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ISOLATION OF BIOLOGICALLY ACTIVE PLANT CONSTITUENTS BY LIQUID CHROMATOGRAPHY

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SUMMARY

Strategies are outlined for the separation of biologically active products of plant origin. The techniques involved include low-pressure liquid chromatography, semi-preparative high-performance liquid chromatography, flash chromatography and droplet counter-current chromatography. Their application to the isolation of compounds from *Sesamum angolense* (Pedaliaceae), *Psorospermum febrifugum* (Guttiferae) and *Cordia goetzei* (Boraginaceae) is described.

INTRODUCTION

The search for new compounds from plant sources is an expanding field of research, which often has as its aim the development of pharmacologically active substances. Whereas these phytochemicals may themselves be of interest, they also provide starting materials for the further investigation (by synthesis or partial synthesis) of other molecules with certain desired activities. The isolation of the pure biologically active constituents responsible for the properties of medicinal plants is essential for structure elucidation, structural modifications and for studying the mode of action of the drug, its side-effects, toxicology, etc. For these investigations, quantities in excess of 10 mg of the relevant compound are generally required. The pathway from the plant to the pure material can be long and tedious. After extraction, the active material must be fractionated, each separation step being guided by a convenient assay. Most important of all during this process is the judicious choice of chromatographic techniques for maximum yield of pure substances with a minimum of effort. Some of the preparative separation techniques available[1] are: paper chromatography, preparative thin-layer chromatography (TLC) (and centrifugal TLC), open-column chromatography, vacuum liquid chromatography, pressure column chromatography [flash, low-pressure liquid chromatography (LPLC), medium-pressure LC (MPLC), high-pressure LC (HPLC)], liquid–liquid chromatography [Craig distribution, droplet counter-current chromatography (DCCC), rotation locular counter-current chromatography (RLCC), centrifugal partition chromatography (CPC)].

Although in some cases a single chromatographic step may suffice to isolate the

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required amount of compound, a combination of methods is most often required. A number of strategies are possible for the separation of lipophilic or hydrophilic substances. Some of these will be illustrated here, with special reference to our on-going programme of research on the biologically active compounds from African medicinal plants. For example, a phytochemical investigation of the plant *Sesamum angolense* Welw. (Pedaliaceae) from tropical Africa is presently being undertaken because of the presence of haemostatic and fungicidal[2] compounds in the root bark. Another plant of current interest is *Psorospermum febrifugum* Spach (Guttiferae), extracts of which are specifically toxic to human colon carcinoma cells[3]. Previous phytochemical investigations led to the isolation of the xanthone psorospermin, an antileukaemic principle of this African plant[4]. However, further examination of the light petroleum (b.p. 60–95°C) extract of the root bark has resulted in the isolation of a series of lipophilic anthranoid derivatives which are responsible for the major part of the cytotoxic activity[3]. Finally, *Cordia goetzei* Gürke (Boraginaceae) is a medicinal plant used in Tanzania for the treatment of leprosy, malaria and abscesses. The stem bark of this tree contains a mixture of fungicidal highly oxygenated polyphenols[5].

EXPERIMENTAL

Iridoid glycosides and a phenylpropanoid glycoside from Sesamum angolense (Pedaliaceae)

Extraction of *Sesamum angolense* root bark from Malawi was carried out first with dichloromethane and then with methanol. DCCC of the methanol extract on a Büchi 670 instrument (Flawil, Switzerland) with chloroform–methanol–2-propanol–water (5:6:1:4) in the ascending mode yielded 15 fractions (I–XV). Preparative LPLC of fraction IV on a Lobar size B column (LiChroprep RP-8, 40–63 μm) (Merck, Darmstadt, F.R.G.), equipped with a Duramat-80 pump (Chemie und Filter, Regensdorf, Switzerland), Uvicord SII detector (254 nm) (LKB, Bromma, Sweden) and LKB 2210 chart recorder, yielded compounds 1 and 2 (Fig. 1) on elution with 10% aqueous methanol. Subsequent elution with 32% aqueous methanol gave compound 3. The same method gave compound 4 from fraction VII and compound 5 from fraction XI, with 15% aqueous methanol as the eluent. Full details of the structure elucidation for compounds 1–5 have been reported elsewhere[6].

