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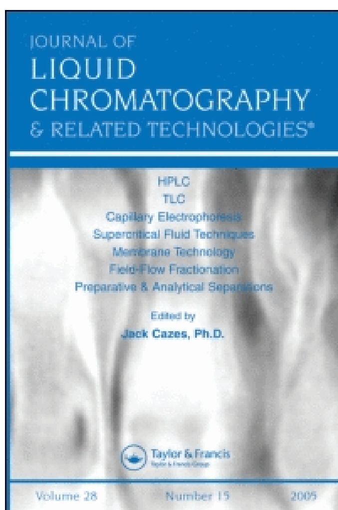

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Preparative Separation of Indole Alkaloids from the Rind of *Picralima nitida* (Stapf) T. Durand & H. Durand by pH-Zone-Refining Countercurrent Chromatography

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Abstract: *Picralima nitida* alkaloids have been shown to possess *in vitro* antimalarial activity comparable to the clinical antimalarial chloroquine and quinine. The *in vitro* IC_{50} values for these alkaloids ranged from 0.01 to 0.9 μg/mL against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum*. As part of our continuing research aimed at identifying new antimalarial leads from African rain forest plants, we have been highly successful in employing pH-zone-refining counter-current chromatography (CCC) to separate and isolate the *Picralima* alkaloids. The separation was performed in two steps with a two-phase solvent system composed of *tert*-butyl methyl ether (MTBE)/acetonitrile/water (2 : 2 : 3, v/v), in which the lower phase was used as the mobile phase at a flow-rate of 2.0 mL min^{-1} in the head-to-tail elution mode pH-zone-refining counter-current chromatography.

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The resulting fractions contained the alkaloids alstonine, akuammigine, akuammine, picraline, akuammine, akuammicine, picratidine were identified using thermospray liquid chromatography-mass spectrometry (LC-MS) and by TLC co-elution experiments with authentic samples. The application of pH-zone-refining CCC to perform successful separations at the multigram level will be discussed. The *Picralima* alkaloids may represent an entirely new antimalarial chemotype with possible pharmacokinetics advantages over the existing drugs.

Keywords: Preparative separation, pH-zone-refining counter-current chromatography, indole alkaloids, *Picralima nitida*, Anti-malarial activity, alstonine

INTRODUCTION

The West African tree *Picralima nitida* Stampf Th. et H.Dur. (Apocynaceae) has a folk reputation as a remedy for chronic malaria.[1] The intensely bitter tasting seeds, sold in markets in Nigeria and Ghana as "akuamma seeds", are used in African traditional medicine for the treatment of fevers, pneumonia, sleeping sickness, and various infectious diseases.[2,3]

Many pharmacological activities have been attributed to the extracts and compounds from this plant including antimalaria,[4] antileishmanial, antitrypanasomal,[5] and analgesic.[6] Different parts of *P. nitida* have been known to contain several indole and dihydroindole alkaloids, of which the major constituents include akuammiline, akuammidine, akuammine, akuammigine, akuammicine, picraline, and alstonine[7,8] (Figure 1). The *in vitro* IC_{50} values against *P. falciparum* for these alkaloids ranged from 0.01 to 0.9 μg/mL against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum.* (Figure 2), with alstonine being the most active compound isolated from the plant. Furthermore, akuammidine has been shown to possess a strong local anaesthetic action and was found to be three times as active as cocaine hydrochloride.[9] Akuammidine also has sympatholytic and a mild, but persistent, hypotensive effect.[10]

Several attempts to isolate large quantities of these pharmacologically active indole alkaloids to be used for further *in vivo* studies and chemical optimization have failed, owing to irreversible adsorption of these alkaloids onto the solid stationary phase. In order to overcome this irreversible adsorption, we employed the support-free technique of preparative countercurrent chromatography (CCC) for the isolation of the Picralima alkaloids.

