Analyses of *Ludwigia alternifolia* L. for Fatty Acids

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Abstract □ Selected extracts of the roots of *Ludwigia alternifolia* L. were examined for fatty acid content. GLC of methyl esters of saturated and unsaturated fatty acids provided qualitative as well as quantitative analyses. No unusual fatty acids were identified that might attribute antitumor activity to these extracts or be responsible for the demonstrated antimicrobial activity of *L. alternifolia* L.

Keyphrases □ *Ludwigia alternifolia* L.—fatty acid content examined □ Fatty acids—*Ludwigia alternifolia* content examined, relationship to antimicrobial activity □ GLC—methyl esters of fatty acids of *Ludwigia alternifolia*

In a separate publication (1), the antimicrobial activity present in the defatted component extracted from the roots of *Ludwigia alternifolia* L.[1] was reported. When the various defatted materials extracted from this plant were tested against an L-1210 lymphosarcoma system, at the Cancer Chemotherapy National Service Center, these materials showed negative results. The lipid fractions were not tested for antitumor activity; however, the lipid fractions were not observed to exhibit antimicrobial activity as did the defatted components. It was felt that since considerable interest has been generated by Tolnai and Morgan (2–4), involved with the *in vitro* studies of antitumor activity of certain fatty acids, further consideration should be given to the fatty acid content of *L. alternifolia* L. Therefore, to determine the fatty acid contents of the fatty materials extracted from the roots of *L. alternifolia* L. for further investigation of any antitumor activity and to provide a more complete analysis of this plant, this study was undertaken.

EXPERIMENTAL

The methyl esters of the fatty acids were prepared from the Rosenthaler Fractions A, B, and C (1, 5). These fractions were obtained by extracting the air-dried, coarsely powdered roots of *L. alternifolia* L. successively with petroleum ether (Fraction A), diethyl ether (Fraction B), and chloroform (Fraction C). The crude plant material was extracted with the aid of a soxhlet extractor for 48 hr. with each solvent, the marc being dried prior to the addition of the succeeding solvent. Each extract was then dried at room temperature and under reduced pressure, resulting semisolid masses being reserved for fatty acid analyses.

The prepared methyl esters were gas chromatographed at 150° using a polar column. The modified method of Goldfine and Block (6), involving preparation of the mercuric acetate adducts of the unsaturated acids and separation of these adducts from the methyl esters of the saturated acids by column chromatography, was employed and the collected eluates were analyzed. The latter procedure was useful in distinguishing the saturated and unsaturated fatty acids. Quantitation as percent of total recovered amounts was achieved by measuring the Disc integration recordings (7).

[1] Identified by John D. Freeman, Assistant Professor of Botany and Curator, Auburn University Herbarium (AUA Nos. 21,495 and 21,496), and George M. Hocking, Professor of Pharmacognosy and Curator, Medicinal Plant Collection (No. 1010), School of Pharmacy, Auburn University.

Table I—Retention Times and Separation Factors at 180 and 150°, Using Polar Column GLC, of Components of Plant Extract

Peak Number	Retention Time, min. 180°	Retention Time, min. 150°	Separation Factor[a] 180°	Separation Factor[a] 150°
I	16.8	18.2	1.35[b]	1.36[b]
II	30.1	33.9	1.36[b]	1.36[b]
IV	53.4	62.8	1.34[b]	1.36[b]
V	61.0	—[c]	1.14	—[c]
VI	72.0	85.9	1.35	1.37
VII	81.9	98.5	1.15	1.15
VIII	95.6	118.0	1.33	1.35
IX	110.0	135.5	1.15	1.15
X	127.6	157.0	1.34	1.36
XI	146.1	175.5	1.14 (1.52)[d]	1.12 (1.49)[d]
XII	172.6	214.2	1.35	1.36
XIII	201.8	249.5	1.17	1.16
XIV	233.0	289.0	1.35	1.37
XV	257.5	315.5	1.11 (1.49)[d]	1.09 (1.47)[d]
XVI	312.2	402.5	1.34	1.37
XVII	343.6	426.5	1.10 (1.99)[d]	1.06 (1.99)[d]
XVIII	413.8	545.0	1.33	1.35

[a] Calculated to two decimal places. [b] Calculated with aid of graph values (Figs. 1 and 2). [c] No value. [d] Modified separation factor.

