A Qualitative and Quantitative Study of The Prominent Phenolic Compounds of *Cannabis sativa* L. And Its Ecological Significance

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INTRODUCTION

An increasing amount of evidence has been reported within the past few years substantiating the existence and interplay of chemical substances which inhibit the establishment, growth, and reproductivity of plant communities. Personal field observations of stands of Cannabis sativa L., commonly called marijuana, indicate that due to decreased numbers of commonly associated weedy plants in and around these stands, some degree of plant inhibition may be occurring that cannot be accounted for by the usual plant competition for minerals, water or light. Although it is commonly found in abandoned fields, roadside ditches, waste areas and so on, Cannabis sativa is cosmopolitan in its habitat and can also occur in disturbed or cultivated areas.

Rice and Parenti (1967) reported their identification of several phenolic inhibitors, isolated from a number of plant species commonly found in abandoned fields, including chlorogenic acid, isochlorogenic acid, ferulic acid, β-resorcylic acid, gentistic acid, a glucose ester of caffeic acid, gallic acid and gallotannic acid. Rasmussen and Rice (1971) concluded that most of the inhibitory effect of Sporobolus pyramidatus is exerted by the decomposition of plant parts, particularly the shoots. After chemical analysis of dead shoot material of S. pyramidatus, they identified the presence of large quantities of two phenolic acids, p-coumaric acid and ferulic acid. Both compounds were able to inhibit seed germination of Aristida palmeri significantly.

Stands of Cannabis sativa exhibit the aggressive behavior of this weedy plant to establish and sustain itself readily and to be more competitive than most common weedy species of abandoned areas. Therefore, this project was undertaken to isolate, identify and quantitate prominent phenolic compounds from extracts of Cannabis sativa and to determine if aqueous extracts of C. sativa would have any inhibitory effects upon the seed germination of commonly associated weedy species.

MATERIALS

Seeds of Cannabis sativa L. were germinated in petri dishes on germination discs for 5 days at 27° C. Seedlings were then planted in sand in 9 inch clay pots, six seedlings per pot. The plants were grown in a growth chamber (Percival, Model PT .80) with a photoperiod of 14 hours light at 75° F and a nighttime temperature of 65° F. Dwarfed and dead plants were removed during the growing period.

The plants were continually supplied with a complete nutrient solution (Hoagland and Arnon, 1950) through a continuous-feed reservoir apparatus throughout growth periods of 3, 7, and 11 weeks. Water and methanolic extracts of leaves, stems, and roots were prepared for each age group by grinding 10 g of fresh plant material with 100 ml distilled water and absolute methanol, respectively, in a Waring Blendor for about one minute and suction-filtered through Whatman No. 1 paper. All extracts were frozen immediately following preparation.

EXPERIMENTATION AND RESULTS

Isolation and Identification of Phenolic Compounds

Preliminary separation of compounds was accomplished by one-dimensional descending paper chromatography. Each extract was streaked along a 34 cm horizonal line on Whatman 3 MM paper; 7 ml of the methanolic extracts and 5 ml of the aqueous extracts were applied. Duplicate pairs of chromatograms were prepared, one pair developed in n-butanol : acetic acid : water (63:10:27, v/v, known as BAW) for 12 hours and the other pair developed in 6% acetic acid (6% AA, v/v) for 5 hours. After development the air-dried chromatograms were examined under long (3360 A) and short (2537 A) ultraviolet light, before and after exposure to NH_3 vapor. Prominent bands were marked and average R_f values calculated.

Further characterization was made possible by color reactions with three phenolic reagents: ferric chloride-ferricyanide reagent, sulfanilic reagent and diazotized p-nitraniline reagent (Smith, 1960). One chromatogram from each pair was cut in four equal lengthwise strips, one strip dipped into one of the above reagents. The fourth strip remained as a control.

