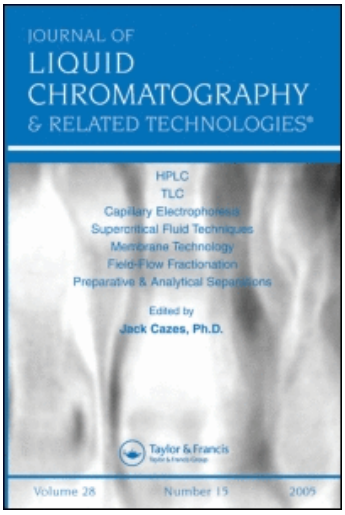

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CHEMICAL ANALYSIS OF THE SEED OF THE RIPE FRUIT OF *TIEGHEMELLA HECKELII*

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ABSTRACT

The seed of *Tieghemella heckelii* was analyzed by various chromatographic methods. The hexane extracts yielded nine fatty acids and sixteen steroid alcohols resolved by GC. These compounds are the main constituents of the solid substance of the hexane extract of the seed used in traditional environment for food and skin disorders. The water extract usually discarded in traditional use of the seed, was found to contain two triterpoid saponins with antiviral activity. Here, we report the isolation and chemical analysis of the hexane extract and water extract.

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INTRODUCTION

Tieghemelia heckelii (Sapotaceae) grows as a large tree found in the rain forest of West Africa. In traditional medicine of the Ivory Coast, the seed is ground and extracted with hot water and cooled off. The fat-enriched solid supernatant is collected and the water is drained and discarded. This fatty substance is used for various purposes including food, as well as medicine for the treatment of skin disorders, such as kwashio-kor, eczema, and children's diseases, such as chicken pox and rubella. The same fatty material is also used as a cosmetic by women for skin and hair treatment. Previous studies have reported the presence of fatty acids and oily substance in the extract of the seed of *T. heckelii*.[1]

The extensive usage in traditional medicine of the water-insoluble substances from the seed of this fruit and the paucity of studies done, prompted a thorough analysis of the hexane extract of the seed. The ground seed was treated with a mixture of hexane : methanol by the Folk method.[2] The two immiscible layers carried different constituents of the seed. In this particular case, the hexane layer of interest was adequately separated from the methanolic layer. The hexane was evaporated and the fatty substances were further dried and analyzed by GC.

Here we report the method of isolation of different constituents of this fraction and their characterization. We conclude that the seeds of *T. heckelii* contains nutritional value and may serve as new cosmetics.[3]

EXPERIMENTAL

Plant Materials

The ripe fruits of *T. heckelii* were collected in the month of November in the Ivory Coast. The fresh pulp (mesocarp) of the fruit was removed and the hard shell (endocarp) was opened to collect the seeds, which were then sun-dried and ground for extraction.

Apparatus

The cross-axis coil planet centrifuge[4] (a prototype fabricated at the National Institutes of Health, Bethesda, MD) 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 shift 15 cm away from the mid point. Each column

consisted of nine layers of left-handed coils of ca 50 m of 2.6 mm I.D. teflon tubing (standard wall size 14, Zeus Industrial Products, Raritan, NJ). 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. The revolution speed was adjusted at 650 rpm with a speed controller (Bodine Electric Company, Chicago, IL) for the present studies.

A Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector (FID) was used for GC analysis of fatty acids. GC separations were performed on a DB Wax capillary column (30 m × 0.32 mm I.D.) (SEG company). The temperature was programmed from 170°C to 280°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 unsaponifiable compounds. GC separations were performed on a COV 1701 capillary column (30 m × 0.25 mm I.D.) (SEG company). The temperature was programmed from 150°C to 280°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.

HSCCC Separation

A two-phase solvent system composed of methyl *t*-butyl ether/1-butanol/acetonitrile/0.5% TFA aqueous solution (1 : 3 : 3 : 5, v/v/v/v) was prepared in a separatory funnel at room temperature, and two phases separated shortly before use. 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 while 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).

RESULTS

First Step of Purification

Isolation of the active principles of the seed extract of *T. heckelii* was started by maceration of 3360 g of seed paste in an equal volume of hexane for

three times, generating 1260 g of fatty acids. The marc of the seed paste was again treated by maceration with distilled water twice and the two extracts were combined, affording 860 g of the dried aqueous extract. The water soluble fraction was further extracted by successive methanol treatments generating 270 g referred to as CC_o, a relatively complex mixture, but substantially enriched with the compounds of interest. This CC_o exhibited 81% of activity at 30 μg/mL in HSV-1 entry assay, whereas the hexane soluble fractions were totally devoid of activity. A sample (18 g) of CC_o was purified by CCC using the solvent system, composed of methyl *t*-butyl ether (MtBE)/1-butanol/acetonitrile/0.5% TFA aqueous solution in a 1 : 3 : 1 : 5 volume ratio. The major fractions containing the interest compounds were pooled, and based on their TLC patterns, gave (7.50 g) of semi-purified residue enriched in Rev. 1 and Gen. 1 (Fig. 1A and B).

