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Chromatographic and spectroscopic studies on the constituents in male and female flowers of *Hagenia abyssinica**

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Abstract: The phenolic constituents of male and female flowers of *Hagenia abyssinica* were examined using chromatographic and spectroscopic techniques. Chromatographic analysis of kosins (phloroglucinols) was carried out using TLC, reversed-phase LC and glass-capillary GLC. Purification of the crude kosins was effected by silica gel column chromatography and purification of phenolic acids using ion-exchange resins and preparative TLC on silica gel. The kosins were characterized by TLC, LC, UV, IR, MS and H^1 NMR spectroscopy while the phenolic acids were characterized by UV-spectroscopy, TLC and LC. The phenolic acids found were protocatechuic acid, *p*-hydroxybenzoic acid and vanillic acid. The content of the kosins and phenolic acids was established quantitatively for both male and female flowers.

Keywords: Hagenia abyssinica; *Rosaceae; Kosso; TLC; LC; GC; analytical spectroscopy; phloroglucinols; phenolic acids.*

Introduction

Hagenia abyssinica (Bruce) Gmel (Rosaceae) is a dioecious plant commonly found at high altitudes in East Africa, especially in Ethiopia and is known locally as "Kosso". A decoction of the female flower is taken as an anthelmintic to expel intestinal worms. A greater emetic activity reported for the male flower discouraged its use in traditional medicine [1]. Phytochemical studies on *H. abyssinica* male and female flowers have been carried out to isolate and identify the kosins and phenolic acids [2, 3]. The present work is a chromatographic and spectroscopic analysis of the phenolic constituents in *H. abyssinica* male and female flowers.

Experimental

Materials

Plant material. Male and female flowers were collected from southern Ethiopia and identity was established by comparison with the herbarium specimens previously deposited in The Department of Traditional Medicine, Ministry of Health (Addis Ababa, Ethiopia). The flowers were sun-dried and powdered before extraction.

Reagents and reference materials. All solvents and reagents used were of AnalaR grade. *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA; Pierce Chemicals Co., Rockford, USA); *N,O*-bis-(trimethylsilyl)acetamide (BSA; Pierce Chemicals Co., Rockford, USA); *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Pierce Chemicals Co., Rockford, USA); Tri-sil/BSA, Formula "D" (in DMF) (Pierce Chemicals Co., Rockford, USA) were used for silylation of kosins. Authentic phenolic acids were purchased from Sigma Chemical Co. (St Louis, MO, USA). The authentic reference kosins and pseudo-aspidinol iB (internal standard) were kindly provided by Professor G.P. Schiemenz (University of Kiel, FRG).

Extraction of kosins

Each type of flower (300 g each) was soaked in peroxide-free diethyl ether for 3 days. The diethyl ether was removed *in vacuo* leaving a dark green oily residue (extract A). The crude extract (extract A) gave an amorphous pale

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yellow solid of crude kosin after treatment with 200 ml saturated solution of $Ba(OH)_2$ followed by acidification with 150 ml 25% acetic acid [4]. The dried mixture of kosins was then purified by column chromatography prior to LC analysis.

Column chromatography

Crude kosins (4 g) were suspended in hexane and chromatographed on 110 g silica gel (60–120 mesh; British Drug House, UK). Fractions (10 ml each) were monitored by TLC on silica gel using the system described below.

Fractions 1 to 20 (hexane) did not contain kosins and were discarded. Each of the fractions from 21 to 60 (hexane–chloroform 1:1, v/v) contained a mixture of protokosin and kosotoxin and the fractions were combined. The solvent was evaporated *in vacuo* leaving a pale yellow amorphous residue which gave protokosin (120 mg) after recrystallization from hot methanol. The mother liquor was evaporated and the residue dissolved in hot hexane from which kosotoxin (150 mg) crystallized out. α-Kosin was obtained by repeated recrystallization from cold acetone [5].

