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Flavonoid Chemotaxonomy of Cacalia (Compositae)

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FLAVONOID CHEMOTAXONOMY OF CACALIA (COMPOSITAE)

by

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INTRODUCTION

The beginnings of plant chemical taxonomy can be traced back to antiquity, for throughout history there are records of man's use and knowledge of plant natural products and their relationship to various groups of plants. More truly scientific applications of chemotaxonomy began several hundred years ago with more detailed studies of natural products, chiefly the oils, medicinals, resins and dyes. It has only been relatively recently, however, with the development of modern chemical technology that analytical methods have been able to yield detailed and accurate information which can be of real value to taxonomy. In the past two decades many areas of chemotaxonomy have been utilized, including the study of such diverse chemical categories as alkanes, lipids, alkaloids, proteins, amino acids, carbohydrates, quinones, terpenoids and phenolic substances, which include the flavonoids. Modern chemical taxonomy seeks to find new and valuable data reflecting the results of biochemical evolution in plants. These data, in conjunction with the morphological criteria now established, can provide the taxonomist with a potentially better set of standards on which to base plant classification.

This report is a study of the flavonoid chemotaxonomy of the eight species of Cacalia occurring in eastern North America, commonly called the Indian Plantains (Table I). The taxonomic status of these plants, now classified in the genus Cacalia, has

Table 1. Location of Cacalia specimens collected for chromatographic analysis.[a]

Species	Location	Voucher Specimen No.
C. atriplicifolia	Michigan, Kalamazoo Co.	R.W. Pippen 380[b]
	Indiana, Monroe Co.	R.W. Pippen 343[b]
C. diversifolia	Florida, Levy Co.	R.W. Pippen 616
C. floridana	Florida, Taylor Co.	R.W. Pippen 615
C. lanceolata	Texas, Brazoria Co.	R.W. Pippen 585
	Texas, Harris Co.	R.W. Pippen 588
	Mississippi, Jackson Co.	R.W. Pippen 597
	Florida, Jefferson Co.	R.W. Pippen 612
C. muhlenbergii	Indiana, Monroe Co.	R.W. Pippen 344
	Tennessee, Cheatom Co.	R.W. Pippen 76[c]
C. sulcata	Florida, Calhoun Co.	R.W. Pippen 605
	Florida, Leon Co.	R.K. Godfrey 64651 FSU[c]
C. suaveolens	Indiana, Fountain Co.	R.W. Pippen 620
	Ohio, Jackson Co.	R.L. Stuckey 3321
C. tuberosa	Indiana, Randolph Co.	R.W. Pippen 346
	Michigan, Barry Co.	R.W. Pippen 369

a. Voucher specimens in the C.R. Hanes Herbarium, W.M.U., unless otherwise stated.

b. Leaves for chromatographic analysis collected at a later date than the voucher.

c. Leaves for chromatographic analysis from plant growing in W.M.U. greenhouse.

long been uncertain because of their close relationship to the large, diversified and cosmopolitan genus Senecio. According to some taxonomists, Cacalia should be considered a part of Senecio, while others maintain that Cacalia is a distinct and separate genus (Pippen, 1968).

The object of this research is to provide information which will help show the relationships of the eight species to each other, and to provide preliminary chemotaxonomic data which can later be used to find the relationship of Cacalia to other closely related groups of plants.

Flavonoid chemical characteristics

Flavonoid pigments are a series of related, water-soluble phenolic compounds. The alcohol extraction procedure used in this research yields not only flavonoids but also related phenolic compounds (Seikel, 1964). In a broader sense, the term "flavonoid compound" encompasses not only the true flavonoids, which are based on the C_{15} skeleton structure of flavone (Figure I), but also closely related compounds such as the chalcones, isoflavones, aurones, stilbenes, cinnamic acids and coumarins (Bate-Smith, 1963).

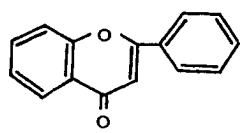

Fig. I. Flavone

Flavonoids occur in their natural state primarily as glycosides, although aglycones may be present (Brehm, 1966). The glycosidation of phenolic compounds is important in providing solubility and stability to light and enzymic degradation (Harborne, 1965). Flavonoids are usually hydroxylated and methylated, showing great variation in the number and position of substitution and thus producing a wide range of variation in the overall structure and chemical reactivity of the compounds (Seshadri, 1962).

