ORIGINAL RESEARCH ARTICLE

Pharmacognostic study and peptidomic analysis of the leaves of Nigerian *Rauvolfia vomitoria* Wennberg (Apocynaceae)

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Abstract
This study was aimed at documenting some pharmacognostic and peptidomic standards on the leaf of Nigerian species of *Rauvolfia vomitoria* Wennberg (RVM). Using standard methods, the fresh and dried pulverized leaves of *R. vomitoria* were standardized macroscopically and microscopically, in addition to the physiochemical evaluation and MALDI TOF/TOF-guided peptidomic screening of the aqueous extracts of the leaf. The leaf is simple, elliptical shaped, crenated margin which is acute. The leaf presented a hexagonal shaped epidermal cell on both sides with anomocytic stomata only on the abaxial surface making it hypostomatous while stomata number and index are 15 – 16 – 18 and 24 – 25 – 26, respectively. The transverse section of the leaf through the midrib suggests that the vascular bundles are conjoint, collateral and closed. Moisture content, total ash, acid insoluble ash, water soluble ash, sulphated ash, water soluble and alcohol soluble extractives were as follows; 7.7 ± 1,5, 4.9 ± 0.7, 1.2 ± 0.8, 4.1 ± 0.2, 4.8 ± 0.9, 3.9 ± 0.9, 8.1 ± 1.7w/w%, respectively. Peptidomic analysis revealed the abundant expression of nature-derived knottin-like peptides with a mass range of 3.1 kDa – 3.7 kDa. Knottin peptides have been reported to perform defense roles in host plants; since they are not ubiquitously distributed *in planta*, their detection in RVM could be useful in the proper identification of the plant and in RVM chemosystematics.

Findings from this study has unveiled some important macroscopic, microscopic and physicochemical standards as well as a new peptidomic phytochemical standard helpful in the proper identification in addition to ensuring the efficacy and safety of the leaf of *R. vomitoria*.

Keywords: *Rauvolfia vomitoria*; Macroscopy; microscopy; physicochemical evaluation; knottin-like peptides

Introduction

The acceptance of the utilisation of herbal medicines is partly dependent on their standardization, on which efficacy as well as safety of medicines largely depend [1]. The quality control of botanicals is vital for assurance of efficacy and quality. Pharmacognostic analysis, which includes macroscopical, microscopical and physiochemical parameters, is one of the simplest and cheapest way of determining the authenticity of botanical drugs [2].

Rauvolfia vomitoria Wennberg, (accepted name: http://www.worldfloraonline.org/taxon/wfo-0000295213) whose old name commonly used in the literature is *Rauwolfia vomitoria* Afzel, belongs to the family Apocynaceae. It is a co-generic species of the popular *R. serpentina* from which reserpine, an important ingredient in antihypertensives was first isolated [3]. The plant is commonly known as 'serpent wood', serpent root, and swizzle stick. It is locally known as 'asofeyeje' (Yoruba) 'wada' (Hausa), akanta' (Igbo) in Nigeria [4]. It is a shrub, which grows well in the rainforest of Pacific, South America, Asia and Africa [3-5]. All the parts of this plant are employed in traditional medicine [3,6]. The roots and the leaves of *R. vomitoria* are consumed as tisane and employed ethno medicinally for hypertension, insanity, depression and cholera [3,7,8]. In Nigeria, it also has reputation for treating fever and as an antidote for snakebite. The powdered root is popular in Diarrhoea and dysentery treatment, while the latex from the leaves is used in management of parasitic skin diseases. The bark is also known for its purgative and emetic properties in Ghana and Nigeria. Other uses include convulsion, jaundice and gastrointestinal troubles [9]. The biological activities reported for this plant include anthelmintic, cytotoxic, anticancer [10,11], antimycobacterial, antioxidant activity [4,12], hepatoprotective [13], antispasmodial [14] and antipyretic [15,16] effects. About 50 alkaloids have been isolated from this plant, among which are reserpine, ajmaline, deserpidine, corynanthine, rescinnamine and yohimbine [3,17,18]. The Genus *Rauvolfia* comprises several species including the African *Rauvolfia vomitoria* which are more distributed across the tropical regions of the World [19]. In Nigeria [20,21] as well as other tropical African countries [22]. Despite the enormous importance of the leaf in traditional medicine as well as its validated biological activities, there lacks well-documented evidence of any study on the pharmacognostic characters and peptidomic data of the leaf of this plant except partially for its root and that of the root of its co-generic species; *R. serpentina* [23,24,25,26]. This study, therefore, provides the first report on the pharmacognostic study and peptidomic analysis of the leaf of the Nigerian species of *R. vomitoria*.