Thin-layer chromatography

Kosins. Precoated silica gel GF_{254} layers (0.2 mm) (Aldrich, Milwaukee, WI, USA) were developed in hexane–chloroform (1:1, v/v) and hexane–chloroform–ethanol (19:19:2, v/v/v). The zones were visualized by irradiation with UV-light and by spraying the plates with Fast Blue Salt B solution (0.25%, w/v, in water) [6].

Phenolic acids. Silica gel GF_{254} (Merck) layers (0.25 mm) were developed in chloroform–acetic acid (9:1, v/v) and benzene–ethyl acetate (11:9, v/v). The zones were located by fluorescence quenching at 254 nm and the colours developed after spraying with an aqueous mixture of 0.1 M ferric chloride and 0.1 M potassium ferricyanide (1:1, v/v) [7]. The phenolic acids were isolated by preparative TLC on silica gel GF_{254} (Merck) (1 mm layer) using chloroform–acetic acid (9:1, v/v) prior to identification by UV-spectrophotometry and LC.

Spectroscopic analysis

The UV spectra were recorded in methanol using a Lambda-5 UV/vis spectrophotometer (Perkin–Elmer, Beaconsfield, UK). IR spectra were recorded on a Perkin–Elmer Infrared spectrophotometer model 297. The mass spectra of the isolated kosins were determined on an A.E.I. MS-9 double-focusing mass-spectrometer (70 eV). FAB–MS data on kosotoxin and protokosin were obtained in the positive ion mode with glycerol matrix, using a VG70 SEQ mass spectrometer. The H^1 NMR spectra of kosins were recorded on a Jeol GX 270 in $CDCl_3$ with trimethylsilane (TMS) as internal standard.

Extraction of phenolic acids

Ion-exchange chromatography. 120 g male or female flower was soaked successively in 600 ml petroleum-spirit (40–60°C) and ethyl acetate for 3 days and filtered. The ethyl acetate extract was evaporated *in vacuo* leaving a dark oily residue. The residue was redissolved in 50% aqueous methanol, filtered and the methanol evaporated *in vacuo*. The resulting aqueous extract after evaporation to 15 ml was passed through a column of Sephadex SP-C25 (H^+ form) (20 × 2 cm i.d.) as previously described [8].

The neutral and acidic phenols were eluted with 200 ml distilled water which was reduced to 15 ml and again transferred to a column of Sephadex DEAE-A25 (acetate form) (20 × 2 cm i.d.). Phenolic compounds without a carboxylic acid function were eluted with 50 ml 0.01 M ammonium acetate (NH_4OAc) (Fraction 1) while phenolic acids were eluted with 0.1 M HCl (50 ml) (Fraction 2). The latter fraction was extracted three times with equal volume of ethyl acetate, the extracts were bulked and evaporated to dryness and redissolved in 3 ml methanol to give Fraction 3. A 0.5-ml aliquot of Fraction 3 was applied to a preparative TLC plate as described below. After development, bands were visualized by UV fluorescence at 254 nm and 354 nm, scraped and extracted with methanol and concentrated *in vacuo* to 3 ml (Fraction 4); this solution was chromatographed by LC.

Liquid chromatography

Kosins. The solvent delivery system consisted of a LKB 2150 pump (Pharmacia LKB Biotechnology, Milton Keynes, UK). Samples were introduced via a Rheodyne 7125 injection valve (Cotati, CA, USA) fitted with a 20-μl sample loop. The photodiode array detector, an HP 1040A (Hewlett–Packard) coupled with an HP 85 personal computer (Hewlett–

Packard) and HP 82901M disk drive, was used for recording chromatograms and the UV-vis spectra.

