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Chromatographic isolation of insecticidal amides from *Piper guineense* root

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ABSTRACT

Fractionation of a light petroleum (b.p. 40–60°C) extract from the male roots of *Piper guineense* Schum and Thonn was carried out by thin-layer chromatography (TLC) followed by reversed-phase high-performance liquid chromatography (HPLC). Fractions were monitored by their insecticidal activity, and five different active amides were purified. The amides were identified by spectral and chemical methods as N-isobutyl-11-(3,4-methylenedioxyphenyl)-2*E*,4*E*,10*E*-undecatrienamide (**1**), N-pyrrolidyl-12-(3,4-methylenedioxyphenyl)-2*E*,4*E*,9*E*,11*Z*-dodecatetraenamide (**2**), N-isobutyl-13-(3,4-methylenedioxyphenyl)-2*E*,4*E*,12*E*-tridecatrienamide (**3**), N-isobutyl-2*E*,4*E*-decadienamide (**4**) and N-isobutyl-2*E*,4*E*-dodecadienamide (**5**). Some of these (**4** and **5**) have not hitherto been identified in *P. guineense*, and one (**2**) appears to be novel from any plant source. TLC–HPLC is suggested to be more suitable than other chromatographic methods used earlier for isolating minor insecticidal components from *Piper* plants.

INTRODUCTION

Most of the earlier work on *Piper* species seem to suggest that the major insecticidally active components are alkamides [1–3]. The isolation of one of these amides (pellitorine) by gas chromatography (GC) from a light petroleum extract of the root of *Piper guineense* was reported earlier [4]. However, GC was found to be unsuitable for the separation and isolation of some of these amides which appear to be unstable at the high temperatures used. There have been few reports on the use of high-performance liquid chromatography (HPLC) for the separation and isolation of amides from *Piper* plant [3,5]. In this study we have used reversed-phase HPLC, preceded by thin-layer chromatography (TLC) for the fractionation of insecticidal amides from a plant extract and for the isolation of these components in sufficient quantities for chemical identification.

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EXPERIMENTAL

The male root of *P. guineense* was used. Details of the origin, processing and extraction of the plant material were described earlier [4]. Briefly, 25 g of dried, powdered root was extracted with 250 ml of light petroleum (b.p. 40–60°C) for 6 h at 20°C and was then filtered.

Bioassays

A Petri dish contact method described earlier [4] was used with adult *Musca domestica* as test insects. Eluents of the TLC and HPLC fractions to be bioassayed were introduced into glass Petri dishes (radius = 4.6 cm; area = 67 cm^2), 2.0 ml of olive oil–light petroleum (0.03%, v/v) were added and the mixture was dried. The olive oil served as carrier to facilitate transfer of test materials to the insects. At least two replicates of ten insects were used at each concentration tested and a set of controls was

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included with each group of bioassays. The insects were supplied with sucrose and water and maintained at 26–28°C. Knockdown (proportion of insects no longer able to upright themselves) was recorded at 2 h and lethal effects (proportion of insects showing no movement even after stimulation by contact) at 24 h.

TLC fractionation of the total extract for insecticidal activity

Silica gel chromatoplates (250 μm, 20 cm × 10 cm, spread on glass plates using Kieselgel 60G, Merck, Darmstadt, Germany) were used for analytical separation and identification of active bands. For quantitative separation of active fractions, however, preparative silica gel chromatoplates (approximately 400 μm, 20 cm × 20 cm of Kieselgel 60G) were used. During a preliminary analytical separation, bands were located both with ultraviolet light at 254 and 365 nm and by iodine staining. In all subsequent TLC separations, bands were only located with UV light. The use of UV light at two wavelengths allowed the borders of the bands to be better defined. The sequence of TLC fractionation of the *Piper* root extract is summarized in Fig. 1. First (TLC I) the concentrated extract was run for 50 min. using chloroform–methanol (10:1, v/v) as developing solvent. The individual fractions obtained from this were eluted and run for 40 min using *n*-hexane–ethyl acetate (3:1, v/v) (TLC II). Each fraction was eluted and re-run under the same conditions as for TLC II, *i.e.* using *n*-hexane–ethyl acetate as solvent (TLC III). In general, R_f values for components after TLC II were lower than after TLC III, presumably because of differences in loading. When monitoring the activity of TLC bands, samples of the extract (4.0 mg dry weight) and the subsequent fractions (0.4 mg) were tested. Bands were recovered by scraping off the appropriate region of silica gel and eluting with acetone–light petroleum (2:1). The eluents were centrifuged at 1000 *g* for 15 min to remove suspended silica gel particles, and then evaporated under partial vacuum and taken up in chloroform. Larger amounts of each of the active crude fractions A, B and C were obtained by separating aliquots (100 mg) of the extract on preparative silica gel chromatoplates (Fig. 1). It should be noted that additional components were obtained during TLC fractionation but were not further examined because of their low insecticidal activity.

