity.

experiment. For instance, D1 was determined to have an MIC = 86 μg/mL in the current experiment, which is several orders of magnitude away from that previously concluded (MIC > 500 μg/mL).

The phytochemicals responsible for the antiviral activity of *Z. americanum* extracts are noticeably photosensitizers, although the contributions of some light independent bioactive antiviral phytochemicals may be present. However, the determination of whether these are visible-light only photosensitizers, UVA-light only photosensitizers or both requires further investigation.

4.3.3 *Selectivity of antiviral activity*

Although the preliminary testing of antiviral activity showed the majority of *Z. americanum* extracts (9 out of 10) and all four phytochemical fractions to be active against HSV-1, only extracts C1, C2, D1 and D2 were evaluated for additional antiviral activity against Sindbis virus (SINV) and poliovirus type-1 (PV-1). These extracts, based on commercial raw materials, were selected for their potent inhibitory effects on HSV-1 and relevance to the assessment of *Z. americanum* preparations as antivirals. The antiviral activities of these extracts are presented in Table 4.8 against all three viruses.

Only two *Z. americanum* extracts (C2 and D1) demonstrated activity against two of the three viruses. In fact, D1 (HSV-1, MIC = 3.1 μg/mL; SINV, MIC = 19 μg/mL) compared favourably with the positive control (HSV-1, MIC = 3.1 μg/mL; SINV, MIC = 6.2 μg/mL). In contrast, C2 had much weaker activities with marginal activity against SINV at 250 μg/mL and an MIC = 62.5 μg/mL against HSV-1. Determination of virus susceptibilities were somewhat limited by the cytotoxicity of the dissolving solvent (DMSO), limiting the maximum testable

Table 4.8 Inhibitory effects of *Z. americanum* extracts on *Herpes simplex* virus type 1 (HSV-1), Sindbis virus (SINV) and Poliovirus type 1 (PV-1) replication in Vero cells. Infected cells were exposed to a combination of fluorescent and long wave UV covering the range 320-600 nm.

	MIC-100 (μg/ml)[a]		
Extract	HSV-1	SINV[b]	PV-1[b]
Root (95% EtOH)	±	>250	>250
Bark (95% EtOH)	62.5	±	>250
Wood 1998 (70% Me_2CO)	3.1	19	>50
Wood 1998/2001 (70% Me_2CO)	125	>500	>500
Flaveria extract (positive control)	3.1	6.2	>50

[a]MIC-100 = minimum concentration required to completely inhibit 100 infectious units of respective virus.
± denotes viral cytopathic effects in < 50% cells (= partial inhibition) at maximum concentration of 250 μg/ml.
[b]> represents highest concentration tested (limited due to cytotoxicity of vehicle-DMSO).

concentration for extracts against both SINV and PV-1 to 250 μg/mL or 500 μg/mL. In spite of that, at the previously mentioned maximum concentrations, none of the tested extracts exhibited great activity against PV-1, despite D1 demonstrating activity as good as the positive control. These results suggest that the three viruses have different susceptibilities to the antiviral constituents of the tested *Z. americanum* extracts, with HSV-1 being most susceptible.

4.3.4 *Methodology used in identification of polyphenols and interpretation of results*

One of the objectives of the present study was to characterize the polyphenolic compositions of *Z. americanum* extracts evaluated for antiviral activities, while testing the hypothesis that antiviral activity *Z. americanum* extracts is dependent on polyphenolic content and composition. This proved to be a larger than expected task and required a number of analytical techniques.

The presence of condensed tannins in crude extracts was determined by a colorimetric assay based on their conventionally conversion to anthocyanidins in hot mineral acid (Porter *et al.* 1986). However, the absence or presence of condensed tannins in crude extracts does not preclude the occurrence of hydrolysable tannins (Tanner *et al.* 2000), as is the case with foliage extracts of the sugar maple, *Acer saccharum* Marsh. (Nicol 1996). Thus, to further corroborate the presence of tannins in extracts LC/MS was employed. To establish the presence of hydrolysable tannins, gallic acid derivatives were monitored at 280 nm, a wavelength commonly used for the detection of phenolic acids due to their absorption maxima (Porter 1989). In contrast, the hydrolysis of proanthocyanidins to anthocyanidins was determined from hydrolyzed crude extracts monitored at 520 nm (Lee *et al.* 2002).

The Folin-Ciocalteau colorimetric assay applied in this study is a simple method suitable commonly used for crude estimation of total phenolic content. However, the usefulness of this assay is limited by its non-specificity (as to type of tannins present), detection of non-tannin phenolics and inability to reflect the diversity in polyphenolic constituents (Hagerman and Butler 1991). Consequently, our efforts to identify and characterize the polyphenolic compositions of *Z. americanum* extracts compared eluting peaks were compared to over 90 authentic standards and about 10 of these were useful in peak identification (Figure 4.3). Nevertheless, it was not always possible to establish the identity of eluting peaks in samples on the basis of UV spectra and retention times alone. In general, compounds that were not unequivocally identified had UV spectra matching that of phenolic standards, but different retention times (Figure 4.4A), due most likely to the occurrence of a derivative of the phenolic standard. Where possible, generated mass spectra were used to aid in identification of compounds (Figure 4.4B).

4.3.5 *Phenolic composition of extracts evaluated for antiviral activities*

As a consequence of the unimpressive antiviral activities of the evaluated *Z. americanum* extracts against the Sindbis virus and poliovirus type-1, the discussion of phytochemical constituents responsible for antiviral activity are offered with respect to activities noted towards HSV-1.

Anti-HSV activity of *Z. americanum* extract could not be attributed to the presence of tannins, since neither proanthocyanidins nor hydrolysable tannins were present in samples (Table 4.9). In addition, there was no apparent correlation between the mean content of total phenolics measured as chlorogenic acid equivalents (CAE) and antiviral activity. For example, D1 (HSV-1, MIC = 3.1 μg/mL; CAE, 186 ± 8), which demonstrated the most superior activity