Metabolic stability of flavonoids

The wide range of flavonoid chemical diversity enhances their value as chemotaxonomic tools, since they may be easily separated by two dimensional paper chromatography. Of even greater value to their usefulness in chemical taxonomy is their apparent metabolic stability. According to Harborne (1965), the only undisputed function of the flavonoid pigments is that they are primarily responsible for flower and fruit color. In some very unusual cases, flavonoid compounds inhibit seed germination and the growth of certain plant pathogens (Alston and Turner, 1963). Overall, however, flavonoid compounds have not been found to have an active part in cellular metabolism and may be considered as stable metabolic byproducts. These generally accumulate at the site of their synthesis which may be in any part of the plant (Towers, 1964). The significance of the relatively inert physiological role of flavonoids to their use in chemotaxonomy is well explained by E.C. Bate-Smith (1963):

> Constituents which are actively concerned in essential metabolic processes may be present in larger or smaller amounts (or even totally absent) depending on the momentary balance of these processes of metabolism in which they are involved. The exceptional usefulness of the flavonoid constituents as taxonomic guides however is due to the fact that they are not actively concerned in cellular metabolic processes. If, subsequent to their formation, they are involved in some normal physiological process, these must proceed at a relatively low rate, so that any particular flavonoid constituent can be relied on to be present in more or less constant amounts in the same tissues of the same species so long as the plants are grown under normal healthy physiological conditions.

Environmental stability of flavonoids

A recent study has found that the qualitative flavonoid composition of one clonal species of Spirodela is affected by artificial variation of certain nutrients and length of light exposure, however two other clonal species cited in the same report showed mainly quantitative variation if any at all (Ball et al., 1967). McClure and Alston (1964,1966) in similar studies on the same species found that variation of such diverse areas as light exposure, nutrient salts and plant hormones produced no significant qualitative differences in the plant's flavonoid composition. It is now generally accepted that any repeatable qualitative variation in the flavonoid composition of plants is indicative of genetic, rather than environmental differences (Brehm, 1966).

METHODS AND MATERIALS

Two dimensional paper chromatograms were made from extracts of the leaves of each of the eight species, and from the flower heads of five of the species. All plant material extractions and chromatograms were run in duplicate and where possible were run for more than one location or population of a species.

Plants were collected and stored according to standard herbarium techniques. The majority of the specimens used in this study were obtained on a collecting trip in the southeastern United States during the summer of 1967.[1] A list of the species used and their collection location is found in Table 1.

The chromatographic method followed was basically that of Alston and Turner (1962). For chromatographic application, 0.5 g of ground, dry leaves were extracted for 10 to 12 hours in the dark with 5.0 ml of 1% HCl in absolute methanol (Mallinckrodt Chemical Works). The extraction mixture was then centrifuged for five minutes at 6000 r.p.m. in a clinical centrifuge after which the supernatant fluid was pipetted off and a 2.0 ml aliquot prepared for immediate use. The extract was applied directly to full sheets ($18\frac{1}{4}$" x $22\frac{1}{2}$") of Whatman 3 MM paper in fifteen consecutive applications of 5.0 ul each. Chromatograms were developed in two directions, the first in tertiary butanol (Matheson, Coleman and Bell), glacial acetic acid (Allied Chemical Co.), and water, 3:1:1: v/v, and the second direction in

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[1]This trip was made possible by a grant from the Faculty Research Fund, Western Michigan University, Kalamazoo, Michigan.

glacial acetic acid and water, 15:85 v/v. The development time for the first direction was approximately 22.5 hours and for the second was about 4.5 hours.

The developed chromatograms were air dried and the spots detected in daylight and ultraviolet light, with and without the presence of ammonia vapor and then were sprayed with a 1% solution of $AlCl_3$ in ethanol. In all cases, reagents used were of reagent or A.C.S. grade, or of the highest grade available commercially. Water used was glass distilled and always taken from the same source. Caution was taken throughout to insure that all equipment was dust, fingerprint and in general, contaminant free.

In addition to the experimental chromatograms, control runs were made periodically, in which normal procedure was followed with the omission of only the leaf material.

Spots were scored according to location and detection characteristics, and those which on different chromatograms shared similar traits, were assumed to be the same (Table 2). The numerical assignment of spots was arbitrary and as new spots appeared on subsequent chromatograms, new numbers were consecutively assigned.

