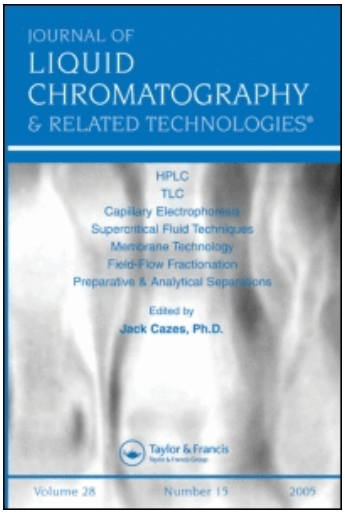

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Bertin G. Kipre[a]; Antoine A. Coffi[b]; Augustin A. Adima[a]; Tea Gokou[b]; Yoichiro Ito[c]; Benjamin K. Gosse[a]

[a] Laboratoire des Substances Naturelles bio - actives, Département de Chimie, Institut National Polytechnique, Yamoussoukro, Ivory Coast [b] Laboratoire de Chimie Organique structurale, Département de Chimie, Université d'Abidjan - Cocody, Abidjan, Ivory Coast [c] Center for Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

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Total Chemical Analysis of the Seed of *Tieghemella heckelii* by Diverse Chromatography Techniques

Bertin G. Kipre,[1] Antoine A. Coffi,[2] Augustin A. Adima,[1] Tea Gokou,[2] Yoichiro Ito,[3] and Benjamin K. Gosse[1]

[1]Laboratoire des Substances Naturelles bio - actives, Département de Chimie, Institut National Polytechnique, Yamoussoukro, Ivory Coast
[2]Laboratoire de Chimie Organique structurale, Département de Chimie, Université d'Abidjan – Cocody, Abidjan, Ivory Coast
[3]Center for Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract: Arganines A, C, D, tieghemelin, fatty acids, and steroid alcohols were isolated and purified in a total study of the seeds of *Tieghemella heckelii* by diverse chromatography methods. The hexane extracts yielded nine fatty acids and sixteen steroid alcohols resolved by gas chromatography, whilst the water extract gave three relatively homogenous compounds using TLC analysis, and further studies with NMR revealed the arganines previously cited. The optimization of saponins was achieved by the HSCCC method. Aside from this, conversion of Tieghemelin to Arganine C has been fulfilled to seek for the increment of the yield of the most active compound and avoidance of separation of both saponins. Here, we report the isolation and the chemical analysis of the named constituents of the seeds.

Keywords: *Tieghemella heckelii*, Saponins, Fatty acids, Steroid alcohols

INTRODUCTION

Tieghemella heckelii Pierre ex. A. Chev. (Sapotacae) is a large rain forest tree.[1] The valorization of the national flora has helped research to identify the fruit of

Correspondence: Yoichiro Ito, Center for Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bldg. 50, Rm 3334, 50 South Drive MSC 8014, Bethesda, Maryland 20892-8014, USA. E-mail: itoy@nhlbi.nih.gov

this tree as a potential source of important therapeutic agents.[2] The dried seeds of the ripe fruit have been reported to contain fatty acids and oily substances;[3] the latter being used for consumption, for cosmetic purposes, and also serves as an effective treatment of nutritional deficiency in children.[4] Extracts of the bark of the tree and the roots are used as treatments for malaria, stomach ulcers, and skin disorders.[5] Findings upon extracts of the fruit to strongly inhibit the growth of HIV and to ascertain accounts of anti-herpetic properties associated with the seed extract have been revealed elsewhere.[6] Nevertheless, for a complete analysis, we sought to isolate the active ingredients vis a vis HIV and HSV and other compounds of medicinal properties in tropical areas. For this purpose high speed countercurrent chromatography (HSCCC) has been used in addition to routine chromatography techniques. High speed countercurrent chromatography (HSCCC) is a support free liquid-liquid partition chromatography.[7] As an important separation technique, it eliminates complications between solutes and solid supports such as adsorptive sample loss and deactivation, tailing of solute peaks, and contamination. It has been used for preparative separation and purification for natural products in recent years.[7] Using a series of chromatographic procedures, four triterpenoid saponins, arganine A, arganine C, arganine D, tieghemelin, and nine fatty acids and sixteen steroid alcohols were isolated from the seed of *Tieghemella heckelli* samples.

