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Nitrification Inhibitors from the Roots of *Leucaena leucocephala* (Lam.) de Wit

presented by

Andrew J. Erickson

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of the requirements for

M.S. degree in Horticulture

Major professor

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NITRIFICATION INHIBITORS FROM THE ROOTS OF *LEUCEANA LEUCOCEPHALA* (Lam.) de Wit

By

Andrew J. Erickson

A MASTERS THESIS

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ABSTRACT

NITRIFICATION INHIBITORS FROM THE ROOTS OF *LEUCEANA LEUCOCEPHALA* (LAM.) DE WIT

By

Andrew J. Erickson

Analysis of soil samples taken from the rhizosphere of *Leucaena leucocephala* (Lam.) de Wit trees showed unusually high levels of ammonia and low levels of nitrate. This led to the hypothesis that the trees were somehow inhibiting nitrification. The methanol extract from *L. leucocephala* roots displayed activity in an in vitro nitrification inhibition bioassay, using *Nitrosomonas europaea*. Bioassay-directed fractionation of the extract on Amberlite XAD-2 resin and preparative HPLC yielded the most active compound, gallocatechin. Gallocatechin displayed NI activity at 12 ppm, compared to the DMSO control. The activity displayed by gallocatechin at 12 ppm is similar to that of nitrapyrin, a commercial nitrification inhibitor, at the 10 ppm concentration. Gallocatechin strongly inhibited the conversion of ammonia to nitrite at 50 ppm concentration. Three other compounds identified from the root extract were epigallocatechin, catechin, and epicatechin. Catechin was not inhibitory to *N. europaea* at 10 ppm concentration. Epigallocatechin and epicatechin were isolated as mixtures and thus were not assayed individually.

The *Leucaena leucocephala* extracts were also evaluated for bioactivity in antimicrobial, mosquitocidal, insect anti-feedant, topoisomerase inhibition, and nematicidal bioassays. The anti-fungal assays were conducted on *Aspergillus*, *Fusarium*,

Rhizoctonia, *Botrytis*, and *Gleosporum* spp., and the anti-bacterial assays included *E. coli*, *Staphylococcus* and *Streptococcus* spp. The mosquitocidal and insect anti-feedant assays were carried out on *Aedes aegyptii* larvae and on *Helicoverpa zea* and *Lymantria dispar* caterpillars, respectively. The anti-cancer screen employed mutant *Saccharomyces cerevisae* strains which were sensitive to topoisomerase I and II poisons. The nematicidal bioassays were conducted on *Caenorhabditis elegans* and *Panagrellus redivivus*. None of the extracts displayed activity in the antimicrobial, mosquitocidal, insect anti-feedant, topoisomerase inhibition, nor nematicidal bioassays.

Dedicated to my Dad

ACKNOWLEDGMENTS

I would like to express my appreciation to my major professor, Dr. Muralee Nair for the support and guidance of this research. I also wish to thank my committee members, Drs. John Kelly and Alvin Smucker for their aid in this process.

Drs. Amitahb Chandra and James Nitao were of great help while they were associated with the Bioactive Natural Products Laboratory. Dr. Russel Ramsewak deserves a great deal of thanks for his continued support of this work, not to mention his generally sunny disposition making the daily grind in the laboratory *fun*. I also wish to thank my fellow graduate students Mark Kelm, Jennifer Miles and Haibo Wang for the good times in the lab. I also wish to thank all my contemporaries in the Horticulture Organization of Graduate Students (HOGS) for their support and camaraderie during this time. I also would like to give much thanks to Drs. Rufus Isaacs and James Miller for the employment opportunity that made the continuation and completion of this research much easier.

Lastly, this work would not have been possible without the help and support from my fiancé, Carmen Giddens and from my Mom, Sharon Erickson.

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LIST OF ABBREVIATIONS

ATCC	.....	American Type Culture Collection
BNPL	.....	Bioactive Natural Products Laboratory
COSY	.....	Correlation spectroscopy NMR
DEPT	.....	Distortionless Enhancement by Polarization Transfer
dd	.....	doublet of doublet
DMSO	.....	Dimethyl Sulfoxide
EDTA	.....	Ethylenediamine tetraacetic acid
FAO	.....	Food and Agriculture Organization
HCl	.....	Hydrochloric acid
HMQC	.....	Heteronuclear Multiple Quantum Correlation
HPLC	.....	High Pressure Liquid Chromatography
Hz	.....	Hertz
KOH	.....	Potassium hyroxide
MeOH	.....	Methanol
mL	.....	Milliliter
MPLC	.....	Medium Pressure Liquid Chromatography
MHz	.....	MegaHertz
MIC	.....	Minimum Inhibitory Concentration
NI	.....	Nitrification Inhibition
^{13}CNMR	.....	Carbon Nuclear Magnetic Resonance
^{1}HNMR	.....	Proton Nuclear Magnetic Resonance
ODS	.....	Octadecyl silane
RI	.....	Refractive index
RO	.....	Reverse osmosis
TLC	.....	Thin Layer Chromatography
UV	.....	Ultra-violet

Chapter I

Introduction and Literature Review

Introduction

Nitrification, the conversion of ammonium (NH_4^+) ion to nitrate (NO_3^-) ion by bacteria, is a widespread process occurring in most soils and in freshwater and marine sediments. Nitrification is of interest to agriculture, since large quantities of nitrogen fertilizer is used globally as ammonium salts. In 1996, farmers in the United States applied over 1.6 million metric tons of ammonium fertilizers (United Nations FAOSTAT web page). Worldwide, over 10 million metric tons of ammonium fertilizers (FAOSTAT web page) were applied in 1996. Not only does nitrification decrease fertilizer application efficiency by lowering cost-effectiveness, the resulting product, nitrate, readily leaches out of soil away from roots and into groundwater. Nitrates in groundwater are of the most concern because of possible adverse human health effects.

A well known problem stemming from nitrate toxicity is methemoglobinemia or "blue-baby" syndrome, which occurs when infants less than four months of age consume too much nitrate (Rosenfield and Huston, 1950). Nitrates also have been implicated as a possible source of carcinogenic N-nitroso compounds in the stomach. This could occur when nitrite (NO_2^-) is formed from nitrate (NO_3^-) in the stomach, and the nitrite reacts with secondary amines from consumed meat. N-nitroso compounds formed in the stomach from nitrates in groundwater is confounded by the fact that N-nitroso

compounds in the stomach are also formed from nitrites used to preserve meats, and groundwater nitrate may not be an important source of these carcinogens. Thus, between making fertilizer applications more efficient and possibly preventing human health problems, there has been considerable interest in the nitrification process.

This project was originated from the observation of unusual levels of ammonium and nitrate in African soils by Drs. Alvin Smucker and Boyd Ellis of Michigan State University, Department of Crop and Soil Sciences. They detected unusually high levels of ammonium ions in the rhizosphere of *Leucaena leucocephala* trees. The trees were being grown as part of an alley-cropping system. Additional research confirmed the results of the initial measurements. *L. leucocephala* plants were grown in the greenhouses of MSU, and initial nitrification inhibition experiments with dried *L. leucocephala* leaves produced similar results as in the case of the African soil. Based on this preliminary data, it was our hypothesis that the *L. leucocephala* tree produces compounds that have the potential to inhibit nitrification.

Past research on nitrification has focused on both natural and synthetic nitrification inhibitors, with several synthetic nitrification inhibitors having been developed for commercial use. The literature review for this thesis is divided into four sections: biology of nitrifying bacteria, natural nitrification inhibitors, synthetic nitrification inhibitors, and the last section pertains to *Leucaena leucocephala*. The literature review for nitrification inhibitors presented in this chapter is focused on naturally occurring nitrification inhibitors.

Literature Review

Biology of Nitrifying Bacteria

The process of nitrification is mediated by two sets of bacteria. The first set, consisting of the bacteria *Nitrosomonas europaea*, *Nitrosolobus multiformis*, and *Nitrospirea* spp., converts ammonium (NH_4^+) to nitrite (NO_2^-). The conversion of nitrite ion to the nitrate (NO_3^-) ion is mediated by *Nitrobacter agilis*. Ammonium ion is converted to nitrite ion in two steps. First, the enzyme ammonia monooxygenase oxidizes ammonium ion to hydroxylamine (I). Then the enzyme hydroxylamine oxidoreductase converts hydroxylamine to nitrite (II), followed by its conversion to nitrate by the nitrite reductase enzyme (III). Most nitrification inhibitors have been found to inhibit the enzyme ammonia monooxygenase (Brock et al., 1994).

I. $NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O$
II. $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$
III. $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$

Most of the bacteria involved in the nitrification process are obligate chemolithotrophs, although *N. agilis* can survive on pyruvate as an energy source. The nitrosofyers producing species rely solely on the oxidation of ammonium ion for their energy source, though the ammonia monooxygenase enzyme can accept several other substrates source (Brock et al., 1994). Drozd (1980) reported that whole cells of *Nitrosomonas europaea* could produce propylene oxide from propylene, phenol from benzene, and cyclohexananol from cyclohexane. Hyman and Wood (1983) showed that *N. europaea* could accept methane as a substrate, with the enzyme converting methane to methanol. Subsequent work by Hyman and Wood showed that *N. europaea* could

convert bromoethane into acetaldehye (1983), and also benzene to phenol, which was further oxidized to hydroquinone (Hyman and Wood, 1985).

Since these bacteria play a significant role in soil nitrogen processes, there has been considerable research conducted on all aspects of nitrifying bacteria biology, with much focus on how to prevent the nitrification process. A great deal of this work has focused on the chemical ecology of the interaction between nitrifying bacteria and plants. As will be shown in the next section, there is considerable evidence that plants produce secondary products that are capable of interfering or inhibiting the nitrification process. Much research has also focused on synthetic compounds which are potential inhibitors to the growth of these bacteria, with the goal of producing inexpensive, safe soil additives which will maintain soil nitrogen levels from applications at higher levels for longer times. This will allow for lower amounts of nitrogen fertilizer applications during the growing season.

Plant Secondary Compounds with Nitrification Inhibition Activity

The inhibition of nitrification by plant products has been known for some time. Lyon et al (1923) reported that wheat grown in containers decreased the level of nitrates in the soil versus the soil in control containers without plants, after accounting for nitrogen intake by the plants. In other experiments, dried, ground oat and corn roots were placed into cans of soil. The cans were leached four times over the course of 90 days, and the exudates were analyzed for nitrates. The exudates from the cans containing oat and corn roots showed progressively less nitrate, the total amount being much less than the control. Richardson (1935) noted that soils under grasslands in England displayed

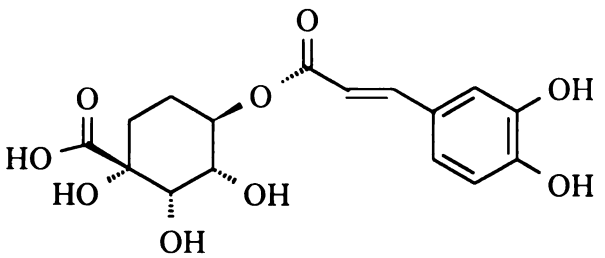
low nitrification rates. He inferred that the presence of the grasses was responsible for the reduced rate of nitrification (Richardson, 1938).

In experiments using African grasses planted in pots, Theron (1951) found that the nitrification process was inhibited by the presence of the grasses. Stiven (1952), using the African grass *Trachypogon plumosus*, observed a lack of nitrification activity in the pots in which they were grown. The water extracts of *T. plumosus* roots displayed antibiotic activity against bacteria, although no nitrifying bacteria were used in the tests. Basabara (1964) reported that tannins retarded production of nitrate in samples of incubated soil, but he was not sure whether low nitrate levels were due to the inhibition of nitrifying bacteria or from the growth of competing organisms.

Root pieces of the grass *Hyparrhenia filipendua* were found to inhibit the growth of nitrifying bacteria (Boughey, 1964 and Munro, 1966a). Further work by Munro (1966b) showed that water extracts of the roots from *H. filipendua* and *Themeda triandra* inhibited the production of nitrate. However, it should be noted that Munro used large amounts of extracts for these experiments, and there were also heterotrophic bacteria present in the cell suspensions. Further evidence of root extracts from grasses inhibiting nitrification was established by Neal (1968). Extracts from *Bouteloua gracilis* and *Taraxacum officinale* inhibited the growth of cultured *Nitrosomonas europaea* and *Nitrobacter agilis*.

Root washings from ryegrass, wheat, salad rape, lettuce and onion growing on quartz chips were studied, and it was found that washings from ryegrass roots had the greatest effect on reducing the rate of nitrification (Moore and Waid, 1971).

Rice (1964) reported that root extracts from the American rangeland plants *Aristida oligantha, Bromus japonicus*, *Cenchrus pauciflorus*, and *Digitaria sanguinalis* also inhibited nitrification. Followup of this work was accomplished by investigating the active compounds in *A. elatior*, *E. corollota*, and *Helianthus annus*. Chlorogenic acid (**1**) and several of its isomers, including isochlorogenic acid (**2**), were found as the active compounds in *A. elatior*, and *H. annus*. In *E. corollota*, the activity was due to the presence of gallotannins (Rice, 1965).



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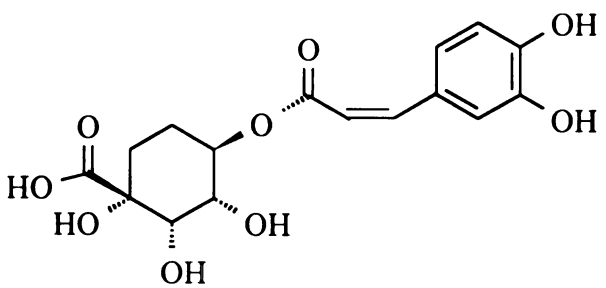

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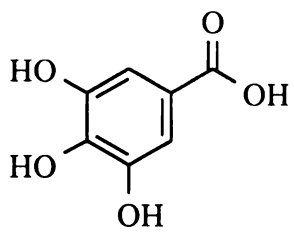
Rice (1965) conducted further work on three *Euphorbia* species, *E. corollata*, *E. marginata*, and *E. supina*. He compared their activities against nitrogen-fixing and nitrifying bacteria, but he only used cultures of *Nitrobacter* for this study. He found that

water extracts of *E. corollata* and *E. supina* both inhibited the growth of *Nitrobacter*, *E. corollata* having the greatest activity. From a positive ferric chloride precipitation test, Rice concluded that the active constituent in *E. supina* were gallotannins. Subsequently, Rice (1969) confirmed the presence of gallotannins in *E. supina*, and he isolated gallic acid (**3**), ellagic acid (**4**) and glucose from purified tannins that had been hydrolyzed.