The prominent bands previously marked were cut from the remaining paper of each pair and eluted for 48 hours in 70% methanol ($R_f \geq 0.50$) or 50% methanol ($R_f < 0.50$). Eluates with similar R_f values were combine

Further isolation of the compounds was accomplished by two-dimensional paper chromatography. Each eluate group was spotted in quadruplicate on Whatman No. 1 paper at a point 8 cm above the bottom and right-hand margin of the paper. Depending on the amount of volume available, 1.0 - 1.5 ml of eluate were spotted per chromatogram. Each paper was developed first in the long phase with BAW and second, in the short phase with 6% AA. Following development in 6% AA the chromatograms were examined as before with long and short UV light, both before and after NH_3 exposure. Dipping of whole chromatograms, as opposed to the dipping of segments of chromatograms as before, into one of the three aforementioned phenolic reagents was performed.

Positive identification was made by selecting previously isolated compounds and co-chromatographing them in one-dimension on Whatman No. 1 paper with possible known compounds. Known compounds were prepared in the concentration of 20 mg / 40 ml absolute ethanol. Chromatograms were prepared in duplicate by alternating known and unknown spots every 1.5 inches along a line 8 cm from the bottom of the paper. 3-4 ml were applied to each spot in order to produce spots visible enough for positive identification. Both solvent systems previously mentioned were again used.

Following development of each paper, R_f values were calculated after observations under long and short UV, before and after exposure to

NH_3 vapor. Further identification was made by dipping the chromatograms in ferric chloride-ferricyanide reagent and sulphanilic reagent.

Unknown A, isolated from 7 week old leaf extracts, produced an R_f=0.79 in BAW, and in 6% AA two spots were observed at 0.43 and 0.67 (Table I). The spots were bright blue under long and short UV light before exposure to NH_3, and an even more intense bright blue fluorescence after exposure to NH_3. The spots produced a light blue coloration upon dipping in ferric cloride-ferricyanide reagent, but were negative in the sulfanilic reagent. Caffeic acid produced a comparable spot to Unknown A in BAW, with an R_f=0.79 (Table I). In 6% AA caffeic acid produced a long streak from the origin to 0.44, with a single spot at 0.67 . The caffeic acid streak and spot fluoresced a very bright blue. A dark blue coloration of the 0.67 spot was observed after dipping in ferric chloride-ferricyanide reagent and dark brown in sulfanilic reagent. Since both Unknown A and caffeic acid chromatographed identically and fluoresced similarly under UV light, Unknown A was identified as caffeic acid. It appeared that caffeic acid was possibly present in 3-week old leaf extract.

Unknown B, isolated from 11-week old leaf extract, was chromatographed beside chlorogenic acid (3-β-caffeoyl-quinic acid). Unknown B produced R_f's = 0.67 in BAW and 0.61 in 6% AA (Table I), fluorescing bright blue under long and short UV before exposure to NH_3 and a very brilliant yellow-green (called duck-egg green, DEG by Rice, 1965). Exposure to ferric chloride-ferricyanide reagent produced a light blue

coloration. Chlorogenic acid chromatographed with R_f's = 0.62 in BAW and 0.61 in 6% AA (Table I). A bright blue, highly fluorescent spot was visible under UV light before exposure to NH_3 turning to a brilliant yellow-green (DEG) under UV light after NH_3 exposure. Upon dipping in ferric chloride-ferricyanide reagent, chlorogenic acid turned dark blue. With the similarity demonstrated by the above data, Unknown B was clearly indicated to be chlorogenic acid. A common spot appeared above both known chlorogenic acid and Unknown B at an R_f = 0.71. This spot appeared dark blue under UV light before NH_3 and DEG after NH_3 exposure. These data indicated that isochlorogenic acid may be present in Cannabis sativa, but its presence could not be confirmed.