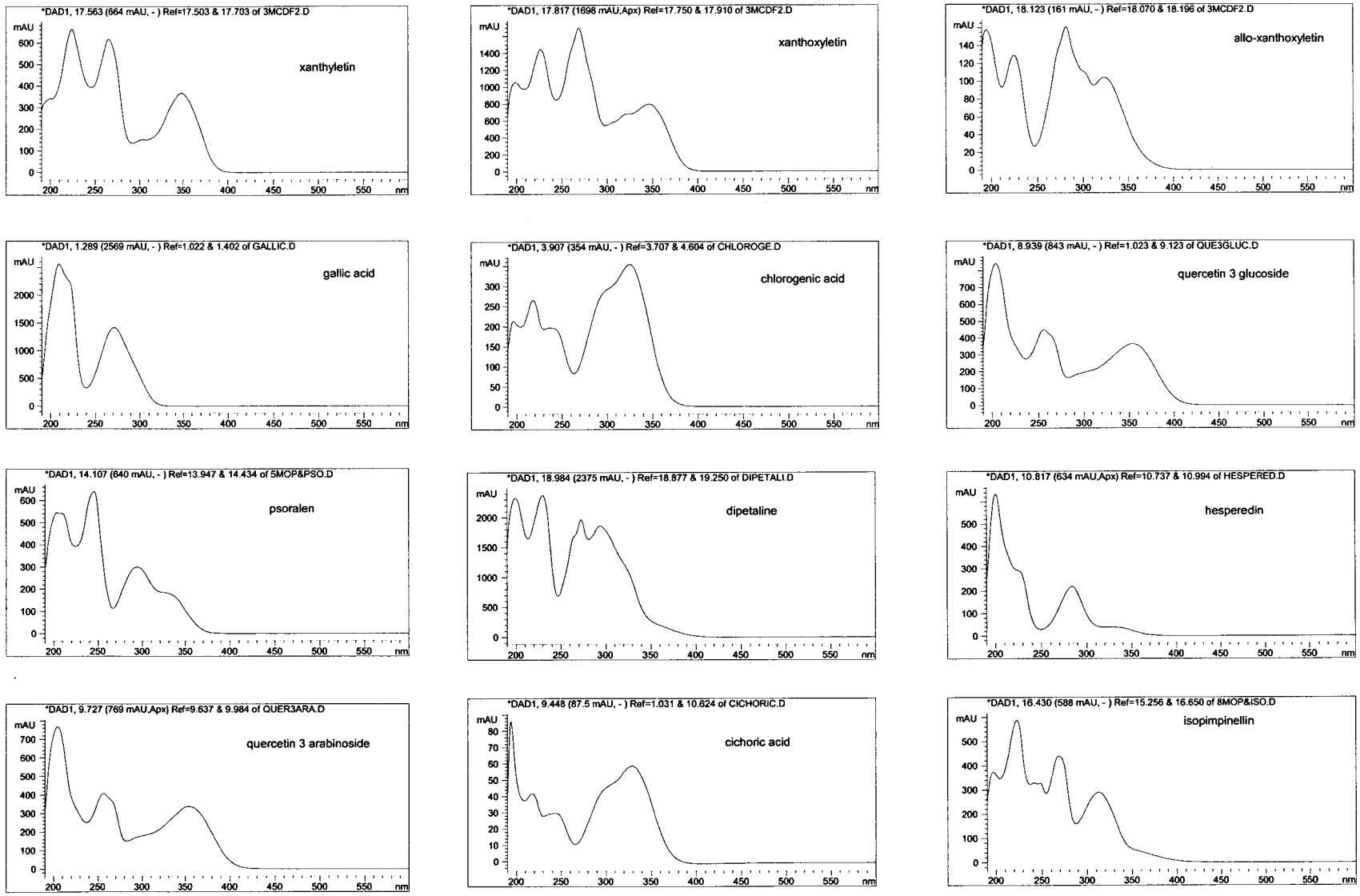

Figure 4.3 Absorption spectra and retention times of some standard polyphenols incorporated into the LC/MS method for the analysis of *Z. americanum* extracts used in antiviral study.

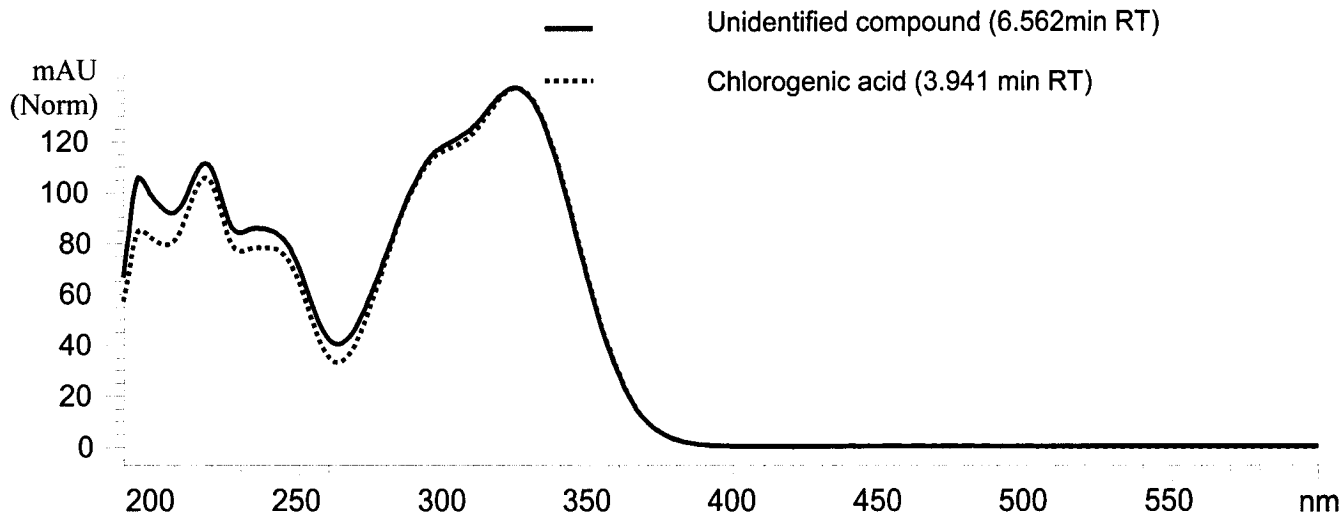

Figure 4.4A Absorption spectra of an unidentified compound and chlorogenic acid.

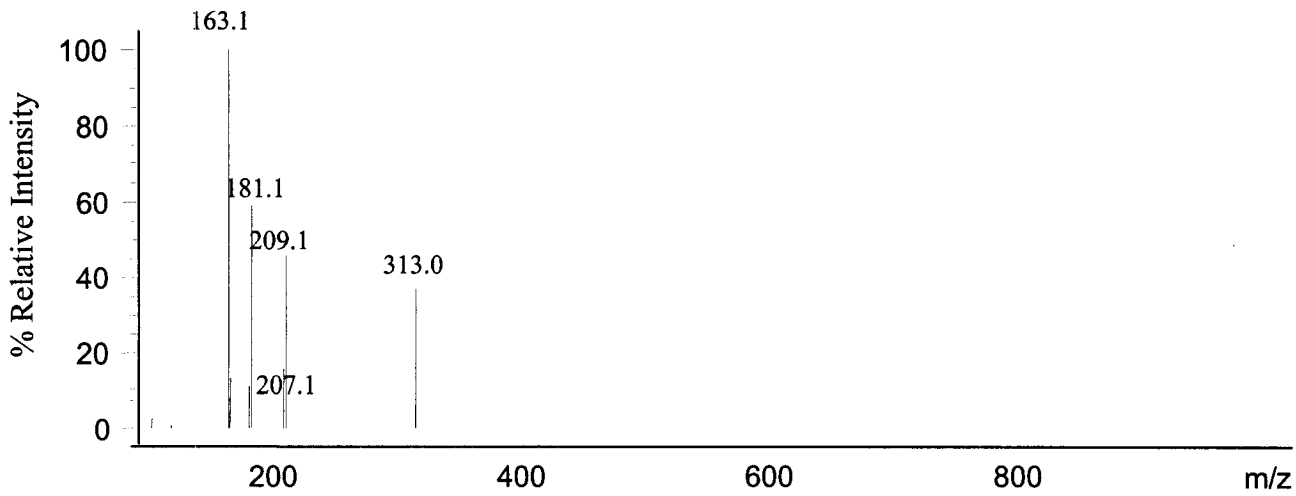

Figure 4.4B Positive ion MS of an unidentified compound acquired during APCI-MS analysis.

Table 4.9 Preliminary phytochemical analyses of *Z. americanum* extracts evaluated for antiviral activities.

	Tannins[b]		Total phenolics (mean ± sd)[1]
Extract	Hydrolysable	Condensed	Content (μg/mg dry wt)
Alkaloid residue	-	-	162 ± 16^{bcd}
Sample 8 (50% MeOH)	-	-	246 ± 17^{a}
Sample 8 (Hot H_2O)	-	-	98 ± 36^{e}
Husk (95% EtOH)	-	-	181 ± 18^{bc}
Husk (EtOAc)	-	-	113 ± 14^{de}
Root (95% EtOH)	-	-	196 ± 8^{ac}
Bark (95% EtOH)	-	-	85 ± 12^{e}
Wood 1998 (70% Me_2CO)	-	-	186 ± 8^{bc}
Wood 1998/2001 (70% Me_2CO)	-	-	135 ± 6^{be}
M1 (95% EtOH)[a]	+	+	519 ± 42
M2 (70% Me_2CO)[a]	+	+	587 ± 46

[a]Foliage extracts of *Acer saccharum* (sugar maple) included to ensure method functionality, but were not evaluated for antiviral activities.
[b]Determined by HPLC analyses extracts after acid treatment (+ = present; – = absent)
[1]Total phenolics expressed as chlorogenic acid equivalents (CAE) using Folin-Ciocalteu colorimetric assay conducted in triplicate. Means with the same letters are not statistically different based on Bonferroni's multiple comparison test performed for *Z. americanum* extracts only ($P < 0.05$).

of all extracts tested was not statistically different from B1 (HSV-1, MIC = 90 μg/mL; CAE, 181 ± 18) (Bonferroni's *t*-test, $p < 0.05$).

