CHEMICAL AND ANTIBACTERIAL PROPERTIES OF KINOS FROM EUCALYPTUS SPP. CITRIODOROL —THE ANTIBIOTIC PRINCIPLE FROM THE KINO OF *E. CITRIODORA*

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ABSTRACT

The kinos of four Eucalyptus species, *viz.*, *E. citriodora*, *E. robusta*, *E. pilularis* and *E. globulus*, have been studied for their general physical and chemical characteristics as well as their antibacterial activity against a few representative micro-organisms. Ellagic acid is present in all the four species while glucose could be detected only in *E. citriodora* and *E. globulus* kino. In combination, possibly as a glucoside of 7-methyl-kæmpferol, glucose was found chromatographically in the former kino. The kino from *E. citriodora* showed considerable activity against Gram-positive organisms which warranted the isolation of the active principle.

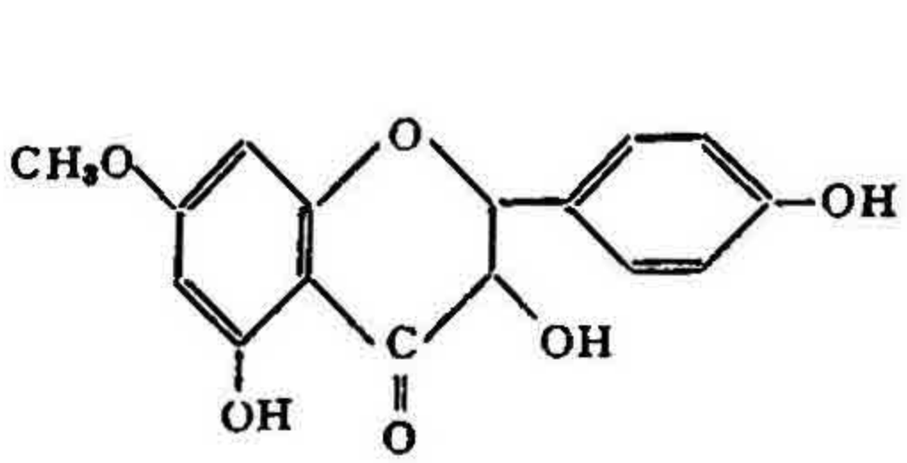
During its purification four crystalline substances were isolated besides a hydrocarbon, which were identified as aromadendrin-7-monomethyl ether, kæmpferol-7-monomethyl ether, ellagic acid and a dimethyl ether of aromadendrin the constitution of which has not been rigorously established. The latter was present only in the kino of a tree growing in a private garden a point which may be of some philogenetic interest in view of the classification of Eucalyptus spp. by Baker and Smith on the basis of the constituents present.

Various procedures like adsorption, partition and paper chromatography failed to yield the antibiotic in a crystalline state. However, a very highly active chromatographically homogeneous substance designated 'Citriodorol' has been isolated and preliminary studies suggest that it might belong to the group of *o*-dihydroxy flavonols.

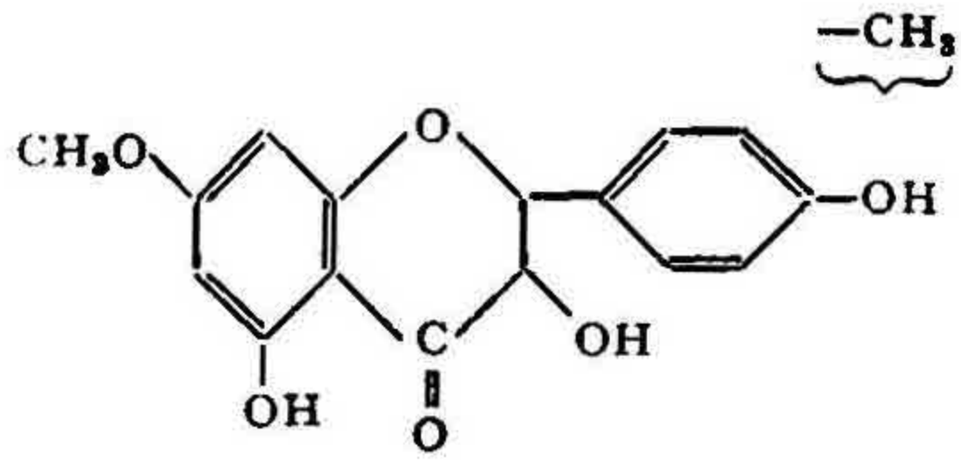
Citriodorol and the other constituents of the kino showed no marked pharmacodynamic effects.

Kinos of various trees and particularly of the Eucalyptus species[1] have long been used empirically for medicinal purposes, but none of them have been examined in any detail in this light. Among the Eucalyptus species that were introduced

in this country four species (*E. citriodora*, *E. robusta*, *E. pilularis* and *E. globulus*) from which kinos could be easily tapped have been investigated by us for their antibacterial property. The kino from *E. citriodora* which appeared to be more promising has been studied in some detail and the results are now reported in this paper. Evidence for the presence of a fairly potent antibiotic substance for which the name "citriodorol" is now suggested, has been adduced and methods for its concentration described. Among the various other constituents present in the kino. the following have been isolated in a pure state: aromadendrin-7-monomethyl ether (I), aromadendrin-dimethyl ether (II), kæmpferol-7-monomethyl ether (III) and ellagic acid (IV). In addition, free glucose and a glucoside of (III) have been detected in the aqueous extracts of the kino. The identity of (I) has



(I)



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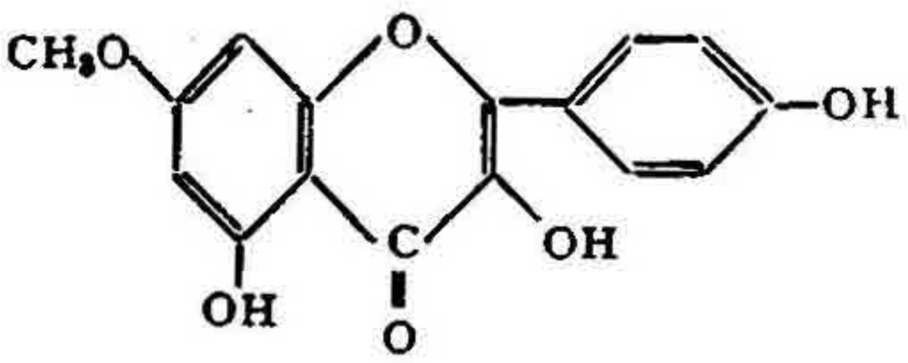

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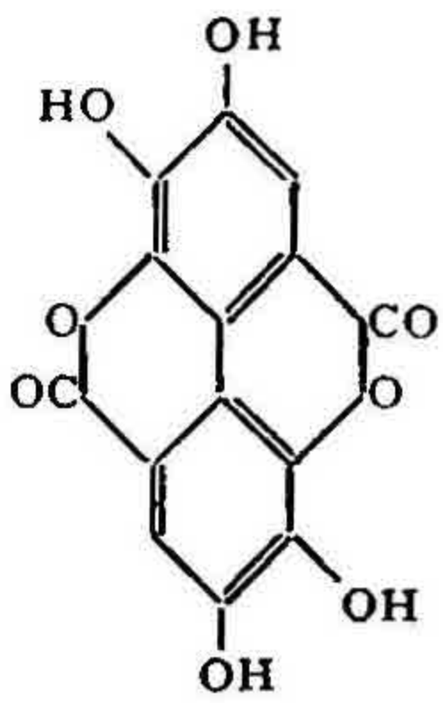
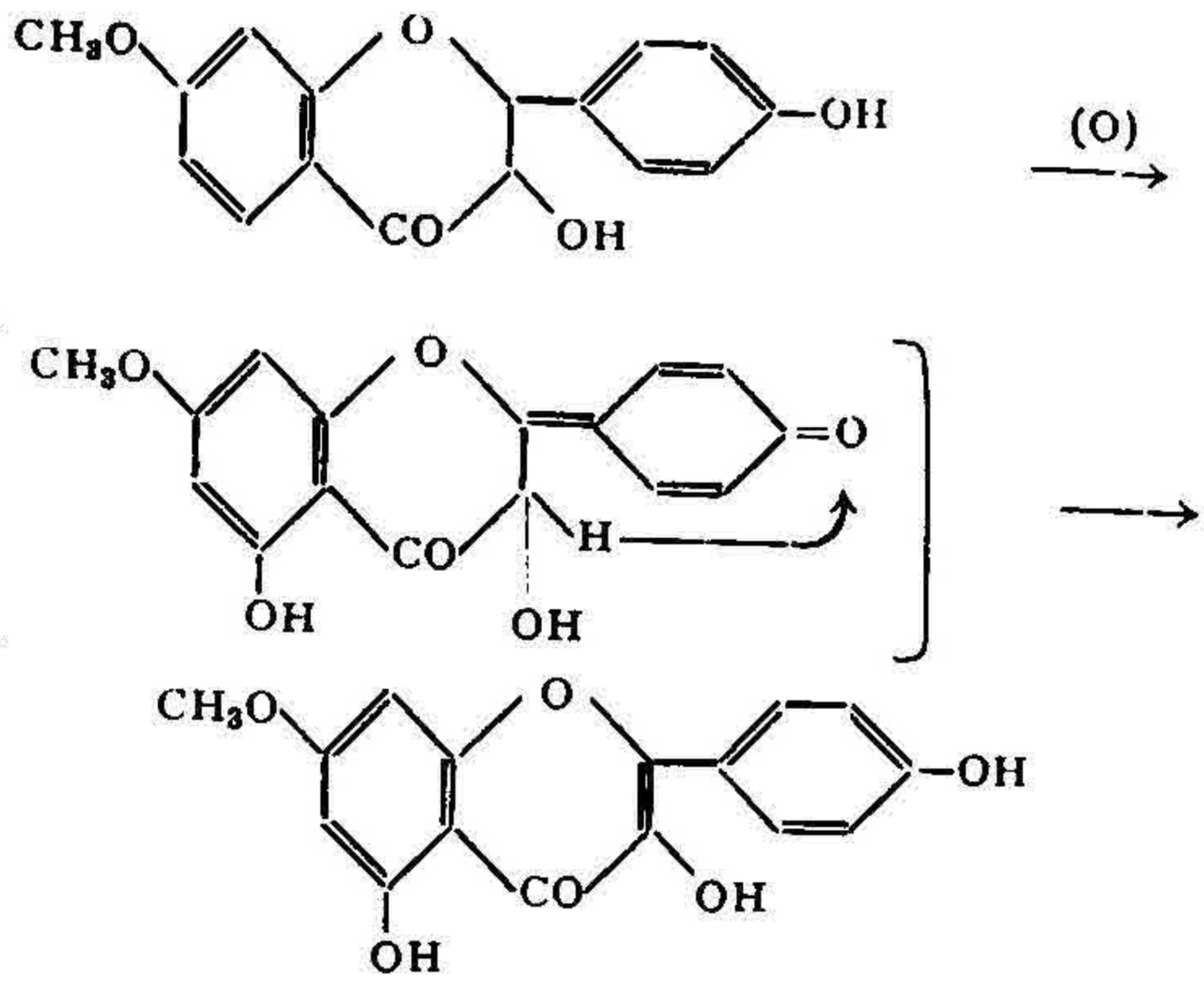