Materıals and Methods

Identification and collection of the leaves of Rauvolfia vomitoria

The leaves of *R. vomitoria* were collected in November 2020 from Medicinal plant garden of the Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, authenticated at the herbarium of the Department of Pharmacognosy and Herbal

Medicine, Faculty of Pharmacy, Niger Delta University, Bayelsa State, with an herbarium specimen voucher number NDUP-2020-06.

Preparation of the leaves of *R. vomitoria*

Fresh leaves of *R. vomitoria* were dried in the oven at 35˚C and thereafter pulverized. This was employed for the evaluation of the physicochemical parameters and powdered microscopy.

Macroscopic Evaluation

The macro morphological description of the fresh leaves of *R. vomitoria* was done with the aid of the naked eye as described in the literature [27].

Microscopic Evaluation

The anatomical description of fresh leaf surfaces, transverse sections and that of the coarse powder was made as earlier reported and visualized under a light microscopy [28]. The pictures were taken using a digital camera attached to the microscope.

Physicochemical parameters

Evaluation of moisture content by loss on drying method, total ash, sulphated ash, acid insoluble ash and water-soluble ash employing a Muffer's furnace at 600 °C, water and alcohol soluble extractives using standard method [2,29] was separately carried out on 1g of powdered sample. Each parameter was carried out in ten replicates.

Knottin Peptide Extraction and Identification

The leaves of *Rauvolfia vomitoria* Wennberg (RVM) (Apocynaceae) were collected, and dried. Powdered RVM plant material was extracted using methanol:dichloromethane (1:1; v/v) for 24 h with occasional shaking at 25˚C. Following filtration, half volume of double distilled water was added to the filtrate and the mixture centrifuged for 3 min at 1000 rpm. The separated aqueous phase was collected using a separating funnel. This resulting aqueous fraction was further diluted to less than 10% MeOH with solvent A which is made up of 100% double distilled water and 0.05% trifluoroacetic acid for C_{18}-packed flash pre-purification. The C_{18}-packed cartridges (Strata Gigatubes C_{18}-E; 5 g, 20 mL, Phenomenex, Germany) used were first preconditioned with one volume of MeOH, followed by activation with one volume of solvent B (10% ddH_2O in acetonitrile and 0.05% trifluoroacetic acid) and equilibration with two volumes of solvent A. To free the C_{18}-packed column from contaminants and phenolic compounds, it was washed with 20% solvent B and putative bound peptides were further eluted with 20 mL of 80% solvent B. Lyophilisation of the eluates was done using the lyophiliser after pipetting out 200 µL each of the 20% and 80% eluates for preliminary crude MALDI-TOF MS analysis of knottin peptide-like masses. To achieve this, 0.5 µL each of the eluates was properly mixed with 3 µL of matrix (alpha cyano hydroxyl cinnamic acid) and spotted on the MALDI target plate. The plate was then allowed to dry well in the dark. MALDI spectra acquired were processed using the 4800 Analyzer [30].

RP-HPLC fractionation of C_{18} peptide-rich eluate

The lyophilised plant material was dissolved in a moderate volume of buffer A (0.05% trifluoroacetic acid prepared in distilled water). The solution was further filtered before purification using semi-preparative RP-HPLC on Dionex Ultimate 3000 HPLC unit (Dionex, Amsterdam, The Netherlands) with the following specification: 250 mm × 10 mm; 5 µm, 100 Å, Kromasil column. A Linear gradient of 0.1 – 2% min^{-1} solvent B at a flow rate of 3 $mLmin^{-1}$. Fractions collected at intervals were analyzed for peptide masses using the MALDI TOF/TOF analyzer following the earlier described procedure. Fraction of interest was freeze dried and reconstituted in solvent A for analytical HPLC (flow rate of $1mLmin^{-1}$) to characterize peptides for hydrophobic behaviour [30].