Isolation of Saponins

A 150 mg amount of the CCC semi-purified fraction enriched in arganine C (Rev. 1)/tieghemelin (Gen. 1) was treated in *n*-butanol : 0.3 M NH_4OHCO_3 solution (pH = 8.9). This solution was stirred for 30 min at room temperature, then, pooled in the separatory funnel. The organic layer was separated from the aqueous phase enriched with the ionized carboxylic tieghemelin. The same volume of *n*-butanol was used again to treat the aqueous phase for tiring out the rest of arganine C (Rev. 1). Each phase was treated by 0.5% TFA until neutral pH = 7.0. After drying, the organic layer afforded 54 mg of arganine C (Rev. 1) enriched residue and 95 mg of tieghemelin (Gen. 1) enriched aqueous residue. The analytical TLC control confirmed the separation of these two compounds. The impurities in each fraction were eliminated by flash chromatography on florisil with the eluent: Chloroform/Methanol/0.5% TFA (6 : 4 : 0.5); after drying, the organic layer gave 35 mg of arganine C (Rev. 1) and the aqueous layer gave 64 mg of tieghemelin (Gen. 1).

Isolation of Fatty Acids

An 8.0 g amount of the hexane soluble fatty acid was hydrolyzed by refluxing it with 50 mL of 1.0 M solution of potassium hydroxide in 95% ethanol for 1 hour. The solution was cooled, and 100 mL of water was added. The solution was then extracted thoroughly three times with 50 mL portions of diethyl ether. The extract was washed three 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 three times with 50 mL portions of diethyl ether. The free fatty acids were recovered after washing the extract with water, drying it

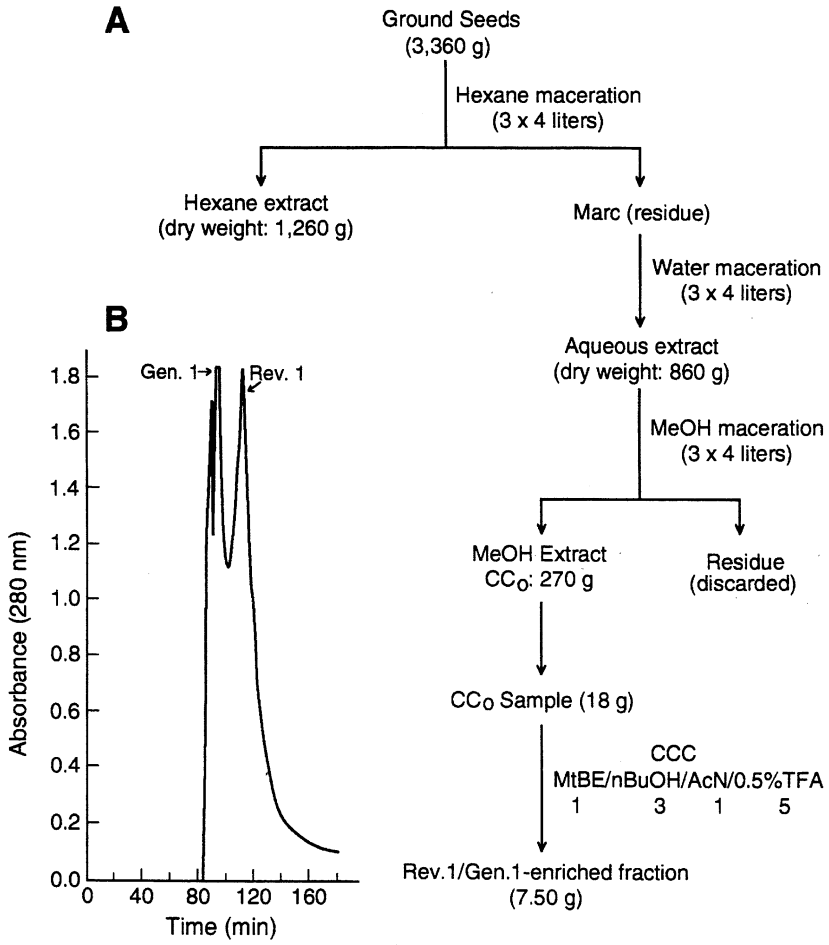

Figure 1. A. The first step purification of the seed of *T. heckelii.* B. CCC separation of 18 g of CC_o fractions from methanol extract. Experimental conditions are as follows: apparatus: cross-axis coil planet centrifuge with 10 cm revolution radius equipped with a pair of multilayer coil of 2.6 mm 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.