(IV)

been rigorously established by its physico-chemical properties, its conversion to the dimethyl ether, oxidation to (III) and comparison with authentic samples. However, clear-cut experimental evidence for the position of one of the methyl groups in aromadendrindimethyl ether (II) is lacking although it has been shown to be identical with the one described by Hillis[2] who prepared it by methylation of aromadendrin. That it is a 7-methyl derivative is clear from its formation from (I) by methylation. In view of its formation from (1) by the action of diazomethane, it is conceivable that the 4′-position is methylated. The mechanism of aerial oxidation of (I) to (III) may then be envisaged as shown below:



(III) has been identified by comparison with an authentic sample. The steam volatile material of the kino, unlike that of the pines,[3] contributes very little to its activity. As all these substances possess low antibacterial activity, whatever there is in the kino is to be ascribed to citriodorol. Further detailed examination of this compound awaits obtaining it in a crystalline state.

It may be mentioned in passing that all these substances, except ellagic acid, have not been previously reported in any kino. The unmethylated derivatives were, however, isolated by Hillis[2] from the *E. calophylla* kino and katuranin[4] (identical with aromadendrin) from *Cercidiphyllum japanicum*. That the monomethyl and the dimethyl derivatives should occur separately in two samples of the kino of the same species, *i.e.*, *E. citriodora* is a point of interest.

Experimental

Materials and Methods

(i) *Collection of kinos.*—The kino from *E. pilularis* was obtained from the State Sylviculturist, Ootacamund; the kino from *E. globulus* through the kind courtesy of Dr. V. N. Patwardhan, Director, Nutrition Laboratories, Coonoor, South India; while the kinos from *E. citriodora* and *E. robusta* were collected locally by the Government Sylviculturist. Another specimen of the kino from *E. citriodora* was obtained from a private garden in Bangalore. Interesting differences in the constituents of the two samples of the latter kino have been found and will be referred to *inter alia*. The samples were not subjected to any heat treatment prior to ascertaining their antibacterial activity.

(ii) *Antibacterial and antifungal assays.*—The antibacterial potency of a fraction was assayed by the routine serial dilution method.[5] Ordinary nutrient broth medium (pH 7·4) was used. Standard strains of (1) *Micrococcus pyogenes* var.

aureus and (2) *Escherichia coli* var. *communis* (both originally obtained from the King Institute, Guindy, Madras), (3) *Mycobacterium phlei* ATCC 3555 have been used as standard strains for assay.

Bioautographic technique[6] was used to find out the position of the active fraction on a circular paper chromatogram (*vide infra*). After developing a chromatogram, rectangular strips (width 1 cm.) were cut off across the bands and placed on seeded plates with a sterile forceps. After incubation, a clear zone of inhibition could readily be seen.

For antifungal assays, Roff and Atkinson's[7] method was used with slight modification, in that potato dextrose agar[8] was used instead of the malt agar medium. The potency was tested against standard strains of (1) *Penicillium notatum* ATCC 9178, (2) *Aspergillus niger* ATCC 9142 (both originally obtained from NURL, Illinois), and (3) *Fusarium oxysporium* No. 413 (originally obtained from the Ministry of Agriculture, Argentina).

All the assays were carried out in duplicate.

(iii) *Chromatography.*—Both adsorption and paper partition chromatography have been applied to separate and characterize various components present in the *E. citriodora* kino.

Out of the adsorbents tried, calcium sulphate was found to give consistent results. To resolve about 1 g. of the crude antibiotic, a column 18″×1·5″ dia. of the adsorbent was found suitable. A mixture of benzene and purified ligroin (1 : 2) was used as a developing solvent. The bands after extrusion were eluted with warm ethanol.

Unidimensional ascending chromatograms (Whatman No. 1, 9″×12″) were developed according to Hillis.[9] Circular paper chromatograms[10] (Whatman No. 1 filter discs 24 cm. dia.) were developed with a mixture of 6% phenol in water (v/v) and absolute ethanol (4 : 1), after saturation over the aqueous phase for 2 hours (*cf.* Table V). In both cases, spots or bands were identified by (1) examination under ultraviolet light; (2) spraying with Tollen's reagent, and (3) spraying with an ethereal solution of ferric chloride (1%).

(A) *Physico-chemical properties of the kinos.*—The characteristics of the kinos are given in Table I.

A preliminary examination of the four kinos showed absence of nitrogen and sulphur-containing substances. All of them seem to contain ellagic acid. The kinos from *E. robusta* and *E. pilularis* appeared, however, to be free from polysaccharides, while the kinos from *E. citriodora* and *E. globulus* gave positive tests for their presence. While the evidence for their presence, besides free glucose, in *E. citriodora* kino could be obtained chromatographically according to the procedure of Giri and Nigam,[12] only glucose could be detected after hydrolysis with acid.

TABLE I

Physical characteristics of the eucalyptus kinos

	Kinos			
	E. citriodora	*E. pilularis*	*E. robusta*	*E. globulus*
Group classification[11] ..	Turbid	Ruby	Gummy	Ruby
Appearance	Pale yellow-dark brown with an empyreumatic odour	Ruby red, hard and brittle break with concoidal fracture		Deep brown, extremely brittle
Ash content (%)	0·36	0·01	0·32	0·10
Moisture content (%) ..	7·8	15·4	24·2	15·4
Fusion point (° C.) ..	155	Decompose and char without fusion.		
% Solubility at room temperature—				
Ethyl alcohol ..	24·2	3·7	0·1	17·3
Ether	0·9	*	11·3	0·1
Benzene	*	*	0·2	*
Chloroform	*	*	*	*
Carbon-tetrachloride ..	*	*	*	*
Ethyl acetate	1·5	0·2	*	0·2
Acetone	0·6	1·1	*	1·7
Petrol (40–60°) ..	*	*	*	*
Water	2·8	11·2	18·9	2·6

* Practically insoluble to sparingly soluble.

(B) *The antibacterial spectrum* of the various fractions of the kinos has been given in Table II.

(C) *The chromatographic behaviour* of the four kinos on unidimensional and circular paper chromatograms is shown in Figs. 1 to 5.

(D) *Constituents of the kino of* E. citriodora.—The results of the preliminary chemical examination of the kino are described below:—

1. *Steam volatile material.*—Powdered kino (400 g.) was suspended in distilled water (800 c.c.) and distilled in steam till ca. 12 l. of the distillate was collected. The distillate was extracted with ether (total 3 l.) which yielded a pale oily brown, part'y crystalline residue (0·5 g.). The crystalline matrix was pressed between folds of filter-paper when it melted at 115–22°. It assayed 1 : 50,000 against *M. aureus* and lost its activity after 2 months.

The aqueous portion was made alkaline with barium hydroxide and filtered. The filtrate (12 l.) was concentrated to about 250 c.c. acidified to Congo red and extracted with ether. The ether extract yielded on evaporation a brown gummy

TABLE II

Antibacterial spectrum of the various fractions of the Eucalyptus kinos

Fraction soluble in	Complete inhibition in μg./c.c.											
	E. citriodora			*E. pilularis*			*E. robusta*			*E. globulus*		
	M. aureus	*E. coli*	*My. pheli*	*M. aureus*	*E. coli*	*My. phlei*	*M. aureus*	*E. coli*	*My. phlei*	*M. aureus*	*E. coli*	*My. phlei*
Water ..	150	1000	100	200	Nil	100	100	Nil	200	1000	Nil	Nil
Ethyl alcohol	100	Nil	50	100	Nil	100	1000	Nil	200	500	Nil	Nil
Ether ..	100	Nil	50	100	Nil	200	Nil	Nil	Nil	Nil	Nil	Nil
Acetone ..	1000	Nil	500	200	Nil	500	1000	Nil	Nil	Nil	Nil	Nil
Chloroform	100	Nil	500	500	Nil	1000	..	..	..	200	Nil	Nil
Carbon tetra-chloride ..	500	Nil	Nil	1000	Nil	Nil	..	..	..	500	Nil	Nil
Benzene ..	Nil	Nil	Nil	..	..	..	500	Nil	Nil	Nil	Nil	Nil
Ethyl acetate	100	Nil	Nil	500	Nil	Nil	1000	Nil	Nil	100	Nil	Nil
Petrol (40–60°) ..	Nil	Nil	Nil	Nil	Nil	Nil	..	..	..	..	..	..

residue, which on trituration four times with petrol (40–60°) (total 20 c.c.) gave a brown insoluble material (0·06 g.). (The antibacterial and antifungal activity, *vide* Tables III and IV). The petroleum ether extract on concentration deposited an inactive colourless crystalline substance (0·12 g.), m.p. 127–8·5°.

These fractions were not examined further.

2. *Isolation of a hydrocarbon m.p.* 61°.—On extraction of air-dried, powdered kino (100 g.) three times with hot petroleum ether (40–60°) (total 250 c.c.) and evaporation of the extract, an oily residue (10 mg.) was obtained. It crystallised from 95% ethanol (charcoal) in colourless, flat needles melting at 61°. It was insoluble in 80% phosphoric acid and in concentrated sulphuric acid.

It was devoid of any antibacterial or antifungal activity and was not examined further.

