1986

Plant Germination and Growth Inhibitors From Ceratiola Ericoides and Calamintha Ashei (Herbicides, Allelopathy, Monoterpenes, Phenolics).

Nesrin Tanrisever
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PLANT GERMINATION AND GROWTH INHIBITORS FROM CERATIOLA ERICOIDES AND CALAMINTHA ASHEI

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PLANT GERMINATION AND GROWTH INHIBITORS FROM *CERATIOLA ERICOIDES* AND *CALAMINTHA ASHEI*

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In partial fulfillment of the
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in
The Department of Chemistry

by

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to Anne and Baba

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ABSTRACT

As part of a chemical-ecological study of the Florida scrub community, *Ceratiola ericoides* (Empetraceae) and *Calamintha ashei* (Lamiaceae) were chemically investigated for natural products with possible allelochemical activities.

The chromatographic separations and chemical investigations were guided by germination and radicle growth bioassays performed on seeds of commercial lettuce (*Lactuca sativa*) and *Schizachyrium scoparium*, a native grass of the Florida sandhill community.

From the dichloromethane extract of aerial parts of *C. ericoides*, 2',4'-dihydroxychalcone, the dihydrochalcones angoletin and 2',6'-dihydroxy-4-methoxy-3',5'-dimethyldihydrochalcone, the flavanones 8-methylpinocembrin, 6,8-dimethylpinocembrin and 7-hydroxyflavanone were isolated along with the triterpenes erytrodiol and ursolic acid.

Significant inhibition on *S. scoparium* radicle growth was observed with 6,8-dimethylpinocembrin when applied in a saturated aqueous solution of ursolic acid.

From the active region of the ethyl acetate extract of *C. ericoides*, catechin, epicatechin, (A-2) dimer and

related proanthocyanidins were isolated. Although mixtures of proanthocyanidins showed activity, A-2 alone exhibited no inhibitory effects on test seeds.

Surface washings of fresh *C. ericoides* leaves provided the novel dihydrochalcone ceratiolin. Ceratiolin decomposes spontaneously to form, besides other unidentified products, hydrocinnamic acid. Pure ceratiolin does not inhibit germination and radicle growth. However, hydrocinnamic acid shows significant inhibitory activity on both, *L. sativa* and *S. scoparium*.

From active chromatographic fractions of the dichloromethane extract of *Calamintha ashei*, two menthofurans, the known evodone and the new monoterpene calaminthone as well as the sesquiterpene caryophyllene oxide were isolated. The above terpene mixture completely inhibited *S. scoparium* germination but bioassays performed with pure compounds exhibited no significant allelopathic activity. Volatility test of evodone showed strong inhibition on germination of *S. scoparium* seeds. A dramatic increase in inhibition of the radicle length of *S. scoparium* by evodone dissolved in a saturated aqueous solution of ursolic acid was observed when compared to a pure aqueous solution of evodone.

Tests for possible micelle formation, using the acridine fluorescence method with natural products and the surfactants ursolic acid and emulphogen indicated that mixtures of natural products in leaf leachates lead to micellar solutions which may aid the dissolution and therefore effectiveness of nonpolar allelochemicals.

CHAPTER I

INTRODUCTION

I-1. Allelopathy.

Allelopathy is defined as the inhibitory or stimulatory biochemical interactions between all types of plants including microorganisms.[1] Although the term literally means "mutual harm", the emitting species generally gains some advantage and the recipient is harmed.

The effects plants have on each other were observed as early as 300 B.C. when Theophrastus stated that chick pea, contrary to other related crop plants, "exhausts the ground" and destroys the weeds.[2] Later, in 1795, Plenk was the first to seriously suggest that plants may interact by means of their chemicals.[3]

The black walnut tree *(Juglans nigra)* is a classical example of allelopathic interactions. In 1881, Stickney and Hoy observed that the vegetation under the black walnut tree was very sparce compared to that of most other common shade trees. Hoy claimed the main reason for this to be the poisonous character of the rain or fog drip from the leaves of *Juglans nigra*.[4] The active constituent was later identified by Davis[5] as juglone (**1**). It was proposed that the toxicity is a result of large amounts of juglone released and the wet nature of the soil that restricts anaerobic metabolism by soil microorganisms, allowing juglone to build up to toxic levels.[6]

I-2. Types of allelochemicals.

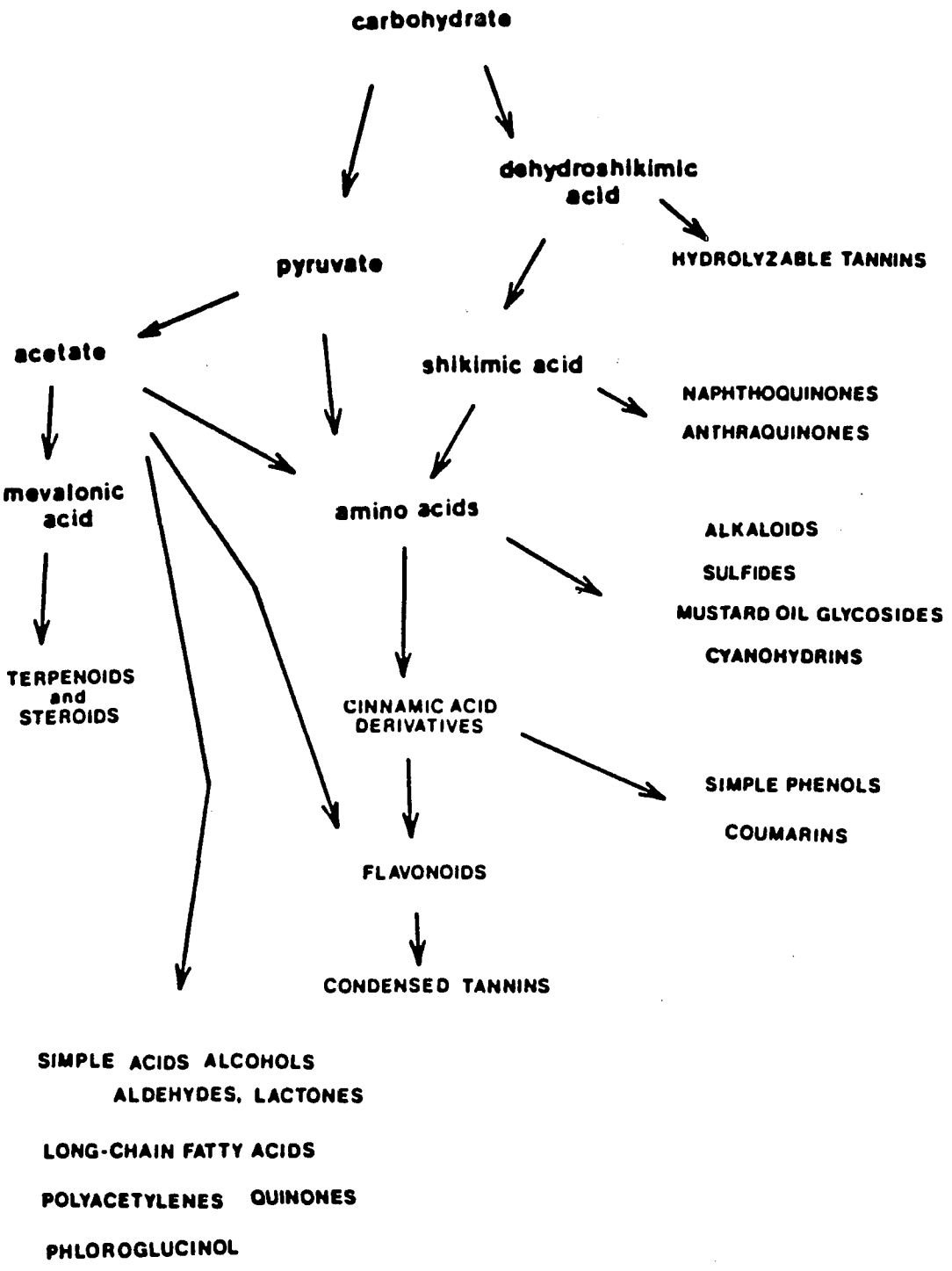
Although by far the most frequently reported allelochemicals are simple phenolic acids[7,8] and volatile monoterpenes,[9,10] many different types of secondary metabolites (Scheme 1) have been implicated in allelopathy.

Some of these allelochemicals are very common, small compounds such as acetaldehyde, acetone, methanol, and ethanol which are emitted as volatile growth inhibitors by beet, tomato, and carrot plants.[11] Many simple acids contained in fruit juices also are inhibitors of seed germination. Malic, citric, tartaric, and acetic acid concentrations in fruits have been reported to be high enough to inhibit germination.[12]

Long chain fatty acids may also be important in allelopathic interactions of higher plants. Al Saadawi *et al.*[13] found nine fatty acids in decomposing residues of *Polygonum aviculare*. Sodium salts of all of these acids were inhibitory to seedling growth of bermuda grass.

Among phenolic acids, p-hydroxybenzoic, vanillic (**2**), ferulic (**3**), and p-coumaric (**4**) acids are the most commonly encountered allelochemicals in the literature.[14,15,16,17]

Coumarins, lactones of o-hydroxycoumaric acids are reported to be potent inhibitors. Rice and Pancholy[18] identified scopolin (**5**) as a strong inhibitor of nitrifying bacteria. A few furanocoumarins such as psoralen (**6**) have



Scheme 1. Formation of secondary metabolites.

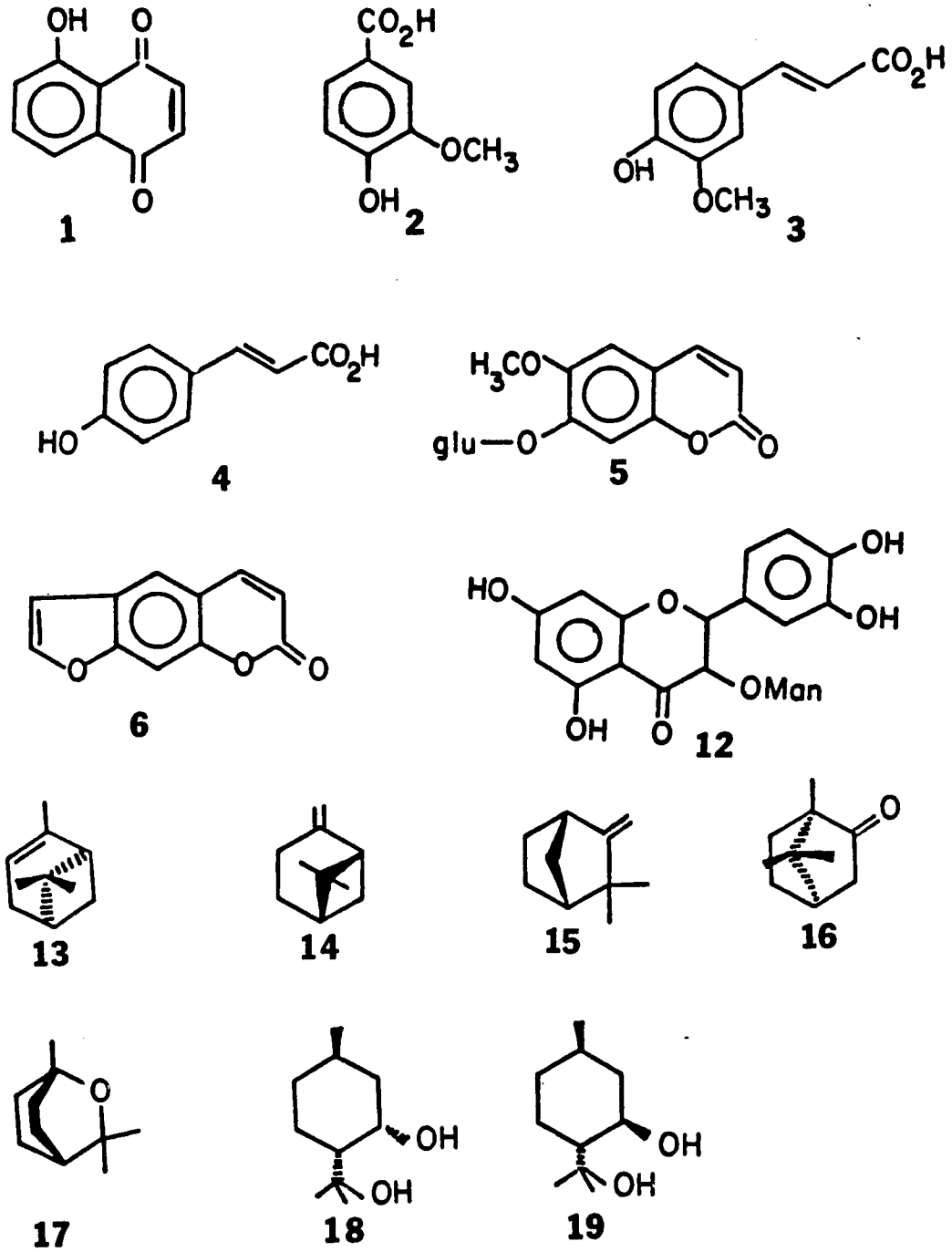
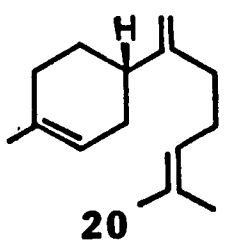


Fig. 1. Natural products suspected of having allelopathic activities.



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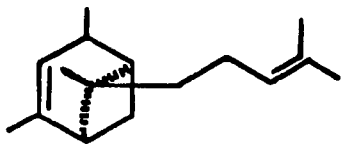

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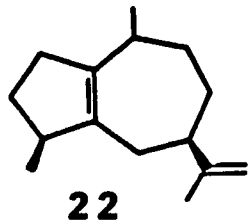

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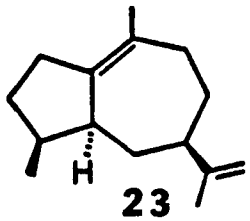

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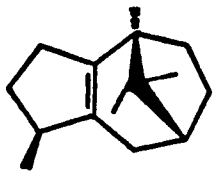

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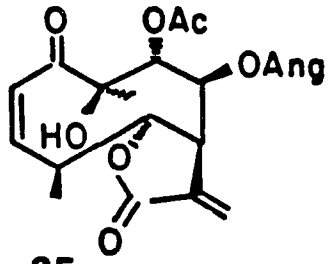

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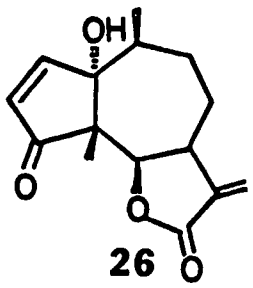

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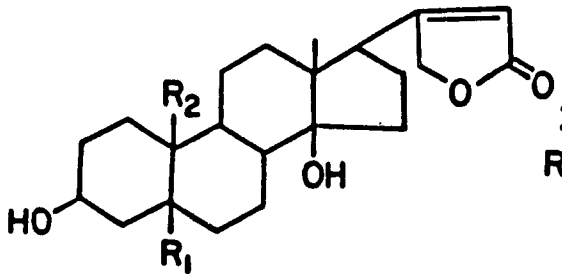

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R_1= H

R_2= CH_3

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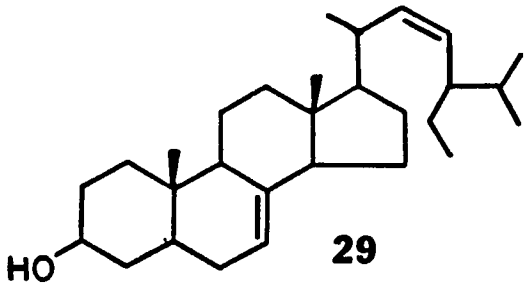
R_1 = OH

R_2= C(=O)—H

Fig. 1. (continued)



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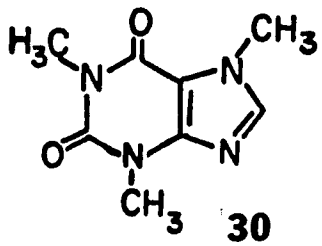

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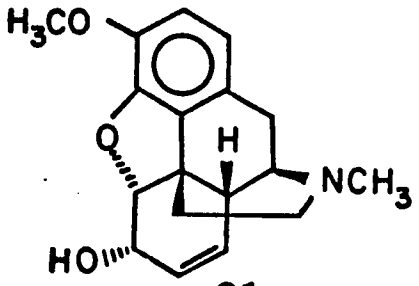

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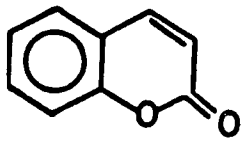

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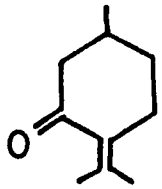

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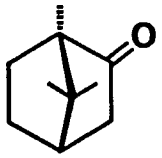

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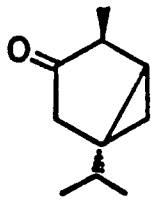

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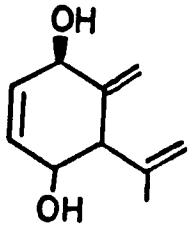

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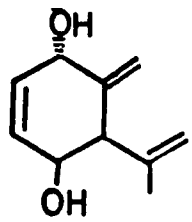

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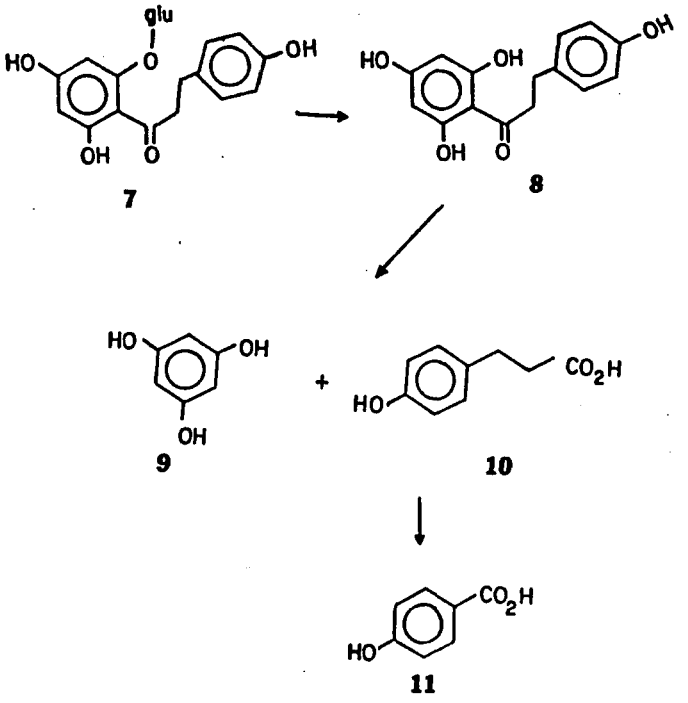
Fig. 1. (continued)

also been identified as plant growth inhibitors.[19]

In spite of the large number and distribution, only a few flavonoids have been associated with allelopathy. Phlorizin in apple root residues was shown to inhibit the growth of apple seedlings.[20] Phlorizin decomposes in the apple root residues forming phloretin (**8**), phloroglucinol (**9**), p-hydroxyhydrocinnamic acid (**10**) and p-hydroxybenzoic acid (**11**). These decomposition products have also been shown to inhibit the growth of apple seedlings. Some other flavonoids such as quercitrin (**12**) were found to be toxic to nitrifying bacteria.[21]

Among terpenoids, numerous monoterpenes have been found to cause allelopathic effects. Muller and coworkers demonstrated the inhibitory activity of the bicyclic monoterpenes α-pinene (**13**), β-pinene (**14**), camphene (**15**), camphor (**16**), and 1,8 cineole (**17**) from *Salvia leucophylla*, *S. apiana* and *S. mellifera* on test seedlings.[22] Camphor (**16**) and 1,**8**-cineole (**17**) were also shown to be the most active constituents of *Artemisia californica*.[23] Nishimura *et al.* isolated two germination and growth inhibitors and identified them to be racemic p-menthane-3,8-cis diol (**18**) and its trans isomer (**19**).[24]

Several sesquiterpenoids have also been implicated in allelopathy. Recently, a mixture of active sesquiterpene hydrocarbons consisting of β-bisabolene (**20**), bergamotene (**21**), α-guayene (**22**), α-bulnesene (**23**), and



Scheme 2. Decomposition of phlorizin.

β-patchoulin (**24**)[25] were isolated from the Louisiana ragweed *Ambrosia artemisifolia*. Specific effects were observed with some sesquiterpene lactones. For instance, the germacranolide calein A (**25**) had an inhibitory effect on *Amaranthus palmeri* but had no pronounced activity on crop seeds. Parthenin (**26**) was shown to inhibit clover and carrot while promoting the growth of wheat, onion and *A. palmeri*.[25]

Only a few triterpenoids have growth inhibitory effects. Chondrillasterol (**27**) exhibited inhibitory effects on some species like onion and wheat while

promoting the growth of carrot seedlings.[26] Two cardiac glycoside aglycones, digitoxigenin (**28**) and strophantidin (**29**) are other examples of active triterpenoids.[12]

Common alkaloids such as caffeine (**30**) and codeine (**31**) suppress seed germination.[12] However alkaloids have not been investigated widely for their allelopathic properties.

Other classes of natural products that have been less investigated for their allelopathic potentials include mustard glycosides,[27] tannins[28] and acetylenic compounds.[29]

I-3. Means of release and activity of allelochemicals

As there is a wide variety of phytotoxins, there is also indication that these allelochemicals can be effective in numerous ways. The means of activity may be through effects on division, elongation, or ultrastructure of root cells, or through effects on hormone induced growth, membrane permeability, mineral uptake, stomatal opening, photosynthesis or respiration.[30] The chemicals may also be indirectly active on the plant through inhibition of beneficial bacteria in the soil.[31]

The toxins may be released from plants by a number of mechanisms.[32] They may be exuded from living tissue such as bark, leaves or fruits. The toxins can then reach the ground with the aid of rain, fog or dew, or volatiles may be transported directly through the air. Direct release of

chemicals from the roots into the soil has also been observed. Other sources of toxins in some instances are decaying plant residues such as leaf litter or dead root tissues. In this case, soil bacteria, fungi or algae may play an important role in synthesizing or releasing the toxic agents from the dead tissues.

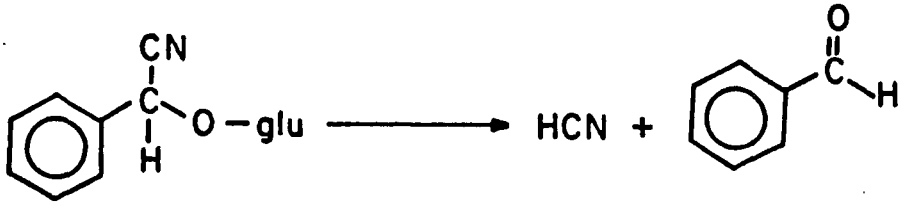
A number of simple experiments have been designed to imitate nature's mild extraction mechanisms and to demonstrate the release of these compounds under natural conditions. Fog drip and rain drip have been simulated under laboratory conditions and were reported by Muller and McPherson[33] to contain the effective toxin.

Tang and Young developed a trapping system for root exudates without disturbing the plant. The system is composed of a column containing an XAD-4 resin attached to the plant system. Water containing mineral nutrients is continuously circulated through the system, eluting extracellular organic material from the sand. Thus hydrophobic exudates are selectively retained by the XAD-4 resin while inorganic nutrients are unaffected.[34] Volatile allelochemicals were detected by sampling the air surrounding the live plant with a large syringe.[35] Another method involved blowing air over the plant and concentrating the volatiles present in a cold trap.[35]

I-4. Factors affecting the extent of toxicity.

Synergistic effects have been detected in a number of allelopathic activities. Williams and Hoagland[36] observed an increase in the inhibition of germination when coumarin (**32**) and p-hydroxybenzaldehyde were applied together in comparison to their individual application. A similar result was observed with the monoterpenes (+)-pulegone (**33**) and (-)-camphor (**34**).[37] A hundred-fold increase in activity was detected when the compounds were administered together than when applied alone.

The released compounds can be nontoxic but degrade to active agents. For instance, the cyanogenic glucoside (**35**) from Johnson grass *(Sorghum halepense)* is decomposed by the action of soil microbes into toxic HCN and benzaldehyde[38] (Scheme 3).



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Scheme 3. Enzymatic breakdown of a cyanogenic glucoside.

The production of allelochemicals may follow a seasonal pattern. Studies on *Artemisia californica* showed a seasonal change in the toxicity of leaf volatiles, rain drip and soil.[39] Detrimental effects increased in late summer and fall and were absent in winter. Paralleling this, quantity of the essential oils increased in late summer and fall. Isothujone (**36**), determined to be moderately toxic, was present in the plant in copious amounts only during this time of activity.[40]

Within species, differences can exist in the amount of toxin produced by different genotypes. Some cucumber *(Cucumis sativus)* accessions appear to greatly inhibit weed germination while others may have no effect.[41]

Plants have also been observed to vary in their production of allelochemicals depending on the environment in which they are grown and in response to the stresses they encounter.[42]

In addition, allelochemicals can have effects only on specific plants or specific parts of plants. Although both piquerol A (**37**) and piquerol B (**38**) have inhibitory effects on growth, piquerol A was shown to be inhibitory mostly on root growth while piquerol B was shown to be more effective in inhibiting stem growth.[43]

I-5. Criteria for the existence of allelopathy.

Although numerous papers have appeared on allelopathy, there still exists considerable scepticism about the existence of the phenomenon since it is difficult to demonstrate this cause-effect relationship in natural situations with multiple variables. It has often been claimed that the harmful effect of one plant on another is due to physical competition for light, soil moisture or mineral nutrients. On the other hand, the general idea of scientists working on allelopathy is that this phenomenon usually interacts with other natural stresses resulting in a synergistic effect.[44,45,46]

To demonstrate the existence of allelopathy, cases were found where the detrimental effects are not caused by any observable physical competitive factors. In Taiwan, the nature of the undergrowth in cypress *(Cryptomeria japonica)* and bamboo *(Phyllostachys edulis)* forests were compared.[47] Studies indicated no differences in pH, moisture, texture, organic matter, total nitrogen and cation exchange capacity in the soils of these forests that exist next to each other. It was noted, however, that there is no undercover in the bamboo forests while cypress forests flourish with undergrowth. Water soluble phenolic acids were detected in bamboo leaves and the soil beneath, whereas they were undetected or detected in very small amounts in cypress leaves and soil. This observation led

the authors to conclude that allelopathic effects are taking place in the bamboo forests.

In another study, laboratory bioassay comparisons were carried out between species that naturally grow under *Adenostoma fasciculatum* and native species that are excluded as undergrowth of *A. fasciculatum*[48]. The species which grow under *A. fasciculatum* were found to be consistently more tolerant of the leaf leachate than were the species not found under this shrub. Removal of the above ground parts of *A. fasciculatum* without disturbing the soil permitted the growth of herbs with a lag period of 10-12 weeks. These observations indicate a direct correlation between the absence of herbs and the toxins in the aerial parts of the plant.

E. P. Fuerst and A. R. Putnam proposed a set of conditions which altogether should constitute proof of allelopathic interference.[49] The proposed criteria include:

i. identification of the symptoms of interference.
ii. isolation, assay, characterization and synthesis of the toxin.
iii. simulation of the interference by supplying the toxin as it is supplied in nature.
iv. quantification of the release, movement and uptake of the toxin.

It was also pointed out that it is desirable to show that the selectivity of the toxin to various species corresponds to the range of species affected by the allelopathic agent.

According to these criteria, it was noted that allelopathic interference has not been unequivocally proven at the present.

I-6. The California Chapparal.

The best documented case of allelopathic interactions among members in a plant community is the Chapparal vegetation of Southern California.[50,51]

A striking characteristic of the chapparal vegetation is the complete lack of herbs beneath shrubs belonging to this vegetation no matter how thin the leafage of the canopy. It was also observed, however, that herbs invade the chapparal when fires recurring at intervals of around 25 years destroy the above ground portions of the dominant shrubs. The herbs disappear when the shrubs regenerate in 5 to 7 years.

The bare zones generated in the presence of shrubs could not be explained by difference in edaphic conditions, differential grazing, seed removal, or competition for light or nutrients. Competition for water could be significant in the production of bare zones. Yet, comparative experiments performed with and without Eucalyptus camaldulensis litter in controlled plots showed

suppression of herb growth in the litter zone although litter helped the conservation of water in the soil.[52] This was taken to be an indication that suppression of herb growth in the litter zone is primarily an allelopathic effect rather than a competitive one.

Chemical investigations of one member of the Chapparal, Salvia leucophylla provided the allelopathic volatile monoterpenes cineole (**17**) and camphor (**16**).[53] Water soluble phenolic toxins like vanillic (**2**), ferulic (**3**), and p-coumaric (**4**) acids were found in A. fasciculatum.[54] Both, volatile terpenes and water-soluble phenolic acids were also detected in Eucalyptus camaldulensis.[55]

I-7. The Florida Scrub.

An ecological situation similar to that of the California Chapparal exists in Florida. This similarity in their vegetation traits is of interest since the areas differ drastically in terms of soil type, topography and climate.[56]

In Florida, the more abundant vegetation type is the SANDHILL community composed mainly of oaks, longleaf pines and a dense herbaceous cover. Throughout the SANDHILL vegetation, there are patches of scrub vegetation composed mostly of sand pine, oaks and evergreen shrubs. Herbaceous

COMMUNITY:	SCRUB	SANDHILL
DOMINANT SPECIES		
PINE:	*Pinus clausa*	*P. palustris* or
OAK	*Quercus chapmannii*	*Q. laevis*
	Q. geminata	*Q. incana*
	Q. geminata	
SHRUB LAYER:	Very dense	Open
HERBACEOUS LAYER:	Nearly none	Complete cover
FOLIAGE PHENOLOGY:	Evergreen	Deciduous
SURFACE LITTER:	Light	Heavy
FIRE FREQUENCY:	20-50 years	3-8 years
PLANT RELATIVE:		
GROWTH RATES:	Slow	Fast
AGE OF PLANTS AT FIRST REPRODUCTION:	Old	Young

Table 1. Contrast in SCRUB and SANDHILL vegetation types.

cover is nearly absent in the SCRUB community. Along with the fact that the communities are composed of different species, the general vegetational characteristics are distinctly different as indicated in Table 1.[55]

In 1895, Nash noted the almost complete floristic difference between the SCRUB and the SANDHILL vegetations, commenting that "the two floras are natural enemies..."[56].

Although the vegetational differences have at times been attributed to lower availability of nutrients in the SCRUB soil, tests for soil differences generally gave negative results.[57,58] At least as far as the macroelements and physical characteristics are concerned, the soil differences are not consistently large enough to account for the sharp boundary between the two plant communities.

Recently, Kalisz and Stone[59] have thoroughly examined the two types of soils. It was concluded that only a few soil traits are different and that these differences are confined to the surface profile, reflecting the different soil forming processes associated with the two vegetation types.

The outstanding characteristics of the SCRUB is its inability to endure fires in contrast to the SANDHILL community that burns over frequently without serious injury to its natural inhabitants.[60] Since the bare soil under and around the SCRUB plants acts as a natural firebreak and

protects the plants from the frequent surface fires, it appears to be to the SCRUB community's advantage to keep the soil devoid of herbaceous cover.

As allelopathy could be a reasonable mechanism for suppressing the growth of herbs, initial investigations to detect presence of allelopathic inhibition were conducted by Dr. G. B. Williamson, Department of Botany, Louisiana State University and his coworkers.[54]

Three types of experiments were performed:

i. Standard bioassays of seed germination and radicle growth with leaf washes of members of the SCRUB.
ii. Greenhouse bioassays on growth of Schizachyrium scoparium, a native grass belonging to the SANDHILL vegetation type.
iii. Reciprocal field transplants.

