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GALLIC ACID AS A NATURAL INHIBITOR OF FLOWERING IN *KALANCHOE BLOSSFELDIANA*

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Abstract—Gallic acid (I) has been isolated as a specific flowering-inhibitory substance from leaves of vegetative *Kalanchoe blossfeldiana*. It is also present in leaves of flowering *Kalanchoe*, apparently in an inactive, non-dialysable form. Gallic acid acts as a flowering inhibitor when applied to *Kalanchoe*, a short-day plant. Its detection and isolation was facilitated by use of a bioassay based on tissue culture of partially induced apices of the long-day plant *Viscaria candida*.

INTRODUCTION

THE MECHANISM of the endogenous regulation of flowering in plants is perhaps one of the most important and tantalising problems in Plant Biochemistry. Several recent reviews[1–6] describe the history and present state of our knowledge. Despite the efforts of various workers no substance from plants has yet been isolated and characterized whose prime physiological effect is one of promoting or inhibiting floral evocation.† The existence of such substances has been postulated for over 30 years and the lack of success in finding examples of either type can be attributed, at least in part, to the lack of convenient and reliable bioassays.

Many experiments, especially the grafting of photoperiodically induced leaves onto non-induced plants to promote flowering, have helped to establish the possible existence of a transmissible flower promoting hormone (florigen). However, it is only more recently that the idea of flowering-inhibitory substances has begun to achieve reality.

Experiments on inhibition of flowering have indicated that, like florigen, leaves might be a source of the flowering inhibitor(s). In a recent paper, Gibby and Salisbury[7] have provided good evidence for the existence of flowering-inhibitory substance(s) or a flowering-inhibitory 'condition', in non-photoperiodically induced leaves of the long-day plant *Xanthium strumarium*. From their experiments they conclude that inhibition of flowering in *Xanthium* is not due to prevention of florigen synthesis, translocational effects, or a translocatable

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† The term, floral evocation, has been suggested by Evans[5] to describe the process at the stem apex that cause the morphological change committing the bud to a state of flowering. This term is intended to distinguish these events from those taking place in the leaf during the act of induction.

[1] A. LANG, in *Encyclopedia of Plant Physiology* (edited by W. RUCKLAND), Vol. 15, p. 1380, Springer, Berlin (1965).
[2] D. J. CARR, *Advmt. Sci.* **23**, 186 (1966).
[3] M. K. CHAILAKHYAN, in *Biochemistry and Physiology of Plant Growth Substances* (edited by F. WIGHTMAN and F. SETTERFIELD), p. 1317, Runge Press, Ottawa (1968).
[4] R. M. SACHS and W. P. HACKETT, *Hortscience* **4**, 103 (1969).
[5] L. T. EVANS (editor), *The Induction of Flowering—Some Case Histories*, Macmillan, Melbourne (1969).
[6] L. T. EVANS, *Ann. Rev. Plant Physiol.* **22**, 365 (1971).
[7] D. D. GIBBY and F. B. SALISBURY, *Plant Physiol.* **47**, 784 (1971).

inhibitory substance. Earlier work, and particularly that of Hodson and Hamner,[8] had demonstrated that extracts possessing flower-promoting activity could be obtained from *Xanthium*. The conclusions of Gibby and Salisbury reiterate and elaborate the idea that the flowering process may be controlled by a balance between as yet unknown inhibitory and promoting agents within plants. More tangible support for this view comes from the recent report by Cleland[9] of the detection of at least one flower-promoting substance along with at least two flowering-inhibitory substances from *Xanthium*. With the short-day plant *Kalanchoe blossfeldiana*, Schwabe[10] has shown that sap from the leaves of long-day, vegetative *Kalanchoe* inhibits flowering when applied to leaves of *Kalanchoe* plants which are undergoing a short-day inductive regime. Significantly, Schwabe was also able to show that sap from leaves of short-day flowering *Kalanchoe* contained neither flowering-inhibitory or -promoting properties. These results point to the existence of flowering-inhibitory substance(s) in leaves of vegetative *Kalanchoe* which are absent or inactive in leaves of the flowering plant.