TABLE III

Antibacterial spectrum of the various constituents of the E. citriodora *kino*

	Complete inhibition in μg./c.c.		
	M. aureus	*E. coli*	*My. phlei*
Citriodorol (various preparations) ..	1·67–2·7	Nil	20
Aromadendrin-7-monomethyl ether (I) ..	1000	Nil	Nil
Aromadendrin-dimethyl ether (II) ..	500	Nil	Nil
Kæmpferol-7-monomethyl ether (III) ..	Nil	Nil	1000
Ellagic acid (IV)	50	200	1000
Steam volatile material	100	Nil	500
Glucoside (crude)	20	1000	100

3. *Extraction of the kino with chloroform.*—Kino (50 g.) was extracted with three changes of boiling chloroform (total 250 c.c.). On concentration *in vacuo*, the clear, colourless extract yielded an oily residue (60 mg.) which did not crystallise. It assayed 1 : 10,000 against *M. aureus*.

4. *Extraction with benzene.*—(*a*) (i) Air-dried powdered kino (100 g.) was refluxed with six changes of benzene (total 500 c.c.) during 6 hours. The combined extracts, on concentration *in vacuo*, deposited a crystalline substance (0·7 g.) which melted at 176–77° after recrystallisation from 50% ethanol.

(ii) A finely ground mixture of the air-dried kino (75 g.) and anhydrous magnesium sulphate (25 g.) was extracted (soxhlet) with benzene for 3 days. On

TABLE IV

Activity of the various fractions of E. citriodora *kino against filamentous fungi*

	Complete inhibition in μg./c.c.					
	Aspergillus niger		*Penicillium notatum*		*Fusarium oxysporium*	
	3 days	8 days	3 days	8 days	8 days	12 days
Citriodorol	20	1000	500	Nil	500	Nil
Aromadendrin-7-monomethyl ether (I)	100	1000	20	500	100	1000
Kæmpferol-7-monomethyl ether (III) ..	500	1000	100	1000	1000	Nil
Ellagic acid (IV)	Nil	Nil	Nil	Nil	200	Nil
Steam-volatile material	1000	1000	1000	1000	1000	Nil
Glucoside (Crude)	500	Nil	20	1000	1000	Nil

concentration of the extract, a pale yellow crystalline matrix separated. On recrystallisation from 50% ethanol, it melted at 178°. The yield was ca. 0·8%.

(iii) The same substance was also isolated from the filtrates after treatment with lead acetate (details given under D 8).

Aromadendrin-7-monomethyl ether (*I*).—The above substance crystallised from 50% ethanol in thin colourless, flat needles and melted at 177–78° (decomp.) in pyrex capillaries. When soda glass capillaries were used, with the same rate of heating, the m.p. was about 4–5° lower. (Found: C, 63·6; H, 4·6%; OCH_3 9·51. $C_{16}H_{14}O_6$ requires C, 63·58; H 4·64%; OCH_3 10·26); $[\alpha]_D^{24°}$ — 20·7 (c. 3·0 in ethanol). The ultra-violet absorption spectrum is given in Graph I. $\lambda_{max} = 290\ m\mu$; $\log \epsilon = 4·2534$; $\lambda_{min} = 247·8\ m\mu$; $\log \epsilon = 3·2790$. It is very similar to that of aromadendrin, also shown in the graph.

It is readily soluble in ethyl alcohol, ethyl acetate, acetone, glacial acetic acid; fairly soluble in hot water, ether, boiling benzene; slightly soluble in cold water. cold benzene and chloroform.

The chromatographic behaviour of the substance is given in Table V. On these chromatograms, a second band (with yellow fluorescence in the ultra-violet light) is invariably present due to aerial oxidation of the substance and not as an impurity which corresponds to that of kæmpferol-7-monomethyl ether (*vide* D 5).

The substance gave a transient purple to brown colour with ferric chloride; crimson with concentrated nitric acid; and a yellow colour when warmed with sodium bicarbonate solution. It reduced hot Fehling's solution and Tollen's reagent; Wilson's boric acid test[13] was negative. An alcoholic solution treated with hydrochloric acid and zinc gave a deep red colour. The material reduced with zinc and acid turned yellow when the solution was neutralised.[14] When an alcoholic solution was reduced with sodium amalgam and the supernatant liquor neutralised with hydrochloric acid, a pink colour was obtained.[15] The antibacterial and antifungal activity of the substance is given in Tables III and IV respectively.

The substance has been identified as aromadendrin-7-monomethyl ether on the basis of the following reactions:—

(i) *Oxidation to kæmpferol-7-monomethyl ether*

(*a*) Oxidation of (I) was carried out according to the conditions described by Hillis[9] for the unmethylated aromadendrin. The product (60% yield) crystallizing from alcohol and melting at 221° was found identical with (III).

(*b*) *By fusion.*—(I) (100 mg.) was heated in a pyrex tube at 260° for 1 minute. in a manner described by Hillis[2] for the conversion to aromadendrin to kæmpferol. The tube was quickly cooled in cold water, when yellow crystals (m.p. 220–21°) were obtained from the tar and had the same R_f value as kæmpferol-7-monomethyl ether.

TABLE V

Chromatographic behaviour of the crystalline constituents of the E. citriodora *kino with different solvent mixtures under various conditions of saturation*

Solvent mixture	Saturation	Kæmpferol-7-monomethyl ether			Aromadendrin-7-monomethyl ether			Aromadendrin-dimethyl ether			Ellagic acid		Citriodorol*
		Day light	U.V. light	Tollen's reagent	Day light	U.V. light	Tollen's reagent	Day light	U.V. light	Tollen's reagent	U.V. light	Tollen's reagent	
1	6 hours (aq. phase)	P.Y.	Y.fl. 0·95	D.B. 0·94	C	Y.fl. 0·95 V.g. 0·83	D.B. 0·95 B 0·83	..	..	..	M.fl. 0·41	D.B. 0·41	0·92
2	2 hours (aq. phase)	P.Y.	Y.fl.t. 0·96	D.B.t. 0·96	C.	Y.fl.t. 0·95 V.g.t. 0·8	D.B.t. 0·96 B.t. 0·8	..	..	..	M.fl. 0·4	D.B. 0·4	0·94
3	do.	P.Y.	Y.fl. 0·38	D.B. 0·39	C	Y.fl. 0·38 V.a. 0·44	D.B. 0·4 B. 0·44	..	..	..	M.fl. Did not move	D.B.	0·42
4	do.	P.Y.	Y.fl. 0·75	D.B. 0·75	C	Y.fl. 0·76 V.a. 0·8	D.B. 0·75 B. 0·8	..	..	..	do.		0·8
5	do.	P.Y.	Y.fl.t. 0·46	D.B.t. 0·45	C	Y.fl.t. 0·45 V.a.t. 0·6	D.B.t. 0·45 B.t. 0·6	..	..	..	do.		0·58

6	2 hours (aq. phase)	P.Y.	Y.fl. 0·46	D.B. 0·45	C	Y.fl. 0·45 V.a. 0·77	D.B. 0·45 B. 0·8	.	..	.	M.fl. D.B. Did not move	0 75
7	do.	P.Y.	Y.fl. 0·46	D.B. 0·47	C	Y.fl. 0·46 V.a. 0·8	D.B. 0·46 B. 0·8	..	..	..	do.	0·72
8	do.	P.Y.	Y.fl. 0·52	D.B. 0·53	C	Y.fl. 0·54 V.a. 0·8	D.B. 0·53 B. 0·8	..	..	.	do.	0·73
9	do.	P.Y.	Y.fl. 0·24	D.B. 0·23	C	Y.fl. 0·23 V.a. 0·75	D.B. 0·22 B. 0·76	C	Bl.fl. 0·48	D.B. 0·48	do.	0·66
10	do.	P.Y.	Y.fl. 0·37	D.B. 0·38	C	Y.fl. 0·38 V.a. 0·76	D.B. 0·38 B. 0·77	..	..	..	do.	0·68

* Identified by bioautographic technique, chocolate pink absorption in the ultra-violet light becomes dark brown with Tollen's reagent. In addition to this band it always gave two bands corresponding to aromadendrin- and kæmpferol–7–monomethyl ethers.

Solvent Mixtures:
1. Phenol: 2 N acetic hydrochloric acids (50 : 50) (Hillis's solvent).
2. Phenol saturated with water.
3. Phenol saturated with water *plus* ethanol (7 : 3).
4. Phenol saturated with water *plus* ethanol (50 : 50).
5. Phenol saturated with water *plus* ethanol (3 : 1).
6. Phenol saturated with water *plus* ethanol (4 : 1).
7. 8% Phenol in water (by weight) *plus* ethanol (4 : 1).
8. 8% Phenol in water (by weight) *plus* ethanol (3 : 1).
9. 6% Phenol in water (by weight) *plus* ethanol (4 : 1).
10. 6% Phenol in water (by weight) *plus* ethanol (3 : 1).

P.Y. = Pale yellow
D.B. = Deep brown
C = Colourless
M.fl. = Mauve fluorescent
/ = tailing.
Y.fl. = Yellow fluorescent
B. = Brown
Bl. = Blue
V.a. = Violet absorbent
V.g. = Yellow green fluorescent

(*c*) *With iodine*.—The oxidation was carried out as described by Seshadri.[15] The crude flavonol so obtained was dissolved in 1% sodium hydroxide and re-precipitated by acidification. It melted at 220–21° after repeated crystallisations from ethanol and was identified as kæmpferol-7-monomethyl ether by mixed m.p. method.

(ii) *Acetylation*

An acetyl derivative, m.p. 133–34°, probably identical with that described by Uoda *et al.*[4] was obtained by pyridine-acetic anhydride method.

(iii) *Methylation*

(*a*) *With diazomethane*.—(II) m.p. 186–87° (confirmed by mixed melting point and absorption curves) was formed by keeping (I) in large excess of diazomethane solution[17] in ether for 4 days.

(*b*) *With methyl iodide*.—Methylation with methyl iodide and anhydrous potassium carbonate in acetone solution for 36 hours yielded mostly (II). The progress of methylation could be readily followed by circular paper chromatography.

Isolation of aromadendrin dimethyl ether (II) from the kino.—Apart from obtaining the dimethyl ether by methylation of aromadendrin-7-monomethyl ether as described above, it was isolated from certain samples of the kino of *E. citriodora* collected from a private garden at Bangalore. In these samples the monomethyl ether was practically absent, and was alsomost replaced by the dimethyl ether. The yield of the purified substance after six crystallisations, however, was about 0·05%.

The substance was isolated from the kino much in the same way as (I). It crystallises from ethanol in thin, glistening colourless needles, melting at 186–87° (decomp.) (Found: C, 64·2, H, 5·0%; $C_{17}H_{16}O_6$ requires C, 64·5, H, 5·1%). The properties agreed very closely to those given by Hillis.[2] The chromatographic behaviour of the substance is given in Table V. The antibacterial activity of the compound has been given in Table III.