Spectrochromatographic data were stored to disk and processed subsequently by HP 82901M disk drive and HP 9000/300 with HP 9133 disk drive and HP 7470A plotter. A stainless-steel column (150 × 4.6 mm i.d.) packed with Partisil-10 ODS (Whatman Clifton, NJ, USA) was used. The mobile phase was pumped at 1.5 ml min^{-1} and consisted of methanol–1% aqueous acetic acid (75:25, v/v; pH* 3.0). Detection was effected at 290 nm.

Phenolic acids. The instrumentation as above was employed, but using a Spherisorb ODS-2 column (250 × 4.6 mm i.d.) (Phase Separations, Deeside, UK) with methanol–5% acetic acid (30:70, v/v; pH* 3.0) at a flow rate of 1.5 ml min^{-1}. Detection was effected at 265 nm.

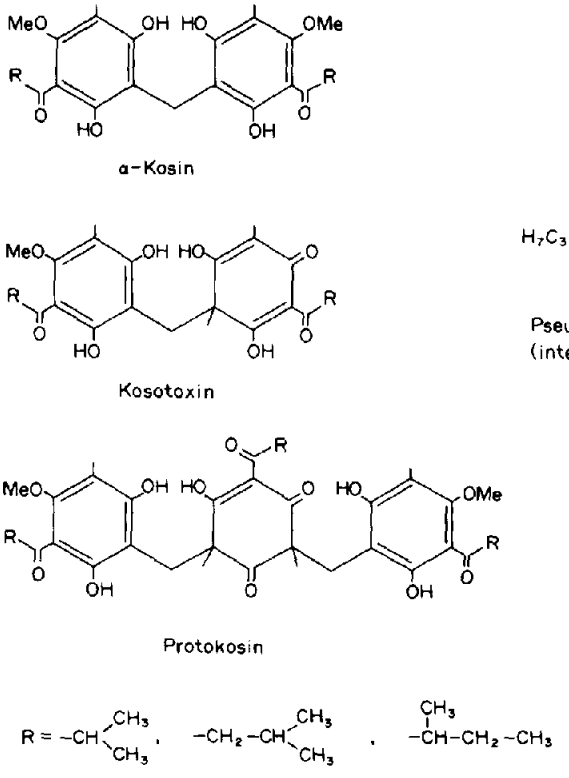
Gas liquid chromatography

Gas chromatographic analysis of the kosins was carried out on a Perkin–Elmer 8320B capillary gas chromatograph equipped with fused silica columns: BP-20 (25 m × 0.33 mm i.d.) (SGE, Australia) or BP-1 (50 m × 0.33 mm i.d.) fitted with Perkin–Elmer GP-100 Graphic Printer. The oven temperature gradient was 70–270°C and 150–200°C at 4°C min^{-1} and 2°C min^{-1}, for BP-1 and BP-20 columns, respectively; the FID detector was maintained at 300°C and injector temperature at 200°C in both columns.

Trimethylsilyl ethers were prepared by treatment of the crude kosins and authentic kosins, previously dried *in vacuo*. A range of silylating agents was used including BSTFA, Tri-sil/BSA in DMF, MSTFA and BSA. Of each sample, 5–6 mg was dissolved in one of the above silylating agents and treated at different temperatures for various reaction times, and then the product injected directly, or after drying in an atmosphere of nitrogen and taken into chloroform.

Results and Discussion

The identity of the isolated kosins: α-kosin (148–150°C), kosotoxin (116–118°C) and protokosin (178–180°C) (Scheme 1) was confirmed by TLC and spectroscopic methods (UV, IR, MS and H^1 NMR) and by comparison



Scheme 1
Structures of the kosins and pseudoaspidinol (internal standard).

with literature data [4]. The kosins of both male and female flowers were found to be similar (Figs 1 and 2).

