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## Note

# Solid-state fluorodensitometric quantitation of arylnaphthalene lignan lactones of *Cleistanthus collinus*

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Arylnaphthalene lignan lactones are naturally occurring dimers in plants and known to have potential use in medicine[1,2]. A few of them are reported to be highly toxic; others find use as antioxidants and insecticides[3].

*Cleistanthus collinus* (Roxb.) Benth. & Hook.f. (family: Euphorbiaceae) is a highly poisonous plant. All parts of it are reported to be toxic and employed as a suicidal, homicidal, cattle and fish poison and for procuring criminal abortion[4–6]. Chemical characterization of different parts of the plant has led to the isolation and identification of certain lignan lactones including diphyllin, cleistanthin A, cleistanthin B and collinusin[7,8]. Although the poisonous nature of the plant has been established[9], the compound(s) responsible for the toxicity is not known. Nevertheless, few reports on the medicinal properties of the lignan lactones have appeared[2,10–12].

While information on the structures and biological activities of the constituents is available, there are little data on the quantitative analytical aspects. Methods reported in the literature cover only the qualitative identification of *C. collinus* from poisoned visceral materials and chromatographic systems for the separation of the lignan lactones[13,14].

Based on the photometric properties (including fluorescence under UV irradiation) of the four compounds mentioned above, we previously developed spectrophotometric and spectrofluorometric methods[15,16]. The present paper deals with an improved technique, namely, the solid-state fluorodensitometric quantitation of diphyllin, cleistanthin A, B and collinusin —the major arylnaphthalene lignan lactones of *C. collinus*— after their thin-layer chromatographic (TLC) separation. While the earlier methods require TLC and subsequent concentration of the compounds, the present method is a direct one. The variation in the fluorescence characteristics of the compounds in solution and on silica gel (TLC) has also been studied and is utilized to enable a better quantitation.

## EXPERIMENTAL

### *Instrumentation*

All densitometric measurements were made with a soft laser (SL-TRFF) scanning densitometer (Biomed., U.S.A.).

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*Reagents*

Chemicals and solvents were of analytical/spectral grade quality.

Authentic samples of diphyllin, cleistanthin A and collinusin were obtained from Ciba-Geigy (Bombay, India) and cleistanthin B from Osmania University, Hyderabad, India. The purity of the samples was verified from their spectral and chromatographic data.

Standard solutions of 500 μg/ml were prepared in ethanol and working standard solutions were obtained by suitable dilution as and when needed.

*Quantitative TLC*

Known volumes of authentic sample solutions of diphyllin, cleistanthin A, B and collinusin were spotted on a TLC plate (Kieselgel 60G, E. Merck, 0.25 mm, activated at 110°C for 30 min) at a distance of 4 cm from the bottom with a microlitre syringe. The plate was developed to a distance of 15 cm with *n*-heptane–chloroform–ethanol (50:50:5). It was then dried and viewed under UV light to ensure the resolution of the compounds.

*Densitometric quantitation*

The UV lamp of the densitometer was allowed to warm up for 15 min prior to use to obtain the best results. The plate was mounted upright on the stage with suitable spacers on the top and bottom of the plate. A low-pressure mercury lamp with an emission peak at 366 nm was used to excite the sample. A cobalt blue filter forms an integral part of the lamp and was used to block emission above 400 nm. The source was placed on the same side of the sample as the detector. The light (fluorescence) emitted from the sample was passed through a collimating slit. A secondary filter that blocks energy below 410 nm was placed between the slit and the detector to prevent UV light from the exciting source from reaching the detector. Thus, only light emitted from the sample is transmitted to the detection and measuring systems. The photomultiplier tube voltage, the analog gain and zero suppression control knobs were adjusted to give 85–90% full-scale deflection when the densest spot was scanned. The scan length was adjusted to a fixed distance by means of a thumb screw provided in the slot of the stage carriage.