Given the limitations of the Folin-Ciocalteau colorimetric assay, particularly at assessing the diversity in polyphenolic constituents, anti-HSV activity of *Z. americanum* extracts were compared with the results of their respective HPLC phytochemical profiles. Among extracts classified as having polar phytochemical constituents, reported anti-HSV activities were marginal at best since these extracts were tested at 100 μg/mL. Because both A1 (alkaloid residue) and A2 (sample 8-50% methanol) showed some activity, where as A3 (sample 8-hot water) did not, these results may be suggestive of the importance of pyranocoumarins to antiviral activity (Table 4.10). This is because xanthyletin, xanthoxyletin and alloxanthoxyletin were all present in significantly higher amounts in both A1 and A2 than in A3. This view is further supported by the notable anti-HSV activities observed for extracts rich in pyranocoumarins (C1, MIC = 150 μg/mL and C2, MIC = 55 μg/mL). As well, isolated pyranocoumarins caused a 50% decrease or less in CPE when tested individually or in combination at concentrations in the range of 6.5-13 μg/mL. Nevertheless, antiviral activity of *Z. americanum* may not always be attributable to the presence of pyranocoumarins, as may be the case with extracts rich in furanocoumarins. For example, 8-methoxypsoralen, a well-known photosensitizing agent with antiviral activity (Hudson *et al.* 1993) was highest in *Z. americanum* husk extracts such as B2 (MIC = 100 μg/mL) (Table 4.10).

The most effective inhibitors of *Herpes simplex* type-1 growth were 70% acetone extracts of *Z. americanum* terminal portions. These extracts (D1 and D2), were not abundant solely in furanocoumarins or pyranocoumarins, but had relatively equal amounts of xanthoxyletin and the antioxidant hesperedin (Haslam 1996).

Table 4.10 Phenolic composition of *Z. americanum* extracts used in antiviral study.

	Content (mg/mg dry weight)[b]																
Extract[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A1	-	-	-	-	-	-	7.4	-	-	-	-	-	44.6	25.7	-	18.5	12.9
A2	9.9	-	0.8	-	-	-	26.4	-	-	-	-	-	34.5	50.1	-	25.6	0.6
A3	11.1	-	0.9	-	-	-	31.5	-	-	-	-	-	4.9	7.8	-	2.1	-
B1	-	-	-	11.9	6.6	47.8	-	19.3	-	40.1	153.3	3.0	-	-	-	-	-
B2	-	-	-	-	-	-	123.6	-	-	54.7	543.1	7.0	-	-	24.2	-	-
C1	-	-	-	-	-	-	-	-	-	-	-	-	81.7	122.1	-	12.6	11.4
C2	-	-	-	-	-	-	-	-	-	-	-	-	98.9	45.5	-	33.2	5.1
D1	7.4	24.0	-	22.6	11.8	10.3	66.4	-	-	-	5.2	-	33.1	67.1	-	11.3	1.8
D2	10.6	-	-	-	-	-	21.2	-	0.8	-	-	-	9.5	19.6	-	5.7	1.4

[a]**A1**: alkalois redisue; **A2**: sample 8 50% MeOH; **A3**: sample 8 hot H_2O; **B1**: husk 95% EtOH; **B2**: husk EtOAc; **C1**: root 95% EtOH; **C2**: bark 95% EtOH; **D1**: wood (1998) 70% Me_2CO and **D2**: wood (1998/2001) 70% Me_2CO.

[b]Phenolic compounds: **1**, chlorogenic acid; **4**, quercetin-3-galactoside; **7**, hesperedin; **10**, psoralen; **11**, 8-methoxypsoralen; **12**, isopimpinellin; **13**, xanthyletin; **14**, xanthoxyletin; **15**, imperatorin; **16**, alloxanthoxyletin and **17**, dipetaline were all unequivocally identified. Unknown compounds tentatively identified as chlorogenic acid derivatives, **2** and **6**; exuletin derivatives, **3** and **8**; quercetin glycoside, **5** and cosmetin derivative, **9** (by LC/MS using UV spectrum, MS data & retention times). Reported amounts based on unreplicated analyses of extracts.

With roughly three times the content of xanthoxyletin and hesperedin found in D2 (MIC = 19 μg/mL) as well as a more complex HPLC profile (Figure 4.5), the potent anti-HSV activity of D1 (MIC < 8.5 μg/mL) may be best attributable to a dose dependant effect as well as the combination of pyranocoumarins, phenolic acids and flavonoids present in this extract, rather than to a single compound or class of compounds.

4.3.6 *Conclusions*

In this study, the therapeutic potential of *Z. americanum* extracts for viral infections was investigated against the *Herpes simplex* virus type 1 (HSV-1), Sindbis virus (SINV), poliovirus type1 (PV-1). Evaluated extracts prepared from four different phytochemical fractions all demonstrated antiviral activities, particularly against HSV-1. Consistent with the tested hypothesis, which was that the antiviral activity of *Z. americanum* extracts is related to the polyphenolic content and composition; antiviral activities were highest (i.e. lowest MICs) with the 70% ethyl acetate extracts (preferentially removing polyphenols) of pooled commercial raw material. Furthermore, the light mediated anti-HSV activity for all extracts were consistently better than their respective dark activities and in the case of both D1 and D2 was substantially enhanced by light.

While a number of mechanisms could account for antiviral activity in virus-cell culture systems, the antiviral assay used in the present study detects principally those phytochemicals that are virucidal in nature, thus inactivating virus particles directly (Hudson *et al.* 2000). Consequently, the potent inhibition of HSV-1 *in vitro* by D1-wood 1998 (70% acetone) may be due to preferentially binding of its polyphenolic constituents to the protein coat of the virus, possibly arresting viral absorption by Vero cells (Haslam 1996).

