ANTIVIRAL ACTIVITIES OF SELECTED CHINESE MEDICINAL PLANTS

by

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Abstract

Medicinal plants in Yunnan Province of China were collected and screened for antiviral activity. Plants that were used to treat diseases that are now known to have viral causes were selected through a systematic survey of information on traditional Chinese medicine and the traditional medicines of ethnic minority groups in the region. Extracts from 31 species in 22 plant families were assayed for inhibition of Sindbis and murine cytomegalovirus infections in mammalian cell cultures. Sixteen of the species showed antiviral activity. *Elsholtzia ciliata* (Thunb.) Hyland of the mint family (Lamiaceae) showed the highest activity. It has more than one active component and one of them was purified using bioactivity-guided phytochemical fractionation. The compound was identified as the polycyclic aromatic hydrocarbon fluoranthene and its activity was enhanced with exposure to long wavelength ultraviolet radiation. It has not been previously reported to have antiviral activity.

Investigations of the mechanism of action were carried out with the known photosensitive antiviral compound hypercin found in medicinal plants of the genus *Hypericum* (Hypericaceae). Three hypercin derivatives and five related quinones were tested in structure-activity relationship studies. The new derivative 2,5,9,12-tetra-(carboxyethylthiomethyl) hypercin showed potent photosensitive virucidal activity against membrane-enveloped viruses. The photoaction was demonstrated to be of the singlet oxygen type that could be reduced by the presence of a singlet oxygen scavenger. In comparisons of mechanisms of action with that of hypercin in the presence and absence of light, the two compounds showed similar potencies in light but hypercin was more potent in the dark. Examination of the effect of these compounds on Sindbis virus structural proteins showed that treatment with the derivative in light caused an alteration of the capsid protein, an effect that was not shown in treatments with hypercin.
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General Introduction

The goal of this project was to conduct a comprehensive study of biologically active compounds from selected plant species, encompassing aspects of ethnopharmacology, botany, microbiology, chemistry and pharmacology. The study included: a) the screening of selected medicinal plants for bioactivity, b) the chemical purification of active compounds, and c) the determination of the nature or mechanism of the activities. The introduction is a perspective on the uses and usefulness of ethnopharmacological information, from traditional medicine, in a screening program for bioactive compounds. The general term 'pharmacognosy' describes the search for bioactive compounds when the context of the research has the potential for developing new therapeutic chemicals. This phase of the study can also be considered as part of ethnobotany since it is a screening of plants used by indigenous peoples. Ethnobotany has been defined by Ford (1978) as "the study of direct interrelations between humans and plants". Ethnopharmacology is more specifically defined by Bruhn and Holmstedt (1981) as "the interdisciplinary scientific exploration of biologically active agents employed or observed by man".

The study of biologically active compounds is a basic part of pharmacology. The pharmacology of traditional medicines is based on the uses of natural products used by cultural groups in the treatment of disease and this body of knowledge can be viewed as the historical result of empirical testing by practitioners of folk medicine. It is my view that this research can provide guides in the search for bioactive natural compounds. The significance of ethnopharmacological research in discovering new therapeutic activities of natural products has been extensively discussed (Schultes and Swain, 1976; Bruhn and Holmstedt, 1981; Delaveau,
According to Farnsworth et al. (1985) 74% of the 119 bioactive plant-derived compounds currently in world-wide use were identified via research based on leads from folk or ethnomedicine. Although historical evidence indicates that traditional medicine has provided good leads to therapeutic chemicals, the question still arises as to whether an ethnopharmacologically guided screening is more effective than random sampling in yielding active compounds. Several comparative studies (Spjut and Perdue, 1976; Verpoorte, 1989; Balick, 1990) support the validity of the approach of using ethnopharmacological information as opposed to random screening.

The information traditional medicine can provide depends on the objective of the screening program and the treatment of the original data. It is important at this point to consider what the ethnopharmacologist should be aware of in the process of eliciting guides to bioactivity screening from traditional medicine information. I use the term 'traditional medicine' to mean a body of historically and culturally transmitted knowledge of medicine based on theories of health and disease. Traditional medicines have culturally based views and principles regarding diagnosis and cure of diseases that may not be directly matched with western scientific research concepts (Elisabethsky, 1986). The uses of plants for healing can often be based on perspectives very different from what the screening program is based on. Therefore, there is an important element of interpretation involved in doing ethnopharmacological research. First of all, the information has frequently to be translated from another language. The interpretation of the meaning of terminology may require an understanding of the framework that they come from, and the treatment of information could call for some careful bridging and correlating of different perspectives.

In studying the activity of chemicals from traditional medicinal plants, there are different extents of correspondence between the effects found or assayed for, and what the
plant was used for. It is a commonly encountered thought that this type of study is testing the efficacy of traditional medicines. There are a number of cases where this is done to some degree because the activity of the isolated plant compound shows a direct reflection of how the plant was used. Morphine, an alkaloid from the opium poppy plant (*Papaver somniferum* L.) that was used to induce euphoria, has been found to be an analgesic that exerts its effect by binding to specific neural receptors (Evans et al. 1988). Similarly, tubocurarine, which was derived from plants of the Loganiaceae used as arrow poisons, has been shown to be effective as such by being a potent muscle relaxant (Bryn Thomas, 1963). Areas where the treatments practiced by both modern and traditional methods are more likely to be aimed at the same targets would probably show higher correlations between ethnopharmacological indications and activity screening results. For example, whether plants used to treat topical infections have antibiotic activity can be ascertained by *in vitro* antibacterial bioassays. Other important drugs from plants, however, have activities that are not directly indicated by their usage in traditional medicine.

The anticancer agents taxol from the Pacific yew tree (*Taxus brevifolia* Nutt.), and vinblastine and vincristin from the Madagascar periwinkle *Catharanthus roseus* (L.) G. Don., were all discovered in laboratory screening programs (Farnsworth, 1984). Even though these plants have been used in traditional medicines, they were not used to treat cancers. There may not be many direct ethnopharmacological indications for cancer treatment due to the complexity and variety of cancers which are being elucidated by western medicine. The number of distinct cancers is estimated at over 100 and up to 300, with no apparent common denominator to the different types (Suffness and Pezzuto, 1991). What can now be diagnosed as cancer may have been more likely diagnosed in folk medicine as an ailment in a particular body organ rather than specifically as a cancer. The discoveries of these drugs demonstrate that medicinal plants present good sources for systematic western scientific screening, even though the target of the screening may not be well indicated due to conceptual differences between the traditional
diagnosis of illnesses and the aims of modern screenings. The chances of finding bioactive compounds are probably higher when there is good correlation between the screening target and traditional usage. However, chances of finding bioactive compounds from a general screening of plants that have histories of being used medicinally are also probably higher than a random screening of plants per se.

Western medical research has also elucidated the microbial agents responsible for many diseases. The importance or relevance of finding new antiviral compounds comes from the fact that although over 400 viruses have been identified in the pathogenesis of a wide range of human diseases (Choppin, 1986), very few antiviral compounds have made their way to clinical use (Prusoff, 1988). The factors that contribute to the slowness in development of antiviral drugs were discussed by Galasso (1988). One of the major challenges in finding effective antiviral compounds is due to the intimate biochemical association between the virus and its host cell, such that it is difficult to damage the virus without damaging its host (Walker, 1988). This close relationship between the virus and cell, however, also makes antiviral compounds potentially useful tools in research on the nature of cells and viruses. Many antiviral compounds have been isolated from medicinal plants (Vanden Berghe et al. 1978; Hudson, 1990), but the applicability of some of them as potential drugs is still at preliminary stages of development and the phytochemical search for antiviral compounds will continue to be a growing area of research (Vlietinck and Vanden Berghe, 1991).

I have presented here the reasoning and considerations for using an ethnopharmacological approach to search for antiviral compounds in medicinal plants. The methodology of research on medicinal plants is usually divided into a series of stages involving the synthesis of work from the disciplines of pharmacognosy, botany, chemistry and pharmacology (Cavé, 1986). It was the goal of this research to conduct a project that encompasses such a synthesis by incorporating the following stages:
1. Systematic search of traditional medicine information to select plants for antiviral screening.

2. Collection and taxonomic identification of plants.


4. Purification of active component(s).

5. Investigation of nature of activity.

Researchers of bioactive plant compounds have frequently expressed the need for good integration between the different disciplines involved, since aspects of the work are often done by different specialists. The best way to understand the interfaces between the different disciplines necessary for strong integrated research is to do a comprehensive investigation that includes all the aspects. It is hoped that good understanding of the research involved in exploring the bioactive natural compounds will contribute to the beneficial management of a rich resource. This is particularly pertinent in view of the fact that we are faced with the loss of both biotic and cultural resources in an era of tremendous social and environmental change.
References


Chapter I.

Collection of Plants for Antiviral Screening
Using Ethnopharmacological Information

Introduction

Medicinal plants in Yunnan Province, People's Republic of China, were selected for antiviral screening using information from traditional medicines. China has one of the oldest continuous and documented systems of traditional medicine. In folklore, the practice of using herbs in healing is attributed to the efforts of the semi-mythological emperor Shen Nong approximately five thousand years ago. There is a written herbal called the Shen Nong Ben Cao Jing that is dated to about 200 A.D. (Huang, L., 1984). The most comprehensive historic compendium of the Chinese medicinal herbs was compiled by Li Shi-zhen in the Ming dynasty in 1578 A.D. This materia medica, the Ben Cao Gang Mu contained 1,892 entries (Li, 1979). An appendix was made later by Zhao Xue-min with 716 more items. A computer database at the Chinese University of Hong Kong lists 4,941 species of higher plants from the traditional Chinese pharmacopoeia (Duke and Ayensu, 1985). The recorded use of these plants presents a large source of organized ethnopharmacological information. In addition, there are over 20 ethnic minority groups in Yunnan. These minority groups include ones like the Dai and the Karen that are of Southeast Asian affinity, and ones with Tibetan links. These groups have established practices of traditional medicine and many of them have historic documents in their own languages. The Dai have their own Institute of Traditional Medicine in Jinghong, where the medicine is practiced as well as researched. Near the town of Dali, there is an annual gathering where people of many different groups travel from long distances to trade, and
medicinal materials are a significant commodity (Towers, personal communication). Ethnobotanical information from the minority groups is in the process of being recorded and it is also translated to Chinese (Yunnan Institute of Medicine Inspection, 1983). The medicinal plants used by these people adds to the pool of candidates for screening. Six hundred and seven plant species are listed in the Chinese Medicinal Plants of Yunnan (Kunming Reserve Unit Health Division, 1970) and its sequel volume (Kunming Institute of Botany, 1978).

Yunnan Province in the southwest of China (lat. 21'10-29'00 N, long. 97'30-106'10 E), is an area known for its botanical diversity (Pei, 1988). The diversity is probably a result of the variety of geo-climatic conditions found in the province. A map showing the location of the province is provided in Figure 1.1. The southern-most region is tropical and includes the Xishuangbanna Prefecture which is reputed to have many unusual and endemic organisms because of the geography of being surrounded by a ring of mountains. Zou (1988) described the composition and features of the Xishuangbanna flora. Over 3800 species of higher plants have been identified in Xishuangbanna, and this number was estimated to represent one sixth of the Chinese flora (Yunnan Institute of Tropical Botany, 1984). The western part of the province has increasingly temperate to alpine conditions due to both gradual and dramatic rises in elevation (Zhang, 1983). There is a plantation where medicinal plants are cultivated in the Cang mountains outside Dali. The diverse flora of this region has also drawn attention from a number of botanical expeditions (Lancaster, 1981; Grey-Wilson, 1988). The rich resources in both plants and ethnobotanical knowledge made Yunnan an ideal area for conducting a screening program.
Figure 1.1: Map of Yunnan Province
The literature of the herbal pharmacopoeia is usually organized by plant names. There are also collections of herbal mixture recipes for the treatment of particular symptoms and illnesses. The Chinese names of the herbs are common names. For this survey, only the literature which includes the corresponding Latin scientific names was used. It was nonetheless necessary to be aware that it is common for a Chinese name to have been applied to more than one species. In many cases, these are very similar species of the same genus that could likely be disputable taxonomic entities. Distinct species or even species in different genera are sometimes given the same name. In some instances because they are found in different regions, they are distinguished with prefixes denoting geographic origins. In many cases the common base name parallels a generic name, and further differences are again denoted by descriptive prefixes. In the uses of the plants medicinally, sometimes only the base name is specified because the different species or varieties are considered to be of the same nature and therefore similarly applicable. It must be remembered that the traditional practice of herbal medicine in China was not developed using the Linnaean system of taxonomic classification. Yet, I find that there is quite an impressive correlation between the traditional Chinese and Linnaean classifications. Credits are due to both the acute observations that form the basis of traditional knowledge, and the work that has gone into correlating that knowledge with the scientific classification system.

The documentation of a Chinese medicinal plant usually includes the following information:

1) name and other common names,
2) morphological characteristics,
3) habitat,
4) part used and collection procedure,
5) nature of medicine,
6) uses in treatment,
7) dosage, methods of preparation and mixture recipes.
The uses in treatment often encompass quite a large array of different symptoms and sicknesses. As an example, the pan-tropical weed *Bidens pilosa* L. (Asteraceae) is cited for use in the treatment of: cold, prevention of influenza, bites by poisonous snakes and insects, hepatitis, bladder infection, intestinalitis, dysentery and rheumatoid arthritis in *Yunnan Chinese Medicinal Plants* (Yunnan Reserve Health Unit, 1970). In the view of traditional Chinese medicine, this apparently diverse range of maladies has certain common characteristics that are affected by the nature of the medicinal herb. The interpretation of what these maladies have in common is based on a theoretical framework of health. The components of the body, medicines and illnesses are all viewed from the perspective of a balance of yin and yang natures. The yin natures are passive, cold and wet and the yang natures are active, hot and dry. The yang nature is associated with the skin, bones and musculature, and the yin nature with the internal organs. The two natures are always coexistent in varying proportions. The heart, for example, is considered yang dominant but has the yin component of an internal organ. Medicines are used to restore the balance of natures disrupted by disease. A simple example of working with the principle of natures is when a medicine of a cold nature is used to counter the hot nature of a fever, or an internal inflammation. Medicines are further designated with specific pharmacological properties (Huang, S., 1984). Many of these properties can be more directly correlated with western pharmacological concepts. Some of these are: the ability to increase circulation (stimulatory), to induce diuretic effects, to cure infections (antibiotic, anti-inflammatory), to calm (sedative), and to dispel parasitic worms (anthelmintic). Other applications are based on more holistic concepts such as detoxification, tonics and general invigoration.

The traditional practice uses a combination of herbs to heal the affected organs as well as achieve an amelioration of the balance of natures. The mixture of plants in a medicinal recipe thus consists of principal acting and supplementary acting ingredients. This leads to the situation that in the compilation of information on its usage, a plant often shows a number of
different applications because it may have been used as the primary ingredient or for supplementary effects in different prescriptions. When addressing this information for leads to bioactivity, what can be used as an indication of what is likely the principal target of a plant's action is to look for citations by more than one source for a particular indication. A plant that is cited for a more specific versus a wide range of uses, is also more likely to be targeted more specifically in its application.

To conduct an antiviral screening of the medicinal plants, I had to establish criteria for the selection of plants for testing from this information base. The interpretation of the information can be complex because it is derived from an elaborate conceptual system of health and medicine. For the purposes of a general search for antiviral activity, the information regarded can nonetheless be obtained with a simpler approach. Some of the diseases that are now linked to viral causes are frequently cited as being treated with medicinal herbs. These diseases are: the cold (rhinovirus), influenza, the flu (influenza virus), and hepatitis or symptoms of liver disorder (hepatitis viruses). The causes of warts are also viral. The approach was thus to systematically survey the literature on the Yunnan pharmacopoiea for plants that have been used in the treatment of these diseases, and to collect the plants for a general screening of antiviral activity.

**Materials and Methods**

Selection of plants to screen for antiviral activity

An initial list was compiled of Yunnan plants that have been used for the treatments of viral diseases from the two volumes of the *Chinese Medicinal Plants of Yunnan*. The plants
on this list were surveyed for citation in other literature on the traditional Chinese
pharmacopoeia. The literature sources surveyed were:

A) in Chinese:

   Kunming Reserve Unit Health Division, 777 pp.

   *Chinese Medicinal Plants of Yunnan*, Sequel. (1978)

   Tiangjing People's Publishing, Tiangjing.

   Yunnan Health Bureau Revolutionary Committee, 213 pp.
   Yunnan People's Publications, Kunming.

   Nanjing Institute of Traditional Medicine, 742 pp.
   Jiangsu People's Publications, Nanjing.

   New Jiangsu Hospital, 2754 pp. + 764 pp. appendix.
   Shanghai Science and Technology Publications, Shanghai.
   (the dictionary information is compiled from other sources
   and includes information from minority tribes)

B) in English:

   Duke, J.A. and Ayensu, E.S., 705 pp.,
   Reference Publications, Algonac


   compiled by Li Shi-Chen, 482 pp.,
   translated from Chinese by F. Porter-Smith and G.A. Strand.
   Georgetown Press, San Francisco.

   Hsu, H.Y., Chen, Y.P., Shen, S.J., Hsu, C.S., Chen, C.C.,
   and Chang, H.C., 932 pp.,
   Oriental Healing Arts Institute, Long Beach.

   Chang, H.M. and But, P.H. (eds.), Vol. 1 and 2,
   CMMRC, Chinese University of Hong Kong,
The list of potential plants for testing was appended with the survey of a number of publications on the traditional medicine of Yunnan ethnic minority tribes, in Yunnan. These are usually small booklets, written in Chinese but some have notations in the minority tribe language. There are no specific authors of these booklets.

   Simao District Traditional Medicine Research Station.

   Xishuangbanna Traditional Medicine Research Office.

   De Hong Prefecture Health Unit Drug Inspection Station.

   Yuxi District Drug Inspection Station.

e. *Herbal Medicines of Lijiang.* (1971)
   Lijiang District Revolutionary Committee Production Division Health Unit.

   Yunnan Institute of Medicine Inspection.

   Vol. 1, 2, 3 and appendix to medicinal recipes,
   Xishuangbanna Prefecture Science and Technical Committee and
   Xishuangbanna Prefecture Health Unit.

   Yunnan Minority Publications.

i. *Naxi Tribe Medicines* (1979)
   Lijiang District Drug Inspection Station.

j. *Yi Tribe Medicines* (1979)
   Lijiang District Drug Inspection Station.

Plants were prioritized in qualification for screening on the basis of specificity of application and frequency of citation for similar uses in different sources of information. A plant was considered to be a good candidate for collection if it did not have a large range of other uses besides those previously mentioned. A plant was also of more interest if it was cited by more than one source for uses in the screening criteria. During the process of plant
collection, this prioritization was used as a guideline as to which plants to look for. Which plants were actually collected was also largely determined by availability.

Plant collection

Plant collection was made in Yunnan Province with the collaboration of the Kunming Institute of Botany (KIB) of the Chinese Academy of Sciences. Plants were collected from three regions of Yunnan during the months of July and August, 1988: Kunming area in central Yunnan; Dali-Lijiang area in northwestern Yunnan; and Xishuangbanna in the south of the province. In each region, local botanists assisted with the location and identification of plants (Wang Zongyu of the KIB in Kunming, Xu Hua and Shi Zhaolong of the Dali Drug Inspection Station, Cui Jingyun of the Yunnan Institute of Tropical Botany in Xishuangbanna). Some plants were collected with the help of herbal doctors that currently use them (Dr. Fan Bingjun in Dali, Drs. He Zhegao and He Shixiu of the Naxi minority in Lijiang). Five samples were from plants already collected in preparation for use by Dr. He Shixiu. Two samples were purchased from the pharmacy of the Dai Traditional Medicine Institute in Jinghong, Xishuangbanna.

Voucher herbarium specimens of the plant species collected have been deposited at the Herbarium of the University of British Columbia (UBC) in Vancouver. Identifications of the voucher specimens were verified by Li Yenhui at the Ethnobotany Laboratory of the Kunming Institute of Botany in Kunming, Yunnan.

Crude extract preparation

Extracts were made in Kunming from air-dried samples of the whole plant or, in a few cases, from the portion specified as being used medicinally. Ten-gram samples of plant
material were percolated exhaustively in 90% ethanol and dried under reduced pressure at below 40° C. The extracts were dried to residues for ease of transportation back to UBC in Vancouver for testing. Official permission was obtained for the export of these samples from the People's Republic of China.

Results

The list of potential plants for antiviral screening is shown as Table 1.1. The viral illnesses for which the plants are used are also listed. The sources for the treatment citations are shown by numbers and letters referring to the publications listed in the previous section. The publications on medicines of minority peoples are designated with letters and on traditional Chinese medicine designated with numbers. In some cases, the pharmacological indications cited for a plant apply to two closely related species and they are listed together. There were 82 species selected, from 69 genera and 43 plant families.