The results indicated a higher inhibition of native grasses than non-native grasses and lettuce which was in some instances stimulated. The production of inhibitory chemicals of SCRUB species was observed to be a seasonal phenomenon occurring primarily during the rainy season (Tables 2,3). As a result of the preliminary investigations, it was concluded that allelopathy is a very likely factor in preventing intrusion of SANDHILL species into the SCRUB

	Bonamia grandiflora	*Calamintha ashei*	*Ceratiola ericoides*	*Ceratiola ericoides litter*	*Conradina canescens*
March	95(83)*	96(95)	88*(144)*	100(210)*	99(132)*
April	86*(66)*	61*(67)*	103(164)*	87(217)*	8*(38)*
May	95(127)*	89*(103)	98(177)*	109(202)*	8*(32)*
June	97(195)*	85*(143)*	93(172)*	92(257)*	6*(52)*
July	93*(122)*	90*(68)*	90*(105)	97(161)*	74*(52)*
August	95(191)*	98(166)*	92*(141)*	100(211)*	100(148)*
September	92*(162)*	6*(40)*	93*(150)*	93*(198)*	10*(27)*
October	84*(154)*	86*(148)*	91*(125)*	86*(208)*	99(147)*
November	82*(118)*	92(102)	99(92)*	97(190)*	87*(77)*

Table 2. Results of bioassays of test washes on *Lactuca sativa* seeds. Numbers are germination as a percent of the control and in parentheses are radicle lengths as percent of the control. An asterix means a significant difference ($p < 0.05$).[54]

	Bonamia grandiflora	*Calamintha ashei*	*Ceratiola ericoides*	*Ceratiola ericoides* litter	*Conradina canescens*
March	74(122)*	66*(133)	80(114)	51*(28)*	74*(126)*
April	81(90)	93(76)	103(91)	133(84)*	112(82)
May	50*(52)*	85(72)*	83(99)	81(81)	83(79)
June	53*(120)	25*(44)*	85(83)	9*(79)	19*(50)*
July	57*(82)	20*(76)*	55*(80)	48*(93)	52*(84)*
August	88(67)*	84(72)*	84(102)	100(108)	104(91)
September	78(98)	103*(104)*	93(129)*	85*(101)*	111(108)
October	47*(114)	63*(83)	74(108)	70(95)	56*(117)
November	121(83)	123(83)	147(84)	95(91)	161*(81)*

Table 3. Results of bioassays of test washes on *Schizachyrium scoparium*. Numbers are germination as a percent of the control and in parentheses are radicle lengths as percent of the control. An asterix means a significant difference ($p < 0.05$).[54]

areas. Thus, Putnam and Fuerst's first criterion for the proof of the existence of allelopathy was fulfilled.

The second criterion, namely attempts towards identification of the toxins, represents a part of this dissertation.

CHAPTER II

INVESTIGATION OF ALLELOCHEMICALS FROM *Calamintha ashei*

II-1. Introduction

The mint family (Lamiaceae) is rich in monoterpenes some of which have been implicated in allelopathy. In a number of genera, notably *Salvia*, allelopathic activity has been documented.[61] Members of this family are particularly rich in flavonoids[62] and derivatives of cinnamic acid,[63] and they often contain the triterpenes oleanolic **(39)** and ursolic **(40)** acid.[64] Chemical studies of the genus *Calamintha* have been limited. Ursolic acid and its derivatives appear to be widespread in this genus[65,66], and some tannins and flavonoids have been reported.[67] Investigation of the volatiles of *C. nepeta* (L.) yielded the monoterpenes cineole **(17)**, menthol **(41)**, isomenthone **(42)** and neoisomenthol as major constituents.[68,69] A recent study of the headspace volatiles of *C. nepeta* subsp. *glandulosa* showed that the major components were piperitone oxide **(43)** and piperitenone oxide **(44)**.[70]

Calamintha ashei, a member of the Florida SCRUB community with documented inhibitory effects on the growth of SANDHILL species,[54] had previously not been chemically investigated.

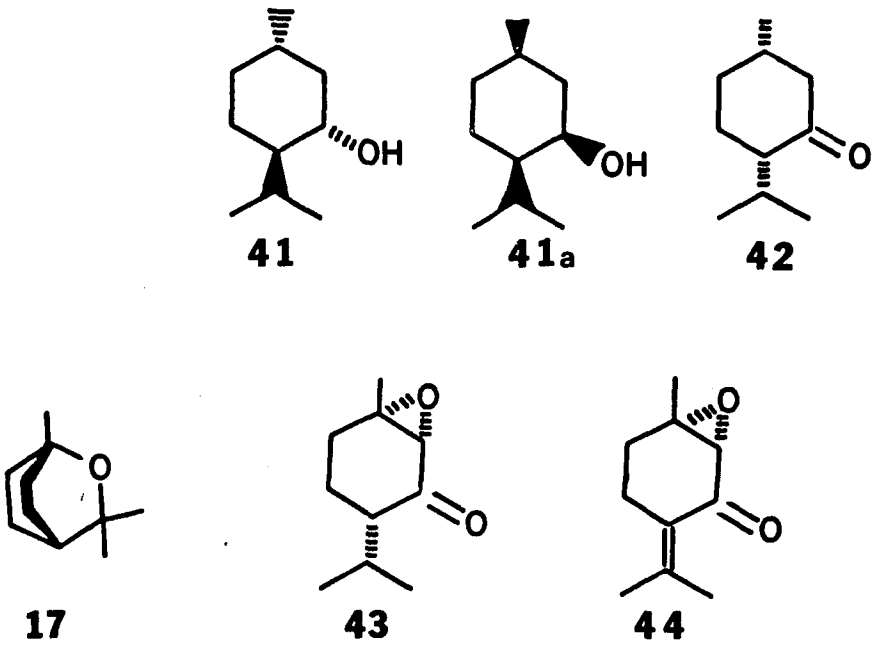


Figure 2. Reported terpenoids of the genus *Calamintha*.

II-2. Results and Discussion.

II-2.1. Chromatographic fractionations of Allelopathic crude extracts of *Calamintha ashei*.

Crude extracts were obtained from ground leaves of *C. ashei* as described in the experimental section. The extracts were tested for germination and radicle growth activity on lettuce *(Lactuca sativa)* and seeds of the native grass *(Schizachyrium scoparium)* the results being summarized in Table 4. The data indicate that the chloroform extract is the only one with significant activity on the native Florida grass, *S. scoparium*, even though no significant germination and growth activity could be observed on *L. sativa*. Methanol and water extracts

inhibited radicle growth of *L. sativa* while the growth inhibitions of *S. scoparium* were not statistically significant. Despite the solubility problems in preparing aqueous test solutions of the petroleum ether extract, significant activity on *S. scoparium* was observed. From these observations, the active constituents appeared to be relatively nonpolar.

Open column chromatography on silica gel was performed on the crude chloroform extract. The results of the bioassays indicated an active zone in nonpolar fractions which eluted immediately before the chlorophyll band (Table 5). As shown in Figure 3, the activity appears to be specific showing greater growth inhibitions of *S. scoparium* than of *L. sativa* radicles. Since the activity of the more polar fractions was mainly stimulatory on *S. scoparium* germination and had only minor effects on *L. sativa*, these fractions were not further investigated. Re-extractions of the aerial parts of *C. ashei* were performed using petroleum ether (P.E.) and dichloromethane mixtures in order to achieve maximal extraction of the components with slightly lower polarity than chlorophyll.

Table 4. Bioassay results on various crude extracts from *Calamintha ashei*.

Ext.	*Lactuca sativa*		*Schizachyrium scoparium*	
	% germination[+]	% radicle length[+]	% germination[+]	% radicle length[+]
P.E.	98	83	100	65
chloroform	102	108	59*	127
methanol	92	76*	81	117
water	101	52*	75	100
10% P.E., 90% CH_2Cl_2 mother liquor	74*	142*	81	136*
10% P.E., 90% CH_2Cl_2 percipitate	106	113	59*	132

[+]Germinations and radicle lengths are expressed as percentages of the control.
*An asterix indicates significant difference from control at $p \leq 0.05$.

Table 5. Bioassay results on chromatographic fractions of the chloroform extract of *Calamintha ashei*.

	Lactuca sativa		*Schizachyrium scoparium*	
Fraction	% germination[+]	% radicle length[+]	% germination[+]	% radicle length[+]
1	100	101	100	28*
3	100	73*	29*	56*
5	115*	111	200*	111
7	100	109	111	114
9	108	113	200*	103
11	89	116	129	93
13	96	94	186*	134
15	104	59*	186*	86

[+]Germinations and radicle lengths are expressed as percentages of the control.
*An asterix indicates significant difference from control at $p \leq 0.05$.

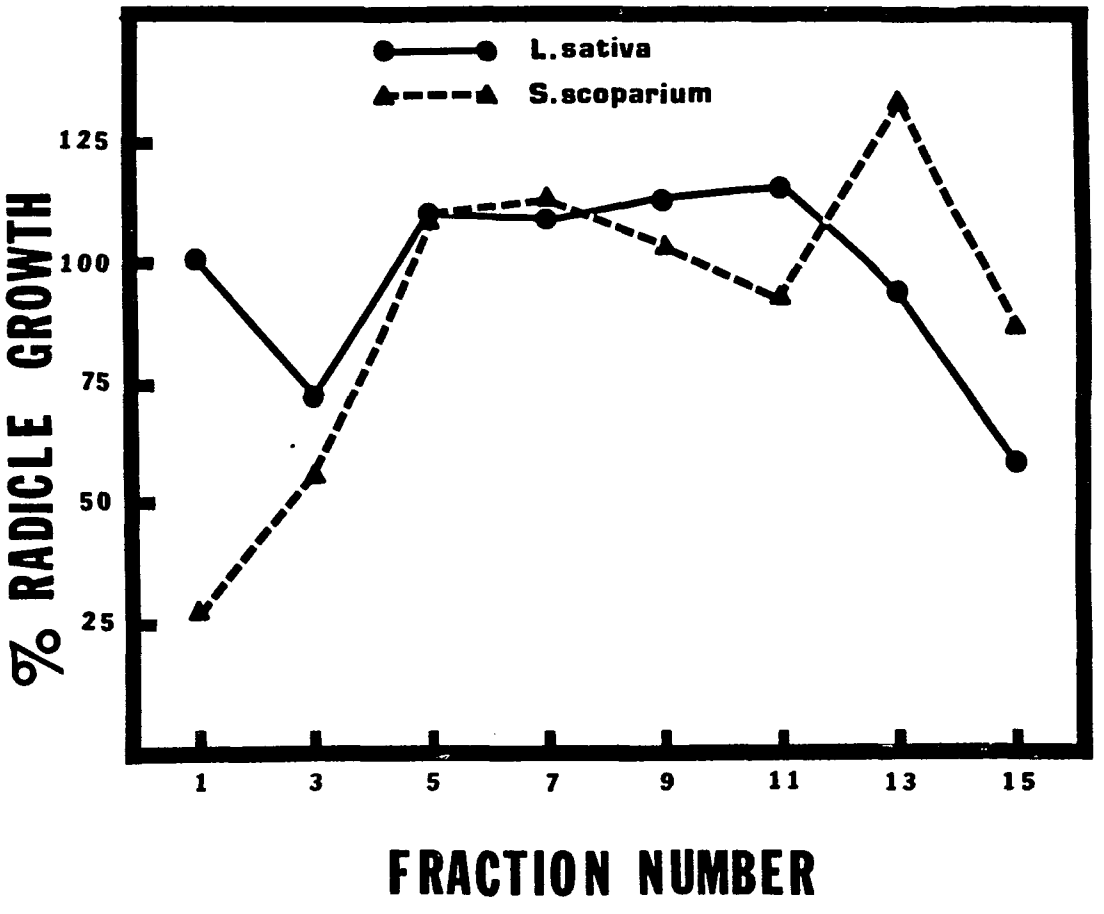


Figure 3. Comparison of the *L. sativa* and *S. scoparium* radicle length inhibitions by diromatographic fractions of the chloroform extract of *C. ashei*.

II-2.2. Identification of ursolic acid (40).

Ursolic acid precipitated along with some monoterpenes and flavonoids from the petroleum ether - DCM extract upon slow evaporation. Successive recrystallizations yielded a pure white solid which could be acetylated as well as methylated, indicating the presence of both a carboxylic acid and an alcohol functionality. The ^{1}H HMR spectrum of the acetate derivative contained signals for an olefinic proton (H-12) at δ 5.23 and for H-3 at δ 4.52. The rest of the spectral absorptions were typical for a triterpenoid with an upfield envelope and one acetyl signal at δ 2.06. A peak at *m/z* 411 in the mass spectrum indicated the presence of an angular carboxylic acid [M-COOH] at C-17. The base peak at *m/z* 248 suggested a Diels Alder type fragmentation typical for molecules of the ursane or oleanane skeleton. The ^{13}C DEPT experiments of the acetate derivative exhibited 8 quaternary, 8 methyl, 9 methylene and 7 methine carbons, excluding oleanolic acid as a possible structure. Comparison of spectral patterns and TLC coelutions in various solvents with a standard of ursolic acid confirmed its identity with this triterpene.

II-2.3. Separation and identification of the components of the allelopathically active fractions.

The mother liquor from the petroleum ether-DCM extract was fractionated on a silica gel column. Franction A-9 contained chlorophyll, which was used as a marker of activity based on the previous bioassay data; therefore, only the fractions eluted before A-9 were bioassayed. Fraction A-6 completely inhibited germination of *S. scoparium* while only radicle growth of *L. sativa* was affected. Fraction A-4 had no effect on test seedlings and Fraction A-8 exhibited significant stimulatory effects which could possibly be caused by very small amounts of allelochemicals in that fraction.[71] The gas chromatography traces of Fractions A-4, A-6, and A-8 were compared. Fraction A-6 was mainly composed of three constituents based on three major peaks. The peak with retention time 6.90 also appeared in the GC trace of Fraction A-4 which implied that, on its own, the compound giving rise to this peak is not active. Peaks with retention times 7.07 and 4.92 appeared only in the *GC* trace of A-6.

II-2.3.1. Identification of caryophyllene oxide (45).

The white crystalline compound obtained from fraction A-5 gave in its mass spectrum a molecular ion at *m/z* 220

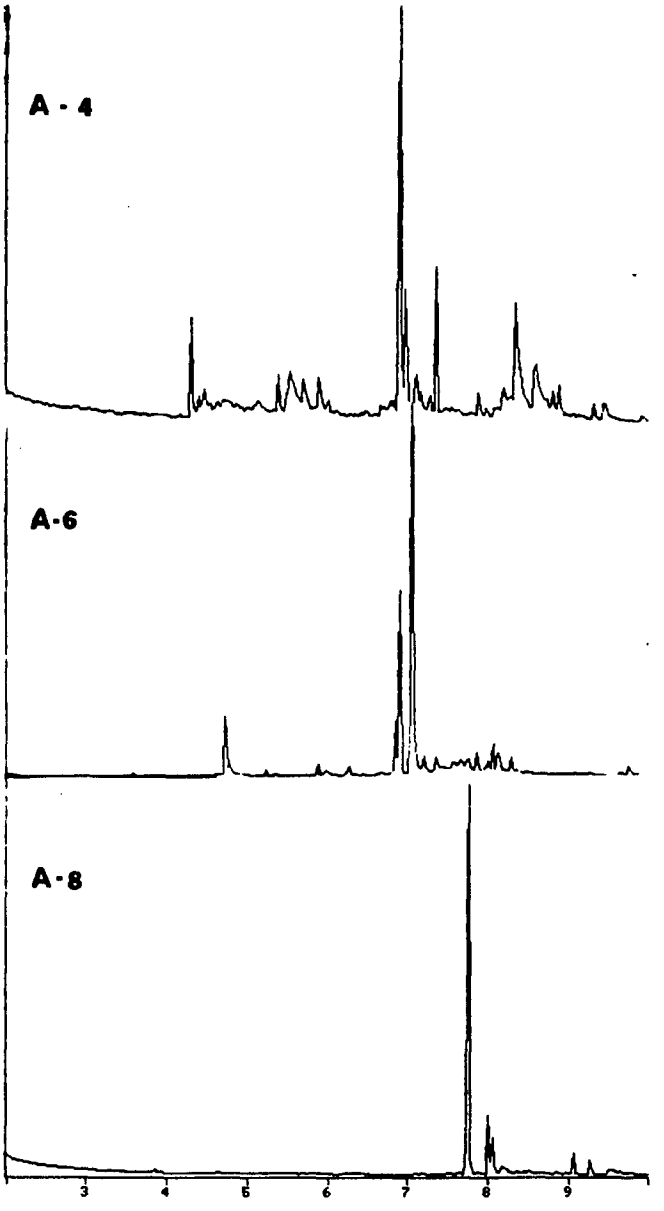


Figure 4. Gas chromatographic comparisons of column fractions 4, 6, and 8 from CH_2Cl_2 - petroleum ether extract of *C. ashei*. GC conditions; injection temp: 250°C, Oven: T_1 = 70°C, T_2 = 25°C, rate 15°/min, column pressure: 15 psig, column flow: 30 ml/min, 2 min delay.

Table 6. Bioassay data for the initial chromatographic fractions of the petroleum ether - dichloromethane extract of *C. ashei*.

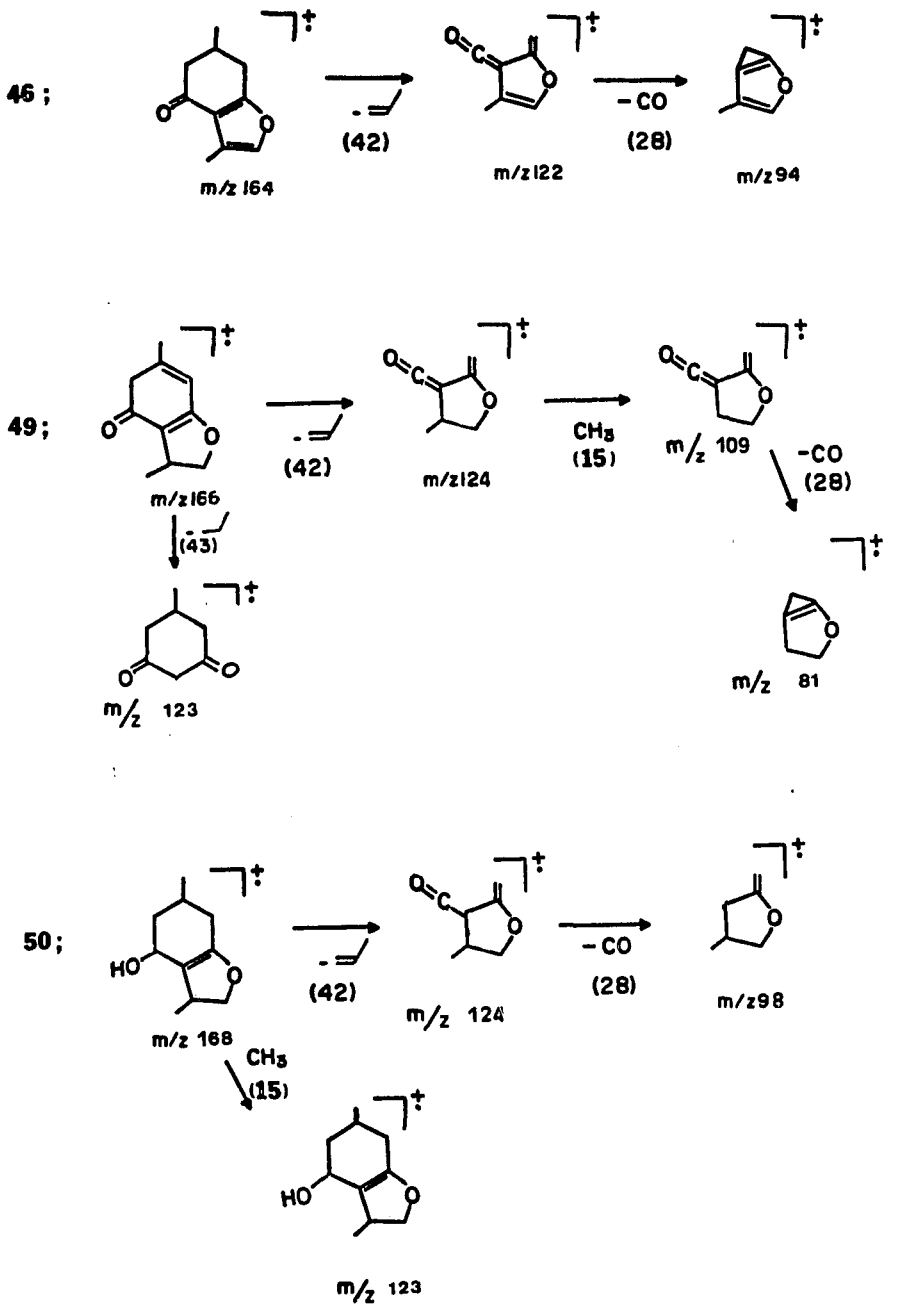
Fraction[≠]	*Lactuca sativa*		*Schizachyrium scoparium*	
	% germination[+]	% radicle length[+]	% germination[+]	% radicle length[+]
A-3	96.7	114.1*	66.1	128.2*
A-4	93.3	118.3*	73.0	90.9
A-6	96.7	61.3*	0.0*	---
A-8	96.7	128.9*	72.8	139.1*

[+]Germinations and radicle lengths are expressed as percentages of the control.
[≠](238 ppm) A-3, (238 ppm) A-4, (285 ppm) A-6, and (380 ppm) A-8 were solubilized.
*An asterix indicates significant difference from control at $p \leq 0.05$.

which could be formulated as a sesquiterpene with the empirical formula $C_{15}H_{24}O$. The ^{1}H NMR spectrum revealed the presence of an olefinic methylene group (H-12a, H-12b) giving rise to allylically coupled broadened singlets at δ 4.97 and δ 4.86 and a doublet of a doublet appeared at δ 2.78 (H-6). The ^{13}C NMR spectrum exhibited a doublet at δ 63.4 and a singlet at δ 59.5 suggesting an epoxide linkage in the compound. ^{1}H and ^{13}C NMR spectral comparisons of the new isolated compound with those of caryophyllene epoxide revealed their identity. GC-MS analysis of epoxide **45** unambiguously established its identity with the compound of retention time 6.90 in the chromatographic fractions A-4 and A-6.

II-2.3.2. Identification of evodone (46)

The white crystalline compound is an uncommon monoterpene first isolated from Evodia hortensis.[72] Evodone exhibits a characteristic mass spectral fragmentation pattern.[73] Major peak assignments are depicted in Scheme 4. The initial loss of *m/z* 42 is due to a Diels Alder fragmentation. According to the fragmentation, the carbonyl group could either be at position 4 or 7. However, as outlined for calaminthone **(48)**, the downfield proton shift of the C-3 methyl absorption indicated that the carbonyl has to be positioned



Scheme 4. Mass spectral fragmentation patterns of menthofuran derivatives **46**, **49**, and **50**.

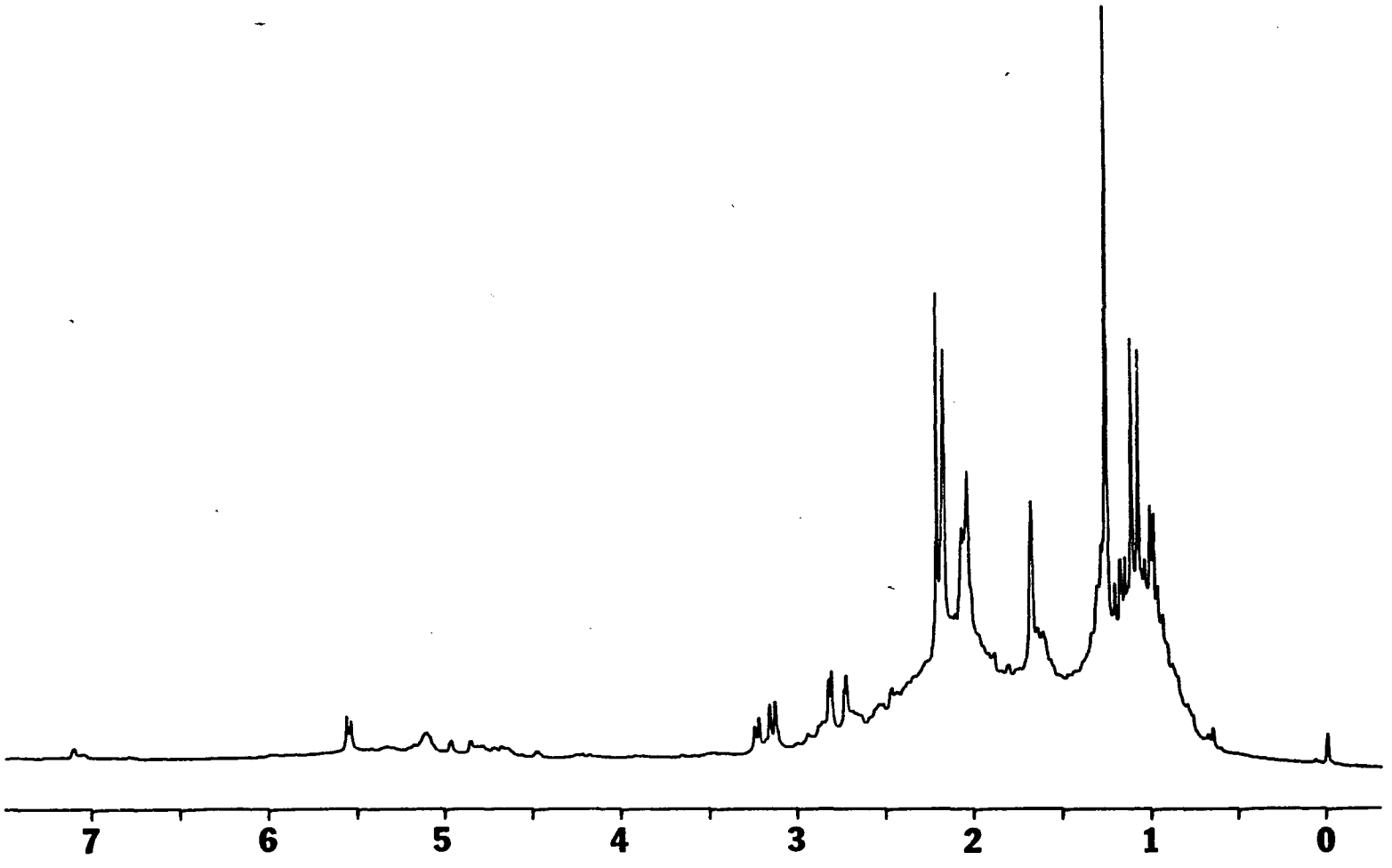


Figure 5. ^{1}H NMR spectrum of fraction A-6 from CH_2Cl_2 - petroleum ether extract of *C. ashei*.

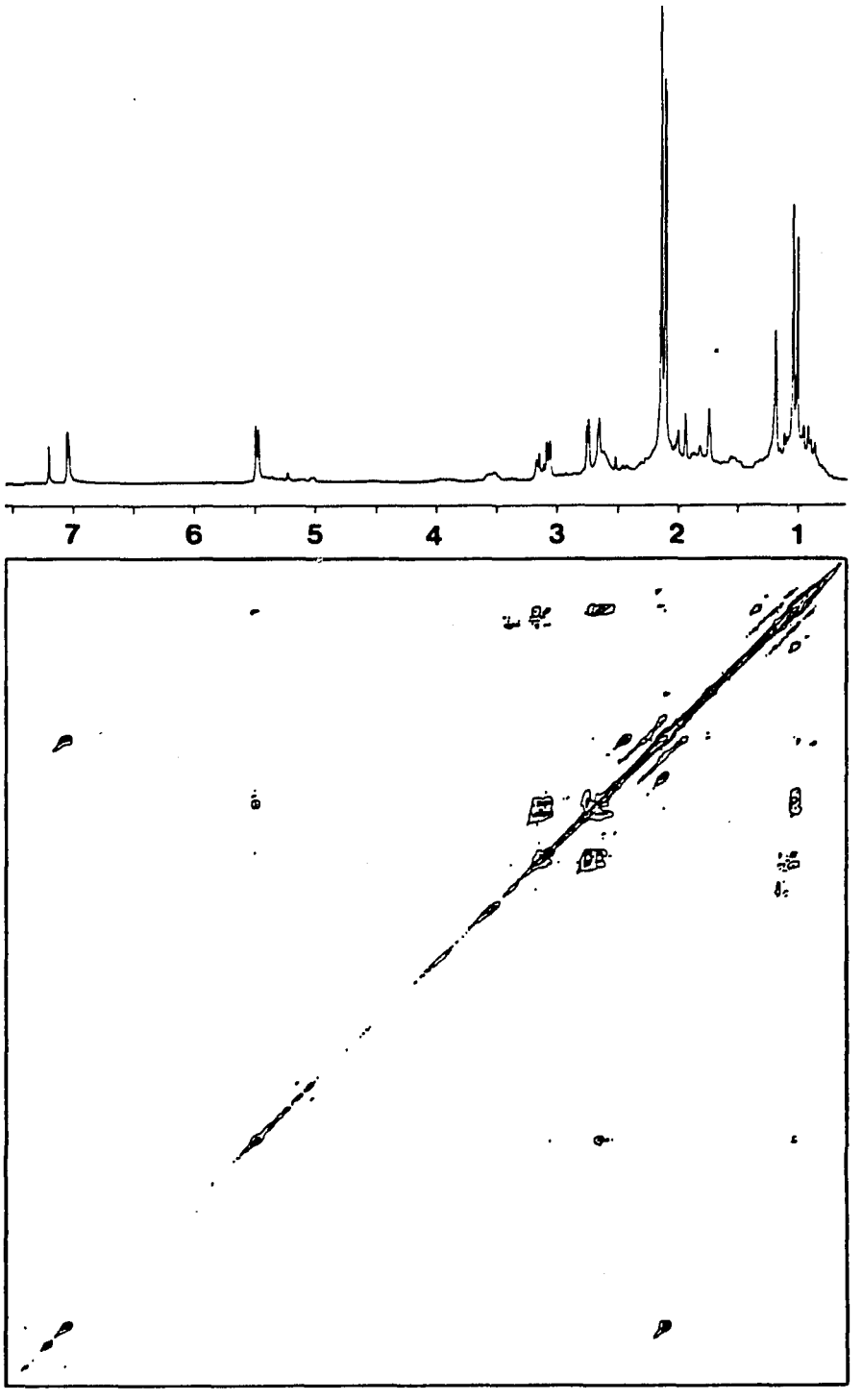


Figure 6. ^{1}H NMR 2D COSY 45 spectrum of calaminthone **(48)**.

at C-4. The ^{1}H NMR values for evodone are in agreement with data reported in the literature.[74] ^{13}C NMR signals were assigned by comparison with data for menthofuran **(47)**, and calaminthone **(48)**, and are summarized in Table 7.

II.2.3.3. Structure elucidation of calaminthone (48)

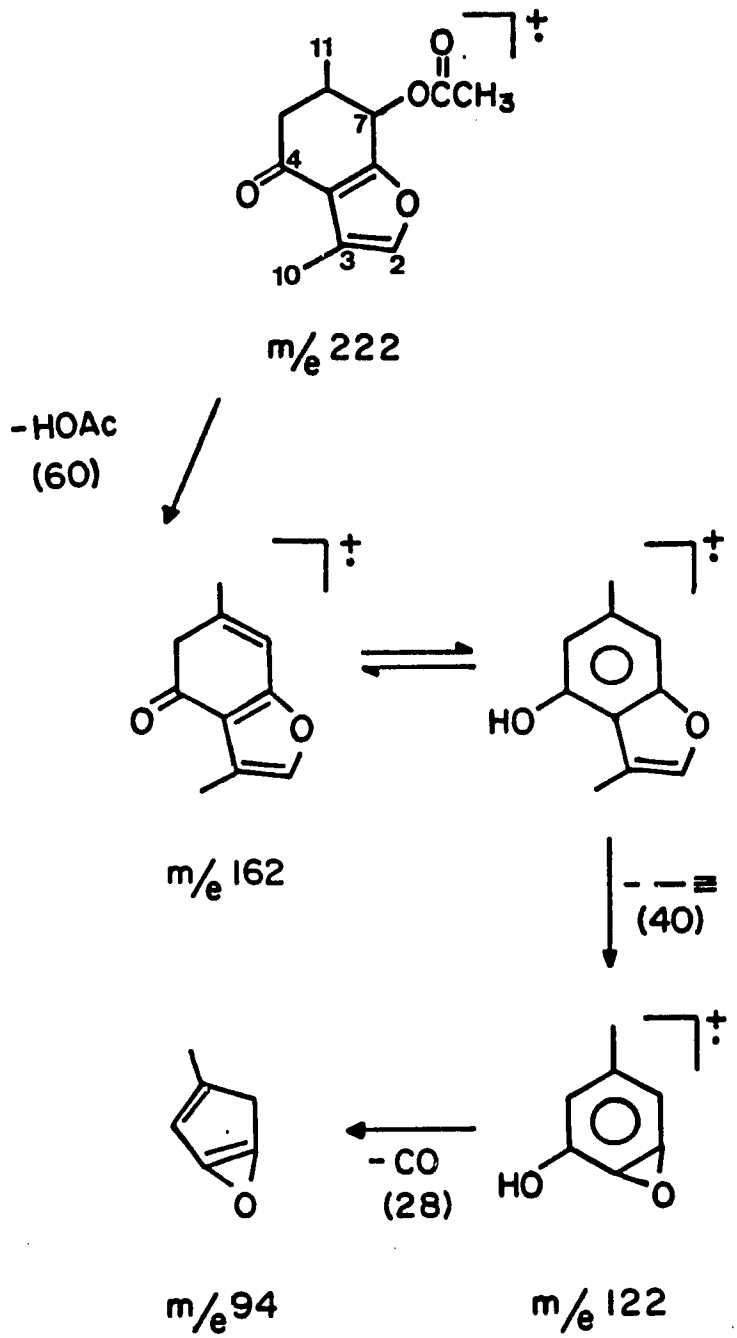
This new compound was isolated as a pale yellow oil. It was the major component of fraction A-6 as determined by comparison of the ^{1}H NMR spectrum of fraction A-6 with that of pure **48** (Figures 5 and 6). The molecular ion at *m/z* 222 in the mass spectrum was consistent with the formula $C_{12}H_{14}O_4$. The base peak at *m/z* 43 along with a prominent peak at *m/z* 162 was indicative of the loss of acetic acid from the parent molecule. The facile loss of *m/z* 60 ($M-CH_3-COOH$) was attributed to the favorable formation of a stable aromatic species as shown in Scheme 5.