Analytical HPLC was performed with 5-μl samples on a 7-μm LiChrosorb RP-8 column (250 mm × 4.6 mm I.D.) (Knauer, Bad Homburg, F.R.G.) with 10% aqueous methanol (Fig. 2A) or 32% aqueous methanol (Fig. 2B) at a flow-rate of 1.5 ml/min (Model 8700; Spectra-Physics, San Jose, CA, U.S.A.). Detection was at 254 nm (LKB Uvicord SII).

Anthracene derivatives of Psorospermum febrifugum (Guttiferae)

Root bark of *Psorospermum febrifugum*, collected in Malawi, was extracted with light petroleum (b.p. 60–95°C). The resulting orange resin was cytotoxic to a Co-115 human colon carcinoma cell line[3] and this assay was employed for subsequent fractionation of the extract. Full details of the isolation and structure determination of compounds 6–11 (Fig. 4) from the light petroleum extract have been given elsewhere[3]. Flash chromatography was performed on silica gel Si 60 (63–200 μm) (Merck) and gel filtration on Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Preparative LPLC

separations of compounds 7, 10 and 11 was carried out on Lobar size B columns (LiChroprep RP-8, 40–63 μm) with 93% aqueous methanol at a flow-rate of 2 ml/min.

Analytical HPLC was carried out with 10 μl of a 5 mg/ml solution of an extract on a 7-μm LiChrosorb RP-18 column (250 mm × 4.6 mm I.D.). The eluent (Fig. 5) was delivered at 1.5 ml/min by a Spectra-Physics 8700 pump. Chromatograms and spectra were recorded with an HP1040A photodiode-array detector (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Polyphenols from Cordia goetzei (Boraginaceae)

Stem bark of *Cordia goetzei* was extracted sequentially with light petroleum (b.p. 60–95°C) chloroform and methanol. Screening for biological activity with a TLC bioassay[7] showed the presence in the methanol extract of compounds active against the fungus *Cladosporium cucumerinum*. Subsequent fractionation of the methanol extract by DCCC on a Büchi 670 apparatus with chloroform–methanol–water (43:37:20) in the descending mode and crystallization of fractions 26–35 from ethyl acetate–hexane gave compound 14. Polyphenol 15 was obtained after subjecting DCCC fractions 39–43 to LPLC on a Lobar size B column of LiChroprep RP-8 (40–63 μm) with 60% aqueous methanol. Full details of the isolation of compounds 14 and 15, together with their structure determination, have been given elsewhere[5]. Compounds 12 and 13 were separated by semi-preparative HPLC of DCCC fractions 161–225 on a 10-μm μBondapak C_{18} column (300 mm × 7.8 mm I.D.) (Waters-Millipore, Milford, MA, U.S.A.) with 40% aqueous methanol at a flow-rate of 5 ml/min. The instrumentation comprised a Waters 6000A pump coupled to a Waters automatic gradient controller. Detection at 254 nm was effected with a variable-wavelength UV detector (Pye-Unicam, Cambridge, U.K.). The sample (150 mg) was dissolved in 1 ml of mobile phase and filtered through Millex HV_4 filter units (0.45 μm; Millipore, Milford, MA, U.S.A.). For each batch, 20–50 μl of solution were injected. The structure determination of compounds 12 and 13 has been given elsewhere[5]. Analytical HPLC was carried out with 5 μl of a 5 mg/ml solution on 7-μm LiChrosorb RP-18 (250 mm × 4.6 mm I.D.) or 10-μm μBondapak C_{18} (300 mm × 3.9 mm I.D.) columns. The eluent (the composition is shown in Figs. 7 and 8) was delivered at 1.5 ml/min by a Spectra-Physics 8700 pump and chromatograms were recorded on an HP1040A photodiode-array detector.