EXPERIMENTAL

HSCCC Apparatus

The cross-axis coil planet centrifuge (A prototype fabricated at the National Institutes of Health, Bethesda, MD, USA) was used for preliminary purification of crude extract. The apparatus holds a pair of multilayer coil separation columns at a distance 10 cm from the central axis of the centrifuge. In order to retain a satisfactory amount of the stationary phase, the column was mounted on the rotary shaft 15 cm away from the mid point. Each column consisted of 9 layers of left handed coils consisting of 50 m long, 2.6 mm i.d; teflon tubing. The beta value varies from 0.5 at the internal terminal to 0.75 to the external terminal. The two columns were connected in series to provide a total capacity of 570 mL.[8]

GC Apparatus

A Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector (FID) was used for GC analysis of fatty acids.[8]

GC separations were performed on a DB Wax capillary column (60 m $\times$ 0.25 mm i.d) (SEG company). The temperature was programmed at 250°C at the rate of 2 mL/min increment, while the temperature at the injector and the detector was kept constant at 280°C. The carrier gas was hydrogen and air.

The same apparatus was used for GC analysis of steroid alcohols compounds, except that analyses were performed on a COV 1701 capillary column (30 m × 0.25 mm i.d.) (SEG Company).

TLC Plates

All fractions were analyzed by silica gel TLC plates (BAKER Si500F) using the solvent system, chloroform:methanol:0.5% TFA (60:40:5). The TLC plates were visualized by cerium sulfate charring [2% $Ce(SO_4)_2$ (w/v) in 5.6% H_2SO_4 (v/v)].[5]

Apparatus for Measuring Physical Properties of Analytes

Melting points were determined on a Thomas–Hoover capillary apparatus. Optical rotations were measured using a Jasco P–1010 polarimeter.[1]

Mass Spectroscopy

Negative ion FABMS were run on Fisons V.C. ZAP. SPEC., Fisons, and JEOL HX110A spectrometers.[1]

HSCCC Separation Procedure

The two phase solvent system composed of methyl *t*.-butyl ether/1-butanol/acetonitrile/0.5% TFA aqueous solution (1:3:1:5, v/v/v/v) was prepared in a separatory funnel at room temperature and the two phases separated shortly before use.[5] The sample solution was prepared by dissolving 18 g of the crude extract in 100 mL of the solvent consisting of about equal volumes of each phase.

The separation was performed as follows: The column was first completely filled with the upper organic phase followed by sample loading from a pressured glass bottle. Then, the apparatus was rotated at 650 rpm with a speed controller (Bodine Electric Company, Chicago, IL, USA) for the present studies. The lower aqueous phase was eluted through the head end of the column at a flow rate of 3 mL/min. The effluent from the outlet of the column was continuously monitored through a UV detector (Uvicord S, LKB Instruments, Stockholm/Bromma, Sweden) at 280 nm and collected into test tubes using a fraction collector (LKB Instruments).

Plant Material

T. heckelii Pierre ex A. locally called «makoré» was taxonomically authenticated by Prof. Laurent Ake Assi, Centre National Floristique, Université de Cocody, Ivory Coast, with a voucher specimen numbered 9749 deposited at the herbarium of Abidjan Botanic Garden. Ripe fruits were collected on August 20, 1999, in Daloa Forest, Haut Sassandra Region, Ivory Coast.[1] The fresh pulp (mesocarp) of the fruit was removed and the shell (endocarp) was opened to collect the seed, which was then sun dried and ground for extraction.

RESULTS AND DISCUSSION

Extraction and Purification of Arganines A, C, D, and Tieghemelin

As shown In Figure 1 (A),the ground seed paste (3.36 kg) was macerated three times with hexane, removing 1.26 kg of hexane soluble materials. Based on the water solubility of the two glycosides, the marc (residue) was extracted with water and the dry aqueous extract was treated with methanol to obtain a MeOH-soluble residue referred to as CCo (860 g). A 18 g sample of CCo was purified by the cross axis coil planet centrifuge, using a solvent system composed of methyl *t*.-butyl ether (MtBE):1-butanol (nBuOH):acetonitrile (AcN):0.5% TFA in a volume ratio of 1:3:1:5 (B). Two peaks enriched in Gen. 1 and Rev. 1 were resolved by this method. These were pooled, yielding a 7.50 g fraction.