The phylogenetic distribution of the plant families according to order and class is shown in Table 1.2.
Table 1.1

Medicinal Plants Selected as Candidates for Antiviral Screening and Their Ethnopharmacological Indications for Treatment

<table>
<thead>
<tr>
<th>Plant Family</th>
<th>Species</th>
<th>Part Used</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apiaceae</td>
<td>Centella asiatica (L.) Urban</td>
<td>** PL</td>
<td>cold 1, infectious hepatitis 1, jaundice 4, hepatitis f, (5).</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td>Apocynaceae</td>
<td>Plumeria rubra var. acutifolia (Poir.) Ball.</td>
<td>** RT</td>
<td>hepatitis a, b, g, (4,f,5-herpes).</td>
</tr>
<tr>
<td>Araceae</td>
<td>Alocasia macrorrhiza (L.) Schott</td>
<td>RT</td>
<td>cold 1.</td>
</tr>
<tr>
<td>Araliaceae</td>
<td>Livistona chinensis R. BR.</td>
<td>* LF</td>
<td>hepatitis b, (4).</td>
</tr>
<tr>
<td>Araliaceae</td>
<td>Hedera nepalensis K. Koch</td>
<td>PL</td>
<td>hepatitis 1.</td>
</tr>
<tr>
<td>Asteraceae</td>
<td>Bidens pilosa L.</td>
<td>** PL</td>
<td>cold 3, f, prevent flu 1, 4, 8, jaundice 1, (5).</td>
</tr>
<tr>
<td>Asteraceae</td>
<td>Blumea balsamifera (L.) DC.</td>
<td>PL</td>
<td>cold 1, flu 1, (4).</td>
</tr>
<tr>
<td></td>
<td>Dichrocephala benthamii C.B. Clarke or D. chrysanthemifolia (Bl.) DC.</td>
<td>** PL</td>
<td>hepatitis 1, 2, f, cold 1, 4, (i). used by Dr. Fan Bingjun for cold</td>
</tr>
<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used #</td>
<td></td>
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<td>-------------</td>
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</tr>
<tr>
<td>Asteraceae</td>
<td><em>Laggera pterodonta</em> (DC.) Benth.</td>
<td>*PL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold 1, 3, 4, prevent flu 1, (5). + used by Dr. Fan Bingjun for cold</td>
<td></td>
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<tr>
<td></td>
<td><em>Spilanthes paniculata</em> Wall.</td>
<td>PL</td>
<td></td>
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<tr>
<td></td>
<td>cold 1, 4.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berberidaceae</td>
<td><em>Mahonia nepalensis</em> DC.</td>
<td>**BR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hepatitis b, used in 4 of 7 mixtures for hepatitis treatment. (purchased from the Dai Traditional Medicine Institute at Jinghong, Xishuangbanna)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brassicaceae (Cruciferae)</td>
<td><em>Rorippa montana</em> (Wall.) Small</td>
<td>PL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>flu 1, 4, jaundice hepatitis 1, warts 1.</td>
<td></td>
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<tr>
<td>Caesalpiniaceae</td>
<td><em>Cassia tora</em> L.</td>
<td>*SD</td>
<td></td>
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<tr>
<td></td>
<td>hepatitis 4, b, supports liver 6, 8, (5,7).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combretaceae</td>
<td><em>Quisqualis indica</em> L.</td>
<td>**PL,FR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hepatitis b, (4,6,7,9)</td>
<td></td>
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<tr>
<td>Convolvulaceae</td>
<td><em>Dichondra repens</em> Forst.</td>
<td>**PL</td>
<td></td>
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<tr>
<td></td>
<td>flu f, jaundice hepatitis 1, jaundice 4, 8, liver diseases 9, (5).</td>
<td></td>
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<tr>
<td>Cornaceae</td>
<td><em>Dendrobenhamii capitata</em> (Wall.) Hutch.</td>
<td>LF,FL,FR</td>
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<tr>
<td></td>
<td>hepatitis 1, 4.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td><em>Helwingia himalaica</em> HK. f. et Thomas</td>
<td>LF,FL</td>
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<td></td>
<td>flu 1, (4).</td>
<td></td>
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<tr>
<td>Cycadaceae</td>
<td><em>Cycas siamensis</em> Miq.</td>
<td>**LF</td>
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<tr>
<td></td>
<td>hepatitis A 1, jaundice hepatitis 1.</td>
<td></td>
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</tr>
<tr>
<td>Dillenaceae</td>
<td><em>Dillenia indica</em> L.</td>
<td>*BK</td>
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<tr>
<td></td>
<td>hepatitis b.</td>
<td></td>
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<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used #</td>
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<tr>
<td>Dipsacaceae</td>
<td><em>Dipsacus asper</em> Wall.</td>
<td>RT</td>
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<tr>
<td></td>
<td></td>
<td>supports liver 3, 4, 8, g, (1).</td>
<td></td>
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<tr>
<td>Ebenaceae</td>
<td><em>Diospyros kaki</em> L.f.</td>
<td>** BK</td>
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<tr>
<td></td>
<td></td>
<td>jaundice hepatitis 8, f, (1,4,7).</td>
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<tr>
<td></td>
<td></td>
<td>(purchased at the Dai Traditional Medicine Institute at Jinghong, Xishuangbanna)</td>
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<tr>
<td>Euphorbiaceae</td>
<td><em>Breynia rostata</em> Merr.</td>
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<tr>
<td></td>
<td></td>
<td>acute hepatitis 1, hepatitis 1, cold 1.</td>
<td></td>
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<tr>
<td></td>
<td><em>Euphorbia prolifer</em> Ehrenb. ex. Boiss.</td>
<td>** PL</td>
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<td></td>
<td></td>
<td>warts 1, f.</td>
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<tr>
<td></td>
<td></td>
<td><em>Euphorbia</em> spp. were frequently cited for the treatment of warts 1, 4, f.</td>
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<tr>
<td></td>
<td><em>Homonia riparia</em> Lour.</td>
<td>* PL</td>
<td></td>
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<td></td>
<td></td>
<td>infectious and chronic hepatitis 1, 4. +</td>
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<tr>
<td></td>
<td><em>Ricinus communis</em> L.</td>
<td>** LF,RT</td>
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<td></td>
<td></td>
<td>jaundice hepatitis f, (1,4,7,8).</td>
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<tr>
<td>Fabaceae (Leguminosae)</td>
<td><em>Desmodium triquetrum</em> (L.) DC.</td>
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<td></td>
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<td>cold 4, 8, b, (2,5).</td>
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<td></td>
<td><em>Kummerowia striata</em> (Thunb.) Schindl.</td>
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<td>cold 1, infectious hepatitis 1,4.</td>
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<td></td>
<td></td>
<td>used by Dr. Fan Bingjun for hepatitis</td>
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<tr>
<td>Gentianaceae</td>
<td><em>Halenia elliptica</em> D. Don</td>
<td>** PL</td>
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<td></td>
<td></td>
<td>hepatitis 1, 2, f, h, (4).</td>
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<tr>
<td></td>
<td><em>Swertia punicea</em> Hemsl. or <em>S. yunnanensis</em> Burkill (common name = hepatitis herb)</td>
<td>** PL</td>
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<tr>
<td></td>
<td></td>
<td>infectious and jaundice hepatitis 1,2, hepatitis 4, acute hepatitis 9. +</td>
<td></td>
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<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used</td>
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<tr>
<td>Hypericaceae</td>
<td><em>Hypericum japonicum</em> Thunb.</td>
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<tr>
<td></td>
<td>infectious and chronic hepatitis $^1$,</td>
<td></td>
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<tr>
<td></td>
<td>hepatitis $^3$, $^4$, $^6$, $^i$, (a).</td>
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<td>Hypericaceae</td>
<td><em>Hypericum patulum</em> Thunb.</td>
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<tr>
<td></td>
<td>infectious and chronic hepatitis $^1$,</td>
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<tr>
<td></td>
<td>hepatitis $^4$, $^i$.</td>
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<tr>
<td>Iridaceae</td>
<td><em>Belamcanda chinensis</em> DC.</td>
<td>** BU</td>
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<tr>
<td></td>
<td>flu $^g$, hepatitis $^f$, $^g$, (4,7)</td>
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<tr>
<td></td>
<td>used by Dr. He Shixiu for flu,</td>
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<tr>
<td></td>
<td>sample collected from Dr. He.</td>
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<tr>
<td>Iridaceae</td>
<td><em>Coelogyne corymbosa</em> Lindl.</td>
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<tr>
<td></td>
<td>flu $^1$, $^4$.</td>
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<tr>
<td>Lamiaceae (Labiatae)</td>
<td><em>Acrocephalus indicus</em> Briq.</td>
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<td></td>
<td>cold $^d$.</td>
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<tr>
<td>Lamiaceae (Labiatae)</td>
<td><em>Elsholtzia blanda</em> (Benth.) Benth.</td>
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<tr>
<td></td>
<td>flu $^1$, $^4$, hepatitis $^4$.</td>
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<tr>
<td>Lamiaceae (Labiatae)</td>
<td><em>Elsholtzia bodneri</em> Vant.</td>
<td>PL</td>
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<tr>
<td></td>
<td>hepatitis $^1$.</td>
<td></td>
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<tr>
<td>Lamiaceae (Labiatae)</td>
<td><em>Elsholtzia ciliata</em> (Thunb.) Hylander</td>
<td>** PL</td>
<td></td>
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<tr>
<td></td>
<td>or <em>E. rugulosa</em> Hemsl.</td>
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<tr>
<td></td>
<td>cold $^1$, $^4$, $^f$, flu $^1$, $^4$.</td>
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<tr>
<td></td>
<td>used by Dr. He Shixiu for flu</td>
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<tr>
<td>Lamiaceae (Labiatae)</td>
<td><em>Elsholtzia densa</em> Briq.</td>
<td>** PL</td>
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<tr>
<td></td>
<td>flu $^4$.</td>
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<tr>
<td></td>
<td>used by Dr. He Shixiu for flu,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sample collected from Dr. He.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamiaceae (Labiatae)</td>
<td><em>Elsholtzia penduliflora</em> W.W. Sim</td>
<td>LF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>or <em>E. flava</em> (Benth.) Benth.</td>
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<tr>
<td></td>
<td>jaundice hepatitis $^1$, flu $^1$, $^4$,</td>
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<tr>
<td></td>
<td>cold $^4$, $^h$.</td>
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<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used</td>
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<tr>
<td>Lamiaceae</td>
<td><em>Perilla frutescens</em> (L.) Britton</td>
<td>**</td>
<td>PL</td>
</tr>
<tr>
<td></td>
<td>cold 6, 8, f, flu 3, (4). used for cold by Dr. He Shixiu, sample collected from Dr. He.</td>
<td></td>
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<tr>
<td></td>
<td><em>Rabdosia phyllostachys</em> (Diels) Hara</td>
<td>**</td>
<td>PL</td>
</tr>
<tr>
<td></td>
<td>cold f, other <em>Rabdosia</em> spp. used for cold 4, f. used by Dr. He Shixiu for cold, sample collected from Dr. He.</td>
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<tr>
<td></td>
<td><em>Scutellaria orthocalyx</em> Hand.-Mazz.</td>
<td>**</td>
<td>PL</td>
</tr>
<tr>
<td></td>
<td>hepatitis 1, (4,f), other <em>Scutellaria</em> spp. used for cold.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td><em>Stachys kouyangensis</em> (Vaniot) Dunn</td>
<td>**</td>
<td>PL</td>
</tr>
<tr>
<td></td>
<td>acute and chronic hepatitis 1, (4). used by Dr. He Zhegao for hepatitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liliaceae</td>
<td><em>Polygonatum kingianum</em> Coll. et Hemsl.</td>
<td>PL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>chronic hepatitis 1, (4).</td>
<td></td>
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<tr>
<td></td>
<td><em>Tupistra chinensis</em> Baker</td>
<td>RH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cold 1, flu 1, (4). +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loganiaceae</td>
<td><em>Buddleja officinalis</em> Maxim</td>
<td>*</td>
<td>PL,FL</td>
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<tr>
<td></td>
<td>hepatitis a, c, supports liver 4, 7, 8.</td>
<td></td>
<td></td>
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<tr>
<td>Malvaceae</td>
<td><em>Sida szechuanensis</em> Matuda</td>
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<td>PL</td>
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<td></td>
<td>hepatitis 4, b, (1)</td>
<td></td>
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<tr>
<td></td>
<td><em>Urena lobata</em> L.</td>
<td>*</td>
<td>LF,RT</td>
</tr>
<tr>
<td></td>
<td>flu 1,5.</td>
<td></td>
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<tr>
<td>Menispermaceae</td>
<td><em>Sinomenium acutium</em> (Thunb.) Rehd. et Wils.</td>
<td>LF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cold 1, bronchial infections 1, (4).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used</td>
<td>Notes</td>
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<td>----------------------------------------------------------------------</td>
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<tr>
<td>Myrsinaceae</td>
<td><em>Ardisia mammilata</em>  Hance</td>
<td>PL</td>
<td>hepatitis 1, 4, a.</td>
</tr>
<tr>
<td></td>
<td><em>Embelia sessiliflora</em> Kurz or E. ribes  Burm. f.</td>
<td>** RT</td>
<td>hepatitis 1, 2, b, f, (4)</td>
</tr>
<tr>
<td>Oleaceae</td>
<td><em>Carissa spinarum</em>  A. DC.</td>
<td>PL</td>
<td>acute and chronic hepatitis 1.</td>
</tr>
<tr>
<td>Oxalidaceae</td>
<td><em>Oxalis corniculata</em> L.</td>
<td>** PL</td>
<td>cold 1, jaundice hepatitis 1, 4, (7,j,g).</td>
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<tr>
<td></td>
<td><em>Passiflora wilsonii</em> Hemsl.</td>
<td>PL</td>
<td>hepatitis 1, a, c, g.</td>
</tr>
<tr>
<td>Plumbaginaceae</td>
<td><em>Plumbago indica</em> L.</td>
<td>* PL</td>
<td>hepatitis b, (4). collected from medicinal plant garden in Dai village, Menglun, Xishuangbanna.</td>
</tr>
<tr>
<td>Poaceae (Graminae)</td>
<td><em>Cymbopogon distans</em> (DC.) Stapf.</td>
<td>PL</td>
<td>hepatitis 1, (3).</td>
</tr>
<tr>
<td></td>
<td><em>Phyllostachys</em> sp. (gold bamboo)</td>
<td>** PL</td>
<td>hepatitis b.</td>
</tr>
<tr>
<td>Polypodiaceae</td>
<td><em>Stenoloma chusanum</em> (L.) Ching</td>
<td>** PL</td>
<td>cold 1, flu 4, infectious hepatitis 1. used by Dr. He Shixiu for cold, sample collected from Dr. He.</td>
</tr>
<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used #</td>
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<tr>
<td>Primulaceae</td>
<td><em>Lysimachia christinae</em> Hance</td>
<td>PL</td>
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<tr>
<td></td>
<td>jaundice hepatitis 1, 4, bronchial infection 1.</td>
<td></td>
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<tr>
<td>Rubiaceae</td>
<td>Hedyotis uncinella Hook. et Arn. or <em>H. capitata</em> Wall.</td>
<td>**</td>
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<tr>
<td></td>
<td>prevent and cure jaundice hepatitis 1, hepatitis 1, f, g.</td>
<td>PL</td>
<td></td>
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<tr>
<td></td>
<td><em>Morinda angustifolia</em> Roxb.</td>
<td>*</td>
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<tr>
<td></td>
<td>hepatitis 1a.</td>
<td>RT</td>
<td></td>
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<tr>
<td></td>
<td><em>Paederia scandens</em> (Lour.) Merr.</td>
<td>*</td>
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<tr>
<td></td>
<td>flu 1, bronchial infection 1, cold g, (a).</td>
<td>PL</td>
<td></td>
</tr>
<tr>
<td>Rutaceae</td>
<td><em>Boenninghausenia sesilicarpa</em> Levl. or <em>B. albiflora</em> (Hk.) Meissn.</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cold f, hepatitis 1, flu g, (a).</td>
<td>PL</td>
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</tr>
<tr>
<td></td>
<td><em>Clausenia excavata</em> Burm. f.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>flu 1, 4, b.</td>
<td>LF</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Evodia lepta</em> (Spr.) Merr.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>prevent and cure flu 1, hepatitis 1, 4, (8).</td>
<td>PL</td>
<td></td>
</tr>
<tr>
<td>Sapindaceae</td>
<td><em>Sapindus rarak</em> DC.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hepatitis 1, b.</td>
<td>LF</td>
<td></td>
</tr>
<tr>
<td>Santalaceae</td>
<td><em>Thesium himalense</em> Royle</td>
<td>PL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cold 1, bronchial infection 1.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saururaceae</td>
<td><em>Houttuynia cordata</em> Thunb.</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cold g, flu 4, f, jaundice hepatitis 1, (6,7,8,9)</td>
<td>PL</td>
<td></td>
</tr>
<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used #</td>
<td>Notes</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------------</td>
<td>-------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Scrophulariaceae</td>
<td><em>Siphonostegia chinensis</em> Benth.</td>
<td>**</td>
<td>PL</td>
</tr>
<tr>
<td></td>
<td>(jaundice hepatitis f, hepatitis 4, (3,7,8) used by Dr. He Zhegao for hepatitis)</td>
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<tr>
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<td><em>Solanum spirale</em> Roxb.</td>
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</tr>
<tr>
<td></td>
<td>(hepatitis a, b, cold 4, (i).)</td>
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<tr>
<td>Verbenaceae</td>
<td><em>Verbena officinalis</em> L.</td>
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</tr>
<tr>
<td></td>
<td>(cold f, flu 3, 4, hepatitis f, (1,5,7,8,g).)</td>
<td></td>
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</tr>
</tbody>
</table>

# Part used: BK, bark; BR, branch; BU, bulb; FL, flower; FR, fruit; LF, leaf; PL, whole plant; RH, rhizome; RT, root; SD, seed.

** Assayed for antiviral activity.

* Sample collected but left in Yunnan.

( ) Species listed in document, but not cited for use in the treatments of cold, flu, or hepatitis.

+ Plant species had a more specific range of treatments.
Table 1.2

Phylogenetic Distribution of Plants Selected as Candidates for Antiviral Screening
(shown by order and family with number of species)

**Magnoliopsida (Dicots)**

<table>
<thead>
<tr>
<th>Order</th>
<th>subclass total</th>
<th>Magnoliidae</th>
<th>Rosidae subclass total</th>
<th>Asteridae subclass total</th>
<th>Class</th>
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<tr>
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<td></td>
<td></td>
<td>Apiales</td>
</tr>
<tr>
<td>Berberidaceae</td>
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<td></td>
<td></td>
<td></td>
<td>(Umbelliferae)</td>
</tr>
<tr>
<td>Piperales</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Araliaceae</td>
</tr>
<tr>
<td>Saururaceae</td>
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<td></td>
<td></td>
<td></td>
<td>Cornaceae</td>
</tr>
<tr>
<td>Dillenidae subclass total</td>
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<td>Fabaceae</td>
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<tr>
<td>Capparales</td>
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<td></td>
<td>(Leguminosae)</td>
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<td></td>
<td>Geraniaceae</td>
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<tr>
<td>Dilleniales</td>
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<td>Myrtales</td>
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<td>Combretaceae</td>
</tr>
<tr>
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<td>Plumbaginaceae</td>
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<tr>
<td>Malvaceae</td>
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<td>Plumbaginaceae</td>
</tr>
<tr>
<td>Violales</td>
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<td>Primulales4</td>
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<tr>
<td>Myrsinaceae</td>
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<td>Sapindales</td>
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<td>Primulaceae</td>
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<td>Sapindaceae</td>
</tr>
<tr>
<td>Theales</td>
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<td>Hypericaceae</td>
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<td>Liliopsida (Monocots)</td>
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<td>Arecidae</td>
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</tr>
<tr>
<td>Arecales (Palmae)</td>
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<td>Arales</td>
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<tr>
<td>Arecidae</td>
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<td></td>
</tr>
<tr>
<td>Areciidae</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liliidae</td>
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<td></td>
</tr>
<tr>
<td>Arecales</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arecales (Palmae)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arales</td>
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</table>

**Cycadophyta (gymnosperm)**  **Pterophyta (fern)**

<table>
<thead>
<tr>
<th>Order</th>
<th>subclass total</th>
<th>Order</th>
<th>subclass total</th>
<th>Class</th>
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</thead>
<tbody>
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<td>Cycadophyta</td>
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<td>Pterophyta</td>
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<tr>
<td>Cycadaceae</td>
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<td>Filicales</td>
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<tr>
<td>Filicales</td>
<td></td>
<td>Polypodiaceae</td>
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</table>

Cycadaceae

Filicales

Polypodiaceae

26
Discussion

The use of an ethnopharmacological approach to screen for bioactive compounds involves extensive surveying of information. The sources often include local publications and oral traditions in many parts of the world. Research on the medicines in turn generates new information. The need to manage this quantity of information for centralized access and cross-referencing has led to the development of large-scale computer databases (Loub, et al., 1985). The data on traditional Chinese medicine are assembled at the Chinese Medical Material Research Centre at the Chinese University of Hong Kong. The Natural Products Alert (NAPRALERT) system at the University of Illinois, Chicago, aims to compile all chemical, pharmacological and ethno-medical data on natural products (Farnsworth, 1990). Ethnobotanical knowledge is a valuable resource that should be preserved and studied and it is important to have the information organized in order to facilitate further research.

The 82 plant species that were selected as potential candidates for antiviral testing are widely distributed in the taxonomic organization of higher plants. The 40 angiosperm families occur in 31 orders following the taxonomic classification scheme of Cronquist (1981). Seventy-two species are in the class Magnoliopsida (dicotyledons), with 34 species in the most represented subclass Asteridae. Eight species are in the class Liliopsida (monocotyledons). In the 31 species that were assayed for activity, 22 plant families were represented. They included a pteridophyte, a gymnosperm cycad, two monocot families (Poaceae, Iridaceae) and 18 dicot families (Apiaceae, Apocynaceae, Asteraceae, Berberidaceae, Combretaceae, Convolvulaceae, Ebenaceae, Euphorbiaceae, Fabaceae, Gentianaceae, Lamiaceae, Oxalidaceae, Myrsinaceae, Rubiaceae, Rutaceae, Saururaceae, Scrophulariaceae, Verbenaceae). The species also show a range of geographical distributions, from cosmopolitan (i.e. Oxalis corniculata) to regionally limited (i.e. Cycas siamensis). The family represented most frequently is the mint family Lamiaceae (Labiatae), from which six species were assayed. The wide distribution
of the plants selected reflects the fact that the pharmacopoeia surveyed includes all major plant
groups and most angiosperm families. Six of the families in this sampling were from families
that have more than 100 species in the Chinese medical flora (Xiao, 1983):

- Apiaceae,
- Fabaceae,
- Asteraceae,
- Lamiaceae,
- Euphorbiaceae,
- Rubiaceae.

The selection criteria used were of an ethnopharmacological rather than taxonomical nature, and
it appears that a wide range of plants were used in the treatment of the viral diseases: the cold,
influenza, and hepatitis.