Isolation and purification of active components from TLC fractions by HPLC

Three active fractions (A, B and C, recovered by TLC) were further separated by reversed-phase HPLC. An LDC Milton Roy (Stone, UK) MP300 multi-processor, consisting of two Constametric III metering pumps, a Spectromonitor III variable-wavelength detector and a printer, all of which were linked to a computer, was used for HPLC separations. A semi-preparative (250 mm × 10 mm I.D.) 5-μm Spherisorb ODS2 C_{18} column (Phase Separa-

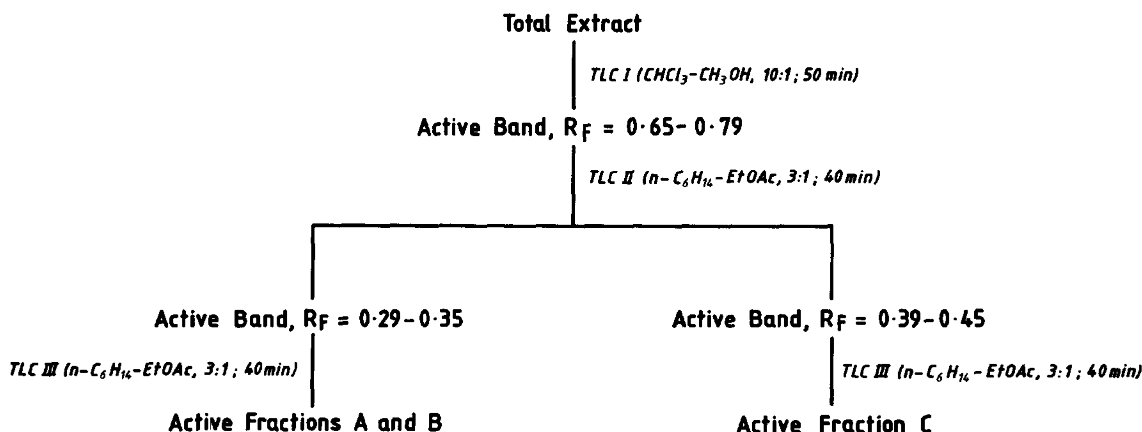

Fig. 1. TLC separation of insecticidal components from *Piper guineense* root extract. For R_F values of active fractions A, B and C after TLC III, see Table I. A, B and C were further separated by HPLC to give components **1**, **2**, **3**, **4** and **5**. EtOAc = Ethyl acetate.

tions, Deeside, UK) was used. The separations were monitored at 254 nm, and the flow-rate was 4.0 ml. A two-solvent gradient elution was carried out using as mobile phase methanol–ammonium acetate buffer (0.05 *M*, pH 5.6). The solvent programme was: 70.0–99.9% methanol over a 15-min period, then 99.9% methanol for another 5 min. Aliquots (50 μg) of each TLC fraction were analysed, and the results were then used to guide in the HPLC fractionation of larger quantities of the TLC fractions. Aliquots of the TLC fractions (0.4 mg) were separated by HPLC and fractions were collected as groups of peaks together (T_1, T_2, T_3, T_4, T_5 and T_6) as shown for the chromatogram in Fig. 2. These HPLC fractions were dried under partial vacuum, taken up in chloroform (1 ml) and bioassayed for insecticidal activity. In order to identify the actual peaks responsible for activity, the HPLC separations of the TLC fractions were repeated and selected individual peaks (areas >8% of the total at 254 nm) were isolated and bioassayed. The active peaks 1, 2, 3, 4 and 5, the elution times for which are shown in Table II, were isolated in sufficient quantities (and then further purified) to allow subsequent structural analysis. For further purification two isocratic solvent systems were used: (i) acetonitrile–ammonium acetate buffer (55:45) for components with elution times of less than 15.0 min during component isolation; (ii) acetonitrile–ammonium acetate buffer (62:38) for components with elution times greater than 15.0 min during component isolation.