Further work by Rice and Pancholy (1972) showed that climax ecosystem vegetation in Oklahoma inhibited nitrification. The stages of vegetation studied ranged from "first successsional" ecosystems to climax vegetation ecosystems. First successional plots were cultivated fields that had been abandoned for a year. The plots then ranged from six, eight, and twenty-five years after abandonment. The last plots studied were from mature pine-oak forest that was defined as the climax ecosystem. It was found that the concentration of ammonium ion increased and the number of nitrifying bacteria decreased moving from first successional stage ecosystems to climax



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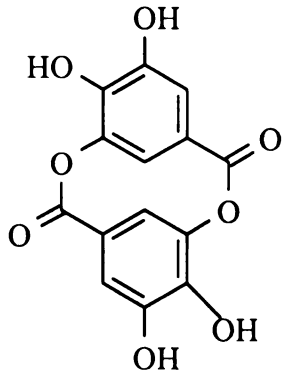

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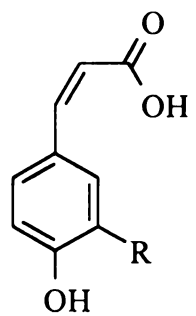
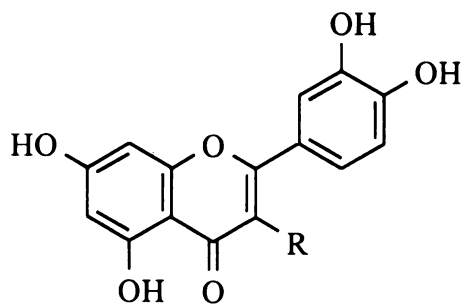
ecosystems. Rice and Pancholy (1973) repeated these experiments, observing the same pattern, and reported that tannins inhibited nitrification. They also found that higher

concentrations of tannins were necessary to inhibit the growth of *N. agiilis* cultures versus *N. europaea*, implying that the first step of nitrification is the easiest to inhibit.

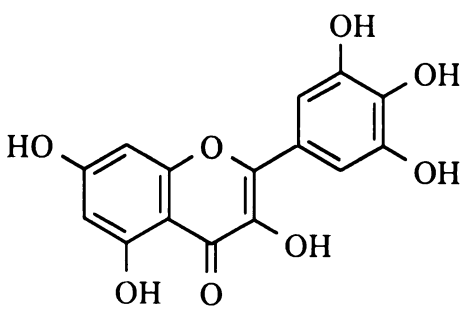
In addition to tannins, caffeic acid (**5**), chlorogenic acid (**1**), ferulic acid (**6**), isochlorogenic acid (**2**), quercetin (**7**), isoquercitrin (**8**), and myricetin (**9**) were found to inhibit nitrification, particularly against *Nitrosomonas* (Rice and Pancholy, 1974).



5. R=OH
6. R=OCH_3



7. R=OH
8. R= rhamnose



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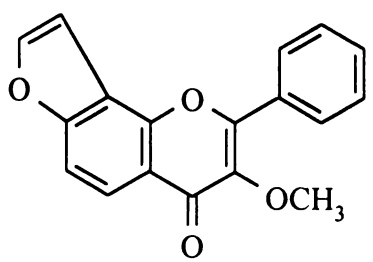

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Extracts from the seeds, bark, and leaves of *Pongamia glabra* Vent. inhibited nitrification in soil bioassays (Sahrawat et al.,1974). In subsequent work, karanjin (**10**), a

furanoflavanoid isolated from the seeds, was shown to be a potent inhibitor of nitrification in a soil-based bioassay (Sahrwat and Mukerjee, 1977).

Products from the neem tree, *Azadirachta indica*, have been studied for nitrification-inhibiting properties. De-fatted neem seed cake is purported to inhibit nitrification (Khandewal et al., 1977). Urea coated with neem cake was shown to hydrolyze slower in soil (Reddy and Prasad, 1975). Extracts from the seeds also have been shown to inhibit nitrification (Sahrawat and Parmer, 1975).

It was found that ammonium levels were considerably higher than nitrate levels in an oak-dominated lowland forest (Lodhi, 1977). In particular, ammonium levels in the soil were much higher than nitrate levels around the oak trees, although this report was not based on work involving nitrifying bacteria. Further research showed that pH ranges in the lowland oak forest were acceptable for nitrifier growth, and that nitrifying bacteria were present in the soils (Lodhi, 1978). Thus, it appeared that the vegetation was having an effect on the nitrification process.

Additional work showed that the soil of ponderosa pine communities in western North Dakota also showed higher levels of ammonium ion versus nitrate. These soils had acceptable pH for nitrifying bacterial growth, and several species of nitrifying bacteria were detected in the soil. It was found that various water and acetone extracts of the needles and bark of ponderosa pine inhibited nitrification in soil bioassays (Lodhi and Killingbeck, 1980). The NI activity was attributed to a variety of polyphenol compounds identified in the extracts, although the individual compounds were not assayed for nitrification inhibition activity. The compounds identified were caffeic acid (**5**), chlorogenic acid (**1**), quercetin (**7**) and large amounts of condensed tannins present in the

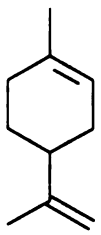
needles. Both caffeic acid (**5**) and quercetin (**7**) were identified as the major NI compounds from the bark. The greatest activity was found in the extracts containing condensed tannins (Lodhi and Killingbeck, 1980).

Waste tea (also called tea fluff), which is high in oxidized polyphenols, was reported to inhibit nitrification (Krishnapillai, 1979). Waste tea was added to samples of field soil, and nitrate levels were measured over a span of 12-16 days. Nitrate levels in the samples with tea fluff were found to increase much slower than in the controls. However, the tea fluff was added in high concentrations (8% w/w) in order to show such high activity (Krishnapillai, 1979). The actual percentage of polyphenols in the waste tea was not measured, and the results could be attributable to other compounds.

Additional evidence is available for the NI activity of phenolic compounds. When the methanol extract of forest floor material containing polyphenols was added to an actively nitrifying layer of soil, the extract significantly reduced the nitrification process (Olson and Reiners, 1983). Fractionation of the extract revealed that tannins and possibly other phenolics were responsible for the nitrification inhibition (Baldwin et al., 1983). Removal of the tannins from the extract eliminated the NI activity. Therefore it was concluded that the NI activity was due to the protein-binding properties of the tannins (Baldwin et al., 1983). But because of the fractionation process used, non-binding phenolics were not tested, and they may have contributed to the NI activity.

White (1986) also hypothesized that volatile plant compounds may be responsible for nitrification inhibition. This hypothesis was formulated from the research on soil conditions of a ponderosa pine forest after the forest had been burned. It was reported that nitrification rates increased after burning, and remained elevated for another ten

months. Further work demonstrated that a mixture of terpenoids and water-soluble compounds from the forest floor caused some nitrification inhibition. However, no specific compounds were characterized from the water-soluble extracts, and these extracts showed only minor NI activity. A mixture of terpenoids was chosen to model the terpenoids commonly found in ponderosa pine resin. The mixture included equal amounts of limonene (**11**), myrcene (**12**), α-pinene (**13**), β-pinene (**14**), and β-phellandrene (**15**). The mixture reduced nitrate production in incubated soil samples (White, 1986). It was speculated (White, 1988) that monoterpenes with a six-carbon ring and a terminal carbon double bond would produce the greatest NI activity. This hypothesis was based on the fact that the most potent known nitrification inhibitors, nitryaprin and acetylene, possessed a six-membered ring and a carbon triple bond, respectively.



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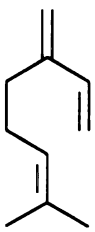

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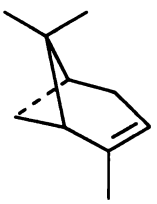

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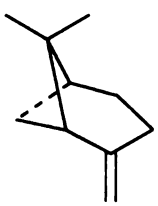

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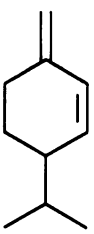

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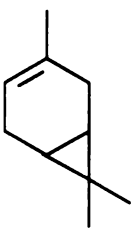

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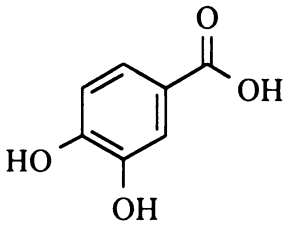
Evidence for this hypothesis was provided by White (1991). Soil was incubated with limonene (**11**), myrcene (**12**), α-pinene (**13**), β-pinene (**14**), α-phellandrene (**15**), and Δ^3-carene (**16**) to study the effects of these compounds on nitrification. Soils from a pine forest and a non-pine forest were incubated in sealed jars stored in the dark. It was observed that the terpenes limited nitrification activity, with greater nitrification rates occurring in the absence of the terpenes (White, 1991). Among the terpenes studied, limonene (**11**), a terpene possessing a six-carbon ring and a terminal double bond, showed the most inhibition, although the difference was small compared to the activity of the other monoterpenes.

Further evidence for the influence of the nitrification process by plant extracts and their components was provided by Howard and Howard (1991), who demonstrated that leaf-litter extracts from oak (*Quercus petraea* (Matt.) Liebl.), larch (*Larix* x *eurolepis* A. Henry), Norway spruce (*Picea abies* (L.) Karsten), beech (*Fagus sylvatica* L.), grand fir (*Abies gradis* (D.Don) Lindley), Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco), lodgepole pine (*Pinus contorta* Douglas ex London), alder (*Alnus glutinosa* (L.) Gaertn), sycamore (*Acer psuedoplatanus* L.), Sitka spruce (*Picea stichensis* (Bongard) Carr.), birch (*Betuola* sp), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), southern beech (*Nothofagus procera* Poepp. & Endl.), western red cedar (*Thuja plicata* D. Don), Corsican pine (*Pinus nigra laricio* (Poiret) Maire), yew (*Taxus baccata* L.), hazel (*Corylus avellana* L.) and Scots pine (*Pinus sylvestris* L.) reduced nitrite production in cultures of *N. europaea* to less than 50% of the control. Norway spruce, Sitka spruce, and western hemlock reduced nitrate production in cultures of *N. agilis* to less than 50% of the control (Howard and Howard, 1991). Treatment of the leaf-litter extracts with

polyvinylpyrrolidone (PVP) to remove polyphenols did not completely remove NI activity. Grand fir, Douglas fir, lodgepole pine, alder, Sitka spruce, western hemlock, southern beech, western red cedar, Corsican pine, hazel and Scots pine reduced nitrite production in cultures of *Nitrosomonas* to less than 50% of controls. However, greater than 1000 ppm concentrations of the leaf-litter extracts were used for these experiments.

The effects of various organic acids on nitrification in soils have also been tested (Karmarkar and Tabatabai, 1991). They found that protocatechuic (**17**), p-coumaric (**18**), vanillic (**19**), caffeic (**5**), and ferulic (**6**) acids reduced nitrification by more than 20% in a soil-based bioassay. The compounds were studied at the 500 ppm concentration.

Limonene (**11**), myrcene (**12**), α-pinene (**13**), sabinene (**20**), and γ-terpinene (**21**), the five most abundant monoterpenes found in coastal redwood (*Sequoia sempervirens*) needles were studied for NI activity (Ward et al., 1997). The compounds were tested in whole-cell cultures of *N. europaea*. They found that myrcene (**12**) and limonene (**11**) displayed inhibiting activity at 1 μg/ml, whereas α-pinene (**13**) and γ-terpinene (**21**) were active at the 10 μg/ml concentration. Sabinene (**20**) displayed no activity.



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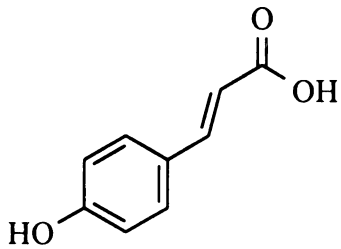

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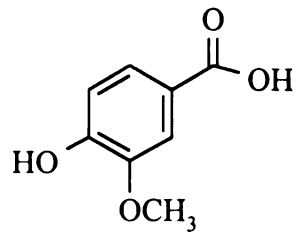

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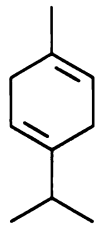

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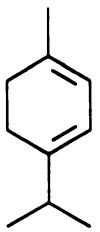
There are also reports that show phytochemicals do not inhibit nitrification. Goring and Clark (1948) studied the differences between soil under growing plants and soil kept in a fallow state. They grew tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill.), wheat (*Triticum* sp.), oats (*Avena sativa* L.), rye (*Secale cereale* L.), bromegrass (*Bromus* sp.), timothy (*Phleum pratense* L.) and Sudan grass (*Sorghum* sp.), and found greater numbers of nitrifying bacteria in the soil with plants. It was concluded that nitrate levels were depressed in the soil containing plants due to mobilization of nitrogen by soil organisms (Goring and Clark, 1948).

Root extracts of four grasses (*Andropogon tectorum*, Schum; *Chloris gayana*, Kunth; *Panicum maximum*, Jacq.; *Pennisetum purpureum*, Schum) and two legumes (*Calopogonium mucunoides*, Desv.; *Stylosanthes gracilis* L.) actually enhanced the rate of nitrification in soil samples (Odu and Akerele, 1973), although the root extracts of *A. tectorum* and *P. purpureum* strongly inhibited the growth of five bacteria isolated from the soil samples.

It was also reported that root exudates from grasses possess little or no nitrification inhibitory activity (Purchase, 1974). *Hyparrhenia filipendula* was studied for its effect on the growth of nitrifying bacteria in the growing medium and the soil

exudate was tested for NI activity. Vigorous bacterial growth in the growing medium suggested that the exudate from the growing medium did not inhibit nitrification.

Bremner and McCarty (1988) reported that the terpenoids limonene (**11**), myrcene (**12**), α-pinene (**13**), β-pinene (**14**), α-phellandrene (**15**), and α-terpinene (**22**) did not



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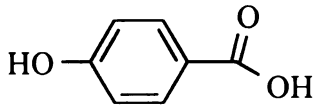

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inhibit nitrification in soil. They hypothesized that the addition of a carbon source to soil induced the uptake of ammonium by heterotrophic soil organisms. Further, most of the recovered nitrogen from the soils they had studied was in the form of nitrate.

Other reports dispute the NI activity of phenolic acids and polyphenols. Bremner and McCarty (1986) tested eight phenolic acids and five tannins for nitrification inhibition, incubating the samples in soil. The phenolic acids tested were chlorogenic (**1**), gallic (**3**), ellagic (**4**), caffeic (**5**), ferulic (**6**), *p*-coumaric (**18**), vanillic (**19**), and *p*-hydroxy-benzoic (**23**) acids. The five tannins were isolated from mangrove (*Rhizophora mangle*), quebracho (*Quebrachia lorentzii*), mimosa (*Albizia julibrissin*), chestnut (*Castanea dentata*) and sumac (*Rhus coriaria*) trees. Among the phenolic acids studied, only ferulic acid showed some activity in one of the soils tested. None of the tannins studied showed activity. Later, McCarty, et al. (1991) tested the activity of caffeic (**5**), ferulic (**6**), and *p*-coumaric (**18**) acids against pure cultures of *Nitrosomonas europaea*, *Nitrospirea* sp., and *Nitrosolobus multiformis*. It was found that these phenolic acids did

not inhibit nitrification significantly. This contradicted the results of various researchers reporting the NI activity of phenolic acids.

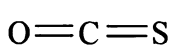
Other research results possibly cast doubt on the ability of phenolic acids to inhibit nitrification at the concentrations present in soil. Levels of ferulic (**6**), *p*-coumaric (**18**), vanillic (**19**), *p*-hydroxy-benzoic (**23**), and syringic (**24**) acids were measured in field soil and compost. The total amounts of acids present were found to range from 1.2 to 10.8 μg/g of soil or compost (Turtura et al., 1989). In the second phase of the experiment, concentrations of acids ranging from 8.3 to 833 μg/g soil were added to a soil sample, and levels of nitrate were determined after ten days. It was found that organic acids significantly inhibited nitrification only at the 833 μg/g concentration (Turtura et al., 1989). These workers concluded that organic acids were not present in soils at amounts that would have any effect upon nitrification.

The effects on NI activity of cotyledon powders from the seeds of *Camellia sinensis* L., *Quercus borealis*, *Quercus petraea*, and *Quercus robur* were evaluated by Kholdebarin and Oertli (1992). These cotyledons are known to be rich in polyphenolic compounds. The addition of the powders to soil samples caused an initial rapid disappearance of ammonium, and also slowed nitrification considerably. They attributed the reduced nitrification rates to immobilization of nitrogen by other microbes, and concluded that cotyledon powders did not significantly affect nitrification.

