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ALLELOPATHIC EFFECTS OF *PARTHENIUM HYSTEROPHORUS* L.

PART IV. IDENTIFICATION OF INHIBITORS*

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KEY WORDS

Allelopathy Fumaric acid Parthenin Phenolics Pollen Leaf Washing Root leachate

SUMMARY

Analysis of the inhibitors from allelopathic species of *Parthenium hysterophorus* L. revealed that sesquiterpene lactones and phenolics formed an important group of water soluble compounds involved in allelopathy. Parthenin was the major sesquiterpene lactone involved though dampsin was also found in traces. Caffeic acid, vanillic acid, ferulic acid, chlorogenic acid, and anisic acid among the phenolics and fumaric acid among the organic acids were the important constituents of the air dried parts of the plant, many of them being traced in the root exudates, leaf washings, pollen and trichome leachates.

INTRODUCTION

Parthenium hysterophorus L. the tropical American weed which has spread to all parts of India forming huge stands exert allelopathic effects. Studies by Sukhada[15] have revealed that the growth inhibitors are exuded to the medium via root exudation, leaching from aerial vegetative parts, through pollen and trichomes which are carried to great distances by wind and also through leaching from the air dried plant parts in the soil. Preliminary analysis of growth inhibitors from the weed revealed the presence of parthenin, caffeic acid and p-coumaric acid in the air dried stems[14]. The present paper reports the analyses of growth toxins released to the substratum via various routes from different parts of the plant.

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MATERIALS AND METHODS

Extraction of the inhibitors

a. Fortnight old Parthenium plants were transplanted one each to earthen pots of 30 cm diameter and 35 cm depth containing soil (compound of 2 part mud + 2 part horse dung manure + 1 part sand) and were grown for 30 days. When the plants were in the flowering stage the pots were drained. In the control, pots without Parthenium plants were used. The pots were arranged in a row and water from a common source was made to trickle into all the pots simultaneously. The leachate that drained though the soil mass was collected through the outlet at the bottom of each pot. One litre of the leachate thus collected was filtered through Whatman No 1 filter paper and used in the analysis.
b. Sand of 120 mesh size was washed as described by Hewitt[8] and was taken in 500 ml beakers and autoclaved at 7 kg pressure for 15 minutes. Parthenium plants were grown in the sterilised sand and were fed daily with 50 ml of quarter strength Hoagland solution. After 30 days growth, the plants were removed carefully from the sand and the sand was washed with 100 ml of distilled water. One litre of the washings was collected, filtered as above and stored at 10°C until further use.
c. Fresh leafy twigs of Parthenium with their cut ends dipped in water taken in 500 ml beakers were arranged so as to get a leafy cover comparable to that in nature. One litre of the deionised water was sprayed on the foliage with a atomiser for half an hour to simulate rain. The foliar drip was collected after the leaves got washed for two minutes, filtered and stored at 10°C.
d. Trichomes scraped off the leaves of Parthenium and pollen collected by gently tapping the fresh capitula of the weed were leached in distilled water in the ratio 1 : 16 and 1 : 64 respectively at 10°C for 72 h.
e. 250 g each of the dry root, stem, leaf, inflorescence and cypsella of Parthenium collected from the natural stand of the weed were surface sterilised with 0.1 per cent mercuric chloride, thoroughly washed with distilled water and soaked in one litre of distilled water at 10°C for 72 h.

All the leachates obtained as above were filtered through Whatman No 1 filter paper and concentrated separately to one tenth their original volume under reduced pressure at 50°C. The concentrates were acidified to pH 2 with concentrated hydrochloric acid and 25 ml aliquotes were repeatedly partitioned against equal volumes of ethyl acetate. The ethyl acetate extracts were distilled under reduced pressure on a water bath at 50°C. The residue was redissolved in 10–15 ml of ethyl acetated and fractionated into acidic and non-acidic fractions by extracting it repeatedly with 2 *N* sodium bicarbonate and sodium carbonate solution. The carbonate-soluble fractions were pooled and acidified to pH 2 and extracted with ethyl acetate. This fraction as well as the non-acidic ethyl acetate fractions were washed thoroughly with water and concentrated *in vacuo* and stored in a desiccator until further use.
f. Soil adhering to root systems of Parthenium and also soil 2 cm away from the plants were extracted separately by the method of Wang, Yang and Chuang[17] which was slightly modified. To 100 g of soil sample 25 ml of 95 per cent ethyl alcohol were added immediately after sampling in order to suppress the activity of microorganisms which might alter the phenolics in soil. 250 ml of 2 *N* sodium hydroxide were added for extraction and the suspension was shaken well and left overnight. The supernatant was collected by centrifugation at 2000 *g* for 10 minutes and the solution was evaporated to one tenth the volume, acidified to pH 2 with hydrochloric acid and extracted with three half volumes of ethyl acetate. The ethyl acetate fraction was evaporated to dryness and stored in a desiccator until further use.