Structure determination

The chemical identities of the isolated compounds were determined by spectral and chemical analysis. The details of these procedures were as outlined earlier [4], except that ^{1}H NMR spectra of components B and F were determined with a JEOL JNM-GX 270 FT NMR spectrometer.

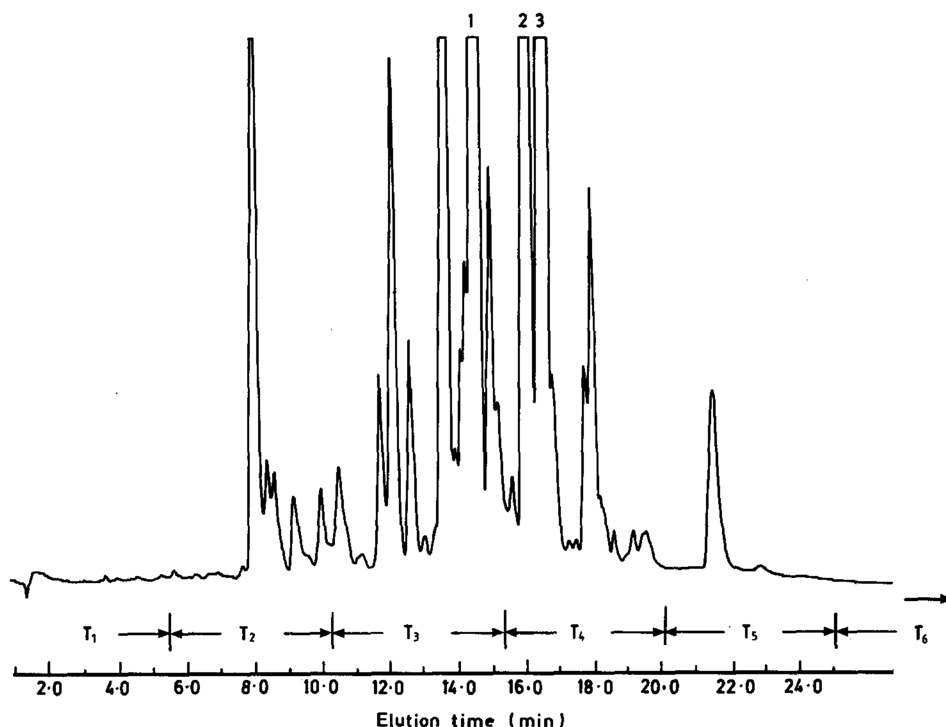

Fig. 2. HPLC separation of components **1**, **2** and **3** from TLC fraction A. Elution times (min) are indicated for the various peaks. Peaks marked 1, 2, 3 were the active components isolated. T_1–T_6 represent groups of peaks collected as fractions for bioassay. Column: Spherisorb 5ODS2; solvent: methanol–ammonium acetate buffer, gradient; flow-rate: 4.0 ml/min; detector: UV, 254 nm.

RESULTS AND DISCUSSION

Many TLC bands with activity were obtained (Fig. 1). However, the main bands recovered in substantial quantities (recovery > 0.005% dry weight of plant material) and which also showed dissimilar activities on bioassay are shown in Table I. Fraction A showed both good knockdown and good lethal toxicity, B showed moderate lethal toxicity but good knockdown whilst C showed poor lethal toxicity but good knockdown.

The chromatograms obtained by reversed-phase HPLC separation of fractions A, B and C, showed that all the fractions contained several closely related components which absorbed at 254 nm. The areas of the integrated peaks of individual active components in each chromatogram were all greater than 15% of the total absorbance for fractions A, B or C (Table II). The peak eluting at approximately 12.0 min constituted nearly 50% of the total, and the peaks eluting at about 15.0–17.0 min constituted between 15 and 23% of the total (Table II). HPLC peaks 1, 2 and 3, which eluted between approximately 15.0 and 17.0 min, were found to cause knockdown and lethal toxicity to 75% or more of the treated insects (Table II). Peak 4, which eluted at about 12.0 min from TLC fraction B, but formed as much as 50.0% of the total integrated peak area, was also found to cause knockdown to over 75% and lethal toxicity to 50% or more of insects when bioassayed. Peak 5 from TLC fraction C on the other hand had an elution time around 14.0 min but showed only moderate activity, with only up to 50% of treated insects being killed in 24 h or being knocked down in 2 h. All the active peaks contained impurities resulting from overlapping of minor