RESULTS

Iridoid and phenylpropanoid glycosides from Sesamum angolense

Following extraction of the root bark of *Sesamum angolense* with dichloromethane, the methanol extract was found to contain a number of iridoid glycosides. In order to obtain the pure glycosides, the methanol extract was first subjected to a DCCC fractionation step. Analytical HPLC of fraction IV on RP-8 with 10% aqueous methanol (Fig. 2A) allowed the separation of the iridoid glycosides phlomiol (1) and pulchelloside-I (2) within 13 min. Elution with 32% aqueous methanol (Fig. 2B) gave an additional peak after *ca.* 12 min, correponding to 3 (verbascoside). However, in this case, compounds 1 and 2 were not separated. The preparative separation of compounds 1–3 was performed by LPLC (Fig. 3). The analytical conditions can be directly applied to the large-scale method. Thus, after injection of

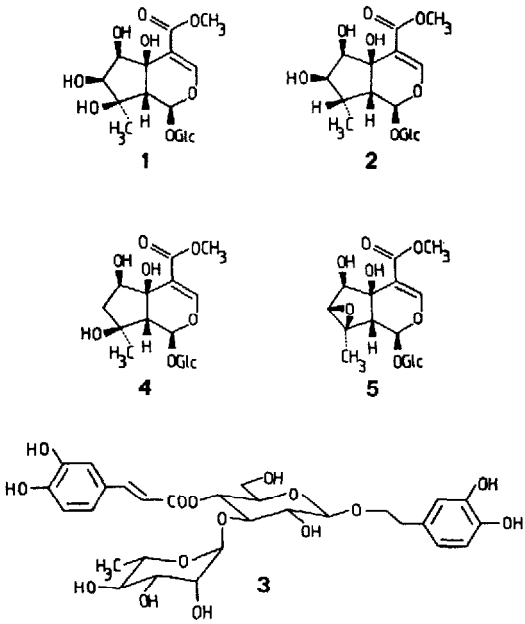

Fig. 1. Glycosidic components of the root bark of *Sesamum angolense* (Pedaliaceae). Glc = Glucose.

130 mg of sample, elution with 10% aqueous methanol enabled the isolation of compounds 1 (71 mg) and 2 (9 mg). Increasing the methanol content of the eluent to 32% gave, in addition, 10 mg of verbascoside. The separation was complete in 20 h.

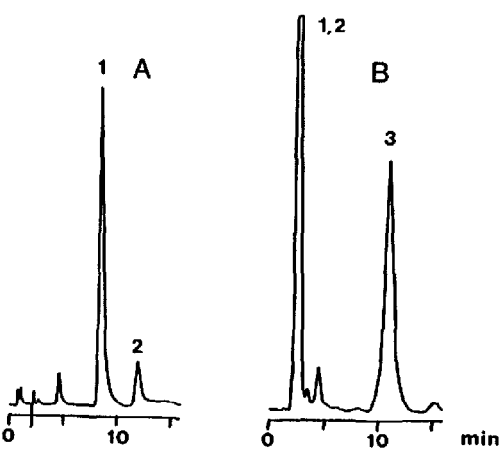

Fig. 2. HPLC analysis of DCCC fraction IV from the methanol extract of *Sesamum angolense* root bark. Column: LiChrosorb RP-8. Detection: 254 nm. Flow-rate: 1.5 ml/min. Sample: 5-μl (25 μg). Eluents: A, 10% aqueous methanol; B, 32% aqueous methanol.

