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Journal of Chromatography A, 673 (1994) 142–146

JOURNAL OF CHROMATOGRAPHY A

Short Communication

High-speed counter-current chromatographic separation of biflavanoids from *Garcinia kola* seeds☆

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(First received November 10th, 1993; revised manuscript received March 8th, 1994)

Abstract

Garcinia kola Heckel (Guttiferae) known in commerce as "bitter kola" is used extensively in Nigeria and Ghana for the treatment of coughs, mouth infection, liver disorders and antidote for arrow poisons. In our studies extracts of seed displayed appreciable activity against several selected microorganisms. To identify the antimicrobial compounds and study other biological effects of *G. kola* and its constituents, we have separated the constituents of the ethyl acetate extract of defatted seeds by high-speed counter-current chromatography. Using this technique with the solvent system *n*-hexane–ethyl acetate–methanol–water (1:4:2.5:2.5, upper phase as the stationary phase and the lower phase as the mobile phase), the ethyl acetate extract provided seven products. Four of these have been characterized as 3–8 linked biflavanoids, kolaflavanone, GB-1, GB-1a and GB-2 by spectral data.

1. Introduction

Garcinia kola Heckel (Guttiferae) commercially known as "bitter kola" is used extensively in the West African traditional medicine for the treatment of various diseases. It is served in Nigerian homes to guests as adjunct to the true kola nuts. The plant is used for the treatment of liver disorders, coughs, mouth infections and also as aphrodisiac, antidiarrheal and antidysentric [1]. Earlier phytochemical work on the various parts of this plant has resulted in the isolation of some flavanoids [1], triterpenoids, biflavanoids [2,3] and polyisoprenyl benzophenone [4].

Kolaviron, a fraction of defatted methanolic extract containing biflavanones of *G. kola* is reported to antagonize lethal poisoning in mice with phalloidin [5]. This fraction and its two compounds, GB-1 and GB-2, have also been shown to have antihepatotoxicity activity in mice in rendering them protection against carbon tetrachloride, galactosamine, α-amanitin and phalloidin [6]. The pharmacological and biological activities of biflavanones have been reviewed in 1986 [7]. Thereafter, kolaviron fraction has been shown to have antidiabetic activity in rabbits and aldose reductase activity in rats [8].

In our work the extracts of the seeds showed appreciable antimicrobial activity against *Bacillus subtilis*, *Mycobacterium intracellulare*, *Staphylococcus aureus* and *Cryptococcus neoformans*

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☆ This work was presented at the *34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18–22, 1993.*

0021-9673/94/$07.00
SSDI 0021-9673(94)00249-9

[9]. To identify the antimicrobial compounds and study other biological effects of *G. kola* and its constituents, we have separated the constituents of the ethyl acetate extract of defatted seeds by high-speed counter-current chromatography (HSCCC). HSCCC is proving to be a very mild but powerful technique which is useful on both the analytical and preparative scale. Using this method, most of the separations are completed within several hours and because no solid support is used in the column, loaded samples are totally recovered without loss or inactivation caused by solid supports [10].

In the work herein reported, as shown in Fig. 1, seven compounds were separated and were designated as GKE-1, -2, -3, -4, -5, -6 and -7. GKE-1, -2 and -3 remain to be identified. GKE-4, -5, -6 and -7 were identified as the biflavanoids GB-2, kolaflavanone, GB-1 and GB-1a (Fig. 2), respectively, by the interpretation of their spectral data [^{1}H and ^{13}C NMR, electron impact (EI) MS and fast atom bombardment (FAB) MS]. This is the first report of the separation of these biflavanoids by HSCCC.

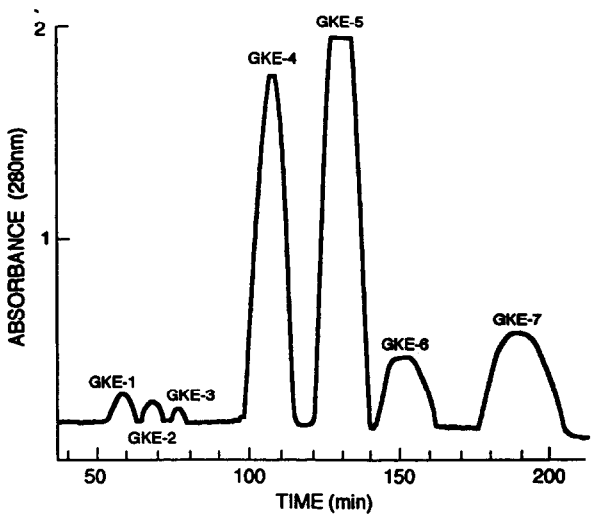
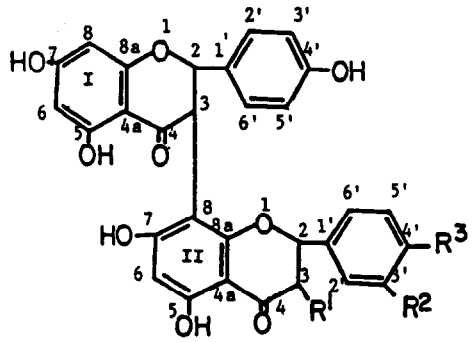


