Screening of Biflavonoid Compounds and British Columbian Bryophytes for Antiviral Activity Against Potato Virus X

by

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B.Sc. (hons.), The University of British Columbia, 2000

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Botany)

We accept this thesis as conforming
to the required standard

The University of British Columbia

April 2003

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Date April 22/2003

DE-6 (2/88)

Abstract

Plant viruses are responsible for causing significant losses to the agricultural industry. Recent investigations of antiviral compounds have suggested that biflavonoids may be a group of compounds which cause powerful inhibition to a broad spectrum of viral pathogens. Due to the high content of biflavonoids reported in bryophytes, this project has screened 50 bryophyte samples which represent 41 species for Potato Virus X (PVX) inhibition using a local lesion assay in *Chenopodium quinoa.* As a result of this screening, bryophytes have been identified as a prominent antiviral group; 29 of the 41 species were shown to cause greater than 75% inhibition when tested at 9.1mg/mL. In addition to screening bryophyte extracts, several biflavonoid compounds were tested directly. When tested at 1.38mg/mL robustaflavone and hinokiflavone where shown to be highly antiviral and exhibited PVX inhibitions of 85% and 78% respectively. Amentoflavone-7,4',4'trimethyl ether showed a marginal PVX inhibition of 29% and amentoflavone did not inhibit PVX at this concentration. Several compounds from the Columbian medicinal plant *Iryanthera megistophylla* have also been screened for inhibition of PVX. Of these compounds, iryanterin K, cinchonain Ib, cinchonain Ia, procyanidin B-2 and cinchonain IIa have been shown to be highly active against PVX when tested at 9.1mg/mL.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I would like to acknowledge the following people who have assisted and supported this project. I am very grateful to Dr. Neil Towers for his mentorship and for making this project possible. I also wish to thank Dr. Chris French for his guidance and funding in this project. Thanks to Dr. Dong Sheng Ming for his friendship, and many lessons in chemistry. Dr. Dong Sheng Ming also played a critical role in the isolation of the *Iryanthera* and *Hylocomium* compounds. Thanks to Mike Boutihillier for providing generous instruction and assistance. Thanks to Olivia Lee, Dr. Wilf Schofield and Shona Ellis for assisting me in my study of bryology and sharing their enthusiasm in this area.

Funding of this project was made possible with contributions from Agriculture and Agri-Food Canada, PhytoDiagnostics Company Ltd. and NSERC.

I would also like to thank the many members of Towers lab including: Keith Pardee, Dr. Eduardo Jovel, Dr. Andrés López and Dr. Kevin Usher. Their friendship, support and guidance made this project a valuable experience.

Thanks to my wife, Beth Hillhouse for her editing assistance and her unconditional support throughout this project. Thanks also to my parents for their assistance and encouragement.

This thesis is dedicated to my parents,
Dave and Marguerite Hillhouse.

1 Introduction

1.1 Viruses

Viruses are quite unlike any other pathogen. The question of whether viruses are living organisms is still frequently debated. Viruses are not capable of motion, nor do they grow or consume nutrients for energy. In fact, viruses are very complex chemical molecules which depend entirely on host cell machinery to reproduce. Webster's New World Dictionary defines a virus as:

> *"Any of a kingdom (Virus) of prokaryotes, usually ultramicroscopic, that consist of nucleic acid, either RNA or DNA, within a case of protein: they infect animals, plants, and bacteria and can reproduce only within living cells so that they are considered as being either living organisms or inert chemicals."*
>
> -Webster's New World Dictionary, 1998.

In comparison with other pathogens, viruses are unique in that their components are intermixed with the cellular components of their host and thus the two organisms are virtually indistinguishable. This intermixed relationship makes it very difficult to eradicate the virus without harming the host.

1.2 Significance of Plant Viruses

Plant diseases cause significant losses to the agricultural industry. It has been estimated that total agricultural losses due to plant disease are as high as US$60 billion per year (Klausner 1987). Fungi and viruses are responsible for the majority of these losses and viruses are reported as being the second most damaging plant pathogen (Mathews, 1991). Currently, viral diseases are a problem for most agricultural crops, but the extent of damage caused by viral infections can vary greatly. Viral infections

typically result in a substantial decrease in crop yield and/or decrease in the quality of a product. Once a crop is infected with a virus there is no means of removing or exterminating that virus from the crop. Because of this, viral infections in fruit trees and other high investment perennial crops can be extremely costly. In addition to the cost of replacing crops, virus infections reduce plant productivity, substantially decreasing crop yields and grower revenues. Some of the more benign viruses are known to cause yield decreases of 25% or more. Some of the most affected crops include potato, tobacco, tomato, cucumber, melons, strawberry, cabbage and radish (Tokuda *et al.* 1981). There is great demand for methods to reduce the damage caused by viruses and controlling viral diseases.

Viral infections in cultivated plants are extremely common. An example of the abundance of viral infection has been shown through a survey of the popular orchid genus, *Oncidium*. Upon examination of this genus, investigators were unable to find any virus-free specimens (Chia *et al.* 1991). Once virus-free orchids were established, it was shown that removal of viruses yielded a 65% increase in inflorescence size and a 21% increase in photosynthetic capacity (Chia *et al.* 1999). Other crops, such as onion and garlic, are also known to be universally infected with viral pathogens. All forms of commercially produced garlic worldwide have been shown to be infected with the onion yellow dwarf virus (Rice *et al.* 1998).

In addition to directly reducing crop yields and damaging plants, viruses can also weaken plants and make them more susceptible to damage caused by other factors. Virus infected plants are more easily attacked by fungal, bacterial and even other viral diseases. Plants that are already weakened by a viral infection are less able to cope with the

secondary infection and the result can be devastating. When two different viruses infect the same host at the same time, the result is known as "plant viral synergism". In this situation the plant may display severe disease symptoms and if the plant survives, the observed damage may be greater than the combined damage (loss of yield) expected from the two separate viruses (Pruss *et al.* 1997). With over 23 viruses that have been reported to cause damage to potato alone (Harvath, 1967), viral synergism can easily occur and when it does, it can render the crop unmarketable.

The ability of plant viruses to be transmitted effectively to other plants causes them to be extremely problematic to plant growers. Currently, the only strategy to reduce the impact of plant viruses is to sow viral free material. Unfortunately, virus-free seed can be very expensive and only provides temporary relief, as viruses are well adapted to re-infect these fields. It is surprising how successful these simple organisms are at dispersing themselves. Some viruses are capable of transmission through various kinds of mechanical disturbances that bring an infected plant or its fluids in contact with another plant. Many viruses take advantage of insects as vectors to spread to other plants; for example the Potato Leaf-Roll Virus is highly adapted to using insects as vectors for dispersal. The Potato Leaf-Roll Virus is capable of proliferating in various aphid species and thus once the virus is present in the insect, it is capable of infecting every susceptible plant that the insect comes in contact with. This highly effective form of dispersal makes it very difficult to keep viruses from entering crops. Insects also make excellent vectors as they often place the viruses directly into the phloem of the plant and thus the virus is capable of rapidly spreading throughout the plant.

Fungi are also significant vectors of viruses. In this case, damage may be caused by both the fungus and the virus. The four most significant fungal vectors are *Olpidium brassicae, Plymyxa graminis, Spongospora subterranea* and the controversial potato wart fungus (*Synchytrium edobioticum*) which was recently found in Prince Edward Island potatoes (Teakle, 1969). Viruses persist inside their fungal hosts and may be transmitted to crops which the fungal host infects or comes into contact with.

1.3 Methods of Controlling Plant Viruses

Unfortunately, there is are few effective methods to prevent or treat viral infections in plants. The most common methods of controlling plant viruses are passive, which do not attempt to directly remove or inhibit viruses. These strategies include: removing infected plants, using insecticides to control vectors, planting barrier crops and protecting crops from damage caused by machinery and the environment. The most effective method of viral control which is available to today's growers is the planting of certified virus-free seed. It is only recently that virus-free seed for most crops has been accessible to growers. Initially, virus-free seed was produced through breeding practices, which simply selected for the most productive plants. Twenty years ago, we still had no means of removing the more persistent viruses such as Potato Virus X (PVX) in potato. As a result of this, until recently, PVX was endemic to all potato growing operations (Klein & Livingston, 1983).

Early methods of producing virus-free seed material took advantage of the discovery that viral concentrations are lower in immature leaves and often absent from the growing meristem (Limasset *et al.* 1949). Thus, in the 50's and 60's, virus-free plants

were produced by growing plants from excised meristems. However, this method was slow and only effective with some viruses.

Another early technique for eliminating viruses from propagative material was through the use of heat treatments. Prolonged exposure to warm growing conditions has resulted in the elimination of several viruses. Potato leaf-roll virus was first eradicated from potato tubes by incubating tubers in air at 37.5°C for at least 25 days (Kassanis, 1949). PVX can be removed from potato by incubating shoots at 37°C for 15 weeks (Mellor and Stace-Smith, 1967); however thermotherapeutic methods are limited as they can require long incubation times and plants already weakened by the virus infection can often be destroyed in the process.

A combination of heat therapy and meristem culturing has resulted in much higher success. But even then only a fraction of the subjected plant material is actually freed of its viral contaminants. For example, the success rate for producing virus-free potato plants infected with PVX is only 4% of the excised plant samples which were subjected to a 23 day treatment (MacDonald, 1973).

The most effective methods of virus elimination involve the use of chemotherapies or antiphytoviral drugs in combination with meristem removal and thermotherapeutic methods. The first available phytoviral drug was dimethyl sulfoxide which was made available in the late 60's (Herschler and Wash, 1967). Although dimethyl sulfoxide is capable of inhibiting viral reproduction and growth, because of its high toxicity it has been of little practical use to the agricultural industry.

The nucleoside analogue ribavirin (Virazole™) is the most commonly used anti-phytoviral drug. Ribavirin was one of the first drugs approved for use in humans for the

treatment of virus diseases. The ability of ribavirin to inhibit plant viruses was first discovered in 1976 (Schuster, 1967) and then further investigated in 1977 (Lerch, 1977) and ribavirin was quickly used for the production of virus-free tissue cultures (Hansen 1979, Klein and Livingston 1982, Cassells and Long 1982). Over the years, ribavirin has been shown to be very useful in the virus elimination from plants. Applying a ribavirin and heat treatment to cultured meristems can increase the incidence of formation of virus-free plants and thus decreases the time and cost of indexing treated plants (Hansen, 1984).

Phytodiagnostics Company Ltd. is a private biotech company based out of Sidney, BC which removes viruses from plant material used by seed potato growers. Researchers of this company have found that treating tissue cultured meristems with high levels of heat and ribavirin is the most versatile and useful means of deriving virus-free propagative material (Ellis, 2001). Once the tissue culture is treated, the plant is rejuvenated and tested for the presence of viruses by means of ELISA (enzyme-lined immunosorbent assay). Once the above methods have been used to produce virus-free plants, tissue culture methods are used to rapidly clone seed plants.

There is still a great demand for a method of directly preventing or eradicating viral infections from plants growing in fields. Dimethyl sulfoxide was also one of the first substances patented and marketed for use as a protective spray against viruses in field crops (Herschler, 1967). This method quickly proved to be impractical as complete coverage of the plant is needed in order to provide protection and the effects of this compound are very short lived (Mikami *et al.* 1993). In 1985, a substance known as "The Marvel of Peru" was patented as being able to provide plants with systemic protection

from viruses. Unfortunately, this substance is a protein which is extremely expensive to make and *in vivo* production has not been approved for agricultural use (Mikami *et al.* 1993).

Recently, fungi in the genus *Fomes* have been shown to produce large amounts of 'acidic high molecular polysaccharides' which have systemic antiviral properties (Mikami *et al.* 1993). Japan Tobacco Inc. and the Institute of Fermentation Organization have developed a strain of the fungus *Fomes fomentarius* known as JTS 3046 which has a remarkably high production of these compounds. When the culture filtrate is applied to plants, plants were shown to acquire systemic protection against TMV (Mikami *et al.* 1993). The only shortcoming of these fungal compounds is that they are specific to the Tobacco Mosaic Virus and apparently not effective on other plant viruses.