Analysis

The acidic and non-acidic fractions of different leachates and ethyl acetate fraction of rhizosphere soil extract were chromatographed using 250 μ thick silica gel (grade H) plates and developed in

benzene: ethyl acetated 7:3 for 90 minutes. The unresolved spot at the origin was eluted with methanol and respotted to the plates and developed in organic layer of pyridine: ethyl acetate: water (1:2:2). The plates were observed under short wave length UV lamp, the fluorescent spots were noted and then developed in iodine chamber. Rf values of different spots were calculated. The plates were also sprayed with alcoholic ferric chloride and diazotised p-nitroaniline. The bright fluorescent spots observed under UV and the spots giving intense brown colour with iodine were also eluted with ethanol and their UV spectra determined.

The non-acidic fractions of different leachates were analysed by column chromatography. The non-acidic fraction was passed through an alumina column and different fractions eluted with petroleum ether: ethyl acetate (70:30) were evaporated to dryness. The acidic fraction was passed through silica gel (mesh size 70–120) column and eluted sequentially with benzene: ethyl acetate 90:10 and benzene: ethyl acetate 90:30. The melting points and the UV absorption spectra of compounds that crystallized out were determined. The NMR spectrum of the crystals from non-acidic fractions that could be obtained in large quantity was determined.

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Different spots on the TLC plates were eluted with methanol and evaporated to dryness. The residues was dissolved in 2.5 ml of 0.05 m phosphate buffer (pH 5.8) and tested for coleoptile growth of *Eleucine coracana* Gaertn. var 'Poorna' following Sukhada[14]. Silica gel scraped off in the blank region of the plate was used as the control.

Yield of inhibitors

Yield of inhibitors from different parts of Parthenium weed was determined by weighing the crystallized samples. In those which could not be obtained in sufficient quantity yield was calculated from the standard curves of authentic samples in the bioassay.

Estimation of total phenolics

The total phenols in the aqueous leachates collected for 72 h from air-dried parts of Parthenium and the soil extracts from the rhizosphere and at 10, 20 and 30 cm from Parthenium plant (following Lodhi[9]) were estimated colorimetrically using Folin-Denis reagent[2].

RESULTS

The inhibitory spots in the acidic and non-acidic fractions of different leachates are given in Table 1. Spot with Rf 0.15 of acidic fraction was prominent in the leachate of all parts of the plant tested and in the leaf-wash. This prominent inhibitory spot exhibited blue fluorescence under UV and turned blue green when viewed after exposure to ammonia. It gave green colour with Gibb's reagent indicating that the compound was a phenolic catechol type unsaturated acid. An ultra violet spectrum of the methanolic eluate of the spot corresponded with that of caffeic acid (Table 2) as also did cochromatography with authentic sample. Spots with Rf 0.31 in roots, 0.32 in stem and 0.39 and 0.42 detected in all the

Table 1. Analyses of inhibitors present in various extracts of *Parthenium hysterophorus* L.

Acidic fraction														
Solvent system I, Rf –	0.04 (a, d g–h)	0.05* (f)	0.06 (i–k)	0.09 (h–k)	0.12 (a–c, g)	0.15 (d, g–k)	0.18 (f)	0.20* (a–c h–j)	0.25 (g, i i–k)	0.31 (g)	0.32 (h)	0.35 (j, h–k)	0.39 (a–k)	0.42 (a, c, i–k)
Coleoptile length (cm) in ragi (*Eleusine coracana* L. Gaertn. var 'Poorna') per cent of control														
	86	109	45	50	63	50	50	100	45	63	81	86	50	45
Solvent system II,	0.76 (i–h)	0.86 (i–h)												
Coleoptile length (cm) in ragi per cent of control														
	82	64	–	–	–	–	–	–	–	–	–	–	–	–
Non-acidic														
Rf –	0.14 (g–k)	0.28 (a–k)	0.30 (i–k)	0.34* (g, i)	0.46 (g–k)	0.53 (g, i–k)	0.64 (h–j)	0.72 (i–h)	0.79 (j)	0.80* (j, i)	0.88 (h)	0.94 (g, i–k)	–	–
Effect on coleoptile length (cm) in ragi per cent control														
	36	2	81	100	63	45	63	113	63	100	68	68	–	–

a, root exudate; b, sand culture extract; c, rhizosphere soil; d, leaf washing, e, f, g, h, i; j, k, leachates of trichome, pollen, root, stem, leaf, inflorescence and fruit respectively.
Solvent I – benzene : ethyl acetate (7 : 3); Solvent II – pyridine : ethyl acetate : acetic acid (1 : 2 : 1).
* non-inhibitory.