MALDI-TOF MS-guided Reduction/Alkylation of knottin peptides

Derivatization of peptides and MALDI TOF/TOF analysis was carried out by first reducing the disulphide linkages within the peptide with 2 μL of dithiothreitol after reconstituting approximately 6 nmol of the lyophilized peptides in 20 μL of 0.1 M ammonium bicarbonate (pH 8.2), and incubating at 65 °C for 10 min. The reduced sample was further alkylated with 4 μL of iodoacetamide. 1 μL of TFA was then used to stop the reaction. MALDI-TOF/TOF analyzer was further used to confirm the chemical modification of the reduced and alkylated peptides. Kalata B1 was used as positive control [30,31].

Enzymatic digestion of peptides and MALDI TOF/TOF analysis

The reduced and alkylated peptides were enzymatically digested with endoproteinase Glu-C and trypsin (Sigma-Aldrich, Austria). To stop the reduction and alkylation reaction prior to enzymatic digest, 1 μL of formic acid was added and incubated for 10 min at 37 °C. Again, 2 μL of the enzyme was further added to the reaction mixture and incubated for 3 – 14 h with gentle shaking and the reaction was quenched with 1 μL of formic acid. To analyse for the modified peptides, 0.5 μL of the sample was mixed with 3 μL of cinnamic matrix and spotted on the MALDI target plate for MALDI TOF analysis. Explorer 4800 software was applied to process all acquired spectra [30,31].

Results

Macroscopic parameters

The macroscopic description of the leaf of *R. vomitoria* is shown on Plate 1 and in Table 1.

a b c

Plate 1: *R. vomitoria* a: shrub, b: branch, c: leaf u: upper and l: lower surfaces growing on the medicinal garden of the Department of Pharmacognosy & Herbal Medicine, Niger Delta University, Wilberforce Island, Bayelsa

Table 1: Macroscopic parameters of the leaf of *R. vomitoria*

Parameter	Description
Petiole	Present
Arrangement	Opposite
Composition	Simple
Shape	Elliptical
Apex	Acute
Base	Acuminate
Margin	Crenate
Texture	Smooth, glabrous
Dimension	13.80 cm x 5.83 cm
Colour	Green

Microscopic parameters

The qualitative and quantitative microscopic descriptions of *R. vomitoria* are as presented on Plate 2 and in Table 2.