An area of plant virology which has seen a lot of recent advances is genetically engineered viral resistance. Despite its initial cost to growers, the use of resistant crops is generally one of the most economical and practical methods of controlling plant viruses, as it requires less protective measures and crops sustain less damage. Resistant crops do not require the controversial application of herbicides, pesticides, or virucides to eliminate weed hosts, insect vectors, or inoculated viruses. Thus, host resistance is one of the most environmentally safe and durable methods for controlling plant diseases. In many plant-virus interactions, resistance is not easily available or cannot be obtained using traditional plant breeding strategies. Recent advances have shown that engineering plants to express viral coat proteins can provide resistance to the introduced virus (Powell-Abel *et al.* 1986, Stubbs and Culver 1998, Watanabe *et al.* 1995). By inserting proteins which are common to several plant viruses, Watanabe *et al.* engineered a tobacco

plant which possessed resistance to Tomato Mosaic Virus, Cucumber Mosaic Virus and Potato Virus Y (Watanabe *et al.* 1995). This high level form of resistance which is caused through transgenic expression of viral proteins is known as 'coat protein-mediated resistance', and has been used to produce effective resistance against Potato Virus X, Potato Virus Y, Potato Virus S, Potato Leaf-Roll Virus and Potato Mop-Top Virus in selected varieties (Spillane *et al.* 1998). Despite the effectiveness of this method and the damage caused by plant viruses, there is considerable consumer opposition to the use of genetically modified crops.

1.4 Potato Virus X

This project focused on finding natural chemicals which can inhibit potato virus X (PVX). PVX is a member of the potexviruses, a group of filamentous viruses with positively wound single stranded RNA genomes. In addition to PVX, this group of viruses contains some other very damaging viruses such as cymbidium mosaic virus, which is a significant virus affecting orchids (Agrios, 1997). PVX is the most widespread of all the potato viruses. It is capable of reducing crop yields by up to 20% before visible symptoms appear and ultimately PVX can reduce the crop yield by as much as 50% (Achultz and Bonde , 1944). Many strains of PVX exist and the virus is known to cause systemic infection in crops within the Solonaceae (including tomatoes, peppers and potatoes) and Cruciferae (including the turnip), and can cause local lesions in members of the Chenopodiaceae (including spinach and beets), Amaranthaceae (including the amaranth), and some legumes.

The agricultural impact currently caused by PVX in potato is not as severe as that of other viruses such as Blueberry Scorch Virus in blueberry or Plum Pox Virus in tree fruit. Damage caused by PVX has been minimized by the use of seed certification programs.

PVX was chosen for use in this study because it is a stable virus which is relatively easy to purify and use experimentally with tests on infectivity. In addition, the consequences of PVX being accidentally released into the environment would not be catastrophic. Due to the nature of viral inhibitors, it is possible that any compounds which are found to be effective inhibitors of PVX will also have inhibitory properties against other plant and animal viruses. This broad spectrum tendency of some antiviral substances makes the use of anti-plant viral assays an extremely cost effective and ethical means of studying antiviral compounds.

1.5 The Importance of Potato Crops

Most of the damage caused by PVX affects potatoes; a crop which suffers from heavy pathogen damage. Not only is potato susceptible to more than a hundred pathogens including, fungi, bacteria, nematodes and mycoplasmas, but it is also susceptible to as many as 23 viruses. Damage caused to the potato by late blight pathogen (*Phytophthora infestans*) in Ireland resulted in the death of 250,000 people in 1845. Currently, diseases affecting this crop are responsible for the hunger of millions living in developing countries. The potato crop is a very important staple, only surpassed in importance by wheat, corn and rice. Although potato crops are presently grown only in temperate areas, cultivation is about to dramatically increase with the introduction of

new tropical varieties that are designed to meet the needs of developing countries in warmer climates. The potato is also of great local importance. The BC Vegetable Marketing Commission lists the potato as the most important vegetable crop in the province. In view of this, we should attempt to increase our potato yields so that we are able to support more of our domestic consumption.

The Canadian potato industry is in need of assistance. Due to the recent emergence of the Potato Wart fungal parasite (*Synchytrium endobioticum*) in Prince Edward Island, Canada was unable to export its potato production to the Eastern United States. We need to invest in the development of cures for potato parasites if we are to keep our potato industry healthy. It has been known for some time that the presence of viruses, no matter how benign they may seem, can lower crop yields and promote occurrence of other disease. Having viral free crops will not only improve our yields, but also promote resistance to contaminants such as the potato wart fungus which was discovered in PEI.

1.6 Flavonoids

Flavonoids are a type of secondary metabolite common to all "higher plants" (Koes *et al.* 1994), and are well known for their antiviral properties against animal viruses (Pietta, 2000, Kaul *et al.* 1985). Flavonoids are characterized as phenolic compounds containing two aromatic rings connected by a three carbon bridge. These compounds are synthesized in plants via the shikimic and malonic acid pathway and are based on the precursor phenylalanine. A simplified depiction of flavonoid synthesis is displayed in Figure 2. Because flavonoids occur almost universally in green land-plants and are not present in algae, flavonoids are thought to have played a fundamental role in the colonization of land habitats (Geiger *et al. 1997*). Current theory is that flavonoids function to protect the plant from damage caused by UV radiation.

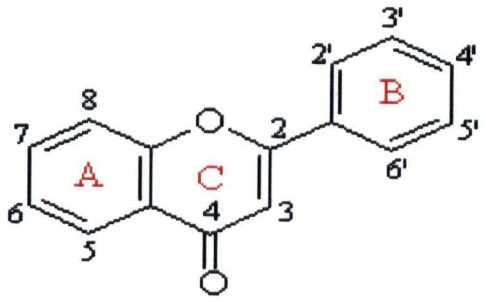


Figure 1: The basic flavonoid unit.
The aromatic rings are labeled with red letters and the positions are numbered. Carbons 2,3 and 4 form a three carbon bridge between the two aromatic rings. These carbons may be arranged to form a third ring structure, or left open to form a group of flavonoids known as chalcone.

Several projects have screened flavonoids for activity against a wide range of animal viruses and currently a large number of flavonoids have been shown to possess antiviral properties. A relatively early flavonoid study by Nagai *et al.* examined 103 different monomeric flavonoids for activity against Influenza A. Their comparison of

different flavonoid structures found that no correlation could be made between certain structures and anti-viral activity, but they did find several flavonoids which demonstrated potent antiviral activity.

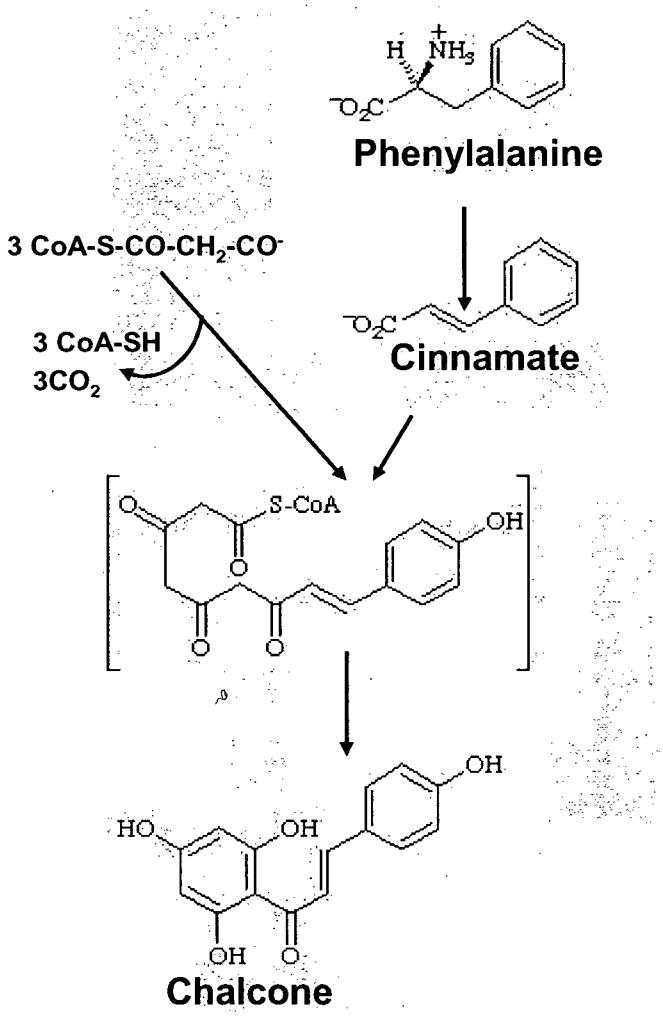


Figure 2: Summary of flavonoid synthesis.
This diagram shows the basic steps in which acetyl-CoA is joined with phenylalanine to form a chalcone, the simplest flavonoid.

The most active structure they found was 5,7,4'-trihydroxy-8-methoxyflavone, see Figure 3 (Nagai *et al.* 1990).

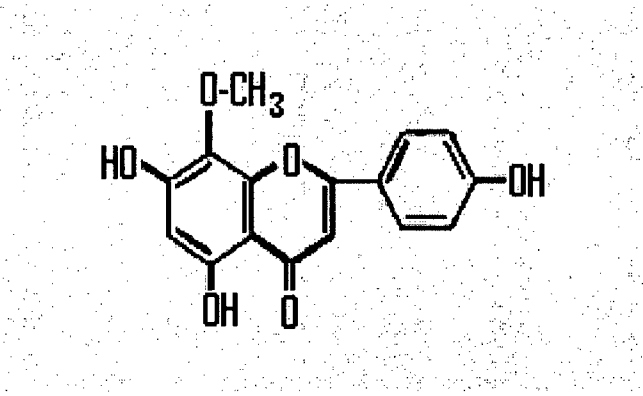


Figure 3: 5,7,4'-trihydroxy-8-methoxyflavone

Collaborations between Dr. French and Dr. Towers have done much of the work studying the effects of flavonoids on plant viruses (French *et al.* 1991, French and Towers 1992, Malhotra *et al.* 1996, Onylagha *et al.* 1997). In these studies, several flavonoids have been identified as exhibiting strong antiviral properties against plant viruses. One of the most notable flavonoids is quercetin (see Figure 4), which even at very low concentrations is a very strong inhibitor of both Tomato Ringspot Virus and PVX (Malhotra *et al.* 1996, French and Towers 1992).

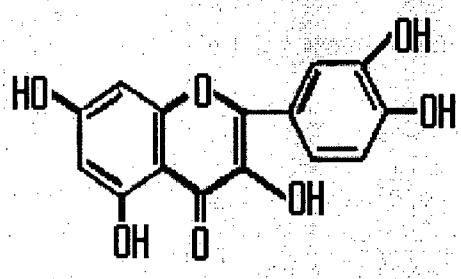


Figure 4: Quercetin

1.7 Biflavonoids

With all the attention that monomeric flavonoids have received for their antiviral properties, it is surprising that little work has been done to investigate the antiviral effectiveness of biflavonoids. Preliminary results indicated that biflavonoids such as robustaflavone effectively inhibit PVX infection in the local lesion assay (French, 2000).

Biflavonoids are dimers made up of two flavonoid units. The best known sources of biflavonoids are the needles, bark and seeds of gymnosperms, the leaves of *Ginkgo biloba* and the nuts and seeds of plants in the family Guttiferae. Biflavonoids are a very consistent character of the Psilotales, Selaginellales, Cycadales and Gymnosperms (with the exception of Gnetales and Pinaceae) (Geiger *et al.* 1997). In these plant groups, almost all synthesized flavonoids are shunted into biflavonoid production and the types of biflavonoids made in these plants are very consistent (Geiger *et al.* 1997). In the Angiosperms, the occurrence of biflavonoids is erratic and thought to have evolved several times independently. The persistence of biflavonoids in the ancient taxa mentioned above and their continued reoccurrence in the more modern taxa suggests that these compounds contribute to the fitness of these plants (Geiger *et al.* 1997). Because biflavonoids have been shown to be most abundant in the cuticle and outer surfaces of plants, it is thought they might provide protection against the invasion of microorganisms or herbivorous insects (Gadek *et al.* 1984).