Quantitation of Identified Phenolic Compounds

The quantitation procedure consisted of establishing a concentration gradient of known compound by 1:2 dilutions of 2.0 mg/ml 70% methanol. A spectrophotometer (Hitachi Perkin-Elmer, UV-Vis spectrophotometer, Model 139) recorded the absorbance standards of the known compounds from 200 nm to 900 nm at 50 nm increments. The absorbance peak occurred at 300 nm and therefore, the absorbance of the unknown compound was recorded at that wavelength. Due to an insufficient volume of Unknown B, identified as chlorogenic acid, it could not be quantitated. However, Unknown A (caffeic acid) was quantitated at a concentration of 0.0113 mg / ml 70% methanol or 0.113 mg caffeic acid / g 7-week old fresh leaf material (Figure 1).

Effects of Cannabis sativa Extracts on Seed Germination

To assay inhibition effects of Cannabis sativa extracts on seed germination, aqueous extracts of 4, 7, and 11 week old leaf, stem, and root material were prepared in the concentration of 10 g plant material / 100 ml distilled water. The extracts were later diluted in a 1:10 ratio with distilled water to increase the total volume.

The tests were constructed by selecting 400 seeds of Bromus japonicus, Helianthus annuus, and Setaria viridis and placing them on germination discs in sterile petri dishes (200 seeds per dish). Each dish was saturated with 5 ml of diluted extract, covered and placed in an incubator at 27° C. Germination counts were made after the first 24 hour period, followed by counts every 12 hours.

There was a reduction in the percent germination of all test seeds exposed to extracts of Cannabis sativa (Tables II, III, IV). Leaf, stem, and root extracts of all three age groups reduced the percent germination of Setaria viridis and Helianthus annuus, with the greatest effect caused by the 7-week stem and 7-week root extracts. Only a slight inhibition could be observed in any test on Bromus japonicus.

Rate of germination was normal for the B. japonicus and S. viridis seeds with the greatest rate increase occurring between the 24 hour and 36 hour stages (Tables II, IV). A stimulatory effect was observed however, in the rate of germination of the H. annuus seeds (Table III).

Table I. Two-dimensional chromatography for positive identification of possible inhibitors.

Compound	R_f's on Whatman No. 1 BAW	R_f's on Whatman No. 1 6% AA	Long & Short UV - NH_3	NH_3 only	Long & Short UV + NH_3	$FeCl_3$-$K_3Fe(CN)_6$	Sulfan. acid
A	0.79	0.43, 0.67	b bl	vis-yel	b bl	lt bl	neg
Caffeic acid	0.79	0.00-0.44 0.67	vb bl	vis-yel	vb bl	dk bl	dk bn
B	0.67	0.61	b bl	neg	yel-gr	lt bl	---
Chlorogenic acid	0.62	0.61	b bl	vis-yel	yel-gr	dk bl	---

(b, bright; bl, blue; bn, brown; dk, dark; gr, green; lt, light; v, very; yel, yellow; vis, visible)