Further work on phenolic compounds by Kholdebarin and Oertli (1994) studied the effects of tea waste, tea seed powder, and specific phenolic acids on nitrification. The phenolic acids tested included chlorogenic (**1**), gallic (**3**), vanillic (**19**), and *p*-hydroxy-benzoic (**23**) acids. Low amounts of nitrogen were recovered from the soil cultures,

suggesting that the phenolic compounds were reacting with the ammonium ion, making it unavailable for nitrification. The overall conclusion of this research was that the phenolics did not inhibit nitrification directly (Kholdebarin and Oertli, 1994).

A recent area of research may provide some answers to how plants inhibit nitrification. In the section describing synthetic nitrification inhibitors, evidence is presented that carbon disulfide and other volatile sulfur compounds exhibit NI activity. Recently, plants have been shown to release sulfur gases. Crushed roots of the plant *Mimosa pudica* was found to produce carbon disulfide (Haines, 1987). Further, live roots, forest-floor litter, and soil samples gathered around *Stryphnodendron excelsum* were incubated in sealed containers, and it was that found the live roots and floor litter both emitted sulfur-containing gases (Haines, 1991). Work was then conducted to determine whether carbon disulfide (**24**) released from *M.pudica* roots had effects on bacteria grown in the rhizosphere, although nitrifying bacteria were not tested in this study. The results of the study showed little evidence of the plant root's releasing enough carbon disulfide to inhibit bacterial growth (Hartel, 1992). It is interesting to note that all *Leucaena* species produce carbon disulfide and carbonyl sulfide (**25**) from crushed roots and shoots (Feng, 1996).



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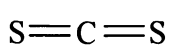

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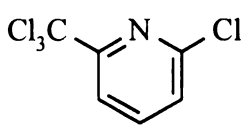
On the whole, there exists considerable evidence for natural control of nitrification, although there is strong debate on their reported activity. It would appear from the published reports that plants and their secondary products exhibit considerable

effect on inhibition of nitrification. However, this uncertainty regarding natural nitrification inhibitors has not prevented researches from developing synthetic nitrification inhibitors.

Synthetic Nitrification Inhibiting Compounds

Synthetic nitrification inhibitors fall into two basic classes: those compounds which contain nitrogen, and volatile compounds. The compounds that are volatile tend to contain carbons with double and triple bonds. Among the nitrogen-containing compounds, only two NI compounds are registered for use in the United States. They are nitrapyrin (**26**) and etridiazole (**27**). Nitrapyrin (N-Serve) was developed by the Dow Chemical Co., Midland, Michigan. Etridiazole is a product of the Olin Corporation (Japan). A third nitrogen containing NI, dicyandiamide (**28**), is available for commercial use in Germany.



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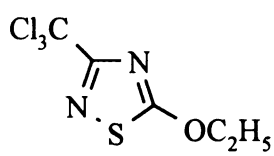

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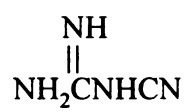

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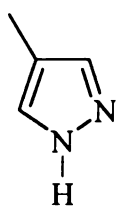
Nitrapyrin is the only nitrification inhibitor currently available commercially in the United States. It was developed and tested in the early sixties (Goring, 1962a, and 1962b). Nitrapyrin is one of the most active nitrification inhibitors known. It is the standard to which other nitrification inhibitors are compared. Considerable work has been done on the effects of nitrapyrin that will not be covered in this review. For further

information, consult Hughes and Welch, (1970); Lewis and Stefanson, (1975); Hendrickson et al., (1978); Roberts, (1979); English et al., (1980); Townsend and McRae, (1980); Westerman et al., (1981); and Chaney and Kamprath, (1982).

As a group, heterocyclic nitrogen compounds display significant NI activity. McCarty and Bremner (1989) tested 45 heterocyclic nitrogen compounds (12 unsubstituted and 33 substituted) for NI activity. Nitrapyrin and etridiazole were included in the study, and both displayed strong NI activities at the 3 ppm level in three types of soil. A number of other compounds also displayed high activity at the 3 ppm level. These compounds included 4-methylpyrazole (**29**), 2-ethynylpyridine (**30**), and 3,4-dichloro-1,2,5-thiadiazole (**31**).



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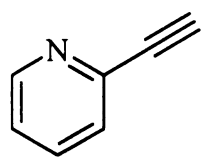

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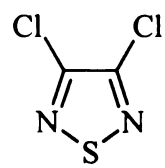

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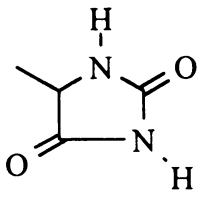
Other heterocyclic nitrogen compounds which exhibit NI activity are the hydantoin derivatives (Shimizu, 1986a, 1986b). In the process of testing hydantoin compounds as slow release fertilizers, it was found that some of the compounds dislplayed NI activity, including 5-methylhydantoin (**32**), 5-ethylidenehydantoin (**33**), ethyleneurea (**34**), and several N-alkylmaleinimides, which included maleinimide (**35**), N-methyl- maleinimide (**36**), and N-ethylmaleinimide (**37**). A soil-based bioassay was used to test these compounds, the compounds being applied at concentrations of 50 and 150 ppm. Dicyandiamide was used as the positive control. The N-alkylmaleinimides



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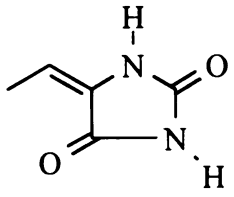

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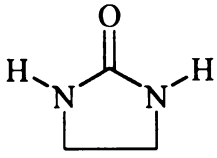

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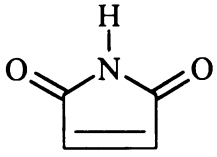

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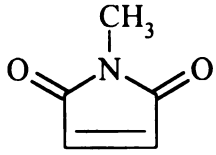

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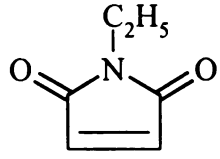

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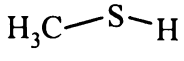
displayed the greatest activity, significantly reducing nitrification at the 50 ppm level (Shimizu, 1986b).

Volatile and gaseous compounds are also known to inhibit nitrification. Carbon disulfide (**24**) has long been known to inhibit nitrification (Gainey, 1914). Other work has shown that carbon disulfide produced from the decomposition of rubber bungs inhibited nitrification (Powlson and Jenkinson, 1971). They showed that a carbon disulfide concentration of 8 ppm completely inhibited nitrification. Other volatile sulfur compounds have been tested for NI activity. Dimethyl disulfide (**38**), methyl mercaptan (**39**), hydrogen sulfide (**40**), and carbon disulfide were studied by Bremner and Bundy (1974). They found that carbon disulfide was the most active among this group of



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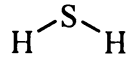

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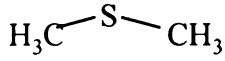

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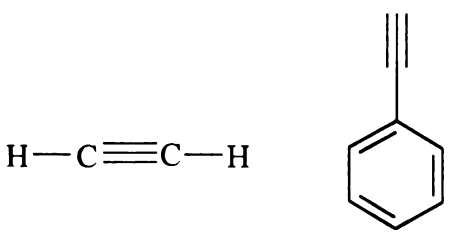
compounds. Dimethyl disulfide displayed some activity, but the rest showed low levels of nitrification inhibition (Bremner and Bundy, 1974). More recent work showed that carbon disulfide acts as an inhibitor of the enzyme ammonia monooxygenase (Hyman et al., 1990).

Acetylene (**41**) and compounds containing carbon triple bonds were also shown to inhibit nitrification. Acetylene was first shown to inhibit nitrification by Hynes and Knowles (1978). Acetylene had been shown to inhibit other reductive and oxidative bacterial processes. Following these leads, Hynes and Knowles tested acetylene for activity against nitrifying bacteria. They found that acetylene completely inhibited nitrification down to a concentration of 10^{-5} atmospheres.

A variety of other compounds containing an acetylene group have been screened or NI activity. Seventeen monosubstituted and seven disubstituted acetylene-containing compounds were tested for NI activity by McCarty and Bremner (1986). They confirmed the activity of acetylene itself, and also found other compounds with significant activity. Propyne (**42**) and 1-butyne (**43**) showed similar activity to acetylene. 2-Ethynylpyridine (**28**) and phenylacetylene (**44**) showed very strong NI activity at the 1, 5, and 10 mg per kg of soil levels. From this work they further evaluated 2-ethynylpyridine as a compound



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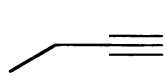

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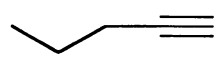

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with potential commercial use (McCarty and Bremner, 1990). They compared 2-ethynylpyridine to compounds in use or patented to be used as nitrification inhibitors in soil. The nitrification inhibition activity of 2-ethynylpyridine was found to compare favorably to both nitrapyrin and etridiazole in the soil types tested.

The compounds described in this section give the scope of the synthetic aspect of the nitrification inhibitor research, although carbon disulfide and some of the other gaseous sulfur compounds exist in nature. There have been other synthetic compounds reported which inhibit nitrification, such as herbicides, but the NI activity is not strong enough to justify their use as nitrification inhibitors.

Botany, Chemistry, and Bioactivity of *Leucaena leucocephala*

Leucaena leucocephala (Lam.) de Wit (formerly *L. glauca*) is a tree in the family Leguminosae, subfamily Mimosoideae. It is one of 16 species in the genus *Leucaena.* It posseses alternate bi-pinnate leaves with ovoid leaflets, and white, globular flowers. The brown, ovoid seeds are produced in pods. The tree originates from southern Mexico and northern Central America (The word Oaxaca translates as "the land where huaxin grows;" huaxin is the Mexican common name for the tree). (New Crops Factsheet, J.L. Brewbaker, author).

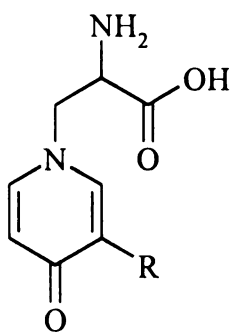
Because *L. leucocephala* is a nitrogen fixer and grows very fast, it has been naturalized all over the tropics and subtropics. It is truly a multipurpose plant; the wood is used for firewood, lumber, pulpwood, craftwood and charcoal; the foliage is used for animal fodder, green manure, and the young shoots are used as food. Foliage intake by

non-ruminant animals must be restricted, because the non-protein amino acid mimosine causes hair loss. The tree also is used extensively in alley-cropping systems.

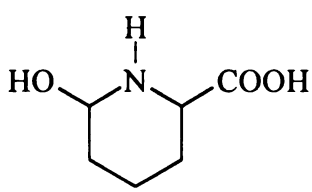
A great deal of research has been conducted on *Leucaena leucocephala*, most of it by agroforestry workers. However, little research has been done on the chemistry of *L. leucocephala*.

The most studied compound isolated from *L. leucocephala* is the non-protein amino acid mimosine (**45**). Mimosine was first isolated from *L. leucocephala* by Mascre (1937). The structure of mimosine was elucidated by Adams et al. (1945). Mimosine gained some notoriety because of its biological activity on mammals, which include hair loss and goiter (Malynicz, 1974; Hegarty et al., 1976; Lopez et al., 1979, and Herrara et a.l, 1980). The presence of this compound limits the use of *L. leucocephala* as a forage plant in some locations. Mimosine was shown to reversibly block cell-cycle progression late in the G1 phase or at the beginning of S-phase in cultured mammalian cells (Hughes and Cook, 1996). Rinsing the cells thoroughly to remove mimosine allows the cells to continue growing.

The presence of mimosine glucoside (**46**) was reported in young seedlings of *L.*



45. R=OH
46. R=glucose



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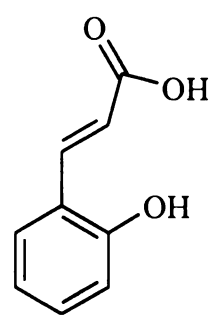

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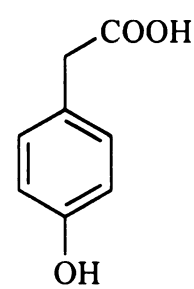

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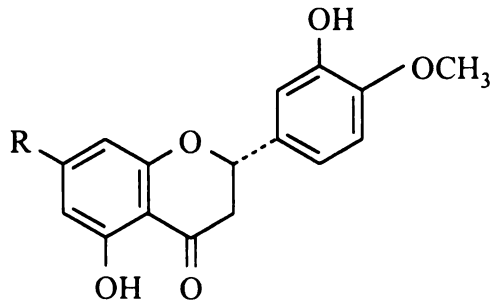
leucocephala, although biological activity was not reported (Tahara et al, 1971). Hegarty (1957), while investigating nitrogen metabolism of subtropical legumes, isolated 5-hydroxypiperidine-2-carboxcylic acid (**47**) from fresh leaves *L. leucocephala.*

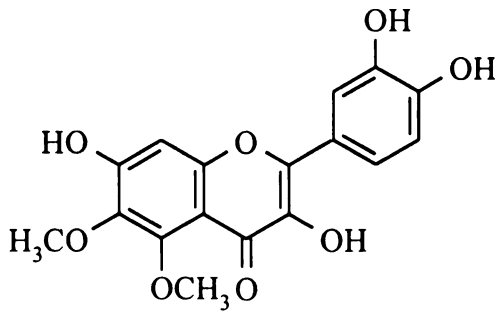
Extracts from the seeds and fruit of *L. leucocephala* have been studied for bioactivity. Alkaloidal extracts from the seeds were tested for analgesic, anthelminthic, mutagenic, antimutagenic and pharmacological activities (Villasensor et al., 1997). This extract exhibited mainly antimutagenic activity (Villasensor et al., 1997). Methanolic extracts of the fruit showed moderate anti-nematicidal activity (Mackeen et al., 1997).

A number of phenolic compounds have been isolated from *L. leucocephala.* Kuo, et al. (1982) studied the allelopathic potential of *L. leucocephala*, using paper and thin-layer chromatography to identify phenolic compounds such as cis-p-coumaric (**18**), o-coumaric (**48**), p-hydroxybenzoic (**23**), p-hydroxyphenyllactic (**49**), and ferulic acids (**6**). Chou and Kuo (1986) further investigated the allelopathic activity of *L. leucocephala* leaves. From aqueous extracts of the leaves they identified mimosine (**43**), quercetin (**7**), and gallic (**3**), protocatechuic (**17**), p-hydroxybenzoic (**23**), vanillic (**14**), and caffeic acids (**5**), along with p-hydroxyphenlyacetic (**49**), ferulic (**6**), and p-coumaric (**18**) acids as the phytotoxic components of the leaves (Chou and Kuo, 1986). Other work showed that the seeds of *L.leucocephala* also exhibit allelopathic activity. Souza et al. (1997) showed that aqueous extracts of the seeds exhibited allelopathic activity against South American pasture weeds. However, they did not identify the active compounds.

Flavonoids isolated from *L. leucecephala* are quercetagetin (**50**) and patuletin (**51**) (Nair and Subramanian, 1962). Also, hesperin (**52**) and hesperidin (**53**) were isolated from the fresh flowers of *L. leucocephala* (Jaswant et al., 1997).