The first report of a biflavonoid containing antiviral activity occurred in 1992 when a screen for antiviral activity in *Cephalotaxus drupacea* indicated that ginkgetin, a biflavonoid originally isolated from *Ginkgo biloba*, was active against Herpes Simplex Virus-1 (Hayashi *et al.* 1992). In 1997, Lin *et al.* published a report indicating antiviral

activity of biflavonoids and characterized this group of chemicals as having antiviral properties against animal viruses. During an examination of the anti-HIV properties of *Garcinia multiflora,* Lin *et al.* noticed a high level of biflavonoids in the active fraction. Upon investigation, eight of the eleven biflavonoids screened were found to be inhibitors of HIV-1 (Lin, Anderson *et al.* 1997). One of these biflavonoids tested, robustaflavone, showed particularly promising results, since it had a much lower IC50 for HIV than for human cells, indicating a selective targeting of the virus. Further investigation showed robustaflavone also to be a potent inhibitor of hepatitis B (Lin, Zembower *et al.* 1997). These results suggested that biflavonoids might effectively inhibit a wide spectrum of viruses. Subsequently, Lin *et al.* again screened these same biflavonoids, this time against 11 different pathogenic viruses, testing for a broader range of antiviral activity (Lin *et al.* 1999). Of the ten biflavonoids they isolated from *Rhus succedanea* and *Garcinia multiflora*, all showed antiviral activity against at least one of the human viruses tested. From this study, robustaflavone, amentoflavone, hinokiflavone and agathisflavone showed high activity against several human viruses including influenza A & B, HSV 1 & 2 and measles (Lin *et al.* 1999). Because of this wide spectrum activity found in so many biflavonoids, it seems logical that other biflavonoids are also active; some biflavonoids may even be more selective (nontoxic to host cells) and more effective than these examined compounds. It also seems likely that these biflavonoids are effective at inhibiting more viruses than the few that were tested.

The first biflavonoid to be discovered was ginkgetin, which was isolated from *Gingko biloba* in 1929 and identified in 1941. As mentioned earlier, this was also the first biflavonoid to be tested and shown to be antiviral. Currently, only approximately

100 biflavonoids have been described; of those tested, most have shown antiviral properties.

1.8 Mechanism of Action of Biflavonoids

Very little work has been done on the mechanism of inhibition caused by antiviral biflavonoids. During an investigation into the ability of robustaflavone to inhibit hepatitis B, Lin *et al.* monitored viral DNA, mRNA and protein antigen levels and found robustaflavone to significantly lower intracellular and extracellular viral DNA levels (Lin, Zembower *et al*, 1997). This decrease in viral DNA suggests that robustaflavone inhibits HBV DNA polymerase. This form of inhibition is most likely to "occur during the early stages of viral genome replication such as during the formation of the primer for negative-strand DNA synthesis" (Lin, Zembower *et al*, 1997). Due to the large size of biflavonoids, it is unlikely that they are capable of penetrating into the core of mature viral particles. More likely biflavonoids inhibit the virus during the replication process, which takes place in the host cytoplasm. Some biflavonoids have been shown to inhibit HIV-1 reverse transcriptase *in vitro* (Lin, Anderson *et al.* 1997). It is also possible that the biflavonoids are encapsulated inside viruses that have managed to undergo genome replication (Lin, Zembower *et al*, 1997).

1.9 Bryophytes

Recent evidence has shown that bryophytes are a rich source of biflavonoids. Bryophytes are a division of small, nonvascular, spore bearing plants with a gametophytic life cycle. The division *Bryophyta* include the liverworts (*Hepaticae*),

hornworts (*Anthocerotae*), sphagnum (*Sphagnopsida*), hair-cap mosses (*Polytrichopsida*), the lantern mosses (*Andreaeopsida*), and the toothed mosses (*Bryopsida*). The biflavonoid content of some mosses has been shown to be as high as 2.5% of their dry weight (Zinsmeister *et al.* 1996). This is somewhat surprising as bryophytes, particularly the true mosses, are thought to produce very few secondary metabolites (Asakawa, 1995), and are rarely studied for their chemical properties. Nevertheless, in 1987, Hans Geiger isolated a new biflavonoid (5'-hydroxyamentoflavone) from the moss *Plagiomnium elatum* and showed biflavonoids to be present in seven unrelated bryophyte taxa (Geiger, 1988). More recently, Tassilo Seeger performed a 2 D-TLC screen of 200 moss species from 61 different families and reported the presence of previously known bi- and triflavonoids in 40 of these families (Seeger, 1992). Unfortunately, Seeger has not been able to publish this work as the method of 2 D-TLC does not provide definitive identification of these compounds. As a result of Seeger's work, Zinsmeister *et al.* have undertaken the task of using NMR to examine these bryophytes for the presence of biflavonoids. Currently, they have tested representative species from six unrelated families of mosses and in each of these families they have found a high occurrence of biflavonoids (Zinsmeister *et al.* 1996). The occurrence of biflavonoids in these unrelated mosses provides evidence that biflavonoids are widespread amongst bryophyte species.

Unlike the biflavonoids found in the non-flowering land plants which are very consistent, the bryophytes contain a very diverse array of biflavonoids, many of which are unique to one species (Geiger *et al.* 1997). Several of these unique bryophyte biflavonoids are listed in Table 1 with the mosses in which they are found.

Table 1: Some unique bryophyte biflavonoids.
This table lists some of the biflavonoids that are reported to be unique to bryophytes.

Biflavonoid	Bryophyte found in	Literature source
5'-Hydroxyamentoflavone	*Plagiomnium elatum*	Geiger, 1988
5'-Hydroxyrobustaflavone	*Rhytidiadelphus squarrosus*	Seeger *et al.* 1990
2,3-Dihydro-3',3'''-Biapigenin	*Homalothecium lutescens*	Seeger *et al.* 1993
3'''-Desoxydicranolomin 2,3-Dihydro-3'''-Desoxydicranolomin 2,3-Dihydro-5',3'''-Dihydroxyrobustaflavone	*Plagiomnium undulatum*	Rampendahl *et al.* 1996
Bartramiaflavone	*Bartramia pomiformis*	Seeger *et al.* 1991
Campylopusaurone 5',3'''-Dihydroxyrobustaflavone 5',3'''-Dihydroxyamentoflavone	*Campylopus clavatus* *Campylopus Holomitrium*	Geiger & Markham,
5',3'''-Dihydroxyrobustaflavone	*Hylocomium splendens*	Becker *et al.* 1986

The bryophyte biflavonoids have yet to be examined for any type of biological activity including antiviral activity. Bryophytes have often been overlooked when it comes to phytochemical studies, as their study poses several unique challenges. Bryophytes do not yield large amounts of chemicals per weight; thus in comparison to vascular plants, larger amounts of plant material is needed. Obtaining adequate amounts of material can be a challenge as bryophytes are small organisms that often grow amongst other species. Also, bryophyte identification can be problematic. Due to limited knowledge of bryophytes, these organisms are often overlooked.

Currently, only one study is known to have investigated the antiviral activity of bryophytes (Kothyari, 1997). This is surprising when we consider the amount of research that has been devoted to the antiviral properties of higher plants. Unlike higher plants, mosses lack advanced structures such as thick outer layers and waxy cuticles, which are capable of providing mechanical defenses against viruses. Despite this apparent

vulnerability, there are no reported cases of viruses capable of infecting bryophytes and thus it seems quite possible that bryophytes contain a chemical defense against viruses. In 1997, Kothyari screened extracts from Himalayan bryophytes for activity against Tobacco Mosaic Virus and Tobacco Ring Spot Virus. Of the 31 species screened, five showed antiviral activity with the extract from *Pellia epipylla* showing the greatest activity (Kothyari, 1997). These findings provide even further motivation for the investigation of bryophyte biflavonoids for antiviral activity.

It is estimated that there are 14,000 bryophyte species belonging to 750 genera growing in British Columbia. Of these 14,000 species, only 320 have been mentioned in chemical studies and there are 580 genera that have yet to be chemically investigated (Asakawa, 1995). With such an abundant and diverse biflavonoid composition produced in these plants which produce very few other secondary metabolites (Geiger *et al.* 1997, Asakawa 1995), bryophytes are an excellent candidate for the study of biflavonoids.

1.10 Objectives

The goal of this project was to identify new sources of antiviral compounds. New antiviral compounds are of great interest to those involved in producing virus-free crops. It is hoped that compounds can be found that are less toxic and less expensive than ribavirin, thus being of greater agricultural use. The ultimate goal is to find an antiviral substance that can be used to exterminate viruses from existing crops in the field. In this project, PVX is used as a model for plant viruses; however, it is very likely that substances identified by this project will be useful against many other viruses, just as ribavirin is quite useful in the treatment of several plant and human viral diseases.

There is mounting evidence that indicates biflavonoids are very strong viral inhibitors and effective against a great many viruses. It was the goal of this project to further study the antiviral properties of biflavonoids as they relate to PVX.

With the goal of identifying new sources of antiviral activity and the high occurrence of biflavonoids present in bryophytes, this project investigated the antiviral properties of extracts from this very interesting, but often understudied group of plants.

2 Methods

2.1 Collection and Identification of Bryophytes

The bryophyte material used for the antiviral screening was collected from various locations throughout British Columbia between August 2000 and September 2001. Collected specimens were rinsed clean, dried thoroughly and stored in paper bags at room temperature. Approximately one to ten grams of dried material were collected for each sample.

Identification was performed using Crum's text "The Mosses of Eastern North America" (Crum, 1981) and Schofield's handbook "Some Common Mosses of British Columbia" (Schofield, 1992). Identified specimens were then verified through comparison with material preserved in the UBC Herbarium. Voucher specimens are stored in the UBC Herbarium, Vancouver, BC.

Table 2: Bryophyte collection.
The following 50 bryophyte samples were used in the anti-PVX assay.

Collec. No.	Name	Date	Location	Collection notes
107	*Andreaea nivalis* Hook.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Growing on rocks on north facing alpine meadow.
128	*Atrichum selwynii* Aust.	May 15, 2002	North Vancouver, BC Mosquito Creek.	Growing near forest trail
129	*Atrichum undulatum* (Hedw.) P. Beauv.	May 15, 2002	North Vancouver, BC Mosquito Creek.	growing along trail
67	*Bryum argenteum* Hedw.	Aug 24, 2000	Prince George, BC	growing on roof top
144	*Bryum capillare* Hedw.	June 22, 2002	Near Port Alberni, BC Kennedy Lake	Growing on rocks
80	*Bryum miniatum* Lesq.	May 7, 2001	Nanaimo, BC. End of cranberry road.	A red moss growing on a very wet rock field in direct sunlight.
85	*Buckiella undulatum* Hedw.	July 31, 2001	Vancouver, BC. Pacific Spirit Park.	Growing amongst large amount of pine needles. Individuals small and flattened.
94	*Buckiella undulatum* Hedw.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Growing on forest floor. Unusually large fluffy appearance.