^{1}H NMR double irradiation of the broad singlet at δ 7.11 (H-2) caused the signal at δ 2.17 (H-10) to sharpen which suggests allylic coupling between H-2 and H-10. Upon irradiation of the doublet at δ 5.56 (H-7), the shape of the complex multiplet at δ 2.66 (H-6) changed. In return, irradiation of this multiplet caused changes in three additional signals. The three-proton doublet at δ 1.09 (H-11) collapsed to a singlet and the two doublet of doublets centered at δ 2.78 and δ 3.20 (H-5) were affected. These

Table 7. ^{13}C NMR chemical shifts of compounds **46, 47, 48.** (50.32 MHz, $CDCl_3$)

C	**47**	**46**	**48**
2	136.60 d	139.04 d	140.21 d
3	119.15 s	119.98 s	119.30 s
4	31.29 t	195.05 s	---- *
5	19.69 t	46.70 t	29.96 t
6	29.45 d	30.77 d	33.79 d
7	31.29 t	31.64 t	76.65 d
8	150.30 s	166.99 s	164.62 s
9	117.03 s	118.90 s	119.00 s
10	7.76 q	8.83 q	8.70 q
11	21.24 q	20.92 q	13.85 q
$-C(=O)CH_3$			170.14 s
$-C(=O)CH_3$			20.77 q

*The carbonyl signal was not detected in the ^{13}C NMR spectrum.



Scheme 5. Mass spectral fragmentation pattern of calaminthone (**48**).

signals collapsed into doublets with typical residual geminal couplings of 17 Hz. The results of these double irradiations were supported by a two-dimensional COSY ^{1}H NMR experiment which graphically displays which proton signals are coupled to each other.

In calaminthone **(48)** the methyl signal at δ 2.17 (H-10) exhibited a chemical shift lower than the equivalent methyl signal of the parent menthofuran **(47)**, which appears at δ 1.89. This downfield shift is generally observed when a carbonyl group exerts a through-space deshielding effect on a methyl group, as exemplified by the methyl shift of C-10-Me in pumilin.[75] Correlation of the coupling constant between H-6 and H-7 ($J_{6,7}$ = 4 Hz) with stereo-models indicated a cis relationship between the protons at C-6 and C-7.[76]

The ^{13}C NMR spectrum of calaminthone contained a doublet at δ 140.2 and a singlet at δ 164.6 which were assigned to oxygen-bearing carbons of the furan ring (C-2 and C-8). A signal at δ 8.7 was typical of a methyl group on a double bond bearing a vicinal oxygen. C-7 appeared at δ 76.7 as a doublet and the signals for the acetate functionality could be observed at δ 170.1 (C=O) and δ 20.8 (CH_3-CO-). The remaining ^{13}C signals were assigned through chemical shift considerations in addition to comparisons with menthofuran **(47)** standard and evodone **(46)** both of which were also found in *C. ashei* (Table 7).

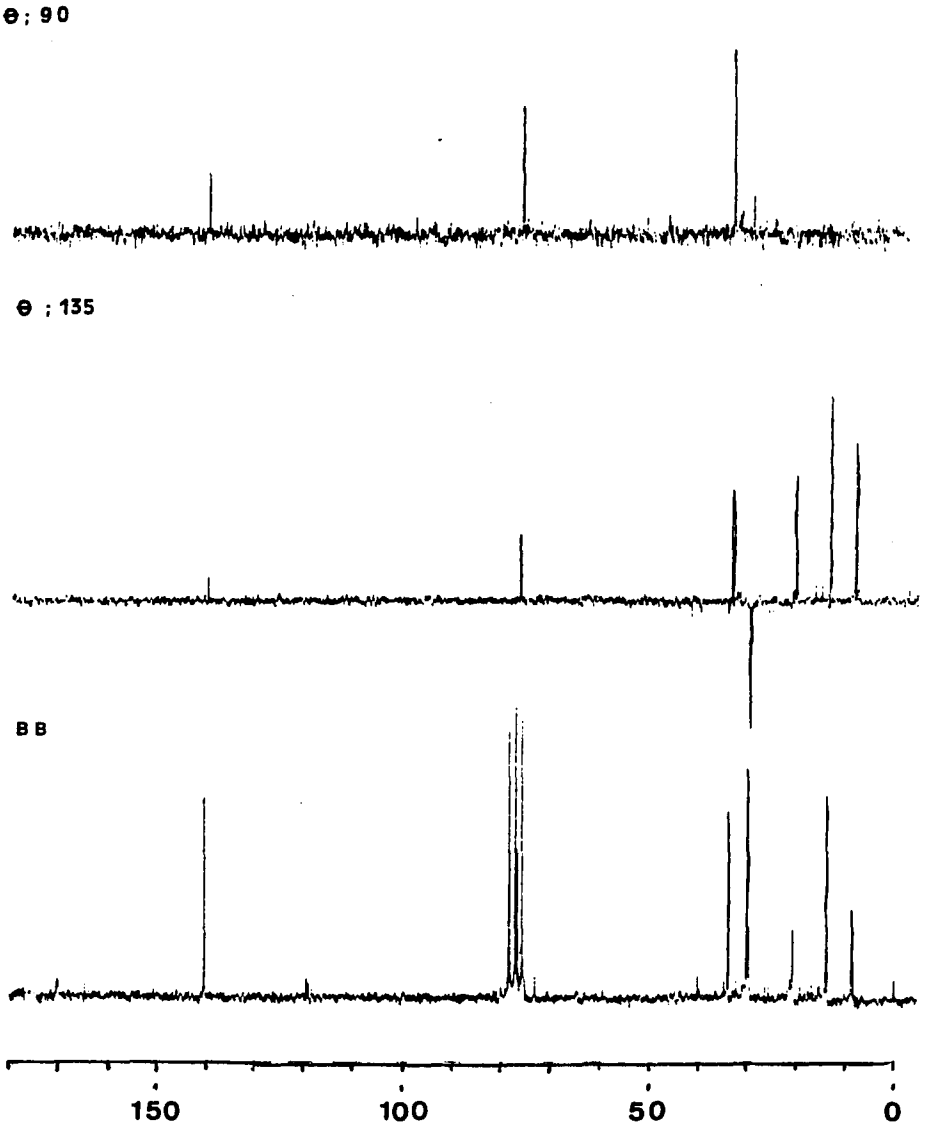


Figure 7. ^{13}C NMR spectrum of calaminthone **(48)**.

II.2.4. Allelopathic activities of the compounds isolated from *Calamintha ashei*.

Although fraction A-6, which represented a mixture of evodone **(46)**, caryophyllene oxide **(45)** and calaminthone **(48)**, had shown complete inhibition of test seed germination, the pure compounds did not exhibit appreciable activity when individually tested. Caryophyllene oxide only inhibited the radicle growth of *L. sativa* while the other compounds stimulated *L. sativa* growth. No effect was observed on *S. scoparium*. This could be attributed to the low solubilities of the pure compounds in comparison with a considerably higher solubility (~ 300 ppm) of the initial mixture.

Volatility tests of evodone **(46)** and calaminthone **(48)** caused significant germination inhibition of *S. scoparium* by **46**. Bioassays with mixtures of ursolic acid and compound **46** as well as caryophyllene oxide **45** were also performed. Evodone dramatically increased the inhibitory activity on *S. scoparium*, whereas *L. sativa* was affected to a lesser extent (Table 8). The possible role of ursolic acid in allelopathic studies will be discussed in Chapter 4.

II-2. 5. Gas Chromatography - Mass Spectral Analysis of Volatiles from *C. ashei*.

II-2.5.1. Volatiles from the steam distillate.

The GC-MS analysis of a steam distillate of the petroleum ether extract yielded the compounds shown in Figure 8. Several of these compounds were identified by spectral comparison with reference spectra in the GC-MS library. Other components were tentatively identified on the basis of their mass spectral fragmentation patterns.

Menthofurans appear to be common in *C. ashei* as a number of menthofuran derivatives **(46, 48, 49, 50)** were detected in the volatiles as well as menthofuran **(47)** itself. The mass spectral fragmentation of **47** was analogous to that of **46** with a favorable loss of 42 mass units. The identity of menthofuran **(47)** was established through a GC-MS comparison to a standard.

Compound **50** showed a molecular ion at *m/z* 168. The loss of a methyl group was more prominent when compared with evodone **(46)**, whereas the loss of *m/z* 42 via a retro Diels Alder fragmentation was less favored (Scheme 4). This phenomenon could be explained by the presence of a hydroxyl group at C-4 which disfavors the retro Diels Alder cleavage. Loss of CO (*m/z* 28) from ion *m/z* 126 formed the radical ion *m/z* 98 as the base peak which must be due to the facile loss of a ring carbonyl rather than cleavage of ketene as is the case with **46**.

Table 8. Bioassay results on pure compounds obtained from *C. ashei*.

Compound	conc. ppm	*Lactuca sativa*		*Schizachyrium scoparium*	
		% G[+]	% RL[+]	% G[+]	% RL
evodone[≠]	50	92	119*	114	94
evodone	250	96	131*	185*	93
evodone + ursolic a.	250	97	81*	57*	33*
evodone volatility test		---	---	22*	72
calaminthone	50	106	116*	100	93
calaminthone	250	113	138*	129	88
calaminthone volatility test		---	---	71	91
menthofuran	50	104	127*	142	71
menthofuran	250	113	127*	100	99
caryophyllene oxide		104	66*	128	91
caryophyllene oxide + ursolic a.		91	80*	171	117
ursolic acid		104	93	86	136

[+]Germinations (G) and radicle lengths (RL) are expressed as percentages of the control.

[≠]The water soluble portion of the maximal amount as indicated was used in the bioassays. Whenever the amount is not given, the amount used was the water soluble portion of 625 ppm maximum concentration.

*An asterix indicates significant difference from control at $p \leq 0.05$.

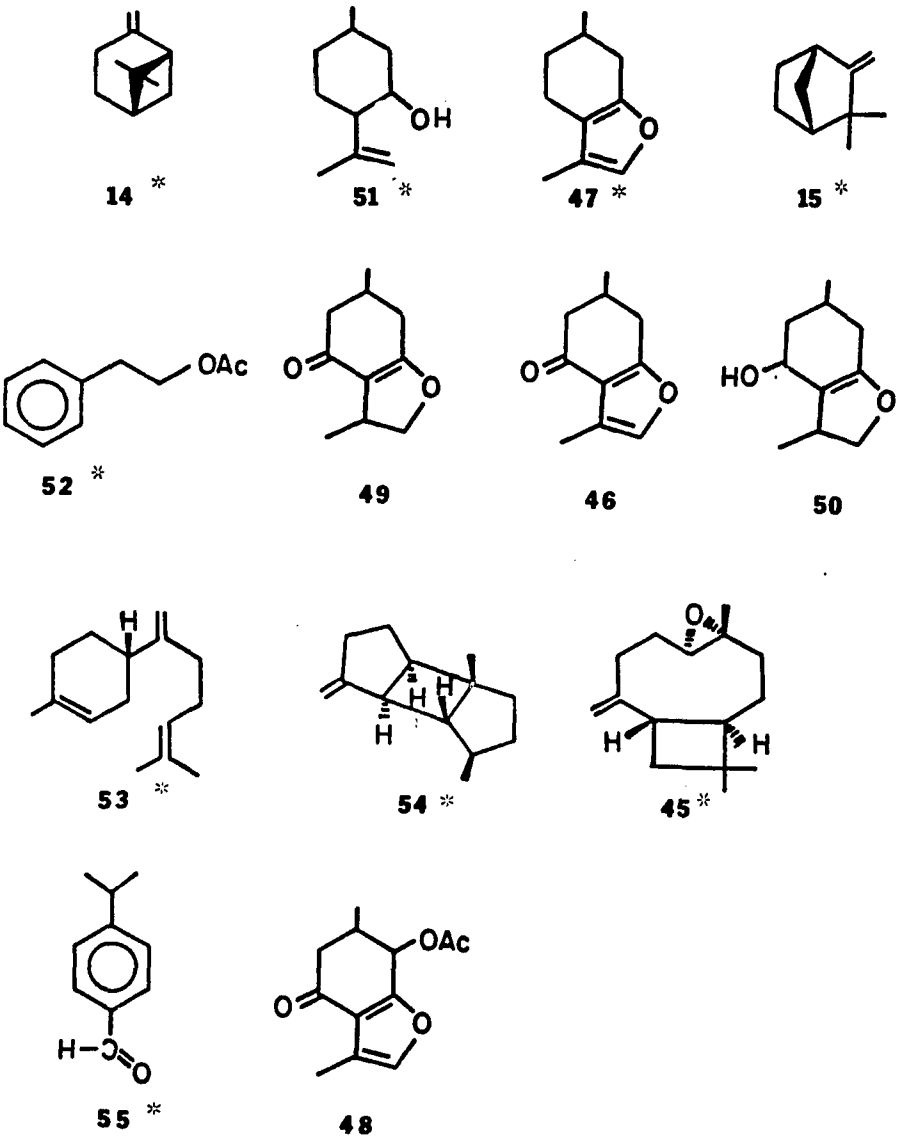


Figure 8. Volatiles from the hexane extract of *C. ashei*. Compounds identified with reference to standards in the mass spectral library disk with a confidence level of above 97% are indicated with an asterix (*).

In the GC trace, the shoulder of the evodone peak seems to be caused by another menthofuran derivative with the parent ion at *m/z* 166 (Scheme 4). The facile loss of 42 mass units could again be explained by a retro Diels Alder cleavage with a carbonyl at C-4. The loss of *m/z* 43 indicated saturation at the isopropyl side chain. Therefore, its structure was tentatively assigned as 2,3-dihydroevodone **(49)**.

II-2.5.2. Volatiles around fresh leaves of *C. ashei*

As the allelopathic activity of *C. ashei* seemed to be concentrated in its essential oils, experiments were conducted to identify the compounds that occur naturally in the air around the plant. The results of various collections are shown in Table 9. It is interesting to note the difference in the monoterpene presence in the atmosphere around the intact leaves. In the fall, only evodone **(46)** was detected while in the spring, α-pinene, β-pinene and limonene were released and evodone **(46)** was not found. The two major sesquiterpene hydrocarbons were the same for both seasons, whereas the two minor sesquiterpene hydrocarbons were detected only in April.

Two collections, one during the day and the other during the night, were carried out in April in order to

Table 9. Volatiles present in the atmosphere surrounding live *C. ashei* branches.

compound	r.t	October night collection	April day collection	April night collection
α-pinene **(13)**	4.08	---	+++	+++
β-pinene **(14)**	4.82	--	+++	+++
limonene **(56)**	6.05	--	++	++
evodone **(46)**		+	---	---
bourbornene **(54)**	15.37	---	+	+
α-humulene **(57)**	16.18	++	++	++
Unknown A*	16.97	---	+	+
Unknown B*	17.65	++	++	++

*Unknown A and B represent sesquiterpene hydrocarbons.

test for qualitative and quantitative differences in the volatiles composition, depending on the light-dark cycle, but no differences were observed. Furthermore, no differences between the volatiles of the whole live plant and those of freshly cut branches could be observed.

II.3. Experimental.

II.3.1. Generalizations.

The following instruments, general experimental procedures and conditions were used for obtaining the various data throughout Chapters II and III unless otherwise indicated.

Instrumentation.

NMR: Bruker WP 200 Fourier Transform NMR spectrometer; ambient temperature; TMS as internal standard.

EIMS: Hewlett Packard 5895 GCMS at 70 eV; source temperature 200°C.

UV: Cary 14 spectrophotometer

IR: Perkin Elmer 621 Infrared spectrophotometer; chloroform as solvent.

MP: Thomas Hoover Capillary Melting Point Apparatus; melting points are uncorrected.

Acetylations were carried out by the addition of excess Ac_2O:Pyridine (2:1) to the compound or mixture dissolved or suspended in CH_2Cl_2. The solution was magnetically stirred at 0°C and left overnight at room temperature. The resultant mixture was extracted with aqueous $NaHCO_3$ (10 %) and subsequently with 2N HCl. After drying *in vacuo*, TLC purification was carried out if needed.

Methylations were performed by dropwise addition of a diazomethane solution in ether into a stirred solution of the compound in CH_2Cl_2 at 0°C. Since the compounds to be methylated were usually yellow, the extent of the reaction had to be monitored by TLC.

Preparation of Diazomethane. Diazomethane was prepared from N-methyl-N'-nitro-N-nitrosoguanidine.[77] To a cooled mixture of 40 mL ether and 15 mL 40% KOH in a

distilling flask, 5 g of reagent dissolved in 15 mL ether, was added dropwise. The yellow diazomethane solution was distilled from the mixture. The procedure was carried out in a special diazomethane distillation apparatus.

Bioassays were carried out on *L. sativa* and *S. scoparium* seeds. The extracts or pure compounds to be tested were vacuum-dried to remove all traces of organic solvents. Unless otherwise stated, the water-soluble portion of 15 mg of test material was taken up in 23 mL distilled water. Following filtration, the pH of the solutions were checked since at pH 4 or below the acidic medium could harm the test seedlings. The solution (5 mL) was added to each sterile petri dish of 10 cm diameter lined with a Whatman #1 filter paper. In the case when volatiles were bioassayed, an aluminum foil boat containing the test compound was placed in the middle of the petri dish while the control contained an empty aluminum boat. Each dish contained 30 seeds of the target plants. Typically, duplicate runs were performed on each target plant. The dishes were covered and kept in the dark for a period of 4 days for *L. sativa* and 15 days for *S. scoparium*. At the completion of each bioassay, the dishes were frozen to terminate growth. After the number of germinated seeds in each dish and the radicle length of the germinated seeds were recorded, the statistical treatment

(ANOVA) was carried out by Dr. G. B. Williamson, Department of Botany, LSU.

II.3.2. Isolation of compounds from *Calamintha ashei*.

C. ashei was first collected in June, 1984 from Sun Ray, Florida. Three subsequent collections were carried out at the same site in October, 1984, October, 1985, and April, 1986.

Ground dry *C. ashei* leaves (125 g) collected in June, 1984 were soaked successively in petroleum ether, $CHCl_3$, MeOH and H_2O. The plant material was left in each solvent for 24 hours using 500 ml solvent. After filtration by suction the plant material was again soaked for 6 hours using 300 ml of each solvent. The procedure yielded the following amounts of extracts: 2.2 g from petroleum ether; 12.7 g from $CHCl_3$ and 6.2 g from MeOH.

From the 12.7 g of the $CHCl_3$ extract, 8.9 g of a precipitate formed upon slow evaporation of the solvent to about 100 ml and the solid was filtered off. Repeated washing of this precipitate with DCM and MeOH yielded 6.5 g of a white solid which mainly consisted of ursolic acid **(40)**. 25 mg of this material was further purified on 1 mm silica gel TLC plate with Et_2O/EtOAc (5:1) as the solvent system. Since **40** was more soluble in most solvents as its acetate, most of its spectral data was obtained from the acetate.

Ursolic acid (**40**). $C_{30}H_{48}O_3$, white solid, m.p. 267-272°.

EIMS *m/z* (rel. int.) 119 (29), 133 (44), 189 (18), 203 (44), 207 (32), 248 (100), 456 (2).

Acetate: ^1H NMR ($CDCl_3$) δ: 2.06 (s, 3H), 4.52 (t,1H), 5.23 (t, 1H). ^{13}C NMR ($CDCl_3$) δ: 15.5q, 16.7q, 17.0q, 17.1q, 18.1t, 21.2q, 21.3 q, 23.3t, 23.6q, 24.0t, 28.0t, 28.1q, 29.7t, 30.6t, 32.8t, 36.8s, 36.7t, 37.7s, 38.2t, 38.8d, 38.9d, 39.5s, 41.6s, 47.4d, 47.8s, 52.5d, 55.3d, 80.9d, 125.7d, 137.9s, 171.3s, 184.2s.

Half of the remaining 3.8 g of the mother liquor of the $CHCl_3$ extract was subjected to silica gel flash chromatography utilizing CH_2Cl_2-acetone mixtures of increasing polarity (10:0, 10:1, 2:1, !;1, 1:2, 1:10, 0:10). Sixteen fractions (150 ml each) were collected. The fractions were tested for allelopathic activity and the results are summarized in Table 5.

A solvent mixture of petroleum ether - CH_2Cl_2 (1:9) was used to obtain more of the biologically active compounds from 100 g of dried ground *C. ashei* leaves collected in October, 1984. This extract (3.4 g) was subjected to silica gel column chromatography utilizing P.E.:CH_2Cl_2 (3:7) as solvent. Eight 75 ml bands were collected before the chlorophyll band eluated. Fraction 6 provided a mixture of **46**, **48**, and **45**, and fraction 5 contained **45** as the major component. Following silica gel

TLC purifications with CH_2Cl_2:acetone (95:5) 112 mg **48** could be obtained. Caryophyllene oxide (10 mg) was purified by prep. TLC on a silica gel plate using CH_2Cl_2 as solvent. For the purpose of obtaining pure **46**, 150 g *C. ashei* leaves collected in October, 1985 were extracted with petroleum ether - CH_2Cl_2 (1:9). The extract was not taken to dryness to avoid possible loss of volatile compounds. About 3 g of this extract was partitioned on a silica gel column with CH_2Cl_2 to yield eight 150 ml fractions before the chlorophyll band. Fraction 5 mainly contained evodone **(46)** as shown by GC-MS analysis. TLC on silica gel with 30% P.E., 68% CH_2Cl_2, 2% acetone yielded 28 mg of pure **46** as the third major band. **46** appears to decompose upon prolonged exposure to air at room temperature.

***Caryophyllene oxide* (45).** Colorless crystals, m.p. 63-65°.

EIMS *m/z* (rel. int.) 220 (not registered), 205 (0.2), 177 (6.5), 161 (8.7), 149 (7.6), 138 (15.2), 121 (32.6), 109 (47.8), 93 (80.4), 79 (100.0), 69 (58.7), 55 (46.5), 43 (97.8), 41 (80.4); ^{1}H NMR ppm: δ 0.98 (3H, s) 1.00 (3H, s), 1.20 (3H, s), 1.05-2.75 (m), 2.78 (1H, dd, $J_{5a,b}$ 10.5 Hz, $J_{5b,6}$ 4 Hz), 4.87 (1H, br s), 4.97 (1H, br s); ^{13}C NMR ppm: δ 16.9q (C-14), 21.5q (C-15), 27.1t (C-9), 29.8 (C-13), 29.8t (C-5/C-8), 30.0t (C-8/C-5), 33.8s (C-10), 39.2t (C-11/C-4), 39.7 (C-4/C-11), 48.6d (C-1), 50.8d (C-2),

59.5s (C-7), 63.5d (C-6), 112.6t(C-12), 151.7s (C-3).

***Evodone* (46)** Colorless crystalline solid. λ_{max}^{MeOH} nm: 261; EIMS *m/z* (rel. int.): 164 (43.2), 149 (3.1), 122 (100.0), 94 (45.9), 77 (3.9), 66 (89.2), 65 (88.4), 41 (6.2); ^{1}H NMR ppm: δ 1.16 (3H, d, $J_{6,11}$ 6 Hz), 2.0-2.9 (m), 2.18 (3H, d, $J_{2,10}$ 1.5 Hz), 2.87 (1H, dd, $J_{5a,5b}$ 16 Hz, $J_{5,6}$ 3.5 Hz).

***Calaminthone* (48)** Pale yellow oil. EIMS *m/z* (rel. int.): 222 (4.5), 162 (55.4), 161 (22.8), 151 (13.9), 133 (10.1), 123 (27.5), 122 (59.1), 94 (29.2), 77 (10.6), 65 (20.7), 43 (100.0), 41 (15.7), 40 (6.6); ^{1}H NMR ppm: δ 1.09 (3H, D, $J^1$6,11 7 Hz), 2.17 (3H, d, $J_{2,10}$ < 1 Hz), 2.21 (3H, s), 2.66 (1H, m), 2.78 (1H, dd, $J_{5a,5b}$ 17 Hz, $J_{5a,6}$ 3Hz), 3.20 (1H, dd, $J_{5a,5b}$ 17 Hz, $J_{5b,6}$ 5 Hz), 5.56 (1H, d, $J_{6,7}$ 4 Hz) 7.11 (1H, br s).

For the COSY 45 N type experiment, 256 FIDs (of 64 scans each) consisting of 1K data points were accumulated; after digital filtering (sine bell) the FID was zero filled to 512 words in the F. dimension. Fourier transformation followed by magnitude calculation with no phase correction yielded a spectrum with 2.55 Hz per point digital resolution in both dimensions. Acquisition parameters were $F_1 = \pm$ 652 Hz and F_2 = 1308 Hz. 2 seconds recycle delay was used.

II.3.3. Detection of the volatiles from *C. ashei*.

A steam distillation was carried out by passing steam over 2 g of the P.E. extract from *C. ashei* leaves collected in June, 1984. The 50 ml distillate collected was extracted with 10 ml of nanograde CH_2Cl_2. The volatiles were analyzed by GC-MS. GC conditions: 30 m silica-bonded FSOT capillary column. Injection temp.: 250°C oven: 45°C for 1 min.; then 5°/min for 15 min and finally 10°/min to 250°C.

***Menthofuran* (47).** r.t. 10.32; EIMS *m/z* (rel. int.): 150 (40.1), 108 (100.0), 91 (8.4), 79 (15.3).

Compound 49. MW 166. r.t. 12.82; EIMS *m/z* (rel. int.): 166 (34.9), 124 (100.0), 123 (44.9), 109 (5.1), 81 (15.4).

Compound 50. MW 168. r.t. 12.24; EIMS *m/z* (rel. int.): 168 (79.1), 153 (30.4), 126 (76.9), 98 (100.0), 84 (30.3), 70 (20.1), 69 (32.2), 43 (23.0).

Volatiles released directly into the atmosphere from intact fresh leaves were trapped from fresh branches collected in October, 1985 and from whole live plant as well as fresh branches collected in April, 1986. An 8 hour overnight collection was performed with the first batch and two 4 hour collections were carried out with the second batch - one during the day and the other during the night. The plant material was placed inside a large

replica of a gas trap through which purified N_2 was passed. Attached to this chamber was a cold-finger in which 1 ml nanograde CH_2Cl_2 was placed. This cold-finger was immersed in a dry ice-acetone bath. The volatiles carried out with N_2 passing through the cold-finger would thus condense at -78°C. The volatiles so obtained were subjected to GC-MS analysis under the given conditions (Table 9); October collection: 30 m silica-bonded FSOT capillary column. Injection temp: 250°C. Oven: 70°C for 1 min then 10°/min to 250°C. Column pressure: 15 psig; column flow: 30 ml/min. April collection: column parameters were the same as in October. Injection temp.: 250°C. Oven: 40° for 1 min; then 5°/min until 250°. Column pressure: 10 psig; column flow: 36 ml/min.

CHAPTER III

INVESTIGATIONS OF ALLELOCHEMICALS FROM *Ceratiola ericoides*

III-1. INTRODUCTION

Ceratiola ericoides of the Empetraceae family is an endemic shrub, a member of the Florida *SCRUB* community, which thrives in the sandy soils of Florida. Although the biological activities of the leaf washes were slight and exhibited erratic seasonal fluctuations as shown in Tables 2 and 3, the plant exhibits extensive inhibitory activity in nature. Greenhouse studies performed on native test seedlings watered with *C. ericoides* leaf washes showed significant decreases in the growths of these seedlings as compared to those of control seedlings.[54] Therefore, an investigation of the inhibitory chemicals of *C. ericoides* was undertaken.

C. ericoides had not been previously investigated for its chemicals except for a taxonomic survey by the use of paper chromatography data.[78] Comparisons with standard phenolic compounds indicated the presence of quercetin-3-rutinoside **(58)**, quercetin-3-galactoside **(59)**, gossypetin-3-galactoside **(60)**, chlorogenic acid **(61)** and p-coumaryl quinic acid **(62)**. The presence of a highly methylated unknown flavanone was also mentioned.

Table 10. Allelopathic effects of *C. ericoides* leaf washes on *Andropogon gyrans* leaf, root and whole plant dry weight (mg) and height (mm). An asterix indicates significant differences between test species and control at $p < 0.05$ level.[54]

	C. ericoides leaf washes	Control
Total leaf dry weight	0.61 ± 0.18*	3.10 ± 0.49
Total root dry weight	0.27 ± 0.05*	0.90 ± 0.21
Total plant dry weight	0.90 ± 0.08*	3.99 ± 0.63
Height	29.7 ± 2.1*	85.7 ± 6.8
n	32	16

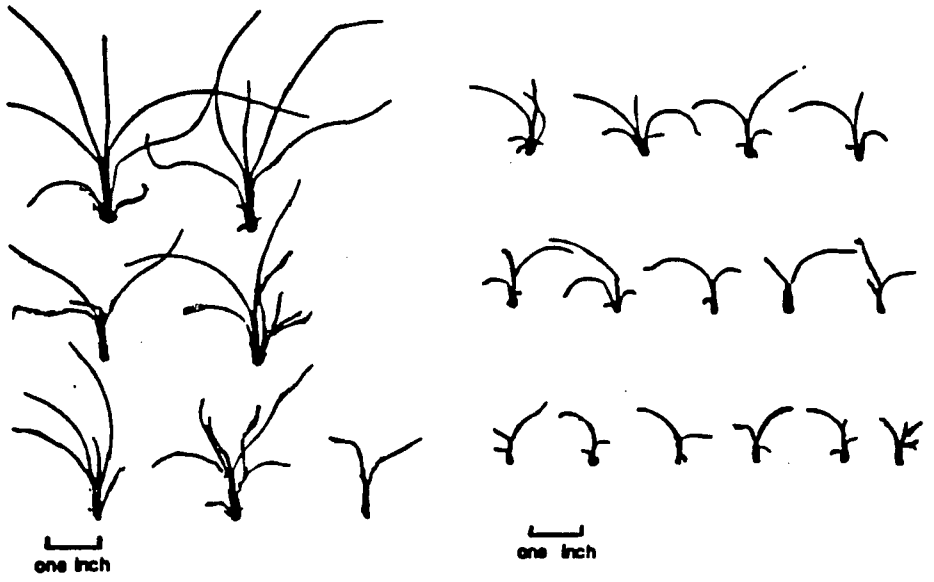
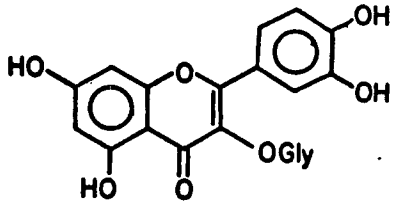
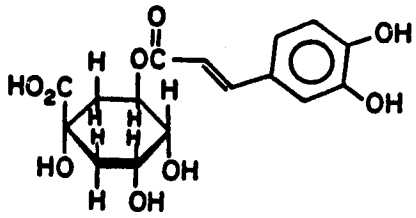


Figure 10. Comparison of the aerial part sizes of control *S. scoparium* (left) and *S. scoparium* treated with *C. ericoides* leaf washes (right).[54]



59 Gly = Gal

58 Gly = Rut



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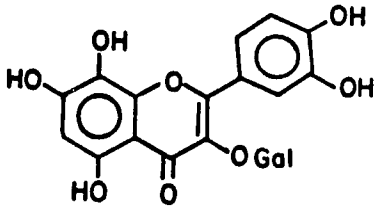

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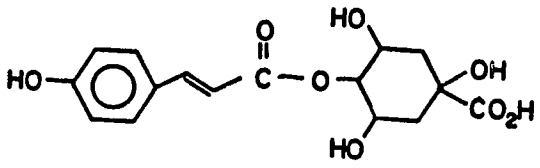

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Figure 9. Natural products reported from *Ceratiola ericoides*.

III-2. RESULTS AND DISCUSSION

III-2.1. Initial extractions.

Crude extracts from dry ground *C. ericoides* leaves were obtained through a sequential use of solvents as

described in Section III-4.2. The bioassays, the results of which are summarized in Table 11, were performed as described in Section II-3.1. Higher activities were observed for the more polar extracts. Germination and growth of the native grass *Schizachyrium scoparium* was affected to a larger extent than the commercial lettuce *(Lactuca sativa)*. This could indicate the presence of specific inhibitor(s) acting as ecological antagonists.