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 55.70% of oleic acid, 36.50% of stearic acid, 4.72% of palmitic acid, 1.15% of linoleic acid, 1.05% of odeolidic acid, and the minor fatty acids are 0.34% of behenic acid, 0.33% of eicosenoic acid, 0.12% of myristic acid, 0.08% of unknown acid, and 0.062% of linolenic acid (Fig. 2 and Table 1).

Isolation of Unsaponifiables

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

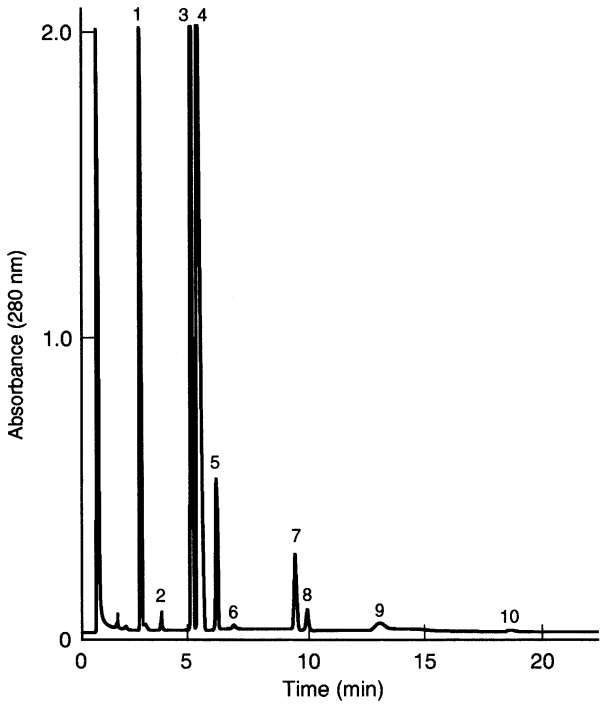

Figure 2. GC analysis of hexane-soluble fractions of the seed of *T. heckelii.* Experimental conditions: apparatus: Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector; column: DB Wax capillary column (30 m × 0.32 mm I.D.); temperature from 170–2800°C at 2 min increment; carrier gas: hydrogen and air.

Table 1. Analysis of Hexane-Soluble Fatty Acids in the Seed of *T. heckelii*

Peak No.	Time	Area	Concentration	Compound Name
1	254	15,907	4.7217	Palmitic acid
2	3.43	400	0.1186	Myristic acid
3	4.887	123,051	36.5249	Stearic acid
4	5.233	187,510	55.658	Oleic acid
5	5.867	3,787	1.1242	Linoleic acid
6	6.635	210	0.0623	Linolenic acid
7	9.394	3,511	1.042	Odeolidic acid
8	9.888	1,127	0.3346	Eicosenoic acid
9	13.113	1,130	0.3353	Behenic acid
10	19.075	264	0.0784	Unknown
Total		336,897	100	

sulphate and evaporated off the solvent. A 1.50 g amount of a white powder was obtained. GC analysis of the unsaponifiable fraction showed that it contained sixteen steroid alcohols. The most abundant are 52.4% of β-sitosterol, 17.50% Δ^7-stigmasterol, 6.50% Δ^7-avenasterol, 6.2% 4-Methylcholest-7-enol and 3.12% Δ^5-campesterol, and the minor compounds are 2.99% cherosterol, 1.49% Δ^7avenasterol, 1.43% cholesterol, 1.31% α-spinasterol, 1.29% brassirasterol, 1.28% α-sitosterol, 1.15% campesterol, 1.079% γ-sitosterol, 1.034% 7, 25 stigmastadienol, 0.72% Δ^7, 22,25 stigmastatrienol, 0.48% Δ^7campesterol (Fig. 3 and Table 2).