In Figure 2, purification of a 100 mg sample by HSCCC using the solvent system methyl *t*.-butyl ether–1-butanol–acetonitrile–0.5% TFA (1:3:1:5) gave 70 mg of 45:55 mixture of arganine C and tieghemelin.[4] One hundred milligram sample of CCo purified by HSCCC with the analytical Ito coil, generated several fractions (F1 to F6) including a reasonably pure and active F5 component of Rf 0.3 found by NMR analysis to be arganine C (Rev. 1). In contrast, fraction F6 was a mixture of two nearly homogenous components of Rf 0.08 and Rf 0.3. This mixture was resolved by preparative TLC with MeOH extraction. NMR analysis proved the Rf 0.08 component to be a new triterpenoid saponin termed tieghemelin (Gen. 1). The same analysis confirmed the second (Rf 0.3) component to be arganine C. An overall yield of 0.38% for Rev. 1 and 0.29% for Gen. 1 was achieved from this pilot study.

In Figure 3, a 3 g sample of CCo (a relatively complex mixture) was partitioned in 1-butanol:0.2M ammonium acetate (NH_4OAc) at pH 6.7. After 10 min of vigorous shaking and centrifugation, the organic layer was carefully separated from the aqueous phase. A 500 mg (dry weight) residue of the organic phase was further purified by CCC using the same two phase system, M*t*BE:1-BuOH:AcN:0.5% TFA (1:3:1:5) but in the presence of 2% NaCl. Here, the organic layer was chosen as a mobile phase while the

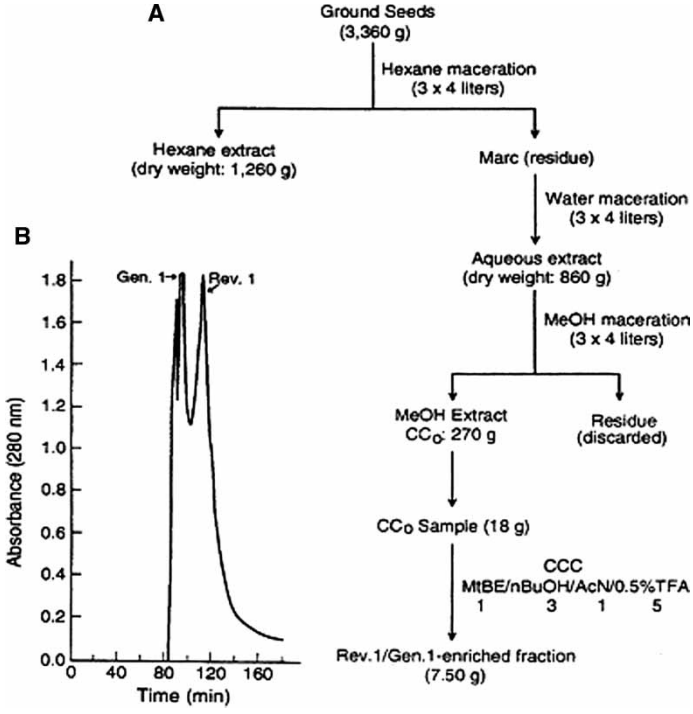

Figure 1. (A) The first step purification of the seed of *T. heckeli* (B). CCC separation of 18 g of CCo fractions from methanol extract. Experimental conditions is as follows: apparatus: cross-axis coil planet centrifuge with 10 cm revolution radius equipped with a pair of multilayer coil of 2.6 mm I.D. and 580 mL capacity; solvent system: methyl *t*.-butyl ether:1-butanol:acetonitrile:0.5% TFA aqueous solution (1:3:1:5); mobile phase: lower aqueous phase; flow rate: 3 mL/min; revolution; 650 rpm.

aqueous layer served as a stationary phase. This operation generated several fractions, which by TLC analysis consisted of three relatively homogenous components (F_a, F_b, and F_c). The NMR studies revealed F_a to be arganine A, F_b to be arganine C (Rev.1), and F_c to be arganine D. These compounds occur in fairly high yield as shown in Figure 3.

