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To the Graduate Council:

I am submitting herewith a thesis written by J. Lena Horst Warren entitled "Effects of nitrogen and calcium fertilization on yield, flavonolignans, and anti-teratoma activity of greenhouse grown "Silybum marianum"." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant, Soil and Environmental Sciences.

Carl Sams, Major Professor

We have read this thesis and recommend its acceptance:

Dennis Deyton, Robert Trigiano

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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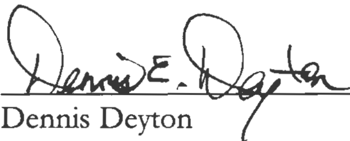

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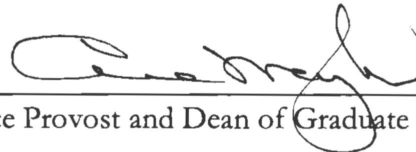

Vice Provost and Dean of Graduate Studies

Effects of nitrogen and calcium fertilization on yield, flavonolignans, and anti-teratoma activity of greenhouse grown *Silybum marianum*

A Thesis
Presented for the
Masters of Science Degree
The University of Tennessee, Knoxville

J. Lena Horst Warren
May 2003

AG-VET-MED.

Dedication

This thesis is dedicated to my husband, Lee Warren, who always has been there to pick me up when I didn't think I could go on. Thank you for your dedicated faithfulness and for teaching me so much about myself.

Acknowledgments

I wish to thank all of the wonderful people who helped me in completing my Master of Science in Plant and Soil Science. Thanks go to Heather Toler and Stephanie Harvey for all of your help with the dirty work – I'm not sure I could have done it all without you. I want to thank my committee members, Dr. Bob Trigiano and Dr. Dennis Deyton for your support and efforts to help me make my thesis something to be proud of. I especially want to thank my advisor, Dr. Carl Sams, for pushing me further than I really thought I could go, for putting up with my many temperaments, and for your support throughout this process. You really have helped to make me a better person – thank you.

Finally and foremost, I want to praise God, who has given me so much – especially the time and talents that he so abundantly has granted to me. All glory and honor to you, Lord, who has made all things (even me!) new.

Abstract

Silybum marianum (L.) Gaertn. plants were grown in a bag culture system in a greenhouse on the University of Tennessee campus during the Spring of 2001 and the Fall of 2001. For the Spring 2001 experiment, calcium concentration was altered to produce three nitrogen to calcium ratio treatments. For the Fall 2001 experiment, nitrogen concentration was altered to produce three nitrogen treatments. Plant samples were analyzed for anti-teratoma activity in a modified potato-disc bioassay and for chemical composition using capillary electrophoresis. Ca:N ratio treatments had no effect on seed yield (15 g/plant) or raw plant extract activity. However, nitrogen treatments had significant effects on both yield and raw plant extract actvity, with plants from the highest N treatment (152 mg/L) producing the greatest biomass (421g of pre-anthesis fresh weight per plant), seed yield (102 g/plant) and seed activity. Ethanolic extracts from roots, stems, and young leaves of pre-anthesis plants inhibited growth of teratomas in the potato disc bioassay. However, only the roots contained the flavonolignan compound silybin, which has been credited for the majority of the bioactivity of seed extracts. Seedlings of *S. marianum* had high levels of activity in the bioassay, matching that of seed extracts, yet only small concentrations of chemical constituents, which were not identified, were found in the extracts.

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Nomenclature

cm	centimeters
g	gram
g/m^2	grams per meter squared
kg/acre	kilograms per acre
KV	kilo-volts
M	molar
m	meters
μA	micro-amps
μm	micrometer
μmol	micro-moles
mg/L	milligrams per milliliter
mL	milliliter
mm	millimeters
mM	milli-molar
mmol	milli-moles
ms	milliseconds
N	normal
nm	nanometers

Abbreviations

B.C.E.	Before the Common Era
cAMP	cyclic adenosine monophosphate
CE	capillary electrophoresis
CFU	colony forming unit
CGE	capillary gel electrophoresis
CTAB	Cetyltrimethylammonium Bromide
CZE	capillary zone electorphoresis
DAD	diode array detector
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
DHAPS	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
DMSO	dimethyl sulfoxide
EOF	electro-osmotic flow
EPSP	5-enolpyruvylshikimate 3-phosphate
GC-FID	gas chromatograph with a flame ionization detector
HPLC	High performance liquid chromatography
I.D.	internal diameter
MECC	micellar electrokinetic capillary chromatography
MEKC	micellar electrokinetic chromatography
NADPH	nicotinamide adenosine dinucleotide phosphate
NB	nutrient broth
NBA	nutrient broth agar

PAL	L-phenylalanine ammonia lyase
PEP	phospoenolpyruvate
SDS	Sodium Dodecyl Sulfate
S3P	shikimate 3-phosphate
Syn	synonyms
UV	ultraviolet
VEGF	vascular endothelial growth factor

I.

Introduction

The use of plants for healing long predates our modern understanding of drug activity and biochemistry. Historically, plant derived medicines have been used to treat innumerable human ailments. Many civilizations have employed and perfected plant remedies in many forms including teas, tinctures, salves and compresses. For instance, the Ebers Papyrus, ca. 1550 B.C.E., describes many diseases that plagued the Egyptian people along with common remedies – many of which were plants. Indian culture has a rich heritage of herbal medicine. Ayruvedic medicine, the ancient system of medical treatment used by the Indian peoples to heal, originated around 2500 B.C.E., is still practiced today(Kapoor, 2001).

Natural plant remedies continue to be employed for various reasons. Many people choose to rely on herbal remedies as a result of religious preference or from a desire for more "natural" methods of healing. A renewed curiosity in plant medicines in the developed world coincides with a growing interest in holistic medicine (Phillipson 1994). People in countries too poor to provide western health care are often forced to use plant extracts to heal (Hersch-Martinez 1995). Various estimates indicate that 70 to 80% of the people who live in developing countries partly or fully rely on herbal remedies (Wijesekera 1991). Continued reliance on traditional herbal remedies, by preference or circumstance, has preserved the knowledge of the value of nearly 21,000 plant species that may have otherwise been lost (Harnischfeger 2000).

Plant extracts are still employed in western medicine when the chemicals they produce are not available synthetically. Some chemicals from plants have not been effectively synthesized. Vinblastine and vincristine, two alkaloids used in the treatment of childhood leukemia, are only available from natural extracts of *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle) (Buchanan et al. 2000). Various synthetic drugs such as chloroquinine and primaquine have become virtually ineffective at controlling strains of *Plasmodium*, the parasite that causes malaria (Yang et al. 1997). The natural chemical, quinine, is still effective, but is only obtained from *Cinchona officinalis* L. Other plants are useful because they produce complex base structures used for further synthesis. For example

Dioscorea floribunda C. Martius & Galeotti produces the steroid diosgenin, the starting point for the chemical synthesis of many oral contraceptives. Sometimes it is less expensive to produce a compound by natural plant synthesis rather than through chemical synthesis. A predominant plant source of analgesics is *Papaver somniferum* L. (Opium poppy), which produces three of the most common prescription painkillers: morphine, codeine, and papaverine more cheaply than they can be produced synthetically (Simpson and Ogorzaly 1995).

Since medicinal plants are the only source for many currently used drugs and many new drugs which are still being elucidated, there is great interest in the research of medicinal plants. There are three areas in which research has been focused: horticulture, pharmacognosy, and phytochemistry (Briskin 2000).

Horticultural research has been concerned with the cultivation of medicinal plants, specifically in optimizing production conditions to produce the highest quality product (Briskin 2000). Research into the cultivation of medicinal plants is critically needed. Despite the huge diversity of plants used for medicinal purposes, only 50 to 100 medicinal species are cultivated; the remainder are harvested from the wild (Harnischfeger 2000). Currently, many plants used in phytomedicinals are not amenable to agriculture because they either grow very slowly, have erratic yields or flowering times, or are simply not in sufficient demand to justify investment in cultivation (Harnischfeger 2000). Also, the volatility of the market concerns many potential growers. However, as the medicinal plant industry grows, demand and over-harvest of many species will increase (Leonhart et al. 2000). The collecting pressure on many wild populations has already caused numerous plant species to become endangered (Nickel and Sennhauser 2000). Examples of species that have become threatened in their native habitats due to exploitation include *Artemisia maritima* L. (Sea Wormwood), *Panax quinquefolius* L.(American Ginseng), *Cassia acutifolia* Del. (Senna), and *Dioscorea deltoidea* Wall. ex Griseb. (Yam) (Gupta 1991). Collecting from wild populations can involve additional complications, such as high costs due to difficulties in transportation and harvesting. Plants in the wild must be hand harvested and are often widely dispersed. Furthermore, wild collections may be substandard in quality and potentially hazardous due to mistaken plant identification or intentional fraud by collectors (Chopra et al. 1991).

Cultivated plants would probably cost less, increase purity, ease standardization, and produce higher yields (Nickel and Sennhauser 2000). *Panax ginseng* C.A. Mey (Asian ginseng), *Piper methysticum* G. Forster (Kava kava), and *Valeriana officinalis* L. (Valerian) have been successfully cultivated. However, there is very little information on production methods for most of the other medicinal species and no published information on the physiology of most medicinals (Gupta 1991). Even breeding of medicinal plants has been minimal (Tetenyi 1991). This makes production more challenging, but enhances research opportunities. Pharmacognostic research focuses on the bioactivity of medicinal plants, using assays to investigate the modes of action and target sites for active phytomedicinal compounds (Briskin 2000). Several criteria that define drug-like properties must be met for a plant extract to be considered medicinally valuable. The compounds must be able to produce the desired effect, be able to reach the target of their activity through biological barriers, be persistent enough to have the desired effect, and be safe (White 2000). Safe chemicals are specific enough so that once administered, they have the intended pharmacological action without significant side effects (White 2000). A major tool for the confirmation of the biological activity of a plant is the bioassay. There are basically two kinds of bioassays – in vivo and in vitro. In vitro methods are the fastest way to screen many different chemicals, but they only test the efficacy and specificity of compounds. In vivo methods can confirm the bioavailability of a given compound and the length of time that it is available in the body (White 2000); however, in vivo bioassays are very time consuming. Thus, due to the very slow rate of in vivo bioassays, in vitro methods must be used for primary screening. There are five criteria that help define a good bioassay. First, the bioassay must be relevant, so that the result of the screen accurately mimics what may happen when the compound tested is applied to the body. Second, it should be effective at eliminating inactive compounds, but should be accurate enough to not eliminate useful compounds. Thirdly, the assay should be fast and, fourthly, versatile so that many different kinds of chemical structures can be tested quickly, and fifthly, a good assay must be reproducible (White 2000). With the large number of screens that a chemical must go through, and the enormous number of chemicals to be assayed, the entire screening process can be very costly. DiMasi et al. (2003) estimated that the development of a new drug costs an average of US$ 802 million, with pre-clinical drug

discovery and screening making up US$ 335 million of that amount. To minimize cost, primary screens need to be inexpensive, fast, easy and have a high accuracy level to quickly eliminate nonactive compounds.

Phytochemical research attempts to structurally identify active compounds by finding plants with biological activity, isolating the active chemicals and then running detailed structural analysis. This knowledge is very important for isolating pure chemicals (Tian et al. 2002), for helping to determine evaluation methods for the quality of herbal extracts (Bergeron et al. 2000) and for testing potential synergistic effects of multiple chemicals found in the plant (Loew and Kaszkin 2002).

In looking for a medicinal plant that would be appropriate to study in production, we searched for the following characteristics: a relatively fast rate of growth, objective medical activity that could be evaluated by bioassay, and a substantive research base verifying the plant's activity. *Silybum marianum* (L.) Gaertn., also known as Milk thistle, met all these requirements.

This study was undertaken to (1) evaluate the effectiveness of producing *S. marianum* in hydroponic systems over multiple seasons of the year; (2) quantify how both seed and vegetative yields of *S. marianum* are affected by nitrogen and calcium concentration applied to the plant in hydroponic growing conditions; (3) examine how the active constituents of *S. marianum* are distributed throughout the plant and when they are produced; (4) discern how the active constituents of *S. marianum* are affected by nitrogen and calcium concentrations; and, (5) evaluate the effectiveness of the active compounds in *S. marianum* as anti-tumor agents.

II.

Literature Review

Production of Medicinal Plants

A significant portion of current medicinal plant research focuses on efficient, sustainable production. Research objectives include optimizing production either in field situations (Rai et al. 2001; Marthur et al. 2000; Pereira et al. 1998), in greenhouse cultivation (Leonhart et al. 2000; Minami et al. 1995), or in tissue culture (Luo et al. 2002; Zhao et al. 2001). Current research also addresses the issue of sustainable harvesting of wild plants since cultivation of certain species is unfeasible (Zschocke et al. 2000) and regular collection methods can have detrimental ecological effects (Schwartz et al. 2001).

Wild Harvest

The cultivation of some medicinally useful species may not be feasible for many reasons. Certain plants, such as *Taxus brevifolia* Nutt. (Pacific yew) and *Dracaena draco* L. (Dragon Tree), may grow extremely slowly and take many decades to reach a harvestable age (Wang et al. 2000; van Rosendaal et al. 2000; Benabid and Cuzin 1997). Also, some endemic species, such as *Ancistrocladus korupensis* D. Thomas & Gereau (a Liana species) , may be adapted to very specialized growing conditions that are difficult to reproduce in cultivation(Foster and Sork 1997). Thus, wild harvesting of some medicinals may be the only alternative. Unfortunately, harvesting of many of these wild species is destructive – bark, roots, and tubers are harvested, which kills the plants.

The combination of destructive harvesting and production limitations creates an unsustainable situation (Zschocke et al. 2000). Schwartz et al. (2001) examined the impact of destructive harvesting of *Pterocarpus angolensis* DC. (Blood wood or wild teak), a hardwood tree species in Tanzania. Based on population size, age, spacial distribution, rate of seed production and the growth rate, as well as herbivory and competition factors, they predicted that the long-term survivability of the species was unlikely if the rapid harvesting of the tree continued.

One solution for the sustainable harvesting of these medicinals involves collecting parts of the plant that are easily regenerated, such as leaves and herbaceous stems. Zschocke et al. (2000) evaluated the use of this technique the on following four threatened native South African species: *Eucomis autumnalis* (Mill.) Chitt. (Pineapple Lily), *Siphonochilus aethiopicus (Schweif.) BL Burt.* (Natal or Wild Ginger), *Ocotea bullata* (Burch.) Baill. (Black Stinkwood) and *Warburgia salutaris* (Bertol.f.) Chiov. (Pepperbark tree). Each of these plants are destructively harvested; *E. autumnalis* and *S. aethiopus* are tuberous plants in which the bulb and rhizome are collected. *Ocotea bullata* and *W. salutaris* are trees whose bark is harvested, often girdling the trees when overharvesting occurs. They found that in all cases, other parts of the plants, including leaves, had comparable or higher activity levels in an anti-bacterial assay than the traditionally harvested plant part. Their research demonstrated the value of using plant part substitution as a way of preserving wild, native populations while continuing to derive benefit from their medicinal content.

Field Cultivation

Ideally, cultivating medicinal plants provides the best situation for both the consumer and the producer. Unfortunately, there is still a major gap between the knowledge necessary to produce an excellent medicinal product and available knowledge of production methods. Research into the production of medicinal plants is a rapidly expanding field of study.

Aloe vera L. (*Aloe barbadensis* Miller) has been used for centuries as a topical healing treatment and continues to be used in many products ranging from hair treatments to sunburn gels. Aloin has been identified as the major active compound in the Aloe extract (Paez et al. 2000). To quantify how light intensity affects *A. vera* in production, Paez et al. (2000) examined the growth, soluble carbohydrates, and aloin concentrations of *A. vera* exposed to the following three light levels: full sunlight, 30% of full sun, or 10% of full sun. Plants exposed to full sunlight yielded the highest total plant dry mass, but sunlight variation did not affect the yields of aloin or other metabolites measured.

Hypericin, a naphthodianthrone, is the dominant medicinal chemical in St. John's wort (*Hypericin perforatum* L.) extract and is believed to have anti-depressant activity (Briskin 2000). Briskin and Gawienowski (2001) grew St. John's wort in a sand culture hydroponic system

and studied how light intensity and root nitrogen supply affected the hypericin concentrations in the leaf. Light intensity was regulated using artificial lighting and was varied from 106 to 402 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Nitrogen levels were varied by diluting the nitrogen level of the stock solution (Gentry and Bellow 1993), which contained 4.42 mM NH_4NO_3 to make treatments of 100, 5, 1, and 0.03% of the original nitrogen concentration. Higher light intensity linearly increased the number of hypericin glands per leaf, where hypericin and other naphthodianthrones are stored (Briskin 2000). Plants exposed to the 5% nitrogen treatment had a significant increase in hypericin glands per leaf, but further decreases in nitrogen had no significant effect.

Several *Echinacea spp.* (Purple cone flower) are believed to contain chemicals that work as immune system boosters (Briskin 2000; Classen et al. 2000; Cheminat et al. 1988) and these species are consumed world wide as a treatment for various diseases, including colds (Briskin 2000) . Hundreds of hectares of *E. purpurea* (L.) Moench. are produced in Europe and the United States (Israelsen 1993). Shalaby et al. (1997) investigated potential field production of *E. purpurea* in Egypt by examining the effects of nitrogen to potassium ratios and plant density per row on yield and growth. The addition of nitrogen improved both yield and growth; however, the addition of small amounts of potassium improved yields more than nitrogen alone. An application of 150 kg of N with 50 kg of K per acre achieved the best yields in their experiment. Plants spaced at a 60 cm between plants, the largest spacing, produced the greatest yields per plant, but the closest spacing of 20 cm between plants produced the highest biomass per area. They did not examine the effects of cultivation on the medicinal quality of *E. purpurea.*

Alkylamides are believed to be one of the most medically active groups of chemicals found in *Echinacea spp.* El-Gengaihi et al. (1998) examined how the alkylamide concentrations varied in various parts of *E. purpurea* as influenced by plant ontogeny and fertilizer regimes. Plants were field grown and plant alkylamide content was determined at the seedling, vegetative, flowering and fruiting growth stages, which were 30, 64, 103, and 195 days, respectively, after transplanting. Ammonium sulfate at rates of 1, 247, 370, or 494 kg/ha and potassium sulfate at rates of 0, 124, or 370 kg/ha were applied in three doses at

two week intervals one month after transplanting. Alkylamide levels dropped in the vegetative portion of the plant as the plants matured, but the concentrations of alkylamides increased in the roots over that same time, reaching maximum concentrations at fruiting. Fertilizer levels affected the total levels of alkylamides, the highest yields obtained with high nitrogen and low potassium levels. Neither fertilizer treatment nor ontogeny effected the alkamide composition.

Mentha arvensis L.f. piperascens Malinv. ex Holmes (cornmint) and *Pelargonium capitatum* (L.) L'Her. ex Ait. (rose-scented geranium) are economically important aromatic species whose essential oils are used in perfumes and food products, but are also important as medicinal plants (Rajeswara Rao 2002). Cornmint is used for soothing skin irritations, pain relief, upset stomach, and several other ailments. Rose-scented geranium is used to relieve menopausal symptoms, skin disorders and tension. Both of these plants are particularly prized in India, though the cultivation of each is insufficient to meet the demands of the population. Rajeswara Rao (2002) examined the effects of spacing of rose-scented geranium and intercropping with cornmint on the essential oil composition of both plants. Row spacing of 60 x 30 cm yielded the highest biomass and essential oil content from the rose-scented geranium. Intercropping with cornmint did not reduce the geranium's overall yield, while allowing a bonus crop to be produced. Essential oils were not altered in either crop by intercropping and noted that this intercropping system reduced weed growth by 40% over rose-scented geranium grown in monoculture.

Based on these and other studies, field production of medicinal plants has great promise as a profitable business in many areas of the world (Israelsen 1993). Understanding the production details enough to manipulate yield and efficiency remains a difficulty, but as more cultivation and research occurs problems will be resolved. Moreover, education and marketing to the growers and the consumers will be critical to success (Dey 2001).

Hydroponic Cultivation

Hydroponic greenhouse cultivation of medicinal plants would allow the production of high quality products year round in a potentially pesticide free environment (Leonhart et al. 2000). Hydroponic greenhouse production can be a very clean system with reduced pest and

pathogen pressure and provides more control for the grower and easy manipulation of nutrients and stress levels. Also, hydroponic production can be utilized almost anywhere, reducing competition for the much needed resource of cultivatable land. Finally, hydroponics uses resources more efficiently and generally has less environmental impact than conventional soil systems. Especially suited for a small grower, hydroponic greenhouse cultivation of medicinals fills a niche and can be profitable (Israelsen 1993). Evaluating medicinal plants for production in hydroponic systems and increasing the understanding of how different nutrients affect the concentrations of beneficial phytochemicals would be of significant scientific and economic value.

Some research has been conducted on the production of medicinal plants in soilless culture systems. Minami et al. (1995) evaluated *Bupleurum falcatum* L. (Chinese Thoroughwax) grown in an ebb and flow hydroponic system and conventional soil production. The systems were compared for differences in morphology and saikosaponin concentration. Plants transplanted into the ebb and flow systems looked very different from plants cultivated in soil conditions, but plants directly seeded into the system were not significantly different from those grown in the soil. Saikosaponin concentrations were not significantly affected by the culture method.

Researchers at the Harrow Greenhouse and Processing Crops Research Centre have recently started research with medicinal plants. Leonhart et al. (2000) examined the effects of germination treatments and float bed production on medicinal plants. Most of the medicinal plants studied were well adapted to float bed production and greenhouse production of these plants had potential.

Cell Culture

Cell culture of medicinal plants, whether for propagation or for mass-producing secondary metabolites is a fascinating and critical field of research in the area of medicinal plants. With the increasing need for medicinal plants, especially rare and endangered species, in vitro propagation of these species may offer a rapid means for the multiplication of critical germplasm (Anand et al. 1998). It also may provide an opportunity to exploit many species without risking their endangerment (Mao et al. 2000). Sometimes, however, merely mass producing a plant will not result in the production of adequate quantities of secondary

metabolites. For example, the Pacific yew (*Taxus brevifolia* Nutt.) which produces paclitaxel, a chemotherapy drug (Wu et al. 2001), is so slow growing (van Rosendaal et al. 2000) and produces such small amounts of paclitaxel (Choi et al. 2000) that sustainable harvesting of paclitaxel from the bark is impossible (van Rosendaal et al. 2000). In addition, even with in vitro propagation of plants, there is still significant variation in the quality and quantity of the product. Thus, cell culture of certain species in a bioreactor system may be the best way to obtain highly standardized secondary metabolite production (Trypsteen et al. 1991).

Anand et al. (1998) successfully used cell culture to propagate *Uraria picta* (Jacq.)DC. (Fabaceae), a threatened herbaceous species native to grasslands from India to Africa. Eighty percent of the plants produced in micro-propagation were successfully established in the greenhouse and about 400 plants were transferred to the field. Mao et al. (2000) also successfully micro-propagated a medicinal plant. They established a rapid clonal propagation system for *Litsea cubeba* (Lours.) Pers. (Exotic verbena), a medicinal tree. They hoped that this propagation method would allow *L. cubeba* to be exploited to its fullest potential without harming the natural local populations.

Cell culture production of secondary metabolites has not been as widely successful as micro-propagation. There are many problems involving the establishment and maintenance of high yielding cell lines, and with perfecting the conditions in which the cells produce high levels of secondary products (Kreis and Reinhard 1989). There are many species in which cell cultures do not produce the compounds of interest that are produced by the whole plant. Currently, the major shortfall of this system is our lack of understanding the pathways, enzymes, regulators and environmental controls involved in production of these secondary compounds. However, recent research has revealing ways around this problem. For instance, *Agrobacterium sp.* mediated cell transformation can be used to produce plant cells with medicinally valuable compounds. Trypsteen et al. (1991) transformed *Echinacea purpurea* with *Agrobacterium rhizogenes* to form hairy roots. The transformed roots grown in vitro were found to have alkamides identical to the ones produced by natural plant sources.

***The Plant* - Silybum marianum**

Taxonomy

Silybum marianum (L.) Gaertner (Syn: *Carduus marianus* L., Milk thistle, Holy thistle, Lady's thistle or Saint Mary's thistle) is classified as follows: Kingdom Plantae, Division Magnoliophyta, Class Magnoliopsida, Order Asterales, Family Asteraceae and Tribe Cardueae. *Silybum eburneum* Coss. Et Durieux is the only other member of the Silybum genus. However, in a genetic study, Hetz et al. (1995) questions whether *S. eburneum* is actually a distinct species. Pointing out only slight morphological variations and citing new evidence of the ease of crossing between the two "species," he contends that *S. eburneum* is only a variant of *S. marinanum.*

Physical Description

The flowers of *S. marianum* are light purple and range in size from 2.5 to 6 cm in diameter. The number of flower heads per plant very significantly with between nine and 50 heads being produced on average. In pot production, the plant may reach a height of 1 m at full bloom. During its vegetative stage, the plant remains a basal rosette 0.75 to 2 m in diameter. *Silybum marianum* leaves are large, approximately 10 cm in width and 30 to 40 cm long. The leaves are obovate with a cuneate base and undulate, spinose-dentate margins. The leaves are glaucous and have a coriaceous texture when grown under water stress. They are predominantly dark green with a glossy sheen. Major leaf veins are highlighted with white marbling. Spines on the leaf range from 1 to 3 cm in length. The flower stalks and heads also possess spines, which are comparable in length. The seeds of *S. marianum* are between 0.5 and 1 cm long and have a large 1 to 2 cm long pappus crown. A single seed head can produce around 100 seeds (Bean 1985).

History

A native to the Mediterranean region, *S. marianum* has spread to most areas of the world. It is considered to be an invasive weed in both Australia and the United States (Austin et al. 1988). Settlers introduced Milk thistle to this continent as a food source (Foster 2000). It's leaves are useful in salads (Foster 2000) and the seeds are particularly high in oils (Hamid et al. 1983).

Silybum marianum has been used as a medicinal plant for centuries. Foster (2000) commented that Pliny the Elder of ancient Rome wrote about using Milk thistle as a vegetable and noted that it was excellent for "carrying off bile." Hildegard von Bingen, who lived between 1098 and 1179, wrote in *Causae et curae* that Milk thistle was good for the cure of ulcers and shingles (Schuppan et al. 1999). Interest in the plant was renewed in 1929 when Schultz noted that Milk thistle preparations improved the symptoms of chronic liver disease, acute hepatitis, and jaundice (Foster 2000).

Chemistry

The collaborative efforts of several German scientists elucidated some of the active components in *S. marianum* (Pelter and Hänsel 1968). Initial investigations identified what was thought to be a single active compound called silymarin (Madus et al. 1969). However, following the invention of thin layer chromatography, it was determined that silymarin is actually a complex mixture of components, (Quercia et al. 1983) consisting mostly of flavonolignans and a few flavonoid compounds (Koch et al. 1985). The biological activity of silymarin is attributed to the following five flavonolignans: silybin (Pelter and Hansel 1968), silychristin (Pelter et al. 1977, Tanaka et al. 1989), isosilybin (Sonnenbichler 1999), silydianin (Abraham et al. 1970), and isosilychristin (Kaloga 1981) and one flavonoid, taxifolin (Quaglia et al. 1999) *(Figure A-1: All figures are located in appendix A)*. In addition to these compounds, it is possible that *S. marianum* contains other novel, medically active compounds. For example, *S. marianum*, like other plants, produces flavonoids that are often free radical scavengers and have other beneficial effects on the mammalian body (Di Carlo et al. 1999). Varma et al. (1980) isolated another compound, betaine hydrochloride, from *S. marianum*, and suggested that it may provide benefit to the liver in addition to that of the purified flavonolignans. There are also many different fatty acids found in the seeds of *S. marianum*, including linoleic, oleic, palmitic, stearic, and arachidic (Hamid et al. 1983), several of which are considered beneficial in the diet (Youdim et al. 2000; Cook and Pariza 1998; Seifert et al. 1997).

Flavonolignans are generally classified under the larger class of compounds called phenols. The name "flavonolignan" was coined in 1968, with the discovery of the first described flavonolignan: silybin (Pelter and Hänsel 1968). The compound and the name were derived from the combination of two subclasses of biochemical compounds: the

flavonoids and monolignols (Bisset et al. 1991). The details of synthesis are lacking, but it is generally agreed that a radical coupling of a flavonoid to a monolignol creates flavonolignans (Hänsel et al. 1972). In the case of the silymarin flavonolignans, the flavonoid taxifolin couples with the monolignol coniferyl alcohol (Hänsel et al. 1972). This was verified in the laboratory synthesis of silybin employing the bimimetic oxidative coupling of 2R, 3R-dihydroquercetin and coniferyl alcohol in the presence of silver oxide (Quercia et al. 1983).

The role of these compounds in the plant is almost completely unknown. Medical studies have demonstrated flavonolignan action and effect on animal cells. However, even in animal cells there are many mechanisms of flavonolignan action that are still unknown. Until future studies elucidate these mechanisms, the scientific community can only speculate about flavonolignan action and role from precursor molecules and the nature of the class of compounds to which they belong.

Phenols are very diverse in form, function, and source, accounting for nearly 40% of all the organic carbon in the biosphere (Buchanan et al. 2000). Phenols are important for structure, defense, color, flavor, and many other plant characteristics (Buchanan et al. 2000). Most phenols are derived from phenylpropanoid and phenylpropanoid-acetate pathways (Buchanan et al. 2000). Structurally, a phenol contains a six carbon aromatic ring and an "acidic" hydroxyl group. Due to the extreme diversity of phenols, it is very difficult to generalize about the chemical activity of the class. However, most phenols are strong antioxidants acting as reducing agents, hydrogen donators, and singlet oxygen quenchers (Rice-Evans et al. 1996).

Flavonoids, the largest single group of oxygen containing ring compounds, are found universally in plants and are a well studied subclass of the phenols (Dillard and German 2000). A flavonoid consists of two aromatic rings connected with a three-carbon chain that is almost always closed to form an oxygen-containing ring (Stafford 1990) *(Figure A-2)*. Flavonoids are very important to the plant even though they are classified as secondary metabolites. They are responsible for a variety of colors found in plant tissues (Herbert 1989) and have significant activity against free radicals (Dillard and German 2000). The flavonoids are divided into several major groups including: chalcones, aurones, biflavonoids, isoflavonoids, flavan-3-ols, proanthocyanidins, anthocyanidins, flavonols, and flavones

(Stafford 1998). All flavonoids are derived from the Shikimate pathway, the pathway responsible for the production of many aromatic compounds including the aromatic amino acids phenylalanine, tryptophan, and tyrosine (Herrmann and Weaver 1999).

The Shikimate pathway is a sequence of seven metabolic steps that begins with phosphoenolpyruvate (PEP) and erythrose 4-phosphate, both from carbohydrate metabolism, and ends with chorismate, the precursor for phenylalanine and tyrosine as well as many aromatic secondary metabolites (Herrmann and Weaver 1999). The steps of this pathway are as follows *(Figure A-3)*: PEP and erythrose 4-phosphate are joined in a condensation reaction by 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase to create 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). Next, the phosphate is removed from DAHP to form 3-dehydroquinate (DHQ) by the enzyme DHQ synthase. The enzyme uses a multi-step chemical process of oxidation, β-elimination, reduction, ring opening, and an intramolecular aldol condensation. DHQ is then dehydrated to give 3-dehydroshikimate (DHS). This reaction is catalyzed by the bi-functional enzyme DHQ dehydratase-shikimate dehydrogenase that also catalyzes the conversion of DHS to shikimate. Shikimate is then formed by the reduction of DHS. Shikimate is converted to shikimate 3-phosphate (S3P) by phosphorilation catalyzed by the enzyme shikimate kinase. Another PEP is added to the pathway and condensed with S3P by 5-enolpyruvylshikimate 3-phosphate synthase to create 5-enolpyruvylshikimate 3-phosphate (EPSP) and an inorganic phosphate. The final step of this pathway involves the creation of chorismate from EPSP by the trans-1,4 elimination of phosphate by chorismate synthase (Herrmann and Weaver 1999). From chorismate, the synthesis of phenylalanine leads to the flavonoids.

Phenylalanine is produced from chorismate via prephenic acid. Prephenic acid is created by a Claisen rearrangement of chorismate by the enzyme chorismate mutase (Herbert 1989). Cinnamic acid is created by the elimination of ammonia from phenylalanine by L-phenylalanine ammonia lyase (PAL). Cinnamic acid is then converted into p-Coumaryl-CoA by the enzymes cinnamic 4-hydrolase, and 4-coumarate: CoA ligase (Stafford 1998), which is condensed with 3-malonyl-CoA to form chalcones. Chalcones are the first intermediates in the flavonoid pathway (Herbert 1989) and they are classified as open chain flavonoids (Stafford 1998). Since p-Coumaryl-CoA is the usual substrate for chalcone

synthase the first intermediate is typically naringenin chalcone (Stafford 1998). Naringenin chalcone is then hydroxylated to close the three-carbon chain and form naringenin (Stafford 1998). Naringenin is hydroxylated to form dihydrokaempferol, which is again hydroxylated to form taxifolin (Herbert 1989), the parent flavonoid to silybin (Bisset et al. 1991).

The main purpose of monolignols in the plant cell is for secondary wall structure and as building blocks for other compounds such as lignins and lignans (Buchanan et al. 2000). Cinnamic acid provides the skeletal structure most monolignols. All monolignols can be formed from cinnamic acid with essentially four different kinds of enzymatic reactions: aromatic hydroxylations, O-methylations, CoA ligations, and NADPH-dependent reductions (Dixon et al. 2001). A monolignol consists of an aromatic ring with a propen-3-ol chain and varying numbers of methoxy groups attached to the ring structure *(Figure A-4)*. The current model of the synthesis is not straightforward; but, instead it is set up in a "metabolic grid," *(Figure A-5)* where reactions producing monolignols can happen in any order (Dixon et al. 2001). For instance to produce Coniferyl alcohol, the monolignol precursor to silybin, cinnamic acid is converted to p-Coumaric acid by cinnamate-4-hydroxylase followed by one of three possible pathways for synthesis (Buchanan et al. 2000). A hydroxylation, O-methylation, and CoA-lignation occur, in differing orders depending on the pathway, to yield Feruloyl-CoA. Feruloyl-CoA is then reduced by two NADPH-dependant reactions forming Coniferyl alcohol (Buchanan et al. 2000).

Medical Benefits

Silybum marianum's diverse medicinal benefits have been the subject of much research. Seed extracts can have protective and regenerating effects on both the liver (Ferenci 1989) and kidney (Sonnenbichler et al. 1999). Extracts have also been effective in the treatment of Death Cap mushroom (*Amanita phalloides*) poisoning, hepatitis (Schuppan et al. 1999), and hypercholesterolemia (Škottavá and Krecman 1998). *Silybum marianum* seed extract and more specifically, the silymarin chemical mixture, has demonstrated many other useful medical qualities in various studies (Chavez 2001, Saller et al. 2001, Alacon de la Lastra et al. 1995).

Silymarin is an effective treatment for Hepatitis C and other liver diseases (Chavez, 2001). In all clinical trails that Chavez (2001) reviewed, noticeable benefit from taking silymarin was observed in some percentage of the patients. In one 1992 study, silymarin was

administered orally to 2637 patients. In the treatment group, there was a 46% improvement in liver function, a significant reduction in liver size and many of the symptoms associated with Hepatitis C were eliminated. Only 1% of the group reported digestive irritation from taking silymarin.

Silybum marianum extract is currently administered in the treatment of Death Cap mushroom poisoning. Floersheim et al. (1978) studied the effects of penicillin and silymarin on Death Cap intoxication in dogs. Both compounds were effective at preventing the rise of liver enzymes in the blood and the decrease of clotting factors. These two variables provide evidence that the liver was not damaged by the Amanita poison when the dogs were treated with either penicillin or silymarin. The authors suggest that silymarin's effects are due to the inhibition of the binding of the toxins in Amanita mushroom to the liver cell membrane. These results corroborated with pre-existing evidence of silymarin's anti-hepatotoxic activities.

Alacon de la Lastra et al. (1995) examined the effect of silymarin on the prevention of ulcers produced in rats by ischemia-reperfusion and on the enzyme myeloperoxidase found in the mucosal lining of the stomach. For two days prior to the experiment, rats were given a treatment of either silymarin in varying concentrations or Alloputinol, an inhibitor of the xanthine oxidase system. Rats were then subjected to ischemia and the stomachs were removed for analysis. The researchers found that silymarin prevented mucosal injury, thus reducing ulcer formation. Silymarin's effects were related to the decrease in the neutrophils in the gastric mucosa, demonstrating that silymarin had an inhibitory effect on neutrophil function.

Breschi et al. (2002) examined the effects of silymarin on bronchoconstriction induced by a hyper-allergenic response in guinea pigs. Silymarin was administered to the guinea pigs intravenously prior to exposure to the antigen. They found that silymarin significantly decreased bronchoconstriction due to the antigen, which may be due to silymarin's general anti-inflammatory action. Silymarin had no effect, however, on the hyperresponsiveness of the guinea pigs. Silymarin, therefore, may be effective at reducing and potentially inhibiting the sudden onset of an allergic asthma attack.

Due to its anti-inflammatory effects, silymarin has been investigated as a potential treatment for arthritis. Gupta et al. (1999) induced arthritis in male wistar rats by injecting a myco-bacteria and paraffin adjuvant in the paw. The animals were given a treatment of varying concentrations of silymarin. Treatments were given one day prior to arthritis induction and continued every second subsequent day for periods of 14 and 48 days. Rats were observed for severity of arthritis on day 14 and 48. Silymarin was effective at inhibiting inflammation and had significant anti-arthritic activity.

Silymarin may also be an effective cholesterol medication. Krecman et al. (1998) studied the effect of silymarin and silybin as compared with the approved cholesterol drug, probucol on rats fed an extremely high fat diet. The rats were given one of three treatments as a dietary supplement. Silymarin was as effective as probucol, at controlling serum cholesterol levels. Silymarin also caused an increase in high density lipoprotein cholesterol and a decrease in the liver cholesterol content, which probucol did not do. Silybin was not as effective as silymarin at controlling cholesterol levels. Silymarin may be more effective than silybin at controlling hypercholesterolemia because of additional bioactivity arising from interactions between the additional flavonolignans found in silymarin.

Alarcon de la Lastra et al. (1991) compared the diuretic effect of silymarin with naringenin and furosemide on isotonic saline loaded rats. The drugs were delivered to the rats through isotonic saline drinking water and the urine from rats receiving each treatment was collected. It was determined that silymarin and naringenin were mild diuretics. Silymarin caused a marked decrease in potassium loss, proving it to be a good potassium sparing diuretic.

Many studies have been done to determine the anti-carcinogenic activity of the flavonolignans found in the plant. Bhatia et al. (1999) examined the inhibition of human carcinoma cell growth and DNA synthesis by silybin and silymarin in cancer assays. Both the mixture and the pure chemical had significant activity against human prostate, breast and cervical carcinoma cells. The drugs were found to be highly inhibitory to cell growth and DNA synthesis of the cancer cell lines, with cell viability being lost only in cervical carcinoma cells. They attributed the anti-carcinogenic effects of silymarin to silybin, the

dominant compound in silymarin, as no additional inhibition was afforded with the use of silymarin over silybin (Bhatia et al. 1999).

Jiang et al. (2000) examined the effects of silymarin on epithelial cancer cells. In previous research(Lahiri-Chatterjee et al. 1999; Zhao et al. 1999), they reported that silymarin inhibited several epithelial cancers. In the current work, silymarin at very low concentrations rapidly prevented the secretion of vascular endothelial growth factor (VEGF) by the epithelial cells. This factor, an angiogenic cytokine, is critical for the growth of a cancer mass by allowing the growth of vascular tissue to feed the tumor growth. Without this essential compound, the tumor cannot grow past a minimal size and will starve. The results of this study suggest that the anti-angiogenic (its ability to stop the excretion of VEGF) potential of silymarin may be critical to its chemopreventive nature.

Kang et al. (2001) studied the dose dependent effect of silybin on cell differentiation in human leukemia cells. They observed that silybin inhibited cell proliferation of the cancer cells and induced cell differentiation. Silybin had an enhancing effect on several other important cancer drugs as well, making it a potentially valuable chemotherapy drug.

Silymarin's wide range of benefits cannot be attributed to one particular effect on the mammalian cell. There are an enormous number of ways that silymarin compounds interact with the cell (Saller et al. 2001). Cell membrane interactions appear to be of particular value. Silymarin is believed to associate with the cell and mitochondrial membrane and increase the membrane stability and reduce the cellular absorption of toxic xenobiotics and other poisons. The exact mechanisms for this interaction have not been fully elucidated though silymarin is thought to react with the membrane bound phalloidin transport system in the cell, which is critically important in the liver for the removal of bile acids, lipophilic hormones, and xenobiotics from the blood stream. Silybin also inhibits the 5-lipoxygenase pathway in kuppfer cells by inhibiting the synthesis of the enzyme leukotriene B_4 at concentrations easily reached in vivo (Dehmlow et al. 1996). This reduces the free radical formation within the cells, lessening damage. Though silybin was found to only inhibit free radical formation at very high concentrations not believed to be achievable in vivo (Dehmlow et al. 1996).

All of the flavonolignans in silymarin: silybin, isosilybin, silychristin, and silydianin, as well as the flavonoid taxifolin, are very effective free radical scavengers. There are many potential uses in the cell for free radical scavengers, including the inhibition of tumor necrosis factors and counteracting alcohol toxicity (Saller et al. 2001). Along with its antioxidant effects, silymarin also increases the cell's natural defenses by up-regulating the expression of superoxide dismutase.

Silymarin appears to affect the up-regulation and down-regulation of many different genes (Saller et al. 2001). The compound appears to down-regulate the genes responsible for hypersensitive response in the liver and activator proteins. The effect on gene expression may be largely indirect, due instead to the action of the chemicals on the compounds that generally up-regulate these genes. For instance, silymarin inhibits the TNFα-induced activation of NF-κB, a gene activator, by inhibiting the phosphorilation and breakdown of the natural inhibitor of NF-κB, υ-κB. Though a Dehmlow et al. (1996) found that silybin, the major flavonolignan in silymarin, did not have any inhibitory effect of the TNFα formation in in vitro rat liver kupffer cells. NF-κB is a key regulator in inflammatory and immune reactions (Saller et al. 2001).