The acetyl derivative on crystallisations successiuvely from ethanol and ether melted at 135–36° which should be identical with that of dimethylkaturanin.[4]

(II) unlike (I) and aromadendrin (Hillis, *loc. cit.*) was found to be very resistant to aerial oxidation under conditions described from the monomethyl ether (*vide supra*). It gives only one band on a chromatogram. However, the optically active isomer was racemised when (II) (100 mg.) was refluxed for 6 hours in 5% dilute sulphuric acid (200 c.c.). The racemised form crystallized readily from dilute alcohol, m.p. 186°.

5. *Isolation of kæmpferol-7-monomethyl ether (III)*.—It was isolated as an insoluble lead complex from an aqueous or alcoholic solution of the kino by addition of a saturated alcoholic solution of lead acetate. On decomposition of the complex by hydrogen sulphide in the usual way it was obtained as yellow amorphous powder in an yield of 0·5% (*vide* D 8).

It readily crystallised from ethanol in bright yellow needles, melting at 220–21° (Found: C, 63·8; H, 4·1%. $C_{16}H_{12}O_6$ requires C, 64·0; H, 4·0%). It was identified as kæmpferol-7-monomethyl ether as described above (the ultra-violet spectrum, the antibacterial and antifungal activity, *vide* Graph II and Tables III and IV).

It gave a colourless, crystalline acetyl derivative, melting at 201° (Hillis[2]).

Methylation of (III) with diazomethane yielded the dimethyl derivative, m.p. 143° (Found: C, 65·3; H, 4·6%. $C_{17}H_{14}O_6$ requires C, 64·97; H, 4·46%). Dimethyl kæmpferol thus obtained was sparingly soluble in alcohol, ether and ethyl acetate. It gave a purple brown colouration with ferric chloride, pink with magnesium and hydrochloric acid. It had an R_f 0·23–0·24 on a circular paper chromatogram with a violet absorption in the ultra-violet light.

However, methylation of (III) with methyl iodide as described under (I) gave a dimethyl compound, m.p. 143–44°, which apparently differed from the above dimethyl derivative in that it possessed R_f 0·48 on a circular paper chromatogram with a sky blue fluorescence under ultra-violet light. Both the derivates were not further examined due to limited quantities available.

6. *Glucoside of kæmpferol-7-monomethyl ether.*—By circular paper chromatographic technique (Giri and Nigam, *loc. cit.*) the presence of glucoside was detected in the aqueous extracts of the kino left after the removal of the lead complex. After hydrolysis of the aqueous solution in the usual manner, the presence of kæmpferol-7-monomethyl ether as well as of free glucose (*vide* Fig. 5) could be detected on paper chromatogram. Further glucose was identified by the preparation of its osazone, m.p. 205°. However, the glucoside as well as the flavonol could not be obtained in a pure state.

7. *Ellagic acid* which forms a sparingly soluble lead salt was isolated from the mother liquors after the removal of kæmpferol-7-monomethyl ether (*vide* D 8).

It crystallises from pyridine in thin, prismatic, fawn coloured needles and does not melt up to 360° (Found: C, 55·2; H, 2·4%. $C_{14}H_6O_8$ requires C, 55·6; H, 2·0%). It was identified as ellagic acid by its properties and also by the formation of the acetyl derivative, m.p. 344–45° (decomp.), which was undepressed when admixed with an authentic sample prepared according to Perkin and Nierrenstein.[18]

The chromatographic behaviour, the antibacterial and antifungal activity, *vide* Tables III, IV and V.

8. "*Citriodorol.*"—A number of procedures were tried to evolve a convenient method of isolation of this compound: (1) Preliminary extraction of the kino with ether. The ether soluble material was fractionated as described below for the alcohol-soluble material; (2) by preliminary extraction with water and removal of the water-soluble impurities (ellagic acid, glucose, glucoside, etc.) before fractionation; and (3) by preliminary removal of small quantities of alcohol-insoluble material. It was found that although the alcoholic extract carried practically all the constituents except cell debris and a little inorganic material, it was the best

starting material as it extracted completely the active substance; while with ether part of the active material was not completely dissolved. Thus only the procedure adopted for fractionation of the alcohol-soluble constituents of the kino is described below. As the amount of steam-volatile material is negligible, it is convenient to omit steam-distillation of the kino prior to its extraction with alcohol.

Fractionation of the kino.—The various steps of the procedure adopted are shown below in the flow sheet.

The crude citriodorol obtained above, gave rise to three different bands on a circular paper chromatogram (Table V) corresponding to (I) (impurity), (II) [due to aerial oxidation of (I)] and the active fraction, identified by its characteristic pale chocolate brown to violet absorption in the ultraviolet (R, 0·66) and bioautographic technique.

Further purification of crude citriodorol

(i) *Fractional extraction with warm water.*—Crude citriodorol (1 g.) was shaken 5 times with warm water (total 25 c.c.) and the aqueous extract evaporated to a pale brown oil (0·6 g.). It was recrystallised from 75% ethanol and identified as (I) (0·25 g.) by a mixed m.p. and its chromatographic behaviour.

Though the activity of the water-insoluble fraction was raised to 1 : 300,000 (I) was still detectable on chromatograms. When the extraction was followed up a 10 mg. quantity of a brownish-yellow residue (1 : 500,000) was obtained. Even this was heterogeneous on a chromatogram. Fractional extraction with chloroform or 50% ethanol did not yield any fraction with increased activity.

(ii) *Purification by chromatography.*—Crude citriodorol (1 g.) was chromatographed on a calcium sulphate column (*vide supra*). About 700 c.c. of the solvent was required for complete development. Two distinct bands (middle M and lower L) were formed apart from some material that was strongly adsorbed at the top (band T). The fractions after extrusion and elution with warm alcohol were evaporated and assayed. Results are presented in Table VI.

Fraction (M) on rechromatography again resolved into three bands, but the activity of the rechromatographed fractions was much lower (1 : 80,000–1 : 160,000).

On rechromatography of Fraction (T), however, a large portion of the material was strongly adsorbed at the top of the column (T_1: yield 82%; activity: 1 : 400,000–450,000; ultra-violet spectrum *vide* Graph III) while lower below a thin band (T_2: yield 10%, activity: 1 : 200,000) was obtained.

(iii) *Purification by paper chromatography.*—As crude citriodorol gave on a circular paper chromatogram only one active band (bioautography), the preparative technique[19] was applied to obtain a chromatographically homogeneous product.

A pale cream coloured amorphous mass (30 mg. from 45 chromatograms), melting between 112–18° (decomp.) active 1 : 500,000 against *M. aureus* (u.v. absorption spectrum *vide* Graph III) was obtained.

Air-dried powdered kino
400 g. (1 : 7000)
Ethyl alcohol, 2 l. refluxed for 1 hour

Filtrate (1 : 10,000)
precipitated with lead acetate in alcoholic suspension ; heated for 15 min. at 70° C. ; left overnight at room temp.

Insoluble matter, 5–7% of the dry weight of the kino, mostly cellular debris, inorganic matter ; give tests for K, Na, Ca, Fe, SiO_2 ; ash 60%.

Filtrate and washings (2·2 l.)—
lead precipitated as lead phosphate by syrupy H_3PO_4 and by Na_2HPO_4 (5%)

Insoluble complex-powdered, suspended in ethanol (2 l.), boiled and decomposed by H_2S when hot, and filtered hot

Filtrate (I) and Washings
Concentrated

Lead phosphate

Filtrate
Concentrated

Lead sulphide

Filtrate (250 c.c.)
Concentrated to 60 c.c. + 60 c.c. water, cooled

Yellow amorphous powder
Ethanol Yellow crystals 0·5 g. m.p. 220–21° (kæmpferol-7-mono-methyl ether)

Mother liquor

Dull, yellow amorphous powder, 3 g., m.p. 190–250° (decomp.)

Brown syrupy filtrate
Further diluted with water, 25 c.c. cooled to 0–5°

Brown gummy residue
Ether 100 c.c. 4 times

Further concentrated

Mother liquor 100 c.c. (not examined)

Yellow amorphous precipitate

Carbon disulphide 100 c.c. boiled

Mother liquor
concentrated further
[Impure aromadendrin-7-monomethyl ether]

Colourless residue, m. p. 160–68°.

Ether extract
completely evaporated
Brownish yellow brittle mass. 8·2 g., m.p. 74–94° (1 : 200,000–250,000)
" Crude citriodorol "

Filtrate contained sulphur

Insoluble portion

Ethanol 100 c.c. boiled

Filtrate
Cooled
Yellow crystals 1·5 g., m.p. 221–22° (kæmpferol–7–monomethyl ether)

Insoluble residue

50% ethanol
colourless crystals 0·1 g., m.p. 177–78° aromadendrin-7-monomethyl ether

Dissolved in hot pyridine (animal charcoal), filtered and cooled, fawn coloured, prismatic needles 0·2 g., m.p. over 360°, ellagic acid

Table VI

Chromatographic purification of crude citriodorol

Fraction	Substance	% Yield Chromatogram No. 1	2	3	Activity (*M. aureus*) 1 in
Top T ..	Brown oil, partially crystalline	32·2	36·3	42·6	400,000
Middle M ..	Brown gum	45·8	44·0	30·0	400,000
Lower L ..	Pale yellow amorphous powder	18·6	16·8	15·5	200,000

(iv) *Adsorption on norit.*—About 20% of the material (u.v. curve Graph III), active 1 in 600,000 could be recovered (by 40% ethanol) from the charcoal after adsorption from a 1% alcoholic solution for 10 minutes.

Properties of citriodorol.—Fraction T, was dried in a desiccator (calcium chloride) under vacuum for a week and the following physical properties were determined.

It was a pale yellowish brown coloured amorphous substance melting at 115–17° with a previous softening at 90°; $[\alpha]_D^{28°}$ + 12·5 (*c*, 2·0 in methanol). It did not give tests for nitrogen and halogens. It is readily soluble in ethanol, methanol, ethyl acetate, acetone, glacial acetic acid, ether, fairly soluble in hot water and very sparingly soluble in hot benzene and cold water. The absorption spectra of fractions T_1 and products obtained from charcoal adsorbates and paper chromatograms (*cf.* Table V) are given in Graph III.