50. R=OH
51. R=glucose



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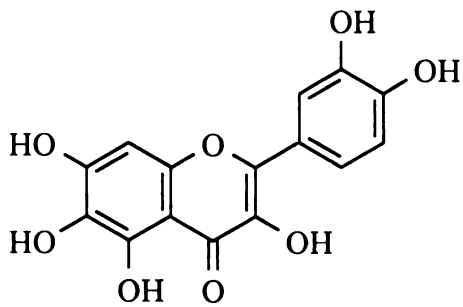
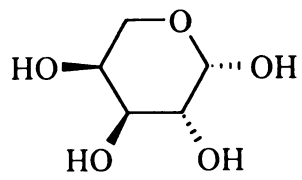


53

Several workers reported the composition of seed and gum oligosacharides. The gum isolated from the seed was composed of arabinose (**54**), galactose (**55**), mannose (**56**), and rhamnose (**57**) (Soni et al., 1984). *L. leucocephala* gum consisted of arabinose (**54**), galactose (**55**), rhamnose (**57**), and glucuronic acid (**58**) during gumosis, a term for gum production from glands on the leaves during fungal attack on the tree (Soni et al., 1991).



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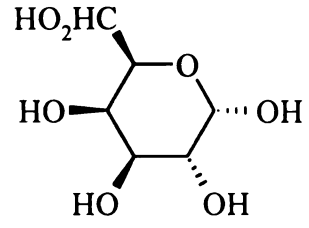

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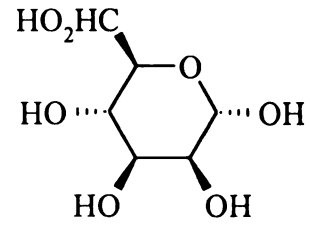

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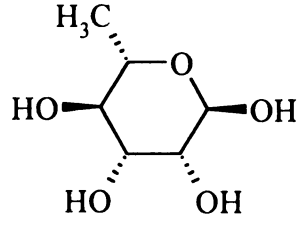

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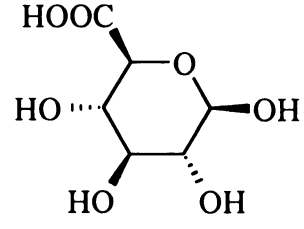
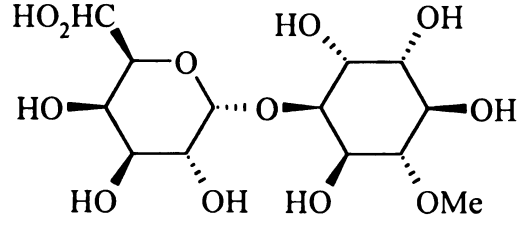


58

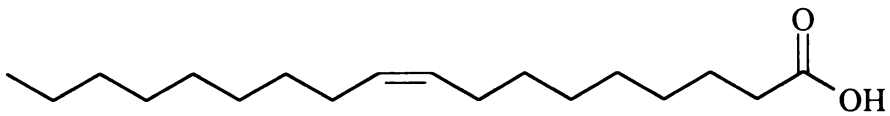
A novel galactopinitol was isolated from *L. leucocephala* seeds, and the structure was confirmed to be O-α-D-galactopyranosoyl-(1→1)-3-O-methyl-D-chiro-inositol (**59**) (Chein et al., 1996). This research implied that the sugar played a role in the desiccation tolerance of the seeds (Chein et al., 1996).



59

The seed oil components of *L. leucocephala* were characterized by Tasneem et al. (1988). The major saturated fatty acids were palmitic ($CH_3(CH_2)_{14}COOH$), stearic

($CH_3(CH_2)_{16}COOH$) and a twenty-two carbon ($CH_3(CH_2)_{20}COOH$) fatty acid. The major unsaturated fatty acids were oleic (**60**), linoleic (**61**), and linolenic (**62**) acids.



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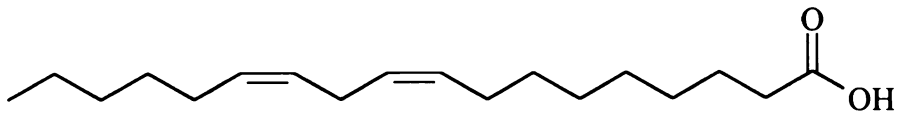

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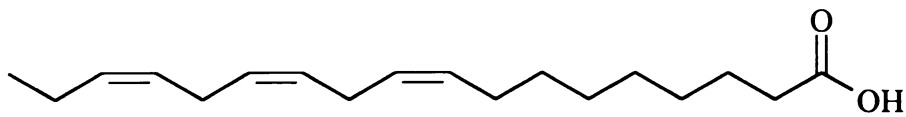
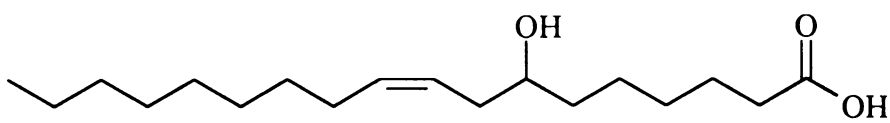


62

Hosamani (1995) reported several unique fatty acids from *L. glauca* seed oil. Ricinoleic (**63**), malvalic (**64**), and sterculic (**65**) acids were reported, in addition to the fatty acids isolated by Tasneem (1988).



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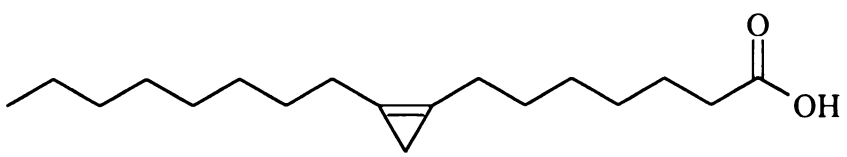

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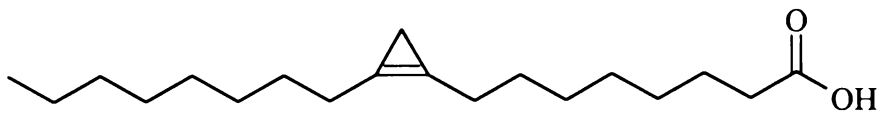


65

There is considerable evidence that plant secondary compounds play an important role in regulating the activity of nitrifying bacteria in the soil. However, it is not clear whether *L. leucocephala* possesses compounds that could inhibit nitrification. The rest of this thesis attempts to answer that question. The second chapter details the results of all the bioassays conducted on *L. leucocephala* extracts. The third chapter discusses the NI bioassay and the chemistry of the active nitrification inhibitory compounds that were isolated. The last chapter summarizes the research work done on *L. leucocephala* and lists the major conclusions.

Chapter II

Extraction of *Leucaena leucocephala* Plant Parts and Preliminary Bioassays

Abstract

The leaves, stems and roots of *L. leucocephala* were sequentially extracted with hexane, ethyl acetate, and methanol. These extracts were tested for anti-fungal, anti-bacterial, insecticidal, nematicidal, and anti-cancer activities. The anti-fungal assays were conducted on *Aspergillus niger*, *Fusarium moniliforme & oxysporum*, *Rhizoctonia solani*, *Botrytis* spp., and *Gleosporum* spp. The anti-bacterial included *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes*. The mosquitocidal assay was carried out on *Aedes aegyptii* mosquito larvae and insect anti-feedant assay on *Helicoverpa zea* and *Lymantria dispar* caterillars. The nematicidal bioassays were conducted on *Caenorhabditis elegans* and *Panagrellus redivivus*. The anti-cancer screen employed mutant *Saccharomyces cerevisae* strains that were sensitive to topoisomerase I and II poisons. Initially, hexane extracts from the leaves showed activity in the anti-feedant bioassay, but it was not reproducible. All other extracts did not show any significant activity in the bioassays performed.

Introduction

Extracts and compounds from *L. leucocephala* have shown a variety of biological activities. The most well known biologically active compound from *L. leucocephala* is mimosine (**45**). Mimosine is a non-protein amino acid that has a variety of effects on animals, notably hair loss in non-ruminant animals (Hegarty, 1964). Alkaloidal extracts from the seeds showed antimutagenic activity (Villasensor et al., 1997). Methanolic extracts from the fruit exhibited moderate activity against the pinewood nematode, *B. zylophilus* (Mackeen et al., 1997). Also, allelopathic activity has been reported on water extracts of *L. leucocephala* leaves (Kuo et al., 1982; Chou and Kuo, 1986). Among the compounds that showed allelopathic activity were gallic acid (**3**), and mimosine (**45**).

Besides the reported activity on mimosine, there has not been much research on biologically active compounds from *L. leucocephala*. Excluding the reports of activity on mimosine, there has been little research on bioactivity from *L. leucocephala* plant parts. Therefore, extracts of *L. leucocephala* plants were investigated for bioactive compounds using the bioassays of the Bioactive Natural Products Laboratory (BNPL) at Michigan State University. The methodology and results of the bioassays conducted on the *L. leucocephala* extracts are presented in this chapter.

Materials and Methods

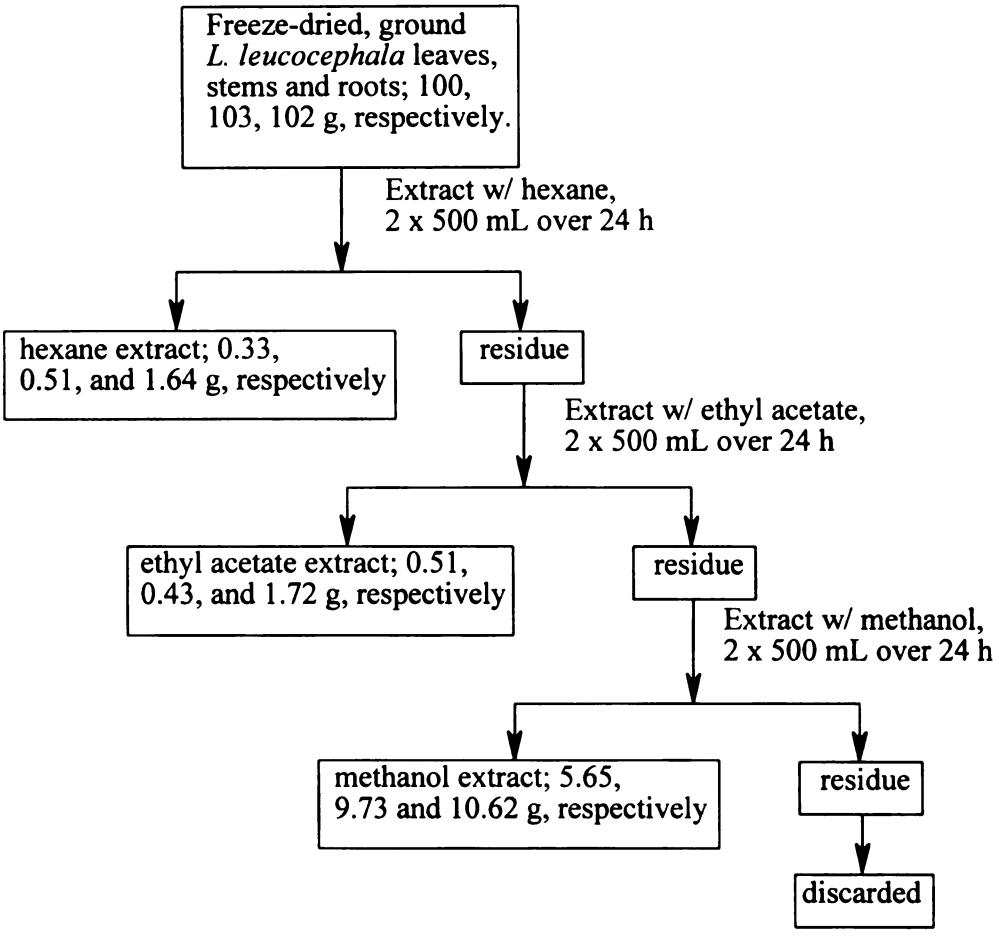
Plant Material: *Leucaena leucocephala* seeds were obtained from Nigeria, Africa. Plants were grown from this seed in the Plant and Soil Sciences Building greenhouses in twelve-inch plastic pots. Plants were later transferred to the Bioactive Natural Products

Laboratory (BNPL) greenhouses. All work was conducted on material from these plants. For initial extractions, whole plants were harvested, and root, stem and leaves were collected separately. The stem collections included all woody portions and the main leaf petioles (*L. leucocephala* is a bi-pinnate plant). A total of 325, 312, and 338 g of fresh leaves, stems and roots, respectively, were collected for the initial extractions. All plant materials were freeze-dried and then ground to a fine powder in a Wiley Mill prior to extraction. Plant parts were harvested as needed.

Initial Extractions: Approximately 100 g of freeze-dried, ground roots, stems, and leaves were extracted separately and sequentially with hexane, ethyl acetate, and methanol (Aldrich Chemical Co., Milwaukee, Wisc.). Two 500 mL aliquots of each solvent were used to extract the plant materials over a 24 h period (Figure 2.1). Solvents were removed from the extract under reduced pressure using a rotary evaporator. The hexane, ethyl acetate, and methanol extracts of the roots weighed 0.33, 0.51, and 5.65 g, respectively. The hexane, ethyl acetate and methanol extracts of the stems weighed 0.51, 0.43, and 9.73 g, respectively. Similarly, the hexane, ethyl acetate and methanol extracts of the leaves weighed 1.64, 1.72, and 10.62 g, respectively. The crude, dried extracts were stored at -20° C until analysis.

Bioassay Organisms: All extracts were assayed for anti-fungal, anti-bacterial, nematicidal, insecticidal/anti-feedant, and for topoisomerase I and II enzyme inhibitory activities. The fungi used were *Aspergillus niger* (MSU-SM 922), *Fusarium oxysporum* (MSU-SM

Figure 2.1: General Extraction Scheme for *L. leucocephala* Plant Parts



1322) and *F. moniliforme* (MSU-SM 1323); and *Rhizoctonia solani*, *Botrytis* spp., *and Gloesporum* spp. Two gram-positive bacteria bioassayed were *Staphylococcus aureus* (ATCC 25923, 29213), and *Streptococcus pyogenes* (MHM[a]-1645, MHM-81-7). One gram negative bacteria, *Escherichia coli* (ATCC 25922) was bioassayed. The yeast *Candida albicans* (MSU-SM 543) was also assayed. Extracts were tested for activity against the nematodes *Caenorhabditis elegans* and *Panagrellus redivivus*. To test for activity against insects, extracts were assayed against the fourth instar larvae of the mosquito *Aedes aegypti*, as well as the caterpillars of *Helicoverpa zea*, *Lymantria dispar*

and *Manduca sexta*. For the topoisomerase assay, three mutant species of *Saccharomyces cerevisae*, JN394 (sensitive to topoisomersase I and II poisons), $JN394_{t-1}$ (sensitive to topoisomerase II poisons), and $JN394_{t2-5}$ (sensitive to topoisomerase I poisons), were used.

Plate bioassays and Media: Petri plate assays using microbes were accomplished by lawning plates with of appropriate media with inoculum containing the test organism being assayed, and then spotting the plates with the test extract in DMSO solutions. All initial bioassays were conducted at 250 ppm, spotted on the plate at 250 $\mu g \bullet 20\ \mu L^{-1}$ DMSO. Fungi were grown on potato dextrose agar (39 $g \bullet L^{-1}$, Difco Laboratories, Detroit, Mich.). Yeast was grown on YMG media (yeast extract 4 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}, maltose 10 $g \bullet L^{-1}$ {Sigma Chemical Co., St. Louis, Mo.}, glucose 4 $g \bullet L^{-1}$ {U.S. Biochemicals, Cleveland, Ohio}, and agar 18 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}). Bacteria were all grown on Emmons media (neopeptone 10 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}, glucose, 20 $g \bullet L^{-1}$ {U.S. Biochemicals, Cleveland, Ohio}, and agar 18 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}). The mutant *S. cerevisae* cultures were grown on YPDA media (bactopeptone 20 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}, yeast extract 10 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}, glucose 20 $g \bullet L^{-1}$ {U.S. Biochemicals, Cleveland, Ohio}, agar 17 $g \bullet L^{-1}$ {Difco Labs, Detroit, Mich.}, and adenine sulfate 2 $ml \bullet L^{-1}$ {ICN Biomedicals, Aurora, Ohio}).