Optimization of Active Saponins Isolation

A small sample (150 mg) residue of the Rev. 1: Gen. 1 enriched fraction was partitioned in 1-BuOH:NH_4HCO_3 (pH 7.9) since ammonium bicarbonate at pH 7.9 proved to be more efficient at ionizing the carboxylic moiety of Gen. 1 than the ammonium acetate (pH 6.7) used initially. The two layers resulting from this partition were separated and dried. The Rev.1 enriched organic phase (54 mg) and the aqueous layer in Gen. 1 (95 mg) were further

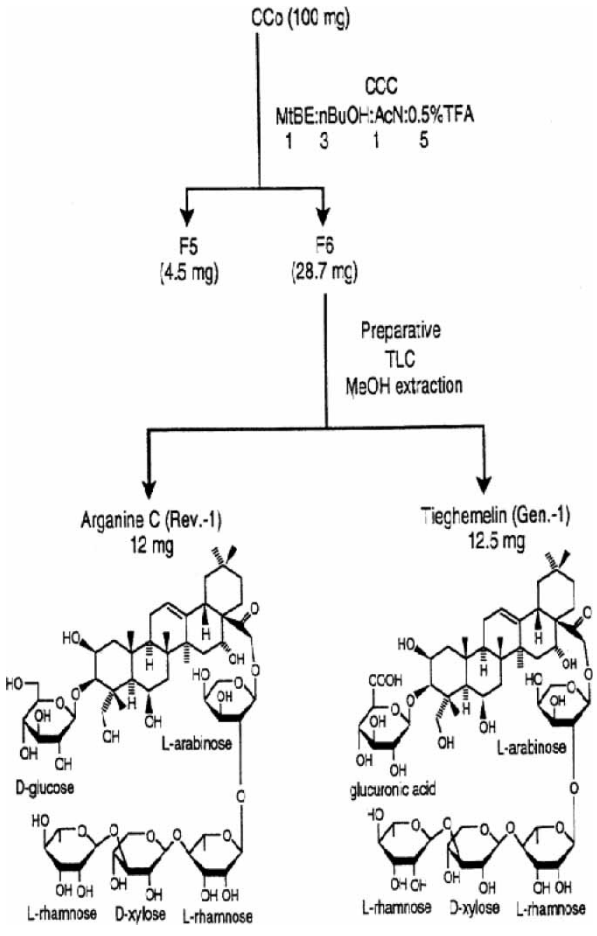

Figure 2. Pilot study for the isolation of viral entry inhibitors from the seeds of *T. heckelii.*

purified by flash chromatography (Figure 4) using florisil as a matrix and chloroform:MeOH:0.5% TFA (6:4:0.5) for elution. This fractionation scheme, remarkable by its simplicity, afforded 35 mg (0.78%) of Rev. 1 and 64 mg (1.43%) of Gen. 1, and was selected for a scale up purification of the rest of the CCC generated fraction enriched in Rev. 1 and Gen. 1.[5]

Conversion of Tieghemelin into Arganine C

A crude saponin fraction from HSCCC (1.5 g), a semi purified crude containing arganine C and tieghemelin at a ratio 1:2, was added to EEDQ

