

CHEMICAL DETECTION OF CYSTEINE-RICH CIRCULAR PEPTIDES IN SELECTED TROPICAL VIOLACEAE AND MORINGACEAE FAMILIES USING MODIFIED G-250 AND MASS SPECTROMETRY

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ABSTRACT

Cysteine-rich circular peptides (CRCs) comprise a large family of gene encoded and low molecular weight polypeptides that has recently engaged the attention of scientists. This class of peptides exhibit a continuous circular configuration and a cystine knot backbone, which defines their resilient nature-directed structural design. Many CRCs have been reported in medicinal plants the first of which is kalata B1 cyclotide from the traditional African plant *Oldenlandia affinis*. Their detection and isolation can be very challenging and evasive. Only about 1% of plant species have so far been reportedly screened. A modified preliminary chemo-microscopic/macrosopic method involving the use of G-250 stain was applied followed by thin layer chromatographic protosite reaction for plant selection. This was followed by the Matrix Assisted Laser Desorption Time of Flight (MALDI-TOF) Mass spectrometry guided experiment for cyclotide discovery. The blue colouration produced upon sample reaction with modified G-250 led to the selection of potential circular peptide containing plant samples. A further MALDI-TOF MS-guided screening resulted in the detection of circular peptides and cyclotides in *Moringa oleifera*, *Rinorea dentata*, *R. oblongifolia* and *R. brachypatela*. *Viola odorata* and *Viola tricolor* (positive controls) indicated the presence of cyclotides. Results from this study can serve as proof-of-concept for plant selection based on preliminary cysteine-rich circular peptide detection in plants especially with the use of G-250 stain.

INTRODUCTION

Peptide-based natural products (PNPs) have received greater research attention following breakthroughs in peptide/protein based therapeutics. For instance, cysteine-rich circular peptides and cyclotides have shown promising potentials for drug development, drug delivery, agricultural plant protection/food security and molecular imaging applications. These classes of peptides are of particular interest due to their occurrence, diversity, abundance, distribution, stability, varied biological activity and sequence plasticity. However, their efficient discovery could be very laborious and time consuming, requiring very sensitive facilities that may not be readily accessible to many laboratories in the tropics where natural biodiversity is high. Thus, a divergent discovery approach can become handy in addressing the above identified short comings.

Drug discovery from natural products has recently received accelerated attention as a result of their pharmacologically enhanced structural complexity and often minimal side effect. The discovery of cysteine-rich cyclotides in plants over a decade has greatly enhanced and diversified the potential for drug discovery from nature beyond protein biologics and secondary compounds. A vast number of peptides and proteins produced in plants are required for daily biological functions. The abundance of these peptides in plants and their physiological roles as signalling molecules is a huge potential source of therapeutic agents or lead compounds; these roles are nevertheless often limited by their widely observed instability. However, cysteine-rich circular peptides/cyclotides (CRCs) are ultra stable and can withstand extreme conditions of temperature, chemicals and enzymatic treatment. Cyclotides are a unique class of cysteine-rich low molecular weight mini-proteins made up of approximately 30 amino acids forming a circular peptide backbone and a knotted

arrangement of three disulphide bonds [1]. Three cystine bridges of six cysteine residues stabilize the structure to form a cyclic cystine knot (CCK) motif. This motif consists of an internal ring resulting from two of the three disulphide bonds, while their connecting backbone segment is threaded by the third disulphide bond [1,2]. The CCK motif is therefore a product of the combination of the cystine knot and cyclic backbone. This motif structure imparts an exceptional stability on cyclotides to withstand extreme conditions of heat, chemical and enzymatic attack [1,2,3,4]. The CRCs have attracted keen research interest as a result of their enormous stability, their intrinsic bioactivity, their natural abundance, sequence diversity and distribution in plants. They have been reported to fulfil the basic drug delivery criteria of stability and bioavailability [5,6]. The reported distribution of Cyclotides in the plant kingdom includes such families as Rubiaceae (coffee), Violaceae (violet) Cucurbitaceae (cucurbit), Solanaceae and Fabaceae (legume). The occurrence and ubiquity of cysteine-rich cyclotides in every Violaceae species so far screened has been reported [7,8]. Over 250 sequences of these ultra stable peptides are available in cybase [9]. Cybase is an online data bank (www.cybase.org.au) dedicated to cystine stabilized circular and linear cyclotides of low molecular weight. The folkloric use of the Rubiaceae plant *Oldenlandia affinis* [10,11] led to the discovery of the first cyclotide. The active uterotonic agent, Kalata B1 was partially characterized and identified as cysteine-rich protein containing about 30 amino acids. The full sequence comprising the cystine knot and macrocyclic structure was determined 25 years later [12,13]. This new family of mini protein was named "Cyclotide" in 1999 as proposed by David Craik and Norelle Daly [14]. Furthermore, recent discoveries of cyclotides from plants result from set out protocols starting from