A B C

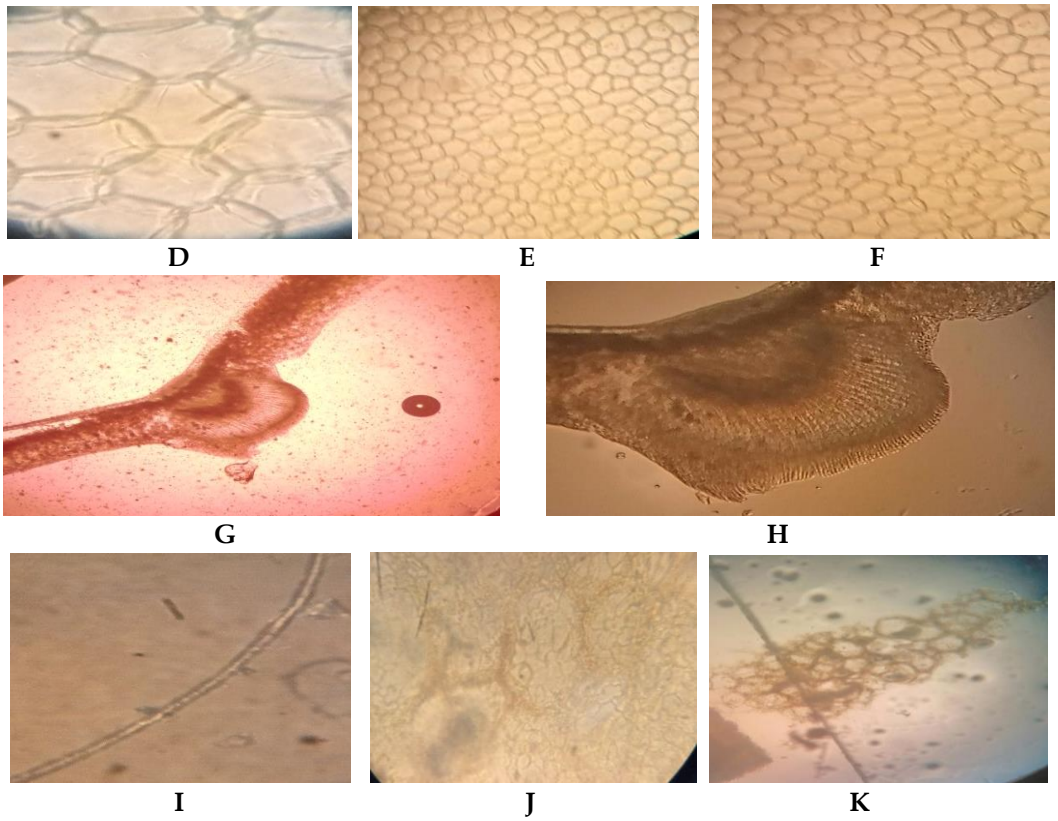

Plate 2: A - C Abaxial epidermis, Plate D - F- Adaxial epidermis, Plate G-H- transverse section (C- collenchyma, XV- xylem vessels, LE/UP- lower and upper epidermis), I - K - powdered leaf (trichome) of *R. vomitoria*.

Table 2: Quantitative microscopy of *R. vomitoria* leaf

Parameter	Minimum	Mean	Maximum
Stomata number	15	16	18
Stomata Index (%)	24	25	26

Physicochemical parameters of *R. vomitoria* leaf

The result obtained from the physicochemical evaluation of the powdered leaf of the plant is as shown in Table 3.

Table 3: Physicochemical parameters of *R. vomitoria* leaf

Parameter	% w/w±SEM
Moisture content	7.7± 1.5
Total ash	4.9 ± 0.7
Acid-insoluble ash	1.2 ± 0.8
Water-soluble ash	4.1 ± 0.2
Sulphated ash	4.8 ± 0.9
Water-soluble extractive value	3.9 ± 0.9
Alcohol-soluble extractive value	8.1 ± 1.7

Knottin Peptide Extraction and MALDI TOF MS-guided Identification

Powdered leaves of RVM was extracted using methanol, dichloromethane and water; the aqueous upper portion was freeze dried to yield the crude peptide-rich aqueous extract while the lower dichloromethane layer was discarded. The knottin-rich peptides in the aqueous extract were obtained by pre-purification using the solid phase extraction and the peptides were identified by the matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS) using their unique isotopic distribution and low mass range. Interesting masses ranging from 3.1 KDa – 3.7 KDa were observed (Figure 1). To identify and group these peptides into the disulphide-rich knottin peptide family, the peptide-rich crude extract was chemically derivatized by deithiothreitol-induced reduction and iodoacetamide-triggered alkylation which resulted in a mass shift of +348 Da (Figures 2). Enzymatic digestion with endoproteinseGluC and trypsin followed to confirm peptide linearity, presence of glutamic acid and arginine residues. This produced fragmentation of peptides (Figure 3) as observed with linear peptides instead of a +18 Da modification observed with cysteine-rich circular peptides. The characteristic hydrophobic behaviour of stable knottin peptides was evident in their late elution profile which fell within 25% - 50% acetonitrile in RP-HLC (Figure 4).