Formation of Methyl Esters of Fatty Acids—The test material (15 mg.) was placed in a Pyrex tube (16 × 150 mm.) with a Teflon-sealed screw cap, and 5 ml. of 2% sulfuric acid in methanol was added. The tube was sealed tightly and placed in a heating block at 105° for 12 hr. The tube was then cooled to room temperature, 5 ml. of distilled water was added, and this mixture was extracted with 2 ml. of redistilled hexane. The hexane extract was removed with the aid of a pasteur pipet and placed in a test tube (15 × 75 mm.). The extracting process was repeated three times with 2 ml. of hexane each time. A small amount of anhydrous sodium sulfate was added to the collected hexane fraction to remove moisture, the supernatant hexane portion was then decanted, and the remaining sodium sulfate was washed with 1 ml. hexane. All hexane fractions were pooled and partially evaporated under nitrogen. The resulting hexane solutions of the methyl esters each were placed in individual airtight containers and stored in the refrigerator for future use.

Separation of Methyl Esters of Saturated and Unsaturated Fatty Acids—A sample (0.5–3.0 mg.) of dry methyl esters of the fatty acids and a portion (10–15 mg.) of mercuric acetate were placed in a culture tube (16 × 150 mm.) equipped with a Teflon-lined screw top, and 1 ml. of a solution containing 5% distilled water and 0.3% glacial acetic acid in methanol was added. The resulting tightly sealed tubes were heated in a water bath at 60° for approximately 3 min. to ensure solution of the mercuric acetate; the tubes then were stored in the dark at room temperature for 24 hr. The solvents and excess acetic acid were removed, and the residue was dried by evaporation under nitrogen gas at room temperature. The dry residue was shaken three times with 2 ml. benzene at 50–60°, and the extracts were filtered through glass wool onto a column of silicic acid (100–300 mesh).

The silicic acid column was prepared from a slurry in benzene which was poured into a pasteur pipet (50 mm. in height) whose tip was first packed with a small quantity of glass wool. The column was eluted with the benzene to a total volume of 20 ml. This eluate contained the methyl esters of the saturated fatty acids, with the

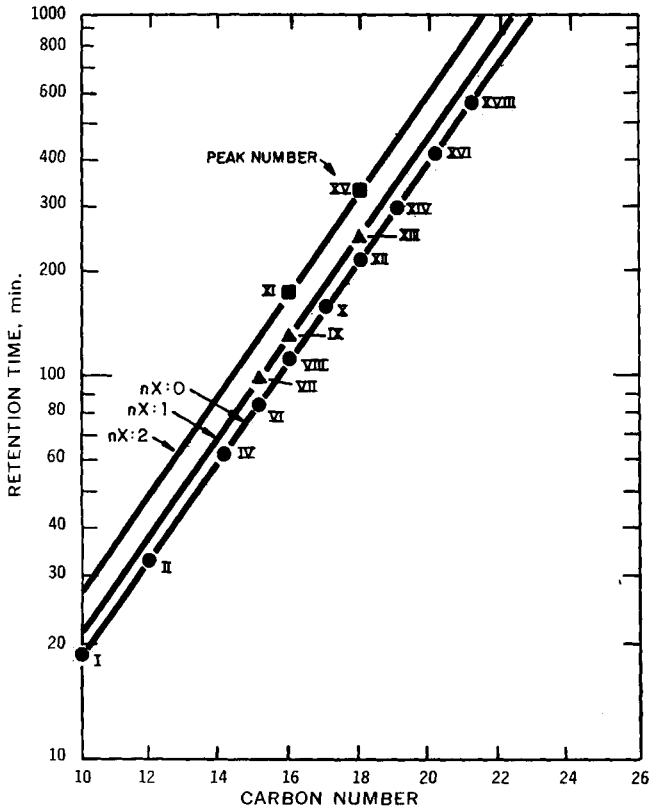

Figure 1—*Retention times of methyl esters of fatty acids derived from* L. alternifolia *L. plotted on a log scale against their carbon numbers. Results obtained at 150° on a polar column.*