The purity and identity of the peaks were examined by diode array detection and by comparison with standards. Their content was analysed by conventional calibration procedures with pseudoaspidinol iB (Scheme 1) as an internal standard using authentic samples in the concentration range 3.6–80 μg ml^{-1}. The phenolic acids from Fraction 3 were separated by preparative TLC (Fraction 4). Analysis by LC, UV-vis-spectroscopy and comparison with authentic samples and published data [9], confirmed the presence of protocatechuic acid, *p*-hydroxybenzoic acid and vanillic acid, respectively. The distribution and type of phenolic acids in both flowers were found to be similar (Figs 3 and 4). It was found that 30% methanol (v/v) gave the optimum separation for the phenolic acids and 75% methanol (v/v) for kosins [Fig. 5(a,b)]. The capacity factor of the phenolic acids and of the kosins decreased above 30 and 75% methanol, respectively. Quantitative analysis was carried out by conventional procedures, using syringic acid (Scheme 2) as internal standard, and the appropriate authentic samples in the concentration range 10–50 μg ml^{-1}. The results of quantitative analyses both of the kosins and the phenolic acids from each flower type are given in Table 1.

The GC analysis of kosins of *H. abyssinica* has not been studied previously, although that

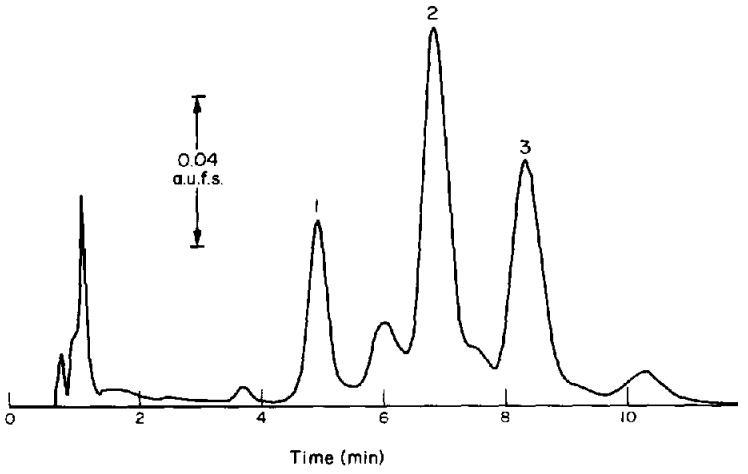


Figure 1
LC separation of (1) α-kosin, (2) kosotoxin and (3) protokosin from the male flower of *H. abyssinica* on Partisil-10 ODS.

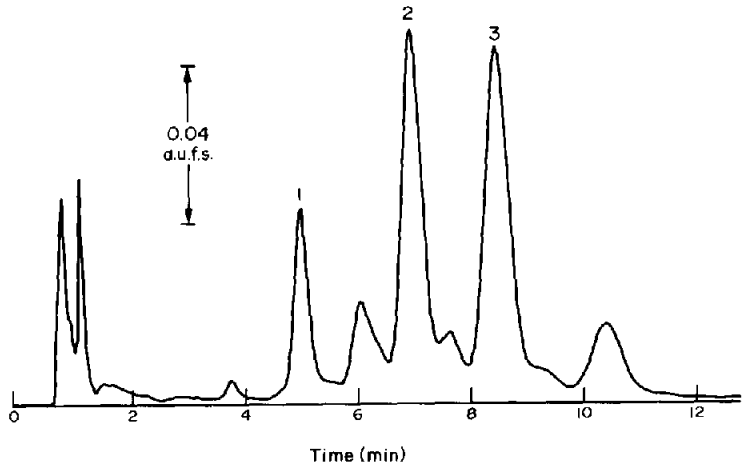


Figure 2
LC separation of (1) α-kosin, (2) kosotoxin and (3) protokosin from the female flower of *H. abyssinica* on Partisil-10 ODS.