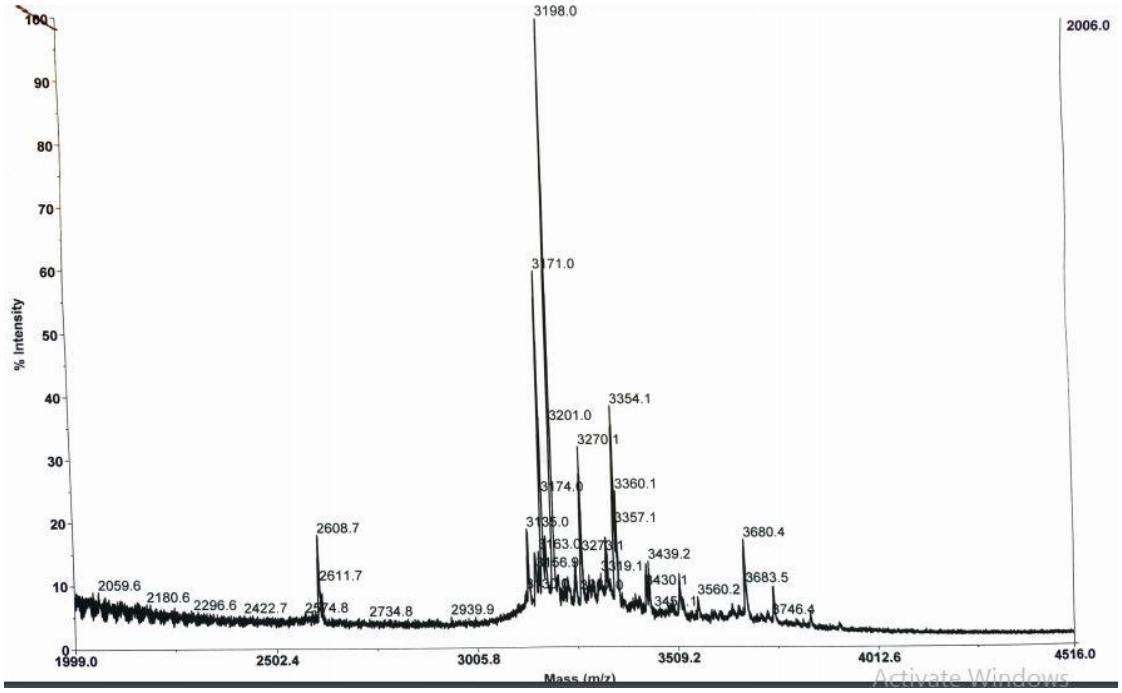

Figure 1: MALDI TOF MS profile of knottin peptide-rich crude extract of *Rauvolfia vomitoria Wennberg (RVM) (Apocynaceae)* showing isotopic distribution of peptide masses within the mass range of 3.1 kDa – 3.7 kDa which has been obtained from the elution of bound peptides using 80% acetonitrile in solvent A (100% double distilled water and 0.05% trifluoroacetic acid).