RESULTS

A total of 27 different phenolic compounds was detected in the leaves of the eight species. The chromatographic data are presented in Table 2 (Rf values and detection characteristics) and Table 3. A composite spot diagram of all eight species is presented in Figure

Table 2. Characteristics of chromatographic spots from leaf extracts of the eastern North American species of Cacalia.

Spot No.	Rf[a]	Rf[b]	UV[c]	Frequency[d]
1	.60	.33	F	4
2	.65	.54	F	1
3	.55	.72	F	8
3a	.55	.81	F	3
4	.50	.59	F	5
5	.40	.55	D	6
7	.32	.59	F	4
8	.31	.72	F	8
9	.33	.80	F	1
10	.25	.78	F	4
11	.39	.71	F	7
12	.45	.71	D	4
13	.56	.65	D	4
14	.62	.16	F	1
15	.03	.07	F	2
16	.29	.65	D	1
17	.71	.82	F	4
18	.66	.55	D	1
19	.37	.60	F	1
21	.76	.44	D	1
22	.59	.31	D	1
23	.32	.66	D	2
24	.49	.31	D	1
25	.47	.40	D	2
26	.59	.75	D	1
27	.46	.79	F	1
28	.14	.65	D	1
29	.74	.71	D	1

a. Rf in first solvent (t-butanol:acetic acid:water, 3:1:1 v/v).

b. Rf in second solvent (acetic acid:water, 15:85 v/v).

c. Ultraviolet light response. D = dark (UV absorbing), F = fluorescent.

d. Indicates the total number of species in which the spot appeared.

2, and spot distribution diagrams for each species in Figures 3 through 10. The flavonoid distribution in the inflorescences is not included in this report because flowers of all of the species were not available and these data are therefore incomplete. In general, however, the data obtained from the floral extracts of those species in which the flowers were available showed a close correlation to the data obtained from leaf extracts of the same species. The floral extracts contained mostly the same phenolic compounds found in the leaves. Among these unique floral spots was a high percentage of aglycones, while in the leaves only one spot (#14) was considered not to be a phenolic glycoside.

In cases where more than one specimen was used for a particular species, the total of all spots found among the specimens was compiled and considered typical of the species, however such action was infrequently requred. This cumulative distribution of the chromatographic spots is given in Table 3.

Data analysis: paired affinity index

The data presented in Table 3 may be analyzed and interpreted to show chromatographic, or presumptive biochemical, relationships of the species to each other and to the whole group. For this statistical analysis of the data, the paired affinity (PA) index and the group affinity (GA) value as presented by Ellison et al. (1962) are used.

Table 3. Chromatographic spot distribution in the eight species of eastern North American *Cacalia*.

	Spot Designation [a]																										
b.	1	2	3	3a	4	5	7	8	9	10	11	12	13	14	15	16	17	18	19	21	22	23	24	25	26	27	28	29
At	x		x			x		x		x	x	x								x	x	x						
Di			x		x			x			x		x		x			x	x								
Fl			x					x			x	x	x	x	x	x											
Ln	x		x	x	x	x	x	x		x	x	x	x				x					x		x			x	
Mu			x	x	x	x	x	x		x							x											
Sl			x		x	x	x	x			x						x											
Su	x	x	x	x		x		x	x	x	x																	
Tu	x		x		x	x	x	x			x	x	x				x						x	x	x	x		x

a. Spots 6 and 20 were omitted from the sequential numbering when it was determined that they were mixtures of already known spots.

b. At = *C. atriplicifolia*; Di = *C. diversifolia*; Fl = *C. floridana*; Ln = *C. lanceolata*; Mu = *C. muhlenbergii*; Sl = *C. sulcata*; Su = *C. suaveolens*; Tu = *C. tuberosa*.

The PA index is expressed as a percentage and defined by the formula:

$$PA = \frac{\text{spots in common for species A and B}}{\text{total spots in A + B}} \times 100$$

Thus the higher the PA value, the closer the correlation of spot patterns for any two species, and theoretically, the closer the degree of assumed relatedness. In using this technique, the fewer the number of spots, the greater the possibility that the results could be misleading. In this study, the average number of spots was 10 per species, which compares to an average of 13 spots per species presented in a report by Ellison et al. (1962) on another group of composites. PA values for all combinations of the eight species were calculated and are found in Table 4.