TABLE 1. FLOWERING INHIBITION BY PRIMARY EXTRACTS OF LEAVES FROM LONG- AND SHORT-DAY *Kalanchoe**; *Viscaria* TISSUE CULTURE BIOASSAY RESULTS

Extract	Inhibition† Long-day	Short-day
Filtered aqueous	+	−
70% aqueous ethanol	+	+
Diffusate from dialysed aqueous	+	−
Non-diffusate from dialysed aqueous	−	−
Ether extract of aqueous extract	+	+
Ether extract aqueous extract	−	−
Ether extract of diffusate of aqueous extract	+	−
Ether extract of non-diffusate of aqueous extract	−	−

* Extracts from equal fresh weights of long- and short-day leaves were used.

† Significant inhibition in *Viscaria* bioassay recorded as +; no inhibition as −.

The present paper described the detection, isolation and characterization of a flowering-inhibitory substance, gallic acid (I), from *Kalanchoe* leaves which inhibits flowering when applied to *Kalanchoe* during an inductive short-day regime. The isolation of this substance was facilitated by use of a new bioassay which uses tissue culture of partially photoperiodically induced apices of the long-day plant *Viscaria candida*.[11–13]

RESULTS AND DISCUSSION

Table 1 shows the flowering-inhibitory properties of some primary extracts of leaves from long- and short-day *Kalanchoe*. Only sap from leaves of long-day *Kalanchoe* was

[8] H. K. HODSON and K. C. HAMNER, *Science* **167**, 384 (1970).
[9] C. F. CLELAND, *Plant Physiol.* **46**, Suppl, 26 (1970).
[10] W. W. SCHWABE, *Planta* **103**, 18 (1972).
[11] J. BLAKE, *Planta* **103**, 126 (1972).
[12] J. BLAKE, *Nature, Lond.* **211**, 990 (1966).
[13] J. BLAKE, *J. Exptl Bot.* **21**, 113 (1969).

inhibitory to flowering when reapplied to *Kalanchoe*.[10] The same result was obtained using the *Viscaria* tissue culture bioassay with filtered aqueous leaf extracts.[11] This agreement between the *Kalanchoe* and *Viscaria* bioassay results was taken as an indication of the validity of using the *Viscaria* bioassay to search for the *Kalanchoe* flowering inhibitor.

When organic solvents were used to obtain inhibitory extracts, such as ether to extract filtered aqueous extracts or aqueous ethanol to extract *Kalanchoe* leaves, no difference between the flowering-inhibitory activity from long- and short-day leaves was observed (Table 1). However, ether extraction of the diffusate obtained by dialysis of filtered aqueous homogenates provided extracts of which only the long-day one was inhibitory (Table 1). Ether extracts of the non-diffusates of aqueous extracts were inactive. The results in Table 1 show that all the flowering-inhibitory properties from aqueous extracts of *Kalanchoe* leaves could be extracted into ether but it was clear from the bioassay results that extraction with 70% aq. EtOH was more efficient for extraction of the inhibitor(s). The flowering-inhibitory substance(s) were apparently quite heat-stable since no loss of activity was observed after autoclaving filtered aqueous extracts in the *Viscaria* tissue culture bioassay medium (pH 5·7) at 120° for 15 min.

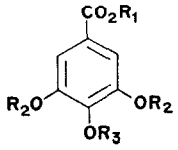
When aq. EtOH extracts of leaves from long-day *Kalanchoe* were fractionated by solvent partition into neutral, basic, water soluble (butanol fraction) and strong, medium and weak acid fractions, only the strong acid fraction showed specific flowering-inhibitory activity in the *Viscaria* bioassay. The only other fraction showing any inhibition of flowering was the neutral fraction which also markedly inhibited growth. Similar fractions of aq. EtOH extracts from short-day *Kalanchoe* leaves gave identical results.

Evidence for the belief that only the strong acid fractions contained specific flowering-inhibitory substance(s) was derived from examination of the activity of various acid and neutral fractions from ether extracts of filtered aqueous extracts and diffusates obtained by dialysis of aqueous extracts. Only the strong acid fractions contained flowering-inhibitory activity; the neutral fractions being completely inactive. It is conceivable that the growth-inhibitory and flowering-inhibitory activities of neutral fractions from the aq. EtOH extract might be associated with the recently discovered, widespread, neutral plant growth inhibitor, xanthoxin,[14,15] which may be an xanthophyll photo-degradation product. Chlorophyllide photo-degradation product(s) have also been found[16] to produce bacterial growth inhibition and it may be significant that neutral fractions obtained from filtered aqueous extracts had no green (chlorophyll) coloration whereas those from aq. EtOH extracts, which inhibited growth and flowering, were very green. Also, chromatography of the latter neutral fractions gave a very wide spread of growth- and flowering-inhibitory fractions all associated with green coloration.