CCC, as an all-liquid chromatographic technique, operates under gentle conditions and allows non-destructive isolation of very chemically sensitive natural compounds. Because the solid stationary phase is absent, adsorption losses are minimized and, hence, a near-100% sample recovery is almost guaranteed.[11] Preparative pH-zone-refining CCC also provides many important advantages over conventional adsorption chromatography that are

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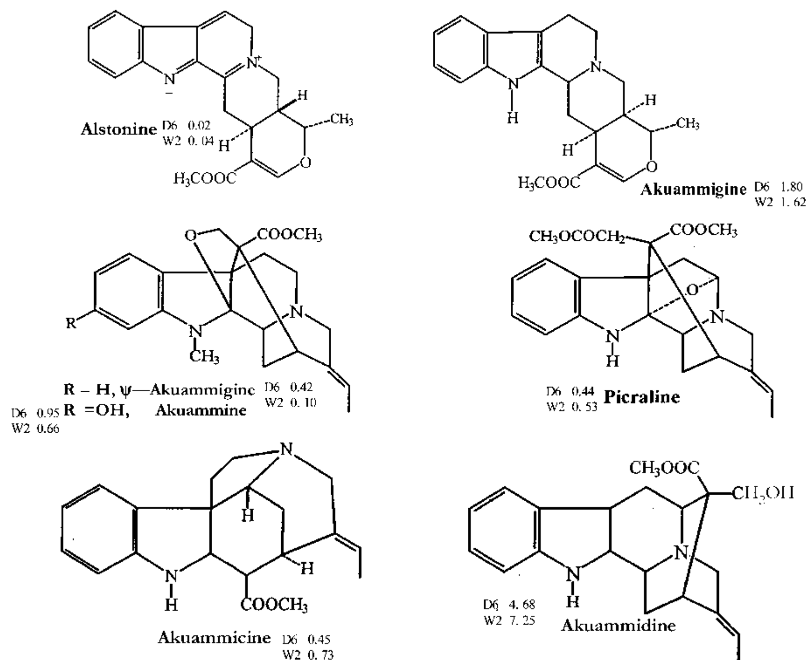

Figure 1. Structures of major alkaloids found in the rind of *P. nitida.*

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commonly used in most natural product laboratories. These include increased sample loading capacity, high concentration of fractions, and concentration of minor impurities. The CCC method has been successfully applied to the separation of numerous alkaloids and other natural products, including the works cited in references.[12–15]

In this study, pH-zone-refining CCC has been successfully applied to the multigram preparative separation of Picralima alkaloids from the crude chloroform : methanol extracts (1 : 1) of the fruit rind.

EXPERIMENTAL

Plant Material

Mature fruits of *P. nitida* were collected by the staff from InterCEDD in February and March 2002, from trees at a homestead at Nnewi, Nnewi Local Government area (Anambra state, Nigeria). The plant material was identified by comparison with a voucher specimen (UNN/83/07) at the

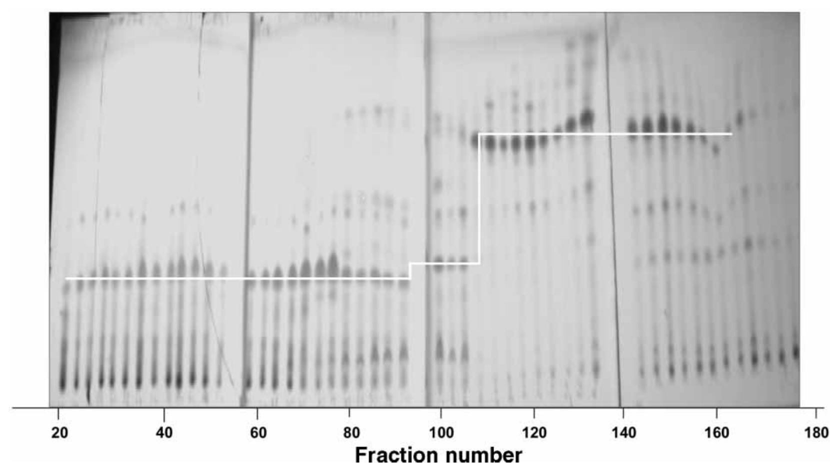

Figure 2. The results of TLC analysis of pH-zone-refining CCC fraction of the crude extract of *P. nitida* fruit rind. The analyses were made by a silica G TLC plate developed with a solvent system composed of benzene-ethylacetate-acetone-25% aqueous ammonium (12 : 6 : 3 : 3 : 1) under a UV lamp. Experimental conditions for the pH-zone-refining CCC is described in the Figure 3 caption.

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Department of Pharmacognosy Herbarium of the University of Nigeria, Nsukka, and authenticated by Mr. A. Ozioko of the Department of Botany of the same university. The fruit-rind was separated, dried, and pulverized. The powdered material was successive extracted with n-hexane, methylene chloride, methylenechloride : methanol (1 : 1), and methanol. Extracts were concentrated in vacuo using the rotary evaporator.