Nematode bioassay: The assay was performed in NG media made without agar. NG media consists of 3 g of sodium chloride (Mallinckrodt Baker, Paris, Ken.), 2.5 g of

bactopeptone (Difco Labs, Detroit, Mich.), 1 mL of 1 M $CaCl_2 \cdot 2H_2O$ (J.T. Baker Chemical Co., Phillipsburg, N.J.) solution, 1 mL of 1 M $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Fair Lawn, N.J.) solution, 25 mL of phosphate buffer (11.968 g KH_2PO_4 {Mallinckrodt, Inc., Paris, Ken.} and K_2HPO_4 {Mallinckrodt, Inc., Paris, Ken.} in 100 mL of water), and 1 mL of cholesterol (Nutritional Biochemicals Corp.) solution (5 mg cholesterol in 1 ml of ethanol) added after autoclaving, all in 1 L of reverse osmosis (RO) water. Nematodes were pipetted from the stock growing solution and added to NG media to make a nematode solution with approximately 25-50 nematodes in a volume of 48 μL. The test extract was applied at 125 μg • 2 μL^{-1} DMSO. The nematodes were checked for mortality at 0, 2, 4, 6, 24, and 48 h.

Mosquitocidal bioassay: Mosquito eggs were obtained from Drs. Alan Raikhel and Alan Hays of the Department of Entomology at Michigan State University. These were kept in a humid environment until needed. The eggs were hatched by placing them in ~500 mL of degassed RO water and incubating at 26° C. At the fourth instar stage, 8-12 larvae were placed in test tubes with 980 μL of water. The extract being tested was added to this solution at the concentration of 250 μg • 20 μL^{-1}, in DMSO. The larvae were then checked for non-motility (judged by putting tube next to bright light; healthy larvae are sensitive to light and will swim away from it) or mortality at 2,4, 6, and 24 h intervals.

Caterpillar bioassay: Each caterpillar assay follows the same general procedure, the only difference being in the media mixtures. Eggs of *H. zea* and *M. sexta* were purchased

from the North Carolina State University Department of Entomology Insectary (c/o Beverley Pagura, NCSU/Dept. of Entomology, 2741 Pillsbury Circle, Rm 108, Raleigh, NC 27695). Eggs of *L. dispar* were obtained from Dr. Bob McCron, Forestry Canada, Forest Pest Management Institute, Sault Ste. Marie, Ontario, Canada. Upon arrival, eggs *H. zea* and *M. sexta* were incubated at 26° C until hatching. *L. dispar* eggs were hatched at room temperature.

When the majority of the eggs hatched, the diet was prepared. Each caterpillar had its own dry diet base, to which the extract solution and liquid agar (Difco Labs, Detroit, Mich.) was added to make a total of 5 g of the final media. The diet was then disbursed into fifteen tubes. The dry diets for *H. zea* and *M. sexta* was purchased from North Carolina State University Insectary. The *L. dispar* dry diet consisted of 36 g of wheat germ (Meijer, Inc, E. Lansing, Mich.), 7.5 g of casein (Sigma Chemical Co., St. Louis, Mo.), 2.4 g of wesson salts (Sigma Chemical Co., St. Louis, Mo.), 0.6 g of sorbic acid (Sigma Chemical Co., St. Louis, Mo.), 0.3 g of methyl paraben (Sigma Chemical Co., St. Louis, Mo.), and 3 g of vitamin mixture for insects (Sigma Chemical Co., St. Louis, Mo.). This mixture was ground to a fine powder in a benchtop coffee grinder.

In each respective diet, 0.845 g of dry diet was used for *L. dispar*, 0.940 g for *H. zea*, and 0.950 g for *M. sexta*. To the dry diet, 1250 μg of extract (for an overall concentration of 250 ppm in the media) and 25 μL of DMSO were added and mixed in thoroughly. For the control set, only 25μL of DMSO was added. Once the dry diet, extract and DMSO were thoroughly mixed together, the diet was ready to be mixed with the liquid agar for distribution into the assay tubes. Each caterpillar diet had a different ratio for determining the appropriate amount of agar. Using the *L. dispar* bioassay as an

example, if five treatments were being tested along with a control, for a total of six media sets, roughly 30 mL of agar would be needed. For a safety margin, 50 mL was prepared. To get the correct amount of water and agar for the mixture, 50 would be multiplied by 0.815 to determine the water amount (42.25 mL of water) and by 0.015 for the agar amount (0.75 g of agar). The agar solution was prepared, autoclaved and then cooled to 50° C before mixing with the dry diet and disbursing into the assay tubes. The agar media preparation ratios for *H. zea* and *M. sexta* were 0.812/0.012 water/agar and 0.794/0.016 water/agar respectively. After the diet in the assay tubes had cooled, one caterpillar was placed in each tube. Since diet containing the extract was prepared on a per unit weight basis, the exact amount placed in each assay tube was unimportant, as long as there was enough media for the caterpillar to survive. The caterpillars were then placed in a 24-26° C environment with a twelve-hour light photoperiod. After six days, the caterpillars were weighed. Extract activity was indicated by stunted or dead caterpillars.

Results and Discussion

The summary of bioassay results from *L. leucocephala* extracts is presented in Table 2.1. None of the extracts from *L. leucocephala* showed any activity against the fungi or bacteria tested. Also, the topoisomerase inhibition and nematicidal bioassays revealed that the extracts were inactive. Initially, the hexane extract from the leaves and stems showed activity against caterpillars and mosquitoes. The methanol crude extract from the roots demonstrated activity against *N. europaea* and *N. multiformis* (see Chapter III for further discussion of this activity).

Bioassay directed fractionation of the leaf extract led to purified fractions with activity against caterpillars. However, there was not enough material from the first extraction to isolate the active compound in quantities necessary for further purification and structural elucidation. In order to obtain more leaf extract, *L. leucocephala* leaves were harvested at various times and freeze dried, ground and extracted with hexane.

Table 2.1: Bioassay Results of *Leucaena leucocephala* Crude Extracts

	Root extracts:			Stem extracts:			Leaf extracts:		
Bioassay Organisms	H	EA	M	H	EA	M	H	EA	M
Aspergillus niger	-	-	-	-	-	-	-	-	-
Fusarium oxysporum	-	-	-	-	-	-	-	-	-
Fusarium monilforme	-	-	-	-	-	-	-	-	-
Gloesporum	-	-	-	-	-	-	-	-	-
Rhizoctonia	-	-	-	-	-	-	-	-	-
Botrytis	-	-	-	-	-	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-
Staphylococcus	-	-	-	-	-	-	-	-	-
Streptococcus	-	-	-	-	-	-	-	-	-
Caenorhabditis elegans	-	-	-	-	-	-	-	-	-
Panagrellus redivivus	-	-	-	-	-	-	-	-	-
Aedus aegyptii	-	-	-	(+)	(+)	-	-	-	-
Helicoverpa zea	-	-	-	(+)	-	-	(+)	-	-
Lymantria dispar	-	-	-	(+)	-	-	(+)	-	-
Manduca sexta	-	-	-	(+)	-	-	(+)	-	-
Saccharomyces 394	-	-	-	-	-	-	-	-	-
Saccharomyces 394(t_{-1})	-	-	-	-	-	-	-	-	-
Saccharomyces 394(t_{2-5})	-	-	-	-	-	-	-	-	-
N. europaea	ns	-	+	ns	(-)	(-)	ns	(-)	(-)
N. multiformis	ns	-	+	ns	(-)	(-)	ns	(-)	(-)

(- indicates no activity; + indicates activity; ns, not soluble; (+) indicates activity from initial extract only; (-) indicates color masking, extract not tested definitively; H,hexane extract; EA, ethyl acetate extract; M, methanol extract)

Fractionation of this extract was guided by thin layer chromatography comparison with the active fractions from the first extract. However, the fractions from the second extract that matched best with the active fractions from the first extract did not show activity against the corn earworms, or any of the other caterpillars. The new extract was not tested against mosquitoes.

The initial extract came from plant material that had been collected just after the plants were moved from the Plant and Soil Science Building (PSSB) greenhouses to the BNPL greenhouses. The BNPL has a strict policy of no insecticide spraying in its greenhouse spaces to prevent detection of false positives. However, the PSSB greenhouses spray regularly. At the time of the move and the subsequent plant harvest, this was not taken into consideration. Thus, it is very likely that when the plants were moved to the BNPL greenhouse, there was an insecticide residue on the leaves and stems. This probably explains the initial mosquitocidal and insect anti-feedant activity observed. Unfortunately, a great deal of effort was directed towards the isolation of an active compound that probably was not there.

Thus, in the BNPL assays, *L. leucocephala* did not show bioactivity. The activity found from the initial screens of the leaf and stem hexane extracts were shown to be false positives, although the mosquitocidal activity was not reinvestigated.

Chapter III

Nitrification Inhibitors from *L. leucocephala*

Abstract

The methanol extract from the roots of *Leucaena leucocephala* displayed nitrification inhibition activity. Initial fractionation of this extract yielded active fractions but not the active compound. Subsequent fractionation of the extract on Amberlite XAD-2 and further purification by preparative HPLC yielded the active compound. The structure of the active compound was elucidated by ^{1}HNMR and ^{13}CNMR, and found to be gallocatechin. Three other catechins also were identified—epigallocatechin, catechin, and epicatechin. Gallocatechin displayed strong nitrification inhibition activity at 50 ppm concentration, as compared to the DMSO control and 10 ppm nitrapyrin, a commercial nitrification inhibitor. Gallocatechin at 6 and 12 ppm displayed similar activity to nitrapyrin at 10 ppm. An authentic sample of catechin did not display activity at the 10 ppm level.

Introduction

There exists considerable evidence that plants produce secondary compounds which inhibit nitrification. Work done in the first half of the 20TH century showed that wheat plants decreased nitrate levels in soil, after nitrate taken up by the plant had been accounted for (Lyon et al, 1923), and that grasslands displayed low nitrification rates (Richardson, 1935 and 1938). Other workers presented evidence that grasses inhibited nitrification as well (Theron, 1951; Stiven, 1953; and Munro, 1966).

Further research began to pinpoint specific plant compounds that showed nitrification inhibition. Tannins and gallotannins were reported to inhibit nitrification by Basabara, 1964, and Rice, 1965 and 1969. A large body of research provided evidence that phenolic acids and some flavonoids inhibit nitrification, including chlorogenic acid (**1**), gallic acid (**3**), caffeic acid (**5**), quercetin (**7**), and karanjin (**10**) (Rice, 1964; Rice, 1965; Rice and Pancholy, 1974; Sahrwat and Mukerjee, 1977). Other work implicated monoterpenes as nitrification inhibitors. Myrcene (**12**), α-pinene (**13**), β-pinene (**14**), and in particular limonene (**11**) were purported to have NI activity (White, 1986; White, 1991).

However, there are published reports questioning the nitrification inhibition activity of naturally occurring compounds. The phenolic acids including gallic and caffeic acid were tested for nitrification inhibition activity in both soil and in pure *N. europaea*, *N. multiformis*, and *Nitrospira* cultures, but were found to be inactive (Bremner and McCarty, 1986; McCarty et al., 1991). It was also shown that phenolic acids only inhibited nitrification at levels much higher than those found in the soil

(Turtura et al., 1989). Also, there was work that questioned the NI activity of terpenes (Bremner and McCarty, 1988).

Thus, questions remain whether plants produce secondary compounds that influence the nitrification process. However, there is much evidence that plants produce secondary compounds that have a variety of roles in protecting the plant. Compounds are produced which fight off insects (Elliott, 1980; Tomizawa and Yamamoto, 1992) , prevent other plants from growing in the area around the plant (Lydon et al., 1997; Macias et al., 1998; Chou et al., 1998), and which fight off microbial infection of plant parts (Pedras et al., 1997; Monde et al., 1998; Papajewski et al., 1998; Iada et al., 1999). Recent evidence shows that plants also produce compounds that are excreted into the soil and influence the growth of mycorrhizal fungi (Siqueira et al., 1991). Thus, it is reasonable to hypothesize that plants produce compounds that also influence the growth of bacteria in the soil. This chapter describes the isolation and characterization of nitrification inhibitory compounds from the roots of *L. leucocephala*, using an in vitro NI assay. Although these compounds were reported earlier as natural products, the NI activity reported for these compounds is novel.

Materials and Methods

Nitrification Inhibition Bioassay

Nitrifying Bacteria and Growth Medium: The *Nitrosomonas europaea* strain used in all bioassay experiments was ATCC 19718, and was supplied by Mary Ann Bruns of Michigan State University, Department of Crop and Soil Sciences. Other nitrifying bacteria used in the bioassay were *Nitrosolobus multiformis* (ATCC 25196) and

Nitrospirea AV (from Apple Valley, Minn.). These bacterial species also were supplied by Mary Ann Bruns. All bacteria were grown using the media described below. Cultures used for bioassays were grown in 2 mL of media in 12 x 75 mm (4 mL) pre-sterilized polystyrene Falcon Tubes.

The growth medium used for this work was a fusion of recipes from American Type Culture Collection (ATCC) and "Nitrifying Bacteria" (Schmidt and Belser, 1982). The growth medium recipe, along with the micronutrient solution was supplied by Mary Ann Bruns of the Michigan State University Department of Crop and Soil Sciences. The elements of the ATCC recipe were taken from #929, the *Nitrosolobus* medium. It consists of (in one liter of RO water) 1.32 g NH_4SO_4 (Mallinckrodt, Inc., St. Louis, Mo.), 380 mg of $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Fair Lawn, N.J.), 20 mg of $CaCl_2 \cdot 4H_2O$ (J.T. Baker Chemical Co., Phillipsburg, N.J.), 87 mg of K_2HPO_4 (Mallinckrodt, Inc., Paris, Ky.), and 0.5% phenol red (Aldrich Chemical Co., Milwaukee, Wisc.) solution. The trace element solution used consisted of 100 mL of RO water, 10 mg of $NaMoO_4 \cdot 2H_2O$ (Mallinckrodt, Inc., St. Louis, Mo.), 20 mg of $MnCl_2$ (MCB Manufacturing Chemists Inc., Cincinnati, Ohio), 0.2 mg of $CoCl_2 \cdot 6H_2O$ (Aldrich Chemical Co., Milwaukee, Wisc.), 10 mg of $ZnSO_4 \cdot 7H_2O$ (Mallinckrodt, Inc., St. Louis, Mo.), and 2 mg of $CuSO_4 \cdot 5H_2O$ (Mallinckrodt, Inc., Paris, Ky.) (solution from Schmidt and Belser, 1982). The chelated iron solution used in the medium consisted of 246 mg of $FeSO_4 \cdot 7H_2O$ (J.T. Baker Chemical Co., Phillipsburg, N.J.), and 331 mg of EDTA disodium (Boehringer Mannheim GmbH, Germany) in 100 mL of RO water (solution from Schmidt and Belser, 1982). For the completed media, the ATCC ingredients were mixed in a flask with 1 mL each of the micronutrient solution and chelated iron solution, and 0.25 mL of the 0.5%

phenol red solution. The final volume was then adjusted to one liter with RO water. The pH of the media was then adjusted to ~7.5 with 0.2 M K_2CO_3 (Aldrich Chemical Co., Milwaukee, Wisc.). The medium was finally filter-sterilized through a 0.2 μm Nalgene bottle-top filter (500 mL) into a sterilized Pyrex storage bottle (500 mL).