During chromatography of the strong acid fraction from aq. EtOH extracts of long-day *Kalanchoe* leaves large losses of flowering-inhibitory activity were incurred as judged by the *Viscaria* bioassay. Losses were particularly marked on silica gel TLC where the mobility of the flowering inhibitor(s) suggested that they were of a very polar nature. In column chromatography on charcoal-celite and silica gel-celite and long-day strong acid fraction showed only one, albeit broad, peak of flowering-inhibitory activity. Ultimately a crystalline flowering-inhibitory substance was obtained from column chromatography of the strong acid fraction on silica gel-celite. This substance was shown, by UV spectroscopy

[14] H. F. Taylor and R. S. Burden, *Nature, Lond.* **227,** 302 (1970).
[15] R. D. Firn, R. S. Burden and H. F. Taylor, *Planta* **103,** 115 (1972).
[16] G. Blaauw-Jansen, *Nature, Lond.* **174,** 312 (1954).

and TLC as the free acid, and by GLC and MS as its fully methylated derivative (II), to be identical with the known plant constituent gallic acid (I). In all thin layer and column chromatography performed with the strong acid fraction it was possible to show that flowering-inhibitory fractions corresponded exactly with gallic acid containing fractions.



(I) $R_1 = R_2 = R_3 = H$
(II) $R_1 = R_2 = R_3 = CH_3$
(III) $R_1 = R_3 = H, R_2 = CH_3$
(IV) $R_1 = H, R_2 = R_3 = CH_3$

In the *Viscaria* bioassay gallic acid produced *ca.* 50% inhibition of flowering compared with controls when applied at 100 μg/ml in the culture nutrient solution. When applied to

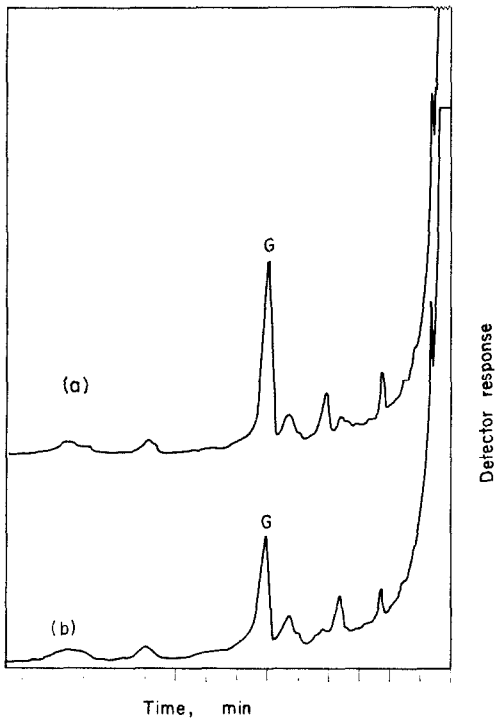


FIG. 1.

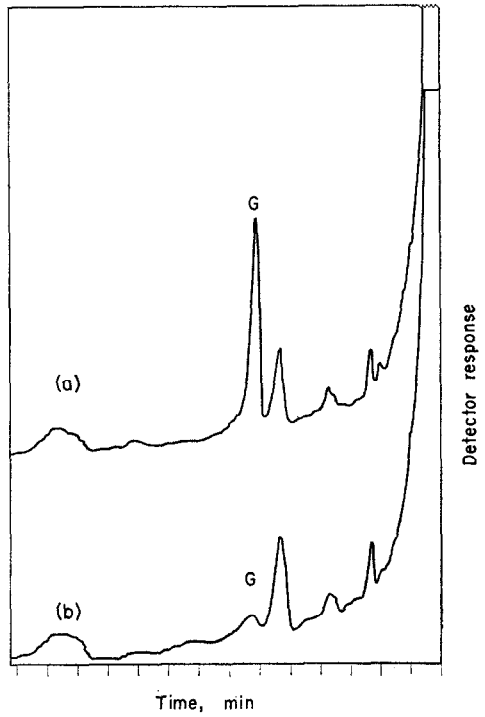


FIG. 2.