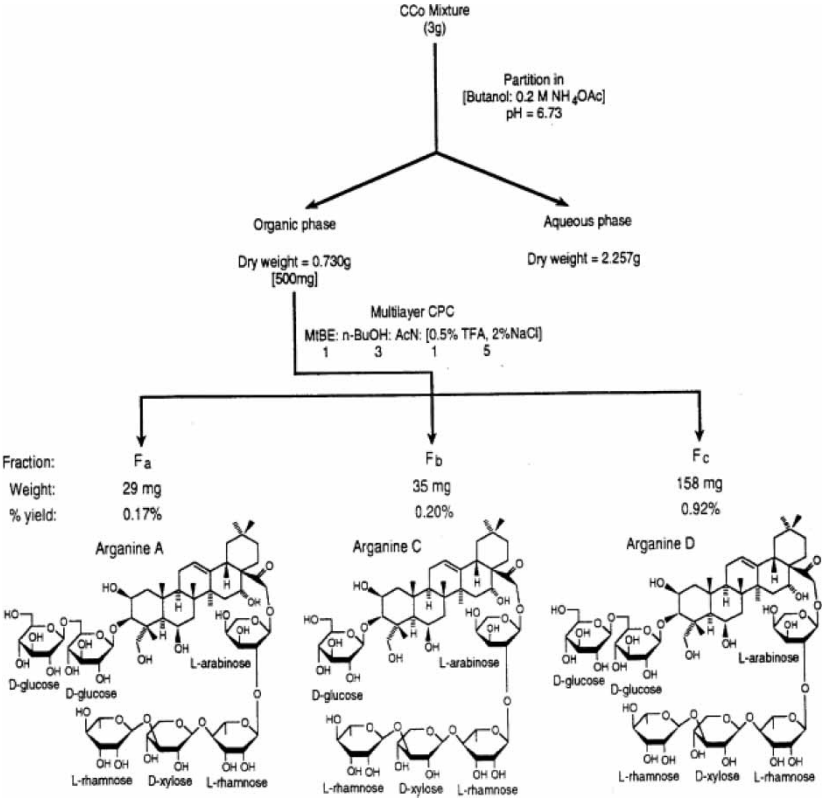

Figure 3. Identification of arganines A, C, and D by partition chromatography of CCo at pH 6.7. A 3 g sample of CCo was partitioned in butanol:0.2 M NH_4OAc at pH 6.7. After 10 min of shaking and centrifugation of the mixture, the organic layer was separated from the aqueous phase. The two layers were dried, yielding 0.730 g of organic residue and 2.257 g of an aqueous brown substance. A 500 mg sample of the organic residue enriched in arganines A, C, D, and deficient in tieghemelin, was further purified by CCC using M*t*BE:nBuOH:AcN:0.5% TFA, 2% NaCl in 1:3:1:5 ratio. Three major fractions (F_a, F_b, and F_c) were pooled, based on their TLC patterns. NMR analysis revealed F_a to be arganine A, F_b to be arganine C (Rev. 1), and F_c to be arganine D.

(2- ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) (150 mg; 1.25 mmol) in DMF (dimethyl formamide) (5 mL), and the mixture was stirred at reflux for 5 h. The solvent was removed using a rotary evaporator, and the brown residue was washed with diethyl ether (5 mL) to yield a brownish oily residue (160 mg) of ethyl ester intermediate. The residue was dissolved in ethanol (10 mL), and $NaBH_4$ (225 mg) was added, with stirring, at 0ºC. After 16 h at 25ºC, methanol was added to decompose unreacted $NaBH_4$. The mixture was filtered and the filtrate evaporated to give a brown solid, which was dissolved in 0.2 M ammonium acetate (50 mL) and extracted with