The antibiotic is not destroyed by heating at 80–90° in aqueous or alcoholic solution between pH 6·8–7·2 nor by long exposure to air. The purest material (from the charcoal adsorbates) assayed 1 : 600,000 against *M. aureus*; 1 : 50,000 against *My. phlei*; but was inactive against *E. coli* even in concentrations of 1 : 10,000. It inhibited mycelial growth (fungistatic) of *Aspergillus niger* in dilutions of 1 : 50,000 but was not so effective against *Penicillium notatum* and *Fusarium oxysporium* (*vide* Tables III and IV).

The colour reactions mentioned below were carried out with the sample separated by preparative chromatograms.

	Reagent	Reaction	Formation of colour
1.	Concentrated sulphuric acid	Bright orange-red colour at the interface.	Immediate
2.	Concentrated hydrochloric acid	Colour brightens a little	5 minutes
3.	Concentrated nitric acid	Develops a brownish tinge	2 minutes
4.	Alc. ferric chloride ..	Dark green colour	
5.	Alc. ferric chloride + ammonium hydroxide	Changed to cherry red.	
6.	Ammonium molybdate solution (1%) ..	Reddish brown colour	
7.	Tollen's reagent ..	Silver deposited	15–20 minutes
8.	Fehling's solution ..	Reddish brown precipitate	
9.	Wilson's boric acid citric acid reagent ..	Yellow colour	Immediate
10.	Sodium amalgam (followed by hydrochloric acid)	No change in colour	
11.	Magnesium amalgam and hydrochloric acid	Pink colour	2–5 minutes
12.	Ware's test ..	Pale purple to green colour	

The positive magnesium amalgam and the negative sodium amalgam tests suggest that the antibiotic might belong to the group of flavonols. Its reactions to ammonium molybdate and to ferric chloride-ammonium hydroxide indicate the presence of ortho-dihydroxy groups.

(E) *Pharmacodynamic studies.*—None of the constituents showed any significant pharmacodynamic reaction. Figures 6 and 7 show the effect of citriodorol, aromadendrin-7-monomethyl ether, ellagic acid and the steam volatile material on the blood pressure, intestines and respiration in dogs. Kæmpferol-7-monomethyl ether (2 mg./kg.) and the glucoside (5 mg./kg.) did not have any effect (not shown). On the other hand, citriodorol, aromadendrin-7-monomethyl ether and kæmpferol-7-monomethyl ether (13 μg./c.c. each) inhibited the acetyl-choline contractions by more than 50% but did not exhibit any antihistaminic property. Ellagic acid and the steam-volatile material had slight stimulatory effect in dilutions of 160 μg./c.c. Glucose produced no effect in this dilution.

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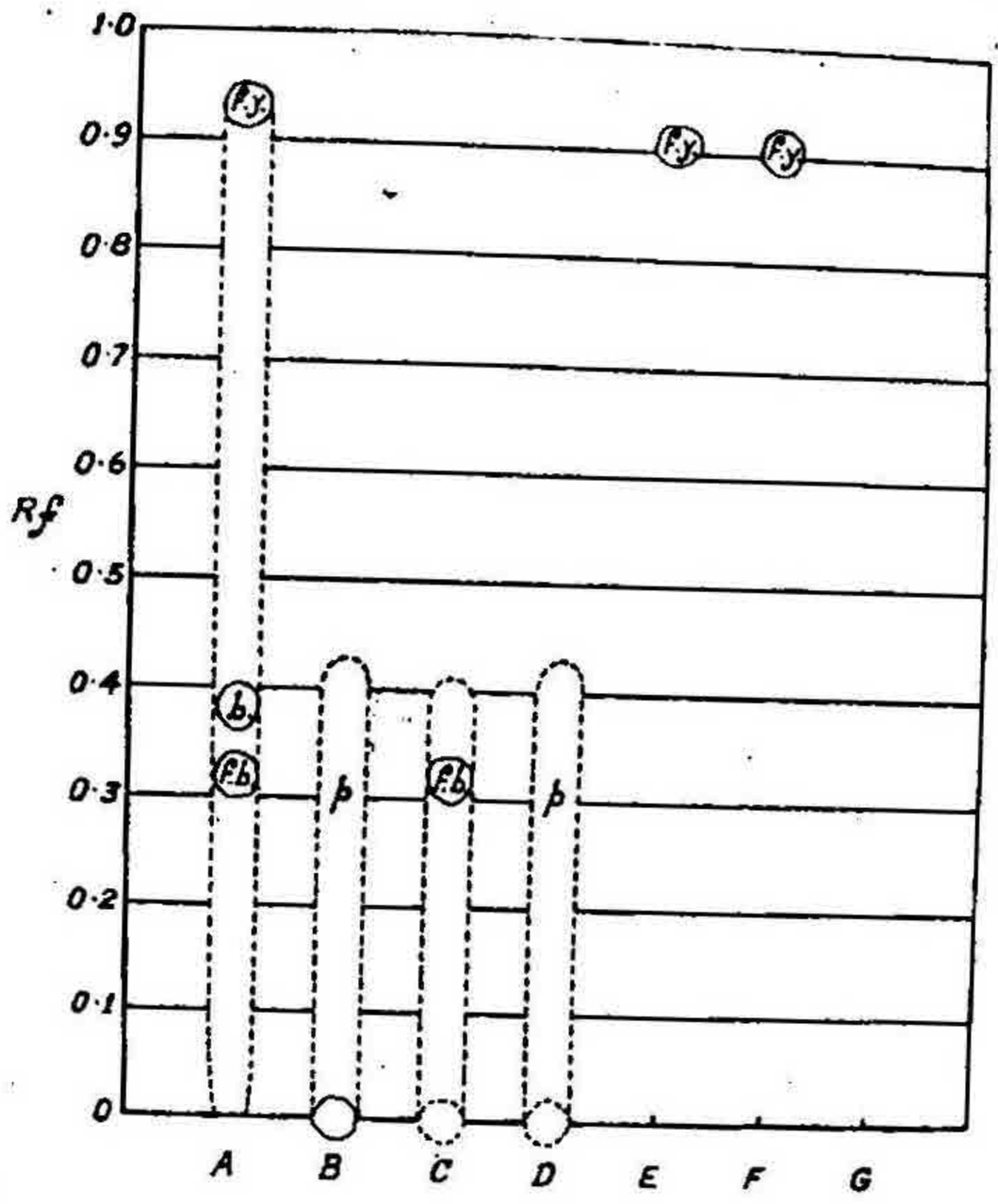