FIG. 1. GLC (XE-60 COLUMN) OF EQUAL ALIQUOTS OF METHYLATED *Kalanchoe* STRONG ACID FRACTIONS FROM AQUEOUS ETHANOL EXTRACTS OF (a) LONG-DAY, VEGETATIVE LEAVES (b) SHORT-DAY, FLOWERING LEAVES.

G marks the peaks of the same retention time as pure methyl gallate trimethyl ether. Both chromatograms were obtained with injections of amounts of extracts corresponding to equal (83 mg) fresh weights of *Kalanchoe* leaves. Recorder sensitivity was half that in Fig. 2.

FIG. 2. GLC (XE-60 COLUMN) OF EQUAL ALIQUOTS OF METHYLATED TOTAL ACID FRACTIONS FROM DIFFUSATES OBTAINED BY DIALYSIS OF AQUEOUS *Kalanchoe* EXTRACTS OF (a) LONG-DAY, VEGETATIVE LEAVES (b) SHORT-DAY, FLOWERING LEAVES.

G marks the peaks of the same retention time as pure methyl gallate trimethyl ether. Both chromatograms were obtained with injections of amounts of extracts corresponding to equal (100 mg) fresh weights of *Kalanchoe* leaves. Recorder sensitivity twice that in Fig. 1.

Kalanchoe leaves during short-day induction, gallic acid produced *ca.* 50% inhibition of flowering at 500 μg/ml in aqueous solution compared with controls. Gallic acid produced no observable growth inhibition in the *Viscaria* bioassay or in *Kalanchoe*.

Figures 1 and 2 show GLC of methylated acid fractions from long- and short-day *Kalanchoe* leaves. Gallic acid is present (Fig. 1) in similar amounts in the strong acid fraction of aq. EtOH extracts from long- and short-day leaves, in agreement with similar flowering-inhibitory activity found in these fractions (see above). In contrast, the total acid fraction obtained from diffusates of dialysed filtered aqueous extracts from leaves of long-day, vegetative and short-day, flowering *Kalanchoe* contain quite different amounts of gallic acid (Fig. 2). There appears to be at least ten times more gallic acid in the long-day than in the short-day diffusate and this can be related to the finding that only the strong acid fraction of the long-day diffusate had flowering-inhibitory properties. These results (Figs. 1 and 2) have been confirmed by chromatography on a different GLC stationary phase, and are essentially reproducible.

The results presented above describe the detection and isolation of gallic acid (I) as a specific flowering inhibitor from leaves of *Kalanchoe blossfeldiana*. It inhibits flowering both in this short-day plant and in apical tissue cultures of the long-day plant *Viscaria candida*. Although gallic acid and its derivatives are known to be of widespread occurrence in plants, especially as components of the hydrolysable tannins and lignins,[17,18] the extent of its flowering-inhibitory properties on plants of differing day-length requirement is as yet unknown. In the two bioassays described here gallic acid was found to be active only at rather high concentrations, 50% inhibition of flowering at 100–500 μg/ml. However, concentrations of this order of magnitude for a biological response are similar to those observed for naturally occurring phenolic plant growth inhibitors, which include gallic acid.[19] In the present work, however, no growth inhibition by gallic acid was detected.

The marked instability of gallic acid at alkaline pH has made estimation of its endogenous concentrations difficult. GLC analysis of a methylated strong acid fraction from the sap of leaves from long-day *Kalanchoe* indicated that gallic acid was present at a concentration of *ca.* 40 μg/ml after taking into account losses from a standard solution of gallic acid subjected to the same extraction procedure. Since Schwabe[10] has observed a greater than 50% inhibition of flowering with the sap of long-day leaves of *Kalanchoe*, a discrepancy may exist between the flowering-inhibitory activity of long-day sap (gallic acid apparently *ca.* 40 μg/ml) and that of pure gallic acid (50% inhibition at 500 μg/ml). *Viscaria* bioassay results may also indicate a discrepancy between the flowering inhibition obtained with crude *Kalanchoe* leaf extracts and pure gallic acid. Three explanations are possible for these observations: gallic acid is present at higher concentration in long-day *Kalanchoe* leaves than determined above, and/or gallic acid is not the only flowering-inhibitory substance present in long-day leaves, and/or there may be some synergistic effect between gallic acid and other components of long-day *Kalanchoe* leaves.

