A SURVEY OF INSECTICIDAL ACTIVITY FROM *DELPHINIUM* X *CULTORUM* CV. MAGIC FOUNTAINS FLOWERS

By

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ABSTRACT

A SURVEY OF INSECTICIDAL ACTIVITY FROM *DELPHINIUM* X *CULTORUM* CV. MAGIC FOUNTAINS FLOWERS

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The genus *Delphinium* (Ranunculaceae) is well known for the production of many structurally complex diterpenoid alkaloids. Numerous publications are available on the discovery, classification and biological activity of these alkaloids. This research highlights compounds isolated by bioassay-directed fractionation of the crude hexane and ethyl acetate extracts of *Delphinium X cultorum* flowers. The bioassays performed include insecticidal assays using 4th instar mosquito larvae (*Aedes aegyptii* L.), neonate gypsy moth (*Lymantria dispar* L.) and corn earworm (*Helicoverpa zea* Boddie) caterpillars, and adult Colorado Potato Beetles (*Leptinotarsa decemlineata* Say), and Japanese Beetles (*Popillia japonica* Newman); nematocidal assays using *Caenorhabditis elegans* Dought and *Panagrellus redivivus* Goody; antimicrobial assays using gram negative and gram positive bacteria, and various plant pathogenic fungi; and topoisomerase enzyme inhibitory assays using mutant *Saccharomyces cerevisiae* strains.

Bioassay-directed fractionation of the hexane extract from *D. X cultorum* flowers led to the isolation of six volatile compounds that were characterized as ethylmethylbenzene, 1-isopentyl-2,4,5-trimethylbenzene, 2-penten-2-ylbenzoic acid, two isomers of 3-butlidene-3H-isobenzofuran-1-one, and a novel compound (2-hex-3-ene-2-one)-phenylmethylketone from GC/MS studies. Bioassay-directed fractionation of the ethyl acetate extract from these flowers led to the isolation of 4-hydroxybenzoic acid and bis-(4-hydroxyphenyl)methanol. Their structures were confirmed by ^{1}H and ^{13}C NMR experiments.

To Grandma Polcik

ACKNOWLEDGEMENTS

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Herner for his support. To my mother, Kay Phares, brother, Nicholas Phares and grandparents, John and Doris Phares and Marjorie Polcik, mother-in-law Rose Miles my love and thanks for their words of encouragement. Lastly, to my husband, Christopher, my love for sharing my triumphs, holding me up during my pitfalls, surviving through my fits, and understanding. I would not have made it without the help from these people.

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LIST OF ABBREVIATIONS

^{13}C-NMR	.....	carbon nuclear magnetic resonance
^{1}H-NMR	.....	proton nuclear magnetic resonance
ANOVA	.....	analysis of variance
bs	.....	broad singlet
CCl_4	.....	carbon tetrachloride
$CHCl_3$	.....	chloroform
CNS	.....	central nervous system
cv.	.....	cultivar
d	.....	doublet
DMSO	.....	dimethylsulfoxide
DNA	.....	dioxyribonucleic acid
eV	.....	electron volts
EI	.....	electron impact
FTIS	.....	Fourier transform infrared spectroscopy
GC	.....	gas chromatography
GC/MS	.....	gas chromatography/mass spectroscopy
GLC	.....	gas liquid chromatography
HPLC	.....	high performance liquid chromatography
IR	.....	infrared

LD_{100}	.....	lethal dose (100%)
LD_{50}	.....	lethal dose (50%)
LSD	.....	least significant difference
m	.....	multipet
m	.....	meta
M	.....	molar
MeOH	.....	methanol
Mhz	.....	megahertz
MLA	.....	methyllycacontine
MPLC	.....	medium pressure liquid chromatography
MSAL	.....	N-methylsuccinyl)-anthranoyllycoctonine
m/z	.....	mass to charge ratio
nAChR	.....	acetylcholine receptor subtypes
NIRS	.....	near infrared reflectance spectroscopy
NMR	.....	nuclear magnetic resonance
p	.....	para
RNA	.....	ribonucleic acid
rt.	.....	retention time
s	.....	singlet
SAR	.....	structure activity relationship
spp.	.....	species
TLC	.....	thin layer chromatography

UV	.....	ultra violet
VLC	.....	vacuum liquid chromatography

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CHAPTER I

Introduction

History and Classification

Greek tradition tells a tale of the hero Ajax related to the origin of the delphinium. In this legend, the mother of the great warrior Achilles expresses a wish that her dead son's armor be presented to the most-worthy warrior in the Achaean host. Of the two claimants, Ajax and Odysseus, Odysseus was the most celebrated and was given the honor. But Ajax, upon hearing the decision, was embittered and took his own life in protest. From that blood-soaked earth, grew a delphinium plant with the Greek letters AI (the first letters of Ajax's name) upon the petals. This gave miraculous support to Ajax's claim and the injustice done to him (Dwyer and Rattray, 1986).

Delphiniums were named by the ancient Greeks due to the flower's apparent resemblance to the dolphin, the Greek word for dolphin being 'delphis.' In 1640, John Parkinson, apothecary to Charles I, cultivated a famous garden in London, and in writing about his plants in *Paradisi in Sole* mentioned *D. elatius flore pleno diversiforum colorum* (Bishop, 1949; Phillips, 1933). This was the first documentation of cultivated delphiniums in England. In Tudor England, akin to the delphinium larkspur was so named because the nectary resembled the spur of a lark's claw.

Delphiniums grew to be a very popular and sought-after garden plant. Their tall stature and often true-blue color was not shared by any other plant in cultivation. Increased interest in breeding delphiniums during the mid-nineteenth century greatly added to the number and quality of available varieties. Work on delphinium breeding into the twentieth century by Blackmore and Langdon afforded gardeners the named varieties of the large delphiniums grown in the present day (Dodeswell, 1997).

Delphinium is a member of the natural order Ranunculaceae. Perennial delphiniums often are referred to as 'true delphiniums'. Related to it is the larkspur (*D. ajacis* L., *D. consolida* L., *etc.*), also a member of the delphinium genus, sometimes called the "annual delphinium" (Puttock, 1959). However, both delphinium and larkspur species contain annual and perennial types, delphinium and larkspur belong to the crowfoot family. This family includes species of anemone, hepatica, buttercup, aconite, and clematis. Some of these species, in addition to delphinium, are notorious for producing poisonous compounds (Kuder, 1947).

Larkspurs are sometimes called wild or naturally occurring delphiniums. Two of the important species of larkspur, *D. consolida* L. and *D. ajacis* L., are collectively called the consolida group (Kuder, 1947). Consolida-type larkspurs have since been classified into their own genus. *D. consolida* L. and *Consolida* is now used interchangeably depending upon the source. These species were introduced to the United States as garden varieties, but have since escaped cultivation and have been naturalized as prairie wildflowers.

The perennial larkspurs are subdivided into two categories, the low larkspurs and the tall larkspurs. Perennial larkspurs colonize stable, closed habitats, and form dense colonies. Vegetative propagation plays the largest role in reproduction because self-fertilization occurs in only 0-1% (Blanche, 1990). Annuals called "plains larkspur" colonize unstable, open habitats. They have a higher germination rate, and do not multiply by vegetative means (Blanche, 1990). Low larkspur, such as *D. nelsonii* and *D. andersonii* A. Gray, are smaller plants, and inhabit dry low-lying areas. Tall larkspur, such as *D. barbeyi, D. occidentale*, and *D. glaucum* S. Wats., are larger plants growing on high mountainous areas, or areas with abundant soil moisture (James and Johnson, 1976). Plains larkspur, such as *D. geyeri* Greene, grows on high windswept short grass plains (Ralphs et al., 1989). All of these larkspurs are toxic.

The cultivated varieties of delphinium are known to horticulturists as "larkspurs" referring to the annual delphinium, and "true delphinium" referring to the perennial type (Kuder, 1947). It is impossible to unmistakably classify delphiniums according to their botanical and ancestral characteristics due to the high level of cross-breeding of these species. The modern garden delphiniums are the result of the hybridization of many species. A brief outline of classification of cultivated delphiniums was published by Robert Kuder (1947).

Cultivated Delphiniums

I. The Annual Larkspurs
   - A. The *Ajacis* type
     1. Tall Rocket Larkspurs
     2. Dwarf Rocket Larkspurs
     3. Hyacinth-flowered Larkspurs
   - B. The *Consolida* type
     1. Double Stock-flowered larkspurs
     2. Emperor or Double Giant Imperial Larkspurs

II. The Perennial Delphiniums
   - A. The *Elatum* type
     1. Candle Larkspurs
     2. Bee Larkspurs
   - B. The *Cheilanthum* type
     1. Garland Larkspurs
     2. Belladonna Larkspurs
   - C. The *Nudicaule* or *Ruysii* type
     1. Red Larkspurs

D. elatum L. and *D. belladonna* Hort. are the parents of all the common garden varieties (Puttock, 1959). *D. elatum* L. originates from the Swiss Alps and can be found in the upper slopes of the mountains of West Virginia and across Europe from the Pyrennes to Siberia (Genders, 1963). The species name is from the Latin 'elatus' meaning tall. The origin of *D. belladonna* Hort. is unknown. Those delphinium species resembling *D. elatum* L. are known as the elatum type (Bishop, 1949). The genus delphinium is distributed almost exclusively in the temperate and cold regions of the Northern Hemisphere, with some from the high mountains of southeast Africa (De la Fuente and Reina, 1990).

Traditional Uses

The delphinium genus has long been known as a group of poisonous plants. Italians called these plants "Little Spur" or "King's Flower" (Phillips, 1933). They were known to the French as "Knight's Spur" or "King's Comfrey". These names refer to the use of this plant to rid oneself of body lice. Sap from larkspur was suggested as a remedy for colic, dropsy, and spasmodic asthma (Cronin and Neilson, 1990). *Delphinium denudatum* Wall. is known as nirbisi, nirvisha, judivar in the following languages; Hindi, Sanskrit, and Punjab, respectively. The root of this plant was used by Indians of southeast Asia to relieve toothaches, as a stimulant (Jain and Defilipps, 1991), alliterative, and tonic (Atta-ur-Rahman et al., 1997). Forking larkspur (*Delphinium consolida* L.) seeds were used in India for their insecticidal properties (Chopra et al., 1969). In ancient times, crude alkaloidal extracts from delphinium plants were used as a poisonous dip for arrowheads. *D. caeruleum* Watt. has been used in traditional Chinese medicine to treat traumatic injuries.

D. elatum L. was classified by Carolus Linneaus in 1753 (Missouri Botanical Garden, 1997). Its seeds were used in Indian medicine as a vermifuge and for their insecticidal, anthelmintic, diuretic, aperient, anti-itch, and emetic properties (Chopra et al., 1956; Beckstrom et al., 1994) The activity, or poisonous nature, of these plants is said to be related to the concentrations of different types of alkaloids.

Background and Project Objectives

Historically, plants have been used for sustenance, pest control, as medicine, and for recreational, and ceremonial purposes. Today, a renewed interest in plants and natural products may be is developing in the light of new knowledge as an alternative to synthetic products, testing the efficacy of the time honored traditional uses. Additionally, it is important to note that natural products are the templates from which these synthetic products created. For these reasons, it is easy to understand the importance of natural

products research. Pure natural products are of interest to those who want a return to the natural, while synthetic derivatives of these natural compounds can be powerful new alternatives for combating disease and infestation problems in agriculture as well as medicine.

Natural products are defined as organic compounds from natural sources, which are unique to one or a small group of related organisms. Natural products are secondary metabolites and are touted as non-essential to the originating organism i.e. plant, animal, or microorganism (Mann, 1987). Natural products from plants are perceived by many as less harmful than their synthetic counterparts. While this idea does not always hold to be true, natural plant products already exist in the environment, and therefore may be broken down, or controlled more readily than synthetic compounds. Natural products may also have more specific modes of action due to the fact that these plants have evolved along with the pests and pathogens that we are attempting to control. The goal of this project was to isolate, purify, and identify the compounds found in *D.* X *cultorum* and *D. bellamosum* that have biologically active properties.

The idea for this project was initiated in 1994 when a high school student from Three Rivers, Michigan, Carrie Scott, wrote to Dr. Lowell Ewart about the Magic Fountain delphiniums that were growing in her garden at home. Carrie reported her observation on plants she believed to be *D.* X *cultorum* cv. Magic Fountains. Carrie noticed that Japanese beetles were feeding on the flowers of these plants, slowly dying, and falling to the ground around these plants.

If accurate, Scott's observation seemed to reflect the findings of some of the toxicology studies done on other delphinium species. Therefore, it was my hypothesis that *Delphinium* X *cultorum*. cv. Magic Fountain contained compounds that have the potential to control Japanese beetles, along with other non-alkaloidal compounds with significant biological activity.

This thesis is comprised of a chapter describing the horticultural aspects of delphinium growth and production, a comprehensive literature review of non-alkaloidal compounds from delphinium species, a chapter on the chemical evaluation of the hexane extract of *D.* X *cultorum* flowers, and finally a chapter on the chemical evaluation of the ethyl acetate extract from *D.* X *cultorum* flowers. Appendix I describes preliminary investigation of *D. bellamosum* flower and stem extracts, in addition to an evaluation of *D.* X *cultorum* L. stem extracts.

CHAPTER II

Horticultural aspects of *D.* X *cultorum* and *D. bellamosum*

The Magic Fountains Series is classified as *D.* X *cultorum*. It is of hybrid origin but directly related to *D. elatum* L. All elatum-type delphiniums are of hybrid origin. Bailey (1976) describes *D.* X *cultorum* Voss (elatum-type) as a garden plant. *D. elatum* L. is a perennial with a knotty crown. Stems are spicatem and can be as tall as 6 feet. The leaves are large, palmate with 5-7 segments that are parted near the base. Also, the leaves can appear palmate with three upper segments parted. Ultimately, the leaf segments can be over 1/4" wide. Racemes are dense and erect. Flowers are up to 1-inch long. The sepals are blue, blunt, glabourous, and 1/2-inch long. The petals are dark shades of dull purple, the two lower petals can be yellow and bearded. The seeds are wrinkled, not scaly (Bailey, 1976).

Elatum-type delphiniums have spire-like flowers which can reach up to six feet tall. The botanical term for this type of flower head is raceme. The flowers are usually blue, white, lavender, purple or pink in color. A mature plant may be anywhere from 1 to 6 feet in height depending on the cultivar. The delphinium cultivar Magic Fountains ranges in height between 1.5 and 3 feet (Genders, 1963). Bee delphiniums such as the Magic Fountains series are so named because early growers of delphinium thought that the off-colored corolla resembled a pollinating bumble bee (Genders, 1963). The "flower" being the calyx.

The elatum-type delphiniums originated in the Pyrennees mountains, and eastwards in Siberia (Wyman, 1977). In the wild, *D. elatum* L. inhabits the clearings, woodland margins, and river valleys in thin birch and aspen forests of Siberia (Everett, 1981).

D. elatum L. is a natural tetraploid. The sterility of these plants was overcome by Dr. Legro (Marshall Cavendish, 1970). He crossed species at the diploid level, which produced tetraploids. The tetraploids were then crossed with hybrid delphiniums. However, elatum-types have four sets of eight chromosomes and therefore, seed development is difficult.

Cheilanthum-type delphiniums, or garland delphiniums have racemes with more sparse florets. The blue varieties are closer to true blue than those of *D. elatum* L. *Delphinium bellamosum* has an average height of 1.5 to 2 feet. They often are referred to as garland delphinium. and appear as 2-3-foot, erect perennial plants. The flower stalks are usually branched. The most popular species in cultivation is *D. formosum* Boiss. & Huet. The horticultural forms are offered as "belladonna" or "bellamosa" (Taylor, 1961). Similarly, garland delphiniums originated in Siberia or eastern Asia (Wyman 1977; Wise 1990). Hortus Third describes *D. bellamosum* as equal to *D.* X *Belladonna* Hort. *D. bellamosum* is similar to *D. elatum* L. but lacking a pronounced central raceme. The sepals are rich blue with a spur over 1-inch long.

Both types of delphiniums provide excellent cut flowers. Delphiniums bloom during the late spring and summer and also in autumn if the flowers are removed before the seed heads are formed. The autumn flowers are smaller than those produced in the spring. Delphiniums grown in a greenhouse may flower more than once if the plants are

cut back after blooming. The second and third crop of flowers will be smaller than the first.

Most delphiniums grow as perennial plants in Hardiness Zones 3-7. To plant delphiniums outdoors in beds, the plants should be placed 12 to 24 inches apart, with the crown 1 to 2 inches below the soil surface. The plants require full sun, and a well-drained soil that has been enriched with compost or manure (Time Life Virtual Garden, 1997). Delphiniums lose vigor after 2 to 3 years and should be replaced with new seedlings. If the plants are grown in a greenhouse, supplemental lighting is recommended. Delphiniums do not have a photoperiodic requirement for flowering. However, the more light they receive over time, the larger the flower head and more often they will flower.

Delphinium seeds should be sown in a disease-free sowing mix. The seeds should be covered lightly with sowing mix and kept watered enough so that the media surface is kept from drying out. The germination container should be maintained in a warm place which receives light and has good air circulation. It is of the utmost importance not to overwater delphinium seeds or plants. Seedlings are especially susceptible to damping off caused by root rot pathogens such as *Phytophthera*, *Rhizoctonia*, or *Pythium* species (Dowdeswell, 1997).

Chilling the seeds for at least one week at approximately 4°C increases the number of seeds that germinate. Germination temperatures between 18 to 24°C are ideal. The temperature should not exceed 26°C. For growing, air and media temperature should be in the range of 15-25 ° C (Dowdeswell, 1997). Delphiniums thrive with a soil pH at 6.0 to 6.5. Delphiniums can be propagated vegetatively by division in spring or fall or by cuttings. Seeds are the more favored means of production.

The diseases to which delphinium are susceptible are as follows: bacterial spot, damping off, mosaic virus, root rot, powdery mildew, black spot, crown rot (Wise, 1990; Genders, 1963). The common insect pests of delphinium in the field or garden are wood-lice, wireworm, Tortrix moth caterpillar, leafminer, cutworm, cockchafers, capsid, thrip, slugs, and leather jacket (daddy long legs larvae) (Genders, 1963). Cyclamen mite and two spotted spider mite are voracious pests in the greenhouse. The damage from mite infestation causes "the blacks," a condition in which the new growth turns black and becomes malformed. This irreversible condition appears mainly at the new growing points.

Carpenter and Boucher's (1992) research provided growers with temperature requirements for storage and germination of *D.* X *cultorum* seed (elatum-type). The most rapid and uniform germination rate was achieved at 20°C. Holding the seeds at 20°C for 3 weeks reduced the time to germination by 50%. A study on the effect of nitrogen level on the vegetative growth and flower production of *D. grandiflorum* L. was reported by Hassan and Khattab (1987). They used nitrogen levels of 0 ,0.5, 1, 1.5, 2, 2.5, and 3 g per plant. The highest leaf number, tallest stem length, and heaviest stem and dry leaf matter were induced at the level of 1.5 g of nitrogen per plant (7.5 g of ammonium sulfate). The authors stated that perhaps these data can be extrapolated to *D. elatum* L. production (Hassan and Khattab, 1987).

Information on growing delphiniums commercially can be accessed on the World Wide Web (www) at http://www.delphinium.co.nz and on delphinium diseases can be obtained on the www at http://msue.msu.edu/msue/imp/mod03/01700333.html.

CHAPTER III

Literature Review

Chemistry of Delphinium

Many classes of compounds have been identified from the *Delphinium* genus. The compounds of interest are generally referred to as secondary metabolites. Secondary metabolites have enormously diverse chemistry and are presumably non-essential to plant growth and development. Their principal function is for ecological interactions (Gershenzon, 1996). This section is organized into specific classes of compounds with some attention being paid to their ecological significance. This review is a compilation of information on compounds isolated from many delphinium species. It shows the breadth of work that has been done on this genus. The alkaloids from this genus have been the primary focus of most of the phytochemical studies, and for this reason only those alkaloids isolated from *D. elatum* L. are discussed. It is important to note that no two plant species have the same profiles of secondary metabolites.

In addition to providing protection to the plant, secondary compounds are often toxic to the plant itself. Therefore, these compounds are either compartmentalized in the plant, or stored in inactive forms. A common way for plants to store secondary metabolites is to add sugar moieties, proteins, or to form salts for the production relatively inert compounds (Bernays and Chapman, 1994).

The initial compounds that an insect, pathogen, herbivore would contact with are the low-molecular-weight, volatile compounds. This class includes a wide variety of

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The initial compounds that an insect, pathogen, herbivore would contact with are the low-molecular-weight, volatile compounds. This class includes a wide variety of

short-chain alcohols, aldehydes, ketones, esters, aromatic phenols, lactones, monoterpenes, and sesquiterpenes. The characteristic odors of some plants often are produced by a group of closely related volatile compounds (Bernays and Chapman, 1994). Volatile compounds produced by many plants are rich sources of secondary metabolites. To date no research on the volatile constituents of delphinium has been reported.

In addition, the epicuticular waxes that accumulate on leaf surfaces are another source for secondary metabolites. The waxy layer or cuticle is made up of complex mixtures of lipophilic compounds such as fatty acids, esters, hydrocarbons, triglycerides, phospholipids, glycolipids, and steroids (Bernays and Chapman, 1994). The cuticular waxes are the next level of plant defense against insects, pathogens, or herbivores.

Acetogenins

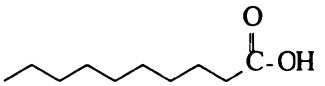
The compounds that comprise the cuticle are biochemically related and are classified as polyketide compounds. They are collectively referred to as acetogenins. These compounds are formed from condensation of acetate molecules (Ikan, 1991). The most common polyketide compounds are lipids. Phenolic compounds fall under the classification of acetogenins, also. Phenolics are characterized by at least one aromatic ring bearing one or more hydroxyl substituents. Phenolic compounds are abundant in plant tissues. There are several important classes of phenolic compounds such as flavonoids, coumarins, phenyl propanoids, tannins which are polymers of phenols, and phenolic acids and their glycosides. Phenolics are derived from the shikimate or malonate biosynthetic pathways (Taiz and Zeiger, 1991).

Lipids. Lipids can be classified broadly into three groups: fats, oils, and waxes. Fats are mixtures of glycerides. Glycerides can be saponified into glycerol backbone molecules, and the salts of the fatty acids. Generally, the fatty acids are from 12 to 34 carbon units in length, and usually contain an even number of carbon atoms. The bulk of the unsaturated fatty acids, including the non-conjugated acids, occur in the *cis* form. Waxes are the esters of fatty acids and monohydric alcohols, containing anywhere from 24 to 36 carbon atoms (Ikan, 1991). They make up the protective cuticle that prevents water loss and inhibits pathogen attack (Taiz and Zeigler, 1991). Fats and oils are storage forms of reduced carbon found in seeds, which are converted into carbohydrates during germination. **Figure 3.1** shows some examples of fatty acids isolated from delphinium species.

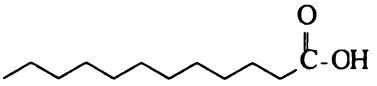
Oil extracted with ether from *D. hybridum* Hort. seed was prepared to form a mixture of methyl esters. The mixture was reported to contain 9,10-dihydroxypalmitic, palmitic, tetrahydroxystearic, dihydroxystearic, stearic, tetrahydroxyeicosanioc, 11,12-dihydroxyeicosanoic, and 11-eicosenoic N-hydroxyamide acids (Chisholm and Hopkins, 1956). This was the first report of C_{20} free fatty acids from the seed oils, i.e. tetrahydroxyeicosanioc and 11-eicosenoic acids, from this plant family.

An unsaturated hydrocarbon, $C_{32}H_{58}$, b.p. 200°/0.1 mm of Hg, $n^{34}{}_D$ 1.4800 with no rotation, and a monohydric unsaturated alcohol, $C_{16}H_{30}O$, b.p 156-160° /0.1 mm of HG, $n^{34}{}_D$ 1.4646 with no rotation were isolated from the petroleum ether extract of the roots of *D. denudatum* Wall. The compounds were purified by adsorption analysis on

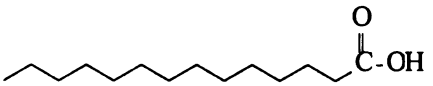
Capric Acid



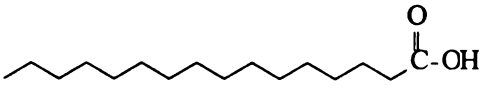
Lauric Acid



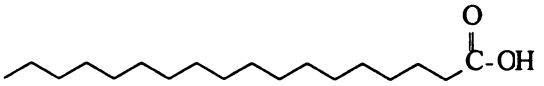
Myristic Acid



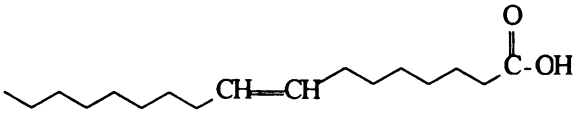
Palmitic Acid



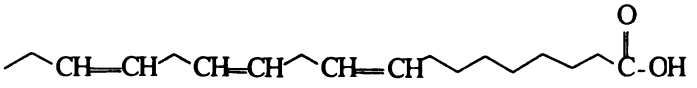
Stearic Acid



Oleic Acid



Linolenic Acid



11-Eicosanoic acid

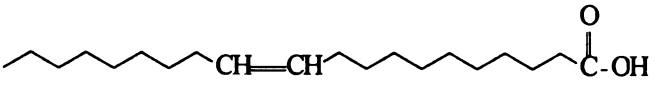


Figure 3.1. Fatty acids reported from *Delphinium* species.

alumina followed by distillation. Purity was checked by thin layer chromatography (TLC) on silica (Qureshi and Ahsan, 1965).

The fatty acid composition of *D. denudatum* Wall. was quantified by separation on TLC and gas liquid chromatography (GLC). The fatty acids identified and quantified by weight were capric (2.3 %), lauric (1.6%), myristic (3.8%), palmitic (16%), palmitoleic (2.5%), stearic (1.3%), oleic (23%), linoleic (30.2%), and linolenic acids (19.3%) (Asif et al., 1981). The seed oil of *D. staphisagria* L. was analyzed for its chemical constituents. Palmitic and steric acids were present in the highest amounts. Fatty acids from C_6 to C_{25} were detected by GC analysis (Costa de Pasquale et al., 1985).

Icosenoic acid was isolated from the seed oil of *C. regalis* S. F. Gray by Dabi et al. (1986). After methylation and analysis of GC/MS, NMR, and IR spectra, it was determined that it was a 20:1 fatty acid. Using two eluent systems, its bromophenacyl ester was identified as 20:1 (*cis* -11) by comparison of HPLC retention times of 20:1 (*cis*-5) as well as several other larger and smaller bromophenacyl esters. The seeds of *C. regalis* S. F. Gray contain 30% of oil, which is comprised of 25% of 20:1 fatty acid. The main fatty acid was 18:1 (Dabi et al., 1986).

Octacosanoic acid and N-acetylanthranilic acid were isolated from the roots of *D. iliense*. These compounds, along with some alkaloids, were identified by chemical and spectral means such as UV, IR, ^{1}H-NMR, and ^{13}C-NMR (Dong et al., 1991).

A study by Hasan and Osman (1993) suggests that *D. ajacis* L. should be considered for use as an agronomic crop due to its seed oil components. It was found to contain linoleic acid (<40%) but no linolenic acid. Oils with this composition belong to the class of non-drying oils. The oil component of these seeds was reported to be 32 %.

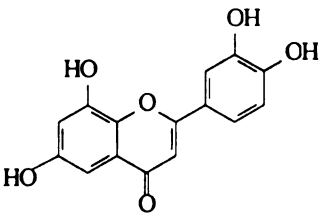
The methyl ester composition of the oils was reported for 16:0, 18:0, 18:1, 18:2, 18:3 and 20:1 as being 6.5, 1.0, 46.5, 18.9, and 27.1 %, respectively. *D. ajacis* L. seeds yielded high oleic content (46.5%) seed oil, as well as, eicos-cis-11-enoic acid (27.1%) (Hasan and Osman, 1993).

Flavonoids. Flavonoids are phenolic compounds. They consist of two aromatic rings, A and B, linked by a three-carbon chain or ring C. They are often present in plant tissue as glycosides. Ring A originates from the malonate pathway whereas the B ring is synthesized from the shikimic acid pathway via phenylalanine (Taiz and Zeigler, 1991). A number of physiological activities in humans and animals have been linked to flavonoids. **Figure 3.2** shows some representative flavonoids isolated from delphinium species.

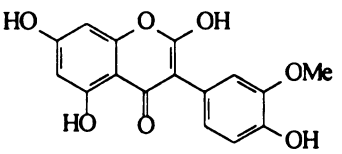
D. zalil Biernert ex. Boiss. is an Indian dye plant known for the production of "gandhaki". The dye was attributed to two coloring substances, one of which was very soluble in alcohol (quercetin) and another which was only sparingly soluble [Quercetin monomethyl ether (iso-rhamnetin)] (Perkin et al., 1898).