samples in the solvent system I and with Rf 0.86 in solvent system II showed characteristics given in Table 2 and were identified as p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, ferulic acid and chlorogenic acid, respectively. This was further confirmed by comparing with the UV spectrum of the pure chemicals. Column chromatography of the acidic fraction of root leachate when eluted with benzene : ethyl acetate (90 : 30) yielded crystals with melting point 182–184°C. This was identified to be anisic acid and was confirmed by comparing its UV spectrum with that of the pure chemical. Sublimation and condensation of the acidic fraction of the leaf, stem and inflorescence yielded two crystalline compounds, one melting at 210 and the other at 286°C. The UV spectrum of the former corresponded with that of fumaric acid.

The column chromatography of the non-acidic fraction that was eluted with petroleum ether : ethyl acetate (70 : 30) yielded crystals with melting point 164–166°C. The UV and NMR spectra corresponded with those of parthenin, a

Table 2. Characteristics of inhibitory spots from the acid fractions of leachates from different parts of *Parthenium hysterophorus* L.

	Rf*	UV	UV + NH_3	Paranitro-aniline (PNA)	PNA + sodium Carbonate	Ferric Chloride	Absorption maxima	Identity
1.	0.15 (d, g–k)	blue	blue-green	yellow-brown	grey blue	green	235, 290–320	Caffeic acid
2.	0.31 (g)	–	–	pale yellow	red	–	265	p-Hydroxybenzoic acid
3.	0.32 (h)	–	blue-violet	yellow-brown	blue	–	222, 290–310	p-Coumaric acid
4.	0.35 (a–c, g)	–	–	yellow-brown	reddish-brown	–	258	Anisic acid
5.	0.39 (a–k)	–	–	yellow	violet	–	260, 290	Vanillic acid
6.	0.42 (a, c, g)	blue	blue-green	pink	pale blue	–	232, 290–317	Ferulic acid
7.	0.38 (i–k)	blue	green	brown	brown	green	330, 300, 245	Chlorogenic acid

* Solvent system for 1–6: Benzene : ethyle acetate (7 : 3); for 7: pyridine : ethyl acetate : water (1 : 2 : 2).
a, root exudate; b, sand culture extract; c, rhizosphere soil; d, leaf washing, e, f, g, h, i, j, k leachate of trichome, pollen, root, stem, leaf, inflorescence and cypsella respectively.

sesquiterpene lactone. The spot with Rf 0.28 on TLC in all non-acidic fractions matched with that of parthenin. A spot with Rf 0.64 in the non-acidic fraction matched with that of dampsin when cochromatographed.

Thus, the root exudates and rhizosphere soil of Parthenium contained anisic acid, vanillic acid, ferulic acid, fumaric acid and parthenin. However, ferulic acid could not be detected in all the samples of rhizosphere soil extract. The leaf washings contained caffeic acid, vanillic acid, ferulic acid and parthenin. Trichomes contained vanillic acid and parthenin. In the pollen vanillic acid and parthenin were among the identified inhibitors. The airdried root extract was rich in anisic acid, vanillic acid and caffeic acid while parthenin, ferulic acid, p-hydroxybenzoic acid were found in traces. The stems were rich in fumaric acid and parthenin. Leaf, inflorescence and seeds contained parthenin as the principal component of the non-acidic fraction though dampsin was also found in small quantity. Fumaric acid, caffeic acid, vanillic acid and chlorogenic acid were among the important components of acidic fraction in the leachates of these

Table 3. Yield of inhibitors in different parts of *Parthenium hysterophorus* L.

Different parts of the weed	Yield per cent on dry weight basis				
	Root	Stem	Leaf	Inflorescence	Cypsella
Parthenin	0.01	0.02	0.30	0.30	0.15
Caffeic acid	0.02	0.015	0.031	0.031	0.025
Vanillic acid	0.018	–	0.030	0.030	0.025
Anisic acid	0.05	–	–	–	–
Fumaric acid	0.01	0.30	0.30	0.30	–
p-Coumaric acid	–	0.10	–	–	–
Total phenolics	1.2	0.04	2.73	2.39	2.6

– undetectable.

parts. The data on the total yield of important inhibitors in different parts (Table 3) revealed that parthenin was the most abundant of the inhibitory constituents in the serial parts followed by caffeic acid and vanillic acid.

The unidentified inhibitory spot could be proved not to be indentical with gentisic acid, gallic acid, o-coumaric acid and quercetin.