Table 4. Paired affinity values.

	At	Di	Fl	Ln	Mu	Sl	Su	Tu
At		33.3	44.4	64.0	44.4	47.1	52.6	48.0
Di	33.3		62.5	43.5	37.5	53.3	35.3	43.5
Fl	44.4	62.5		43.5	25.0	40.0	35.3	43.5
Ln	64.0	43.5	43.5		69.6	63.6	58.3	73.3
Mu	44.4	37.5	25.0	69.6		80.0	58.8	52.2
Sl	47.1	53.3	40.0	63.6	80.0		50.0	63.6
Su	52.6	35.3	35.3	58.3	58.8	50.0		41.7
Tu	48.0	43.5	43.5	73.3	52.2	63.6	41.7	

Data Analysis: group affinity value

These PA values were used to calculate the GA value of each of the species concerned. The GA value is the average of the sum of

all the PA values for one species, and therefore may be interpreted as showing how well one particular species pairs with all of the others. The GA value for each species was determined and presented on a 0 to 100 scale in which the maximum possible value of 100 would show perfect pairing with all other species and a value of zero would show that none of the compounds of this species were found in any of the others. The GA value for each of the eight species is found in Table 5.

Table 5. Group affinity and spot value indices.

	GA		SV
Sl	65.89	Sl	75.00
Ln	59.39	Mu	65.62
Mu	52.49	Su	58.33
Tu	52.24	At	56.25
At	47.70	Di	56.25
Su	47.43	Ln	55.00
Di	44.13	Fl	54.69
Fl	42.02	Tu	50.00

Data analysis: spot value index

Another method of determining a particular taxon's relatedness to a group is the spot value (SV) index, presented here for the first time. This method employs the concept that within a particular group of related plants, certain spots (or phenolic compounds) occur more frequently than do others and therefore the presence of one of these spots within a species may be interpreted as being more indicative of group affinity than the presence of those spots which rarely occur. A numerical value for this interpretation can be derived from the

data in Table 3, in which each spot is given a numerical value equal to the total number of times it occurs in the group. Spot number 1 therefore, has a value of 4, and spot number 3 has a value of 8, since it is found in all of the species. For a given species, the SV index can be derived by dividing the total values of all of its spots by the total number of its spots, or:

$$SV = \frac{\text{total value of spots}}{\text{total \# of spots}}$$

The SV value is presented on a 0-100 scale so that a species which had only spots found in all other species of the group would have a value of 100. The SV indices of the eight species are given in Table 5. A comparison of the rankings (highest to lowest) of the species according to the two criteria is given in Table 6.

Table 6. Comparison of GA and SV rank.

GA	Rank	SV
Sl	1	Sl
Ln	2	Mu
Mu	3	Su
Tu	4	At
At	5	Di
Su	6	Ln
Di	7	Fl
Fl	8	Tu

Confirmation of environmental stability

A series of chromatograms was run on specimens of C. lanceolata collected at three separate locations to determine if Cacalia con-

formed to a qualitative flavonoid stability in different environments, as reported by McClure and Alston (1964,1966). Two of the collection sites were in Texas, (Webster and Arcadia) roughly 20 miles apart, and the third was in Mississippi, approximately 600 miles from the Texas group. PA values were determined for all combinations, and their values ranged from 93.4 to 95.0. This data confirms the validity of flavonoid application in the chemotaxonomy of Cacalia. The most important PA index derived was that for the comparison of the Arcadia, Texas plants to the Mississippi plants. The PA of this comparison was 95.0 as was the PA for the comparison of floral extracts from the same plants.

DISCUSSION

It is not possible at this time to use these data as a source of comparing Cacalia to other related groups, because similar chromatographic information on Senecio and the Mexican senecioid composites is not now available. Some meaningful and interesting observations on the interspecific relationships of the eight species in this group can, however, be made.

Although the GA and SV indices both measure how well a particular species fits into the group, each shows this relationship in a different way. The GA value indicates how well a species pairs or matches with all of the others, while the SV shows the proportion of "major" or "minor" phenolic compounds in a species. The different interpretations of group relatedness shown by these two methods of

analysis is indicated by the differences in rank for C. lanceolata and C. tuberosa in Table 6. Although the overall spot pattern of C. lanceolata generally pairs quite well with the other species in this group as shown by its GA rank, its low SV rank indicates that it also possesses a fairly large number of spots which are either unique or rarely shared by the other species.