Several Ranunculaceae plants were investigated to determine the types of flavonol glycosides they contained. Glucose usually was not found in the seven position of the flavonol glycosides. However, rhamnose, and to a lesser extent xylose, were found to be the sugar moiety at the C-7 position. The flavonol glycosides of Ranunculaceae are used as chemotaxonomic tools for this family (Egger and Keil, 1965).

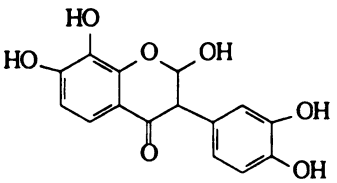
Different parts of *D. consolida* L. were monitored for their flavonoid content during growth and development. The typical flavonoid, isorhamnetin, was absent



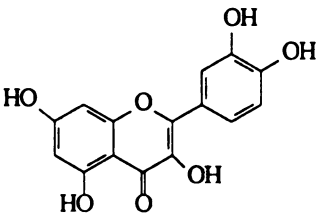
Rutin



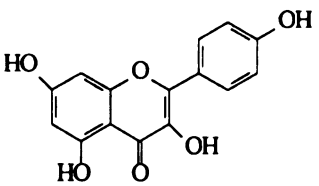
Isorhamnetin



Isoquercitrin



Quercetin



Kaempferol

Figure 3.2. Flavonoids isolated from *Delphinium* species.

throughout the study. For this reason, it was recommended that this plant be classified into a new genus, *Consolida* (Mel'nichuk et al., 1971).

Leaves of *D. flexiosum* and *D. elisabethae* yielded quercetin, isoquercetin, and 7--L-rhamnofuranoside (Arazashvili et al., 1973). Arazashvili et al. (1974a) analyzed flavonoids from the leaves of four species of delphinium. All four contained rutin. In addition, leaves of *D. freynii, D. smalhausenii, D. tamarae* contained quercetin, hyperoside, and kaempferol and astragalin, respectively. Similarly, *D. dzawachischwilii* flowers contained kaempferol-rutinosides.

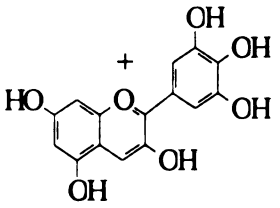
Strzelecka (1976) reported four flavonoid compounds from *D. grandiflorum* L. In three of the flavonoid glycosides, the aglycone was kampferol. The carbohydrate constituents were rhamnose and glucose, rhamnose and arabinose, and arabinose and glucose, respectively. In the fourth glucoside, the aglycone was quercetin. The flavonoids quercetin, rutin, astragalin, and kaempferol-3-rutinoside were isolated from the aerial parts of *D. peregrinum* (Mericli et al., 1991b).

The flavonoids quercetin (3'''-benzoyl-2''-glucosyl)-3-glucoside-7-rhamnoside and partially characterized quercetin (xylosyl, benzoyl)-3-glucoside, and quercetin(benzoyl, glucosyl)-3-glucosides were isolated from *D. carolinianum* Walter during a systematic study of the genus. Several known compounds such as galactosides, glucosides , rhamnosides and xylosides of quercetin and kaempferol were isolated, also (Warnock et al., 1983).

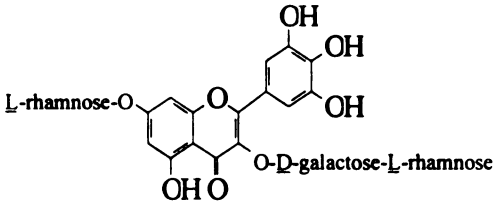
Anthocyanins. Anthocyanins are the most widespread group of pigmented flavonoids, followed by carotenoids. They occur as glycosides having a sugar moiety at the three-position. These secondary products apparently function as attractants of animals and insects for pollination and seed dispersal. The color of an anthocyanin is influenced by the number and position of the hydroxyl and methoxy substituents on the B ring, the presence of chelating metals, the presence of flavone or flavonol copigments and the pH of the cell vacuole in which these compound are stored. The anthocyanin, delphinin, was discovered in *D. consolida* L. by Willstater and Mieg (1915) for the first time. **Figures 3.3** and **3.4** show some anthocyanins isolated from delphinium species.

Anthocyanins were of primary interest as dyes before more easily obtained synthetic dyes were available. Lushchevs'kaya (1937) studied *D. consolida* L., the red delphinium, as a dye-yielding plant. Galfayan (1938) extracted the pigment from *D. orientale* flower petals. It was tested for its use an indicator for alkalimetry and acidimetry. The pigment was pink in color in acid solution, blue in neutral solution, and green in alkaline solution. The pH interval for the blue color is 4.2 to 6.3. Ringer (1950, 1951) measured the absorption of dyes in solution from several plants including *D. consolida* L.

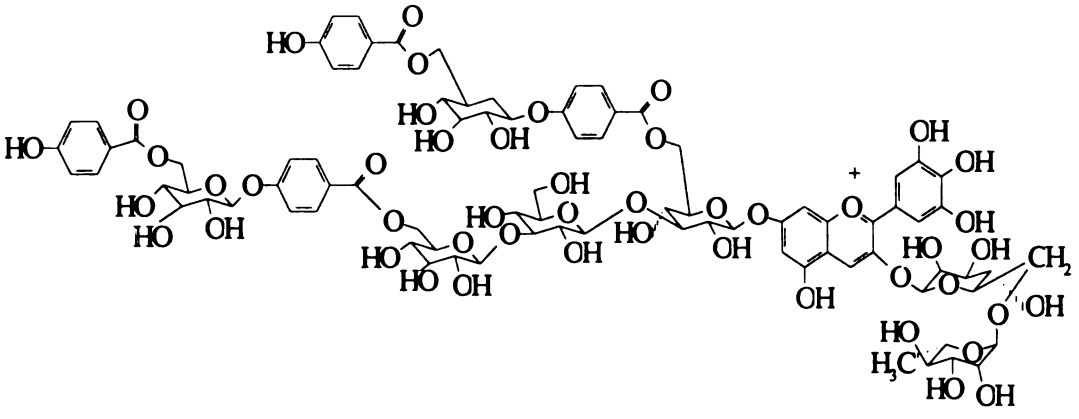
Later, delphinidin diglycoside was isolated from *D. ajacis* L. (Krishnamoorthy et al., 1962). In 1975, Asen and colleagues re-examined the pigment, delphinidin, from from blue larkspur cv. Dark Blue Supreme and reported the structure as delphinidin 3-di-(*p*-hydroxybenzoyl)-glucosylglucoside. Reddish-purple flowers of this cultivar showed distinctive in vivo absorption bands which were found to be related to pH (Asen et al., 1975). The pH of epidermal peels from young, moderate, reddish-purple larkspur flowers



Delphinidin

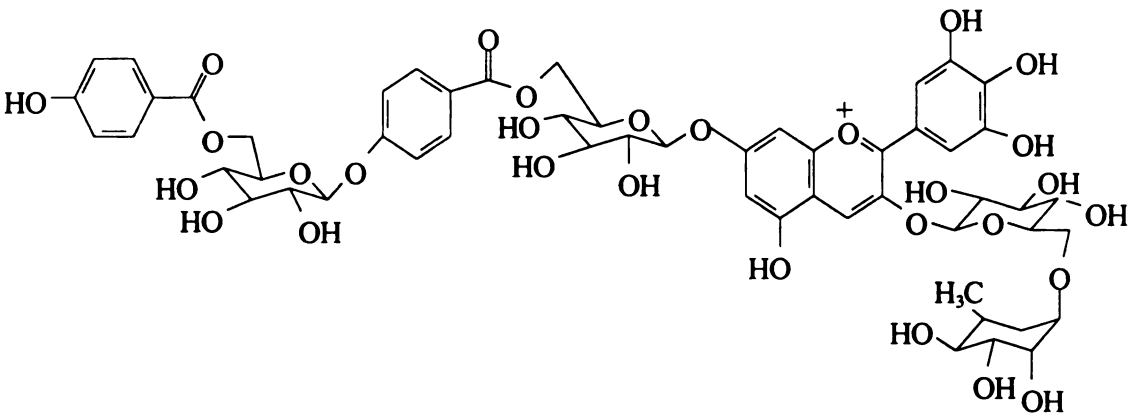


Robinin



Cyanodelphin

Figure 3.3. Anthocyanins isolated from *Delphinium* species.



Viodelphin

Figure 3.4. Another anthocyanin isolated from *Delphinium* species.

was 5.5. As the flowers aged, the pH increased to 6.6 and the color changed to light purplish blue to light blue.

Many older cells contained strong blue crystals of anthocyanin. The anthocyanin and flavonol glycosides in this species are delphinidin, 3-di (*p*-hydroxybenzoyl) glucosylglucoside,kaempferol 3-galactoside-7-rhamnoside (robinin), kaempferol 3-rutinoside, kaempferol 7-rhamnoside, and kaempferol 3-(caffeylgalactosylxyloside)-7-rhamnoside. Also, absorbance of buffered solutions containing these compounds were measured. Delphinidin 3-di (*p*-hydroxybenzoyl) glucosylglucoside at pH 5.6 was reddish-purple in color and was not stable. Addition of kaempferol glycoside copigments resulted in a stable complex, a slight bathochromic shift, and sharpening of the visible absorption maxima. The absorbance maxima of senescent light purplish-blue cells could not be matched by the absorption curves of a 10^{-2} M solution of delphinidin 3-di (*p*-hydroxy-benzoyl)glucosylglucoside in a pH range of 6.6 - 7.2 (Asen et al., 1975).

An anthocyanin from *D. hybridum* Hort. cv. Black Night, viodelphin, was isolated and characterized by Kondo et al. (1990). It is a delphinidin derivative containing two molecules of *p*-hydroxybenzoic acid (Kondo et al., 1990). Kondo et al. (1991) reported the structure of cyanodelphin, a tetra-*p*-hydroxybenzoated anthocyanin from *D. hybridum* Hort. cv. Blue Springs.

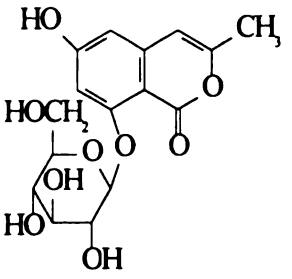
Coumarins and other phenolics. Coumarins generally are found throughout all plant parts. Some coumarins are thought to protect against herbivory, while others are thought to be responsible for attracting pollinators. Coumarins are synthesized from phenyl-

alanine from the shikimic acid biosynthetic pathway. **Figure 3.5** shows a range of phenolic compounds isolated from delphinium species.

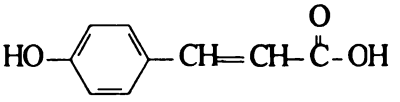
Delphoside, an isocoumarin glucoside, was isolated from the leaves of an unspecified species of delphinium (Arazashvili et al., 1974b). Lomadze et al. (1976a) used polargraphic methods to determine coumarins in *D. flexuosum* and *D. speciosum* Bieb. Delphoside and flexidine were detected before, during and after flowering in *D. flexuosom*. These were detected in *D. speciosum* Bieb. as well, but concentration varied with geographic location. The level of rutin in the leaves of *D. speciosum* Bieb. was found to be 0.58-2.5 (Lomadze et al., 1976b).

Cis-*p*-coumaric acid, α-amyrin (**Figure 3.6**), and α-amyrin acetate were isolated from aerial parts of *D. peregrinum* (Mericli et al., 1991b). The aerial parts of *D. venulosum* Boiss yielded five aromatic compounds including one new compound, 2,5,6 trihydroxypiperonylic acid methyl ester. The other four were reported as *cis* and *trans p*-coumaric acids *m* and *p*-hydroxybenzoic acids, and protocatechuic methyl ester (Mericli et al., 1991a).

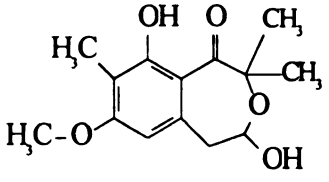
The ethanolic extract from *D. formosum* Bioss.& Huet. afforded a new benzoxepine derivative oxformasine (1,1',7-trimethyl-2-oxo-3,8-dihydroxy-6-methoxy-benztetrahydrooxepine) (Mericli et al., 1996). The roots of *D. denudatum* Wall. yielded 3-hydroxy-2-methyl-4*H*-pyran-4-one (Attar-ur-Rahman et al., 1997).



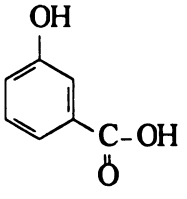
Delphoside



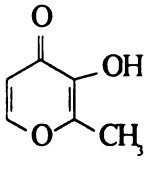
p-coumaric acid



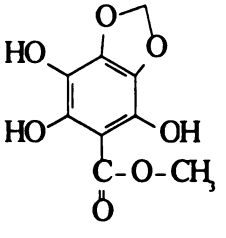
1,1',7-trimethyl-2-oxo-3,8-dihydroxy-6-methoxy-benztetrahydrooxpine



m-hydroxybenzoic acid



3-hydroxy-2-methyl-4H-pyran-4-one



2,5,6-trihydroxypiperonylic acid methyl ester

Figure 3.5. Phenolic compounds isolated from *Delphinium* species.

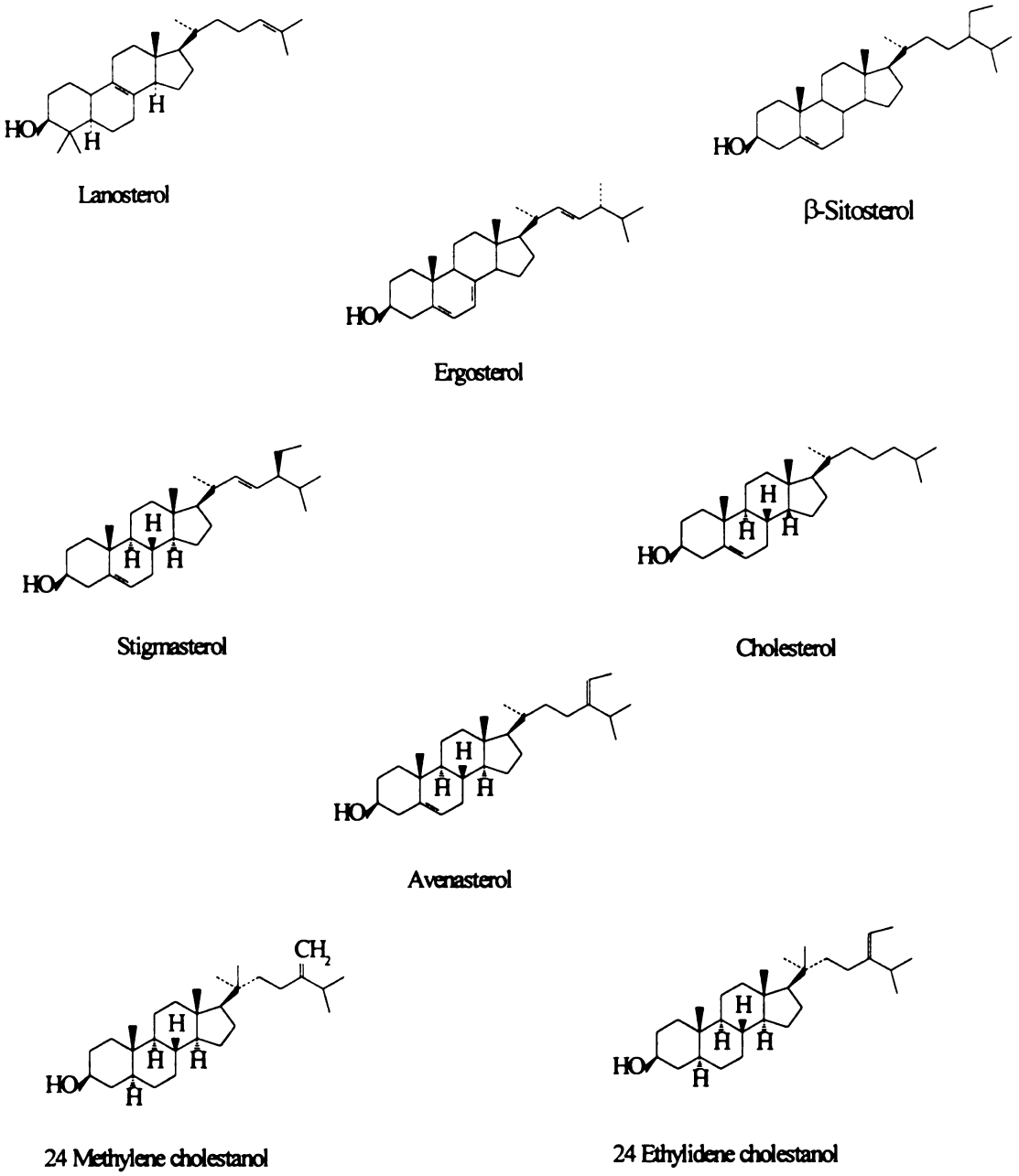


Figure 3.6. Sterols isolated from *Delphinium* species.

Carbohydrates. Carbohydrates are one of the most abundant groups of plant constituents. For this reason, they generally are not considered secondary metabolites. Carbohydrates play important multiple rolls in all life forms. They are the energy stores in plant tissue, and the structural framework of DNA, RNA, and cell walls. Most of the reports on carbohydrates from delphinium are the glycosides of true secondary metabolites such as flavonoids, anthocyanins, coumarins, etc. However, one paper reported the isolation of D-mannitol (**Figure 5.1**) from *D. flexiosum*, *D. elisabethae*, *D. tamarae*, and *D. dzawachischvili* (Dekanosidze et al., 1974).

Terpenoids

Terpenoids form the largest and most diverse class of compounds found in plants. They range from constituents of essential oils such as monoterpenes and sesquiterpenes to nonvolatile triterpenes, sterols, and carotenoids. They are generally lipophilic compounds. Terpenes often serve the plant as defensive compounds against herbivores. They are derived from acetyl-CoA via the mevalonic acid pathway. Steroids are widely distributed in nature. Most steroids are hydroxylated at C-3, and other sites. Sitosterol and stigmasterol are the most abundant plant sterols (Ikan, 1991). A number of plant steriods have pharmacological activity on humans and animals.

Sterols and triterpenes. It is not surprising that β-sitosterol, being one of the ubiquitous sterols, has been reported from many delphinium species. (Qureshi, 1965; Mericli et al., 1991a). In addition to β-sitosterol, many other sterols and triterpenes have been isolated

from plants of this genus. **Figure 3.6** shows some representative sterols isolated from delphinium species.

Trace amounts of cholesterol and Δ^5-avenasterol were detected in *D. denudatum* Wall. roots along with characteristic higher plant fatty acids and the ubiquitous sterols, i.e. β-sitosterol, stigmasterol, etc. (Asif et al., 1981).

Sterols, 4-methylsterols, 4,4-dimethylsterols, and triterpenes from whole nonsterile *D. ajacis* L., and from sterile cultures of callus tissues of germinated seedlings were analyzed by Waller and Mangiafico (1978). Among the 26 sterols and triterpenoids identified, Δ^{22}-stigmasterol was identified in both tissue types, whereas, 24-ethylidenecholestanol was tentatively identified in callus tissue and 24-methylenecholestanol only in non-sterile tissue. Several other differences also were observed. Qualitatively the contents of sterile and non-sterile tissue was similar, suggesting that these sterols fit into a metabolic role in which enzymatic reactions proceed from cycloartenol through 4,4 dimethylsterols and 4-methylsterols to sterols. The difference in quantity may be due to lack of organogenetic controls in the rapidly proliferating tissue grown aseptically, in comparison with the slow-growing nonsterile plants (Waller and Mangiafico, 1978).

Later, whole plants and sterile callus tissue of *D. ajacis* L.were studied for production and the metabolic relationship of sterols. These studies began as an attempt to produce diterpenoid alkaloids in tissue-culture specimens. However, these cultures produced large amounts of sterols rather than alkaloids. The major sterols in the whole-plant tissues were β-sitosterol, campesterol, and stigmasterol. The major sterols in the callus tissue were stigmasterol, 24-ethylidenelophenol, and Δ^{22}-stigmasterol. Five of the

sterols reported from callus tissue were not present in the whole plant. A partial metabolic pathway was proposed for sterol production in *D. ajacis* L. by combining the data from this study and from others (Waller et al., 1981).

Sterols, such as cholesterol, campesterol, stigmasterol, Δ^7-campesterol, β-sitosterol, Δ^5-avenasterol, and Δ^7-avenasterol, were isolated from the fatty seed oil of *D. staphisagria* L.(Costa de Pasquale, 1985).

Nitrogen-Containing Compounds

Diterpenoid and Norditerpenoid Alkaloids. Alkaloids form one of the largest classes of plant secondary metabolites. Alkaloids occur in concentrations of more than 0.01% dry weight, and are found in at least 15% of vascular plants (Bernays et al., 1994). The concentrations of N-containing compounds are usually highest in young tissue. As the plants mature, the concentrations of these compounds slowly decline.

The classic definition of a "true" alkaloid is a compound that has a heterocyclic ring containing a basic nitrogen, is related to amino acids, has a complex molecular structure, exhibits significant, often toxic, pharmacological activity and is produced by members of the plant kingdom (Pelletier et al., 1983). A modern and more appropriate definition for an alkaloidal compound was proposed by S. W. Pelletier in 1983, as a cyclic organic compound containing nitrogen in a negative oxidation state, which is of limited distribution among living organisms. This description encompasses a larger number of pharmacologically active, nitrogen-containing compounds, including the diterpenoid alkaloids, previously referred to as psuedoalkaloids (Pelletier et al., 1983).

Diterpenoid alkaloids are the most extensively investigated compounds from delphiniums due to their extreme toxicity. A summary of the procedure for isolating delphinium alkaloids is published in Chapter 12 of "Toxicants of Plant Origin," (Olsen and Manners, 1989). At least 150 diterpenoid alkaloids have been identified in delphinium (Olsen, 1990). In addition to *Delphinium* (Ranunculaceae), these types of alkaloids have been investigated in *Aconitum* (Ranunculaceae), *Garrya* (Cornaceae), and *Inula* (Compositae) species (Waterman, 1993). As of 1990, 40 % of the diterpenoid alkaloids isolated from larkspur are exclusive to this family (Blanche, 1990). Furthermore, 30% of the lycoctonine-type, 50 % of the hetisine-subtype and 50 % of the aconitine-type are exclusive to the annual delphinium (Blanche, 1990). The classification of diterpenoid and related alkaloids follows:

I. Diterpenoid alkaloids
   - A. Atisine-type
   - B. Veatchine-type
   - C. Delnudine-type

II. Norditerpenoid alkaloids
   - A. Aconitine-type
     1. Aconitum-type
     2. Pyrodelphine-type
     3. Heteratisine-type
   - B. Lycoctonine-type

III. Bisditerpenoid alkaloids

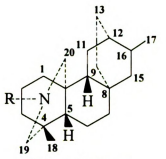
Diterpenoid alkaloids are distributed throughout all the parts of delphinium plants. Alkaloids accumulate in the vacuoles and periplasmic space in plants. The alkaloidal content of most delphiniums is up to about two percent (Kuder, 1947).

Diterpenoid alkaloids are divided into two categories, a hexacyclic C-19 skeleton (norditerpenoid), and those based on C-20 skeletons (diterpenoid). Pelletier (1980a)

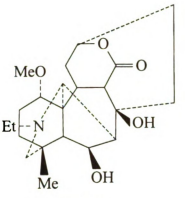
suggested that these two types are derived from tetracylic or pentacyclic diterpenes in which the nitrogen atom of methylamine, ethylamine, or β-aminoethanol is linked to the C-17 and C-19 in the C-19 skeleton (e.g. lycoctonine) and to C-19 and C-20 in the C-20 skeleton (e.g. atisine). These linkages form a substituted piperidine ring.

Diterpenoid alkaloids are referred to as isoprenoid alkaloids. Unlike the classical alkaloids, biogenetically related to amino acids, diterpenoid alkaloids are thought to be formed from the precursor, acetyl CoA, in the mevalonic acid pathway. Specifically, diterpenoid alkaloids of the lycoctonine , heteratisine, veatchine, and atisine types are thought to be formed from the precursors primaiadiene and phyllocladine type intermediates with the incorporation of nitrogen (**Figure 3.7.**) (Gross et al, 1985; Figuernido et al. 1995).

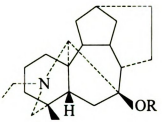
Early attempts to test whether or not specific delphinium alkaloids were of terpenoid origin was demonstrated by the incorporation radiolabled mevalonic acid into the alkaloid structure. Herbert and Kirby (1963) fed *D. elatum* L. plants with DL- [2-^{14}C] mevalonic acid through the cut ends of leaf stalks. The plant was found to contain a non-saponifiable extract which exhibited 1.3% incorporation of radioactivity. The radioactive component was shown to be β-sitosterol. Delpheline isolated from the same plant showed no significant radioactivity. This experiment was conducted during the rapid growth phase of the plant, also known to be the time during the delphinium life cycle to produce the highest level of alkaloids. The authors suggested that delpheline lacks the incorporation of mevalonic acid, because the precursor is converted into the non-basic plant terpenoid before it reaches alkaloid synthesis (Herbert and Kirby, 1963).



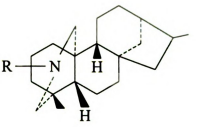
Atisine Skeleton



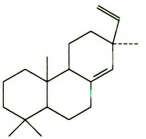
Heteratisine



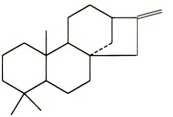
Lycoctonine Skeleton



Veatchine Skeleton



Primaradiene

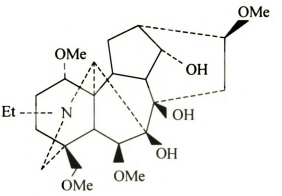


Phyllocladene

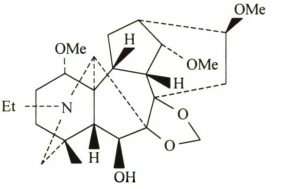
Figure 3.7. Diterpenoid alkaloids and their proposed biosynthetic precursors.

A later communication from Benn and May (1964) reported that browniine and lycoctonine became radioactive in twelve specimens after being supplied with mevalonate-2-^{14}C dibenzylethylenediamine by stem injection (**Figure 3.8**). Sodium acetate - 1-^{14}C, -2-^{14}C were incorporated to a lesser degree. This experiment was carried out twice as long as the previous experiment, and radioactive incorporation was low. However, these results were consistent with those expected for normal terpene biosynthesis. Benn and May (1964) suggested that the unsuccessful incorporation of radio-labelled mevalonate in delphiline by Herbert and Kirby occurred because they used detached leaves. The primary site for alkaloid biosynthesis is in the roots. Furthermore, there may have been significant competition for incorporation by non-alkaloidial terpenes.

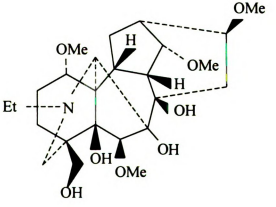
A study by Ovezov (1965) showed that $^{14}CO_2$ was incorporated into elatine. The incorporation of radiolabelled compounds was greater in the roots, supporting Benn and May's (1964) previous idea. Ovezov (1965) suggested that diterpenoid alkaloids were synthesized from several precursors, and the rate of synthesis was different for each alkaloid observed. A later report supported to the claim that diterpenoid alkaloids are synthesized at different rates. Acetate- 2-^{14}C was incorporated mainly into elatinic acid and to a lesser degree in to elatidine and other alkaloids. The low inclusion of acetate- 2-^{14}C into elatidine was explained by the competition for incorporation of the radiolabelled substance into non-alkaloidal terpenes (Ovezov, 1966). Methanol-^{14}C was fed to roots of *D. elatum* L. plants, and this produced a lower level of alkaloids in the plant. However, the ^{14}C was incorporated into the alkaloids, elatine and



Brownine



Delpheline



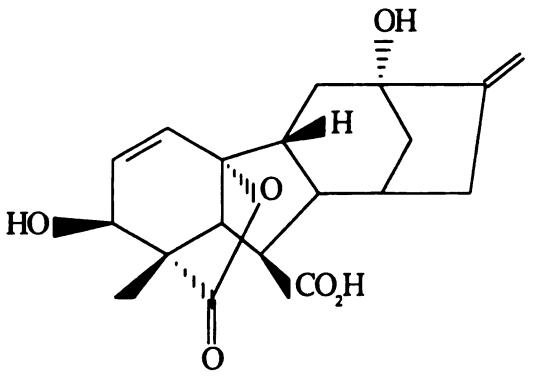
Lycoctonine

Figure 3.8. *Delphinium* alkaloids studied to determine their biosynthesis.

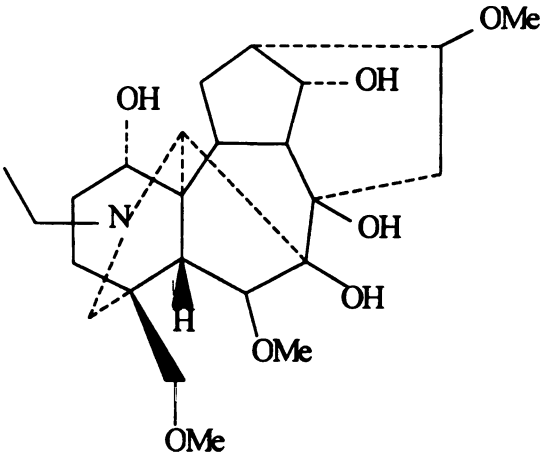
methyllycaconitine from elatinic acid. Methanol-^{14}C was not shown to be a direct precursor of the methoxy groups in alkaloids (Ovezov, 1967).