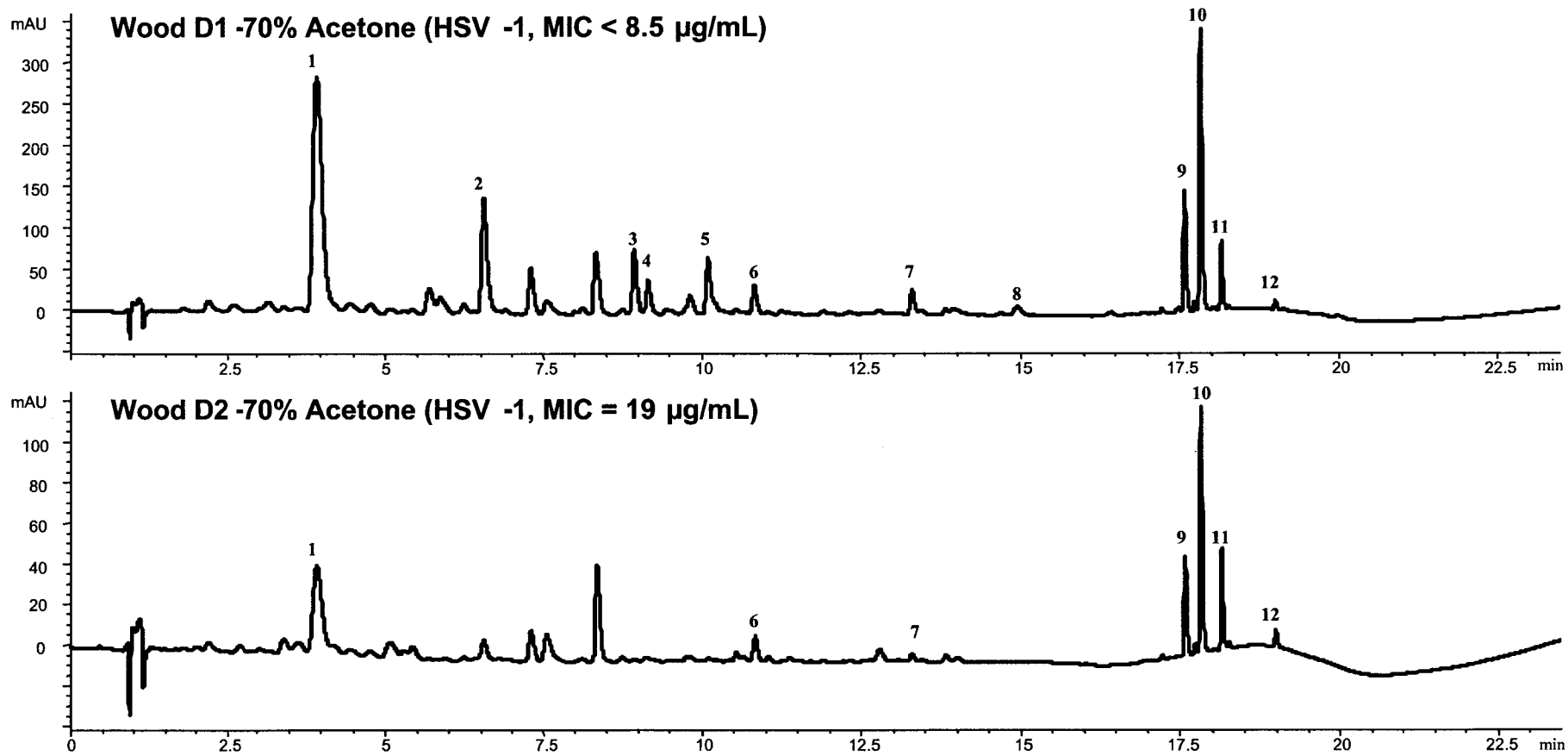

Figure 4.5 HPLC chromatographs of the ethyl acetate extract of commercial raw materials of *Z. americanum* samples acquired by HPLC-DAD at 325 nm. **1**, chlorogenic acid; **3**, quercetin-3-galactoside; **6**, hesperedin; **8**, 8-methoxypsoralen; **9**, xanthyletin; **10**, xanthoxyletin; **11**, alloxanthoxyletin and **12**, dipetaline were all unequivocally identified. Unknown compounds tentatively identified as chlorogenic acid derivatives, **2** and **5**; quercetin glycoside, **4** and cosmetin derivative, **7** (by LC/MS using UV spectrum, MS data & retention times).

Despite the majority of polyphenols being virucidal in nature, antiviral phytochemicals could offer cytoprotective actions against viral infection, by means of an interferon-like effect (Hudson *et al.* 2000) or by inhibiting some stage of the viral replication cycle in infected cells (Vlietinck and Vanden Berghe 1991). The latter could potentially act as a possible site of action for antiviral compounds since compounds such as flavonoids are known to act intracellularly at the level of virus replication (Vlietinck and Vanden Berghe 1991) and the Vero cell line are incapable of producing interferon (Hudson *et al.* 1999).

In addition to reporting for the first time on the antiviral activities *Z. americanum* extracts, this is the first in depth study as to the phenolic acids and flavonoids present in this species, but is by no means a complete catalog of all the potential antiviral phytochemicals present in this plant or evaluated extracts. Nonetheless, the results presented here, clearly provide the pharmacological basis for the traditional uses of this plant for infectious diseases and implicate the polyphenolic constituents of *Z. americanum* extracts in anti-HSV activities. The antiviral activity of commercially pooled material, D1 (HSV-1, MIC = 3.1 μg/mL; SINV, MIC = 19 μg/mL) deserves further investigation and is a good candidate for use as a phytomedicine against *Herpes simplex* type-1 virus and compares favourable with *Echinacea pallida* var. *sanguinea* crude (70% ethanol) inflorescence extract (HSV-1, MIC = 26 μg/mL) determined using the same method as the current study (Binns 2001).

Despite the extensive phytochemical work presented in the current study, further phytochemical investigations are required to identify several unknown compounds that may have contributed to antiviral activity. Unfortunately, due to time constraints, the usefulness of API-MS as a complimentary detection method to DAD-UV/Vis, could not be fully exploited, since conditions were not always optimized for all eluting compounds. Future studies should

determine the antiviral activities of wild *Z. americanum* populations and the phytochemical differences that may be responsible for variations in antiviral activities. Animal models are a logical next step.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Main Conclusions

This thesis is, at the time of writing, the most comprehensive examination of *Zanthoxylum americanum* Mill. (Rutaceae) available. The objectives of this study were to 1) assess the antifungal properties of *Z. americanum* plant extracts, 2) identify and quantify phytochemicals suitable for the authentication and standardization of commercial raw materials of *Z. americanum*, and 3) determine the antiviral activities of various phytochemical fractions prepared from *Z. americanum* extracts. Implicit in each of the stated objectives was an attempt to provide the pharmacological basis for the ethnomedicinal uses of this plant via biological and scientific evaluation of its chemical constituents and assess its potential use as a phytomedicine.

Biological screening of six crude extracts of different *Z. americanum* parts against a broad range of clinical relevant pathogenic fungi showed that antifungal activity differed according to plant part (Chapter 2). However, studies with fractionated extracts indicated that the highest activity (i.e. inhibition of fungal growth) is in the lipophilic fractions for all plant parts, suggesting one class of compounds as being important in antifungal activity. Similarly, Islam *et al.* (2001), showed the chloroform bark extract of *Zanthoxylum budrunga* Wall. to be considerably more active than the methanol extract. Despite reporting a high concentration of flavonoids as well as trace amounts of steroids and alkaloids in their phytochemical screening of the chloroform bark extract, Islam *et al.* (2001) failed to identify the bioactive antifungal constituents.

All extracts demonstrated light-mediated inhibitory effects towards a minimum of seven out of the 11 fungal species tested against. Fruit and husk extracts had the most extensive spectrum of activities with successful inhibition of ten fungi, including the causal agents in candidiasis, cryptococcosis and many dermatophytic infections. The antifungal properties of *Z. americanum* were positively correlated ($r^2 = 0.8610$, $P < 0.001$) to the presence of furanocoumarins. The reported antifungal activity of polar benzophenanthridine-like alkaloids in other *Zanthoxylum* spp., such as *Z. leprieurri* and *Z. xanthoxyloides* (Ngane *et al.* 2000), was not supported by my evaluation of *Z. americanum* phytochemistry and may reflect the dynamics of chemical evolution and resulting variation in plant chemistry.

The results of this study suggest that the traditional uses of this plant for skin infections and sores have a pharmacological basis. The allocation of furanocoumarins to the aerial parts of the plant explain the preferentially use of these plant parts. This finding suggests that traditional healers had some knowledge of the chemical ecology of the plant, without a detailed appreciation of their function as photogenotoxins. The present investigation is the first study to demonstrate antifungal activity in *Z. americanum*.