Schwabe[10] found that only sap from long-day, vegetative *Kalanchoe* and not sap from short-day, flowering *Kalanchoe* leaves inhibited flowering when applied to *Kalanchoe*. The same results are reproduced with filtered aqueous extracts in the *Viscaria* bioassay (Table 1). However, organic solvent extracts of either leaves or aqueous extracts from long- and short-day leaves are all inhibitory to flowering. When aqueous extracts are subjected to dialysis,

[17] J. B. HARBORNE (editor), *Biochemistry of Phenolic Compounds*, Academic Press, London (1964).
[18] E. HASLAM, *Chemistry of Vegetable Tannins*, Academic Press, London (1966).
[19] V. I. KEFELI and C. SH. KADYROV, *Ann. Rev. Plant Physiol.* **22**, 185 (1971).

flowering-inhibitory activity is found only in the diffusate from long-day leaves. Ether extraction of these diffusates gives extracts of which only those from long-day leaves are inhibitory. These results can be interpreted in terms of a free and physiologically active flowering inhibitor (gallic acid) being present in leaves of long-day, vegetative *Kalanchoe* and deactivation of the inhibitor by its being bound to a large molecule or subcellular particle in leaves of short-day, flowering *Kalanchoe*. This bound form of the inhibitor must be readily broken down in contact with organic solvents to give free gallic acid. The observation recorded in Table 1, that no flowering-inhibitory activity could be extracted with ether from the non-diffusate of a dialysed aqueous extract from short-day leaves, may cast some doubt on the above rationale but it is conceivable that changes occurred in the non-diffusate during the time of dialysis at room temp. On the basis of the present results it is therefore tempting to suggest that the agent which binds and deactivates the flowering inhibitor in leaves of flowering *Kalanchoe* may be a florigen—flowering might be the result of inhibitor decactivation. During the present work and earlier work with crude extracts of leaves from short-day, flowering *Kalanchoe*[10,11] no significant promotion of flowering could be detected. In terms of the hypothesis above this could be due to the relatively large amounts of gallic acid in *Kalanchoe* saturating the flowering promotive effects of the inhibitor-deactivating agent which is considered to be present in short-day leaves.

EXPERIMENTAL

Kalanchoe blossfeldiana plants were grown from cuttings in compost in a glasshouse under continuous illumination with tungsten lights to supplement natural daylight. Flowering *Kalanchoe* plants were produced by transferring long-day plants to short-days of 8 hr natural daylight in a glasshouse.

M.ps were measured on a Kofler block and are uncorrected. Solvents used for extraction and chromatography were purified by fractional distillation. Mass spectrometry was performed with an A.E.I. MS902 high resolution instrument. UV spectra were determined with a Pye Unicam SP1800 spectrophotometer.