The composition of the sampling was nevertheless influenced by two factors. Plants
were screened as candidates for collection from a survey of traditional medicine used in the
treatment of specific diseases. Actual collections were made on the basis of availability of
quantities for bioactivity assays, and there may therefore have been an inherent bias against the
collection of rare or of very small plants. The frequent usage of Lamiaceae species (of the mint
family) as medicinal plants could contribute to their high occurrence in such a survey-based
selection. It is possible that mint plants may have been also used more frequently to treat one
of the diseases used in the survey. In folk use, mints seem to have a history of uses in the
treatment of colds generally associated with the aromatic nature of the herbs having an effect on
the respiratory system. Since this survey used the indication for treatment of colds as a
selection criterium, the taxonomic distribution of the plants selected could be influenced by any
tendencies for a plant family to be used more commonly in folk medicine. The preponderance
of certain families of plants in traditional medicine is probably based on observations of
particular properties of the plants. These properties can in turn be attributed to characteristics in
the chemistry of the plant family (Schultes and Swain, 1976). Swain (1972) has discussed
how the origin of modern taxonomy can be traced to the practice of medieval medicine.
In this survey, a plant was considered for antiviral screening if the treatment of one of the viral diseases was one of its uses. Most of the plants surveyed were not used only for these diseases. It is difficult to make a direct link between the traditional uses of plants and the specific viruses that are now known to cause the diseases they are used to treat. The records of hepatitis treatment are variable, with several different types of inflammation of the liver being distinguished. The term for 'hepatitis' in Chinese is 'liver inflammation'. This is not inconsistent with the western diagnosis of hepatitis which also acknowledges several types of affliction of the liver. There are at least five different types of viruses causing hepatitis and the causative agent is not distinguishable by symptoms or even biochemical tests (Hoofnagle and Di Bisceglie, 1990). All uses of plants for treating liver inflammation and jaundice were taken as an indication of treatment for hepatitis. Han et al. (1988) conducted a survey of the effectiveness against viral hepatitis of 97 Chinese medicines. The natural materials selected as potential candidates includes species of nine plant genera (Elsholtzia, Halenia, Houttuynia, Lysimachia, Morinda, Polygonatum, Scutellaria, Swertia, Verbena). Species of these genera have all been cited for the treatment of hepatitis in Yunnan. Swertia spp. and Halenia spp. are reported to show effectiveness in clinical trials. Common colds are considered to be most frequently caused by rhinoviruses, but coronaviruses and enteroviruses may cause similar symptoms; just as flu-like syndromes can be caused by influenza and parainfluenza, as well as other respiratory tract viruses (Lycke, 1983). Plants used solely for treatments of symptoms relating to cold and flu afflictions, such as cough suppression or reduction of phlegm, were rejected for the screen, but those used in the treatment of respiratory tract infections were considered. On the basis that the use of a plant for treatment of cold, flu or hepatitis was an indication for potential antiviral activity, 69 plant genera occurring in Yunnan were identified as candidates for screening.

Representatives of 48 plant genera were collected during two months in Yunnan. Eighteen of the species collected were not assayed for antiviral activity. This was due to
unforeseen limitations imposed by a contract which was drawn up between the Ethnobotany Laboratory at the Kunming Institute of Botany and another institution preventing further research collaborations between the Laboratory and me on specified plant species for a period of time. This unexpected restriction is a reminder that factors of commercial or proprietary interest may arise in the course of research on the traditional medicinal knowledge and the natural resources of any culture or countries. It is integral to the ethnobotanical research approach to develop and maintain good collaborative relationships with the people whose knowledge and resources are involved in the research, and this unexpected development was accepted.
Chapter I. References


Chapter II.
Screening for Antiviral Activity

Introduction

Progress in the discovery of chemical agents for the treatment of viral diseases has in some cases reached the stages of clinical use and trials (Becker, 1984; Allison, 1986). One aim of antiviral research is toward the development of compounds that can inhibit viral infection selectively without being harmful to the virus host. A few mostly synthetic compounds have been approved for therapy, and several natural products have been found to be promising leads as antiviral agents and are under further study (Hudson, 1990, Vlietinck and Vanden Berghe, 1991). The studies of antiviral extracts and compounds derived from plants are summarized by Che (1991). About 4000 plants are estimated to have been screened for antiviral effects, and over 450 species that have shown in vitro activity are listed. Zheng (1988) tested 472 Chinese medicinal herbs for activity against herpes simplex virus type 1 and 31 (7.2%) of the alcoholic extracts of these species was found to be active in in vitro tests. The pandemic threat of the retrovirus-linked acquired immunodeficiency syndrome (AIDS) has given impetus to efforts to find antiviral agents that inhibit the human immunodeficiency virus (HIV), and many compounds have been tested for this effect (De Clercq, 1987). In a screening of 27 Chinese medicinal herbs, extracts of Viola yedoensis Maxim showed inhibition of HIV (Chang and Yeung, 1988). In addition, several compounds of different chemical classes from medicinal plants have shown anti-retroviral activity. These include: sulfated polysaccharides from the Chinese medicinal plant Prunella vulgaris L. (Tabba, et al., 1989), the naphthobianthrone hypericin from Hypericum spp. (Meruelo et al., 1988), the indolizidine alkaloid castanospermine from the seed of the Australian tree Castanospermum australe A Cunn. et Fraser (Walker et al., 1987), and the protein trichosanthin from the tuber of another Chinese medicinal plant Trichosanthes kirilowii Maxim (McGrath et al., 1989). Methoxyflavones are
active against picornavirus. These compounds were isolated from both the Chinese medicinal plant *Agastache rugulosa* O. Kuntze (Ishitsuka et al., 1982) and the African medicinal plant *Euphorbia grantii* Oliver (Van Hoof et al., 1984). These examples of antiviral plant compounds demonstrate the effectiveness of screening medicinal plants in the search for antiviral agents. An overview of the process of developing antiviral agents from plants was presented by Vanden Berghe et al. (1985).

The virus is an obligate intracellular parasite. It differs from other parasitic organisms in that it is dependent on the host at the genetic level by requiring the use of cellular molecular mechanisms in its replication. It is basically composed of nucleic acid genetic material enclosed within a protein nucleocapsid. The nucleic acid can be a single or double strand, consisting of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). The protein capsid is either icosahedral or helical in shape but a few, such as the poxvirus, have more complex symmetry (Choppin, 1986). The capsid can be nonenveloped or membrane enveloped. The nucleocapsid is encased by a lipid bilayer membrane in about half of known animal viruses (Hudson, 1990). The enveloped virus has been defined by Matthews (1983) as "having a bounding lipoprotein bilayer membrane necessary for infectivity in normal conditions".

Virus taxonomy is complicated and the grouping of a virus often reflects its host and/or associated disease. Viruses are often very host specific. Most generally, viruses can be characterized by nucleic acid and particle types (Norrby, 1983). Changes in classification have evolved with the advancement of knowledge and technology that have led to the examination of relationships according to antigenicity (van Regenmortel, 1982), replication strategies (Hershey and Taylor, 1986) and phylogeny (Goldbach, 1990). Matthews (1983) reviewed the history and status of virus taxonomy. Viruses appear to have been very successful in exploiting all forms of life, being ubiquitous in animals, plants and bacteria. Viruses have developed many different molecular strategies of replication and many mutate rapidly. They can persist within
the host and manifest chronic symptoms or remain latent until an episode of reactivation. Viruses have also adapted to numerous vectors of transmission between hosts. All these features about viruses contribute to the complexity of the task of controlling the spread of disease-causing viruses.

The different possible sites of action for antiviral agents are related to the different stages of viral replication. The sequence of events have been comprehensively described by Mitchell (1973). Briefly, virus infection and reproduction include the following stages:

1. adsorption to and penetration of the host cell,
2. uncoating of the viral particle,
3. transcription and translation of viral nucleic acids,
4. replication of genome,
5. assembly and release of progeny virus.

The antiviral agent could target any of these stages. As viral replication depends on cellular molecular mechanisms to some extent, the ideal antiviral agent would have an irreversible effect only on a virus-specific component in one of these stages (Sim, 1990). The majority of antiviral drugs in use are nucleoside analogs that interfere in viral replication by disrupting nucleic acid polymerization, but they have toxic side effects due to the similarity between the viral and the normal cellular process (Montgomery, 1989). The chemical interferences at these different stages will be further discussed in Chapter V which concerns the nature of antiviral action.

For the evaluation of the antiviral potentials of an extract or a chemical, in vitro antiviral assays using animal cell culture systems have become established procedures and are reviewed by Hu and Hsiung (1989). Tests for antiviral effects are based on measurements indicative of virus replication in a suitable host cell system. Antiviral activity can be seen as the inhibition of cytopathic effects (CPE) caused by the virus in the presence of the agent tested.
This method is applicable to viruses that cause a visible change in appearance of infected cells and is useful for testing a range of concentrations. The effective concentration is determined by the absence of visible CPE. A method using a colored reagent has been developed for the in vitro CPE inhibition assay of HIV (Weislow et al., 1989). This allows for the quantification of drug effects with colorimetric techniques. With viruses that form plaques on cell monolayers indicating units of infection, the reduction of plaque numbers in a treated versus a control cell layer gives a quantitative assay of antiviral activity. This method can be used to obtain a dose-response curve by comparing the plaque reductions with different concentrations of compound tested (Hudson et al., 1991). Similarly, in the virus yield reduction assay, virus infected cells are treated with a range of concentrations of the agent tested. The virus yields are then assayed after a suitable incubation time (Boyd et al., 1987). Hu and Hsiung (1988) compared plaque reduction and virus yield reduction assays and found the results to be similar.

Not all viruses form plaques or induce visible CPE. Other methods have been used that monitor viral replication by measuring a virus-specific effect or product. Reverse transcriptase is a RNA-directed DNA polymerase that is virus coded and necessary in the replication of several viruses (Doolittle et al., 1989). The inhibition of reverse transcriptase activity can be used as an indication of an antiviral effect. This method has been used in studies with HIV (Nakashima et al., 1987; Nishizawa et al., 1989) and RNA tumor viruses (Sethi, 1985). Viral antigens in cell cultures can be detected with enzyme-linked immunosorbent assays (ELISA) or immunofluorescence assays (Rabalais et al., 1987; Farber et al., 1987). Effect on viral replication can be detected with the quantification of viral nucleic acids using molecular biology techniques such as RNA-DNA hybridization (Lin et al., 1987). Virus particles can be observed under an electron microscope. Absence of virions or defective virions can also demonstrate antiviral activity (Fong et al., 1987). A tabular summary of the differences between applicability of in vitro antiviral assays is found in Vlietinck and Vanden Berghe (1991).
The biological activity of many plant constituents can be dependent on, or enhanced by, simultaneous irradiation with light (Towers, 1980). Antiviral plant photo-sensitizers, particularly those activated by long wavelength ultraviolet (UVA, 320-400 nm) radiation, have been reviewed by Hudson (1989). To test for the possibility that the activity of some plant extracts might be light-mediated, the bioassays were done in the presence and absence of UVA radiation.

The crude extracts of 31 medicinal plant species from Yunnan, China, were assayed for antiviral activity. These species are distinguished on the list of plants selected as potential candidates for testing which is in Table 1.1 of Chapter I. They are also listed separately in Appendix I. The extract determined to be the most active was also tested for antibacterial and antifungal activities.

Material and Methods

Cell culture

Two monolayer-forming mammalian cell lines from the American Type Culture Collection were used for the bioassays. The 3T3-L1 is a mouse fibroblast cell line and Vero is an African green monkey kidney cell line. The cells were grown in a 5% carbon dioxide atmosphere at 37°C, in Dulbecco's Modified Eagle A Medium (DMEM) with 10% fetal bovine serum (Gibco) and 25 µg/ml gentamicin sulfate (Sigma).
Cytotoxicity Assays

Cell layers of 3T3-L1 were grown in 96 well microtiter plates (Falcon 3072). Cytotoxicity of plant extracts was first tested by exposure of the cells to dilutions of the extracts in the cell culture medium. Two treatments were done: with the extracts added when the cells were still in suspension prior to monolayer formation, and when the cells had formed confluent monolayers. The highest concentration of extract tested was the equivalent of 100 mg of starting plant material per ml, from the ethanol soluble portion of crude ethanolic extract residues. Two-fold serial dilutions were made from this concentration and added to the cells. The treated cells were incubated and observed for cytopathic effects.

Assay Viruses

Two animal viruses were used to screen for antiviral activity. Sindbis (SINV) is a single-strand RNA virus of the Togaviridae, size ~ 40 nm. Murine cytomegalovirus (MCMV) is a double-strand DNA virus of the Herpesviridae, size ~ 200 nm. Both of these are membrane-enveloped viruses. Some species that were active in the screen were assayed against a nonenveloped virus with human polio virus 1. Polio is a single-strand RNA virus of the Picornaviridae, size ~ 25 nm. All three viruses have icosahedral capsid symmetry.

Antiviral Screening Assays

Two-fold serial dilutions of the plant extracts were tested starting with non-cytotoxic concentrations. The infectivities of the two viruses were assayed qualitatively through microscopic observations of characteristic viral cytopathic effects (CPE). Controls of cells only and untreated virus infections were done with each assay for comparison. The viral effects radiate from centers of infection. The CPE of SINV is seen as shrunken and highly
refractive cells, and MCMV causes cell swelling. Antiviral activity was determined by the absence of CPE in a treatment. The minimum active concentration was the lowest concentration of extract at which the absence of CPE was observed. The known UVA-enhanced antiviral plant compound α-terthienyl (Hudson et al., 1986) was used as an antiviral control.

In a comprehensive assay, three separate segments of the viral life cycle were exposed to the plant extracts successively. The cell preparations were incubated with the extract dilutions in culture medium for 24 hrs during monolayer formation. The viruses, at a concentration of 1000 plaque forming units (pfu) per ml, were exposed to the same extract dilutions in phosphate buffered saline (PBS) at 4°C for 30 min. The cell layers were then exposed to the virus/extract mixture for 1 hr of adsorption time at 37°C. After removal of the virus mixture, the cells were incubated in medium with the same extract dilutions. Two sets of control cells were subject to the same manipulations: one set without virus or the plant extracts (cell control), one set exposed to the virus but without plant extracts (virus control). Control assays were done with dilutions of all the solvents used in the addition of the extracts to determine the concentrations below which there were no solvent effects. The plant extracts were usually prepared with methanol or ethanol.

In the multiple-treatment assay, 16 plant extracts showed inhibition of viral infection. These extracts were assayed again following the same procedure but with three parallel preparations of cells. Instead of one set of cells being subject to three successive extract treatments, each set was exposed to only one of the treatments. Each treatment was done in parallel with and without exposure to UVA radiation. The time of extract treatment for each set is shown in the following scheme:
Further assays were repeated with extracts of eight species. The respective extracts in ethanol were filtered (Whatman paper No. 1) to remove undissolved crude residues, evaporated to dryness and the residues taken up in 100% methanol back to the same volumes. The methanol fractions were assayed following the same procedure, with extract treatment of the virus suspension and the cells during virus adsorption.

**Plaque Reduction Assays**

Cell monolayers were formed in petri dishes (Corning 25010, 60mm diameter, for SINV and polio assays; Corning 25000, 35 mm diameter, for MCMV assays). The cells were exposed to the extract / virus mixture for 1 hr of adsorption time, with 1000 pfu of virus per treatment. After removal of the virus mixture, the cells were overlain with medium in 0.5% agarose and incubated until viral plaques were visible. The cells were sometimes fixed with 3.7% formaldehyde in PBS and stained to facilitate plaque counting (crystal violet for SINV and polio, methylene blue for MCMV). Cell and virus controls were done with each assay. Plaque reduction is calculated as a percentage from the number of plaques compared to the virus-only control. An example of the plaque reduction assay is shown in Figure 2.1.
Figure 2.1: Plaque Reduction Assay of Antiviral Activity.
UVA Irradiation

The UVA radiation was provided in a temperature-controlled chamber (Environ-Shaker 3597, Lab-Line Instruments) by a bank of 6 Philips F20T12/BLB light tubes, with peak emission at 350 nm and an incident energy of 270 $\mu$W/cm$^2$. An extract was considered to show enhancement of activity by UVA radiation when antiviral effects were observed at lower concentrations of the extract, compared to the parallel culture kept in the dark.

Antibiotic assays

Bacterial and fungal cultures in liquid media were spread on nutrient agar plates (Muehler-Hinton medium for bacteria, yeast nitrogen base medium for Saccharomyces). Plant extracts were pipetted in 20 $\mu$l aliquots on sterile filter paper disks 6 mm in diameter (Schleicher & Schuell 740-E). The plates were incubated for 24 hrs at 37° C. Parallel plates were incubated in the dark and with exposure to UVA radiation. Antibiotic effects were seen as clear zones of culture inhibition surrounding the paper disks.

Results

Antiviral Activity Screening

The highest concentration used for each extract was determined by the cytotoxicity assays. Extract concentrations used for the antiviral assays were in the range equivalent to 30 $\mu$g/ml to 1.0 mg/ml of original plant material. The crude extracts of 31 plant species were assayed and 16 of them showed antiviral activity. The results are shown in Table 2.1. The concentrations shown were calculated from weight of dry plant material extracted.
Table 2.1

Antiviral Activity of Crude Extracts of Yunnan Medicinal Plants Against Murine Cytomegalovirus (MCMV) and Sindbis Virus (SINV)

<table>
<thead>
<tr>
<th>Plant species listed by family</th>
<th>Minimum Active Concentration (µg/ml)#</th>
<th>UVA enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apiaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Centella asiatica</em> (L.) Urban</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bidens pilosa</em> L.</td>
<td>125</td>
<td>65</td>
</tr>
<tr>
<td>Combretaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Quisqualis indica</em> L.</td>
<td>---</td>
<td>125</td>
</tr>
<tr>
<td>Convolvulaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dichondra repens</em> Forst.</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>Cycadaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cycas siamensis</em> Miq.</td>
<td>---</td>
<td>160</td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Euphorbia prolifera</em> Ehrenb. ex. Boiss.</td>
<td>---</td>
<td>65</td>
</tr>
<tr>
<td>Iridaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Belamcanda chinensis</em> DC.</td>
<td>---</td>
<td>125</td>
</tr>
<tr>
<td>Lamiaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acrocephalus indicus</em> Briq. *</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td><em>Elsholtzia densa</em> Briq.</td>
<td>---</td>
<td>125</td>
</tr>
<tr>
<td><em>Elsholtzia ciliata</em> (Thunb.) Hylander *</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td><em>Rabdosia phyllostachys</em> (Diels) Hara</td>
<td>---</td>
<td>125</td>
</tr>
<tr>
<td><em>Stachys kouyangensis</em> (Vaniot) Dunn *</td>
<td>---</td>
<td>65</td>
</tr>
<tr>
<td>Myrsinaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Embelia sessiliflora</em> Kurz *</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Rutaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Boennninghausenia sessilcarpa</em> Levl. *</td>
<td>---</td>
<td>80</td>
</tr>
<tr>
<td>Scrophulariaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Siphonostegia chinensis</em> Benth. *</td>
<td>---</td>
<td>40</td>
</tr>
<tr>
<td>Verbenaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Verbena officinalis</em> L. *</td>
<td>65</td>
<td>30</td>
</tr>
</tbody>
</table>

* further assay results shown in Table 2.2.  # based on dry wt. of plant material extracted
The 16 active extracts were assayed again when the cell cultures were exposed to extract treatments separately at the pre-infection, virus / infection, and post-infection stages. The antiviral activity in all cases occurred only upon treatment of the virus and of the cells during infection time. Eight extracts of higher activity were selected for further assays. The results from the assays of the filtered methanolic fractions are shown in Table 2.2.

Activity of *Elsholtzia ciliata* and other *Elsholtzia* species

The highest activity in the CPE inhibition assay of methanolic fractions was shown by *Elsholtzia ciliata* with the minimum active concentration of 2 µg/ml. The assay of a separate preparation of crude extract was against Sindbis virus at 0.3 µg/ml with exposure of the virus / extract to UVA. The UVA light enhancement of activity of *E. ciliata* occurred even if only the virus / extract mixture was exposed to light and the virus adsorption period on the cell monolayer was in the dark. One sample of dried plant material obtained from the Ethnobotany Laboratory of KIB was not active. The sample was much more woody than the other collections. Crude extract and solvent fractions of *E. ciliata* had no activity against polio virus in plaque reduction assays. Alcoholic crude extracts of *E. splendens* Nakai ex F. Maek. (from Kunming) and *E. blanda* (Benth.) Benth. (from Nepal) were active against SINV in plaque reduction assays at a concentration equivalent to 2 mg/ml of dry plant material, with UVA light. A sample of the essential oil of *E. ciliata* previously distilled by the Kunming Institute of Botany showed a narrow range of marginal activity in plaque reduction assays between its cytotoxic and inactive concentrations (1:1000 to 1:1500 dilutions).

Antibiotic assays

The results of the antibiotic assay of the *E. ciliata* crude extract are shown in Table 2.3. The extract concentration was the equivalent of 40 µg of dry plant material.
### Table 2.2

**Minimum Active Concentrations of Methanolic Fractions Against Murine Cytomegalovirus (MCMV) and Sindbis Virus (SINV)**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Minimum Active Concentrations (µg/ml)#</th>
<th>Light Mediation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCMV</td>
<td>SINV</td>
</tr>
<tr>
<td><strong>Acrocephalus indicus</strong></td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td><strong>Boenninghausenia sessilicarpa</strong></td>
<td>---</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>---</td>
<td>500</td>
</tr>
<tr>
<td><strong>Elsholtzia ciliata</strong></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td><strong>Embelia sessiliflora</strong></td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td><strong>Euphorbia prolifera</strong></td>
<td>---</td>
<td>30</td>
</tr>
<tr>
<td><strong>Siphonostegia chinensis</strong></td>
<td>---</td>
<td>65</td>
</tr>
<tr>
<td><strong>Stachys kouyangensis</strong></td>
<td>---</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>---</td>
<td>1mg</td>
</tr>
<tr>
<td><strong>Verbena officinalis</strong></td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

# concentrations calculated from weight of dry plant material extracted
Table 2.3

Antibiotic Activity of the Crude Extract of *Elsholtzia ciliata*

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Diameter of Inhibition Zone (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UVA</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> sensitive strain</td>
<td>10</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> resistant strain</td>
<td>12</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> methicillin resistant</td>
<td>(--)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DC2</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>(--)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>10</td>
</tr>
<tr>
<td>methanol control</td>
<td>(--)</td>
</tr>
<tr>
<td>gent. control (<em>E. coli</em>)</td>
<td>16</td>
</tr>
<tr>
<td>gent. control (<em>S. aureus</em> sens.)</td>
<td>25</td>
</tr>
<tr>
<td>fung. control (<em>S. cerevesiae</em>)</td>
<td>12</td>
</tr>
</tbody>
</table>

* diameter of assay disk = 6 mm
(--) = no inhibition zone

gent. = antibiotic gentamicin sulfate (Sigma), 10 µg/disk.
fung. = antifungal fungizone (Gibco), 10 µg/disk.
Extract concentration = 40 µg of dry plant material.
Incubation 24 hrs. in UVA or in dark at 37°C.
Discussion

The screening method used here comprised a general antiviral screening approach rather than one designed to assay specifically for the viruses related to the illnesses that the plants were used to treat. The design of the screening program included a feasibility aspect. Compared to in vivo assays, in vitro bioassays are a cost and effort effective approach to antiviral screening, although there are differences in the ease and viability of assaying different viruses, such that in practicality not all viruses of chemotherapeutic interest for investigation are amenable to this system. Vanden Berghe and Vlietinck (1991) described the composition of a battery of six viruses that represent a range of virus morphology and diseases that they used in screening of plant extracts (adeno-, measles, Coxsackie, herpes simplex, polio-, Semliki forest). The last four are from the same families as the three used in this study.