Fig. 1. Chromatogram of the ethyl acetate fraction of defatted *G. kola* seed extract using the upper phase of *n*-hexane–EtOAc–MeOH–water (1:4:2.5:2.5) mixture as the stationary and the lower phase as the mobile phase. GKE-1, GKE-2 and GKE-3 are unidentified compounds, GKE-4 is identified as the biflavanoid GB-2, GKE-5 as kolaflavanone, GKE-6 as GB-1 and GKE-7 as GB-1a.



	R^1	R^2	R^3
GB-1	OH	H	OH
GB-2	OH	OH	OH
GB-1a	H	H	OH
Kolaflavanone	OH	OH	OMe

Fig. 2. Biflavanoids separated by HSCCC from the ethyl acetate extract of defatted seeds of *G. kola*.

2. Experimental

2.1. General experimental procedures

IR spectra were determined in KBr on a Perkin-Elmer 2811B spectrometer. UV spectra were measured in MeOH on a Perkin-Elmer Lambda 3B UV–Vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were taken in [$^2H_6$]dimethyl sulfoxide with tetramethylsilane as an internal standard on a Varian XL-300 spectrometer (^{1}H at 300 MHz and 75 MHz for ^{13}C). EI-MS spectra were measured at 70 eV on a Finnigan MAT-95. FAB-MS spectra were recorded on a VG7070E-HF spectrometer using Xe gas at 8 kV and *m*-nitrobenzyl alcohol as matrix. HSCCC was performed on high-speed counter chromatograph CCC-2000 with multilayer coil planet centrifuge (Pharma-Tech Research, Baltimore, MD, USA). Total capacity of the column was 260 ml, I.D. 1.6 mm. Revolution was 1400 rpm with a flow-

rate of 1 ml/min. Compounds were detected by a Shimadzu SPD-6A UV detector (Columbia, MD, USA) at 280 nm and each run consisted of 100 mg of the ethyl acetate extract from which the biflavanoids were separated and characterized.

2.2. Plant material

The seeds of *G. kola* were bought from a local market at Ife, Nigeria, and were identified by Mr. S.A. Adesakin, Department of Pharmacognosy, Olafemi Awolowo University. Voucher specimens were deposited in the herbarium of the Faculty of Pharmacy, Obafemi Awolowo University, Nigeria.

2.3. Extraction and isolation

Dried and powdered seeds (1.067 kg) were defatted with light petroleum (b.p. 35–60°C). The extract was divided into light petroleum-soluble (6.65 g) and 95% methanol-soluble (6.95 g) fractions. The defatted seeds were extracted with CH_2Cl_2 (35 g), followed by extraction with 95% EtOH. The EtOH extract was concentrated under reduced pressure to 100 ml and 100 ml water were added. The aqueous mixture was extracted with ethyl acetate (3 × 100 ml) which was evaporated to dryness to yield the ethyl acetate-soluble fraction (22.82 g). This fraction was extracted with *n*-BuOH to provide, on removing the solvent, 9.63 g *n*-BuOH fraction. Although biflavanoids were detected in the CH_2Cl_2 and ethyl acetate fractions, in the present study only the ethyl acetate extract was fractionated by HSCCC.

2.4. HSCCC of ethyl acetate fraction

The solvent used for HSCCC comprised of *n*-hexane–EtOAc–MeOH–water (1:4:2.5:2.5). The lower phase was used as the mobile phase. For chromatography, the ethyl acetate fraction was dissolved in the mobile phase (1 ml). As shown in Fig. 1 seven peaks were observed. On pooling the solutions under each of the seven peaks and evaporation of the solvents, the products obtained were: GKE-1 (14 mg), GKE-2 (8 mg), GKE-3 (6 mg), GKE-4 (11 mg), GKE-5 (42 mg), GKE- 6 (5 mg) and GKE-7 (7 mg).