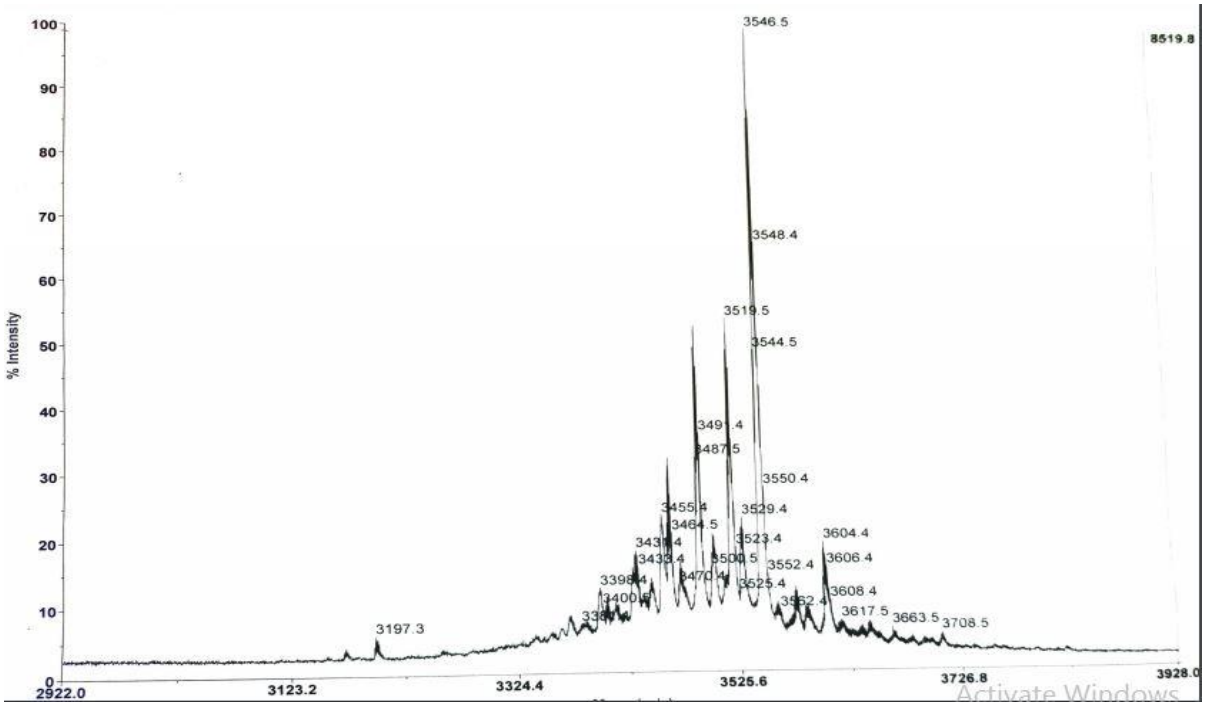

Figure 2: MALDI TOF MS profile of knottin peptide-rich crude extract of *Rauvolfia vomitoria Wennberg (RVM) (Apocynaceae)* after reduction and alkylation showing a typical mass shift of +348 Da following chemical modification achieved by the incubation of peptides with dithiothreitol and iodoacetamide

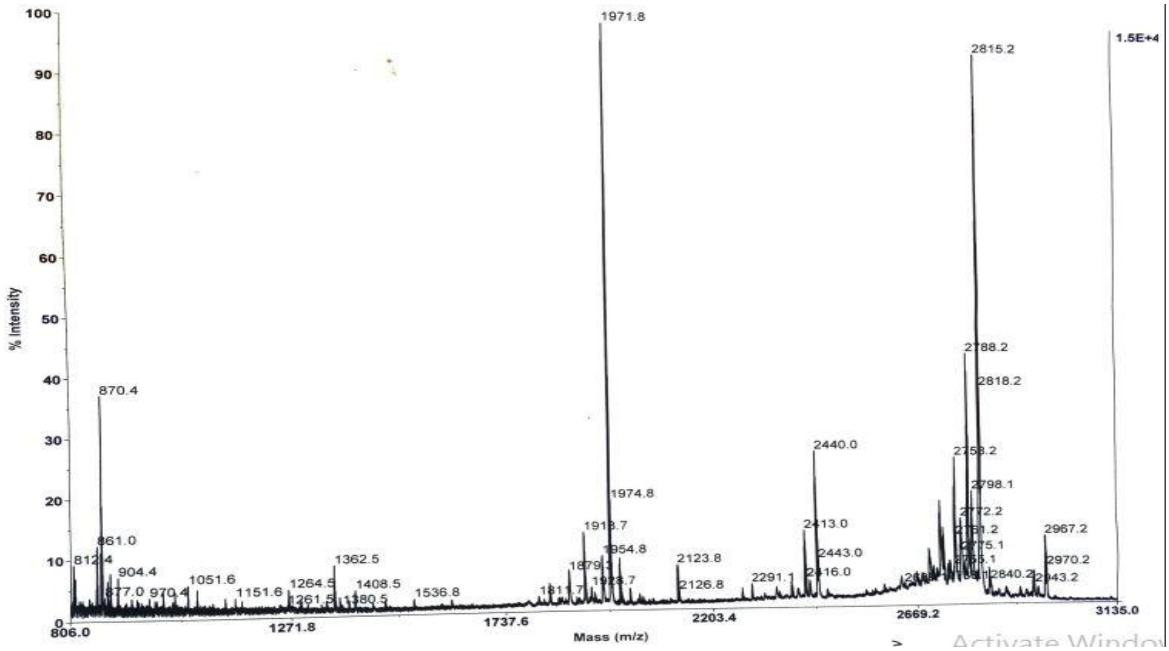

Figure 3: MALDI TOF MS profile of knottin peptide-rich crude extract of *Rauvolfia vomitoria Wennberg (RVM) (Apocynaceae) after digestion*; showing heavy fragmentation of the earlier reduced and alkylated peptide masses; an addition of +18 Da was absent indicative of the possible linearity of the peptide and if circular, the putative presence of more than one glutamic acid in the digested peptides