Production of **Silybum marinaum**

Field Cultivation

Silybum marianum is one of the 50 to 100 medicinal plants that are cultivated (Stieber 1973; Omer et al. 1993). However, little is known about the effects of cultivation practices on the yield and active constituents of the plant (Omer et al. 1993).

A field trial conducted in Hungary by Stieber (1973) indicated that field production of of *S. marianum* was plausable and the entire production process except for weeding and thinning was mechanizable. Stieber noted varietal differences between plants from Europe and plants from the Mediterranean. Plants from the Mediterranean region did not flower in a single season whereas the European thistle did.

Hamid et al. (1983) tried cultivating *S. marianum* in Pakistan as a potential oil crop. The seeds of the plant contain a high, 26%, oil content (Hamid et al. 1983). Oils were extracted

using a soxhlet extraction and analyzed on a gas chromatograph with a flame ionization detector (GC-FID). The analysis of the fatty acid composition of the oil from *S. marianum* seeds is as follows (percentage by weight): 42% linoleic acid, 36% oleic acid, 10% palmitic acid, 7% stearic acid, 3% arachidic acid and 3% behanic acid. Seed yields of *S. marianum* produced in Pakistan, 1483 kg/ha, were comparable to the yields obtained from sunflower and safflower. Since silymarin and the oil from the seeds can be extracted separately, it is possible to obtain simultaneous benefits from a single crop.

In an attempt to maximize productivity of *S. marianum* as a crop in New Zealand, Martin et al. (2000) examined the effect of different sowing times on reproductive development of the thistle. They sowed seeds at monthly intervals for twelve months in two trials. Plants sown between November and May had no buds until the following spring, but then quickly flowered and seeded. Plants sown in the winter months were slow to emerge, but then quickly grew to flowering and seed production. Spring sowing produced highly variable results with poor emergence or rapid development to flower and seed by fall or winter. Martin et al. conclude that the length of time between emergence and flowering is a function of temperature modified by day-length. They suggested that the plants required around 2000 degree-days over 5°C and up to 13 hours day-length to flower. They recommended that *S. marianum* be grown as a biennial in New Zealand, planted in the late summer and harvested the following summer.

Omer et al. (1993) looked at the effects of spacing and potassium and nitrogen fertilization on yield and active constituents in *S. marianum.* Application rates of 55 kg of K_2O/ha yielded the highest percent concentration of the active compound silybin in seed, though the increase was minimal. The largest yields of *S. marianum* seed and percent silymarin occurred at potassium application rates of 115 kg K_2O/ha and nitrogen rates of 140 kg N/ha applied as ammonium nitrate, which were the highest rates tested. This may mean that even greater yields could be achieved at higher fertilizer rates. The closest row spacing of 25 cm yielded the highest seed yields, however the oil and flavonolignan content was decreased when compared with the seed from the wider row spacing of 50 cm.

Omer et al. (1995) further researched the production of *S. marianum* in Egypt on reclaimed lands. They evaluated how nitrogen, potassium and plant spacing influenced

yields, oil content, and silymarin content of the seeds. Maximum yield was obtained by a compact spacing of 20 cm between rows, and that fertilization of about 476 kg N/ha and 238 kg K_2O/ha increased yield. The soil was 98% sand and as a result, yields were highly sensitive to small changes in fertilizer.

Hammouda et al. (1993) found that maximum yields of silymarin were obtained with the application of 375 kg/ha of nitrogen (the highest nitrogen treatment tested) and a 60% field capacity water regime (the medium water level tested). In another study Hammouda et al. (1994) examined the oil content of the *S. marianum* seeds. They compared a white flowered variant of the species with and a purple flowered variant, as well as wild type versus cultivated plants for seed oil content. The cultivated purple flowered plant had the highest level of oil at 34.0% of seed weight, with 96% of the oil in the form of unsaturated fatty acids. The white flowered thistle produced the lowest oil content per seed at 27.8% of seed weight with the lowest unsaturated fatty acid content of 83%. The Sixty percent field capacity irrigation treatment gave the maximal oil yield. Nitrogen treatment did not appear to have any effect under these conditions.

Zalecki and Gorna (1983) examined the effects of desiccation on the yield and quality of *S. marianum* seed using field experiments conducted in 1981 and 1982 in Poland. They found that any desiccation of the plants significantly reduced yields and that avoiding desiccation entirely produced the highest quality seeds.

A few studies have addressed the elemental composition of *S. marianum*. Szentmihalyi et al. (1998) looked at 23 elements in the leaves and seeds of *S. marianum* grown in Hungary *(Table B-1: All tables are located in Appendix B)*. They found that *S. marianum* is an accumulator of aluminium and iron, containing high concentrations of each in all vegetative parts of the plant. They noted, however, that when the plant is brewed as a tea, these compounds are significantly decreased in concentration.

Greenhouse Cultivation

Few experiments have been reported on greenhouse production of *S. marianum* aside from a few studies interested in controlling the invasive character of the species. In one study, Austin et al. (1998) grew *S. marianum* is sand culture to evaluate how plant density and fertilizer regime effected the stem yield of the plant. They also evaluated how *S. marianum*

performed in a competitive setting with *Cirsium vulgare*, another invasive weedy thistle. Both plants were planted 1, 2, 4, 8, or 16 plants per 18 cm diameter pot in monoculture or 1+1, 2+2, 4+4, 8+8 or 16+16 plants per pot in combined plantings. *Silybum marianum* was a very competitive plant, nearly smothering out *C. vulgare* in high density mixed plantings. Shoot yield reached a maximum for *S. marianum* at four plants per container. Shoot yield increased asymptotically with increased planting density. Yield increased with increasing fertilizer concentrations, peaking at 4 times the normal concentration of Hoagland No. 2 solution. At 16 times the concentration of Hoagland's solution, the yields dropped markedly, signifying toxicity symptoms.

Young et al. (1978) examined the germination requirements for *S. marianum* seeds. They discovered that an after-ripening requirement of up to 5 months was necessary for emergence, depending on germination temperature. After the after-ripening requirements were met, the seed would germinate optimally with alternating cold and warm periods between 2 to 15°C and 10 to 30°C, respectively. Highest germination rates were achieved with between 1 mm and 3 cm planting depth, and emergence declined with increased depth. However, significant emergence , 28%, was still achieved at 8 cm in depth. Interestingly, litter cover significantly inhibited seed emergence, with only 2% of seeds emerging when covered with 1 cm of leaf litter. Also, potassium nitrate (KNO_3) added to the germination substrate enhanced germination significantly between the incubation temperatures of 2 to 5°C.

Cell Culture

There is also an interest in the cell culture of *S. marianum*, especially with respect to the influence of nutrient concentration on the active components in the plant. Efforts to produce the flavonolignan mixture, silymarin, in cell culture have largely been ineffective. In vitro cultures that do produce flavonolignans do so in very low concentrations, with production disappearing completely in sustained cultures (Cacho et al. 1999). In an attempt to optimize in vitro methods of producing silymarin, Cacho et al. (1999) manipulated the medium composition and examined its influence on cultured cells of *S. marianum*. The concentrations of KNO_3, KH_2PO_4, iron and calcium were examined. The removal of

calcium from the medium promoted flavonolignan production in culture; however, this also greatly reduced cell growth.

Becker and Schrall (1977) were the first to study cell culture production of *S. marianum* with the goal of producing silymarin. In the first stages of their work they successfully cultured *S. marianum* on a hormone-supplemented Murashige and Skoog (1962) medium *(Table B-2)*. However, their initial work produced no new silymarin in the cultures and the silymarin that was present in the tissue to start with in initial culture disappeared in subcultures. In subsequent work Becker and Schrall (1977b) found that by feeding taxifolin and coniferyl alcohol, the precursors for silybin, to the culture, silybin could be isolated. They also found that by feeding the cell culture luteolin and coniferyl alcohol, it would produce hydnocarpin, a flavonolignan found in an entirely different plant species, *Hydnocarpus wightiana* (Chaulmoogra tree or gum tree), which was an original treatment for leprosy.

Alikaridis et al. (2000) successfully produced silymarin in cell culture. They used explants from seeds that were then transformed using *Agrobacterium rhizogenes*. Both transformed "hairy" roots and untransformed roots were then grown on hormone free agar. After 1.5 months, the roots were harvested and analyzed for flavanolignans. The untransformed root cultures produced all four flavanolignans and the transformed root cultures produced only isosilybin, silychristin and silydianin.

Potato Disc Bioassay

Accurately assessing the bioactivity of extracts is a serious problem in medicinal plant research. Evaluation of medicinal plants with more subjective activity (i.e. St. John's wort) are highly problematic, and even objective activity can be difficult to assess. Bioassays, an objective screening method, can be expensive, particularly in the case of cancer bioassays. Bioassays are indispensable in isolating and characterizing compounds. One alternative to traditional methods may be the potato-disc bioassay that uses potato cells and *Agrobacterium tumefaciens*. This bioassay is cheap, easy to preform, and quick, taking only twenty days to achieve results.

Agrobacterium tumefaciens

The *Agrobacterium* genus contains four identified species that form a truly unique group of bacteria, because they are the only living things to regularly transfer DNA from prokaryotes to eukaryotes. *Agrobacterium tumefaciens* may be the most familiar member of the genus, being the species that causes crown gall disease in many plant species (Stafford 2000). *Agrobacterium tumefaciens* is also the only species in the group that permanently mutates cells so that they divide rapidly without additional plant hormones or in the presence of the bacteria.

Crown gall disease proceeds in several steps *(Figure A-6)*. First, the plant is physically damaged in some way – something as minor as insect damage is all that is needed. When the plant is damaged, nearby cells begin to divide to heal the wound. The damaged cells also excrete phenolic defense compounds to stop invading pathogens. The phenols and other exudates of the dividing cells signal the *A. tumefaciens*. The *A. tumefaciens* then transports a T-strand, a single strand of DNA duplicated from its Ti-plasmid, into a plant cell. The T-strand inserts into the plant DNA and when replicated causes uncontrolled cell division of the mutated plant cells by overproduction of auxins and cytokinins. The oncogenes, as the T-strand is called, also causes the production of opines, which are kinds of amino acids that feed the bacteria. Though not technically a cancer, crown gall disease can be used as a model for cancer and neoplastic tumors in mammalian cells (Stafford 2000).

The Bioassay

The crown gall bioassay was initially investigated in an attempt to understand tumor formation and *A. tumefaciens* transformation of the plant host. Many attempts were made to perfect an easily reproducible bioassay that would allow variable manipulation to study the natural process. Kalanchoë stems (Lipetz 1965), bean and tobacco leaves (Lippincott and Heberlein 1965), and carrot roots (Klein and Tenenbaum 1955) were used to perfect the technique. Problems with standardization, inoculation, plant cell type complexity, experiment length and storagability created difficulties in all of these tissue types. In 1977, Anand and Heberlein discovered that potato tubers behavior was ideal. Potato tuber tissue was uniform, easy to store and sterilize and very susceptible to transformation, producing tumors readily.

It was not long before the potato disc bioassay was recognized as relevant to general cancer research. Researchers began looking at it as a potential prescreen for compounds with anti-carcinogenic potential. In 1981, Galsky et al. conducted a large screening with the bioassay and tested twenty-one known anti-carcinogenic compounds. Their work demonstrated a high correlation between the potato-disc bioassay and the mouse P388 leukemia protocol, a more common prescreen. Of the twenty-one compounds tested, the potato-disc bioassay gave only two false-positives and one false-negative.

The bioassay is becoming more popular as a prescreen for raw plant extracts, since regular mammalian cell bioassays are too expensive for this kind of screening. Haque et al. (2000) took ethanolic extracts from 12 plant species native to Bangldesh with traditional medicinal value and screened them using the potato-disc bioassay. They found that ten of these extracts had significant tumor inhibitory activity. Kundu et al. (2000) used the potato disc bioassay to demonstrate activity of epifriedelanol, a compound extracted from *Vitis trifolia* L. Moreno-Murillo et al. (2001) attempted to isolate the active extract from several Solanaceae species. They used the bioassay as one screen in a battery of tests including the brine shrimp test and the inhibition of cell division test on sea urchin eggs. Through the bioassays, they narrowed seven plant extracts to three, whose activity at inhibiting cell growth was credited to a common chemical, epi-katonic acid.

Harvey et al. (2002) used the potato bioassay to test the anti-teratoma activity of multiple glucosinolate containing seeds. While some seed extracts inhibited tumor formation, others did not and that the activity could not be attributed to the total number of glucosinolates found in the seeds, but rather the type of glucosinolates in the extracts.

Capillary Electrophoresis

Capillary electrophoresis (CE) is a highly versatile separation technique that is extremely useful for the separation of natural products (Issaq 1999). Capillary electrophoresis can be used to separate everything from small biomolecules to proteins and DNA. The matrices of separation can be simple buffers or highly modified buffers using mixtures of organic modifiers, chiral separators and surfactants or gels. Capillary

electrophoresis is becoming an essential tool for separation and identification because of its versatility as well as its use of small sample size and sensitivity.

The Basics of CE

Capillary electrophoresis is comparable to gel electrophoresis, in that molecules are separated by differential response to charge (Heiger 1997). However, CE is far more versatile than gel electrophoresis because many different kinds of media, solid gels to free solutions, can be used within a column. The CE apparatus is simple in principle. It contains a very thin and long, hollow column with buffer reservoirs on both ends. To separate a sample, charge is applied to the column so that one end is positively charged (often the inlet) and the other end is negatively charged (often the outlet). The sample is injected and molecules are then separated by their repulsion and attraction to one end or the other (Heiger 1997). The buffer forces the movement of all molecules toward the outlet, essentially pushing them along, so that, theoretically, all molecules will eventually pass by the detector *(Figure A-7)*. This "buffer push" is called the electro-osmotic flow (EOF); and occurs because the applied charge affects the affinity of the buffer to the capillary wall.

The complications to this simple system occur in the perfecting the separation of various molecules. There are many ways to improve the separation-ability of CE (Issaq 1999). Focusing on the most basic separation method, capillary zone electrophoresis (CZE), the adaptations are nearly innumerable (Heiger 1997). The major method for changing the separation using CZE is to change the EOF. This can be done by changing the applied voltage, the buffer pH or concentration, the temperature, using capillary coatings or by putting additives into the buffer. Buffer additives include organic modifiers (i.e. ethanol or methanol) which change the viscosity, generally decreasing the speed of the EOF. For example, the addition of methanol to the buffer reduces the EOF in the capillary by changing the zeta potential (z^0) and viscosity of the buffer, which can be necessary when separation occurs because the solute elutes too rapidly. The weaker the EOF, the longer the samples will take to move through the column, and therefore a better separation of peaks will result (Heiger 1997). Surfactants such as sodium dodecyl sulfate (SDS) or cetyltrimethylammonium bromide (CTAB) can be added to alter selectivity, change the speed or the direction of the EOF. Zwitterions can be added to the buffer to increase ionic

strength without increasing the buffer conductivity and are particularly useful in protein selection. Chiral selectors such as cyclodextrins can be added to the buffer as well making it possible to separate enantiomers (Issaq 1999).

Besides these buffer additives, other modifications are possible. These include several other separation techniques such as micellar electrokinetic chromatography or MEKC, capillary gel electrophoresis, isoelectric focusing and isotachophoresis (Heiger 1997). Micellar electrokinetic chromatography is particularly useful for the separation of neutral solutes. This is possible because of the use of high concentrations of surfactants, which create micelles that attract non-polar molecules. Since the micelles are charged, they migrate with or against the EOF dragging the non-polar molecules with them, allowing separation (Heiger 1997). Capillary gel electrophoresis (CGE) is largely used for size-based separation of proteins and nucleic acids. In CGE, polymers are added to the running buffer, which acts like a sieve slowing large molecules while allowing smaller ones to continue, successfully separating compounds that are very similarly charged (Heiger 1997). Isoelectric focusing is a well-established technique in gel electrophoresis that has been adapted to CE. Isoelectric focusing applies a pH gradient by using zwitterionic molecules called ampholytes. The column is filled with a mixture of ampholytes and sample, then a charge is applied and the molecules migrate until they become uncharged, causing separation (Heiger 1997). Isotachophoresis (ITP) uses two buffers for separation. The sample is surrounded by two different buffers that keep everything migrating at the same rate. This method keeps individual ions moving in discrete zones but all at the same speed (Heiger 1997).

Benefits of CE

Capillary electrophoresis has many benefits over other analysis techniques. It works with very small amounts of solvent and sample, reducing waste significantly and reducing the amount of sample necessary for useful quantification. Capillary electrophoresis is also less expensive, not using complicated columns or large amounts of solvent (Suntornsuk 2002). It is a very fast method, with average runs taking between 10 and 15 min (Suntornsuk 2002). The CE is also a non-destructive separation technique that does not modify or destroy the sample being analyzed, making recollection possible. Due to the highly versatile nature of the CE, it also has a high tolerance for complex matrices of the sample so that

samples do not have to be very clean in order to run (Timerbaev 2001). Simply filtering a raw extract through a 0.4 μm filter is sufficient cleaning to keep from damaging a column, and with the right buffers, these extracts will separate into their constitutive parts for easy quantitative analysis. Since samples do not need to be as clean, volatile components of the sample are less likely to be degraded by extraction procedures, yielding better results.

Drawbacks of CE

Capillary electrophoresis has several drawbacks. Reproducibility of peak shape and size between runs is not as high as in other systems (Timerbaev 2001). Migration times of peaks can change significantly with only minor variations in the runner buffer or sample matrix, making identification more challenging. In addition, minor fluctuations in charge, temperature or buffer volume can significantly shift chromatograms. Careful monitoring of every variable is essential for consistent results. For certain compounds, sensitivity is lower in the CE than other instrumentation (Suntornsuk 2002).

CE Methods

Flavonoid separation using CE is well documented (Issaq 1999), producing good precision and excellent linearity, making quantitative analysis possible (Suntornsuk 2002). Bjergegaard et al. (1993) used micellar electrokinetic capillary chromatography (MECC) to separate flavonoid glycosides. They looked at the effects of temperature, voltage, electrolyte, CTAB and organic modifier concentration of the migration order, time and peak areas of the flavonoids. Cetyltrimethylammonium bromide (CTAB) and the cholate-taurine system were effective for flavonoid separation, but the cholate-taurine system gave far better separation. Different variables had an effect on the migration order of the flavonoids, which were due to differences in the hydrophobicity and charge of the compounds. Their study demonstrates that this separation method has valid application for practical analysis of samples of flavonoids from plant extracts.

Fonseca et al. (2001) attempted to identify the best conditions for the separation of several phenolic compounds, among them a few flavonoids suspended in methanol. The best condition for quantitative work on the tested phenols was 20 mmol/L tetraborate buffer with a pH of 10 and direct detection at 337 nm. Precision of migration and peak area using this system with an electrokinetic rinse prior to injection was better than 4 and 2%,

respectively. Though these separation conditions may not be ideal for the separation of all flavanoids, they provide a good baseline for further research.

Lee and Ong (2000) examined the differences between high-performance liquid chromatography (HPLC) and capillary electrophoresis in determination of catechins and theaflavins from green and black teas. Catechins and theaflavins are polyphenols. Capillary electrophoresis was two thirds faster at separation than HPLC. Both methods were repeatable and comparable. Capillary electrophoresis proved less prone to day-to-day variation, with <2% variation of migration time. Though they report that the CE proved five times less sensitive than HPLC using their methods, this seems highly unlikely due to the high level of sensitivity reported using CE. Problems with their detector, their buffer, or their injection method may have reduced the ability of the CE to detect the polyphenols at low concentrations.

Liu and Sheu (1994) used CE as a method for determining the flavonoids in the medicinal root of *Scutellaria baicalensis* Georgi. A MECC method was employed and it required a buffer composed of 20 mM sodium dodecyl sulphate, 10 mM sodium dihydrogenphosphate and 12.5 mM sodium borate to achieve optimum separation of the six flavonoids of interest. The run time was 25 min long. A pH of 9.7, a SDS concentration of 0.02 M, and a temperature of 20°C were found to be optimum for separation in the shortest amount of time. They isolated six flavonoids from the root samples, oroxylin A 7-O-glucuronide, baicalein, wogonin 7-O-glucuronide, baicalin, wogonin, and oroxylin A at concentrations of 14.46, 28.52, 33.01, 122.13, 7.25, and 4.58 mg/g, respectively.

Quaglia et al. (1999) contributed the first published technique for the CE separation of flavonolignans. They compared the separation of the methanolic extract from *S. marianum* fruits on CE and HPLC. The separation on the HPLC was carried out using the following two different stationary phases: a Purospher C18 (250 x 4 mm I.D., 5 micron) and a Lichrosphere C8 (250 mm x 4 mm I.D, 5 micron). For the Purospher C18 stationary phase, water, acidified until pH 2.6 with 10% H_3PO_4, was mixed with acetonitrile in the ratio of 62:38 in isocratic mode. The Lichrosphere C8 stationary phase was used with a mobile phase of water, acidified at pH 2.3 with 10% H_3PO_4 , mixed with acetonitrile and methanol, which were used to form a gradient elusion. The photodiode array detector conditions were: a λ

value of 289 nm, an acquisition rate of spectra 1600 ms, a spectral bandwidth for each channel 4 nm, and a wavelength range of 220-350 nm. The CE method was preformed using an uncoated silica capillary with a total effective length of 35 cm. The buffer consisted of an equal mixture of 100 mM borax solution and 100 mM boric acid solution, pH 9 with the addition of 15% methanol as an organic modifier. A β-cyclodextrin was also added to separate silybin and isosilybin. Quercetin was used as an internal standard. Injection was 2 sec and analysis was carried out at a λ value of 200 nm. Although repeatability and results were comparable for both separation methods, HPLC allowed for better separation of silybin and isosilybin. However, the best separation of silychristin, silydianin, taxifolin and silybin was achieved using CE. Concentrations of the flavonolignans when calculated from each method were nearly identical. In one sample, silybin concentrations were 11.40 or 11.42 mg/g, silychristin concentrations were 6.60 or 7.50 mg/g, silydianin concentrations were 7.50 or 6.01 mg/g, and taxifolin concentrations were 3.30 or 3.30 mg/g when anlysed using HPLC or CE, respectively.

III.
Materials and Methods

Silybum marianum (L.) Gaertn. plants were grown in a hydroponic system utilizing perlite for root support. Three nutrient treatments were applied in a randomized block design to account for the effects of shading across the greenhouse. Plant samples were taken at the four leaf stage, the 15 to 20 leaf stage, and at senescence. Three experiments were conducted in spring 2001, fall 2001, and summer 2002. Plant samples were analyzed for differences in bioactivity. Water, ethanol, methanol, acetone and chloroform were used to extract compounds from the samples. The extracts were first run on a potato disc tumor bioassay to determine which extracts were active. Active extracts were then analyzed using HPCE to determine chemical composition.

Experiment 1 – Spring 2001

Hydroponic Production

Silybum marianum seed was purchased from Johnny Selected Seeds® (955 Benton Avenue, Winslow, Maine, U.S.A., 04901) and planted in 20 cm^3 (8 in^3) Oasis® cubes on February 26, 2001. The Oasis® cubes were soaked prior to planting to ensure even moisture levels throughout the medium. Seeds were covered with 0.5 cm of loosely packed crumbled Oasis® material, and placed under an overhead sprinkler system. Seeds germinated between 6 to 12 days. The first set of true leaves emerged on day 18 after planting, after which a 10% diluted hydroponic nutrient solution *(Table B-3)* was applied three times a week until planting.

When the seedlings had reached between 4 and 6 leaves, 27 uniform plants were selected and transplanted on day 59 into five-gallon trade pots (with an actual volume of 25.5 L) filled with a 75% perlite and 25% peat mixture. The layout of experiment 1 is shown in Figure A-8.

The transplanted seedlings were placed on an automatic watering system and were also watered by hand twice a day until they were established. On day 67, three separate fertilizer treatments with varying nitrogen to calcium ratios were added and applied for the remainder

of the experiment. Calcium content was manipulated to produce treatments with nitrogen to calcium ratios of 2:1 (257.3 mg/L N: 129.0 mg/L Ca), 1:1 (257.3 mg/L N: 257.3 mg/L Ca), and 1:2 (257.3 mg/L N: 514.1 mg/L Ca). The full nutrient solutions supplied to the plants are shown in Table B-4. Nutrients were mixed to actual dilution in 50 gallon barrels and supplied to the plants using Roberts® green medium flow spray stakes that supply 0.7 L per min in a 160° spray pattern. A solar clock controlled the frequency and length of watering by "collecting" 0.6 moles of sunlight using a solar sensor, and then triggering a solenoid valve to water for 40 sec. As the weather got hotter the amount of light necessary to trigger watering was decreased and the length of watering was increased to prevent water stress.

One fully expanded leaf from the lower half and one partially expanded leaf (40 to 60% expanded) from the upper quarter of the plant were taken from each plant during anthesis, on day 96. The samples were put in whirl-pac® bags and stored on ice until they could be placed into storage at -30°C. Plants began to bloom on day 90 and were allowed to self-pollinate. Seed heads were allowed to mature and were individually harvested as the heads began to release seeds. On day 107, the first seed head was harvested. When all the seed heads had been harvested (50 days later), collected seed heads were mechanically threshed. Seed was sifted, and stored in whirl-pac® bags at 4°C. A total seed weight per plant was then taken. After senescence, the stems and roots were harvested over a nine day period from July 24 (day 148) to August 2 (day 157). Stem and root samples were collected and stored in the same manner as the leaf samples.

Potato Disc Bioassay

A potato disc bioassay as described by Galsky et al. (1981) was preformed to test the anti-teratoma activity of the extracts from sampled *S. marianum* tissue. The plant extracts were applied to the potato discs at the time of infection by *Agrobacterium tumefaciens* (C58) to evaluate the extracts' ability to suppress tumor growth. Silymarin, silybin, and taxifolin standards were also utilized in the bioassay.

Extractions

Initial extractions were performed on seed samples to determine which solvents would extract the active compounds. Three unique seed samples were taken from each fertilizer treatment (Calcium:Nitrogen ratios 2:1, 1:1, 1:2). Five subsamples were taken from each of the 9 seed samples, weighed into 0.5 g amounts, and placed into 45 test tubes. Each subsample in every set of five subsamples was treated with a different 5 mL aliquot of solvent (water, ethanol, methanol, chloroform or acetone). Each sample was ground using a Kinematica Polytron (model # PT10/35, Brinkmann Industries, Wesbury, NY) for one min at speed "6". The extraction procedure *(Procedure C-1: All procedures are in appendix C)* then proceeded as follows: samples were shaken on a vortexer (Small Vortexer, Model PV6, Glas-Col Terre Haute, IN) for 30 sec at maximum speed, sonicated for 20 min, and vortexed again for 30 sec at maximum speed. The mixtures were then centrifuged (centrifuge model Centra MP4R International Equiptment Co. Needham Heights, MA 02494 using rotor #224) at 1000 RCF at 5°C for 10 min. The supernatant was removed, and 5 mL of fresh solvent was added back to each sample. This process was repeated two additional times. Following the extraction procedure the supernatants were pooled and dried under forced air in a 30°C water bath(Multivap Analytical Evaporator, model # 8105 Organomation Ass. Inc. Berlin, MA, 01503). Once dried, the residue was redissolved in 5 mL of 12.5% dimethyl sulfoxide (DMSO) by sonication for 40 min and by vortexing for three 30 sec intervals over 6 hours and then stored at 4°C until the bioassay. Twenty-four hours prior to the bioassay, extracts were sterile filtered using a 0.2 µm-pore nylon sterile filter *(Procedure C-3)*.

Seed samples were also sequentially extracted with water, (highest polarity) methanol, ethanol, acetone and chloroform (lowest polarity). Three seed samples from each fertilizer treatment were weighed into nine, 0.5 g samples. Samples were then suspended in water, ground as previously described, and treated with the extraction procedure *(Procedure C-1)*. Following the third extraction with water, the samples were dried overnight under forced air. After drying, 0.5 mL of methanol was added to the sample pellet and procedure 3.1 was repeated. This process was repeated for each of the three remaining solvents: ethanol, acetone, and chloroform. At each stage in the sequential extraction, supernatants for each solvent were pooled, dried, and redissolved as previously described in procedure C-3.

Extractions were also done on root and leaf samples. The vegetative tissue extraction procedure *(Procedure C-2)* was preformed as follows: three 0.5 g samples of each frozen tissue type (roots, partially expanded and fully expanded leaves) from each fertilizer treatment were taken and ground in 10 mL of ethanol by mortar and pestle before the sample could thaw. The sample and the solvent were then transferred into a test tube and capped. Samples were then vortexed for 30 sec at maximum speed and sonicated for 50 min. Samples were again vortexed for 30 sec at maximum speed, sonicated for an additional 15 min, and vortexed for another 30 sec at maximum speed. After allowing the samples to settle for one hour, the supernatant was removed from the tissue. The tissue was then resuspended in 5 mL of ethanol and the process described above was repeated two additional times. The tissue was then dried overnight under forced air and then resuspended in 5 mL of chloroform. This extraction procedure was then repeated three times using chloroform as the solvent. The supernatants were pooled, dried, and redissolved as described previously in procedure C-3.

Standards

Silymarin, silybin, and taxifolin were used as standards in the bioassay at concentrations of 1000, 750, 500, 250, 100, 50, 10, and 1 µM. Each compound was weighed out and suspended in 10 mL of 3.125% DMSO, to make a 1000 µM solution. Silymarin, a mixture of compounds, was assumed to have a molecular weight similar to silybin, its major constituent. Each mixture was then diluted with 3.125% DMSO to make 2 mL of each concentration. These standards were then filter sterilized using a 0.2 µm-pore nylon sterile filter and assayed.

Bioassay Preparation – Media

Agrobacterium tumefaciens was grown first on nutrient broth agar (NBA) to maintain single colonies of bacteria. Nutrient broth agar was composed of 0.5% sucrose, 0.8% nutrient broth, 0.1% yeast extract, and 1.5% granulated agar. All constituents were added to distilled water, then stirred and heated until the solution clarified. After clarification, the agar was autoclaved (Autoclave, model # SR-24E, Consolidated Stills and Sterilizers, Boston, MA) on a liquid cycle to kill potential bio-contaminants. The autoclave cycle was a total of 30 min long, including the pressurization and depressurization of the autoclave

chamber, at a temperature of 121°C. The autoclaved solution was poured into sterile petri plates using an auto-plater (Omnispense, Cat. # 375010, Serial # 0824, Wheaton Instruments, Milleville, NJ) in a negative pressure, clean hood. Fifteen milliliters of NBA was poured into each petri plate. Nutrient broth agar plates were stored in plastic bags at 4°C for up to two months pending use.

Nutrient broth (NB) was used to make liquid cultures for the bioassay. Nutrient broth was composed of 0.5% sucrose, 0.8% nutrient broth, and 0.1% yeast extract. The NB was made as described above, except that prior to autoclaving, NB was divided into 100 mL amounts poured into Erlenmeyer flasks and 9 mL amounts poured into test tubes. Nutrient broth was stored wrapped in aluminum foil at 4°C for up to one month prior to use.

Potato discs were maintained on water agar with ampicillin for the bioassay. Water agar was made composed of 1.5% granulated agar in distilled water. The agar was prepared as described above. After autoclaving the agar was then cooled to 60°C and filter sterilized ampicillin was added at the rate of 100 mg/L. After stirring, the agar was then plated as described above. The water agar plates were stored in plastic bags at room temperature for up to two months or until needed.

Culturing *A. tumefaciens* – Streak Plates

To maintain single colonies of *A. tumefaciens* (C58), streak plates were maintained. Streak plates were made on NBA. Under a negative pressure hood, a sample of the bacteria was taken with an sterile inoculation loop from a previously grown culture (either in liquid or on agar) and spread back and forth across 1/3 of a clean plate. The loop was then sterilized by heat in until red hot in a Bacti-Cinerator III (Product # 889-001007, Oxford Labware, St. Louis, MO). The inoculation loop was then dragged across the plate at a 90° angle from the previous streaking for four or five straight lines. The loop was again sterilized and the last streaked line was then streaked out again at a 90° angle from its edge in three or four straight lines. Plates were then incubated at room temperature (25°C) in the dark for 48 hours. After 48 hours the plates were sealed with parafilm and stored at 4°C until they were needed. Streak plates were used within one month of creation.

Culturing *A. tumefaciens* – Inoculum Preparation

A liquid suspension of *A. tumefaciens* was used to infect the potato discs. 50-100 mL of NB was inoculated under a negative pressure hood with one colony of *A. tumefaciens* taken from a streak plate. The NB was placed on a shaker and agitated for 48 hours. The concentration of bacteria in the NB ranged from 1×10^9 to 7×10^9 colony forming units (CFU)/mL. A serial dilution was preformed to determine the exact number of CFU after each bioassay. Serial dilution was done by adding 1 mL of the culture to 9 mL of NB, and shaking. The mixture was then diluted the same way eight more times. The 10^{-6}, 10^{-7}, and 10^{-8} dilutions were then spread in 100 μL aliquots onto NBA plates. The NBA plates were then incubated at room temperature for 48 hours and the numbers of colonies were counted. The concentration was then calculated as the average of the number of colonies on each plate times the inverse dilution of each plate.

The Bioassay

Potatoes were purchased from a local grocery store. For most assays, *Solanum tuberosum* L. 'Russet Burbank' potatoes were used; however, *S. tuberosum* L. 'Russet Norkotah' was substituted when the 'Russet Burbank' could not be obtained. Potatoes were used within two days of purchase from the grocery store. Potatoes were thoroughly scrubbed in tap water and then submerged in a 20% hypochlorite (Clorox bleach, the Clorox company, Oakland, CA) solution for 20 min to surface sterilize the potatoes. Using a sterile (bleached) knife, both ends of the potato were then removed and the potato soaked in a new bleach for an additional 10 min. The potatoes were then transferred to a laminar flow hood. An autoclaved and surface sterilized (in 98% ethanol) cork borer 1.5 cm in diameter was used to extract cores from the potato. The extracted core was cut into 0.5 cm thick discs, with 1.5 cm from each end being discarded. A sterile autopsy knife (Bard-Parker® Rib-back® Carbon Steel no. 60 autopsy blade with a #8 scalpel handle, Becton, Dickson & Co, Franklin Lakes, NJ) was used for this procedure. The potato discs were placed onto the ampicillin water agar petri plates with five discs per plate. To account for potential variation between potatoes, potato discs from the same potato were spread evenly across all of the plates in a block. Inoculation occurred when three blocks were completed.

Inoculation

Agrobacterium tumefaciens C58 was used to infect potato discs. Plant extracts were diluted by adding 0.5 mL of extract to 1.5 mL of sterile water. 2 mL of *A. tumefaciens* liquid culture was then added to the diluted extracts. 100 μL of this mixture was spread on each potato disc. Standards were applied in the the same way. Sterile water, filter sterilized DMSO, water with *A. tumefaciens*, DMSO with *A. tumefaciens*, and cyclic adenosine monophosphate (cAMP) with *A. tumefaciens* were used in all bioassay to allow comparisons across assays.

After inoculation, the plates were wrapped in parafilm and incubated at 25°C for 20 days. The potatoes were kept at a high humidity (not regulated) environment by putting pans of water in the incubator with the potatoes. After 20 days the potato discs were stained with Lugol, a solution made of potassium iodide and iodine diluted in water. The tumors, which did not stain, were counted. Potato discs that were contaminated or dehydrated were not counted.

Capillary Electrophoresis

Extractions

A second set of extractions were prepared for analysis using capillary electrophoresis (CE). Samples of the seeds, leaves, and roots were analyzed on the CE. Three samples of each type of vegetative tissue (roots, partially expanded and fully expanded leaves) from each fertilizer treatment and all seed samples were used. The CE extraction procedure *(Procedure C-4)* was done as follows: 0.5 g of each sample was taken and ground in 9 mL of ethanol by mortar and pestle before the sample could thaw. Before grinding, 1 mL of 0.01 M quercetin suspended in ethanol was also added to the sample as an internal standard. The sample and the solvent was then transferred into a test tube and capped. Samples were then vortexed for 30 sec at maximum speed and sonicated for 50 min. After that the samples were vortexed again for 30 sec at maximum speed and sonicated for an additional 15 min. After allowing the samples to settle for 1 hour, the supernatant was removed from the tissue. The supernatant was then filtered to 0.4 micron and run on the CE.

Standards

Silymarin. silybin, and taxifolin were run on the CE at concentrations of 1000, 750, 500, 250, 100, 50, 10, and 1 μM for peak identification and confirmation of linearity within this concentration range. Each compound was weighed and suspended in 50 mL of ethanol to make three 1000 μM standards. Silymarin was again assumed to have a molecular weight similar to silybin, its major constituent. Each mixture was then diluted with ethanol to make 10 mL of each concentration. Each of these standards was run immediately on the CE and what was not used initially was stored at 4°C.

Instrumentation

The analysis of the samples was carried out on a HP 3-D CE (model # 61600AX, Hewlett Packard, Palo Alto, CA). An uncoated silica capillary with a total length of 43 cm, an effective length of 35 cm, and an interior diameter of 0.05 mm was used for the separation. The capillary column was maintained at a temperature of 30°C, and the voltage was applied at a constant 20 KV at a current of 60 μA. A diode array detector (DAD) was set to record all wave lengths from 190 to 400 nm. Initial analysis of electropherograms took place at 200 nm. Peaks were further identified using their absorption profiles and by spiking the samples with standards. The run time of the separation was 20 min. Before each run, the column was flushed with buffer for five min and after every third run the column was flushed with 1 N NaOH for 10 min, 0.1 N NaOH for 10 min, water for 10 min and buffer for 20 min. Before each flush, the buffers were replenished. Samples were introduced by hydrodynamic injection for 2 sec then were run using simple capillary zone electrophoresis.

Buffer

A borate buffer solution made by mixing a 20 mM borax solution equally with 20 mM boric acid. This solution was adjusted to pH 9 using 1 N NaOH. Methanol was added to the buffer at a rate of 1.5 mL to each 9.5 mL of buffer to produce a 15% methanolic solution as modified from Quaglia et al. (1999).

Statistics

The greenhouse experiment was designed as a randomized complete block design. The bioassay was a randomized complete block design with three replications. All statistical analysis was done using SAS (2002, version 8) and Sigma Plot 2000.

Experiment II – Fall 2001

Hydroponic Production

Experiment II was started in fall 2001. *Silybum marianum* seed that were purchased from Johnny Selected Seeds® and stored for six months at 4°C was planted as in the previous experiment on September 10. Germination occurred between day 4 and 9. When the first set of true leaves started to emerge on day 14, a 10% diluted hydroponic nutrient solution *(Table B-3)* was applied three times a week until planting. One hundred and eight uniform plants were transplanted on day 42, when they had reached between 3 and 5 leaves. The plants were transferred into five-gallon trade pots filled with a 75% perlite and 25% peat mixture. The plants were laid out in pairs so that half the plants could be destructively harvested prior to bloom. The experimental design in the greenhouse is shown in Figure A-9. Seedlings not planted were collected, frozen and stored at -30°C. The transplanted seedlings were watered by an automatic watering system until they were established. On day 53, the respective fertilizer treatments were applied for the remainder of the experiment. Nitrogen was altered to produce low (47.1 mg/L), medium (100.6 mg/L), and high (151.8 mg/L) nitrogen fertilizer treatments *(Table B-5)*. Nutrients solutions were applied to the plants from concentrated solutions diluted by in-line injector systems. A base fertilizer was mixed using Hydrogardens® tomato formula and $MgSO_4$ *(Table B-6)*. The fertilizer flow from the main line was then split into three separate flows and the treatments were added by injectors from concentrated base solutions made of $CaCl_2$ and $CaNO_3$ *(Figure A-10)*. The frequency and length of watering was controlled as described in experiment 1.

Half of the plants were harvested on day 101, between the 8-10 leaf stage. Anthesis had not occurred. One of the oldest non-senescent leaves (between leaf 1 and 4), one leaf from the middle third of the plant (between leaf 4 and 7), and one of the most immature

leaves (between leaf 8 and 10) were taken from each plant as well as stem and root samples. The samples were put in whirl-pac® bags and stored on ice until they could be placed into storage at -30°C. The plants began flowering on day 172. Flowers were hand pollinated once a day using a small camel hair brush. Seed heads were allowed to mature and were bagged when the inflorescence had past anthesis. The seed heads were harvested when the plants died. Plants were harvested from day 284 until day 314, June 21, 2002.

Potato Disc Bioassay

A potato disc bioassay as described by Galsky et al. (1981) was preformed to test the activity of the extracts from sampled *S. marianum* tissue. The plant extracts were applied at the time of infection to the potato discs by *Agrobacterium tumefaciens* (C58) to evaluate the extracts ability to suppress tumor growth.

Extractions

Seed samples were sequentially extracted as described in the previous experiment *(Procedure C-1).* Except that only ethanol and chloroform were used as solvents and that six seed samples from each fertilizer treatment were used. Extractions preformed on the roots, stem and leaves were the same as described in the vegetative extraction procedure *(Procedure C-2).* However, six samples of each tissue type (roots, young, mid-aged, and old leaves, and stem) from each fertilizer treatment were taken.

The Bioassay

All preparations and procedures for the bioassay were the same as described in the previous experiment.

Capillary Electrophoresis

Extractions

Extractions were done as described in the previous experiment for sample analysis by CE *(Procedure C-4).* Samples of the seeds, leaves, stems, and roots were analyzed. Six samples of each type of tissue (roots, young, mid-aged, and old leaves, stem and seeds) from each fertilizer treatment were used.

Instrumentation

All preparations and procedures for running the CE were the same as described in the previous experiment.

Statistics

The greenhouse experiment was as a randomized complete block design with six replications. The bioassay was a randomized complete block design with three replications. All statistical analysis was done using SAS (2002, version 8) and Sigma Plot 2000.