In the second experimental study, phytochemical analyses of the non-aerial parts of *Z. americanum* lead to the isolation and identification of four pyranocoumarins as distinct phytochemical markers for the wood and bark. The natural range of chemical variation for xanthyletin, xanthoxyletin and alloxanthoxyletin were determined from 34 different wild populations of *Z. americanum* across Eastern Ontario, which provides benchmark data for comparison of future commercial preparations (Chapter 3). The studies presented here also provided for the first time a validated HPLC based method to authenticate and standardize commercial raw material of *Z. americanum*. In addition, the current study provided tools

suitable for the assurance of high quality and safe phytomedicine based on preparations of *Z. americanum* wood.

In our assessment of the antiviral properties (Chapter 4), various phytochemical fractions prepared different *Z. americanum* plant parts all showed some antiviral activity (Chapter 4). However, wood extracts of commercially available raw materials, drawn from natural populations previously surveyed were the most potent inhibitors of viral growth with the order of viral susceptibilities being HSV-1 >> SINV > PV-1. Although antiviral activity was correlated to the presence of several phytochemicals, including furanocoumarins, more potent anti-HSV activity was evident for extracts containing pyranocoumarins, phenolic acids and flavonoids. This is the first study to demonstrate a potential correlation between the marker constituents of Northern prickly ash and anti-HSV activity. In addition, this study provides scientific rationale for the ethnomedicinal uses of *Z. americanum* preparations to treat infectious conditions such as mouth sores.

5.2 Regulation of phytomedicines

The current study made significant contributions to the study of *Z. americanum* and the biological basis for some of its ethnomedicinal uses. As a whole, work in this thesis has demonstrated both the potent antifungal activities as well as biologically significant HSV-1 inhibitory actions of *Z. americanum* extracts. Furthermore, the current research addresses the potential utilization of *Z. americanum* as a phytomedicine for human and animal health markets worldwide and provides an HPLC based method for the assurance of quality and safety of commercial materials.

Considering the potential risks of the pharmacologically active furanocoumarins and the absence of these compounds from extracts demonstrating potent antiviral activities, *Z. americanum* preparations clearly have greater potential for ethical use as antiviral as opposed to antifungals. Yet, despite an increased interest in green medicines or plant-based formulations and active principles to treat an extensive range of ailments throughout the world (World Health Organization 2001), the major dilemmas associated with the regulation of phytomedicines are still a concern. At issue, is the treatment of phytomedicines as food or drugs.

In the United States of America, phytomedicines are regulated under the Dietary Supplemennts Health and Education Act of 1994 (DSHEA) within the framework of the Federal Food, Drug and Cosmetic Act (FCDA). Because *Z. americanum* preparations will contain ingredients of botanical origin, they would be categorically identified as dietary supplements. Under DSHEA, such preparations would be exempt from regulation as drugs (Bidlack and Wang 2000) and preclude claims of its use to diagnose, prevent, mitigate, treat, or cure the various ailments established in ethnobotanical reports. However, statements describing the supplement's effects on the "structure and function" or general "well-being" of the body would be admissible as long as these are truthful and bear the statement

> *"This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease."*

Despite these provisions, consumers may still be left unsure as to safety, quality and usefulness of the product. More importantly, there may be little incentive for use of phytomedicines with promising results for the treatment of ailments like skin diseases or mouth sores.

Since the inception of the German Commision E in 1978, herbal products and conventional drugs have essentially been treated alike in Germany and Europe generally. In

contrast to the United States of America, European guidelines for registration would require proof of pharmaceutical quality for preparations of *Z. americanum*, with evaluations of safety and effectiveness being established from monographs published by the Commision E or the European Scientific Cooperative on Phytotherapy (ESCOP). By evaluating herbal products according to similar procedures required for new drug approval, much needed proof of quality, safety and effectiveness of the herbal product is assured. However, the European system raises issues of affordability and accessibility, for generally regarded as safe materials. Acknowledging the need to address consumer concerns for safety and product quality without stifling the development of the natural health industry, Canada has proposed an entirely different approach to the regulation of phytomedicines which has now reached the stage of draft legislation (Canada Gazette 2001; World Health Organization 2001).

The Natural Health Products Regulations recognizes a reduced registration requirement in cases of long and safe use of traditional medicines and allows for a full range of health claims, including structure-function, risk reduction, prevention or treatment claims (Canada Gazette 2001). Under this framework, several provisions including site licensing, good manufacturing practices, clinical trials among others are made ensuring that the standards of evidence are in accordance with the claim (Canada Gazette 2001). Ultimately, consumers may benefit from safe, effective and quality phytomedicines arising from an appropriate regulatory environment for the natural health product industry.

The Canadian regulatory system provides opportunity for phytomedicines like prickly ash to move forward to commercialization, where as they might be less successful in the European or American system.

5.3 Future studies

The antiviral activity of *Z. americanum* wood can be developed into an effective HSV-1 phytomedicine. An animal model is now needed to determine anti-HSV activity and a more detailed assesment of all the antiviral compounds is needed. The mode of action for pyranocoumarins activity is not well defined and remains to be determined. Pyranocoumarin chemistry is not well understood with respect to habitat variability. A seasonal study as to the chemical variation of identified pyranocoumarin markers phytochemicals within and among natural population sites is the next step. Stability of marker compounds during storage or in preparations should be addressed. Phytochemical analysis of the polar constituents, in particular benzophenanthridine and aporphine alkaloids need to be undertaken. Evaluation of various phytochemical fractions of the wood may further provide justification for the many other traditional uses of this plant. It is hoped that this study will provide impetus to investigate this species and its antiviral application in human medicine.

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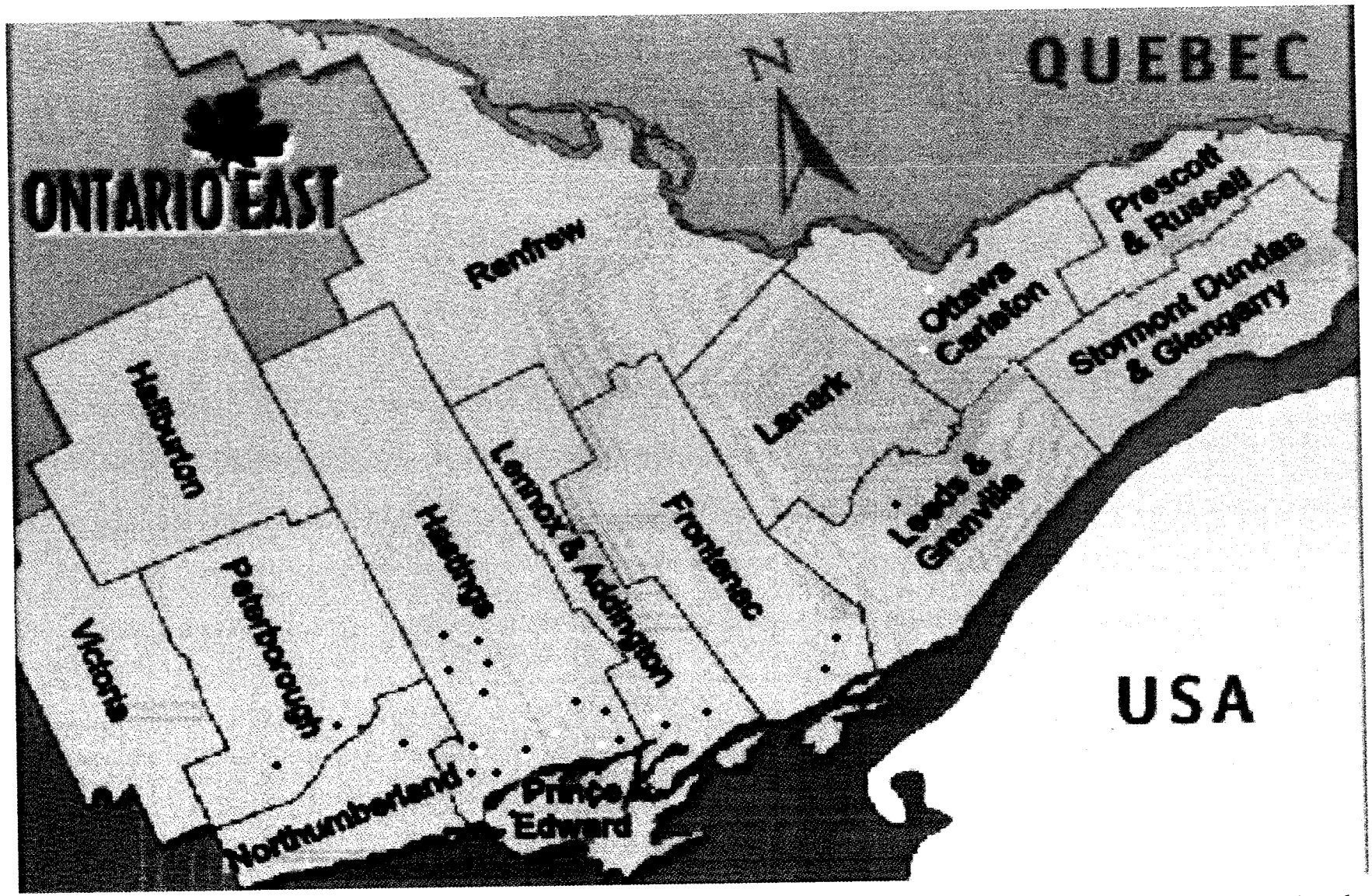
APPENDIX