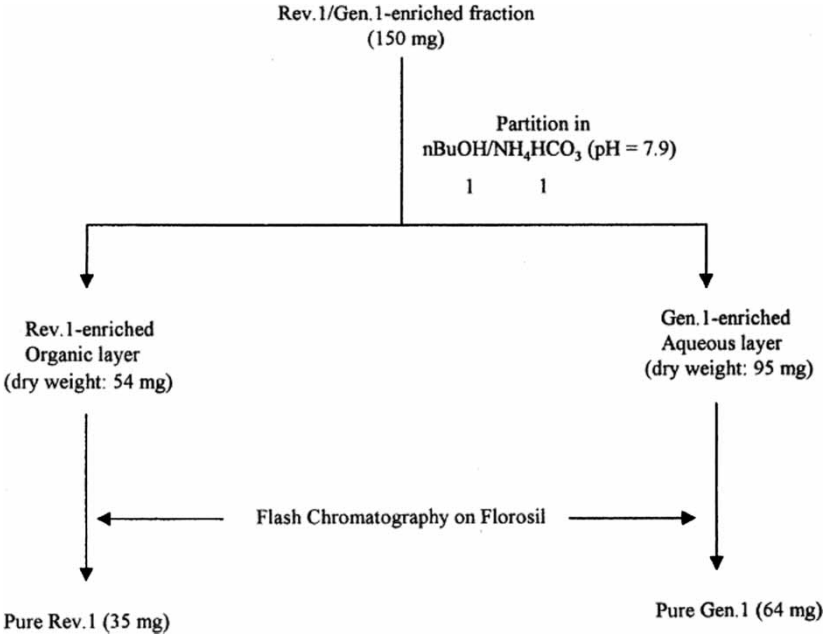

Figure 4. Isolation of Rev. 1 and Gen. 1 by liquid partition and flash chromatography. Rev. 1: Gen. 1 enriched fraction, A 150 mg (dry weight) residue of the Rev. 1: Gen. 1 enriched fraction was partitioned in 1-BuOH:NH_4HCO_3 at pH 7.9. The two non-miscible layers were separated and dried. The Rev. 1 enriched organic phase (54 mg) and Gen. 1 enriched aqueous layer (95 mg) were separately purified by flash chromatography on florisil matrix using chloroform:MeOH:0.5% TFA (6:4:0.5) as an eluant. This operation afforded 35 mg of Rev. 1 and 64 mg of Gen. 1 from 150 mg sample, which represents an overall yield of 0.78% and 1.43%, respectively, based on the original weight of the plant seeds.

butanol (2×50 mL). After drying the organic phase over Na_2SO_4, the solvent evaporation left 750 mg of white solid, in which the 1:2 ratio between the above two components was increased to >100:1 by TLC analysis. This reaction mixture was subjected to CCC purification as described below.[8]

Purification of Arganine C

A sample (750 mg) of the reaction crude mixture was purified by HSCCC using a two phase solvent system composed of methyl *t.*-butyl ether (M*t*BE)/1-butanol/acetonitrile/0.5% TFA aqueous solution at a 1:3:1:5 volume ratio. The multilayer coil separation column was eluted with the lower aqueous phase at a flow rate of 3 mL/min under 650 rpm.[8] The CCC separation yields a single peak of Arganaine C as illustrated in Figure 5.

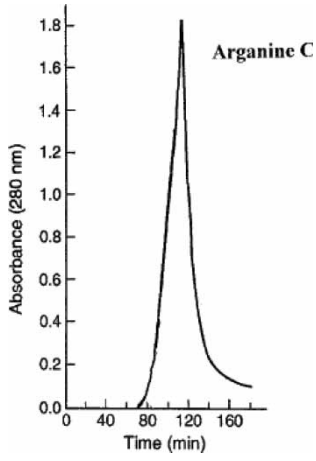

Figure 5. HSCCC separation of the reaction products of the crude saponin. Experimental conditions areas follows: Apparatus: cross-axis coil planet centrifuge with 10 cm revolution radius; column: a pair of multilayer coils of PTFE (polytetrafluoroethylene) tubing, 2.6 mm I.D. with a total capacity of about 570 mL; sample: 750 mg of crude saponin mixture; solvent system: methyl *t*.-butyl ether/1-butanol/acetonitrile/0.5% aqueous TFA (1:3:1:5, v/v/v/v); mobile phase: lower aqueous phase eluted head to tail; flow rate: 3 mL/min; detection: 280 nm.

Physical Properties and Structure Elucidation of Arganine and Tieghemelin

Arganine C (1)

mp 181-183 °C; $[\alpha]^{24}D$ −37° (c 1, MeOH); Negative ion FABMS: m/z 1237 [M - H]-, 1091 [M - Rha - H]-, 681 [M - 2Rha - Xyl - Ara - H]-.

Tieghemelin (2)

mp 197-199°C; $[\alpha]^{24}D$ -26° (c 1, MeOH); Negative ion FABMS: m/z 1251 $[M - H]^-$, 1105 $[M - Rha - H]^-$, 1075 $[M – Glc A - H]^-$, 973 $[M - Rha - Xyl - H]^-$, 827 $[M - 2Rha - Xyl - H]^-$, 695 $[M - 2Rha - Xyl - Ara - H]^-$. Details on NMR spectroscopy about these molecules have been reported in early studies.[1]

Isolation and Analysis of Fatty Acids

An 8.00 g amount of the hexane soluble oil was hydrolyzed by refluxing it with 50 mL of 1.0 M solution of potassium hydroxide in 95% ethanol for 1 h. The solution was cooled, and 100 mL of water was added. The