FIG. 1

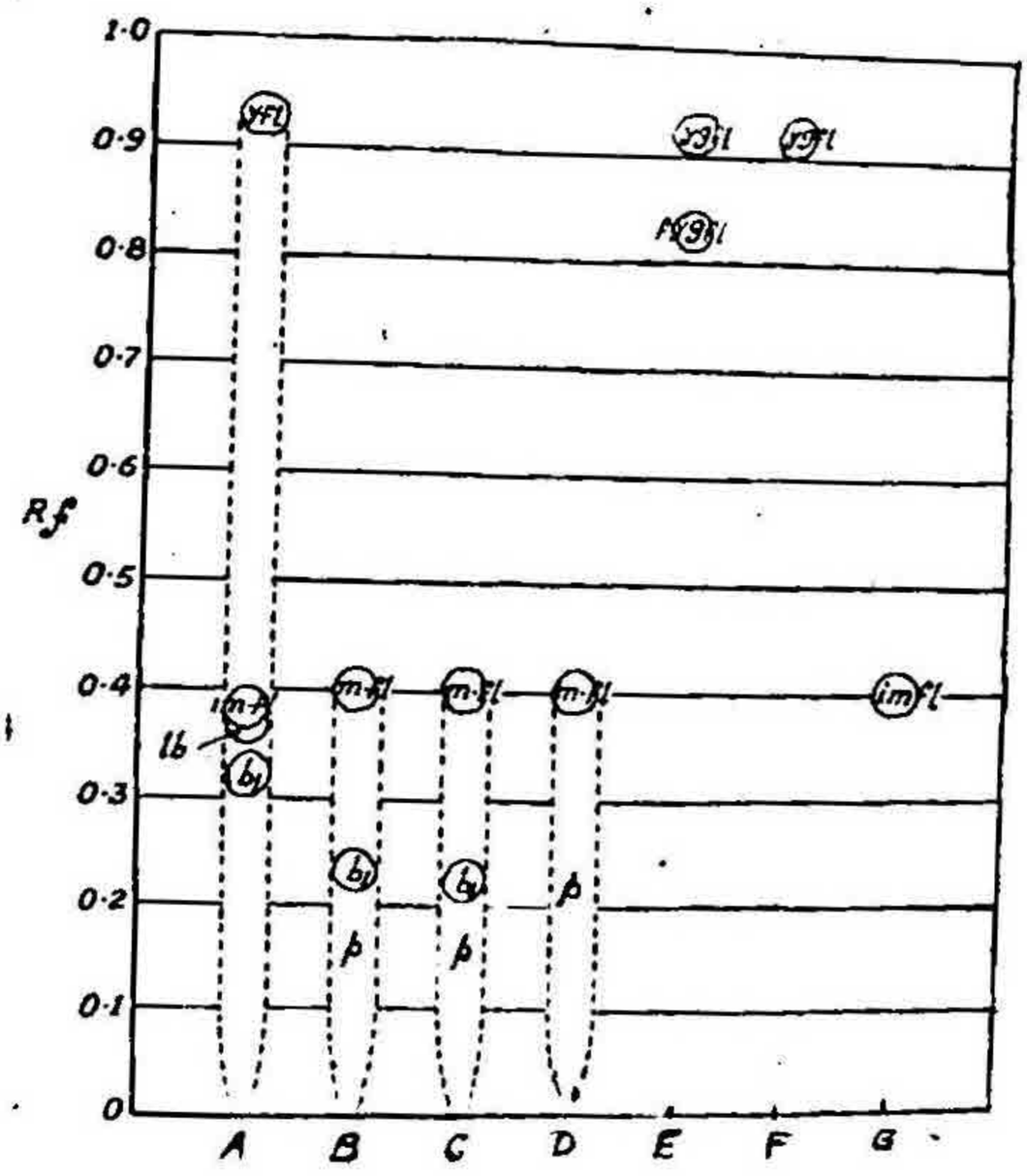


FIG. 2

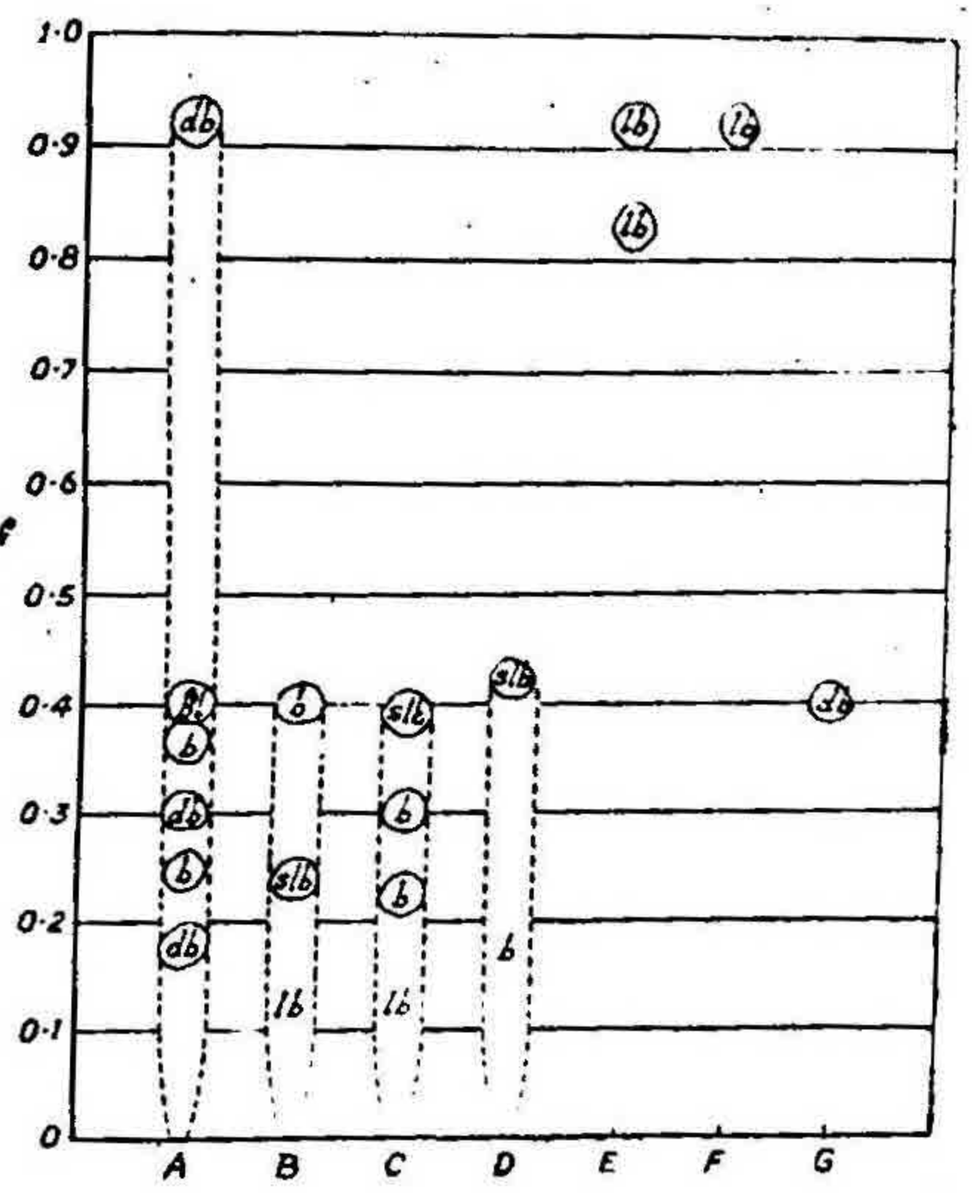


FIG. 3

Chromatographic behaviour of the constituents of the kinos.

FIGS. 1–3. Solvent system No. 1 (ascending technique).

FIG. 1. Seen in daylight ; FIG. 2. Seen in ultra-violet light ; FIG. 3. Seen after spraying with Tollen's reagent.
A = *E. citriodora*; B = *E. pilularis*; C = *E. globulus*; D = *E. robusta*; E = Aromadendrin-7-monomethyl ether;
F = Kæmpferol-7-monomethyl ether; G = Ellagic acid.
b = brown; b_1 = blue; Bl = black; *d* = dark; *f* = faint; *fl* = fluorescence; *g* = green; *i* = intense; *l* = light;
m = mauve; *p* = pink; *sl* = slightly; *y* = yellow.