GA rank and geographical range

A comparison between the GA rank of a species and the range of its geographical distribution may be made, and here shows an interesting correlation. In their geographical ranges, C. sulcata, C. diversifolia and C. floridana are quite restricted, being endemic to certain confined habitats in Florida (Kral and Godfrey, 1958). C. atriplicifolia has the widest range, occurring throughout eastern North America, followed by C. lanceolata which occurs throughout the Gulf and Atlantic coastal plains from Texas to the Carolinas. The other species are more moderate in the range of their occurrence, with C. suavolens being sparce to rare within its range.

Allowing for only a minor switch in the GA rank for C. suaveolens and C. atriplicifolia (they differ by only 0.27 on the scale) the correlation between GA rank and geographical range may be determined and is shown graphically in Figure 11.

endemic	wide	moderate	wide	endemic
High		GA value		Low

Fig. 11. Correlation of GA rank to geographical range.

In this relationship, the middle GA rankings are occupied by the species of moderate range, the fairly high and fairly low rankings by the widely ranging species, and the extremes of GA rank by the endemics. Although this correlation may be purely coincidental, it is assumed at this time that it may be significant. Similar treatment of data from future studies on related taxa will be required, however, before any meaningful conclusions may be drawn.

Of the four specimens of C. lanceolata studied for this report, three were C. lanceolata var. lanceolata, while the fourth, and only one from Florida, was C. lanceolata var. elliottii. Chromatographic analysis of the specimens disclosed that the Florida plants lacked three spots of major occurrance in the other specimens, and in addition possessed a major spot found elsewhere only in the Florida endemics. The PA values between the Florida variety and the others averaged 47.1, as compared to an average PA value of 57.1 when compared to the Florida endemics. These results indicate that in this case there is a definite positive correlation between morphological and biochemical diversity.

SUMMARY

This report represents a pilot study to determine the practicality of flavonoid chemotaxonomic research of the cacalioid genera of Compositae. In the opinion of the author, the results of this study are promising enough to suggest further work on this group.

Within the group of plants studied in this report, a possible

relationship between the GA rank and the range of geographical distribution has been shown and a new method of group affinity analysis, the SV index, has been presented.

In future flavonoid chemotaxonomic investigations with the cacalioid genera, use of floral extracts would allow much more detailed analyses of data because of the larger number and greater diversity of phenolic compounds found in the flowers as compared to those found in leaves. Also, the use of the SV index will serve well, and possibly better than the use of the GA value, in establishing inter-group relationships.

The results of this research have shown a positive correlation between morphological and biochemical diversity and a negative correlation between environmental and biochemical diversity.