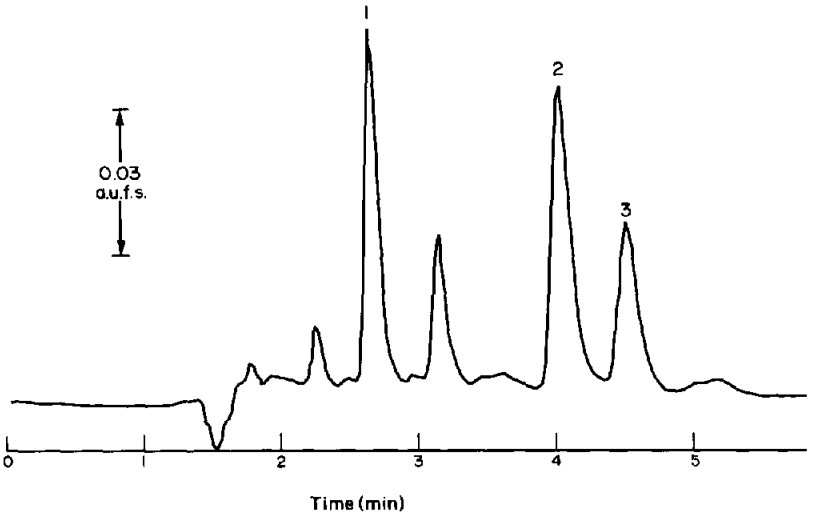


Figure 3
LC separation of (1) protocatechuic acid, (2) *p*-hydroxybenzoic acid and (3) vanillic acid from the male flower of *H. abyssinica* on Spherisorb-ODS-2 column.

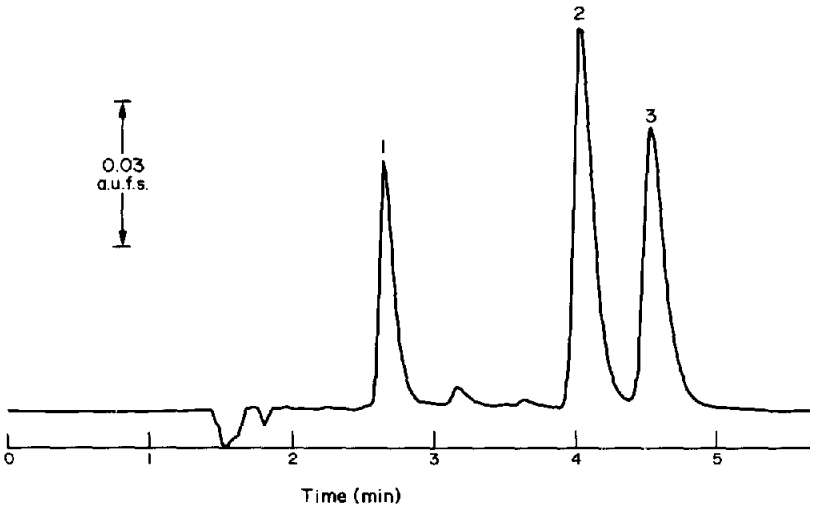
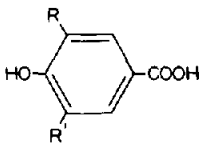


Figure 4
LC separation of (1) protocatechuic acid, (2) *p*-hydroxybenzoic acid and (3) vanillic acid from the female flower of *H. abyssinica* on Spherisorb-ODS column.



R = H, R' = H, *p*-hydroxybenzoic acid

R = OH, R' = H, protocatechuic acid

R = OCH_3, R' = H, vanillic acid

R = OCH_3, R' = OCH_3, syringic acid (internal standard)

Scheme 2
Structures of the phenolic acids.

of *Dryopteris* phloroglucinols in general has been discussed by other workers [10, 11]. When the derivatized kosins were analysed, many products were observed, possibly due to decomposition reactions at the methylene bridge and side chains, as previously reported in cases of *Dryopteris* phloroglucinols [11]. Incomplete derivatization may also have been a factor.