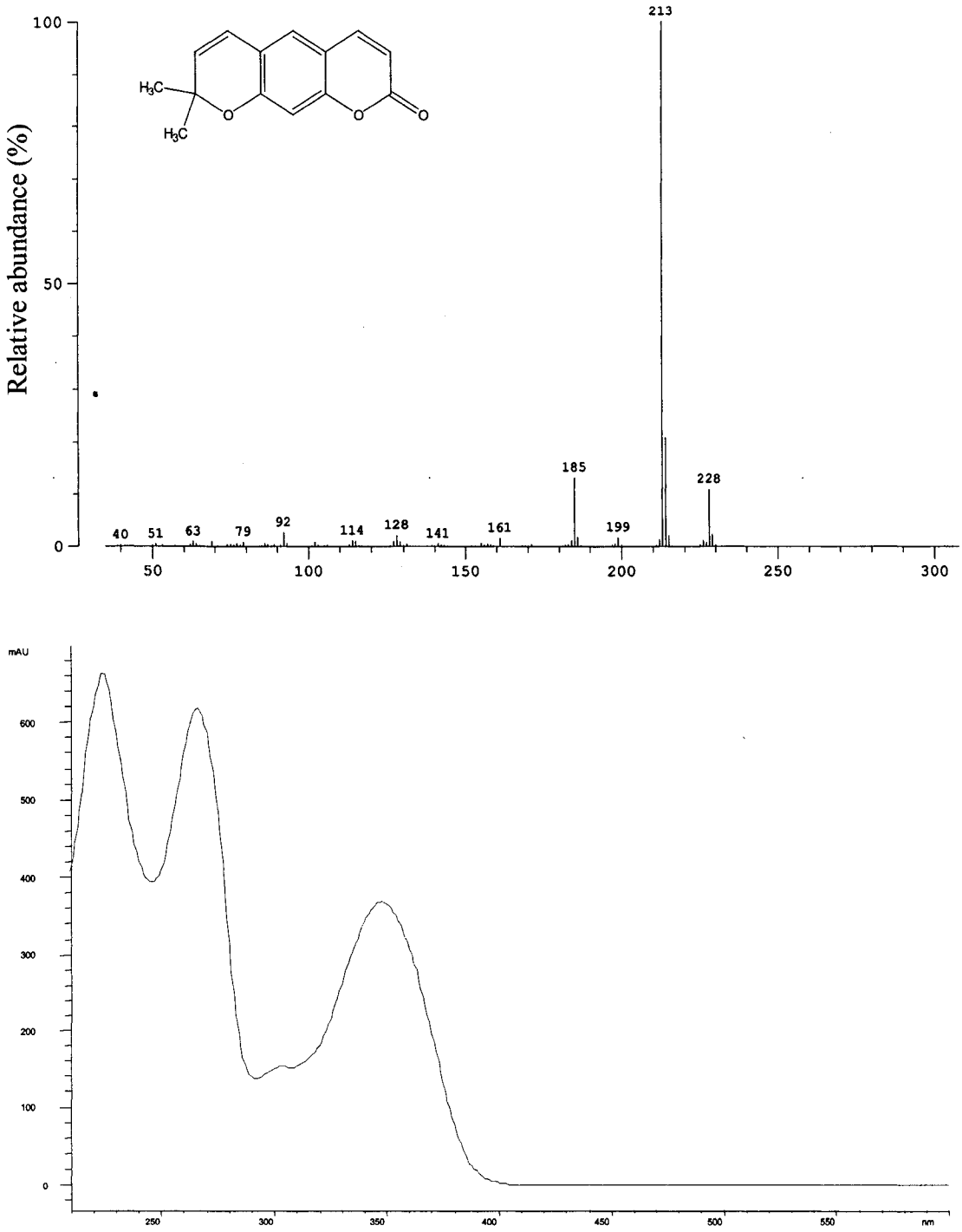
Appendix 1.1 Map of *Z. americanum* populations studied. Authentic plant materials were collected from 27 sites in 1998 (green) and 7 sites in 2001 (yellow).

