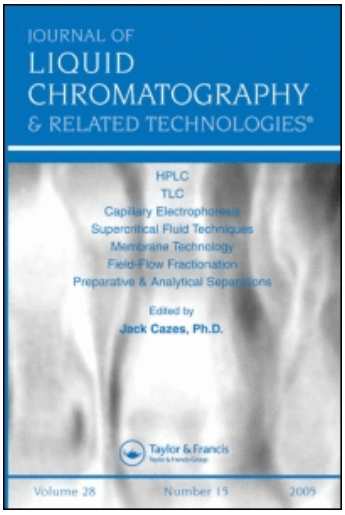

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Analysis of Components of Neem (*Azadirachta indica*) Oil by Diverse Chromatographic Techniques

Benjamin Gossé[a]; Adima A. Amissa[a]; Félix Anoh Adjé[a]; Florence Bobélé Niamké[a]; Denis Ollivier[b]; Yoichiro Ito[c]

[a] Laboratoire de Substances Naturelles Bio-actives, Département de Chimie, Institut National Polytechnique, Cote d'Ivoire, West Africa [b] Laboratoire Interregional de la Repression des Fraudes, Marseille, France [c] National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

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Analysis of Components of Neem (*Azadirachta indica*) Oil by Diverse Chromatographic Techniques

Benjamin Gossé, Adima A. Amissa, Félix Anoh Adjé, and Florence Bobélé Niamké
Laboratoire de Substances Naturelles Bio-actives, Département de Chimie, Institut National Polytechnique, Cote d'Ivoire, West Africa

Denis Ollivier
Laboratoire Interregional de la Repression des Fraudes, Marseille, France

Yoichiro Ito
National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract: The seed of *Neem Azadirachta indica* (Meliaceae) was analyzed by various chromatographic methods.[1] The hexane extract yielded six fatty acids and six unsaponified compounds resolved by high speed countercurrent chromatography (HSCCC) and confirmed by GC. These compounds are the main constituents of the solid substance of the hexane extract of the seed used in traditional medicine since ancient times. In 1968 Butterworth and Morgan isolated azadirachtin as the main insecticidal component of Neem.[2] To date, more than 300 natural products have been isolated from different parts of the tree, with new compounds added to the list every year.[3] Here, we report the isolation and chemical analysis of the hexane extract whole components.

Keywords: Neem, *Azadirachta indica*

Address correspondence to Yoichiro Ito, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 50, Room # 3334, 50 South Drive, Bethesda, MD 20892, USA. E-mail: itoy2@mail.nih.gov

INTRODUCTION

Azadirachta indica (Meliaceae), originating from India, is one of the richest sources of secondary metabolites in nature.

It grows as a tree found in the temperate woodlands of southern West Africa. In traditional medicine of the Ivory Coast, the leaves, bark, or seed is ground and extracted with hot water and cooled off. The same oily material is also used as a cosmetic by women for their skin and hair treatment. Previous studies have reported the presence of fatty acids and oily substance in the extract of the neem seed.[4] Indigenous to India, the neem tree is mostly grown as a shade tree in a variety of habitats. Fruit from the tree contains kernels that are rich in oil (up to 40%). From these seeds, a unique, cold press oil is expelled, which contains natural constituents which have specific benefits for skin care. Scientific research has confirmed that neem seed oil is non-toxic to mammals and may be a very effective organic antiseptic, antifungal, antibacterial, dermatological, and dental agent. It is also widely hailed as a natural insect repellent. This oil has been used for centuries in traditional Indian medicine to aid in the healing of topical skin disorders such as eczema, psoriasis, rashes, burns, and acne. It is rich in fatty acids and glycerides and together with its healing properties, provides an excellent natural moisturizing base for skin care formulations.

Neem oil can be applied inside the vagina and it will work as a spermicide. It cures vaginitis and it prevents the transmission of sexual transmission diseases, possibly including HIV. The extensive usage of the water-insoluble substances from the seed of this fruit in traditional medicine, and the paucity of studies done, prompted a thorough analysis of the hexane extract of the seed. The ground seed was treated with hexane by the maceration method. The hexane solution of interest was adequately separated from the marc. The hexane was evaporated and the fatty substances were further dried and analyzed by GC. Here, we report the analysis of different constituents of hexane extract and their characterization. We concluded that the neem seed contains nutritional value, and may serve as a contraception and as an ingredient for cosmetics.[5]

EXPERIMENTAL

Apparatus

The cross-axis coil planet centrifuge[6] (a prototype fabricated at the National Institutes of Health, Bethesda, MD, USA) was used for preliminary purification of the 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 9 layers of left-handed coils of ca 50 m of 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. The revolution speed was adjusted at 650 rpm with a speed controller (Bodine Electric Company, Chicago, IL, USA) for the present studies.