Viscaria candida *tissue culture bioassay*. The bioassay was carried out essentially as described by Blake[11] but with the following differences. *Viscaria* plants were grown from seed in compost in a glasshouse in short-days of 8 hr daylight. When plants had three or four expanded leaf pairs they were transferred to a controlled temperature room (14–17°) where they were illuminated for 8 hr per day with an equal number of tungsten (75 W) and fluorescent (Philips TLS 40 W/33) lights (*ca.* 4000 lx). When these vegetative plants had four to six expanded leaf pairs or after 1 week, whichever was the sooner, they were transferred to another room (16–25°) where they were given 5 days continuous light induction prior to excision of the apex for tissue culture. This was given with tungsten (60 W) lights (*ca.* 1600 lx) supplemented for 8 hr per day with an equal number of fluorescent (Luxram warm white 60/85 W) lights (tungsten plus fluorescent illumination *ca.* 16 000 lx). This induction period gave between 80 and 90% flowering after tissue culture of the excised apices. Excised apices were each grown on a filter paper bridge dipping into Millipore filter-sterilized nutrient solution (6 ml) in disposable, sterile, screw-top plastic tubes (Sterilin Ltd.). Aqueous extracts for bioassay were used as diluent in the preparation of the nutrient solution. Other extracts were added in an appropriate solvent, generally MeOH or acetone, to the filter paper bridges and the solvent was evaporated under high vacuum prior to adding the nutrient solution. Preliminary extracts from *Kalanchoe* leaves were usually bioassayed at amounts equivalent to between 1 and 3 g fr. wt of leaves per tube per apex. Chromatographic fractions were tested at somewhat higher fresh weight equivalent amounts (*ca.* 5 g fr. wt of *Kalanchoe* leaves per tube per apex). The bioassay tissue cultures were grown in a controlled temperature room (14–17°) under 8 hr short-day illumination with an equal number of fluorescent (Philips TES 40 W/33) and tungsten (75 W) lights (*ca.* 4000 lx). After 3–4 weeks growth tissue cultures were analysed for inhibition of flowering by assessing the vegetative, transitional or flowering state of the apex as described elsewhere.[11] Usually ten replicates of each treatment and an untreated control were used in these experiments. At this time cultures had *ca.* 4 expanded leaf pairs and growth inhibition was indicated when this number was significantly below that of the controls.

Chromatography. TLC was carried out on layers (250 μ) of either silica gel H or cellulose (Whatman CC41). Extracts of fractions from TLC plates for bioassay were obtained by saturating the adsorbant with water and washing with methanol. Column chromatography was carried out as described with packings made up from activated charcoal silica gel and celite 545.

GLC was carried out with a Pye 104 dual column chromatograph fitted with dual flame ionization detectors and dual injector heaters. Silanized glass columns (1·5 m × 4 mm i.d.) were packed with either 1·5%

XE-60 or 1% OV-17 adsorbed on Gas-Chrom Q (80–100 mesh). In all cases nitrogen carrier gas flow rates were 60 ml/min, injector heaters were set at 250° and the oven temp. was 149°. Under these conditions methyl gallate trimethyl ether (II) had retention times of 6·1 min on the XE-60 column and 8·1 min on the OV-17 column. Gallic acid and *Kalanchoe* fractions for GLC were methylated. Quantitative estimation of gallic acid present in *Kalanchoe* fractions was made by comparison of the peak heights produced with standard injections of methyl gallate trimethyl ether and heights of the corresponding peaks in aliquots of methylated *Kalanchoe* fractions.

Aqueous ethanol extraction of Kalanchoe *leaves.* Leaves from long- and short-day *Kalanchoe* were extracted in a similar manner. Deep frozen long-day leaves (5·2 kg fr. wt) were macerated and soaked in 70% aq. EtOH (7 l.) for 2 days after which time they were filtered off and resoaked in fresh 70% aq. EtOH (5·5 l.) for a further 3 days. After a final filtration, the combined filtrates and washings were concentrated *in vacuo* at 35° to 1·2 l. The concentrate was adjusted to pH 8 with saturated Na_2CO_3 before extracting it with EtOAc (4 × 400 ml) to give the neutral plus basic EtOAc fraction. This fraction was extracted with 2 N HCl (6 × 400 ml) then washed with 5% $NaHCO_3$ and brine before drying (Na_2SO_4) prior to evaporation to dryness *in vacuo* to give the *neutral fraction* (23·6 g). The 2 N HCl extract was adjusted to pH 9·5 with saturated Na_2CO_3 and extracted with EtOAc (4 × 400 ml) and this EtOAc extract was dried (Na_2SO_4) prior to evaporation to dryness *in vacuo* to give the *basic fraction* (0·7 g). The aqueous remainder after the initial EtOAc extraction at pH 8 was adjusted to pH 3 with 4 N HCl and extracted with EtOAc (8 × 400 ml) giving the EtOAc *total acids* fraction then the remaining aqueous (pH 3) was extracted with *n*-BuOH (5 × 400 ml). This *n*-BuOH extract was dried (Na_2SO_4) prior to evaporation to dryness *in vacuo* giving the *butanol* fraction (19·0 g). The EtOAc-*total acids* fraction was dried (Na_2SO_4) before concentrating *in vacuo* to 250 ml, then extracted with phosphate buffer, pH 6·3 [10 × 50 ml of a solution of KH_2PO_4 (27·2 g) and KOH (48 g) in water (2 l.)]. The buffer extract was adjusted to pH 3 with 4 N HCl and extracted with EtOAc (8 × 100 ml). This latter EtOAc extract was dried (Na_2SO_4) prior to evaporation to dryness *in vacuo* to give the *strong acid fraction* (2·6 g). The remaining EtOAc after the buffer extraction above was extracted with 5% $NaHCO_3$ (6 × 50 ml) and then with 1 N NaOH (6 × 50 ml). Both these extracts were adjusted to pH 3 with 4 N HCl and extracted with ethyl acetate (4 × 100 ml) which was dried (Na_2SO_4) prior to evaporation to dryness *in vacuo* to give respectively the *medium acid fraction* (2·5 g) and the *weak acid fraction* (0·2 g).