Table 1. Analysis of hexane-soluble fatty acids in the seed of *T. heckelii* [4]

Peak No.	Time (min)	Area	Conc. (%)	Name
1	2.54	15907	4.72	Palmitic acid
2	3.43	400	0.12	Myristic acid
3	4.887	123051	36.52	Stearic acid
4	5.233	187510	55.65	Oleic acid
5	5.867	3787	1.12	Linoleic acid
6	6.635	210	0.06	Linolenic acid
7	9.394	3511	1.04	Odeolidic acid
8	9.888	1127	0.33	Eicosenoic acid
9	13,113	1130	0.34	Behenic acid
10	19,075	264	0.08	Unknown
	Total	336897	100	

solution was then extracted thoroughly (3 times) with 50 mL portions of diethyl ether. The extract was washed 3 times with water. The water washings were added to the aqueous layer. This was then acidified with 6.00 M hydrochloric acid (in slight excess) and extracted 3 times with

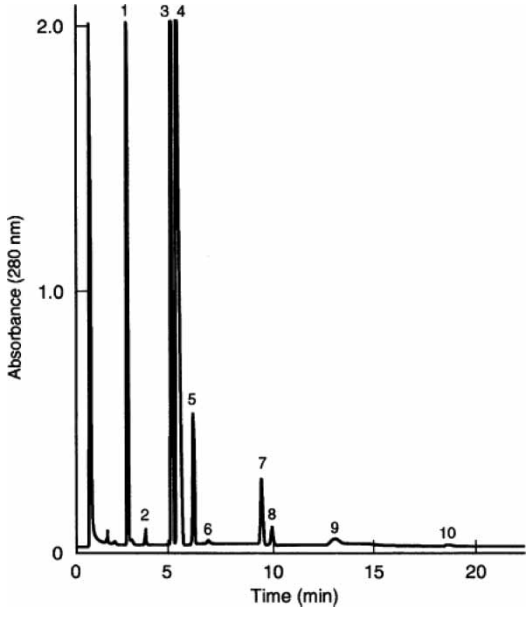

Figure 6. GC analysis of hexane-soluble fractions (fatty acids) of the seed of *T. heckelii.* Experimental conditions: apparatus: Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector; column: DBWax capillary column (30 m long, 0.32 mm I.D.); temperature from 170–280ºC at 2 min increment; carrier gas: hydrogen and air.[4]

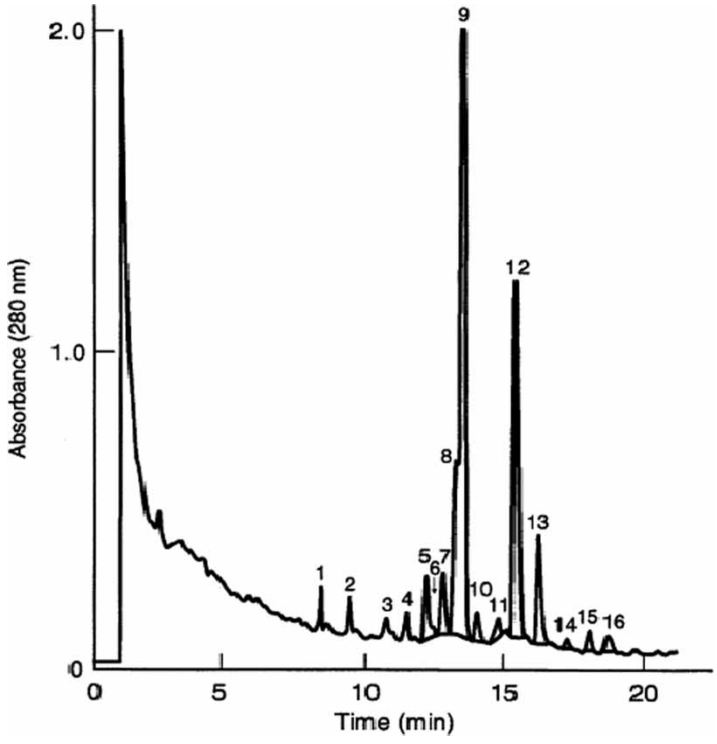

Figure 7. GC analysis of hexane-soluble fractions (Steroid Alcohols) of the seed of *T. heckelii.* Experimental conditions: apparatus: Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector; column: DBWax capillary column (30 m long, 0.32 mm I.D.); temperature from 170–280ºC at 2 min increment; carrier gas: hydrogen and air.[4]

50 mL portions of diethyl ether. The free fatty acids were recovered after washing the extract with water, drying it over anhydrous sodium sulphate 10.0 g and evaporating off the solvent. A 5.30 g amount of a mixture of free fatty acids was obtained.