Appendix 1.2 Road Locations of *Z. americanum* populations studied

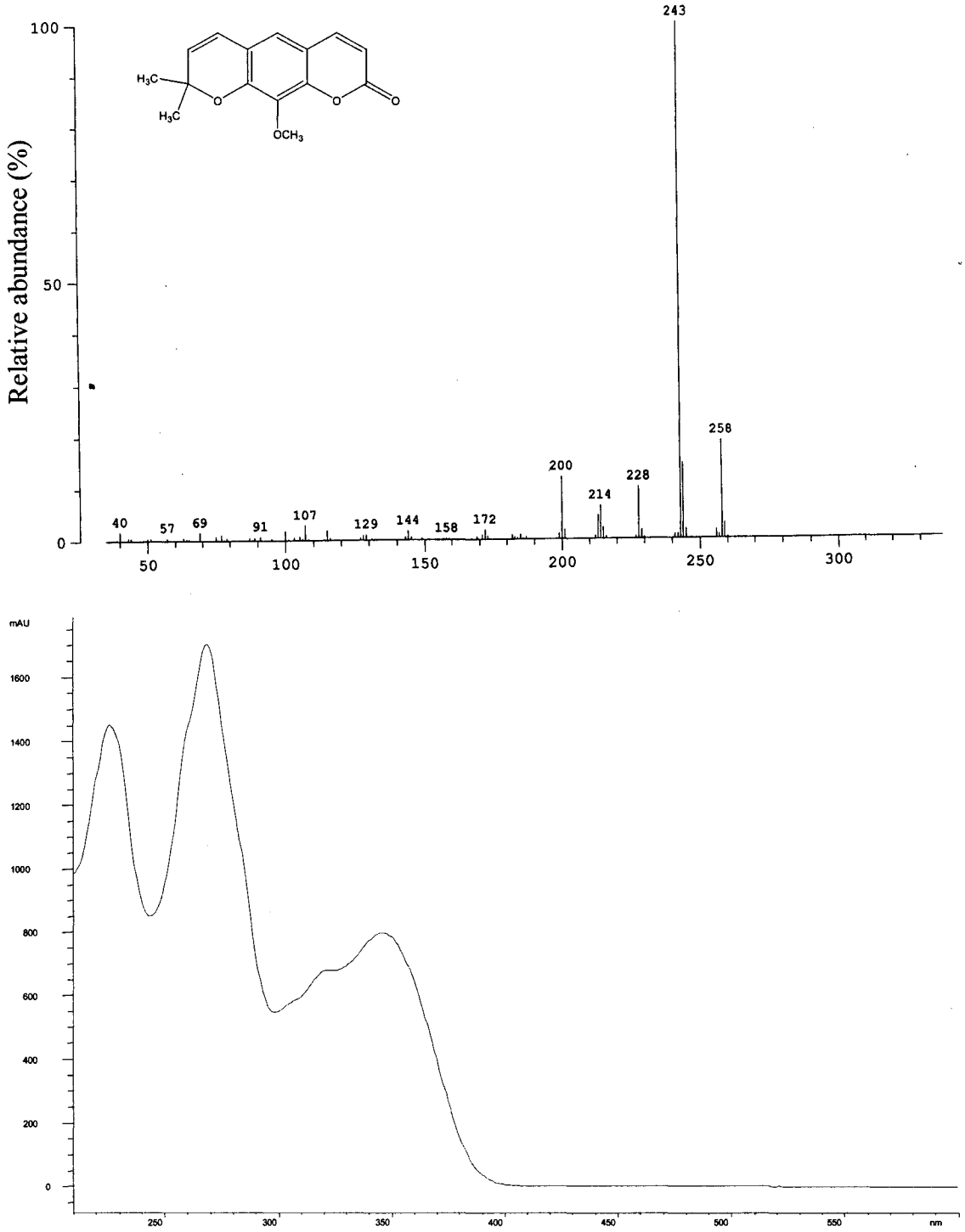
Year-Site	Road location	County
1998-1	Eggleton Rd @ Galivan Rd N.W corner	Hastings
1998-2	4th of Thurlow I/4 mile East of Shannonville Rd. (Beside Bridge and Creek)	Hastings
1998-3	1/4 mile N of Melrose on McFarlane Rd	Hastings
1998-4	5th L Tyendaga 1/2 km W of Marysville Rd	Lennox & Addington
1998-5	401 NW Corner of Salmon River	Hastings
1998-6	Hwy 401 before Hwy 37 at mile post #545.5	Hastings
1998-7	Odessa Rd - 2 km north of 401	Lennox & Addington
1998-8	Hwy 15 N to Frontenac 16 (Joyville Rd) in N.E corner of intersection.	Frontinac
1998-9	Pine Grove Rd - 2 km east of Hwy 15	Frontinac
1998-10	Hwy 15 - 2 km north of Portland across from fire #2827	Leads & Grenville
1998-11	South side of Hoover Rd , 1/2 mile W of Rainee Rd (Roden Twsp)	Hastings
1998-12	Bronson Rapid SE corner where bridge crosses Moira River	Hastings
1998-13	Springbrook Rd, 2 km west of Hwy 62, North side.	Hastings
1998-14	Hwy 62 @ Reid settlement Rd. NW corner.	Hastings
1998-15	Stirling - Campbellford Rd @ entrance to Ferris Prov. Pk	Northumberland
1998-16	Hwy 21east of Hwy 28 besides bridge (Peterborough County)	Peterborough
1998-17	County Rd 2, 3 km W of village of Hastings(Peterborough County)	Peterborough
1998-18	Glen Miller Rd 3 km South of Frankford	Hastings
1998-19	Glen Miller Rd,@ Selter Lane Kernels	Hastings
1998-20	English Settlement Rd, 1/4 mile west of Wooler Rd	Hastings

Appendix 1.2 (Cont'd)

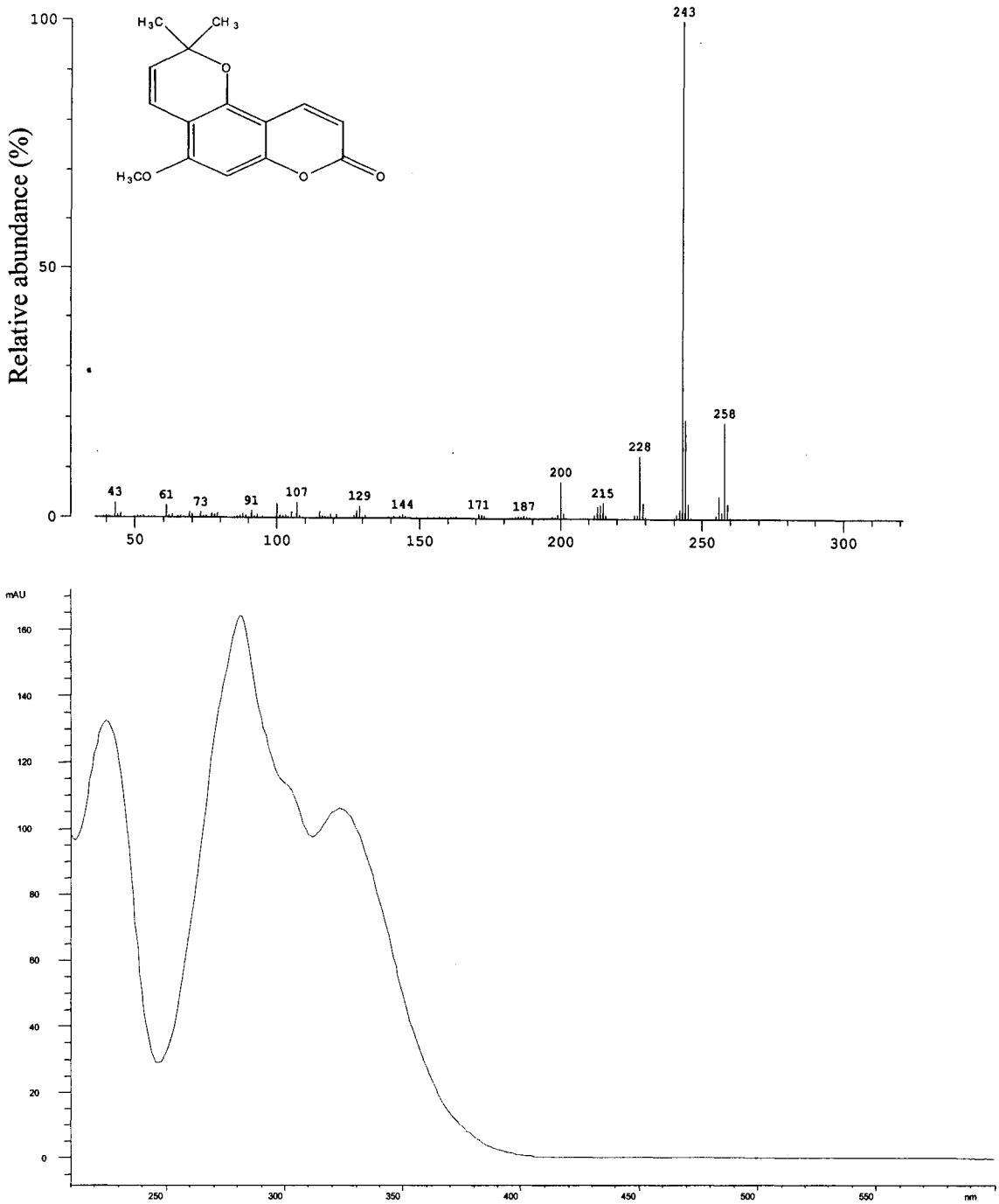
Site	Road location	County
1998-21	2nd Line Ameliasburg @ Bryants Rd	Prince Edward
1998-22	Victoria Rd, 1/4 mile east of Lyle Vanclief's house	Prince Edward
1998-23	Hwy 62 @ the Valley Rd	Prince Edward
1998-24	Demorestville Rd, 1.5 km east of Hwy 62	Prince Edward
1998-25	DemorestvilleConservation area ~1/2mile south of Village	Prince Edward
1998-26	N E corner of Big Island - on North Shore Rd.	Prince Edward
1998-27	North/South Rd, on East End of Big Island	Prince Edward
2001-1	HWY 401 North side 5 Km West Napanee (GPS - 44°14.97N X 77 °22.17W)	Lennox & Addington
2001-2	North side of Hwy 401, Approx. 546.5 mile post (GPS - 44° 12.42N X 77° 21.35W)	Hastings
2001-3	North west corner HWY 401 & 62 (GPS - 44° 11.62N X 77 °24.03W)	Hastings
2001-4	Eggleton Rd/Galivan Rd, NW Corner (GPS - 44° 17.83N X 77° 33.61W)	Hastings
2001-5	Glen Miller Rd, 300 ft South of 4th Sydney	Hastings
2001-6	Mud Lake, Britannia beach	Ottawa-Carleton
2001-7	Approx. 1.2 Km from McCaffrey Trial & Dwyer Hill Road	Ottawa-Carleton