TABLE I

R_F VALUES, RECOVERY AND TOXICITY OF MAIN ACTIVE FRACTIONS OBTAINED FROM THE SEPARATION OF TLC III FRACTIONS

TLC III, dose = 6.0 $\mu g/cm^2$; solvent, *n*-hexane-ethyl acetate (3:1, v/v). Degree of activity (percentage of test insects responding: + + + + = 100–95%; + + + = 95–75%; + + = 75–50%; + = 50–25%; − = 0–25%. Each fraction was tested on twenty insects. KD = Knockdown after 2 h; M = mortality after 24 h. The proportion recovered was expressed relative to total dry weight of plant material (710 g).

TLC fraction	R_F of band	Proportion recovered (%)	Degree of activity	
			KD	M
A	0.23–0.37	0.018	+ + + +	+ + + +
B	0.37–0.42	0.005	+ + +	+
C	0.51–0.69	0.022	+ + + +	−

TABLE II

ELUTION TIMES AND ACTIVITIES OF PEAKS ISOLATED FROM TLC FRACTIONS BY REVERSED-PHASE HPLC

Percentage % total int. peaks = % total absorbance for fractions A/B/C (= 100) detected at 254 nm. A 0.4-mg amount of each TLC fraction was separated. Twenty insects were used for the bioassays. Degree of activity (percentage of test insects responding): + + + = 95–75%; + + = 75–50%; + = 50–25%. KD = knockdown after 2 h; M = mortality after 24 h.

TLC fractions	Active HPLC peak(s)	Elution time (min)	Percentage % total int. peaks	Degree of activity	
				KD	M
A	1	14.6	17.4	+ + +	+ + +
	2	16.1	15.3	+ +	+ + +
	3	16.8	22.8	+ + +	+ + +
B	4	11.9	49.6	+ + +	+ +
C	5	14.3	15.3	+	+

peaks adjacent to the major peaks (Fig. 2). On changing from methanol–ammonium acetate buffer to acetonitrile–ammonium acetate buffer as eluting solvent and rechromatography, much better resolution was obtained enabling large quantities of material to be purified. For example, components **2** and **3**, which were isolated together (Fig. 2) and were barely separable with methanol–ammonium acetate buffer, were completely resolved when acetonitrile–ammonium acetate buffer (62:38) was used (Fig. 3). The peaks were therefore purified isocratically using acetonitrile–ammonium acetate buffer of appropriate strength. In this way pure components **1, 2, 3, 4** and **5** were obtained for determination of their chemical identities. The components were identified as follows.

*N-Isobutyl-11-(3,4-methylenedioxyphenyl)-2E,4E,10E-undecatrienamide (component **1**, 2.1 mg)*. Mass spectrum (*m/z*, % intensity): 355 (M^+, 68), 220 (56), 161 (15), 135 (100), 57 (42), 40 (65). ^{1}H NMR (δ, deuterochloroform, 270 MHz): 0.93 (d, J = 6.7 Hz, 6H), 1.47 (m, 4H), 1.80 (m, 1H), 2.18 (m, 4H), 3.17 (t, J = 6.4 Hz, 2H), 5.44 (br s, 1H), 5.74 (d, J = 15.6 Hz, 1H), 5.94 (s, 2H), 6.01 (dt, J = 15.9, 7.0 Hz, 1H), 6.07 (m, 1H), 6.12 (dd, J = 15.3, 9.8 Hz, 1H), 6.28 (d, J = 15.6 Hz, 1H), 6.73 (br s, 2H), 6.87 (br s, 1H), 7.19 (dd, J = 10.1, 15.0 Hz, 1H). IR (γ, NaCl flat, neat, cm^{-1}): 3305, 2930, 1660, 1630, 1620, 1550, 1260, 1000, 925. UV (λ_{max}, methanol): 260, 305 nm.