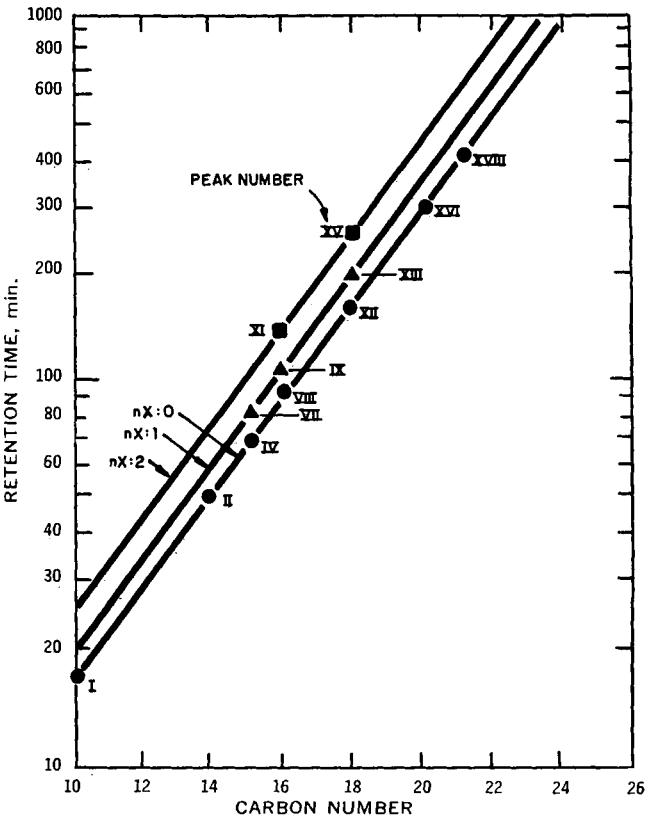

Figure 2—*Retention times of methyl esters of fatty acids derived from* L. alternifolia *L. plotted on a log scale against their carbon numbers. Results obtained at 180° on a polar column.*

methyl esters of the unsaturated fatty acid mercuric acetate adduct remaining at the top of the column as indicated by a yellow band. The benzene eluate was evaporated to dryness under nitrogen gas and stored under refrigeration as previously described.

The mercuric acetate adducts were eluted with 10 ml. of 5% glacial acetic acid in absolute ethanol. To recover the methyl esters of the unsaturated fatty acids, this eluate was treated with 1 ml. of 6 *N* hydrochloric acid and 5 ml. of water in a culture tube (25 × 150 mm.) with a Teflon-lined cap. After 5 min., this mixture was diluted with 10 ml. water and extracted with 2 ml. of hexane. The hexane layer was removed with the aid of a pasteur pipet. This extraction was repeated three times, using 1 ml. hexane each time. All hexane solutions were combined in a culture tube (16 × 150 mm.) with a Teflon-lined cap and washed twice with 2 ml. water. The resulting hexane fractions were dried with anhydrous sodium sulfate as previously described. The total hexane solution was placed in a test tube (13 × 100 mm.), evaporated under nitrogen gas, and stored.

The chromatographic assay was carried out with the aid of a gas chromatograph[2], equipped with a flame-ionization detector, and a recorder[3] with Disc chart integrator. A polar column [15% EGSS-X on Chromosorb W (AW) 80–100] and a nonpolar column (5% SE-30 on Chromoport 80–100), each using a 1.5-m. (5-ft.) by 0.63-cm. (0.25-in.) glass U-shaped column, was used with helium as the carrier gas. The retention time (R.T.) for each peak on the recorded chromatograph was measured to the nearest 0.1 mm. from the origin of the solvent peak to the maximum of the respective peak.

The separation factor (S.F.) was calculated for each component relative to the preceding saturated component, as described by Landowne and Lipsky (8), according to the respective retention time for each component used:

$$\text{S.F.} = \frac{\text{R.T. of component}}{\text{R.T. of preceding saturated component}} \quad \text{(Eq. 1)}$$

[2] Fand M model 402, Hewlett Packard.
[3] Honeywell, model 227.

The separation factor increases as separation temperature increases for the methyl esters of the unsaturated straight chain fatty acids, but decreases as separation temperature increases for the saturated straight and branched chain components (8).

The semilog plot of the log of the retention time *versus* the carbon number of a homologous series was constructed for the identification of various components as previously described by Ruseva-Atanasona and Janak (9), Murray (10), and James and Webb (11).

RESULTS AND DISCUSSION

The retention times and separation factors of the methyl esters of the fatty acids of *L. alternifolia* L. at 150 and 180°, using a polar column in GC analysis, are listed in Table I. The semilog graphs of the retention time *versus* carbon number for the methyl esters are shown in Figs. 1 and 2. The standard mixtures of methyl esters of fatty acids used in this study are listed in Table II. The retention times, separation factors, and graphic analyses for the methyl esters of the saturated and unsaturated fatty acids of *L. alternifolia* L., which were separated by the mercuric acetate method, were in agreement with similar experiments carried out with the standard mixtures. The identification of the respective methyl ester of the fatty acid and the average percent composition recovered for each component are listed in Table III.

Landowne and Lipsky (8) showed that the separation factor for the methyl ester of a saturated fatty acid is inversely proportional to the temperature, whereas the reverse is true for the unsaturated acid esters. This observation was confirmed in this analysis (Tables I and III).

A modified separation factor was used as a means of identifying the di- and triunsaturated fatty acid esters:

$$\text{S.F. (modified)} = \frac{\text{R.T. of unsaturated acid ester}}{\text{R.T. of parent-saturated acid ester}} \quad \text{(Eq. 2)}$$

These values are listed in Table I and agree with similar calculations with a standard mixture (K108) (Table II).