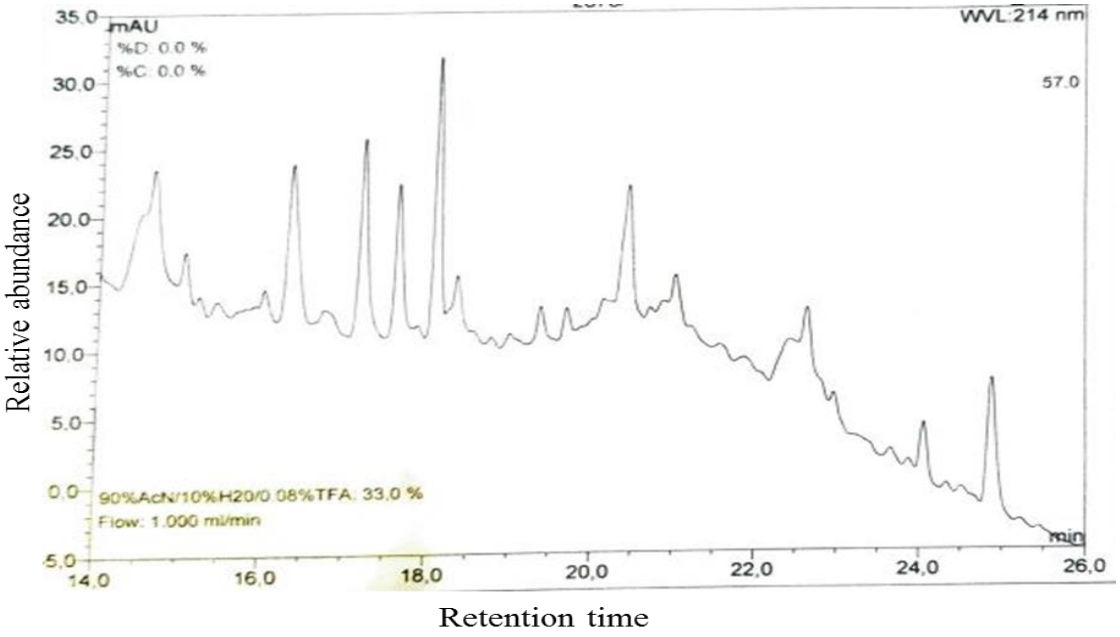

Figure 4: Analytical RP-HPLC of crude knottin-rich peptide extract of *Rauvolfia vomitoria* showing the hydrophobicity behaviour of the peptides and late elution profile of the 80% peptide optimised aqueous fraction obtained by solid phase extraction of C_{18}-bound peptides using 80% buffer B (10% ddH_2O in acetonitrile and 0.05% trifluoroacetic acid

Discussion

The plant is a shrub with simple leaves which are almost elliptical in shape, acute apex, acuminate base and crenate margin. Anomocytic stomata are present only on the lower epidermis (Plate 2(A-C)), which then makes the leaf hypostomatous. Nearly all species of the plant have stomata only on the abaxial surface. The presence of anomocytic stomata has also been reported in other plant species such as *Catharanthus roseus* (L.) G. Don and *Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult. from the Apocynaceae family [32]. Both surfaces show the presence of straight walled hexagonal epidermal cells (Plate 2(A-F)). The transverse section of the leaf through the midrib and lamina shows that the vascular bundles are conjoint, collateral, closed with numerous uniseriate trichomes present. The presence of trichomes is also shown in the powdered leaf (Plate 2(I-K)). The values of the total and acid insoluble ash (4.9 and 1.2% w/w, respectively) are below the maximum limit of 14 and 2%w/w stipulated for total and acid insoluble ash, respectively by the European Pharmacopeia [33]. Low acid -insoluble ash value indicates that a very small amount of the inorganic component is insoluble in acid. This indicates that adulteration by materials such as silica and stones, is low, and a low value may also affect the amount of the component absorbed in the gastrointestinal tract when taken orally. The moisture content of the powder plant is within the acceptable maximum limit of 10% [33], thus implying that the powder drug should not be susceptible to endogenous enzymatic attack and should withstand a long period of storage without microbial attack. Alcohol-soluble (8.1%w/w) and water-soluble (3.8%w/w) extractive values obtained in this study (Table 3) show that alcohol is a better solvent of extraction for the

leaf of this plant. Furthermore, both values generally help to give an idea about the quality of the plant material.