The structure of diterpenoid alkaloids have been compared to that of the gibberellins (**Figure 3.9**). Diterpenoid alkaloids and gibberellins may share similar early biosynthetic pathways (Waller et al., 1969). The C-19 or norditerpenoid alkaloids have been subdivided into two groups,the aconitine-type and the lycoctonine-type (**Figure 3.10**). These two groups share some common elements. Both possess a hexacyclic skeletal system comprised of one 7-membered, three 6-membered, and two 5-membered rings. Both contain a tertiary nitrogen substituted with an ethyl or methyl group (Keith et al.,1970). The C-1 and C-8 are attached to an oxygen-containing moiety which can be hydroxyl, methoxy, or ester functionalities. The C-14 has an α-oxygen functional group, C-16 a methoxy group in the β-configuration. The C-18 usually possesses either a methoxy or an N-substituted ester of anthranilic acid (Keith et al., 1970).

Lycoctonine-type alkaloids are medially toxic, methoxyl monoesters of the alkaloidal amino alcohol bearing benzoic acid or anthranilic acid ester substituents. In general, C-7 is always oxygenated, and these types of alkaloids are widely distributed in nature (Stern, 1954). Aconitines are acutely toxic with a lethal dose on the order of 2-5 mg for human. They are heavily substituted with methoxy groups, two of the alcoholic hydroxyl groups being esterified, one by acetic acid and the other by an aromatic acid (e.g. benzoic acid or a *p*-methoxy- or 3,4-dimethoxy derivative) (Stern, 1954). Usually, C-7 is not oxygenated when compared to the lycoctonine-type alkaloids. Those lycoctonine-type alkaloids with N-(methylsuccinyl)anthranilic acid at the C-18 position

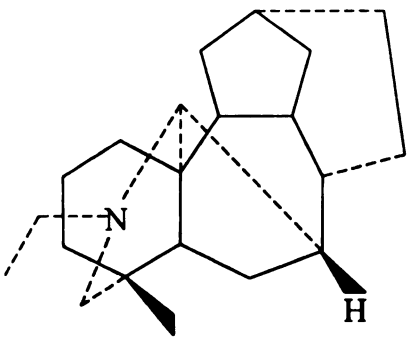


Gibberellin A_3

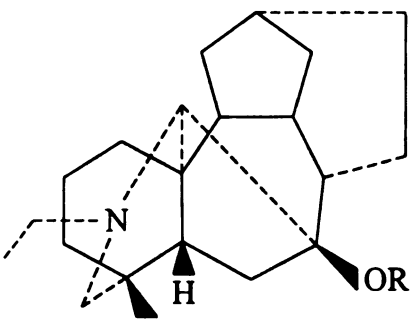


Delcosine

Figure 3.9. Gibberellin and delcosine.



Aconitine-type Skeleton



Lycoctonine-type Skeleton

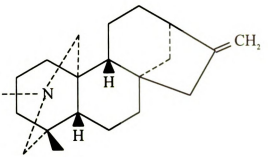
Figure 3.10. Norditerpenoid alkaloid skeletons.

have been reported to be the most toxic of this type. The LD_{50} values were between 2.7 and 10 mg/kg in mice. Structure-activity relationship studies show that toxic norditerpenoid alkaloids must contain a tertiary nitrogen and an anthranilic acid ester (Manners et al., 1995).

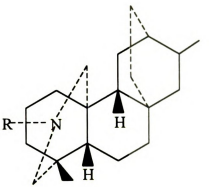
Aconitines can be subdivided into three groups based on their different skeletal arrangements, such as (1) Aconitum-type in which the C-7 is either not substituted or is substituted with anything other than oxygen, (2) Pyrodelphinine-type in which there is a double bond between C-8 and C-15, and (3) Heteratisine-type in which there is a lactone moiety present (Pelletier et al., 1980a). The chemistry of C-19 diterpenoid alkaloids was reviewed by Pelletier and coworkers (1980a).

The C-20 diterpenoid alkaloids can be divided into three groups, the atisine-type, veatchine-type, and the delnudine-type (**Figure 3.11**) (Pelletier and Mody, 1981). Atisines are simple non-toxic amino alcohols which are not extensively oxygenated and which contain at most one methoxy moiety (Stern, 1954). The three different C-20 skeletons isolated differ in attachment of the C-15 and C-16 bridge at either C-11, C-12, or C-13. The chemistry of the C-20 diterpenoids alkaloids is reviewed by Pelletier (1980a).

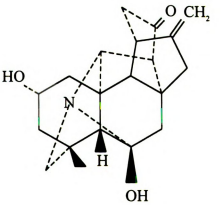
The largest and most complicated diterpenoid alkaloids are the bis-diterpenoid alkaloids isolated from *D. staphisagria* L. (**Figure 3.12**) (Pelletier et al., 1980a). Bisditerpenoids consist of two C-20 diterpenoid alkaloids attached at the C-16 position (Pelletier and Mody, 1981).



Veatchine-type Skeleton



Atisine-type Skeleton



Delnudine

Figure 3.11. Diterpenoid alkaloids.

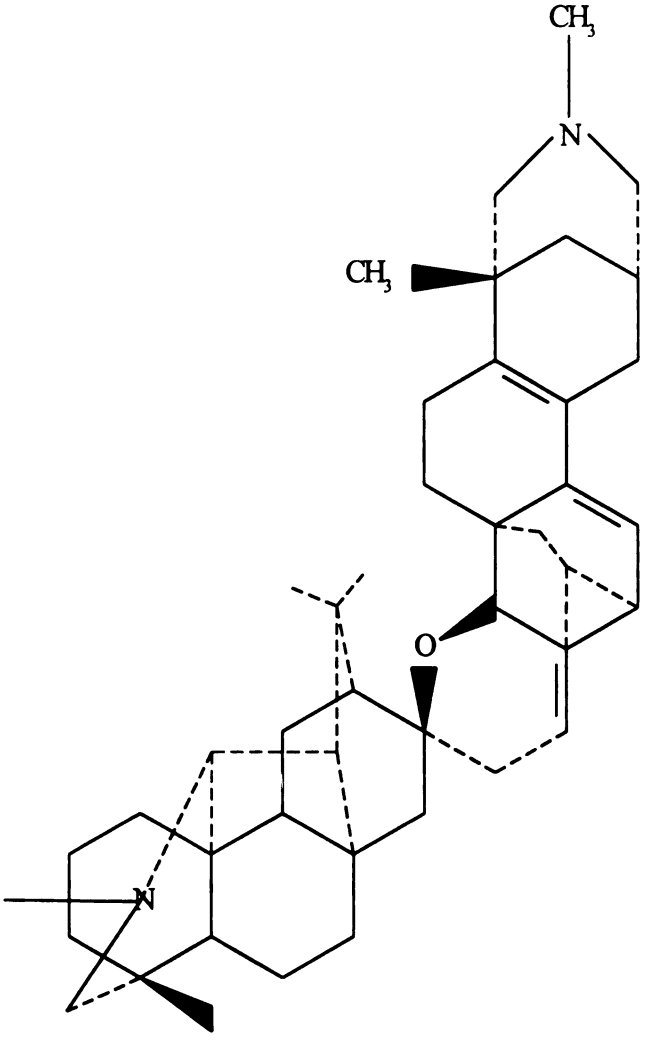


Figure 3.12. Bisditerpenoid alkaloidal skeleton.

Alkaloids are by-products of plant metabolism, i.e. secondary metabolites. It has been conjectured that alkaloids are reserve materials for protein synthesis, supporting the earlier definition of an alkaloid. A more popular and modern view is that alkaloids are defense compounds and serve to protect the plants from pathogen infection, insect infestation, or mammalian herbivores. Countless alkaloids have been found in numerous species of delphinium. The alkaloids reported from *D. elatum* L. are listed in **Table 3-1**.

The only work reported on *D. belladonna* Hort., a close relative of *D. bellamosum* was a study of the quantitative estimation of the alkaloids in the different organs of this plant (Hashem et al., 1974a). Total alkaloid content was measured in roots, stems, leaves, flowers, fruits, and seeds before flowering, during flowering, and during fruiting. The highest alkaloid content was in the leaves before and during flowering, and in the fruits during fruiting. The highest overall level was in the seeds during fruiting. Ajacine was identified, and its levels were monitored in this study as well. Ajacine's highest level was found in the seeds of this plant. The trend for the alkaloid level in this plant during growth and development was the alkaloid percentage increased in stems, leaves, and roots during the flowering stage and decreased in the fruiting stage (Hashem et al., 1974a). This plant was reported to have insecticidal properties due to the alkaloid content. Phytochemical screening was carried out on the different organs of this plant. Alkaloids were detected in the root, stem, leaf, flower, pericarp and seeds. These alkaloids were identified by IR spectral studies. The fingerprint regions for ajacine, delphamine, and ajaconidine were identified by comparison to previously reported literature values (Hashem et al., 1974b).

Table 3.1 Alkaloids Isolated from *D. elatum* L. and *D. bellamosum*

Alkaloid	Species*	Plant part **	References
andersonidine	e	s	Wada et al., 1992;Atta-ur-Rahman et al., 1995
ajacine	b	s	Hashem et al., 1974b; Pelletier and Page, 1978
ajaconine	e	s	Pelletier et al., 1990;Yunusov, 1993
ajacinoidine	b	s	Hashem et al., 1974b; Pelletier and Page, 1978
blacknidine	e	w	Park et al., 1995
blacknine	e	w	Park et al., 1995
delatine	e	s	Goodson, 1943; Stern, 1954
delatisine	e	s	Ross et al., 1991; Atta-ur-Rahman et al., 1995
delcorine	e	s	Wada et al., 1992;Atta-ur-Rahman et al., 1995
delcosine (delphamine)	b	s	Hashem et al, 1974b; Pelletier and Page, 1978
delectinine	e	w, s	Vaospv, 1993; Park et al., 1995;Yunusov, 1991
delelatine	e	w, s	Park et al., 1995; Pelletier et al., 1989; Yunusov, 1991
deltaline	e	w, s	Rabinovich, 1954; Kuzovkov, 1956; Feofilaktov, 1954
delphelatine	e	w	Feofilaktov, 1954
delpheline	e	w, s	Goodson, 1943; Cookson, 1954; Herbert et al., 1963; Pelletier et al., 1989; Bando et al., 1989; Park et al., 1995; Stern, 1954; Olsen and Manners, 1989
eladine	e	s	Pelletier et al., 1989; Wada et al., 1992; Yunusov, 1991; Attar-ur-Rahman, 1995
elanine	e	s	Pelletier et al., 1990; Yunusov, 1993
elasine	e	s	Pelletier et al., 1990; Yunusov, 1993
elatine	e	w, s	Rabinovich, 1952, 1954; Dozortseva, 1956; Kuzovkov, 1956; Ovezov, 1966,1967; Pelletier et al., 1989; Ross et al., 1991; Olsen and Manners, 1989
elatidine	e	w	Ovezov, 1966
eldelidine (deltamine)	e	w	Rabinovich, 1952; Strzelecka, 1968

Table 3.1 cont. Alkaloids Isolated from *D. elatum* L. and *D. bellamosum*

Alkaloid	Species*	Plant Part**	References
eldeline (deltaline)	e	w	Rabinovich, 1952,1954; Kuzovkov et al., 1956; Strzelecka, 1968
isodelpheline	e	s	Pelletier et al., 1989; Wada et al., 1992; Yunusov, 1991; Attar-ur-Rahman, 1995
lycoctonine	e	s	Pelletier et al., 1989; Yunusov, 1991
methyllcaconitine	e	w, s, r	Goodson, 1943; Strzelecka, 1966, 1967,1968; Ovezov, 1967; Pelletier et al., 1989; Stern, 1954
monoacetyl-delcosine	b	s	Hashem et al., 1974b; Pelletier and Page, 1978
nudicaulidine	e	w	De la Fuente, 1990; Yunusov, 1993
14-deacetyl-nudicauline	e	w, s	Vaospv, 1993; Park et al., 1995; Pelletier et al., 1989; Yunusov, 1991
nudicauline	e	w	Vaospv, 1993; Pelletier et al., 1989; Yunusov, 1991
pacidine	e	s	Wada et al., 1992; Att-ur-Rahman et al., 1995
pacifiline	e	s	Wada et al., 1992; Att-ur-Rahman et al., 1995
pacifidine	e	s	Wada et al., 1992; Att-ur-Rahman et al., 1995
paciline	e	s	Bando et al. ,1989
pacifinine	e	s	Wada et al., 1992; Att-ur-Rahman et al., 1995
pacinine	e	s	Pelletier, et al., 1990; Bando et al., 1989; Yunusov, 1993
yunnadelphinine	e	s	Wada et al., 1992; Atta-ur-Rahman et al., 1995

*e= *D. elatum* L., b= *D. bellamosum*
**s=seeds, w= whole plant, r=roots

Biologically Active Compounds Reported From Delphinium

Many secondary metabolites are known poisons for insects, mammals, and microorganisms. Some types of secondary metabolites protect the plants from abiotic factors or competitive interactions against other plants. These poisonous attributes can be exploited for use as drugs, pesticides, herbicides, antibiotics, antifungals, etc. All of the biological activities reported from delphinium species are due to norditerpenoid and diterpenoid alkaloids produced by these plants. The review chapter titled "The Toxicology and Pharmacology of Diterpenoid Alkaloids" by Benn and Jacyno (1983) in Volume four of Alkaloids Chemical and Biological Perspectives reviews the activities of diterpenoid alkaloids from *Delphinium*, *Aconitum*, and *Garrya* species.

Toxicity and Pharmacological Studies. Delphiniums produce cardiac poisons possessing a depressant action (Martin, 1953). *Delphinium* species contain many complex and biologically active diterpenoid alkaloids. At least 150 diterpenoid alkaloids have been identified in delphinium (Olsen, 1990). Several investigators found that alkaloid concentration varies as a function of environmental conditions, and stage of plant growth. The apex or reproductive raceme retains higher concentration of alkaloids than other parts (Ralphs et al., 1988). Of the wild range plants, tall larkspurs have the highest concentration of alkaloids during their vegetative growth stages, whereas low larkspurs display a constant level of alkaloids (Manners et al., 1993).

Delphiniums grow wild in pastures, are consumed by stock, and are the leading cause of cattle loss in the western rangelands. As expected, the toxicity of delphinium plants on cattle is attributed to their complex diterpenoid alkaloids. Toxicity decreases as

the plants mature, but increases again during seed development. The most highly substituted alkaloids produce the highest toxicity. Autopsies of animals killed by larkspur showed inflammation of the stomach, small intestine, and the lining of the windpipe. The blood vessels on the surface of the body and the kidneys were extremely congested (Marsh et al., 1918). The alkaloids in these plants produce central vagal stimulation with slowing of the heart rate. This is followed by a sudden increase in the rate and force of the heartbeat due to a central sympathetic stimulation. There is a direct increase of excitability of heart muscle which in fatal cases leads to death from ventricular fibrillation (Martin, 1953). This condition is referred to as "Delphinosis". The common names of some delphiniums reflect this effect (e.g. poison weed and staggerweed) (Kuder, 1947).

Alkaloid concentration is inversely correlated with palatability in some plants. Alkaloids are considered to be bitter due to their basic nature. However, cattle readily graze on delphiniums under conditions of low availability of fodder. Rabbits, guinea pigs, cats, and rats were used in many experiments to outline the parameters of larkspur toxicity, determine the symptoms, and identify the types of compounds responsible for this phenomena (Atianasova-Shopova, 1969). Subcutaneous injection of delphinium ethanolic extracts at different levels allowed researchers to determine at what time during the growing season certain species were most toxic, and which species were most toxic overall (Crawford, 1907).

To better coordinate cattle grazing with areas having high populations of wild larkspurs, Olsen (1977b) reported the toxicity of three tall larkspurs, *D. barbeyi, D. glaucescens, and D. occidentale*. Olsen (1977a) developed a rat bioassay that would

estimate the toxicity of *D. barbeyi*. This assay was designed so that the toxicity of the injected solution was expressed in terms of the equivalent amount of dried plant material. Median lethal dose (LD_{50}) was determined by rats injected subcutaneously with ethanol extracts dissolved in saline solution from the aerial delphinium parts prior to flowering. This stage of growth produced median lethal doses ranging form 5.0 to 6.7 mg/g of body weight. The initial effects were central nervous system depression and an increased respiratory rate. Loss of motor function followed in the cervical muscles and then the thoracic limbs. Periods of apparent excitement and sudden exaggerated muscle activity occurred, also. Finally after which the rats became paralyzed and died. Death from larkspur poisoning occurred from 1.5 to 18 h after injection. From this study, it was determined that larkspur's toxicity was due to total alkaloid concentration. *D. barbeyi* was about four times more toxic than *D. glaucescens*, and approximately 10 times more toxic than *D. occidentale* (Olsen, 1977b). *D. barbeyi* has been classified as one of the most dangerous species related to forage poisoning in cattle (Keeler, 1975).

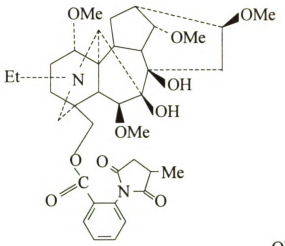
Clark et al. (1987) published a study designed to determine the total alkaloid concentration in larkspur and lupine with Near Infrared Reflectance Spectroscopy (NIRS). Total alkaloid concentration is used as a preliminary indicator of plant toxicity. NIRS was the first reliable method for monitoring total alkaloid levels when ranchers were trying to determine safe or dangerous ranging periods.

The most recent and perhaps most reliable method of quantitative analysis of norditepenoid alkaloids in larkspur, Fourier Transform Infrared Spectroscopy (FTIS), was reported by Gardener and coworkers (1997). Plant samples with known concentrations of alkaloids determined by high-pressure liquid chromatography (HPLC),

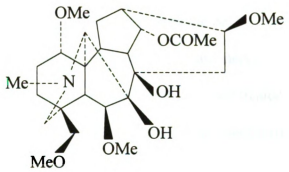
and gravimetric methods were used to construct a calibration curve. The measurements of toxic and total alkaloid concentrations were shown for *D. barbeyi, D. occidentale, and D. glaucesens*. Olsen (1978) compared the toxicity of larkspur on sheep and cow by determining LD_{50} for both types of animals. The results showed that sheep tolerate about four times more larkspur than cattle.

Ingestion of wild delphiniums by cattle can produce teratogenic effects, as well as death (Keeler, 1969). Larkspur toxicity is usually acute, resulting in death 3 to 8 h after an animal ingests a lethal dose. Death generally occurs from paralysis followed by respiratory failure. The clinical signs of larkspur poisoning in cattle are as follows: restlessness, stiff gate, stradle-stance followed by collapse, nausea, and abdominal pain (Ralphs et al., 1989). Other signs have been described as loss of muscle control, salivation, trembling, rapid and weak respiration and heart action, bloating, and ultimately respiratory paralysis (Keeler, 1975).

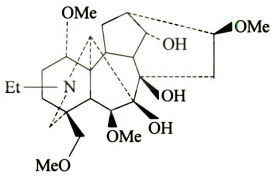
Aiyar and colleagues (1979) collected leaves of *D. brownii* Rybd. and tested extracts from this material, attempting to determine which of the chemical constituents were responsible for cattle deaths due to poisoning. The primary symptom of *D. brownii* Rybd. poisoning is paralysis. Pharmacological research on some diterpenoid alkaloids suggests that a primary site of action of these toxins is the neuromuscular junction. Purification of the alkaloid fraction from the methanol extract yielded three major constituents, MLA, browniine and browniine acetate (**Figure 3.13**). The depressant effects of these alkaloids was tested on the responses of the electrically-induced muscle twitch of the isolated rat phrenic nerve-diaphragm preparation and an electrically stimulated longitudinal muscle strip of guinea pig ileum. The individual alkaloids did not



Methyllycaconitine



14-Acetylbrowniine



Browniine

Figure 3.13. Alkaloids toxic to cattle.

exhibit activity when tested on the ileum preparation. However, the crude tertiary alkaloid fraction showed significant nicotinic blocking action in the phrenic nerve-diaphragm prepartion. MLA was the most effective, and most of the activity on the nerve-diaphargm test was attributed to this compound. A dose response curve comparing MLA to reference compounds such as, aconitine hydrochloride and (+)-tubocurarine further illustrated this conclusion.

Preliminary assays using sciatic nerve-sartorius muscle preparation further supported the conclusion that MLA produces competitive blockage at nicotinic receptors, as well as effecting sodium channels (Aiyar et al., 1979). The blockage by MLA was at least partially reversed by eserine. The alkaloid could slowly be washed out on equilibration of the paralyzed tissue with fresh physiological solution. Sciatic nerve-sartorius preparations from frog (*Rana pipiens*) were stimulated electrically and treated with MLA (10^{-7} M). This concentration produced a 50 % inhibition of post-synaptically recorded action potentials within 20 sec. A concentration of 10^{-8} M the 50 % inhibition was obtained after 50 sec (Aiyar et al., 1979).

A follow-up study to the in vitro mammalian assays was done in vivo on calves to see if the site of action was the same. Calves injected with MLA showed signs of its having a neuromuscular site of action as expected from the previous assays (Nation et al., 1982). Physostigmine was tested and it proved to be an effective antidote when MLA injections were at lethal levels. This further supports that MLA has a neuromuscular site of action (Nation et al., 1982).

A study of the relationship of relative total alkaloid concentration and toxicity of *D. occidentale* Wats., tall duncecap larkspur, on mouse bioassay over the entire growing

season showed that mouse bioassay is better correlated to cattle toxicity rather than to total alkaloid content (Olsen, 1983). Saline solutions of the alkaloid mixtures were administered by subcutaneous injection between the scapula (Olsen, 1983). Later, *D. barbeyi*, *D. occidentale*, and *D. glaucescens* were evaluated for total alkaloid concentration. The variation in concentration of individual alkaloids across the three species was analyzed by GC. Of all the different alkaloids isolated, MLA was shown to be the most toxic using, the mouse bioassay developed earlier by Olsen in 1983 (Manners, 1991). The authors stated that MLA was only one of the toxic alkaloids present in these plants.

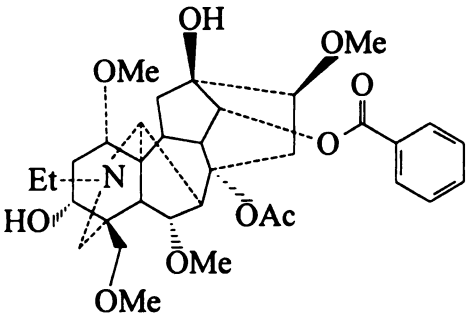
In order to find a suitable laboratory animal model to test larkspur toxicity, researchers tested the toxicity of larkspur extracts on mice, hamsters, rats and sheep. The extracts were given orally or by subcutaneous injection (Olsen et al., 1991). By comparing LD_{50}s and susceptibility to oral dosage versus subcutaneous injection, the authors concluded that, of the animals tested, mice would be the best choice for testing toxicity of larkspur. They had high susceptibility, rapid response time, and a small dose requirement (Olsen et al., 1991).

The arrhythmogenic and heart rate effects of several diterpenoidal alkaloids from *D. cashmirianum* Royle have been measured on isolated guinea atria. Aconitine produced a dose-related increase in heart rate previous to the arrhythmias. Arrhythmias were produced at concentration of 3×10^{-5}M. Only lappaconitine showed an arrhythmogenic effect at 1×10^{-4}M, without a prior increase in heart rate. An in vivo study with rabbits showed that lappaconitine produced a decrease in heart rate, followed by arrhythimias. N-deacetyllappaconitine produced arrhythimias in conjunction with a

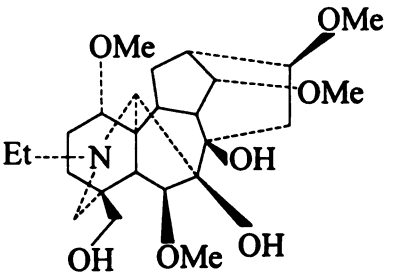
decrease in blood pressure. Lycaconitine produced a small increase in blood pressure while the alkaloid cashmiradelphine produced a small decrease. However, these responses were not dose- related. Aconitine caused an increase in heart rate followed by arrhythimias and decreasing blood pressure, which was reversible at 0.005 mg/kg. These activities were limited to compounds having an anthranoyl or N-substituted anthranoyl group in position 4 (**Figure 3.14**) (Shamma et al., 1979).

Tulyaganov and coworkers (1976) published the pharmacology of some aconite alkaloids. Lycoctonine, delcorine, and eldeline showed a myorelaxant, antidepolarizing effect on nerve muscle transmission, low toxicity, did not produced cardiac arrhythmias, did not stimulate different nerve receptors, and did not affect the central nervous system (CNS). Delphinine administered at (0.01 mg/kg) I.p. showed significant deleterious effects on liver function in adult male rats (**Figure 3.15**) (El-Maraghy, 1995). Elatine from *D. elatum* L., condelphine from *D. confusum*, and MLA and delsemine form *D. dictyocarpum* are referred to as curariform alkaloids (**Figure 3.16**).

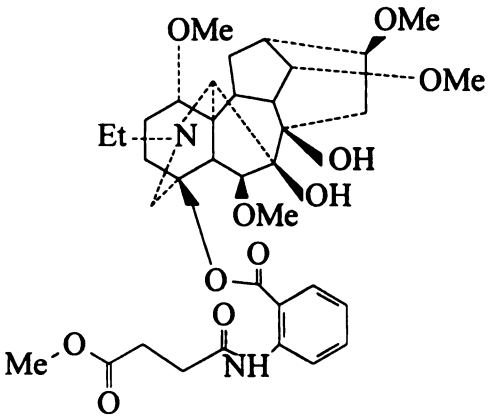
The effects of Jadwar or *D. denudatum* Wall. were investigated by testing on rats. The rats were treated with CCl_4 (3.0 mg/kg) which caused a severe depletion of glycogen and adrenal ascorbic acid. It caused an increase in levels of serum-free fatty acids, bilirubin, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, cholesterol, triglycerides, and blood urea. Oral administration of the aqueous extract (1g/ kg on three consecutive days) of *D. denudatum* Wall., prior to CCl_4 treatment antagonized the effect of CCl_4 by significantly elevating the levels of liver glycogen and adrenal ascorbic acid, and depletion of the levels of the other metabolites, thus showing a protective effect on the liver against CCl_4-induced hepatoxicity (Khan et al., 1981).



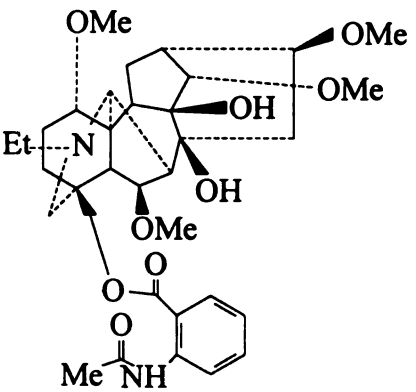
Aconitine



Lycoctonine

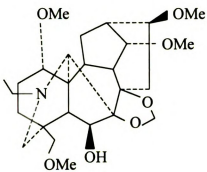


Cashmiradelphine

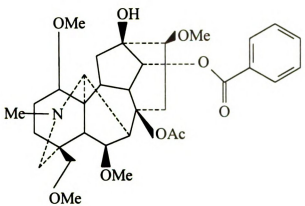


Lappaconitine

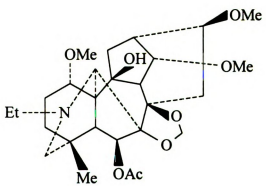
Figure 3.14. Alkaloids from *D. cashmirianum* Royle. which produce arrhythimia.



Delcorine

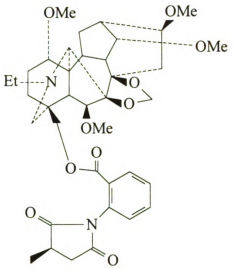


Delphinine

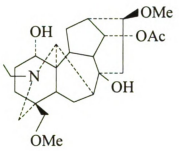


Deltaline

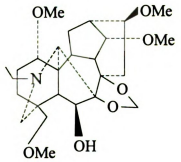
Figure 3.15. Alkaloids with pharmacological activity.



Elatine



Condelphine



Delcorine

Figure 3.16. Curariform alkaloid.

Studies by Alkondon and colleagues (1992) showed that MLA is a highly potent, yet reversible inhibitor of the ^{125}I- α bungarotoxin binding site in adult rat hippocampal membranes. It is comparable to or more effective than other α-toxins from snakes. For this reason the authors suggested it be considered for use in the characterization of neuronal nicotinic receptors.