Reagents

Tert-Butyl methyl ether (MTBE, Sigma-Aldrich, St Louis, MO, USA), acetonitrile, chloroform $CHCl_3$, Fisher), methanol (MeOH, Fisher), hydrochloric acid (HCl 37%, Sigma-Aldrich), and triethylamine (TEA, Sigma-Aldrich). The pure alkaloids (ψ-akuammigine, picraline, akuammicine, picranitidine, akuammine, akuammiline, akuammidine, akuammigine, and alstonine) were gift samples from the International Center of Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria.

Mass Spectrometry

A Finnigan ion-trap mass spectrometer (LCQ) was used for the analysis of alstonine. The mass spectra were acquired with a LCQ ion trap mass

spectrometer (Finnigan Mat, ThermoQuest, San Jose, CA, USA). The instrument was fitted with an electrospray (ESI) source. Samples were dissolved in acetonitrile/water (1 : 1) to which was added 0.1% formic acid, and infused at a rate of 3 μL/min. The LCQ was interfaced to a computer workstation running Finnigan Mat LCQ Navigator software.

Nuclear Magnetic Resonance Spectrometry

The ^{1}H and ^{13}C NMR spectra of alstonine in deuteriochloroform were recorded on a Bruker Avance 300 spectrometer at the frequency of 300.1 and 75.5 MHz, respectively.

Chemical shifts are reported in parts per million (ppm) and coupling constants in hertz (Hz).

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Antimalarial Screening

The *in vitro* antimalarial assays were performed by using a modification of the semi-automated microdilution technique described earlier.[16,17] Two *P. falciparum* malaria parasite clones, designated as Indochina (W-2) and Sierra Leone (D-6), were used in susceptibility testing. The W-2 clone is resistant to chloroquine, pyrimethamine, sulfadoxine, and quinine, and the D6 clone is sensitive to chloroquine but resistant to mefloquine. The test extracts and compounds were dissolved in DMSO and serially diluted using malarial growth medium. Drug-induced reduction in the uptake of tritiated hypoxanthine was used as an index of parasite growth inhibition (Table 1).

pH-Zone-Refining Countercurrent Chromatography

The pH-zone-refining CCC separations were performed with a CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corporation, Baltimore, MD) equipped with three coils connected in series (inner diameter of tubing = 2.6 mm, total volume 325 mL).

The separations were performed using a modification of the previously described operating conditions.[18] Briefly, the two-phase solvent system used consisted of *tert*-butyl methyl ether (MTBE)-acetonitrile-water (2 : 2 : 3, v/v). The solvent system was equilibrated in a separating funnel, and the two phases were separated before use. The organic upper layer (UP) was rendered basic by addition of triethylamine (TEA) resulting in a pH ∼10.7). The aqueous lower layer (LP) was acidified with HCl conc. to pH ∼1.7. The basic organic phase was used as the stationary phase and the

Table 1. *In vitro* antimalarial activity of *Picralima nitida* alkaloids against D-6 and W-2 Clones of *Plasmodium falciparum*

	IC_{50} (g/mL)	
Alkaloid	D-6 Clone	W-2 Clone
Akuammicine	0.45	0.73
Akuammine	0.95	0.66
Alstonine	0.017	0.038
Picraline	0.44	0.53
Picratidine	0.80	0.92
Picranitidine	0.04	0.03
Akuammidine	4.68	7.25
Akuammigine	1.80	1.62
ψ-Akuammigine	0.42	0.10
Melinonine A	>10.00	>10.00
Chloroquine	0.003	0.084
Artemisinin	<0.001	<0.001
Quinine	0.027	0.097

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acidic LP was used as the mobile phase. The separation was initiated by filling the entire column with the stationary phase using the LC pump, and then loading the sample. The sample solutions were prepared by dissolving the crude alkaloid extract of *P. nitida* in 100 mL of a phase mixture consisting of equal volumes of each phase. The sample solutions were sonicated for several minutes before injecting onto the column, and dissolved in a mixture of stationary and mobile phases at the ratio of 1 : 1, e.g., 50 mL : 50 mL for a 15 g of CH_2Cl_2 : MeOH extract of *P. nitida.* The pH of sample was adjusted to ~10.13 by addition of 5.2 mL of triethylamine to the sample solution. The mobile phase was then pumped into the column at 2 mL/min while the column was rotated at 834 rev./min in the combined head to tail elution mode.[19,20] The absorbance of the eluate was continuously monitored at 280 nm and 4-mL fractions were collected. The pH of each eluted fraction was measured with a pH meter. The fractions collected were brought to dryness using a Speed Vac concentrator and were analyzed by TLC as shown in Figure 3.