The total phenolic acid content was highest in leaves and inflorescences followed by that of seeds, roots and stems. In soil from rhizosphere and in that at 10, 20 and 30 cm radial distance from Parthenium, the total phenolic acid content was respectively 0.025, 0.02, 0.015 and 0.011 per cent on dry weight basis.

DISCUSSION

Sesquiterpene lactones and phenolics form the main water soluble inhibitory constituents of *Parthenium hysterophorus*. Parthenin was the major sesquiterpene lactone though dampsin was also present in small quantity. Rodriguez[13] reported the presence of parthenin in *Ambrosia psilostachya* another species known to exhibit allelopathic influence[10] and belonging to the subtribe Ambrosinae which also includes Parthenium. He also reported the presence of *ambrosin* in *Parthenium hysterophorus* collected from different parts of India but he could not detect the same in Bangalore populations (personal communication to the author, 1976). Among the phenolics detected in Parthenium weed were caffeic acid, vanillic acid, anisic acid, chlorogenic acid, ferulic acid, p-hydroxybenzoic acid and p-coumaric acids which are the common phenolics known to be involved in allelopathy towards other species[12]; as an exception anisic acid has not been reported as allelopathic. This compound has been isolated from *Eupatorium odoratum*[1] another member of the Compositae which is shown to exert

allelopathic effects from studies conducted in our laboratory (unpublished data). Kaemferol and quercetin-3-0-glycosides have also been detected in *Parthenium hysterophorus* L.[13]. However, the inhibitory nature of these glycosides remains to be asertained. Free quercetin could not be detected in the present analysis.

The occurrence of fumaric acid in the stems and leaves of Parthenium in large quantity is interesting to note. In the stem and young seedlings of *Helianthus annuus*, another species which has allelopathic potentialities[19], this acid is reported to be plentiful[11]. In the present study even a one per cent solution of fumaric acid did not cause growth inhibition. However, its effect in combination with that of other inhibitors needs to be found out. A comparison of the analysis of rhizosphere soil, root exudates and air dried root leachate clearly evidenced the exudation and accumulation in soil of phenolic inhibitors like anisic acid, vanillic acid, ferulic acid and parthenin together with fumaric acid from the roots. Though parthenin content in root is low as compared to that in aerial vegetative parts, occurrence of parthenin in rhizosphere soil in sufficiently high concentration may be due to its translocation from aerial parts. Bonner and Galston[4] isolated transcinnamic acid in crystalline form from the root exudates of *Parthenium argentatum* and proved its inhibitory action. However, this compound could not be detected in root exudate or in leachate from any part of *Parthenium hysterophorus*. Considerable amounts of phenolic acids at 10 and 20 cm distance of Parthenium plants shows the extention of the inhibitory zone. As these phenolics decline in concentration with increasing distance from the root system, the origin of these phenolics most probably is the root system of the Parthenium plant. Although caffeic acid and ferulic acids are found in different parts of Parthenium, failure to detect the compound in soil extract may be due to their conversion in soil to other forms by the soil bacteria. Such a conversion of furulic acid to vanillic acid by *Rhodotorula rubra* was demonstrated by Turner and Rice[16]. However, though many of the inhibitory compounds could not be extracted in the soil they may be still effective there in inhibiting the plant growth as Blum and Rice[3] found that 33–300 ppm concentrations of tannic acid which could not be recovered from soil were still effective in reducing nitrogen fixation.

Parthenin, caffeic acid, vanillic acid and ferulic acid are also rain washed from Parthenium leaves to soil and may accumulate to toxic level. Leaching of chlorogenic acid, p-coumaric acid, and ferulic acid from the leaves of *Eucalyptus camaldulensis* has been reported by del Moral and Muller[6]. Chou and Muller[5] detected tannic, gallic, vanillic, p-hydroxybenzoic, chlorogenic acids, hydroquinone, arbutin and two other phenolics in the artificial rain drip of *Artostaphylos glandulosa*. Wilson and Rice[19] detected scopoletin and a-naphtol accumulation from foliar leachate of *Helianthus annuus*, which reduced the per-

centage of seed germination and oven dry weights of many species. Hair and pollen of Parthenium transport parthenin and vanillic acid over long distances.

Air-dried plant parts contribute large amount of phenolics and sesquiterpene lactones to the soil by rain-wash and by death and decay of plant residue. Wang *et al.*[17] identified p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, ferulic acid and syringic acid released from plant residue in soil and quantified and demonstrated their inhibitory effect under cultural condition. Whitehead[18] isolated p-hydroxybenzoic, vanillic acid and p-hydrozybenzoic acid to soil during their decay.

Thus addition of large amounts of sesquiterpene lactones and phenolics to the soil by various routes from Parthenium weed render the soil highly unsuitable to the growth of other species.

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