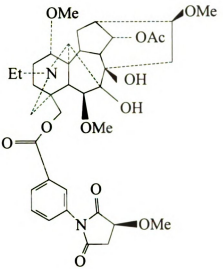
Now, MLA is used as a ligand for distinguishing neuronal nicotinic acetylcholine receptor subtypes (nAChR). These receptors are the focus for pharmaceuticals, because of their involvement in Alzheimer's Disease (Jacyno, 1996). MLA has high affinity for α7 type nAChRs. Lycaconitine differs by the absence of methyl substituent from the succinimide ring. The pharmacology or toxicology of the two have been virtually unstudied. An LD_{50} (mouse, iv) of ca 15 mg/kg of lyaconitine is less toxic than MLA (LD_{50} mouse iv 3 mg/kg). MLA was compared to lyaconitine in rat brain preparations. Lyaconitine is half as potent as MLA in the inhibition of binding of [3H]-(-)cytisine in its affinity for α4β2 nAChR subtypes. Lyaconintine displayed higher activity in the inhibition of binding of [^{125}I]-α-bugarotoxin (α7 nAChR subtype) but less than MLA. MLA is a potent and specific antagonist of the αBGT sensitive nAChR of cultures hippocampal neurons in rat. Its action is reversible (Jacyno, 1996). In addition, Wonnacott and colleagues (1993) showed that MLA is able to discriminate among nicotinic acetylcholine receptor subclasses. Yum and colleagues (1996) demonstrated that MLA was effective in dinstinguishing between certain subtypes of nicontinic receptors in chicks.

A study by Hardick and Co-workers (1996) compared the binding activities of nudicauline, elatine, and MLA as well many other norditerpenoid alkaloids (**Figure**

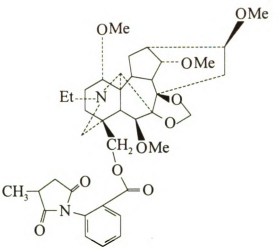
3.17). From this study these workers established further support for the necessity of the methylsuccinimido ring for potency of these type of alkaloids on binding with the mammalian neuronal nAChR.

The tertiary nitrogen on MLA was reported to have a conformation suitable to fit the nicotinic pharmacophore, similar to that of the quanternary nitrogen present in acetylcholine. Semi-synthetic drugs with quanternary nitrogen moieties such as delphinine methochloride and delphonine methochloride were shown to have greater reactivity, further supporting the evidence of the alkaloid nitrogen as the binding site for interaction with the nicotinic receptor site (Manners et al., 1995).

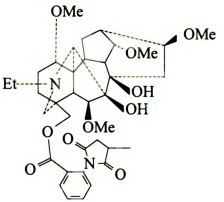
MLA is known as delatine, delsemidine, and mellictine (Coates et al., 1994). It finds continued use in Russian medicine as a muscle relaxant for surgery. MLA was detected in *D. grandiflorum* L., *D. crassifolium*, and *D. triste*. All three of these species have been shown to produce curare-like effects, *D. crassifolium* having the most activity (Mats, 1972). MLA is referred to as the most potent, non-proteinaceuos, neuronal nicotinic acetylcholine receptor antagonist found to date (Hardick et al., 1994). By regioselective anthranoylation of demethylated aconitine, a novel analogue of MLA was prepared. This novel semi-synthetic alkaloid was synthesized for use in the study of the roles of the anthranilate and succinimide moieties in the selective biochemical pharmacology of MLA at protein receptors (Hardick et al., 1994). An evaluation of toxic N- (methylsuccinyl)-anthranoyllycoctonine (MSAL) norditerpenoid alkaloids determined that the presence of the methylsuccinimide group and probably the ester carbonyl and the lycoctonine nitrogen are primary factors in establishing toxicity of the norditerpenoid alkaloids in the mammalian system (Manners et al., 1994).



Nudicauline



Elatine



Methyllycaconitine

Figure 3.17. Alkaloids studied for binding activity with nAChR.

A few of the alkaloids from *D. elatum* L.have been studied for their pharmacological activity. Elatine has curare-like action on the central nervous system (Chopra et al., 1969) (**Figure 3.17**). Deltaline (eldeline) has been isolated from *D. elatum* L. among other delphinium species. It is an example of 10-hydroxylated diterpenoid alkaloids which have been reported as relatively uncommon (**Figure 3.18**). It showed weak effects on smooth muscle using rabbits and guinea pigs. Another alkaloid isolated from *D. elatum* L., delcorine, was shown to depress blood pressure and respiratory rate in cats and dogs (**Figure 3.18**). It showed inhibiton of contractions in smooth muscle tissue in rat and rabbit, and produced contractions in smooth muscle tissue of guinea pig. Also, it proved to be a ganglionic blocking agent (Benn and Jacyno, 1983).

Alellopathic Studies. Alkaloids isolated from *D. ajacis* L., delcosine, ajaconine, and an unknown designated as LBA-III, were tested on pea plants to observe their affect on plant growth (**Figure 3.19**) (Waller et al., 1969). Segments of 4-day, old pea cambium were attached with tubing containing a lanolin +1/20 M sucrose solution. Auxin was applied to these segments either above or below the lanolin, sucrose, alkaloid solution. Kinetin was added to the auxin in some treatments, but had no effect. Evaluation of the treatments was made by weighing the top 10 mm of the segments and comparing them to the control weights, or by preparing microscopic sections from below the tip and projecting them onto a planimeter to measure the surfaces of xylem and phloem (Waller et al., 1969). Delcosine and LBL-III produced inhibition to both xylem and phloem. Ajaconine showed no significant effect on growth (Waller et al., 1969). It was proposed that delcosine and LBL-III may compete with the gibberellic acids for enzyme active sites or that they might

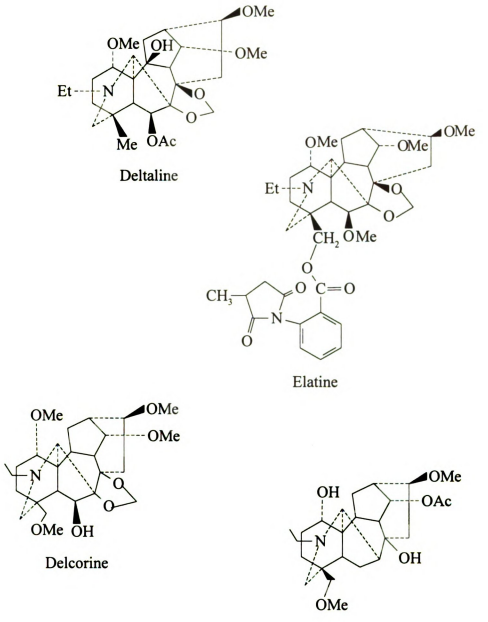
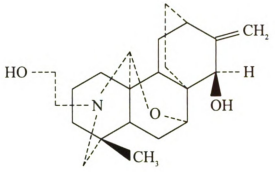
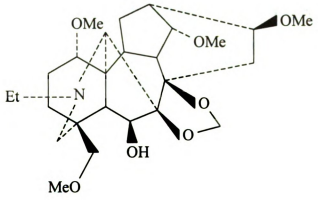


Figure 3.18. Alkaloids with pharmacological activity.



Ajaconine



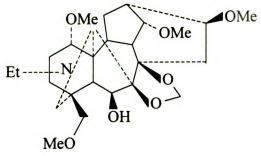
Delcosine

Figure 3.19. Alkaloids studied for allelopathic activity.

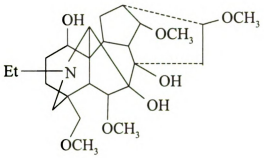
be a feedback control of gibberellic acids in some manner (Waller et al., 1969).

Insecticidal Activity. Some of the earliest work on the biological activity of the delphinium species was reported by W. M. Davidson (1929). The oils from *D. consolida* L. and Stavesacre (*D. staphisagria* L.) were prepared as soap emulsions. The *D. consolida* L. emulsion was effective against *Tetranychus telarius* L., *Paratetranychus pilosus* C.& F., and a variety of aphid species (*Aphis rumicis, Myzus persicae, M. rosea, M. cerasi, A. pomi* DeGeer). The *D. staphisagria* L. emulsion was found to give effective control (>95%) of red spider mites (*T. telarius*), a number of aphid species (*A. rumicis, M. persicae, M. rosea, M. cerasi, A. pomi* DeGeer), and whitefly (*A. vaporariorum).* The alkaloids present in this emulsion, delcosine, delsoline, and delphinine were identified as both stomach and contact poisons on some insect species (**Figure 3.20**). Delphinine-HCl tested up to 0.16% concentration was inefficient against the green peach aphid (*M. persicae*), mealy bugs, red spider mites, *Heliothrips femoralis* and the fall webworm (*Hyphentria cunea*). However, at a concentration of 0.085% Delphinine-HCl killed onion thrips (*Thrips tobaci*) and cabbage worm. Delcosine (0.63 %) was effective against *A. rumicis, M. persicae, T. tabaci, H. cunea, P. rapae*. Delsoline (0.042%) was effective against *A. rumicis, M. persicae*. A 0.085% solution of this alkaloid with soap was effective against *T. tabaci* (Davidson, 1929).

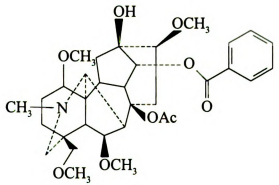
Plants used in traditional medicine were tested by Soviet scientists in 1945. Among them six species of delphinium including, *D. elatum* L. were tested. *D. elatum* L. was the most effective against a variety of insect pests (Petrischeva, 1945). The



Delcosine



Delsoline



Delphinine

Figure 3.20. Stomach and contact poisons on insects from *Delphinium* species.

delphinium plants showed to possess potent insecticidal activity even after 2-3 years of freezer storage. Later, Gatty-Kostyal and Stawowczyk (1956) compared the quality, quantity, and content of active substance in above- and below-ground plant parts of *D. elatum* L. with *D. oxysepalum* Borb. & Pax. The insecticidal activity of *D. consolida* L. and *D. orientale* was proposed to be due to their alkaloids (Avramova et al., 1964). *D. ajacis* L. a.k.a *C. ambigua* L. is known to posses insecticidal and growth inhibitory activities (Pelletier et al., 1980b). *D. crassifolium*, a Russian folk-remedy plant was among the plants collected during the Transbaikal medical expedition of 1936. This plant was used as a fly repellent in old Russia (Efros, 1946). Another delphinium species found to have insecticidal activity was *D. delavayi*. Whole plants were extracted with a variety of organic solvents and tested on several species of insects (Lee et al., 1943).

Extracts from five species of delphinium were tested for their effectiveness against bedbugs (*Cimex lectularius*) and lice (*Pediculus humanus* L.). The extracts were tested as sprays, dips, and droplets applied directly to the insects by a micropipet. The Delphinium alkaloids were active on contact and produced paralysis. The activity was attributed to the alkaloids. However, it was not considered that the alkaloids are general contact insecticides, but rather stomach posions. *D. elatum* L. was one of the most potent of the species tested. The treatment was lethal to the lice, but the bedbugs were able to recover from the treatment (Busvine, 1946).

Seeds of *D. hybrid* Hort. cv. Pacfic Giant, King Arthur were investigated for insecticidal properties. Chloroform extracts of crushed seeds were tested against armyworm (*Spodoptera eridania),* bean aphid *(A. fabae),* mite *(T. urticae),* spotted cucumber beetle larvae *(Diabrotica undecimpunctat howardi),* malaria mosquito

(Anopheles quadrimaculatus), *Empoasca abrupts, Heliothis virescens*, and the common housefly *Musca domestica*. In all cases, the extract produced either mortality or feeding inhibition (Jennings et al., 1986).

The same extract was tested for nicotinic activity against insect cholinergic receptors. The inhibition of [^{3}H]-propionyl-α-bungarotoxin binding to *M. domestica* head homogenate. α- bungarotoxin is an antagonist of nicotinic acetylcholine receptors in insects. The seed extract displayed potent inhibition of [^{3}H]-propionyl-α-bungarotoxin (Jennings et al., 1986). The compound responsible for this activity was MLA showing the highest level of inhibition. In fact, the level of cholinergic activity of this alkaloid at the insect nicotinic receptor is much more potent than that reported for the rat muscle receptor. Lycoctonine inhibited [^{3}H]-propionyl-α-bungarotoxin binding to the insect cholinergic receptor at higher concentration than MLA. Lycoctonine is an analogue of MLA lacking the aromatic ester function. However, the investigators surmised that *T. urticae* and *A. quadrimaculatus* were killed by saponins in the extract, rather than by the alkaloids. The authors proposed that MLA may have evolved in Ranunculaceae species as a highly specific ligand for the insect nicotinic receptor and acts as a antifeedant/insecticide through its action on this target site in the insect (Jennings et al., 1986).

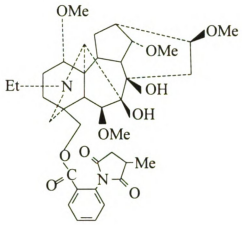
The cholinergic activity of this alkaloid at the insect nicotinic receptor is much more potent that that reported for the rat muscle receptor, where the ED_{50} was determined to be 2.3×10^{-6} M. Aconitine, which is more potent on the rat muscle nicotinic receptor, was found to be less active. The rank in order of potency for inhibition of α-bugarotoxin binding in the insect preparation is MLA>lycoctonine>aconitine and aconitine>MLA>

lycoctonine in the rat phernic nerve-diaphragm prepartion (**Figure 3.21**) (Jennings, 1986).

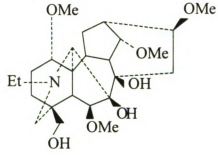
The inhibition reported for MLA in the fly head nicotinic receptor assay is the most potent ever reported for non-proteinaceous toxin at this site in insects (Jennings, 1986). Later review of this work by the authors brought them too the conclusion that the previous experiments on the vertebrate preparations were performed at levels to high to affect the housefly receptor. They concluded that the acetylcholine receptors of insects and vertebrates have significant differences, and they should be studied further (Jennings et al., 1987). They proposed that MLA may be useful for studying these differences. Knowledge of these differences could aid in the development of more effective new pesticides.

Seeds of garden hybrid delphinium closely related to the cultivar Pacific Giant and the species *D. elatum* L. have been used as a source for homogenous MLA for structure activity relationship (SAR) studies. Investigators were able to confirm the absolute stereochemistry of MLA as S (Coates et al., 1994). The biological activity of this pure compound was tested by ligand-binding assays on rat membranes. The nACHR subtype was identified by [^{125}I]-α-bungarotoxin labelling. The pure MLA showed high affinity for inhibiting [^{125}I]-α-bungarotoxin-binding. These values were similar to those found with the assay testing the citrate salt of MLA. This study was the beginning of the modeling of the nicotinic pharmacophore to determine SAR of MLA (Coates et al., 1994).

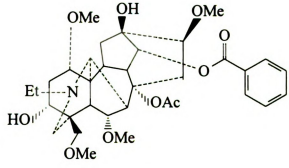
Later, 17 C_{19}-diterpenoid alkaloids isolated from *Delphinium spp.* were tested for inhibition of radiolabelled αBGTx binding to rat and housefly neural tissue



Methyllycaconitine



Lycoctonine



Aconitine

Figure 3.21. Insecticidal and mammalian alkaloid toxins.

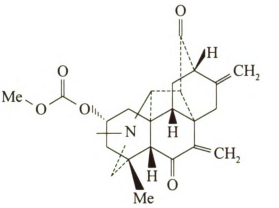
(Kukel, 1994). This series of alkaloids exhibited a range of inhibitory potencies (IC_{50}) from 42 pM in the house fly head prepartion to in excess of 100 μM in the rat brain preparation. The most potent alkaloids in the series tested, all possessed the C_{18} succinimide moiety functionalities of MLA, confirming that the succinimide group contributes to the potent activity seen with these alkaloids (Jennings, 1986). These results are consistent with previous assays by Jennings (1986).

D. staphisagria L. has been used since ancient times to kill and prevent infestation of body parasites. It goes by many common names such as Lousewort, Stavesacre, Lice-Grains, and Stephans Grains. Seeds from this plant were mixed into oatmeal bait to kill rats, and were sprinkled around the bases of fruit trees to ward off ants (Smith et al., 1981). The insecticidal nature of this plant was thought to be related to its alkaloids, as are the activities shown by many *Delphinium* species. Trexler (1960) patented the insect repellents or deterrents containing Staphisagria (U.S. 2,939,816).

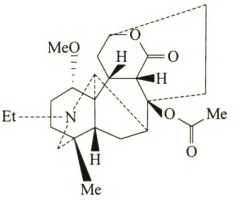
An alkaloid insecticide from the extract of *D. consolida* L. was active against head lice and nits (Strzelecka et al., 1957). In 1966, Strzelecka showed that the benzene and chloroform ($CHCl_3$) extracts containing total alkaloid of *C. regalis* L. produced 100% mortality of lice and nits in 2 h. Extracts without alkaloids showed no activity. The alkaloid delsoline and the alkaloid mixture produced 100% mortality at a 1:2000 dilution ratio after 1 h.

Antimicrobial Activity. The alkaloids from *D. flexuosum* were shown to have bactericidal effect in a 1:10,000 dilution when tested on gram positive rather than gram negative bacteria (Zolotnitskaya et al.,1962). A bacteriostatic effect was observed on *Staphylococcus aureus* at $1:10^4$-10^7 dilution and *Escherichia coli* at $1:10^4$-10^5 (Zolotnitskaya et al., 1962).

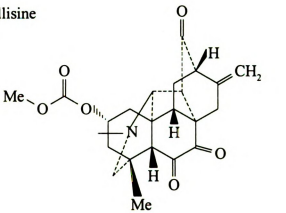
Siddiqui and co-workers (1990) demonstrated that *D. denudatum* Wall. had antimicrobial and immuno-modulating properties. Organic extracts were tested against 30 gram-negative and 10 gram-positive bacterial strains. These extracts showed activity against gram negative bacteria such as *Pseudomonas*, *Yersinia*, and *Plesionmonas* strains. Gram-positive bacteria, *Staphylococcus aurues* and *Bacillus suvtilus*, were inhibited by these extracts. Ethanolic extracts from the roots of *D. denudatum* Wall. exhibited antifungal activity against *Stachybotrys atra, Trichophyton longifusus, Curvularia lunata, Drechslera rostrata, Epidermophyton flocosum, Microsporum canis, Nigrospora oryzae, Ganoderma applanatum, Allescheria boydii, E. floccosum Dutarium rotatum, Pleutortus ostreatus,* and *Dutarium rotatum (*Atta-ur-Rahman, 1997*)*. 8-acetylheterophyllisine and vilmorrianone displayed antifungal activity against *A. boydii, E. floccosum,* and *A. niger*. Panicutine exhibited antifungal activity against *A. boydii, S. atra, P. ostreatus, N. oryzae,* and *D. rotatum (*Atta-ur-Rahman, 1997)(**Figure 3.22**).



Panicutine



8-Acetylheterophyllisine



Villmorrianone

Figure 3.22. Antifungal alkaloids from *D. denudatum* Wall.

It is clear from this review published on the phytochemistry of the *Delphinium* genus, that the bulk of research has focused on the norditerpenoid and diterpenoid alkaloids produced from these plants. The biological activity associated with delphiniums is for the most part due to these alkaloidal compounds. The following chapters report new non-alkaloidal compounds isolated from the hexane and ethyl acetate extracts from *D.* X *Cultorum* flowers using bioassay-directed fractionation and purification procedures. There are no phytochemical reports available for *D.* X *Cultorum*, a hybrid of *D. elatum* L.

CHAPTER IV

Chemical Evaluation of the Hexane extract from *D.* X *cultorum* Flowers

Abstract

Delphinium species are known for producing complex norditerpenoid and diterpenoid alkaloids, which show a wide range of biological activities. We have isolated six compounds from the mosquitocidal fraction resulting from the hexane extract of *D.* X *cultorum* flowers. It is our hypothesis that this biological activity is due to one or more of these non-alkaloidal compounds. Although compounds ethylmethylbenzene (**1**), 1-Isopentyl-2,4,5-trimethylbenzene (**2**), and 2-Penten-2-ylbenzoic acid (**6**) were reported earlier, they have not been isolated from plant tissue, until now. This is the first report of 3-Butylidene-3H-isobenzofuran-1-one (**4**) and an isomer (**5**) thereof being isolated from any delphinium species. All of these compounds and a novel derivative of acetophenone, 2-(hex-3-ene-2-one)-phenylmethylketone (**3**) were characterized from their GC-EIMS spectral data. In addition, β-sitosterol (**7**) was isolated and characterized by spectral methods.

Introduction

The temperate plant, *Delphinium elatum* L. (Ranunculacae) native to East Asia, is known for its diterpenoid and norditerpenoid alkaloid constituents. Many traditional uses of delphinium species are related to their insecticidal activity (Efros, 1946; Smith et al., 1981; Chopra et al., 1969). More recently, investigators linked this insecticidal activity to the diterpenoid and norditerpenoid alkaloids produced by these plants (Coates et al., 1994; Jennings et al., 1986; 1987; Kukel, 1994). In addition to insecticidal activity, these alkaloids possess allelopathic activity (Waller et al., 1969), antimicrobial activity against gram-negative and gram-positive bacteria, and a variety of fungi (Atta-ur-Rahman, 1997; Siddiqui et al., 1990 Zolotnitskaya et al., 1962). Also, some of these compounds are being considered for pharmacuetical applications (Jacyno, 1996). In this chapter, we report that *D.* X *cultorum*, a hybrid species from *D. elatum* L. possesses insecticidal compounds that are not related to these alkaloidal components. Volatile components characterized by GC-EIMS are reported from a mosquitocidal fraction isolated from the hexane extract of *D.* X *cultorum* flowers.

Experimental

Plant Material

D. X *cultorum* seeds cv. Magic Fountians Dark Blue/ White Bee were donated by Bodger Seed, Ltd. South El Monte, CA. The seeds were germinated in high porosity, peat-based growing mix in styrofoam trays (4"x 6" x 2") and grown until the first leaf appeared. The seedlings then were planted in pots (4"x 4" x 6") containing high porosity, peat-based growing mix and grown to the five-leaf stage. At that point, the plants were transplanted

into large clay pots containing high porosity, peat-based growing mix and were grown until maturity. The plants were grown year round in the Pesticide Research Center Greenhouses at Michigan State University.

Plant parts were harvested when 75-85% of the florets were fully expanded on each raceme. The florets and leaves were removed from each stem and the stem was cut into two to 3.5 cm sections. The separate plant parts were then packed into reclosable plastic bags and stored at -20°C. Frozen plant parts were weighed on a Mettler BD601 balance (Mettler-Toledo, Inc., Hightstown, NJ). The separated plant material was spread evenly onto three metal lyophilizer trays (29x 31.5 x 3.4 cm) and lyophilized using a bulk tray lyophilizer (FTS Systems, Inc., Stone Ridge, NY) at 5°C for 48h. The dried plant material was macerated into a fine powder in a commercial Waring blender, packed into reclosable plastic bags and stored at -20°C until analysis. The water content of the fresh *D.* X *cultorum* flowers was approximately 80%.

General Experimental

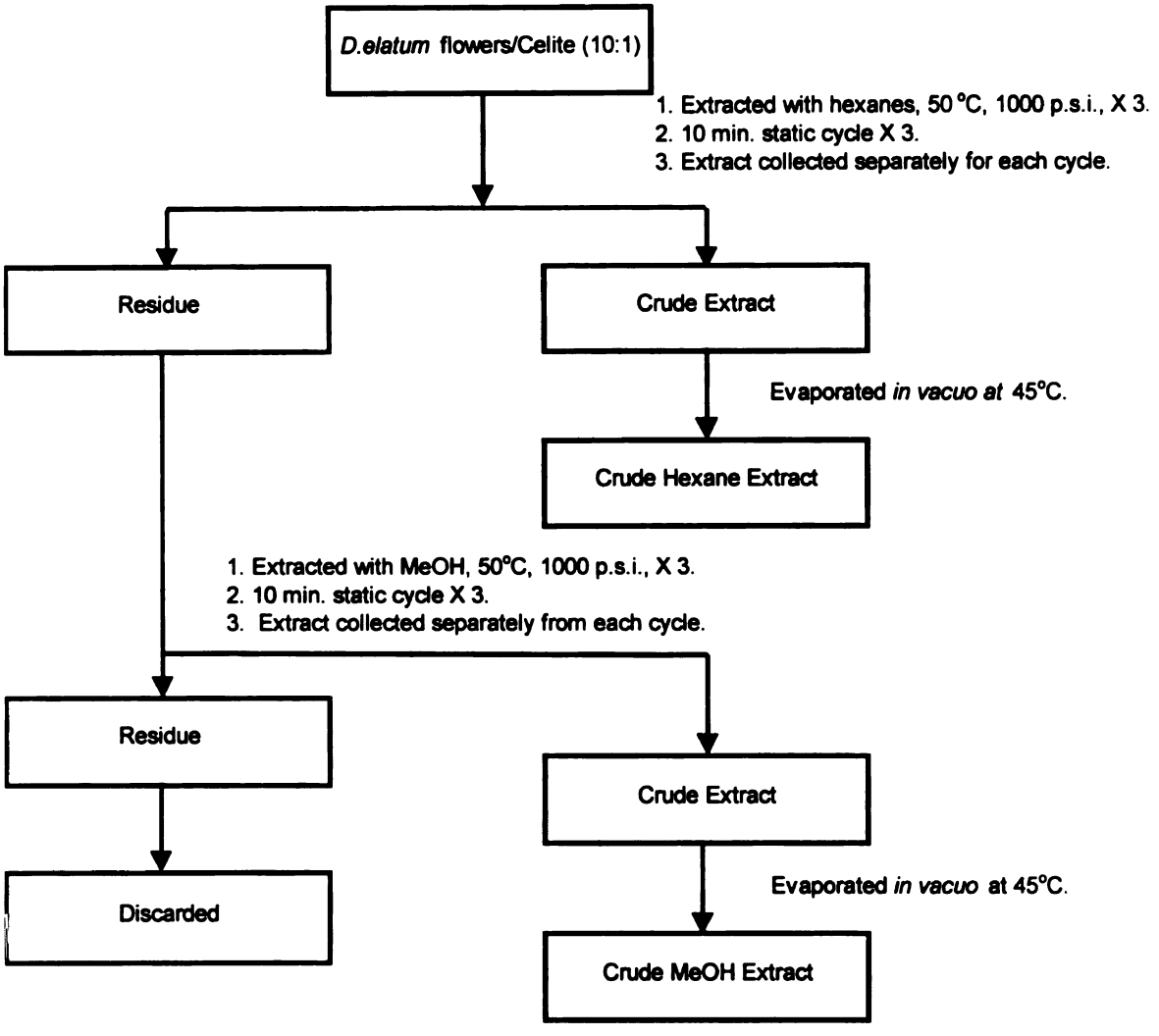
The components of the hexane extract of *D.* X *cultorum* flowers were analyzed by thin layer chromatography (TLC). Concentrated solutions of the crude extracts were prepared in $CHCl_3$. The solutions then were applied to silica plates (20 x 5 cm, 250 μm with inorganic binder and UV 254) with 5 μL capillary pipettes. The spots were allowed to dry. The TLC plates were placed into solvent chambers equilibrated with 15 mL of the following solvent systems: $CHCl_3$/ MeOH (4:1), (10:1), and (1:1), and hexane/ acetone

(4:1). TLC plates (250 μ) and preparative TLC plates GF (20 x 20 cm^2, 1000 μ) were purchased from Analtech Inc. (Newark, Delaware).

GC-EIMS was performed using a 30 m DB5 column (0.32 x 0.25 mm) on a JEOL-JMS-AX505H mass spectrometer. The column temperature was set at 50 °C and programmed to increase to 190°C at 10 °C /min. The ionization energy was 70 eV. The solvent was acetone and the carrier gas was helium. Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for the Research Resources, National Institutes of Health. NMR spectra were recorded on a Varian VXR 300 MHz spectrometer (Varian, Palo Alto, CA) at room temperature at the Max T. Rogers NMR facility, Chemistry Department, Michigan State University.

Extraction

Accelerated Solvent Extraction. An ASE 200 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA) was used to extract the plant material. The dry, macerated plant material was mixed with diatomaceous earth to yield a 10:1 mixture by weight. This mixture was hand-packed tightly into 33 mL cells which contained a cellulose filter in the bottom. The extraction was done under 1000 psi and 60°C. The extraction solvent (10 mL per cycle) was collected in 60 mL vials. The plant material was extracted sequentially with hexane and MeOH (Aldrich, Milwaukee, WI) (**Scheme 4-1**.).



Scheme 4-1. Accelerated solvent extraction procedure.

Large-scale extraction of D. X cultorum flowers. A large glass column was used for the extraction of *D.* X *cultorum* flowers to obtain a sufficient quantity of crude hexane extract. Lyophilized and macerated flowers (409 g) were packed in the column that contained a cotton plug at the bottom. The plant material was extracted sequentially with hexane (3.5 L x 3) and ethyl acetate (2.0 L x 3). The plant material was soaked in solvent for at least 72 h, with exchange of fresh solvent every 12 h. Solvent was removed *in vacuo* at 40°C on a rotary evaporator yielding crude hexane extract (8.68 g) and ethyl acetate extract (3.61 g). The dried extract was stored at -20°C until bioassay and purification. The residual plant material was discarded.