Experiment III – Summer 2002

Hydroponic Production

Experiment III was conducted in summer 2002. *Silybum marianum* seed that were harvested from the spring 2001 experiment and stored for six months at 4°C were planted as in the previous experiment on May 3. When the first set of true leaves started to emerge on day 14, a 10% diluted hydroponic nutrient solution *(Table B-3)* was applied three times a week until planting. One hundred and eight plants were transplanted on day 57, when they had reached between 4 and 6 leaves. The plants were transferred into five-gallon trade pots filled with a 75% perlite and 25% peat mixture. The experimental design was the same as the fall 2001 experiment and is shown in Figure A-9. All seedlings had to be used in the experiment and none were left to be collected for analysis due to poor germination. The transplanted seedlings were supplied with base fertilizer (Hydrogardens® tomato formula and $MgSO_4$) for three weeks. On day 74, the respective fertilizer treatments were applied for the remainder of the experiment. Fertilizer treatments *(Table B-5)* and application methods *(Figure A-10)* were the same as used in fall 2001 experiment. The frequency and length of watering was controlled as described in experiment 1. The plants did not flower and the experiment was terminated on December 1.

IV.

Results

Experiment 1 – Spring 2001

Hydroponic Production

Silybum marianum was successfully grown in the hydroponic system and grew aggressively in the greenhouse environment. Calcium to nitrogen fertilizer ratio treatments did not significantly effect the seed yield which averaged 33.25 g/plant across all treatments *(Figure A-11)*. The plants began producing seed 107 days after planting and finished by 157 days after planting. The plants averaged a height of 120 cm from pot rim to primary flower tip. Basal plant diameter averaged 70 cm.

Potato Disc Bioassay

The solvents methanol, ethanol, acetone, and chloroform removed tumor inhibitive compounds from seeds, inhibiting tumor formation on average 43, 53, 45, and 49% respectively *(Figure A-12)*. Water extracts were inactive in the potato-disc bioassay, with tumor counts insignificantly different from the control. When the seed tissue was sequentially extracted with all the solvents in order from high polarity to low – methanol, acetone, and chloroform were found to remove active compounds. They inhibited tumor formation by 50, 30 and 37% respectively. The water and ethanol sequential extracts did not produce results significantly different from the controls *(Figure A-13)*.

Seeds from calcium to nitrogen ratio treatments in the spring 2001 experiment were analyzed in the bioassay. Calcium to nitrogen ratios had no effect on extract activity in the bioassay ($P > 0.05$). Ethanol and chloroform used in the extractions were effective at controlling tumor growth, inhibiting tumor formation by 51 and 43% respectively *(Figure A-14)*.

Leaves collected from the spring 2001 crop and extracted with ethanol and chloroform exhibited no tumor inhibitive properties when applied to the bioassay ($P > 0.05$). The ethanolic extract of the upper leaves inhibited teratoma growth by 21%, which was not significant ($P = 0.22$). Calcium to nitrogen ratios had a significant effect on the

activity levels of both the chloroform or ethanol extracts ($P = 0.02$), though the effect did not increase the leaf activity to a significant level, with the 1:2 Ca:N ratio increasing activity to 23% over 18 and 9% from the 1:1 and 2:1 Ca:N ratio treatments, respectively.

All standards run in the potato disc bioassay were effective at preventing tumor growth. Silymarin, the mixed flavonolignan standard did not produce linear control of tumor formation. Instead the relationship between concentration and tumor control appeared sigmoidal ($r^2 = 0.95$):

$$y = 64.59\, e^{-e^{\frac{x - 60.59}{55.20}}}$$

Where y is the percent control and x is the concentration of silymarin in µM *(Figure A-15)*. Silymarin at 750 µM concentration inhibited tumor formation by 65%. The highest concentration tested in the bioassay, 1000 µM was not able to be counted due to contamination.

Silybin, the major flavonolignan in silymarin, produced a somewhat linear response when run in the bioassay and was fit to the following equation ($r^2 = 0.68$):

$$y = 0.04x - 30.48$$

Where y is the percent control and x is the concentration of silybin in µM *(Figure A-16)*. Silybin at highest concentration, 1000 µM inhibited tumor formation by 65%.

Taxifolin proved effective at inhibiting tumor production in the potato bioassay as well. The bioassay responded in a quadratic manner to the concentrations of taxifolin tested. The bioassay response to taxifolin was fit to the following equation ($r^2 = 0.91$):

$$y = -(7.69 \times 10^{-5})x^2 + 0.15x + 8.58$$

Where y is equal to percent control and x is equal to taxifolin concentration in µM *(Figure A-17)*. The highest concentration of taxifolin tested, 1000 µM, inhibited tumor formation by 72%.

Capillary Electrophoresis

Standards were run at concentrations of 1000, 750, 500, 250, 100, 50, 10, and 1 µM to determine the CE response to each compound. Good linearity in response was achieved for both silybin ($r^2 = 0.99$) *(Figure A-18)* and taxifolin ($r^2 = 0.98$) *(Figure A-19)*. Taxifolin was not

detectable at 10 or 1 μM. Silybin was not detectable at 1 μM, but was detected at 10 μM. Peak areas for the different compounds were not equal and equal concentrations. Silybin gave far larger peaks than taxifolin.

Seed extracts run using CE exhibited peak separation between the flavonolignan compounds silybin, silychristin, isosilychristin, silydianin, and the flavonoid taxifolin *(Figure A-20)*. Identification of these peaks was done by spiking the samples with standards *(Figure A-21)* and comparing the absorption spectra *(Figure A-22-A-27)*.

Leaf extracts analyzed using CE exhibited three peaks, one of which matched the standards. These peaks were labeled by time of appearance, unknown peak 1 (u1), unknown peak 2 (u2), and silydianin (sd) *(Figure A-28 and A-29)*. Peak areas were compared with samples from the fall 2001 experiment and are further described below.

Experiment II – Fall 2001

Hydroponic Production

Flowering was significantly delayed in the Fall 2001 plants when compared with the Spring 2001experiment. Plants did not begin flowering until 172 days and did not finish seeding until 314 days after seeding. Vegetative yields from the fall 2001 experiment were significantly affected by nitrogen treatments ($P < 0.01$), with plants from the lowest nitrogen treatment (47.1 mg/L N) producing the lowest vegetative yield which was less than half that of the plants from the two higher nitrogen treatments (100.6 and 151.8 mg/L N) *(Figure A-30)*. Seed yield was also significantly affected by nitrogen treatment ($P < 0.01$) *(Figure A-30)*. The plants from the high N treatment had the highest seed yields, which averaged 104.7 g/plant. Plants from the middle N treatment produced the second largest yield, which averaged 50.8 g/plant. The plants on the low N treatment yielded an average of 33.4 g/plant, which was not significantly different from plants exposed to the middle N treatment ($P > 0.05$). Harvest date was not significantly affected by nitrogen treatments ($P > 0.05$), which was probably due to small sample size since the high N treatment appeared to be delayed slightly from the other two treatments by an average of eight days.

Potato Disc Bioassay

Seeds from plant treatments in the fall 2001 experiments were run in the potato bioassay. Nitrogen treatments from the greenhouse experiment had a significant effect on the activity of the ethanolic extractions ($P = 0.03$). The average inhibition of tumor growth by the ethanolic extracts across fertilizer treatments was 58%; however the high N, medium N, and low N treatments had tumor suppression activity of 64, 56, and 53%, respectively *(Figure A-31)*. The medium N treatment was not significantly different from either the high or low N treatments, which were significantly different from each other. The chloroform seed extracts, which inhibited tumor growth in the potato disc bioassay by 50% *(Figure A-31)* were not significantly affected by the nitrogen treatments ($P = 0.48$).

Fall 2001 *S. marianum* root ethanol and chloroform extracts had significant activity in the potato disc bioassay *(Figure A-32)*. The ethanolic extracts had the greater level of tumor inhibition, at 45%. Nitrogen treatments had no effect on the activity levels of the root ethanolic extracts ($P = 0.94$). The chloroform root extract had slightly less activity than the ethanol extract, at 30% tumor control. Nitrogen concentration had no effect on the activity levels of the chloroform root extracts ($P = 0.75$).

Stem ethanolic extracts taken from fall 2001 samples also had significant tumor inhibitive activity, at 43%. Chloroform extracts of the stems, however, had no activity in the bioassay *(Figure A-33)*. Nitrogen treatments had no significant effect on the tumor inhibitive qualities of either the chloroform or ethanol extracts of the stems ($P = 0.22$).

Leaves from the fall 2001 experiment, extracted with ethanol, exhibited significant levels of tumor inhibition when applied to the potato disc bioassay *(Figure A-34)*. The level of control was significantly affected by leaf age ($P < 0.01$), fertilizer treatment ($P = 0.09$), and the interaction of these two variables ($P < 0.01$).

Ethanolic extracts of the immature leaves harvested from the top of the plant inhibited tumor formation. Nitrogen treatments, however, significantly affected that activity. The high N treatment produced the lowest activity levels in the immature leaf extracts, inhibiting only 17% of tumor growth, which was not significantly different than the controls. The medium N treatment produced the highest activity levels in the immature leaf extracts, inhibiting 51% of tumor growth in the potato disc bioassay. The low N treatment

produced significant, though lower, activity in the immature leaf extracts, which inhibited 42% of tumor growth.

The ethanol extract of the leaves from the middle third of the plant had significant tumor inhibition, which varied depending on nitrogen treatment. The low N treatment produced the highest activity levels in the "middle leaf" extracts, inhibiting 45% of tumor growth. The high and medium N treatments did not produce significantly different results from one another ($P > 0.05$), producing activity levels in the "middle leaf" extracts of 36 and 35% inhibition of tumor growth.

The ethanolic extracts of the oldest leaf samples harvested from the bottom of the fall 2001 plants had essentially no activity in the bioassay. Nitrogen concentration did not cause significant differences among treatments, however, the leaves from plants grown in the high N treatment produced significant tumor inhibitive activity, with 30% control, whereas those grown in the medium and low N treatment did not. The low and medium N treatments produced 27% and 26% inhibition of tumor growth from the oldest leaf extracts.

Seedlings collected from the fall 2001 experiment were also run in the bioassay. Ethanolic extracts had significant ($P < 0.01$) tumor suppression activity, inhibiting 54% tumor growth. Chloroform extracts of the seedlings also had significant tumor suppression activity, inhibiting 30% of tumor growth *(Figure A-35).*

Capillary Electrophoresis

Extracts from seed samples collected from the fall 2001 experiment contained silybin, silychristin, isosilychristin, silydianin, and the flavonoid taxifolin and electropherograms were not significantly different from the spring 2001 seed samples *(Figure A-20).* Statistical analysis of the effects of nitrogen treatment on peak area showed no significant differences ($P = 0.8696$). However, a trend was observable. All of the compounds increased slightly with decreasing nitrogen concentrations. The low N fertilizer treatment had slightly, though not significantly, higher levels of silybin, silychristin, silydianin, and taxifolin than the other two treatments.

Ethanolic extracts of roots from the fall 2001 experiment were run on the CE. Three peaks of significant area were selected for statistical analysis. Nitrogen treatment had no

significant effects on peak area. Two of the peaks were identified as silybin and silydianin *(Figure A-36)* by spiking the samples *(Figure A-37)* and comparing the absorption spectra *(Figure A-38)*.

Stems from the fall 2001 experiment run on the CE showed no dominant compound. Only tiny peaks, slightly larger than noise, were observed on electropherograms *(Figure A-39 and A-40)*.

Electropherograms of the ethanol extracts of immature leaves harvested from the fall 2001 experiment showed three distinct peaks. These peaks were labeled by time of appearance as unknown peak 1, unknown peak 2, and unknown peak 3 *(Figure A-41)*. None of these peaks could be identified as any of the standards *(Figure A-42 and A-43)* Peaks 1 and 2 were significantly affected by fertilizer concentration. Unknown peak 1 was significantly lower concentrations in the spring 2001 plant samples than any of the plant samples from the nitrogen treatments from the fall 2001 experiment (P = 0.0033). Unknown peak 2 was in the highest concentration in leaf extracts from the low N treatment used in the fall 2001 experiment and in the lowest concentration in the leaf extracts from the high N treatment used in the fall 2001 experiment and all treatments used in the spring 2001 experiment. The leaf extracts from plants exposed to the medium N treatment from the fall 2001 experiment had statistically similar concentrations of unknown peak 2 when compared with the leaf extracts from the low N treatment.

Leaves collected from the middle third of the plant, when run on the CE, had two dominant compounds in the ethanolic extract *(Figure A-44)*. Neither of these peaks were significantly affected by nitrogen treatment. The second peak was identified as taxifolin by spiking samples *(Figure A-45)* and looking at the absorption spectra *(Figure A-46)*.

Oldest leaf samples taken from the fall 2001 experiment had low levels of compounds in the ethanol extract *(Figure A-47)*. None of the peaks could be identified as a standard *(Figure A-48)*, however "unknown peak 2" from the other leaf samples was present in lower concentrations in the oldest leaf samples *(Figure A-49)*. An additional peak, labeled "unknown peak 5", appeared in many of the lower leaf samples, but was suspected to be a contaminant due to it's erratic appearance in not only these lower leaf samples, but in other samples as well. Peak 1, labeled "unknown peak 2," was significantly affected by nitrogen

treatment ($P < 0.01$), being the highest in leaves from the medium N treatment used in the fall 2001 experiment and the lowest in leaves from the high N treatment used in the fall 2001 experiment. Peak 2, labeled "unknown peak 4," was not significantly affected by nitrogen treatment ($P = 0.45$), though a trend toward higher concentrations of this peak with increased nitrogen levels was observable.

Seedling samples collected from the fall 2001 experiment were analyzed using CE . The electropherograms showed three dominant peaks *(Figure A-50).* None of the peaks could be identified as a flavonolignan or taxifolin through spiking the samples *(Figure A-51)* or through analysis of their absorption spectra *(Figure A-52).* However, the three peaks appear to have the same compounds as found in the immature leaf samples, and were labeled "unknown peak" 1, 2, and 3 in order of appearance.

Experiment III – Summer 2002

Seeds planted for summer 2002 had very poor germination rates, around 60%. When the seedlings were planted into the hydroponic system heat stress killed about a third of the plants. The plants that survived grew very slowly and were evidently stressed by the heat. It became evident that they were not going to bloom quickly and the plants were destroyed.

V.

Discussion

Hydroponic Production

Silybum marianum (L.) Gaertn. can be grown in a greenhouse environment and can be produced in hydroponic culture. The plants did attract many pests, among them, ants, lepidoptera larvae, white fly, aphids, spider mites, sooty old, powdery mildew, and mice. Sprays such as cinamate, diazanon, pyrethrum, *Bacillus thuringiensis*, powdered sulfur immersed in water and 'Safer soap,' an oil emulsion, were effective at controlling most of the pests without damaging the plants. None of these pesticides are registered for use on *S. marianum* but appear to be safe for use on these plants. Since there are no registered pesticides for *S. marianum*, organic production seems a viable option.

Commercial Viability

Based on the design of the fall 2001 experiment, the yields that were achieved were suboptimal. Hamid et al. (1983) reported a seed yield of 148.3 g/m^2. If the maximum average seed yield had been achieved from all plants allowed to seed, yield per area used in the greenhouse would have only been 0.5 g/m^2. Vegetative yields would have only amounted to 19.6 g/m^2. Yield per plant in the hydroponic system was very good. Work done by Adzet et al. (1987) who evaluated 58 populations of *S. marianum* claims that the plants produced between 3 to 5 inflorescences, each of which yielded an average of 3.9 g per florescence. At maximum, based on these numbers, their yields were around 19.3 g per plant. Even the plants in the greenhouse subjected to the low N treatment produced higher yields of 33.7 g/plant. The problem is rather, that there were not enough plants. The density chosen for the greenhouse experiments was intentionally chosen to prevent any effects due to crowding. For production plants need to be grown at a higher density for viabile production. Austin et al. (1999) noted that *S. marianum* produced substantially higher number of stems with increased density per pot in monoculture. Competition for space may increase the amount of energy that the plant puts into seed production, increasing yield per area. It is difficult to give a cost value to the crop, no references were located on the cost of seed going to the medicinal industry; however, several seed companies were selling *S.*

marianum seeds online in the 2002 for about $3.00/g. Using the seed yield per area figured above, the fall 2001 crop would have made $1.41/m^2, though each plant, using average total yield across all nitrogen treatments, would have made $153.88/plant.

Triggering Anthesis

The extreme differences between the length of the spring 2001 experiment and the fall 2001 experiment as well as the failure of the summer 2002 experiment point to the need for a greater understanding of the mechanism that triggers flowering in *S. marianum.* Martin et al. (2000) suggested that the flowering of *S. marianum* may be triggered by a combination of temperature and day length. They found a nearly linear relationship between these two factors reporting that for every hour that day length is increased above 13.3 hours, the thermal time requirement (where the temperature was above 5°C) for the plants decreased by 454 degree days. Martin et al. (2000) suggested, however, that the correlation of these two factors and the length of the vegetative stage was not very high and suggested that other factors, such as vernilization may be involved. The spring 2001 experiment demonstrates evidence against the need for vernilization prior to *S. marianum* blooming. The greenhouse temperatures during the entire experiment did not drop below 15°C, nor were there long periods of time where the temperatures were below 20°C *(Figure A-53*). This temperature could conceivably be cool enough to vernilize the plants, which are native to a warm Mediterranean climate, but is seems unlikely. However, the summer 2001 experiment may support such a supposition. The summer 2002 plants did not bloom even though they were transplanted into the hydroponic system at nearly the longest day of the year and grew for nearly 1 ½ months at day lengths longer than 13 hours *(Figure A-56*). However, temperatures in the greenhouse were never below 20°C for the entire length of the summer 2002 experiment and may have somehow inhibited bloom formation.

Day length seems more critical for blooming than temperature. In the spring 2001 experiment, the plants were never exposed to day lengths shorter than 11 hours *(Figure A-55)* whereas in the fall 2001 experiment, shortly after transplant into pots the day length dropped below 11 hours and the plants did not reach anthesis until the following year when day length had increased past 11 hours *(Figure A-55 and A-56).* Induction of bloom would have probably occurred before 11 hours, since the plants bloomed so quickly following that

day length. The summer 2002 experiment clearly demonstrates that day length over a certain number of hours is not the only requirement for bloom induction. Perhaps decreasing light levels over the course of the experiment inhibited flowering. Slafer et al. (1994) found that wheat cultivars responded to different rates of increasing photoperiod when they varied the speed at which they increased light levels supplied the wheat seedlings. The wheat grew faster from the seedling emergence phase to the terminal spikelet initiation phase, increasing growth rate in conjunction with the rate of change of photoperiod. If plants respond to increasing day length, they likely respond to decreasing day length as well. *Silybum marianum* grown in the summer 2002 experiment may have responded to the decreasing light levels, preparing for overwintering instead of flowering. More work needs to be done to determine the timing of both day length and temperatures and how they relate to bloom time.

Post Anthesis

Temperature may have played more of a role in the rate of senescence. The length of time that the plants in the spring 2001 experiment bloomed may have been closer to the plants in the fall 2001 experiment if the temperatures in the greenhouse had not reached the extremes that they did in 2001. The temperatures during the bloom period of the fall 2001 experiment did not exceed 33°C *(Figure A-54)*, whereas the temperatures during the bloom period of the spring 2001 experiment spiked several times above 40°C *(Figure A-53)*. The extremely high temperatures may be responsible for the more rapid decline of the plants in the spring 2001 experiment and may account for some of reason the yields were smaller in the first experiment.

Effects of Fertilizer Treatments

The calcium to nitrogen ratios used as treatments in the spring 2001 experiment had no effect on yields. There were relatively high concentrations of all the elements in the nutrient solution given to the plants *(Table B-4)*. There may well have been luxurious amounts of nitrogen being applied to the plants so that a shortage of calcium as an accompanying cation to nitrate was not an issue. However, calcium may have been supplied in sufficient quantities to satisfy the nutritional needs of *S. marianum* plants at the lowest concentration supplied to the plants (128 mg/L).

The response of the plants in the fall 2001 experiment to the nitrogen treatments supplied to them implies that 151.80 mg/L, the high N treatment may not be sufficient to maximize yields. All nitrogen treatments increased both the seed and vegetative yields suggesting that these nitrogen treatments used did not exceed the critical nitrogen level for maximal yields suggesting higher nitrogen amounts may be necessary.

Bioassay Activity and Plant Chemistry

As described in the materials and methods section, a crown gall bioassay was preformed. Crown gall is caused by *A. tumefaciens*, bacteria that attacks plant cells by inserting a Ti plasmid and causing uncontrolled, tumor-like growth in the transformed cells (Stafford 2000). This bioassay takes advantage of this natural interaction, inoculating potato with the bacteria and then applying extracts to see if the tumor growth can be prevented. Though the exact action of inhibition by various chemicals on this bioassay have not yet been elucidated, and probably vary with chemical, the bioassay has been shown to be very accurate when compared with mouse P388 leukemia protocol assays, only demonstrating two false positives and one false negative in a twenty-one compound study (Galsky et al. 1981). This being said, the results of the assay preformed on *S. marianum* extracts are very promising. Not only did seed extracts have activity, which coordinates with previous anti-cancer studies on silymarin (Bhatia et al. 1999; Jiang et al. 2000; Kang et al. 2001), but other parts of the plants had activity as well. This does not agree with general assumption that other parts of *S. marianum* do not have medicinal activity.

The seed extract activity was positively affected by increased nitrogen treatment in the fall 2001 experiment. Comparing the results of the spring 2001 bioassay with the fall 2001 bioassay, it appears that even higher nitrogen treatment (257.3 mg/L) may have detrimental effects on the anti-teratoma activity exhibited by the vegetative tissues in *S. marianum.* Nitrogen affects the composition of plants more than any other mineral nutrient. Marschner (1995) suggested that nitrogen supply effects the competition for photosynthates among different metabolic pathways. When nitrogen is suboptimal, as may have been the case with the fall 2001 crop, more carbon is directed down pathways for the production of more carbohydrates, storage lipids, and oils, which causes the total production of plant

compounds to increase by plant area (i.e. leaf area, shoot height). Increasing nitrogen levels, however causes more sequestration of nitrogen, diverting carbon away from carbohydrate and lipid pathways (Marschner 1995). Since the active compounds in the seeds are flavanolignans, which are produced from a branch of the shikimate acid pathway which does not require nitrogen assimilation, they may be positively affected by slight levels of nitrogen deprivation. It has also been demonstrated that DAHPS, an enzyme in the shikimate pathway, is induced by nitrogen starvation and that the production of phenols from this pathway is a normal stress response in most plants (Herrmann and Weaver 1997). However, at more extreme levels of nitrogen starvation, the compounds may be negatively affected due to part of their structure coming from lignans, which actually increase, rather than decrease with increasing nitrogen supply, since the precursors of the compounds are tyrosine or phenylalanine (Marschner 1995).

Interestingly, the CE data does not correlate with the nitrogen effects on activity. None of the concentrations of silybin, silychristin, isosilychristin, silydianin, or taxifolin were significantly altered by nitrogen concentration. These results correlate with much of the published research on *S. marianum*. Omer et al. (1995) found that the silymarin content (the mixture of the aforementioned compounds) in the seeds were not significantly affected by the nitrogen or potassium treatments applied. Hammouda et al. (1993) reporteded that nitrogen treatment made a difference in silymarin content in field trials, but the differences were very small compared to the concentration differences caused by watering treatments, despite very large changes in total nitrogen added to the soil. The affect of nitrogen on bioassay activity of the seed extracts may be due to very small changes in all of the compounds, that when combined produce a significant effect. Another possible explanation for activity variation may be due to additional compounds being dissolved in the raw ethanol extract that do not appear when using traditional extraction procedures. The traditional extraction procedure for isolating silymarin involves a soxhelet extraction in petroleum ether for at least four hours and then another extraction in boiling methanol for two five hour periods. The methanol extracts are then dried to give silymarin (Quaglia et al. 1999). This procedure could easily destroy or remove the active compounds that may be showing the additional activity demonstrated in the raw ethanol extract. Which appears to be the case

with isosilychristin, which appears in the raw ethanolic extract of the seeds *(Figure A-20)* but does not appear in the silymarin standard. Such an active compound may be found in particularly low levels and show up on the electopheragram as one of the many small peaks that were not closely examined due to the extremely low concentration. If this hypothetical compound was extremely different in structure from the flavonolignans, the CE may not have detected the compound because analysis was only done using the UV range (190 – 400 nm).

Root tissue from the fall 2001 experiment also demonstrated activity in the bioassay. This is a very interesting finding, since no other work on root activity of *S. marianum* has been done. If the flavanolignans that are present in the roots can be harvested effectively, production of these plants may become more profitable since the useable portion of the plant would be greater. It makes sense that silybin, at least, would be found in the roots since it has been found to be an iron chelator (Borsari et al. 2001) and would be beneficial in roots and root exudates for this purpose. The greatest amounts of mRNA for the inducible isoenzymes of the shikimate pathway are found in the roots as well as the flowers (Herrmann and Weaver 1997), which increases the likelihood of finding some secondary metabolites in the roots even if they were not silymarin compounds. From the CE data it appears that there may be an additional compound besides the six standard chemicals found in silymarin that may be bioactive, however isolation of the peaks needs to be done in order to fully test its activity.

Stem ethanolic extracts from the fall 2001 experiment were also active in the bioassay. However, no peaks were identifiable above the baseline noise when the samples were run on the CE. This lends credibility to the hypothesis of the presence of a compound in the plant that is either active in extremely low concentrations or that is not being detected by the DAD detector in the CE in the absorption spectra used. It may also be that the compounds that are active adhered to the column and did not elute until the 5 min buffer flush that was run prior to the next sample run. Potentially, the compounds did not separate in the chemistry that was used for analysis; however, a large peak will generally appear on the electropherogram if compounds are just co-eluting.

The immature and middle leaf samples taken from the fall 2001 experiment showed activity in the potato bioassay. This is a different result than the spring 2001 experiment, where neither the upper or lower leaves had activity in the assay. This is likely due to the differences in the stage at which the leaves were harvested. The leaves from the spring 2001 experiment were harvested during anthesis where as the leaves harvested from the fall 2001 experiment were harvested pre-anthesis. The physiological difference between pre-anthesis and anthesis is very notable, with the plant producing many different chemicals from one growth stage to the next. The largest peaks found in the immature leaves of the fall 2001 plants, which were not any of the silymarin compounds, were found in lower levels in the spring 2001 immature leaf samples and silydianin was found in the spring 2001 immature leaf samples, but not the Fall 2001 ones *(Figure A-57).* The activity of these compounds appears to be highly variable depending on nitrogen treatment, as seen in the bioassay from fall 2001. The interesting thing, however, is that none of the three peaks in the immature leaf samples or the two peaks in the middle leaf samples correlated to the changes in activity of the samples by fertilizer treatment. Enough samples may not have been run to see significant differences since the CE has a higher level of variability between electropherograms. The peaks may not be representative of the active compounds, which for some reason are not showing up on the electropherograms. The additive changes of many compounds may alter the bioactivity of samples. The only way to confirm or discredit the activity of these compounds is to fraction collect them and run them in a bioassay.

One peak that seems particularly interesting is "unknown peak 1." This peak appeared in all of the active extracts from the vegetative portion of the plant except for the stem extract. The 3D electropherogram of the samples show it to be the same compound. It appeared to absorb the most at wave lengths shorter than 220 nm – into vacuum UV wavelengths. This compound may be fairly high in concentration, but since the analysis was done at short wavelengths, where its absorption is not great, it could be overlooked easily. Another peak that does not absorb significantly at 220 nm, and therefore does not appear above the noise on the line electropherograms is a peak that elutes at around 9.6 min. It is present in the immature and middle leaf samples and the seedling samples. It appears that the maximum absorbance of that compound is between 240 and 280 nm. "Unknown peak

2" and "Unknown peak 3" appear to have very similar UV absorption patterns as taxifolin and may be flavonoids, which increases their likelihood of being biologically active, since so many flavonoids act as free radical scavengers, UV protectants, antifeedants, and in other biologically important roles (Buchanan et al. 2000). Also, many flavonoids, have been shown to have anti-cancer activity (Kobayashi et al. 2002).

The ethanol extracts of seedlings harvested from the fall 2001 experiment had excellent activity levels, on average at 55% tumor control. This activity is not as high as the average activity of the seed extracts, but is still extremely high. Due to the far higher biomass produced by the seedlings, the overall yield of activity may be higher in the seedlings than the seeds. Producing seedlings instead of seeds would be better for the grower because of the much larger amount of useful biomass that could be produced and the speed at which it could be produced. Even though the compounds that make the seedlings active are not the traditional silymarin compounds, these compounds appear as affective at controlling tumor growth and could be just as useful.

Conclusions

Silybum marianum grows well in a greenhouse environment and can be produced in hydroponic culture. Hydroponic cultivation produced higher seed yields per plant than reported in field trials. However, many details still need to be evaluated to make production economically feasible including planting density and triggering anthesis. High levels of nitrogen are clearly needed to produce maximum yields with high levels of extractable activity. Seeds of *S. marianum* are not the only parts of the plant that produce active compounds. Activity appears to be confined to areas of new growth and the roots. Vegetation after anthesis does not appear to retain active compounds, whether it is due to compound degradation or migration to the flower head and seeds. The distribution of active compounds mimics closely the distribution of shikimate pathway activity in the plant and may give a clue as to what the active compounds are, since most of them are not the traditional compounds of interest found in silymarin. Further work needs to be pursued on the action of the compounds as anti-cancer agents as each holds potential to be an effective treatment.

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Appendix A - Figures

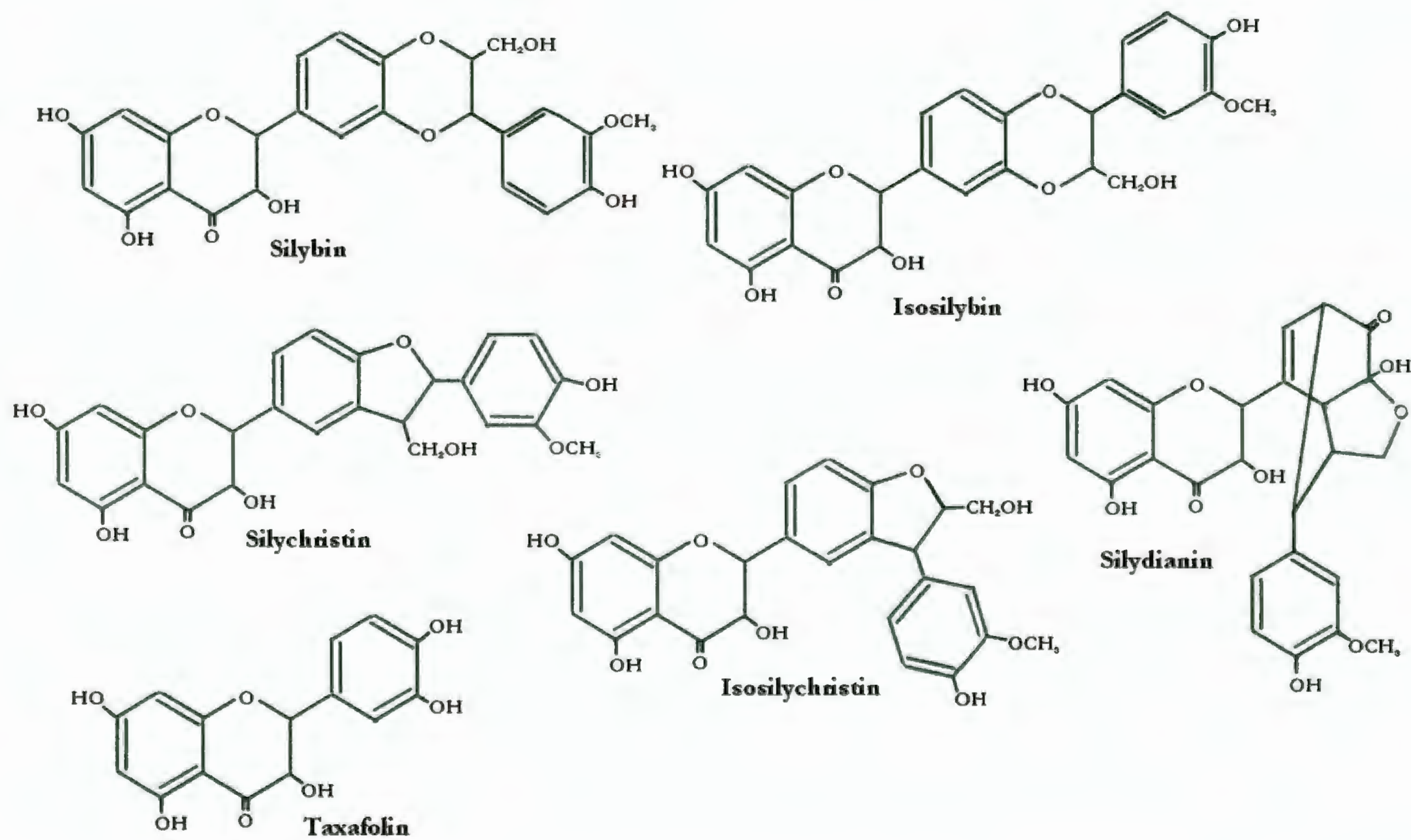
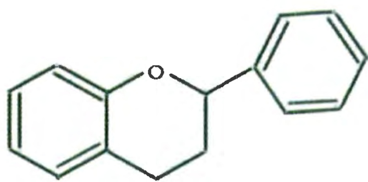
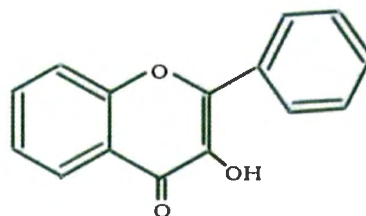
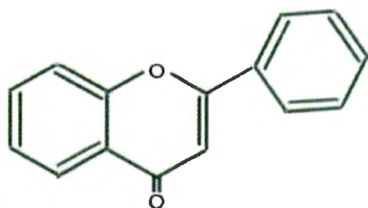


Figure A-1 - The active compounds found in silymarin: the active extract of *Silybum marianum*







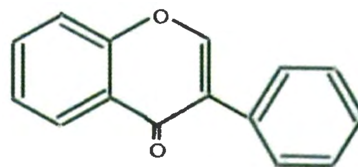


Figure A-2 - Base flavonoid structures

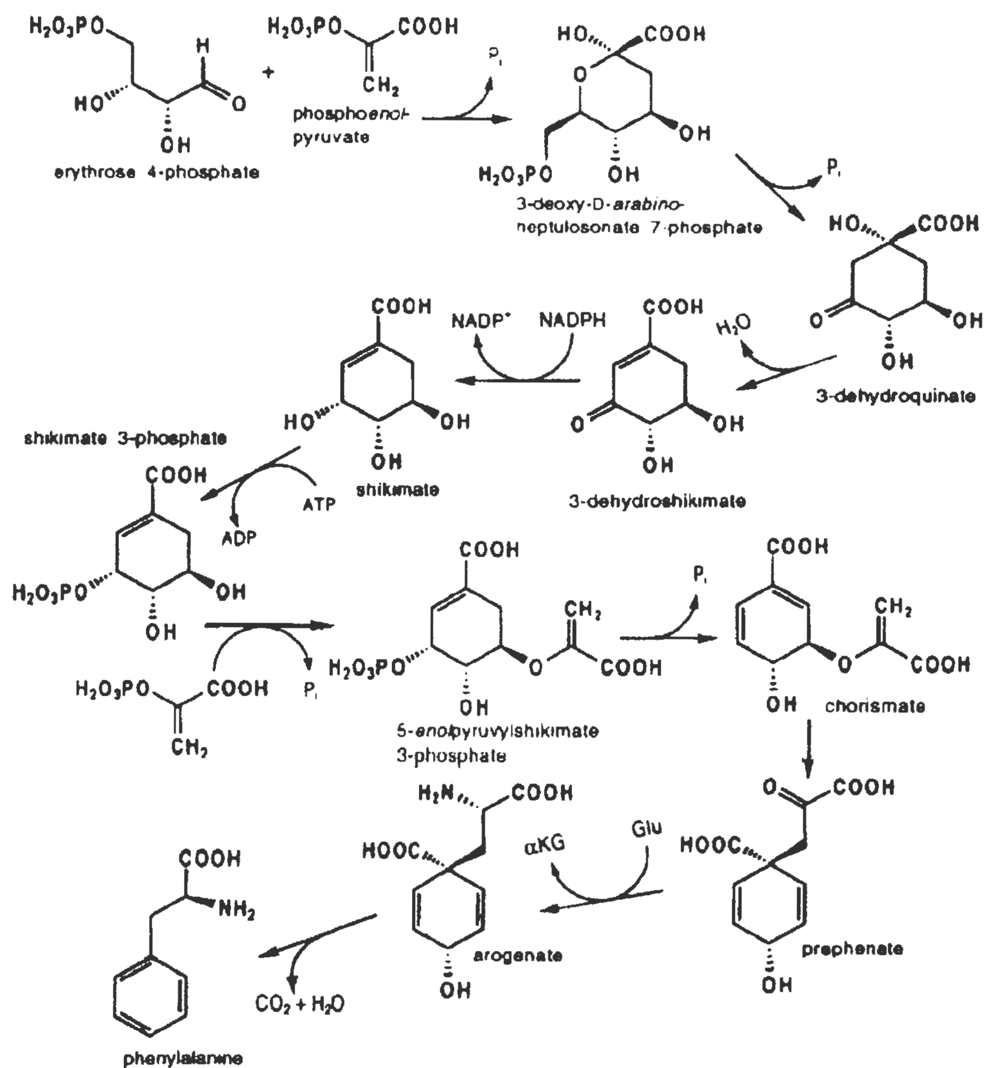


Figure A-3 - The shikimate acid pathway from erythrose 4-phosphate to phenylalanine

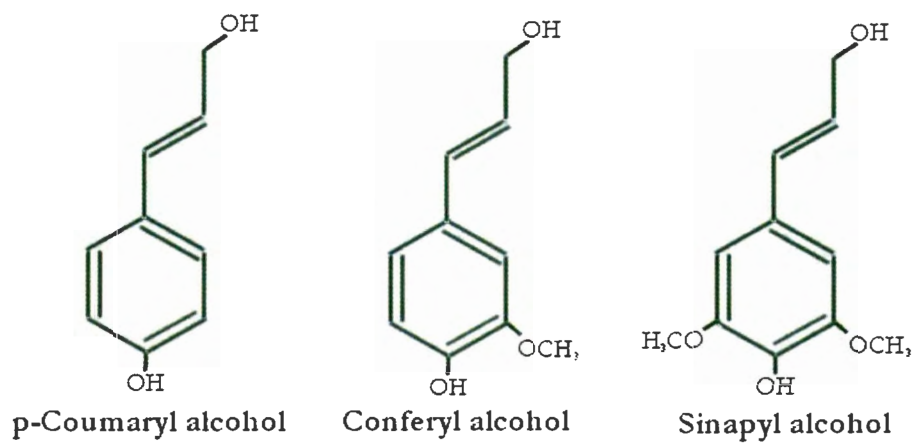


Figure A-4 - Three examples of monolignol structure.