Chromatography, isolation and identification of the flowering inhibitor. (a) *TLC.* Fractions from thin layer chromatograms of strong acid fractions from long-day *Kalanchoe* leaves were extracted and assayed for flowering-inhibitory activity using the *Viscaria* tissue culture bioassay. The flowering inhibitor(s) remained on or near the orgin (R_f 0·0–0·1) on silica gel plates eluted with EtOAc–$CHCl_3$–HOAc (15:5:1) while, under identical conditions, abscisic acid and gibberellins A_1, A_3, A_4, A_5, A_7 and A_9 had R_f values > 0·4. On cellulose plates, eluting with *iso*PrOH–15 M NH_4OH–H_2O (10:1:1), flowering-inhibitory activity was detected between R_f 0·0 and 0·2. Silica gel plates eluted with water gave one broad flowering inhibitory region at R_f 0·6–1·0. Aliquots of fractions from these chromatograms were later methylated for GLC analysis (XE-60 and OV-17 columns) and the flowering-inhibitory fractions were found to correspond with gallic acid containing fractions.

(b) *Column chromatography.* Strong acid fraction (610 mg) from an aqueous ethanol extract of long-day *Kalanchoe* leaves was adsorbed on a minimum quantity of celite from methanol solution and placed on top of a column of celite–silica gel (2:1, 60 g, 20 × 3·3 cm). The column was made up in light petroleum (b.p. 60–80°) and was eluted in 150 ml fractions of $CHCl_3$ containing increasing amounts of EtOAc in 5 or 10% steps. *Viscaria* tissue culture bioassay of aliquots of the column fractions indicated that flowering-inhibitory activity was eluted in one broad peak in fractions 7–12 which had been eluted with 50–90% EtOAc in $CHCl_3$. Fractions 7 and 8 were crystalline solids and fractions 9–12 were semisolid fractions. Fraction 7 (9 mg) was recrystallized from acetone-light petroleum (b.p. 60–80°) to give a crystalline substance m.p. 200–220° (decomposition) which was active as a flowering inhibitor in the *Viscaria* bioassay. After methylation of this inhibitor, GLC on XE-60 and OV-17 columns showed only one peak with the same retention time as methyl gallate trimethyl ether. In TLC on silica gel, solvent system benzene–MeOH–HOAc (90:16:8), the inhibitor showed a single spot of the same R_f (0·17) and deep purple colour reaction with a ferric chloride spray as pure gallic acid. These two observations distinguish the flowering inhibitor from two known naturally occuring gallate methyl ethers, syringic acid (III) and eudesmic acid (IV), which would have higher TLC R_f's[20] and different ferric chloride colour reactions. The UV spectrum of the inhibitor was identical with that of a pure sample of gallic acid: λ_{max}^{EtOH} 217 (ε, 27 500), 272 (ε, 9630) nm. The MS of the methylated inhibitor showed a molecular ion at *m/e* 226·0838, $C_{11}H_{14}O_5$ requires 226·0841. This molecular formula corresponds to that of methyl gallate trimethyl ether (II) and the mass spectral fragmentation pattern of the methylated flowering inhibitor was identical to that obtained with pure methyl gallate trimethyl ether. GLC analysis (XE-60 and OV-17 columns) of the remaining flowering-inhibitory column fractions, 8–12, showed them all to contain gallic acid with a maximum amount in fraction 9; the distribution of gallic acid in these fractions corresponded to the distribution of flowering-inhibitory activity in the *Viscaria* bioassay.