The results also suggest the possible presence of a heat sensitive phytotoxin, as the soxhlet extration with methanol showed no activity while the methanol wash of the XAD-4 resin did. However differences in the procedures for obtaining these extracts may have also played a role. Nevertheless, the soxhlet extract had a much darker, tar-like appearance indicating possible decomposition of one or more of its constituents.

Bioassays were also performed on root and litter extracts (Table 12). The germination and radicle growth inhibitions of *S. scoparium* were statistically insignificant for both roots and litter extracts, although the dichloromethane (DCM) extract of litter showed marginal activity. Significant stimulation of germination and radicle growth was observed for *L. sativa* when treated with the litter extracts. Since litter showed high activities at certain times in the year, this lack of inhibition was attributed to the low level of toxins in autumn.

The ^{1}H NMR spectrum of DCM litter extract contained mainly a large hydrogen envelope in the aliphatic region which is typical for triterpenoids. No further chemical studies were done on these extracts.

III-2.2. Chemical investigations on the dichloromethane wash of the aerial parts of *C. ericoides*

The DCM extract was chromatographed on a Sephadex LH-20 column as described in Section III-4.3. Recombined fractions 1-7 consisted of a mixture of nonpolar compounds, fractions 8-9 contained two triterpenes as the major constituents, fractions 10-17 consisted mainly of flavonoids, and fractions 18-35 were mostly polar residues eluted during final column washes with polar solvents. Table 13 displays the results of bioassays performed on these fractions. There was no appreciable activity for any fraction although combined fractions 10-17 showed the highest activity. In order to check for possible influences on activity due to insolubility of the test sample, fractions 10-17 were predissolved in dimethyl sulfoxide (DMSO) and then added to water. The results indicated an increase in the activity of fractions 10-17 where *L. sativa* radicle growth was stimulated and *S. scoparium* radicle growth was inhibited which compares favorably with the initial bioassays for the leaf leachates in water.

Table 11. Germination and growth inhibitions causaed by crude extracts from the aerial parts of *C. ericoides*.

	Lactuca sativa		*Schizachyrium scaparium*	
Extract	% germination[+]	% radicle[+] length	% germination[+]	% radicle[+] length
Hexane	99	89	125	87
CH_2Cl_2	96	63*	63	34*
EtOAc	96	78*	83	37*
MeOH XAD-4 wash	100	73*	50*	28*
MeOH soxhlet	98	85	88	104

[+]Germination and radicle lengths are expressed as percentage of the controls.

*An asterix indicates significant difference from the control at $p \leq 0.05$.

Table 12. Germination and radicle growth inhibitions caused by *C. ericoides* root and litter extracts.

Extract	*Lactuca sativa*		*Schizachyrium scaparium*	
	% germination[+]	% radicle[+] length	% germination[+]	% radicle[+] length
ROOTS :				
CH_2Cl_2	108	92	171	91
MeOH	108	63*	57	124
H_2O	96	54*	100	70
LITTER :				
CH_2Cl_2	126*	137*	69	66
H_2O	58*	132*	90	117

[+]Germination and radicle lengths are expressed as percentage of the controls.

*An asterix indicates significant difference from the control at $p \leq 0.05$.

Table 13. Germination and growth inhibitions of fractions of *C. ericoides* DCM extract on a Sephadex LH 20 column.

Fractions	*Lactuca sativa*		*Schizachyrium scaparium*	
	% germination[+]	% radicle[+] length	% germination[+]	% radicle[+] length
1-7	89*	93	100	106
8-9	96	100	100	93
10-17	93	99	117	83
10-17 (DMSO)[≠]	100	132*	50*	69
18-35	100	106	100	94
DMSO	83*	93	100	141

[+]Germination and radicle lengths are expressed as percentage of the controls.

[≠]Sample was initially dissolved in 0.5 ml dimenthyl sulfoixide (DMSO) and subsequently taken up in 25 ml H_2O.

*An asterix indicates significant difference from the control at $p \leq 0.05$.

III-2.2.1. Identification of ursolic acid (40) and erythrodiol (63).

Chromatographic fractions 8-9 from the Sephadex LH-20 column of the DCM extract showed no activity. Nevertheless, the structures of the triterpenes in fractions 8-9 were identified since they were the major constituents of the DCM extract (62%). Silica gel column observations done on the acetates of the two triterpenes showed the relative abundance by weight of the triterpenes **63** and **40** to be 1:4. Ursolic acid **(40)** was identified by comparison of its spectral data (NMR and MS) with an authentic sample obtained from *C. ashei* (Section II-2.2.).

Erythrodiol **(63)** exhibited a mass spectral fragmentation pattern similar to that of **40**. Retro-Diels-Alder fragmentation, typical of oleananes and ursanes containing a 12(13)-double bond,[79] caused the ion at *m/z* 234. Facile loss of the angular $-CH_2OH$ manifested itself as the base peak at *m/z* 203. The ^1H NMR spectrum contained signals for H-3 at δ 3.22, for H-12 at δ 5.19 and for H-28 at δ 3.55 and δ 3.21. The ^{13}C NMR spectrum displayed the characteristic signals for C-3 at δ 79.2, C-12 at δ 124.8, C-13 at δ 138.1, and C-28 at δ 69.6. Comparison of the ^1H NMR spectrum and coelutions on TLC of **63** with an erythrodiol standard established their identity.

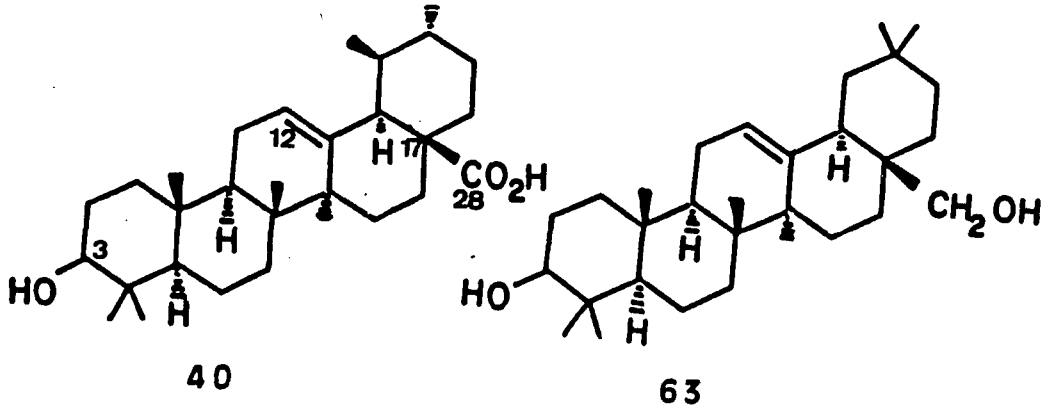


Figure 11. Triterpenes in *Ceratiola ericoides*.

III-2.2.2. Identification of flavonoids 64 to 69.

The distinct increase in activity of fractions 10-17 in the presence of DMSO made it desirable to learn about their constituents. ^1H NMR analysis of a portion of these fractions suggested the presence of flavonoids. Bioassays of the flavanoid mixture were performed in saturated aqueous solutions of ursolic acid (**40**). Reasons for this experiment will be discussed in Chapter IV. The bioassays indicated an increase of activity on *S. scoparium* seeds in the presence of ursolic acid (**40**) as seen in Table 14.

Silica gel column chromatography of 150 mg of fractions 10-17, which had been obtained from the Sephadex LH-20 column provided six compounds. Their structures,

which were determined by extensive NMR analysis combined with mass spectral data, are discussed below.

***Angoletin* (64)** exhibited a ^{1}H NMR spectrum with methylene proton signals centered at δ 3.02 and δ 3.41 and a five-proton absorption appearing as a broad singlet at δ 7.30 typical of aromatic protons. Strong mass spectral peaks at *m/z* 91 and *m/z* 168 indicated the presence of a dihydrocinnamyl moiety. The ion at *m/z* 195 was attributed to the favorable formation of an oxonium ion on the phloroglucinol ring. The ^{1}H NMR spectrum also exhibited two three-proton singlets at δ 2.10 and δ 2.12 which were assigned to two methyl groups on an aromatic ring. The difference in their chemical shifts suggested an unsymmetrical substitution pattern at this ring. A three-proton singlet at δ 3.67 was suggestive of a methoxy group on an aromatic ring. A one-proton singlet at δ 13.46 was attributed to a strongly hydrogen-bonded proton. The UV spectrum exhibited two maxima, one at 291 nm for the phloroglucinol ring and the other at 335 nm for the cinnamate ring. Addition of the weak base NaOAc to **64** during UV measurements caused a small shift of the maximum for the phloroglucinol ring to 293 nm. This is expected from a compound containing a free hydroxyl group at C-4'. The above data were in agreement with structure **64** which has been reported as angoletin.[80] The ^{13}C NMR data

Table 14. Biological activities of a solution of fraction 10-17 in water compared with data of a solution of fraction 10-17 in a saturated aqueous solution of ursolic acid (40).

	Lactuca sativa		*Schizachyrium scoparium*	
Sample	%germination[+]	% radicle[+] length	%germination[+]	% radicle[+] length
portion of fractions 10-17 with ursolic acid	102	104	88	69*
portion of fractions 10-17 without ursolic acid	92	144	96	103

[+]Germinations and radicle lengths are expressed as percent of their controls.

*An asterix indicates significant difference from the control of $p \leq 0.05$.

reported[80] for **64** contained a number of interchangeable assignments for the carbons of the phloroglucinol ring which were assigned by comparison with the ^{13}C NMR data of its isomer **65** as shown in Table 20.

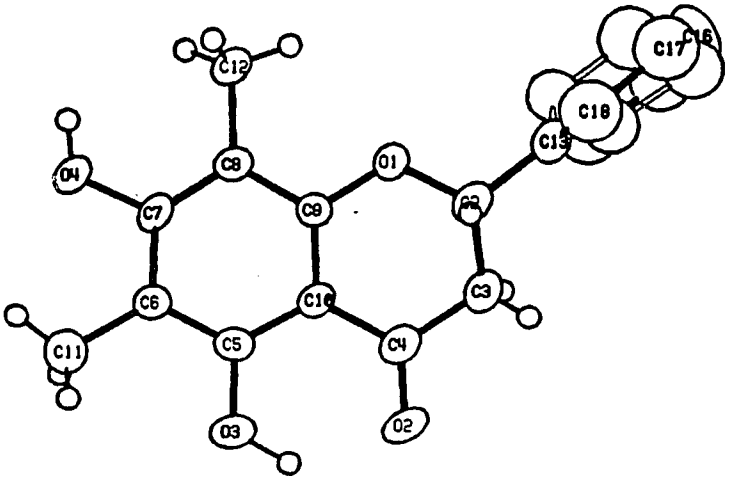
2',6'-dihydroxy-4'-methoxy-3',5'-dimethyldihydrochalcone (65).[81,82]

The ^{1}H NMR and mass spectral data of this compound were nearly identical with those of **64**. The main difference was the appearance of a six-proton singlet at δ 2.11 which must be caused by two magnetically equivalent methyl groups. The presence of a methyl group resulted in a three-proton singlet at δ 3.71. Two magnetically equivalent hydroxyl groups on the phloroglucinol ring gave rise to a single two-proton signal at δ 9.41. The above data indicated the presence of a symmetrically substituted phloroglucinol ring with the methoxy substituent at C-4'. As expected, neither the UV maximum for the phloroglucinol ring at 281 nm nor the maximum for the cinnamate ring at 350 nm gave a shift upon the addition of NaOAc. The ^{13}C NMR data which had not been reported previously was analyzed in comparison to the assignments made for its isomer **64** (Table 20). Two peaks appeared around 8 ppm for the methyl groups. The methoxy carbon gave a signal at δ 60.3 and the methylene carbons showed inverted

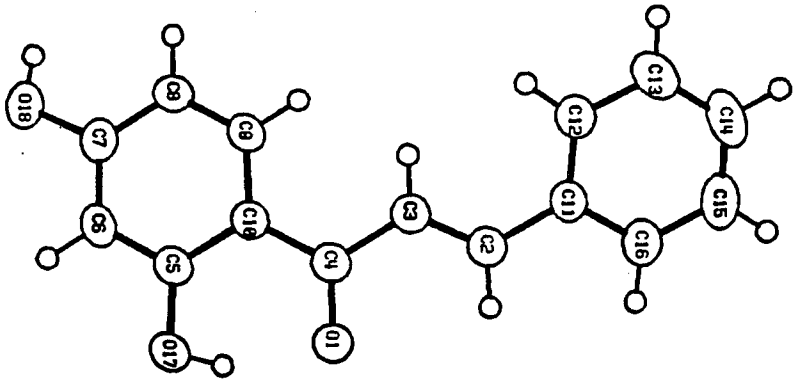
^{13}C-DEPT 135° signals at δ 30.6 and δ 46.1. The carbonyl gave a singlet with the expected chemical shift δ 205.7.

6, 8-dimethylpinocembrin (66). The structure of compound **66** was deduced by comparison of its ^{1}H NMR data to that reported in the literature.[83] The symmetrical methyl groups at C-6 and C-8 appeared as a six-proton singlet at δ 2.08. The protons on C-3 gave rise to characteristic doublet of doublets at δ 2.84 and δ 3.05. The coupling constant between H-3a and H-3b which was not reported previously was found to be 17.5 Hz, a value typical for such geminal couplings in flavonoids. H-2 appeared as a doublet of a doublet at δ 5.41. The mass spectral fragmentation pattern was typical for flavanones as shown in Scheme 6. Retro Diels-Alder fragmentation followed by the loss of carbon monoxide resulted in the base peak at *m/z* 152. Single crystal x-ray diffraction analysis of **66** confirmed the structure. It is interesting to note that the stereochemistry at C-2 was found to be R,S while only one isomer is usually found in enzymatic transformations of chalcones to flavanones.[84] This suggested that compound **66** must be spontaneously formed from a chalcone precursor without the involvement of an enzyme and therefore represents an artifact.

8-methylpinocembrin (67)[83]. The ^{1}H NMR spectrum was nearly identical with that of **66** except for the appearance of a one-proton singlet at δ 6.0 along with the loss of a

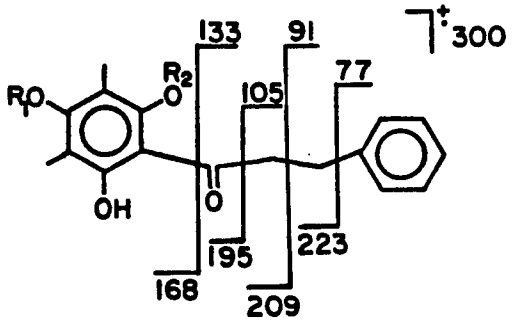


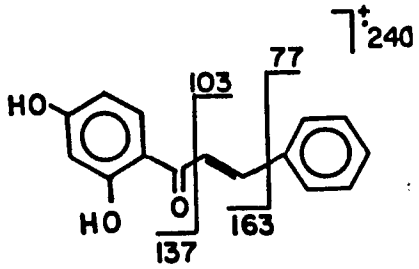
66



69

Figure 12. Single crystal x-ray structures for compounds **66** and **69**.





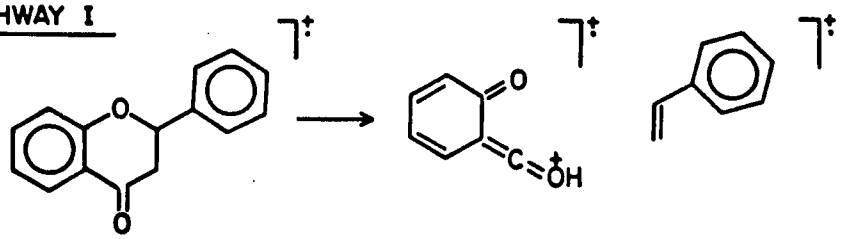
69

64; R_1 = H R_2 = Me

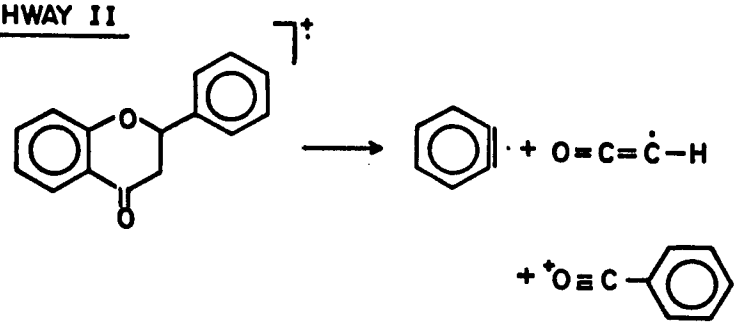
65; R_1 = Me R_2 = H

FLAVANONES:

PATHWAY I



PATHWAY II



Scheme 6. Mass spectral fragmentation patterns of flavonoids in *Ceratiola ericoides*.

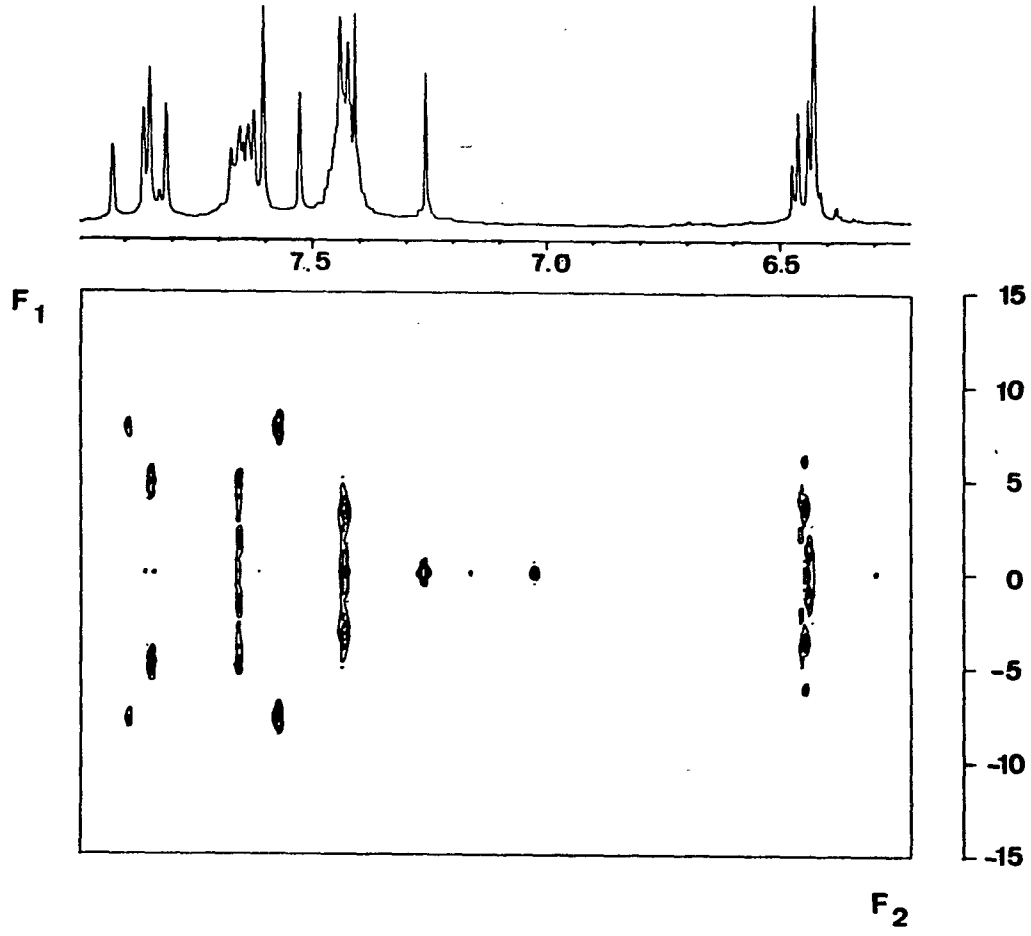
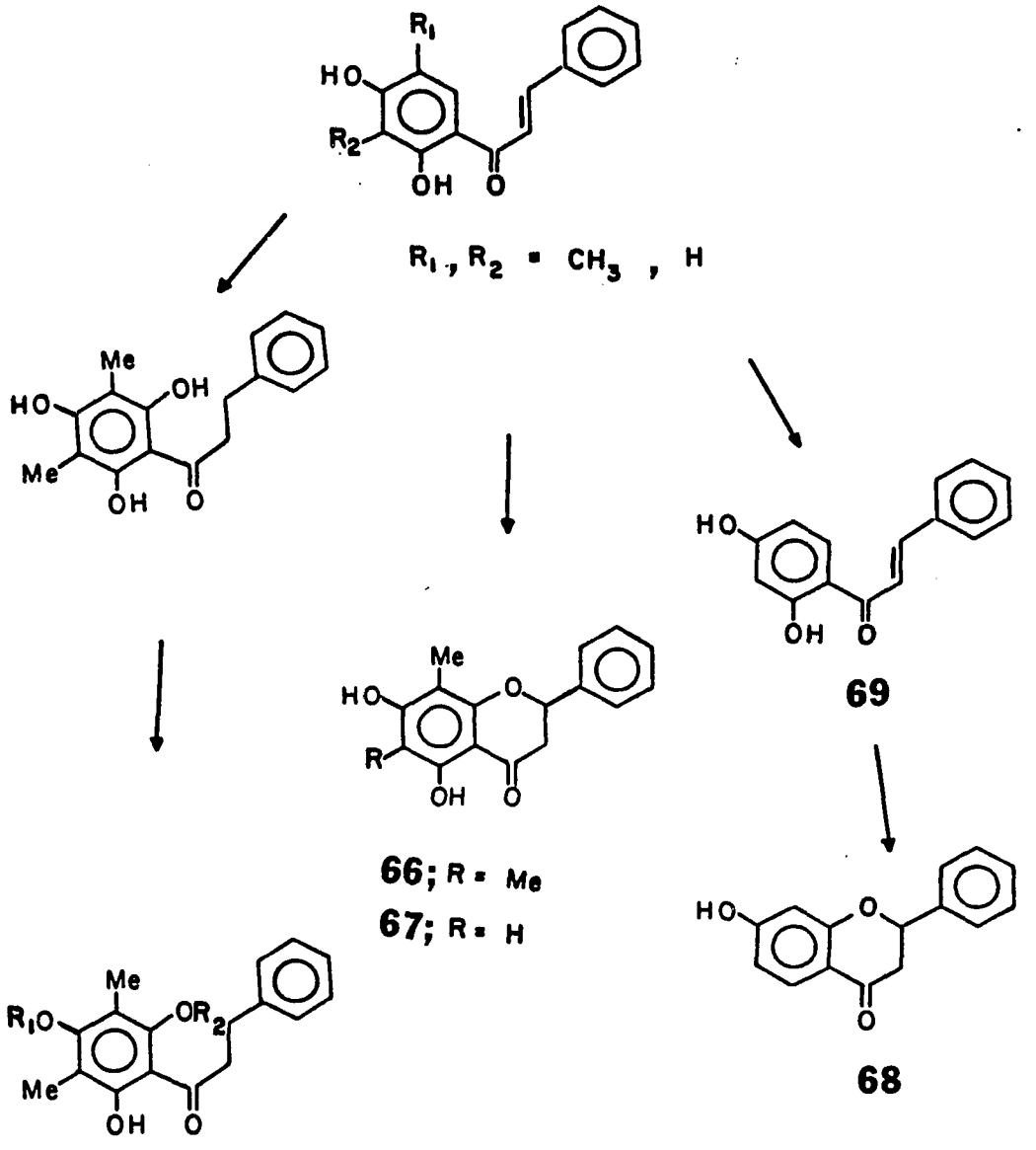


Figure 13. Two dimensional J-Resolved spectrum of 2', 4'-dihydroxy- chalcone **(69)**.

methyl signal. The ultraviolet spectrum had maxima for the phloroglucinol ring at 290 nm and for the cinnamate ring at 335 nm. Upon addition of the complexing agent $AlCl_3$, these maxima shifted to 317 nm and 370 nm, respectively, similar to that reported for pinocembrin.[85] This suggested the absence of a sterically hindering alkyl substitution at C-6,[86] leaving C-8 as the only site for methyl substitution.

7-hydroxyflavone (68).[87] Compound **68** had a ^1H NMR spectral pattern similar to those of **66** and **67** with differences restricted to the phloroglucinol ring proton resonances. A doublet at δ 6.48 with a coupling constant of 2 Hz was assigned to H-8 and the doublet of a doublet of δ 6.56 (J = 8.5; 2 Hz) to H-6. The doublet at δ 7.87 with a coupling constant 8.5 Hz was assigned to H-5. Mass spectral fragmentation of **68** was typical for a flavanone, the base peak at *m/z* 136 being due to a Retro Diels-Alder fragmentation (Scheme 6).

2', 4'-dihydroxychalcone (69).[88] This chalcone exhibited a complicated ^1H NMR spectrum in which all of the olefinic and aromatic signals in the region δ 6.4 - 7.9 overlapped. Therefore the coupling constants could only be determined from a two-dimensional J-resolved ^1H NMR spectrum which gives chemical shifts in the F_2 dimension and the coupling values (Hz) in the F_1 dimension (Figure 13). The ^{13}C NMR spectrum was in accordance with the data reported in the literature[89] with the carbonyl resonance



Scheme 7. Biogenetic pathways to the flavonoids in *Ceratiola ericoides* which all lack B ring oxygenation.

resonance at δ 192.0, C at δ 120.2, C at δ 144.6 and the hydroxyl-bearing C_2' and C_4' at δ 163.2. The molecular structure of **69** was confirmed by single crystal x-ray diffraction. In the solid state the carbonyl function was found to be *s-cis* to the *trans* carbon carbon double bond and *syn* to a phenolic hydroxyl, forming the expected intramolecular hydrogen bond.

The biogenetic relationships between **64** to **69**, which all belong to the less common flavonoid group lacking B ring oxidation, are depicted in Scheme 7.

III-2.2.3. Biological activities of flavonoids 64-69.

Biological activities of compounds **64-69** had not been previously reported except for assessments of the cytotoxic and antimicrobial potential of angoletin **(64)** and its benzyl derivatives.[80] No activity was observed in the case of **64**.

Five of the six known flavonoids isolated from *C. ericoides* were tested only in the presence of saturated aqueous solutions of ursolic acid and erythrodiol mixture as there was not enough material for tests without the triterpene mixture. Comparison of the radicle growth activities of each compound in solution with the triterpene mixture with that of a solution of the triterpene mixture alone yielded opposite effects on *L. sativa* and

S. scoparium. The compounds stimulated growth of *L. sativa* while inhibitions as low as almost 50% were observed for *S. scoparium*. When compared with water controls, which could be a better comparison for assession the behavior of the natural system, 6,8-dimethylpinocembin **(66)** was the only compound that showed a statistically significant inhibition. Although the other flavanoids caused some decrease in the radicle lengths of *S. Scoparium* test seedlings, none of these activities observed were statistically significant. *S. scoparium* germination was not significantly affected by the flavanoids with or without the triterpene mixture.

III-2.3. Volatiles from *C. ericoides*.

A steam distillation was carried out with the hexane extract of *C. ericoides* and the distillate was analyzed by GC-MS. Along with a number of unidentified mono- and sesquiterpenes, caryophyllene **(89)**, humulene **(57)**, γ-amorphene **(70)**, faresol **((71)**, geraniol propanoate **(72)**, β-ionone **(73)**, γ-ionone **(74)**, eugenol **(75)**, salicylic acid methyl ester **(76)**, camphor **(16)**, and α-pinene (13) were detected with a confidence level of greater than 97% by comparison of their mass spectra with those of standards stored in the mass spectral library. In the same distillate, 4-phenyl,2-butanone **(68)**, benzaldehyde

Table 15. Results of bioassays with *C. ericoides* flavonoids dissolved in a saturated aqueous solution of an ursolic acid-erythrodiol mixture.

	L. sativa				*S. scoparium*			
	% germination[+]		%radicle length[+]		%germination[+]		% radicle length[+]	
	H_2O[≠]	triterpene mixture[≠]	H_2O	triterpene mixture	H_2O	triterpene mixture	H_2O	triterpene mixture
angoletin **(64)**	97	97	90*	101	81	86	85	66*
2',6'-dihydroxy-4' methoxy-3',5'-dimethyldihydrochalcone **(65)**	100	100	107	120*	89	94	90	70
2',4'-dihydroxychalcone **(69)**	88*	88*	121	136*	100	106	91	71
6,8-dimethylpinocembrin **(66)**	86*	86*	103	115	92	97	66*	52*
8-methylpinocembrin **(67)**	90*	90*	117	132*	95	100	73	57*
triterpene mixture	100	---	89*	-----	95	---	128	---

[+]Germination and radicle lengths are expressed as percentages of their controls.
[≠]Each sample was compared to a H_2O control as well as a triterpene mixture control.
*An asterix indicates significant difference from the control at $p \leq 0.05$.

(69) and angoletin **(64)** or its isomer **65** have been identified by their characteristic mass spectral fragmentation patterns.

It was observed that fresh *C. ericoides* leaves are odorless, but when dried they emanate an odor much like that one encounters near plants in the natural environment. To trap the volatiles in the atmosphere around the dry leaves, nitrogen was passed over dried leaves for eight hours as described for another similar experiment in Section II-3.3. Sampling the air around dry *C. ericoides* leaves indicated the presence of angoletin **(64)**, 2',6'-dihydroxy -4'-methoxy -3',5'-dimethyldihydrochalcone **(65)** and 2',4'-dihydroxychalcone **(69)** as major constituents which were ealso isolated from the dichloromethane extract of *Ceratiola ericoides*. It is interesting to note that 6,8-dimethylpinocembrin **(66)** which exhibited biological activity in the presence of ursolic acid was also detected in the atmosphere around the dry leaves.

II-2. 5. Analysis of the ethyl acetate extract of *C. ericoides*

The ethyl acetate extract of the water-methanol (10%) wash of dried ground *C. ericoides* leaves (Scheme 14) showed appreciable activity as seen in Table 11. A portion of this extract was chromatographed over silica gel. Fraction 19 yielded a white crystalline material which decomposed to

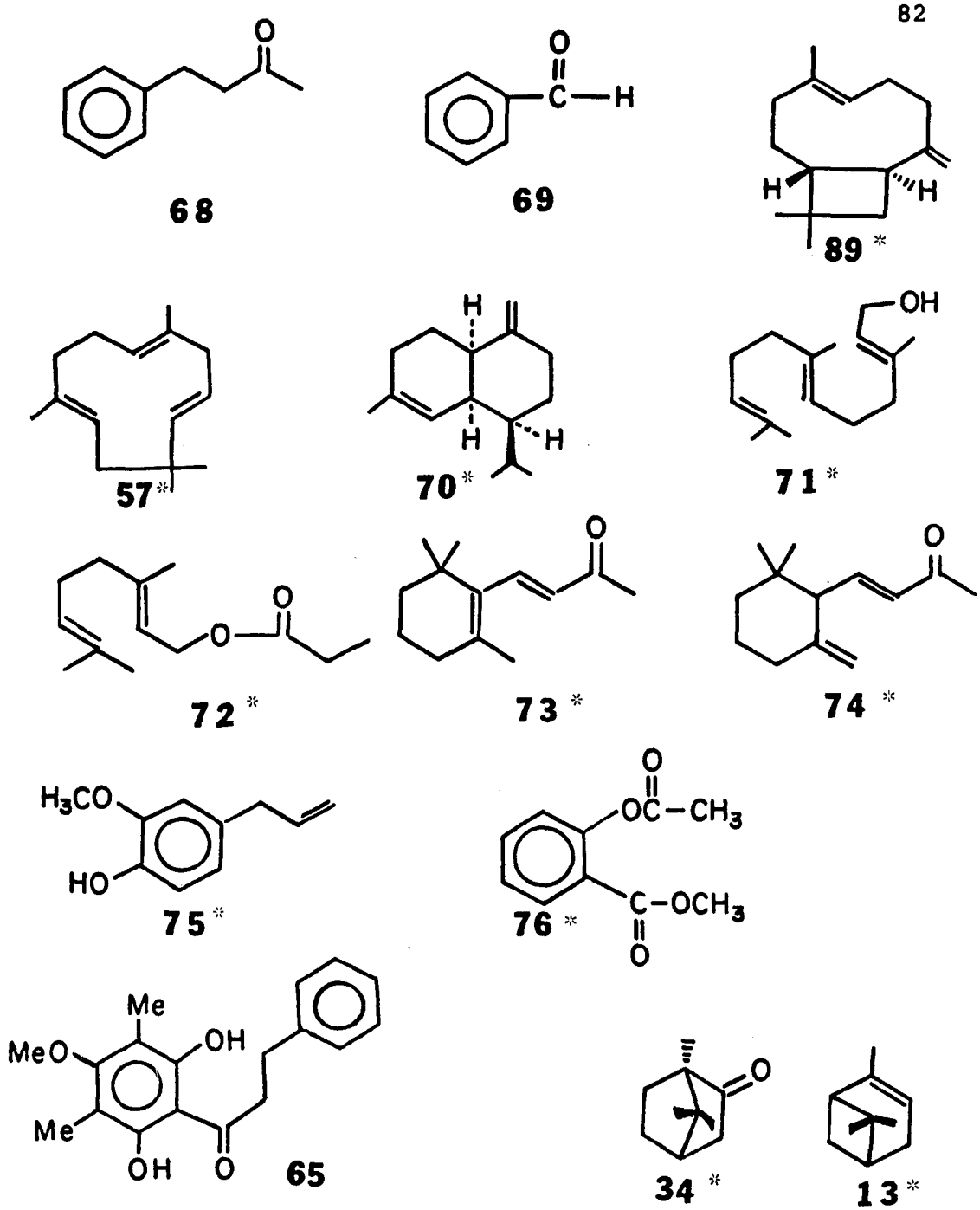
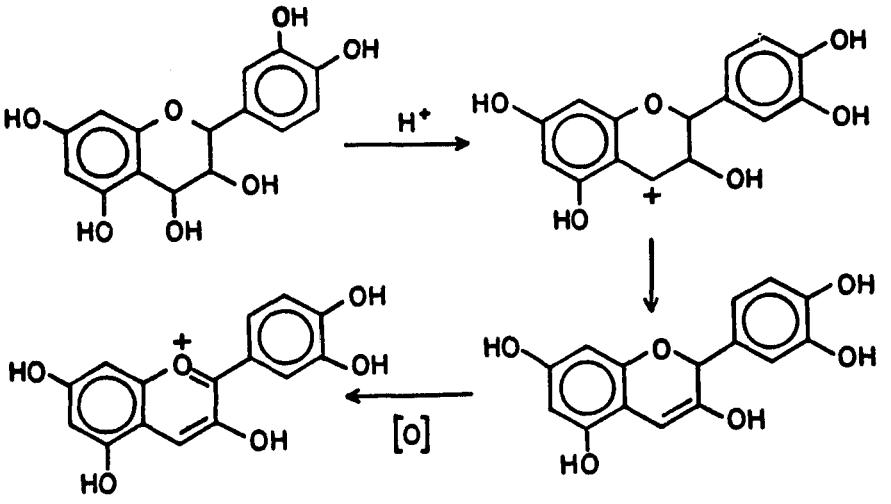


Figure 14.