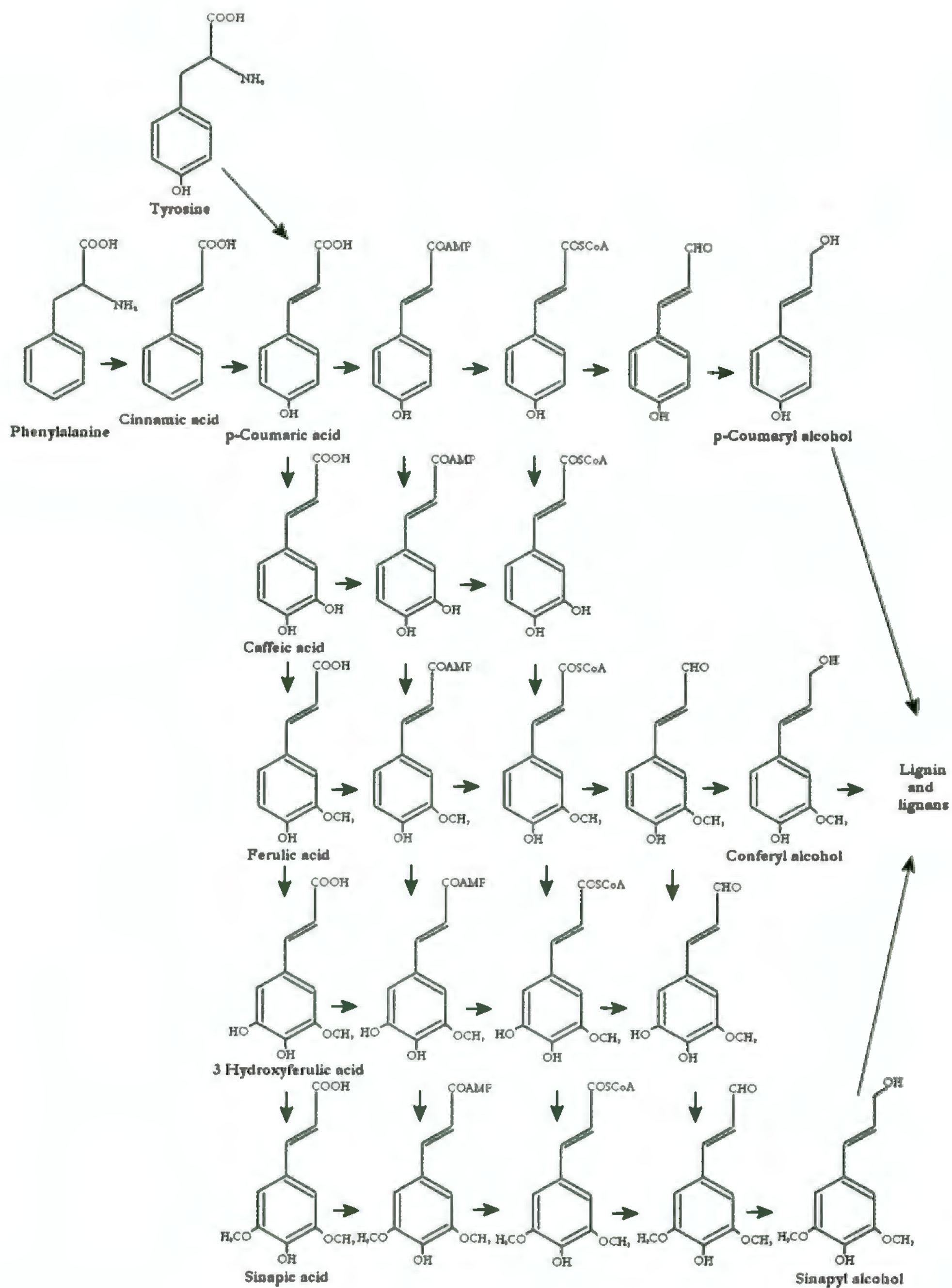


Figure A-5 - The metabolic grid synthesis of monolignols
(Interpreted from Buchanan et al. 2000)

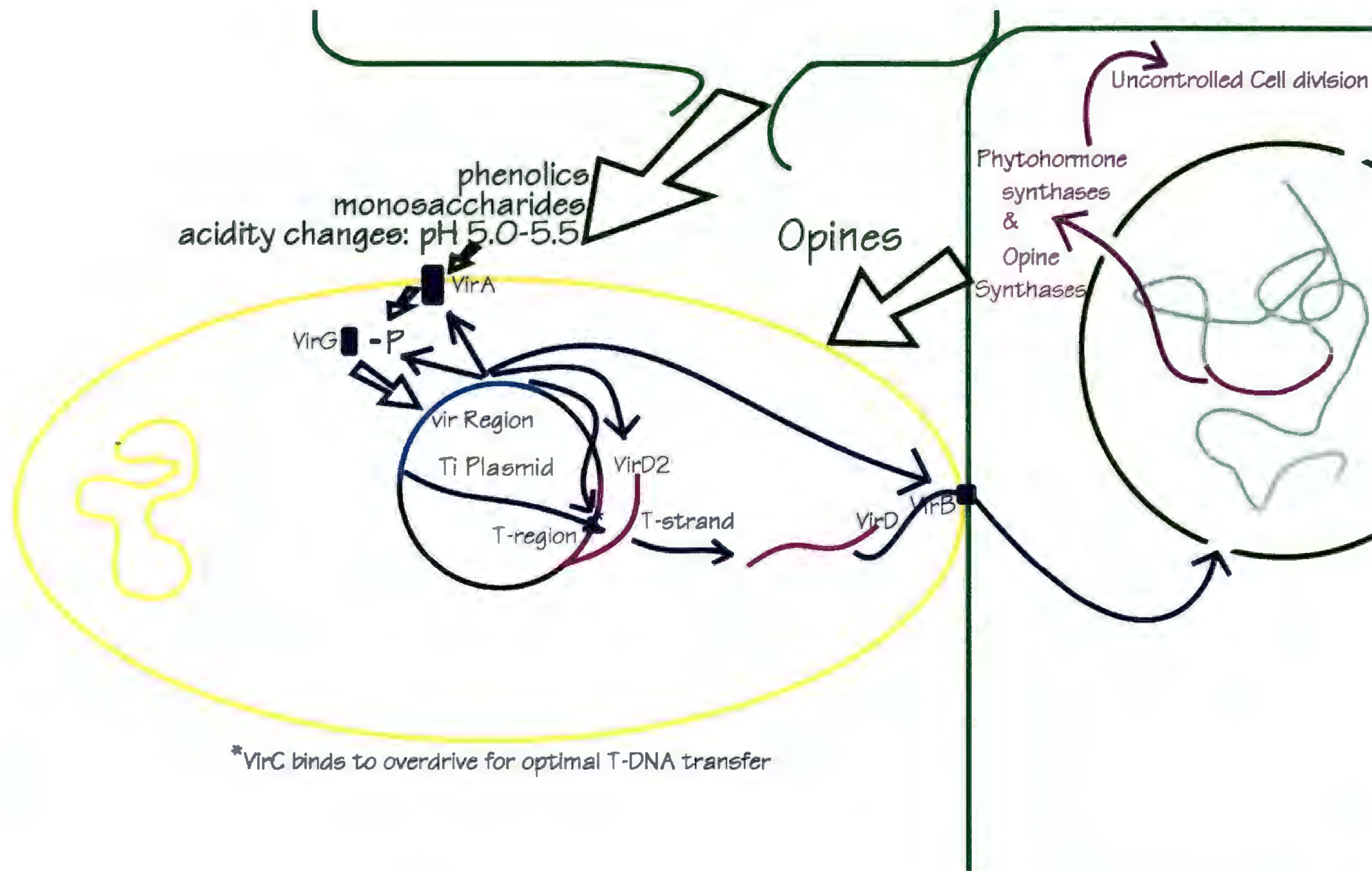


Figure A-6 - The steps involved in the signaling of *Agrobacterium tumefaciens* and the subsequent transformation of the plant cell.

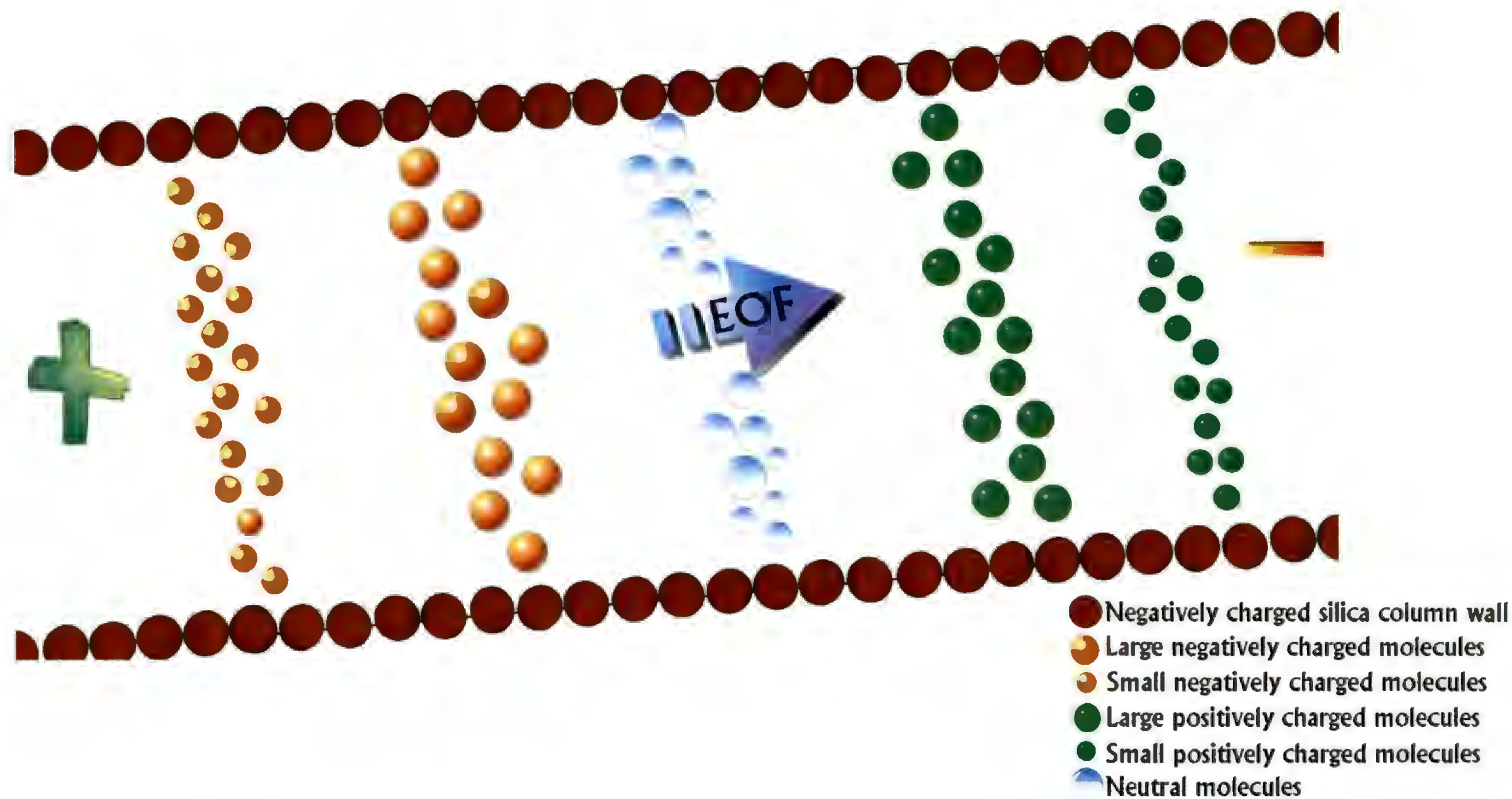


Figure A-7 - Solute migration in Capillary zone electrophoresis. The neutral and negatively charged particles are pulled toward the anode by the electro-osmotic flow (EOF). (Interpreted from Heiger, 1992)

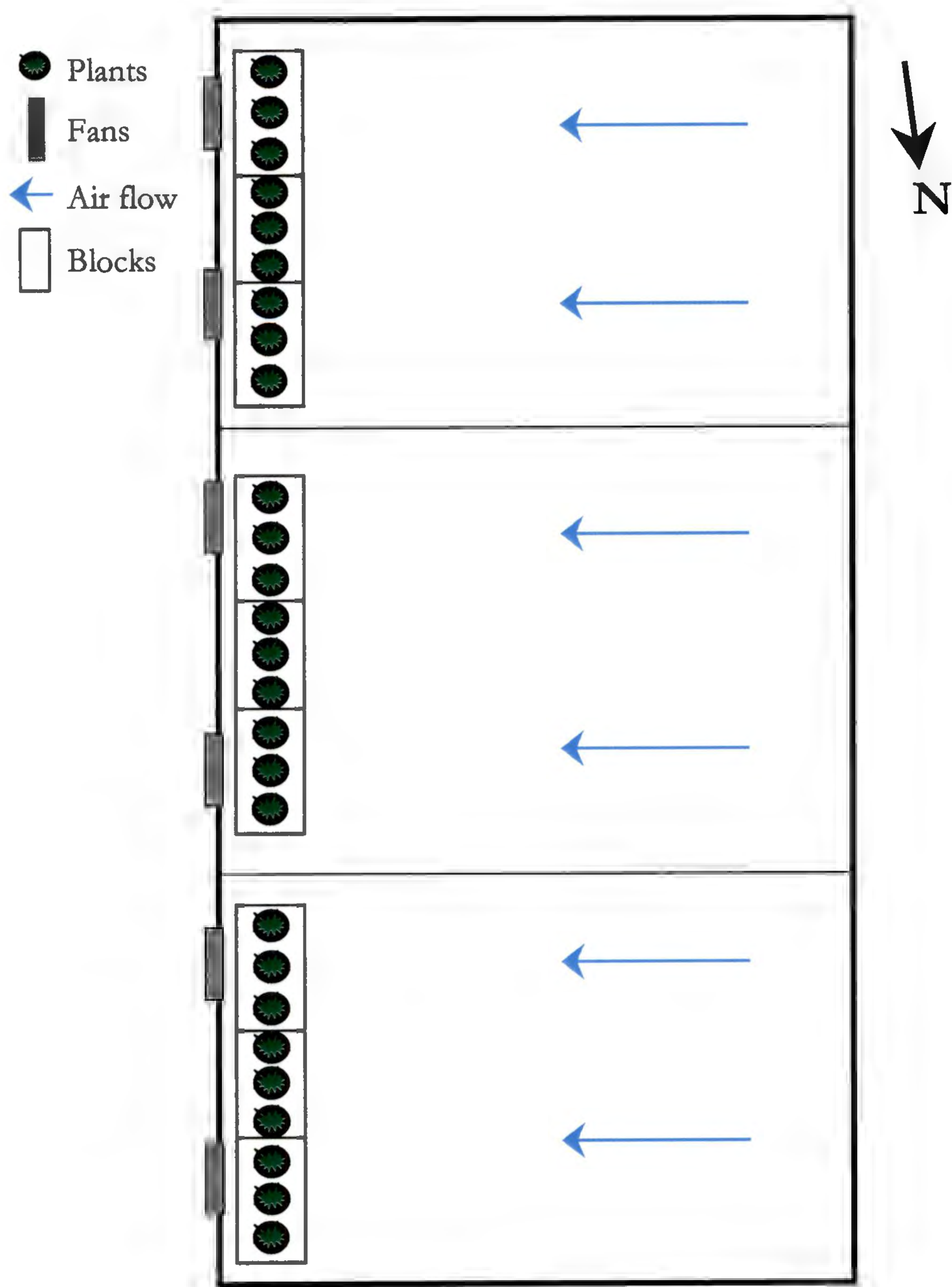


Figure A-8 - Arrangement of the spring 2001 greenhouse experiment. Plants were blocked by proximity to the cooling fans.

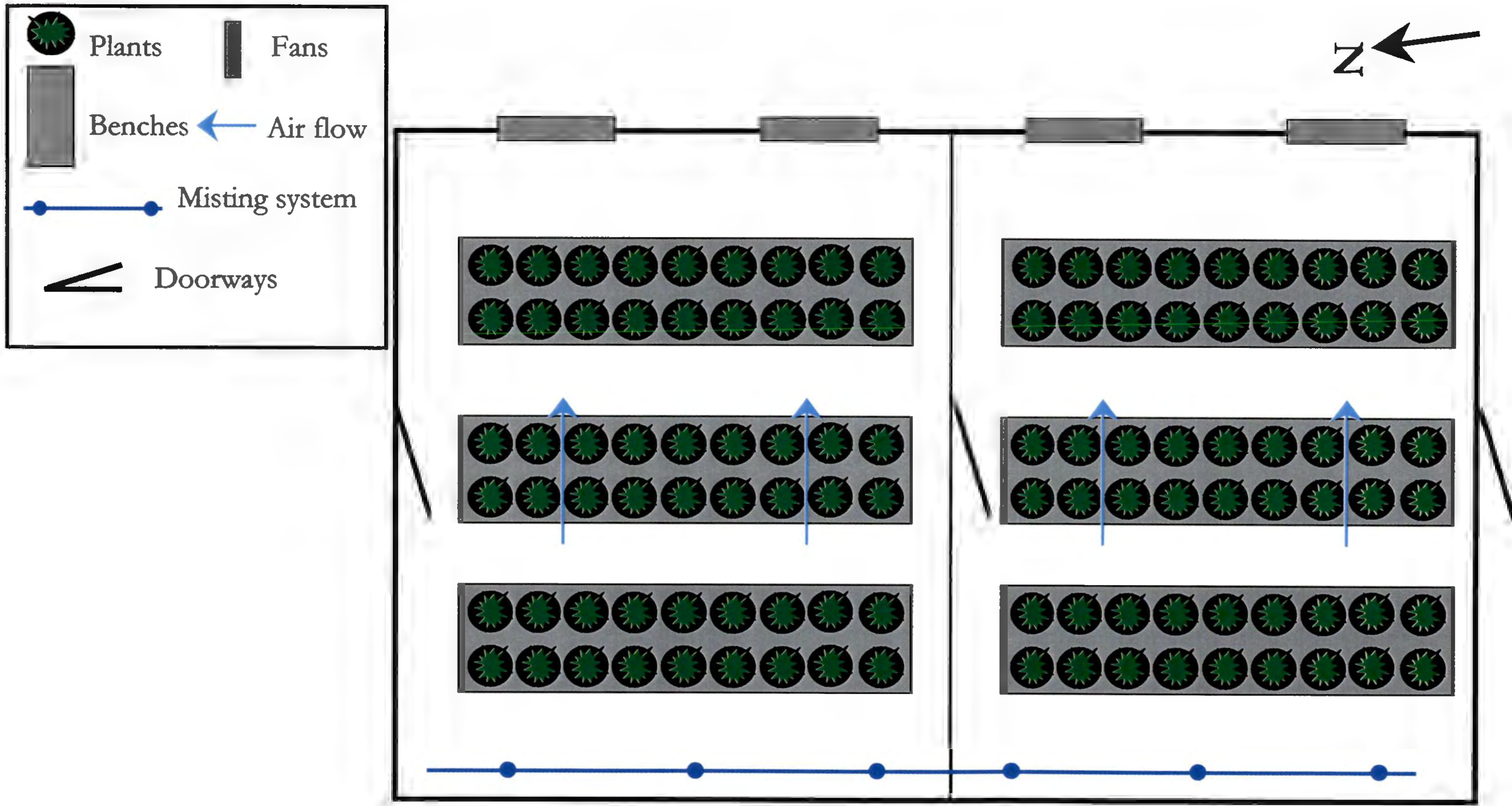
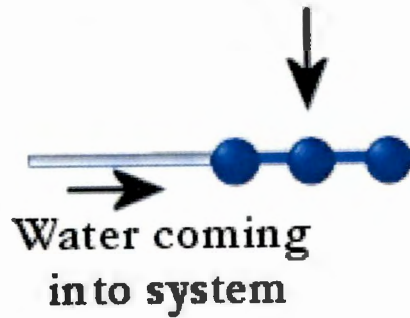


Figure A-9 - Arrangement of the fall 2001 greenhouse experiment. Plants are blocked by bench.



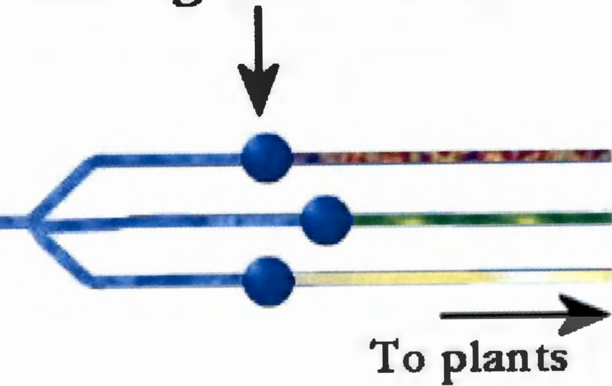


Figure A-10 - Fertilizer Injection layout.

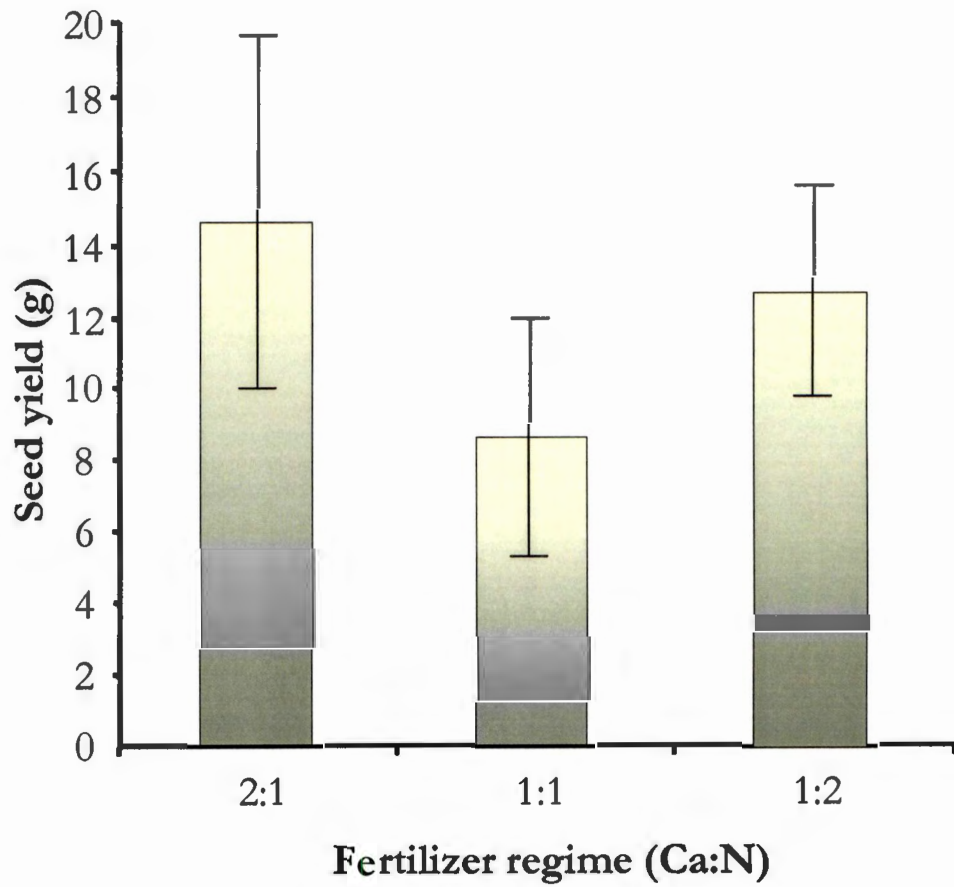


Figure A-11 - Seed yields from Spring 2001 experiment in grams.

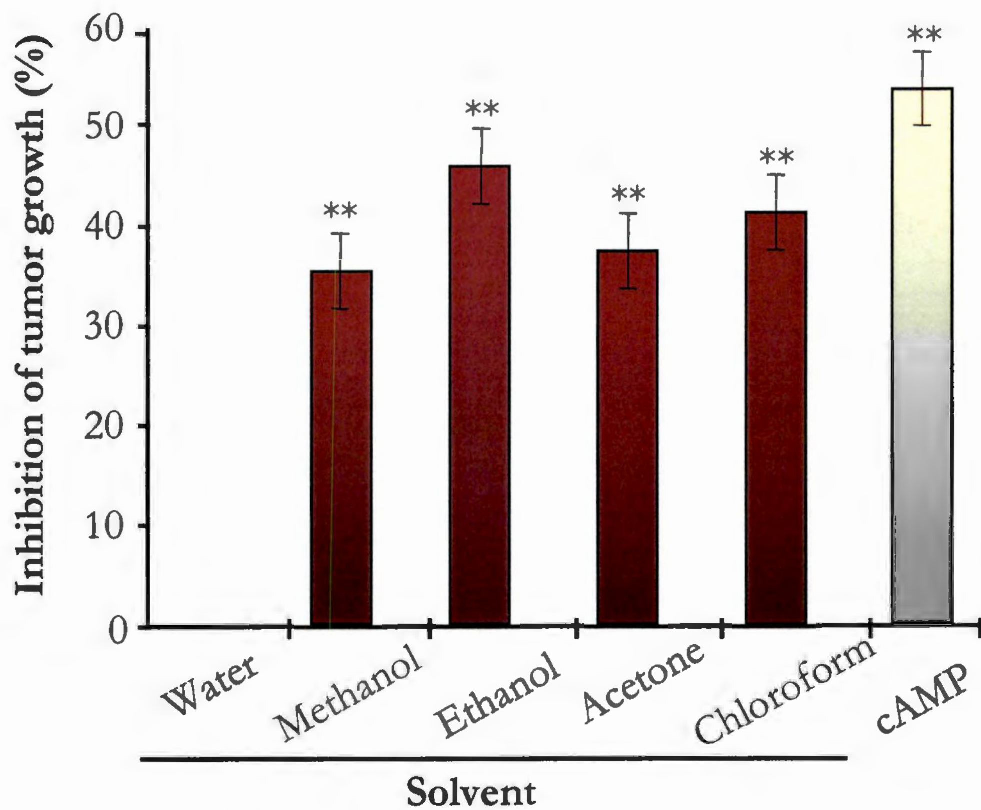


Figure A-12 - Percent tumor inhibition of all extracts with a single solvent extraction. Adenosine cyclic monophosphate (cAMP) is a known anti-carcinogen. ** denotes tumor formation significantly different from controls ($P<0.01$).

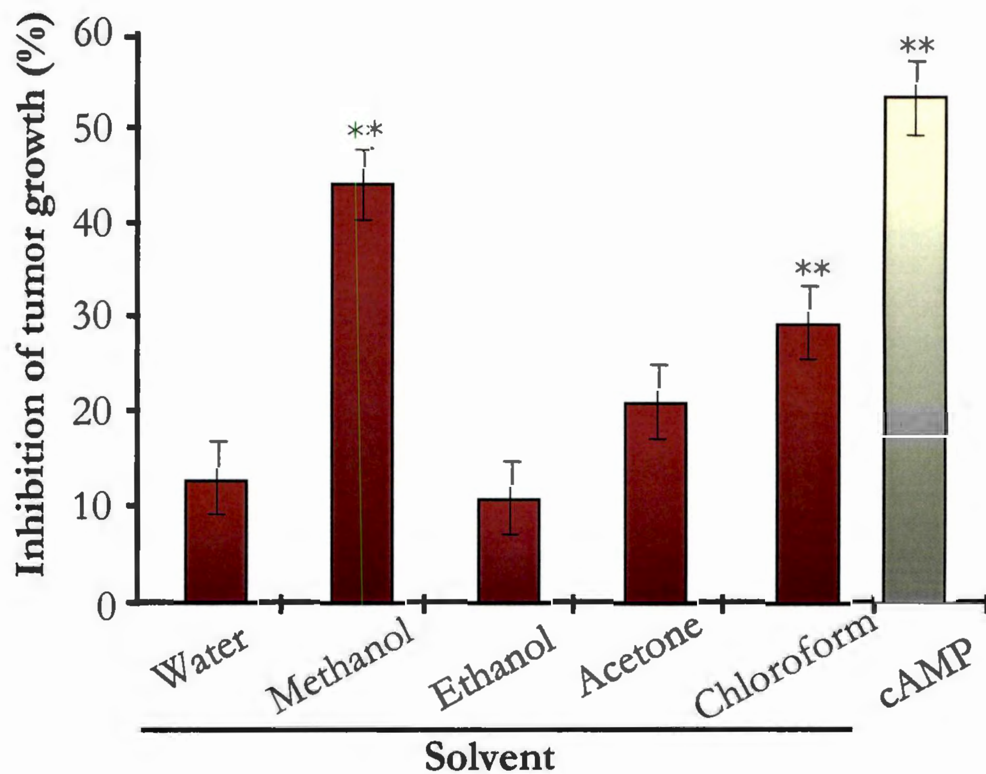


Figure A-13 - Percent tumor inhibition of all extracts with a sequential solvent extraction from highest polarity solvent to lowest polarity solvent. Adenosine cyclic monophosphate (cAMP) is a known anti-carcinogen. ** denotes tumor formation significantly different from controls ($P<0.01$).

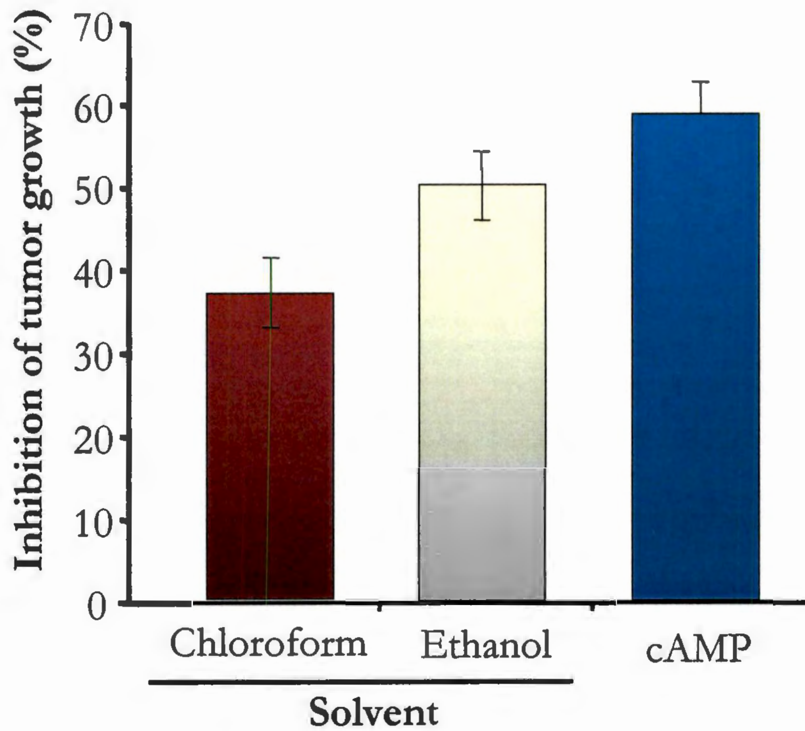


Figure A-14 - Percent tumor inhibition of seed extracts from the spring 2001 experiment. Ethanol and chloroform extracts were significantly different (P <0.05).

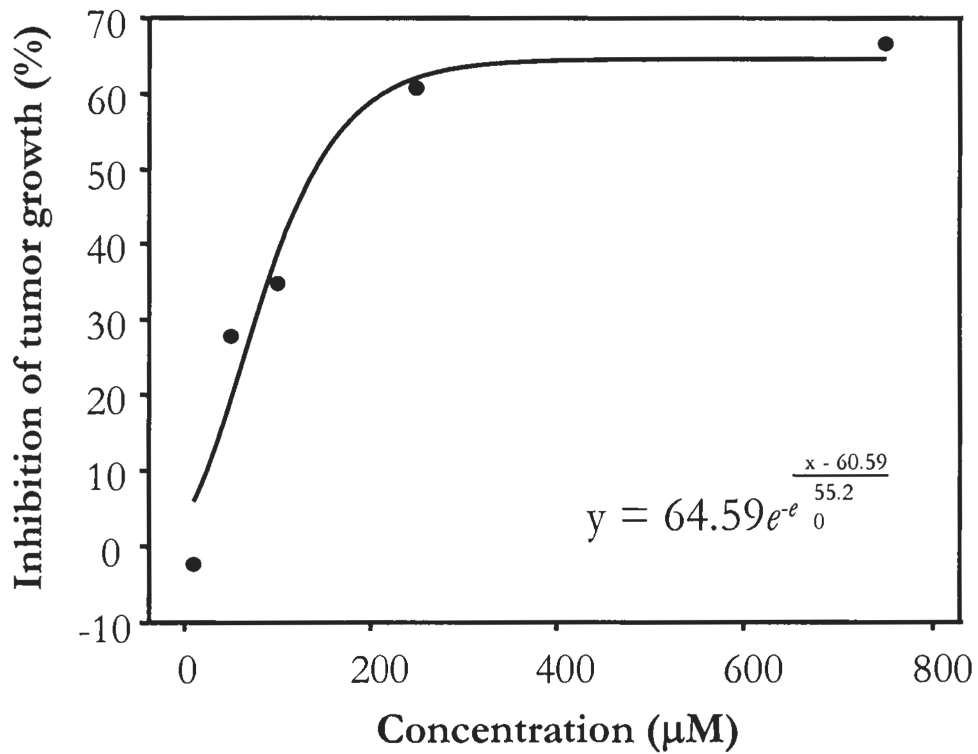


Figure A-15 - Percent inhibition of tumor growth by silymarin as related to concentration in μM (r^2=0.95).

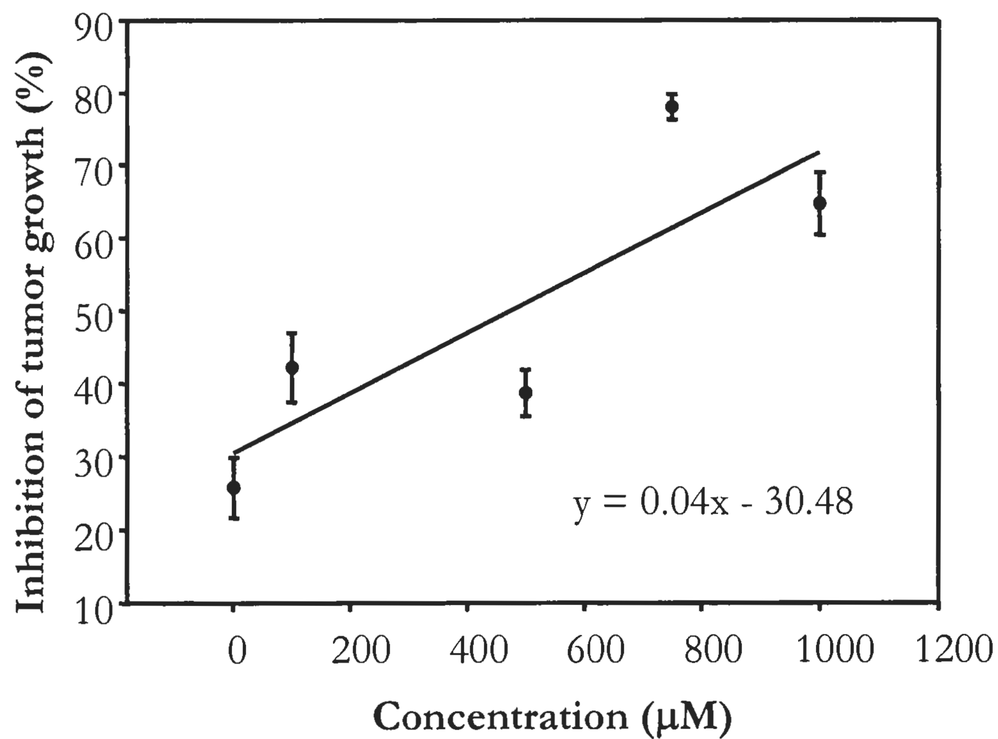


Figure A-16 - Percent inhibition of tumor growth by silybin as related to concentration in μM (r^2=0.6841).

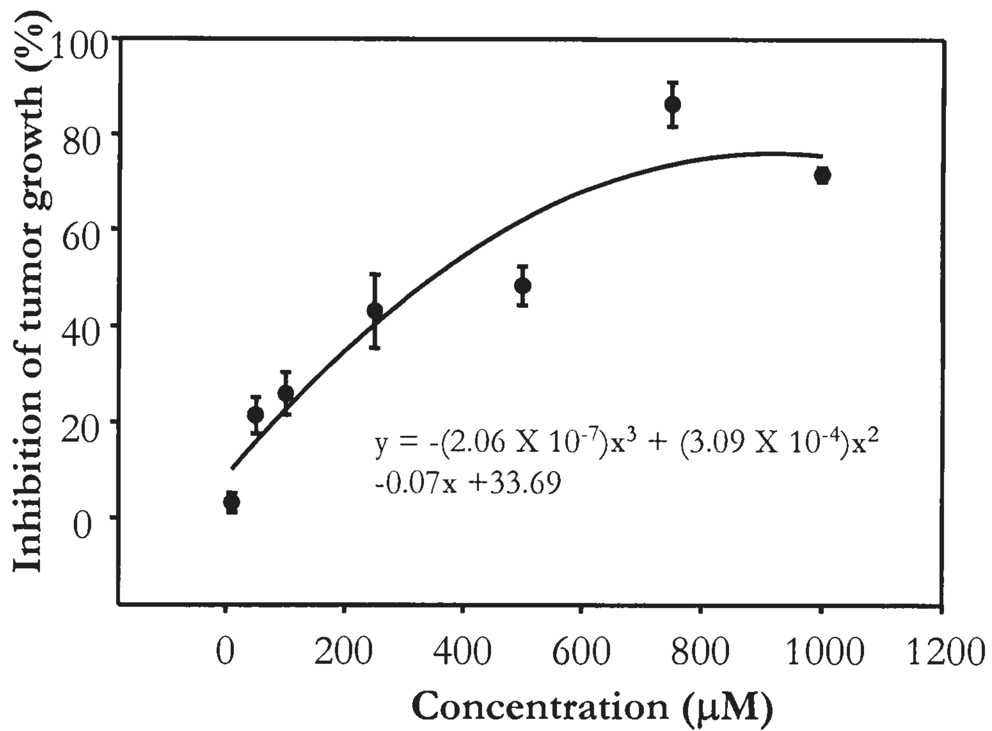


Figure A-17 - Percent inhibition of tumor growth by taxifolin as related to concentration in μM (r^2=0.9099).

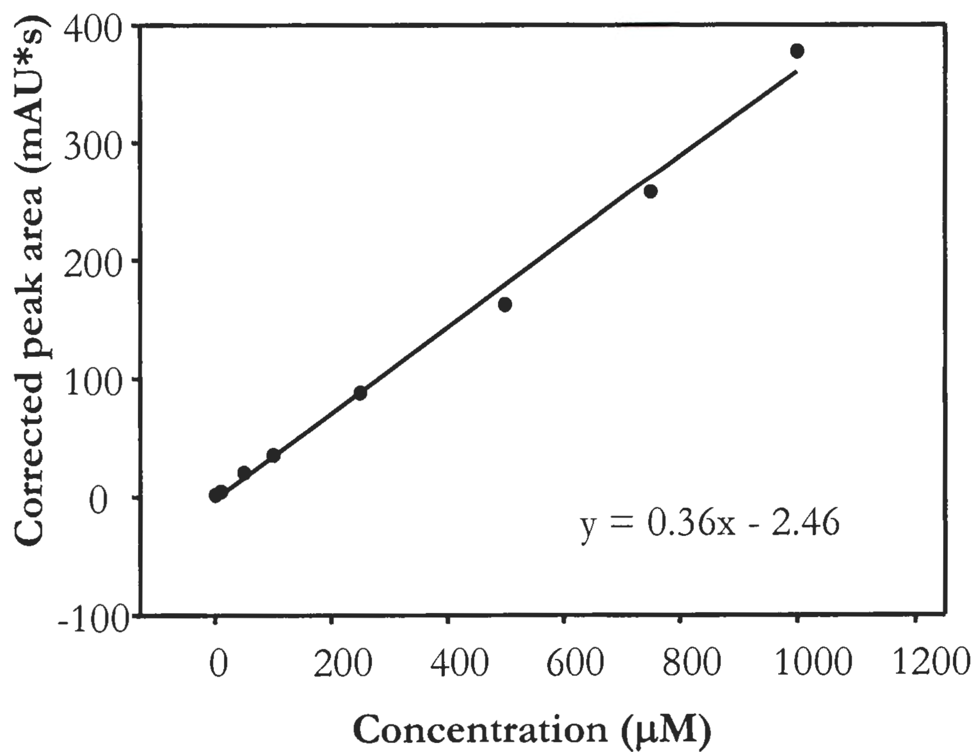


Figure A-18 - Capillary Electrophoresis response to silybin concentration in μM as represented by peak area (r^2=0.99). Peak area was corrected by elution time.

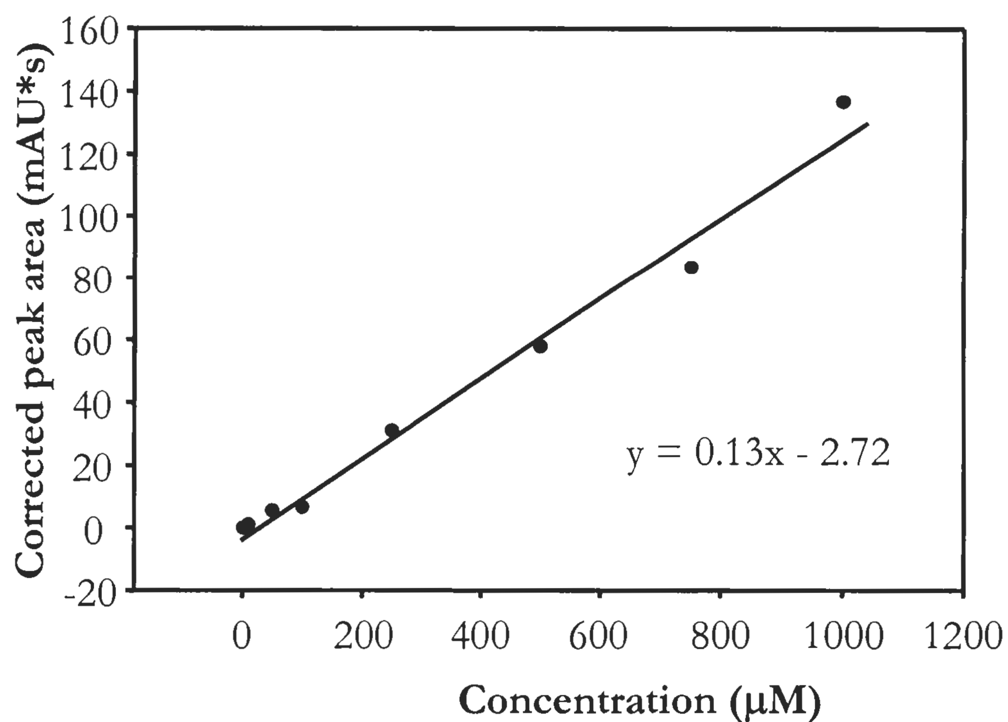


Figure A-19 - Capillary Electrophoresis response to taxifolin concentration in µM as represented by peak area (r^2=0.98). Peak area was corrected by elution time.

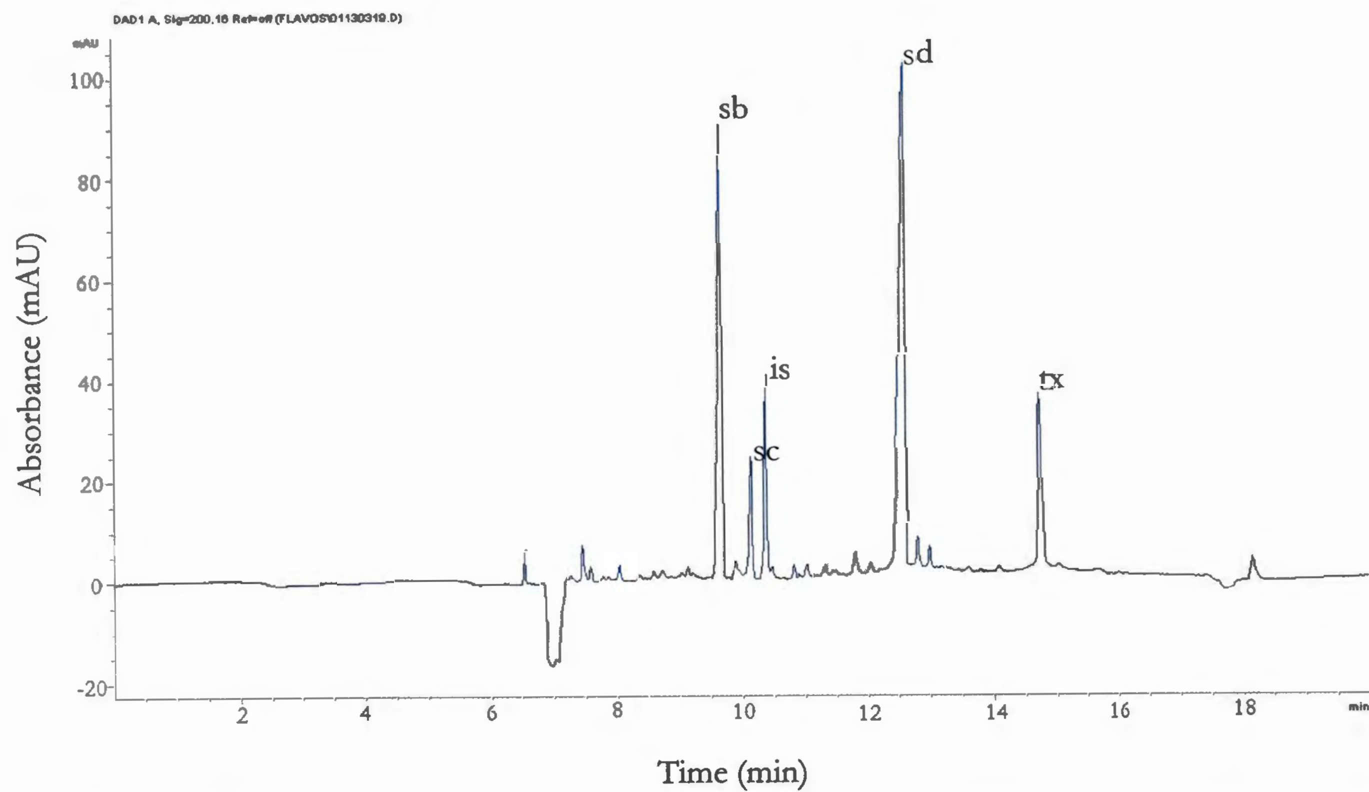


Figure A-20 - Standard electropherogram of seed samples. Labeled peaks from left to right are (sb) silybin, (sc) silycristin, (is) isosilychristin, (sd) silydianin, and (tx) taxifolin.