The phenol red in the media was used to indicate the growth of the bacteria. The culture tubes containing the bacteria were seeded when the pH was ~7.5. The optimum pH range for the bacterial growth is about 6.0-7.5. One of the byproducts of the nitrification process is protons, and this makes the solution become acidic. As the solution becomes acidic, the phenol red shifts in color from pink to yellow. This was used as an indication of growth of *N. europaea*. When the media had changed color to yellow, dropwise addition of 0.5% K_2CO_3 (Aldrich Chemical Co., Milwaukee, Wisc.) from sterilized, cotton plugged Pasteur pipettes was used to shift the pH back to ~7.5.

Bioassay Procedure: For the bioassay, extracts were filter-sterilized through a pre-sterilized Millex GV 0.22 μm filter unit whenever possible (depending on solubility and amount of bioassay solution). For use in the bioassay, the pH of the bacterial cultures was adjusted several times, usually four times. All initial assays were qualitative in nature, that is, the inhibition was measured only by lack of color change. All crude extracts were assayed at 250 ppm, and purified fractions were assayed at 100 ppm. Twenty microliters of the test sample bioassay solution was added for each milliliter of bacterial media solution. Controls with 20 μL of dimethyl sulfoxide (DMSO) per mL of media were employed, as were media blanks with no bacteria in them. Some select

assays were quantitated for nitrite using the Greiss-Ilosvay method (Keeney and Nelson, 1982).

Greiss-Ilosvay Method: This method was designed for measuring nitrite in a soil matrix, but it can be adapted for measuring nitrite in any non-soil matrix as well. The method used in this work was taken from Keeney and Nelson (1982). This assay is based on the fact that NO_2^- reacts with primary aromatic amines (diazotizing reagents) in acidic solution to produce diazonium salts. These salts then couple with aromatic compounds which contain specific amino or hydroxyl groups to form colored azo compounds which can be detected by spectroscopic methods. Specifically, the nitrite in solution reacts with sulfanilamide in the diazotizing reaction, and then is coupled with N-(1-napthyl)-ethylenediamine to form reddish purple colored solutions.

Reagents used in this assay were: Diazotizing reagent, 0.5 g of sulfanilamide (Sigma Chemical Co., St. Louis, Mo.) in 100 mL of 2.4 N hydrochloric acid (HCl), stored at 4° C; coupling reagent, 0.349 g N-(1-napthyl)-ethylenediamine dihydrochloride (Aldrich Chemical Co., Milwaukee, Wisc.) in 100 mL of 0.12 N HCl, stored in an amber bottle at 4° C; standard nitrite (NO_2^-) solution, 0.247 g of sodium nitrite ($NaNO_2$) (Aldrich Chemical Co., Milwaukee, Wisc.) in water, brought to 1000 mL in a volumetric flask. This solution contained 50 μg of NO_2^- nitrogen/mL when completed, and was stored at 4° C.

To prepare samples for analysis, an aliquot of test sample was placed in a 50 mL volumetric flask. Reverse osmosis water was added to adjust the volume to ~45 mL. One mL of the diazotizing reagent was added to that solution and followed by 1 mL of

the coupling reagent. The solution was then kept at room temperature for 20 minutes. The solution was then adjusted to 50 mL, mixed thoroughly, and the color intensity was measured at 540 nm against a blank solution. All samples were analyzed on a Shimadzu UV-260 UV-Visible Recording Spectrophotometer.

A standard curve was prepared by analyzing reference samples containing 0, 1, 2, 3, 4, 5, and 6 μg of NO_2^- in 50 mL volumetric flasks. To analyze nitrite content in culture tubes containing nitrifying bacteria, 30-40 μL aliquots were removed daily and placed in 50 mL volumetric flasks, and the samples were analyzed as described above. Nitrite content of the 30 or 40 μL aliquots from the test samples were calculated from the standard curve. The nitrite content of the entire microbial solution was then calculated per mL of media.

Fractionation of Methanolic Root Extract

For preliminary fractionation, the extract described in the Materials and Methods section of Chapter II was used. The initial separation was accomplished via medium pressure liquid chromatography (MPLC). Thin layer chromatography (TLC) experiments on the root crude methanol extract using both silica (Uniplate Silica Gel GHLF TLC plates, inorganic binder, UV254, scored 20 x 20 cm, 250 microns) and reverse phase (C18) plates (Whatman $MKC_{18}F$ Reversed Phase TLC plates, 1 x 3 in., 200 microns) did not achieve good separation. Since the extract was very polar in nature, it was decided to use C18 MPLC for the initial fractionation. The solvent system used was MeOH:H_2O 60:40. However, initial attempts to dissolve the extract in the MeOH:H_2O 60:40 solvent system was met with the appearance of insoluble material. A MeOH:H_2O 70:30 solvent

system then was used to treat the sample before MPLC. The insoluble fraction was centrifuged, the supernatant was recovered and evaporated to dryness. The dried supernatant (0.83 g) was then purified by MPLC (35 x 4 cm, 6.5 cm tapered ends, 125 g of C18, using a Chemco 81-M-2 Low Prep Pump), using MeOH:H_2O 60:40 mobile phase. Column was equilibrated with ~300 mL of MeOH:H_2O 60:40. Fractions (30 mL) were collected after discarding 100 mL of the mobile phase, although UV-active bands were collected separately. Fractions II, III and IV were UV-active. Altogether, 20 fractions were collected, and after TLC analysis were combined to yield a total of 9 fractions. Bioassay of these fractions showed that fraction II was the most active. Three more MPLC purifications were conducted to gather a total of ~1 g fraction II.

Attempts to purify the active fractions by LC-20 HPLC were not successful. Column used for the analyses were the dual Jaigel ODS columns (20 x 250 mm i.d., 15 micron particle size). However, separation of the active compound was not achieved at this stage.

This fraction was evaluated to determine its organic nature with the material being subjected to a melting point test using a Bristoline Bristolscope melting point apparatus. Small amounts of the material were dissolved in RO H_2O in two test tubes, and one test tube made acid with HCl, and the other basic with KOH. The fraction also was subjected to flame test.

Desalting Column Separations: Results of chemical tests indicated that fraction II may contain salts. Thus, removing the salt compounds on a desalting column was attempted. A Bio-Rad 10DG EconoPac column (10 mL capacity, 12 x 1.5 cm, with ~6 cm of

polyacrylamide gel) was used. Fraction II (203.5 mg) from the MPLC was dissolved in two mL of RO H_2O and applied on the column, and another mL of RO H_2O was used to transfer the remainder. The column was eluted with 3 mL of water, and the elution volume was discarded. The solvent used for fraction elution was RO H_2O, and six one mL fractions were collected. Two more fractions were collected which were 23 and 40 mL in volume, respectively. The yield from these eight fractions were as follows: fraction 1 (light brown)-4.7 mg, fraction 2 (light brown)-9.4 mg, fraction 3 (light brown)-13.6 mg, fraction 4 (orange-brown)-7.3 mg, fraction 5 (pink)-6.9 mg, fraction 6 (pink)-9.0 mg, fraction 7 (reddish-brown)-59.4 mg, and fraction 8 (brown)- 23.3 mg. A total of 133.6 mg was collected off the column, for a yield of 65.7%. After HPLC analysis, fractions were combined to yield a total of five fractions. Bioassay of the fractions showed that fraction 4 was the most active, with fractions 1-3 showing moderate activity.

Three more desalting separations were performed, with a total of 541.8 mg of fraction II being applied to the column. A total of 28.9 mg of fraction 4 was collected. The crystalline compound yielded from this fractionation then was subjected to ^{1}H and ^{13}C NMR experiments and was found to be sucrose by NMR comparison with an authentic sample.

Purification of Active NI Compounds

Two additional extractions were carried out to yield an ample supply of extract for the purification of the NI compound. In the first extraction, 165 g of freeze-dried, ground *L. leucocephala* roots were extracted sequentially with hexane, ethyl acetate, and methanol. For each solvent, two 500 mL aliquots were added and drained over 24 h. The

hexane and ethyl acetate extractions were conducted to emulate the conditions of the initial extraction. The methanol extract was the extract of interest. The solvent was removed under reduced-pressure rotary evaporation. A total 7.76 g of methanol extract was obtained from the first extraction.

For the second extraction, 244 g of freeze-dried, powdered *L. leucocephala* roots were extracted sequentially with hexane, ethyl acetate and methanol. For the hexane and ethyl acetate, two 500-mL aliquots of solvent were added and drained over 24 h. For the methanol extract, four 500-mL aliquots of solvent were added and drained over 24 h, and the solvent was removed under reduced-pressure rotary evaporation. A total 24.74 g of methanol extract was obtained from the second extraction.

Isolation of NI Compound: Isolation of the active compound was achieved in two steps. In step one, the methanol extract was first processed to obtain the water-soluble fraction. A total of 7.71 g of the extract was dissolved as much as possible in 45 mL of H_2O. All insoluble material was centrifuged to yield 5.40 g of water-soluble portion. This water-soluble material was then fractionated on Amberlite XAD-2 (51 g, Supelco, Inc., Bellefonte, Penn.). The XAD-2 was placed in a 23.5 x 3.5 cm glass column with a teflon stopcock, and secured with a wad of cotton. The XAD-2 resin was washed thoroughly with MeOH and H_2O and finally was equilibrated with water.

The water-soluble fraction (~1 g) was applied to the XAD-2 in 6-10 mL of H_2O. First the column was eluted with 125 mL of 100% H_2O to yield the first fraction. Then the column was eluted with 75 mL of H_2O:MeOH 50:50. The first fraction was collected until ~15 mL of the H_2O:MeOH 50:50 was applied to the column, at which point the

collection of fraction II started. Finally, the column was eluted with 175 mL of 100% MeOH. As with the first fraction, the second fraction was collected until ~25 mL of the 100% MeOH was applied. After that, the third fraction was collected until all the MeOH had been applied and until ~25 mL of the H_2O re-equilibration solvent had been applied. In the first fractionation, 1.01 g of water-soluble material was purified on the XAD-2 column. Fractions I-III yielded 877, 45.9, and 75.1 mg, respectively. These fractions were assayed for NI bioassay, and the methanol fraction (fraction III) was found to be active. Thus, fraction III was the focus of further collection. In total, 453.6 mg of fraction III was collected from a total of 5.31 g of the water-soluble material applied to the XAD-2.

In the second step, further purification of the XAD-2 methanol fraction was attempted by HPLC. The methanol fraction was profiled on an LC-20 Preparative HPLC (Japan Analytical Instruments). Two different column systems were used. The first was two Shodex GS-3102F columns (20 x 300 mm i.d., 3 micron pore size, 40,000 m.w. exclusion) connected in tandem; the mobile phase used was MeOH:H_2O 70:30, 4 mL/min flow rate, and the peaks were detected at 210 nm. The second was two Jaigel ODS columns (20 x 250 mm i.d., 15-micron particle size) connected in tandem, with the same mobile phase, flow rate and UV wavelength as the GS-3102F system. The GS-310 columns gave the best separation. Two other solvent systems were tried with the GS-310, which were MeOH:H_2O 60:40 and MeOH:H_2O 80:20, respectively, at 4 mL/min flow rate, but the MeOH:H_2O 70:30 gave the best separation.

The XAD-2 methanol fraction was then subjected to preparative HPLC separation on LC-20 using the GS-3102F columns. For the initial work on the HPLC, 126 mg was

separated in five runs, each injection consisting of 31.5 mg. Seven fractions were collected during each injection, with the combined fractions giving 52.6, 6.0, 3.2, 18.9, 4.1, 11.9, and 5.3 mg respectively (Figure 3.1). All fractions were then bioassayed, with fractions V-VII showing the best activity.

Repeated preparative HPLC purification was carried out in six injections on 283.8 mg of the XAD-2 methanol fraction, and 21.3, 24.5, and 17.3 mg of active fractions V-VII, respectively, were collected. Fraction VI was further purified by preparative HPLC, using the ODS columns, and it yielded 24.1 mg of compound **1**.

Compound **1** ^{1}H and ^{13}C NMR. ^{1}H NMR (500 MHz, d_6-DMSO): δ 2.33 (dd, J_1=16 Hz, J_2=8 Hz, 1H, H-4a), 2.59 (dd, J_1=16 Hz, J_2=5.5 Hz, 1H, H-4b), 3.77 (m, 1H, H-3), 4.41 (d, J=7 Hz, 1H, H-2), 4.83 (d, J=4.5 Hz, 1H, 3-OH), 5.67 (d, J=2 Hz, 1H, H-7) 5.87 (d, J=2 Hz, 1H, H-5), 6.23 (s, 2H, H-2', 6'), 8.00 (bs, 1H, 6- OH), 8.75 (bs, 2H, 3'-, 5'- OH) 8.91 (bs, 1H, 4'- OH) 9.14 (bs, 1H, 8- OH). ^{13}C NMR (75.5 MHz, CD_3OD): δ 28.2 (C-4), 68.9 (C-3), 83.0 (C-2), 95.6 (C-6), 96.4 (C-8), 100.8 (C-4a), 107.3 (C-2', 6'), 131.7 (C-1'), 147.0 (C-3', 5'), 157.0 (C-8a), 157.7 (C-5), 158.0 (C-7).

Several low-intesity peaks in the ^{1}H and ^{13}C spectra of compound **1** indicated the presence of a closely related compound. These peaks were determined to be significant, and compound **2** was identified (only peaks that showed up in the NMRs are listed). Important peaks for determining compound **2**, ^{1}H NMR (500 MHz, d_6-DMSO): δ 4.09 (m, 1H, H-3), 4.64 (s, 1H, H-2), 6.36 (s, 2H, H-2', 6'). ^{13}C NMR (75.5 MHz, CD_3OD): δ 29.3 (C-4), 67.6 (C-3), 80.0 (C-2), 107.1 (C-2', 6'), 134.1 (C-1'), 146.8 (C-3', 5').

Additional quantities of the HPLC fractions were prepared from 20.24 g of the methanol root extract following the same general purification protocol. However, several

changes were made to make the separation process more efficient. For each XAD-2 separation, two grams of water-soluble fraction from the root methanol extract was applied to the resin. For the preparative HPLC work, ~100 mg of XAD-2 methanol fraction was injected for each purification. Also, the flow rate was changed to 3 mL/min and the refractive index detector was used (Figure 3.2). From 17.22 g of water-soluble material applied to XAD-2, a total of 1096.6 mg of the MeOH fraction was collected. Approximately one gram of the MeOH fraction was purified by preparative HPLC to yield 24.7 mg of compound **1**.

Fraction VII was collected in enough quantity to be analyzed by NMR. A total of 12.3 mg of fraction VII was recovered after re-injection on the LC-20. Although the chromatogram only showed one peak, the proton NMR spectrum indicated that the fraction was a mixture of compounds **3** and **4**. Notable peaks from the proton NMR are as follows (500 MHz, CD_3OD): δ 2.50 (dd, J_1=16.5 Hz, J_2=8.25 Hz, H-4), 2.74 (dd, J_1=16.5 Hz, J_2=3.25 Hz, H-4), 2.85 (dd, J_1=16 Hz, J_2=5.25 Hz, H-4), 3.98 (m, H-3), 4.18 (m, H-3), 4.56 (d, J=7.5 Hz, H-2) 4.81 (s, H-2), 5.85 (d, J=2.5 Hz, H-7), 5.91 (d, J=2 Hz, H-8), 5.92 (d, J=2.5 Hz, H-5), 5.94 (d, J=2 Hz, H-6), 6.69 (s), 6.71 (s), 6.74 (s), 6.76 (s), 6.80 (d), 6.83, (d, J=1.5 Hz), 6.97 (d).