The two viruses used in the general screening assays represent two major divisions in virus classification based on the nature of the nucleic acids: RNA vs. DNA, and single-strand vs. double-strand (SINV = ssRNA, MCMV = dsDNA), but these are both membrane enveloped. The third virus used in further assays is a nonenveloped virus (polio = ss RNA). The characteristics of SINV will be discussed in more detail in Chapter V in the examination of antiviral mechanisms. Regarding the viruses that cause the diseases considered in the plant selection: rhinovirus (cold) is also a picornavirus and influenza viruses are ss RNA orthomyxoviruses, with membrane envelopes. Not all of the different hepatitis viruses have been fully characterized but they include the nonenveloped ss RNA hepatitis A picornavirus. Hepatitis B is a dsDNA hepadnavirus. It is not membrane enveloped but has instead an envelope consisting of the viral coat protein, lipids and carbohydrates (Summer et al., 1983). By comparing the overlap in virus grouping using more than one morphological feature, Matthews (1983) has proposed that using the presence or absence of the membrane envelope would be a more "informative and predictive" primary division of the virus groups than the one using the RNA vs. DNA criterion. From the perspective of studying the bioactive nature of
compounds, the presence or absence of a membrane envelope would also be an important feature to consider because the envelope could be a different site of action. The extract of *Elsholtzia ciliata*, which was active against the two membrane bound viruses, was not active against the nonenveloped polio virus. This suggests that the antiviral action of this particular extract could involve the virus membrane.

Six of the extracts active against SINV were also active against MCMV. None were active against MCMV only. In general, activity was observed at lower extract concentrations against SINV than against MCMV. Besides the differences in the nature of their nucleic acids, MCMV is also a much larger virus in size and genome. A simple explanation for the difference in sensitivity could be that it was due to the size difference, especially possible if the virus membrane was the site of antiviral action.

The antiviral activity shown in these assays could have taken place at several different stages of the viral cycle. The effective treatment included exposure of the virus first to the extract and the exposure of the cells to the same virus/extract mixture. The extract could have inhibited viral infection by affecting the virus particle itself, by interfering with the virus-host cell recognition and entry process, or by interfering with the early steps of intracellular viral reproduction. Antiviral activity from a pre-infection treatment would indicate an interferon-stimulatory type effect. Wachsman et al. (1987) have reported this type of effect against Sindbis virus from plant extracts of the Meliaceae. Activity from a post-infection treatment would suggest interference in the later steps of viral replication, or in viral progeny packaging and release.

In the screening process, the active concentrations of the extracts were used more as indications of relative activity. There was slight variability in the exact minimum active concentrations between repeated assays. An effort was made to assay the extracts
simultaneously to minimize the variability. In the assays of the crude extracts brought back from Yunnan, the concentrations were calculated back to the original amount of plant material extracted. This was done because the residues brought back from Yunnan redissolved incompletely and to different extents amongst the extracts. Only the soluble portions were tested. To continue screening assays, these portions from eight active species were separated from the remaining residues, dried and taken up in methanol to the same volume.

The plant extract that showed the highest antiviral activity was from *Elsholtzia ciliata* (Thunb.) Hylander of the mint family Lamiaceae. In the general screening assay, the extract was active against SINV to 30 μg (of original plant sample extracted) per ml, which was the lowest concentration tested, and against MCMV to 65 μg/ml. In the methanolic fractions assay, *E. ciliata* was again the most active, showing activity against both SINV and MCMV at 2 μg/ml, with UVA. In assays of larger samples obtained for purification of active components, it was active against SINV at 0.3 μg/ml (actual concentration of extract) with exposure to UVA light. On this basis, *E. ciliata* was selected as the most active plant for the isolation of an active component using bioassay-guided fractionation. The activity occurred even if only the virus/extract mixture was exposed to light and the virus adsorption period on the cell monolayer was in the dark. This indicates that the action inhibited the ability of the virus to penetrate and infect the cell. It is likely that the virus particle was directly inactivated.

The chemistry of the purification process of the active component from *E. ciliata* is the subject of the next chapter. In a follow up study of the bioactivity, *Elsholtzia* extract also showed some UVA-enhanced antibacterial and antifungal activity. The activity was not dramatic and this aspect was not further examined. The essential oil of *Elsholtzia* species has been reported to have antibacterial and antifungal activity (Bisht et al., 1985; Chaturvedi and Saxena, 1985). It has also been reported to have insect growth retarding activity (Bhattacharya and Bordoloi, 1986) The oil sample assayed in this study showed only weak antiviral activity
and the purification of its constituents was not pursued. The sample that contained more shoot and root material was also not active, suggesting the activity may be more abundant in the leaf material, but since sample quantities were always limited, leaves and twigs were not separated in further samples.

The Lamiaceae was the most highly represented of the 22 plant families screened, and five of the six species examined showed activity. Two of the active species were from the genus *Elsholtzia* (*E. ciliata* and *E. densa*), and two other *Elsholtzia* species acquired later (*E. blanda* and *E. splendens*) were also active. Seven *Elsholtzia* species were selected as potential candidates for this screening from the pharmacopoeia of Yunnan, which was the highest number of species for one genus. A table of the Chinese medicinal *Elsholtzia* species is presented at the beginning of Chapter III. The active species *E. splendens* was not on the candidates list (Table 1.1) because it had been not cited by its Latin name in the sources on Yunnan plants. It was cited in *Chinese Herbal Pharmacology* (Nanjing Institute of Pharmacology, 1980) that an aqueous extract of *E. splendens* inhibited echovirus. Since it occurs in Yunnan, it probably was used there medicinally because the Chinese name for *E. splendens* can be used for several species, including: *E. blanda, ciliata, densa* and *rugulosa*. This common nomenclatural overlap or discrepancy becomes evident when the attempt is made to match organisms designated by traditional names with scientific taxonomic groups. Nonetheless, the high percentage of bioactivity from the genus that was found to be most cited in this information survey supports the hypothesis that there is a good correlation between bioactivity and plant groups frequently used in traditional medicines.

Three species in the antiviral screening showed UVA-enhanced activity. *Elsholtzia ciliata* and *Stachys kouyangensis* are mint plants and the other was *Boenninghausenia sessilicarpa* of the Rutaceae. The Rutaceae is known to contain furanocoumarins which are photosensitive compounds (Towers, 1980). There have been no reports of photosensitizers
from the Lamiaceae. The four *Elsholtzia* species tested all showed activity and the activity was enhanced by UVA except for the case of *E. densa*. This suggests that there are interesting phytochemical questions concerning the genus *Elsholtzia*, as well as the family Lamiaceae, which require further investigation.

Ethnopharmacological information from Yunnan was used as a guideline in the selection of plants to screen for antiviral activity. The fact that 16 out of 31 plants tested demonstrated activity supports the validity of the approach of searching for bioactive compounds in medicinal plants. The 16 active species are in 12 plant families. In addition, five of the active species were selected for the screening based on their documented usage by the ethnic minority tribes of Yunnan (see citation notations in Table 1.1, Chapter I). These five species are:

- *Acrocephalus indicus* Briq.,
- *Belamcanda chinensis* DC.,
- *Quisqualis indica* L.,
- *Siphonostegia chinensis* Benth.,
- *Stachys kouyangensis*. (Vaniot) Dunn.

The species *Belamcanda chinensis*, *Quisqualis indica*, and *Siphonostegia chinensis* are also documented in the general literature on traditional Chinese medicine, but they are not cited as treatment for any of the viral diseases used as the screening selection criteria. Therefore, it was from the minority groups' ethnopharmacological information that these plants were selected for screening. The fact that five of sixteen active species were selected because of the examination of additional culturally-based information concerning the same flora, indicates the importance of both researching and preserving ethnobotanical knowledge.
Chapter II. References


Chapter III

Purification and Identification of Active Component from *Elsholtzia ciliata*

Introduction

In the screening of 31 medicinal plants for activity against the enveloped viruses Sindbis and murine cytomegalovirus in mammalian cell cultures, the crude extract from the leaves and twigs of *Elsholtzia ciliata* (Thunb.) Hylander of the Lamiaceae showed potent UVA-enhanced activity. This species was selected as the species to purify the active component from because it showed the highest activity amongst 16 active species. It would also be of additional phytochemical interest since there are no previous reports of phototoxins or photosensitizers from the mint family. In choosing a plant from a screening program for the purification of the active component, the availability of the plant in quantity is again a factor.

Samples of *E. ciliata* could be obtained in quantity as it is a commonly occurring shrub in Yunnan province and other parts of southern China (Steward, 1958).

The Chinese name for *Elsholtzia* is 'xiang ru' (香薷). It was cited in *Ben Cao Gang Mu* (Li, 1979), the first Chinese materia medica, and the illustration is shown in Figure 3.1. In traditional medicine, the plant was used as a tonic against general alterations to the body nature such as suffering from the chills (Huang, 1984). The common use of the plant in treating the cold and flu is probably related to this view.
The genus *Elsholtzia* has an Old World distribution with approximately 35 species of annuals, perennials and shrubs (Everett, 1981). Ten species and six synonyms were entered in the *Dictionary of Chinese Medicinal Herbs* (New Jiangsu Hospital, 1977). These are listed in Table 3.1.

In the original plant collection, a sample of *Elsholtzia* was collected as *E. rugulosa* because it is cited for use in the treatment of the cold and flu. Leaves and twigs were collected from a shrub that was not in flower. This sample proved to be the most active in the antiviral screening. Later collections of larger quantities of the plant material for chemical purification were made by the Phytochemistry Laboratory of Kunming Institute of Botany. With the plant in flower, the identification was revised to *E. ciliata*. Both species are shrubs, with *E. ciliata* described as being from 30-100 cm in height and *E. rugulosa* being 50-100 cm. The chemical purification process of the active compound was carried out with samples identified as *E. ciliata*. This species is also documented for usage in the treatment of cold and flu (Jiangsu New Hospital, 1979). Judging from the number of synonyms in the botanical literature on *Elsholtzia*, it seems that the taxonomy within the genus is not well defined. Considering that in the medicinal literature survey, the name 'xiang-ru' applies to *Elsholtzia* in general, and the extracts of three species (*E. blanda, E. ciliata, E. splendens*) showed similar UVA-enhanced antiviral activity, the question of the taxonomic distinction between *E. ciliata* and *E. rugulosa* was not considered to be critical in the search for an antiviral compound.
Table 3.1
Species of *Elsholtzia* Used in Chinese Medicine

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>shrub, 100-150 cm</td>
<td>E. <em>bodneri</em> Vant.</td>
<td>shrub, 30-100 cm</td>
<td>E. <em>cypriani</em> (Pavlov.) Wu et Chow</td>
<td>annual, 10-50 cm</td>
<td>shrub, 100-200 cm</td>
<td>E. <em>fructicosa</em> (D. Don) Rehd.</td>
<td>shrub, 100-200 cm</td>
<td>shrub, 50-150 cm</td>
<td>perennial, 30-50 cm</td>
</tr>
<tr>
<td>'dien xiang ru' (dien = colloquial name for Yunnan)</td>
<td></td>
<td></td>
<td></td>
<td>'tu xiang ru' (tu = earth, soil)</td>
<td>'da yeh xiang ru' (da yeh = big leaf)</td>
<td></td>
<td></td>
<td>'xi zhou xiang ru' (xi zhou = fine wrinkle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>= E. <em>cristata</em> Willd.</td>
<td></td>
<td>= E. <em>communis</em> (Coll. et Hemsl.)Diels</td>
<td></td>
<td></td>
<td>= E. <em>polystachya</em> Benth.</td>
<td></td>
<td></td>
<td>= E. <em>haichowensis</em> Sun</td>
</tr>
</tbody>
</table>

** assayed for and showed antiviral activity

* potential assay candidate in Yunnan pharmacopoeia but not collected.
The chemical nature of the active component of *E. ciliata* was unknown and the approach to the isolation was to use the process of bioassay-guided phytochemical fractionation. There have been previous analyses of the chemistry of *Elsholtzia* species. The literature reporting the chemistry of *Elsholtzia* is listed in Appendix II. The majority of the studies were done in India, China and Russia. In the NAPRALERT database (NAPRALERT, 1992), over 90% of compounds reported from *Elsholtzia* are mono- and sesquiterpenes from the essential oil. Bisht et al. (1985) summarized the components of the essential oils of various *Elsholtzia* species and these include: terpenoids, unsaturated alcohols, carboxylic acids, and esters. Major components of the oils have been reported to be acylfuran derivatives such as elsholtzia ketone and dehydroelsholtzia ketone (Kobold et al., 1987). One report (Park et al., 1985) cited in NAPRALERT identified four flavonoid compounds from *E. ciliata* in Korea. Two other Korean reports (Chi and Lee, 1981; Han et al., 1981) were cited with conflicting results regarding the absence or presence of saponins, and sterol and/or triterpene moieties in the entire plant. Alkaloids are reported to be absent from *E. patrini* (syn. *ciliata*) (Woo et al, 1978) and *E. splendens* (Chi and Lee, 1981). Since antiviral activity has been found in every major chemical group of compounds (Vanden Berghe et al., 1985; Roberts, 1988; Hudson, 1990), the isolation of an active component has to take the empirical approach of identifying an unknown, by determining which fraction(s) contains the active component through bioassay after each chemical separation. Hostettmann et al. (1991) generally summarized the process of isolating bioactive constituents from medicinal plants in their review of the developments in preparative separation techniques.

The activity-guided fractionation of *Elsholtzia ciliata* extracts was divided into two parts. The initial fractionation indicated the presence of more than one active component and a second procedure was developed for isolating one of them.
Materials and Methods

Antiviral bioassays

All fractions obtained from separation procedures were assayed for activity against Sindbis virus in the presence and absence of UVA light following the procedure described in the Materials and Methods section of Chapter II. The virus / extract mixture was exposed to 30 min of UVA prior to addition to the cell layer.

Fractions were dried to residues first under reduced pressure at below 40°C then under a stream of nitrogen gas. The residues were weighed and redissolved in methanol to known concentrations for assay. With fractions that were not completely soluble in methanol, acetonitrile was used instead, or dimethyl sulfoxide (DMSO) was added. The fraction was diluted into PBS for assay such that the solvent concentration was never above 5% and the DMSO 0.1%. These concentrations have been shown to have no effect on the cells or virus.

Crude extract preparations

Air-dried leaves and twigs of Elsholtzia ciliata (Thunb.) Hylander were obtained from the Kunming area of Yunnan. The first two samples were collected from the Forestry Institute Reserve at An-Ning 40 km. from Kunming. The latter samples were collected at Heilongtan Park in Kunming. The plant material was ground to a powder using a Wylie mill. The powder was extracted exhaustively with methanol, with cold extraction followed by repeated percolations. Extracts were concentrated under reduced pressure at below 40°C. The crude extract used in the second separation described (B) was obtained as a freshly extracted residue from the Phytochemistry Laboratory of the KIB. It was air-shipped from Hong Kong to Vancouver.
Chemical Separations

Liquid-liquid partition chromatography

- crude methanolic extract : 5x volume H\textsubscript{2}O
- partition successively in order of increasing polarity with:
  - hexanes
  - diethyl ether
  - chloroform
  - ethyl acetate
  - butanol

Flavonoid extraction

Ethanolic crude residue was washed with boiling H\textsubscript{2}O, filtered and separated with H\textsubscript{2}O saturated butanol. The butanol fraction was dried and taken up with methanol for assay.

Column liquid chromatography (CC) and thin layer chromatography (TLC)

Separation of active solvent fractions were tried with chromatographic systems using the following stationary phases, and different organic solvent gradients:

- silica column (Silica gel 60, 70-230 and 230-400 mesh)
- TLC (Merck Kieselgel 60)
- alumina TLC (Eastman Kodak 6062)
- polyamide TLC (MN-Polyamid DC 6.6)
- Sephadex LH20 column (Pharmacia)
- acetylated polyamide column (MN-Polyamid SC6-AC)
Charcoal separation

An active column fraction was swirled with activated charcoal powder and washed repeatedly over celite through a sintered glass funnel. The eluted fraction was concentrated for assay.

High performance liquid chromatography (HPLC)

Active column fractions were further separated on HPLC using a Waters 660E controller, and the Waters 994 photodiode array detector (Millipore). The UV detector was set to scan for absorption at 330nm, as UVA-enhanced active column fractions have shown absorption at this wavelength. Normal phase separations were done using an analytical Merck Hibar Lichrosorb Si60 column (5 μM particle size), with chloroform as the major eluting solvent. Methanol was added to increase polarity for both isocratic and gradient systems in the process of optimizing separation. Up to 0.2% triethylamine or up to 1% acetic acid were added as weak ionic buffers. Reverse phase separations were done using an analytical Varian MCH10 column (10 μM particle size), with acetonitrile (MeCN): water isocratic or gradient elution systems. In buffered systems, 1% acetic acid was added. Flow rate was 1 ml/min.

Structural Analyses

Ultraviolet Spectroscopy

UV spectra of liquid column fractions were obtained on the Pye Unicam SP8-100 UV spectrophotometer. Spectra from HPLC fractions were obtained during the separations on the Waters 994 photodiode array detector.
Infrared Spectroscopy (IR)

IR spectra were obtained by C. De Soucy-Brau on the Perkin-Elmer 1710 infrared spectrophotometer with Fourier transform, using chloroform as solvent, in the Chemistry Department, UBC.

Mass spectroscopy (MS)

Electron impact mass spectra were obtained using both gas chromatography (GC) electron impact MS, and from solid probe samples on a Finnigan 1020 automated GC/MS.

High resolution mass spectroscopy

High resolution MS was done by the Mass Spectrometry Centre at the Chemistry Department of UBC and the fragmentation analyzed with the DS-55 MS data system.

Proton nuclear magnetic resonance spectroscopy (\(^1H\)-NMR)

Proton NMR spectra were obtained at 400 MHz, using CDCl\(_3\) or CD\(_2\)Cl\(_2\) as solvent and tretramethylsilane (TMS) as internal standard, on the Brucker instrument at the NMR facilities of the Chemistry Department of UBC.
Results

Chemical separation and purification

Liquid-liquid solvent partitioning

In solvent partitioning of the crude extract, there was activity in the three least polar solvent fractions. The results are shown in Table 3.2

Table 3.2

Concentrations of Solvent Partition Fractions Active Against Sindbis Virus with UVA Light

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Active Concentration $\mu$g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanolic crude</td>
<td>0.3</td>
</tr>
<tr>
<td>hexane</td>
<td>$&lt; 0.15$</td>
</tr>
<tr>
<td>ether</td>
<td>$&lt; 0.15$</td>
</tr>
<tr>
<td>chloroform</td>
<td>0.65</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>not active</td>
</tr>
<tr>
<td>butanol</td>
<td>not active</td>
</tr>
</tbody>
</table>

Flavonoid extraction and charcoal separation

The fractions obtained from these procedures were not active.
Fractionation procedures

A. Starting with crude extraction of 400 g of dry plant material, followed by solvent partitioning.
Fractionation procedure A was developed from empirical trials for better separation of active components. A single active component was not isolated from the procedure. Two active HPLC fractions were obtained with normal phase buffered conditions. The retention times (RT) of these two fractions differed by 18 minutes, indicating a difference in polarity. The two active peaks representing these fractions are indicated in the HPLC chromatograph in Figure 3.2. It was seen on TLC that these fractions had multiple components, but further separation was stopped at this point as the yield of this active material was less than 1 mg from the 400 g dry plant sample. Any further separations would result in insufficient quantities for further structural analysis.

B. Starting from methanolic crude extract residue from 8 kg of fresh plant material obtained from the Phytochemistry Laboratory of KIB.
**Figure 3.2:** Normal Phase HPLC Chromatograph of *Elsholtzia* Fraction
(Peaks representing fractions showing antiviral activity indicated by arrows)
Fractionation procedure B was developed using a much larger quantity of material for separation. It resulted in the isolation of an active compound from the most nonpolar fraction of the extract. The active components that exhibited a more polar nature have not been purified.

A single peak indicated by the HPLC chromatograph was collected and found to be active (RP MCH10 column, 7:3 CH$_3$CN : H$_2$O, 1 ml/min flow rate, RT 13 min, UV 350 nm detection). The purification of the peak by repeated HPLC injection is shown in the chromatographs in Figure 3.3. The yield was 700 µg from 8 kg of fresh plant material, or 87 parts per billion (ppb).
Figure 3.3: Separation of Active Compound By Reverse Phase HPLC

A. UV absorption peak of compound targetted for isolation in HPLC chromatograph (indicated by arrow).

B. UV spectra of individual compounds from series of chromatographic peaks.

C. Chromatograph of isolated compound (UV spectrum shown in Figure 3.5).
Structural identification

The active compound was identified as the polycyclic aromatic hydrocarbon fluoranthene, \( \text{C}_{16}\text{H}_{10} \). The chemical structure is shown in Figure 3.4.