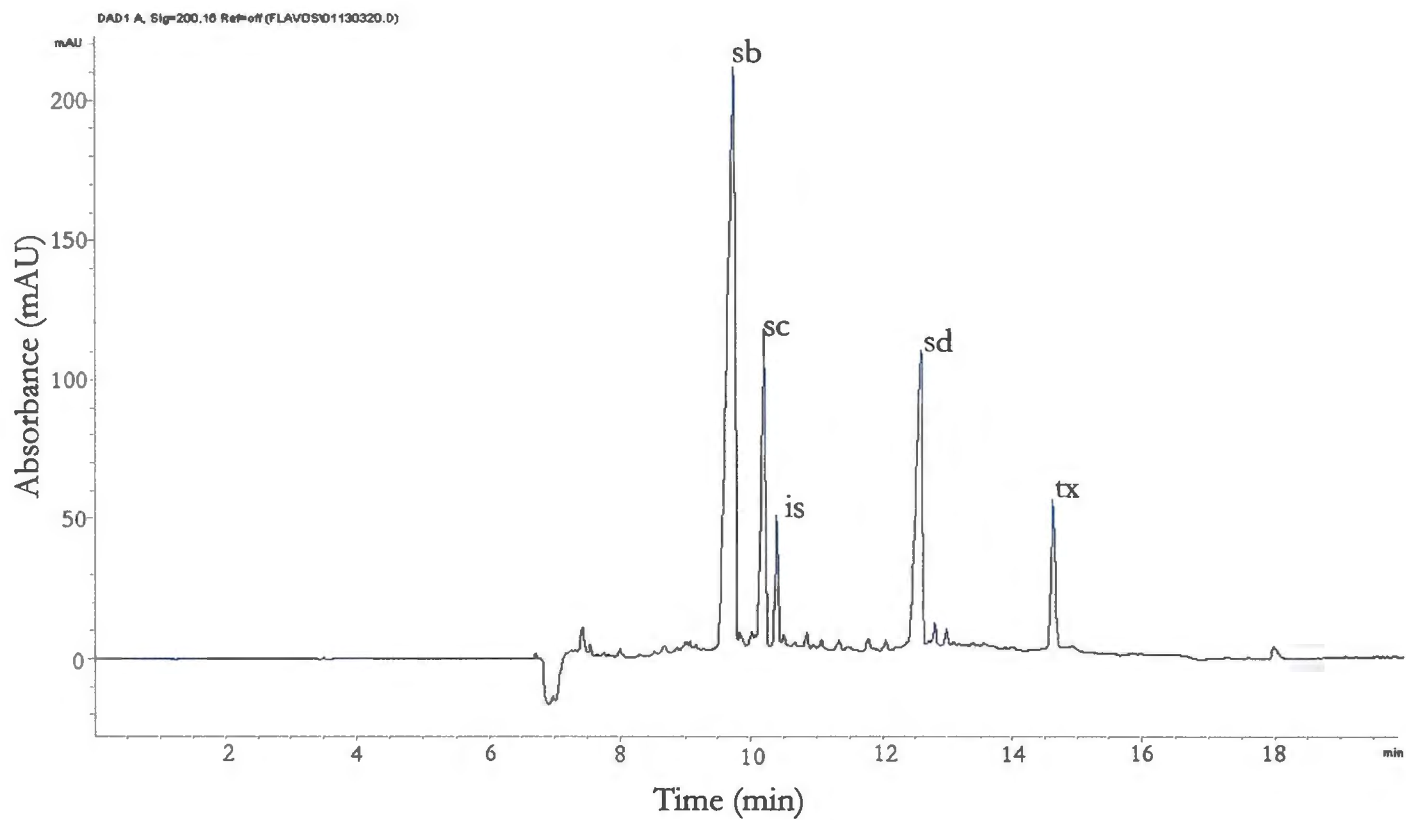


Figure A-21 - Electropherogram of seed samples spiked with silymarin. Labeled peaks from left to right are (sb) silybin, (sc) silycristin, (is) isosilychristin, (sd) silydianin, and (tx) taxifolin.

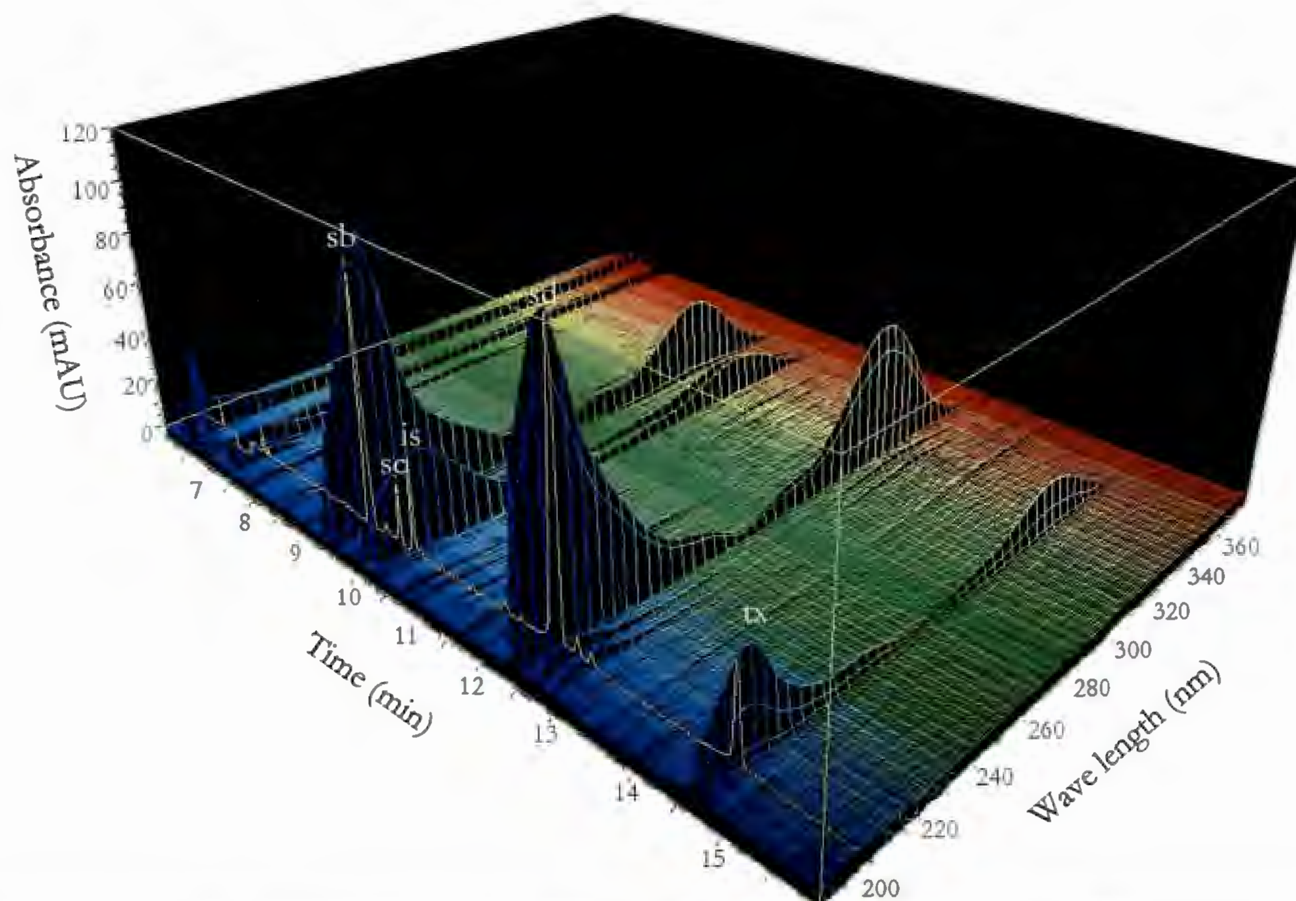


Figure A-22 - 3D Electropherogram of seed samples. Labeled peaks from left to right are (sb)silybin, (sc)silycristin, (is)isosilychristin, (sd)silydianin, and (tx)taxifolin.

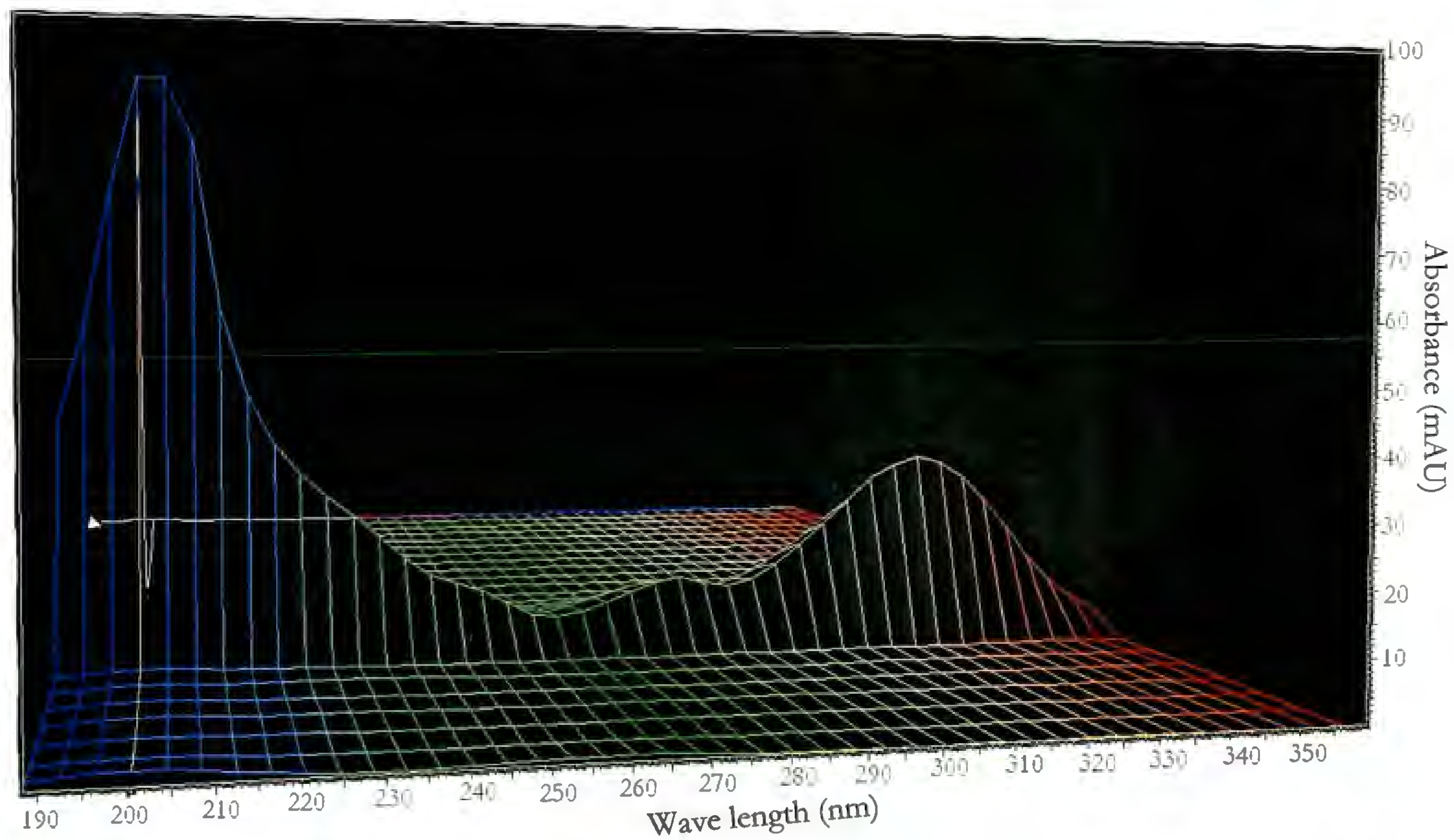


Figure A-23 - 3D Electropherogram of silybin.

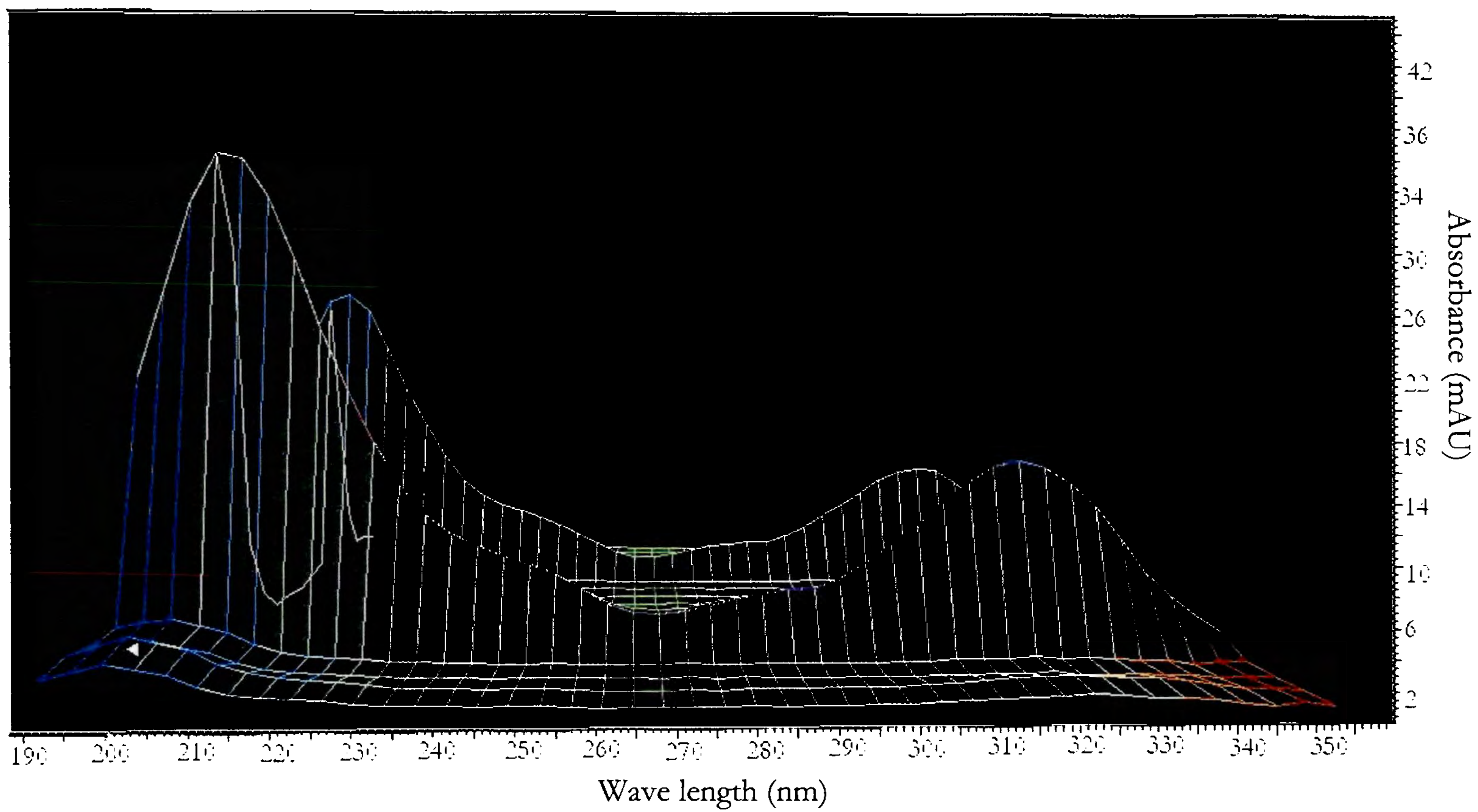


Figure A-24 - 3D Electropherogram of silychristin and isosilychristin

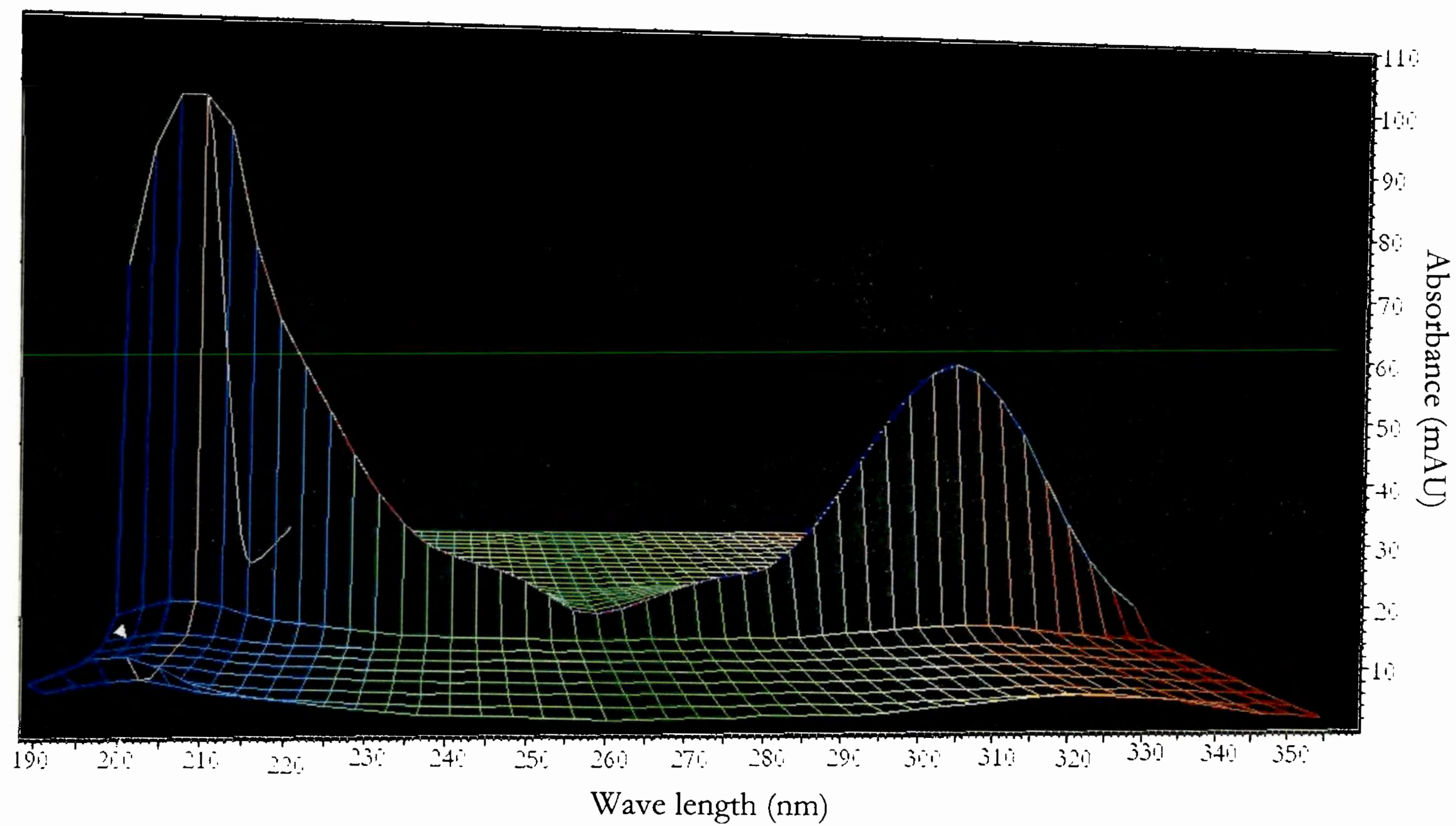


Figure A-25 - 3D Electropherogram of silydianin.

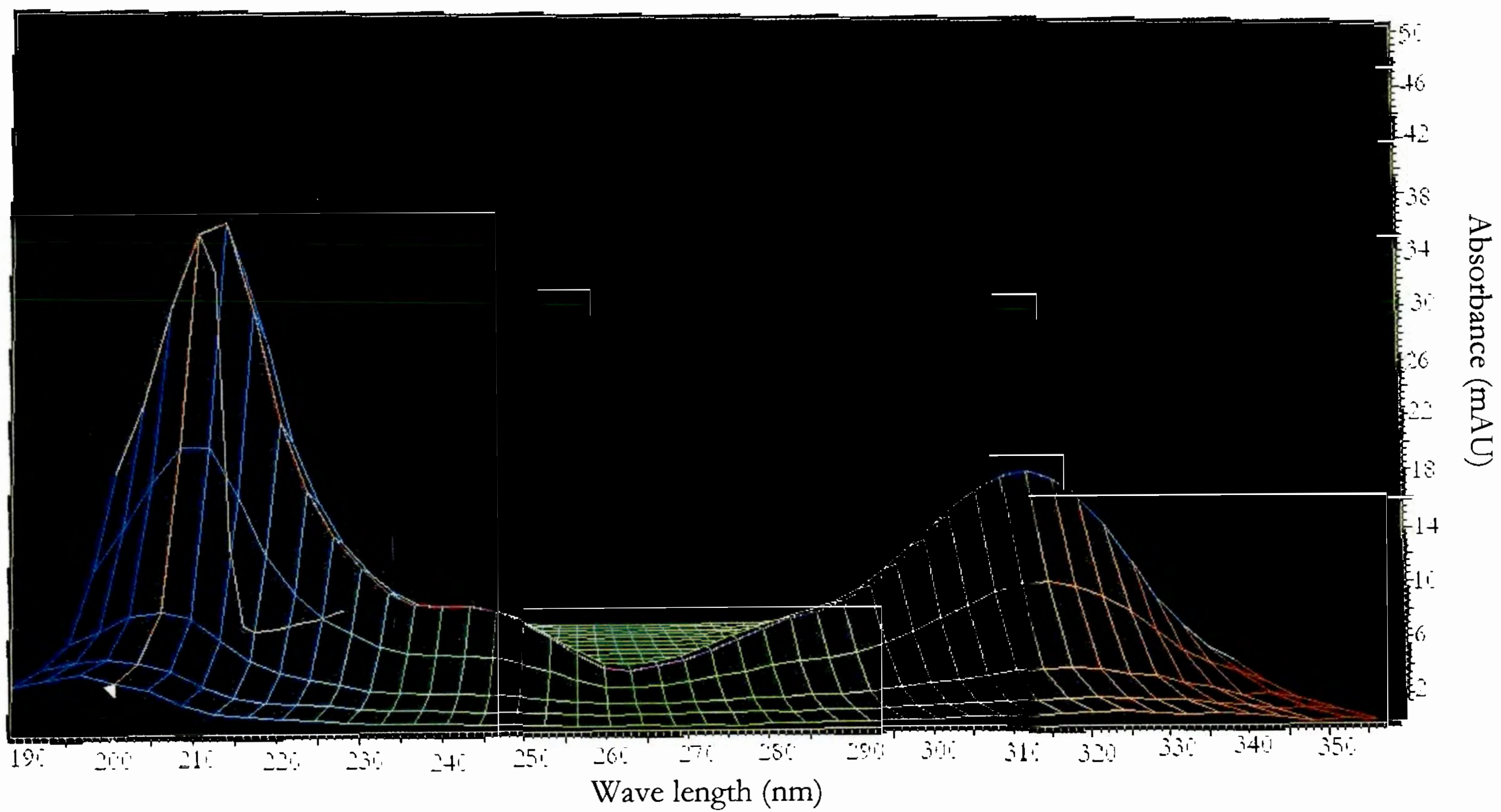


Figure A-26 - 3D Electropherogram of taxifolin.

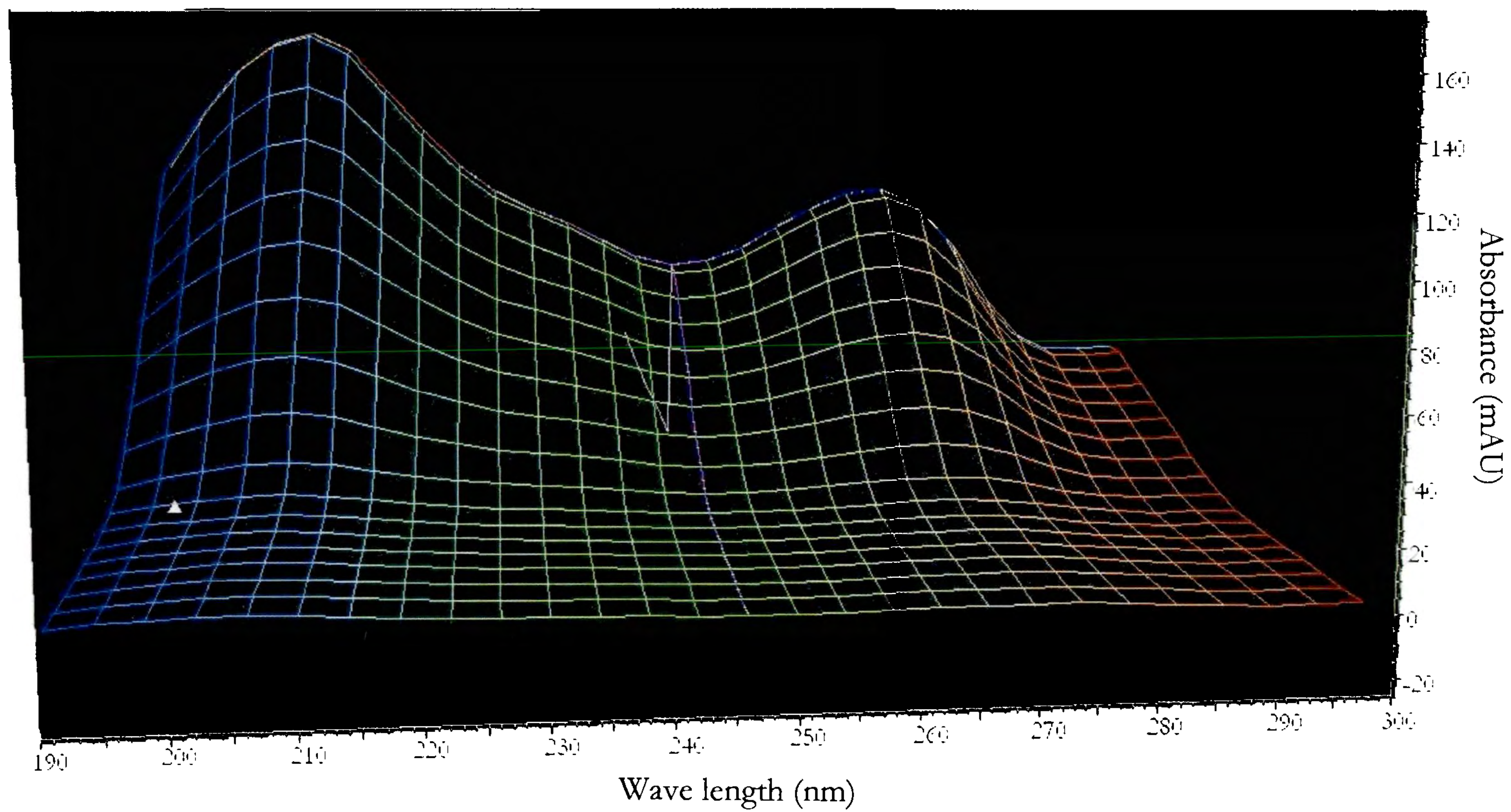


Figure A-27 - 3D Electropherogram of quercetin.

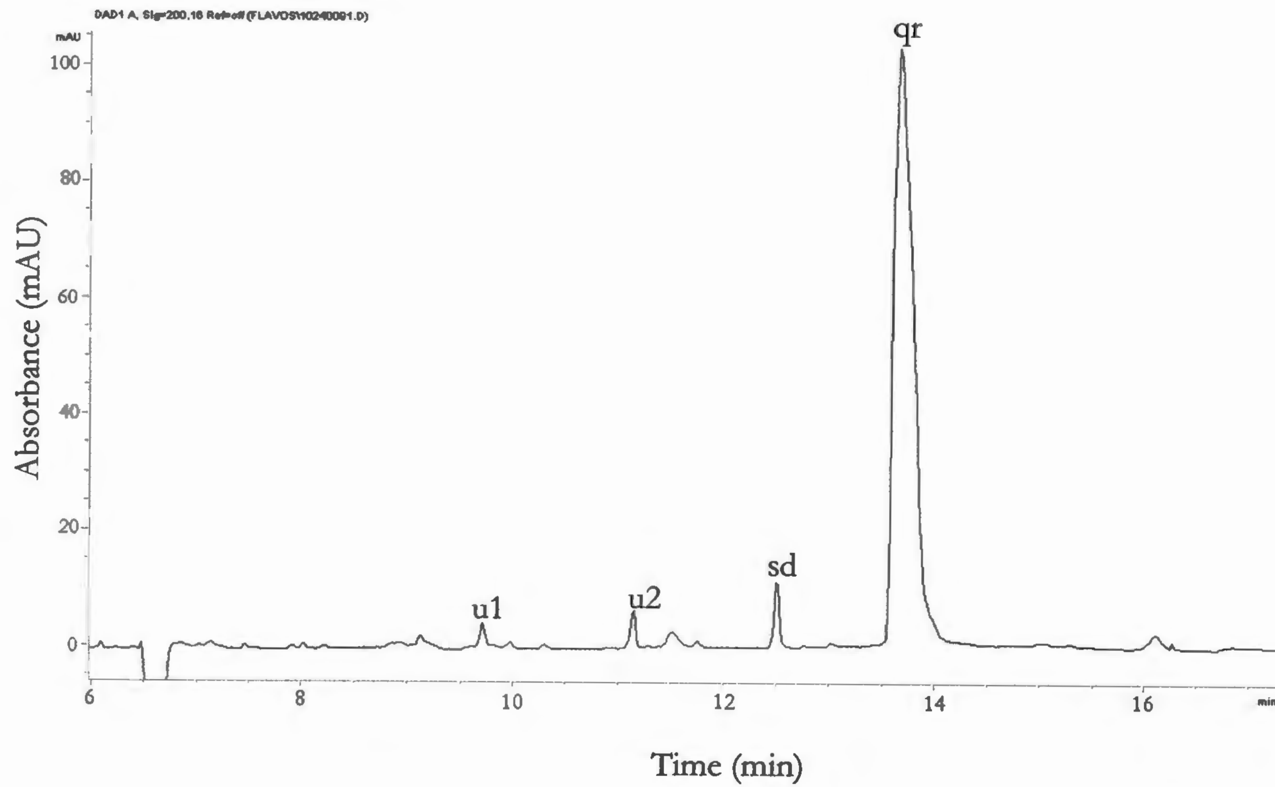


Figure A-28 - Standard electropherogram of upper leaf samples from the spring 2001 experiment. Labeled peaks are (u1) unknown peak1, (u2) unknown peak 2, (sd) silydianin, and (qr) quercitin, the internal standard.

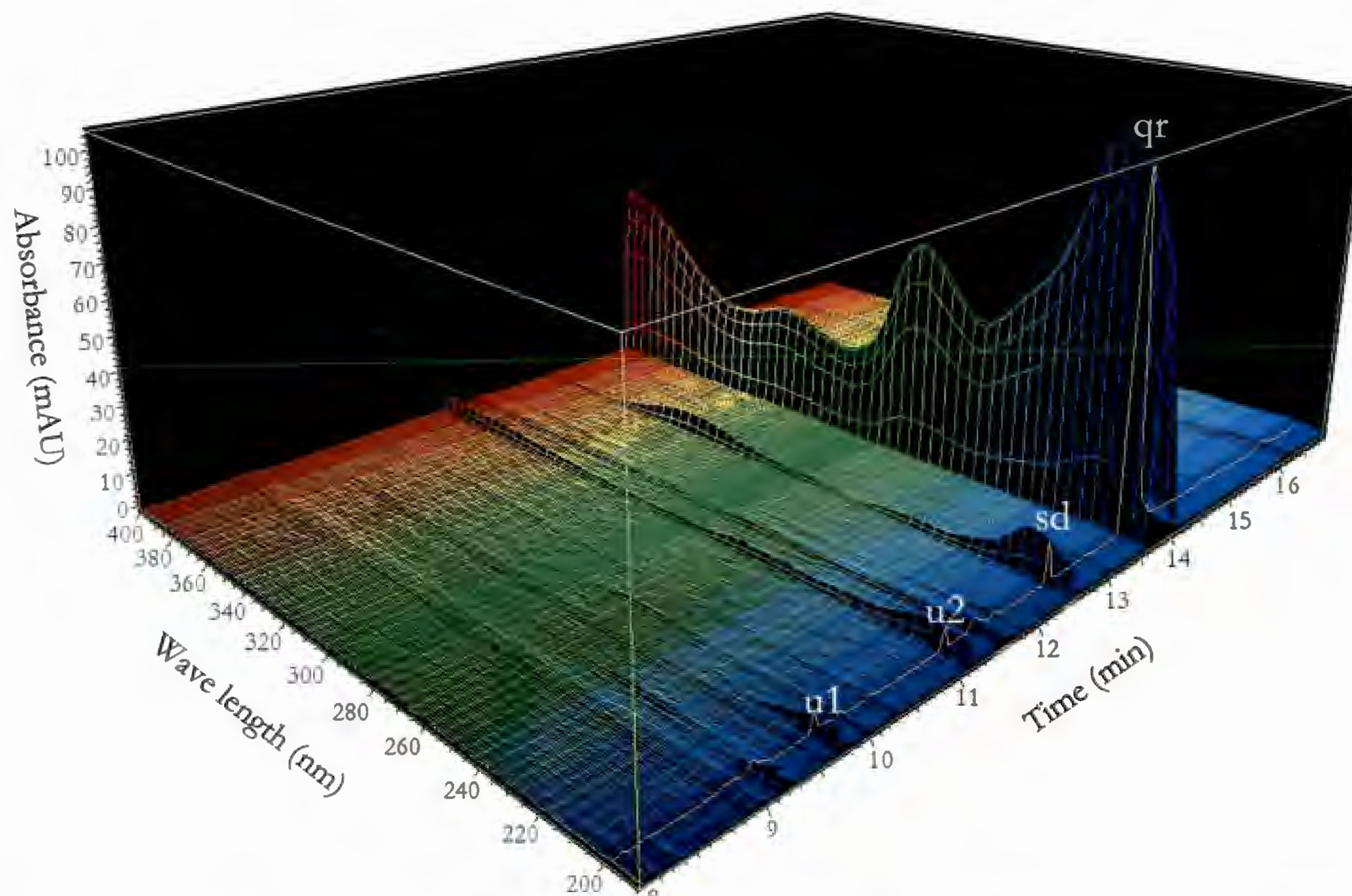


Figure A-29 - 3D Electropherogram of upper leaf samples from the spring 2001 experiment. Labeled peaks from left to right are (u1) unknown peak 1, (u2) unknown peak 2, (sd) silydianin and (qr) quercitin.

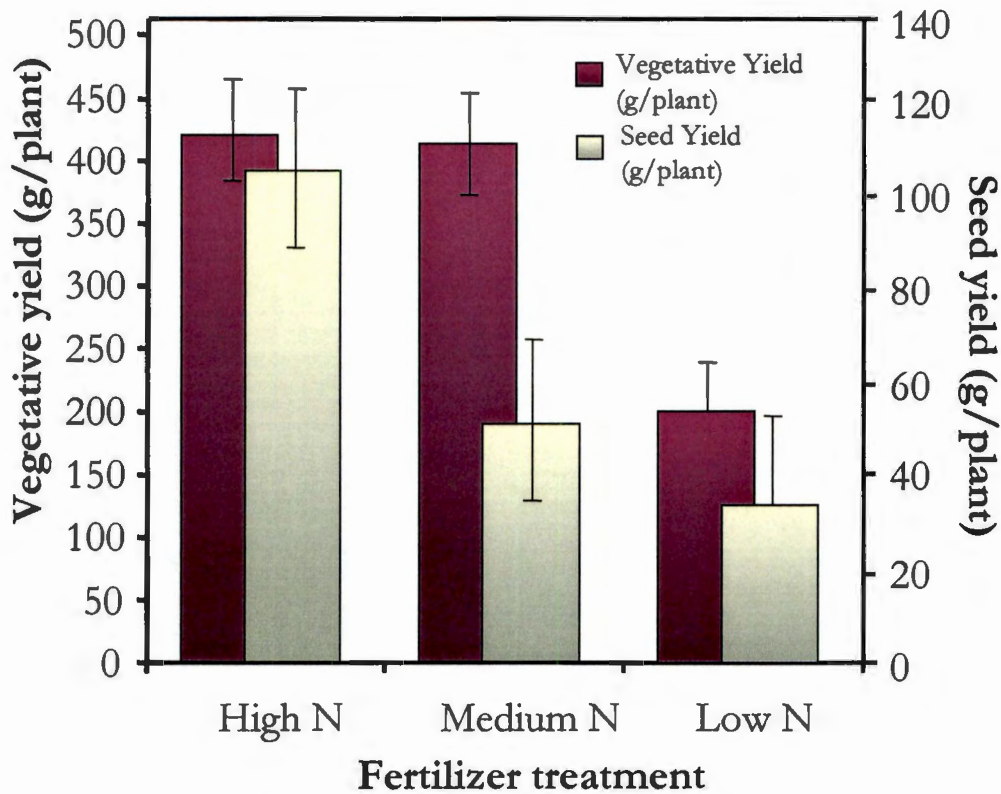


Figure A-30 - Vegetative and seed yields from Fall 2001 experiment in grams. The vegetative yield is represented on the left y-axis and the seed yield is represented on the right y-axis.

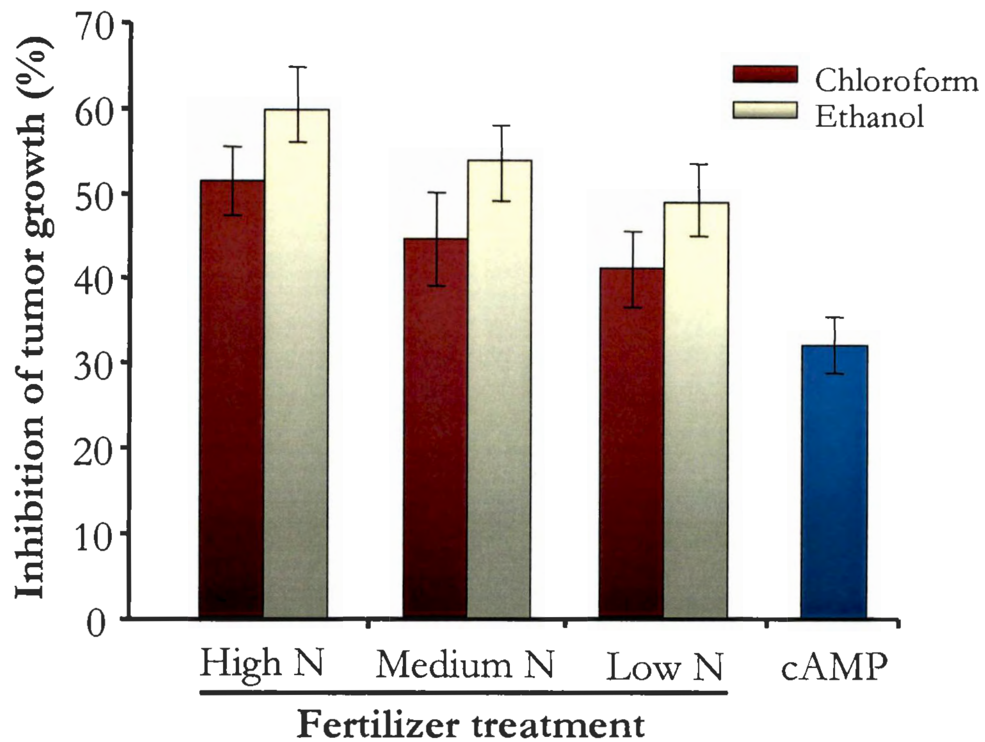


Figure A-31 - Percent tumor inhibition of seed extracts from fall 2001 experiment. Low (47.13 mg/L) and high N (151.80 mg/L) ethanol extracts are significantly differed from one another ($P<0.05$).