Insecticidal Assays

Corn Earworm Assay. Corn earworm eggs (*Helicoverpa zea* Boddie) and dry corn earworm diet was purchased from North Carolina State Insectory, Department of Entomology, North Carolina State University, Raleigh, North Carolina. The eggs were hatched in an incubator at 27°C. The dry diet was dispensed into scintillation vials (940 mg) for each treatment. Crude extracts or purified compounds were dissolved in DMSO to give a concentration of 1250 $\mu g \cdot 25\ \mu L^{-1}$, unless otherwise stated. Twenty-five μL of these stock solutions were mixed thoroughly with the portions of dry diet. A 25 μL of DMSO was used as a control. Agar solution (1.4%) was mixed and autoclaved for 5 min at 15 psi and 125°C to melt the agar. This solution was held in a water bath at 45°C, and added to the dry diet until the total diet weighed 5 g. The final concentration of test extracts and compounds was 250 ppm. The wet diet was mixed thoroughly, and 3-4 drops

of diet were dispensed into 3.5 mL polystyrene vials. The freshly poured portions of diet were allowed to cool and dry for at least 1 h. After drying, one neonate larvae was placed in each vial, and the vials were capped. The treatment and control vials were held in a growth chamber at a photoperiod of 16 h day and 8 h night with day temperature at 28°C and night temperature at 24°C. Each treatment had 15 replicates. The treatments were arranged in a completely randomized design. The larvae were weighed (mg) on a Mettler AE163 analytical balance (Mettler-Toledo, Inc., Hightstown, NJ) after six days. Statistical analysis was conducted using ANOVA and the F test. Least Significant Difference (LSD) was calculated for those treatments with significant F values, and used to compare treatment mean weights with controls.

Gypsy Moth Caterpillar Assay. Gypsy moth eggs (*Lymantria dispar* L.) were obtained from the Insect Production Unit of the Canadian Forest Service, Sault Ste. Marie, Canada. The dry diet was mixed in the Bioactive Natural Products Laboratory, Department of Horticulture, Michigan State University. The recipe was as follows: wheat germ (36 g), casein (7.5 g), Wesson's salt mix (2.4 g), sorbic acid (0.6 g), methylparaben (*p*-hydroxy-benzoic acid methyl ester) (0.3 g), Hoffman-Larouche #26862 vitamin mixture (Hoffman-Larouche, Inc., Nutley, NJ) (3.0 g). The eggs were hatched in an incubator at 27°C. The dry diet was dispensed into scintillation vials (845 mg) for each treatment. Crude extracts or purified compounds were dissolved in DMSO to give a concentration of 1250 μg · 25 μL^{-1}, unless otherwise stated. Twenty-five μL of these stock solutions were mixed thoroughly with the aliquots of dry diet. A 25 μL aliquot of DMSO was used as a control. Agar solution (1.4 %) was mixed and autoclaved for 5 min at 15

psi and 125°C to melt the agar. This solution was held in a water bath at 45°C, and added to the dry diet until the total diet weighed 5 g. The final concentration of test extracts and compounds was 250 ppm. The wet diet was mixed thoroughly, and 3-4 drops of diet were dispensed into 3.5 mL polystyrene vials. The freshly poured portions of diet were allowed to cool and dry for at least 1 h. After drying, one neonate larvae was placed in each vial, and the vials were capped. The treatment and control vials were held in a growth chamber at a photoperiod of 16 h day and 8 h night with day temperature at 28°C and night temperature at 24°C. Each treatment had fifteen replicates. The treatments were arranged in a completely randomized design. The larvae were weighed (mg) on an analytical balance after six days. Statistical analysis was conducted using ANOVA and the F test. LSD was calculated for those treatments with significant F values, and used to compare treatment mean weights with controls.

Mosquito Assay. First instar mosquito larvae (*Aedes aegypti* L.) were provided by Drs. Alexander Raikel and Alan Hays, Department of Entomology, Michigan State University. The larvae were raised in 500 mL beakers containing 150-250 mL of reverse osmosis water in an incubator at 26°C, for three days. Ten to 12 fourth instar larvae were placed in 980 μL of reverse osmosis H_2O in 12 x 75 mm borosilicate glass test tubes. Crude extracts or purified compounds were dissolved in DMSO, unless otherwise stated, to give a concentration of 250 μg · 20 μL^{-1}, unless otherwise stated. A 20 μL aliquot of DMSO was used as a control. Treatments and controls were covered and left at room temperature. There were three replicates per treatment. The number of dead larvae were recorded at 2 h intervals, up to and including 24 h. Statistical analysis was conducted

using ANOVA and the F test. LSD was calculated and used to compare treatment means with controls.

Japanese Beetle Assay. Adult Japanese beetle (*Popillia japonica* Newman) were collected for 8 h on sunny, calm days at the Michigan State University Tennis Facility with a hanging floral lure/ pheromone trap provided by Dr. Dave Smitley, Department of Entomology, Michigan State University. The beetles were collected 1-2 days prior to the assay. They were kept in plastic containers (1L) with perforated lids. The beetles were fed leaves from a climbing rose tree or a grapevine. Test solutions for the micro-application, direct dip, and leaf assays were prepared at 250 and 100 $\mu g \cdot mL^{-1}$ concentrations of crude delphinium flower extracts in acetone. The micro-application assay was performed by treating the insects, held upside down, with 1 μL of the test solution on the abdomen. The droplet was allowed to dry. Twenty beetles per treatment were placed into 150 x 15 mm polystyrene disposable petri plates containing two pieces of filter paper. Each treatment and the control was replicated three times. The beetles were fed with either grape or rose leaves depending on what they were fed when captured. The petri plates were kept in a growth chamber at 25°C and a photoperiod of 16 h day, 8 h night. The leaves and filter paper were changed daily. Mortality was recorded first at 2 h followed by 24 h intervals for 96 h.

For the direct dip assay, five beetles were placed in a covered strainer. The strainer was dipped into the solution for 5 s. The beetles were removed from the strainer and allowed to dry. The strainer was rinsed with acetone between treatments. Twenty beetles per treatment were placed into 150 x 15 mm polystyrene disposable petri plates

containing two pieces of filter paper. Each treatment and the control were replicated three times. The beetles were fed either grape or rose leaves, depending upon what they were fed when captured. The petri plates were kept in a growth chamber at 25°C and a photoperiod of 16 h day, 8 h night. The leaves and filter paper were changed daily. Mortality was recorded first at 2 h followed by 24 h intervals for 96 h.

For the leaf dip assay, grape leaves were dipped into the treatment solutions and allowed to dry. The leaves were fitted with small glass vials containing reverse osmosis water and stoppered with cotton. One leaf was added to each 150 x 15 mm polystyrene disposable petri dish containing two pieces of filter paper and 5 beetles. Each treatment and the control was replicated three times. After 24 h the remaining treated foliage was removed and replaced with fresh untreated foliage. The petri plates were kept in a growth chamber at 25°C and a photoperiod of 16 h day, 8 h night. The leaves and filter paper were changed daily. Mortality was recorded first at 2 h followed by 24 h intervals for 96 h.

A no-choice feeding assay was performed on 20 beetles placed into two 150 x 15 mm polystyrene disposable petri plates containing two pieces of filter paper. One large mature leaf and one small new leaf and flowers from *D. X cultorum* flowers were fitted with small glass vials containing cotton, fitted with cotton plugs and placed into the dishes. One dish contained leaves, the other flowers. The petri plates were kept in a growth chamber at 25°C and a photoperiod of 16 h day, 8 h night. Mortality was recorded first at 2 h followed by 24 h intervals for 96 h.

Colorado Potato Beetle Assay. Adult Colorado potato beetles (*Leptinotarsa decemlineata* Say) were collected from Michigan State University Entomology Research

Station, courtesy of Dr. Ed Grafius. The potato beetles were fed potato leaves from plants grown in the Pesticide Research Center Greenhouses at Michigan State University. Test solutions for the micro-application, direct dip, leaf assays were prepared at 250 and 100 $\mu g \cdot mL^{-1}$ concentrations of crude delphinium flower extracts in acetone. The micro-application, leaf dip, and direct dip assays were performed exactly like that of the Japanese beetle assay, except potato leaves were used instead of grape or rose leaves.

Nematocidal Assay

This assay was conducted on two nematode cultures, *Caenorhabditis elegans* Dought and *Panagrellus redivivus* Goody kept in culture at the Bioactive Natural Products Laboratory, Michigan State University. C. *elegans* Dought was kept on NG agar media in disposable petri plates wet with 2-4 mL of physiological saline solution. *C. elegans* Dought was grown with a strain of *Escherichia coli* on which it fed. P. *redivivus* Goody was kept in axenic, liquid Basal-Heme media (5 mL) in scintillation vials. The cultures were stored at room temperature and subcultured every four weeks. Appendix II lists the recipes for NG and Basal-Heme media.

The nematodes were added to 1 mL of physiological saline solution in a scintillation vial. This solution was diluted until the nematodes numbered 15-20 in a 48 μL aliquot. Forty-eight μL of dilute nematode solution was delivered to each of three wells per treatment in a polystyrene 96 well plate. Crude extracts or purified compounds were dissolved in DMSO, to give a concentration of 12.5 $\mu g \cdot 2\ \mu L^{1}$, unless otherwise stated. Two μL of the test solution or DMSO alone, as a control was added to each well

bringing the final concentration to 250 ppm. The plate was covered, sealed with parafilm, and kept in a humid chamber. The number of dead nematodes was recorded every two h up to and including 24 h.

Anti-microbial and Topoisomerase Inhibitory Assays

Micro-organisms (*Aspergillus parasiticus, Candida albicans, Escherichia coli, Staphylococcus epidermidis, Streptococcus aureus, Fusarium moniliform, Fusarium oxysporum, Rhizoctonia solanii, Saccharomyces cerevisiae* strain 394, *S. cerevisiae* strain 394_{t-1}, and *S. cerevisiae* strain 394_{t2-5}) were stored at -80°C in 1.2 mL Nalgene cryogenic vials containing a 70 % glycerol solution. The cryogenic culture was thawed and 50 µL of the suspension was spread onto a 100 x 15 mm disposable polystyrene petri plate containing an agar media specifically for that organism. (Appendix II lists the recipes used for each micro-organism.). The transfer was performed in a laminar air-flow hood (Contamination Control, Inc. Lansdale, PN) to maintain sterile conditions. The cultures were grown in an Equatherm incubator (Curtin Matheson Scientific, Inc., Kennesaw, GA) at 27.5°C until the organism covered the plate, approximately 24 h for bacteria and yeast, and 48 to 72 h for fungi. Two to 3 mL of physiological saline solution (8.5 g/L) was used to dislodge the colonies or hyphal mat from the petri plate. This solution was placed into a 16 mm O.D. x 125 mm long, 19 mL, disposable, borosilicate glass test tube and diluted to 10 mL with saline solution. These cultures were stored at 4°C until the assay.

The culture suspensions were mixed on a Vortex Genie mixer (Fisher Scientific, Norcross, GA) until all cells or hyphae were suspended uniformly. Fifty µL of the

suspension was spread onto a petri plate containing the designated agar media in a laminar air-flow hood to maintain sterile conditions. The plates were left in the hood to dry for 30 min. With a permanent marker, the plate was divided into 3 segments by drawing lines on the outer-underside of the plate. Crude extracts or purified compounds were dissolved in DMSO, to give a concentration of 250 $\mu g \cdot 20\ \mu L^{-1}$, unless otherwise stated. These spots were allowed to dry for 30 min. After drying, the petri plates were sealed with parafilm and placed in an incubator at 27.5°C. The zone of inhibition was measured in cm after 24, 48, and 72 h.

Purification of Hexane Extract

A preliminary separation of the crude hexane extract from *D.* X *cultorum* was performed by solvent-solvent partition. A solution of the crude hexane extract (4 g) in hexane (200 mL) was extracted in a 1L separatory funnel with MeOH: H_2O (95:5) (300 mL). The hexane layer was removed, and the aqueous MeOH layer was extracted twice with hexane (200 mL). The combined hexane extracts were evaporated *in vacuo* at 45°C, yielding a yellow-green hexane-soluble fraction (3.8 mg). Similarly, the aqueous MeOH (95:5) extract afforded a green-waxy fraction (283 mg). This green-waxy fraction (283 mg) was purified further using preparative TLC on silica plates developed with $CHCl_3$. This yielded fractions A-E with weights of 3.4, 4.6, 9.9, 3.4, and 177.4 mg, respectively. The bands were removed after viewing under UV light at 254 and 366 nm, and then eluted with MeOH. Fraction B was dissolved in acetone ($2\ mg \cdot mL^{-1}$) and analyzed by GC-EIMS. Fifteen peaks were obtained in the Total Ion Current (TIC) chromatogram.

Six compounds with retention times of 3.00, 6.98, 13.11, 13.22, 13.75, 13.90 min and relative abundances of 5, 9, 100, 15, 33, %, respectively were identified from the TIC chromatogram.

Compound **1**: m/z values (% rel. int.): 55(6), 91(12.5), 105(100), 120 (M^+, 28.75). These m/z values are identical to the reported values (National Institutes of Standards and Technology, 1998) for the isomers of ethylmethylbenzene.

Compound **2**: m/z values (% rel. int.): 51(3.75), 77 (6.25), 105 (26.25), 133 (100), 190(M^+, 10.63). This fragmentation pattern matches that reported by Kingston et al. (1988) for 1-isopentyl-2,4,5-trimethylbenzene.

Compound **3**: m/z values (% rel. int.): 55 (16.25), 65 (2.5), 77 (3.75), 83 (100), 91 (12.5), 97 (7.5), 105 (8.75), 119 (67.5), 132 (21.25), 145 (2.5), 173 (2.5), 201 (18.75), 216 (M^+, 47.5).

Compound **4**: m/z values (% rel. int.): 55 (3.75), 77 (11.25), 89 (2.5), 103 (18.75), 131 (27.5), 146 (30), 159 (100), 188 (M^+, 28.75). This fragmentation pattern matches that of the published data for 3-Butylidene-3H-isobenzofuran-1-one (Uozumi et al., 1989; Ogawa et al., 1995; Watanabe et al., 1993).

Compound **5**: m/z values (% rel. int.): 50 (5), 77(7.5), 103 (16.25), 131(20), 146(36.25), 159(100), 188 (M^+, 26.25). This fragmentation pattern matches that of the published data for 3-Butylidene-3H-isobenzofuran-1-one (Uozumi et al., 1989; Ogawa et al., 1995; Watanabe et al., 1993).

Compound **6**: m/z values (% rel. int.): 55 (27.5), 63 (3.75), 91 (12.5), 106 (32.5), 120 (11.25), 134 (21.25), 148 (78.75), 161 (100), 190 (M^+, 72.5). This fragmentation pattern was identical to that of the published MS data by Li et al. (1993).

Fraction C obtained from the purification of the aqueous methanol extract was recrystallized from MeOH to yield compound **7** (6.2 mg). Compound **7** was characterized by ^{1}H and ^{13}C NMR analysis.

Compound **7**: ^{1}H NMR ($CDCl_3$): δ0.68(3H, *s*, 18-H X 3), 0.81(6H, *d*, 27-H X 3, 26-H X 3), 0.92(3H, *m*, 21-H X 3), 1.0(3H, *bs*, 19-H X 3), 1.12(6H, *m*), 1.24(3H, *m*), 1.51(10H, *m*), 1.82(9H, *m*), 1.97(2H, *m*), 2.25(2H, *m*) 3.54 (1H, *m*, 6-H X 1,), 4.76(1H, *m*, 3-H X 1), 5.35(1H, *m*, 5-H X 1). ^{13}C NMR ($CDCl_3$): δ11.86(C-18), 11.98(C-29), 18.77(C-27), 19.02 (C-21), 19.40(C-19), 19.82 (C-26), 21.08(C-11), 23.05 (C-28), 24.29 (C-15), 26.04 (C-4), 28.25(C-16), 29.13(C-23), 29.70 (C-25), 31.60(C-2), 31.90 (C-7,C-8), 33.93 (C-22), 36.15(C-20), 36.51(C-10), 37.23(C-1), 39.76 (C-12), 42.23 (C-13), 45.82 (C-24), 50.12 (C-9), 56.03 (C-17), 56.75 (C-14), 71.68(C-3), 121.72 (C-6), 140.73(C-5). The ^{1}H and ^{13}C NMR chemical shift values are in agreement with the published values for β-sitosterol (Slomp and MacKeller, 1962; Akihisa et al., 1986; Bisht et al., 1978; Shoolery and Rogers, 1958; Holland et al., 1978).

Results and Discussion

Flowers of *D.* X *cultorum* were extracted sequentially with hexane and MeOH. Preliminary bioassays were performed on these extracts for insecticidal, mosquitocidal, antimicrobial, nematocidal, and topoisomerase enzyme inhibitory activities. The assays revealed that the hexane extract exhibited potent mosquitocidal activity at 10 ppm concentration; 100 % mortality was obtained in 2 h. The MeOH extract exhibited 100 % mortality in 2 h at 250 ppm. Both extracts showed antifeedant activity on corn earworm

(**Table 4.1**) and gypsy moth caterpillars (**Table 4.2**). Neither of the extracts showed any insecticidal activity against the Colorado potato or Japanese beetles. Similarly, these extracts did not show antimicrobial, nematocidal or topoisomerase enzyme inhibitory activities.

Bioassay-directed fractionation of the hexane extract was conducted using mosquito larvae and corn earworm neonates to monitor biological activity. The hexane- and MeOH:H_2O(95:5)-soluble fractions were tested for biological activity against mosquito larvae. Both exhibited LD_{100} in 2 h at 100 ppm. In addition, these fractions were tested against corn earworm (**Table 4.3**). Both showed significant antifeedant activity, however the MeOH:H_2O(95:5)-soluble fraction was the most active.

The mosquitocidal fraction B from the purification of the MeOH:H_2O(95:5)-soluble fraction was analyzed by GC-EIMS. The TIC profile indicated 15 peaks; six of the major peaks were characterized and assigned structures based on their fragmentation patterns. Peak **1,** at 3.00 min., was assigned to have a structure identical to compound **1**, an isomer of ethylmethylbenzene as shown in **Figure 4.1**. The base peak in its EIMS spectrum at m/z 105 was due to a homolytic cleavage of the methyl group. The peak at m/z 91 is due to homolytic cleavage of the ethyl group. However, it is not clear whether this compound is the *o*, *m*, or *p* isomer of ethylmethylbenzene.

The peak at 6.98 min in the TIC chromatogram of the mosquitocidal fraction is due to compound **2**, 1-Isopentyl-2,4,5-trimethyl benzene shown in **Figure 4.2**. The base peak in its EIMS spectrum at m/z 133 is due to the homolytic cleavage of the isopentyl moiety.

Table 4.1. Average weights of *H. zea* Boddie larvae after 6 days. Treatments were tested at 250 ppm. * $p \leq 0.01$ (compared to control).

Treatment	Average Weight (mg)
Control	15.60
Hexane Extract	0.85*
Methanol Extract	8.57*

Table 4.2. Average weights of *L. dispar* L. larvae after 6 days. Treatments were tested at 250 ppm. *$p \leq 0.01$ (compared to control).

Treatment	Average Weight (mg)
Control	18.05
Hexane Extract	8.04*
Methanol Extract	8.75*

Table 4.3. Average weights of *H. zea* Boddie larvae after 6 days. Treatments were tested at 100 ppm. *$p \leq 0.01$ (compared to control).

Treatment	Average Weight (mg)
Control	37.22
Hexane Soluble Fraction	29.97*
Methanol:Water (95:5) Fraction	0.83*

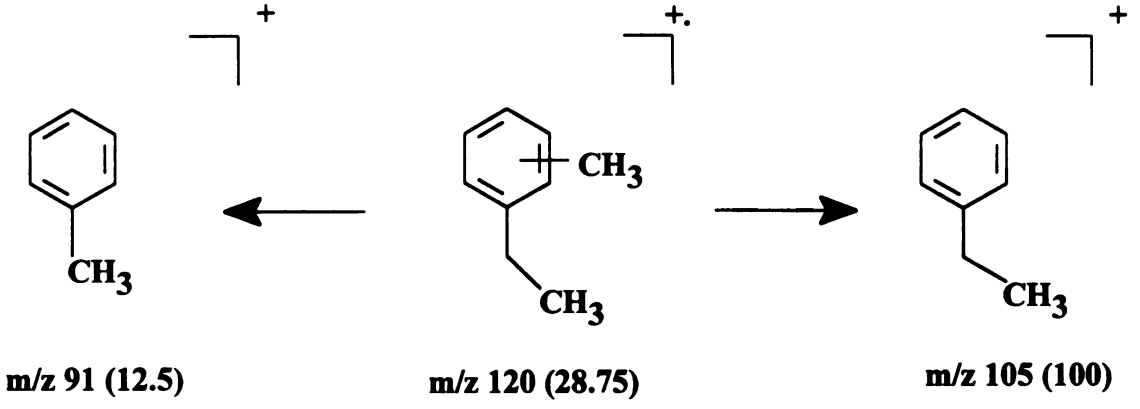
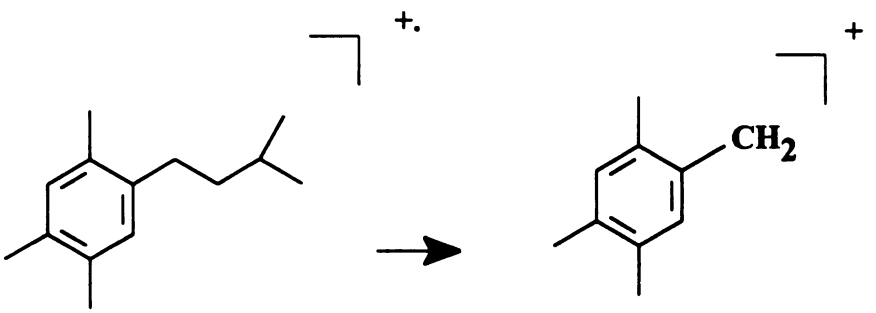


Figure 4.1. Ethylmethylbenzene and MS fragmentation pattern.



m/z 190 (11) **m/z 133 (100)**

Figure 4.2. 1-Isopentyl-2,4,5-trimethylbenzene and its MS fragmentation pattern.

Compound **3**, as indicated by the TIC peak at 13.11 min., was characterized from its EIMS fragmentation pattern as 2-(Hex-3-ene-2-one)-phenylmethylketone. **Figure 4.3** depicts a plausible fragmentation pattern for this compound. The peak at m/z 83 was resulting from a heterolytic cleavage at C-11 (**Figure 4.4**). The peak at m/z 201 was due to the methyl group loss at C-13. Similarly, the peak at m/z 173 was formed by the acetate group loss at the C-1. The peak at m/z 119 with a relative abundance of 67.5% was due to the heterolytic cleavage of the hex-3-ene-2-one side chain at C-2. A higher abundance of this peak compared to the loss of an acetate at m/z 173 (2.5) was indicative of a stable radicle. Similarly, the peak at m/z 105 was assigned to a homolytic cleavage of the fragment ion m/z 173 at C-11. The ion at m/z 132 was resulting from a hydrogen rearrangement followed by the cleavage at C-9.

The TIC profile gave peaks at 13.22 and 13.75 min. in the chromatogram that were assigned to compounds **4** and **5**, respectively. However, the MS analysis revealed that both **4** and **5** showed identical fragmentation profiles. Indicating that both of these compounds are isomers of 3-Butylidene-3H-isobenzofuran-1-one (**Figure 4.5**). The base peak at m/z 169 was the result of a homolytic cleavage of the ethyl group in the butylidene side chain. The fragment ion at m/z 146 was due to a γ-hydrogen rearrangement followed by a homolytic cleavage of the propylene group in the butylidene side chain. The GC-MS fragmentation pattern was insufficient to determine the structures of **4** and **5**.

Compound **6** was identified from the TIC at. 13.90 min. as 2-pent-2-ylbenzoic acid (**Figure 4.6**). The base peak at m/z 161 was due to homolytic cleavage of an ethyl

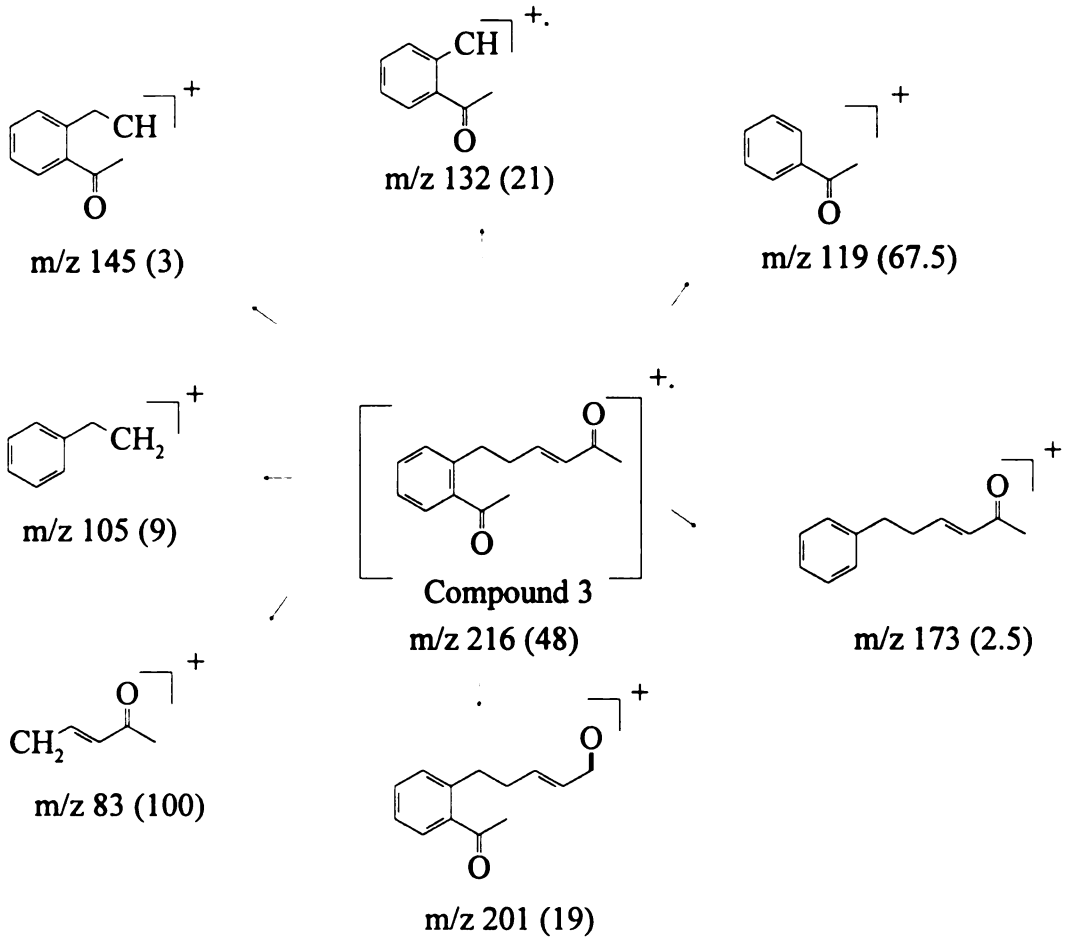


Figure 4.3. 2-(Hex-3-ene-2-one)-phenylmethylketone and its MS fragmentation pattern.

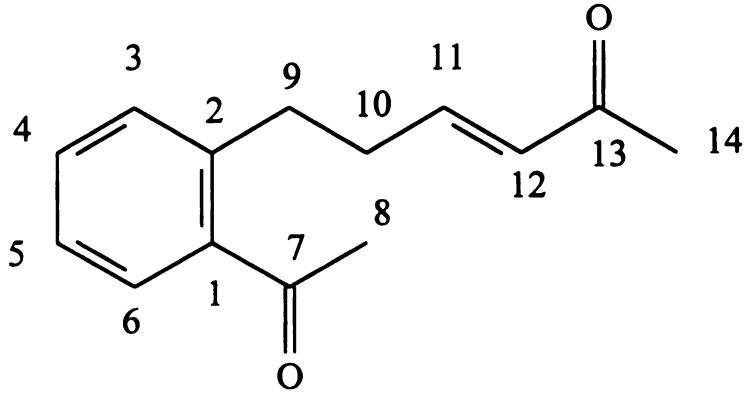


Figure 4.4. 2-(Hex-3-ene-2-one)-phenylmethylketone.