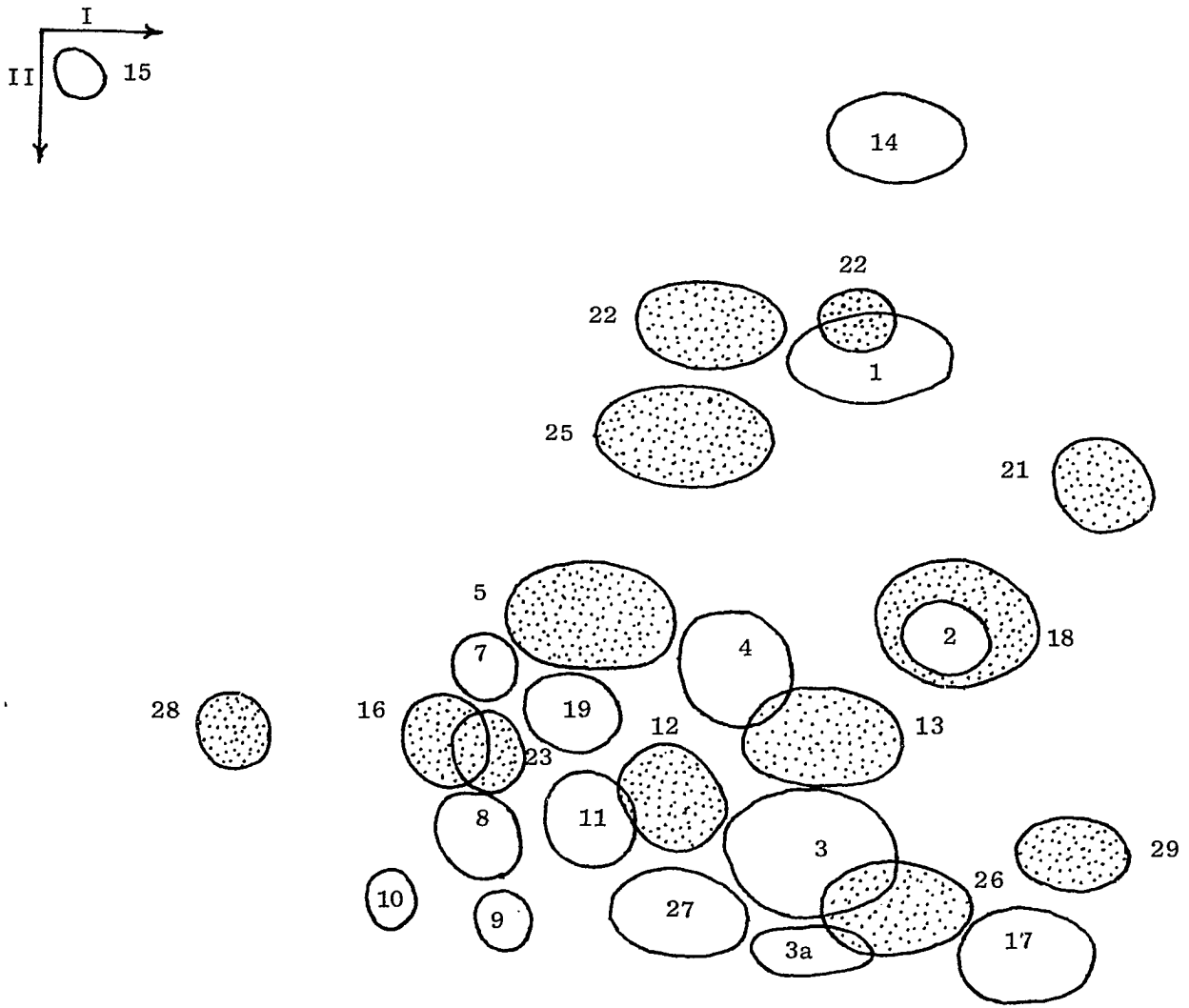


Fig. 2. Composite two-dimensional chromatographic pattern for the leaves of eight species of Cacalia. Shaded spots represent UV absorbing (dark) compounds, open spots represent fluorescent compounds.

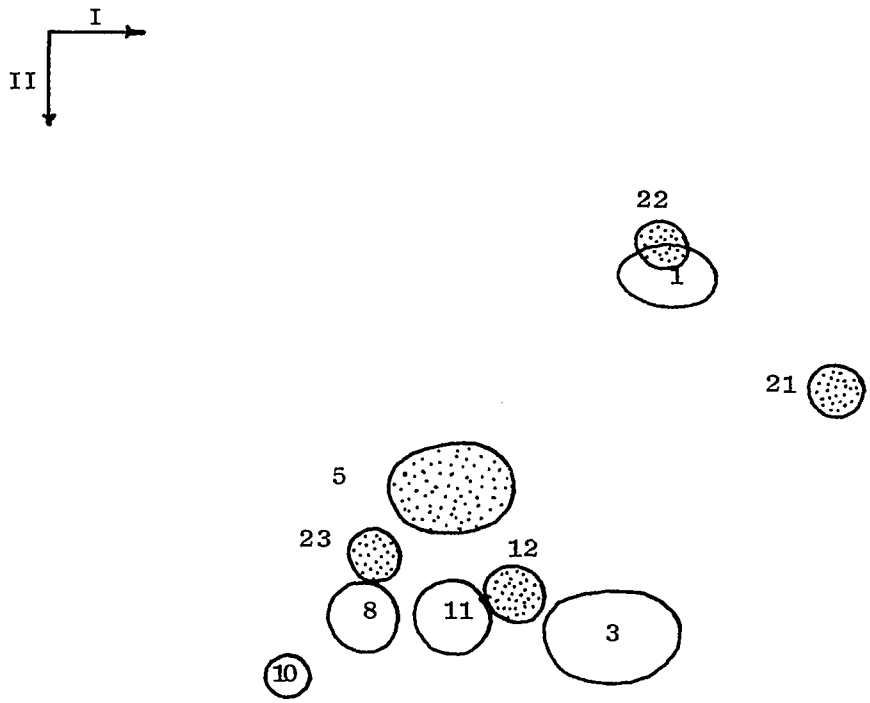
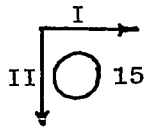


Fig. 3. Representative two-dimensional chromatographic pattern of the leaf extracts of C. atriplicifolia.