plant selection and Mass spectrometry-guided phytochemical screening experiments for the identification of these macrocyclic mini-proteins in plant families [7,15]. This could be laborious and time consuming. Even more recently, a combination of genomic, transcriptomic and peptidomic analyses are applied to identify, characterize and document cyclotide distribution in plant families [16,17,18,19]. Based on an estimate, cyclotides occurrence in plants may be in their thousands [7]. Even more recently combined laboratory and bioinformatic approaches have been applied to conservatively predict more than 150,000 cysteine-rich cyclotides in Violaceae alone [25]. Moreover, cyclotides are not limited to some environment, geographies or climates; they are widely distributed in plants across the globe from the rain forest in Hawaii to the desert in Australia, Alps in Sweden [27] and tropical forest of Africa. Tropical Africa is host to one of the world's richest plant biodiversity. There is therefore a potential for a higher positive hits during cyclotides screening experiments in this region. However, to minimise waste, ensure efficiency and protect tropical plant biodiversity, a divergent discovery approach is desirable. This study adopted the divergent approach including macroscopic, microscopic and thin layer chromatographic chemical detection methods aimed at identifying these cysteine-rich polypeptides in host plants. Further, this preliminary step was followed by a mass spectrometry (MALDI-TOF)-guided experiment to partially characterise cysteine-rich circular peptides in selected plants.

RESULTS/DSICUSSION

Macroscopic/Microscopic tissue staining

The macroscopic staining of selected plant samples viewed with the aid of the hand lens enabled the observation of regions within

the leaf vasculature, which picked up the G-250 stain (Figure 1). Unlike *Vernonia amygdalina*, *Ocimum gratissimum* and *Duranta repens*, which did not pick up any observable dye stain of G-250, *Rinorea dentata* and *Rinorea oblongifolia* leaves both picked up the stain with different intensities. The intensity of the blue colour produced is directly proportional to the different time points as the experiment progressed. The colour produced was similar to the cyclotides-rich standards -*Viola odorata* and *Viola tricolor* suggesting the presence of circular peptides (Figure 1). In particular, the upper surface of the tested leaves appeared to respond faster to colour reaction as compared to the lower surface, which may be indicative of a higher expression of the circular peptides. The distribution of the observed blue colour on the leaf architecture is irregular and random. For instance, in *R. dentata*, one of the tested leaf samples, the modified dye was picked up in a unique manner lining the leaf from one side of the leaf blade towards the midrib. This may result from the difficulty of the stain to evenly penetrate the epidermal cells to locate the peptides. It may also result from the uneven expression and distribution of the peptides in the samples. In order to evaluate the possible masking effect of chlorophyll on visual judgement, samples were boiled in absolute ethanol and further softened in distilled water prior to dye application. Although visual identification of stained regions improved, it was not quite significant. Moreover, ethanol could extract some of the soluble peptides especially during the decolourization process. The duration for the commencement of colour reaction was from 20 minutes after immersion of the leaf samples into the dye vessel. Colour development proceeded until 60 minutes post immersion.