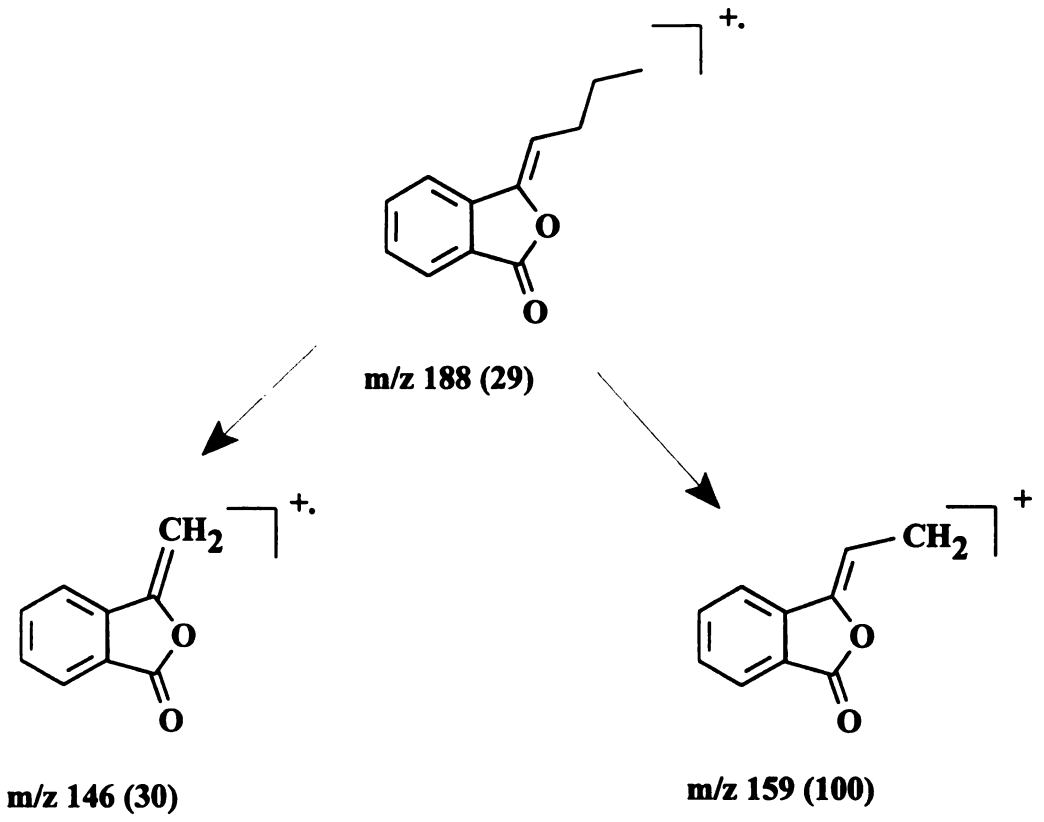


Figure 4.5. 3-Butylidene-3H-isobenzofuran-1-one and its MS fragmentation pattern.

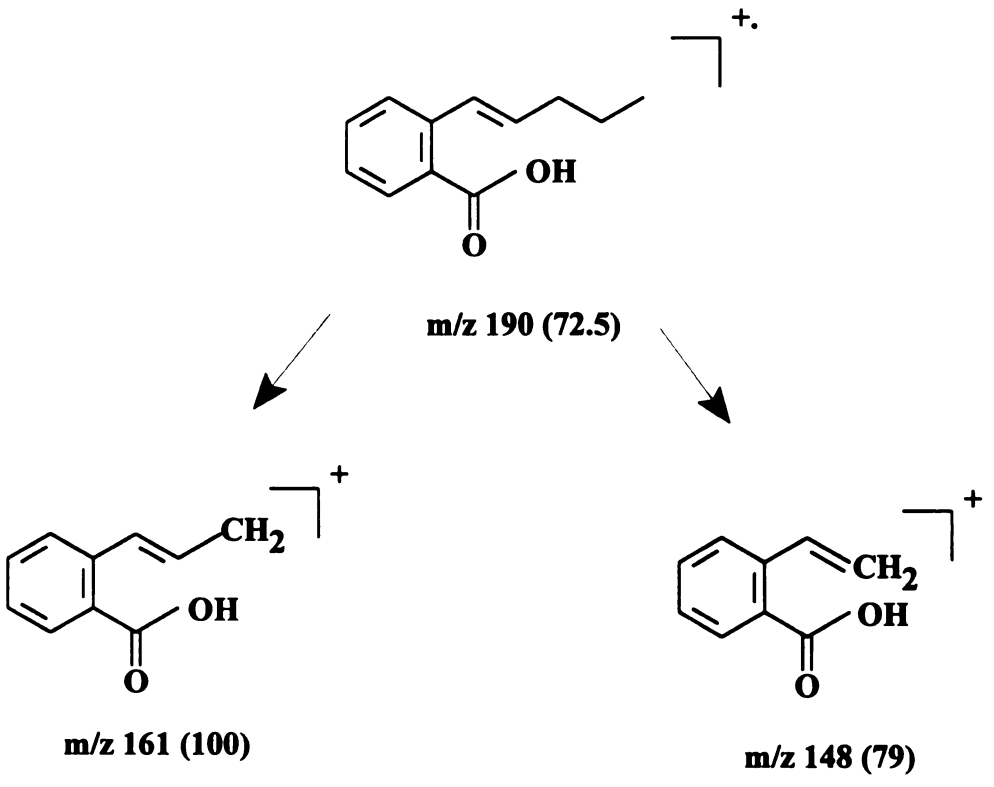


Figure 4.6. 2-Pentylbenzoic acid and its MS fragmentation pattern.

group from the pentyl side chain in compound **6**. The peak at m/z 148 was resulting from a γ-hydrogen rearrangement and loss of a propylene group.

The MeOH:H_2O (95:5)-soluble fraction afforded a mosquitocidal fraction with an LD_{100} on mosquito larvae in 2 h at 1 ppm, and compound **7**. Compound **7** (**Figure 4.7**) was characterized from its ^1H and ^{13}C NMR spectral data. The spectral data indicate a chemical formula of $C_{29}H_{50}O$ for compound **7**. There was an exchangeable proton at δ1.82 that was assigned to the 3-OH, and was embedded in a multiplet with 4-CH_2 protons. The δ values at 3.54 and 5.35 were assigned to the allylic protons at C-6 and C-5, respectively. Both the ^1H and ^{13}C NMR spectra of this compound revealed that it is identical to the NMR data of β-sitosterol.

A very low yield of the mosquitocidal fraction containing compounds **1-6** did not allow further purification of it to yield pure compounds in order to test them for individual

insecticidal activity. Further investigation of these compounds is necessary to determine which of them is responsible for the insecticidal activity. In addition, ethylmethylbenzene, 1-Isopentyl-2,4,5-trimethylbenzene, and 5-Penten-2-ylbenzoic acid have never been reported from natural sources. 3-Butylidene-3H-Isobenzofuran-1-one and its isomers are commonly found in essential oils of *Angelica acutilboa* (Takano et al., 1990), *A. glauca* Edgew (Dai and Qui, 1996), *A. tenuissima* (Kim et al., 1989), *Apium graveolens* var. graveolens (Ferh, 1979), *A. graveolens* var. rapaceum (Gijbels et al., 1985), *Levisticum officinale* (Gijbels et al., 1980), *Ligusticum actiloba* var. sugiyamae

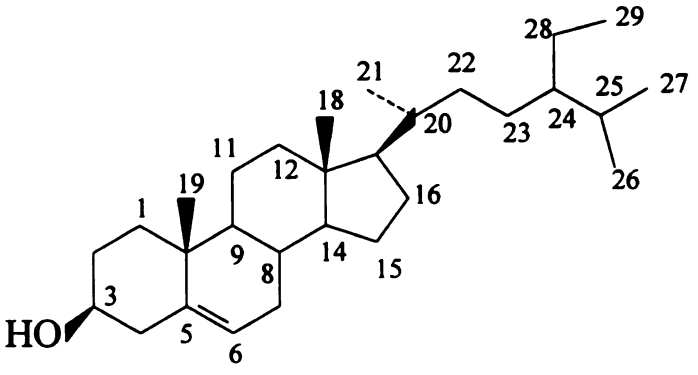


Figure 4.7. β-sitosterol.

(Yamagishi et al., 1974), *L. acuminatum* (Huang and Pu, 1989), *L. acutilobum* (Kariyone et al., 1937), *L. chuanxiong* Hort. (Yu, 1983; Kimura et al., 1989), *L. porteri* (Delgado et al., 1992), *L. sinense* Oliv cv. Chaxiong (Luo et al., 1996; Xi et al., 1987), *L. wallichii* Franch (Ko et al., 1977; Ko et al., 1980), *Opopanax chironium* (Gijbels et al., 1983). This is the first report of ethylmethylbenzene, 1-Isopentyl-2,4,5-trimethylbenzene, 3-Butylidene-3H-Isobenzofuran-1-one, and 5-Penten-2-ylbenzoic acid from *Delphinium* species. Also, 2-(Hex-3-ene-2-one)-phenylmethylketone is isolated as a natural product for the first time.

CHAPTER V

Chemical Evaluation of the Ethyl Acetate Extract from *D.* X *cultorum* Flowers

Abstract

Little chemistry has been reported for *Delphinium* species, other than diterpenoid or norditerpenoid alkaloids. In this chapter, we report the isolation of compounds **1-3** from the ethyl acetate extract of *D.* X *cultorum* flowers. The known compounds, D-mannitol (**1**) and 4-hydroxybenzoic acid (**2**), have been isolated previously from other *Delphinium* species. This is the first report of the isolation of compound **3**, bis-(4-hydroxyphenyl)methanol, from a natural source. These compounds were characterized from their ^{1}H- and ^{13}C NMR spectral data. Compounds **1-3** were not active when tested against mosquito larvae (*Aedes aegyptii* L.) and corn earworm neonates (*Helicoverpa zea* Boddie).

Introduction

This Siberian native species, *D. elatum* L., is known predominately for its norditerpenoid and diterpenoid constituents. However, some interesting phenolic compounds have been isolated previously from species other than *D. elatum* L. Mericli, A. et al.(1991) isolated 2,5,6-trihydroxypiperonylic acid methyl ester from the aerial parts of *D. venulosum* Boiss in addition to *cis* and *trans p*-coumaric acids *m* and *p*-hydroxybenzoic acids, and protocatechuic acid methyl ester. *D. formosum* Bioss afforded a new benzoxepine derivative oxformasine (1,1',7-trimethyl-2-oxo-3,8-dihydroxy-6-methoxy-benztetrahydrooxepine) (Mericli et. al, 1996). 3-hydroxy-2-methyl-4*H*-pyran-4-one was isolated from *D. denudatum* Wall. (Attar-ur-Rahman et. al, 1997). No biological activity was reported for any of these compounds.

In this chapter and for the first time, we report insecticidal activity of the crude ethyl acetate extract from *D.* X *cultorum* flowers, purified using bioassay directed fractionation. With this procedure, we isolated two of the major constituents, which are phenolic compounds, present in a corn earworm antifeedant fraction. When tested, none of these compounds **1-3** showed activity in our other bioassays. There are no reports of compounds **1-3** being isolated from *D.* X *cultorum* flowers, until now.

Experimental

Plant Material

D. X *cultorum*. seeds cv. Magic Fountians Dark Blue/ White Bee were donated by Bodger Seed, Ltd. South El Monte, CA. The seeds were germinated in high porosity,

peat-based growing mix in styrofoam trays (4"x 6" x 2") and grown until the first leaf appeared. The seedlings then were planted in pots (4"x 4" x 6") containing high porosity, peat-based growing mix and grown to the five-leaf stage. At that point, the plants were transplanted into clay pots (1 gal.) containing high porosity, peat-based growing mix and were grown until maturity. The plants were grown year round in the Pesticide Research Center Greenhouses at Michigan State University.

Plant parts were harvested when 75-85% of the florets were fully expanded on each raceme. The florets and leaves were removed from each stem and the stem was cut into two to 3.5 cm sections. The separate plant parts were then packed into reclosable plastic bags and stored at -20°C. Frozen plant parts were weighed on a Mettler BD601 balance (Mettler-Toledo, Inc., Hightstown, NJ). The separated plant material was spread evenly onto three metal lyophilizer trays (29x 31.5 x 3.4 cm) and lyophilized using a bulk tray lyophilizer (FTS Systems, Inc., Stone Ridge, NY) at 5°C for 48h. The dried plant material was macerated into a fine powder in a commercial Waring blender, packed into reclosable plastic bags and stored at -20°C until analysis. The water content of the fresh *D.* X *cultorum* flowers was approximately 80%.

General Experimental

The components of the crude ethyl acetate extract were analyzed initially by thin layer chromatography (TLC). A concentrated solution of the crude extract was prepared in $CHCl_3$. The solution was applied to silica plates (20 x 5 cm, 250 μm with inorganic binder and UV 254) with a 5 μL capillary pipette. The spot was allowed to dry. The TLC

plate was placed into a solvent chamber equilibrated with 15 mL of $CHCl_3$:MeOH. TLC plates were purchased from Analtech Inc. (Newark, Delaware, USA).

NMR spectra were recorded on a Varian VXR 300 MHz spectrometer (Varian, Palo Alto, CA) at room temperature in the Max T. Rogers NMR facility at Michigan State University.

Extraction

Extraction of D. X cultorum flowers. A large glass column was used for the extraction of *D. X cultorum* flowers to obtain a sufficient quantity of crude hexane extract. Lyophilized and macerated flowers (409 g) were packed in the column that contained a cotton plug at the bottom. The plant material was extracted sequentially with hexane (3.5 L x 3) and ethyl acetate (2.0 L x 3). The plant material was soaked in solvent for at least 72 h, with exchange of fresh solvent every 12 h. Solvent was removed in vacuo at 40°C on a rotary evaporator yielding crude hexane extract (8.68 g) and ethyl acetate extract (3.61 g). The dried extract stored at -20°C until bioassay and purification. The residual plant material was discarded.

Insecticidal Assays

Corn Earworm Assay. Corn earworm eggs (*Helicoverpa zea* Boddie) and dry corn earworm diet was purchased from North Carolina State Insectory, Department of Entomology, North Carolina State University, Raleigh, North Carolina. The eggs were hatched in an incubator at 27°C. The dry diet was dispensed into scintillation vials (940 mg) for each treatment. Crude extracts or purified compounds were dissolved in DMSO

to give a concentration of 1250 μg · 25 μL^{-1}. Twenty-five μL of these stock solutions were mixed thoroughly with the portions of dry diet. A 25 μL of DMSO was used as a control. Agar solution (1.4%) was mixed and autoclaved for 5 min at 15 psi and 125°C to melt the agar. This solution was held in a water bath at 45°C, and added to the dry diet until the total diet weighed 5 g. The final concentration of test extracts and compounds was 250 ppm. The wet diet was mixed thoroughly, and 3-4 drops of diet were dispensed into 3.5 mL polystyrene vials. The freshly poured portions of diet were allowed to cool and dry for at least 1 h. After drying, one neonate larvae was placed in each vial, and the vials were capped. The treatment and control vials were held in a growth chamber at a photoperiod of 16 h day and 8 h night with day temperature at 28°C and night temperature at 24°C. Each treatment had 15 replicates. The treatments were arranged in a completely randomized design. The larvae were weighed (mg) on a Mettler AE163 analytical balance (Mettler-Toledo, Inc., Hightstown, NJ) after six days. Statistical analysis was conducted using ANOVA and the F test. Least Significant Difference (LSD) was calculated for those treatments with significant F values, and used to compare treatment mean weights with controls.

Mosquito Assay. First instar mosquito larvae (*A. aegyptii* L.) were provided by Drs. Alexander Raikel and Alan Hays, Department of Entomology, Michigan State University. The larvae were raised in 500 mL beakers containing 150-250 mL of reverse osmosis water in an incubator at 26°C, for three days. Ten to 12 fourth instar larvae were placed in 980 μL of reverse osmosis H_2O in 12 x 75 mm borosilicate glass test tubes. Crude extracts or purified compounds were dissolved in DMSO, unless otherwise stated, to give a concentration of 250 μg · 20 μL^{1}, unless otherwise stated. A 20 μL aliquot of

DMSO was used as a control. Treatments and controls were covered and left at room temperature. There were three replicates per treatment. The number of dead larvae were recorded at 2 h intervals, up to and including 24 h. Statistical analysis was conducted using ANOVA and the F test. LSD was calculated and used to compare treatment means with controls.

Preparative High Performance Liquid Chromatography

Active fractions were subjected to preliminary separation on a recycling preparative HPLC Model LC-20 (Japan Analytical Industry, Dichrom, Santa Clara, CA) with a 2-Jaigel-GS310-F columns (i.d. 20 x 300mm) connected in series. The mobile phase, MeOH:H_2O (90:10), was used under isocratic conditions at a flow rate of 5 mL · min^{-1}. The samples were filtered through a 0.22 μ filter (Millipore Corp, Bedford, MA), prior to injection. A variable-wavelength UV detector S-310A Model II (Soma Optics, LTD., Dichrom, Santa Clara, CA) was used to collect data at 210 nm. Fractions were purified with two Jaigel S-343-15 ODS (15μ) columns (i.d. 20 x 250 mm) connected in series. The mobile phase, MeOH:H_2O (70:30), was used under isocratic conditions at a flow rate of 3 mL · min^{-1}. All fractions were dried under vacuum on a Buchii RE111 Rotovapor (Brinkman, Westbury, NY) at 45°C.

Purification of Ethyl Acetate Extract

A preliminary separation of the crude ethyl acetate extract from *D. X cultorum* was performed by solvent-solvent partition. Crude ethyl acetate extract (1.09 g) was dissolved in hexane (200 mL), and MeOH: H_2O (90:10) (300 mL) was added to this

solution. The mixture was poured into a 1L separatory funnel and shaken well. The hexane layer was removed, and the MeOH:H_2O layer was extracted twice with hexane (200 mL X2). The hexane extracts were combined and evaporated *in vacuo* at 45°C to afford fraction I (395.2 mg). Similarly, removal of solvent *in vacuo* at 45°C gave a MeOH:H_2O (90:10) soluble fraction II (703 mg).

Fraction II (2 g), accumulated from several solvent-solvent partitions, was dissolved in MeOH:H_2O (90:10) at a concentration of 45 mg/mL. This solution was fractionated further on a sep-pak C_{18} cartridge (Millipore Corp., Bedford, MA) using MeOH: H_2O (90:10) as the eluting solvent. Four fractions (1.25 mL each) were collected and removal of solvent afforded 1012.1, 174.1, 78.5, and 47.9 mg, respectively, for fraction A-D. Following this, the sep-pak cartridge was eluted with 5 ml of MeOH. The combined MeOH yielded fraction E (399.2 mg). Finally, the sep-pak cartridge was stripped with $CHCl_3$ (2 mL) and the $CHCl_3$ evaporated to dryness to give fraction F (156.1 mg).

Fractions A-D were combined based on their biological activity against corn earworm (*H. zea* Boddie) to give fraction G (1.2 g). It was then dissolved in MeOH:H_2O (90:10) at a concentration of 205 mg/mL and purified by preparative HPLC on Jaigel GS310-F columns. This yielded six fractions A-F weighing 21.8, 323.6, 621.5, 10.2, 0.6, 123.0 mg at retention times of 0-25, 25-28, 28-35, 35-38, 38-46, 46-110 min., respectively. Fraction C was recrystallized from MeOH and yielded compound **1** (10.1 mg). Compound **1** was characterized by ^1H and ^{13}C NMR experiments.

Compound **1**: ^1H NMR (d_6-DMSO): δ 3.34 (4H, *dd*, J=6 Hz,1-H X 2, 6-H X 2), 3.44 (2H,*dt*, J=3 Hz, 2-H, 5-H), 3.55 (2H, *dd*, J=10.5 Hz, 3-H, 4-H), 4.13 (2H, *d*, J=2.4

Hz, 1-OH, 6-OH), 4.33 (2H, *m*, 2-OH, 5-OH), 4.41 (2H, *d*, J=2.4 Hz, 3-OH, 4-OH). ^{13}C NMR ($(CD_3)_2SO$): δ 63.92 (C-1, C-6), 69.78 (C-2, C-5), 71.43 (C-3, C-5). These values were analogous to those reported in Breitmaier et al. (1987) for D-mannitol.

Fraction F (R_t. 46-110) dissolved in MeOH:H_2O (70:30) at a concentration of 40 mg/mL and purified using the Jaigel S-343-15 ODS column. Three injections yielded six fractions I-VI weighing 4.6, 23.9, 9.6, 49.7, 4.2, 1.7 mg. These were collected at retention times 22-34, 34-37, 37-42, 42-47, 47-50, 50-90 min., respectively. Fraction II and VI were purified further on the same column using MeOH:H_2O (60:40) as the mobile phase at a flow rate of 3 mL/ min. Compound **2** (13.9 mg) was obtained from the HPLC purification of fraction II (R_t. = 52 min). Compound **3** (25.6 mg) was obtained from the HPLC purification of fraction VI (R_t. = 57min). Compounds **2** and **3** were characterized by ^1H- and ^{13}C NMR experiments.

Compound **2:** ^1H NMR (d_6-DMSO): δ 6.85 (2H, *d*, J= 7.8 Hz, 3-H, 5-H), 7.85 (2H, *d*, J=7.8 Hz, 2-H, 6-H), 11.2 (1H, *bs*, 4-OH). ^{13}C NMR (d_6-DMSO): δ 110.97 (C-2, C-3, C-5, C-6), 127.50 (C-4), 157.23 (C-7). Both ^1H and ^{13}C NMR chemical shift values were identical to the published spectral data for 4-hydroxybenzoic acid (The Aldrich Library of ^{13}C and ^1H FT NMR Spectra, 1993).

Compound **3**: ^1H NMR (d_6-DMSO): δ 3.72 (1H, *s*, 1-H), 6.67 (4H, *d*, J=8.7 Hz, 2-H, 2'-H, 6-H, 6'-H), 7.74 (4H, *d*, J=8.4 Hz, 3-H, 3'-H, 5-H, 5'-H). ^{13}C NMR (d_6-DMSO): δ 55.64 (C-7), 114.17 (C-2, C-2', C-3, C-3', C-5, C-5', C-6, C-6'), 131.27 (C-1, C-1'), 159.03 (C-4, C-4').

Methylation of Compound 3.

Diazomethane was prepared by dissolving 0.5 N-nitroso-N-methylurea in 10% KOH solution (50 mL) with ether (50 mL) over an ice bath. The mixture was stirred gently for a few minutes and poured into a 250 mL separatory funnel. The KOH solution was removed and the diazomethane solution was then washed with 25 mL of ice cold water. The diazomethane solution was poured into a brown bottle containing KOH pellets and stored at -20°C. The diazomethane solution and a small amount of MeOH was added to a sample of compound **3** (8 mg). The sample was capped, and allowed to sit in the hood for 2 h. The ether was evaporated with N_2 gas, and removed the MeOH *in vacuo* at 30°C.

Compound **4**: ^{1}H NMR (d_6-DMSO): δ 3.72 (1H, *s*, 1-H), 3.84(6H, *s*), 6.67 (4H, *d*, J=8.7 Hz, 2-H, 2'-H, 6-H, 6'-H), 7.74 (4H, *d*, J=8.4 Hz, 3-H, 3'-H, 5-H, 5'-H).

Acetylation of Compound 4.

To compound **4** a solution of pyridine (2 mL) and acetic anhydride (200 μL) was added. The clear solution was allowed to sit for two days in the dark. The solvent solution was removed *in vacuo* at 50°C.

Compound **5**: ^{1}H NMR (d_6-DMSO): δ 2.35 (3H, *s*), 3.72 (1H, *s*, 1-H), 3.84(6H, *s*) 6.67 (4H, *d*, J=8.7 Hz, 2-H, 2'-H, 6-H, 6'-H), 7.74 (4H, *d*, J=8.4 Hz, 3-H, 3'-H, 5-H, 5'-H).

Results and Discussion

D. X *cultorum* flowers were extracted sequentially with hexane and ethyl acetate. A preliminary bioassay performed on the ethyl acetate extract revealed that the average weights of the treated caterpillars (*H. zea* Boddie) were significantly smaller than the controls when tested at 250 ppm ($p \leq 0.01$). After partitioning, the aqueous MeOH-soluble fraction possessed a slightly higher level of activity than the hexane soluble fraction on *H. zea* neonates when tested at 250 ppm ($p \leq 0.01$). Therefore, the aqueous MeOH-soluble fraction was chosen for further bioassay-directed purification.

Separation of the aqueous MeOH-soluble fraction was performed on a C_{18}-sep-pak cartridge to remove as much of the non-polar compounds from this fraction as possible. The fractions obtained from this separation (A-F) were tested against corn earworm (*H. zea* Boddie) to determine their activity (**Figure 5.1**). All of the fractions exhibited high activity. However, the MeOH- and $CHCl_3$-eluted fractions showed slightly less activity and were not studied further.

Compounds **2** and **3** did not show activity against either corn earworm neonates or mosquito larvae when tested at 250 ppm concentration. Compound **1** was isolated from a non-active fraction in crystalline form and was not expected to posses biological activity against these organisms, and therefore was not tested.

The ^1H NMR spectral data for compound **1** showed some interesting features. The three peaks at δ 4.13, 4.33, and 4.41, integrated for 2 H each and exchanged with D_2O, indicate a polyhydroxylated molecule. The peak at δ 3.34 integrated for 4 H was assigned to an aliphatic CH_2. The doublets at δ 3.55 indicated a CH adjacent to another CH moiety, both bearing OH groups. The triplets δ 3.44 indicated a CH adjacent to a CH_2

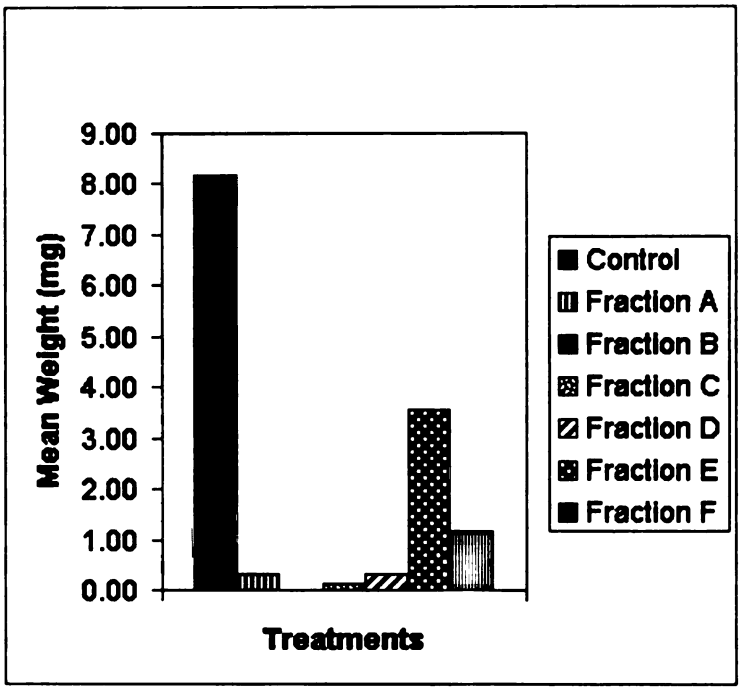


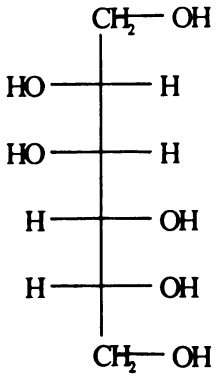
Figure 5.1. Average weights of *H. zea* Boddie larvae after six days. Treatments were tested at 250 ppm. $p \leq 0.01$.

moiety, both bearing OH groups. The carbon data suggested the presence of three sets of carbons appearing at δ 39.2, 69.78, and 71.43. This implied that compound **1** was symmetrical. The NMR spectral data for Compounds **1** (**Figure 5.2**) were in agreement with those values reported in the literature for D-mannitol (Brietmaier, 1987).

Some features of the ^{1}H NMR spectral data for compound **2** (**Figure 5.2**) were the peaks at δ 6.85 and 7.85, both integrating for 2 H with a coupling constant of 7.8 Hz. This is typical of an AB system indicating para-substituents on an aromatic ring. The peak at δ11.2, with a downfield chemical shift value, was indicative of a desheilded proton. Peaks in this region of a ^{1}H spectrum were indicative of a phenolic or an acidic proton. Both ^{1}H and ^{13}C NMR spectral data revealed that **2** is identical to the spectral data of *p*-hydroxybenzoic acid.

Compound **3** **(Figure 5.2)** is reported as a synthetic product (Elliger, 1985). However, its spectral data is not available in the literature. This is the first report of the spectral characterization of this compound. The ^{1}H NMR of pure compound **3** indicated an AB system as evidenced by the doublets at δ 6.85 and 7.85 with a J value of 7.8 Hz. Both of these peaks integrated for four protons each. This suggested symmetry in the molecule that contains two aromatic rings. The singlet peak at δ 3.72 integrating for 1-H was assigned to a CH group. The fact that this peak was a singlet further supported that **3** is symmetrical.

Features of the ^{13}C NMR spectral data that should be noted are the chemical shifts of the three carbons bearing hydroxyl moieties at δ 55.6 for C-7, and δ 159.03 for C-4 and C-4'. The downfield chemical shifts clearly indicated oxygen functionalities present



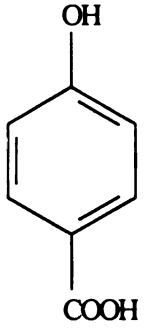
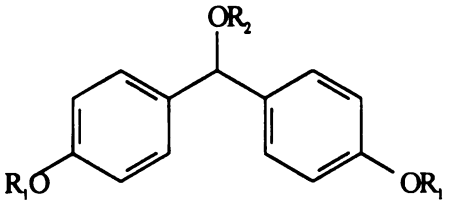


Figure 5.2. D-mannitol (**1**) and *p*-hydroxybenzoic acid (**2**).