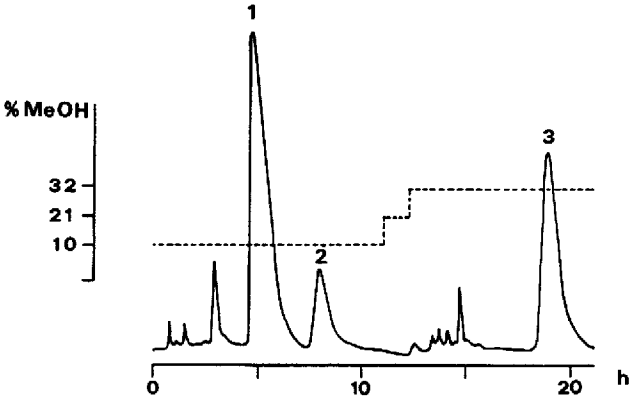

Fig. 3. LPLC separation of DCCC fraction IV from the methanol extract of *Sesamum angolense* root bark. Column: Lobar RP-8 (310 mm × 25 mm). Eluent: aqueous methanol (MeOH, 10 to 32%) step gradient; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 130 mg (3 ml).

Compound 4 (6β-hydroxyipolamiide) and the new iridoid glycoside sesamoside (5) were similarly obtained by the combination of DCCC with LPLC.

Cytotoxic anthracene derivatives from Psorospermum febrifugum

An analytical separation of the major anthranoid pigments occurring in the root bark of *Psorospermum febrifugum* is shown in Fig. 5. Fractionation of the light petroleum extract gave a total of six yellow compounds 6–11 (Fig. 4). Compounds 6,

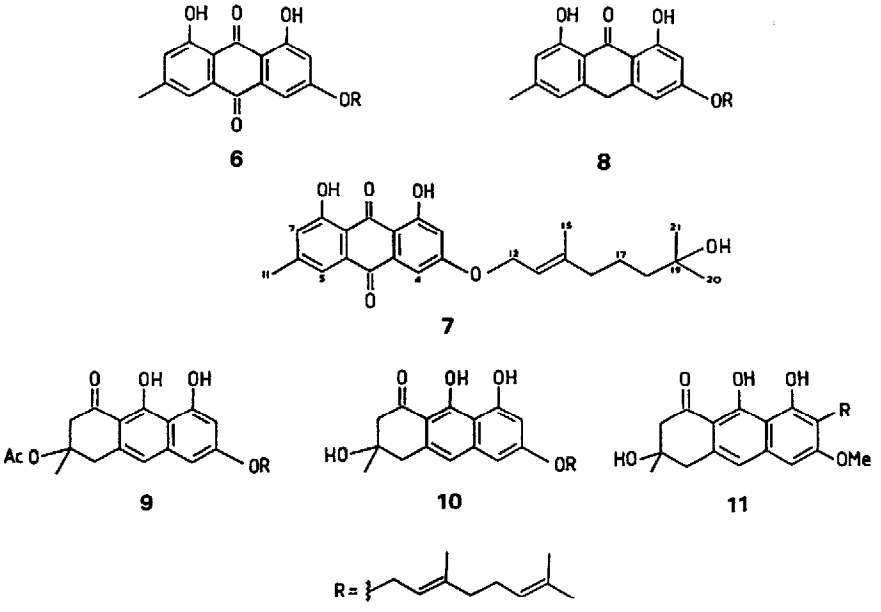

Fig. 4. Anthranoid pigments obtained from *Psorospermum febrifugum* (Guttiferae).