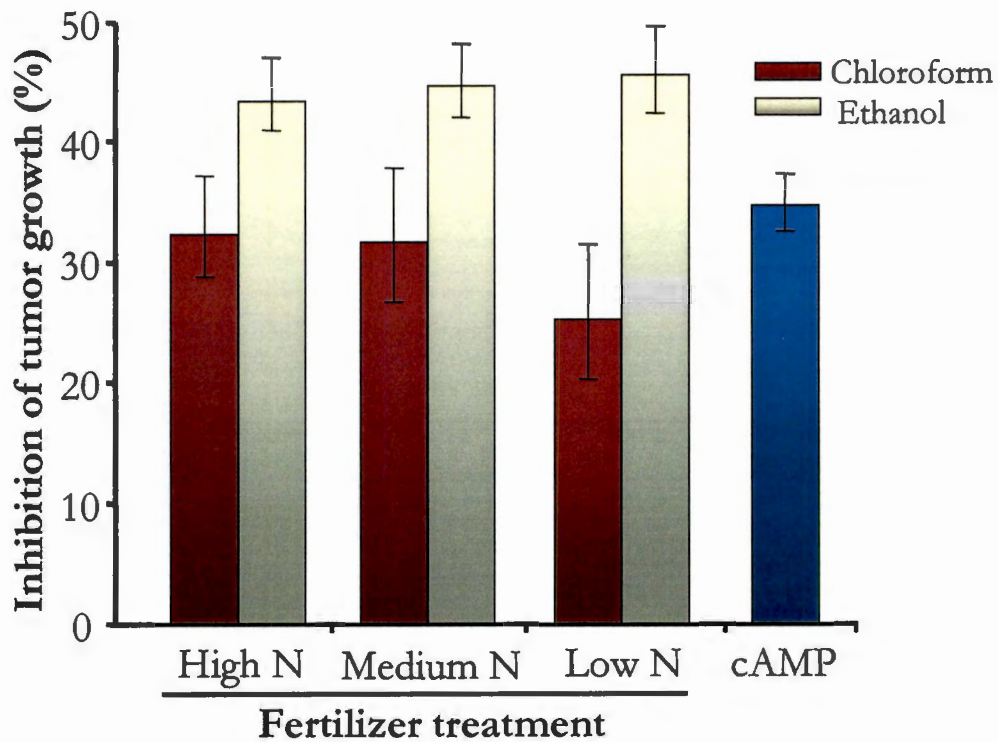


Figure A-32 - Percent inhibition of tumor growth by root extracts from the fall 2001 experiment. Nitrogen had no significant effects on tumor inhibition of either the ethanol or chloroform extracts ($P>0.05$).

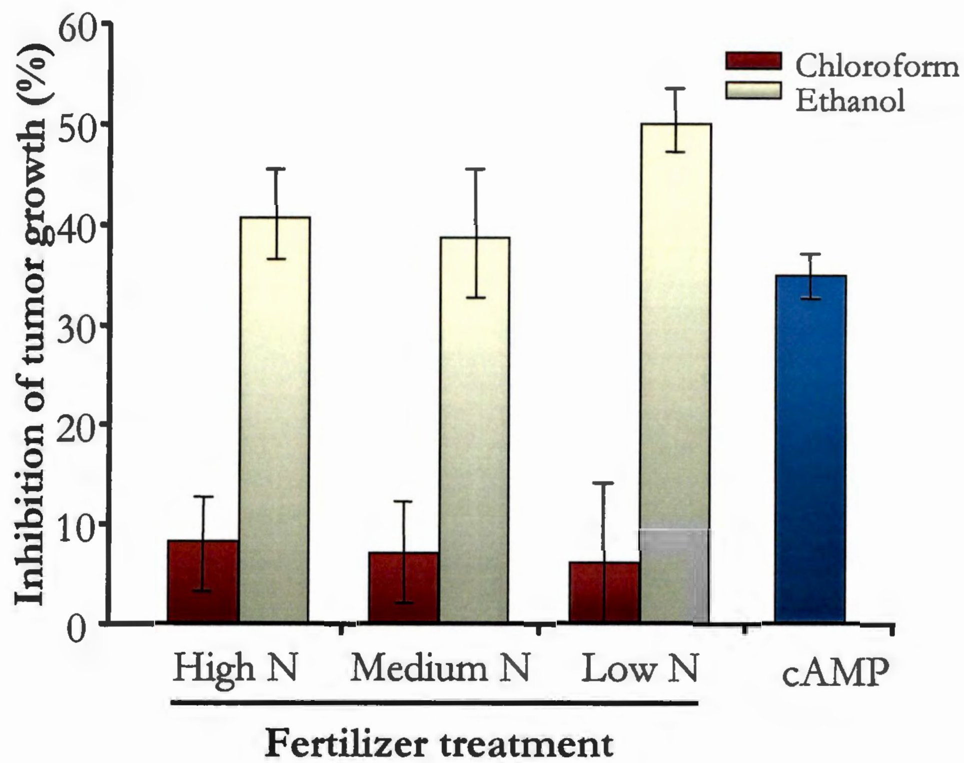


Figure A-33 - Percent inhibition of tumor growth by stem extracts from the fall 2001 experiment. Nitrogen had no significant effects on tumor inhibition of either the ethanol or chloroform extracts (P>0.05).

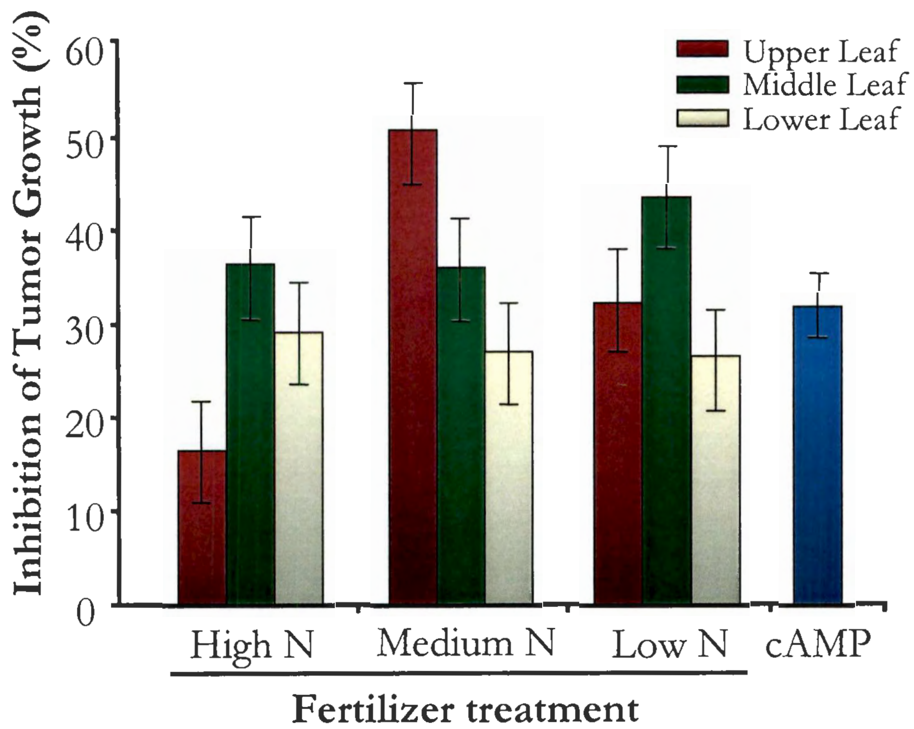


Figure A-34 - Percent inhibition of tumor growth by leaf extracts from the fall 2001 experiment. Upper, middle, and lower leaf activities were affected differently by fertilizer treatment.

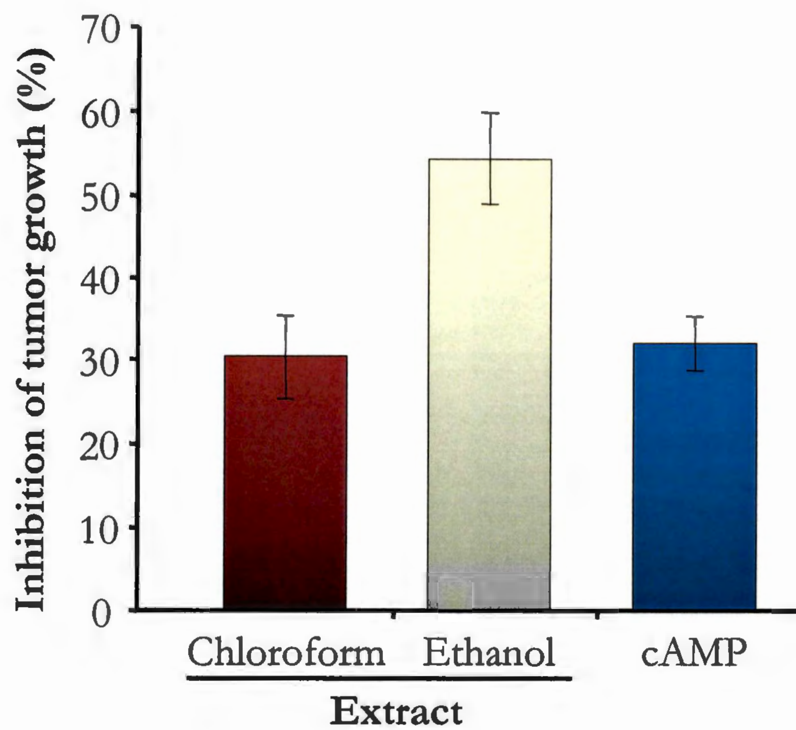


Figure A-35 - Percent inhibition of tumor growth by seedling extracts from the fall 2001 experiment.

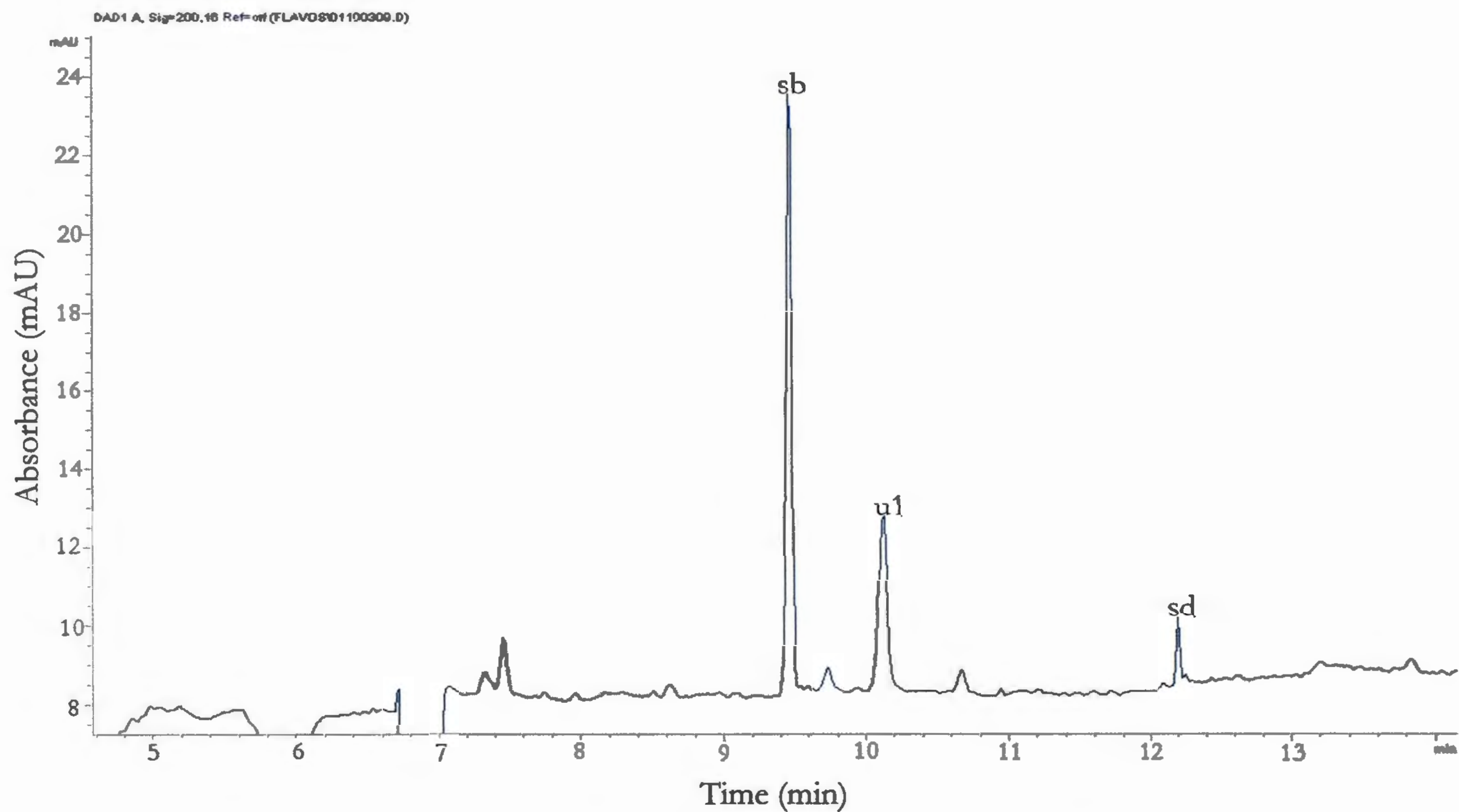


Figure A-36 - Standard electropherogram of root samples. Labeled peaks from left to right are (sb) silybin, (u1) unknown peak 1, and (sd) silydianin

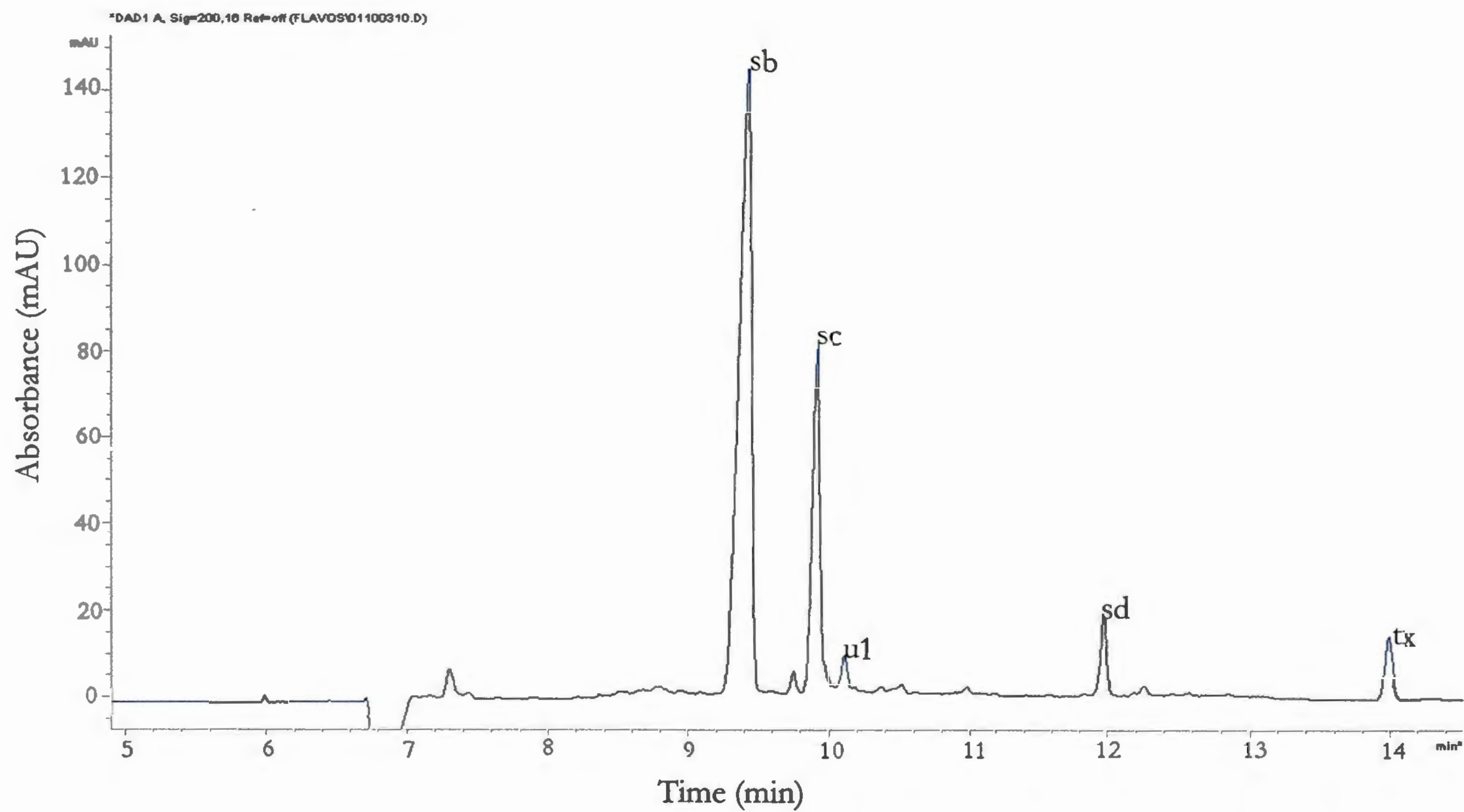


Figure A-37 - Electropherogram of root sample spiked with silymarin. Labeled peaks from left to right are (sb)silybin, (sc)silycristin, (u1) unknown peak 1 (sd)silydianin, and (tx)taxifolin.

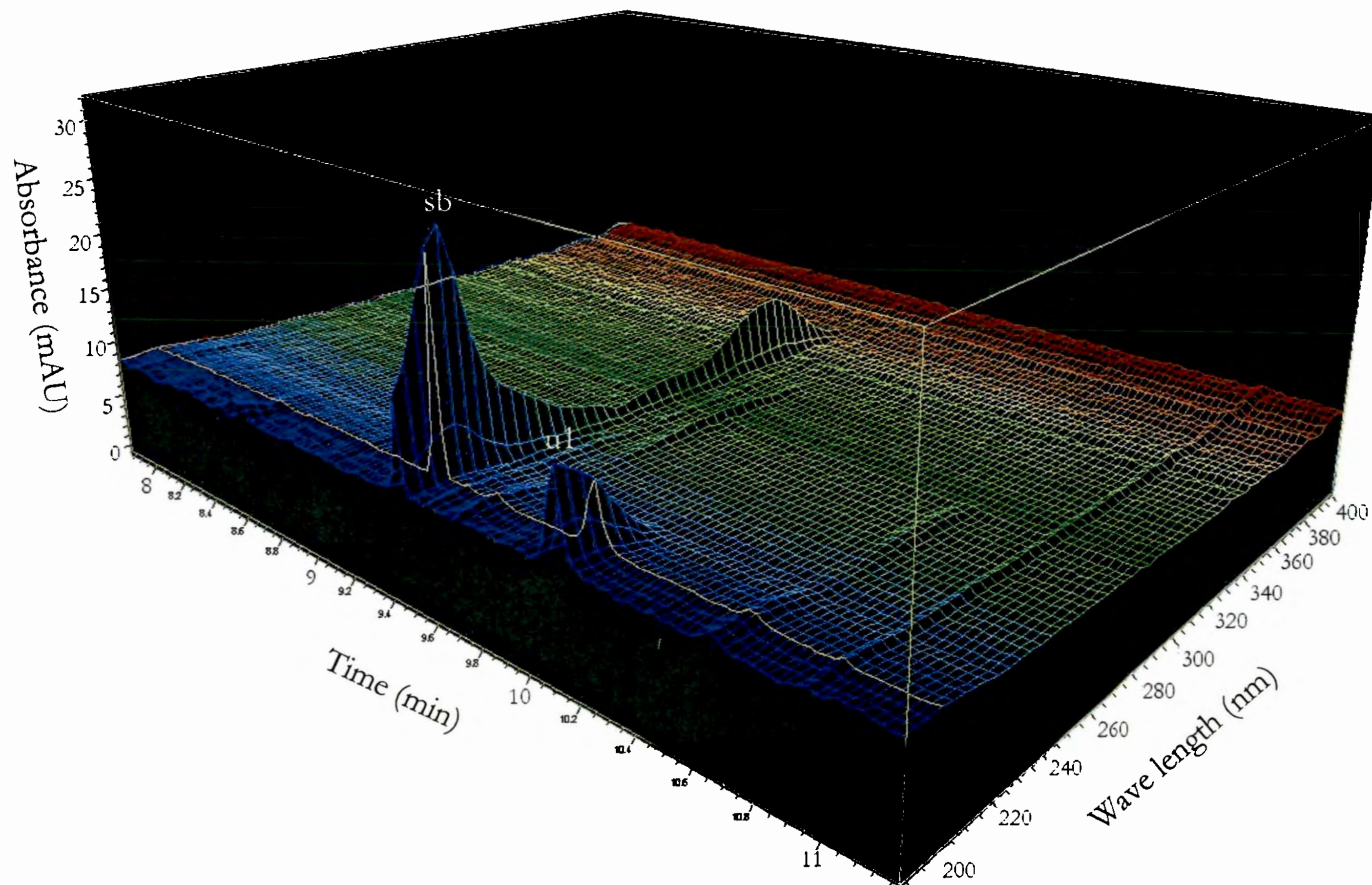


Figure A-38 - 3D Electropherogram of root samples. Labeled peaks from left to right are (sb) silybin, (a) unknown peak 1. The third peak, silydianin, was cut off.

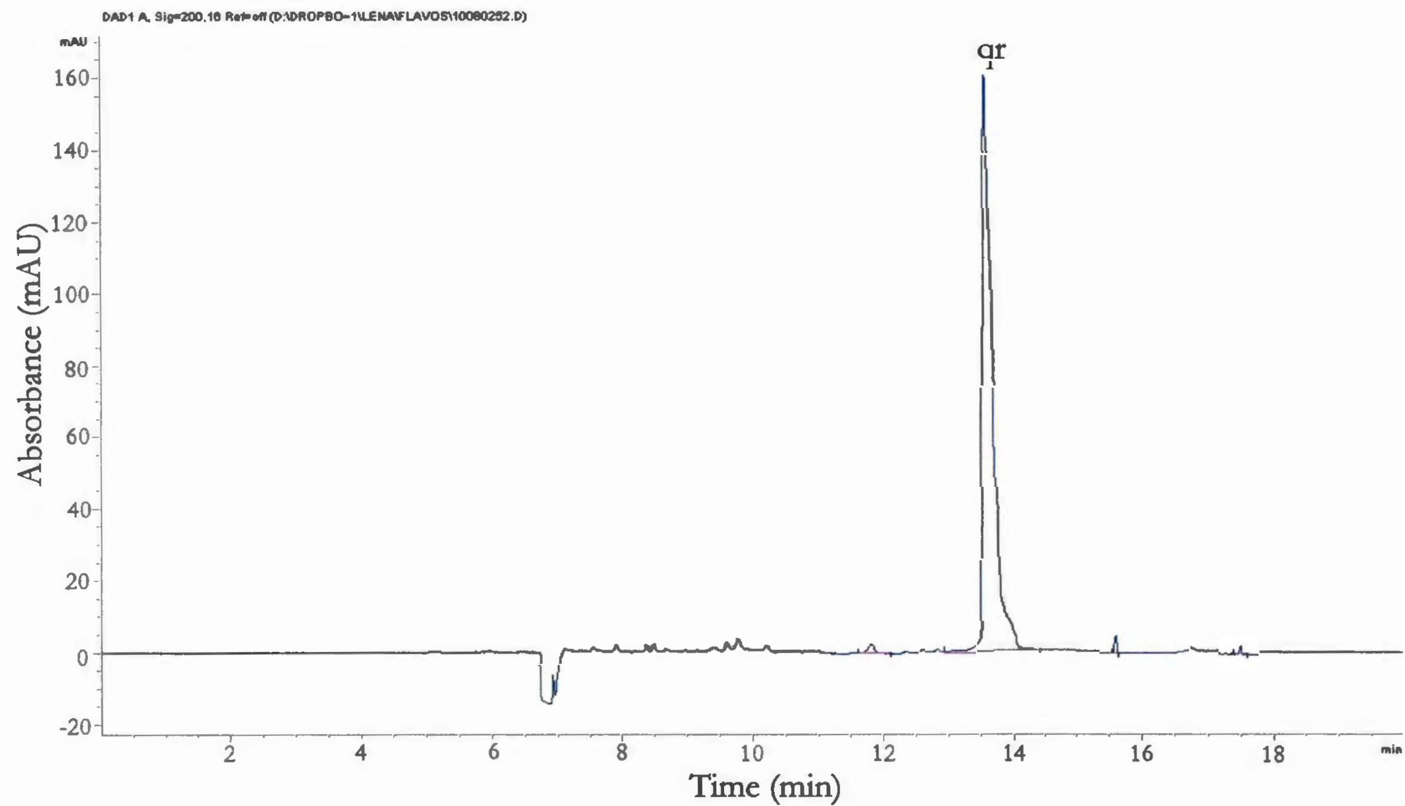


Figure A-39 - Standard electropherogram of stem samples. Peak qr is quercetin, the internal standard. No other peaks could be identified

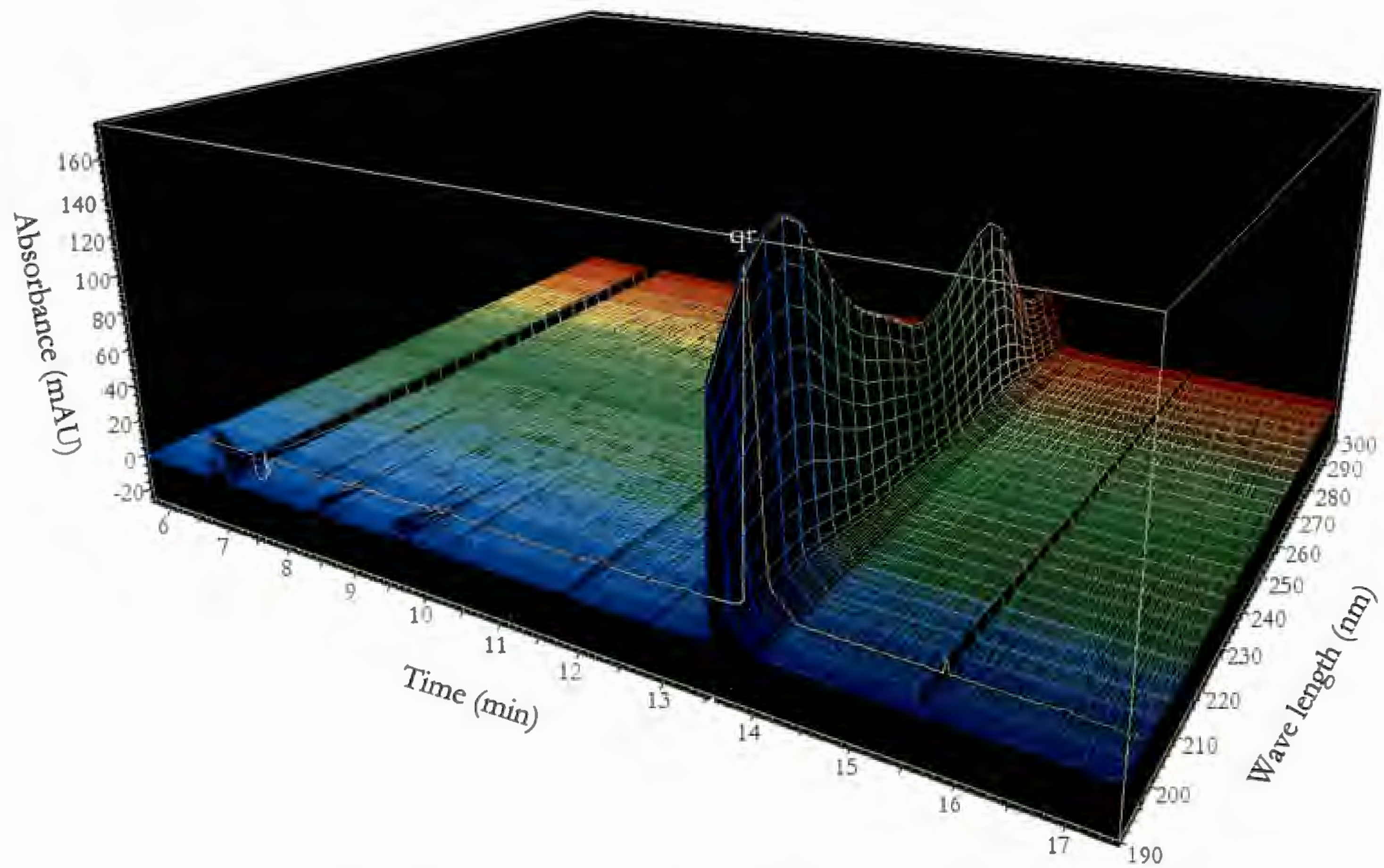


Figure A-40 - 3D Electropherogram of stem samples. Peak (qr) is quercetin.

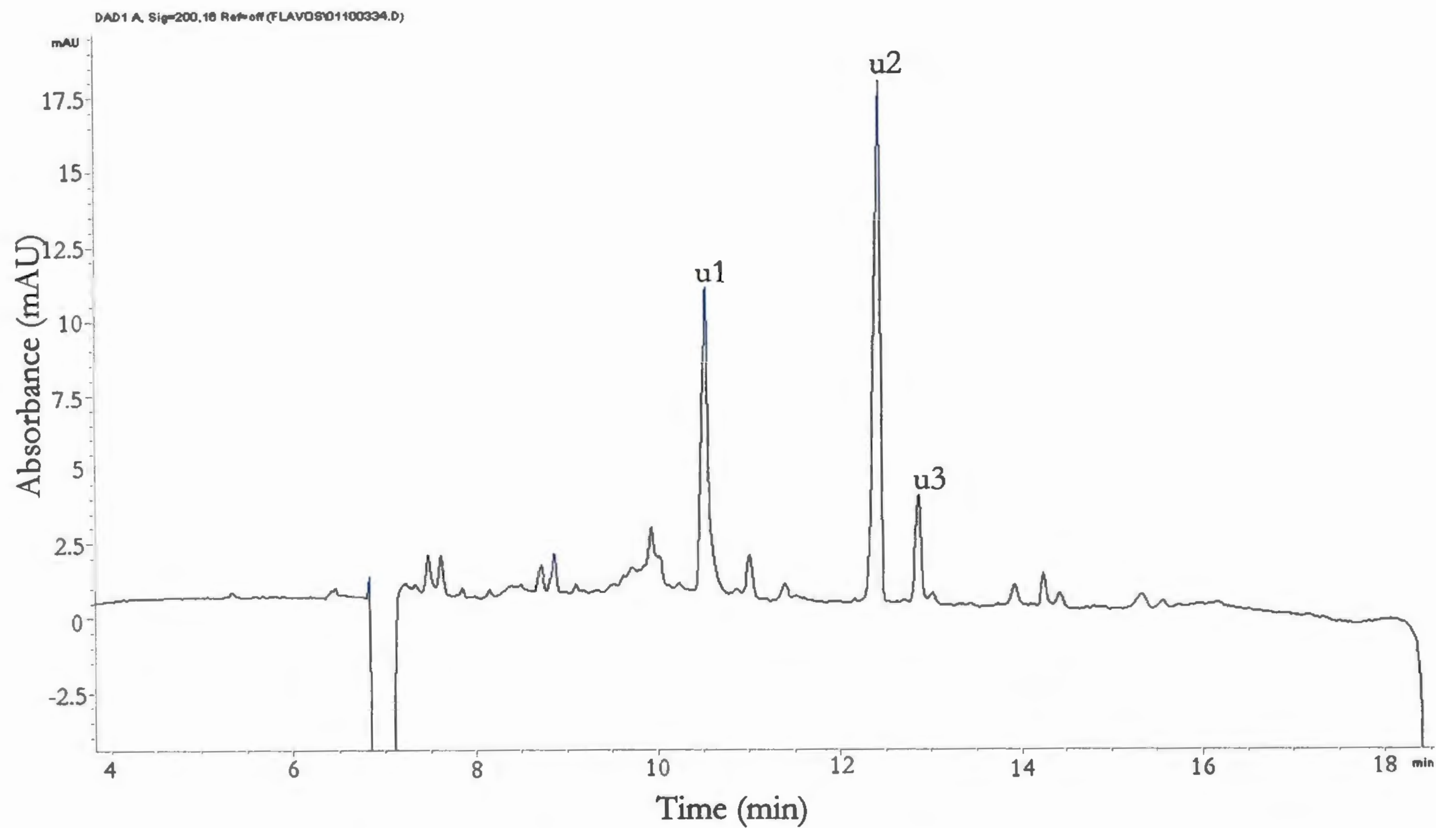


Figure A-41 - Standard electropherogram of upper leaf samples from the fall 2001 experiment. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2, (u3) unknown peak 3

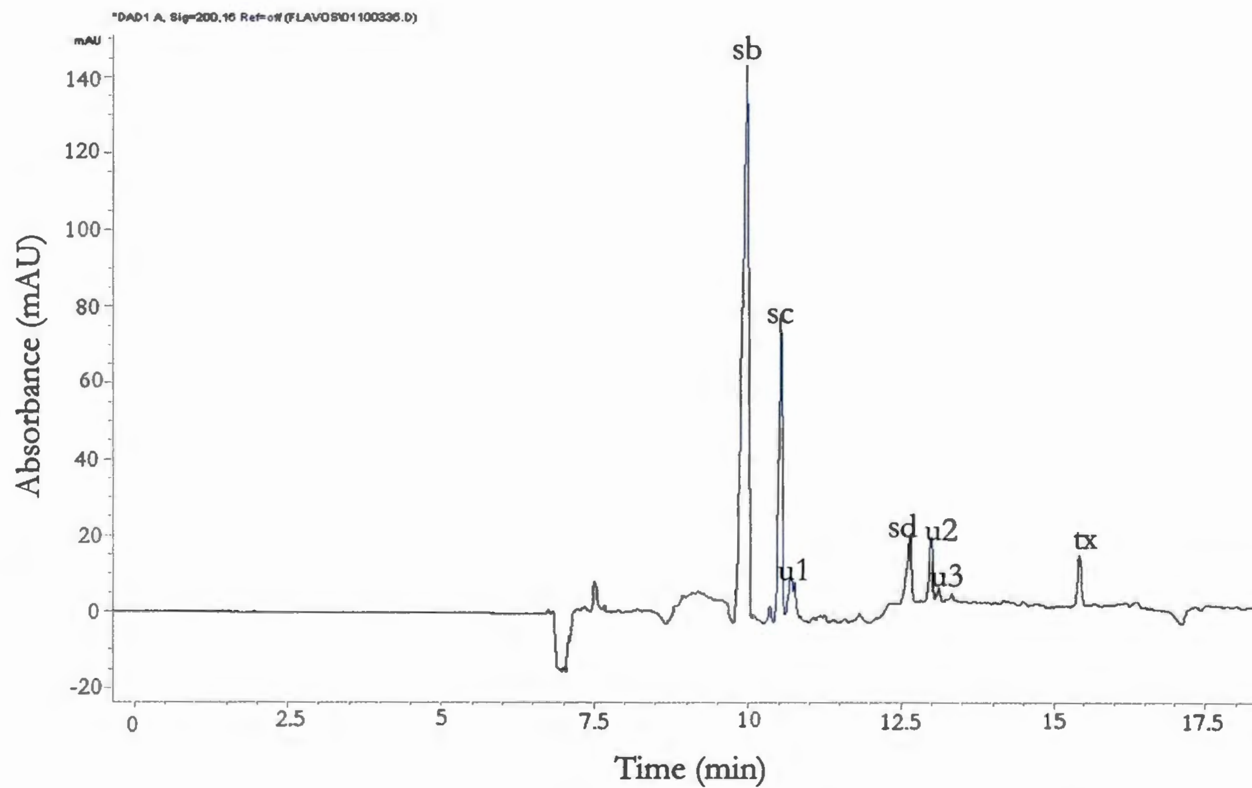


Figure A-42 - Electropherogram of upper leaf sample spiked with silymarin. Labeled peaks from left to right are (sb) silybin, (sc) silycristin, (u1) unknown peak 1, (sd) silydianin, (u2) unknown peak 2, (u3) unknown peak 3, and (tx) taxifolin.

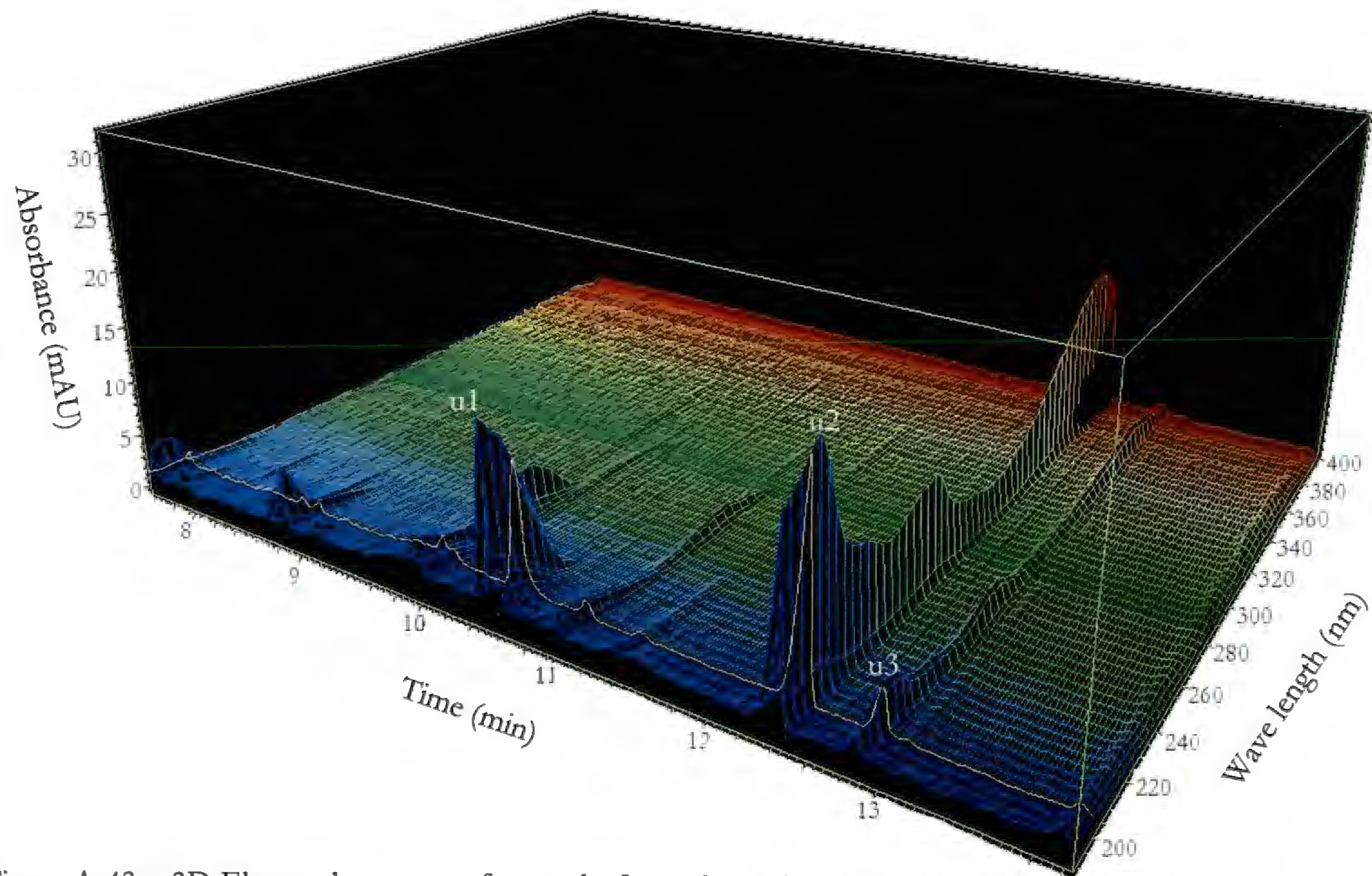


Figure A-43 - 3D Electropherogram of upper leaf samples. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2, (u3) unknown peak 3

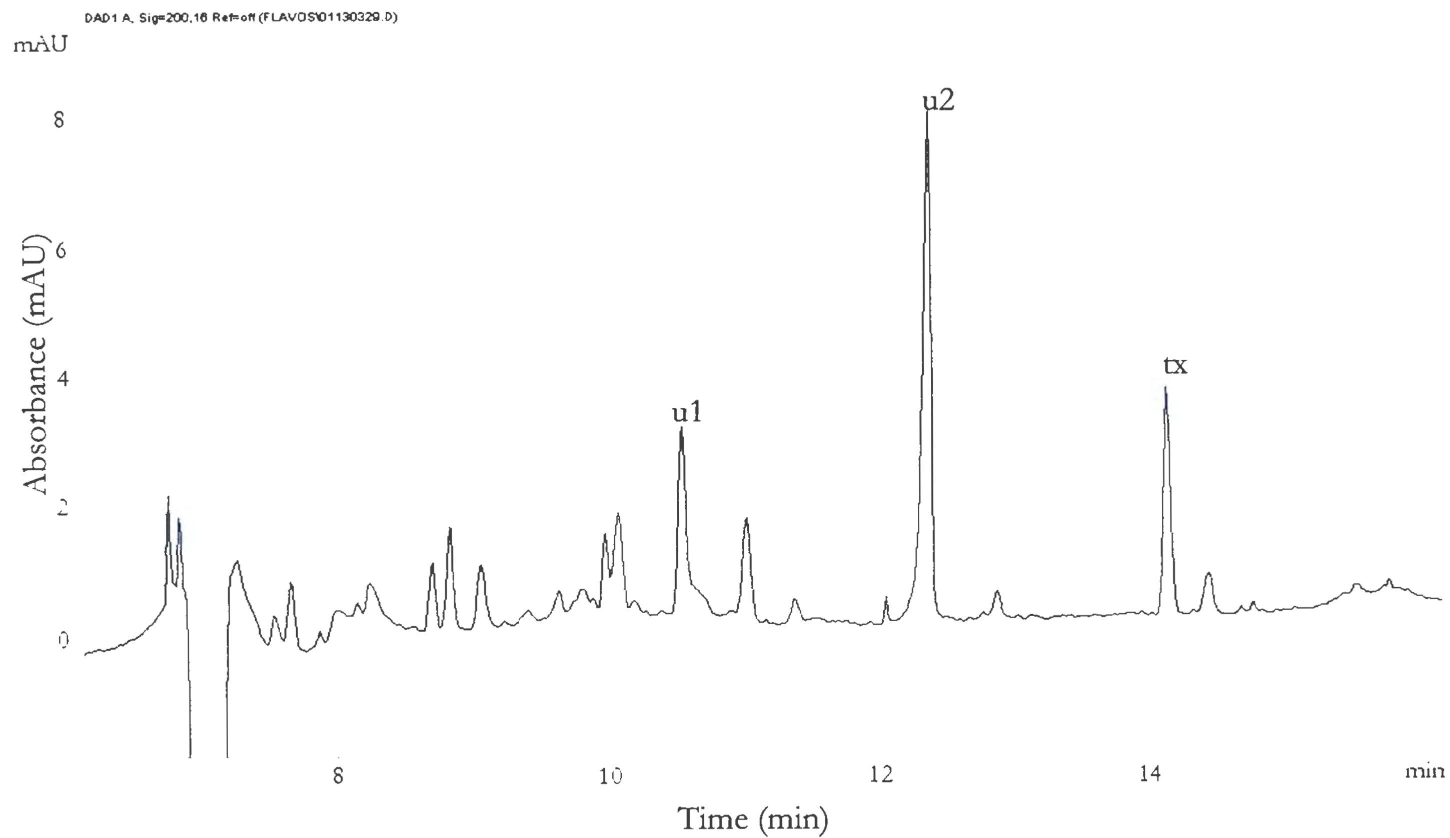


Figure A-44 - Standard electropherogram of middle leaf samples. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2, (tx) taxifolin

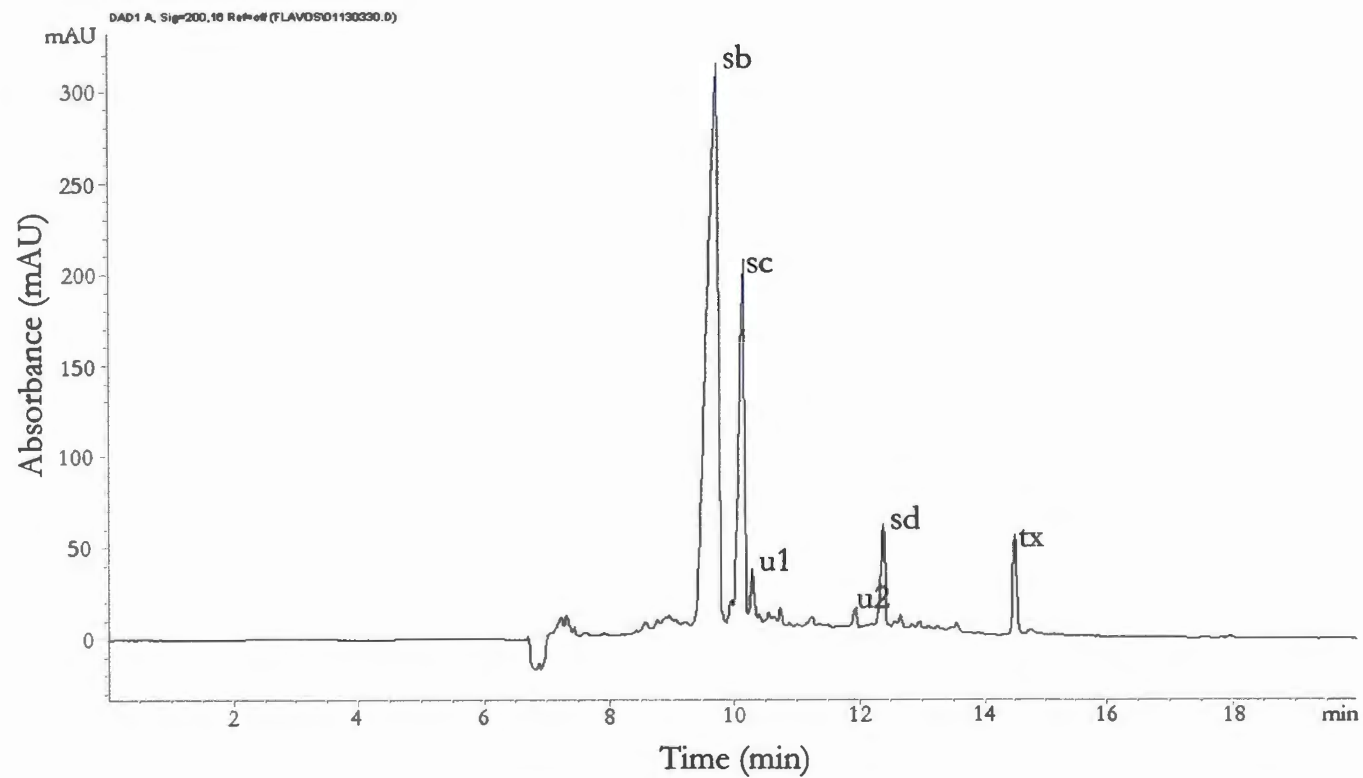


Figure A-45 - Electropherogram of middle leaf sample spiked with silymarin. Labeled peaks from left to right are (sb) silybin, (sc) silycristin, (u1) unknown peak 1, (u2) unknown peak 2, (sd) silydianin, and (tx) taxifolin.