Table II—Contents of Standard Mixtures of Methyl Esters of Fatty Acids[a]

Methyl Esters of[b]	KE	EF	K108	Branched Mix L	PO & O
n8:0	x				
n10:0	x				
n12:0	x				
i14:0				x	
n14:0	x	x		x	
a15:0				x	
n15:0				x	
i16:0				x	
n16:0	x	x	x	x	
n16:1					x
a17:0				x	
n18:0	x	x	x		
n18:1			x		x
n18:2			x		
n18:3			x		
n20:0		x			
n22:0		x			
n24:0		x			

[a] The standard mixtures used were obtained from Applied Science Labs, Inc., State College, PA 16801. [b] Shorthand designation of the fatty acids represented: n8:0 is normal octanoic acid and n + normal straight chain, i = iso-branched chain, and a = anteiso-branched chain. The unsaturation is indicated by the unit to the right of colon, *i.e.*, x:1 is a monoene.

Table III—Identification of Respective Methyl Esters of Fatty Acids and Average Percent Composition Recovered from Each Fraction

Peak Number	Fatty Acid Identification	Percent Composition[a] A	B	C
I	n10:0	—[b]	—[b]	—[b]
II	n12:0	—[b]	—[b]	—[b]
IV	n14:0	2.3	2.7	2.6
V	n14:1	—[b]	—[b]	—[b]
VI	n15:0	1.9	1.9	3.2
VII	n15:1 and i16:0	2.3	1.8	3.0
VIII	n16:0	27.1	29.0	25.7
IX	n16:1	5.4	5.4	8.3
X	n17:0	2.2	2.6	2.1
XI	n16:2	4.2	8.3	14.6
XII	n18:0	9.4	6.1	5.1
XIII	n18:1	20.9	10.4	9.1
XIV	n19:0	1.5	1.2	—[b]
XV	n18:2	16.1	20.4	20.0
XVI	n20:0	5.5	2.6	2.4
XVII	n18:3	2.3	2.6	2.6
XVIII	n21:0	1.42	1.7	—[b]
XIX	n22:0	—[b]	—[b]	—[b]
XX	n24:0	—[c]	—[b]	—[b]

[a] A = petroleum ether fraction, B = diethyl ether fraction, and C = chloroform fraction. [b] Less than 1% of total. [c] Average of three determinations.

There is indication that peak VII is a combination of i16:0 and n15:1. This hypothesis was supported by the chromatogram of the unsaturated and saturated esters after separation by the use of mercuric acetate. The semilog plots of the homologous series also support this conclusion.

There were many very small peaks, representing not more than 0.1% of the total percentage composition, which were not positively identified. The following components were tentatively estimated: i8:0, n8:0, n9:0, i10:0, n10:0, and a17:0; they are not listed in Table III.

The peak estimated to be a17:0 was, for the most part, masked by n16:1, peak IX. There was difficulty in separating these saturated and unsaturated esters using mercuric acetate because of the relative minute quantity of a17:0. However, by comparison with the standard, Branched Mix L, the calculated separation factors, and the semilog graphics, peaks of this nature could be estimated.

The other minor peaks mentioned appeared early on the chromatograph where resolution was not as discrete or was masked by the solvent peak. In several cases, there was good resolution, but the peak was too minor to be considered.

The use of a nonpolar column provided confirmation for the qualitative aspect of this study. A reversal of the unsaturated fatty acid peaks was observed. The unsaturated fatty acid methyl esters were eluted from the column before the saturated components. However, in many cases the peaks of the unsaturated esters will mask those of the branched chain saturated esters. In addition, the mono-, di-, and trienes are grouped under one peak, rather than under separate peaks, as was observed with the polar column. This nonpolar column was not considered necessary for the quantitative studies of this sample.

The quantitative results are listed in Table III. The average percentage composition of the recovered sample is listed for each component present in quantities greater than 1%. These results involved only the polar column chromatographs.

No attempt was made to explain the differences observed in the fatty acids and their respective concentrations found in the Rosenthaler Fractions A, B, and C from the roots of *L. alternifolia* L.

SUMMARY AND CONCLUSIONS

The identification of the respective methyl esters of the fatty acids of *L. alternifolia* L. and the average percent composition recovered from each fraction (A, B, and C) are listed (Table III). Although the fatty acids determined are predominately the common C-16 and C-18 saturated and unsaturated ones, small percentages of the less common C-15, C-17, and C-19 acids were identified. Those acids found in significant proportions could be expected in higher plants and could not be expected to possess antitumor activity nor to be responsible for the antimicrobial activity demonstrated to be present in *L. alternifolia* L.

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