Volatiles from the hexane extract of *C.ericoides*. Compounds identified with reference to standards in the mass spectral library disk with a confidence level of above 97% are indicated with an asterix (*).

a reddish mass upon heating with MeOH during recrystallization efforts. Its co-occurrence with catechins as well as the facile decomposition behavior implied that this fraction may be composed of 3,4-dihydroxyflavone type compounds.[90]



Scheme 8. Formation of anthocyanidins from leucoanthocyanidins.

The biological activities on *L. sativa* and *S. scoparium* were caused by compounds present in different regions in the chromatogram as shown in Table 16. Germination of *L. sativa* was completely inhibited by decomposed fraction 19 while *S. scoparium* was mostly inhibited by the more polar fractions.

In order to obtain more of the active material, a parallel separation was carried out by partitioning a crude methanol extract of *C. ericoides* leaves between ethyl acetate and water. The glycosides remaining in the aqueous

phase were trapped through absorption by charcoal as described in Section III-4.5.[85] Comparison of the activities of these extracts showed that the activity is much higher in the EtOAc extract (Table 17).

The constituents of the ethyl acetate layer were chromatographed over silica gel. Based on ^1H NMR and TLC comparisons, fractions 11-14 roughly corresponded to the active region of column E-1. Results of the bioassay of fractions 11-16 indicated moderate activity which was more pronounced on *S. scoparium* seeds.

III-2.4.1. Identification of the constituents of ethyl acetate extract fractions 11, 13, and 14.

Major portions of these fractions were acetylated with Ac_2O-pyridine since separations of the free phenols proved difficult.

Epicatechin (77) was the major component of fraction 11. The mass spectrum of the free phenol showed a molecular ion at *m/z* 290. Characteristic fragmentation of the pyran ring gave the ions *m/z* 152 and *m/z* 139. The ^1H NMR spectrum of the acetate had two typical doublet of doublets at δ 2.89 and δ 3.00 for the H-4 protons. The very small coupling between H-2 at δ 5.07 and H-3 at δ 5.37

Table 16. Bioassay results for column fractions of ethyl acetate extract (E_1).

	L. sativa		*S. scoparium*	
Fraction	%germination[+]	length[+]	%germination[+]	length[+]
1-9	103	87*	100	157
10-11	99	75*	125	112
12-16	103	100	100	67*
17-18	95	12*	150	11*
19 (degraded)	0*	--	125	83
20-21	114	31*	100	27*
22-23	103	31*	50	11*
24-29	107	43*	0*	--
30-36	103	42*	0*	--
37-42	103	69*	25	27*
43-46	110	101	175	100
47	99	58*	175	93

[+]Germinations and radicle lengths are expressed as percentages of their controls.

*An asterix indicates significant difference from the control at $p \leq 0.05$.

Table 17. Comparison of the activities from the ethyl acetate extract and the glycosides from *C. ericoides*

Sample	*Lactuca sativa*		*Schizachyrium scoparium*	
	% germination[+]	% radicle length[+]	% germination[+]	% radicle length[+]
EtOAc phase	64*	23*	30*	18*
Glycosides in H_2O phase	79*	72*	90	94

[+]Germination and radicle lengths are expressed as percentages of the control.
*An asterix means a significant difference from the control at $p \leq 0.05$.

which led only to the broadening of signals was in accordance with a *syn* substitution of H-2 and H-3 on the pyran ring. The doublets at δ 6.55 and δ 6.67 were assigned to H-6 and H-8, respectively. The ^{13}C NMR spectrum gave a triplet at δ 25.4 for C-4, a doublet at δ 66.1 for C-3, a doublet at δ 76.0 for C-2 along with the expected signals for the two aromatic rings. The structure of **77** was confirmed by TLC and ^{1}H NMR comparisons with an acetylated epicatechin standard.

The minor component, the acetate of catechin **(78)**, had a similar ^{1}H NMR spectrum. The major difference was the large coupling between H-2 and H-3 (6 Hz) in this case which would be expected from the anti substitution of H-2 and H-3 on the pyran ring. The structure of **78** was also confirmed by a ^{1}H NMR comparison with a standard of catechin acetate.

Acetylation of a mixture of fractions 13 and 14 yielded three other components along with the acetate of epicatechin **(77)**. These compounds gave spectra similar to epicatechin but contained several extra signals. Inspection of the COSY ^{1}H NMR spectrum of the aromatic region of compund **79**, which is almost identical to compounds **80** and **81**, indicated the presence of four aromatic rings per molecule. Excluding the acetyl carbons,

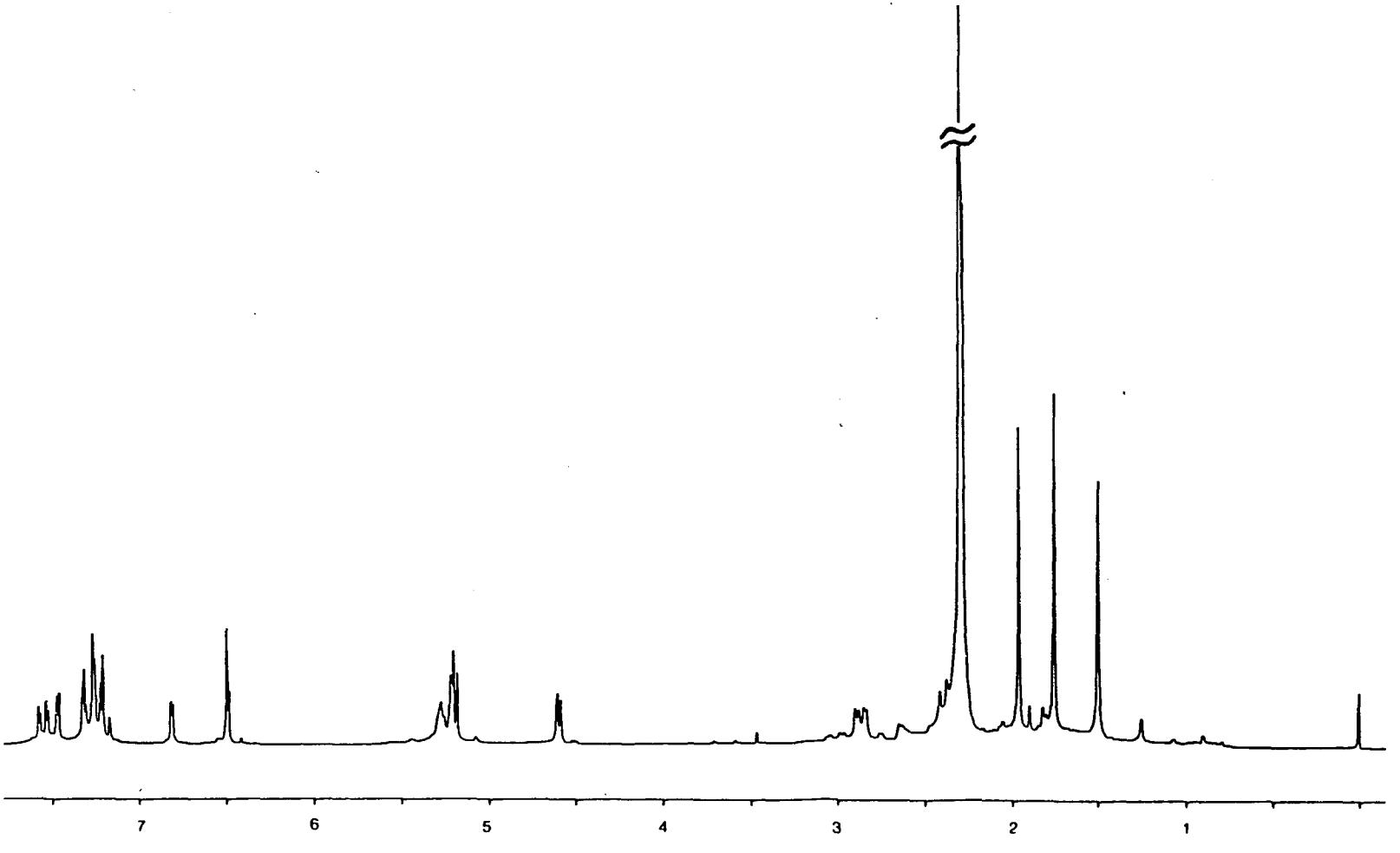


Figure 15. ^{1}H NMR spectrum of **79**.

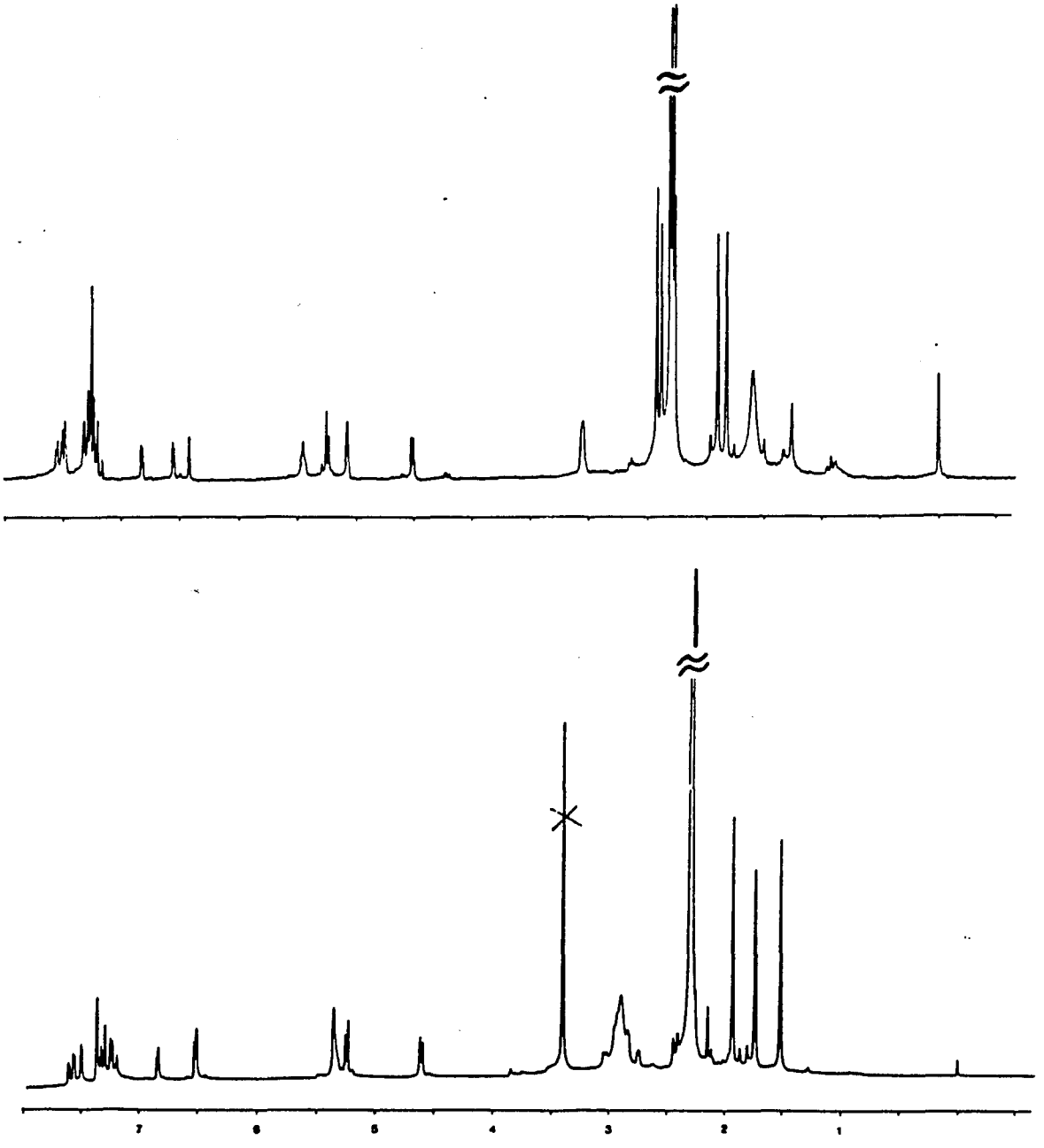


Figure 16. ^{1}H NMR spectrums of unidentified A-type dimers.

thirty carbons were observed for **70** in its ^{13}C NMR spectrum. These observations suggested that these compounds might represent dimeric proanthocyanidins.

According to available literature,[91] oligomers of catechin (**78**) and epicatechin (**77**) exist as two types. Type B consists of units attached by one C-C bond ($4_u \rightarrow 8_l$ or $4_u \rightarrow 6_l$). Due to slow rotation in such systems,[92,93] the ^1H and ^{13}C NMR signals appear as partially resolved, broad peaks at ambient temperature. In contrast, compounds of the A-type typically have two bonds of attachment of the two units ($4_u \rightarrow 8_l$; $2_u \rightarrow \underline{O} \rightarrow 7_l$). This locks these molecules into rigid conformations exhibiting sharp ^1H NMR signals.

The dimeric compounds isolated from *C. ericoides* had sharp ^1H NMR signals implying the presence of rigid structures of the A-type. The ^1H NMR spectrum of **79** contained only one H-8 signal. The ^{13}C NMR spectrum contained a methine as well as a methylene signal in the region of the C-4 resonance. The signal for one C-2 was a singlet at δ 97.7 which is in accordance with a ketal functionality.[94] From these observations, it was deduced that C-2_u, C-4_u and C-8_l were three of the four points of attachment. Since in the ^{13}C NMR spectrum, only nine acetyl carbonyl absorptions were available in the acetyl

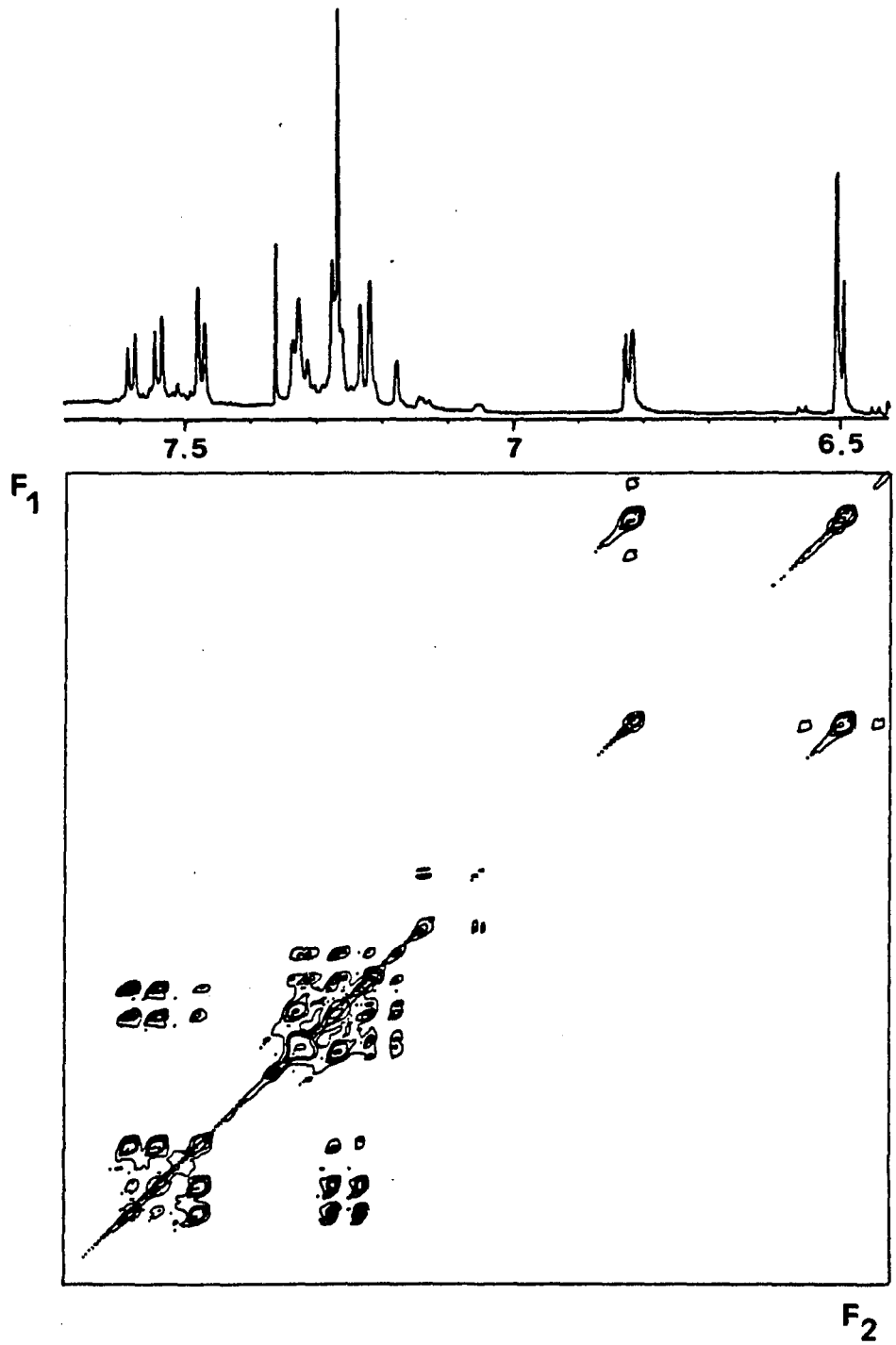


Figure 17. Two dimensional ^{1}H NMR COSY 45 spectrum of the aromatic region of **79**.

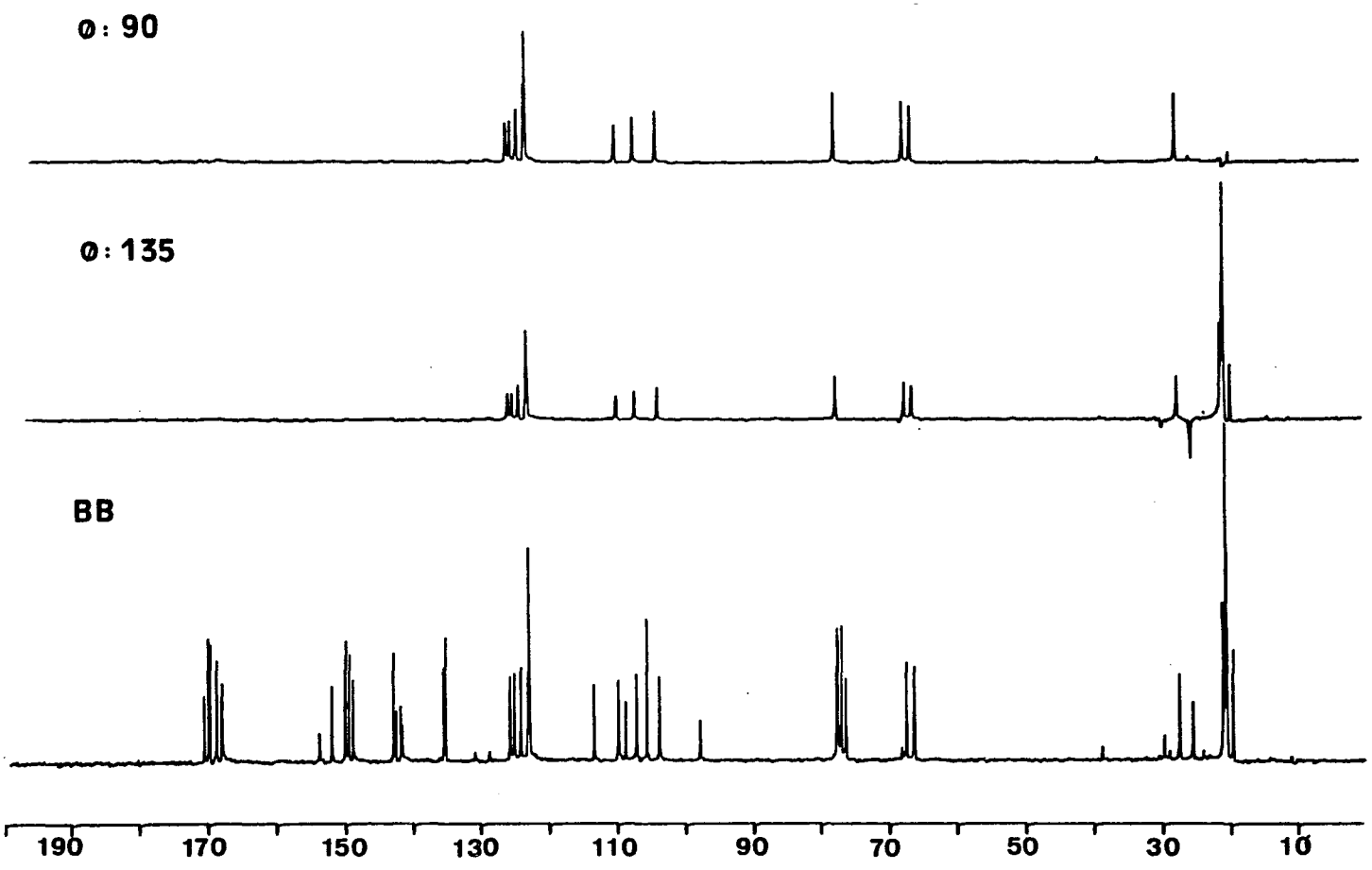
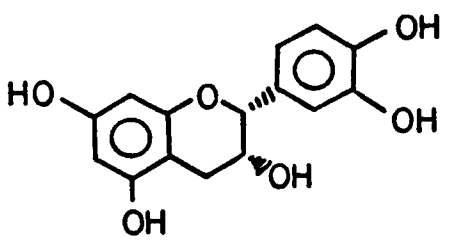


Figure 18. ^{13}C NMR spectrum of **79**.

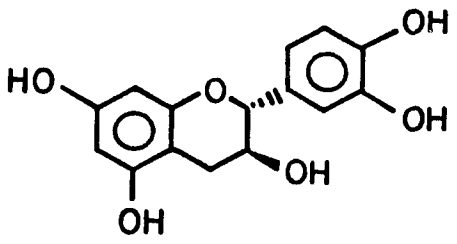
carbonyl region (δ 167 - 170) the final attachment had to be through one of the oxygen functions in the lower flavan unit. ^{1}H and ^{13}C NMR values of epicatechin ($4_u \rightarrow 8_l$; $2_u \rightarrow \underline{O} \rightarrow 7_l$) epicatechin (A-2) were in best agreement with those of the dimer in question. However, since not all values agreed with data reported in the literature and the coupling constants were not available for all proton absorptions[96], other structural alternatives had to be considered. A Dreiding model of a molecule with the acetal bond between C-2_u and C-$3'_l$ seemed to be much less strained than the A-2 dimer itself. This molecule required close proximity between H-2_l and H-6_u. However no ^{1}H NMR NOE effects could be observed between H-2_l and H-6_u at double irradiation powers 51L-31L. Therefore the possibility of the proposed structure was precluded for **79** although it still remains as one of the possibilities for **80** and **81**. Co-TLC and ^{1}H NMR comparisons of dimer **79** with an authentic sample of the acetate confirmed the structural identity.

The A-2 dimer was first isolated by Mayer and coworkers from *Aesculus hippocastanum*.[95] The structure was solved later by Haslam and coworkers.[96] Recently other papers have appeared on this and related compounds.[97]

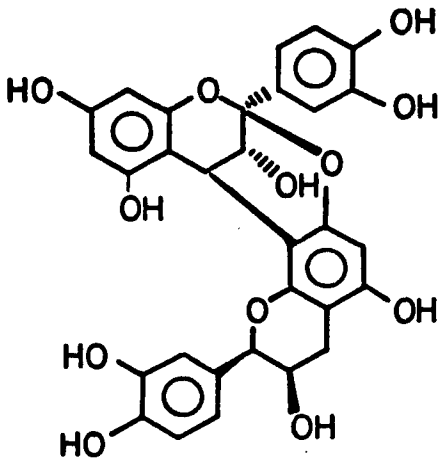
Compounds **80** and **81** gave ^{1}H NMR signals similar to those of **79** with small differences in the chemical shifts of the proton absorptions. This implied that compounds **80**



77

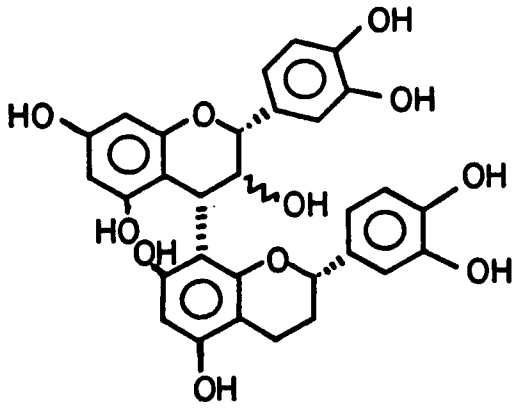


78



79

A - type
dimer



B - type
dimer

Figure 19. Catechins and proanthocyanidins.

Table 18. Growth and germination inhibitions of the column fractions from *C. ericoides* ethyl acetate partition of the methanol extract.

Fraction	*L. sativa*		*S. scoparium*	
	% germination[+]	% radicle length[+]	%germination[+]	% radicle length[+]
E-3; 11-50 ppm	102	95	104	62*
E-3; 11-250 ppm	102	70*	88	36*
E-3; 13-50 ppm	93	86*	73	100
E-3; 13-250 ppm	100	72*	58*	44*
E-3; 14-50 ppm	100	75*	73	43*
E-3; 14-250 ppm	100	66*	100	47*
E-3; 16-50 ppm	102	97	96	59*
E-3; 16-250 ppm	95	82*	62*	54*

[+]Germinations and radicle lengths are expressed as percentages of the controls.

*An asterix indicates significant difference from the control at $p \leq 0.05$.

and **81** are isomers of A-2. Interestingly, decomposition of their acetate occured upon standing at ambient temperature for two weeks while the A-type dimers are reported to be stable under these conditions.[91]

III-2.4.2. Biological activities of proanthocyanidins

Proanthocyanidins have been reported to show various biological activities. The tannins in mature peanut skins, which are mostly of the type A, are credited with fungistatic properties.[91] Reports have also appeared on the inhibitory activities of proanthocyanidins on bacteria.[98] The inhibition of bacteria by proanthocyanidins becomes significant when the effects on nitrifying bacteria are considered. As little as 2 ppm condensed tannins added to soil completely inhibited oxidation of NH_4^+ to NO_2 within three weeks.[99] The concentrations of condensed tannins in the top 15 cm of soils under study were quantified as varying between 8 and 93 ppm. This would indirectly inhibit the growth of plants such as grasses with roots near the soil surface.

Synergistic effects from tannins have been observed from the inhibition of growth of *Avena sativa* coleoptile segments. Condensed tannin alone caused slight inhibition while condensed tannin mixed with small concentrations of indole acetic acid completely blocked root segment elongation.[100]

Monomeric catechins found in *Ceratiola ericoides* exhibited small to moderate growth inhibitions on *L. sativa* while only catechin **(78)** itself exhibited a slight germination inhibition on *S. scoparium* as seen in Table 19. The A-2 dimer **(79)** did not exhibit activity when tested alone while the mixture of A-1 epicatechin ($4_u \rightarrow 8_\ell$; $2_u \underline{O} \rightarrow 7_\ell$) catechin and A-2 showed an activity equivalent to the activity of the fraction containing A-type dimers. Although the presence of A-1 in *C. ericoides* has not been confirmed, this result is significant in demonstrating that mixtures of A-type dimers can be selectively active on the native grass which is also the case with *C. ericoides's* ethyl acetate fractions that contain proanthocyanidins (Fractions 13-14 in Table 18).

III-2.5. Water washes of fresh whole *C. ericoides* leaves.

When fresh whole leaves of *C. ericoides* were dipped into water at ambient temperature, the ethyl acetate-chloroform extract of the water layer almost exclusively contained one major compound, which was named ceratiolin, as can be seen in the crude ^1H NMR spectrum (Figure 20). The compound was suspected to have ecological significance for the following reasons: a compound on the surface of a leaf is more likely to be washed into the soil with

Table 19. Germination and growth inhibitions by standards of the catechins found in *Ceratiola ericoides*.

Compound	conc. (ppm)	*Lactuca sativa* % germination[+]	*Lactuca sativa* % radicle length[+]	*Schizachyrium scoparium* % germination	*Schizachyrium scoparium* % radicle length[+]
catechin (78)	62	95	87*	66*	122
catechin (78)	620	97	92*	84	107
epicatechin (77)	62	94	75*	77	136*
epicatechin (77)	620	97	64*	73	113
A-2 dimer (78)	62	85	96	114	102
A-2 dimer (79)	620	92	70*	114	76
A-1 + A-2	62	110	98	97	126
A-1 + A-2	620	98	67*	100	34*

[+]Germination and radicle lengths are expressed as percentages of the controls.

*An asterix indicates significant difference from control at $p < 0.05$.

rainfall than an internal leaf constituent. Unless a plant possesses special release mechanisms for an active constituent, or the active constituent is formed when plant tissue decomposes on the ground, external constituents seem to play a more important role in allelopathy. It was for this reason and the availability of a larger quantity of this new compound that this natural product was investigated more extensively.

III-2.5.1. Structure elucidation of ceratiolin (82)

Ceratiolin **(82)**, which is a novel natural product, was obtained as a relatively unstable pale yellow solid. Elemental analysis along with the molecular ion observed in the GC-MS spectrum of the diacetylated derivative at *m/z* 386 indicated a molecular formula $C_{17}H_{18}O_5$ for the parent molecule. A broadened aromatic singlet at δ 7.26 in the ^1H NMR spectrum accounted for five protons. Two adjacent groups were indicated by the coupling in the ^1H NMR spectrum with signals at δ 2.99 and δ 3.25 as well as inverted ^{13}C NMR DEPT signals obtained at $\Phi = 135°$. Mass spectral peaks at *m/z* 77, 91, 105, and 133 supported the foregoing data indicating the presence of a β-phenylpropionyl moiety.