GC analysis of the fatty acids showed that it contained nine fatty acids. The most abundant are 55.7% of Δ^1- oleic acid, 36.5% of stearic acid, 4.7% of palmitic acid, 1.12% of Δ^2- linoleic acid, 1.04% of odeolidic acid and the minor fatty acids are 0.34% of behenic acid, 0.34% of eicosenoic acid and 0.12% of myristic acid (Table 1 and Figure 6).

Isolation and Analysis of Steroid Alcohols

The aqueous solution was extracted thoroughly (3 times) with 50 mL portions of dichloromethane. The organic layer was dried over anhydrous sodium

Table 2. Analysis of unsaponifiable fraction of the seed of *T. heckelii*

Peak no.	Time (min)	Area	Conc. (%)	Name
1	8.632	1541	1.43	Cholesterol
2	9.684	1383	1.28	Brassirasterol
3	10.925	1240	1.15	Campesterol
4	11.647	1409	1.31	α-Spinasterol
5	12.3	3353	3.12	Δ5-Campesterol
6	12.613	512	0.48	Δ7-Campesterol
7	12.899	3217	2.99	Cherosterql
8	13.26	6703	6.23	24-Methylcholest-7-enol
9	13,452	56339	52.38	β-Sitosterol
10	13,95	1600	1.48	Δ5-avenasterorl
11	14,657	1112	1.03	Δ7,25 Stigmastadienol
12	15,273	18821	17.50	Δ7 Stigmasterol
13	16,058	6995	6.50	Δ7 Avenasterol
14	17,085	773	0.72	Δ7,22,25 Stigmastatrienol
15	17,929	1161	1.08	γ-Sitosterol
16	18,524	1381	1.28	α-Sitosterol
	Total	107539	100	

sulphate and evaporated off the solvent. A 1.50 g amount of a white powder was obtained.[4] GC analysis of the non saponified fraction showed that it contained sixteen steroid alcohols. The most abundant are 52.40% of β- sitosterol, 17.50% Δ^7-stigmasterol, 6.50% Δ^7-avenasterol, 6.23% 4- Methylcholest-7- enol, 3.11% of Δ^5-campesterol, 2.99% of cherosterol, 1.49% of Δ^5 avenasterol, 1.43% cholesterol, 1.31% of α-spinasterol, 1.29% of brassirasterol, 1.28% of α-sitosterol, 1.153% of campesterol, 1.08% of γ-stisterol, 1.034% of 7,25 stigmastadienol and the minor compounds are 0.72% Δ^7- 22, 25 stigmastatrienol and Δ^7- campesterol (Table 2 and Figure 7).

CONCLUSION

Countercurrent chromatography is a form of liquid liquid partition chromatograph, which separates soluble substances such as natural biochemical products according to their partition coefficient or differential solubility, in two immiscible solvents. In this study, the CCC method used gave a broad spectrum of separations encompassing arganines, steroid alcohols, and fatty acids. It provides an excellent mixing efficiency and avoids adsorption, denaturation, hydrolysis, or change of configuration of target compounds.

The total chemical analysis of the seeds of *Tieghemella heckelii* using this method has shown the richness of this plant starting from the master of tropical viral diseases to the discovery of a promising Anti HIV drug. It is, therefore, a

powerful tool of a qualitative and quantitative analysis that could help industry to set up a cost effective preparative technology and give common sense comprehensive information about the significant progress in the quest of eradicating AIDS, a serious handicap to sustainable development in West African countries, especially in Cote d'Ivoire. This paper is, therefore, an underground work, which needs to be polished and improved to scale up the benefits of the plant for the preservation of humanity.

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