134	*Claopodium crispifolium* (Hook) R. & C.	May 16, 2002	North Vancouver, BC Mosquito Creek.	Growing on side of Maple tree
103	*Dicranum fuscescens* Turner.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Found on Ground and stumps
83	*Eurhynchium oreganum* (Sull.) Jaeg.	July 31, 2001	Vancouver, BC. Pacific Spirit Park,16th Ave.	A feather moss growing on a large rock.
150	*Eurhynchium praelongum* (Hedw.) Br.Eur.	July 31, 2001	Vancouver, BC. Pacific Spirit Park.	Growing on log.
160	*Eurhynchium praelongum* (Hedw.) Br.Eur.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	On boulders by stream.
68	*Grimmia pulvinata* (Hedw.) Sm.	Aug 28, 2000	Stein Valley, BC	Growing on alpine boulders.
88	*Hylocomium splendens* (Hedw.) B.S.G.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Plants growing to very large size, dominant ground cover.
127	*Hylocomium splendens* (Hedw.) B.S.G.	May 15, 2002	North Vancouver, BC Mosquito Creek.	Dominant ground cover. Collected for chemical study
84	*Hypnum circinale* Hook.	July 31, 2001	Vancouver, BC. Pacific Spirit Park,16th Ave.	A small moss growing flattened along decaying log.
105	*Hypnum circinale* Hook.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Shaded area, on log near trail.
109	*Hypnum circinale* Hook.	Sep 5, 2001	Vancouver, BC. Lighthouse Park.	Growing on dry rock, exposed area.
91	*Isothecium stoloniferum* Brid.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Horizontal form. Found growing on top of a log. Feathery growth form.
92	*Isothecium stoloniferum* Brid.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Vertical form. Found hanging in hair like form off of deciduas trees.
89	*Leucolepsis acanthoneuron* (Schwaegr.) Lindb.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Growing to very large size.
136	*Leucolepsis acanthoneuron* (Schwaegr.) Lindb.	May 16, 2002	North Vancouver, BC Mosquito Creek.	Growing on soil in shaded area.
124	*Oligotrichum aligerum* Mitt.	May 15, 2002	North Vancouver, BC Mosquito Creek.	Found on ground near trail.
137	*Plagiomnium insigne* (Mitt.) TJ Kop.	May 16, 2002	North Vancouver, BC Mosquito Creek.	Growing on shaded forest floor.
110	*Pleurozium schreberi* (Brid.) Mitt.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Growing on dead wood, near lake.
106	*Pogonatum contortum* Menzies ex Brid.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Growing on forest floor.
146	*Pogonatum contortum* Menzies ex Brid.	June 22, 2002	Sutton Pass Vancouver Island.	Growing on stream bank and in water.
69	*Polytrichastrum alpinum* (Hedw.) GL Sm.	Aug 28, 2000	Stein Valley, BC	Found in clearing next to logging road on sand and pebbles
139	*Polytrichum commune* Hedw.	May 16, 2002	North Vancouver, BC Mosquito Creek.	Growing on forest floor.

145	*Polytrichum juniperinum* Hedw.	June 22, 2002	Near Port Alberni Kennedy Lake	Growing beside logging road, in exposed area
123	*Polytrichum piliferum* Hedw.	May 15, 2002	North Vancouver, BC Mosquito Creek.	Growing on forest floor
100	*Racomitrium canescens* (Hedw.) Brid.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Found on ground beside trail
71	*Racomitrium canescens* var. *ericoides* (Brid.) B.S.G.	Aug 29, 2000	Pemberton, BC. Duffy Lake Rd. Lillooet Lake.	Growing on rock in very dry dusty area. Distinctly grey when dry. Bushy with many leaves, very full appearance.
121	*Racomitrium lanuginosum* (Hedw.) Brid.	April 15, 2002	Vancouver, BC Cypress Park	Growing on rocks.
131	*Rhizomnium glabrescens* (Kindb.) TJ Kop.	May 16, 2002	North Vancouver, BC Mosquito Creek.	Growing on Rotten logs
126	*Rhytidiadelphus loreus* (Hedw.) Warnst.	May 15, 2002	North Vancouver, BC Mosquito Creek.	Growing on old log.
120	*Rhytidiadelphus squarrosus* (Hedw.) Warnst.	April 13, 2002	Vancouver, BC UBC	Growing amongst grass.
95	*Rhytidiadelphus triquetrus* (Hedw.) Warnst.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Forming dense mats on forest floor. Shaded area.
70	*Rhytidiadelphus triquetrus* (Hedw.) Warnst.	Aug 28, 2000	Stein Valley, BC	Growing on rocks
101	*Rhytidiopsis robusta* (Hook.) Broth.	Sep 1, 2001	Squamish, BC. Garibaldi Park.	Forming dense mats on Forest floor. Dominant species in Subalpine forest.
93	*Scapania bolanderi* Austin.	Aug 6, 2001	Port Alberni, BC. Sproat Lake.	Growing on vertical surface of a stump.
57	*Sphagnum capillifolium* (Ehrh.) Hedw.	Aug 21, 2000	Near Prince George, BC Ness Lake	Forming a floating mat in a marsh,
61	*Sphagnum fuscum* (Schimp.) Klinggr.	Aug 21, 2000	Near Prince George, BC Ness Lake	Growing in spruce bog.
58	*Sphagnum pacificum* Flatberg.	Aug 21, 2000	Near Prince George, BC Ness Lake	Margin of pool in pine forest.
63	*Sphagnum papillosum* Lindb.	Aug 21, 2000	Near Prince George, BC Ness Lake	Growing in a wet bog area among other species
62	*Sphagnum rubellum* Wils.	Aug 21, 2000	Near Prince George, BC Ness Lake	Growing in a wet bog area among other species
64	*Sphagnum russowii* Warnst..	Aug 21, 2000	Near Prince George, BC Ness Lake	Growing on refuse in Bog
132	*Tetraphis pellucida* Hedw.	May 16, 2002	North Vancouver, BC Mosquito Creek.	Growing on rotting stump
119	*Tortula muralis* Hedw.	April 13, 2002	Vancouver, BC UBC	growing on exposed concrete

2.2 Extraction of Bryophytes

Clean air-dried plant samples were ground in liquid nitrogen using a mortar and pestle. The resulting fine ground powder underwent three consecutive two day extractions at room temperature using approximately 40mL of ethanol per extraction. Upon each extraction the supernatant was vacuum filtered and dried *in vacuo*. The resulting crude extracts were stored at -20°C.

2.3 The Antiviral Assay

Extracts and compounds were tested for antiviral activity using a local lesion assay with PVX infection of *Chenopodium quinoa,* as described previously by French *et al.* 1991. In this assay the bryophyte extract or other test substance is incubated with PVX for 30 minutes before being used to inoculate one side of a *C. quinoa* leaf. The remaining half leaf is then inoculated with a control solution containing an equivalent PVX concentration. After a week of incubation in a growth chamber (20°C, cool white light, 16h light:8h dark) the plant develops lesions in response to PVX infections. The percent inhibition caused by the test substance can then be calculated by comparing the number of lesions on each side of the leaf.

Test extracts were prepared as solutions containing 10mg/mL of dried extract in 5% Ethylene Glycol Monomethyl Ether (EGME). EGME was used to improve the solubility of the extract in the solution; at these concentrations this solvent has been shown to have no effect on the number of lesions produced by PVX (French and Towers, 1992). To avoid precipitation in water, the dried extract was first dissolved in EGME and then distilled water was added.

To incubate the virus with the extract solution, 20μL of a 1.15mg/mL PVX solution was added to 200μL of the 10mg/mL extract solution, resulting in a final viral concentration of 0.10mg/mL and a final extract concentration of 9.1 mg/mL. A control solution was prepared in a similar manner, except the extract solution was replaced by 200μL of 5% EMGE. Both the control and test solutions were incubated for 30 minutes at room temperature.

Following incubation of the test and control solutions, the solutions were used to inoculate five week old *C. quinoa*. Plants which were older than five weeks were no longer suitable for use in the assay as they possessed a higher level of resistance to viral infection and thus did not produce enough lesions to yield consistent results. Prior to inoculation, plants were powdered with carborundum in order to abrade the leaf during the inoculation process, thus allowing transmission to occur. The inoculation was performed using a freshly gloved finger. The control side of the leaf was chosen at random and then marked by punching a small hole on that side of the leaf tip. On each side of the leaf, 10uL of the corresponding solution was rubbed into the leaf. Typically each plant possessed 4-7 leaves suitable for inoculation.

Figure 5: Five week old *Chenopodium quinoa.*

Following one week of incubation in an environmental chamber the resulting lesions on each half leaf were counted. Leaves that did not produce a minimum of 15 lesions on the control side were not counted, as often their results were not consistent. The percent inhibition for each leaf was calculated and the average percent inhibition and standard deviation on all leaves was also calculated.

Figure 6: *Chenopodium quinoa* leaf showing lesions.
PVX induced lesions are present on control side of leaf after one week of incubation.

Each sample was tested on at least three separate occasions, each time using a minimum of four leaves on each of three plants. Leaves failing to produce 15 lesions on the control half were not counted. If multiple leaves of a plant failed to produce this minimum level of infection, then the results of the plant were ignored, as it is likely the plant was overly mature and possessed an increased resistance to infection. The results of samples which did not yield a minimum of three replications involving three plants each, have been deemed unreliable and are not reported.

C. quinoa plants were grown from seed in an environmental growth chamber with 16 hour photosynthetic periods at 25°C. Approximately 50 seeds were germinated in a three inch plastic pot filled with Terralite® Readi-Earth® potting soil. One week after seeds were sown, seedlings were transplanted into individual three inch plastic pots.

2.4 Biflavonoid Testing

Four purified biflavonoid compounds: robustaflavone, hinokiflavone, amentoflavone and 7,4',4'-amentoflavone were obtained and tested for antiviral activity using the local lesion assay. The robustaflavone sample was provided by Yuh-Meei Lin and the MediChem research group. The remaining three biflavonoids were purchased from Apin Chemicals Ltd., U.K.

Due to the small amount of material available, samples were only successfully tested at a final concentration of 1.38mg/mL.

2.5 Fractionation of the Bryophyte *Hylocomium splendens*

A large quantity of *H. splendens* was collected from North Vancouver highlands. The sample was dried and ground to a fine powder using a mechanical grinder. The resulting 600 gram sample was subjected to four consecutive three day extractions in boiling ethanol. The resulting dark green coloured extract was then dried down *in vacuo* at 40°C to yield 56.1 grams of powdered crude extract.

A liquid-liquid partition was performed in order to separate the crude extract into four fractions based on solubility of the different components within the crude extract. This partition was performed by dissolving the crude extract in water, placing the solution in a two liter separatory funnel and washing several times with 95% hexane,

followed by several washings with ethyl acetate and finally the remaining solution was washed with butanol.

Thin Layer Chromatography (TLC) was used to analyze the composition of each fraction. Due to similar TLC profiles, the hexane and the ethyl acetate fractions were amalgamated. The resulting three fractions were then tested for anti-PVX activity using the local lesion assay as was described previously.

Following the results of the local lesion assay, further separation of the hexane-ethyl acetate fraction was achieved through the use of a dry loaded silica gel column. 24.16g of the hexane-ethyl acetate fraction were dissolved in acetone and dried onto 67g of SiliCycle® silica gel 60 (230-400 mesh). The sample was then loaded on to a 3.81cm diameter column which was packed with 400g of the same silica gel. The column was then washed with solvent beginning with 100% hexane and the polarity of the solvent was gradually increased by diluting the hexane with acetone in 5-10% increments. Upon addition of 100% acetone, the column was terminated and allowed to run dry. The resulting 115 fractions were then analyzed using thin layer chromatography and similar fractions were amalgamated to yield 30 unique groups of fractions. Based on the polarity, colour and quantity, seven of the amalgamated groups were then chosen for antiviral testing. The three adjacent groups that were identified as being active were amalgamated and with the assistance of Dr. Dong Sheng Ming, separated in a Sephadex™ LH-20 column eluted with methanol.