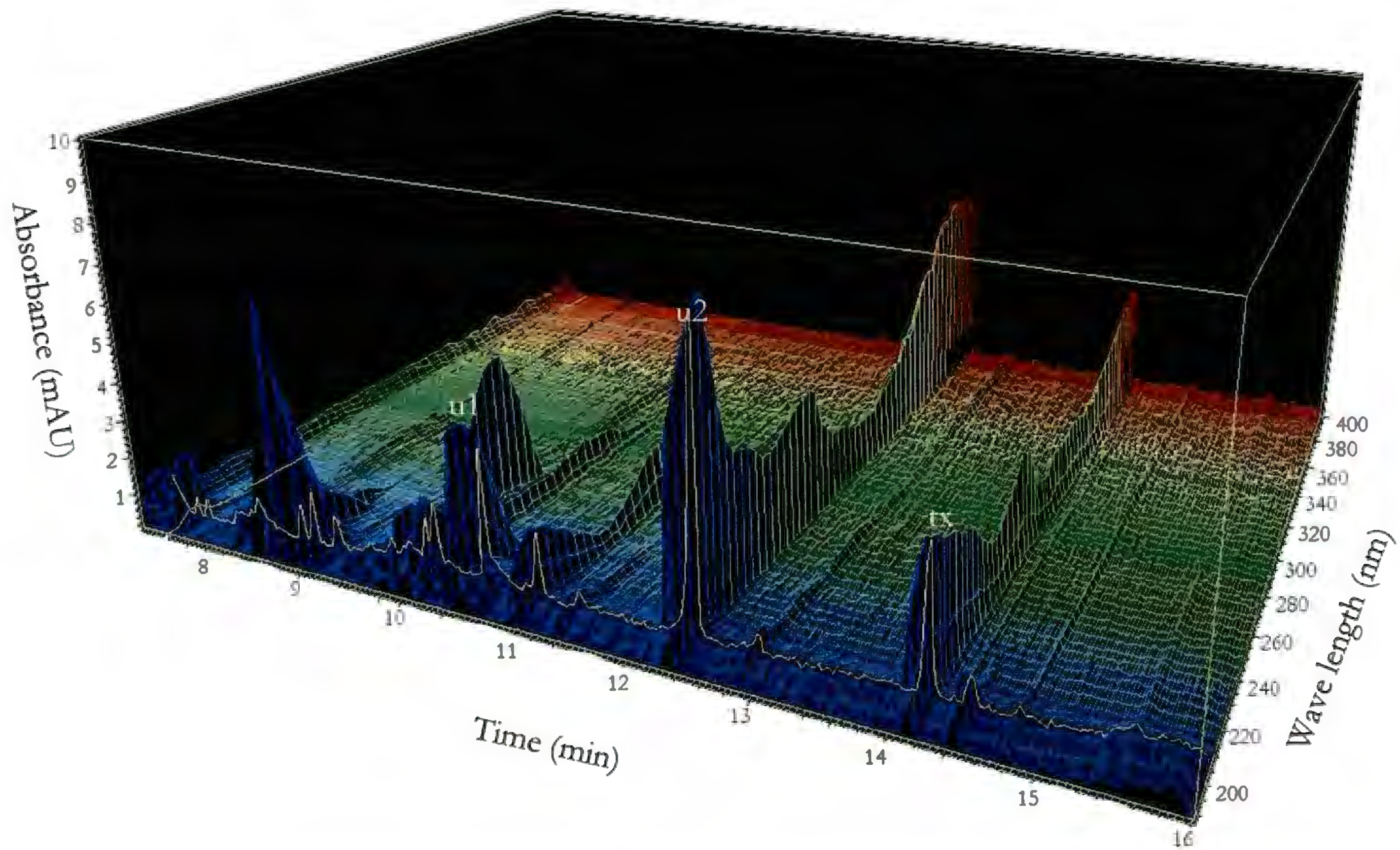


Figure A-46 - 3D Electropherogram of middle leaf samples. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2 (tx) taxifolin

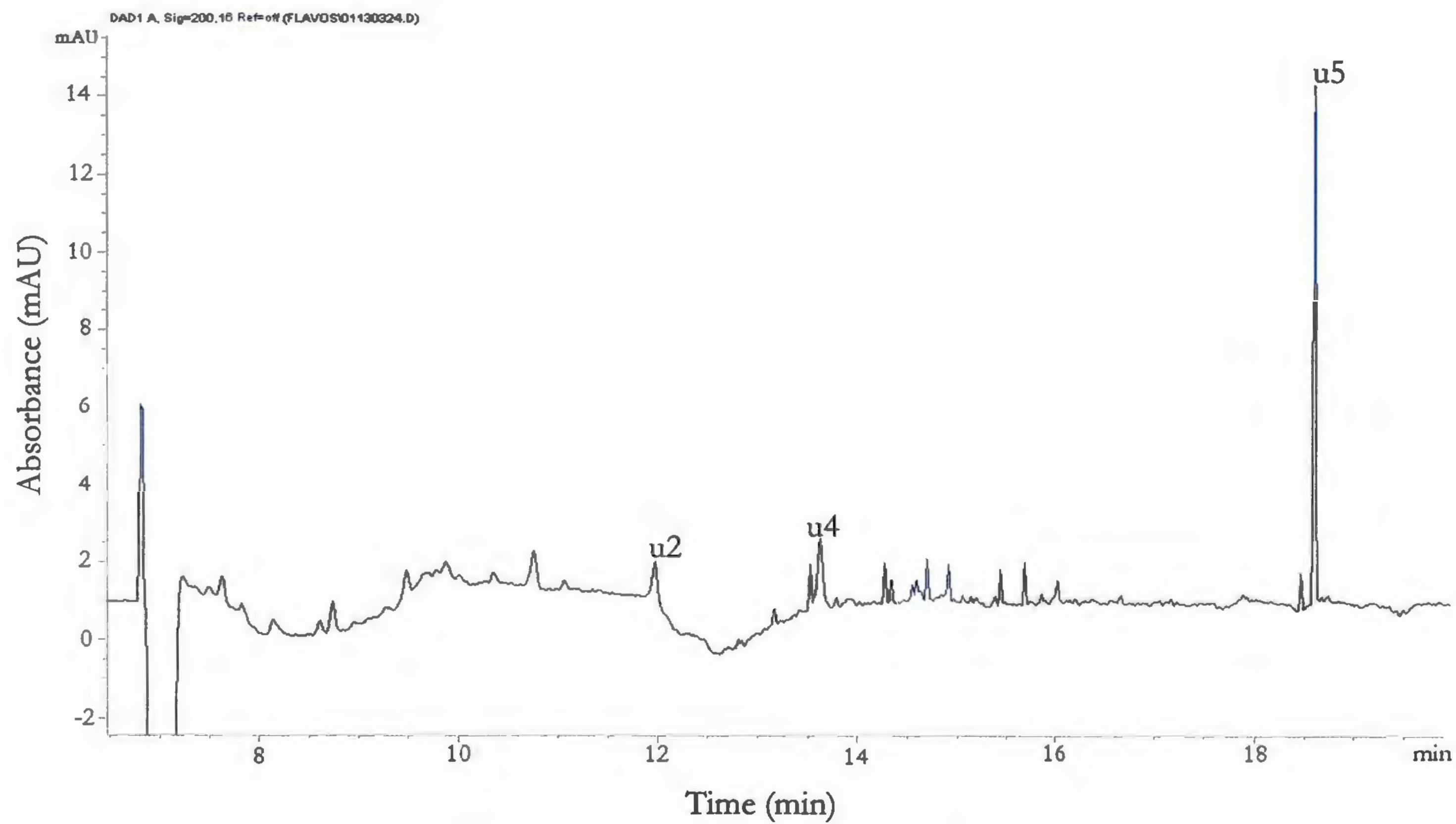


Figure A-47 - Standard electropherogram of lower leaf samples. Labeled peaks are (u2) unknown peak 2, (u4) unknown peak 4, and (u5) unknown peak 5.

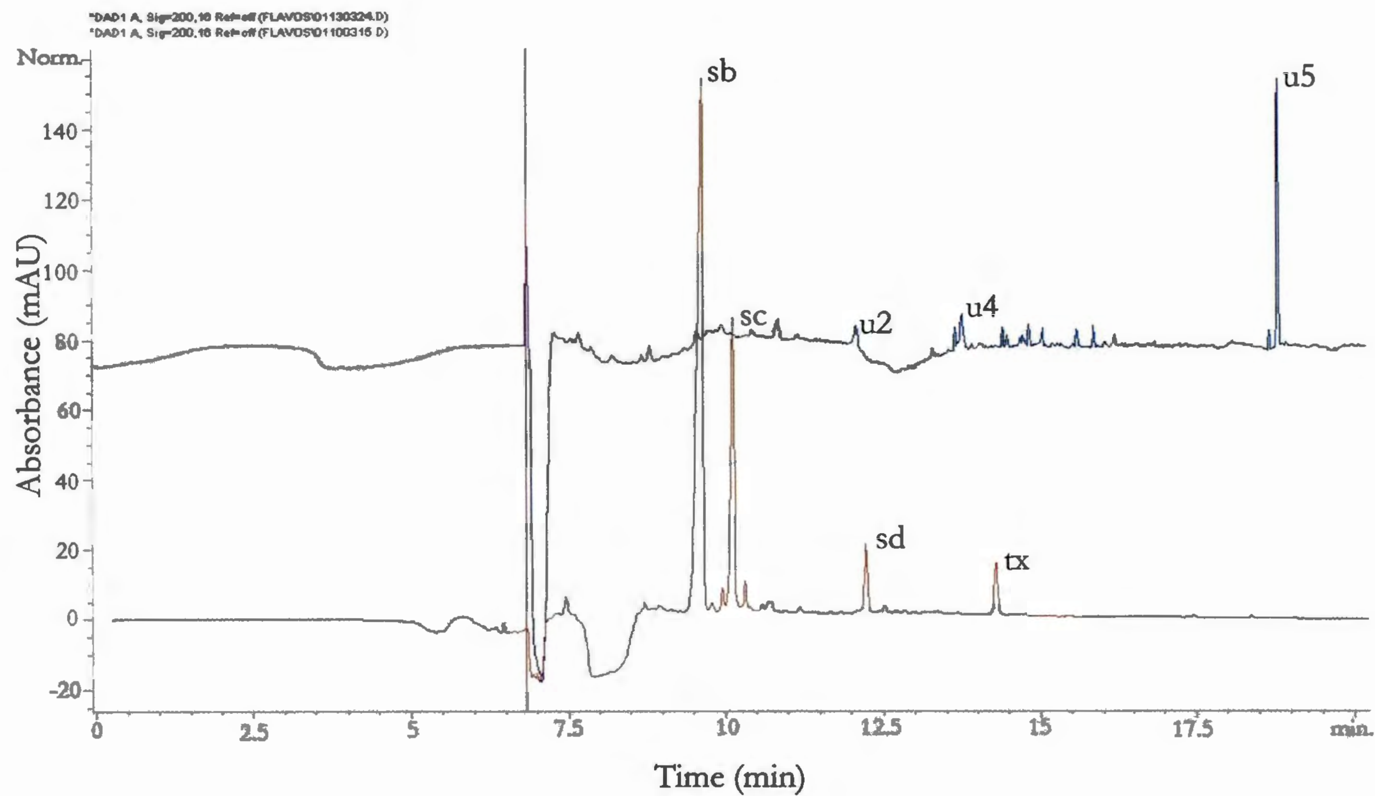


Figure A-48 - Electropherogram of lower leaf sample (upper line) compared with electopherogram of silymarin (lower line). Labeled peaks are identified as (sb) silybin, (sc) silychristin (sd) silydianin, (tx) taxifolin (u2) unknown peak 2 (u4) unknown peak 4 (u5) unknown peak 5 .

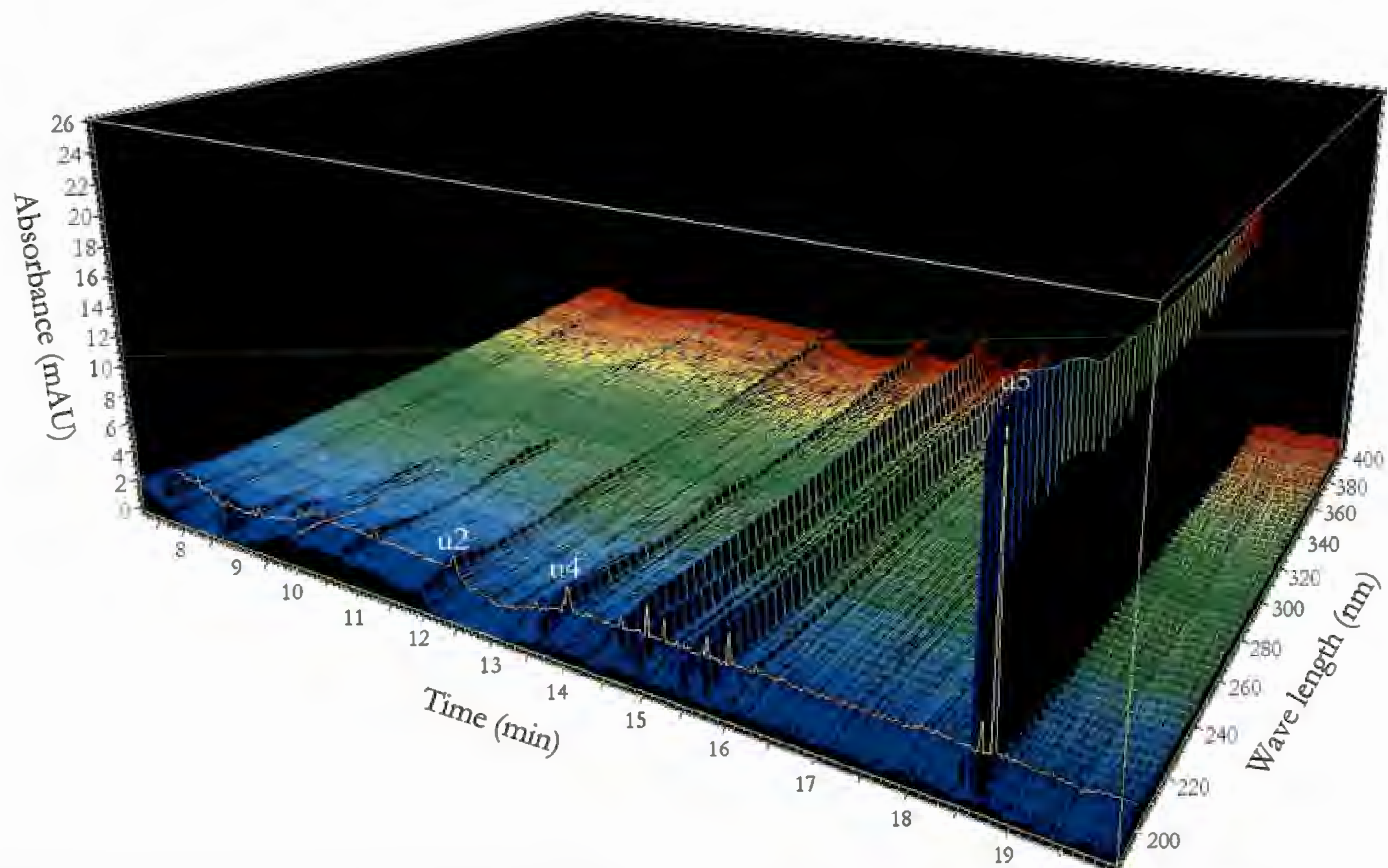


Figure A-49 - 3D Electropherogram of lower leaf samples. Labeled peaks are (u2) unknown peak 2, (u4) unknown peak 4, (u5) unknown peak 5

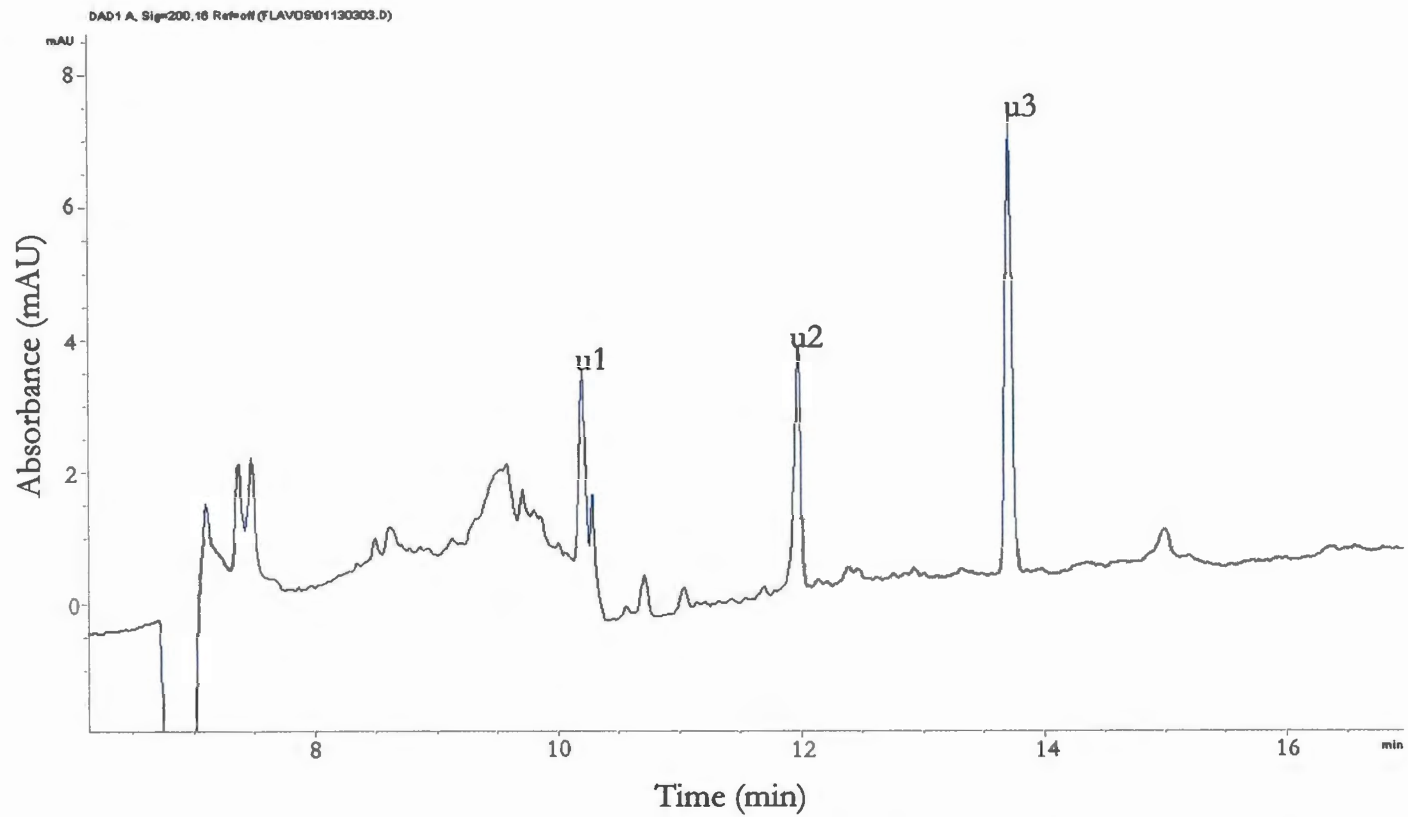


Figure A-50 - Standard electropherogram of seedling samples. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2, and (u3) unknown peak 3.

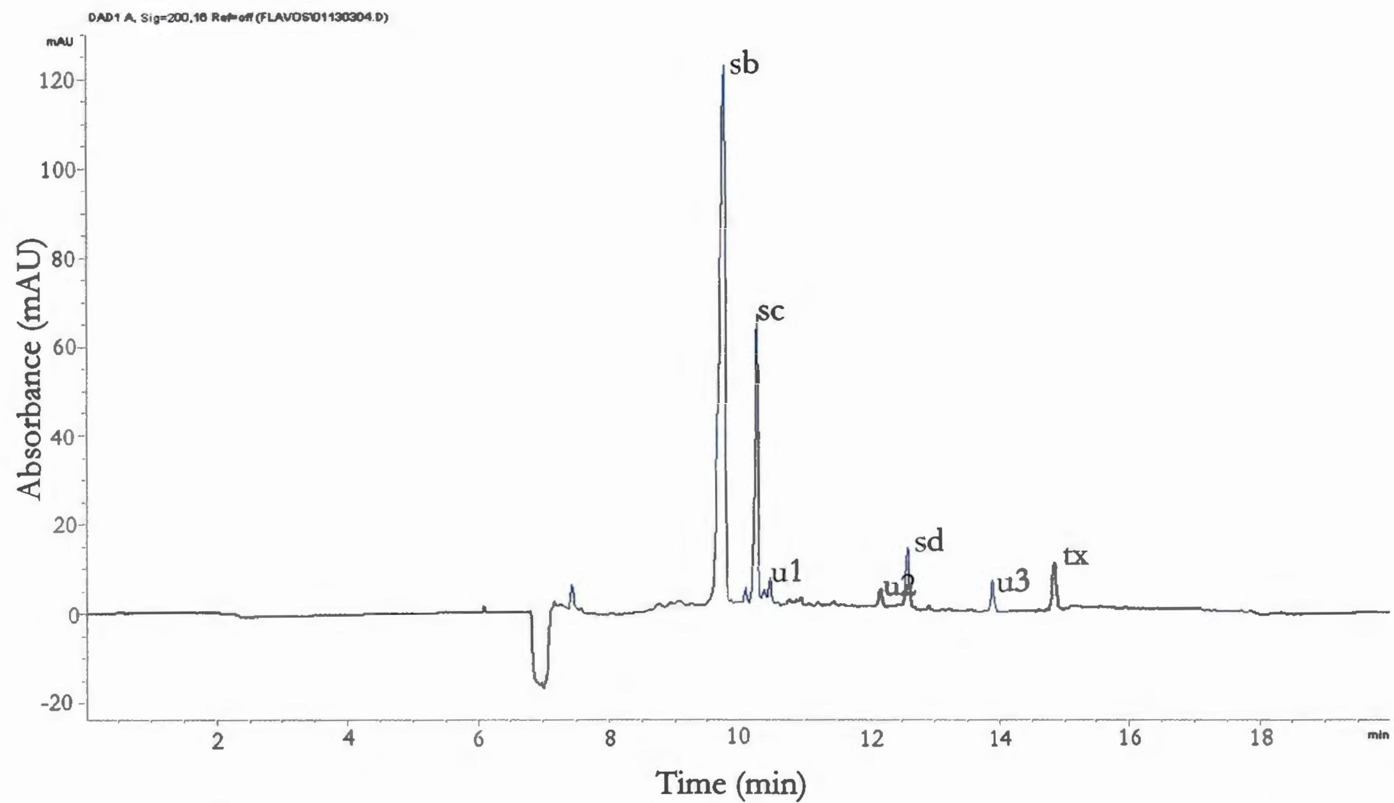


Figure A-51 - Electropherogram of seedling sample spiked with silymarin. Labeled peaks from left to right are (sb) silybin, (sc)silycristin, (u1) unknown peak 1, (u2) unknown peak 2, (sd) silydianin, (u3) unknown peak 3 and (tx)taxafolin.

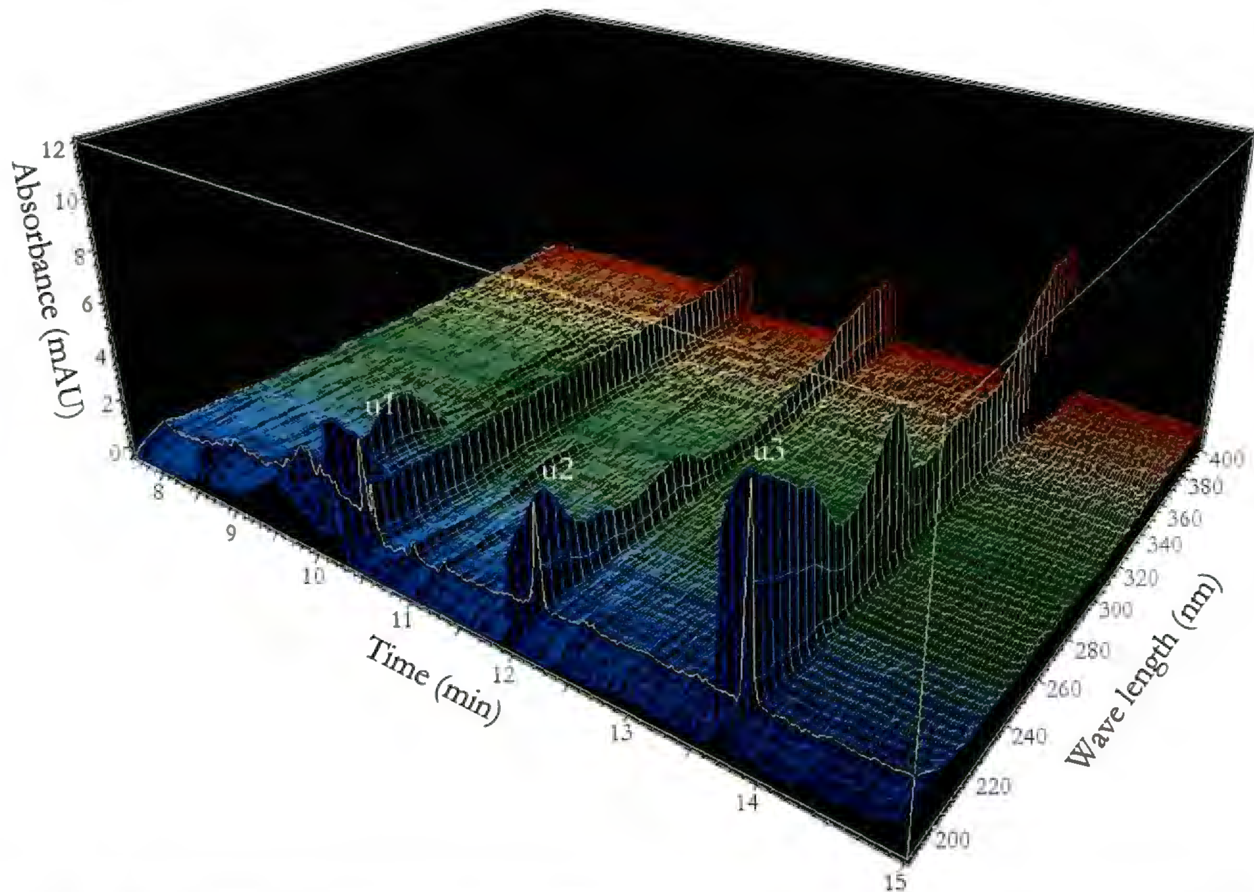


Figure A-52 - 3D Electropherogram of seedling samples. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2, and (u3) unknown peak 3.

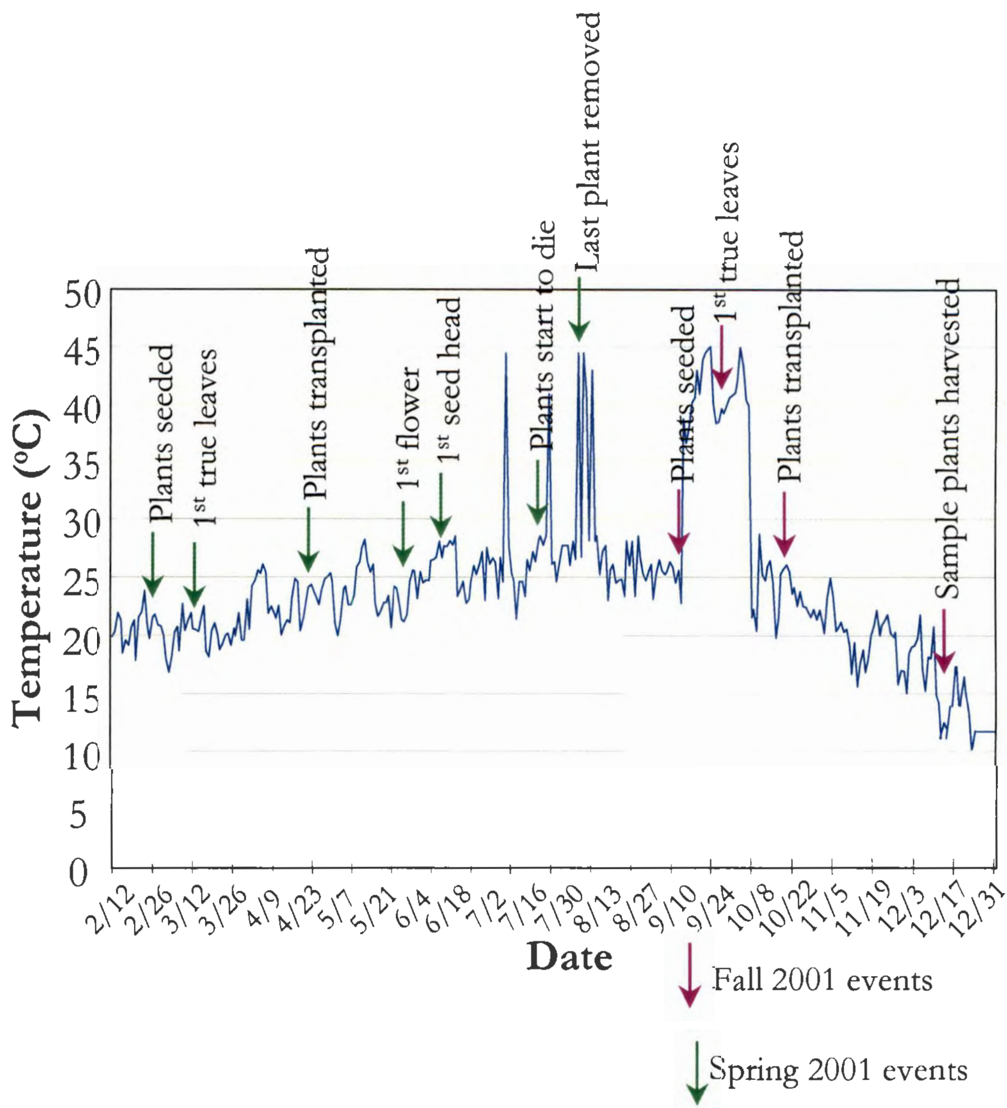


Figure A-53 - Greenhouse temperatures for 2001 with important horticultural dates.

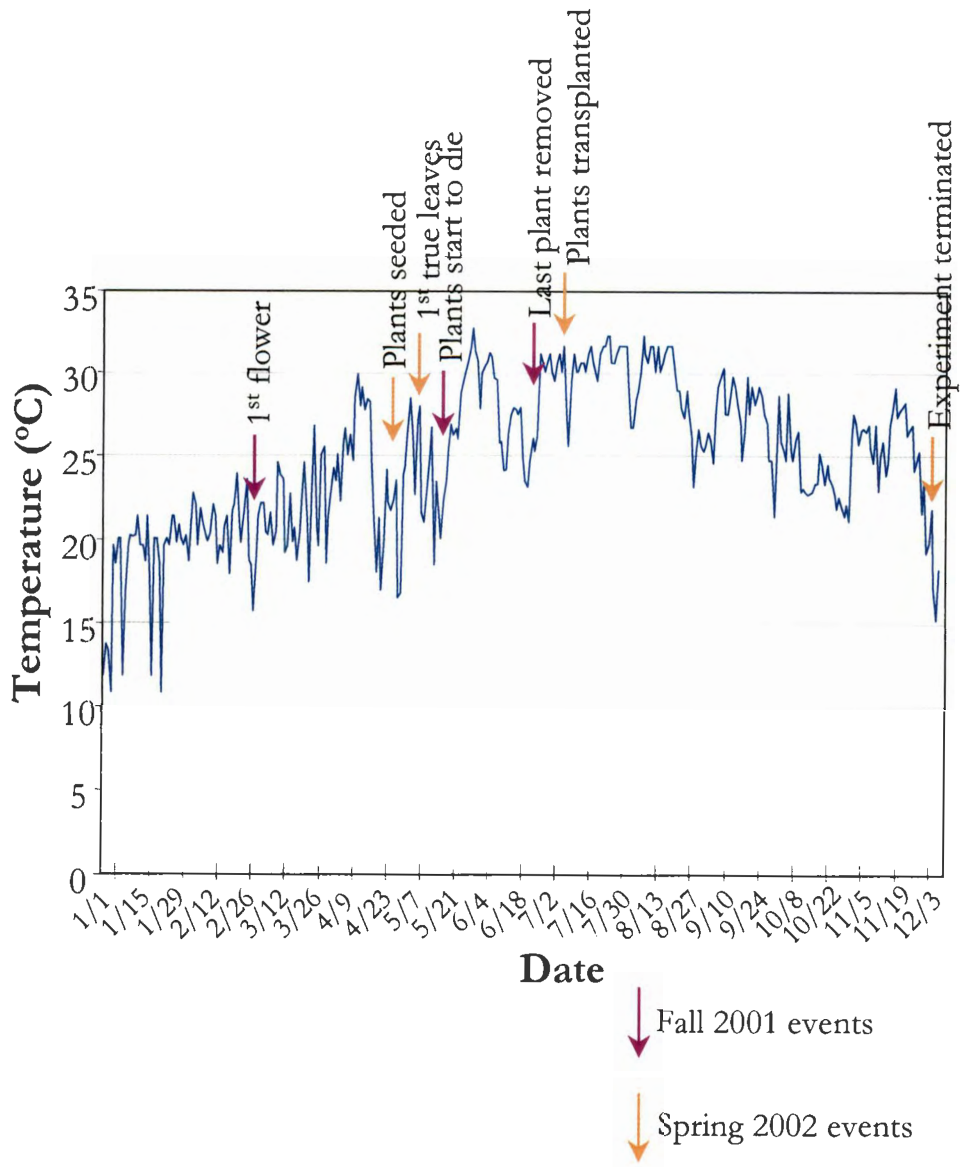


Figure A-54 - Greenhouse temperatures for 2002 with important horticultural dates.

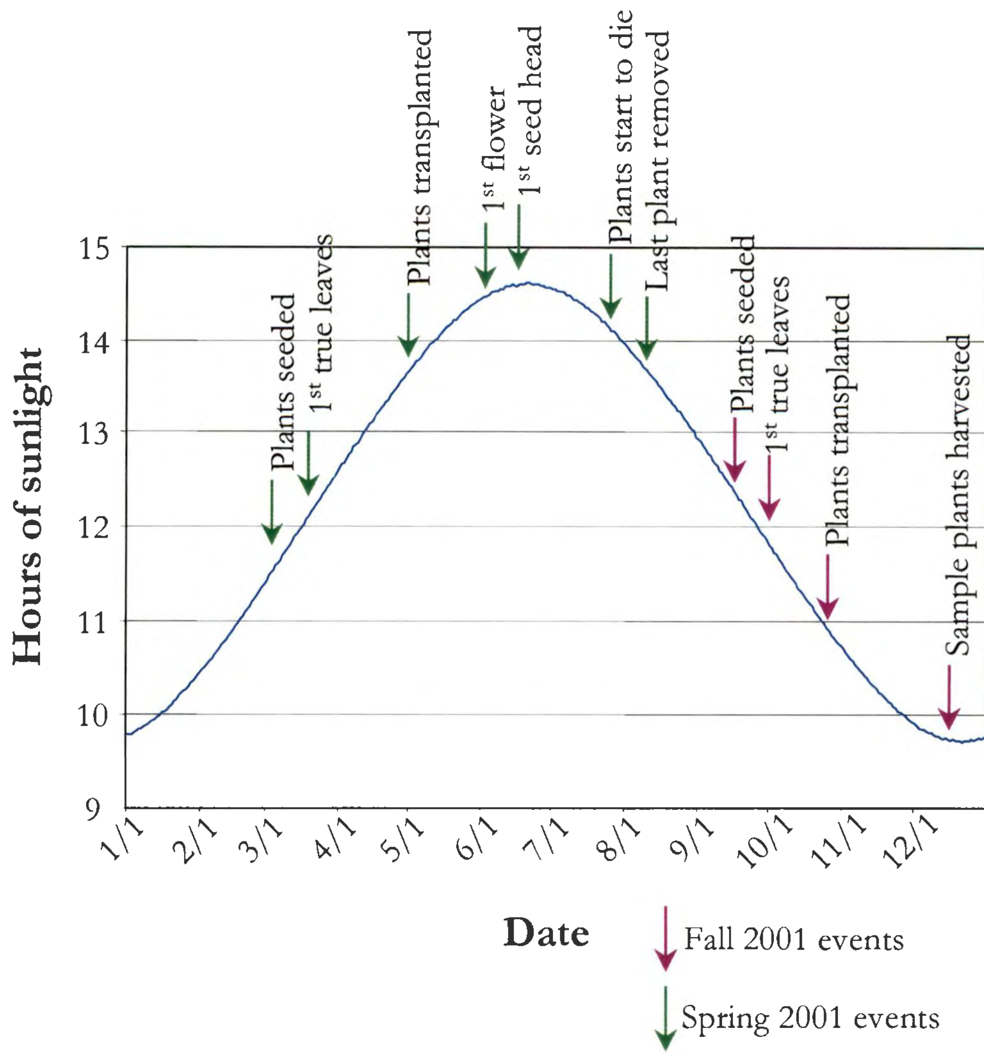


Figure A-55 - Day-length in Knoxville for 2001 with important horticultural dates.