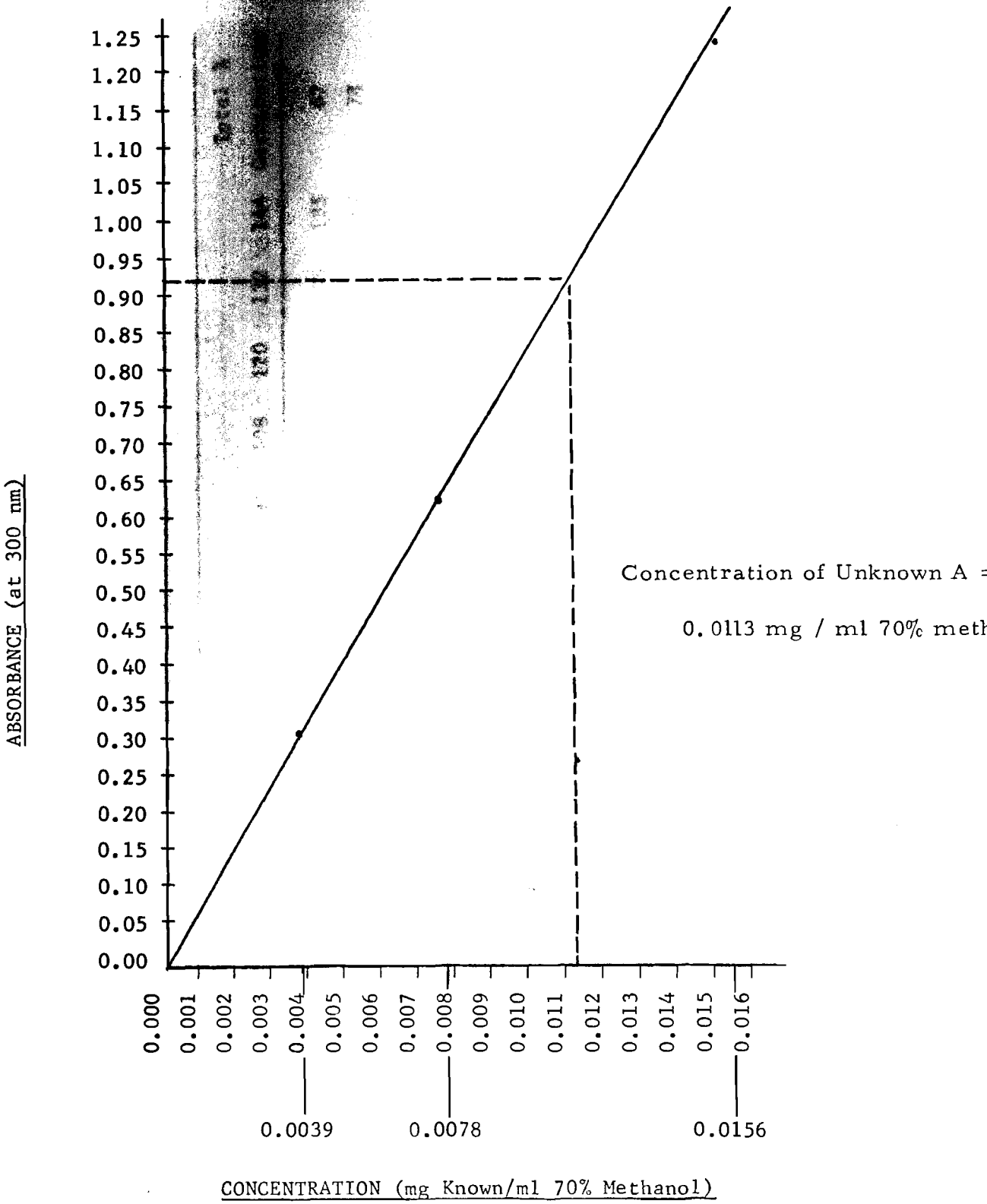

Figure 1. Determination of the quantity of Unknown A (caffeic acid) extract from 7-week old fresh leaf material of Cannabis sativa L.

Table II. Effects of aqueous extracts of Cannabis sativa L. on rate and per cent germination of selected seeds of Bromus japonicus.

Extract	Number of seeds germinated/time in hours											
	24	36	48	60	72	84	96	108	120	132	144	Germination Seed %
4 week leaf	16	108	127	130	131	134	134	134	135	135	135	67
7 week leaf	32	122	138	149	152	155	155	155	157	157	157	78
11 week leaf	26	95	106	121	124	126	127	127	128	128	128	64
Control	52	159	169	173	174	176	177	177	178	178	178	89
4 week stem	38	136	154	156	157	158	158	159	159	159	159	79
7 week stem	10	111	122	128	130	133	133	133	133	133	133	66
11 week stem	12	115	144	149	151	152	152	152	152	152	152	76
Control	52	159	169	173	174	176	177	177	178	178	178	89
4 week root	46	122	127	129	132	133	133	135	136	136	136	68
7 week root	23	154	168	171	174	175	175	175	175	175	175	87
11 week root	38	140	151	151	153	154	154	154	154	154	154	77
Control	52	159	169	173	174	176	177	177	178	178	178	89

germination of selected seeds of Helianthus annuus.