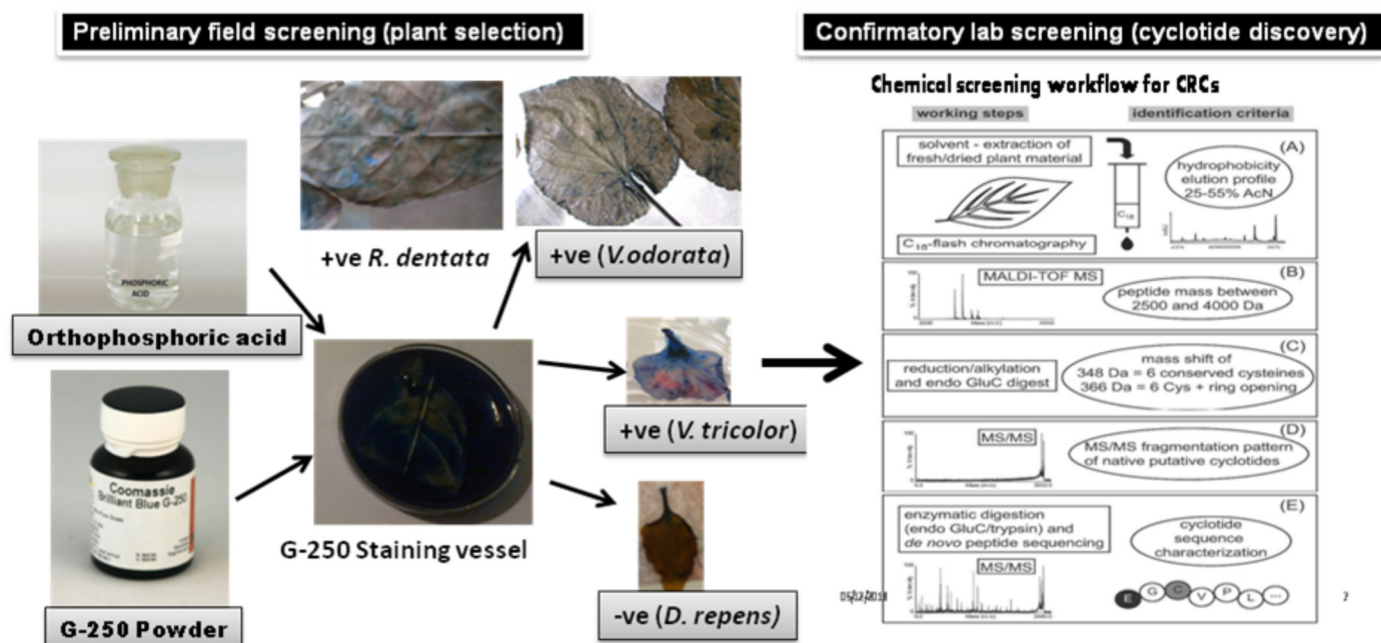


Figure 1: Preliminary Phytochemical screening involving a new on-site staining method (left) and an established discovery workflow (right) for cysteine-rich circular peptides and cyclotides from selected Nigerian plants [19]

Microscopic tissue preparation and staining

The microscopic colour reaction involved the epidermal peels as well as the transverse sections through the midrib of the tested leaf. Cells revealed by the surface preparation were independently stained with the G-250 stain and Safranin O. Few cells found within the epidermal layer of *R. dentata* leaves picked up the stain in a similar manner to that of *V. odorata*. Regions that picked up the stain were similarly randomly distributed as observed in the macroscopy. Epidermal cells of *O. gratissimum* and *D. repens* did not pick up the stain, which may signify absence of circular peptides. Cells lining the conducting vessels picked up more of the G-250 stain, while some isolated portions of the cells equally picked up the stain (Figure 2). The transverse section showed the

presence of covering trichomes, cortex, phloem and xylem vessels whose responses to G-250 varied (Figure 3). Unlike Safranin O, which poorly stained parenchymatous cells and cells lining the phloem vessels, the blue dye strongly stained the cells lining the conducting xylem vessels. However, the G-250 dye did not stain the parenchyma cells and the pith but strongly stained both cells of the phloem and the xylem vessels of *R. dentata* section (Figure 2). Thus, the epidermal cells and cells surrounding conducting vessels tend to pick up more G-250 stain than other parts of the section. This gives an idea of the distribution of the circular peptide molecules within the leaf with emphasis on the midrib region.