3 Results & Discussion

3.1 Anti-viral Activity of Bryophytes.

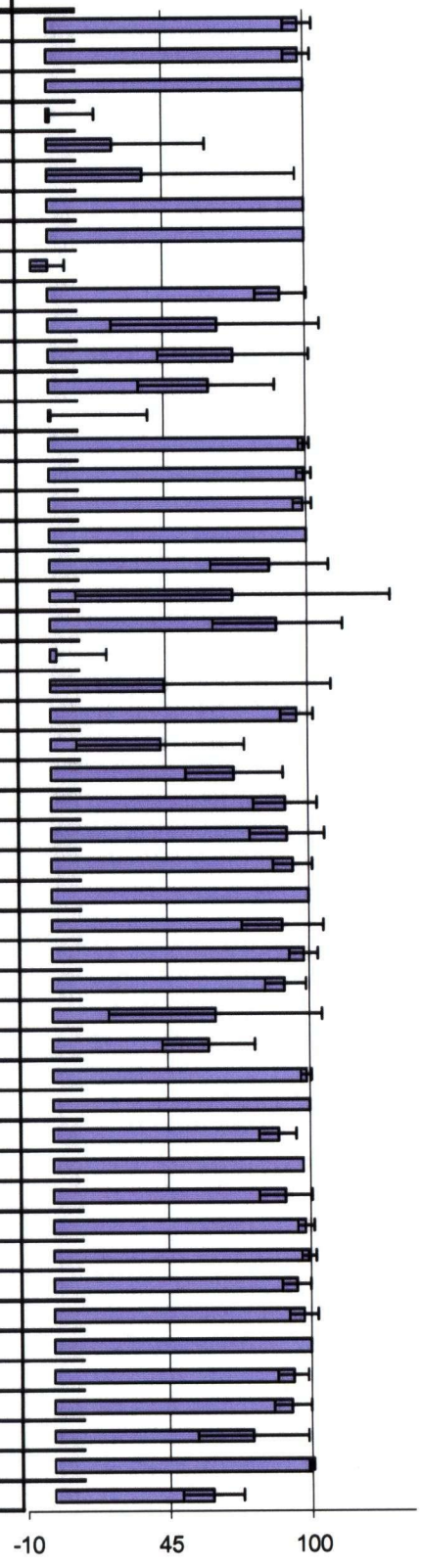
Table 3 provides an alphabetical listing of the percent inhibition of PVX caused by each bryophyte sample that yielded consistent results. The percent inhibition has been calculated as an average of all testing for each sample. The percent error has been calculated as the standard deviation among all leaves tested for each sample.

3.1.1 Results were not obtained from all samples collected.

Of the 70 samples that were collected and identified, reportable data was only obtained from 50 of the samples. Leaves which did not produce a minimum of 15 lesions on the control side were not counted, as the results were too easily distorted by a small number of inconsistent lesions; as well, the standard deviation amongst these leaves was very high. Data was also not taken from plants that contained multiple leaves that failed to develop adequate lesions. It was noted that the lesion counts from the remaining leaves on these plants did not correspond to data taken from other plants. The failure of these plants to produce adequate lesions is most likely due to the plants being at a higher maturity level and thus having a greater resistance to infection. Ideal growing conditions and experience are required in order to grow large numbers of *C. quinoa* all possessing the exact same fitness and maturity. In many cases, experiments failed to produce adequate numbers of lesions and had to be repeated. These additional repetitions were not anticipated at the time of collection and thus several samples were depleted before useful data could be obtained.

Table 3: The anti-PVX activity of tested bryophyte species.
All samples were tested at 9.1mg/mL using the local lesion assay. The bars on the right are a graphical representation of the levels of inhibition and error for each sample.

Collection #	Bryophyte Species	% inhibition	% error
107	Andreaea nivalis	98	6
128	Atrichum selwynii	98	5
129	Atrichum undulatum	100	0
67	Bryum argenteum	2	17
144	Bryum capillare	26	36
80	Bryum miniatum	38	59
85	Buckiella undulatum	100	0
94	Buckiella undulatum	100	0
134	Claopodium crispifolium	-6	14
103	Dicranum fuscescens	91	10
83	Eurhynchium oreganum	65	40
150	Eurhynchium praelongum	62	26
160	Eurhynchium praelongum	72	29
68	Grimmia pulvinata	1	38
88	Hylocomium splendens	99	3
127	Hylocomium splendens	99	2
84	Hypnum circinale	100	0
105	Hypnum circinale	99	3
109	Hypnum circinale	86	23
91	Isothecium stoloniferum	89	25
92	Isothecium stoloniferum	71	61
136	Leucolepsis acanthoneuron	3	19
89	Leucolepsis acanthoneuron	44	64
124	Oligotrichum aligerum	96	6
137	Plagiomnium insigne	43	32
110	Pleurozium schreberi	71	19
146	Pogonatum contortum	91	12
106	Pogonatum contortum	92	14
139	Polytrichum commune	94	8
123	Polytrichum piliferum	100	0
69	Polytrichastrum alpinum	90	16
145	Polytrichum juniperinum	98	6
71	Racomitrium canescens	64	41
100	Racomitrium canescens	91	8
121	Racomitrium lanuginosum	61	18
131	Rhizomnium glabrescens	99	2
126	Rhytidiadelphus loreus	100	0
120	Rhytidiadelphus squarrosus	87	7
95	Rhytidiadelphus triquetrus	90	10
70	Rhytidiadelphus triquetrus	98	0
101	Rhytidiopsis robusta	98	3
93	Scapania bolanderi	99	3
63	Sphagnum papillosum	94	5
64	Sphagnum russowii	97	5
57	Sphagnum capillifolium	100	0
61	Sphagnum fuscum	93	6
58	Sphagnum pacificum	93	7
62	Sphagnum rubellum	77	21
132	Tetraphis pellucida	100	1
119	Tortula muralis	61	12



3.1.2 The majority of species caused a high level of inhibition.

Of the 41 species successfully tested for antiviral activity, 29 resulted in greater than 75% inhibition against PVX. Thus, 71 % of the bryophyte species examined were highly effective at inhibiting PVX infections in *C. quinoa* when tested at 9.1mg/mL. In comparison to another antiviral screening of this nature, this study has identified a very high proportion of its samples as being antiviral. In a very similar screening performed in this lab, 31 algal species were tested for PVX inhibition at a concentration of 10mg/mL in a similar local lesion assay. Of the species tested only seven species (23% of those tested) were found to cause greater than 75% PVX inhibition (Pardee, 2001).

3.1.3 The correlation between activity and error.

Despite the fact that 46 of the 50 samples listed in Table 3 caused greater than 25% inhibition against PVX, only 29 have been labeled as highly active. The labeling of samples as "highly active" was restricted to samples exhibiting greater than 75% inhibition, as samples causing less than this level of inhibition exhibited a greater level of experimental error. By rearranging the samples in terms of their reported antiviral activity, as is done in Figure 7, it becomes apparent that there is a definite inverse correlation between the degree of inhibition and the level of uncertainty in a sample. This relationship between error and inhibition is a common characteristic of the local lesion assay (Pardee, 2001). As the level of inhibition decreases, the outcome becomes more random and the deviation among the results increases.

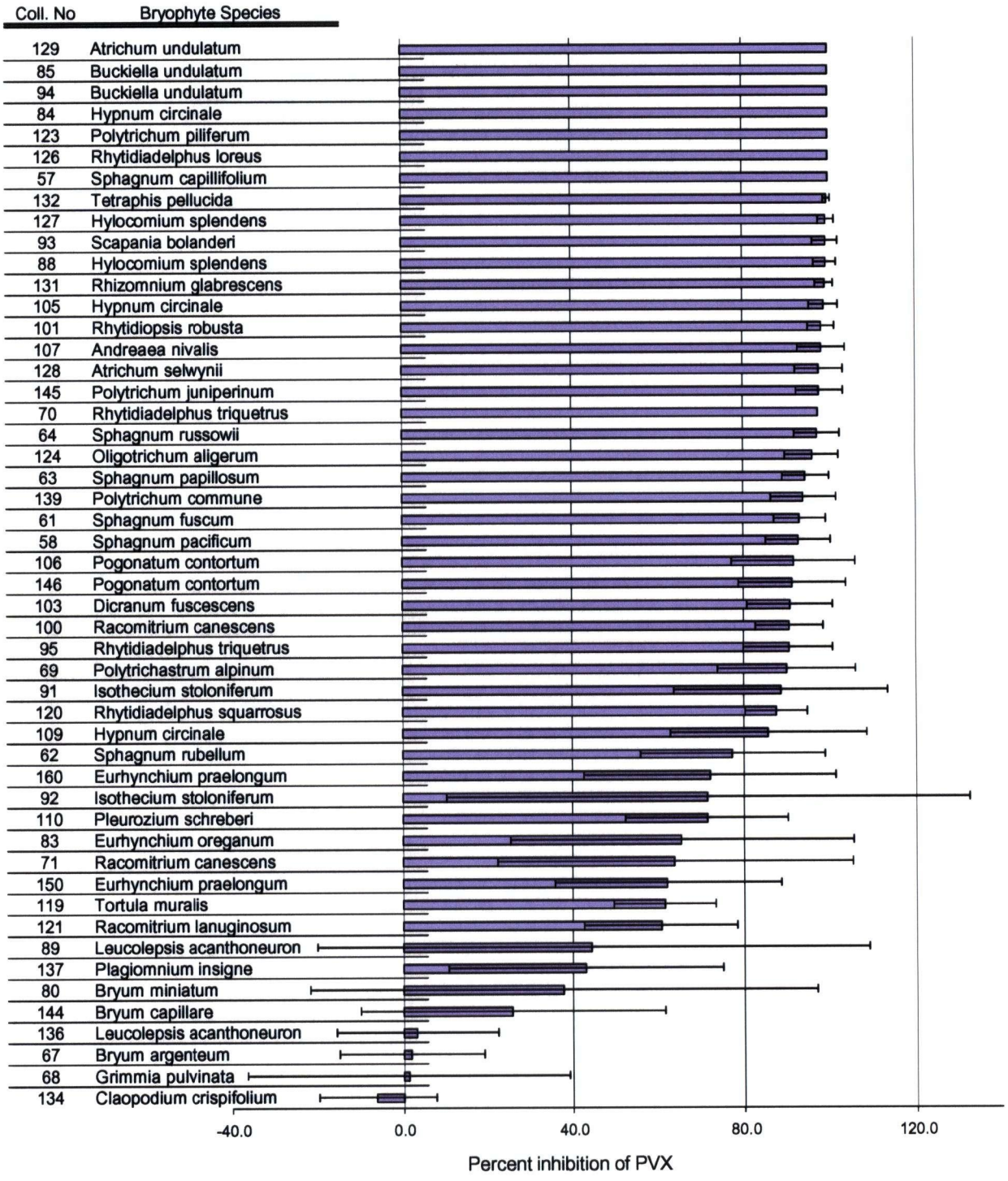


Figure 7: Relationship between inhibition and uncertainty in bryophyte samples.
Data from Table 3 is arranged in order of antiviral activity. As activity decreases uncertainty increases.

3.1.4 High error is a result of inconsistent infection production.

The high level of error present in the less active samples indicates the inconsistency in the number of infections which occur upon each inoculation. Table 4 illustrates this point by displaying the local lesion assay results of sample 127 *Hylocomium splendens* (Hedw.) B.S.G. The total inhibition for this sample was calculated as 99.5% with a standard deviation of 1.8%. It would appear that the results of the experiments for this sample are very consistent; however, if we compare the number of lesions on each control for this sample, we see that they have a standard deviation of 21.9%. Thus the inoculation of the same control solution has a fairly inconsistent outcome. This same inconsistency is noted among all other data obtained from the local lesion assay. Therefore, in the case of a non-active sample, we obtain a high level of uncertainty, as the number of lesions produced by each inoculation is not consistent. Despite this inconsistency in the number of lesions caused by an inoculation, highly antiviral substances still produce very consistent results as they prevent the formation of lesions at any level.

Table 4: The local lesion assay results of sample 127 *H. splendens*.
The number of lesions counted on the control and test side of each leaf are listed. The percent inhibition of each leaf has been calculated. The results of all leaves have been averaged and listed as the average percent inhibition. The standard deviation of all controls has been calculated to show the variable levels of lesion development. Leaves producing less than 15 lesions were omitted.