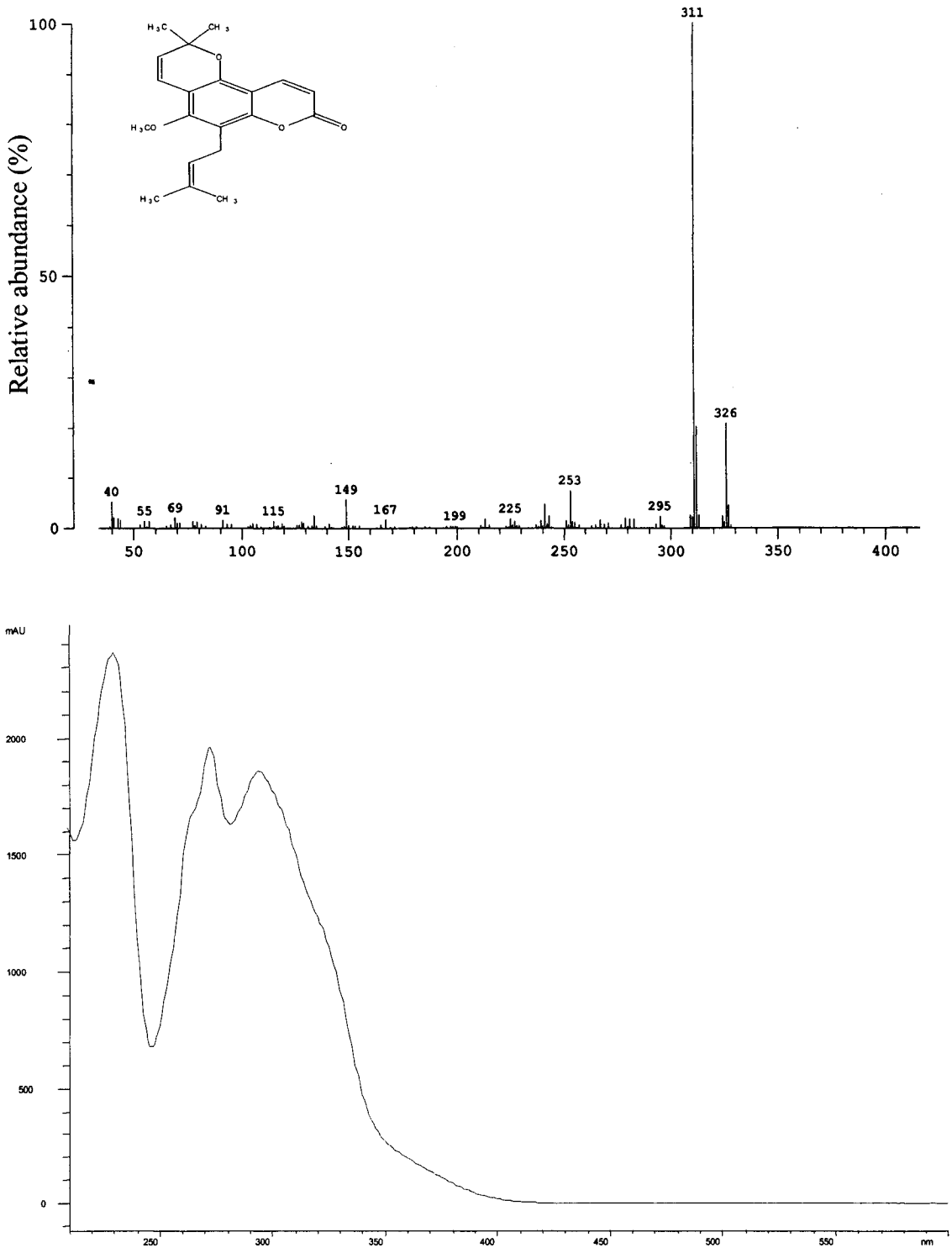
Appendix 2.1 MS and UV spectral data for xanthyletin.



Appendix 2.2 MS and UV spectral data for xanthoxyletin.



Appendix 2.3 MS and UV spectral data for alloxanthoxyletin.



Appendix 2.4 MS and UV spectral data for dipetaline.

Appendix 3.1 Phytochemical analysis of crude extracts used in recovery experiments. Based on duplicate analysis of separately extracted samples.

Extract	Compound content (µg/mg used)		
	Xanthyletin	Xanthoxyletin	Alloxanthoxyletin
Sample 24-70% ethanol	24.69	73.96	8.34
Sample 24-90% ethanol	26.10	63.42	9.17
Sample 8-50% methanol	23.95	29.65	17.29
Sample 8-75% EtOH	32.29	40.48	26.79

Appendix 4.1 List of autehntic standards used in the characterization of polyphenolic constituents of *Z. americanum* extracts evaluated in antiviral study (Chapter 4).

3 hydroxybenzoic acid
3,3',4'hydroxyflavone
3,4 dihydroxybenzoic acid
3,7,4' trihydroxyflavone
5-methoxypsoralen
7 hydoxyflavone
8-methoxysoralen
alloxanthoxyletin
angelicin
apigenin
bresoreylic acid
caffeic acid
caffeoyl tartate
caffeoyl putresci
catechol
chlorogenic acid
cichoric acid
cinnamic acid
cinnamoyl putrescine
cosmetin
coumarin
coumaroyl spermidine
delphinidin 3,5 diglucoside
dihydroxynaphtoquinone
dihydroxyquercetin
dipetaline
diphenylboric acid
echinacoside
ellagin
epicathechin
eriodictyol
exuletin
ferulic acid
feruloyl putrescine
feruloyl spermidine
flavanone
fusaric acid
gallic acid
genistein
gentisic acid
gibberellic acid
hesperedin
hesperetin
hydrocaffeic acid
imperatorin
isopimpinellin
kaempferol
kaempferol 3 glucoside
kaempferol 3 rutinoside
lucenin
luteolin
malvidin 3,5 diglucosside
morin
myricetin
myricetrin
n-digallic acid
naringenin
naringin
n-hydroxyphenylacetic acid
orsellinic acid
ortho coumaric acid
para coumaric acid
para hydroxyphenyl propionic acid
para hydroxyphenylacetic acid
pelargon
phdoridzin
pinocembrin
protocatechuic acid
psoralen
quercetin
quercetin 3 arabinoside
quercetin 3 galactoside
quercetin 3 glucoside
quercetin 3 O rhamnoside
quercetin 3 rhamnoside
rhamnetin
rosmarinic acid
rutin
salicylic acid
sinapic acid
syringic acid
tannic acid
taxifolin
trans cinnamic acid
trans para hydroxycinnamic acid
umbelliferone
vanillic acid
veratric acid
vitexin
xanthoxyletin
xanthyletin