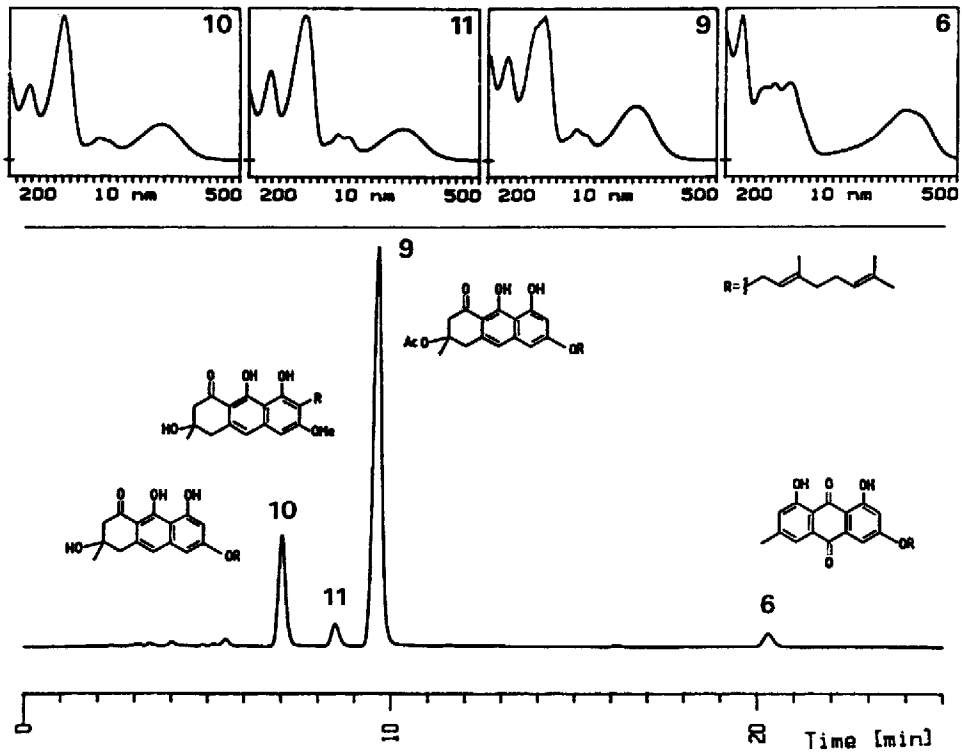

Fig. 5. HPLC–UV analysis of a light petroleum extract of *Psorospermum febrifugum* root bark. Column: LiChrosorb RP-18. Eluent, 90 to 100% methanol (+ 0.5 ml/l phosphoric acid) over 15 min, then 100% methanol for 10 min; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 10 μl (50 μg). Me = Methyl; Ac = acetyl.

8 and 9 were crystallized directly from the relevant flash chromatography fractions[3]. Anthrone (8) although previously described as occurring in the plant[8], was obtained only as an artefact of isolation and was not observed in the HPLC analysis of the extract. Traditional chromatographic methods, such as open-column chromatography, led to decomposition and irreversible adsorption of the sensitive pigments. It was necessary, therefore, to use rapid (flash and low-pressure liquid chromatography) or weakly interactive (gel filtration) methods for the successful isolation of the cytotoxic compounds. The tetrahydroanthracenes 9 and 10 were the major cytotoxic constituents of *P. febrifugum* root bark. Tetrahydroanthracene (11)[9] was also active but anthrone (8) only exhibited marginal cytotoxicity. Anthraquinone (7) was isolated in only small amounts from the extract and, like anthraquinone 6, was biologically inactive.

Antifungal polyphenols from Cordia goetzei

The antifungal methanol extract of *Cordia goetzei* stem bark was found to be a very complex mixture when analysed by HPLC-UV (Fig. 7). Preliminary fractionation by DCCC, together with a bio-autographic TLC assay for fungicidal activity[7], enabled the attribution of activity to a certain number of defined components of the

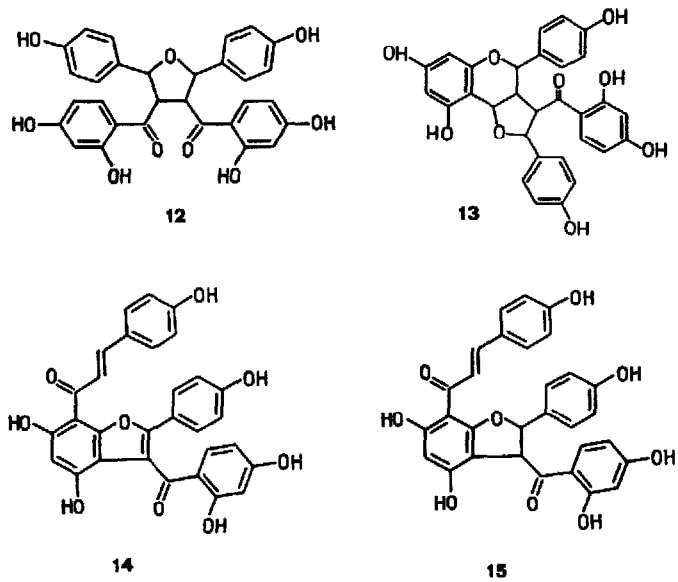

Fig. 6. Antifungal polyphenols from *Cordia goetzei*.