Results and Discussion

One of the early goals of this project was to develop a nitrification inhibition bioassay with rapid turnaround time. An important limiting factor in culturing nitrifying bacteria is their slow growth. Thus, mature cultures of *N. europaea* have only a small amount of deposited bacteria at the bottom of the tube and the growing medium remained

clear. To indicate the growth of bacteria in small test tubes, a pH indicator was added. Therefore, an extract with potential for nitrifying bacterial growth inhibition was indicated by the lack of color change. Once the pH of the medium was adjusted, it took 3-5 days for the color to change from pink to yellow. The bacterial growth inhibition was confirmed further by measuring the change in nitrite levels over time. However, the innate usefulness of the color-based assay is in the fact that quick results can be discerned without a great deal of analysis or cost. This is one of the benefits when screening plant extracts for activity in a bioassay-directed fractionation, since numerous fractions will be screened during the purification process. However, the bioassay did not lend itself to screen highly colored extracts from leaves and stems. Accordingly, activity was not detected in any of the extracts from leaves or stems from *L. leucocephala* in the initial assays. The methanol crude extract from the roots showed activity in the initial screening, and was studied further using bioassay-directed fractionation and purification.

The crude methanol extract was subjected to medium-pressure liquid chromatography (Figure 3.1). Bioactivity was shown in the second fraction. The active fraction was a complex mixture and was too polar to separate using reverse phase HPLC. Repeated MPLC fractionation yielded 1.0 g of combined active fractions, which was subject to some chemical tests. The melting point determination of the fraction revealed that it might contain inorganic salts, because it did not melt at temperatures greater than 350° C. Therefore, further purification of the MPLC active fraction involved "de-salting" using a Bio- Rad Econo-Pac 10DG column (Bio-Rad), designed for desalting solutions containing proteins (Figure 3.2).

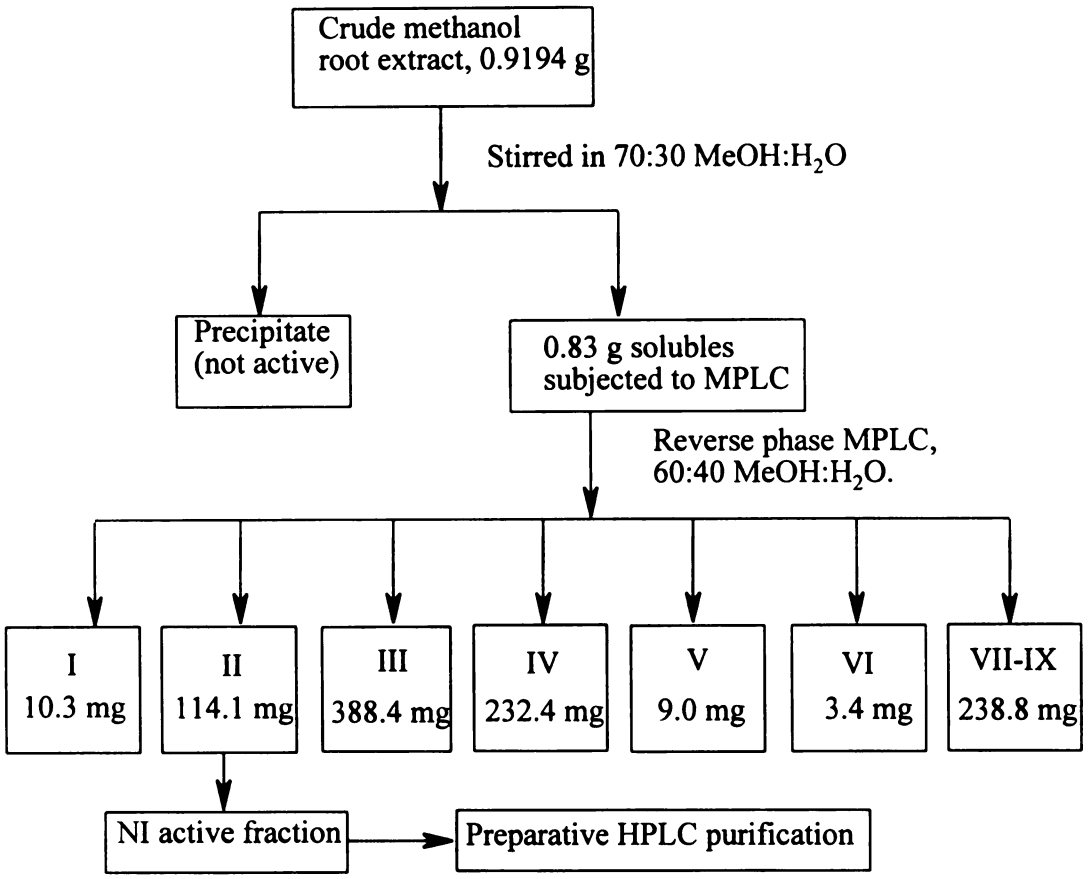


Figure 3.1: MPLC fractionation of root methanol extract.

The fractions from the desalting column were collected on the basis of volume and color. Initially, 1 mL fractions were collected, but as the eluant color, bands were collected as separate fractions. The colors of the bands collected were brown, to orange-brown, orange, and pink. Although fractions from the desalting process showed activity in the qualitative NI bioassay, fractions assayed later and analyzed for nitrite content did not display activity. The desalting step yielded an active fraction containing sucrose,

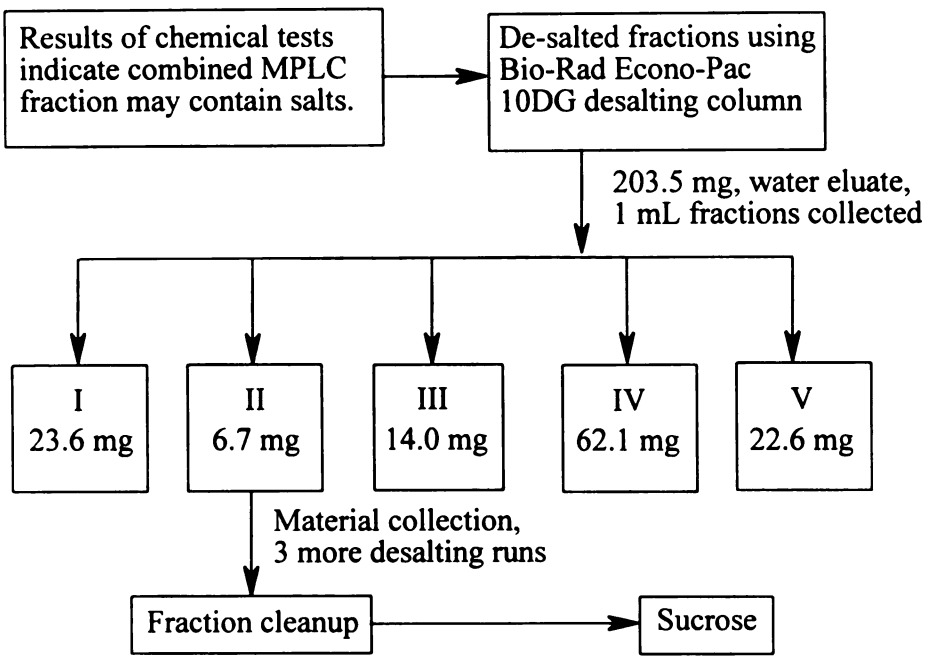


Figure 3.2: Desalting separation of the active fraction from the MPLC separations.

and the fraction displayed activity in the qualitative NI bioassay. However, a sucrose standard did not show NI activity. Further purification yielded only sucrose and the activity of the material was lost in the desalting step. Therefore, the desalting step was not used for further purification of the NI active compounds.

A new method was developed which used Amberlite XAD-2 as an absorbing agent for the NI compounds, followed by preparative HPLC for final isolation. Methanol extracts of fresh roots and the water-soluble portion of the MeOH extract was fractionated over XAD-2 resin by eluting with water, H_2O:MeOH and finally MeOH. Only the MeOH fraction from the XAD-2 purification showed activity (Figure 3.3).

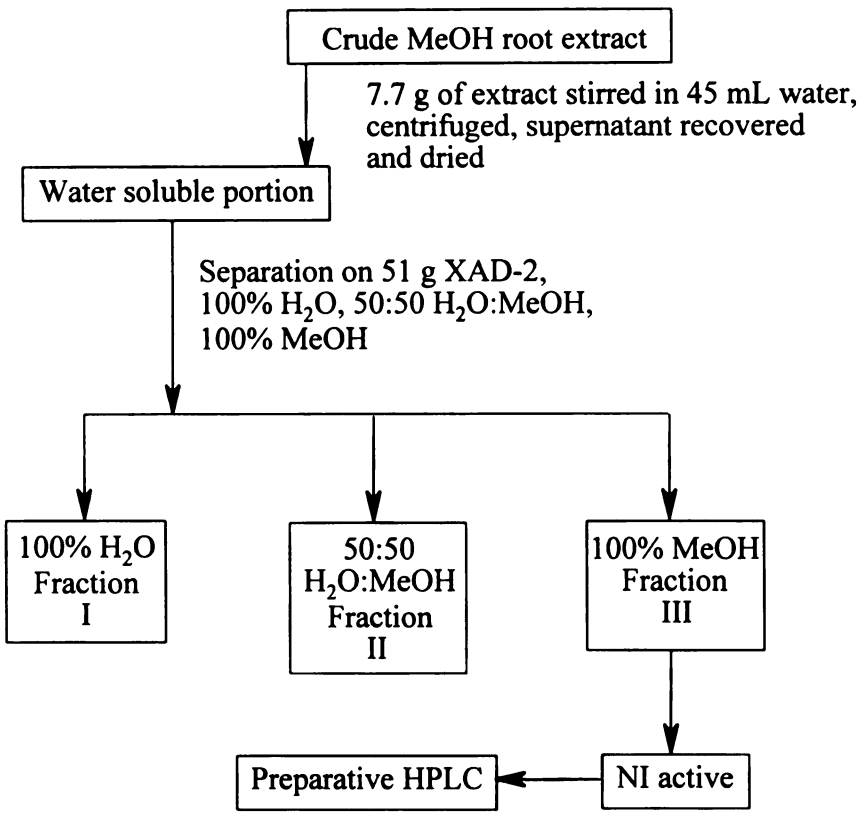


Figure 3.3: XAD-2 fractionation process.

The MeOH fraction then was purified by preparative HPLC using GS-3102F columns, seven fractions being collected. Fractions V-VII showed activity, fraction VI being the most active. Fraction VI was pure enough to conduct spectral studies and was designated compound **1**. Proton, carbon, and HMQC NMR experiments were conducted on compound **1**. Unfortunately, while removing d_6-DMSO (used for NMR experiments) under high vacuum and moderate heat-water bath, compound **1** decomposed. This prompted a repeated isolation of compound **1**. The structure of compound **1**, along with

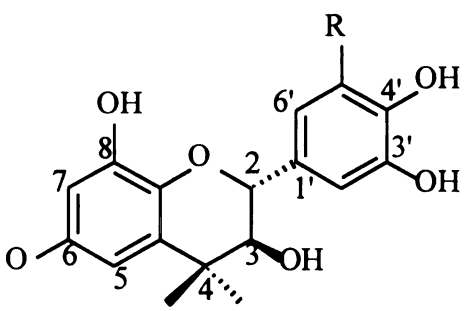
compounds **2, 3** and **4** were elucidated by ^{1}H, ^{13}C and 2-D NMR experiments in conjunction with study of the literature.

The ^{1}H NMR for compound **1** showed five exchangeable peaks with D_2O. The peak at 8.75 ppm integrated for two protons and exchanged with D_2O. The peak at 4.83 ppm appeared as a doublet that exchanged with D_2O. There were two sets of doublets of doublets, occurring at δ 2.33 and 2.59 ppm, showing geminal coupling of 8 and 5.5 Hz, respectively, and each set integrating for one proton each. The HMQC experiment correlated these proton signals to the carbon at 28.2 ppm. In the COSY spectrum, the doublet of doublets correlated strongly to each other, and they also correlated to the multiplet at 3.77 ppm. From the HMQC spectrum, the multiplet at δ 3.77 correlated to the carbon at 66.3 ppm. From the COSY spectrum, the multiplet at δ 3.77 also correlated to the doublet at 4.41 ppm. The doublet at δ 4.41 correlated to the carbon at 81.1 ppm in the HMQC spectrum.

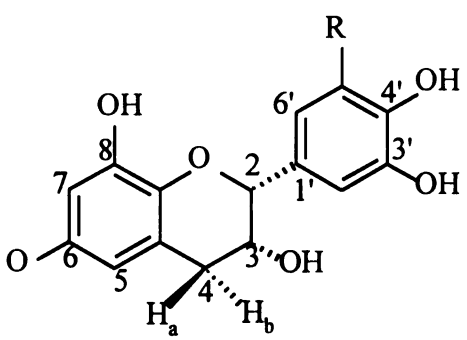
There were several other peaks which showed correlation in the HMQC and COSY experiments. The two doublets at 5.67 and 5.87 ppm correlated to each other in the COSY spectrum, and in the HMQC spectrum they matched up with the signals at 95.6 and 96.4 ppm, respectively. The singlet at δ 6.23, which integrated for two protons, did not show any correlations in the COSY experiment. In the HMQC spectrum it matched up with the carbon signal at 106 ppm.

The ^{13}C NMR spectrum showed 12 peaks, the most intense peaks appearing at δ 106 and 130, respectively. The DEPT spectrum showed there were six quaternary carbons at 100.8, 131.7, 147.0, 157.0, 157.7, and 158.0 ppm, five methine carbons at 68.9, 83.0, 95.6, 96.4, and 107.3 ppm, and one methylene carbon at 28.2 ppm.

Using XAD-2 and preparative HPLC to purify the *L. leucocephala* root methanol extract was successful. The most active NI compound isolated was determined to be gallocatechin (compound **1**), although a mixture of catechin and epicatechin was also found to exhibit activity in the initial bioassays (compounds **3** and **4**). The NMR data of this compound confirmed that it is identical to the existing spectral data for gallocatechin. Gallocatechin is a fifteen-carbon flavonoid, lacking the carbonyl group at C-4. The term gallo discerns the three hydroxyl groups on the B ring at C-3', C-4', and C-5'. Catechins also possess hydroxyls at C-3, C-5, and C-7 and have no double bond between C-2 and C-3. Catechins where the B-ring at C-2 and the hydroxyl at C-3 are cis to each other are designated as *epi*. Catechins where C-2 and C-3 are trans to each other are not given a particular designation.



Compound **1**: R= OH
Compound **3**: R=H



Compound **2**: R=OH
Compound **4**: R=H

The doublet of doublets in the ^{1}H NMR of **1** at 2.33 and 2.59 ppm were assigned to the C-4 protons. The multiplet at 3.77 ppm couples with both C-4 and C-2 protons was designated to the C-3 proton. The C-2 proton, a doublet at 4.41 ppm, was coupled to the C-4 proton. The three exchangeable peaks at 8.75, 8.91, and 9.14 ppm confirmed phenolic groups. The peak at δ 8.75 integrated for two protons, and explained the

magnetic equivalence of the two hydroxyl groups on the B-ring at C-3' and C-5'. The C-4' and C-8 hydroxyl groups were assigned to peaks at 8.91 and 9.14 ppm, respectively. The C-3 and C-6 hydroxyl groups appeared at 4.83 and 8.00 ppm, respectively.