Figure 3.4: Chemical Structure of Fluoranthene

Ultraviolet spectroscopy

The UV spectrum was obtained from the HPLC photodiode array detector and shown in Figure 3.5. Molecular extinction coefficients (\( \varepsilon \)) were calculated for the absorption maxima (\( \lambda_{\text{max}} \)) from absorption units using Beer's Law and compared with published values for fluoranthene in Table 3.3.
Figure 3.5: UV Spectrum of Purified Fluoranthene

Table 3.3

Extinction Coefficients from Ultraviolet Spectra of Fluoranthene

<table>
<thead>
<tr>
<th>( \lambda_{\text{max}} \text{ nm} )</th>
<th>( \text{AU} )</th>
<th>( \text{calculated} \ \varepsilon )</th>
<th>( \lambda_{\text{max}} \text{ nm} )</th>
<th>( \varepsilon )</th>
</tr>
</thead>
<tbody>
<tr>
<td>236</td>
<td>0.510</td>
<td>50000</td>
<td>235</td>
<td>39800</td>
</tr>
<tr>
<td>277</td>
<td>0.224</td>
<td>21960</td>
<td>276</td>
<td>22400</td>
</tr>
<tr>
<td>287</td>
<td>0.328</td>
<td>32160</td>
<td>287</td>
<td>31600</td>
</tr>
<tr>
<td>323</td>
<td>0.060</td>
<td>5880</td>
<td>323</td>
<td>5750</td>
</tr>
<tr>
<td>343</td>
<td>0.080</td>
<td>7840</td>
<td>342</td>
<td>7760</td>
</tr>
<tr>
<td>359</td>
<td>0.080</td>
<td>7840</td>
<td>358</td>
<td>7950</td>
</tr>
</tbody>
</table>

\( \text{AU} = \) absorption unit from Waters 994 photodiode array detector,
(concentration of sample injected = 1 mg/ml)

* from Morton (1975) *Biochemical spectroscopy.*

\( \varepsilon = \) extinction coefficient \( \lambda_{\text{max}} = \) absorption maximum
IR Spectroscopy

The IR spectrum indicated the presence of aromatic components in the region of 700 - 800 cm\(^{-1}\) and 1200 cm\(^{-1}\). The spectrum is shown in appendix III.

Mass spectroscopy

The EI spectrum of a solid probe sample with the relative abundance of fragments is shown in Figure 3.6.

High resolution mass spectroscopy

The molecular ion mass of 202.0786 was matched to the mass of 16 carbon and 10 hydrogen atoms, with 0.4\% deviation. The fragment analysis is shown in Appendix IV.

Proton nuclear magnetic resonance spectroscopy

The \(^1\)H-NMR spectra of the purified sample and the authentic sample from Aldrich Chemicals of fluoranthene are shown in Figure 3.7.
Figure 3.6: El Mass Spectrum of Purified Fluoranthene
(Solid probe sample on Finnigan 1020 automated GC/MS)

E/I MS m/z (relative abundance): 204 (1.47), 203 (17.84), 202 [M+ C_{16}H_{10}] (100), 201 (14.45), 200 (25.46), 174 (5.91), 150 (5.98), 111 (5.22), 101 [C_{8}H_{5}] (72.28), 100 (60.28), 99 (20.05), 88 [C_{7}H_{4}] (57.74), 87 (32.31), 75 (23.25), 55 (28.76), 49 (62.49), 41 (31.11).

The fragment assignments were obtained from the high resolution MS fragment analysis shown in Appendix IV.
Figure 3.7: NMR Spectra of Purified (A) and Authentic (B) Samples of Fluoranthene.
Spectral assignments are shown in the following table:

**Table 3.4**

**1H-NMR Spectral Assignments of Fluoranthene**

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shift (ppm)</th>
<th>Coupling</th>
<th>Coupling Constants (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ 1 = δ 6</td>
<td>7.97</td>
<td>d</td>
<td>J = 7.00</td>
</tr>
<tr>
<td>δ 2 = δ 5</td>
<td>7.66</td>
<td>dd</td>
<td>J = 7.00, 8.13</td>
</tr>
<tr>
<td>δ 3 = δ 4</td>
<td>7.87</td>
<td>d</td>
<td>J = 8.13</td>
</tr>
<tr>
<td>δ 7 = δ 10</td>
<td>7.95</td>
<td>dd</td>
<td>J = 3.12, 5.51</td>
</tr>
<tr>
<td>δ 8 = δ 9</td>
<td>7.40</td>
<td>dd</td>
<td>J = 3.12, 5.51</td>
</tr>
</tbody>
</table>

Carbon positions are shown in Figure 3.4.  
$d$ = doublet  
$dd$ = doublet of doublet  
Coupling constants were calculated from a 10Hz/cm expansion.
Antiviral activity of fluoranthene

The antiviral activities of fluoranthene with and without exposure to UVA are shown in Table 3.5. Both purified and authentic samples were tested and showed the same potency.

Table 3.5

Minimum Active Antiviral Concentrations of Fluoranthene

<table>
<thead>
<tr>
<th>Virus</th>
<th>Active Concentration μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SINV</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>MCMV</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>polio</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>N.A.</td>
</tr>
</tbody>
</table>
Discussion

The bioactivity-guided empirical approach was used to separate the unknown active components. The basic scheme was to separate the plant extract by chemical nature and pursue further separation of the most active fraction after each step. Preliminary separation by nature of polarity was done with successive partitioning of crude extract against solvents of increasing polarity. The antiviral activity was found to be located in the three least polar solvent fractions. Column chromatography was continued with one fraction and in a number of trials, the activity was found to spread over a series of fractions with several different chromatographic systems and conditions. Procedure A was developed first in the chemical separation of the active fraction in *Elsholtzia ciliata*. Silica is the most commonly used chromatographic stationary phase material. It is applicable in the separation of a wide range of compounds but its adsorptive nature will tend to retain some chemical types and give poor separation. Compounds of ionic nature or those with polar functional groups are known to separate poorly and give tailing bands in liquid-solid chromatography (Snyder and Kirkland, 1979). In an effort to achieve better resolution of the activity than that obtained using silica systems, other stationary phase sorbent materials were selected to cover a range of characteristics that are considered suitable for the separation of different types of chemical compounds (Touchstone and Dobbins, 1983). Alumina, like silica, separates by order of polarity but can give better separation of some compounds such as bases and quinolines. Polyamide and acetylated polyamide are considered to have less adsorptive properties and may give better resolution of compounds that have a tendency to be retained. Sephadex LH20 is a chemically modified dextran polysaccharide used in gel filtration chromatography which separates more by molecular size than by adsorption. Other chromatographic conditions such as column dimensions and solvent elution gradients were also varied. Significantly better separation of the active fraction was not obtained by modifying the adsorbent natures and solvent polar strengths.
The approach of testing fractions obtained from following established separation procedures for a group of potential bioactive compounds was tried. The flavonoid fraction was not found to be active. The active fractions from solvent partitioning and preliminary column separations were strongly pigmented by chlorophylls. Treatment with charcoal was used for the removal of pigments, but assay of the remaining fraction showed that the active component was retained by the charcoal. Therefore charcoal treatment could not be used as a separation step.

It was considered that the lack of resolution in chromatography was due to the chemical nature of the active components. Since ionic compounds are known to give poor liquid-solid chromatographic resolution, the extract was separated into acidic, basic and neutral portions following classic organic chemical separation methods (Shriner et al., 1980). The activity was found to be in the acidic portion. Chromatography of the acidic portion showed better resolution with the addition of a weak base or weak acid as a buffer in the solvent system. This supported the hypothesis that ionic components contributed to the poor chromatographic resolution. HPLC separations indicated that there was more than one active component. As it stands, the active constituents in the acidic fraction following procedure A have not been isolated. It has been shown that ionic compounds inhibit Sindbis virus infection by affecting its binding to cell membrane receptors (Mastromarino et al., 1991). This inhibition would take place in the adsorption process. The *Elsholtzia* fractions were active in treatments prior to cellular entry of the virus that would include the adsorption process. Since the active fraction exhibited ionic properties, it is possible that the active component exhibits this type of action.

When it was determined that the plant sample used to develop procedure A would be insufficient to yield the active compounds in enough quantity for structural identification, a larger quantity of crude extract was obtained from Yunnan. With a larger quantity of extract, a nonpolar active fraction in a workable quantity could be separated from the hexane portion of
the initial solvent partition. Procedure B was developed for the isolation of an active compound and it did not require the pH partitioning and buffering treatments of procedure A. It is likely that the more polar active component that has yet to be purified was responsible for the ionic behavior of the active fraction in procedure A. The development of procedure A was the result of addressing the chemical nature of this more polar component because it was present in a higher concentration in comparison to the less polar active component. The presence of a very nonpolar active fraction had been observed in some of the chromatographic trials but its concentration in the smaller sample really did not allow for further purification. As it turned out, the nonpolar active compound identified represented only 87 ppb of plant material.

The purified active compound was identified as the polyaromatic hydrocarbon (PAH) fluoranthene, with the structural formula of $C_{16}H_{10}$. The fragmentation pattern of the mass spectrum gave a 98% match with that of fluoranthene in the computerized National Bureau of Standards Mass Spectra Library. High resolution MS was done to confirm the atomic composition. Since PAHs are not known plant compounds, other possible molecular compositions containing nitrogen and/or oxygen were considered. The atomic formula of fluoranthene gave the closest fit to the molecular ion mass of 202.0786. The other major fragments also showed the closest fit with a compound composed of carbon and hydrogen.

The low field shifts in the proton NMR spectrum indicated the presence of aromatic components. The behavior in chromatography and the characteristics of the UV spectrum suggested the possibility of photoactive plant compounds such as thiophenes, which are sulfur heterocycles. The UV spectra of acetylenic compounds often exhibit a series of peaks in the 300-400 nm range with a maximum between 220-280 nm (Bohlmann et al., 1973). The characteristic series of peaks in the acetylenes is attributed to the series of conjugated bonds. The spectra of PAHs, however, exhibit a very similar pattern. Aromatic rings have series of conjugated bonds that have the same UV absorption characteristics (Morton, 1975).
max and extinction coefficients calculated from absorption units of the spectrum from a purified sample matched published values for fluoranthene. Since fluoranthene has no substitution functions, the IR spectrum only indicated the presence of aromatic groups.

The chemical shifts and coupling constants obtained for the purified sample could not be matched to any published NMR spectra for thiophenes or other aromatic structures. When other data indicated the structure of fluoranthene, the spectrum was compared to those of fluoranthene in *The Aldrich Library of NMR Spectra* (Ponchart and Campbell, 1973) and *The Sadtler Handbook of PNMR* (Simmons, 1978). The chemical shifts were in the same field region between δ 7.15-8.0 ppm, the multiplets were more confused in the published spectra. The splitting patterns were also not the same as those analyzed by Heffernan et al. (1967). However, their spectrum was obtained at 100 MHz whereas the purified sample spectrum was obtained at 400 MHz. A sample of fluoranthene was purchased from Aldrich Chemicals (F80-7) and subjected to the same 400 MHz NMR scan. A spectrum with the same shifts and splitting patterns between δ 7 and 8 ppm as the purified sample spectrum was obtained (Figure 3.7). Fluoranthene is a bilaterally symmetrical molecule, therefore the spectral assignments showed only five types in the ten protons.

With the match of the NMR, Mass, and UV spectra and HPLC chromatograms of an authentic fluoranthene sample to those of the purified compound, no further analyses were done. Antiviral components from the more polar active fraction of the *Elsholtzia* extract have yet to be isolated and identified. Their identification is also an interesting question in regard to whether photo-active plant compounds occur in *Elsholtzia* and the Lamiaceae.

Fluoranthene is not known as a plant constituent but it was isolated from a plant extract using bioassay-guided fractionation. It was found as a light-mediated antiviral activity target compound because it showed potent UVA-enhanced activity against Sindbis virus (10 ng/ml
with UVA and 5 µg/ml in dark). There were several other HPLC peaks within a 20 minute retention range of fluoranthene (see Figure 3.3). They were not purified for identification but those HPLC fractions also showed antiviral activity. The PAHs phenanthrene and pyrene (obtained from Dr. J. Kagan, Department of Chemistry, University of Illinois) were injected on HPLC for comparison. They could be matched by retention time and by UV spectra to peaks in the active HPLC fractions. It is probable that a group of PAHs are present in the plant extract, and they could have also contributed to the light-mediated antiviral activity. The antiviral mechanism of fluoranthene will be discussed in the next chapter, but how this compound came to be isolated from a plant sample has to be considered.

It is possible that the source of the PAHs was from contamination in the purification procedure. Large volumes of solvents were used to process a large sample of plant extract and a compound present in less than 1 part per million (ppm) was isolated. With separation and analytical techniques capable of purifying trace components, it has become more of a possibility that impurities from materials used in the processing can be accumulated and be found in an identifiable quantity. The major solvent used in this procedure consisted of a mixture of n-hexanes. The impurities remaining after the evaporation of 4 liters of hexanes under reduced pressure was submitted for NMR (De Soucy-Brau, Chemistry Department UBC, unpublished data). While there was a peak in the aromatic region of the spectrum, it did not match any of those obtained for fluoranthene, nor was the spectrum complex enough to indicate the possible presence of PAHs. As another check, a small amount of the crude extract (60mg) was processed using only 60 ml of hexanes. The fluoranthene peak was obtained on the HPLC trace from this treatment, further indicating that it was not from the accumulation of impurities from hexanes. Another piece of evidence supporting this conclusion is that UV fluorescent nonpolar active fractions had been detected in earlier chromatographic trials using other solvent fractions of the plant sample. Even though they were not chemically identified, it is likely that they consisted of the PAHs and were not solvent-related.
Polyaromatic hydrocarbons have been found in foliage as well as in crops (Buckley, 1987). PAHs are generated by the incomplete combustion of fossil fuels, especially coal (Williamson, 1973), and are found throughout the environment in air, water, sediment and soil. The levels in the environment can be correlated to levels of human activities (Jones et al., 1989). Analyses of plant foliages have been used to monitor levels of anthropogenic chemicals present in the environment (Eriksson et al., 1989). Wild and Jones (1991) extracted total PAH contents in the range of 5 - 16 µg/kg fresh weight from carrots. Jones et al. (1992) archived annual vegetation samples from 1965 to 1989 for analysis of levels of polychlorinated biphenyls and PAHs. They found total level of 13 PAHs to range from 187 to 472 ng/kg dry weight. The level of fluoranthene ranged from 9 to 31 ng/kg dry weight. In comparison, the yield from *Elsholtzia ciliata* of 87 µg/kg of fresh plant material was higher but of a similar magnitude to PAH levels reported from plant sources.

If the explanation for the occurrence of PAHs in the *Elsholtzia* samples involves accumulation of environmental pollutants, then such compounds might be expected to be found ubiquitously in the other species tested. In a study of chlorinated hydrocarbons, Calamari et al. (1991) found them to be present in foliage in all parts of the world. Furthermore, there is a linear relationship between concentrations of chemicals in foliage and in air. The question then arises as to why the antiviral activity shown by the PAHs in *Elsholtzia ciliata* was not seen in all the Yunnan plants screened, or at least from samples collected in the same area. The level of PAHs present generally in plants may not be high enough to generate antiviral activity. In *E. ciliata*, besides the PAHs, there was another active component that has not been identified. The high activity of the plant extract in the screening program was probably from the combined effect of several compounds, and the first compound to be identified from bioassay-guided fractionation was a PAH. Another mint plant (*Stachys*) also showed UVA-enhanced activity in the antiviral screening; the active component(s) had not been identified. It would be
interesting to see if there is also a combination of PAHs and other active components present in *Stachys kouyangensis*.

It is also possible that *Elsholtzia* accumulates a higher concentration of PAHs. Buckley (1982) had found variation between species when examining the accumulation of PCBs in foliage. In a review, Schönherr and Riederer (1989) discussed the properties of chemicals and plant cuticles affecting the foliar uptake of chemicals. They considered the leaf surface to be a major interceptor of airborne pollutants by calculation of geometric area alone. Riederer (1990) followed with a theoretical model of the foliage / atmosphere system to calculate the partition coefficients for reference chemicals with different components in the air-to-vegetation transfer of persistent organic chemicals. The values show that the lipophilic portion of the foliage, representing the cuticle in the model, can effectively scavenge lipophilic molecules from the surrounding atmosphere. It is thus possible that foliage with more lipophilic components will also have a greater tendency to accumulate airborne lipophilic molecules like the PAHs.

Plants of the mint family often have high contents of lipophilic molecules in trichomes, as demonstrated by the fact that they are the sources of many aromatic oils (Baumgardt, 1982). This family characteristic may lead to a higher accumulation of PAHs, and other pollutant molecules in *Elsholtzia*. Four species were assayed and three of them showed UVA-enhanced antiviral activity. The species *E. densa* showed more moderate activity which was not UV-mediated. This is an annual plant whereas the other three are perennial shrubs. It is likely that this difference arises from greater sequestering of environmental compounds by perennial plants. Since many PAHs have been shown to be carcinogenic (Jones, 1982), their possible accumulation in the Lamiaceae might raise health-related concerns regarding the popular consumption of herbs from the mint family. The topic of differential accumulation needs further investigation.
It was an unexpected outcome that the compound isolated from this bioassay-guided search for active molecules in a medicinal plant species is probably not synthesized by the plant. This occurred because the photo-activity of fluoranthene met the selection criteria of the UVA-mediated antiviral screening program. The pharmacognosist should be aware of that the process of isolating a bioactive natural products can lead to the identification of anthropogenic compounds. The presence of anthropogenic contaminants in the environment and in organisms has been of concern as possible threats to human health because of their known or potential toxicity and carcinogenicity. Since many of these chemicals display bioactivity, it is perhaps not surprising to find a "pollutant" in a search that targets by detection of bioactivity. As many plants provide food and medicines, the distinction between environmental contaminants and phytochemicals in plants has significant implications in human health. Plants are also an important component of the environment as a whole, and further investigations into the relationship between environmental chemicals and phytochemistry may contribute to our understanding of human impact on the global environmental quality.
Chapter III. References


NAPRALERT database (1992) issue 2, maintained by the Program for Collaborative Research in the Pharmaceutical Sciences, Dept. of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago.


Chapter IV.

Structure - Activity Relationship Studies of the Antiviral Compound Hypercin

Introduction

The aim of this project was to conduct a thorough study of a biologically active compound, beginning from a screening search and continuing with the isolation of the active compound to the investigation of its nature of action. Medicinal plants from Yunnan, China, were screened for antiviral activity and the species *Elsholtzia ciliata* was selected for the isolation of an active component. Ideally, the nature of action studies would have been carried out with the purified compound. However, during the empirical development of the purification process, it appeared that the time it would take to follow the original plan would be of concern. It was decided to conduct the nature of action studies with an alternative known antiviral plant compound while the purification of the *Elsholtzia* component was still in progress. Hypericin is a photosensitizer antiviral which has been isolated from *Hypericum* (Hypericaceae). Since the activity of *Elsholtzia* was shown to be light-mediated, there is a similarity in the photosensitization nature of the activity. *Hypericum japonicum* Thunb. and *H. patulum* Thunb. are both used in Yunnan to treat hepatitis and were on the list compiled of candidates for antiviral screening (Table 1.1, Chapter I) even though plants were not collected from Yunnan in this survey. In view of the photo-active nature of the compounds, it is useful to begin with a general discussion of photosensitizer compounds.
**Photosensitizer Compounds**

A photosensitizer is a chemical that can have an electron promoted to a higher energy orbital with the absorption of a photon. The excited state molecule in turn reacts with a second molecule. Reactions where electronically excited molecules generate radicals which act as intermediates are classified as type I, and reactions where only electronically excited molecules act as intermediates are classified as type II (Gollnick, 1968). Frequently in biological systems the intermediate reacts with oxygen and a reactive oxygen species is generated. Singlet oxygens are reactive oxygens with promoted electron energy states, and they can be generated by an input of energy (Halliwell and Gutteridge, 1989). Other reactive oxygens are generated via free radicals, the addition of a single electron generates the superoxide anion, and the addition of one more electron produces the peroxide ion. These two types of reactions do not necessarily occur exclusive of each other. Plant photosensitizers have commonly exhibited type II reactions with the formation of singlet oxygen (Hudson and Towers, 1991). In the cascading process, when the reactive molecules affect a target molecule and produce a biological effect, the result is considered a photodynamic reaction (Towers, 1984; Spikes, 1989). The activity can be mediated by long wavelength ultraviolet UVA (320-400 nm) or visible light. Some common photosensitizers are pigments, ketones, quinones and aromatic molecules (Foote, 1987). The requirement of light for the activity or enhancement of activity for these compounds can be used as a point of experimental manipulation in studying their modes of action.

Free radical-mediated reactions are part of normal cellular metabolic functions but these type of reactions can also cause harmful biological effects in the form of membrane or protein damage, or lipid peroxidation (Fantone and Ward, 1985). The biological activity of the photo-active compounds has potential therapeutic application when these types of tissue damage are selectively targeted at micro-organisms, pests or tumor cells. Viruses in
particular are also susceptible to effects of reactive molecules on the nucleic acid strands. Photosensitizers have been shown to have antimicrobial, antiviral and insecticidal effects (Marchant, 1987). Many different classes of chemicals have been found as naturally occurring phototoxins. The major classes are: the polyketides (polyyynes, thiophenes, quinones and chromenes), cinnamate derivatives (coumarins and furanyl compounds), and alkaloids (derivatives of tryptamine, phenylalanine, tyrosine and anthranilic acid). The actions and therapeutic prospects of plant photosensitizers were reviewed by Hudson and Towers (1991). The antiviral properties of three groups of compounds were reviewed by Hudson (1989). The activities of thiophenes and polyyynes are attributed to singlet oxygen damage to viral membranes (Hudson and Towers, 1988). The generation of singlet oxygen in UVA by thiophenes and related compounds has been demonstrated (Garcia et al, 1984). Membrane damage by the acetylenic compounds α-terthienyl and phenylheptatriyne has also been shown (MacRae et al., 1980). Furanyl compounds such as furanocoumarins, appear to act through the formation of covalent adducts with viral nucleic acids (Hradecna and Kittler, 1982; Hudson et al., 1988). The compounds are thought to intercalate with the nucleic acids at the pyrimidine bases. The resulting adducts would prevent the execution of normal genetic transcription or translation (Song and Tapley, 1979; Cimino et al., 1985). The action of the furanoquinoline alkaloids is similar to that of the furanocoumarins (Pfyffer et al., 1982). The β-carboline alkaloids, however, do not cause cross-linking with viral DNA (Altamirano et al., 1986). They probably do act on the nucleic acids as they have been shown to have interactions with DNA in studies that did not consider the role of light (Duportail and Lami, 1975; Hayashi, et al., 1977), and to cause UVA-mediated chromosomal damage in cells (Towers and Abramowski, 1983).