The densitometer is equipped with a time-based electronic integrator which relates position and time in the quantitation of the sample, and was used to quantitate the area under the curve.

*Calibration curve*

The linearity between the fluorescence intensity and concentration was established by constructing calibration curves for all the four compounds.

Dried leaves of *C. collinus* were extracted with alcohol after defatting with hexane as reported earlier[16]. An aliquot of the final extract was chromatographed and subjected to densitometric measurements.

*Recovery after spiking*

Known amounts of the authentic samples (5 μg in ethanol) of the lignan lactones were added to blood and tissue homogenate (prepared by homogenization of 1 g rat stomach with 1 ml of water) and processed for densitometric quantitation following the procedure reported elsewhere[16].

RESULTS AND DISCUSSION

The chemical structures of the four compounds studied are shown in Fig. 1.

The chromatographic resolution of the four compounds along with a leaf extract of *C. collinus* is shown in Fig. 2. Typical densitograms of the mixture of authentic samples of the four compounds and that of the leaf extract are given in Figs. 3 and 4, respectively. As shown, the leaf extract contains a few more fluorescent compounds apart from the lignans, the identification of which remains to be established.

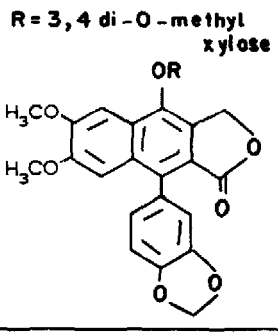
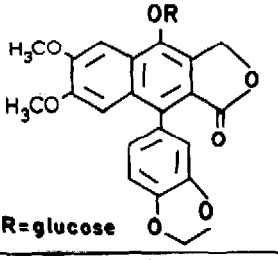
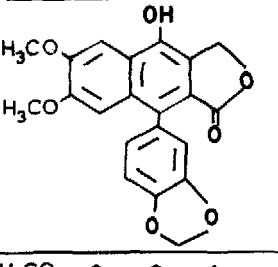
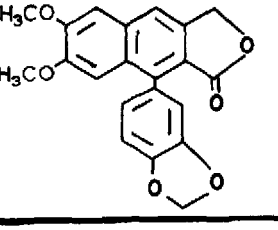
| COMPOUND | STRUCTURE | $R_F$ VALUE* |
|---|---|---|
| CLEISTANTHIN A |   | 0·37 |
| CLEISTANTHIN B |   | 0·01 |
| DIPHYLLIN |   | 0·22 |
| COLLINUSIN |   | 0·55 |

Fig. 1. Chemical structures and $R_F$ values of the lignan lactones from the leaves of *C. collinus*. TLC was carried out on Kieselgel 60G with *n*-heptane–chloroform–ethanol (50:50:5) as mobile phase.