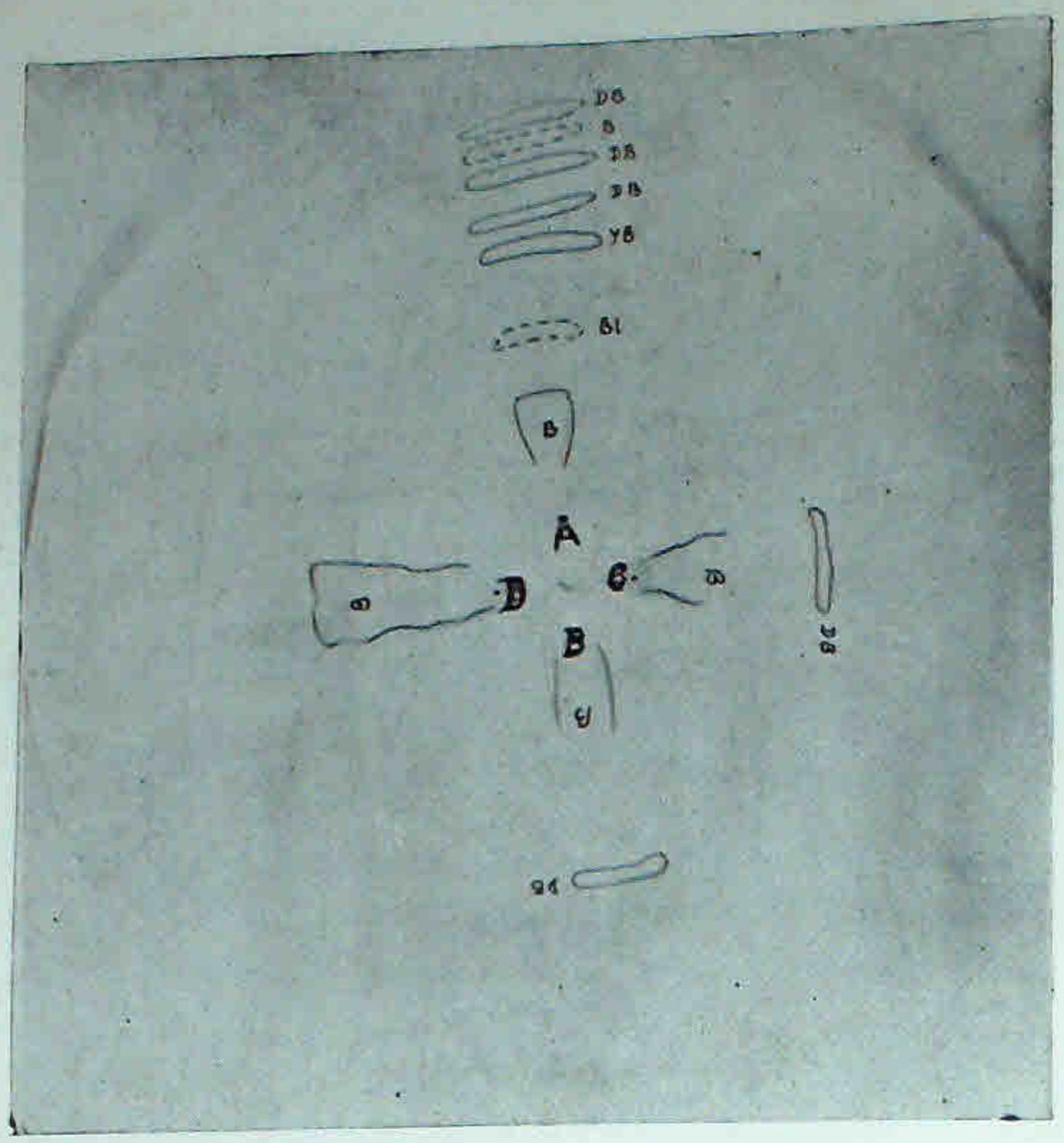


FIG. 4

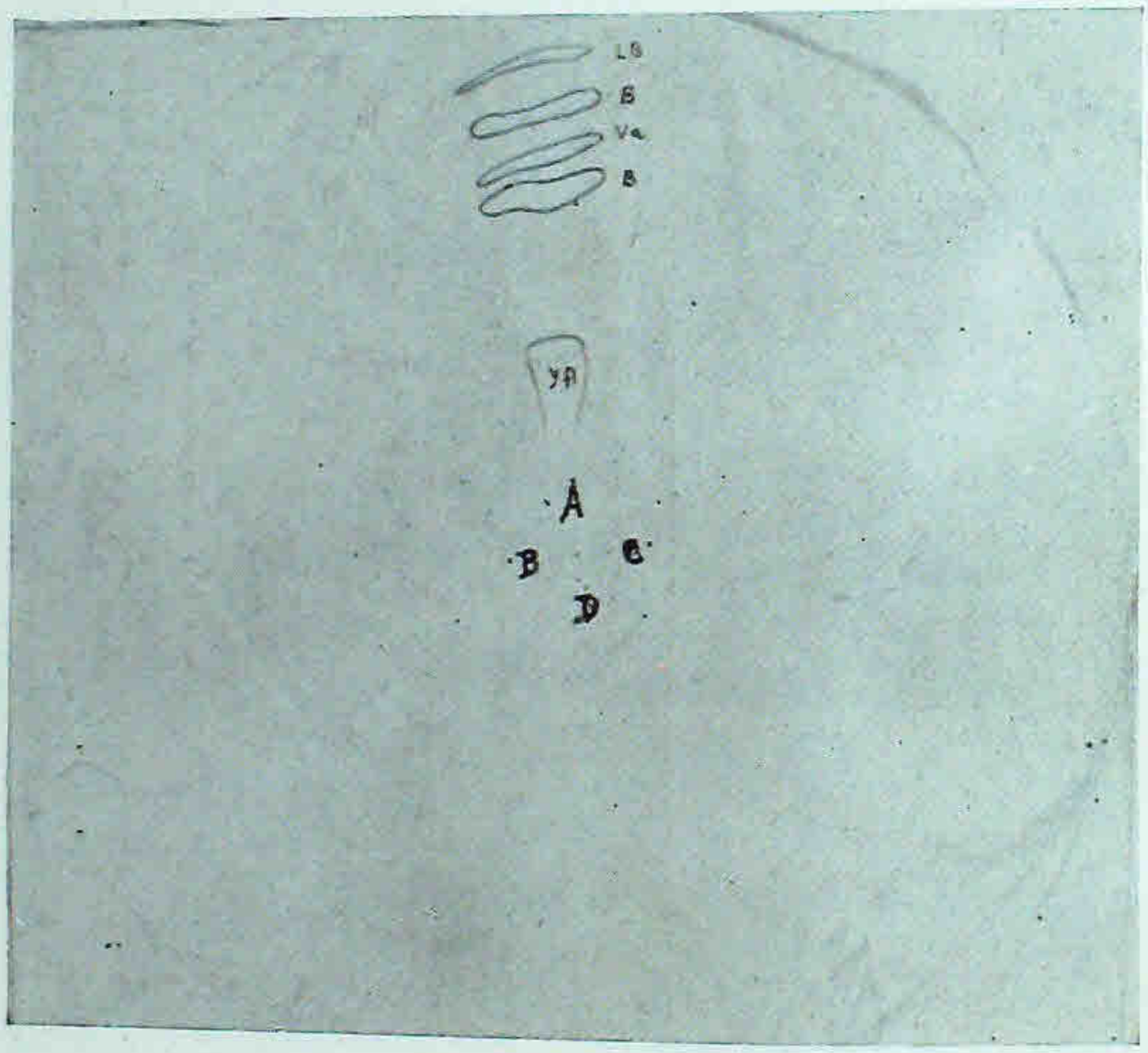


FIG. 5

Chromatographic behaviour of the constituents of the kinos

FIGS. 4 and 5. Solvent System No. 9 (Circular).

A = *E. citriodora*; B = *E. pilularis*; C = *E. globulus*; D = *E. robusta*.

FIG. 4. Seen after spraying with Tollen's reagent.

B = brown; DB = dark brown; *Bl* = black; YB = yellowish brown.

FIG. 5. Seen in ultra-violet light.

B = blue; LB = light blue; *Va* = violet absorption; *Yfl* = yellow fluorescence.

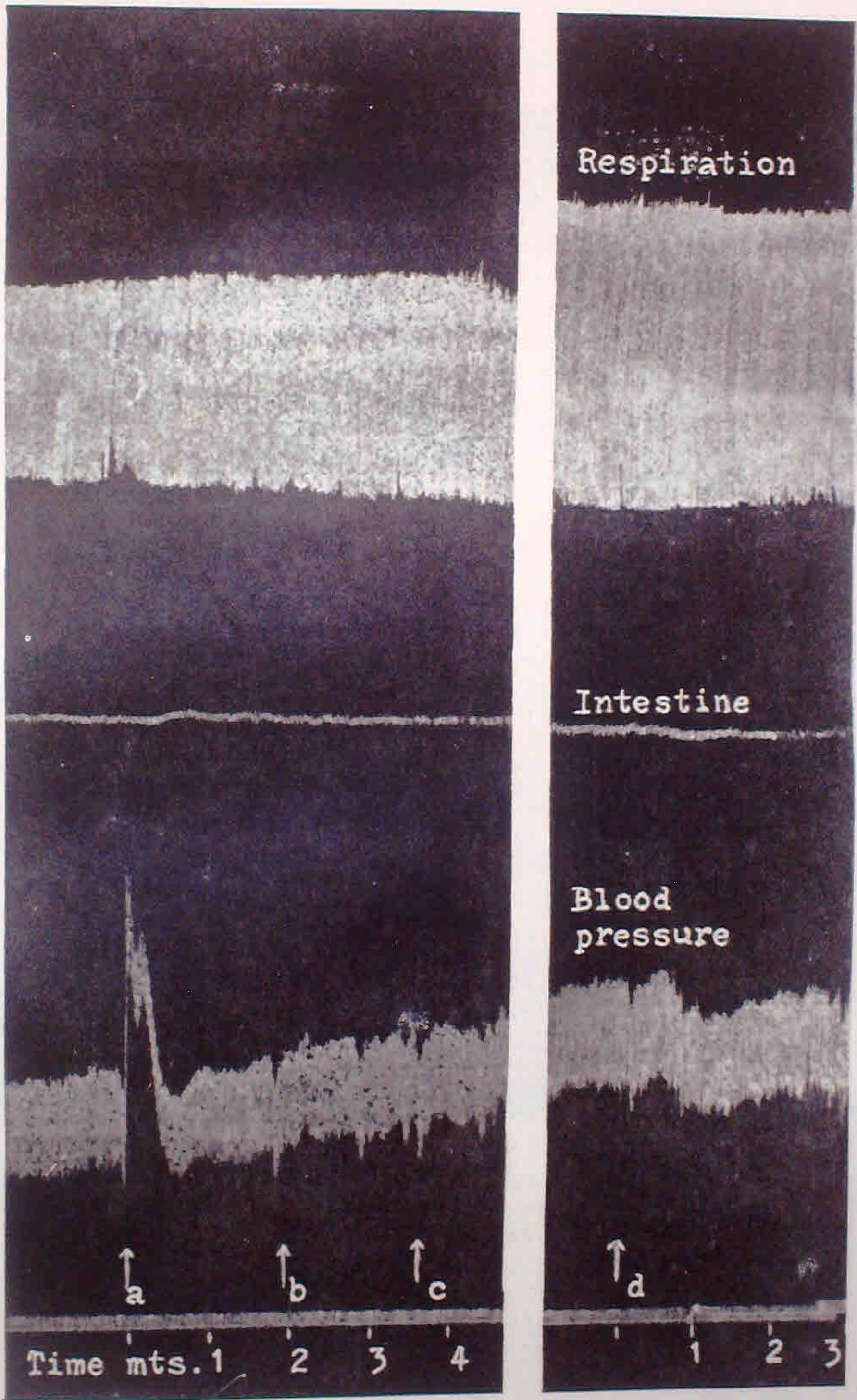
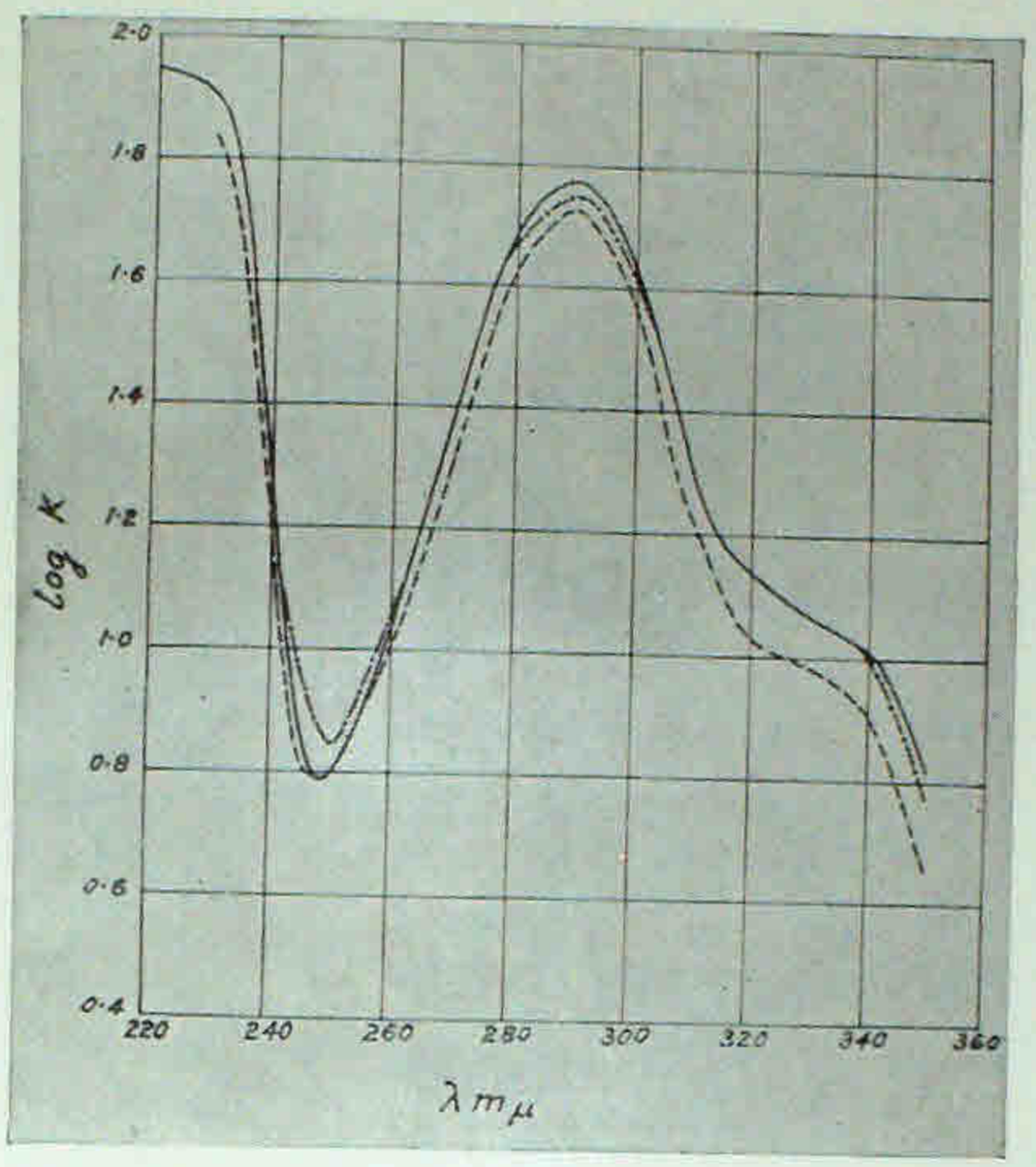