Compound **3** $R_1 = R_2 = H$

Compound **4** $R_1 = Me$, $R_2 = H$

Compound **5** $R_1 = Me$, $R_2 = OCMe$

Figure 5.3. Bis-(4-hydroxyphenyl)-methanol and its analogues.

at these positions. Furthermore, the peak at δ 114.17 representing eight identical carbon atoms indicated that this molecule is symmetrical.

It was evident from the ^{1}H-NMR of **3** that it contained a total of 3-OH groups, one each at 4 and 4' positions. The third hydroxy group was assigned to C-7. To confirm the presence of the phenolic hydroxyls, **3** was methylated using CH_2N_2. The ^{1}H-NMR of the methylated product, **4**, gave a sharp singlet for 6 H and confirmed that the 4 and 4' hydroxy moieties were methylated. Also, the ^{1}H-NMR of **4** gave a singlet at δ 3.91 indicating the presence of the third hydroxyl group. In order to confirm the third hydroxy group in **3**, the dimethyl product, **4**, was acetylated using acetic anhydride in pyridine.

The ^{1}H-NMR analysis of the acetylated product, **5**, gave a singlet integrating for 3 H, confirming the presence of a hydroxy group at C-7. Therefore, compound **3** was assigned the structure bis-(4-hydroxyphenyl)-methanol from the ^{1}H and ^{13}C NMR spectra of the parent compound and its derivatives.

This is the first report of D-mannitol (**1**), 4-hydroxybenzoic acid (**2**) and bis-(4-hydroxyphenyl)methanol (**3**) from *D.* X *cultorum* flowers. Compound **2**, 4-hydroxybenzoic acid was isolated from *D. venulosum* Boiss (Mericli et. al, 1991). D-mannitol (**1**) was isolated from *D. flexiosum*, *D. elisabethae*, *D. tamarae*, and *D. dzawachischvili* (Dekanosidze et. al, 1974). However, bis-(4-hydroxyphenyl)-methanol (**3**) has not been reported from any natural source, until now. While the ethyl acetate extract yielded new chemistry from this plant, further investigation is warranted to identify those compounds responsible for its biological activity against corn earworm neonates.

CHAPTER VI

Summary and Conclusions

Delphinium X *cultorum* cv. Magic Fountains flowers were investigated for insecticidal, nematocidal, antimicrobial, and topoisomerase enzyme inhibitory assays. Lyophilized and macerated *D.* X *cultorum* flowers were sequentially extracted with hexane, ethyl acetate, and MeOH. The crude extracts were assayed at 250 ppm concentrations against 4th instar mosquito larvae (*A. aegyptii* L.), corn earworm (*H. zea* Boddie) and gypsy moth *(L. dispar* L.) neonate larvae, Japanese beetle (*P. japonica* Newman), Colorado potato beetle (*L. declimenta* Say), nematodes (*C. elegans* Dought) and (*P. redivivus* Goody), microbial pathogens *(A. parasiticus, C. albicans, E. coli, S. epidermidis, S. aureus, F. moniliform, F. oxysporum, R. solanii),* and a topoisomerase enzyme inhibitory assay using *S. cerevisiae* strain 394, *S. cerevisiae* strain 394 t_1, *S. cerevisiae* strain 394 $t_{2\text{-}5}$. Based on the results of these initial bioassays, the hexane and ethyl acetate extracts were chosen for further purification.

The hexane extract showed mosquitocidal activity against fourth-instar *A. aegyptii* larvae. A 50 ppm concentration gave 100% mortality in 2h. Also, it showed antifeedant activity on corn earworm (*H. zea* Boddie) and gypsy moth (*L. dispar* L.) neonate larvae at 250 ppm concentrations. Similarly, the ethyl acetate extract showed antifeedant activity against corn earworm neonate larvae (*H. zea* Boddie) at 250 ppm concentration.

The hexane extract from *D. X cultorum* flowers was separated using solvent-solvent partitioning and purified by preparative TLC on silica gel plates. It yielded a mosquitocidal fraction showing 100% mortality in 2 h at 1 ppm concentration. From this fraction, 6 volatile, non-alkaloidal compounds were characterized using GC-EIMS experiments. The compounds characterized were ethyl-methyl benzene, 1- Isopentyl-2,4,5-trimethylbenzene, and 2-Penten-2-ylbenzoic acid, 2 isomers of 3-Butylidene-3H-isobenzofuran-1-one and a novel derivative of acetophenone, 2-(Hex-3-ene-2-one)phenylmethylketone. However, 2-(Hex-3-ene-2-one)- phenylmethylketone was the most abundant constituent of this fraction. Due to the low yield of these compounds, we were unable to determine which of these compounds was responsible for the mosquitocidal activity observed. In addition, β-sitosterol was isolated from the hexane extract was confirmed from its ^{1}H and ^{13}C NMR spectral data.

In addition to the mosquitocidal fraction, initial bioassays with corn earworm (*H. zea* Boddie) showed that the hexane extract possessed antifeedant activity. Further, investigation of the hexane extract is necessary to determine which of the reported compounds is producing mosquitocidal activity, as well as separation of the active fraction to yield pure compounds with antifeedant activity.

The ethyl acetate extract was separated by solvent-solvent partitioning and purification using a C_{18} sep-pak cartridge. This extract yielded a fraction, which displayed corn earworm antifeedant activity at 250 ppm concentration. The active fraction was purified using a preparative HPLC with size exclusion and reverse phase C_{18} columns. Two compounds were isolated from this fraction, 4-hydroxybenzoic acid and bis-(4-hydroxyphenyl)-methanol. Neither of these compounds displayed corn earworm

antifeedant or mosquitocidal activity when tested at 250 ppm concentration. In addition, D-mannitol was also isolated from this extract.

This is the first report of the isolation of these compounds from *D.* X *cultorum*. D-mannitol, 4-hydroxy benzoic acid, and β-sitosterol have been isolated from other delphinium species such as *D. felxiosum*, *D. elisabethae*, *D. tamarae*, and *D. dzawachischilli*; *D.venulsoum*; and *D. ajacis* and *D. staphisagria*, respectively. 3-Butylidene-3H-isobenzofuran-1-one is a common constituent of celery, lovage and angelica, but has never been isolated from delphinium. The compounds ethyl-methyl benzene, 1- Isopentyl-2, 4,5-trimethylbenzene, and 2-Pentenylbenzoic acid, and bis- (4-hydroxyphenyl) methanol have been reported as synthetic products and have not been isolated from plant tissues until now. Finally, 2-(Hex-3-ene-2-one)-phenyl methyl ketone is a novel compound and has not been reported until now.

As stated previously, delphiniums are well known for their diterpenoid and norditerpenoid alkaloidal constituents. The phytochemical research reported from delphiniums reflects this fact. To the present time, delphinium research is still focusing on the isolation, identification, and biological activities associated with these alkaloids. A select few researchers have published new or novel chemistry from delphinium species, other than alkaloids. The results presented in this thesis show that delphinium should be considered as a potential source for novel compounds with biological activities that are not related to alkaloids.

APPENDICES

Appendix I

SURVEY OF *D. BELLAMOSUM* FLOWER AND STEM, AND *D. X CULTORUM* STEM AND LEAF EXTRACTS FOR BIOLOGICAL ACTIVITY

This appendix briefly describes the extraction, biological screening and preliminary fractionation of crude extracts from flowers, stems, and leaves of *D.* X *cultorum* cv. Magic Fountains Dark Blue/ White Bee, Dark Blue Dark Bee, and White/ Dark Bee. These cultivars were grown as described in chapter IV. *D. bellamosum* plants were donated by Walters Gardens, Michigan. The dormant plants were planted in Bacto mix in clay pots and grown to maturity in the Pesticide Research Center Greenhouses at Michigan State University. The plant material was lyophilized and processed in the same manner described in chapter IV. The bioassays referred to in this section are described in chapter IV. The procedure for extraction of the plant material is outlined in **Scheme 4.1.**

The hexane and MeOH extracts of *D. bellamosum* flowers and stems were tested against mosquito larvae (*A. aegyptii*) and showed no activity. These extracts were investigated primarily for potential mosquitocidal compounds.

The hexane extract from *D. X cultorum* stem showed antifeedant activity against corn earworm (*H. zea* Boddie) and gypsy moth (*L. dispar* L.) neonates at 250 ppm concentration ($p \leq 0.01$), and mosquitocidal activity on fourth instar mosquito larvae (*A. aegyptii*) at 250 ppm concentration in 1 h. The MeOH extract from *D. X cultorum* stem showed 100 % mortality on fourth instar mosquito larvae (*A. aegyptii* L.) at 250 ppm concentration in 4 h and showed antifeedant activity against gypsy moth (*L. dispar* L.)

neonates at 250 ppm concentration ($p \leq 0.01$). However, it was not active on corn earworm neonates at 250 ppm concentration. The MeOH extract from *D. X cultorum* leaves showed antifeedant activity against gypsy moth neonates (*L. dispar* L.). However, the hexane and methanol leaf extracts from *D.* X *cultorum* did not show any other insecticidal activity. The hexane and MeOH extracts of the *D.* X *cultorum*. stem and leaf showed no activity against the following organisms at 250 ppm concentration: nematodes (*P. redivivus* Goody and *C. elegans* Dought),and microorganisms (*A. parasiticus, C. albicans, E. coli, S. epidermidis, S. aureus, F. moniliform, F. oxysporum, R. solanii, S. cerevisiae* strain 394, *S. cerevisiae* strain 394 t_{-1}, *S. cerevisiae* strain 394 t_{2-5}).

Separation of the hexane-soluble fraction from D. X cultorum flowers. The hexane-soluble fraction from solvent -solvent partition of hexane extract from *D. X cultorum* flowers as described in chapter IV, showed 100 % mortality on fourth instar mosquito larvae at 2 h at 250 ppm concentration. This fraction (3.6 g) was purified using vacuum liquid chromatography (VLC) followed by medium-pressure liquid chromatography (MPLC) methods to yield seven fractions A-G weighing 38.8, 682.5, 289.0, 225.0, 26.7, 15.7, and 20.5 mg, respectively. Purification of fraction C (115.8 mg) by PTLC on silica plates yielded seven fractions A-G weighing 17.9, 18.6, 17.2, 2.1, 2.6, 0.5, 28.8 mg, respectively. Fractions A, C, and G showed 100 % mortality at 25 ppm in 12, 2, and 4 h, respectively. While these fractions appeared as only one spot in many TLC analyses, ^{1}H NMR showed them to be spectroscopically impure.

Purification of the Hexane Extract from D. X *cultorum Stem.* The crude hexane extract (603 mg) was subjected to fractionation using MPLC on silica. Six fractions, A-F were collected with weights of 83.2, 103.7, 65.2, 37.4, 39.1, and 136.9 mg, respectively. These fractions were tested for biological activity against mosquito larvae (*A. aegyptii* L.) and nematodes (*C. elegans* Dought). Fractions A-C showed 100 % mortality on fourth instar mosquito larva at 100 ppm concentration after 4, 2, and 3 h, respectively. However, these fractions were not active against *C. elegans* Dought.

The fraction C (37.8 mg) was purified using PTLC on silica plates. Four bands were collected, A-D with weights of 21.9, 10.7, 1.9, 5.5, respectively. Fraction B and C showed 100 % mortality on fourth instar mosquito larvae at 50 ppm after 3 and 21 h, respectively.

Separation of the Crude Ethyl Acetate Extract from the Stem. Lyophilized and macerated stem material (105 g) was extracted sequentially with hexane and ethyl acetate, similar to the flower material in Chapter V. Crude ethyl acetate extract from the stem (2.29g) was subjected to solvent-solvent partitioning. As described in chapter V, two fractions yielded from this separation, were a hexane-soluble fraction (1.26g) and MeOH:H_2O (95:5)-soluble fraction (1.00g). The aqueous MeOH (95:5)-soluble portion was eluted through C_{18} sep-pak cartridge, similar to that of the ethyl acetate flower extract in chapter V. The fraction eluted with MeOH:H_2O (672 mg) was subjected to MPLC separation on silica. Twenty-one fractions A-U were collected based on UV absorbance and they weighed 1.3, 4.3, 6.3, 6.6, 7.3, 21.5, 106.6, 139.2, 57.8, 202.3, 70.3, 72.0, 48.3, 39.0, 46.6, 39.5, 45.3, 5.3, 5.9, 8.7, 4.3 mg, respectively. Some of these fractions were tested against corn

earworm (*H. zea* Boddie). Fractions E, G-J, O showed excellent antifeedant activity against corn earworm neonates.

Time constraints and lack of plant material in some of these cases did not allow the fractions described here to be investigated further for their active constituents. The work outlined in this appendix indicates that there are additional active constituents in *D.* X *cultorum* stem. Further investigation of the stem fractions to obtain pure and active compounds, was not conducted, partly because this is not within the scope of this thesis research.

Table A.1. Mosquitocidal assay of MPLC fractions.

Concentration and time at which 100 % mortality was reached in 24 h. n = no activity. $p \leq 0.1$.

Treatment	125 ppm
Fraction A	n
Fraction B	n
Fraction C	2h
Fraction D	16h
Fraction E	n
Fraction F	n
Fraction G	n

Table A.2. Average Weight of Corn Earworm (*H. zea* Boddie) after six days when tested against MPLC fractions.

Treatments were tested at 250 ppm. * $p \leq 0.01$, ns = not significant.

Treatment	Average Weight (mg)
Control	8.17
Fraction A	8.53 ns
Fraction B	10.95 ns
Fraction G	0.0*
Fraction H	0.03*
Fraction I	0.07*
Fraction J	0.03*
Fraction T	9.49 ns
Fraction U	9.34 ns
Control	6.61
Fraction E	2.04*
Fraction O	1.18*

APPENDIX II

RECIPES FOR MEDIA USED IN BIOASSAYS

Table A.3. YMG Agar Media Recipe.
YMG was used to grow *Candida albicans.*

Ingredient	Amount
yeast extract	4 g · L^{-1}
malt extract	10 g · L^{-1}
dextrose	4 g · L^{-1}
agar	18 g · L^{-1}

Table A.4. Emmons Agar Media Recipe.
Emmons was used to grow, *Escherichia coli, Staphylococcus epidermidis* and *Streptococcus aureus.*

Ingredient	Amount
neopeptone	10 g · L^{-1}
glucose	20 g · L^{-1}
agar	18 g · L^{-1}

Table A.5. YPDA Agar Media Recipe.
YPDA was used to grow *Saccharomyces cerevisiae* strain 394, *S. cerevisiae* strain 394 t_{-1}, and *S. cerevisiae* strain $394_{t2\text{-}5}$.

Ingredient	Amount
Bacto peptone	20 g · L^{-1}
yeast extract	10 g · L^{-1}
dextrose	20 g · L^{-1}
agar	17 g · L^{-1}
adenine sulfate solution (0.5%)	1 mL · L^{-1}

Table A.6. PDA Agar Media Recipe.
PDA was used to grow *Aspergillus parasiticus, Fusarium moniliform, Fusarium oxysporum*, and *Rhizoctonia solanii.*

Ingredient	Amount
potato dextrose agar	29 g · L^{-1}

To prepare agar plates, the ingredients of appropriate medias were weighed and added to 1-L Erlenmeyer flasks. Five-hundred mL of deionized water was added to the flask, and the solution was mixed well. The flasks were stoppered with a foam plug and the tops were covered with foil. The media were autoclaved for 20 min at 15 psi at 125°C. After removal from the autoclave, the flasks were allowed to cool until they could be handled. Twenty-five mL of agar solution was poured into petri plates in a laminar airflow hood. The agar was allowed to cool and set. The plates were stored in plastic bags at room temperature until further use.

Table A.7. NG Media Recipe.
NG media was used to culture *Caenorhabditis elegans* Dought nematodes.

Ingredient	Amount
NaCl	3 g · L^{-1}
Bacto peptone	2.5 g · L^{-1}
Agar	17 g · L^{-1}
Phosphate Buffer	25 mL · L^{-1}
1M CaCl2	1 mL · L^{-1}
1M MgSO4	1 mL · L^{-1}
cholesterol 5 mg/ mL EtOH	1mL · L^{-1}

Table A. 8. Phosphate Buffer Recipe

Ingedient	Amount
KH_2PO_4	11.968 g · 88 mL^{-1} H_2O
K_2HPO_4	2.088 g · 12 mL^{-1} H_2O

Table A.9. Basal Media Recipe.
Basal-heme media was used to culture *Panagrellus redivivus* Goody.

	Basal Media
Bacto Soytone	16 g · L^{-1}
Yeast extract	12 g · L^{-1}
Heme solution	8 mL

Basal media was mixed and autoclaved for 20 min at 15 psi and 125°C.

Heme solution was prepared by the following method:

In a 50 mL beaker, 0.75 g hemoglobin was mixed with 15 mL of freshly prepared 0.1 N NaOH. This mixture was stirred on a magnetic stirrer at speed 4.5 for 1 h. After mixing, this solution was sterile-filtered and added to the basal media. Three mL of this sterile basal-heme media was dispensed into a sterile scintillation vial. Vials containing this media were stored at 4°C.

LITERATURE CITED

Literature Cited

Aiyar,V. N.; Benn, M. H.; Hanna,T.; Jacyno, J.; Roth, S. H.; Wilkens, J. L. The principal toxin of *Delphinium brownii* Rydb., and its mode of action. *Experientia*, **1979,** *35*, 1367-8.

Akihisa, T.; Thakur, S.; Rosenstein, F. U.; Matsumoto, T. Sterols of Cucurbitaceae: the configurations at C-24 of 24-alkyl-$^{5}\Delta$,$^{7}\Delta$,$^{8}\Delta$-sterols .*Lipids*, **1986,** *21*, 39-47.

Alkondon, M.; Pereira, F. R.; Wonnacott, S.; Albuquerque, E. X. Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. *Mol. Pharmacol.* **1992,** *41*, 802-8.

Arazashvili, A. I.; Moniava, I. I.; Kemertelidze, E. P. Flavonoids of *Delphinium flexiosum* and *Delphinium elisabethae*. *Khim. Prir. Soedin.***1973**, *9*, 556-7; Chem. Abstr. **1974,** *80*,118204z.

Arazashvili, A. I.; Moniava, I.I.; Kemertelidze, E. P. Flavonoids from some species of Delphinium. *Khim. Prir. Soedin.***1974,** *10*, 251.Chem.Abstr. **1974,** *81*, 13277r.

Arazashvili, A. I.; Nikonov, G. K.; Kemertelidze, E. P. Structure of delphoside, a new isocoumarin glycoside. *Khim. prir. Soedin.***1974**, *6* ,705-7.Chem. Abstr. **1975,** *82*, 67466m.

Asen, S.; Stewart, R. N.; Norris, K. H. Anthocyanin, flavonol copigments, and pH responsible for larkspur flower color. *Phytochemistry*. **1975,** *14*, 2677-82.

Asif, M.; Tariq, M.; Tamanna, S. A.; Ahmad, M. U. The sterols and fatty acids of *Delphinium denudatum* roots. *Fette Seifen Anstrichmittel*. **1981,** *83*, 323-324.

Atanasova-Shopova, S.; Rusinov, K.; Markova, M. Pharmacological studies of Bulgarian plants with a view of their anticonvulsive effect. *Izv. Inst. Fiziol., Bulg. Akad. Nauk*. **1969**, *12*, 205-16. Chem Abstr. **1970,** *72*, 98942t.

Atta-ur-Rahman; Choudhary, M.I. Diterpenoid and steroidal alkaloids. *Nat. Prod. Reports*. **1995**, *12*, 361-379.

Atta-ur-Rahman; Nasreen, A.; Akhtar, F.; Shekhani, M. S.; Clardy, J.; Parvez, M.; Choudhary, M.I. Antifungal diterpenoid alkaloids from *Delphinium denudatum*. *J. Nat. Prod.* **1997,** *60*, 472-4.

Avramova, S.; Surbova, S. Alkaloids of some Bulgarian plants and their insecticidal activity II. *Epidemiol. Mikrobiol. Infektsiozni Bolesti* **1964,** *1*, 23-6. Chem. Abstr. **1965,** *62*, 16628a.

Bailey L. H., *Hortus Third*: a concise dictionary of plants cultivated in the United States and Canada. 1976, Macmillan, NY. 367-369.

Bando, H.; Wada, K.; Tanaka, J.; Kimura, S.; Hasegawa, E.; Amiya, T. Two new diterpenoid alkaloids from Delphinium pacific giant and revised ^{13} C-NMR assignment of delpheline. *Heterocycles.* **1989,** *29*, 1293-1300.

Beckstrom-Sternberg, S. M.; Duke, J. A.; Wain, K. K. A. The Ethnobotany Botany Database.[Online] Available. http://probe.nalresda.gov:8300/cgi-bin/browse/ethnobotdb. ACEDB version 4.0-data version, July 1994.

Benn, M. H.; Jacyno, J. M. The Toxicology and Pharmacology of Diterpenoid Alkaloids. In: *Alkaloids: Chemical and Biological Presepectives*, Pelletier, S. W. ed.; John Wiley and Sons: New York, 1983; 1; 153-209.

Benn, M. H.; May, J. The biosynthesis of diterpenoid alkaloids. *Experentia*, **1964,** *20*, 252-3.

Bernays, E. A.; Chapman, R. F. Host-Plant Selection by Phytophagous Insects. *Contemporary Topics in Entomology*, Chapman and Hall: New York, 1994; 2, 14-59.

Bishop, F. A. *The Delphinium; A Flower Monograph*, Collins: London, 1949.

Bisht, N. P. S.; Singh, R.; Bansal, R. K.; Singh, P. Isolation of n-Triacontane, aliphatic alcohols and sitosterols from *Convolvulus Microphyllus* Sieb. *Z. Naturforsch.* **1978,** *33b*, 249.

Blanche, C. Delphinium L. Subgen. Delphinium: origin and evolutionary trends. *Collect.Bot.* **1990,** *19*, 75-95.

Breitmaier, E.; Voelter, W.*Carbon-13 NMR Spectroscopy High-Resolution Methods and Applications in Organic Chemistry and Biochemistry.* Weinhiem: New York, 1987; 3, 379-99.

Busvine, J. R. The comparative toxicity of various contact insecticides to the louse (*Pediculus humanus* L.) and the bed-bug (*Cimex lectularius* L.). *Ann. Applied Bio.* **1946,** *33*, 271-9.

Carpenter, W.J.; Boucher, J. F. Temperature requirements for the storage and germination of Delphinium x cultorum seed. *HortSci.* **1992,** *27*, 989-92.

Chisholm, M.J.; Hopkins, C.Y. Composition of Delphinium seed oil. *Can. J. Chem.* **1956,** *34*, 459-64.

Chopra, R. N.; Chopra, I. C.; Varma, B.S.; *Supplement to Glossary of Indian Medicinal Plants*, Council of Scientific and Industrial Research: New Dehli, India, 1969; xii.

Chopra, R. N.; Nayar, S. L.; Chopra, I. C.; *Glossary of Indian Medicinal Plants,* Council of Scientific and Industrial Research: New Dehli, India, 1956; xx.

Clark, D. H.; Ralphs, M. H.; Lamb, R. C. Total alkaloid determination in larkspur and lupine with near infrared reflectance spectroscopy. *Agron. J.*, **1987**, *79*, 481-5.

Coates, P.A.; Blagbrough, I.S.; Rowan, M.G.; Potter, B.V.L.; Pearson, D.P.J.; Lewis, T. Rapid and efficient entry to substituted 2-succinimidobenzoate-3 azabicyclo[3.3.1] nonanes: AE-bicyclic analogues of methyllycaconitine. *Tetrahedron Lett.* **1994,** *35*, 8709-12.

Cookson, R. C.; Tevett, M. E. The functional groups of delpheline. *Chem. Ind.* **1954,** 1324-5.

Costa de Pasquale, R.; Controneo, A.; Lauk, L.; Dugo, G. Composition and biological activity of the fatty seed oil of *Delphinium staphisagria*. Note I. *Int. J. Crude Drug Res.* **1985,** *23*, 5-11.

Crawford, A.C. Larkspur as Poisonous Plants. *USDA Bureau of Plant Industry,* Bull. III, 1907, 5-12.

Cronin, E. H.; Nielsen, D. B. Larkspurs a deadly beauty. *Utah Science*. **1990,** 8-9.

Dabi, E.; Hethelyi, E.; Zambo, I.; Tetenyi, P.; Simonidesz, V. Unsaturated fatty acid from seed oil of *Consolida regalis*. *Phytochemistry*. **1986,** *25*,1221-2.

Dai, B.; Qiu, C. Study on chemical constituents of essential oil of Huiluye Danggui (*Angelica glauca* Edgew.).*Zhong-caoyao*. **1996,** *27*, 77-8.Chem. Abstr. **1996,** *125*, 67124m.

Davidson, W. M. Insecticidal tests with oil and alkaloids of larkspur (*Delphinium Consolida*) and stavescare (*Delphinium Staphisagria*). *J. Econ. Ent.* **1924,** *22*, 226-34.

De la Fuente, G.; Gavin, J. A.; Reina, M.; Acosta, R. D. The structures of Cardionine and 11-acetylcardionine, new C_{20} diterpenoid alkaloids from the selective INAPT NMR techniques. *J. Org. Chem.* **1990,** *55*, 342.

De La Fuente, G., S. E.; Reina M. Some phytochemical studies of the genera Aconitum, L. and Consolida (D.C) S.F. Gray. *Collect. Bot.***1990,** *19*, 129-40.

Dekanosidze, G. E.; Arazashvili, A. I.; Turabelidze, D. G.; Kemertelidze, E. P. D-Mannitol from Peucedanum and Delphinium. *Khim. Prir. Soedin.***1974,***10,* 79-80. Chem Abstr. **1974,** *80,* 121237t.

Delgado, G.; Reza-Garduno, R. G.; Rios, M. Y.; Del Rio, F. Phthalides and monoterpenes of the hexane extract of the roots of *Ligusticum porteri. Planta Med.* **1992,** *58,* 570-1.Chem. Abstr. **1993,** *118,* 77131f.

Dong, Y.; Wang, R.; Chen, Y. Studies on the chemical constituents of *Delphinium iliense* Huth. *Gaodeng Xuexiao Huaxue Xuebao.* **1991,** *12,* 1490-2. Chem. Abstr. **1992,** *117,*167723j.

Dowdeswell, T. Dowdeswell's Delphiniums. [Online] Available http://www.delphinium.co.nz/, Jan. 1998.

Dozortseva, P.M. Elatin *Med. Prom.* **1956,** *10,* 31-32.Chem. Abstr. **1957,** *51,* 5367f.

Dwyer, J.; Rattray, D. *Magic and Medicine of Plants,* 1; Reader's Digest General Books: New York, 1986; 232.

Efros, R. S. Finding alkaloid bearing folk remedy plants in the Transbaikal region. *Farmatsiya.***1946,***9,* 22-3. Chem Abstr. **1947,** *41,* 7673.

Egger, K.; Keil, M. Flavonol glycosides in Ranunculaceae, distribution of the glycoside types. *Ber. Deut. Botan. Ges.***1965,** *78,* 418-22.Chem. Abstr.**1966,** *65,* 1034b.

Elliger, C. A. Deoxygenation of aldehydes and ketones with sodium cyanoborohydrate. *Synth. Comm.* **1985,** *14,* 1315-24.

El-Maraghy, N. N.; Sarhan, R. Effect of delphinine alkaloid isolated from *Delphinium ajacis* on liver and kidney functions of adult male rats. *Zagazig J. Pharm. Sci.* **1995,** *4,* 109-115.Chem. Abstr. **1996,***125,* 160812u.

Everett, T.H. *The New York Botanic Garden Illustrated Encyclopedia of Horticulture.* 1981, Garland Publishing, Inc.: New York, 1029-1034.

Fehr, D. Study of the aroma substances of celery (*Apium graveolens* L.) *Pharmazie* **1979,** *34,* 658-62.Chem. Abstr. **1980,** *92,* 213657f.

Feofiliaktov, V. V.; Alekseeva, L. D. Composition of alkaloids of *Delphinium elatum* L. *J. Gen. Chem. USSR.* **1954,** *24,* 747-51.

Figueiredo, M. R.; Gottlieb, O. R.; Kaplan, M. A. C. Evolution of diterpene alkaloids. *An. Acad. Bras. Cienc.* **1995,** *67,* 425-9.

Galfayan, G. T. A new indicator for alkalimetry and acidimetry. *J. Applied Chem.* **1938,** *11*, 110-2. Chem. Abstr. **1938,** *32*, 4459.

Gardner, D. R.; Manners, G. D.; Ralphs, M. H.; Pfister, J. A. Quantitative analysis of norditerpenoid alkaloids in larkspur (*Delphinium spp.*) Fourier transform infrared spectroscopy. *Phytochemical analysis*, **1997,***8*, 55-62.