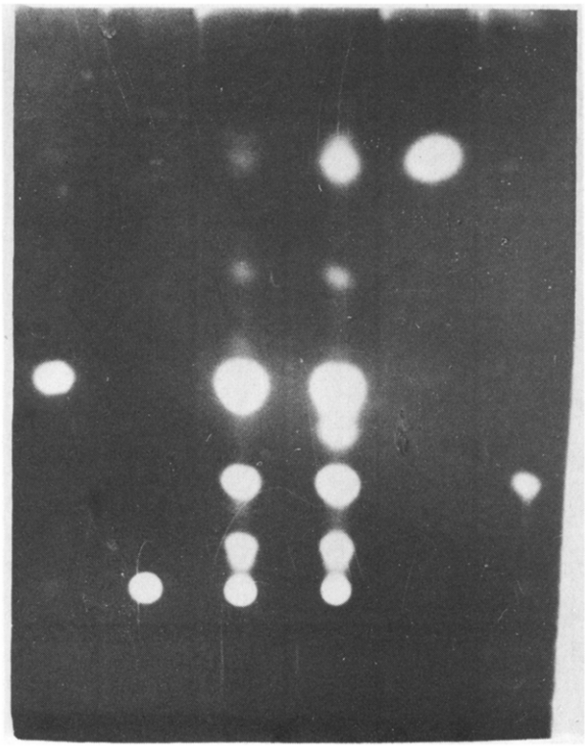


Fig. 2. UV photograph of the TLC resolution of *C. collinus* lignan lactones: from left to right, cleistanthin A ($R_F$ 0.37), B ($R_F$ 0.01), leaf extracts of *C. collinus* (collected from two different places separated by a distance of 200 km), collinusin ($R_F$ 0.55) and diphyllin ($R_F$ 0.27).

Calibration graphs were constructed for all the four compounds individually by plotting the percentage peak area against concentration. The linearity extends over the range of 0.25–1.50 μg for diphyllin, cleistanthin A and collinusin and 0.10–1.0 μg for cleistanthin B (Fig. 5).

*Intensity of fluorescence with time*

In order to monitor the change, if any, in the intensity of fluorescence with time, the compounds after chromatography were subjected to densitometric evaluation at intervals over a period of 24 h. The intensity of fluorescence of cleistanthin A, B and collinusin remains constant, while that of diphyllin decreases after 12 h, the effect being predominant at low concentrations.

*Sensitivity*

The lowest limit of sensitivity of the proposed method is 0.25 μg of diphyllin, cleistanthin A and collinusin and 0.10 μg of cleistanthin B. A comparison of sensitivity obtainable by the above method and that by spectrofluorometry[16] is provided in Table I.

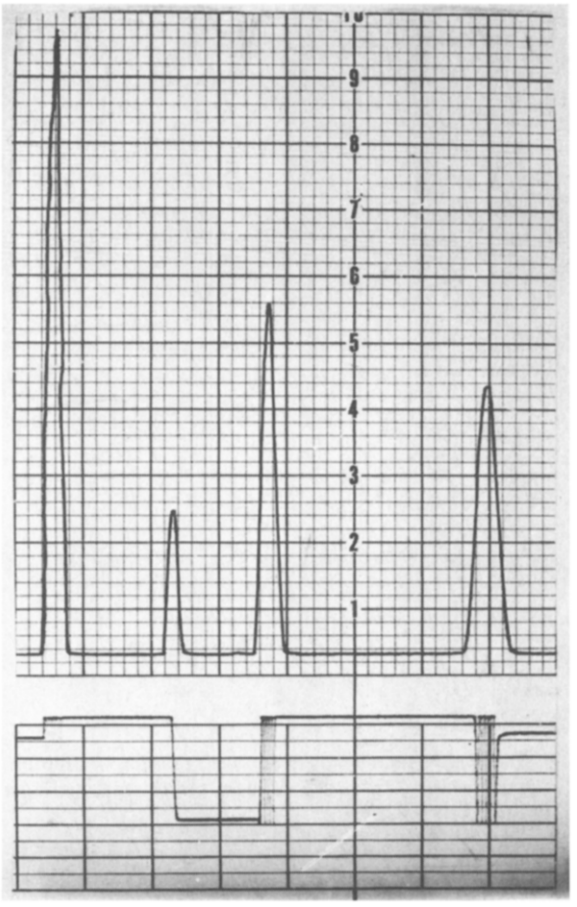



Fig. 3. Densitogram of a mixture of four lignan lactones (each 1 μg in ethanol) of *C. colinus* after TLC. Peaks (left to right): cleistanthin B, diphyllin, cleistanthin A and collinusin.