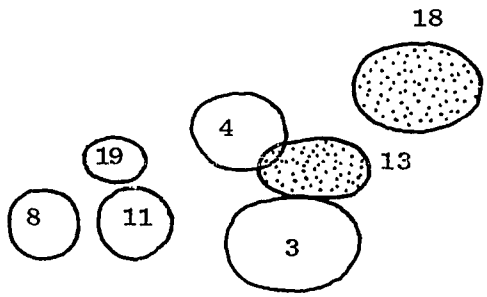
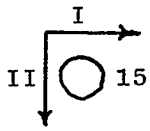


Fig. 4 Representative two-dimensional chromatographic pattern of the leaf extracts of C. diversifolia.



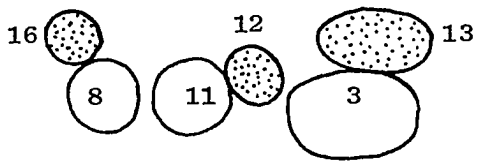
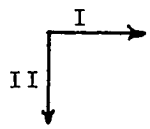


Fig. 5. Representative two-dimensional chromatographic pattern of the leaf extracts of C. floridana.



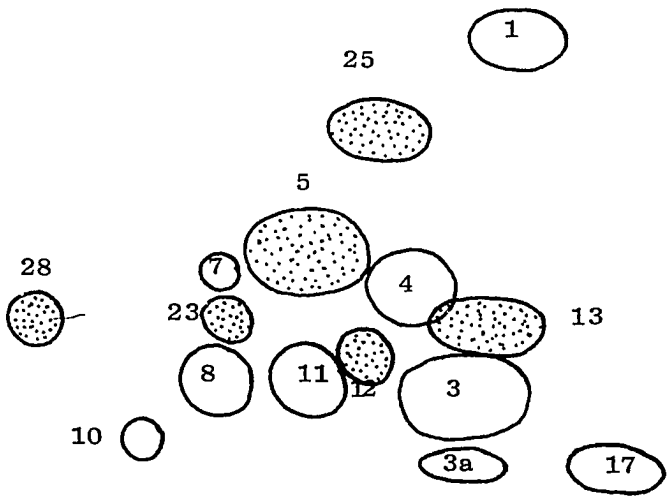
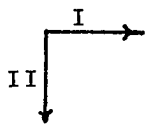


Fig. 6. Representative two-dimensional chromatographic pattern of the leaf extracts of C. lanceolata.



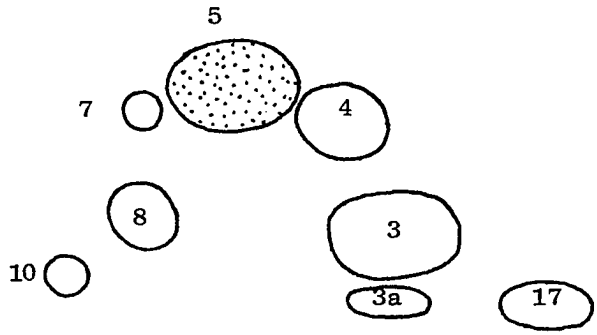
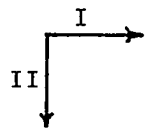


Fig. 7. Representative two-dimensional chromatographic pattern of the leaf extracts of C. muhlenbergii.



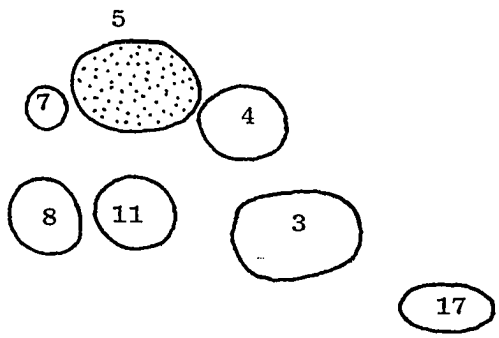


Fig. 8. Representative two-dimensional chromatographic pattern of the leaf extracts of C. sulcata.