Gatty-Kostyal, M.; Stawowczyk, A. The content of active substances in the Tatra larkspur (*Delphinium oxysepalum*) and their toxicity. *Diss. Pharm.* **1956,** *8*, 257-69; Chem Abstr. **1956**, *51*, 10744d.

Genders, R. *Delphiniums*, J. Gifford: London, 1963.

Gershenzon, J. Introduction to Plant Secondary metabolism. Seminar, Michigan State University. 1996, unpublished 1-21.

Gijbels, M. J. M.; Bos, R.; Scheffer, J. J.; Svendsen, A. B. Pthalides in roots of *Opopanaax chironium. Planta Med.* **1983**, *41*, 3-6; Chem Abstr. **1983,** *98*, 157869m.

Gijbels, M. J. M.; Fischer, F. C., Scheffer, J. J. C. Phthalides in roots of *Apium graveolens*, *A. graveolens* var. rapaceum, *Bifora testiculata* and *Petroselinum crispum* var. tuberosum *Fitoterapia* **1985,** *56*, 17-23. Chem. Abstr. **1985,** *103*, 138590u.

Gijbels, M. J. M.; Scheffer, J.J.C.; Svendsen, A. B. Phthalides in the essential oil from roots of *Levisticum officinale. Planta Med.* **1982,** *44*, 207-211.

Gijbels, M. J.; Scheffer, J. J. C.; Svendsen, A. B. Z-butylidenephthalide in the essential oil from roots of *Levisticum officinale. Planta Med.* **1980**, 41-7. Chem. Abstr. **1981,** *94*, 127132r.

Goodson, J. A. The alkaloids of the seeds of *Delphinium elatum*, L. *J. Chem. Soc.***1943,** 139-41.

Gross, D.; Schutte, H. R.; Schreiber, K. Isoprenoid Alkaloids. In: *Biochemistry of Alkaloids.* Mothes, K; Schutte, H. R.; Luckner, eds., M. VEB Deutscher Verlag der Wissenschaften: Berlin/DDR, 1985; 361-362.

Hardick, D. J.; Blagbrough. I. S.; Wonnacott, S.; Potter, B. V.L. Regioselective anthranoylation of demethylated aconitine: novel analogues of aconitine, inuline, and methyllycaconitine. *Tetrahedron Lett.* **1994,** *35*, 3371-4.

Hardick, D. J.; Blagbrough, I.S.; Cooper, G.; Potter, B. V. L., Critchley, T.; Wonnacott, S. Nudicauline and elatine as potent norditerpenoid ligands at rat neuronal α-bungarotoxin binding sites: importance of the 2-(methylsuccinimido)benzoyl moiety for neuronal nioctinic acetylcholine receptor binding. *J. Med. Chem.* **1996,** *39*, 4860-4866.

Hasan, S. Q.; Osman, S. M. Studies on indigenous seed oils. *J. Oil Technol. Assoc.* India (Bombay). **1993,** *25*, 47-9.

Hashem, F. M.; Abdel-Wabab, S. M.; Ahmed, M. S.; Seida, A. A. Quantitative estimation of the alkaloids in the different organs of *Delphinium ajacis* L. and *D. belladonna* Kelw. grown in Egypt. *Bull. Fac. Pharm.*, Cairo University, **1974**, *13*, 217-25.

Hashem, F. M.; Abdel-Wabab, S. M.; Salah, A. M.; Seida, A. A. Phytochemical screening and isolation of the main active constituents of *Delphinium ajacis* L. and *D. belladonna* Kelw. growing in Egypt. *Bull. Fac. Pharm.*, Cairo University, **1974,** 13, 205-15.

Hassan, M. R.; Khattab, M. Effect of nitrogen level on the vegetative growth and flower production of *Delphinium grandiflorum* L. plants. Alexandria J. Ag. Res. **1987,** 32, 265-71.

Herbert, E. J.; Kirby, G. W. Mevalonic acid and delpheline biosynthesis. *Tetrahedron Let.* **1963**, *23*, 1505-6.

Holland, H. L.; Diakow, P. R. P.; Taylor, G. J. ^{13}C nuclear magnetic resonance spectra of some C-19-hydroxy, C-5,6 epoxy, C-24 ethyl, and C-19-norsteroids. *Can. J. Chem.* **1978,** *56*, 3121-3127.

Huang, Y.; Pu, F. The analysis of the chemical component of essential oils from three Ligusticum species. *Yaowu Fenxi Zazhi.* **1989**, *9*, 147-51; Chem. Abstr. **1990**, *112*, 33325u.

Ikan, R. *Natural Products*, Academic Press, Inc.: San Diego, CA, 1991.

Jacyno, J. M.; Harwood, J. S.; Lin, N. H.; Campbell, J. E.; Sullivan, J. P.; Holladay, M. W. Lycaconitine revisited: partial synthesis and neuronal nicotinic acetylcholine receptor affinities. *J. Nat. Prod.* **1996,** *59*, 707-9.

Jain, S. K.; DeFilipps, R. A. *Medicinal Plants of India,* Reference Publications: Algonac, Michigan 1991; 2, 501.

James, L. F.; Johnson, A. E. Some major plant toxicities of the western United States. *J. Range Manage.* **1976,** *29*, 356-363.

Jennings, K. R. Methyllycaconitine. A potent natural insecticide active on the cholinergic receptor. *ACS Symp. Series* **1987**, *356*, 274-82.

Jennings, K. R.; Brown, D. G.; Wright, D. P. Jr. Methyllycaconitine, a naturally occurring insecticide with a high affinity for the insect cholinergic receptor. *Experentia*, **1986,** *42*, 611-3.

Khan, A. B.; Taiyab, H. M. Effect of "jadwar" *Delphinium denudatum* Wall on experimental liver damage in rats. *Indian J. Pharm Sci.* **1981**, *43*, 120-2. Chem Abst. **1982**, *96*, 156830p.

Kariyone, T.; Kotani, M. Constituents of the fruits of *Ligusticum acutilobum. J. Pharm, Soc. Jpn.* **1937,** *57*, 183-4.Chem. Abstr. **1937,** *32*, 33617.

Keeler, R. F. Toxins and teratogens of higher plants. *Lloydia*, **1975,** *38,* 56-86.

Keith, L.H.; Pelletier, S.W. The C_{19}-Diterpene Alkaloids. In: *Chemistry of the Alkaloids*. Pelletier, S. W., ed., Van Norstrand Reinhold: NewYork.1970; 549-90.

Kim, H. S.; Chi, H. J. Studies on essential oils of plants of Angelica genus in Korea (II). Essential oils of the root of *Angelica tenuissima. Saengyak Hakhoechi* **1989,** *20*, 13-20. Chem. Abstr. **1989,***111*,140298p.

Kimura, M.; Harada, M.; Sekida, S.; Yuda, M. Pharmaceuticals containing butylidenephthalide senkyunolide. Jpn. Kokai Tokkyo Koho JP 01,207,233 1989, JP 01,207,233. Chem. Abstr. **1989,** *111*, 239574n.

Kingston, E. E.; Eichhozler, J. V.; Lyndon, P.; MacLeod, J. K. An unexpected γ-hydrogen rearrangement in the mass spectra of di-ortho-substituted alkylbenzenes. *Organic Mass Spec.* **1988**, *23*, 42-7.

Ko, W. C. A newly isolated antispasmodic-butylidenephthalide. *Jpn. J. Pharmacol.* **1980,** *30*, 85-91; Chem. Abstr. **1980,** *92*, 208863r.

Ko, W. C.; Lin, S. C.; Ye, C. Y.; Wang, Y. T. Alkylpthalides isolated from *Ligusticum wallichi* Franch and their in vitro inhibitory effect on rat uterine contraction induced by prostiglandin $F_{2\alpha}$. *T'ai-wan I Hsueh Hui Tsa Chih.* **1977**, *76*, 669-77; Chem Abstr. **1977**, *88*, 130721p.

Kondo, T.; Suzuki, K.; Yoshida, K.; Oki, K.; Ueda, M.; Isobe, M.; Goto, T. Structure of cyanodelphin, a tetra-*p*-hydroxybenzoated anthocyanin from blue flower of *Delphinium hybridum. Tetrahedron Lett.* **1991,** *32*, 6375-8.

Kondo, T.; Oki, K.; Yoshida, K.; Goto, T. Structure of viodelphin, an anthocyanin from violet flower of *Delphinium hybridum. Chem. Lett.***1990**, 137-8.

Krishnamoorthy, V.; Seshadri, T. R. Survey of anthocyanins from Indian sources III. *J. Sci. Ind. Res.* 1962, 21B 591-3. Chem. Abstr. **1962,** 58, 8235e.

Kuder, R. C.; Larkspurs, Delphiniums, and chemistry. *J. Chem. Educ.* **1947,** 418-22.

Kukel,C.F.; Jennings, K.R. Delphinium alkaloids as inhibitors of α-bungarotoxin binding to rat and insect neural membranes. *Can. J. Phys. Pharm.* **1994**, *72*,104-7.

Kuzovkov, A. D. Aconite alkaloids VIII. Identity of delphelatine with eldeline *Zhur. Obshchei Khim.* **1956**, *26*, 2063-6. Chem. Abstr. **1956**, *51*, 5099h.

Lee, C. S.; Hansberry, R. Toxicity studies of some Chinese plants. *J. Econ. Ent.* **1943,** *36*, 915-21.

Li, S.; Wang, Z.; Fang, X.; Li, Y. Synthesis of (Z)-Ligustilide. *Syn. Comm.* **1993**, *23*, 2909-13.

Lomadze, I. A.; Arazashvili, A. I.; Turabelidze, D. G. Polarographic determination of some coumarins in plants. *Soobshchc. Akad. Nauk Gruz.* SSR **1976**, *81*, 93-6. Chem. Abstr. **1976,** *84*, 176082b.

Lomadze, I. A.; Dzhgamadze, n. N.; Moniava, I. I.; Bostoganashvili, V. S. Quantitative determination of some flavonoids in plants. *Soobshch. Akad. Nauk Gruz. SSR* **1976**, *84*, 369-71. Chem. Abstr. **1977**, *86*, 86157p.

Luo, Y.; Pan, J.; Ding, K.; Yan, Z. Anticonvulsive constituents in the essential oil of Chaxiong (*Ligusticum sinense* Oliv cv. Chaxiong).*Zhongcaoyao.***1996,** *27*, 456-7.Chem. Abstr. **1996,** *125*, 309171u.

Lushchev'kaya, G. Dyestuff-yielding plants in the Ukrainian S. S. R. Mem. *Inst. Chem. Tech. Acad. Sci., Ukrain S. S. R.* **1937**, 13-9.Chem. Abstr **1937**, *31*, 68837.

Mann, J. *Secondary Metabolism*, 2; Clarendon Press: Oxford, 1987.

Manners, G. D.; Panter, K. E.; Ralphs, M. H.; Pfister, J. A.; Olsen, J. D.; James, L. F. Toxicity and chemical phenology of norditerpenoid alkaloids in the tall larkspurs (Delphinium species). *J. Ag. Food Chem.* **1993**, *41*, 96-100.

Manners, G. D.; Panter, K. E.; Ralphs, M. H.; Pfister, J. A.; Olsen, J. D.; James, L. F. The toxic evaluation of norditerpenoid alkaloids in three tall larkspur (Delphinium) Species. In: *Plant-associated Toxins Agricultural, Phytochemical, and Ecological Aspects.* Colegate, S. M., Dorling, P. R., eds., CAB International: Wallingford, UK 1994, 178-83.

Manners, G. D.; Panter, K. P.; Pelletier, S. W.; Structure-activity relationship of norditerpenoid alkaloids occurring in toxic larkspur (Delphinium) species. *J. Nat. Prod.* **1995,** *58*, 863-9.

Manners, G.D.; Pfister, J.A.; Ralphs, M.H.; Panter, K.E.; Olsen, J.D. Larkspur chemistry: toxic alkaloids in tall larkspurs. *J. Range Management.* **1991,** *45*, 63-7.

Marsh, C.D., Clawson, A.B., Marsh, H. Larkspur or poison weed. *USDA Farmer's Bulletin* **1940**, *988*, 1-12.

The Marshall Cavendish Encyclopedia of Gardening. Garrod and Lofthouse International Limited: Crawley, England. 1970; 449-454.

Martin, F. N. Jr. M. D. Poisonous cultivated plants. *Bull. Tulane University of Louisiana Med. Fac.* **1953,** *12*,159-172.

Mats, M.N. New curare-like agents of plant origin. *Rast. Resur.* **1972,** *8*, 249-52.Chem. Abstr.**1972**, *77*, 79509u.

Mel'nichuk, G. G. *Consolida regalis* flavonoids. *Ukr. Bot. Zh.* **1971**, *28*, 525-7. Chem. Abstr. **1972,** *76*, 32232p.

Mericli, A. H.; Mericli, F.; Ulubelen, A.; Ilarslan, R. Aromatic compounds from *Delphinium venulosum. Phytochemistry*, **1991**, *30*, 4195-6.

Mericli, F.; Mericli, A. H.; Ulubelen, A.; Ilarslan, R. Nonalkaloidal constituents of *Delphinium peregrinum. Fitoterapia* **1991**, *62*, 460.Chem. Abstr.**1992**,*117*, 4210a.

Mericli, F.; Mericli, A. H.; Becker, H.; Ulubelen, A.; A benzoxepine derivative from Delphinium formosum. *Phytochemistry*, **1996**, *42*, 1257-8.

Missouri Botanical Garden. [Online] Available http://www.mobot.org/MOBOT/TROPICS/China/ Delphinium.html, 1997.

Nation, P. N.; Benn, M. H.; Roth, S. H.; Wilkens, J. L. Clinical signs and studies of the site of action of purified larkspur alkaloid, methyllycaconitine, administered parenterally to calves. *Can. Vet. J.* **1982**, *23*, 264-6.

National Institutes of Standards and Technology. NIST Chemistry Webbook.[Online] Available. http://webbook.nist.gov/chemistry, 1998.

Ogawa, Y.; Maruno, M.; Wakamatsu, T. Silver catalyzed cyclization of alkonoic acids: efficient synthesis of 3-alklidenephthalides, γ-alkylidenebutenolides, and g-alklidenebutyrolactones. *Heterocycles.* **1995**, *41*, 2587-99.

Olsen, J. D. Relationship of relative total alkaloid concentration and toxicity of duncecap larkspur during growth. *J. Range Management*, **1983,** *36*, 550-2.

Olsen, J. D. Rat bioassay for estimating toxicity of plant material from larkspur (*Delphinium sp.*). *Am. J. Vet. Res.* **1977,** *38*, 277-9.

Olsen, J. D. Tall larkspur poisoning in cattle and sheep. *J. Am. Vet. Assoc.* **1978,** *173*, 762-5.

Olsen, J. D. Toxicity of extract from three larkspur species (*Delphinium barbeyi, D. glaucescens, D. occidentale*) measured by rat bioassay. *J. Range Management.* **1977,** *30*, 237-8.

Olsen, J. D.; Ralphs, M. H.; Lane, M. A. Aversion to eating poisonous larkspur plants induced in cattle by intraruminal infusion with lithium chloride. *J. Animal Sci.* **1989,** 67,1980-5.

Olsen, J. D.; Sisson, D. V. Toxicity of extracts of tall larkspur (*Delphinium barbeyi*) in mice, hamsters, rats and sheep. *Toxicol. Lett.* **1991,** 56, 33-41.

Olsen, J. D.; Manners, G. D. Toxicology of diterpenoid alkaloids in rangeland larkspur (*Delphinium spp.*).*Toxicants of Plant Origin,* Cheeke, Peter R., ed.;CRC Press, Inc.: Boca Raton, FL, 1989; 292; 308-9; 313-21.

Olsen, J. D.; Manners, G. D.; Pelletier, S.W. Poisonous properties of larkspur (*Delphinium spp.*) *Coll. Botan.***1990,** *19*,141-51.

Ovesov, K. Participation of acetate-2-^{14}C in the biosynthesis of *Delphinium elatum* alkaloids. *Izv. Akad. Nauk Turkm. SSR., Ser. Biol. Nauk* **1966,** 56-60. Chem. Abstr. **1967,** *66*, 102541g.

Ovezov, K. Dynamics of accumulation and kinetics of metabolism of *Delphinium elatum. Izv. Akad. Nauk Turkm. SSr, Ser. Biol. Nauk* **1965,** 66-9.Chem. Abstr. **1966,** *64*, 3971a.

Ovezov, K. Methanol-^{14}C as a precursor of diterpene alkaloids. *Izv. Akad. Nauk Turkm. SSR, Ser. Biol. Nauk* **1967**,12-18. Chem. Abstr. **1967,** *67*, 61665a.

Park, J. C.; Desai, H. K.; Pelletier, S. W. Two new norditerpenoid alkaloids from *Delphinium elatum* var. "Black Night." *J. Nat. Prod.* **1995,** *38*, 291-5.

Pelletier, S. W.; Mody, N. V. Developments in the chemistry of diterpenoid alkaloids. *J. Nat. Prod.* **1980,** 43, 41-71.

Pelletier, S. W.; Ross, S. A.; Desai, H. K. A norditerpenoid alkaloid from *Delphinium elatum. Phytochemistry,* **1990,** 29, 2381-3.

Pelletier, S. W.; Mody, N. V. The Chemistry of C_{20}-Diterpenoid Alkaloids. In: *The Alkaloids Chemistry and Physiology.* Manske, R. H. F., Holmes, H. L., eds. Academic Press: New York, 1981; 18, 100-211.

Pelletier, S. W.; Page, S. W. Diterpenoid Alkaloids. *Natural Product Report.* **1986,** *3,* 281-306.

Pelletier, S. W.; Page, S. W. Diterpenoid Alkaloids. *Natural Product Reports.* **1978,** *8,* 219-29.

Pelletier, S.W.; Sawhney, R. S.; Desai, H. K.; Mody, N. V. The diterpenoid alkaloids of *Consolida ambigua. J. Nat. Prod.* **1980,** *43,* 395-406.

Pelletier, S.W.; Ross, S.A.; Kulanthaivel, P. New Alkaloids from *Delphinium elatum* L. *Tetrahedron.* **1989,** *45,*1887-92.

Pelletier, S.W. The nature and definition of an alkaloid. *Alkaloids Chemical and Biological Perspectives*, John Wiley & Sons: New York, 1983,1-32.

Perkin, A. G.; Pilgrim, J. A. The colouring matters of the Indian dye stuff Asbarg, *Delphinium zalil. J. Am. Chem. Soc.* **1898,** *83,* 267-74.

Petrischeva, P. A. Plant insecticides. *Amer. Rev. Soviet Med.* **1945,** *2,* 471-3.

Phillips, G. A. *Delphiniums Their History and Cultivation,* 1; The Macmillan Company: New York, 1933; 17-45.

Puttock, A. G.; *Delphiniums and Campanulas*, J. Gifford: London, 1973.

Qureshi, A. W.; Ahsan, A. M. Studies on *Delphinium denudatum.* Examination of the petroleum ether extract of the root. *Pakistan J. Sci. Ind. Res.* **1965,** *8,* 173-5.

Rabinovich, M. S. Aconite alkaloids. V. Eldeline and eldelidine. *Zhur. Obshchei Khim.* **1952,** 1702-10. Chem Abstr. **1952,** *47,* 9336e.

Rabinovich, M. S. Aconite alkaloids. VI. Elatine. *Zhur. Obshchei Khim.* **1954,** *24,* 2242-5. Chem. Abstr. **1956,** 50, 378h.

Ralphs, M. H.; Olsen, J. D.; Pfister, J. A.; Manners, G. D. Plant-animal interactions in larkspur poisoning in cattle. *J. Animal Sci.* **1988,** *66,* 2334-43.

Ralphs, M. H.; Pfister, J. A.; Olsen, J. D.; Manners, G. D.; Nielsen, D. B. Reducing larkspur poisoning in cattle on mountain ranges. *Utah Science* **1989,** *4,* 110-5.

Ringer, A. Measurements of absorption in solutions of red and yellow dyes. III. Plant dyes. *Pharmazie* **1950,** *5,* 385-91. Chem. Abstr. **1951,** *45,* 2632b.

Ringer, A. Measurements of absorption in solutions of green, blue, and violet dyes. Pharmazie **1951**, *6*, 44-8,103-7, 156-61.Chem. Abstr. **1951**, *45*, 10053h.

Ross, S.A.; Joshi, B.S.; Desai, H.K.; Pelletier, S. W.; Newton, M. G.; Zhang, X.; Snyder, J.K. Delatisine, a novel alkaloid from *Delphinium elatum* L. *Tetrahedron* **1991**, 47, 9585-98.

Seymour, E. L. D., ed. *The Wise Garden Encyclopedia.* Harper Collins Publishers: New York 1970; 322-4.

Shamma, M.;Chinnasamy, P.; Miana, G. A.; Khan, A.; Bashir, M.; Salazar, M.; Patil, P.; Beal, J.L. The alkaloids of *Delphinium cashmirianum. J. Nat. Prod.* **1979**, *42*, 615-23.

Shoolery, J. N.; Rogers, M. T. Nuclear Magnetic Resonance Spectra of Steriods. *J. Am. Chem. Soc.* **1958,** 80, 5121-5135.

Siddiqui, R.; Shahana, K.; Shekheani, S. Antimicrobial and immunomodulating properties of *Delphinium denudatum* extracts. *Abstracts of the Annual Meeting*, The American Society of Microbiology. 1990, 384.

Slomp, G.; MacKeller, F. A. Nuclear Magnetic Resonance Studies on Some Hydrocarbon Side Chains of Steriods. *J. Am. Chem. Soc.* **1962,** 84, 204-206.

Smith, A. E.; Secoy, D. M., Plants Used for agricultural pest control in Western Europe before 1850. *Chem. Ind.* **1981**, *10*, 12-7.

Stern, E. S. The Aconitum and Delphinium Alkaloids. In: *The Alkaloids Chemistry and Physiology* Manske, R. H. F. ; Holmes, H. L., eds.1954, 4, 275-333.

Strzelecka, H. Sterile cultures of *Delphinium elatum* roots. *Abh. Deut. Akad. Wiss. Berlin, Kl. Chem. Geol. Biol.* **1966**, 603-5; Chem. Abstr. **1967,** *67*, 8644s.

Strzelecka, H. Diterpene alkaloids of *Delphinium elatum* I. Chromatographic studies *Diss. Pharm. Pharmacol.* **1967**, *19*,81-4; Chem. Abstr. **1967,** *67*, 36360q.

Stzelecka, H. Diterpene alkaloids of *Delphinium elatum.* II. Studies on cultures of isolated roots. **1967**, *19*, 85-9;Chem. Abstr. **1967**, *67*, 41011f.

Strzelecka, H. Alkaloids of *Delphinium elatum.* III. Quantitative and qualitative investigations of alkaloid complex during growth. *Diss. Pharm. Pharamacol.* **1968,** *20*,319-24; Chem. Abstr. **1968,** *68*, 17541v.

Strzelecka, H.; Wojciak, Z. Insecticide for head lice. *Przeglad Epidemiol.* **1957,** *11*, 183-8. Chem. Abstr. **1957**, *52*, 2324d.

Strzelecka, H.; Dobrowolska, B. Flavonoid compounds in *Delphinium grandiflorum*. *Acta. Pol. Pharm.* **1976,** *33*, 135. Chem. Abstr. **1976,** *85*, 189225w.

Taiz, L.; Zeiger, E. *Plant Physiology*.The Benjamin/Cummings Publishing Company, Inc.: Redwood, CA 1991, 318-345.

Takano, I.; Yasuda, I.; Takahashi, N.; Hamano, T.; Seto, T.; Akiyama, K. Analysis of essential oils in various species of Angelica root by capillary gas chromatography. *Kenkyu Nenpo-Tokyo-toritsu Eisei Kenkyusho* **1990,** *41*, 62-9. Chem. Abstr. **1991,** *114*, 171399p.

Taylor, N., ed. *Taylor's Encylopedia of Gardening*. 1961, Houghton Mifflin Company, Boston 314-7.

The Aldrich library of ^{13}C and ^{1}H FT NMR spectra Ed. 1 Milwaukee : Aldrich Chemical Co., 1993; 1084.

Time Life Virtual Garden [Online] Available www.pathfinder.com/vg/Timelife/CG/Books/EOI/Html/E01046X.html, 1997.

Trexler, H. L. Insect repellents or deterrents containing Staphisagria. U.S. 2,939,816 1960. Chem. Abstr. **1960,** *54*, 20064c.

Tulyaganov, N.; Dzhakhangivov, F. N.; Sadvitdinov, F. S.; Khamdamov, I. Pharmacology of some aconite alkaloids. *Farm. Rastit. Veshchestv.* **1976**, 76-91; Chem. Abstr. **1976**, *89*, 140208m.

Uozumi, Y.; Kawasaki, N.; Mori, E.; Mori, H.; Shibasaki, M. *J. Am. Chem. Soc.* **1989**, *111*, 3725-7.

Vaospv, Z. M.; Tel'nov, V. A.; Bessonova, I. A.; Gorelova, A. P. Alkaloids from the cultivated species *Aconitum tauricum* and *Delphinium elatum. Khim. Prir. Soedin* **1993**, 82. Chem. Abstr. **1995,** *123,* 222862e.

Wada, K.; Yamamoto, T.; Bando, H.; Kawahara, N. Four diterpenoid alkaloids from *Delphinium elatum. Phytochemistry*, **1992,** *31*, 2135-8.

Waller, G. R.; Mangiafico, S. Sterol and triterpenes of *Delphinium ajacis*; a comparison of whole nonsterile plants versus sterile callus tissue culture- a possible industrial use. *Symp. Pap.- IUPAC Int. Symp. Chem. Nat. Prod.*11th **1978,** *2,* 394-5.Chem Abstr.**1979,** 91, 207423q.

Waller, G.R.; Burstrom, H. Diterpenoid alkaloids as plant growth inhibitors. *Nature*, **1969**, *222*, 576-8.

Waller, G.R.; Mangiafico, S.; Foster, R.C.; Lawrence, R.H., Jr. Sterols of *Delphinium ajacis*; production and metabolic relationships in whole plants and callus tissue. *J. Med. Plant Res.* **1981,** *42*, 344-55.

Warnock, M. J.; Lui, Y.; Mabry, T. J. Acylated flavonols from *Delphinium carolinianum. Phytochemistry.* **1983,** *22*, 1834-5.

Watanabe, M.; Ijichi, S.; Morimoto, H.; Nogami, K.; Furukawa, S. Wittig-Horner reaction of dimethyl phthalide-3-phosphonates with aldehydes: synthesis of 3-ylidenephthalides and characterizations of their E-and Z-isomers. *Heterocycles.* **1993**, *36,* 553-63.

Waterman, P. G. ed., Alkaloids and sulfur compounds. *Methods in Plant Biochemistry-Alkaloids and Sulfur Compounds*. Dey, P. M., Harborne, J. B. eds., Academic Press, San Diego, CA, 1993, 8, 453, 457, 459.

Willstater, R.; Meig, W. *Liebig's Ann. Chem.*1915, 408, 61.In: Harbourne, J. B. Plant Polyphenols-XI. The Structure of Acylated Anthocyanins. *Phytochemistry,* **1964,** *3*, 151-60.

Wonnacott, S.; Albuquerque, E. X., Bertrand, D. Methyllycaconitine: A New Probe That Discriminates Between Nicotinic Acetylcholine Receptor Subclasses. In: *Methods in Neurosciences Receptors: molecular biology, receptor subclasses, localization, and ligand design.*12, Conn, P.M., ed.,1993, 263-275.

Wyman, D. *Wyman's Gardening Encyclopedia*. Macmillan, NY. 1977, 303-5.

Xi, Y.; Sun, M.; Li, W. Chemical constituents of Gaoben (*Ligusticum sinense*). *Zhongcaoyao* **1987**, *18*,54-5.Chem. Abstr. **1987,** *107*, 93547z.

Yamagishi, T.; Kaneshima, H.; Kinoshita, Y., Mori, M. Standardization of crude drugs produced in Hokkaido 5. Existence of ligustilide in *Angelica radix. Hokkaidoritsu Eisei Kenkyusho Ho.***1974**, *24*, 47-51.Chem. Abstr. **1975,** *83*, 84751v.

Yu, C. Preliminary study if the constituents of essential oil from *Liguisticum chuanxiong Hort. Beijing Yixueyuan Xuebao* **1983,** *15*, 217-19. Chem. Abstr. **1985,** *103,* 27055d.

Yum, l.; Wolf, K. M.; Chiappinelli, V.A. Nicotinic acetylecholine receptors in separate brain regions exhibit different affinities for methyllycaconitine. *Neuroscience* **1996,** *72*, 545-55.

Yunusov, M. S., Diterpenoid Alkaloids. *Nat. Prod. Rep.* **1991, 8**, 499-526.

Yunusov, M. S., Diterpenoid Alkaloids. *Nat. Prod. Rep.* **1993**, 10, 471-86.

Zolotnitskaya, S. Y. Melkumyan, I. S.; Voskanyan, V. E. The antimicrobial activity of some alkaloid-containing plants of the Armenian flora. *Izv. Akad. Nauk Arm. SSR, Biol. Nauki* **1962**, *15*, 33-8. Chem. Abstr. **1963,** 59, 1742a.