Experiment 1

Plant 1	control	test	% inhibition
leaf 1	20	0	100.0
leaf 2	20	0	100.0
leaf 3	75	5	93.3
leaf 4	15	0	100.0
leaf 5	70	5	92.9
leaf 6	20	0	100.0

Plant 2	control	test	% inhibition
leaf 1	35	0	100.0
leaf 2	60	0	100.0
leaf 3	15	0	100.0
leaf 4	20	0	100.0
leaf 5	18	0	100.0
leaf 6	15	0	100.0

Plant 3	control	test	% inhibition
leaf 1	16	0	100.0
leaf 2	15	0	100.0
leaf 3	20	0	100.0
leaf 4	16	0	100.0
leaf 5			
leaf 6			

Experiment 2

Plant 1	control	test	% inhibition
leaf 1	25	0	100.0
leaf 2	77	3	96.1
leaf 3	55	0	100.0
leaf 4	20	0	100.0
leaf 5	72	0	100.0
leaf 6			

Plant 2	control	test	% inhibition
leaf 1	45	0	100.0
leaf 2	43	0	100.0
leaf 3	17	0	100.0
leaf 4	80	0	100.0
leaf 5	16	0	100.0
leaf 6			

Plant 3	control	test	% inhibition
leaf 1	70	0	100.0
leaf 2	20	0	100.0
leaf 3	44	0	100.0
leaf 4	20	0	100.0
leaf 5	18	0	100.0
leaf 6			

Experiment 3

Plant 1	control	test	% inhibition
leaf 1	50	0	100
leaf 2	68	0	100.0
leaf 3	65	0	100.0
leaf 4	15	0	100.0
leaf 5	38	0	100.0
leaf 6	24	0	100.0

Plant 2	control	test	% inhibition
leaf 1	43	0	100.0
leaf 2	67	5	92.5
leaf 3	15	0	100.0
leaf 4	25	0	100.0
leaf 5			
leaf 6			

Plant 3	control	test	% inhibition
leaf 1	17	0	100.0
leaf 2	34	0	100.0
leaf 3	25	0	100.0
leaf 4	60	0	100.0
leaf 5	22	0	100.0
leaf 6			

Average percent inhibition	99.5 %
Standard deviation of inhibition	1.8 %
Standard deviation of control	21.9 %

3.1.5 Eight species with multiple samples.

Only 41 different bryophyte species were represented by the 50 samples which yielded results. Additional samples of a particular species were chosen due to observable differences within samples of the same species. These samples may have differed in growth form, habitat, or samples were different varieties of a species. Table 2 shows specific details or differences for each sample.

Despite the observed phenotypical differences between the repeated samples, they produced very similar results. The two samples of *Racomitrium canescens,* were an exception to this as they yielded very different results one producing 63.6% inhibition and the other producing 90.6%. Unlike other repeated samples, the *Racomitrium canescens* samples were identified as different varieties of the species. The less active sample was identified as *Racomitrium canescens* var. *ericoides* (Brid.). It is possible that the differences separating these two varieties are greater than they appear and go beyond physical appearances. The other repeated samples that produced similar results provide evidence to support the reproducibility of the methods used in this study. The fact that different samples of the same species which were collected during different seasons and from very different habitats still produced the same effect, increases the likelihood that the reported results are indicative of the true antiviral properties of these species.

3.1.6 Relatedness among bryophyte samples.

Figure 8 shows the phylogeny of the bryophyte samples with their reported antiviral activity. The phylogenetic tree lists the 41 species and the corresponding 26 genera and 14 families to which they belong. This sample is small when compared to the estimated 400 families, 750 genera and 14000 species of bryophytes found in British Columbia.

When the relationships between bryophyte species are taken into consideration, it becomes apparent that the activity of the samples in Table 3 is concentrated among groups of related species. The majority of the active samples belong to the three families: Hylocomiaceae, Polytrichaceae and Sphagnaceae. All of the samples from these families caused high levels of viral inhibition. Because a minimum of six different species exhibited very high levels of viral inhibition, it is possible that anti-viral properties are common throughout these families. Other bryophyte families tested may also have widespread anti-viral activity, but due to poor representation of their families in this study, such predictions cannot be made at this time. The phylogenetic tree also indicates several families that contain poor antiviral activity. The Mniaceae, Bryaceae and Brachytheciaceae all contain several species with little antiviral activity and do not seem to be noteworthy in terms of their antiviral properties.

Bryophytes were initially chosen for this study due to their abundance of biflavonoids; however, not all bryophytes produce these compounds. In fact, it seems that the presence of biflavonoids is restricted to the *Bryopsida* or toothed mosses. The *Sphagnopsida* and the *Andreaeopsida* are quite unique in that they do not produce any flavonoids (Geiger *et al.* 1997). While the *Polytrichopsida* have not been as intensely studied, as of yet, no flavonoids have been found in this group either (Geiger *et al.* 1997). In terms of chemistry, the *Hepaticopsida* are the most thoroughly studied of the bryophytes. While this group is known for its diverse secondary metabolites; no biflavonoids have been found to be present (Asakawa 1995). In view of the theory that the bryophytes would contain antiviral activity because of their abundance of biflavonoids, we would not expect the *Andreaeopsida, Sphagnopsida, Hepaticopsida and*

possibly the *Polytrichopsida* to be active. Despite their lack of biflavonoids, all representatives of these groups caused extremely high levels of PVX inhibition. It is possible that this antiviral activity in these latter groups is due to other antiviral compounds such as tannins, which are present in these groups, but absent from the *Bryopsida*. The *Sphagnopsida* are well known for their tannin content and it is not surprising that they contain these large antiviral compounds, as there is great selective pressure from their aquatic habitat for antiviral compounds such as this.

Despite their reported abundance of biflavonoids, several groups of the *Bryopsida* did not exhibit antiviral activity. Many of the non active species have been explicitly cited to contain biflavonoids and thus it is possible that not all of these compounds are active against PVX. However it is also possible that some of the biflavonoids present in these plants could not be extracted by the methods used. Several mosses have been shown to contain a high content of biflavonoids which are bound within the cell wall; these compounds cannot be extracted with alcohol (Geiger *et al.* 1997). It is also possible that the bryophytes were harvested during a time in which they possessed extremely low levels of biflavonoids. Studies have shown that the flavonoid content of mosses undergo considerable seasonal variation (Brinkmeier, 1999). In view of this, it is possible that the non-active species did not possess considerable quantities of biflavonoids at the time these samples were harvested.

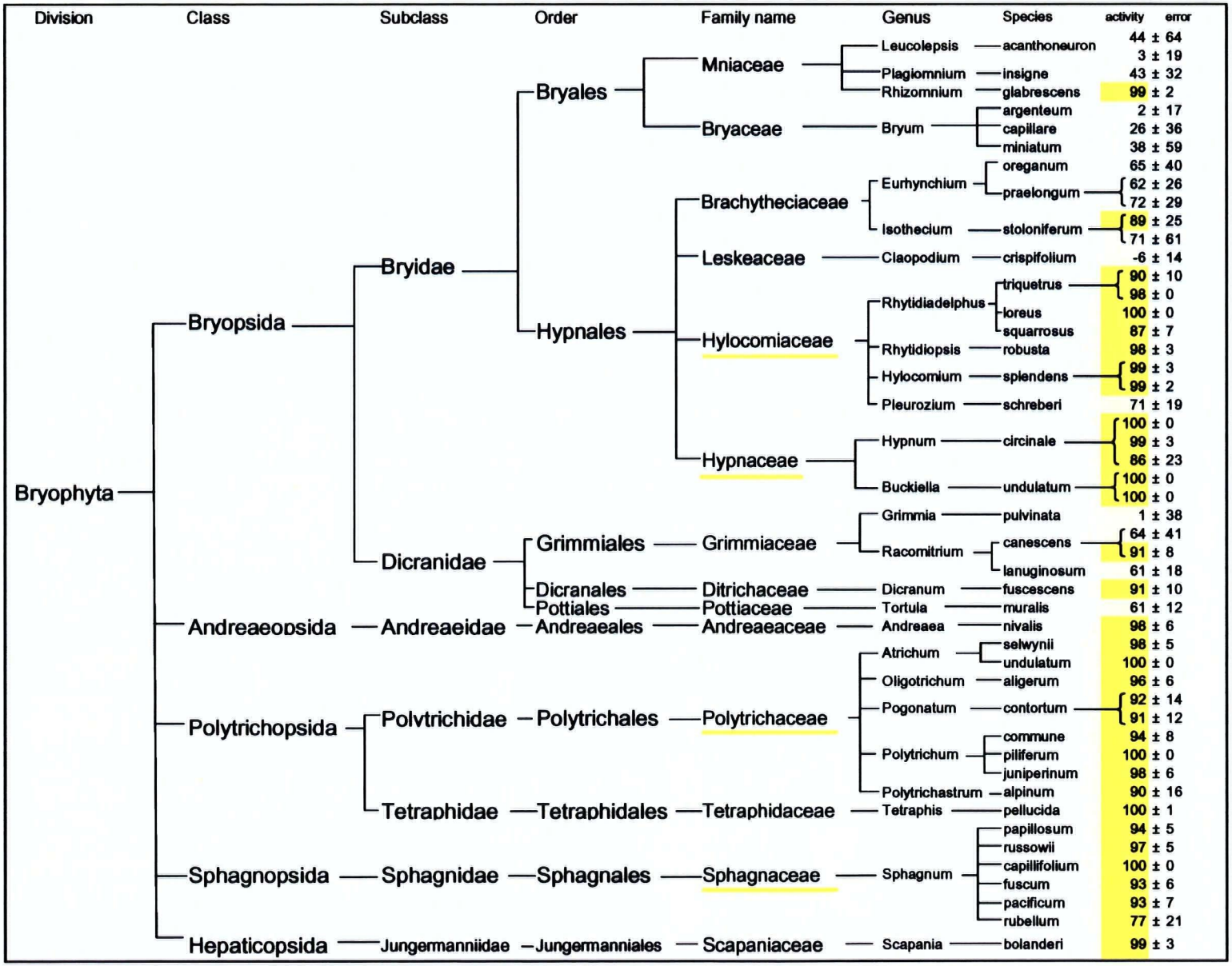


Figure 8: The phylogeny of the bryophyte samples tested.

Through the arrangement of species in terms of their relatedness, it becomes apparent that the antiviral activity is clumped among related species.

3.2 Anti-viral Activity of Biflavonoids.

Four isolated biflavonoids compounds were obtained and tested for antiviral activity using the local lesion assay and *C. quinoa*. These compounds are known to be present in the vascular plants *Rhus succedanea* and *Garcinia multiflora* and have previously been tested against human pathogenic viruses (Lin, Anderson *et al.* 1997). The compounds were only available in very small quantities and thus were first tested at the low concentration of 1.38mg/mL; a concentration at which other pure compounds are commonly tested using the local lesion assay (French and Towers, 1992). Once data was successfully obtained at this low concentration, an attempt was made to concentrate the remaining biflavonoids solutions and test them at 13.8mg/mL. Unfortunately this data could not be reported in this study as several trials did not produced adequate lesions and the limited material did not allow for further testing. However, preliminary results were very unusual; at higher concentrations these compounds appeared to increase the level of PVX infection.

Table 5 reports the results of testing the biflavonoid compounds at 1.38mg/mL. Robustaflavone caused the greatest level of viral inhibition of the biflavonoids tested. Hinokiflavone was also shown to exert a very high level of inhibition. Amentoflavone-7,4',4'trimethyl ether produced minimal levels of inhibition and amentoflavone was not active.

Table 5: The anti-PVX activity of the biflavonoid compounds.
The following four biflavonoids were obtained as pure isolated compounds and tested at 1.38mg/mL using the local lesion assay. Robustaflavone and Hinokiflavone showed high levels of inhibition for this low concentration.

Biflavonoid	Percent inhibition
Robustaflavone	85.1 ± 22.5
Hinokiflavone	78.3 ± 19.3
Amentoflavone	-10.5 ± 47.2
Amentoflavone-7,4',4'trimethyl ether	29.0 ± 46.6

The first three compounds in Table 5 have previously been tested for antiviral activity against animal viruses. In their 1997 study, Lin *et al.* tested 14 biflavonoids for *Rhus succedanea* and *Garcinia multiflora* for their ability to inhibit HIV-1 reverse transcriptase (RT). Of these biflavonoids, robustaflavone, hinokiflavone and amentoflavone were all found to cause a very high level of HIV-1 RT inhibition (97-90%) when tested at the rather high concentration of 200μg/mL. The reported activity of these compounds against HIV-1 RT does not correspond to their activity against PVX in Table 5 as amentoflavone did not demonstrate any inhibition of PVX infection. While amentoflavone-7,4',4'trimethyl ether was not tested in Lin, Anderson *et al.*'s 1997 study, other methoxylated biflavonoids were. Lin *et al.* noticed that by methylating hydroxyl groups on these biflavonoids the compounds lost antiviral activity (Lin, Anderson *et al.* 1997). Based on these observations, we would not have expected amentoflavone-7,4',4'trimethyl ether to have caused the 29.0% inhibition against PVX, which was observed in this study.