The signals at 5.67 and 5.87 ppm were assigned to C-8 and C-6 protons, respectively. They are somewhat downfield because of shielding from the neighboring hydroxyl groups. The singlet at 6.23 ppm, which integrates for two protons, corresponded to the C-2' and C-6' protons. Because of the symmetry present in the B ring, they resonated at the same frequency.

Characteristic low intensity peaks in the gallocatechin ^{1}H and ^{13}C spectra established the designation of epigallocatechin (compound **2**) as the minor component. Notably, the multiplet at δ 4.01 (corresponding to H-3), the singlet at δ 4.64 (corresponding to H-2), and the singlet at δ 6.36 (corresponding to H-2' and H-6') provided the strongest evidence for the presence of epigallocatechin. Although the chemical shifts recorded for compounds **1** and **2** differed from published data, the relative positions are consistent with the literature values. Davis et al. (1996) published data for gallocatechin and epigallocatechin, reporting H-2, H-3, and H-2', 6' for epigallocatechin at δ 4.82 (s), 4.19 (m) and 6.57 (s), respectively, and δ 4.51, 3.97 and 6.46, respectively for gallocatechin in $(CD_3)CO$. The singlet at 4.01 ppm is distinctive for the *epi* catechins, and the singlet at δ 6.36 is distinctive for the gallocatechins. The doublet of doublets for epigallocatechin were not resolved, although the signals were partially visible in the spectrum.

Several minor peaks at 29.3, 67.4, 80.0, 107.1, and 134.1 ppm in the ^{13}C NMR spectrum of gallocatechin also provided evidence for the presence of epigallocatechin.

These peaks correspond to C-4, C-3, C-2, C-2', 6', and C-1' of epigallocatechin, respectively. Davis et al. (1996) reported chemical shift values for C-4, C-3, C-2, C-2', 6', and C-1' of *epi*gallocatechin at 28.8, 67.0, 79.5, 107.0, and 130.8 ppm, respectively, and at 28.5, 68.4, 82.8, 107.3, and 131.6, respectively, for gallocatechin.

Fraction VII was determined to be a mixture of catechin (compound **3**) and epicatechin (compound **4**), as evidenced by the presence of characteristic peaks in the ^1H NMR spectrum. From the relative peak intensity, it appeared as though catechin was the major component. The first characteristic signals for catechins are indicated by the doublet of doublets at 2.50, 2.74, and 2.85 ppm, respectively. The peaks at δ 2.50 showed a geminal coupling of 8.25 Hz, while the signals at δ 2.85 gave a 5.25 Hz geminal coupling. Both coupling values are characteristic of the non-*epi* catechin configuration. The set of peaks at δ 2.74 showed a geminal coupling value of 3.25 Hz, which is very close to what Chien-Chang Shen et al. (1993) found for the H-4b proton at C-4 for (-)-epicatechin. The set of dd matching with those at δ 2.50 was hidden in the dd at 2.85 ppm, and could not be analyzed for shift or coupling constant.

The peaks at δ 4.56 and 4.81, corresponding to the C-2 proton, gave the strongest evidence for a mixture of *epi* and non-*epi* catechins, respectively. The peak at δ 4.56 is a doublet with a coupling constant of 7.5 Hz. This is very similar to the coupling constant reported for the signal at δ 4.41 (J_1=7 Hz) for gallocatechin. Literature values reported for the C-2 proton on catechin include 4.51 ppm (d, J_1=7.3 Hz, d_6-DMSO) by Chien-Chang Shen et al. (1993); 4.56 ppm (d, J_1=7.4 Hz, $(CD_3)_2CO$) by Davis et al. (1996); and 4.53 ppm (d, J_1=8 Hz, $(CD_3)_2CO$) by Dübeler et al. (1997). The singlet which appeared at 4.81 ppm is indicative of an epicatechin. A singlet at δ 4.75 was reported by Chien-

Chang Shen et al (1993) for the C-2 proton in epicatechin (in d_6-DMSO). This peak was reported as a singlet at δ 4.88 for epicatechin in $(CD_3)_2CO$ by Davis et al (1996). Davis et al (1996) also reported a singlet at δ 4.82 for the C-2 proton for epigallocatechin.

The two multiplets at 3.98 and 4.18 ppm, corresponding to the C-3 protons, also indicated the presence of an epi and non-epi catechin. Chien-Chang Shen et al (1993) reported multiplets at 3.84 and 4.03 ppm for catechin and epicatechin, respectively. Davis et al (1996) reported H-2 peaks at δ 3.99 and 3.97 for catechin and gallocatechin, respectively (in $(CD_3)_2CO$). Also, they found that the H-2 peaks for epicatechin and epigallocatechin resonated at 4.21 and 4.19 ppm, respectively in $(CD_3)_2CO$.

Further, compounds **3** and **4** are suspected of not having the gallo configuration on the basis of the peaks corresponding to the protons on the A- and B-rings. There are four sets of doublets at δ 5.85, 5.91, 5.92, and 5.94 corresponding to the H-8 and H-6. The set of 5.85 and 5.92 have coupling constants of 2.5, and the set of 5.91 and 5.94 have coupling constants of 2.0. The peaks which give the best indication that there are no gallo type catechins present is the set at ~6.7 to ~7.0, corresponding to the protons on the B-ring, which consists of several doublets and singlets. The gallo type catechins don't have any peaks above ~6.5 ppm, as shown by the isolation of gallocatechin in this work, and also as seen by Davies et al (1996). The gallo type catechins show a singlet for the protons on the B-ring because of the symmetry present.

Gallocatechin was analyzed for the minimum inhibitory concentration (MIC) in the NI bioassay, along with an authentic sample of catechin, nitrapyrin (a commercial nitrification inhibitor), and fraction V from the HPLC purification. Gallocatechin was assayed at 50, 12.50, 6.25, and 3.12 ppm, while all other samples were tested at 10 ppm.

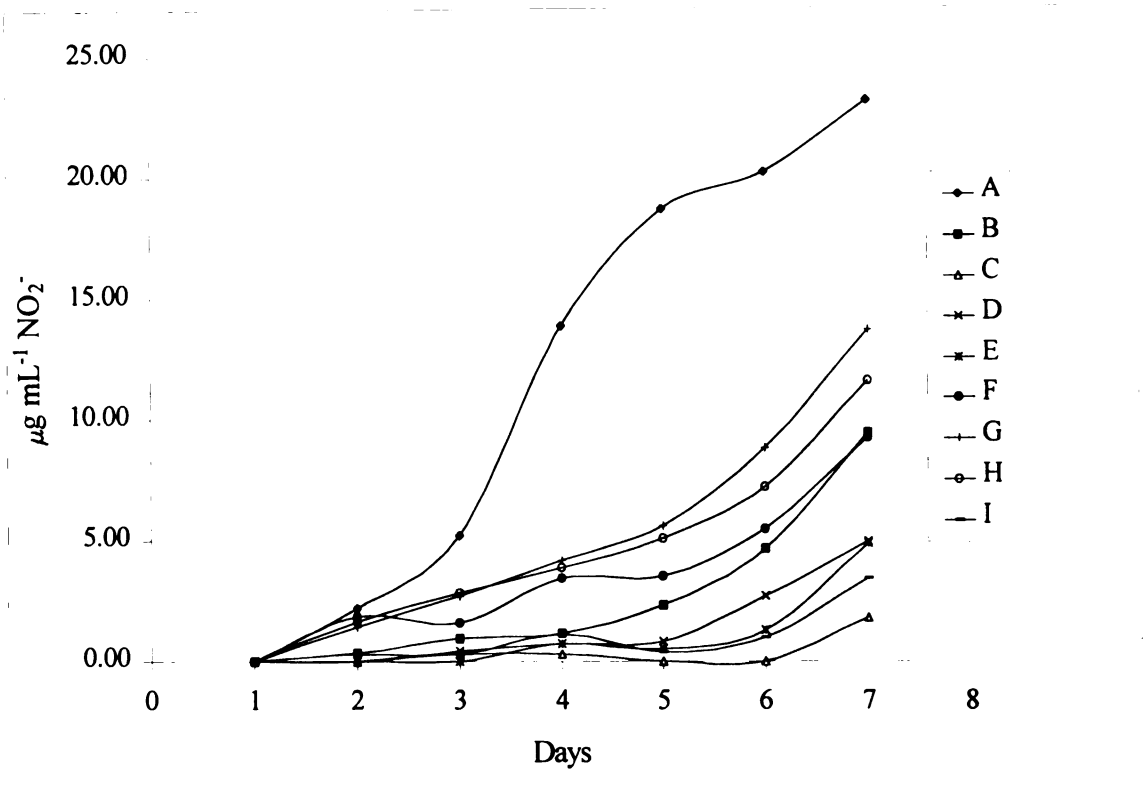


Figure 3.4: Nitrite levels measured in MIC measurement for gallocatechin and several other samples. A--non-DMSO control; B--DMSO control; C--50 ppm gallocatechin (g.c.); D--12.5 ppm g.c.; E--6.25 ppm g.c.; F--3.12 ppm g.c.; G--10 ppm HPLC fraction 5; H--authentic sample of 10 ppm (+)-catechin; I--10 ppm nitrapyrin. Negative values were normalized to zero. Data was subjected to analysis of variance and found to be highly significant at the 0.01 level.

Nitrapyrin showed strong nitrification inhibition at 10 ppm concentration compared to the DMSO control. Gallocatechin was found to be highly active at 50 ppm concentration, as compared to nitrapyrin and the DMSO control (Figure 3.4). The 12.50 and 6.25 ppm concentrations of gallocatechin showed activity similar to nitrapyrin. Gallocatechin was not active at the 3.12 ppm level, compared to the DMSO control. Catechin and HPLC fraction V did not display activity at the 10 ppm level.

The class of catechin compounds display a wide spectrum of biological activity, including apoptosis of stomach cancer cells (Hibasami et al., 1998), inhibition of telomerase (Naasani et al., 1998), accelerated healing of acid induced ulcerative colitis in rats (Sato et al., 1998), prevention of skin cancer (Barthelman et al., 1998), and inhibition of chitin synthase II(Kim et al., 1999). Numerous reports have characterized the potent antioxidant activities of the catechins (Lotito and Fraga, 1998; Vinson and Dabbagh, 1998; Plumb et al., 1998; Nakao et al., 1998; Kashima, 1999; Kondo et al., 1999). Much of this work has not focused specifically on gallocatechin, the studies usually focusing on (+)-catechin, and the gallates, in particular epigallocatechin gallate. Nonetheless, the antioxidant activity of both the native molecules and the gallate esters is well established. Some of the natural phenolic compounds discussed in the literature review also have been reported as antioxidants, including caffeic and chlorogenic acid (Wang et al, 1999), quercetin, and myricetin (Gordon and Roedig-Penman, 1998; Hopia and Heinonen, 1999). Thus, considering that the conversion of ammonia to nitrite is an oxidation process, the mechanism of inhibition involving gallocatechin and the other compounds may be through their anti-oxidant activities. This hypothesis has not been established by experimental evidence.

From this work, gallocatechin was shown to inhibit nitrification at 6 and 12 ppm concentrations, as compared to the DMSO control and 10 ppm nitrapyrin. Gallocatechin strongly inhibited nitrification at 50 ppm concentration. The mixture of catechin and epicatechin displayed activity at the 50 ppm level during the fractionation process; an authentic sample of catechin did not display activity at the 10 ppm level. Although

gallocatechin is a well characterized compound and widespread in plants, the NI activity reported here for gallocatechin is novel.

Chapter IV

Summary and Conclusion

Analysis of soil samples taken from the rhizosphere of *Leucaena leucocephala* trees showed unusually high levels of ammonia and low levels of nitrate. This led to the hypothesis that the trees were somehow inhibiting nitrification. Hexane, ethyl acetate, and methanol extracts from *Leucaena leucocephala* leaves, stems, and roots were tested for NI and other bioactivities. The NI bioassays were conducted in vitro against *Nitrosomonas europaea.*

The root methanol extract from *L. leucocephala* showed activity in the NI bioassay. Initial bioassay-directed fractionation of the root methanol extract yielded active fractions, but not pure active compounds. During this process, fractions containing sucrose were found to be active in the NI bioassay. An authentic sample of sucrose tested in the NI bioassay did not show activity.

Bioassay-directed fractionation of the root methanol extract on Amberlite XAD-2 resin and preparative HPLC yielded three active fractions. The most active fraction was found to contain gallocatechin. The structure of gallocatechin was elucidated by ^{1}H and ^{13}C NMR. Also, three other catechin compounds were identified during this process. These compounds were epigallocatechin, catechin, and epicatechin. Epigallocatechin was identified as a minor constituent of the HPLC fraction containing gallocatechin.

Catechin and epicatechin were found to be a near equal mixture in an NI-active HPLC fraction. The third HPLC fraction (fraction V) with minor activity was not pursued.

Gallocatechin displayed activity in the NI bioassay at the 12 ppm concentration, as compared to nitrapyrin, a commercial nitrification inhibition compound, and the DMSO control. The activity of gallocatechin at 12 ppm was approximately the same as that of 10 ppm of nitrapyrin. Gallocatechin showed strong NI activity at the 50 ppm concentration. An authentic sample of catechin was assayed at 10 ppm, but compared to the DMSO control it did not display activity. The HPLC fraction V was assayed at 10 ppm, but it did not display activity compared to the DMSO control or nitrapyrin.

Although gallocatechin and the family of catechin compounds are widespread in the plant kingdom, the activity reported here for gallocatechin is novel. Tannins have been reported to have nitrification inhibitory activity, but there are no reports of individual catechins that are capable of inhibiting nitrification. Catechins have been widely reported as potent antioxidants. The conversion of ammonia to nitrite is an oxidative process. Therefore, the antioxidant capacity of gallocatechin may be playing a role in the mechanism of nitrification inhibition. However, this hypothesis needs to be substantiated by further research.

All of the extracts were tested in anti-fungal, anti-bacterial, insecticidal, nematicidal, anti-cancer and NI bioassays. The anti-fungal assays were conducted on *Aspergillus*, *Fusarium*, *Rhizoctonia*, *Botrytis*, and *Gleosporum* spp. The anti-bacterial included *E. coli*, *Staphylococcus* and *Streptococcus*. The mosquitocidal assay was carried out on *Aedes aegyptii* mosquito larvae and insect anti-feedant assay on *Helicoverpa zea* and *Lymantria dispar* caterillars. The nematicidal bioasasys were

conducted on *Caenorhabditis elegans* and *Panagrellus redivivus*. The anti-cancer screen employed mutant *Saccharomyces cerevisae* strains that were sensitive to topoisomerase I and II poisons. No activity was detected in the antimicrobial, mosquitocidal, nematicidal or anti-cancer assays. Anti-feedant activity was initially detected in the leaf hexane extracts. However, this activity was later shown to be attributable to insecticide residues on the leaves from greenhouse spraying where the plants were initially grown.

In conclusion, gallocatechin, a novel nitrification inhibitor, was isolated from the roots of *Leucaena leucocephala*. Nitrification inhibition was detected using a simple in vitro bioassay developed for this work. Since gallocatechin possesses NI activity, it is possible that other catechins may possess this activity. Further research is necessary to prove that other catechins possess NI activity, and to provide evidence for the possible antioxidant mode of action of gallocatechin as a nitrification inhibitor.

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