*N-Pyrrolidyl-12-(3,4-methylenedioxyphenyl)-2E,4E,9E,11Z-dodecatetraenamide (component **2**, 3.4 mg)*. Mass spectrum (m/z, % intensity): 367 (M^+, 56), 232 (50), 192 (30), 164 (70), 135 (100), 98 (25), 70 (43). ^{1}H NMR (δ, deuterochloroform, 270 MHz): 1.48 (m, 2H), 1.55–1.64 (m, 4H), 2.17 (m, 4H, 3.61 (br, 4H), 5.95 (s, 2H), 5.95 (d, J = 10.9 Hz, 1H), 6.02 (m, 2H), 6.09 (m, 1H), 6.18 (dd, J = 15.1, 10.6 Hz, 1H), 6.24 (dd, J = 16.0, 7.7 Hz, 1H), 6.30 (d, J = 8.6 Hz, 1H), 6.75 (br s, 2H), 6.88 (br s, 1H), 7.25 (dd, J = 10.1, 14.6 Hz, 1H). IR (γ, NaCl flat, neat, cm^{-1}): 2920, 1650, 1620, 1595, 1500, 1440, 1245, 1033, 1000, 925. UV (λ_{max}, methanol): 259, 296 nm (inflexion).

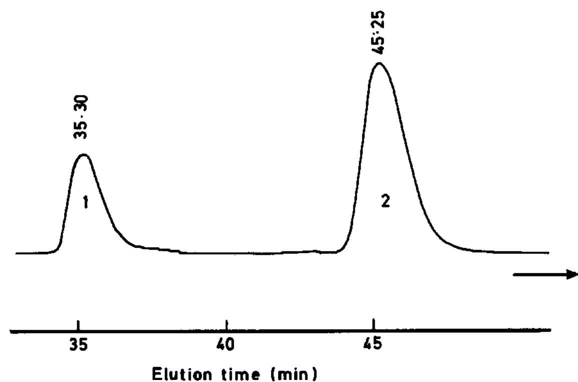

Fig. 3. HPLC purification of components **2** and **3**. Elution times (min) are indicated for the active peaks (1 and 2). Column: Spherisorb 50DS2; solvent: acetonitrile–ammonium acetate (62:38), isocratic; flow-rate: 4.0 ml/min; detector: UV, 254 nm.

*N-Isobutyl-13-(3,4-methylenedioxyphenyl)-2E,4E,12E-tridecatrienamide(component **3**, 7.8 mg)*. Mass spectrum (m/z, % intensity): 383 (M^+, 100), 369 (35), 248 (39), 180 (21), 152 (38), 135 (70), 57 (12). ^{1}H NMR (δ, deuterochloroform, 270 MHz): 0.93 (d, J = 6.7 Hz, 6H), 1.30 (m, 4H), 1.42 (m, 4H), 1.80 (m, 1H), 2.14 (m, 4H), 3.17 (t, J = 6.4 Hz, 2H), 5.45 (br, 1H), 5.74 (d, J = 14.7 Hz, 1H), 5.92 (s, 2H), 6.01 (dt, J = 15.6, 7.7 Hz, 1H), 6.07 (m, 1H), 6.14 (dd, J = 15.3, 9.9 Hz, 1H), 6.28 (d, J = 15.6 Hz, 1H), 6.73 (br s, 2H), 6.88 (br s, 1H), 7.20 (dd, J = 15.0, 10.1 Hz, 1H). IR (NaCl flat, neat, cm^{-1}): 3305, 2920, 1645, 1630, 1605, 1545, 1250, 1030, 1000, 920. UV (λ_{max}, methanol): 260, 304 nm.

*N-Isobutyl-2E,4E-decadienamide (component **4**, 15 mg)*. The spectral data obtained were identical to those reported earlier for the component isolated by gas–liquid chromatography [4]. The identity of this compound was also confirmed by ^{3}H reduction followed by hydrolysis and identification of the hydrolysis product [4].

*N-Isobutyl-2E,4E-dodecadienamide (component **5**, 2.1 mg)*. Mass spectrum (m/z, % intensity): 251 (M^+, 40), 179 (100), 166 (12), 152 (36), 96 (34), 57 (8), 41 (8). ^{1}H NMR (δ, deuterochloroforum, 270 MHz): 0.87 (t, J = 7.7 Hz, 3H), 0.93 (d, J = 6.7, 6H), 1.26-1.30 (m, 8H), 1.42 (m, 2H), 1.83 (m, 1H), 2.10–2.18 (q, 2H), 3.17 (t, J = 6.4 Hz, 2H), 5.44 (br, 1H), 5.74 (d, J = 15.1 Hz, 1H), 6.06–6.14 (m, 2H), 7.19 (dd, J = 10.1, 15.8 Hz, 1H). IR (γ, NaCl flat, neat, cm^{-1}): 3295, 2950, 2920, 1645, 1625, 1605, 1550, 1530, 990. UV (γ_{max}, methanol): 258 nm.