2.5. GKE-4 (GB-2)

t_R 108 min; IR: 3340, 1634, 1515, 1280, 1179, 1090, 837 cm^{-1}; FAB-MS: *m/z* 575 $[M+H]^+$, 573 $[M-H]$, $C_{30}H_{22}O_{12}$; EI-MS: M^+ absent, 556, 430, 296, 270, 126, 123, 107; ^1H NMR:(300 MHz) Part I δ 5.41 (1H, d, $J = 12$ Hz, H-2), 4.39 (1H, d, $J = 12$ Hz, H-3), 5.82 (1H, d, $J = 2$ Hz, H-6), 5.76 (1H, d, $J = 2$ Hz, H-8), 7.03 (2H, d, $J = 8$ Hz, H-2′, 6′), 6.54 (2H, d, $J = 8$ Hz, H-3′,5′) 12.0 (1H, s, chelated OH), Part II δ 4.73 (1H, d, $J = 12$ Hz, H-2), 3.93 (1H, d, $J = 11$ Hz, H-3), 5.84 (1H, s, H-6), 6.80 (1H, d, $J = 2$ Hz, H-2′), 6.84 (1H, d, $J = 8$ Hz, H-5′), 6.70 (1H, d, $J = 8$ Hz, H-6′), 11.5 (1H, s, chelated OH); ^{13}C NMR; Part I δ 79.6 (C-2), 45.2 (C-3), 194.5 (C-4), 105.7 (C-4a), 160.2 (C-5), 97.5 (C-6), 160.7 (C-7), 96.6 (C-8), 162.7 (C-8a), 125.9 (C-1′), 126.9 (C-2′,6′), 115.8 (C-3′,5′), 155.7 (C-4′), Part II δ 80.8 (C-2), 70.0 (C-3), 195.5 (C-4), 106.0 (C-4a), 161.7 (C-5), 97.8 (C-6), 164.6 (C-7), 101.2 (C-8), 164.4 (C-8a), 126.1 (C-1′), 118.4(C- 2′,5′), 142.8 (C-3′), 143.6(C-4′), 124.4 (C-6′).

2.6. GKE-5 (Kolaflavanone)

t_R 130 min; IR: 3360, 1640, 1520, 1280, 1170, 1091, 840 cm^{-1}; UV: 292, 329 nm; FAB-MS: *m/z* 589 $[M+H]^+$, 587 $[M-H]$, $C_{31}H_{24}O_{12}$; EI-MS: M^+ absent, 570, 444, 296, 270, 137, 126, 107; ^1H NMR: Part I δ 5.50 (1H, d, $J = 12$ Hz, H-2), 4.49 (1H, d, $J = 12$ Hz, H-3), 5.82 (1H, d, $J = 2$ Hz, H-6), 5.76 (1H, d, $J = 2$ Hz, H-8), 7.03 (2H, d, $J = 8$ Hz, H-2′,6′), 6.65 (2H, d, $J = 8$ Hz, H-3′,5′), 11.90 (1H, s, chelated OH), Part II δ 4.90 (1H, d, $J = 12$ Hz, H-2), 4.04 (1H, d, $J = 12$ Hz, H-3), 5.84 (1H, s, H-6), 6.81 (1H, d, $J = 2$ Hz, H-2′), 6.83 (1H, d, $J = 8$ Hz, H-5′), 6.71 (1H, d, $J = 8$ Hz, H-6′), 11.5 (1H, s, chelated OH), 3.73 (3H, s, OMe); ^{13}C NMR: Part I δ 82.7 (C-2), 47.3 (C-3), 197.4 (C-4),

105.5 (C-4a), 166.4 (C-5), 101.3 (C-6), 164.6 (C-7), 101.8 (C-8), 163.7 (C-8a, 129.8 (C-1'), 128.8 (C-2',6'), 114.8 (C-3',5'), 157.7 (C-4'), Part II δ 82.7 (C-2), 70.1 (C-3), 196.5 (C-4), 106.0 (C-4a), 163.1 (C-5), 97.8 (C-6), 162.7 (C-7), 105.7 (C-8), 162.4 (C-8a), 126.7 (C-1'), 111.8 (C-2'), 140.5 (C-3'), 146.2 (C-4'), 114.8 (C-5'), 128.8 (C-6'), 55.6 (OMe).