In considering the therapeutic applicability of photosensitizers, the question arises as to how the light is administered to activate the compounds. The situation is simple in the case of topical applications. Psoralen-UVA therapy has been used in the treatment of the
skin affliction psoriasis (Parrish et al., 1974; Gupta and Anderson, 1987). A photochemotherapy system was also developed for the treatment of cutaneous T-cell lymphoma with methoxypsoralen with extracorporeal photo-activation of patients' blood fractions (Edelson et al., 1987). It is now also possible to direct light to specific internal tissues with the use of laser fiber-optic technology. Coupled with the ingestion of a photosensitive chemical, the requirement of light for activation then becomes a tool for selectively targeting tumor tissues for sites of action (Douglas et al., 1981). A number of studies have also looked at the use of photosensitizers in the sterilization of blood products, especially with regard to the inactivation of viruses which may not be removed by filtration methods applicable to other micro-organisms (Matthews et al., 1988; Nyendorff et al., 1990). The sterilization may be achieved with a lower concentration of a photosensitive than a non-photosensitive compound because the potency is enhanced by light. The presence of a compound that is also inactive in the dark in low concentration may be considered clinically more suitable for blood products used in transfusion. Controlling the time or location of activity by manipulating the application of light will likely lead to the development of more innovative therapeutic applications with photosensitizers.

The antiviral compound fluoranthene, isolated from *Elsholtzia*, has been shown previously to have photo-activity against a number of aquatic organisms such as fish, mosquito larvae and brine shrimp (Kagan, 1985), indicating a general toxicity to eukaryote cells. It causes hemolysis of human erythrocytes with UVA exposure through action on the cell membrane (Wang and Kagan, 1989). It also inactivates *Escherichia coli* in the presence of UVA. Tuveson et al. (1987) tested its effect on DNA repair deficient mutant *E. coli* strains and on the transforming activity of *Haemophilis influenzae* transforming DNA to show that DNA can be a target of action. Induced mutation was not shown with a histidine locus reversion assay, but this does not necessarily rule out the possibility of mutagenic activity. In the same study, photo-oxidation experiments showed the generation in light of
singlet oxygen by fluoranthene in both organic and aqueous media. There is also evidence
for the formation of superoxide anions, which is known to lead in turn to the generation of
reactive hydrogen peroxides. The exact mechanism of the photodynamic action of
fluoranthene is thus complex, and it is possible that both singlet oxygen and superoxide
production take place. Because of its phototoxicity and possible mutagenicity, the antiviral
activity of fluoranthene may not have therapeutic potential. While the activity shown was
significant, it was likely to have arisen from a non-virus specific oxygen-mediated attack on
membranes. Even though fluoranthene is regarded as one of the more benign PAHs in regard
to carcinogenicity, its role as an environmental pollutant is still to be estimated because of its
phototoxicity and undetermined mutagenicity.

Investigations of the mechanism of action of a photosensitive antiviral plant
compound was continued with the quinonic compound hypericin. The studies concerning
structure-activity relationships is covered in this chapter. The studies concerning the site and
molecular target of action is covered in the following chapter.

**Hypericin**

Species of the genus *Hypericum* have been documented as traditional medicinal
plants in several areas of the world besides China (Blanchan, 1922; Satyavati et al., 1987).
Some physiological effects could undoubtedly be ascribed to a photosensitive component.
In a folk prescription of a *Hypericum* extract as an antidepressant, there is a warning to fair
complexioned people about possible side effects of skin irritation (Moore, 1979). Certain
species of *Hypericum* have a photosensitive effect when ingested by grazing animals and the
polycyclic quinone, hypericin, is considered to be responsible for this effect (Giese, 1980).
There are about 200 species of *Hypericum* and the compound hypericin was first isolated
from the common European species *Hypericum hirsutum* L. It is a fluorescent red pigment.
The structure of the naphthaquinone system that forms the hypericin skeleton is shown with the numbering of the carbons in Figure 4.1.

Hypericin causes hemolysis of erythrocytes with exposure to light (Pace and Mackinney, 1941). Hypericin has been shown to have antiviral activity against a number of viruses. These include: radiation leukemia virus (Meruelo et al., 1988), Friend leukemia virus, murine immunodeficiency virus (Lavie et al., 1989), equine infectious anemia virus (Kraus, et al., 1990), human immunodeficiency virus type 1 (HIV-1) (Schinazi et al., 1990), Moloney murine leukemia virus, influenza virus A, (Tang et al., 1990), vesicular stomatitis virus, herpes simplex virus types 1 and 2, parainfluenza virus, and vaccinia virus (Andersen et al., 1991), Sindbis virus, murine cytomegalovirus (Hudson et al, 1991), human cytomegalovirus (Barnard et al., 1992), and duck hepatitis B virus (Moraleda et al., 1993). In only three of these studies was the role of light taken into account (Carpenter and Kraus, 1991; Hudson et al., 1991; Moraleda et al, 1993).
Structure-activity studies elucidate the molecular geometry and chemical nature of active compounds that are necessary for activity or which affect the potencies. Chemical derivatives, relatives and analogues of an active compound are tested and compared for activity in order to discern the pertinent features. Several studies treated antiviral structure-activity relationships of hypericin and related quinonic compounds in the anthraquinone and anthrone groups. Pseudohypericin, which has a hydroxymethyl substitution instead of one of the methyl groups, shows diminished activity (Meruelo et al., 1988). By testing quinones containing parts of the hypericin ring system, Kraus et al. (1990) propose that the complete ring structure is required but is not sufficient for antiviral activity. Andersen et al. (1991) found activity against enveloped viruses with derivatives of emodin. Emodin can be thought of as the top portion of the hypericin ring system. The activity increases, though, with expansion of the ring system to a bianthrone and to the full hypericin. Barnard et al. (1992) found differences in activity between anthrones and anthraquinones with different hydroxyl, methyl and carboxyl substitution patterns. Schinazi et al. (1990) tested anthraquinones substituted with hydroxyl, amino, halogen, carboxylic acid, substituted aromatic, and sulfonate groups against HIV-1. They found the polyphenolic and/or polysulfonate substituted ones to be most potent. The carbonyl function of quinones is considered to be essential to the antiretroviral activity of hypericin and analogues (Kraus et al., 1990; Lavie et al., 1990). The replacement of methyl side chains with more polar groups also results in diminished activity. The influence of light was never considered in these studies.

In this study, three synthetic derivatives of hypericin and five bianthrones were screened for light-mediated activity against the membrane enveloped Sindbis virus (SINV). Sindbis virus is sensitive to hypericin activity. This activity is shown when the virus/compound mixture is exposed to fluorescent light prior to addition to cells (Hudson, et al., 1991, Lopez-Bazzochi et al., 1991). Natural compounds with similar structures were also assayed for activity against SINV and murine cytomegalovirus (MCMV).
Buckwheat, or *Fagopyrum* plants (Polygonaceae) have also been reported to cause photosensitive reactions when consumed by livestock. The compound fagopyrin has the same naphthabianthrone central ring structure as hypericin (Thomson, 1971) but it has not been tested for antiviral activity. Three fungal compounds with a polyaromatic nucleus were also tested in my studies. The perylenequinone cercosporin, first isolated from the soybean blight, *Cercospora kikuchii* (Matsumoto et Tomoyasu) Gardner, has light-sensitive activity to mice and bacteria (Yamazaki et al., 1975). Its plant pathogenic effect is lipid peroxidation resulting in leaf membrane damage. It also has antifungal effects and has been shown to produce both singlet oxygen and superoxide (Daub, 1987). Lavie et al. (1991) have reported its antiretrovirus activity. Hypocrellin is another perylenequinone isolated from *Hypocrella bambuase* (B. et Br) sacc.. In their review of the hypocrellins Zhenjun and Lown (1990) discussed reports of its photosensitive antibacterial and antitumor effects. Cercosporin, hypocrellin A and fagopyrin are all deep red fluorescent pigments like hypericin. Duclauxin is a secondary metabolite in the Talaromyces (Frivad et al., 1990). It has also been isolated from *Penicillium stipitatum* Thom (Kuhr and Fuska, 1973). It has a complex polycyclic central skeleton that is dimerized from oxaphenalenones (Chexal et al., 1979).
Materials and Methods

Chemicals

Hypericin, three derivatives of hypericin, and five bianthrones were synthesized in the laboratory of Dr. L.H. Zalkow at the Georgia Institute of Technology, Atlanta, Georgia (Gruszecka-Kowalik and Zalkow, 1991; Zembower, 1990). The chemical structures are shown in Figures 4.2 and 4.3.

The crude extracts of *Fagopyrum esculentum* Moensch flowers and of *Hypericum perforatum* L. were provided by Z. Abramowski (Botany Dept. UBC). Dry residues were dissolved in ethanol for the bioassays. The structure of the compound fagopyrin found in *Fagopyrum* is shown in Figure 4.4.

The compound duclauxin was provided by Dr. J. Jacyno (Microbial Products Research Unit, South Atlantic Area Agricultural Research Center, U.S. Department of Agriculture, Athens, Georgia). Cercosporin was provided by Dr. M. Daub. (Department of Plant Pathology, North Carolina State University, Raleigh). Hypocrellin A, hypocrellin peroxide and crude extract residue of *Hypocrella bambuase* were provided by Prof. J. Zhou (Phytochemistry Laboratory, Kunming Institute of Botany). The structures of these compounds are shown in Figures 4.5-4.7.
Figure 4.2: Chemical Structure of Hypericin and Derivatives

Hypericin and Derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>R</th>
<th>R₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypericin</td>
<td>CH₃</td>
<td>H</td>
</tr>
<tr>
<td>EGK-138</td>
<td>COOH</td>
<td>H</td>
</tr>
<tr>
<td>EGK-149</td>
<td>CH₃</td>
<td>CH₂SCH₂CH₂CO₂H</td>
</tr>
<tr>
<td>EGK-162</td>
<td></td>
<td>Hypericin glucoside</td>
</tr>
</tbody>
</table>

Figure 4.3: Chemical Structure of Bianthrone

Bianthrone

<table>
<thead>
<tr>
<th>Derivative</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZ-IV-48</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>DZ-IV-54</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>DZ-IV-55</td>
<td>OH</td>
<td>CH₃</td>
</tr>
<tr>
<td>DZ-IV-64</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>DZ-IV-65</td>
<td>H</td>
<td>CO₂H</td>
</tr>
</tbody>
</table>
Figure 4.4: Chemical Structure of Fagopyrin

Figure 4.5: Chemical Structure of Duclauxin
Figure 4.6: Chemical Structure of Cercosporin

Figure 4.7: Chemical Structures of Hypocrellin Compounds
Antiviral Assays

The extracts or chemical compounds were assayed for inhibition of viral cytopathic effects in serial dilutions following the same protocol described in the Material and Methods section of Chapter II. One set of the compound/virus mixtures were exposed to 30 min of visible light before adding to the cell layers for infection. A parallel set was kept in the dark. For cytotoxicity assays, the compounds were exposed to 30 min of light. The adsorption time on the cells take place in the dark in all the treatments. Experimental manipulations with the photosensitive compounds were done in dim light. Light was provided by a bank of six fluorescent tubes (General Electric F20/CW) at the incident energy of 1.5 W/cm². The peak emission range of the fluorescent light was at 570-595 nm, and one of the major absorption peaks of hypericin is at 588 nm (Giese, 1980).

Screening of hypericin analogues was done first against Sindbis virus. The active derivative was then tested against MCMV and poliovirus. *Fagopyrum*, cercosporin and duclauxin were tested against both enveloped viruses. The hypocrellin compounds were assayed against MCMV.

Results

Screening of quinonoid compounds

The antiviral activities of eight hypericin derivatives and related quinones by microtiter assay using 2-fold serial dilutions from 10 to 0.003 μg/ml, in comparison to that of hypericin is shown in Table 4.1.
Of the three hypericin derivatives, compound EGK-149 showed significant activity which was in the same range as that of hypericin. It was then tested against MCMV and was active in the plaque assay at 1.25 µg/ml in light.

Fagopyrum Assays

The crude extracts of Hypericum and Fagopyrum were assayed in 2-fold serial dilutions starting from the highest concentration of 100 µg/ml. The results are shown in Table 4.2.

Fungal Compounds Assays

In 2-fold serial dilution assays starting from the highest concentration of 100 µg/ml, the compound duclauxin showed no activity. The activity of cercosporin is shown in Table 4.3. Cercosporin showed a cytotoxic effect beginning at 5 µg/ml and higher concentrations. At the concentration effective in the dark against SINV, the cells appeared abnormal even though there was no virus infection.

The activity of the hypocrellin compounds are shown in Table 4.4. The Hypocrella extract and hypocrellin A both showed light-enhanced activity but the endoperoxide of hypocrellin did not. However, hypocrellin A began to show cytotoxicity at a concentration of 10 µg/ml.
Table 4.1

**Minimum Active Concentrations of Hypercin Compounds Against Sindbis Virus**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>Hypericin</td>
<td>0.02</td>
</tr>
<tr>
<td>EGK-138</td>
<td>5.0</td>
</tr>
<tr>
<td>EGK-149</td>
<td>0.02</td>
</tr>
<tr>
<td>EGK-162</td>
<td>5.0</td>
</tr>
<tr>
<td>DZ-IV-48</td>
<td>N.A.</td>
</tr>
<tr>
<td>DZ-IV-54</td>
<td>N.A.</td>
</tr>
<tr>
<td>DZ-IV-55</td>
<td>* 5.0</td>
</tr>
<tr>
<td>DZ-IV-64</td>
<td>N.A.</td>
</tr>
<tr>
<td>DZ-IV-65</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A. = no activity  
* = no viral infection but visible cell effect

Table 4.2

**Minimum Active Antiviral Concentrations of Hypericum and Fagopyrum Crude Extracts**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Minimum Active Concentration, µg/ml</th>
<th>Fagopyrum</th>
<th>Hypericum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>SINV</td>
<td></td>
<td>1.0</td>
<td>50.0</td>
</tr>
<tr>
<td>MCMV</td>
<td></td>
<td>10.0</td>
<td>&gt;100.0</td>
</tr>
</tbody>
</table>
Table 4.3

**Minimum Active Antiviral Concentration of Cercosporin**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Active Concentration (μg/ml)</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>SINV</td>
<td>0.3</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>MCMV</td>
<td>2.5</td>
<td>10*</td>
<td></td>
</tr>
</tbody>
</table>

* No viral infection but showing cytotoxic effects.

Table 4.4

**Minimum Active Antiviral Concentrations of Hypocrella Compounds**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Hypocrella extract</th>
<th>Hypocrellin A</th>
<th>Hypocrellin peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCMV</td>
<td>0.30</td>
<td>0.15</td>
<td>10*</td>
</tr>
</tbody>
</table>

* No viral infection but showing cytotoxic effects.

(Assays for SINV not interpretable due to loss of infectivity in virus stock)
Discussion

In the screening of hypericin relatives and similar compounds, light-mediated antiviral activity was found in the crude extract of the buckwheat plant *Fagopyrum*, and four of the synthetic hypericin analogues. The *Fagopyrum* extract was active in a range of concentrations similar to the *Hypericum* extract. Hypericin has been shown to be a photosensitive antiviral compound from *Hypericum*. Since the two plants have a similar history of causing photosensitive reactions, and *Fagopyrum* contains the compound fagopyrin which is structurally similar to hypericin, it is reasonable to attribute the antiviral effect of *Fagopyrum* to fagopyrin. Fagopyrin differs from hypericin only in the substitution groups at positions 3 and 4. This would suggest that the necessary structure for activity is the central naphthabianthrone ring system, or some portion of it. Lavie et al. (1990) found that the elimination of the carbonyl functions on the ring system greatly reduces reverse transcriptase inhibition.

The three synthetic hypericin derivatives (EGK compounds) tested, all differed from hypericin in only the non-hydroxyl substitution groups on the naphthabianthrone center. The three derivatives showed different degrees of potency. The compound EGK-149, 2,5,9,12-tetra(carboxyethylthiomethyl) hypericin, showed significant antiviral activity with similar potency to that of hypericin. Kraus et al. (1990) suggested that the hydroxyl groups may be important in antiretroviral activity, because the naphthabianthrone system alone is not sufficient for activity against equine infectious anemia virus (EIAV). The lesser potency of the two other derivatives found in this present study indicated that the activity from the presence of both of those features could still be greatly influenced by other substitution groups. Substitution groups of EGK-149 contain sulfur. Schinazi et al. (1990) found anthraquinones with sulfonate substitutions to be among the more active of an array tested against HIV-1, even though the potencies are much less than that of hypericin. The role of
sulfur in the molecule would be an interesting area for further structure-activity investigations. The nature of action of EGK-149 is the subject of Chapter V.

Compound EGK-138 is hypericin dicarboxylic acid. It had been found to be ineffective in reducing the production of radiation leukemia virus (RadLV) in mice by Lavie et al. (1990). They associate the reduction in activity to the increase in polarity of side chains at positions 3 and 4. Analogues with acetoxy (hypericin diacetate) and hydroxy (hydroxy-desmethylhypericin) groups at those positions also have diminished activity. Desmethyl hypericin, lacking the 2 methyl groups, has good activity in the direct inactivation of virions but is ineffective in *in vivo* assay. Meruelo et al. (1991) proposed that the methyl groups affect the retention of the molecule by cells. Compound EGK-162 is a glucoside that was not completely characterized. It is a green pigment, unlike the intense red of hypericin and EGK-149. Its different absorption spectrum may be one of the reasons for its lesser activity shown in the assay conditions.

While comments on the activity of fagopyrin should be made from studies made with the purified compound, it is interesting to note that fagopyrin and EGK-149 both have large side chains on the hypericin ring system. EGK-149, especially, has these groups at four positions that are not substituted in hypericin. All active hypericin compounds reported thus far have hydroxyl groups at carbons 1,6,8 and 13, as well as at 3 and 4 (or 10 and 11). How these substitution groups relate to the other molecular elements of hypericin deemed important for action remains to be determined.

The assay of the bianthrones relates to the effect of the nature of the central ring skeleton on activity. Bianthrones do not have the two central (naphtha) rings of the hypericin skeleton. Only one of the bianthrones assayed, DZ-IV-55 was active. Bianthrone with no side chains is not effective in inactivation of RadLV (Lavie et al., 1990). Emodin bianthrone,
however, showed activity against five viruses (vaccinia virus, parainfluenza, herpes simplex virus types 1 and 2, and vesicular stomatitis virus) in a study comparing the activity of several anthraquinones (Andersen et al., 1991). The activity is not as great as that of hypericin but the D,L-diastereoisomers of emodin bianthrone are consistently and significantly more active than the meso-isomer. This difference is attributed to the closer resemblance in three dimensional structure of the planar extended ring of the D,L-isomer to the hypericin ring, whereas the meso-isomer ring is more bent across the molecule between the two anthrone groups. Compound DZ-IV-55 in this study is an emodin bianthrone, but it is not known whether it is a mixture of isomers. It showed some activity, but the active concentrations caused an alteration to the assay cells even though they remained viable. Looking at the differences in substitution groups on the bianthrones tested, DZ-IV-55 has the same pattern as that of hypericin. This corroborates the interpretation that the substitution groups at positions 3,4 and 9,10 are significant in the structure-activity relationship.

Hypericin (8-ring) can be synthesized via emodin bianthrone (6-ring) from the monomer, emodin (3-ring), as depicted in Andersen et al. (1990). Emodin or emodin anthrone can be considered to be one of the anthrone portions of the hypericin skeleton. Andersen et al. (1991) found aloe emodin to be inactive against six viruses tested, including herpes simplex virus (HSV) types 1 and 2. Sydiskis et al. (1991) found it to inactivate HSV-1 and -2, as well as three others: varicella-zoster virus, pseudorabies virus and influenza virus. Both groups found it to be inactive against rhinovirus. Carpenter and Kraus (1991) found emodin to be inactive against EIAV, even with exposure to light. Both emodin and anthrone are inactive against RadLV (Lavie et al., 1990).

The differences in reported results is a reminder that there are many elements that can make comparisons between antiviral evaluations challenging. Assays can be done using a number of very different methods (as discussed in Chapter 2), and assays may be conducted
with a variety of viruses, and viruses may differ between strains. Stereochemistry of antiviral compounds has to be considered, and the influence of light must be incorporated into the testing strategy.

The structures of cercosporin and hypocrellin are similar to hypericin, but represent an extended quinone originating from naphthalene. They have in common the central 5-ring perylenequinone skeleton and two methoxyl substitution groups (C2, C11). Cercosporin has a methylene-dioxy bridge between C6 and C7, and two alcohol side chains (C1, C12). It did show light-mediated activity against membrane enveloped viruses in this assay, corroborating its previously reported light- and oxygen-mediated activity on membranes. Hypocrellin A has a different substitution pattern with a 7-membered ring formed between C1 and C12 and methoxyl groups at C6 and C7. It showed more potent light-mediated activity than cercosporin, but it showed cytotoxicity at concentrations higher than 10 μg/ml. The endoperoxide of hypocrellin was only marginally active. This inactivity could be explained by the fact that endoperoxides are often the adduct products of a singlet oxygen reaction and would probably not produce another photodynamic effect. It is possible for a photo-active reaction to result in an active end product. If this is not the case, it indicates that the activity occurred during the short-lived excited singlet oxygen generation state. The requirement for oxygen for the photo-action of cercosporin has been demonstrated (Yamazaki et al., 1975). It appears that the original configuration of the center ring where the peroxide formed was important in generating the activity. The formation of the peroxide function disrupts the aromatic extension between the two anthraquinone groups. It was shown in the studies of anthraquinones by Andersen et al. (1991), that antiviral activity increased with the expansion of the ring system from emodin to bianthrone to hypericin. It is likely that the same reduction in the number of extended aromatic rings would result in a loss of activity. The electrons in the p-orbitals of conjugated bonds extending over several ring systems are easily excited by visible light (Hader and Tevini, 1987) and would generate the photo-activity. The
structure-activity relationships of these compounds with perylenequinone skeletons corroborates with the conclusion that the extended quinone central skeleton is important in photodynamic reactions. In a screening for protein kinase C inhibitors, the perylenequinone calphostin C and hypericin and pseudohypericin were found to be active (Takahashi et al., 1989). The quinone skeleton was considered to be important in this similarity even though the role of light was not taken into account.