DISCUSSION

The two major components of the seed extract of *T. heckelii* in the water extract, which is present in a relatively large semi-purified fraction could not be resolved by HSCCC, despite several attempts of modification of the reference solvent system. The TLC method used in the final purification step of two major components proved to be cumbersome and is inherently associated with sample loss. This prompted the efforts for optimization of the isolation conditions of our two interest compounds present in CC_o parent fraction, still taking advantage of the highly efficient HSCCC method. Furthermore, the remarkable key difference in the structures of the two compounds at the glucosyl group on the position-C3 of the triterpenoid moiety, made it clear that glucuronic acid in the tieghemelin (Gen. 1) could be ionized weakly, without affecting the glucose residue in arganine C (Rev. 1). Tieghemella oil could be classified as a pharmaceutic acid, such as olive

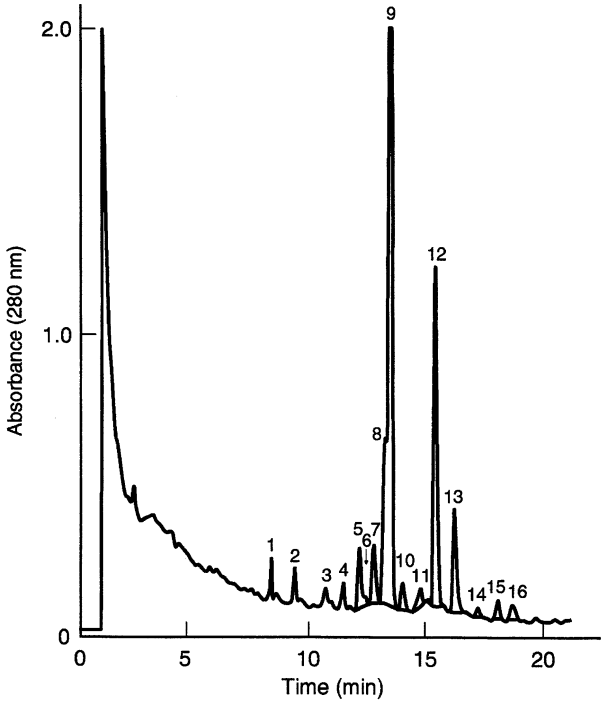

Figure 3. GC analysis of unsaponifiable fractions of the seed of *T. heckelii.* Experimental conditions: apparatus: Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector; COV 1701 capillary column (30 m × 0.25 mm); temperature: from 150–2800°C at 2 min increment; carrier gas: hydrogen and air.

oil, used as a setting retardant for dental cement and in the preparation of soaps, plasters, and liniments. It could also be used as an ademulcent, an emollient, and a laxative.[4] Its oil is utilized for the treatment of children suffering from nutritional deficiency. The oil has wound healing and cosmetic properties. The tieghemella oil is a nutrient and is widely used as cooking oil or a food additive, for human consumption by the native people in West Africa.[5]

Various seed products, such as vegetable oils, are rich in unsaturated fatty acids. The acid part of the glycerides consists mainly of various unsaturated fatty acids. Most of these fatty acids enhance transdermal and buccal drug delivery. In addition, the fatty acids possessed a notable activity against various bacteria and viruses.[6] Long-chain fatty acids are the most important substrates for the heart. In addition, they have been shown to affect signals in pathways and gene expression.

Table 2. Analysis of Unsaponifiable Fraction of the Seed of *T. heckelii*

Peak No.	Time	Area	Concentration	Compound Name
1	8.632	1,541	1.4326	Cholesterol
2	9.684	1,383	1.286	Brassirasterol
3	10.925	1,240	1.1527	Campesterol
4	11.647	1,409	1.3102	α-Spinasterol
5	12.3	3,353	3.1184	Δ^5-Campesterol
6	12.613	512	0.476	Δ^7-Campesterol
7	12.899	3,217	2.9913	Cherosterol
8	13.26	6,703	6.2335	4-Methylcholest-7-enol
9	13.452	56,339	52.3895	*β*-Sitosterol
10	13.95	1,600	1.4875	Δ^5-Avenasterol
11	14.657	1,112	1.0338	7,25 Stigmastadienol
12	15.273	18,821	17.5012	Δ^7-Stigmasterol
13	16.058	6,995	6.5047	Δ^7-Avenasterol
14	17.085	773	0.7187	Δ^7, 22,25 Stigmastatrienol
15	17.929	1,161	1.0794	γ-Stisterol
16	18.524	1,381	1.2844	α-Sitosterol
Total		107,539	100	

CONCLUSION

From the preliminary results, we obtained on the seed of *T. heckelii*, a full investigation of the plant seed is needed on the various stages of development of the fruit, to identify all its active ingredients. Therefore, the present results are only a beginning of the work.

Like all vegetable fats, tieghemella butter globally has softening, anti-drying, and protective effects. These activities are even more noticeable due to the high content in unsaponifiables.

The *T. heckelii* Project will be an integrated, long-term effort to preserve the ecological integrity of forest woodland in western, eastern, and southern Ivory Coast, through reinforcement of the economic importance of the *T. heckelii* tree, which is a source of the food oil known as tieghemella butter, and environmental protection.

ACKNOWLEDGMENT

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the fruit of the tree was identified as a potential source of important therapeutic agents.

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