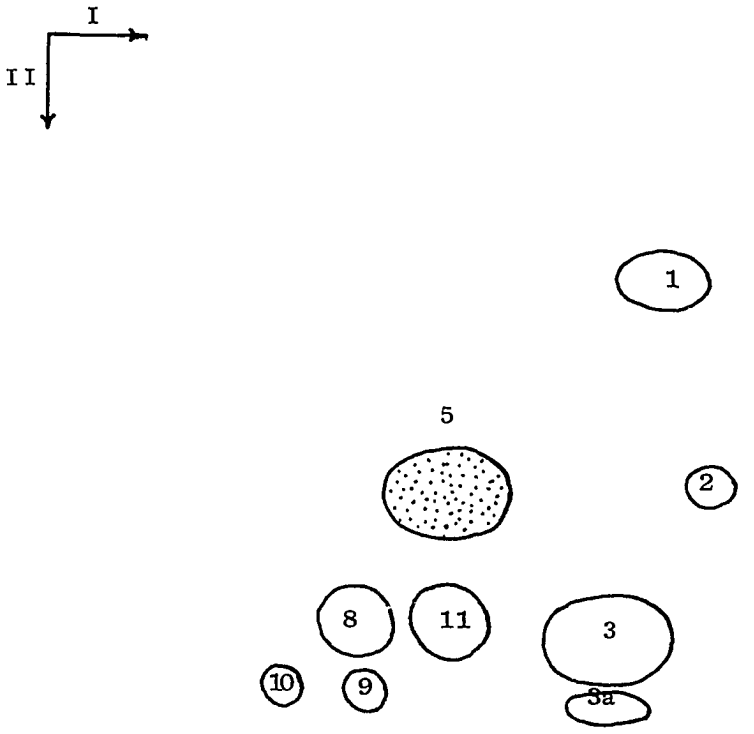


Fig. 9. Representative two-dimensional chromatographic pattern of the leaf extracts of C. suaveolens.

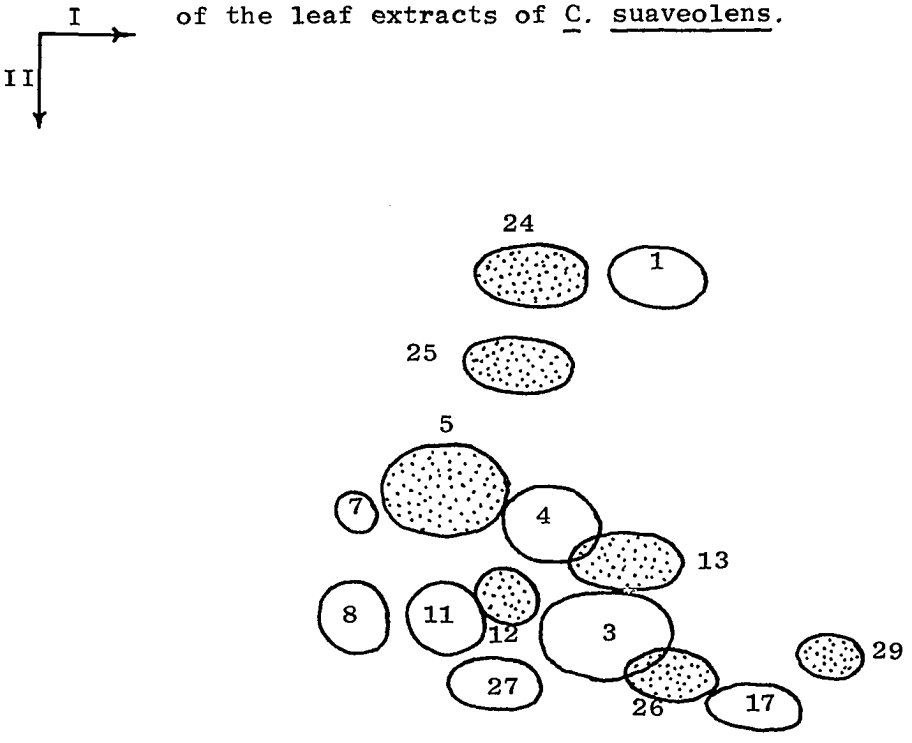


Fig. 10. Representative two-dimensional chromatographic pattern of the leaf extracts of C. tuberosa.

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