On ^{3}H reduction and hydrolysis [4], component **5** gave as its main hydrolysis product a fatty acid which eluted from HPLC at 22.5 min compared

with the elution times of 4.0, 10.0 and 16.0 min for hexanoic acid, octanoic acid and decanoic acid, respectively, which were run as standards. Thus hydrolysed reduced **5** probably contained twelve carbon atoms and was dodecanoic acid. The λ_{max} from the UV spectrum of **5** suggests the presence of a conjugated diene system adjacent to an amide carbonyl in the molecule, and the IR spectral data is characteristic to the system, $-CH_2-CH=CH-CH=CH-CONHR$ [6]. The fact that the UV and IR spectra of component **5** suggests it is a 2*E*,4*E*-dienamide, the presence of dodecanoic acid as a major component of its reduced hydrolyzed products, and the spectral data from mass spectrometry (MS) and ^1H NMR, shows that component **5** is *N*-isobutyl-2*E*,4*E*-dodecadienamide which is being named here kalecide after one of the local Ghanaian names of *P. guineense*, Kale. This compound has not been previously isolated from *P. guineense*. The only reported isolation of this compound from a plant source (*P. peepuloides)* was by Dhar and Raina [7]. The spectral data of component **3** are consistent with those reported for guineensine from *P. attenuatum* by Dasgupta and Ray [8]. The isolation of guineensine from *P. guineense* has been reported previously by Okogun and Ekong [9]. The spectral data of component **1** were consistent with those obtained for pipercide isolated from *P. nigrum* [3]. However, pipercide has so far only been detected in *P. guineense* as a lower homologue and a contaminant in the mass spectrum of guineensine [9]. The present work is the first report of the actual isolation of pipercide in a reasonably pure form from *P. guineense*. The isolation of N-pyrrolidyl-12-(3,4-methylenedioxphenyl)-2*E*,4*E*,9*E*,11*Z*-dodecatetraenamide, *i.e.* component **2**, has not been previously reported. This amide is being given the trivial name guineensinamide. Okogun and Ekong [9] suggested from GC–MS of a hydrogenated mixture of amides, the presence of an amide with M^+ 367, which was indicated to be an isobutylamide of eicosanoic acid. The IR and ^1H NMR spectral data obtained for the pure compound isolated with M^+ 367 in the present work shows this compound lacked an –NH group and hence could not be an isobutyl amide. Furthermore, the fragmentation pattern obtained from the mass spectrum was not compatible with the structure of the compound being an isobutylamide of eicosanoic acid. The UV (λ_{max}) at 259 and 296 nm obtained for component **2** is also indicative of the presence of a *Z*-configuration [10].

Isolation procedures involving solvent extraction, column chromatography, TLC and preparative TLC have so far been employed in phytochemical work on *P. guineense*. Most of the studies undertaken which were aimed at the isolation of insecticidal principles from *Piper* species have concentrated on extracts from the fruit. The fact that the isolation of guineensinamide, kalecide and pellitorine from *P. guineense* has hitherto not been reported (pipercide was only reported as a contaminant of guineensine) may partly be due to the relatively low content of these compounds in the plant materials examined. The use of HPLC enabled such components to be isolated in the present work. It must be noted, however, that Addae-Mensah *et al.* [11] did not report the presence of pellitorine in the light petroleum extracts of the root, though the present work shows it is present in the root extract in substantial quantities. Considering the extensive phytochemical work done on *P. guineense*, the absence of any reports on the isolation of pellitorine, kalecide and guineensinamide is significant since unlike previous work on this plant, the present work involved bioactivity-directed isolation of the compounds. The two pairs of homologues isolated and the presence of several successive peaks in all the HPLC chromatograms may suggest the presence of several homologous series of compounds. TLC–HPLC has therefore facilitated the isolation of a number of amides which have hitherto not been reported in *P. guineense*, hence may be the most suitable method for isolating components, particularly the minor yet insecticidally active components from *P. guineense* and related species.

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