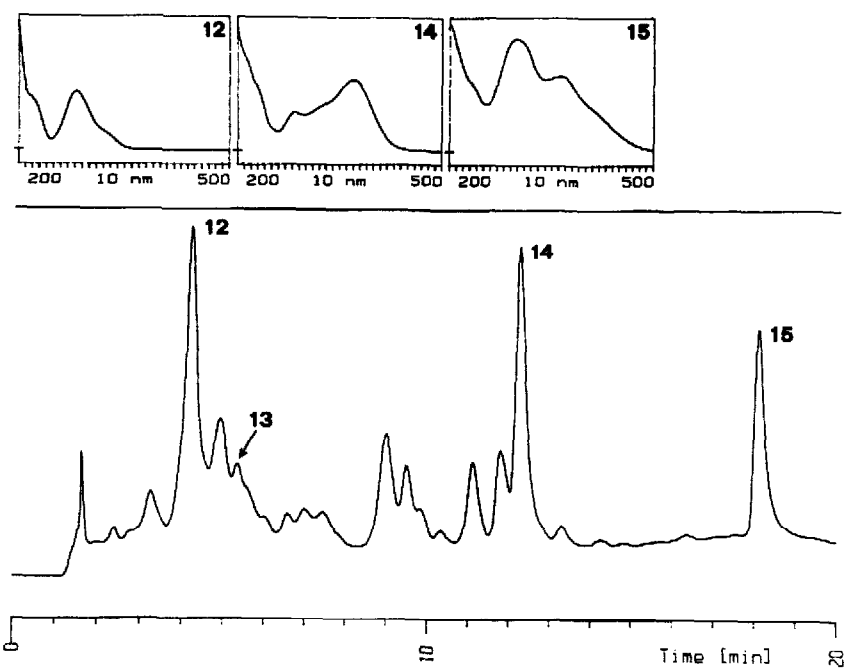

Fig. 7. HPLC–UV analysis of a methanol extract of *Cordia goetzei* stem bark. Column: LiChrosorb RP-18. Eluent: 55 to 75% methanol over 15 min, then 75% methanol for 5 min; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 5 μl (50 μg).

mixture. Thus, DCCC fractions 161–225 were shown to contain two novel major antifungal compounds, 12 and 13. An analytical HPLC separation of compounds 12 and 13 on an octadecylsilyl column is shown in Fig. 8, together with the corresponding UV spectra, recorded on-line with a photodiode-array detector[10]. The analytical HPLC separation was used as the basis for determining semi-preparative HPLC separation conditions. A typical semi-preparative HPLC chromatogram on a μBondapak C_{18} column with 40% aqueous methanol as eluent is shown in Fig. 9. Baseline separation of compound 12 occurred, but 13 had to be collected by heart-cutting in order to obtain the pure product. By repetitive injections, a total of 83 mg of compound 12 and 29 mg of compound 13 were obtained. These two compounds were indistinguishable by TLC on silica gel in several solvent systems.

In addition, two known benzofuran derivatives, 14 and 15, were isolated from the stem bark[5]. The antifungal compound 14 was purified by direct crystallization after DCCC, whereas the antifungal dihydrobenzofuran 15 required an additional LPLC step on an RP-8 column for final purification.