The remainder of the molecule mainly consisted of quaternary carbon atoms which made it difficult to

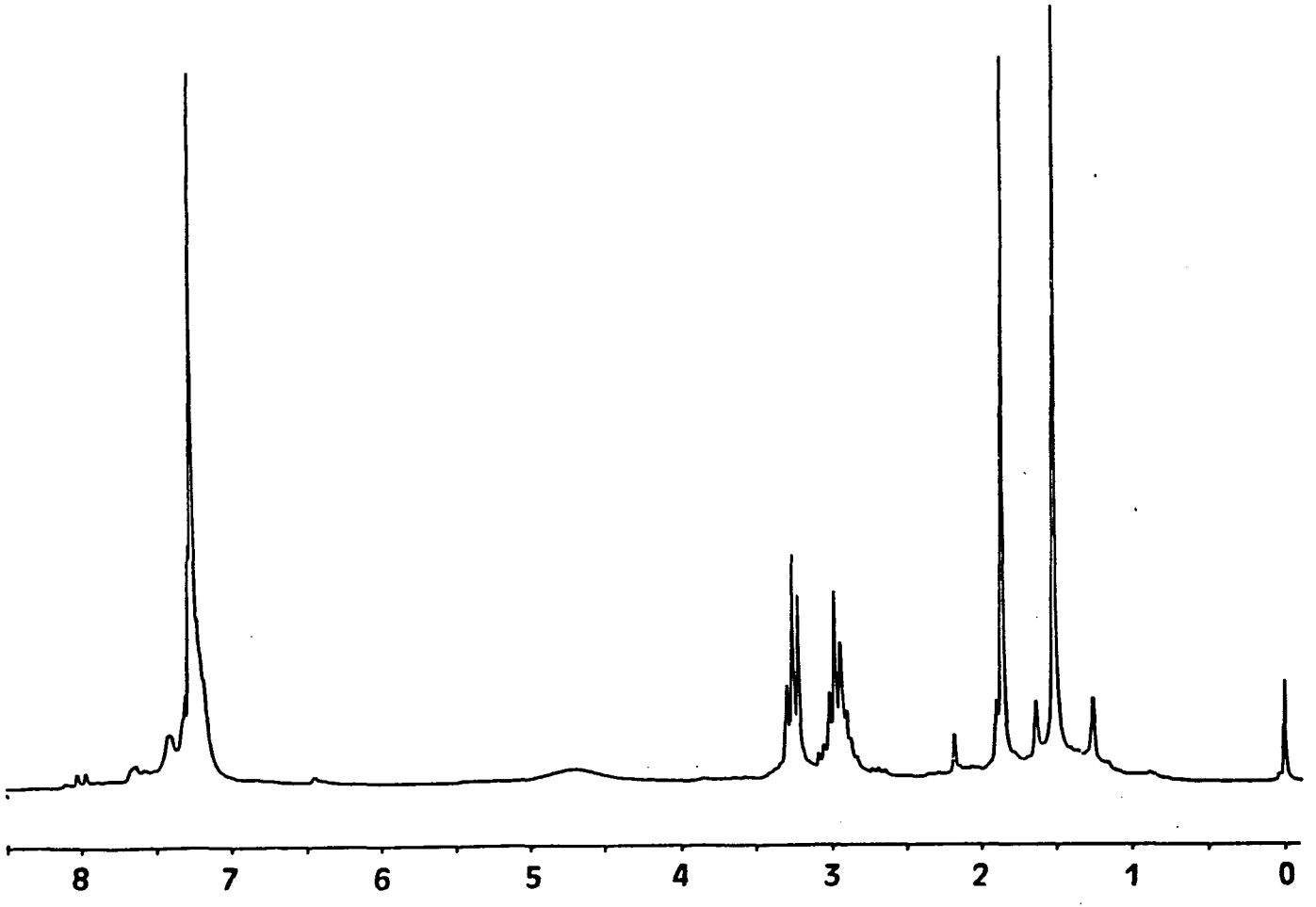


Figure 20. ^{1}H NMR spectrum of the EtOAc-chloroform extract of water washes of *Ceratiola ericoides* leaf surfaces.

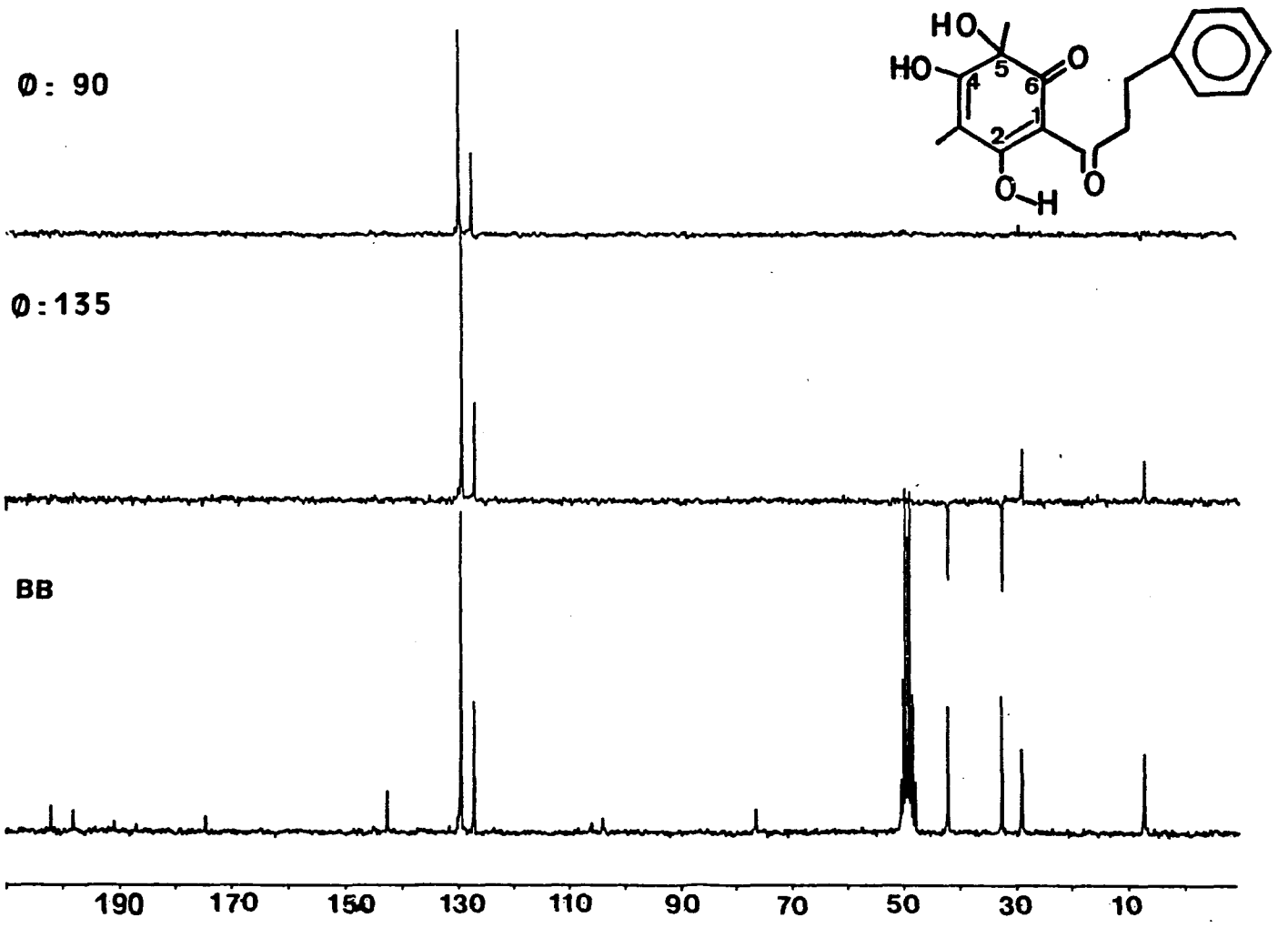


Figure 21. ^{13}C NMR spectrum of 82.

determine the connectivity of the carbon skeleton through ^{1}H NMR analysis. The compound did not dissolve in the common NMR solvents in large enough quantities to enable a ^{13}C NMR INADEQUATE experiment, which would have shown which carbons are coupled to each other. Efforts to grow single crystals of ceratiolin failed as well as attempts to prepare crystals from the copper II complex of the molecule. Therefore more indirect reasoning was utilized in determining its structure.

Formation of two 2,4-dinitrophenylhydrazone derivatives, as detected by TLC, implied the presence of at least two carbonyl functionalities in the molecule. The carbonyl functions appeared to have either cross-conjugation or extended-conjugation, as indicated by the strong infrared signals at 1668 cm^{-1} and 1640 cm^{-1}.[88]

A sharp IR peak at 3460 cm^{-1} and a broad shallow band between 3400 cm^{-1} and 2700 cm^{-1} indicated two types of hydroxyl groups in the molecule, without and with hydrogen bonding, respectively. Acetylation of the compound with acetic anhydride-pyridine yielded two ^{1}H NMR acetyl signals at δ 2.1 and δ 2.4. These absorptions corresponded to acetylations of a hydroxyl group on a saturated carbon and a hydroxyl group on a double bond, respectively. A three-proton ^{1}H NMR singlet at δ 1.50 and a ^{13}C NMR quartet at δ 28.8 were in agreement with a methyl group on a carbon bearing a hydroxyl group. The three-proton singlet at δ

1.85 indicated the presence of a methyl group on a double bond. The appearance of a ^{13}C NMR quartet at δ 7.0 was typical of a methyl group on a double bond which is part of an enol moiety.[80,101]

An *in situ* derivatization with trichloroacetylisocyanate (TAI)[104] (Scheme 9) with ^{1}H NMR spectra obtained at 0, 20, 50 minutes and 5 hours, revealed the presence of two reactive hydroxyl groups. The tertiary hydroxyl group, which reacted more slowly, caused an expected β-shift of $\Delta\delta$+0.14 for the methyl signal at δ 1.50. Two effects were observed on the methyl group at the double bond, one of which was due to the hydroxyl group on the double bond. The total effect was $\Delta\delta$ +0.07. Clear effects on the methylene hydrogens of the β-phenylpropionate portion of the molecule were observed, indicating through space effects of the TAI derivatives.

A positive $FeCl_3$ test (green color) indicated the presence of an enolic or a phenolic system. Tripling the spectral window to 30 ppm revealed a D_2O exchangeable ^{1}H NMR singlet at δ 18.86 which is indicative of a very strongly hydrogen-bonded enol signal. Comparative studies described in the literature on keto-enol equilibria of β-diketones indicated that the substituent α to both carbonyl groups has a very marked influence on the keto-

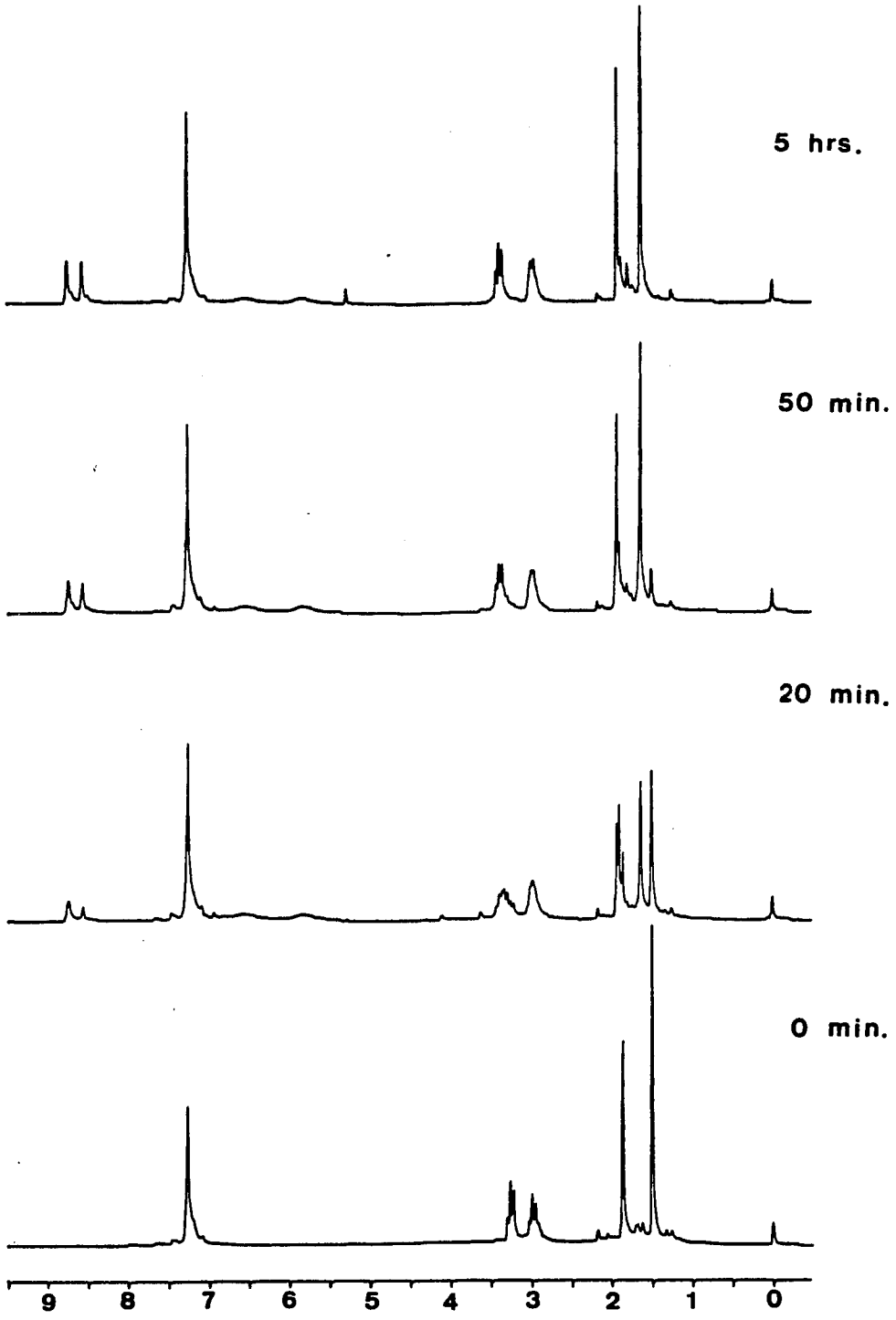
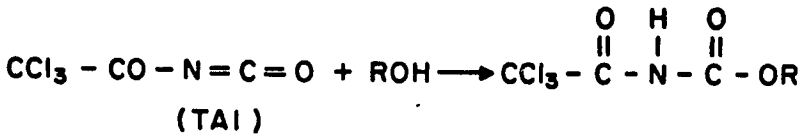


Figure 22. ^{1}H NMR spectrum of 82 with *in situ* TAI derivatization.

Table 20. ^{13}C NMR data* of compounds **64**,+ **65**, and **82** (50,32 MHz)

Carbon	**64**	**65** ($CDCl_3$)	**82** ($MeOH-d_4$)
C_1	141.7 s	141.6 s	142.5 s
C_2	128.5 d	128.4 d	129.4 d
C_3	128.5 d	128.4 d	129.4 d
C_4	126.0 d	125.9 d	127.0 d
C_5	128.5 d	128.4 d	129.4 d
C_6	128.5 d	128.4 d	129.4 d
C'_1	106.6 s	----- s	105.9 s≠
C'_2	159.2 s	158.2 s	191.0 s
C'_3	109.0 s	108.6 s	104.0 s≠
C'_4	159.2 s	162.7 s	174.5 s
C'_5	108.6 s	108.6 s	65.7 s
C'_6	161.5 s	158.2 s	198.0 s
C_α	44.7 t	46.1 t	41.9 t
C_β	31.0 t	30.6 t	32.4 t
CO	205.2 t	205.7 s	201.9 s
OMe	61.9 q	60.3 q	-----
C-3'-Me	7.5 q	8.2 q	7.0 q
C-5'-Me	8.6 q	8.8 q	28.8 q

*Peak multiplicities of compounds **65** and **82** were determined by heteronuclear multipulse programs (DEPT).
+Data obtained from reference 80.
≠Assignments are interchangeable.



Scheme 9. Formation of a derivative with trichloroacetyl-isocyanate (TAI).

enol equilibrium.[103] In the presence of electronegative substituents in this position, the degree of enolization increases dramatically resulting in a sharp enol hydrogen signal which appears at the very downfield end of the ^{1}H NMR region. Tricarbonyl methine compounds which fall into this category give such sharp downfield signals near δ 18 to 19.[104] Accordingly, two sets of three tautomeric forms shown in Figure 23 were the possible structures for ceratiolin. Each one of these structures contain a tricarbonyl methine system and a methyl group or a double bond which is a part of an enol moiety.

In order to investigate the possibility of the existence of a rapidly exchanging equilibrium mixture of tautomers manifesting themselves as a single peak at ambient temperature, ^1H NMR temperature studies were carried out. At -80° in CD_2Cl_2, the only change in the enol signal was a shift from δ 18.86 to δ 19.28 indicating an even stronger hydrogen bonding at low temperature. Due to slower exchange rates at low temperatures, it was also possible to observe the other two hydroxyl protons, one at δ 8.52 for the enol hydroxyl proton and the other at δ 5.09 for the tertiary hydroxyl proton. At elevated temperatures up to 100°C in CD_3-NO_2, the enolic signal shifted upfield to δ 18.68 with accompanied broadening. This could either be due to a decrease of the hydrogen bond strength or indicate formation of an equilibrium between the tautomeric enols. These data at low and high temperatures indicate the existence of one stable tautomer at room temperature in the ceratiolin molecule.

As structures **c** and **f** were not expected to be major tautomers due to the higher energy of the exocyclic double bond, they were not considered as possibilities and structures **a** and **d** were compared with **b** and **e** to determine which tautomer exists at ambient temperature.

Calculations using Woodward-Fieser rules for ultraviolet addition maxima yielded approximately 350 nm as

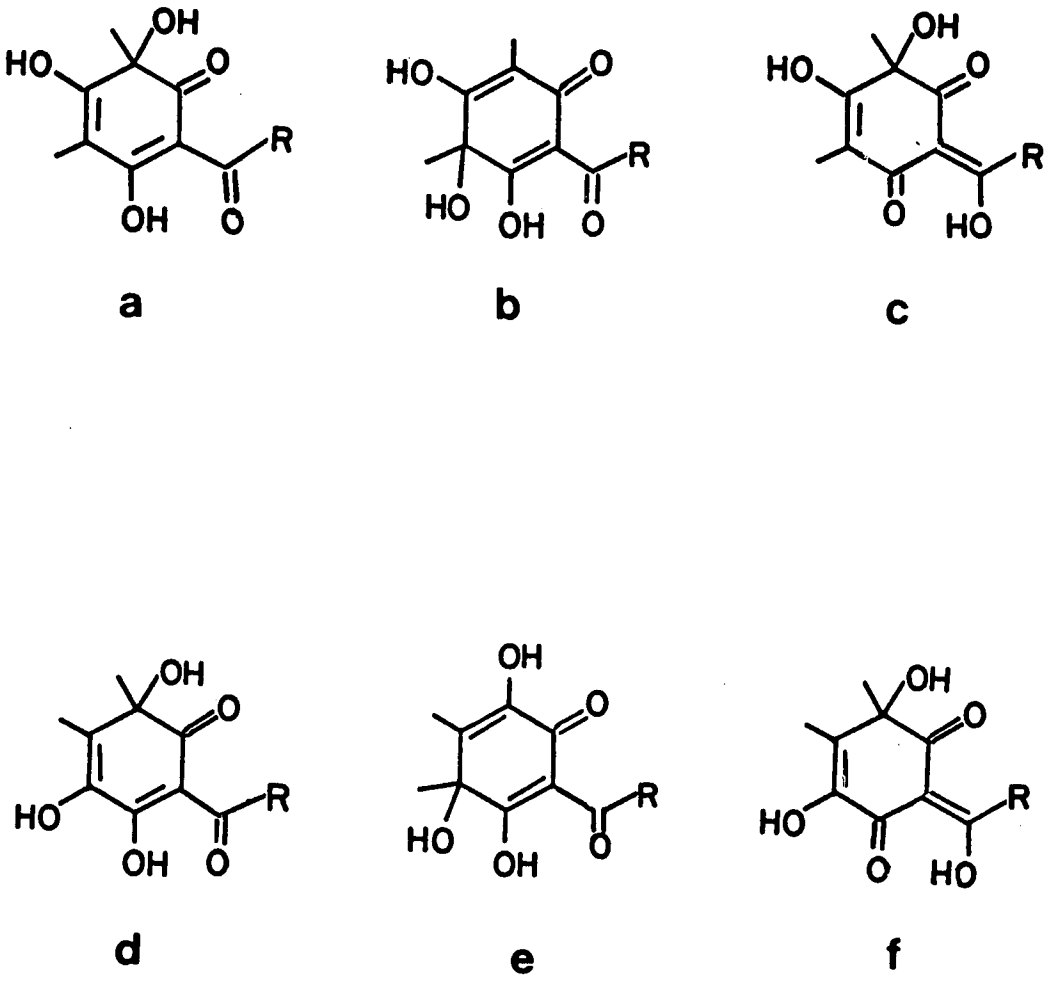


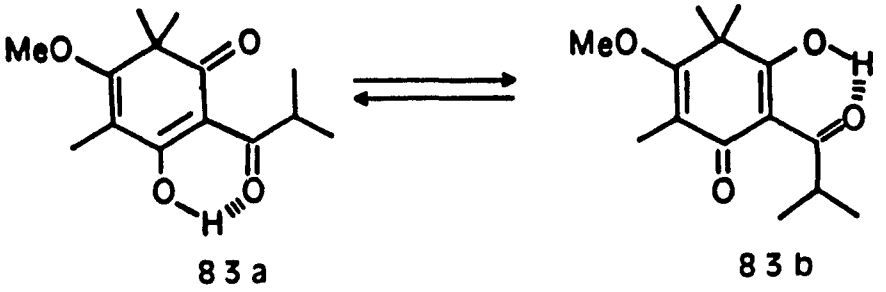
Figure 23. Possible structures for ceratiolin based on basic ^{13}C, ^{1}H NMR and MS arguments.

the maximum for the tautomer with extended conjugation, which is near the experimental values at 358 nm. Calculations for the tautomer with cross-conjugation gave a value around 275 nm. In accordance with this information, the tautomer with the extended conjugation was found to be the more favored form in systems similar to ceratiolin. However the preference is not exclusive. For instance, tasmanone **(83)** exists as a 2:1 mixture of **83a** and **83b** (Figure 24). Despite the similarity of the compounds, ceratiolin **(82)** surprisingly exists as the single tautomer **a** or **d** as indicated by the ^{1}H NMR studies.[105]

With structures a and b, the exclusive preference for the tautomer with extended conjugation could be explained through two effects. In b, less stable cross conjugation replaces the extended conjugation in a and the electron-rich tertiary alcohol group is adjacent to the enol carbon which would somewhat destabilize the system.[106] Furthermore, in tautomer a, a second hydrogen bonding exists between the hydroxyl at C-5' and the oxygen at C-6' which would contribute to an increase in stability of tautomer a. The first argument for tautomers a and b could also be used for explaining the preference of d over e, however the second argument is not valid.

This absence of the additional strengthening argument for d over e along with biogenetically unfavored positions of the hydroxyl group at C-3' and the methyl at C-4' rule

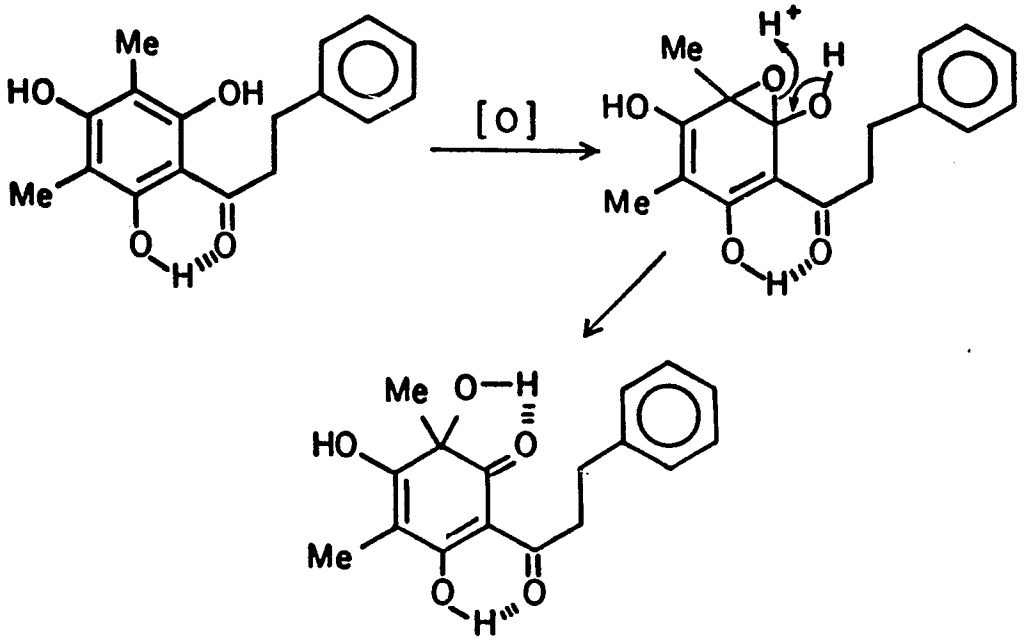
out **d** and **e** as possible structures. The structure of ceratiolin was therefore deduced to be **a** with possible traces of **b**. Scheme 10 shows how ceratiolin **(82)** can be formed biogenetically from the type of flavonoids present in *C. ericoides*. Biological epoxidation of the aromatic ring leads to the formation of a ketone since the molecule does not possess hydrogens at the site of oxidation thus precluding the NIH shift.



83a **83b**

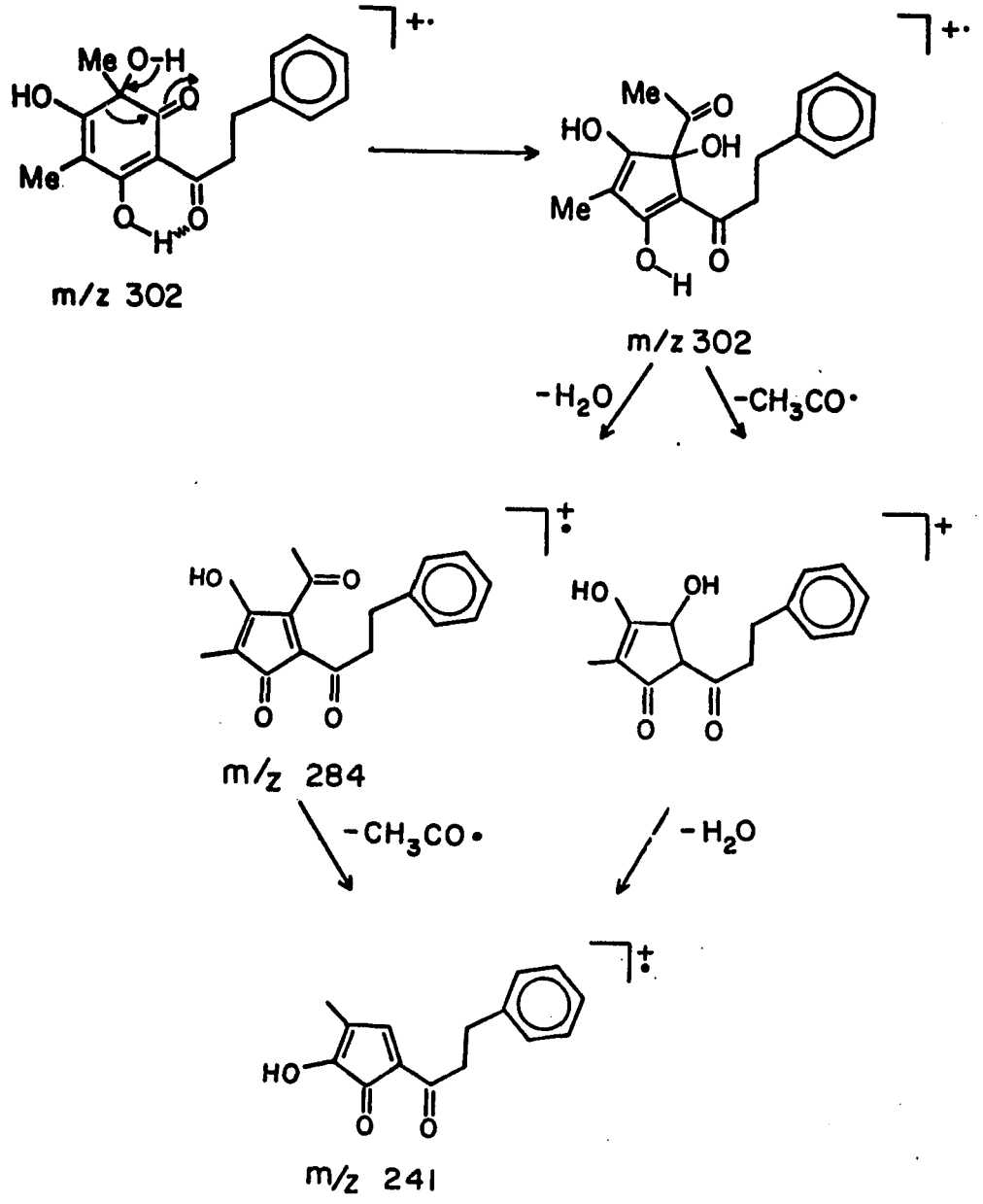
Figure 24. Tautomeric forms of tasmanone **(83)**.

The successive losses of an ethyl group (*m/z* 43) and water (*m/z* 18) or *vica versa* in the mass spectrum of **82** could only be explained by a benzilic acid type rearrangement of the six-membered ring to a five membered ring as shown in Scheme 11. The presence of these mass units in the mass spectrum of **82** confirmed the position of the tertiary hydroxyl group to be adjacent to the carbonyl at C-6'. Rearrangements of this type from a six-membered ring to a five-membered ring have been shown to occur in

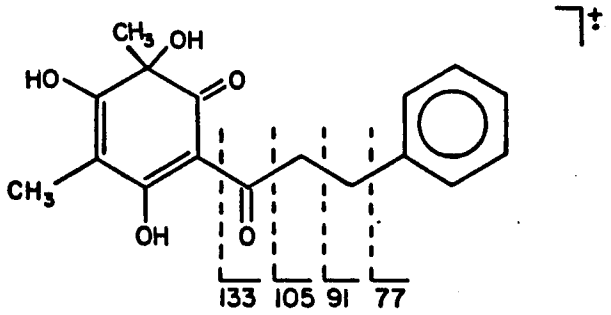


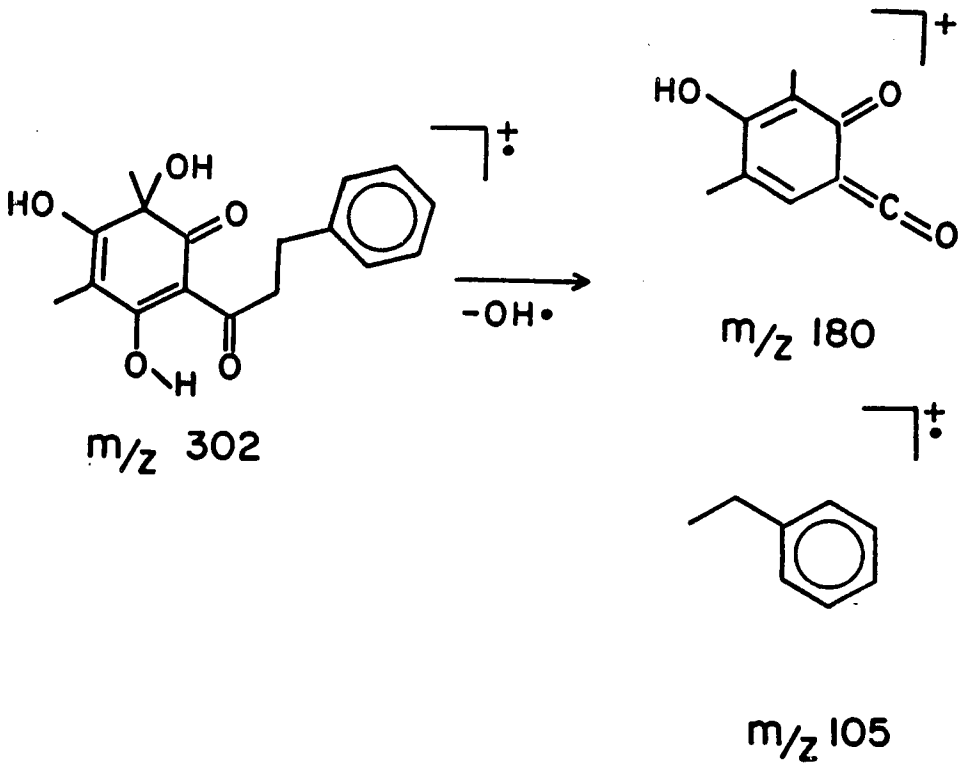
82

Scheme 10. Possible biogenesis of Ceratiolin (82).



Scheme 11. Mass spectral fragmentation of Ceratiolin (82).





Scheme 11. (continued)

basic medium.[107] Variations of such rearrangements have been observed in flavonoid constituents of *Myrica gale*.[82] Biogenetic pathways involving such rearrangements have also been proposed.[108]

III-2.5.2. Degradation products of ceratiolin (82).