The importance of the extended quinone skeleton to photo-activity is also supported by the fact that duclauxin did not show any activity. It has a different type of polycyclic skeleton even though carbonyl functions are present and excited triplet state carbonyl groups can play a role in the transfer of energy in the promotion of a molecule like oxygen to a singlet state (Cilento, 1980). The oxaphenalenone-derived skeleton of duclauxin does not have the same degree of extension of aromatic rings as hypericin and the perylenequinones.

In summary, the structural characteristics significant to the activity of hypericin appear to be: a) the extended quinone central skeleton, b) the quinone carbonyl functions, and c) hydroxyl substitution groups at certain positions. These features are necessary for activity but the activity can be affected by other substitution groups as well.
Chapter IV References


Chapter V

Nature of Antiviral Action of the Hypericin Derivative EGK-149

Introduction

The new synthetic derivative of hypericin, 2,5,9,12-tetra (carboxyethylthiomethyl) hypericin (EGK-149) showed potent photosensitive antiviral activity in the screening of hypericin analogues. The nature of its action was compared with that of hypericin. To elucidate the nature of action of a compound, one has to consider the range of potential target sites for the action. The possible sites of action for an antiviral agent in the viral infection cycle are: a) direct inactivation of virion, b) attachment to and entry of host cell, c) uncoating of virus genome, d) viral protein synthesis transcription and translation, e) replication of genome, and f) assembly and release of progeny virus. The functions that are potential targets at each one of these stages will be briefly described, with examples of known inhibitors and known or proposed mechanisms of actions.

Direct inactivation of the virus particle takes place extracellularly. Many compounds such as acids, urea, phenolics, and detergents can have such virucidal effects but they are not considered therapeutically useful because of their cytotoxicity (Vanden Berghe et al., 1985). Compounds such as hypericin and fluoranthene are considered virucidal because they can cause a reduction in viral infectivity with treatment of the virus prior to addition to the cell. This was demonstrated in the treatment exposing only the virus / compound mixture to UVA prior to cellular infection. However, the fact that an antiviral effect is seen with a pre-infection treatment of the virus does not necessarily mean that the virus was rendered non-infectious. The effect can still be due to inactivation of a viral component that is part of a
latter stage of replication. The therapeutic potential of an extracellular virucidal effect would be in the prevention of the spread of infection.

One of the factors that results in the restriction of many viruses to specific hosts is the requirement of specific receptors on the cell surface for recognition and attachment. The antiviral activity of sulfated polysaccharides such as dextran sulfate has been attributed to inhibition of the binding of HIV to its cellular receptor (Mitsuya et al., 1988). Other forces such as electrostatic interactions, hydrogen and hydrophobic bondings are also thought to influence the viral binding (Lonberg-Holm, 1980). Polyionic compounds have been shown to inhibit Sindbis virus binding to cellular receptors. Polyanions appear to act on the virus particles while polycations bind to the cell membrane receptors (Mastromarino et al., 1991). The penetration of the cell by the virus is thought to take place via one of the cellular endocytic processes like receptor-mediated endocytosis, or direct translocation with non-enveloped viruses (Dimmock, 1982). With enveloped viruses, fusion occurs between the cell and envelope membranes. The fusion of paramyxovirus has been shown to be inhibited by synthetic peptides (Richardson and Choppin, 1983).

The process of uncoating releases the viral genome from the protein capsid. It appears that the process can involve multiple stages and that greater time is required for the release of nucleic acid from more complex viruses (Matthews, 1973). The capsid structure of some picornaviruses has been determined to the three-dimensional atomic level, and contains hydrophobic pockets in one protein located within the 'canyon' depressions on the capsid surface. Compounds synthesized by the Sterling-Winthrop Research Institute have been shown to prevent the uncoating of rhinoviruses by binding to these hydrophobic pockets (Smith et al., 1986). These 'WIN' compounds consist of isoxazole and oxazolinyphenoxyl groups linked by aliphatic chains. It is thought that the mechanism of action is the inhibition of capsid disassembly through decreasing the flexibility of the protein. The 'canyon
hydrophobic pocket' structure is found in the icosahedral capsid proteins of a number of plant and animal viruses. As this structure is generally lacking in cell proteins, it has been proposed that this is a target site for which selective antiviral agents could be designed to be applied to viruses with this feature (Rossman, 1989).

Virus replication involves the synthesis of viral proteins and nucleic acids. The majority of antiviral agents approved for clinical use, such as idoxyuridine, acyclovir (ACV) and azidothymidine (AZT), are nucleoside analogues that interfere with these replication processes. Such analogues can act through being incorporated in a genetic process by mimicking the normal nucleosides but in turn disrupt the normal function (Montgomery, 1989). The drug ACV inhibits virus induced DNA polymerase activity (Furman et al, 1979), and AZT triphosphate inhibits reverse transcriptase (Furman et al, 1986). Guanidine, 3-methyl quercetin, and the methoxyflavone isolated from a Chinese medicinal plant have all been shown to inhibit RNA synthesis (Tershak, 1982; Castrillo and Carrasco, 1987; Ishitsuka et al., 1982). The methoxyflavone does not directly inactivate the RNA polymerase complex but appears to prevent its formation in the infected cell. Polynucleotides have been shown to inhibit influenza viral transcriptase (Smith et al., 1980), and the nucleoside ribavirin triphosphate inhibits influenza RNA polymerase (Eriksson et al., 1977). The molecular approach to interference with genetic expression has also been taken. Oligonucleotides antisense to messenger RNAs (mRNA) have been shown to inhibit viral protein synthesis (Smith et al., 1986; Lemaitre et al., 1987). Sim (1990) summarized the current knowledge of virus-specific inhibitors and discussed the future potential of using the molecular approach in the discovery of antiviral agents. It is proposed that the identification of virus-coded proteins necessary in viral replication will indicate targets for the selection and design of antiviral agents that do not impede host functions.
Viruses produce structural and non-structural functional proteins such as polymerases. In many viruses, proteins are synthesized as large precursors that are then cleaved by proteases. Protease inhibitors have been shown to suppress the production of infectious influenza virus but the enzymes affected could be of cell origin (Zhirnov et al., 1982). The virus envelope is composed of cell membrane components and viral glycoproteins. A number of antiviral compounds, like tunicamycin and the plant alkaloid castanospermine, can inhibit glycosylation and exhibit antiviral effects (Klenk and Schwartz, 1982; Walker et al., 1987). The assembly of virus particles is considered to be a generally self-regulated process where the capsid is constructed from basic subunits but the genome also has to be packaged within the capsid to produce an infectious virion. The progeny assembly and release processes are not very well understood.

A summary of the different sites of antiviral action should also include the activity of interferon which is a cellular product and not virus-specific. A virus infected cell is stimulated to synthesize and export interferon. Interferon signals other cells to modulate cell functions in a protective manner and to produce an antiviral protein. It is thought that the protein impedes the transcription of viral mRNA (Jackson, 1986). Interferon has been used in treatment to induce this effect. The extract of *Melia azedarach* L. has also shown the ability to induce an antiviral state (Wachsman et al., 1987). The nature of the effect of the isolated glycopeptide meliacin resembles that of interferon but it does not appear to be interferon-mediated (Andrei et al. 1988). The mechanism of this meliacin protective effect and the role of this protein in the plant remain to be elucidated.

The scope of the potential sites of antiviral activities in the virus infection cycle is wide and far from being fully understood or explored. For most antiviral compounds, the mechanisms of action have not yet been elucidated in full detail. Proceeding from what is known and proposed about the nature of the activity of hypericin, the mechanism of action of
its active derivative EGK-149 was investigated. The activity of hypericin has been examined in a number of studies and there is probably more than one mechanism involved. Hypericin was reported to have antiretroviral activity by direct inactivation or interference with the assembly and release process (Meruelo et al., 1988). The study also showed suppression of reverse transcriptase (RT) activity in infected cell cultures. Lavie et al. (1989) documented abnormal viral cores using electron microscopy. It is suggested from comparison with reports concerning other viral core formations that hypericin may disrupt the polyprotein or proteins essential in the assembly process, and infectivity is lost when the RNA is not incorporated into the progeny particles. The suppression of RT activity does not take place when tested with purified enzymes.

Hypericin is active against several retroviruses, including the immunodeficiency and leukemia viruses (listed in Chapter IV). Hypericin also inhibits the activity of the enzyme protein kinase C (Takahashi et al., 1989). Phosphorylation activity is part of the reverse transcription process and its inhibition could result in the inhibition of reverse transcriptase activity reported by Meruelo et al. (1988) that is not from direct inactivation of RT. Schinazi et al. (1990), however, showed hypericin inhibition of the HIV-1 RT, even though this effect can be negated with the addition of bovine serum albumin (BSA). They suggested that this effect of hypericin on the enzyme may be from a non-specific binding to proteins. Carpenter and Kraus (1991) showed the complete inactivation of equine infectious anemia virus reverse transcriptase activity by hypericin with 60 minutes of exposure to light. Viral infectivity, however, can be eliminated with only 10 minutes of exposure to light indicating that RT inhibition is not the only mechanism of action. Hudson et al. (1991) reported the light-enhanced inhibition of MCMV occurring at an early stage of the replication cycle and likely from action on an intracellular target. Moraleda et al. (1993) proposed that the activity of hypericin against duck hepatitis B virus (DHBV) takes place in morphogenesis steps late in the replication process.
Tang et al. (1990) demonstrated that hypericin is active against membrane enveloped viruses but not against nonenveloped viruses. Exposure of the compound and virus to light greatly enhanced the antiviral effects against enveloped viruses, even though there is still a significant effect in the dark (Lopez-Bazzochi et al., 1990; Hudson et al., 1991). Meruelo et al. (1988) found that the compound was localized in the surface membrane when incubated with cells. These findings about hypericin indicate that the viral membrane is a site for the photodynamic mechanism of antiviral action. These studies show that there is probably not a simple explanation for the mechanism of action(s) of hypericin. The role of light was also not considered in all of these studies. While hypericin has been shown to have activity in the dark, its photosensitivity makes it important to control light conditions in studies to elucidate the mechanism of action.

A probable mechanism for action on membranes is via singlet oxygen produced by the promotion of hypericin to a reactive state by the absorption of photons. It has been shown that hypericin does generate singlet oxygen in visible light (Duran and Song, 1986). To confirm that the membrane was the likely site of action, hypericin and EGK-149 were tested against a nonenveloped virus (polio) and another enveloped virus (MCMV). Since the possibility of other mechanisms of action that may be independent of light have been proposed in previous studies, different stages of the virus infection cycle were treated with and without light to better elucidate the site of activity.

The singlet oxygen generated by photosensitized hypericin can react in two ways with other molecules. It can form a new product with them such as in the formation of endoperoxide compounds, or it can transfer its energy to excite another molecule returning to the ground state itself. This phenomenon is called quenching (Hader and Tevini, 1987). Singlet oxygen commonly reacts with conjugated double bonds, aromatics and heterocycles and many such bonds are found in biological molecules. For example, lipid peroxidation is
the formation of hydroperoxides with the fatty acid side chains of membrane lipids that causes membrane damage. Well known quencher molecules include: the β-carotenes, α-tocopherol (vitamin E), phenols and azide compounds (Halliwell and Gutteridge, 1989). A compound with a high rate of reaction with singlet oxygen can be considered to be a 'singlet oxygen scavenger'. To test if singlet oxygen is involved in a photodynamic reaction, a quencher or singlet oxygen scavenger compound can be added to the system to see if the effect is reduced or eliminated (Foote, 1987).

To investigate if there is indeed a singlet oxygen effect in the antiviral activity of compound EGK-149, it and hypericin were assayed in the presence of cholesterol as a singlet oxygen scavenger. Cholesterol has been used as a trap for singlet oxygen because the two molecules react to form 3β-hydroxy-5α-cholest-6-ene-hydroperoxide (Kulig and Smith, 1973). The viral membrane of the Sindbis virus used in the assay is a lipid bilayer derived from the host plasma membrane with a representative sample of cellular membrane lipids and cholesterol is a known component (Lenard, 1980). Adding cholesterol to the assay therefore would not raise the question of toxicity as would the use of some other effective quenchers like sodium azide.

The molecular target of the singlet oxygen photodynamic effect can be part of the membrane system. Damage to membrane liposomes by photosensitizers has been demonstrated (McRae et al., 1985). The viral membrane is composed of a host-derived lipid bilayer and viral coded membrane proteins (Marsh, 1987). The host membrane proteins are excluded and the viral envelope spike glycoproteins are involved in the receptor binding and fusion processes of host cell entry. When the membrane is the proposed site of photodynamic reactions, both lipid components and the proteins are possible targets of action. The structure of the Sindbis virus particle has been well-characterized. The viral RNA is packaged in an icosahedral nucleocapsid composed of one 30 kilodalton (kD) capsid
protein (Fuller, 1987). The surrounding envelope has two glycoproteins that span and protrude from the lipid bilayer; they are arranged in trimers in an icosahedral lattice (Harrison, 1986). The spike proteins, designated E1 and E2, have their major domains external to the membrane (Schlesinger and Schlesinger, 1986). It was hypothesized in the present study that the protein domains exterior of the envelope are potential targets of photodynamic action. Schinazi et al. (1990) described a non-specific binding to proteins shown by hypericin. The photosensitive compound α-terthienyl was reported to cause a cross-linking of membrane proteins in bacteria in UVA (Downum, et al., 1982). It also has potent antiviral activity (Hudson et al., 1986). Moraleda et al. (1993) observed that hypericin caused a cross-linking of the preS envelope protein of DHBV surface antigen particles and inferred that it also occurs with virus particles. To see if the antiviral action could be taking place on the viral proteins, the proteins from Sindbis virus particles treated with hypericin, EGK-149, and α-terthienyl, in light and dark, were separated by gel electrophoresis in order to discern any alterations.

Material and Methods

Time of treatment antiviral assays

Antiviral assays with Sindbis virus were done using the plaque reduction method (described in Material and Methods of Chapter II). The virus / cell system was treated with hypericin and compound EGK-149 , and exposed to light at different periods in relation to the virus infection process. The treatments consisted of the following stages, with a parallel treatment done in the dark:
1) cell layer only treated for 1 hr and compound removed prior to virus adsorption,
2) virus particles only for 30 min, compound remained during adsorption in the dark,
3) virus particle only for 30 min, compound removed before the adsorption period in the dark,
4) virus and cell during 1 hr of adsorption,
5) cell layers post-adsorption, at 0-1 hr, 3-4 hr and 5-6 hr.

The incident energy of the fluorescent light source was 1.5 W/cm$^2$. The exposure to light took place in the Environ-Shaker cabinet, at 4°C with virus particles only and at 37°C with cells and virus. For exposing the virus particles only to the compounds (as in treatment 3), the virus suspensions of 1000 pfu/ml were treated with the compounds for 30 min in light or dark. The suspensions were concentrated in Centricon-30 miniconcentrators (Amicon) which have filters with a 30,000 molecular weight (MW) cutoff by centrifuging for 10 min at 5000 G in a Sorval SS-34 rotor at 4°C. The particles were washed twice this way with PBS and then resuspended in culture medium for assay.

Time of treatment assays were limited with MCMV. The use of the murine cell line (3T3-L1) that was necessary for its assay was discontinued due to observable changes in monolayer properties.

Singlet oxygen mechanism assays

Cholesterol (Sigma C3137) was dissolved in 95% ethanol and added to SINV plaque reduction assays in light and dark as a singlet oxygen scavenger at concentrations between 0.1 and 100 µg/ml. The compound / virus mixture solution was essentially saturated with cholesterol at 50 µg/ml. In addition to cell only and virus only controls, virus was treated with the same concentrations of cholesterol only.
Viral protein separation

Sindbis virus particles at $5 \times 10^5$ pfu/ml were treated with one of the three compounds, EGK-149, hypericin or $\alpha$-terthienyl for 30 min in light or dark. The compound concentrations tested were: 0.1, 1.0, 10 and 100 $\mu$g/ml. The suspension was added to equal volume of 20% polyethylene glycol (PEG) and the proteins were precipitated in the centrifuge (Sorval) by spinning at 15,000 G for 2 h. The pellet was resuspended in 1 ml of distilled water and respun in a microcentrifuge (Eppendorf 5415C) for 20 min at 12,000 G. The proteins were separated by 10% sodium dodecylsulfate protein agar gel electrophoresis (SDS-Page). Proteins were quantified with the Bradford Protein Quantification Assay and 25 or 50 $\mu$g were loaded per lane to gels on a vertical slab gel unit (Mighty Small SE 200, Hoefer Scientific). Control lanes were run simultaneously with untreated virus proteins. Since the virus stock was purified from cell cultures, controls were also run with 3T3-L1 cell proteins. The gels were stained with Coomassie Blue. The molecular weights (MW) of the proteins were calculated in reference to standard markers (Sigma MW SDS-70L Kit). The MW of the envelope proteins are 50 kD for E₁ and 60 kD for PE₂. The capsid protein has a MW of 30 kD (Bonatti et al., 1979).

Results

Time of Treatment Activity Assays

Plates with the fixed and stained cell layers of a Sindbis virus plaque reduction assay where the cell / virus system were treated at different times of the infection process, in light and dark, are shown in Figure 5.1. The percentages of plaque reduction are listed in Table 5.1. The effects of EGK-149 against MCMV are shown in Table 5.2.
Figure 5.1: Sindbis Virus Antiviral Plaque Reduction Assay Plates from Treatments with Compounds at Different Stages of Viral Infection Cycle.
Table 5.1: Percentage of Sindbis Virus Plaque Reduction From Treatments at Different Stages of Viral Infection Cycle

<table>
<thead>
<tr>
<th>Compound</th>
<th>A) Pre- Infection</th>
<th>B) Virus Only</th>
<th>C) Virus and Infection</th>
<th>D) Infection Time Only</th>
<th>E) Post Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypericin 0.05 µg/ml</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>EGK-149 0.05 µg/ml</td>
<td>0</td>
<td>100</td>
<td>81</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>EGK-149 0.1 µg/ml</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>Hypericin 0.05 µg/ml</td>
<td>0</td>
<td>62</td>
<td>62</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EGK-149 0.05 µg/ml</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>EGK-149 0.1 µg/ml</td>
<td>0</td>
<td>51</td>
<td>56</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Stage of treatment with compound in light and dark:

A) cell layer only for 1 hr and compound removed prior to virus addition.
B) virus only for 30 min (compound remained during infection in the dark).
C) virus only for 30 min and with cell layer during 1 hr of infection.
D) virus and cell layer during 1 hr of infection.
E) cell layer only post-infection after removal of virus.

0 % = no antiviral effect 100 % = total elimination of viral infection
Table 5.2

Percentage of Murine Cytomegalovirus Plaque Reduction from Treatment with Compound EGK-149 at Different Stages of Viral Infection Cycle

<table>
<thead>
<tr>
<th>Stage of Infection Process Treated with Compound *</th>
<th>A) Pre-infection</th>
<th>B) Virus Only</th>
<th>C) Virus and Infection</th>
<th>D) Infection Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>0</td>
<td>0</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>Dark</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

Concentration of compound EGK-149 = 0.1 µg/ml.
* same as defined in Table 5.1.
0 % = no antiviral effect 100 % = total elimination of viral infection

The virus particles that were treated with the compounds and recovered after washing before adding to cells were not infective.

Singlet oxygen mechanism assays

The effects of the presence of cholesterol as a singlet oxygen scavenger on the antiviral activity of hypericin and EGK-149 are shown in Figures 5.2 and 5.3.

Effect of hypericin and EGK-149 on Sindbis virus proteins

The structural proteins of Sindbis virus are indicated on the SDS-Page gel shown in Figure 5.4. The 30 kD capsid protein showed a retardation shift when the virus particles have been treated with 10 or 100 µg/ml of EGK-149 in light. No other protein alterations were seen, with the EGK-149 treatments in dark or with the hypericin and α-terthienyl treatments in light or in dark.
Figure 5.2: Percentage of Sindbis Virus Plaque Reduction by Compound EGK-149 in the Presence of Cholesterol

Percentage
Plaque
Reduction

Concentration of cholesterol \( \mu g/ml \)

- o = cholesterol control, in light and dark
- + = EGK-149 0.05 \( \mu g/ml \), in light
- * = EGK-149 0.05 \( \mu g/ml \), in dark
- \( \Delta \) = EGK-149 0.10 \( \mu g/ml \), in light
- • = EGK-149 0.10 \( \mu g/ml \), in dark

Each symbol point represents the result from one assay.
Figure 5.3: Percentage of Sindbis Virus Plaque Reduction by Hypericin in the Presence of Cholesterol

Percentage Plaque Reduction

Concentration of cholesterol µg/ml

Each symbol point represents the result from one assay.
Figure 5.4: Electrophoretic Gel (SDS-PAGE) of Sindbis Virus Proteins Treated with Compound EGK-149 and Hypericin.

L = treatment in light  D = treatment in dark

Capsid protein in lane 2 showed retarding shift.
Discussion

To determine the site of antiviral action of EGK-149, the virus / cell system was treated with the compound and with hypericin at different times which encompass different stages of the virus infection cycle. It has been shown that the hypericin has an extracellular virucidal activity against enveloped viruses. This action appears to be from a light-mediated singlet oxygen effect on the virus membrane. The novel hypericin derivative EGK-149 has also exhibited this light-mediated virucidal activity. The time of treatment assays were also to compare the actions of the two compounds. The action of hypericin appears to be complex and likely entails more than one mechanism. Comparison of the nature of action with a derivative would continue the studies to elucidate and distinguish these mechanisms.

In addition to the light-mediated effect on membranes, hypericin has also exhibited significant activity in the dark (Lopez-Bazzochi et al., 1990). It inhibits the activities of monoamine oxidase (Suzuki et al., 1984) and protein kinase C (Takahashi et al., 1989) in enzyme assays; and the release of reverse transcriptase in infected cells (Meruelo et al., 1988). Sites of actions in the intracellular viral replication process have been proposed for its activity. Lavie et al. (1989) found disruption of the assembly of radiation leukemia progeny virions. This event would take place late in the replication process at some time after the cell has been exposed to the virus. Hudson et al. (1991) suggested the possibility of interference in MCMV transcriptional events early in the replication cycle. In the treatment of the cells after exposure to SINV with hypericin or EGK-149, there was no reduction in plaque formation. This indicates that there was no interference with the intracellular replication events with SINV. It is unfortunate that time of treatment assays were only conducted with EGK-149 against MCMV for technical reasons and there were no post-infection treatment assays. There appeared to be an inhibition effect with the treatment of the cell and virus during the adsorption time when the virus infects the cell. Since the cells were exposed to
virus for 1 hour, it is possible that the compound was acting on early intracellular events besides the membrane disruption. The action of hypercin and EGK-149 on the early intracellular events of MCMV infection definitely needs further investigation. It had shown virucidal activity at the higher concentration of 1.25 µg/ml in light (Results, Chapter IV) where the cells were not exposed to light. That concentration in light would be cytotoxic as well.