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).

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 ID.) (SEG Company). The temperature was programmed from 170°C to 280°C at the rate of 2 mL/min increments, 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 ID.) (SEG Company).

The temperature was programmed from 150°C to 280°C at the rate of 2 mL/min increments, while the temperature at the injector and the detector was kept constant at 280°C. The carrier gas was hydrogen and air.

Plant Materials

The ripe seeds of neem were collected in the month of June in the Ivory Coast. 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.

HSCCC Solvent System

The two-phase solvent system composed of hexane/ethyl acetate/methanol/1% TFA aqueous solution (4:5:4:5, 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 1 g each of the crude extract from hexane extract in 10 mL of the solvent consisting of about equal volumes of each phase.

Extraction

The dried neem seed was ground in a screen Wiley mill and 50 g of the seed powder was macerated 3 times with a mixture of hexane. This generated 15 g of hexane soluble oil.

RESULTS

Isolation of Fatty Acids

The hexane soluble oil (8.00 g) 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 (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 M hydrochloric acid (in slight excess) and extracted 3 times with 50 mL portions of diethyl ether. The free fatty acids were recovered after washing the extract with water, drying it over 10.0 g anhydrous sodium sulfate, and evaporating off the solvent. A mixture of free fatty acids (2.20 g) was obtained. GC analysis of the fatty acids showed that it contained nine fatty acids. The most abundant are 43.1% of Δ^1-oleic acid, 19.4% of palmitic acid, 17.6% of Δ^2-linoleic acid, 16.4% of stearic acid, 1.3% of arachidic acid; the minor fatty acids are 0.6% of odeolidic acid, 0.3% of Δ^3-α- linoléic acid, 0.2% of margaric acid, 0.2% of behenic acid, 0.2% of lignoceric acid, and 0.1% of Δ^1-gadoléic acid (Figure 1 and Table 1).

Isolation of Unsaponifiables

The aqueous solution was extracted thoroughly (3 times) with 50 mL portions of dichloromethane. The organic layer was dried over anhydrous sodium sulphate and evaporated off the solvent. A white powder in the amount of 1.50 g was obtained. GC analysis of the unsaponifiable fraction showed that it contained sixteen steroid alcohols. The most abundant are 62.92% of β-sitosterol, 19.17% Δ^7-stigmasterol, 15.65% Δ^7-avenasterol, 1.18% 4-methylcholest-7-enol, and the minor compounds are 0.80% Δ^5- campesterol and 0.20% cholesterol (Figure 2 and Table 2).

Isolation of Phenolic Compounds

Methanol/water (80:20, v/v, 2 × 10 mL) was added to 10 g of neem oil and homogenized for 2 min with a polytron. The two phases were separated by centrifugation at 3,800 rpm fro 15 min. Hydroalcoholic extracts were