CONCLUSIONS

Some of the strategies employed for the separation of biologically active substances from medicinal plants have been described. A liquid–liquid partition technique (DCCC), followed by LPLC on reversed-phase columns, enabled the

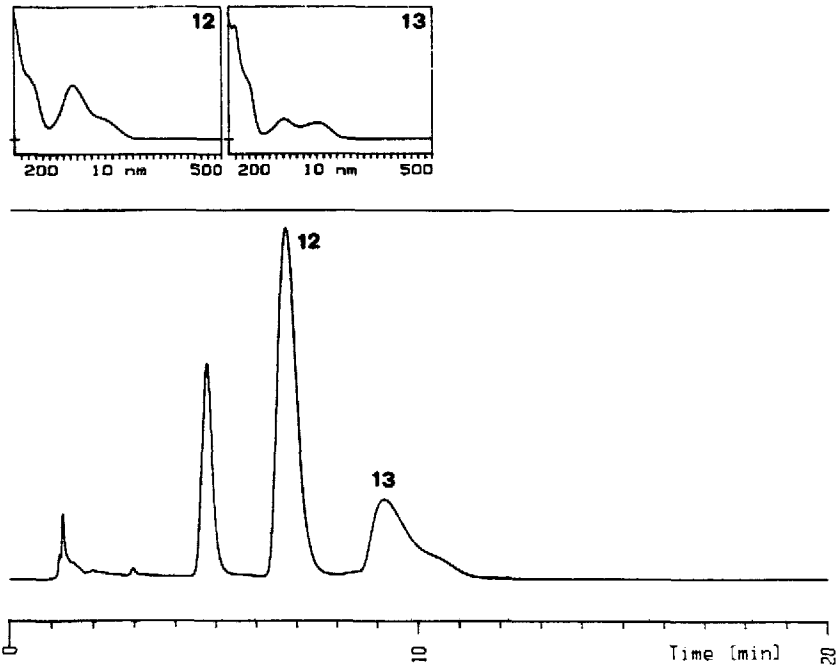

Fig. 8. HPLC–UV analysis of DCCC fractions 161–225 from the methanol extract of *Cordia goetzei* stem bark. Column: μBondapak C_{18} (300 mm × 3.9 mm). Eluent: 50% methanol; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 5 μl (25 μg).

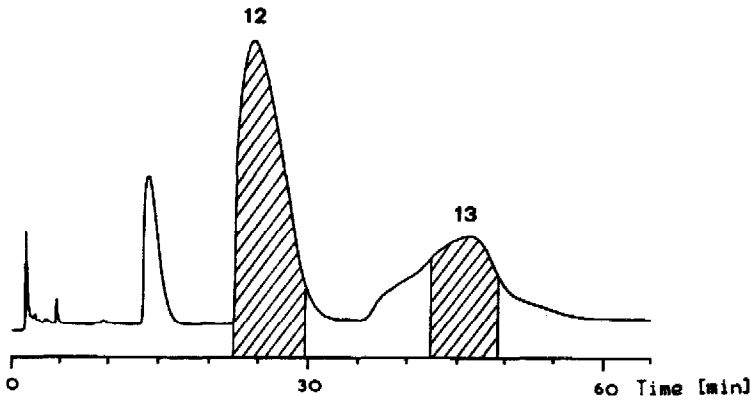

Fig. 9. Semi-preparative HPLC separation of compounds 12 and 13 from *Cordia goetzei*. Column: μBondapak C_{18} (300 mm × 7.8 mm). Eluent: 40% methanol; flow-rate, 5 ml/min. Detection: 254 nm. Sample: 20 μl of a 150 mg/ml solution.

isolation of iridoid and phenylpropanoid glycosides, and of complex polyphenols. In addition, chalcone-derived polyphenols from *Cordia goetzei* have been separated by semi-preparative HPLC, a chromatographic technique used previously by us in, *e.g.*, the separation of isomeric saponins and closely related chromenes and dichromenes[11]. For more lipophilic compounds, a combination of flash chromatography and LPLC has been used with success for the separation of sensitive cytotoxic constituents of *Psorospermum febrifugum*. Traditional open-column methods led to irreversible adsorption and decomposition of the readily oxidized tetrahydroanthracene derivatives.

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