After the desired peaks were eluted, the rotation and elution were stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line pressurized at approximately 80 psi. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

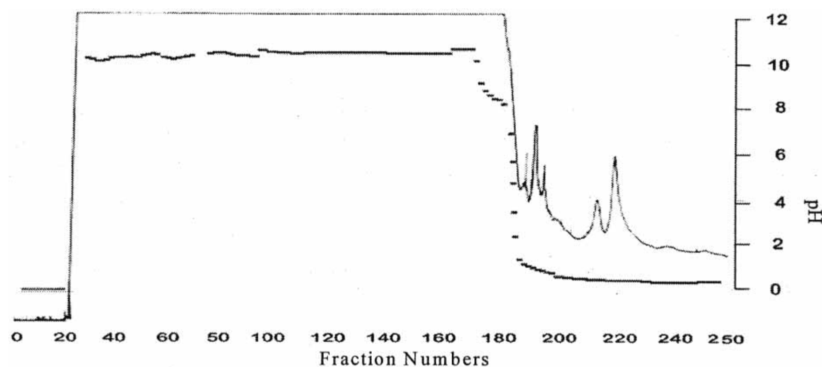

Figure 3. pH-Zone-refining CCC chromatogram for the separation of 15 g of crude alkaloid extract from *Picralima nitida.* Experimental conditions are as follows: solvent system: tert-butyl methyl ether/acetonitrile/water (2 : 2 : 3, v/v/v); stationary phase: upper organic phase (pH 10.7 with triethylamine); mobile phase: aqueous HCl (pH 1.7); flow rate: 2 mL/min; sample size: 15 g dissolved in a mixture of both phases, 50 mL each; revolution speed: 830 rpm; retention of the stationary phase: 50%. Fractions 21-95 contained mainly alstonine with minor spots below. Subsequent pH-zone-refining CCC separation of this sub-fraction yielded pure alstonine. The chromatogram has the broad rectangular shape characteristic of pH-zone-refining CCC.

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Analysis of Fractions

All collected fractions were analyzed by using silica gel G thin-layer chromatography Analtech normal phase 10 × 20 cm scored. Plates were developed using the following solvent systems: i. C_6H_6-EtOAc-CH_3OH-i-PrOH-25% aqueous NH_3 12 : 6 : 3 : 3 : 1, v/v/v/v/v, ii. $CHCl_3$: CH_3OH (4:1) saturated with 25% aqueous NH_3, iii. Toluene-EtOAc-diethylamine (7:2:1) and spread with a Dragendorff reagent to detect the alkaloids. Alkaloids were identified by co-TLC and comparison of their ^{1}H and ^{13}C NMR data with those of reference compounds.

RESULTS AND DISCUSSION

The pH-zone-refining CCC chromatograms of alkaloids are shown in Figure 3. The chromatogram has the broad rectangular shape characteristic of pH-zone-refining CCC.[21,22]

A total of 251 fractions (2 mL each) were collected and analyzed by TLC as shown in Figure 2. Seven subtractions were obtained. The separation was completed in ~8.5 h. However, all the alkaloids were eluted within 3.7 hr (3 hr 44 mins). The chromatogram and the elution profiles for alstonine is presented in Figure 2. Fractions 21-95 gave the highest yield of mixtures

enriched with alstonine (1115 mg). Subsequent pH-zone-refining CCC separation of this sub-fraction yielded pure alstonine. The retention times, molecular ion peak, and major diagnostic fragment ions obtained from the LC-MS of this sub-fraction confirmed the presence of alstonine (Iwu patent).

It is important to note, that the separation and purification of indole alkaloids from the crude extract of *P. nitida* was initially performed by several steps using conventional silica gel column chromatography and recrystallization.[23] The conventional method yielded a small amount of these alkaloids. In order to separate larger quantities, a different approach from the conventional methods became necessary. Attempts to separate larger quantities of this extract by conventional HSCCC also failed, mainly due to the poor retention of the stationary phase. pH-zone-refining CCC was implemented successfully to separations at the multigram level using a HSCCC centrifuge. This was achieved with two steps, with a two-phase solvent system composed of *tert*-butyl methyl ether (MTBE)-acetonitrile-water (2 : 2 : 3, v/v).

The results of our studies have demonstrated that multigram quantities of pure indole alkaloids can be obtained from crude extracts of *P. nitida* using pH-zone-refining CCC.

The present method may be applied to the purification of other alkaloids used in commerce even at pilot-scale level.

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