In the present work, the purification of the extracts of kosins and phenolic acids by silica gel column chromatography and ion-exchange chromatography, respectively, was found to

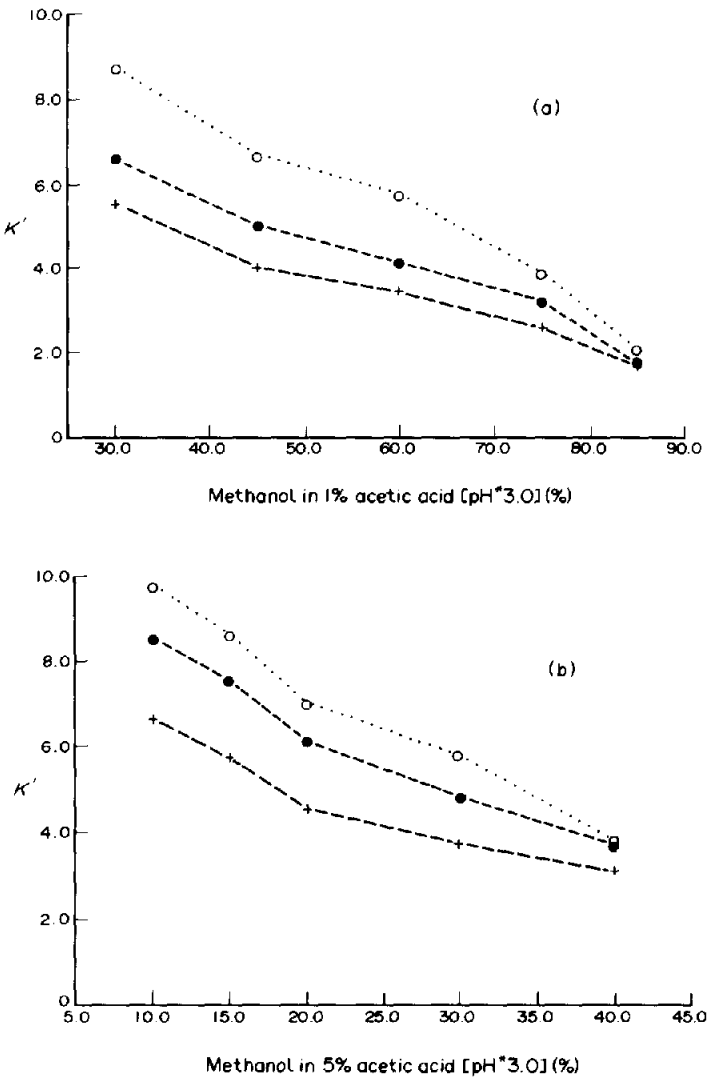


Figure 5
(a) Effect of solvent strength in 1% acetic acid on capacity factor of kosins; +, α-kosin; ●, kosotoxin; and ○, protokosin. (b) Effect of solvent strength in 5% acetic acid on capacity factor of phenolic acids. +, protocatechuic acid; ●, *p*-hydroxybenzoic acid; and ○, vanillic acid.

Table 1
Concentration of kosins and phenolic acids in male and female flowers of *H. abyssinica*

	Flower	Content (μg g^{-1} dry wt) α-Kosin	Kosotoxin	Protokosin
Kosins	Male	220	1080	760
	Female	240	960	720
		Protocatechuic acid	*p*-OH benzoic acid	Vanillic acid
Phenolic acids	Male	4.51	8.92	9.93
	Female	4.86	4.27	5.39

yield an effective separation and so allow quantification of the individual kosins and phenolic acids in each flower type. From the chromatographic and spectroscopic methods employed, it has been established that both male and female flowers are similar with respect to their content both of phloroglucinols and phenolic acids.

Their low-volatility and tendency to decompose impose limitations on the analysis of the kosins by GC.

Given these results, it would appear that the differences in toxicity reported between the male and female flowers of *H. abyssinica* are not attributable to their content of kosins or phenolic acids.

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