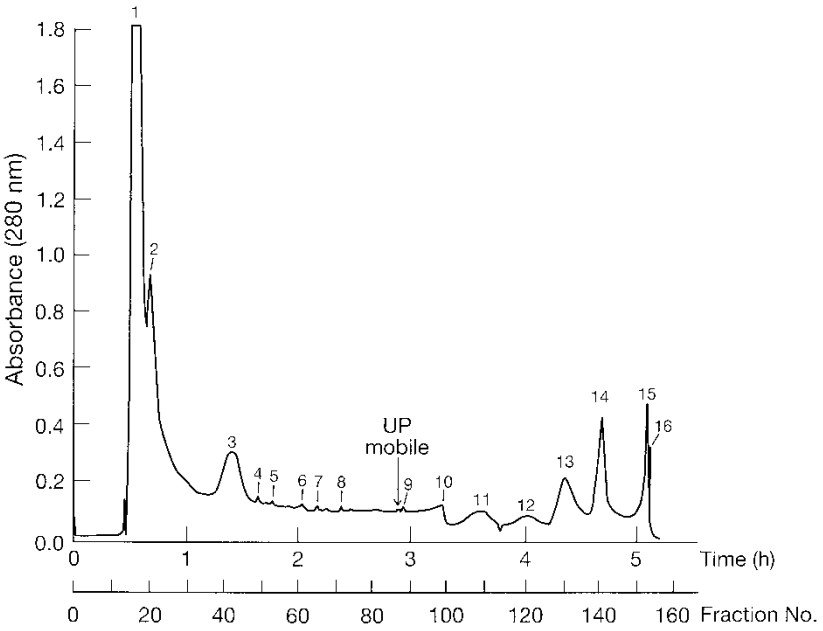

Figure 1. HSCCC analysis of hexane-soluble fractions of neem oil. Experimental conditions: apparatus: Pharma-Tech HSCC unit with 325 mL capacity; solvent system: hexane/ethyl acetate/methanol/1% TFA (4 : 5 : : 4 : 5, v/v/v/v); sample: 1 g of neem oil dissolved in 10 mL of solvents consisting of about equal volumes of each phase; mobile phase: lower phase; flow rate: 2 mL/min; retention of stationary phase: about 60%.

combined and concentrated in a vacuum at 35°C until a syrup consistency was reached. Acetonitrile (5 mL) was added to the extract and it was washed with 3×20 mL of hexane. The nonpolar phases were also purified with 5 mL of acetonitrile. The resulting acetonitrile solution was evaporated under vacuum and dissolved in 5 mL of acetontrile. Finally, an aliquot of 2 mL was evaporated under a stream of nitrogen.

A series of simple phenols was found, such as hydroxy-tyrosol, tyrosol, vanilic acid, and p-counaric acid. Vanillin 4-(acetoxyethyl)-1,2-dihydroxybenzene were also found. But other phenolic compounds were identified such as caffeeric acid, syingic acid, ferulic acid, and homovanillic acid, lueoline, vitamins D + E, and unknown phenolic compounds.

The second part of the chromatogram is more complicated because of the presence of a great number of peaks, some of them related to phenols with high molecular weights such as the dialdehydic form of elecolic acid linked to hydroxytyrosol, the dialdehydic form of elenolic acid linked to tyrosol, and the oleuropein aglycon, respectively, because they have been previously described[7] (Figure 3 and Table 3).

Table 1. Analysis of hexane-soluble fatty acids of neem oil

Peak #	Time (min)	Conc. (%)	Fatty acids
1	4.75		
2	5.63		
3	6.81	17.6	Linoleic acid
4	7.24	0.2	Margaric acid
5	7.34	0.2	Behenic acid
6	8.61		
7	9.14		
8	9.75		
9	11.02	18.6	Palmitic acid
10	11.66		
11	11.82	43.1	Oleic acid
12	13.08	17.7	Stearic acid
13	13.2		
14	13.45		
15	15.38	0.6	Odeolidic acid
16	17.78	1.4	Arachidic acid
17	18.37	0.1	Gadoleic acid
18	23.62	0.3	α-Linoleic acid
19	31.98		
20	32.9	0.2	Lignoceric acid
Total		100	

DISCUSSION

The major components of the neem seed hexane extract could be resolved by HSCCC, HPLC, and GC of the reference solvent system. Neem oil could be classified as a pharmaceutic acid just like olive 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 emollient and a laxative. This oil is utilized for the treatment of children suffering from nutritional deficiency. It has wound healing, cosmetic, and pesticides properties. The neem oil is a nutrient and is widely used as a food conservation additive for human consumption by the people in India.[8–10]

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 possess a notable activity against various bacteria and viruses. Long-chain fatty acids are the most important substrates for the heart. In addition, they have been shown to affect signs in pathways and gene expression.