Neither of the compounds exhibited any pre-infection treatment antiviral effects. In inhibition of viral cytopathic effects assays with serial dilutions, hypercin showed approximately twice the potency as EGK-149 in the dark (see Table 4.1, Chapter IV). This difference is seen again in effects of treating SINV particles only and treating both the virus and the cell during infection. In the dark, hypercin at 0.05 µg/ml caused 62 % plaque reduction as compared to 51 and 56 % by EGK-149 at 0.1 µg/ml. It should be noted that in many reports concerning the activity of hypercin, the concentrations used (greater than 1 µg/ml) would probably be cytotoxic if cells were used in assays and were exposed to light in the presence of the compound. Light-induced cytotoxicity would not affect assays of purified enzyme activity or direct virion inactivation, but as the role of light is important in the activity of hypercin compounds it should be taken into consideration in all studies.

Inhibition of infection was complete with treatment of the virus in light. The extent of inhibition was reduced when the virus was not treated prior to adding to the cell layer. In this treatment where the virus and compound were added simultaneously to the cell layer, there was essentially no activity in the dark. This suggests that with SINV the action of hypercin and EGK 149 was directly against the virus particle. This result is in accordance with the report by Tang et al. (1990) of the virucidal activity of hypercin against enveloped viruses. Since the compounds remained with the virus suspension during the infection time, even though it was in the dark it was not certain that the site of action was limited to the virus
particle. It would be interesting to see if the infectivity is the same if only the virus was exposed to the compounds. The procedure to remove the compounds from the virus suspension before addition to the cells, however, was not developed completely. There was no infectivity from the virus after removal of the compounds but there was also only marginal infectivity from the control cells, showing that there was damage to the virus from the manipulations. I believe it is possible to refine the protocol to accomplish this assay. The loss of infectivity with exposure of only the virus to the compound would confirm the indication from the present evidence that the antiviral effect is from the direct inactivation of the virus.

The effect in the dark from the prior treatment of the virus indicates that the activity is light-enhanced but not entirely light-dependent. The photosensitive activity is attributed to the generation of singlet oxygen which damages the membrane. It is likely that the singlet oxygen effect took place much faster than the dark effect. Longer time requirement for the occurrence of the dark effect could explain why there was no activity in the dark shown without prior treatment of the virus particles. The effect in the dark could be independent of oxygen but this has not been demonstrated. The membrane envelope is a feature shared by many groups of viruses, but viruses also differ greatly in other aspects of molecular composition. It is reasonable to conclude that hypericin compounds have a light-enhanced singlet oxygen type effect against membrane-enveloped viruses, but there is evidence for actions interfering with intracellular replicative events of specific virus groups.

Addition of cholesterol as a singlet oxygen scavenger was used to see if singlet oxygen participates in the photodynamic reaction causing the antiviral effect. A high concentration of a scavenger is expected to inhibit a singlet oxygen dependent reaction. The antiviral effect of EGK-149 in light was reduced in a dose-dependent manner by the presence of cholesterol (Figure 5.2). This indicates that singlet oxygen is involved in the reaction.
The effect was not as apparent with hypercin (Figure 5.3) at the concentrations tested. Hypericin has a stronger effect in the dark than EGK-149 and this is evident from the reduction in viral plaque formation caused by hypericin in the dark at the same concentration. While there was a slight plaque reduction effect, the dark effect appears to be sufficient to maintain significant activity was not influenced by the presence of cholesterol. Since the dark effect of 0.05 µg/ml of hypericin was approximately 50% plaque reduction, it is likely that at an even lower concentration the dark effect would be eliminated. If the light-enhanced effect is isolated from the dark effect, the influence of a singlet oxygen scavenger may be more evident. The indication seen of slight reduction in the light activity at this concentration is consistent with the model that hypericin has both light-enhanced membrane-directed activity and significant activity in the dark. The singlet oxygen-mediated photactivity appears to be a more important component in the action of the derivative EGK-149, even though it has also shown some activity in the dark.

Reduction of activity in the presence of cholesterol indicates but does not prove the necessity of singlet oxygen in the reaction. More elaborate techniques can be used to ascertain the role of singlet oxygen. Cholesterol as a scavenger can produce a number of oxidation products from free-radical or peroxide reactions (Halliwell and Gutteridge, 1989), but it produces primarily the 5-α-hydroperoxide from reacting with singlet oxygen (Kulig and Smith, 1973). This molecule can be chemically isolated to be sure that this was the indeed the end product of the scavenger reaction. Singlet oxygen can also be detected by electron spin resonance measurements, or its effects be shown to potentiated when applied in deuterium oxide which prolongs its lifetime compared to an aqueous medium. It is interesting that hypericin and its derivative have shown a difference in sensitivity to modulation of photo-activity by a singlet oxygen scavenger. It seems more likely, however, that this difference is due to the contributing effects of components of the activities that were
independent of light and not due to differences in the singlet oxygen mechanism of action. Therefore, further efforts to confirm the involvement of singlet oxygen were not undertaken.

The photodynamic oxygen-mediated antiviral effect is considered to target the membrane system. As the Sindbis virus membrane is derived from the host cell membrane, the virion envelope and the cell membrane that the virus has to penetrate would present similar sites of action. However, a non-cytotoxic concentration of the compounds was capable of inactivating the virus or inhibiting the infection process. It is possible that the virus is more sensitive due to the difference in magnitude of size between a cell and a virus particle, especially since SINV is only ~40 nm in diameter. It is also possible that more specific interactions involving membrane components are involved, and the viral membrane is not identical to the plasma membrane. A major difference is in the protein composition of the membranes as the protein components of the viral envelope are entirely virus-coded. In the examination of viral proteins for specific effects on membrane components by the compounds, there appeared to be no direct alteration of the coat proteins that span the lipid bilayer of the envelope. Neither was a cross-linking of proteins such as that reported as being caused by hypericin on DHBV or by α-terthienyl on bacterial proteins observed. The protein banding showed a retarding in the electrophoretic mobility of the 30 kD capsid core protein after exposure to EGK-149 in light. Moraleda et al. (1993) observed some aggregation effect on the DHBV core protein from treatment with hypericin in the dark. The change in the capsid protein probably contributed to the overall photodynamic activity of EGK-149, even though the capsid is surrounded by the membrane envelope. The membrane integrity was likely to have been disrupted through a characteristic photosensitizer action on the lipid components such as peroxidation of fatty acids. In the time of treatment assays, EGK-149 did not show a post-infection effect, which would potentially result from defective capsid proteins interrupting the progeny virus production. The change in the protein was found in treatment of large numbers of virus particles ($5 \times 10^5$ pfu/ml) with 10 and 100 μg/ml of the
compound. The antiviral assays were conducted using 0.5 and 1.0 μg/ml of compound against virus at 10^3 pfu/ml, and a concentration as high as 10 μg/ml would be cytotoxic in light. The specific protein-altering photo-active effect of EGK-149 is however intriguing because it was not observed with the other photosensitizers hypericin and α-terthienyl. It showed that there are different molecular events involved in what is generically described as a photosensitizer effect, even with closely related molecules. The complex sulfur-containing substitution groups on EGK-149 may account for this difference in effect with hypericin.

The activity of a novel derivative of hypericin brings new information to be considered in the saga of deciphering the complex mechanisms of action of hypericin. The identification of a light-dependent action on a specific protein is also interesting in terms of the molecular mechanism of photodynamic reactions. Studies on the nature of the specificity can be pursued as the composition of the capsid protein is already known. Investigations have shown diverse bioactive effects produced by the plant compound hypericin and efforts to clarify the mechanisms of action continue because of its antiretroviral therapeutic potential. It is a good example of how the elucidation of bioactive natural products can lead to potential therapeutically useful compounds as well as generate research toward the understanding of molecular actions.
Chapter V. References


Summary Discussion

The scope of this project included the search and isolation of a biologically active compound and investigations of the nature of action of another bioactive plant compound. In a brief summary, this work consisted of the following stages:

1. Systematic search of ethnopharmacological information to select medicinal plants for antiviral screening.

   Documentations of the traditional medicines in Yunnan province of China were screened for plants that have been used to treat diseases that are now known to have viral causes.

2. Collection and identification of plant specimens.

   Medicinal plants were collected from three areas of Yunnan to test for antiviral activity.


   In assays of 31 plant species against two membrane-enveloped viruses, 16 showed activity. The most active species selected for isolation of active component(s) was *Elsholtzia ciliata* of the mint family. This and two other species showed activity enhanced with exposure to long wavelength ultraviolet radiation.

4. Purification of active component(s) from active species through bioactivity-guided fractionation.

   One active compound isolated from *E. ciliata* was identified as the polycyclic aromatic hydrocarbon fluoranthene. The presence in the plant of this anthropogenic compound was probably from environmental accumulation. Other active components of a more polar and possibly ionic nature remain to be identified.
5. Investigation of nature of activity.

Investigation of the mechanisms of action was carried out with the photosensitive antiviral compound hypericin from medicinal plant of the genus *Hypericum*. In structure-activity relationship experiments testing derivatives and relatives of hypericin, a new derivative, 2,5,9,12-tetra(carboxyethylthiomethyl) hypericin (EGK-149), showed potent photosensitive virucidal activity against enveloped viruses. The activity of EGK-149 was shown to be of the singlet oxygen type that could be reduced by the presence of cholesterol as a singlet oxygen scavenger. It also showed the ability to alter the Sindbis virus capsid protein with exposure to light which was not shown by hypericin.

The study covered the different aspects of research in the elucidation of a bioactive compound even though there was a change from the original plan to find, isolate and investigate the mechanism of action of one type of compound when the latter studies were done with the known plant compound hypericin. As it turned out, the hypericin compounds probably presented a better system of study as the compound fluoranthene purified from *Elsholtzia* is probably not a plant constituent. Its isolation from a bioactivity screening of medicinal plants raised instead quite a different set of questions regarding both phytochemical methodologies and implications relating to environmental quality. While it was the aim of the project to be comprehensive of the different aspects of natural product research, it was understood that in order to do so the work in each area would have to be limited to a certain extent. Each of the aspects covered have the potential of being done more extensively and they have each raised the need for further research.

The importance to preserve ethnopharmacological information was demonstrated by the fact that five of the sixteen active antiviral species were only selected on the basis of their
uses in the traditional medicine of ethnic minority tribes in Yunnan. Plants are obviously rich resources of bioactive compounds and have played a significant role in providing useful drugs or prototypes of drugs as well as experimental research tools. The progress in the understanding of the causes of diseases is leading to the refinement of the bioassays to target for curative agents. New \textit{in vitro} assay systems such as ones based on measuring receptor binding or enzyme activity allow for the relatively rapid screening with small amounts of plant material for specific activities. Plants remain a wealth of potential for new bioactivity screening approaches but the threatened loss of biodiversity from the massive destruction of ecosystems like the tropical rainforest imparts urgency to promote conservation.

Knowledge about viruses is in the process of rapid development. It is likely that viral assay systems are also undergoing developments and more standardization that will lead to more effective antiviral screening programs allowing for testing against a larger battery of virus types or more specific disease viruses. It would interesting to examine the efficacy of plants used in traditional medicine by testing them against the causative agents of the diseases they were used to treat. However, the protocols of testing against specific human disease viruses was beyond the scope of this project.

The results on the chemistry of plants of the genus \textit{Elsholtzia} have raised some questions to be answered. As there is another UV-active fraction in \textit{E. ciliata}, it remains to be determined if the plant contains any photo-active compounds of plant origin. This is interesting because photosensitizers have not been reported from the family Lamiaceae. The possibility that some plants have a scavenging effect of lipophilic environmental compounds is worthy of consideration because of the roles many plants have as food and medicine and as a major component of the global environment.
The mechanisms of action of hypericin are not fully elucidated. Similarities and differences between the activities of hypericin and the novel derivative EGK-149 add more data to be considered in the process of resolving the mechanisms. The work to distinguish the mechanism of action of a compound is complicated as demonstrated by the fact that few have been precisely identified to the molecular level. It was probably advantageous to work with a compound like hypericin for which there is a foundation of existing information on the nature of activity. The quantity and variations of the reported information, however, illustrate again the complexity of the task.

It was my goal to conduct a project that combined the different disciplines involved in the process of identifying a biologically active molecule and understanding how it works. These disciplines of pharmacognosy are: ethnobotany, microbiology, chemistry and pharmacology. I believe that the five aspects of this project have accomplished this goal and that in addition to my gain in knowledge in each of the fields involved, the integrated perspective of the whole endeavor was an important education.
Appendix I

**Medicinal Plants Assayed for Antiviral Activity**
and Their Ethnopharmacological Indications for Treatment

<table>
<thead>
<tr>
<th>Plant Family</th>
<th>Species</th>
<th>Part Used</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apiaceae</td>
<td><em>Centella asiatica</em> (L.) Urban *</td>
<td>PL</td>
<td>cold, infectious hepatitis</td>
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<td>Apocynaceae</td>
<td><em>Plumeria rubra</em> var. <em>acutifolia</em> (Poir.) Ball.</td>
<td>RT</td>
<td>hepatitis</td>
</tr>
<tr>
<td>Asteraceae</td>
<td><em>Bidens pilosa</em> L. *</td>
<td>PL</td>
<td>cold, prevent flu, hepatitis, jaundice</td>
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<tr>
<td>Berberidaceae</td>
<td><em>Mahonia nepatensis</em> DC.</td>
<td>PL</td>
<td>hepatitis</td>
</tr>
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<td>Combretaceae</td>
<td><em>Quisqualis indica</em> L. *</td>
<td>PL</td>
<td>hepatitis</td>
</tr>
<tr>
<td>Convolvulaceae</td>
<td><em>Dichondra repens</em> Forst. *</td>
<td>PL</td>
<td>flu, hepatitis, jaundice</td>
</tr>
<tr>
<td>Cycadaceae</td>
<td><em>Cycas siamensis</em> Miq. *</td>
<td>LF</td>
<td>hepatitis, hepatitis A, jaundice</td>
</tr>
<tr>
<td>Ebenaceae</td>
<td><em>Diospyros kaki</em> L.f.</td>
<td>BK</td>
<td>hepatitis, jaundice</td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td><em>Euphorbia prolifer</em> Ehrenb. ex. Boiss. *</td>
<td>PL</td>
<td>hepatitis</td>
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<tr>
<td></td>
<td><em>Ricinus communis</em> L.</td>
<td>RT</td>
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<td>Fabaceae</td>
<td><em>Kummerowia striata</em> (Thunb.) Schindl.</td>
<td>PL</td>
<td>cold, hepatitis A</td>
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<tr>
<td>Gentianaceae</td>
<td><em>Halenia elliptica</em> D. Don</td>
<td>PL</td>
<td>hepatitis</td>
</tr>
<tr>
<td>Iridaceae</td>
<td><em>Belamcanda chinensis</em> DC. *</td>
<td>BU</td>
<td>flu, hepatitis</td>
</tr>
<tr>
<td>Plant Family</td>
<td>Species</td>
<td>Part Used #</td>
<td>Part Used</td>
</tr>
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<tr>
<td>Lamiaceae</td>
<td><em>Acrocephalus indicus</em> Briq.* cold</td>
<td>PL</td>
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<tr>
<td></td>
<td><em>Elsholtzia densa</em> Briq. * flu</td>
<td>PL</td>
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<tr>
<td></td>
<td><em>Elsholtzia ciliata</em> (Thunb.) Hylander *</td>
<td>BR</td>
<td>BR</td>
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<tr>
<td></td>
<td><em>Perilla frutescens</em> (L.) Britton cold, flu</td>
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<td><em>Rabdostia phyllostachys</em> (Diels) Hara * cold, hepatitis, jaundice</td>
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<td><em>Scutellaria orthocalyx</em> Hand.-Mazz. cold</td>
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<td><em>Stachys kouyangensis</em> (Vaniot) Dunn * hepatitis A and B</td>
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<td>Myrsinaceae</td>
<td><em>Embelia sessiliflora</em> Kurz.* hepatitis</td>
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<tr>
<td>Oxalidaceae</td>
<td><em>Oxalis corniculata</em> L. cold, hepatitis, jaundice</td>
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<tr>
<td>Poaceae</td>
<td><em>Phyllostachys</em> sp. (gold bamboo) hepatitis</td>
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<tr>
<td>Polypodiaceae</td>
<td><em>Stenoloma chusanum</em> (L.) Ching cold, flu, infectious hepatitis</td>
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<td>Rubiaceae</td>
<td><em>Hedyotis uncinella</em> Hook. et Arn. prevent and cure jaundice hepatitis</td>
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<td>Rutaceae</td>
<td><em>Boenninghausenia sessilicarpa</em> Levl. * cold, hepatitis</td>
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<td>Sauruaceae</td>
<td><em>Houttuynia cordata</em> Thunb. cold, flu, jaundice hepatitis</td>
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<td>Scrophulariaceae</td>
<td><em>Siphonostegia chinensis</em> Benth. * hepatitis A and B</td>
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<tr>
<td>Verbenaceae</td>
<td><em>Verbena officinalis</em> L. * infectious hepatitis</td>
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# Part used: BK, bark; BR, branch; BU, bulb; LF, leaf; PL, whole plant; RT, root.

* Showed antiviral activity in bioassay.
Appendix II.

Literature on the Chemistry of *Elsholtzia*

**On essential oil components**

In English:

Composition of *Elsholtzia polystachya* leaf essential oil (1988)

Terpenoids from *Elsholtzia* species. III. New constituents of essence oil from *Elsholtzia pilosa* (1988)
Bestmann et al., Z. Naturforsch. C: Biosci. 43 (5-6), 370-372.

Terpenoids from *Elsholtzia* species. II. Constituents of essential oil from a new chemotype of *Elsholtzia cristata* (1987)

* Constituents of essential oil of *Elsholtzia strobilifera* (1985)

Volatile constituents from *Elsholtzia polystachya* (1984)

* Gas chromatographic examination of the essential oil of *Elsholtzia blanda* (1983)

Composition of essential oil of *Elsholtzia strobilifera* Benth. (1980)

* Essential oil of *Elsholtzia pilosa* (1976)

Chemical studies on the essential oil of *Elsholtzia densa* (1971)
Vashist, V.N. and Atal, C.K., Flavour Ind. 2 (1), 47-48.

* Caryophyllene epoxide from the oil of *Artemesia scoparia, Elsholtzia polystachya, Piper hookeri,* and *Piper brachystachyum.* (1970)
Thappa et al., Curr. Sci. 39 (8), 182-183.

Essential of *Elsholtzia polystachya* (1967)
Vashist et al., Indian J. Chem. 5 (3), 130.

Elsholzidiol and Elsholtzione (chemistry data)

In Chinese:

Cheng et al., Yunnan Zhiwu Yanjiu 11 (1), 91-6.
* Composition of the essential oil of *Elsholtzia blanda* (1989)

Components of essential oils of *Elsholtzia stautonii* Benth. (1989)

* Studies on the components of essential oils of *Elsholtzia splendens* and *Origanum vulgare* (1983)

In Russian:
Characterization of essential oils of different *Elsholtzia ciliata* biotypes (1988)

Composition of *Elsholtzia stautoni* essential oil (1987)

* Accumulation of essential oils in *Elsholtzia patrinii* and *Lophanthus anisatus* (1987)

* Essential oil of *Elsholtzia patrinii* (1984)

Study of some biological characteristics of new essential oil producing plants, *Elsholtzia patrinii* and *Lophanthus anisatus* in the Crimea (1980)
  Barannikova, T.A., Dokl. TAKha, 266, 56-61.

In Polish:
Composition of *Elsholtzia patrinii* oil (1969)

In Japanese:
Essential oil of *Elsholtzia ciliata* (1967)

In German:

*Elsholtzia*
  In: *Chemotaxonomie der Pflanzen* Vol. IV (1966),
  Hegnauer, R., Birkhauser Verlag, Basel, pp. 311-312.

On other chemical components

Six flavonoids in *Elsholtzia densa* Benth. (1991)
Flavonoids of *Elsholtzia cristata* (1988)

* Flavonoids of *Elsholtzia ciliata* (1985)

* Phytochemical screening of Korean medicinal plants. (III) (1981)

* Screening of saponins in the plants (1981)


* Screening of Indian plants for biological activity. Part VIII. (1978)

* Occurrence of alkaloids in Korean medicinal plants (1978)
Woo et al., Soul Taehakkyo saengyak yonguso opjukjip 17, 17-19.

The occurrence of iridoid glycosides in the Labiatae (1972)

Structure of elsholtzidiol, a new bisubstituted furan of *Elsholtzia densa* (1970)
Vashist, V.N. and Atal, C.K., Experientia, 26 (8), 817-818.

Phytochemical investigation of *Elsholtzia cristata* (1966)

Pharmacobotanical analysis of species of the genus *Elsholtzia*. II. (1964)
Swieboda, M., Diss. Pharm. 16 (1), 121-128. (in Polish)

On chemical synthesis

A new and short synthesis of dehydroelsholtzione (Naginata ketone) and isoegomaketone (1980)
Pilot et al., Tetrahedron Lett. 21 (49), 4717-20.

New synthesis of elsholtzia ketone (1977)

* = cited and % of compounds present calculated in NAPRALERT database, issue 2, 1992.
(Program for Collaborative Research in the Pharmaceutical Sciences, Dept. of Medicinal Chemistry and Pharmacognosy, College of Pharmacy at the University of Illinois, Chicago).

(This information is compiled from the following sources: the NAPRALERT database, computer literature searches through the UBC library system and literature seen.)
Appendix IV

High Resolution Mass Spectrum Fragment Analysis of Purified Fluoranthene

(By UBC Department of Chemistry Mass Spectroscopy Centre DS-55 MS data system)
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