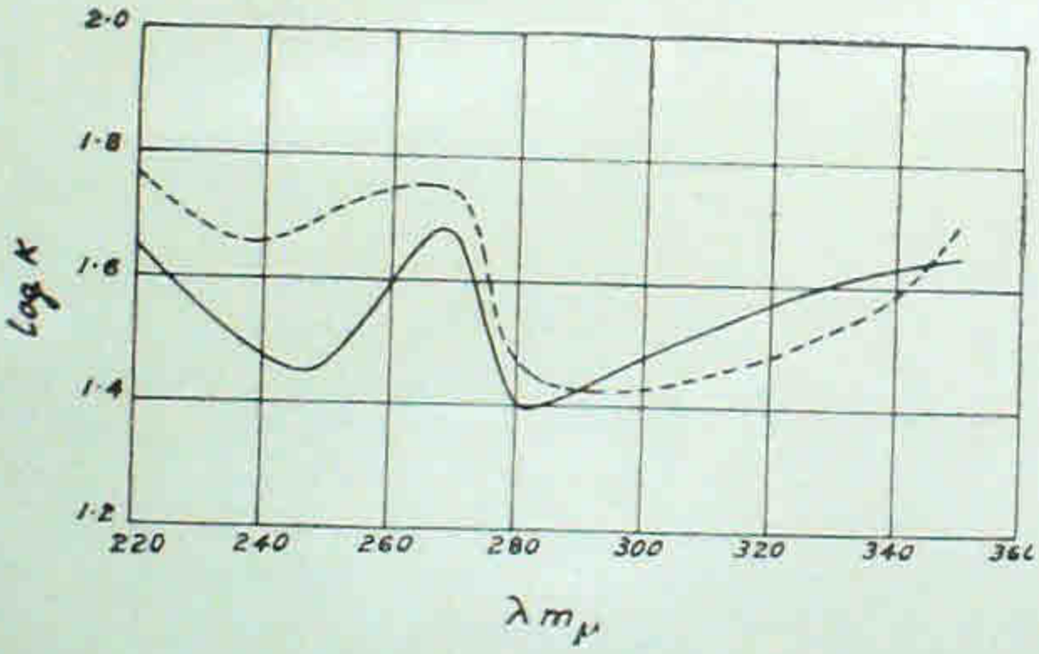
FIG. 6

Effect on blood pressure, intestines and respiration of (*a*) adrenaline 3 μg./kg.; (*b*) alcohol 0·5 c.c.; (*c*) citriodorol 2 mg./kg.; (*d*) citriodorol 10 mg./kg.



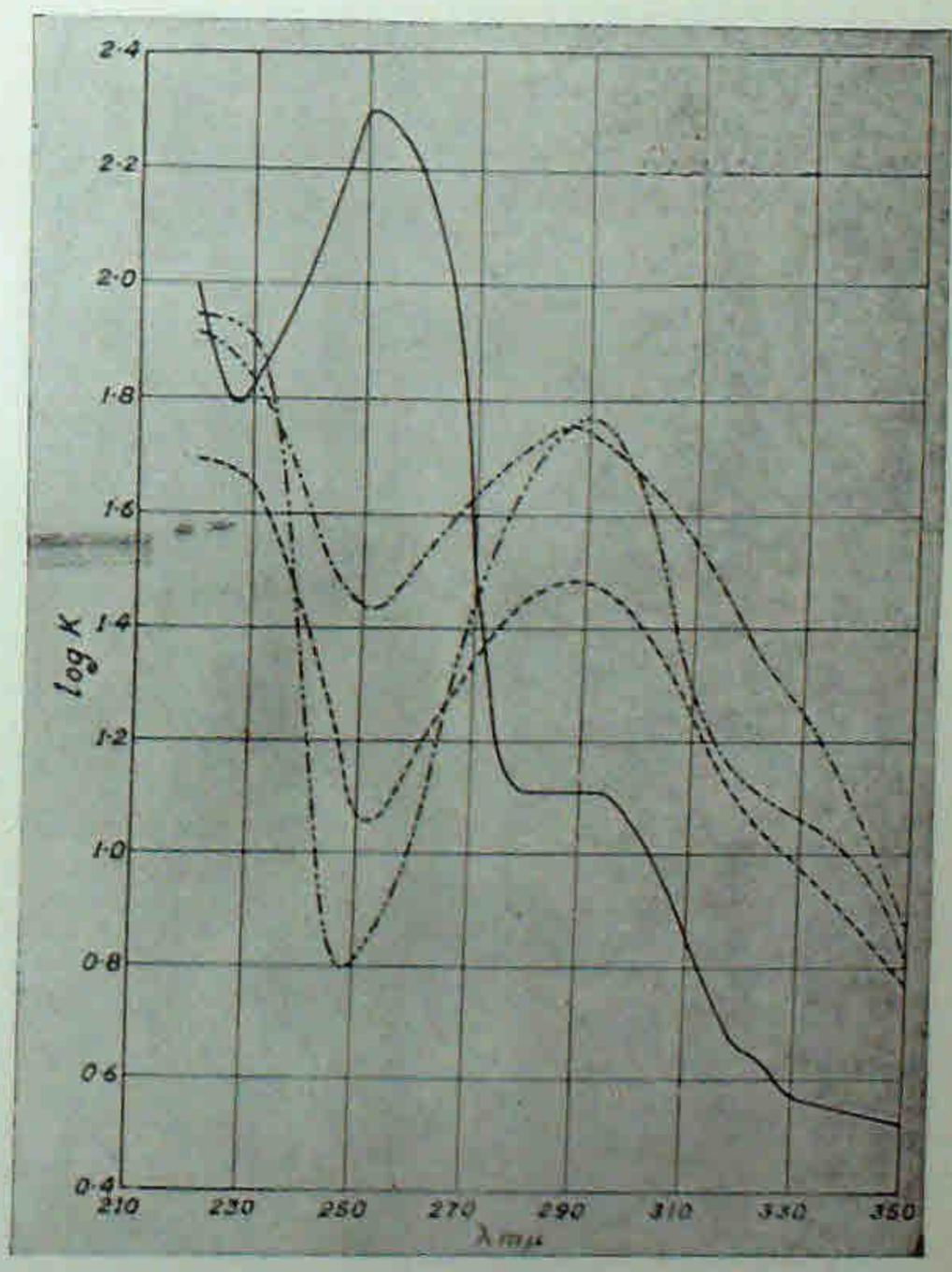
GRAPH I

——— Aromadendrin 7-Monomethyl Ether
–·–·– Aromadendrin Dimethyl Ether
– – – – Aromadendrin Dimethyl Ether (Hillis)



GRAPH II

– – – – Kaempferol 7-Monomethyl Ether
——— Kaempferol Dimethyl Ether



GRAPH III

Active Fractions
–·–· adsorbed on charcoal
——— from Paper Chromatograms
– – – Fraction T
–··– Aromadendrin 7-Monomethyl Ether

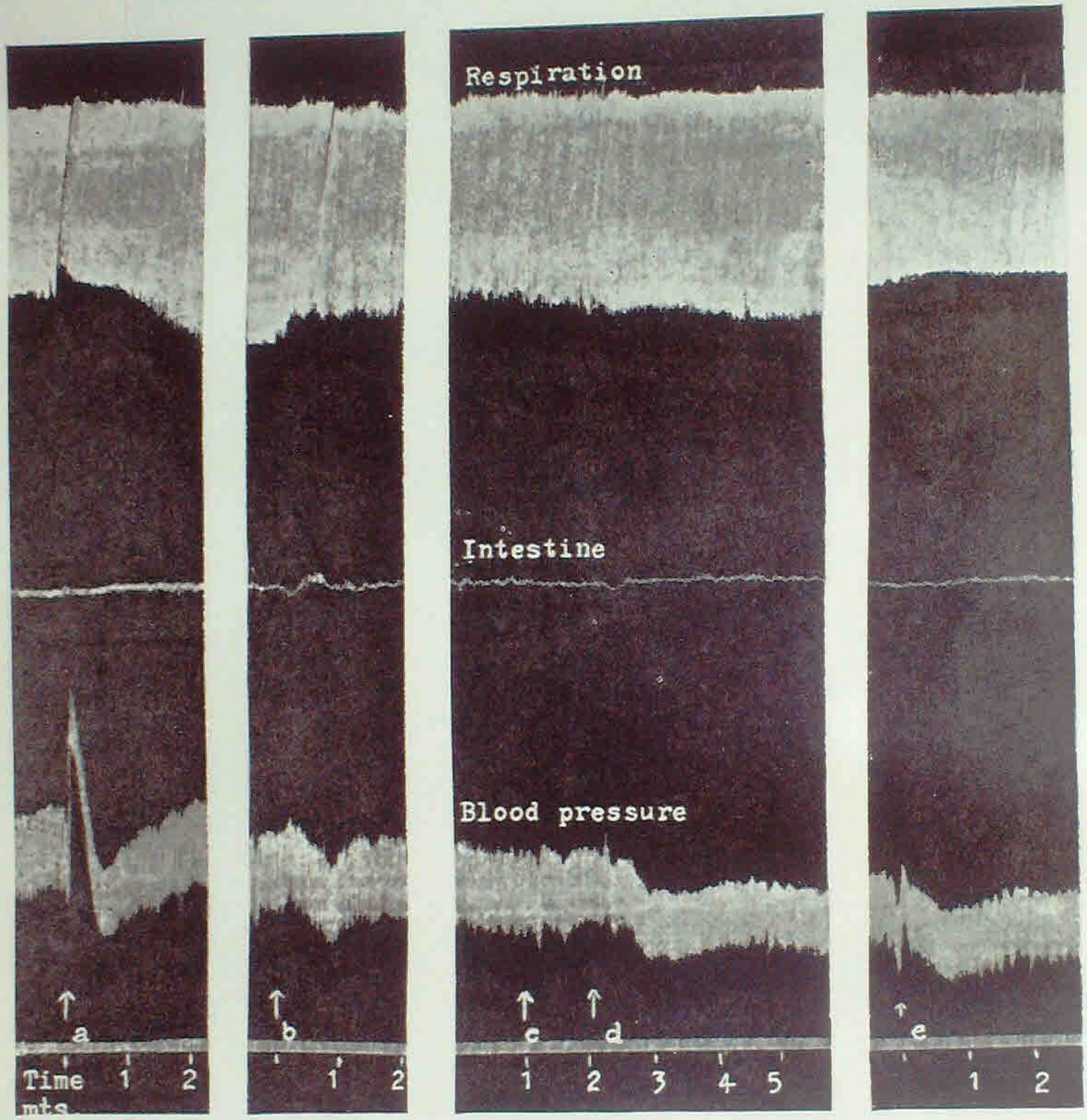


FIG. 7

Effect on blood pressure, intestines and respiration of (*a*) adrenaline 3 μg./kg. ; (*b*) steam volatile material 4 mg./kg. ; (*c*) gum acacia 1·6 c.c. ; (*d*) ellagic acid 1·2 mg./kg. ; (*e*) aromadendrin 7-monomethyl ether 3 mg./kg.