2.7. GKE-6 (GB-1)

t_R 152 min; IR: 3360, 1638, 1520, 1270, 1170, 1091, 838 cm^{-1}; UV: 293, 328 nm; FAB-MS: m/z 559 $[M+H]^+$, 557 $[M-H]$, $C_{30}H_{22}O_{11}$; EI-MS: M^+ absent, 540, 414, 296, 270, 126, 107; ^1H NMR: Part I δ 5.38 (1H, d, $J = 12$ Hz, H-2), 4.38 (1H, d, $J = 12$ Hz, H-3), 5.76 (1H, d, $J = 2$ Hz, H-6), 5.72 (1H, d, $J = 2$ Hz, H-8), 7.02(2H, d, $J = 8$ Hz, H-2',6'), 6.54 (2H, d, $J = 8$ Hz, H-3',5'), 12.1 (1H, s, chelated OH), Part II δ 4.86 (1H, d, $J = 12$ Hz, H-2), 3.98 (1H, d, $J = 12$ Hz, H-3), 5.80 (1H, s, H-6), 7.0 (2H, d, $J = 8$ Hz, H-2',6'), 6.68 (2H, d, $J = 12$ Hz, H-3',5'), 11.7 (1H s, chelated OH); ^{13}C NMR: Part I δ 80.0 (C-2), 47.3 (C-3), 196.4 (C-4), 104.0 (C-4a), 164.5 (C-5), 97.5 (C-6), 163.0 (C-7), 98.5 (C-8), 165.0 (C-8a), 128.0 (C-1'), 126.5 (C-2',6'), 116.0 (C- 3',5'), 158.0 (C-4'), Part II δ 80.0 (C-2), 70.2 (C-3), 196.8 (C-4), 105.5 (C-4a), 164.0 (C-5), 97.8 (C-6), 164.0 (C-7), 102.0 (C-8), 165.5 (C-8a), 128.0 (C-1'), 128.5 (C-2',6'), 115.1 (C-3',5'), 158.0 (C-4').

2.8. GKE-7 (GB-1a)

t_R 189 min; IR: 3350, 1640, 1520, 1168, 1092, 838 cm^{-1}; FAB-MS: m/z 543 $[M+H]^+$, 541 $[M-H]$, $C_{30}H_{22}O_{10}$; ^1H NMR: Part I δ 5.42 (1H, d, $J = 12$ Hz, H-2), 5.18 (1H, d, $J = 12$ Hz,H-3), 5.82 (1H, d, $J = 2$ Hz, H-6), 5.71 (1H, d, $J = 2$ Hz, H-8), 7.03 (2H, d, $J = 8$ Hz, H-2',6'), 6.65 (2H, d, $J = 8$ Hz, H-3',5'), 12.5 (1H, s, chelated OH), Part II δ 5.29 (1H,d, $J = 12$ Hz, H-2), 2.56 (2H, m, H-3), 5.76 (1H, s, H-6), 7.20 (2H, d, $J = 8$ Hz, C-2',6'), 6.70 (2H, d, $J = 8$ Hz, C-3',5'), 11.50 (1H, s, chelated OH); ^{13}C NMR: Part I δ 78.3 (C-2), 47.4 (C-3), 195.9 (C-4), 105.7 (C-4a), 162.2 (C-5), 97.5 (C-6), 162.7 (C-7), 96.6 (C-8), 164.8 (C-8a), 128.0 (C-1'), 126.6 (C-2',6'), 115.8 (C-3',5'), 157.5 (C-4'), Part II δ 81.3 (C-2), 47.4 (C-3), 196.7 (C-4), 106.0 (C-4a), 163.1 (C-5), 97.8 (C-6), 164.7 (C-7), 105.7 (C-8), 164.9 (C-8a), 128.0 (C-1'), 128.9 (C-2',6'), 115.1 (C-3',5'), 157.5 (C-4').

3. Results and discussion

Seeds of *G. kola* were extracted in sequence with light petroleum, CH_2Cl_2 and 95% ethanol. The ethanol extract was fractionated into ethyl acetate and *n*-BuOH fractions. Biflavanoids detected in CH_2Cl_2 and ethyl acetate fractions were separated in the present study from the ethyl acetate fraction. As shown in Fig. 1, seven products were isolated (GKE-1–GKE-7) from this fraction. The products GKE-1, -2 and -3 consisted of unidentified compounds. Four biflavanoids present, in GKE-4, -5, -6 and -7, were characterized by spectral analyses as GB-2, kolaflavanone, GB-1 and GB-1a, respectively. Of the four biflavanoids and the other three unidentified products, kolaflavanone was found in the ethyl acetate fraction to be present in the highest amount. Earlier biflavanoid separation of *G. kola* has been reported using droplet countercurrent chromatography and $CHCl_3$–MeOH–water as the solvent system (the more polar layer as the mobile phase [5]. However, separation of three products, GB-1, GB-2 and kolaflavanone, in unspecified amount was reported. Furthermore, no details concerning the spectral characterization of the compounds was presented.

Acknowledgements

This study was supported by an USAID grant DAN-5053-G-00-1071-00. The authors wish to thank Drs. Y. Ito and R.J. Highet, Laboratory of Biophysical Chemistry, National Heart, Blood and Lung Institute, National Institutes of Health, Bethesda, MD, USA and Dr. Edward Chou, Pharma-Tech Research, Baltimore, MD, USA for helpful discussions.

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