As ceratiolin **(82)** decomposes slowly upon standing, experiments were performed to determine what kind of degradation products are formed under natural conditions.

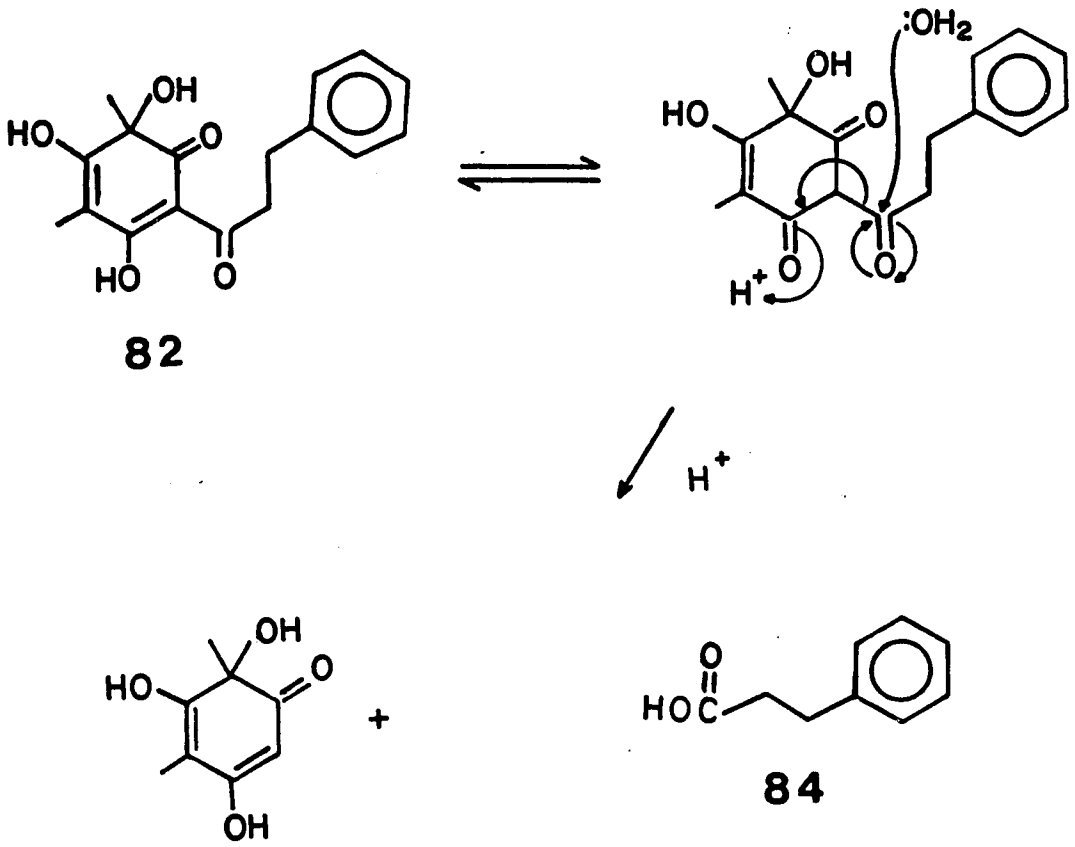
The soil in *Ceratiola ericoides* ecosystem is acidic with pH levels dropping to 4 in some areas. To observe the effects of acid on the compound, an acid degradation was carried out by refluxing **82** in 4N HCl. Hydrocinnamic acid **(84)** and its methylester were formed under the experimental conditions as determined by ^{1}H NMR spectral analysis of the product mixture as well as GC-MS comparisons to standards. Scheme 12 displays the proposed route to the formation of hydrocinnamic acid **(84)** from **82**. The rest of the degradation products appeared to be a brownish polymerized material, degraded beyond identification.

It is interesting to note that ceratiolin **(82)** was not encountered in the crude extracts of *C. ericoides'*, but only in the surface washes with water or 5 minute surface dippings in methanol which contain **82** as the major component. The amount of ceratiolin extracted from dry

plant material stored at room temperature for six months is about 20 times less than the amount obtained from fresh plant material. It follows that **82** is a labile compound which may decompose in crude extracts in the presence of acidic compounds. Hydrocinnamic acid **(84)** which is present in the volatiles of polar fractions of the dichloromethane extract could be one of the degradation products of ceratiolin **(82)** which was originally extracted with CH_2Cl_2.

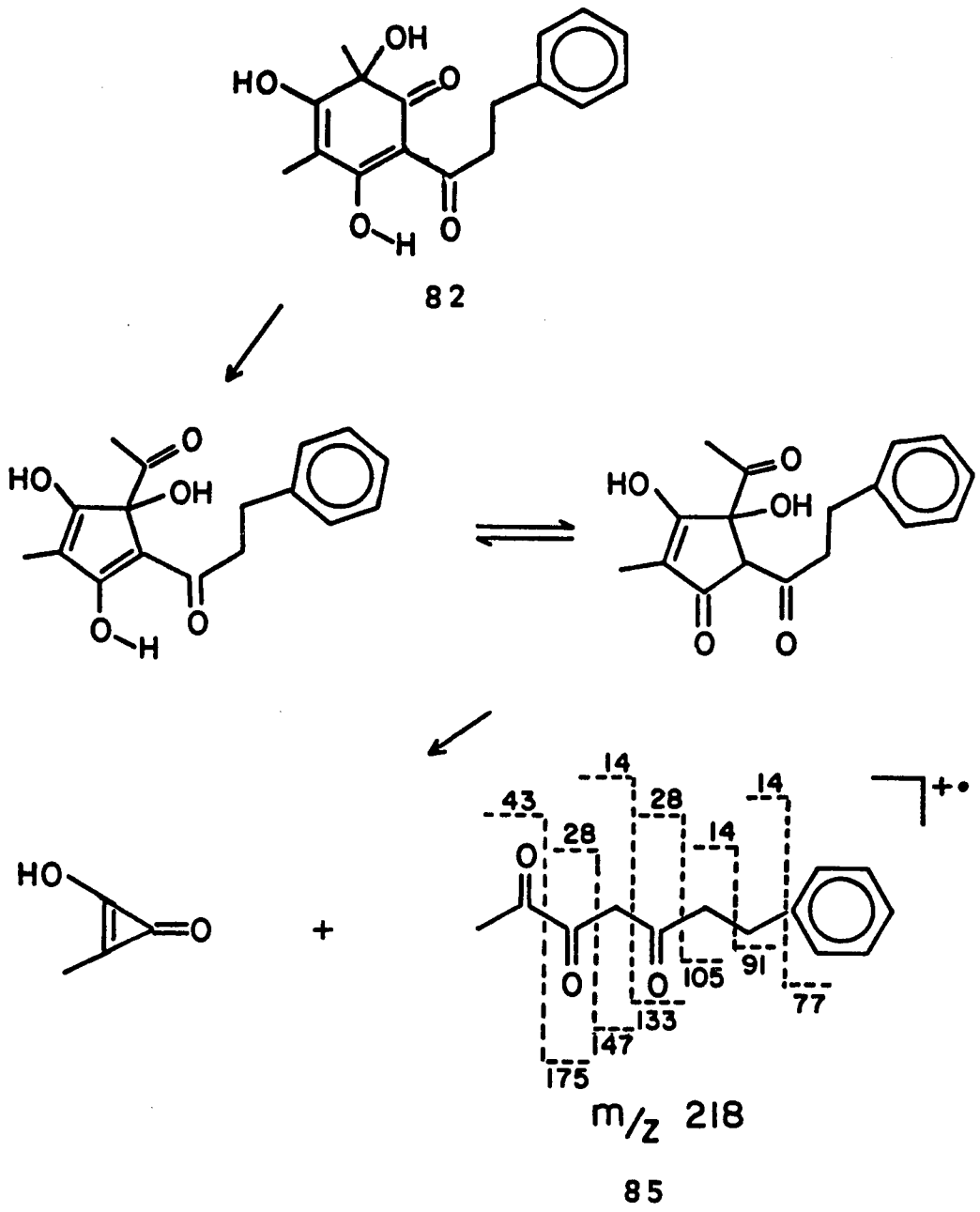
A decomposition was carried out in $CHCl_3$ under sun radiation in a quartz cell for one week. As a result, a small amount of hydrocinnamic acid was formed along with a triketone formed from the five-membered intermediate **(85)** and was identified by its distinct mass spectral fragmentation pattern shown in Scheme 13. Formation of **85** from the five-membered intermediate should be favorable since an aromatic species is expelled from the molecule during the process. The other decomposition products were not identified.

In a second experiment, parallel decompositions were carried out in water where one sample was exposed to sunlight and the other was kept in the dark. Both samples contained hydrocinnamic acid when checked after 3 days. The sample kept in the dark contained trace amounts of the triketide **(85)** while the one exposed to sun radiation contained comparable quantities to that of hydrocinnamic



Scheme 12. Formation of hydrocinnamic acid (84) from 82.

acid (84). Since the formation of these compounds was fast and facile in water, such decomposition products should be forming under natural conditions, especially in the decaying litter on the ground.



Scheme 13. Proposed formation of 85 from 82.

III-2.5.3. Biological activities of ceratiolin (82) and its degradation product hydrocinnamic acid (84).

As can be seem from Table 21, ceratiolin which was tested as a saturated water solution does not exhibit inhibitory activity against test seeds. Moreover, a growth stimulatory effect was observed with ceratiolin on *S. scoparium*. On the other hand, hydrocinnamic acid which forms in water solutions of ceratiolin, shows a high inhibition on both test seeds. At 63 ppm, hydrocinnamic acid has significant activity while equimolar ceratiolin at 125 ppm shows no activity. This may partially explain why the litter washes of *Ceratiola ericoides* show activity while leaf washes show smaller degrees of activity. Since soil under *Ceratiola ericoides* is acidic and would promote the degradation of ceratiolin into hydrocinnamic acid, it is likely to find more activity on the ground rather than in the leaf rain leachates. Other degradation products have not been isolated and tested and more activity may possibly be contributed from those species.

III-3. EXPERIMENTAL

III-3. 1. Plant material.

Aerial parts of *Ceratiola ericoides* (Empetraceae) were collected in September 1983 from Sun Ray, Florida; in June

Table 21. Comparative effects of hydrocinnamic acid **(84)** and ceratiolin **(82)** on germination and radicle growths of test seeds.

Compound	conc. (ppm)	*L. sativa*		*Schizachyrium scoparium*	
		germination[+]	% radicle length[+]	%germination[+]	% radicle length[+]
hydrocinnamic acid	1000	0*	---	0*	---
hydrocinnamic acid	500	0*	---	2*	17
hydrocinnamic acid	250	4*	9	50	32
hydrocinnamic acid	125	95	40	87	53
hydrocinnamic acid	63	109	56	74	65
hydrocinnamic acid	31	100	72	113	88
hydrocinnamic acid	16	104	76	109	85
ceratiolin	125	102	83	102	145

[+]Germinations and radicle lengths are expressed as percentages of the controls.

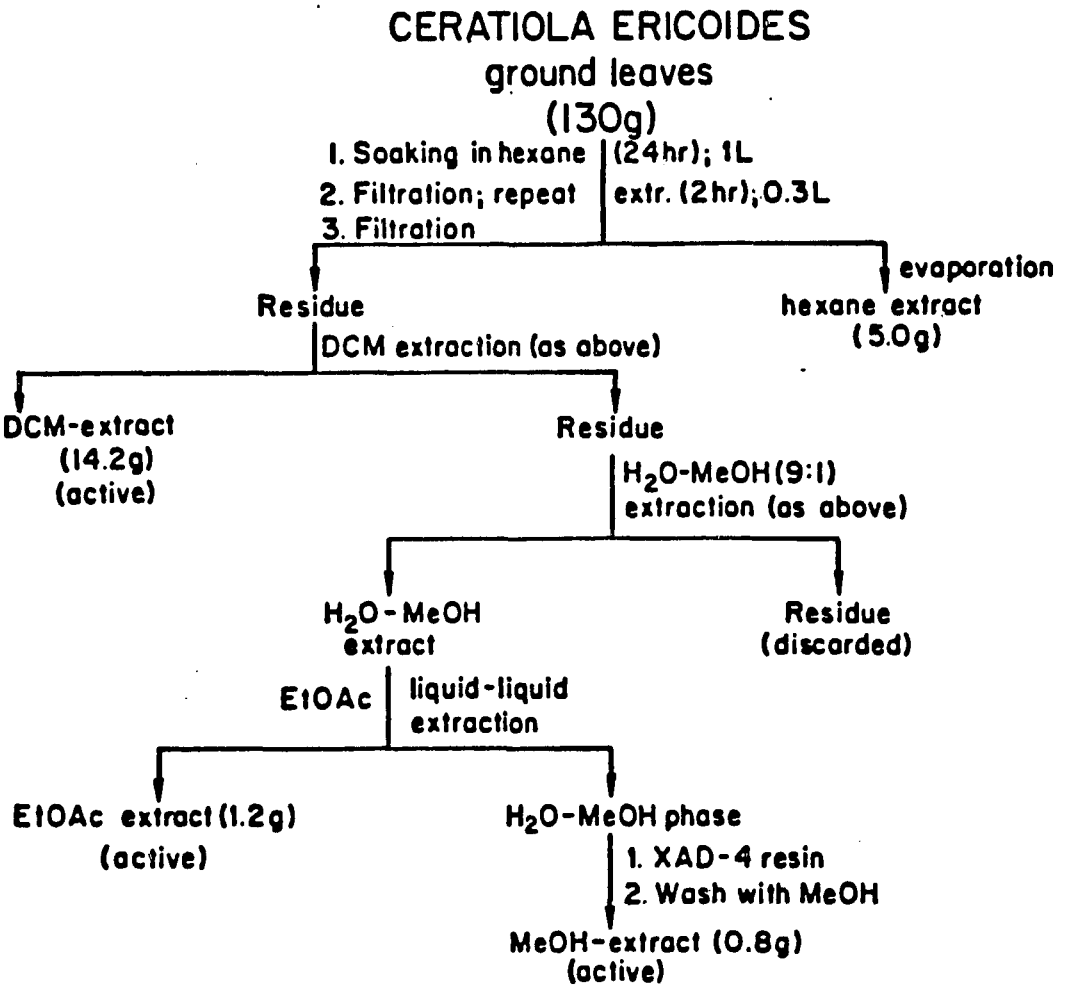
*An asterix indicates significant difference from the control at $p \leq 0.05$.

from Sun Ray, Florida; in September 1984 from Perdido Key, Florida; in October 1985 from Sun Ray, Florida. The plant was identified by Dr. G. B. Williamson, Botany Department, Louisiana State University.

III-3.2. Initial extractions.

The initial crude extractions of leaves collected in September 1983 were extracted as shown in Scheme 14. Bioassays were done on thee crude extracts by taking a saturated solution of 15 mg extract and going through the assay as described in Section II-3.1. As a parallel experiment, 15 g of fresh leaves were extracted with 200 ml MeOH for eight hours in a soxhlet extractor. The extract obtained had a tarry dark appearance.

Roots collected in September 1984 were extracted as follows; 100 g dried roots cut to 1 cm pieces were soaked in 1 L CH_2Cl_2 for 24 hours. 2.1 g of extract was obtained after evaporation of the solvent under vacuum. The mark was then resoaked in 1.5 L MeOH for 24 hours. After evaporation of the solvent, 3.2 g of MeOH extract was obtained. 5 g dried roots cut to 1 cm pieces were separately soaked at 7-8°C in 50 mL distilled H_2O for 2 days. The extracts were bioassayed in the same manner as for the leaf extracts. The H_2O wash was used as is after the root pieces were filtered out.



Scheme 14. Crude extractions of *Ceratiola ericoides* aerial parts.

Litter collected in October 1985 below *C. ericoides* plants was initially soaked in 360 ml distilled H_2O for 24 hours. After filtration, the mark was dried at 35°C for 24 hours. It was subsequently soaked in two portions of 350 ml CH_2Cl_2 (12 hours each).

III-3.3. Isolation of the constituents of the CH_2Cl_2 extract of *C. ericoides* leaves.

III-3.3.1. Isolation of triterpenes 40 and 63.

7.8 g of the 14.2 g CH_2Cl_2 extract precipitated when the solvent was evaporated to 200 ml. The remaining 6.4 crude CH_2Cl_2 extract was chromatographed over Sephadex LH-20 (200 g) using a mixture of $CHCl_3$:MeOH (1:1). 36 fractions of 50 ml each were obtained. Fractions 8-9 (1.1 g) contained two triterpenes, of the same composition as the initial precipitate, and fractions 10-17 (2.3 g) were mainly composed of flavonoids and were recombined.

When fractions 8-9 were stirred with ether: EtOAc (1:1), **63** was solubilized to a much greater extent than **40**. Therefore, after repetitive dissolutions in this solvent mixture, pure **63** was obtained for spectroscopic identification of the compound. In order to separate the triterpenes, 0.5 g of the mixture was acetylated with excess Ac_2O and pyridine as described in Section II-3.1. The mixture was then chromatographed over silica gel using CH_2Cl_2: Acetone (10:1). Fifteen 75 ml fractions were

obtained. Fractions 3-6 contained 142 mg of diacetylated **63** and 7-10 gave 413 mg acetylated **40**.

***erythrodiol* (63).** White solid. IR V_{max} cm^{-1}: 2900, 1440, 1360; EIMS *m/z* (rel. int.): 442 (M, (1.8), 424 (M-H_2O, (0.6), 411 (2.1) [M-CH_2OH], 234 [$C_{16}H_{24}O$] (13.3), 207 [$C_{14}H_{23}O$] (14.9), 203 [$C_{15}H_{21}$] (100), 189 [$C_{14}H_{21}$] (6.5), 107 [C_8H_4] (4.3). ^1H NMR ppm: δ 3.21 d, 3.22 dd, 3.55 d, 5.19 t. ^{13}C NMR ppm: δ 69.6 t, 79.2 d, 124.8 d, 138.1 s. The spectral data of ursolic acid acetate was described in Section II-3.2.

III-3.3.2. Isolation of flavonoids 64 - 69.

The combined fractions 10-17 (150 mg) were chromatographed over silica gel with increasingly polar mixtures of CH_2Cl_2:MeOH (1:0, 10:1, 10:2, 10:3, etc.). 12 fractions (40 ml each) were taken. Fractions 2 and 3 contained **64** (5 mg) and **65** (8 mg) which were separated on a 1 mm silica gel TLC plate utilizing 10% petroleum ether 90% CH_2Cl_2 as the solvent system. Fractions 4-6 yielded **66** (9 mg), **67** (12 mg) which were separated on a 1 mm silica gel TLC plate utilizing CH_2Cl_2 as solvent. Fraction 9 gave **68** (1 mg) and **69** (13 mg), after prep. silica gel TLC separation with CH_2Cl_2: acetone (19:1).

***Angoletin* (64).** Yellow solid; λ_{max} nm (log ε): 291 (4.16) (293 NaOAc); 335 (3.60); ; ^1H NMR ppm: δ 2.10 (3H,s), 2.12 (3H,s), 3.02 - 3.41 (4H,m); 3.67 (3H,s), 5.38

(1H,bs), 7.30 (5H,bs), 13.46 (1H,s).

EIMS *m/z* (rel. int.): 300 (12.1), 283 (2.3), 195 (100.0), 168 (18.2), 91 (22.7).

***2',6'-Dihydroxy-4'-methoxy-3',5'-Dimethyldihydrochalcone* (54).** Yellow solid; λ_{max} nm (log ε): 281 (4.37), 350 (3.62).

EIMS *m/z* (rel. int.): 300 (24.8), 283 (5.7), 195 (100), 168 (19.3), 91 (26.6); ^{1}H NMR ppm: δ 2.1.1 (6H,s), 3.03 and 3.46 (4H,m), 3.71 (3H,s), 7.26 (5H,bs), 9.41 (2H,bs).

***6,8-Dimethylpinocembrin* (66).** White crystals.

EIMS *m/z* (rel. int.): 284 [M^+] (71), 266 [M-H_2O] (3), 207 [M-C_6H_5]$^+$ (45), 180 [M-C_8H_8]$^+$ (75), 152 [M-C_8H_8-CO]$^+$ (100); ^{1}H NMR ppm: δ 2.08 (6H,s), 2.84 (1H, dd, $J_{3a,3b}$ 17.5 Hz), 3.05 (1H, dd $J_{2,36}$ 13 Hz, $J_{3a,3b}$ 17.5 Hz, 5.41 (1H, dd, $J_{2,3a}$ 3 Hz, $J_{2,3b}$ 13 Hz), 7.44 (4H,m).

***8-Methylpinocembrin* (67).** White solid. λ_{max} nm MeOH: 225 sh, 290 (317 w. $AlCl_3$), 335 sh (370 w. $AlCl_3$).

EIMS *m/z* (rel. int.): 270 [M]$^+$ (96), 269 [M-H]$^+$ (49), 193 [M-C_6H_5]$^+$ (100), 166 [M-C_8H_8]$^+$ (48), 138 [M-C_8H_8-CO]$^+$ (64); ^{1}H NMR ppm: δ 2.06 (3H,s), 2.82 (1H, dd, $J_{2,3a}$ 3 Hz, $J_{3a,3b}$ 17.5 Hz), 3.09 (1H, dd, $J_{2,3b}$ 13 Hz, $J_{3a,3b}$ 17.5 Hz), 5.37 (1H, dd, $J_{2,3a}$ 3 Hz, $J_{2,3b}$ 13 Hz), 6.01 (1H,s), 7.44 (4H,bs), 12.31 (1H,s).

***7-hydroxyflavanone* (68).** White solid.

EIMS *m/z* (rel. int.): 240 $[M]^+$ (91), 239 $[M-H]^+$ (64), 163 $[M-C_6H_5]^+$ (73), 136 $[M-C_8H_8]^+$ (100), 108 $[M-C_8H_8-CO]^+$ (51), 104 $[C_8H_8]^+$ (56); ^{1}H NMR ppm: δ 2.84 (1H, dd, $J_{2,3a}$ 3 Hz, $J_{3a,3b}$ 17.5 Hz), 3.06 (1H, dd, $J_{2,3b}$ 13 Hz, $J_{3a,3b}$ 17.5 Hz), 5.48 (1H, dd, $J_{2,3a}$ 3 Hz, $J_{2,3b}$ 13 Hz), 6.48 (1H, d, $J_{6,8}$ 2 Hz), 6.56 (1H, dd, $J_{5,6}$ 8.5 Hz, $J_{6,8}$ 2 Hz), 7.44 (4H,m), 7.87 (1H, d, $J_{5,6}$ 8.5 Hz).

***2',4'-Dihydroxychalcone* (69).** Yellow crystals.

EIMS *m/z* (rel. int.): 240 $[M]^+$ (12), 239 $[M-H]^+$ (77), 163 $[M-C_6H_5]^+$ (100), 137 $[M-C_8H_7]^+$ (77), 77 $[C_6H_5]^+$ (47); ^{1}H NMR ppm: δ 6.44 (1H, d, $J_{6,8}$ 1.5 Hz), 6.45 (1H, dd, $J_{6,8}$ 1.5 Hz, $J_{8,9}$ 7.9 Hz), 7.03 - 7.74 (5H,m), 7.58 (1H, d, J 9.4 Hz), 7.85 (1H, d, $J_{8,9}$ 7.9 Hz), 7.90 (1H, d, J 9.4 Hz); ^{13}C NMR ppm: δ 103.7 d, 108.0 d, 114.2 s, 120.2 d, 128.5 d (2C), 128.9 s (2C), 130.7 d, 132.0 d, 134.6s, 144.6 d, 163.2 s, 166.3 s, 192.0 s.

The ^{1}H NMR 2D-J-RESOLVED spectrum of 2',4' dihydroxychalcone **(69)** was computed from 0.5 K points in the F_2 dimension and 128 points in the F_1 axis. The FID was zero filled to 256 words in the F_1 dimension. 400 Hz x 50 Hz spectral widths were used. Acquisition time was 0.64 s. There was a 2 second delay between acquisitions. 64 transients were completed for each of the 128 traces. After ourier transformation followed by magnitude calculation, the matrix was tilted. The spectrum was symmetrized about the $F_1 = 0$ axis.

To bioassay the flavonoids, initially an excess of the triterpene mixture was dissolved in H_2O with the aid of a sonicator for an hour to obtain a saturated solution. After filtration of undissolved triterpenes, saturated solutions of the flavonoids were prepared by adding 5 mg of each flavonoid to 22 ml of triterpene solution and sonicating this mixture for an hour. After filtration, bioassays were performed as described in Section II-3.1.

III-3.4. Volatiles of *Ceratiola ericoides*.

Two methods were utilized to analyze the volatiles. Volatiles from the hexane extract of *C. ericoides* were obtained by passing steam over 3 g hexane extract coated on the inside walls of a distillation column. The 30 ml aqueous distillate thus obtained was extracted with 10 ml nonagrade CH_2Cl_2 and was subjected to CG-MS analysis. 30 m FSOT column was used for the GC analysis. Injection temperature: 250°; Oven: 45° for 1 min, then 5°/min until 250°; Column pressure: 12 psig. Column flow rate 40 ml/min. Volatiles were also collecIted directly from the atmosphere around intact dry leaves with the method utilizing a large replica of a gas trap as described in Section II-3.3 for *Calamintha ashei* volatiles. GC conditions; Injection temperature: 250°; Oven: initially 70°, then 10°/min to 250°. Column pressure 15 psig and column flow 32 ml/min.

III-3.5. Separations of the ethyl acetate extract.

1200 ml H_2O:MeOH (9:1) wash of 130 g dried, ground *C. ericoides* leaves (Scheme 15) was extracted with 6 x 400 ml of EtOAc. Upon evaporation of the solvent under vacuum, 1.2 g EtOAc extract was obtained.

A silica gel column was run with 600 mg of the EtOAc extract. Elutions were carried out with increasingly polar mixtures of CH_2Cl_2-acetone (10% acetone to 20% acetone) 47 fractions of 50 ml each were collected. Fractions with similar TLC patterns were recombined and each group was bioassayed after preparation of saturated solutions from 15 mg of each fraction in 22 ml H_2O.

To obtain more material in the biologically active region, a parallel extraction was done on plant material collected in June 1984. Dried, ground *C. ericoides* leaves (500 g) were soaked successively in 2 x 2L hexane and 2 x 2L CH_2Cl_2 in order to remove nonpolar compounds. The mark was then soaked in 2 x 2L MeOH initially for 24 hours and then for 6 hours to yield 17.2 g extract after evaporation of MeOH under vacuum. The extract was partitioned between EtOAc-H_2O (2L each). Upon evaporation of EtOAc, 4.1 g of crude extract was obtained. This extract was chromatographed over silica gel. Elutions with increasingly polar mixtures of CH_2Cl_2-acetone (10% acetone, to 20% acetone) provided 18 fractions (100 ml) each. Bioassays were performed on saturated solutions from 1.5

mg/23 ml and 15 mg/23 ml of the fractions that corresponded to the active region of the previous EtOAc column.

Fractions 11-14 were acetylated with Ac_2O-pyridine. Silica gel TLC (1 mm) with ether - 5% EtOAc as solvent yielded 1 mg catechin **(78)** pentaacetate, 24 mg epicatechin **(77)** pentaacetate from 39 mg of Fr 11. 35 mg of an acetylated mixture of Fr. 13-14 yielded 19 mg of the nonaacetate of A-2 **(79)** along with smaller quantities of other unidentified dimers and *epicatechin* **(77)** *pentaacetate*, which was a white semicrystalline solid.

λ_{max} nm MeOH: 227, 270; EIMS (of free phenol) (rel. int.) 290 (13.9), 272 (2.5), 152 [$C_8H_8O_3$] (38.5), 139 [$C_7H_7O_3$] (100.0), 123 [$C_7H_7O_2$] (52.4); ^{1}H NMR ppm: δ 1.90 (3H,s), 2.25 (3H,s), 2.27 (9H,s), 2.89 (1H, dd, $J_{4a,4b}$ 17.5 Hz), 3.00 (1H, dd, $J_{3,4a}$ 2 Hz, $J_{4a,4b}$ 17.5 Hz), 5.07 (1H, bs), 5.37 (1H, m), 6.55 (1H, d, $J_{6,8}$ 2 Hz), 6.67 (1H, d, $J_{6,8}$ 2 Hz) 7.13 - 7.37 (3H, m); ^{13}C NMR ppm: δ 19.9 q, 20.0 q, 25.4 t (C-4), 66.1 d (C-3), 76.0 d (C-2), 107.4 d, 108.2 d, 109.3 d, 121.5 d, 122.6 d, 123.9 d, 135.5 s (C-1'), 141.4 s, 141.5 s, 149.2 s, 149.3 s, 154.5 s, 154.5 - 169.7 s.

Catechin (78) pentaacetate. White solid.

EIMS *m/z* (rel. int.): 290 (14.2), 272 (2.1), 152 [$C_8H_8O_3$] (33.4), 139 [$C_7H_7O_3$] (100.0), 123 [$C_7H_7O_2$] (54.8); ^{1}H NMR ppm: δ 1.99 (3H,s), 2.28 (12H,s), 2.66 (1H, dd, $J_{3,4a}$ 6 Hz, $J_{4a,4b}$ 17.5 Hz), 2..87 (1H, dd, $J_{3,4b}$ 5 Hz,

$J_{4a,4b}$ 17.5 Hz), 5.14 (1H, d, $J_{2,3}$ 6 Hz), 5.25 (1H,m), 6.59 (1H, d, $J_{6,8}$ 2 Hz), 6.66 (1H, d, $J_{6,8}$ 2 Hz), 7.17 - 7.28 (3H,m).

Epicatechin ($4_u \rightarrow 8_\ell$; $2_u \rightarrow \underline{O} \rightarrow 7_\ell$) epicatechin acetate (79). White solid.

λ_{max} nm MeOH: 227, 270; ^{1}H NMR ppm: δ 1.50-2.30 (27H, CO-CH_3), 2.80 (1H, dd, $J_{3,4b}$ 4 Hz, $J_{4a,4b}$ 17.5 Hz), 2.94 (1H, dd, $J_{3,4a}$ 2 Hz, $J_{4a,4b}$ 17.5 Hz), 4.61 (1H, d, $J_{3,4b}$ 4 Hz), 5.20 (1H, d, $J_{3,4}$ 4 Hz), 5.30 (1H, brs), 5.23 (1H, brs), 6.50 (1H, d, $J_{6,8}$ 2 Hz), 6.51 (1H,s), 6.83 (1H, d, $J_{6,8}$ 2 Hz), 7.14 - 7.59 (6H,m); ^{13}C NMR ppm: δ 19.8 - 21.2 q, 25.9 t (C-4), 27.9 d (C-4_u), 66.1 d [C-3), 67.4 d (C-3), 77.7 d (C-2), 98.1 s (C-2_u), 103.9 d, 105.6 s, 107.1 d, 109.0 s, 109.9 d, 113.5 s (C-8), 122.5 d, 122.6 d, 122.7 d, 123.9 d, 125.2 d, 125.6 d, 135.2 s, 135.4 s, 141.3 s, 141.9 s, 142.6 s, 142.9 s, 148.7 s, 149.4 s, 149.9 s, 150.5 s, 151.6 s, 153.9 s, 167.1 - 170.3.

For the ^{1}H NMR COSY 45 N type experiment, 1128 FIDS (of 48 scans each) consisting of 0.5 K data points were accumulated; after digital filtering (sine bell) the FID was zero filled to 256 words in the F_1 dimension. Fourier transformation followed by magnitude calculation with no phase correction yielded a spectrum with 1.02 Hz per point digital resolution in both dimensions. Acquisition

parameters were $F_1 = \pm 130$ Hz and $F_2 = \pm 260$ Hz. Two seconds recycle delay was used.

III-3.6. Recovery of the glycosides from the water solution.

Granular coconut charcoal (60 g) was added to the H_2O solution that remained after it was extracted with EtOAc as described in Section III-4.4. This mixture was left to stand overnight. After the charcoal was filtered, it was washed successively with 250 ml cold H_2O, 250 ml boiling MeOH, 500 ml boiling H_2O. 300 ml of 7% phenol in H_2O was then added to the charcoal and this mixture was left to stand overnight. The phenol-H_2O azeotrope (b.p. 99°C) was distilled off under vacuum (80° C). The concentrated aqueous solution was extracted with $CHCl_3$ to remove the last traces of phenol. The glycosides were stored as a concentrated solution in H_2O. The concentrated solution was used as is when preparing samples for bioassay.

III-3.7. Isolation of ceratiolin (82).