TABLE I

ARYLNAPHTHALENE LIGNAN LACTONES OF *C. COLLINUS*: COMPARISON OF SENSITIVITY OBTAINED IN SPECTROFLUOROMETRY AND FLUORODENSITOMETRY

| *Compound* | *Spectrofluorometry** (μg/ml) | *Fluorodensitometry* (μg) |
|---|---|---|
| Diphyllin | 0.1 | 0.25 |
| Cleistanthin A | 1 | 0.25 |
| Cleistanthin B | 1 | 0.10 |
| Collinusin | ** | 0.25 |

* Minimum of 3 ml solution (0.3, 3, 3 μg) is required for measurement.
** No response even at a concentration of 600 μg/3 ml in ethanol.

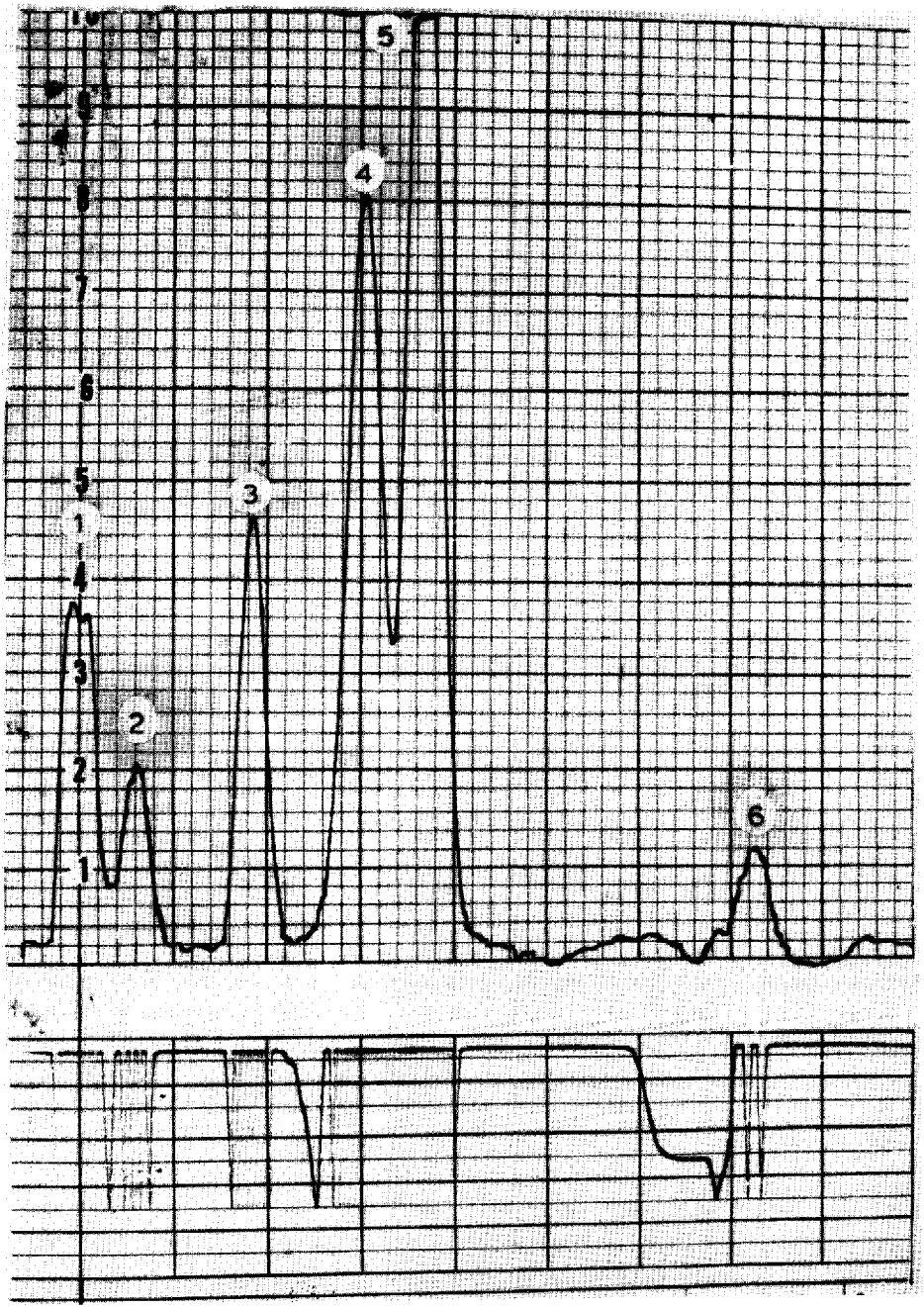



Fig. 4. Densitogram of *C. collinus* leaf extract[16] after TLC. Peaks: 1 = cleistanthin B; 3 = diphyllin; 5 = cleistanthin A; 6 = collinusin.

*Reproducibility*

The precision of the method using densitometric analysis was evaluated by carrying out the experiments with a sample containing all the four lignan lactones in

TABLE II

REPLICATE ANALYSIS OF MIXTURES OF FOUR LIGNAN LACTONES OF *C. COLLINUS* OVER A PERIOD OF 9 DAYS

| *Compound* | *Amount present (μg)* | *Percentage peak area* | | | | | | | | | *Mean* | *S.D.* | *C.V. (%)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | | | |
| Diphyllin | 0.50 | 11.875 | 11.744 | 11.736 | 10.528 | 12.347 | 11.354 | 10.515 | 11.355 | 10.310 | 11.29 | 0.56 | 5.03 |
| Cleistanthin A | 1.25 | 35.608 | 37.072 | 34.576 | 36.623 | 36.267 | 35.281 | 35.43 | 36.706 | 37.548 | 36.123 | 0.97 | 2.68 |
| Cleistanthin B | 0.50 | 12.316 | 13.438 | 12.417 | 13.341 | 12.586 | 12.146 | 12.162 | 12.248 | 12.571 | 12.56 | 0.502 | 3.99 |
| Collinusin | 0.25 | 5.835 | 6.480 | 6.826 | 6.392 | 6.415 | 5.828 | 5.704 | 6.415 | 5.725 | 6.18 | 0.41 | 6.65 |