Recently, Zhan and colleagues [34] characterzed a cytotoxic yohimbine-type alkaloid from the aerial parts (leaf) of *Rauvolfia vomitoria*. These alkaloids-rich extract from the plant has demonstrated potent antimicrobial activities and cytotoxic potentials. However, it is not known if knottin peptides identified in this plant play a synergistic role via a different mechanism for the observed antimicrobial and cytotoxic activity of the leaves. Further research is therefore needed to unveil this possibility. The aqueous extracts of *R. vomitoria*, which is rich in these reported knottin peptides, demonstrated antickling activity and very poor *in vivo* oral acute toxicity [17] which may suggest target specific activity and selectivity commonly known with peptide therapeutics. Although Gruber et al. [35] reported the presence of cyclotide-like peptides in pressed herbarium samples of *Rauwolfia vomitoria*. This is the first report on the identification of linear disulphide-rich knottin-like peptides in field collections of Nigerian *Rauwolfia vomitoria* species. This class of peptides whose defence roles in host plant has been established also contributes to the structural resilience of the leaf vasculature where they are most often unevenly distributed [35,36].

Knottin peptides, which are also classified as cysteine-rich peptides (CRPs), particularly those of plant origin are emerging pharmacological tools whose molecular weights are lower than typical protein therapeutics. Bioactive knottin peptides are structurally and functionally endowed with an optimized on-target specificity and a lower off-target toxicity [38,37]. In this study, we applied MALDI TOF MS-led peptidomic analysis of peptide optimized aqueous fraction of RAV to detect peptide masses whose reduction and alkylation produced a mass shift of 348 Da indicating cystine bonds comprising 6 cysteine residues in 3 disulphide linkages following reduction with dithiothreitol and iodoacetamide alkylation of six reduced sulfhydryl radicals [39]. Following the biophysical properties of these peptides, in particular, their hydrophobic behaviour on RP-HPLC, cysteine content, mass range and interesting stability profile, these peptides appear to be members of the cystine knot peptides which may be knottins or hevein-like peptides, having a knotted disulfide linkage bridging the cysteine residues at position I−IV, II−V, and III−VI (11, 13, 20). This study applied MALDI TOF MS-guided peptidomic analysis to identify cysteine-rich knottin-like peptides in RAV whose thiol bonds confers additional stability for application in peptide drug discovery. While the enzymatic digestion of native peptides with endoproteinase Glu-C was effectively resisted, the digestion following reduction and alkylation of peptides resulted in their fragmentation. This suggests that the peptides may belong to the cysteine knot inhibitor (ICK) family [30], which Gressent et al, [40] reported as bioactive and linear, containing six cysteine residues and showing high stability profiles after boiling and rigorous solvent extraction steps [40, 41]. Since *Rauvolfia vomitoria* leaf nature-derived peptides are not ubiquitously

distributed in plants, their detection in the leaf could be useful in plant identification and chemosystematics [42].

Conclusıon

Standardization of medicinal plants will continue to be increasingly relevant in an attempt to ensure safety and efficacy of phytomedicines [43]. This study provides some acceptable macroscopic, microscopic and physicochemical standards as well as a uniquely new peptidomic data that are vital for the correct identification of the leaf of *R. vomitoria*. These standards may be incorporated into the Nigerian Herbal and West African Herbal pharmacopeias.

Findings from this study have unveiled for the first time the occurrence of low molecular weight (<4000 Da) and highly stable linear knottin peptides in a medicinally important species of the Apocynaceae family of flowering plants collected from Nigeria. Therefore, a further in-depth and elaborate knottin peptide mapping, abundance and distribution in *R. vomitoria* leaf vasculature as well as their bioactivities following their ethnophamacological data including antisickling bioactivity will be required for the clinical application of the plant.

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