[20] E. STAHL and P. J. SCHORN, in *Thin-Layer Chromatography*—A *Laboratory Handbook* (edited by E. STAHL), p. 384, Springer, Berlin (1965).

The *Viscaria* bioassay indicated that flowering-inhibitory activity of a strong acid fraction from long-day *Kalanchoe* leaves was eluted from a column of charcoal–celite (1:2) with water containing 30–60% acetone. GLC analysis (XE-60 and OV-17 columns) of methylated aliquots of the column fractions showed that this peak of flowering-inhibitory activity corresponded to gallic acid elution from the column.

Aqueous extraction of Kalanchoe *leaves.* Fresh long- and short-day leaves were treated similarly. Aqueous extracts were prepared by macerating fresh leaves with an equal weight of distilled water. The macerate was filtered twice [filter paper, then a Millipore filter (0·45 μ pore size), both under reduced pressure] to give the *filtered aqueous extract* (long-day extracts *ca.* pH 5·2, short-day extracts *ca.* pH 4·7).

Filtered aqueous extracts were dialysed in Visking tubing against three changes of distilled water for 24 hr at room temp. The combined aqueous diffusates were concentrated *in vacuo* at 35°. The non-diffusate was collected. The pH of diffusates and non-diffusates was always between 4 and 5.

The filtered aqueous extracts, diffusates and non-diffusates of aqueous extracts were directly extracted with ether. The ether extracts were dried (Na_2SO_4) prior to evaporation to dryness *in vacuo*. Residual ether in the ether extracted aqueous fractions was removed *in vacuo* at 35° before the fractions were used in the *Viscaria* bioassay. Ether extracts of filtered aqueous extracts and the dialysis diffusates could be further fractionated into neutral and various acid fractions as indicated by the following example. The combined and concentrated diffusate (500 ml) obtained by dialysis of a filtered aqueous extract of long-day leaves (300 g fr. wt) was extracted with ether (4 × 100 ml). This ether extract was dried (Na_2SO_4) prior to concentration *in vacuo* to 250 ml and was then extracted with 5% $NaHCO_3$ (4 × 75 ml) followed by 1 N NaOH (4 × 65 ml). The remaining ether was washed with 2 N HCl, 5% $NaHCO_3$ and water before drying (Na_2SO_4) prior to evaporation to dryness *in vacuo* to give the *neutral fraction* (4 mg). The $NaHCO_3$ and NaOH extracts were separately adjusted to pH 3 with conc. HCl and extracted with ether (3 × 75 ml) which was dried (Na_2SO_4) before evaporation to dryness *in vacuo* to give respectively *strong acid* (56 mg) and *weak acid* (35 mg) fractions. In experiments where a total acid fraction was required it was obtained by directly extracting the initial ether extract of the aqueous extract with 1 N NaOH. The NaOH extract was adjusted to pH 3 with conc. HCl and extracted with ether which was dried and evaporated as before to give the *total acid fraction.*

Estimation of gallic acid in long-day leaf sap. Sap from fresh leaves of long-day *Kalanchoe* (228·2 g fr. wt, 25·1 g dry wt, pH 5·6) was expressed using a plant press. An aliquot (20 ml) was adjusted to pH 2 with 4 N HCl and extracted with EtOAc (4 × 5 ml). This EtOAc extract was extracted with 5% $NaHCO_3$ (4 × 5 ml). The $NaHCO_3$ extract was acidified with conc. HCl and extracted with EtOAc (4 × 5 ml) which was dried (Na_2SO_4) prior to evaporation to dryness *in vacuo*. The strong acid fraction thus obtained was methylated for GLC analysis. A control extraction of an aqueous gallic acid solution (4 mg/20 ml) was carried out exactly as above. Quantitative GLC analysis (XE-60 column) of the control methylated strong acid fraction showed that 48% of the gallic acid had been lost during the extraction. After correction, GLC analysis of the leaf sap strong acids indicated that gallic acid was present at *ca.* 40 μg/ml of sap, 35 μg/g fr. wt of leaf or 320 μg/g dry wt of leaf.

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Key Word Index—Kalanchoe blossfeldiana; *Viscaria candida*; gallic acid; flowering inhibitor; tissue culture bioassay.