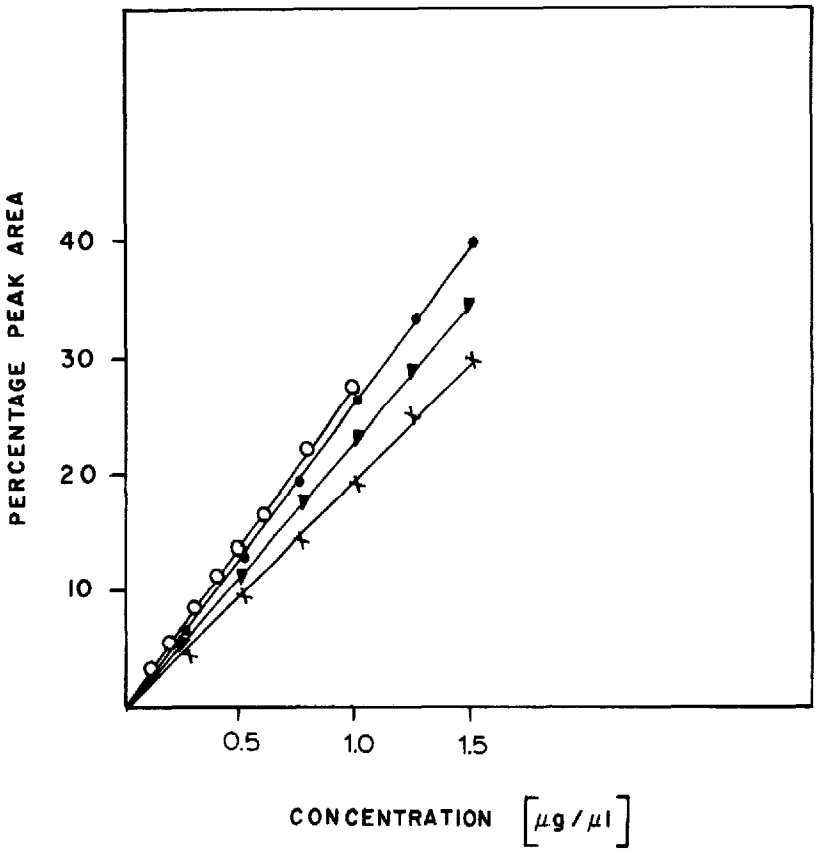



Fig. 5. Fluorodensitometric calibration curves for lignans from *C. collinus:* ×—×, diphyllin; ●—●, cleistanthin A; ○—○, cleistanthin B; ▼—▼, collinusin.

known quantity over a period of 9 days. The results presented in Table II indicate a precision of measurement of about ± 2.68–6.65% as determined by the coefficient of variation (c.v.) for the four compounds. However, to obtain better results it is suggested that a calibration curve be constructed for each plate.

Recovery data from spiked biological samples of blood and tissue homogenate are given in Table III.

An interesting observation during the course of our investigation is that collinusin (a structural analogue of diphyllin), which could not be estimated by spectrofluorometry even at a concentration of 600 μg/3 ml, was quantitatively analysed by the present fluorodensitometric method at a concentration of 0.25 μg; also the intensity of luminescence on chromatograms remains unaltered for more than a fortnight. It is also interesting to note that diphyllin, which offered the maximum spectrofluorometric turn-out with unaltered luminescence in solution over a number of days, occupies the last place in the series when scanned for fluorodensitometry (Table I). As shown in Fig. 3, the relative order of solid state fluorescence intensity is cleistanthin B > A > collinusin > diphyllin, whereas in spectrofluorometry, it is di-

TABLE III

RECOVERY OF LIGNAN LACTONES OF *C. COLLINUS* FROM SPIKED RAT STOMACH HOMOGENATE

Each value is the average from three determinations.

| *Specimen* | *Diphyllin* | | *Cleistanthin A* | | *Cleistanthin B* | | *Collinusin* | |
|---|---|---|---|---|---|---|---|---|
| | *Amount added (μg)* | *% Recovery* | *Amount added (μg)* | *% Recovery* | *Amount added (μg)* | *% Recovery* | *Amount added (μg)* | *% Recovery* |
| Blood | 5 | 93.7 | 5 | 94.5 | 5 | 92.8 | 5 | 92.7 |
| Tissue homogenate | 5 | 90.3 | 5 | 90.7 | 5 | 88.5 | 5 | 88.1 |

phyllin > cleistanthin B > cleistanthin A. Similar observations have been reported for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$[17].

## CONCLUSIONS

Many reports on the medicinal utility of lignan lactones have appeared in the literature. Compounds like podophyllotoxin, α- and β-peltatin have been found to be active against tumours[3]. Similarly, diphyllin which occurs also in *Diphyllia grayii* and *Haplophyllum hispanicum* was found to possess cytotoxic properties and is twice as potent as 6-mercaptopurine, a well known cytotoxic agent[2]. Anticancer screening tests with whole plant extracts of *C. collinus* on human epidermoid carcinoma of the nasopharynx in tissue culture, Walker carcinosarcoma 256 in the rat and L-1210 lymphoid leukaemia in the mouse have confirmed the anticancer activity of the plant. Cleistanthin A, another lactone glycoside of diphyllin, was found to possess remarkable neutrophilic granulocystosis activity in the rat, mouse, cat and monkey during drug-induced granulocytopenia[10,11]. The biological activity of other compounds, namely cleistanthin B and collinusin, are under study.

Due to the toxic nature of the plant, clinicians and forensic toxicologists often encounter the problem of determining the active constituents in body fluids and tissues. A simple and at the same time precise protocol is therefore essential. The method reported here meets these requirements.

## ACKNOWLEDGEMENTS

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