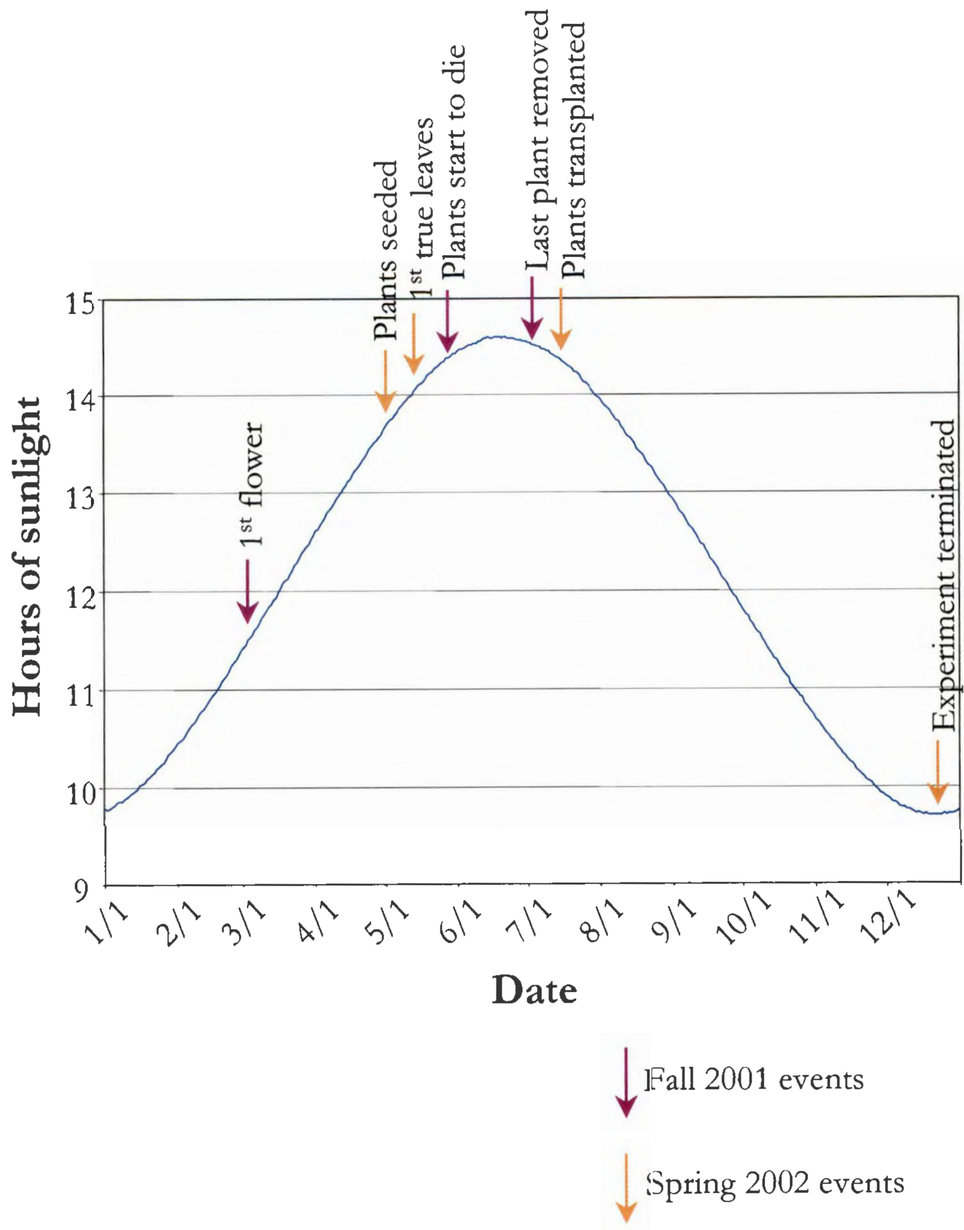
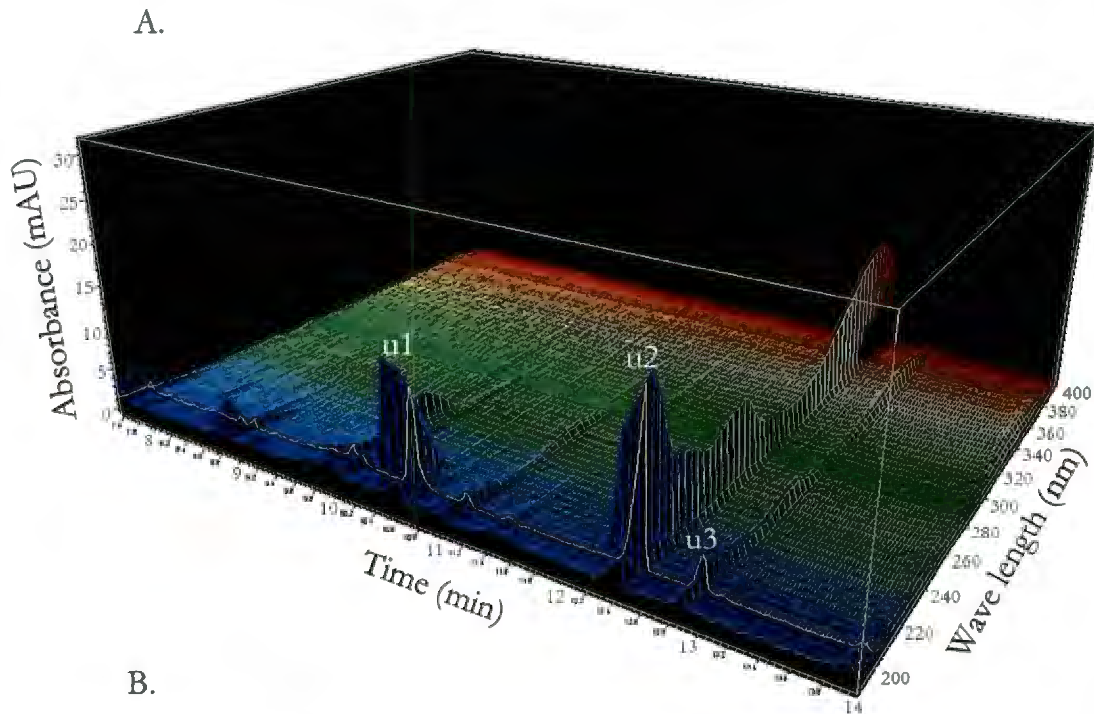


Figure A-56 - Day-length in Knoxville for 2002 with important horticultural dates.

A.



B.

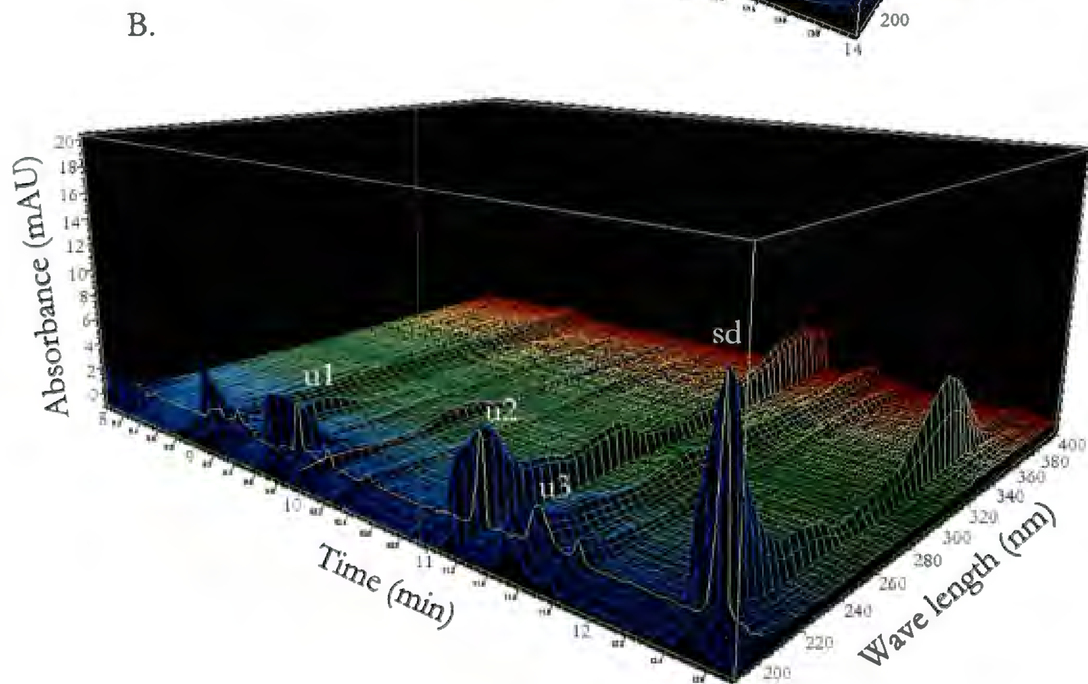


Figure A-57 - 3D electropherograms of upper leaf samples from (A) the fall 2001 experiment and (B) the spring 2001 experiment. Labeled peaks are (u1) unknown peak 1, (u2) unknown peak 2, (u3) unknown peak 3, and (sd) silydianin,

Appendix B - Tables

Table B-1 – Elemental composition of *Silybum marianum* grown in Hungary. As analyzed by Szentmihályi et al. (1998).

Element	Concentration*	Standard Deviation
Al	3470	175
As	< dl**	–
B	32.64	1.68
Ba	31.99	2.84
Ca	37268	355
Cd	0.33	0.034
Co	3.75	0.72
Cr	13.48	0.009
Cu	13.62	0.58
Fe	3109	102
Hg	< dl	–
K	45870	620
Li	4.64	0.62
Mg	5051	86
Mn	140.7	12.9
Mo	0.22	0.057
Na	5584	43
P	3408	96.5
Pb	3.46	0.11
S	2089	109
Ti	66.33	6.49
V	6.64	0.28
Zn	28.65	0.57

*Concentration = μg/g of macerated dried tissue
**< dl = below detection limits

Table B-2 - Murashige and Skoog (1962) formula

MS Salts	*mg/L*
MAJOR SALTS	
Ammonium nitrate (NH_4NO_3)	1650
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	440
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	370
Potassium nitrate (KNO_3)	1900
Potassium phosphate (KH_2PO_4)	170
MINOR SALTS	
Boric acid (H_3BO_3)	6.2
Cobalt Chloride ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric sulfate ($CuSO_4 \cdot 5H^2O$)	0.025
Manganese sulfate ($MnSO_4 \cdot H_2O$)	16.9
Potassium iodide (KI)	0.83
Sodium molybdate ($NaMoO_4 \cdot 2H_2O$)	0.25
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	8.6
IRON	
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	27.8
Na_2EDTA	37.3
Total mg/L	*4627.93*

Table B-3 - Elemental breakdown of full strength hydroponic solution given to *Silybum marianum* seedlings at a 1:10 diluted rate

Elements	mg/L
Total Nitrogen	23.91
Ammonia Nitrogen	0
Phosphorus	46.95
Potassium	188.53
Calcium	0
Magnesium	2.92
Sulfur	0
Iron	2.39
Manganese	1.20
Zinc	0.30
Boron	1.20
Copper	0.30
Molybdenum	0.06
Chlorine	11.95

Table B-4 - Hydroponic solution applied to the Spring 2001 greenhouse experiment in mg/L.

	Treatments (Ca:N)		
Elements	Treatment 1 (1:2)	Treatment 2 (1:1)	Treatment 3 (2:1)
Total Nitrogen	257.26	257.26	257.26
Ammonia Nitrogen	7.75	7.75	7.75
Phosphorus	71.70	71.70	71.70
Potassium	496.00	496.00	496.00
Calcium	128.97	257.29	514.13
Magnesium	114.64	114.64	114.64
Sulfur	151.18	151.18	151.18
Iron	2.99	2.99	2.99
Manganese	3.14	3.14	3.14
Zinc	0.45	0.45	0.45
Boron	1.49	1.49	1.49
Copper	0.45	0.45	0.45
Molybdenum	0.15	0.15	0.15
Chlorine*	32.15	259.28	713.92
Total mg/L	1260.56	1615.20	2325.05

*Chlorine levels are exaggerated because these figures do not take into consideration the volatilization of this element.

Table B-5 - Hydroponic solution applied to the Fall 2001 and Summer 2002 greenhouse experiment in mg/L.

	Treatments		
Elements	Treatment 1 *(Low N)*	Treatment 2 *(Medium N)*	Treatment 3 *(High N)*
Total Nitrogen	47.13	100.63	151.80
Ammonia Nitrogen	1.94	6.40	10.66
Phosphorus	46.89	46.89	46.89
Potassium	188.30	188.30	188.30
Calcium	188.83	187.64	188.87
Magnesium	26.04	26.04	26.04
Sulfur	30.49	30.49	30.49
Iron	2.39	2.39	2.39
Manganese	1.19	1.19	1.19
Zinc	0.30	0.30	0.30
Boron	1.20	1.20	1.20
Copper	0.30	0.30	0.30
Molybdenum	0.06	0.06	0.06
Chlorine*	296.92	182.01	76.29
Total mg/L	830.01	767.42	714.11

*Chlorine levels are exaggerated because these figures do not take into consideration the volatilization of this element.

Table B-6 - Elemental composition of Hydrogardens® tomato fertilizer

Elements	mg/L
Total Nitrogen	23.91
Ammonia Nitrogen	0
Phosphorus	46.95
Potassium	188.53
Calcium	0
Magnesium	2.92
Sulfur	0
Iron	2.39
Manganese	1.20
Zinc	0.30
Boron	1.20
Copper	0.30
Molybdenum	0.06
Chlorine	11.95

Appendix C - Procedures

Procedure C-1 – Extraction of seed samples

After samples were weighed and ground, the following extraction procedure was followed:

1. Vortex sample (30 sec, max speed)
2. Sonicate (20 min)
3. Vortex (30 sec, max speed)
4. Centrifuge (1000 RCF, 5°C, 10 min)
5. Remove supernatant
6. Add 5 mL of solvent back to samples
7. Repeat steps 1 through 6 two additional times, pooling supernatant from each sample

If extracting with solvents sequentially: the following steps were added

8. Dry sample pellets under forced air (room temperature)
9. Re-suspend tissue in next solvents
10. Repeat steps 1 through 6 three times

Procedure C-2 – Extraction of vegetative tissue samples

Samples were treated as follows:

1. Weigh each sample into one 0.5 g amount
2. Grind in 10 mL of ethanol using a mortar and pestle
3. Transfer samples to test tubes
4. Vortex (30 sec, max speed)
5. Sonicate (50 min)
6. Vortex (30 sec, max speed)
7. Sonicate (15 min)

8. Vortex (30 sec, max speed)
9. Let stand 1-2 hours
10. Remove supernatant
11. Add 5 mL of solvent back to samples
12. Repeat steps 1 through 11 two additional times, pooling supernatant from each sample
13. Dry sample pellets under forced air (room temperature)
14. Re-suspend tissue in next solvents
15. Repeat steps 1 through 6 three times for each solvent

Procedure C-3 – Preparing samples for the bioassay

After completing procedure 3.1 or 3.2 the following procedure was followed:

1. Dry pooled supernatants (overnight, under forced air, 30°C)
2. Add 5mL of 12.5% DMSO to residue
3. Sonicate (40 min)
4. Vortex over the course of the day, allowing time between each vortexing for residue to dissolve
5. Store samples at 4°C for no longer than 1 week
6. 24 hours before the bioassay, sterile filter using a 0.2 μm-pore nylon sterile filter under a laminar flow hood.

Procedure C-4 – Capillary Electrophoresis Extractions

Samples were treated as follows:

1. Weigh each sample into one 0.5 g amount

2. Grind in 9 mL of Ethanol and 1 mL of 0.01 M quercetin using a mortar and pestle
3. Transfer samples to test tubes
4. Vortex (30 sec, max speed)
5. Sonicate (50 min)
6. Vortex (30 sec, max speed)
7. Sonicate (15 min)
8. Let stand 1-2 hours
9. Remove supernatant
10. Filter supernatant into CE vials (0.4 micron)

Procedure C-5 – Performing the potato disc bioassay

Culturing *Agrobacterium tumefaciens* – Streak Plates

1. Prepare nutrient broth agar (NBA)
2. Pour plates using 20 mL of NBA per plate
3. Allow plates to cool, solidify, and dry slightly
4. Using an inoculation loop, take a sample of *A. tumefaciens* from a stock and inoculate the plates using streaking techniques to obtain single colonies
5. Seal plates and incubate in the dark at room temperature for 48 hours
6. Store at 4°C. Use the plates within one month of creation

Inoculum preparation

1. Inoculate 50 ml of NB with 1 loop (inoculation loop) of *A. tumefaciens* single colony from a streak plate
2. Shake the culture for 48 h at room temperature
3. Use immediately

Determination of bacterial count of Inoculum

1. Serial dilute bacterial culture in test tubes containing NB

   - Add 1 mL of bacterial culture to 9 ml of NB

   - Mix using vortexer on low speed

   - Add 1 mL of diluted culture to 9 ml of NB

   - Mix using vortexer on low speed

   - Repeat 3 and 4 until bacterial culture has been diluted eight times

2. Make plates using dilutions

   - Spread 100 µl of last three dilutions on NBA plates

   - Incubate at room temperature in the dark for 48 hr

   - Count colonies and multiply by 10^7, 10^8, and 10^9 depending on dilution to get CFUs /ml

Bioassay Preparation

1. Foil and autoclave all needed equipment:

   beakers, pipette tips (5mL and 1000 µl), aluminum foil, cork borers (2, one slightly smaller than the other), scalpel, large tweezers, test-tubes

2. Make water agar plates

3. Filter sterilize samples and prepare samples and standards (dilutions)

Bioassay

1. Prepare potatoes

   - wash and scrub lightly medium sized potatoes

   - Soak potatoes by immersion in 20% clorox for 20 min

   - Cut the ends off of the potatoes using a bleached knife

- Soak potatoes for 10 min in a new 20% clorox solution

2. Add *A. tumefaciens* bacterial culture to diluted samples
3. Extract the core of the potatoes using a sterile cork borer
4. Remove 1 cm from each end of core and discard
5. Cut the remainder of core into 0.5cm thick discs and place on plates, putting 5 discs per plate.
6. Inoculate discs with 1000 µl of sample and bacteria aliquot.
7. Wrap plates with parafilm
8. Incubate for 20 days at 25°C in the dark

Counting the tumors

1. Stain potato discs using a diluted (10:1) lugol solution
2. Count tumors
3. Do not count tumors on discs that are too dehydrated or contaminated

(Anand and Heberlein 1977, Chardonnett unpublished work)

Procedure C-6 – Running CE for flavonolignan separation

Preparation of buffer

1. Mix 20 mM borax solution equally with 20 mM boric acid.
2. Adjust to pH 9 using 1 N NaOH.
3. Add methanol to produce a final 15% methanolic solution.

Preparation of the CE

1. Replace column: uncoated silica capillary with a total length of 43 cm, an effective length of 35 cm, and an interior diameter of 0.05 mm

2. Add buffer to the replenishment system and condition as described in the manual
3. Set up the following for the runs: Temperature of 30°C, Voltage a constant 20 KV, and a current of 60 μA
4. Flush and condition the column as follows (1st use of column): 1 N NaOH for 10 min, 0.1 N NaOH for 10 min, water for 20 min and buffer for 30 min.

Running samples

1. Prepare samples as described in Procedure C-4.
2. Set each run for 20 minutes
3. Record all wavelengths from 190 to 400 nm.
4. Set primary wavelength to 200 nm.
5. Prior to each run, flush the column with running buffer for 5 minutes
6. After every third run flush and recondition the column with: 1 N NaOH for 10 min, 0.1 N NaOH for 10 min, water for 10 min and buffer for 20 min.
7. Before the reconditioning, buffers should be replenished and the waste vial emptied using the replenishment system.

(Modified from Quaglia et al. 1999)

Appendix D – Statistics Tables

Spring 2001 Statistics Tables

Statistical analysis of calcium treatment effects on seed yield in the Spring 2001 experiment. Fertilizer treatments are defined as (B) 2:1 Ca:N, (R) 1:1 Ca:N, and (Y) 1:2 Ca:N.

The Mixed Procedure

Data Set	WORK.SPRING2001YIELDS
Dependent Variable	TotalSeedweight

Class	Levels	Values
Treatment	3	b r y
Block	9	1 2 3 4 5 6 7 8 9

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	16	0.98	0.3954

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Treatment ----------------

Obs	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	b	14.7478	3.4576	16	4.27	0.0006	A
2	r	8.6622	3.4576	16	2.51	0.0234	A
3	y	12.7544	3.4576	16	3.69	0.0020	A

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.923755	Pr < W	0.0487
Kolmogorov-Smirnov	D	0.14187	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.108563	Pr > W-Sq	0.0851
Anderson-Darling	A-Sq	0.677691	Pr > A-Sq	0.0721

Statistical analysis of differences between the solvent's effect on seed extract activity from the spring 2001 experiment using single-solvent extraction. The dependent variable is total number of tumors.

```
                Model Information

Dependent Variable            tumors
Covariance Structure          Variance Components
Estimation Method             REML
Residual Variance Method      Profile
Fixed Effects SE Method       Model-Based
Degrees of Freedom Method     Containment

            Class Level Information
Class        Levels    Values
Extract          11    A C DMSO DMSO+Bac E Extract M
                       W water water+Ba
Treatment         5    Blue Red Treatment Yellow
                       control

                  Dimensions
         Covariance Parameters             2
         Columns in X                     11
         Columns in Z                      5
         Subjects                          1
         Max Obs Per Subject             751
         Observations Used               406
         Observations Not Used           345
         Total Observations              751

              Covariance Parameter
                   Estimates
             Cov Parm       Estimate
             Treatment             0
             Residual        28.6624

           Type 3 Tests of Fixed Effects
                   Num      Den
    Effect          DF       DF    F Value    Pr > F
    Extract          9      394      16.00    <.0001

         Mean separation for tumors
------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Extract -----------------
                                      Standard                                  Pr >     Let
       Obs    Extract      Estimate     Error         DF     t Value        |t|     Grp

         9    water         26.8000     2.3943       394       11.19      <.0001     A
         3    DMSO          16.1818     1.6142       394       10.02      <.0001     B
        10    water+Ba      10.9167     1.5455       394        7.06      <.0001     C
         7    W              9.6364     0.6590       394       14.62      <.0001     C
         1    A              5.9853     0.6492       394        9.22      <.0001     D
         2    C              5.6222     0.5643       394        9.96      <.0001     D
         5    E              5.1757     0.6224       394        8.32      <.0001     D
         6    M              6.1846     0.6640       394        9.31      <.0001     D
         8    cAMP+Bac       4.4444     1.7846       394        2.49      0.0132     D
```

Statistical analysis of differences between solvent effect from the sequential extraction on seed extract activity from the spring 2001 experiment. The dependent variable in number of tumors.

Dependent Variable: Number of Tumors

Class	Levels	Values
Treatment	11	A C DMSO DMSO+Bac E M W Waste Water Water+Ba cAMP
Plant	9	2.4 2.5 2.6 3.2 3.8 3.9 4.2 4.5 4.6

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	10	1396	85.38	<.0001

Mean separation for tumors

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Treatment ----------------

Obs	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
4	DMSO+Bac	27.5714	0.7760	1396	35.53	<.0001	A
8	Waste	22.9902	1.3601	1396	16.90	<.0001	B
5	E	21.8840	0.7622	1396	28.71	<.0001	B
10	Water+Ba	21.5000	0.6596	1396	32.60	<.0001	B
7	W	21.3187	0.8472	1396	25.17	<.0001	BC
1	A	19.3095	0.7649	1396	25.24	<.0001	CD
2	C	17.3769	0.7649	1396	22.72	<.0001	D
6	M	13.6850	0.7595	1396	18.02	<.0001	E
11	cAMP	11.3571	0.7760	1396	14.64	<.0001	F
9	Water	8.2000	0.6596	1396	12.43	<.0001	G
3	DMSO	6.2000	0.7517	1396	8.25	<.0001	H

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.959951	Pr < W	<0.0001

Statistical analysis of calcium treatment effects on the activity of the leaf extracts from the Spring 2001 experiment in the bioassay. Fertilizer treatments are defined as (1) 1:1 Ca:N, (2) 2:1 Ca:N, and (3) 1:2 Ca:N.

The Mixed Procedure

Data Set	WORK.LEAVESEXP1
Dependent Variable	control

Class	Levels	Values
ferttreat	3	1 2 3
part	2	lowleaf upperlea
extract	2	chloroform ethanol

Effect	Num DF	Den DF	F Value	Pr > F
ferttreat	2	359	4.14	0.0166
extract	1	359	0.01	0.9326
ferttreat*extract	2	359	0.00	0.9967

Mean Separation for control

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=ferttreat ----------------

ferttreat	extract	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1		17.5381	3.5959	359	4.88	<.0001	AB
2		8.9973	2.9468	359	3.05	0.0024	B
3		22.7797	4.0750	359	5.59	<.0001	A

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=2 Effect=extract ----------------

ferttreat	extract	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
_	chloroform	16.2641	2.8062	359	5.80	<.0001	A
_	ethanol	16.6126	3.0185	359	5.50	<.0001	A

-------------------- ADJUSTMENT=LSD(.05) BYGROUP=3 Effect=ferttreat*extract ------------

ferttreat	extract	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	chloroform	17.1422	4.6873	359	3.66	0.0003	AB
1	ethanol	17.9340	5.4545	359	3.29	0.0011	AB
2	chloroform	8.9738	3.9615	359	2.27	0.0241	B
2	ethanol	9.0208	4.3636	359	2.07	0.0394	AB
3	chloroform	22.6761	5.7629	359	3.93	<.0001	AB
3	ethanol	22.8832	5.7629	359	3.97	<.0001	A

Statistical analysis of differences between solvent, and plant part effect on extract activity from the spring 2001 experiment. The dependent variable is percent control.

Data Set	WORK.LEAVESEXP1
Dependent Variable	% control

Class	Levels	Values
part	2	lowleaf upperleaf
Plant	8	2.5 2.8 3.4 3.5 3.6 4.2 4.3 4.9
extract	2	chloroform ethanol

Effect	Num DF	Den DF	F Value	Pr > F
part	1	360	0.17	0.6797
extract	1	360	0.07	0.7976
part*extract	1	358	3.23	0.0730

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=part -----------------

Obs	part	extract	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	lowleaf		15.0300	3.8773	11.3	3.88	0.0025	A
2	upperleaf		16.6825	4.0906	13.1	4.08	0.0013	A

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=2 Effect=extract ----------------

Obs	part	extract	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
3		chloroform	15.3457	3.8868	11.2	3.95	0.0022	A
4		ethanol	16.3669	4.0712	13.4	4.02	0.0014	A

---------------------- ADJUSTMENT=LSD(.05) BYGROUP=3 Effect=part*extract -------------

Obs	part	extract	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
5	lowleaf	chloroform	18.0746	4.5633	21.5	3.96	0.0007	A
6	lowleaf	ethanol	11.9854	4.8551	27.1	2.47	0.0202	A
7	upperleaf	chloroform	12.6168	4.8806	25.5	2.59	0.0158	A
8	upperleaf	ethanol	20.7483	5.1747	32	4.01	0.0003	A

Fall 2001 Statistics Tables

Statistical analysis of differences between the nitrogen effect on seed yield in the fall 2001 experiment. Fertilizer values are (1) low N, (2) medium N, (3) high N.

Model Information

Data Set	WORK.SEED_YIELD20012002
Dependent Variable	yield
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Treatment	3	1 2 3
Block	6	1 2 3 4 5 6

Dimensions

Covariance Parameters	2
Columns in X	4
Columns in Z	6
Max Obs Per Subject	53
Observations Used	30
Observations Not Used	23
Total Observations	53

Covariance Parameter Estimates

Cov Parm	Estimate
Block	540.04
Residual	1454.32

Fit Statistics

-2 Res Log Likelihood	284.9
AIC (smaller is better)	288.9
AICC (smaller is better)	289.4
BIC (smaller is better)	288.5

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	22	6.59	0.0057

Mean separation for yield

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Treatment ----------------

Obs	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	1	33.7155	14.5360	22	2.32	0.0300	B
2	2	51.2468	16.6387	22	3.08	0.0055	B
3	3	101.63	17.2706	22	5.88	<.0001	A

Statistical analysis of differences between the nitrogen effect on vegetative yield in the fall 2001 experiment. Fertilizer values are (y) low N, (r) medium N, (b) high N.

Model Information

Data Set	WORK.VEG_YIELD
Dependent Variable	PlantW
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Comments	3	b r y
Rep	6	1 2 3 4 5 6

Dimensions

Covariance Parameters	2
Columns in X	4
Columns in Z	6
Subjects	1
Max Obs Per Subject	54
Observations Used	53
Observations Not Used	1
Total Observations	54

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Comments	2	45	9.22	0.0004

Mean separation for plantW

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Comments -----------------

Obs	Comments	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	b	421.39	40.6740	45	10.36	<.0001	A
2	r	414.72	40.6740	45	10.20	<.0001	A
3	y	200.06	41.8532	45	4.78	<.0001	B

Statistical analysis of nitrogen treatment effects on average number of days from transplant to harvest. Fertilizer treatments are defined as (1) high N (2) medium N, and (3) low N.

```
Data Set                      WORK.SEED_YIELD20012002
Dependent Variable            days

 Class         Levels    Values
 Treatment          3    1 2 3
 Block              6    1 2 3 4 5 6

                  Num      Den
    Effect         DF       DF     F Value     Pr > F
    Treatment       2       14        1.91     0.1847
```

Mean separation for days

------------------------ ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Treatment ----------------

Obs	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	1	259.38	4.1601	14	52.74	<.0001	A
2	2	258.76	4.4211	14	49.48	<.0001	A
3	3	265.33	4.7454	14	47.48	<.0001	A

Statistical analysis of differences between Nitrogen treatment effect on seed extract activity from the fall 2001 experiment. Fertilizer treatments are: 1 Low N, 2 Medium N, 3 High N, and 4 cAMP.

```
Dependent Variable          Control

   Class    Levels    Values
   Fert          4    1 2 3 4

                   Num     Den
    Effect          DF      DF    F Value    Pr > F
    Fert             3     232       2.94    0.0340

--------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert ------------------
```

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	1	52.6057	3.7018	232	14.21	<.0001	B
2	2	56.0931	3.5763	232	15.68	<.0001	AB
3	3	63.8105	3.5296	232	18.08	<.0001	A
4	4	41.2011	8.2775	232	4.98	<.0001	B

Statistical analysis of differences between Nitrogen treatment effect on root extract activity from the fall 2001 experiment. The first table examines the chloroform extract; the second the ethanolic extract.

```
Data Set                      WORK.CHLOROFORMROOT
Dependent Variable            control
   Class    Levels    Values
   fert          3    HighN MediumN LowN
   Rep           3    1 2 3
                  Num      Den
    Effect         DF       DF    F Value    Pr > F
    fert            2       87       0.29    0.7476
```

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=fert ------------------

Obs	fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	HighN	33.4884	15.5833	87	2.15	0.0344	A
2	MediumN	29.3119	15.8893	87	1.84	0.0685	A
3	LowN	27.6860	15.5072	87	1.79	0.0777	A

```
Data Set                      WORK.ETHANOLROOT
Dependent Variable            control

   Class    Levels    Values
   fert          3    HighN MediumN LowN
   Rep           3    1 2 3

                  Num      Den
    Effect         DF       DF    F Value    Pr > F

    fert            2      125       0.06    0.9421
```

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=fert ------------------

Obs	fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	HighN	43.4825	5.1066	125	8.51	<.0001	A
2	MediumN	44.9047	4.8528	125	9.25	<.0001	A
3	LowN	45.6936	4.8528	125	9.42	<.0001	A

Statistical analysis of differences between Nitrogen treatment effect on stem extract activity from the fall 2001 experiment.

Data Set	WORK.STEMS
Dependent Variable	control

Class	Levels	Values
fert	3	Blue Red Yellow
Rep	3	1 2 3

Effect	Num DF	Den DF	F Value	Pr > F
fertilizer	2	130	1.51	0.2248

Obs	fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	High N	40.6969	4.9162	130	8.28	<.0001	A
2	Medium N	38.7245	4.9162	130	7.88	<.0001	A
3	Low N	50.0329	4.9162	130	10.18	<.0001	A

Statistical analysis of differences between Nitrogen treatment and plant part on extract activity from the fall 2001 experiment. Also analyzed against cAMP, a control treatment. Fertilizer treatments are defined as (Blue) High N, (Red) Medium N, and (Yellow) Low N.

Dependent Variable %control

Class	Levels	Values
Fertilizer	4	Blue Red Yellow non
Rep	3	1 2 3
Part	4	CAMP Lowerleaf Midleaf Upperleaf

Effect	Num DF	Den DF	F Value	Pr > F
Fertilizer	2	581	4.72	0.0092
Part	2	581	4.76	0.0089
Fertilizer*Part	4	581	5.67	0.0002

-------------------- ADJUSTMENT=LSD(.05) BYGROUP=3 Effect=Fertilizer*Part -------------

Obs	Fertilizer	Part	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
14	Red	Upperleaf	51.1810	6.8923	6.51	7.43	0.0002	A
16	Yellow	Midleaf	45.0939	8.0577	12.1	5.60	0.0001	AB
17	Yellow	Upperleaf	42.2752	6.4298	4.94	6.57	0.0013	AB
13	Red	Midleaf	36.2427	7.4045	8.66	4.89	0.0010	BC
10	Blue	Midleaf	34.9261	7.7883	10.6	4.48	0.0010	BC
9	Blue	Lowerleaf	30.2047	6.3567	4.72	4.75	0.0059	C
12	Red	Lowerleaf	27.2872	6.2765	4.49	4.35	0.0094	CD
15	Yellow	Lowerleaf	26.4941	6.3962	4.84	4.14	0.0096	CD
11	Blue	Upperleaf	17.0922	6.6201	5.55	2.58	0.0448	D
18	non	CAMP	31.6948	12.8043	70.5	2.48	0.0157	ABCD

Statistical analysis of differences between the solvent's effect on seedling extract activity from the fall 2001 experiment.

```
Dependent Variable          control

  Class       Levels    Values
  Extract          2    chloroform ethanol
  Rep              3    1 2 3

                    Num      Den
    Effect           DF       DF    F Value     Pr > F
    Extract           1       75      14.59     0.0003

------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Extract -------------------

                                   Standard                              Pr >      Let
 Obs    Extract       Estimate      Error         DF     t Value         |t|       Grp
  1     chloroform    30.3507       5.0355        75        6.03       <.0001       B
  2     ethanol       54.3452       5.4776        75        9.92       <.0001       A

                          Tests for Normality
    Test                    --Statistic---     -----p Value------
    Shapiro-Wilk            W      0.946686    Pr < W      0.0024
```

Statistical analysis of differences between Nitrogen treatment and peak areas of immature leaves. Fertilizer treatments are defined as (B) high nitrogen, (R) medium nitrogen, (Y) low nitrogen, and (F) is samples from the Spring 2001 experiment.

Peak 1:

Data Set	WORK.PEAK1
Dependent Variable	CorrectedArea

Class	Levels	Values
Fert	4	B F R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	3	20	6.36	0.0033

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	5.5180	1.2915	20	4.27	0.0004	A
2	F	1.8113	0.6204	20	2.92	0.0085	B
3	R	5.8532	1.1184	20	5.23	<.0001	A
4	Y	5.9730	1.1184	20	5.34	<.0001	A

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.971017	Pr < W	0.6921
Kolmogorov-Smirnov	D	0.099071	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.027448	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.209689	Pr > A-Sq	>0.2500

Peak 2:

The Mixed Procedure

Model Information

Data Set	WORK.PEAK2
Dependent Variable	CorrectedArea

Class Level Information

Class	Levels	Values
Fert	4	B F R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	3	19	13.30	<.0001

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	3.4261	0.9119	19	3.76	0.0013	BC
2	F	1.9615	0.4560	19	4.30	0.0004	C
3	R	5.1499	0.7897	19	6.52	<.0001	AB
4	Y	7.3973	0.7897	19	9.37	<.0001	A

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.970496	Pr < W	0.7009
Kolmogorov-Smirnov	D	0.122046	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.057604	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.32809	Pr > A-Sq	>0.2500

Peak 3:

Model Information

Data Set	WORK.PEAK3
Dependent Variable	CorrectedArea

Class	Levels	Values
Fert	4	B F R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	3	20	5.84	0.0049

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	0.8336	1.7720	20	0.47	0.6431	B
2	F	7.1375	1.0231	20	6.98	<.0001	A
3	R	1.1271	1.7720	20	0.64	0.5320	B
4	Y	1.3024	1.7720	20	0.73	0.4709	B

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.878067	Pr < W	0.0076
Kolmogorov-Smirnov	D	0.238879	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.235628	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	1.285447	Pr > A-Sq	<0.0050

Statistical analysis of differences between Nitrogen treatment and peak areas of leaves harvested from the middle portion of the plant. Fertilizer treatments are defined as (B) high nitrogen, (R) medium nitrogen, (Y) low nitrogen.

Peak1:

The Mixed Procedure

Data Set	WORK.PEAK1M
Dependent Variable	Carea

Class	Levels	Values
Fert	3	B R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	2	9	0.75	0.4996

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	1.7207	0.7888	9	2.18	0.0571	A
2	R	1.8037	0.7888	9	2.29	0.0480	A
3	Y	0.5808	0.7888	9	0.74	0.4803	A

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.902182	Pr < W	0.1692
Kolmogorov-Smirnov	D	0.157998	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.052873	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.396822	Pr > A-Sq	>0.2500

Peak 2 (Taxifolin):

Model Information

Data Set	WORK.PEAK3M
Dependent Variable	Carea

Class	Levels	Values
Fert	3	B R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	2	9	0.27	0.7696

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	3.7740	0.9078	9	4.16	0.0025	A
2	R	4.5391	0.9078	9	5.00	0.0007	A
3	Y	3.6792	0.9078	9	4.05	0.0029	A

Statistical analysis of differences between Nitrogen treatment and peak areas of seeds. Fertilizer treatments are defined as (B) high nitrogen, (R) medium nitrogen, and (Y) low nitrogen. All peaks are labeled in "Data Set."

```
                              The Mixed Procedure

                Data Set                         WORK.SILYBIN
                Dependent Variable               Carea

                  Class     Levels     Values
                  Fert           3     B R Y

                                    Num      Den
                    Effect           DF       DF     F Value     Pr > F
                    Fert              2        8        0.14     0.8696

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------
                                   Standard                                  Pr >      Let
           Obs      Fert     Estimate      Error           DF      t Value        |t|      Grp

            1        B        21.2727       5.1254          8         4.15     0.0032       A
            2        R        23.9683       5.1254          8         4.68     0.0016       A
            3        Y        25.2719       5.9183          8         4.27     0.0027       A

                              Tests for Normality

              Test                      --Statistic---       -----p Value------
              Shapiro-Wilk              W      0.955436      Pr < W        0.7138
              Kolmogorov-Smirnov        D      0.212801      Pr > D       >0.1500
              Cramer-von Mises          W-Sq   0.055375      Pr > W-Sq    >0.2500
              Anderson-Darling          A-Sq   0.297511      Pr > A-Sq    >0.2500


                              The Mixed Procedure

                Data Set                         WORK.SILYCHRISTIN
                Dependent Variable               Carea

                  Class     Levels     Values
                  Fert           3     B R Y

                                    Num      Den
                    Effect           DF       DF     F Value     Pr > F
                    Fert              2        8        0.07     0.9366

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert -------------------
                                   Standard                                  Pr >      Let
           Obs      Fert     Estimate      Error           DF      t Value        |t|      Grp

            1        B         6.6654       1.4585          8         4.57     0.0018       A
            2        R         6.6092       1.4585          8         4.53     0.0019       A
            3        Y         7.3532       1.6841          8         4.37     0.0024       A

                              Tests for Normality

              Test                      --Statistic---       -----p Value------
              Shapiro-Wilk              W      0.941206      Pr < W        0.5348
              Kolmogorov-Smirnov        D      0.168813      Pr > D       >0.1500
              Cramer-von Mises          W-Sq   0.067172      Pr > W-Sq    >0.2500
              Anderson-Darling          A-Sq   0.367578      Pr > A-Sq    >0.2500
```

The Mixed Procedure

Data Set	WORK.SILYDIANIN
Dependent Variable	Carea

Class	Levels	Values
Fert	3	B R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	2	8	0.29	0.7528

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert --------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	27.7449	7.2091	8	3.85	0.0049	A
2	R	33.5576	7.2091	8	4.65	0.0016	A
3	Y	35.6626	8.3243	8	4.28	0.0027	A

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.943559	Pr < W	0.5632
Kolmogorov-Smirnov	D	0.186173	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.061681	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.333439	Pr > A-Sq	>0.2500

The Mixed Procedure

Data Set	WORK.TAXIFOLIN
Dependent Variable	Carea

Class	Levels	Values
Fert	3	B R Y

Effect	Num DF	Den DF	F Value	Pr > F
Fert	2	8	0.75	0.5027

-------------------------- ADJUSTMENT=LSD(.05) BYGROUP=1 Effect=Fert --------------------

Obs	Fert	Estimate	Standard Error	DF	t Value	Pr > |t|	Let Grp
1	B	5.2758	1.5544	8	3.39	0.0094	A
2	R	5.0733	1.5544	8	3.26	0.0115	A
3	Y	7.7455	1.7949	8	4.32	0.0026	A

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.904152	Pr < W	0.2076
Kolmogorov-Smirnov	D	0.174293	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.062774	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.434593	Pr > A-Sq	0.2475

Vita

Jennifer Lena Horst Warren was born in Warwar, Nigeria on December 23, 1978. She was fortunate enough to have lived many places while growing up, including Camrose, Alberta, Canada; London, Ontario, Canada; and Newberry, SC, USA. Lena graduated from Newberry High School in 1996. From there she went to Newberry College and graduated Magna cum laude with a B.S. in Environmental Science with a minor in mathematics in 2000. After that, Lena moved to the University of Tennessee to pursue a Master of Science in Plant and Soil Science, which she received in May 2003.

Lena intends on pursuing her Ph.D. and eventually teach at the university level.

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