Further work by Lin *et al.* tested 10 of the same biflavonoids for antiviral activity against 11 different viruses: influenza A/Texas/36/91, influenza A/Beijing/32/92, Influenza B/Panama/45/90, Measles, Adenovirus Type 5, Parainfluenza Type 3,

Respiratory Syncytial Virus, Herpes Simplex Virus Type 1, Herpes Simplex Virus Type 2, Human Cytomegalovirus, and Varicella Zoster Virus (Lin *et al.* 1999). Again in this study robustaflavone, hinokiflavone and amentoflavone were among the biflavonoids tested. In all cases these three biflavonoids caused viral inhibition against the 11 human pathogenic viruses. The effectiveness of each biflavonoid varied for each virus. In view of this observation, we should not be surprised that all three of these biflavonoids were shown to be active against HIV-1 RT while only two were active against PVX. Of these three biflavonoids, amentoflavone was the least active against the 11 viruses. This corresponds with the results of the biflavonoids tested against PVX.

It should also be noted that in their 1999 study Lin *et al.* demonstrated that in all 11 cases the biflavonoids exhibited greater inhibition against the virus while still possessing lower toxicity to the host cells than the available prescription drugs (ribavirin, 9-(3-hydroxy-2-phophonylmethoxypropyl)adenine, acyclovir and gancyclovir) (Lin *et al.* 1999).

The structures of the four biflavonoids listed in Table 5 are provided in Figure 9 - Figure 12 on page 44. All of these biflavonoids are dimers of the flavonoid apigenin. Robustaflavone and hinokiflavone are very similar compounds; their only difference is the way in which the apigenin units are joined. Robustaflavone has a 3'-6 carbon-carbon linkage and hinokiflavone has a 4'-6 ester linkage. This difference in attachment did not seem to correspond with any significant difference in the anti-PVX properties of these compounds; however these different structures did exhibit different levels of inhibition against several of the human viruses mentioned earlier (Lin *et al.* 1999). Both robustaflavone and amentoflavone are different dimers of apigenin; amentoflavone has a

5'-8 linkage. Because of this difference in attachment, these compounds will have a different three-dimensional shape which is most likely the cause for their different anti-viral activities. This difference in attachment to the first subunit is minor as both the 5' and 3' position are meta positions have similar properties. However, the position of attachment to the second subunit will have an impact on the electron densities throughout the surrounding pi-orbitals of the compounds, as the 6 and the 8 position have different properties. The 5'-8 linkage in amentoflavone will cause the oxygen forming the cyclic ether bond in C-ring of the second apigenin subunit to possess a lower electron density when compared to that of robustaflavone. It has been noted that electron dense units such as hydroxyl groups are a key component of all active biflavonoids (Lin, Anderson *et al.* 1997) and thus it is possible that the decrease in electron density of the second cyclic ether in the amentoflavone molecule may contribute to its lower activity.

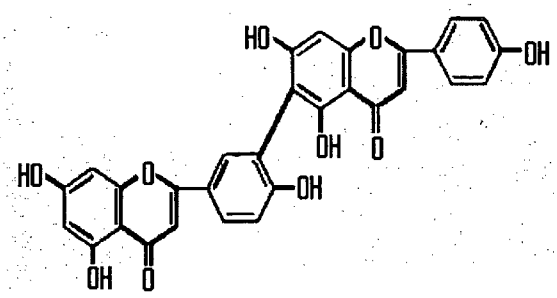


Figure 9: Robustaflavone

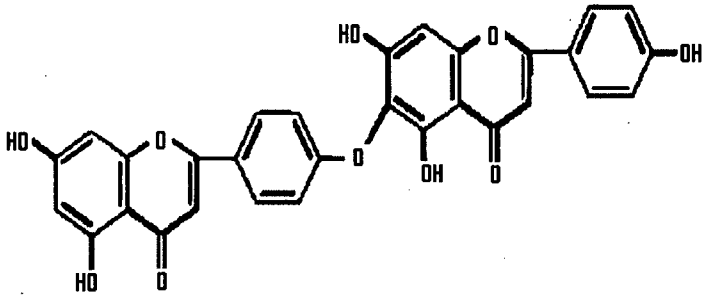


Figure 10: Hinokiflavone

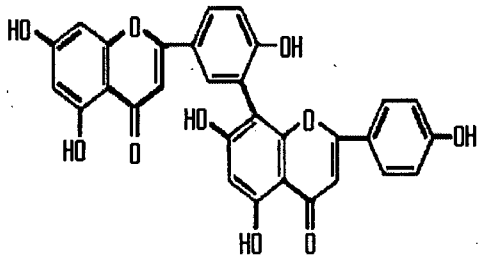


Figure 11: Amentoflavone

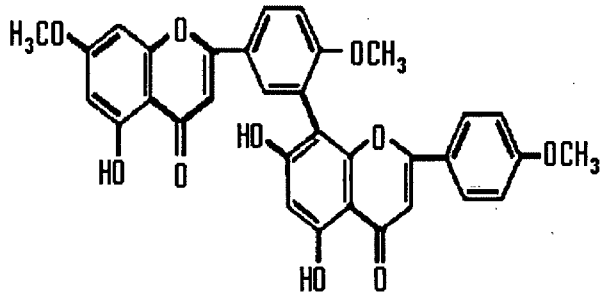


Figure 12: Amentoflavone-7,4',4'trimethyl ether

3.3 The Chemistry of *Hylocomium splendens*

Hylocomium splendens was selected for further chemical investigation. In addition to anti-viral activity, this species was selected because of its abundance and large plant size which made collection of large amounts of material possible. This plant was also chosen because it is a member of the *Bryopsida*, the largest and yet least chemically studied of the bryophyte groups. In addition, this plant has been very poorly studied in terms of its organic chemistry and thus any information gained would be of value. It is surprising that so little work has been done in terms of this plant's organic chemistry, when this plant has been so widely used as an environmental indicator and a great deal of investigation has been done into this plant's inorganic chemistry. Becker *et al.* are responsible for the only published information regarding this species' organic chemistry (Becker *et al.* 1986). In a very brief study of the chemistry of *H. splendens* Becker *et al.* isolated only one compound from the plant. This highly abundant compound which they isolated was identified as a new biflavonoid, 5',3'''-dihydroxyrobustaflavone (Becker *et al.* 1986). In addition to this biflavonoid, Becker *et al.* observed six other flavonoid compounds to be present in the plant; however they were unsuccessful in isolating and identifying these compounds.

The chemical study of *H. splendens* was particularly interesting as there has been very little work done in this area and the plant is known to possess large amounts of one biflavonoid. The main purpose of investigating the chemistry of this plant was to determine if biflavonoids were responsible for the anti-PVX activity; however with such

a limited understanding of the chemistry of these mosses, any chemical information gained would be valuable.

Upon liquid-liquid fractionation of the *H. splendens* extract, fractions were compared using thin layer chromatography. The chromatograms of the hexane and ethyl acetate fractions exhibited a very similar spotting pattern as is shown in Figure 13. In view of the similarities of these two fractions, they were amalgamated into one fraction.

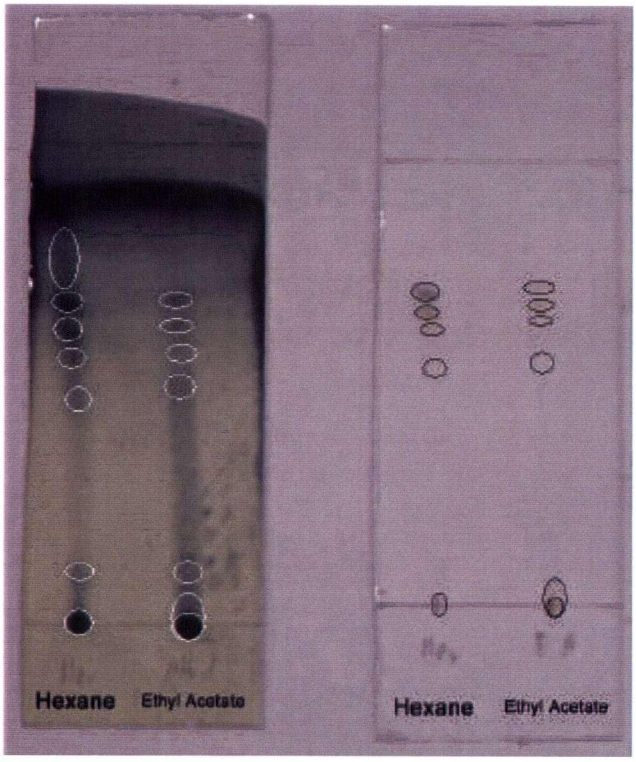


Figure 13: Comparison of the hexane and ethyl acetate fractions of *H. splendens*.
The silica plates were developed in a solution which was three parts hexane and two parts acetone. The plate on the left has been stained with15% phosphomolybdic acid. The plate on the right has not been stained. Spots visible under normal light have been circled. The chromatographs of the hexane fraction and the ethyl acetate fractions both possess a similar spotting pattern.

The three unique fractions produced by the liquid-liquid partition were tested for activity using the local lesion assay. The hexane and ethyl acetate fractions were considerably more active than the butanol or water fractions and thus were selected for further separation using a silica column. The hexane-ethyl acetate fraction was separated

into 115 fractions and these fractions were again compared using thin layer chromatography. Using the chromatograms, the fractions were grouped according to their chemical similarities. Seven fractions were then selected for antiviral testing using the local lesion assay. The groups were selected as they represented the different groups which were likely to contain activity (i.e. extremely non-polar groups were not considered). The results of the antiviral assay for the different fractions are listed in Figure 14 and indicate that most of the activity in the plant is localized between fractions 74-95 of the column. Coincidently, these fractions were also unique in that they were the only fractions to possess a golden colour, which is a common characteristic of flavonoids.

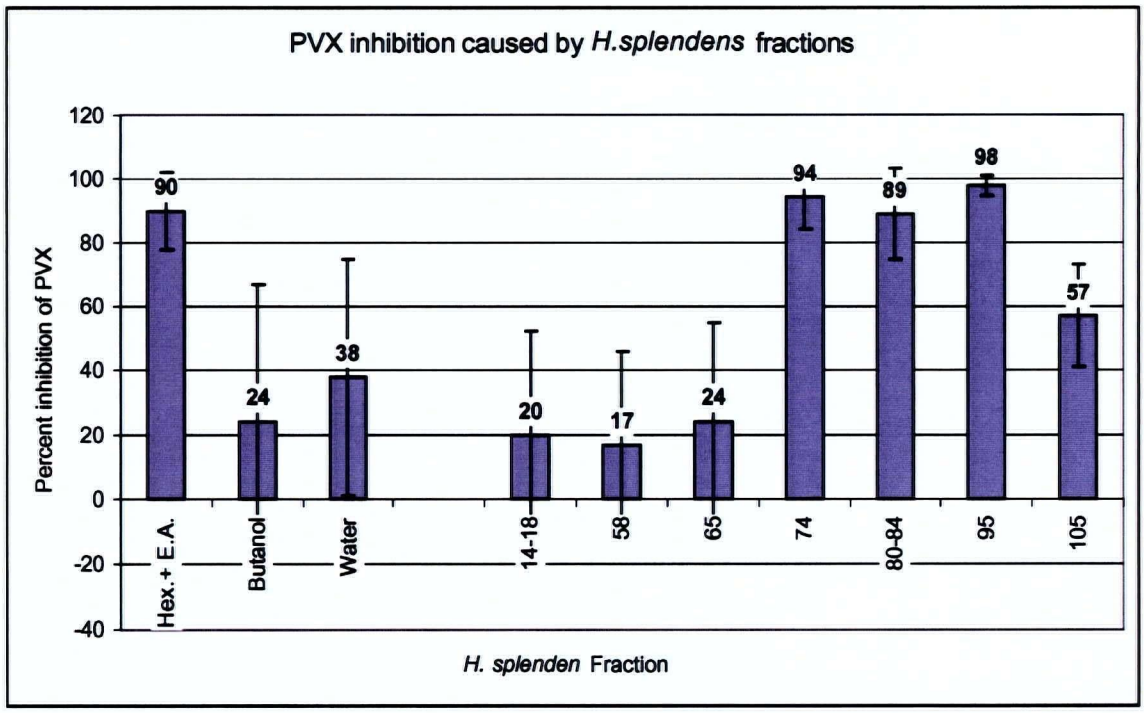


Figure 14: PVX inhibition caused by *H. splendens* fractions.
The combined hexane and ethyl acetate fraction possessed the highest activity of the liquid-liquid partition. Fractions 74-95 showed the highest activity of the silica column fractions which were tested.