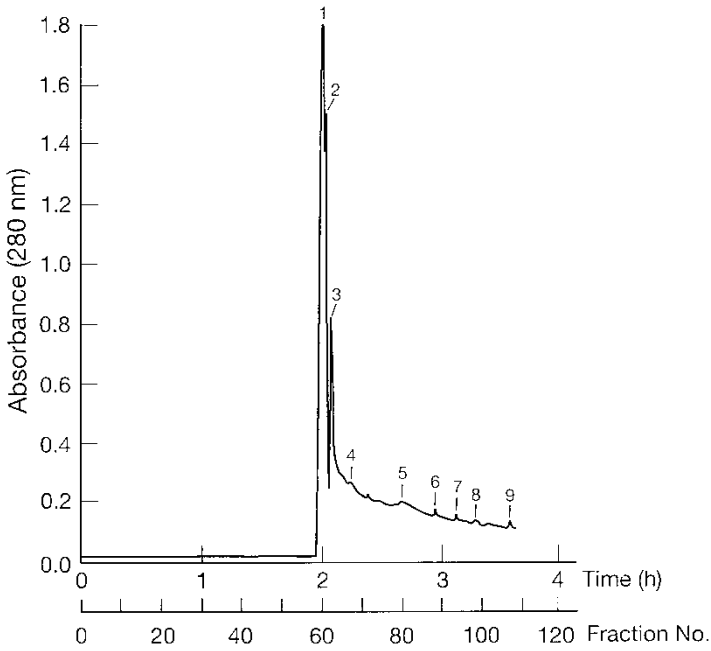

Figure 2. HSCCC analysis of unsaponifiable fractions of neem oil. Experimental conditions: apparatus: Pharma-Tech HSCCC unit with 325 mL capacity; solvent system: hexane/ethyl acetate/methanol/1% TFA (4:5:4:5, v/v/v/v); sample: 1 g of neem oil dissolved in 10 mL of solvents consisting of about equal volumes of each phase; mobile phase: lower phase; flow rate: 2 mL/min; retention of stationary phase: about 60%.

CONCLUSION

From the preliminary results we obtained on the seed of *Azadirachta indica*, a full investigation on the plant seed is needed in the different regions of Cote d'Ivoire to identify all its fatty acids. Therefore, the present results are only the beginning of our work.

Table 2. Analysis of unsaponifiable fraction of neem oil

Peak #	Time (min)	Conc. (%)	Phytosterols
1	14.57	19.17	Δ^7-stigasterol
2	15.26	1.18	4-methylcholest-7-enol
3	16.38	62.92	β-sitosterol
4	16.535	15.65	Δ^7-avenasterol
5	16.67	0.27	Δ^5 -campesterol
6	18.324	0.8	cholesterol
Total		100	

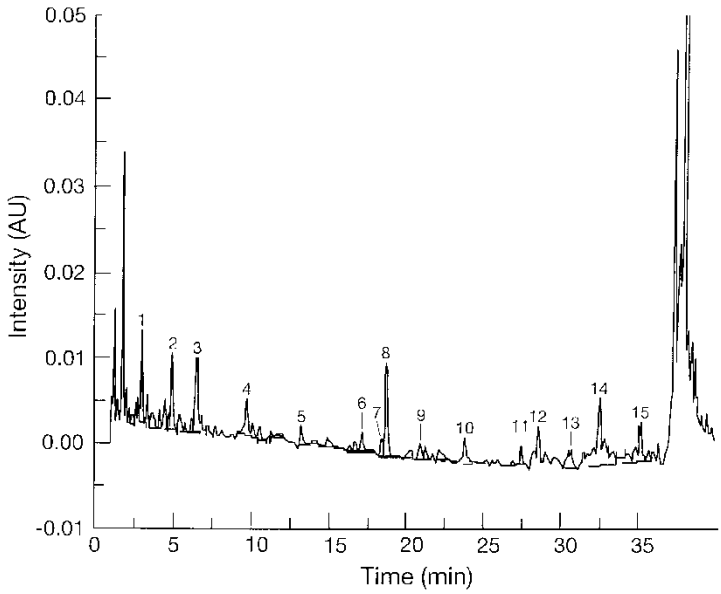

Figure 3. HPLC analysis of phenolic fractions of neem oil. Experimental conditions: apparatus: HPLC system consisited of D-7000 HPLC system, a pump L-7100, a Chromolit-Guard cartridge RP-18^{e} Merck (4.6 mm × 100 mm).

Table 3. HPLC analysis of phenolic compounds of neem oil

Peak #	Time (min)	Conc. (%)	Phenols
1	2.94	4.88	Hydroxy-tyrosol
2	4.89	4.74	Tyrosol
3	6.45	7.08	Vanillic acid
4	9.68	3.38	Caffeic acid
5	13.16	7.7	Vanillin
6	16.67	0.88	p-Coumaric acid
7	17.11	1.47	Vitamin D
8	18.68	7.3	Vitamin E
9	20.87	1.83	Ferulic acid
10	23.8	2.36	Luteolin
11	28.29	1.81	Pinresinol
12	28.53	4.05	Oleuropein aglycon
13	32.15	4.2	Ligstroside aglycon
14	32.53	6.34	Unknown
15	35.2	3.56	Unknown
Total		62	

Like all vegetable fats, *Azadirachta indica* oil globally has softening, anti-drying, and protective effects. These activities are even more noticeable due to the moderate content in unsaponifiables.

The *Azadirachta indica* project will be an integrated, long-term effort to preserve the ecological integrity of forest woodland in the western, eastern, and southern Ivory Coast through reinforcement of the economic importance of the *Azadirachta indica* tree, source of the pesticides formulation ingredients known, and environmental protection.

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