	Number of seeds germinated/time in hours											
Extract	24	36	48	60	72	84	96	108	120	132	144	Total % Germination
4 week leaf	1	3	6	8	50	50	51	51	52	53	53	26
7 week leaf	1	1	2	2	50	50	52	60	62	62	62	31
11 week leaf	3	5	5	5	26	28	30	30	46	46	46	23
Control	1	2	5	6	90	91	92	97	97	97	107	53
4 week stem	2	4	6	7	28	28	30	32	32	32	32	16
7 week stem	1	2	4	4	11	11	11	11	11	11	11	5
11 week stem	1	9	11	11	27	35	36	39	49	49	49	24
Control	1	2	5	6	90	91	92	92	97	97	107	53
4 week root	6	10	12	12	44	44	45	46	46	46	46	23
7 week root	1	9	11	11	21	21	21	21	21	21	21	10
11 week root	2	5	6	6	46	48	48	49	52	52	52	26
Control	1	2	5	6	90	91	92	92	97	97	107	53

Table IV. Effects of aqueous extracts of Cannabis sativa L. on rate and per cent germination of selected seeds of Setaria viridis.

	Number of seeds germinated/time in hours											
Extract	24	36	48	60	72	84	96	108	120	132	144	Total % Germination
4 week leaf	2	37	40	54	61	61	61	61	61	61	61	30
7 week leaf	3	32	33	71	78	78	78	78	78	78	78	39
11 week leaf	2	9	10	47	50	50	50	50	50	50	50	25
Control	3	104	122	133	167	167	167	167	167	167	167	83
4 week stem	2	23	24	31	38	38	38	38	38	38	38	19
7 week stem	3	5	6	8	17	17	17	17	17	17	17	8
11 week stem	2	31	32	39	54	54	54	54	54	54	54	27
Control	3	104	122	133	167	167	167	167	167	167	167	83
4 week root	4	48	49	49	49	49	49	49	49	49	49	24
7 week root	1	17	18	29	32	32	32	32	32	32	32	16
11 week root	2	35	36	50	63	63	63	63	63	63	63	31
Control	3	104	122	133	167	167	167	167	167	167	167	83

DISCUSSION

Paper chromatography of extracts of Cannabis sativa L. resulted in the identification of two phenolic acids, caffeic acid and chlorogenic acid. Both compounds are derivatives of cinnamic acid, and are well documented in the literature as plant inhibitors (Varga and Koves, 1959; Rice, 1965a, 1965c; Abdul-Wahab and Rice, 1967; Parenti and Rice, 1969).

Of particular interest is the inhibition occurring in plant species associated with abandoned fields. Seed germination analyses on three such species, Bromus japonicus, Helianthus annuus, and Setaria viridis, have definitely indicated the presence of toxic substances in aqueous extracts of C. sativa.

Two extracts in particular had tremendous influences on the percent germination of S. viridis and H. annuus. They were 7-week stem and 7-week root extracts. Additional inhibition was also caused by the 4-week and 11-week stem and root extracts on these two plant species.

Of the three species tested Bromus japonicus was only slightly affected by the C. sativa extracts, whereas the other two species were greatly affected. Parenti and Rice (1969) found that seed germination of B. japonicus was initially affected by aqueous extracts of Digitaria sanquinal but final germination percentages were not affected appreciably. However, these crabgrass extracts did significantly reduce the oven-dry weight of 14-day old Bromus japonicus seedlings. Rasmussen and Rice (1971) showed

a similar behavior of B. japonicus. Their soil extracts, prepared from soil collected from or adjacent to stands of Sporobolus pyramidatus, caused an initial reduction in the percentage of B. japonicus seeds from germinating although recovery occurred within 2 weeks. Seedlings of B. japonicus were definitely affected by these soil extracts. Rhizome and leaf extracts of Johnson grass produced identical results upon the seed germination and seedling growth of Bromus japonicus (Abdul-Wahab and Rice, 1967). These data point out that Bromus japonicus is one of the pioneer species which is not inhibited by competitors during seed germination, but can be and often is affected by inhibitors after leaf production is well underway and the seedlings are promoting the photosynthetic process.

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