The three groups of fractions which were represented by fractions 74, 80-84 and 95 were amalgamated and with the assistance of Dr. Dong Sheng Ming, were further separated using a column of Sephadex™ LH-20. The resulting separation yielded only

one pure compound which was of a quantity large enough to be analyzed. This compound has a distinct golden colour which is characteristic of flavonoids. This sample has subsequently been sent for NMR analysis. Currently, the NMR results have not been received and the identity of this compound is not known.

Unfortunately, time and materials did not allow for further investigation into the chemistry of *H. splendens.* Due to the small amount of compound isolated from the active fractions, this compound could not be tested further for antiviral activity. Based on the abundance of 5',3'''-dihydroxyrobustaflavone reported to be present in *H. splendens* (Becker *et al.* 1986) and based on the colour of the isolated compound, it is quite possible that the isolated compound is indeed 5',3'''-dihydroxyrobustaflavone.

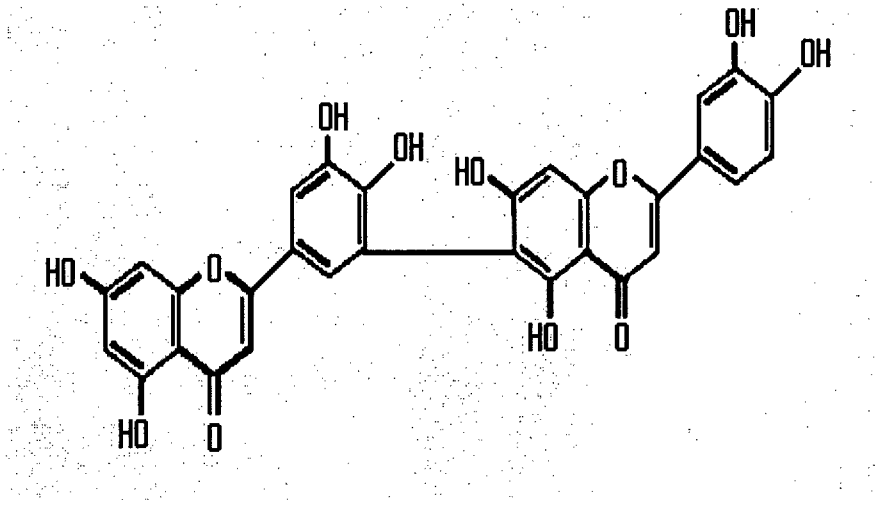


Figure 15: 5',3'''-Dihydroxyrobustaflavone

The structure of 5',3'''-dihydroxyrobustaflavone is displayed in Figure 15. In comparison to the other biflavonoids examined, this compound has two additional hydroxyl units on the 3' positions of each monomeric unit. The free hydroxyl units are thought to be involved in viral particle interaction (Lin, Anderson *et al.* 1997), thus the

added hydroxyl units may increase the compound's ability to interact with viral particles. In all of the biflavonoids tested for antiviral activity by Lin *et al.,* no compound has possessed as many hydroxyl groups as are present on this *H. splendens* biflavonoid. 5',3'''-Dihydroxyrobustaflavone possess a 5'-6 linkage between its monomeric units. This type of linkage is somewhat similar to the 3'-6 linkage of robustaflavone as the second subunits are both attached at the 6^{th} position. As a result, the cyclic ether of the second subunits will have a more negative character than that of compounds such as amentoflavone which are bound at 8^{th} position. Based on this comparison, it seems likely that 5',3'''-dihydroxyrobustaflavone might have a strong antiviral activity similar to that of robustaflavone.

3.4 Anti-viral Compounds from *Iryanthera megistophylla*

As a result of collaborations with Dr. Dong Sheng Ming and Dr. Andrés López, newly isolated compounds from the Columbian vascular plant *Iryanthera megistophylla* became available for anti-PVX testing. *Iryanthera megistophylla* was one of several medicinal Columbian plants which was collected and studied by Dr. Andrés López as part of his doctoral degree. Nine compounds isolated from this plant by Dr. Dong Sheng Ming were tested for PVX inhibition using the local lesion assay. The results of this study are listed in Table 6 and have also been recently published in *The Journal of Natural Products* (Ming *et al.* 2002).

Table 6: Anti-PVX activity of *Iryanthera megistophylla* compounds.
Compounds were tested at 9.1mg/mL. Results have been published in Ming *et al.* 2002. Quercetin and Ribavirin are positive controls.

Compound	Percent PVX Inhibition
1) Megislignan	0 ± 60
2) Megislactone	30 ± 37
3) Grandinolide	30± 43
4) Iryantherin K	88± 18
5) Iryantherin L	67± 39
6) Cinchonain Ib	98± 5
7) Cinchonain Ia	100± 0
8) Procyanidin B-2	98± 7
9) Cinchonain IIa	100± 0
Quercetin	100± 0
Ribavirin	94± 7

The results of the antiviral testing in Table 6 indicate that the following five *Iryanthera megistophylla* compounds are highly antiviral against PVX: iryantherin K, cinchonain Ib, cinchonain Ia, procyanidin B-2 and cinchonain IIa. The latter three compounds displayed an antiviral effect that was greater than the commonly used antiviral compound, ribavirin. The structures of these compounds are displayed along with their activities in Figure 16-Figure 22. As is indicated by the structures, several of the compounds tested are diastereoisomers, or compounds with chiral centers which are not mirror images. Because of their different tree-dimensional shape, one would not necessarily expect these diastereoisomers to have the same level of antiviral activity. The two diastereoisomers iryantherin K & L differed in their degree of PVX inhibition

(88±18% and 67±39% respectively); however, the other diastereoisomers tested, cinchonain Ia & Ib, exhibited nearly identical antiviral activities.

When we compare the structures of the compounds with their antiviral activities in Figure 16-Figure 22, we see that megislignan, megislactone and grandinolide are the compounds with the least number of hydroxyl units and are also the least active of these compounds. As has been mentioned earlier, in the case of the biflavonoids, a correlation has been noted with the amount of hydroxyl units and the antiviral activity (Lin, Anderson *et al.* 1997).

Of the nine *Iryanthera megistophylla* compounds tested, only the six flavonoids compounds exhibited antiviral activity. Procyanidin B-2 and Cinchonain IIa are both biflavonoids and as a result of this study are the first to be published as having anti-plantviral inhibition.

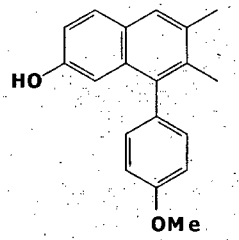


Figure 16: Megislignan
(0% inhibition)

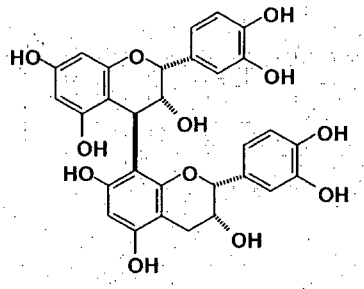


Figure 17: Procyanidin B-2
(98% inhibition)

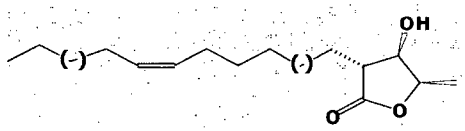


Figure 18: Megislactone
(30% inhibition)

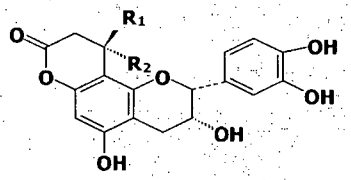


Figure 19: Cinchonain
Cinchonain Ib: R1 = 3,4-dihydroxyphenyl, R2 = H
(98% inhibition)
Cinchonain Ia:R1 = H, R2 = 3,4-dihydroxyphenyl
(100% inhibition)

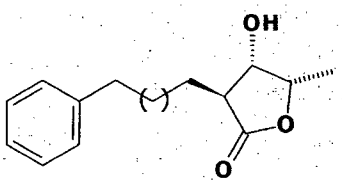


Figure 20: Grandinolide
(30% inhibition)

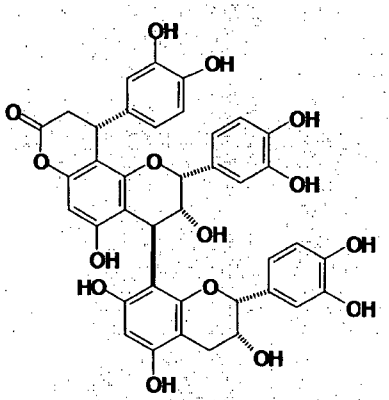


Figure 21: Cinchonain IIa
(100% inhibition)

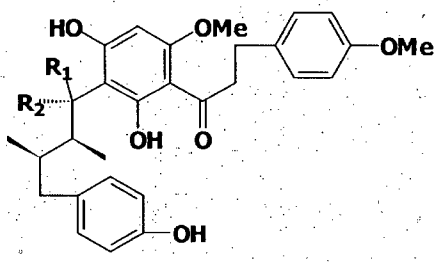


Figure 22: Iryantherin
Iryantherin K: R1 = H, R2 = 4-hydroxyphenyl
(88% inhibition)
Iryantherin L: R1 = 4-hydroxyphenyl, R2 = H
(67% inhibition)

3.5 Summary

A large majority of the bryophyte species screened have been identified as exhibiting considerable antiviral activity against PVX and as a result, the bryophytes have been identified as a new source of antiviral activity. The bryophytes were originally selected for this study because it was thought that the biflavonoid content might confer antiviral activity; however, many of the active bryophyte species are from groups which are known not to contain flavonoids and thus we cannot conclude that biflavonoids are responsible for the antiviral activity of extracts from these organisms. A study of the chemistry of *Hylocomium splendens* was performed in order to gain a greater understanding of the cause of the antiviral activity of the biflavonoid-containing mosses. While activity was observed in a fraction of the compounds which resemble the flavonoids components of the plant, attempts to isolate and identify these compounds were unsuccessful and as it stands, we do not know which compound(s) are responsible for the antiviral properties of these plants.

Four biflavonoids were obtained for antiviral testing. Of these compounds, robustaflavone and hinokiflavone have been identified for the first time as being highly antiviral against PVX at very low concentrations. It is encouraging that these biflavonoids which were previously shown to be very effective at inhibiting several human viruses are now shown to be capable of inhibiting PVX. This implies that other compounds identified as being antiviral with the local lesion assay may also be effective against human viruses. In view of these results, it is also likely that other antiviral biflavonoids will contain activity against PVX.

Nine compounds from the Columbian plant *Iryanthera megistophylla* were for the first time tested for anti-PVX activity. Of these compounds the flavonids: iryanterin K, cinchonain Ib, cinchonain Ia and the biflavonoids: procyanidin B-2 and cinchonain IIa have been shown to be highly active against PVX. Several of these compounds have been shown to be more effective than ribavirin at inhibiting PVX infections. These compounds should be tested for phytotoxic effects, as it is possible that they are less toxic than ribavirin and they may greatly facilitate virus elimination in tissue culture.

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