Fresh leaves (500 g) collected in September 1984 were soaked at ambient temperature in 2L H_2O for 6 hours and resoaked for 20 hours. The combined washes were extracted with 500 ml EtOAc: $CHCl_3$ (1:1). Concentration of the extract in vacuo yielded 400 mg of a crude mixture with as the major constituent as determined by ^1H NMR. A

solution of this mixture in MeOH (5 ml) when kept at -40°C for several days provided 140 mg of ceratiolin **(82)** which precipitated from the syrup and was recrystallized twice from MeOH, m.p. 148-149°. A parallel extraction of the water phase with $CHCl_3$ - EtOAc was also done after adding an equivalent volume of 4 N HCl solution in H_2O to the aqueous layer. This increased the amount of matter extracted, however only about half the amount of ceratiolin precipatated when the extract was stored in MeOH in the freezer. Another extraction was done on the same batch of plant material which was dried and kept at ambient temperature for 6 months. However, although some quantities were used, only 9 mg ceratiolin **(82)** precipitated after several days at -40°C.

***Ceratiolin** (82).* Yellow solid, m.p. 148-149°. λ_{max} nm MeOH (log ε): 228 (4.12), 325 (3.99), 358 (3.97), IR^{KBr} cm^{-1}: 3450, 1668, 1639, 1578.

EIMS *m/z* (rel. int.): 302 $[M^+]$ (3.7), 284 $[M-H_2O]^+$ (2.2), 259 $[M - C_2H_3O]^+$ (40.5), 241 $[M - C_2H_3O - H_2O]^+$ (11.6), 180 $[M - C_8H_9 + H]^+$ (16.0), 133 $[C_9H_9O]^+$ (18.0), 105 $[C_8H_9]^+$ (57.3), 91 $[C_7H_7]^+$ (100)1 ^1H NMR ppm: δ 1.50 (3H,s), 1.85 (3H,s), 2.99 and 3.25 (4H,m), 7.26 (5H, brs), 18.86 (1H, s, ex. D_2O).

Elemental analysis: found, C: 67.25, H: 6.25 ($C_{17}H_{18}O_5$ requires: C: 67.54, H: 5.96).

TAI derivatization: ^{1}H NMR ($\Delta\delta$) : 1.50 ($\Delta\delta^+$ 0.14), 1.85 ($\Delta\delta^+$ 0.07), 2.99 ($\Delta\delta^+$ 0.14), 3.25 ($\Delta\delta^+$ 0.04).

III-3.8. Degradation experiments on ceratiolin (82).

Acid degradation of 20 mg ceratiolin was carried out by preparing a solution of 20 mg ceratiolin **(82)** in a mixture of 10 ml 12 N HCl, 10 ml H_2O and 10 ml MeOH. The solution was refluxed for two hours while being stirred with a magnetic stirrer. Methanol was evaporated with a rotatory evaporator and the remaining aqueous solution was extracted twice with 10 ml portions of CH_2Cl_2.

Compound **82** (10 mg) was placed in a sealed quartz cell in 2 ml $CHCl_3$. The solution was exposed to sun radiation and total decomposition took place in one week as determined by the ^{1}H NMR spectrum of the crude mixture. Two 10 mg samples of **82** were dissolved in 2 ml distilled H_2O each and as in the previous experiment, one sample was exposed to sunlight while the other was kept in the dark. The samples were subsequently extracted into 2 ml $CHCl_3$ each at the end of 3 days. The products were analyzed by GC-MS.

Triketone (85). $C_{13}H_{14}O_3$. GC r.t. 7.53.

EIMS *m/z* (rel. int.): 218 (9:1), 175 (42.9), 147 (5.2), 133 (8.1), 105 (33.3), 91 (100.0), 77 (11.4), 43 (20.1).

Hydrocinnamic acid (84). G.C. r.t. 5.02.

EIMS *m/z* (rel. int.): 150 (32.2), 105 (12.8), 104 (40.2), 91 (100.0), 77 (12.4), 65 (7.1), 51 (7.0); ^{1}H NMR ppm: δ 2.78 (4H,m), 7.28 (5H,m), 11.36 (1H,brs).

Methyl hydrocinnamate (86). G.C. r.t. 3.69.

EIMS *m/z* (rel. int.): 164 (26.6), 133 (9.1), 105 (36.3), 104 (100.0), 91 (60.6), 77 (15.8), 65 (8.5), 51 (8.9).

CHAPTER IV

PRELIMINARY STUDIES ON THE ROLE OF NATURAL SURFACTANTS IN THE ACTION OF NONPOLAR ALLELOCHEMICALS

IV-1. Introduction.

Non-polar natural products such as mono- and sesquiterpene hydrocarbons are not sufficiently water-soluble to reach high enough concentrations to exhibit inhibitory effects on target species. Therefore, if such nonpolar natural products are biologically active in nature, mechanisms of transport other than just simple dissolution into water must be operative.

A proposed mechanism for volatile monoterpenes involves adsorption of the monoterpenes that exist in the atmosphere surrounding the plant by dry soil colloids.[52] Seedling growth is claimed to be inhibited when adsorbed terpenes are dissolved in the cutin of seedlings in contact with the soil colloids.

Alternately, it has also been observed that when some compounds are initially taken up in small amounts of organic solvents to facilitate solubilization in water they exhibit higher germination and growth regulation activities than when they are directly dissolved in water. A similar phenomenon has been observed for a mixture of flavonoids obtained from *Ceratiola ericoides* (Table 14). In this case when the compounds were predissolved in dimethysulfoxide, activity levels were significant while in water alone significant levels would not be reached.

An alternate proposed mechanism involves the solubility enhancement of nonpolar allelochemicals by natural surfactants which are frequently present in the plant along with the allelochemicals.[25] This mechanism, if operative, would allow the release and transport of water-insoluble allelochemicals from the plant surface into the soil through natural rain leaching. Triterpene acids and fatty acids have been cited as possible candidates for natural surfactants as they contain a polar functional group as well as a large nonpolar portion, which are the requirements for a surfactant.

All three scrub plants investigated in our laboratories, namely *Conradina canescens*,[109] *Calamintha ashei* and *Ceratiola ericoides* contain the triterpene ursolic acid **(40)** in copious amounts. When the biological activities of the crude extracts from *Calamintha ashei* were examined (Table 4) it was found that the petroleum ether-dichloromethane extract's precipitate, which was enriched in ursolic acid, was more active than the mother liquor even though ursolic acid itself is not active. Since ursolic acid is reportedly used as a commercial surfactant, experiments were performed to determine if and how ursolic acid is effective in enhancing regulatory effects of nonpolar allelochemicals.

Table 22. Comparative bioassays on *Lactuca sativa* using monoterpenes in water, in aqueous emulphogen and in aqueous ursolic acid.

Test solution	% germination[≠] Compared to: water control	emulphogen control	ursolic acid control	% radicle length[≠] Compared to: water control	emulphogen control	ursolic acid control
camphene, E[+]	108	110	---	75*	86*	---
camphene, U	112	---	97	91*	---	97
camphene, W	104	---	---	100	---	---
borneol, E	0	0	---	---	---	---
borneol, U	0	---	0	---	---	---
borneol, W	56	---	---	40*	---	---
carvone, E	0	0	---	---	---	---
carvone, U	0	---	0	---	---	---
carvone, W	0	---	---	---	---	---
limonene, E	102	104	---	79*	91*	---
limonene, U	108	---	93	100	---	106
limonene, W	112	---	---	103	---	---

Table 22. (continued)

Test solution	% germination≠			% radicle length≠		
	Compared to:			Compared to:		
	water control	emulphogen control	ursolic acid control	water control	emulphogen control	ursolic acid control
camphor, E+	96	92	---	53*	51*	---
camphor, U	0	---	0	---	---	---
camphor, W	104	---	---	76*	---	---
citronellol, E	0	0	---	---	---	---
citronellol, U	0	---	0	---	---	---
citronellol, W	0	---	---	---	---	---

+E stands for emulphogen solution, U for ursolic acid solution, W for water solution.

≠Germination and radicle lengths are expressed as percentages of the perspective controls.

*An asterix indicates significant difference from the control at $p < 0.05$.

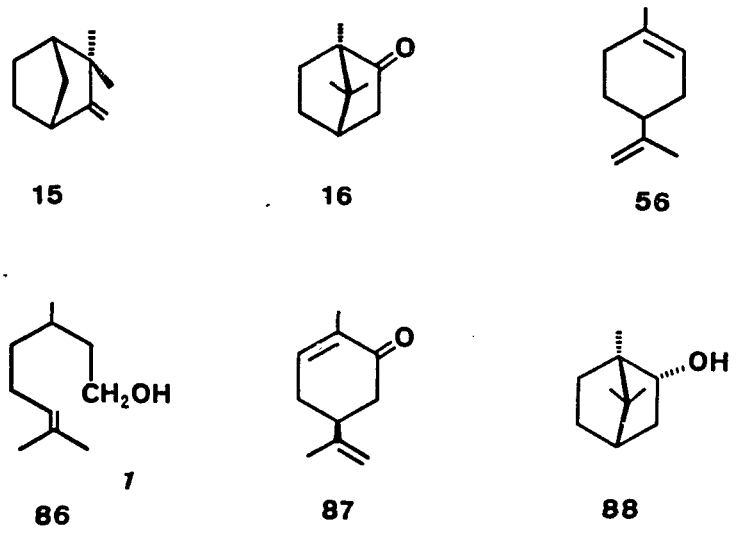


Figure 25. Monoterpene allelochemicals tested in (Chapter IV).

IV-2. Germination and growth bioassays with ursolic acid solutions of test compounds versus surfactant solutions of test compounds.

A number of monoterpenes were bioassayed as solutions of the surfactant tridecyloxypolyoxyethylene ethanol (emulphogen); ursolic acid and pure water. Emulphogen was the surfactant of choice since it is a gentle nonionic detergent with no adverse effects at the (500 ppm) levels or below in contrast to denaturing detergents such as sodium dodecylsulfate which completely inhibited germination of test seeds. The results are displayed in Table 22. In the case of the monoterpenes citronellol **(86)** and carvone **(87)**, differences could not be observed since

the compounds were highly active completely inhibiting seed germination with all test solutions. Limonene **(56)** and camphene **(15)** showed very low activities. Significant inhibitory effects were observed with the compounds **56** and **15** in emulphogen solutions when compared to emulphogen controls as well as to pure water controls. The presence of a surfactant appears to enhance the activities of these nonpolar natural products.

The effects of compounds **56** and **15** are insignificant for aqueous ursolic acid and pure water solutions, when compared with their respective controls. Borneol **(88)** completely stopped germination when tested in aqueous solutions of ursolic acid and emulphogen while the water solution showed 50% germination and radicle growth inhibition. In the case of camphor **(16)**, the results were dramatic with total germination inhibition observed for the ursolic acid solution of camphor while the emulphogen solution of **16** caused decreases only in the mean radicle length and the effect of **16** in pure water was less significant.

Since higher effects were observed for both aqueous solutions of test compounds with emulphogen and ursolic acid solutions, experiments were carried out to determine if the presence of natural surfactants could be effective by solubilizing the nonpolar allelochemicals in water.

IV-3. Investigations for possible micelle formation with natural surfactants.

To investigate natural leachates of test plants for possible formation of micelles which would indicate the presence of surfactants in the test solution, a method using the fluorescence yield of acridine was applied.[110] This method is based on the fact that below the critical micelle concentration (CMC), the fluorescence of acridine is independent of surfactant concentrations while above the CMC, quantum yields decrease drastically with increasing surfactant concentrations. This is due to the behavior of acridine which only fluoresces in protic solvents and is sufficiently hydrophobic to be mainly located within the hydrocarbon-like interior of the micelles.

Figure 25 displays distinct differences between the behavior of ethyl acetate which does not form micelles, thus giving 100% fluorescence yield at a wide range of concentrations versus the surfactants which display the characteristic curves of micelle-forming tensides.

Natural products phenol, hydrocinnamic acid and benzaldehyde gave positive micelle tests indicating a possible decrease in the CMC with increasing size of the hydrocarbon portion. The results shown in Figure 27 indicate that a mixture of a wide variety of natural products can lead to a solution with surfactant

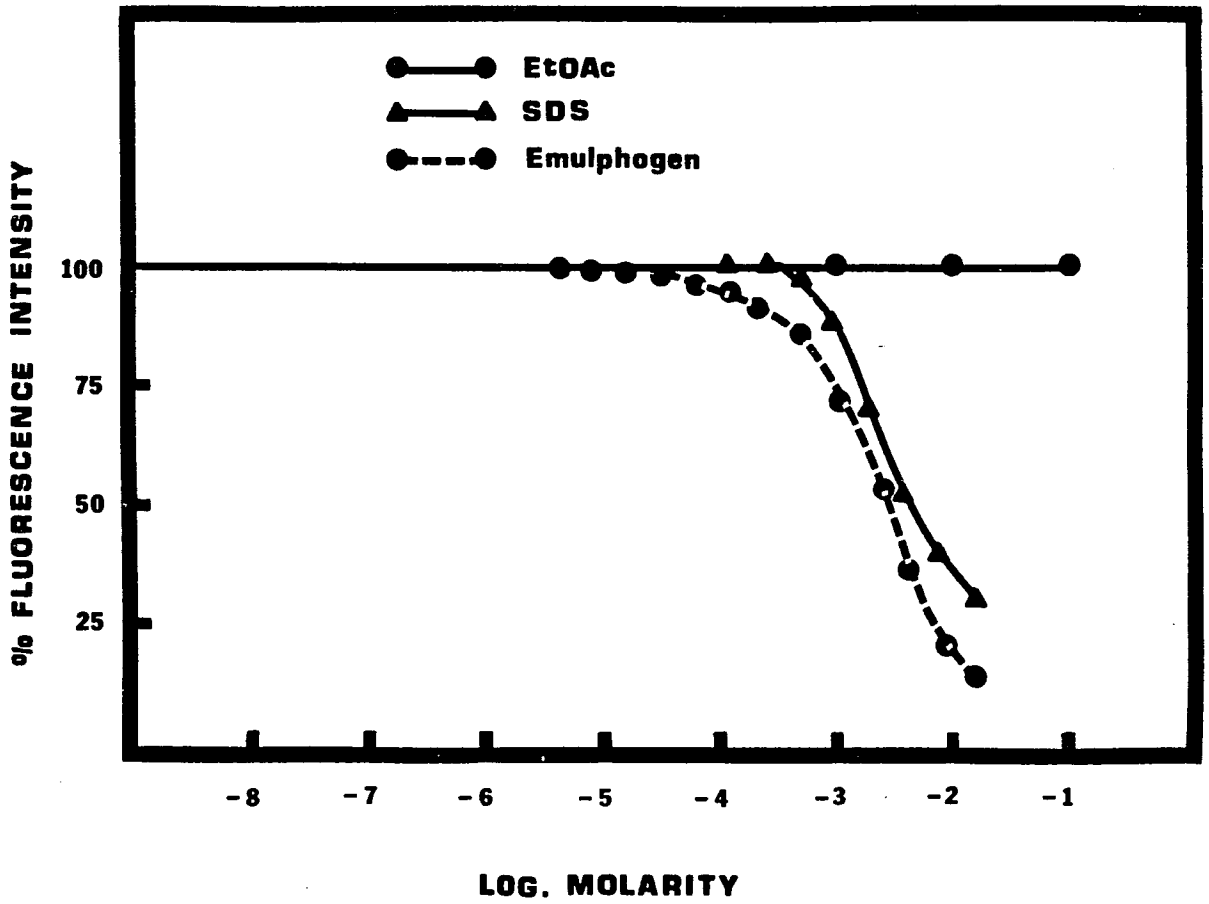


Figure 26. Dependence of relative fluorescence intensity of 1.2×10^{-5} M acridine solution vs. concentrations of SDS, emulphogen and ethyl acetate.

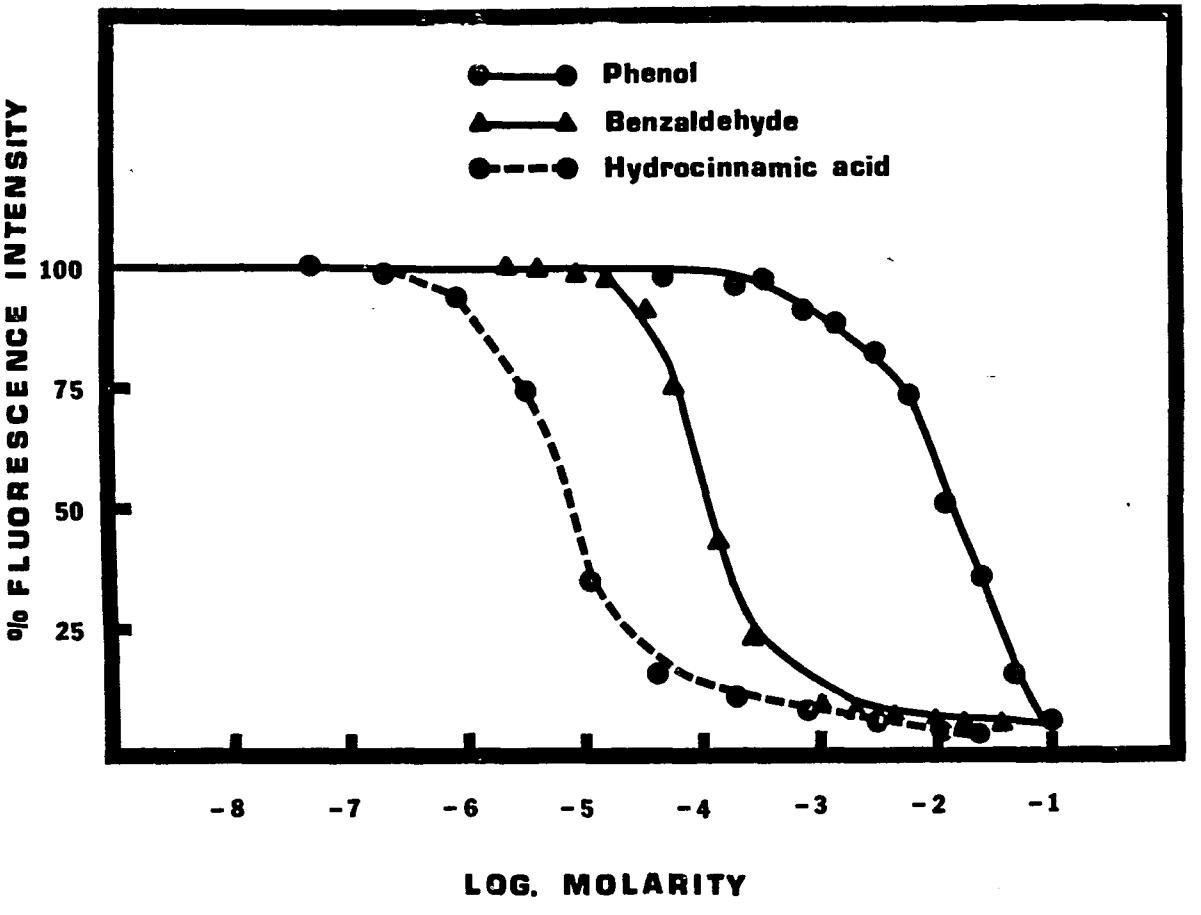


Figure 27. Dependence of relative fluorescence intensity of 1.2×10^{-5} M acridine solution vs. concentrations of phenol, hydrocinnamic acid and benzaldehyde.

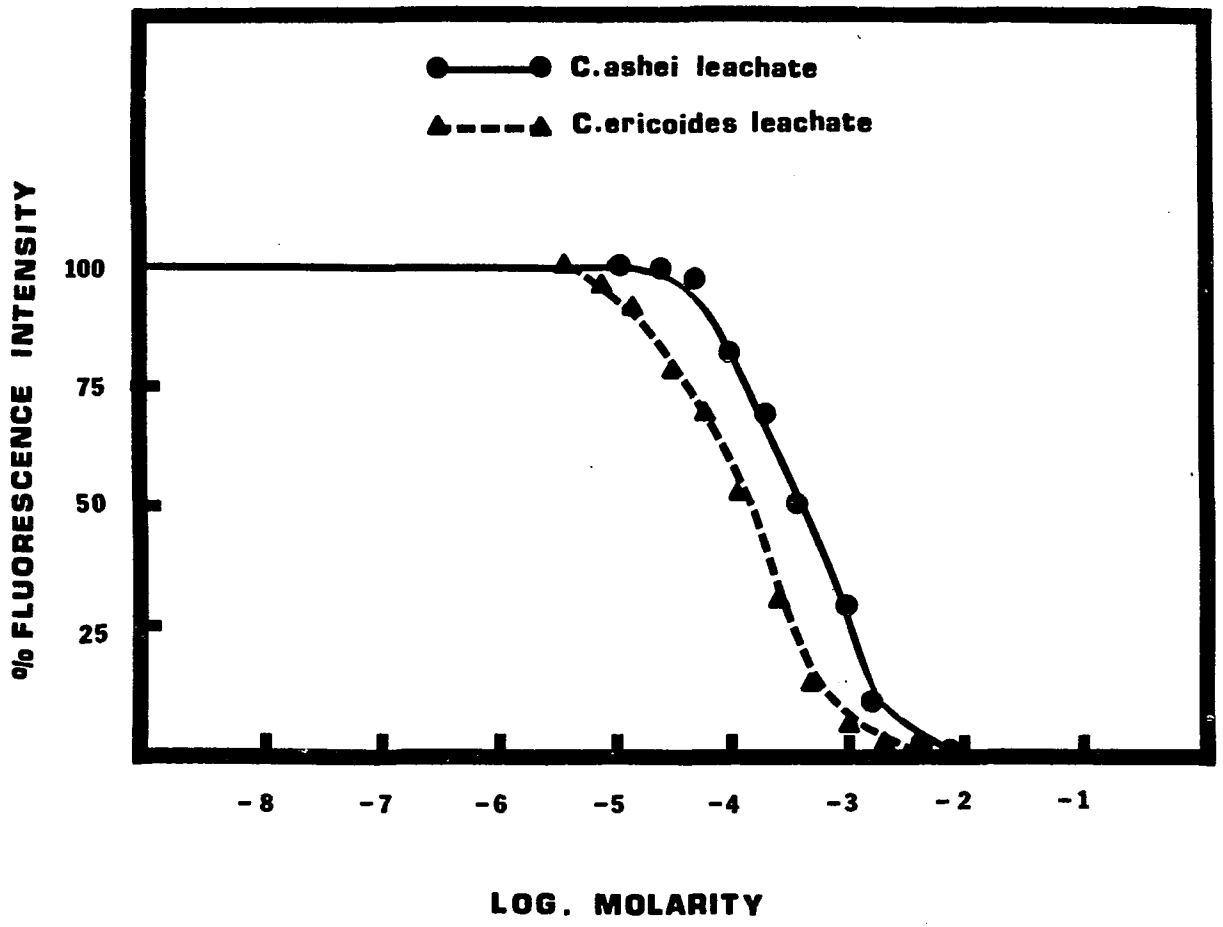


Figure 28. Dependence of relative fluorescence intensity of 1.2×10^{-5} M acridine solution vs. concentrations of leachates from *Calamintha ashei* and *Ceratiola ericoides*.

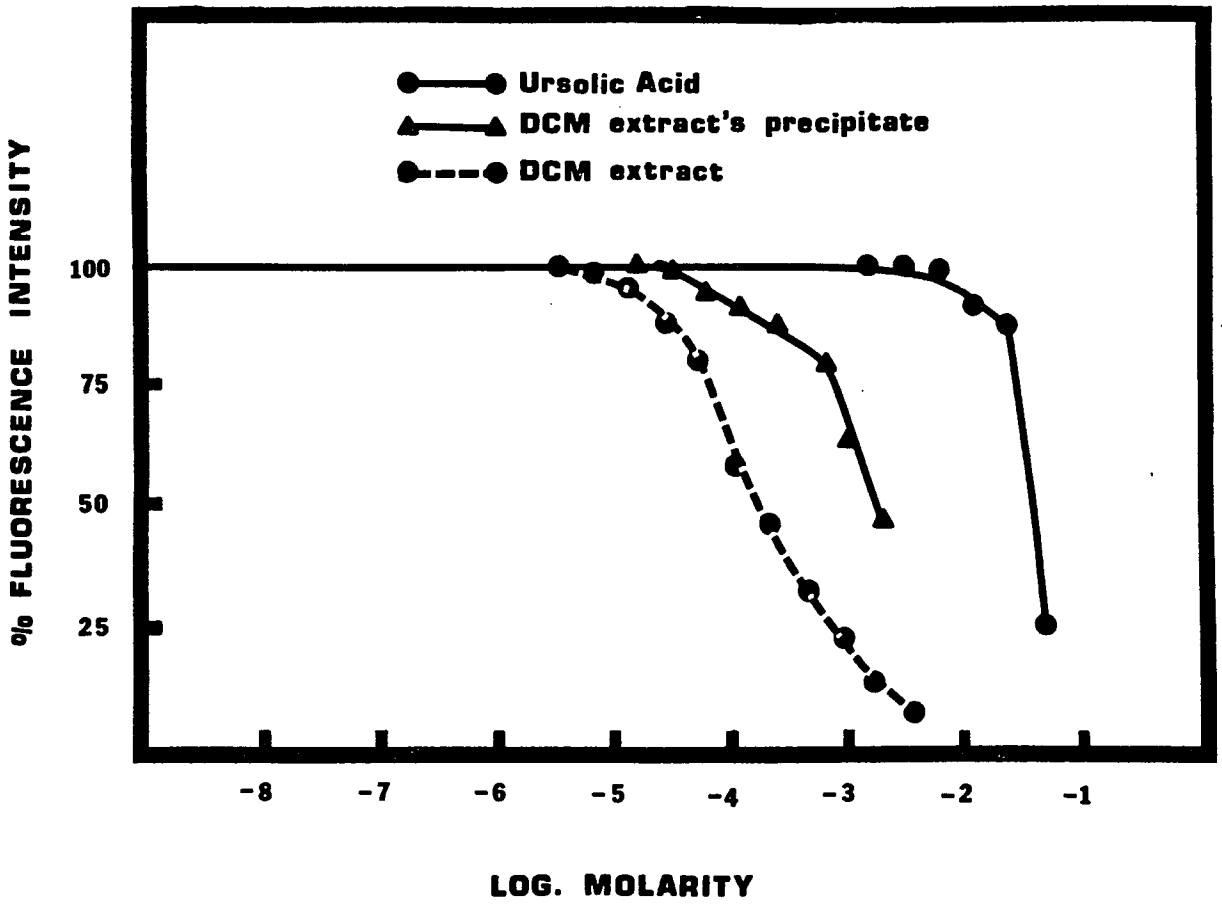


Figure 29. Dependence of relative fluorescence intensity of 1.2×10^{-5} M acridine solution vs. concentrations of *Calamintha ashei* constituents.

characteristics. Using the acridine method, aqueous leachate from leaves of another scrub member, *Conradina canescens*, was reported to form a micelle solution.[109] Similar results were obtained with aqueous leachates of *Ceratiola ericoides* and *Calamintha ashei* (Figure 28). To calculate concentration, the average molecular weight in the leachate was assumed to be 300. To assess the role of ursolic acid in solubilizing nonpolar natural products, the mother liquor of the 10% petroleum ether-dichloromethane extract of *C. ashei*, the precipitate of this extract and pure ursolic acid were tested (Figure 29). The mother liquor, which contained traces of ursolic acid, showed micellization at lower concentrations; the precipitate enriched in ursolic acid had a CMC at higher concentrations and only the most concentrated solution of pure ursolic acid was above the CMC even though extended sonication was used in dissolving the compound. These results indicate that ursolic acid is less likely to be a means of increasing the solubility of nonpolar allelochemicals under natural conditions.

However inspection of test seeds treated with ursolic acid solutions indicated the release of a pink pigment from the seeds into the solution. Therefore, it is possible that ursolic acid aids nonpolar allelochemicals by possible destruction of and/or transport through the membranes of a target seed.

IV-4. Comparative dilutions of ursolic acid, allelochemical solutions for bioassays.

The following dilution experiments were performed to test if an increase in biological activity is due to a possible increase in concentration of an allelochemical solubilized by ursolic acid or the increase in bioactivity might be due to another mechanism such as membrane destruction and/or membrane transport effects.

Two kinds of solutions were prepared for each monoterpene used in the experiment. One was a saturated solution of ursolic acid and monoterpene while the other was a mixture of separately prepared saturated solutions of ursolic acid and of the monoterpene. The second type of solution should not have increased levels of the monoterpene due to dissolution by ursolic acid. The first solution, which was diluted by half before the onset of the bioassay could have increased monoterpene levels due to micelle formation and dissolution in ursolic acid solution.

The results shown in Table 23 indicated low levels of activity for both types of solutions. While limonene and to a lesser extent camphor gave results that indicated a higher stabilization of these monoterpenes in ursolic acid, borneol showed a slight tendency in the opposite direction.

Table 23. Comparative bioassays on *Lactuca sativa* for monoterpene-ursolic acid solutions.

Test solution	% germination[+] Compared to: water control	% germination[+] Compared to: ursolic acid control	% radicle length[+] Compared to: water control	% radicle length[+] Compared to: ursolic acid control
borneol, ursolic acid solution[≠]	97	106	28*	28*
borneol solution + ursolic a. solution[#]	90	98	22*	22*
camphor, ursolic acid solution[≠]	81*	89	28*	28*
camphor solution + ursolic a. solution[#]	73*	80*	35*	35*
ursolic acid solution[i]	92	---	---	101

[i]For borneol and camphor bioassays this ursolic acid solution was used.
*An asterix indicates significant difference from control at $p < 0.05$.

Table 23. (continued)

Test solution	% germination[+] Compared to: water control	% germination[+] Compared to: ursolic acid control	% radicle length[+] Compared to: water control	% radicle length[+] Compared to: ursolic acid control
limonene, ursolic acid solution[≠]	92	79	72*	77
limonene solution + ursolic a. solution[#]	106	91	95	101
camphene, ursolic acid solution[≠]	112	97	98	104
camphene solution + ursolic a. solution[#]	116*	100	99	105
ursolic acid solution[ii]	116*	---	94	---

[+]Germination and radicle lengths are expressed as the percentages of the indicated controls.

[≠]This is a saturated solution of the monoterpene in ursolic acid solution which is subsequently diluted by one half.

[#]This is made by mixing a saturated solution of the monoterpene in water with a saturated ursolic acid solution.

[ii]This solution has been used for camphene and limonene bioassays.

It was concluded that other methods of testing which do not involve dilution are necessary to be able to assess if the increase in activities is due to an increase in the solubility of a bioactive compound or whether other factors play a more important role.

IV-5. Conclusions.

Since fluorescence measurements indicate that micelles form in the aqueous leachates from the leaves of scrub species and since the surfactant emulphogen does increase the activity levels of monoterpenes in bioassays, it can be concluded that with the help of other solubilizing natural products in a leachate, nonpolar natural products may exhibit higher activities than when tested in the pure form under laboratory conditions. This effect is complementary and not an alternative to the "volatility" mechanism which also appears to be operative as seen by the dramatic effects observed for evodone volatiles (Table 8).

Ursolic acid shows a synergistic effect as exemplified by a number of experiments in this dissertation although the nature of the effect is, at present, not clear.

IV-6. Experimental.

IV-6.1. Measurement of relative acridine intensity.

Acridine, which was purified on a silica gel column using dichloromethane as eluent, was dissolved in distilled water with magnetic stirring for 4 hours, providing a solution of 6.0×10^{-4} M concentration. An aqueous surfactant solution was prepared by dissolving each test compound in water with extended sonication (2 hours). The initial test concentration was the one obtained by mixing the surfactant and acridine solutions in a 4:1 ratio. For dilution, aqueous acridine solution (1.2×10^{-5} M) was added in a 1:1 ratio to the test solution after each measurement. The fluorescence yield obtained for various test solutions was compared to pure acridine solution to determine the relative fluorescence intensity.

A SLM 4800 spectrofluorimeter with an excitation slitwidth of 8 nm and an emission slitwidth of 2 nm was used. Emission wavelength was chosen as 425 nm. While the excitation wavelength was 360 nm for compounds that did not absorb UV radiation at 360 nm, in cases where compounds did absorb at 360 nm, 395 nm was used as the excitation wavelength.

Freshly prepared solutions of surfactants were used for each experiment.

IV-6.2. Bioassays performed with surfactant and ursolic acid solutions.

Stock solutions of emulphogen at 50 mg/100 ml and ursolic acid 2 mg/100 ml were used. 15 mg of each monoterpene was dissolved by sonication (2 hours) in each of these solutions and a water solution. The undissolved monoterpenes were filtered out through a fine fritted funnel in the case where the monoterpene was a solid. In the case of a liquid monoterpene, after the solution was let to settle for 2 hours, the bioassay solution was obtained by sampling the stock solution at its middle point with a pipette.

The solutions for the comparative ursolic acid solutions were prepared as described at the bottom of Table 23.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Nesrin Tanrisever

Major Field: Chemistry

Title of Dissertation: Plant Germination and Growth Inhibitors from Ceratiola ericoides and Calamintha ashei

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination: July 17, 1986