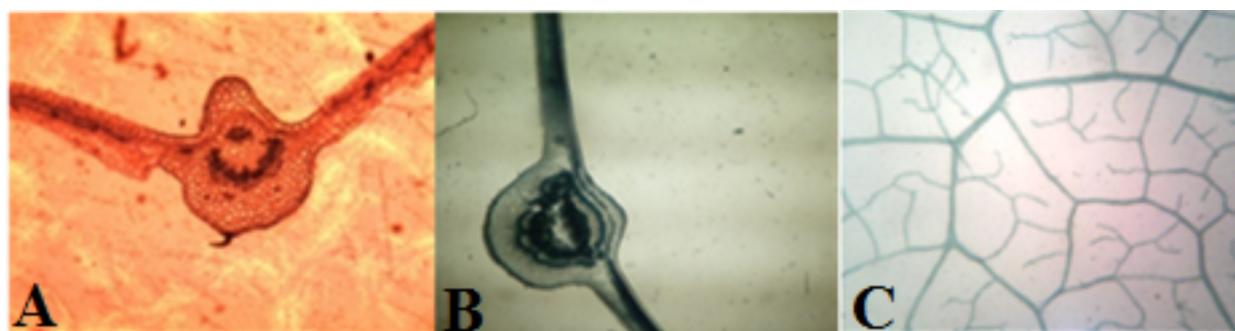


Figure 2 : Responses of *Rinorea dentata* (RD) cells to Safranin stain and modified G-250; (A) Transverse section(TS) of RD stained with Safranin O; (B) TS of RD stained with G-250; (C) Upper surface of RD showing intensely coloured veins

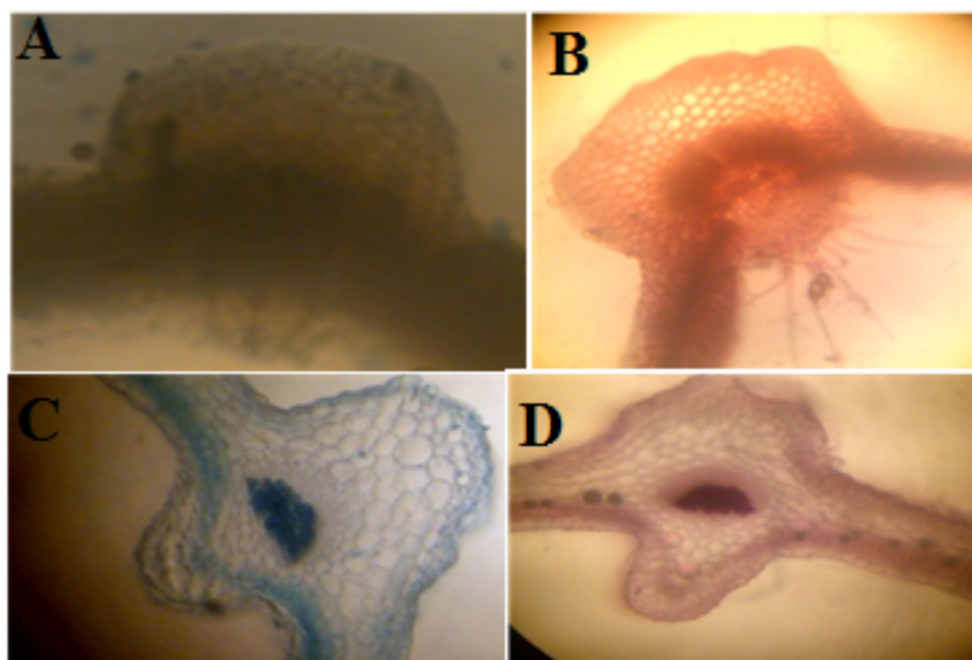


Figure 3: Differential responses of *Ocimum gratissimum* (A) and *Viola odorata* (B) to G-250 (A, C) and Safranin O (B, D) stains.

Thin Layer Chromatography

Isolated circular peptides from RD, *V. odorata* and *Moringa oleifera* produced a blue colouration on TLC plate upon reaction with modified G-250. Gluthathione (GSH: a linear peptide) did not produce such colour change. *Cajanus cajan* seed aqueous extracts

and *Saba comrensensis* extracts did not give this colour change. With ninhydrin, purple to red colouration was not observed with isolated circular peptides; however, purple to red colouration characterised GSH and *Cajanus cajan* isolated peptides (Plate 1).

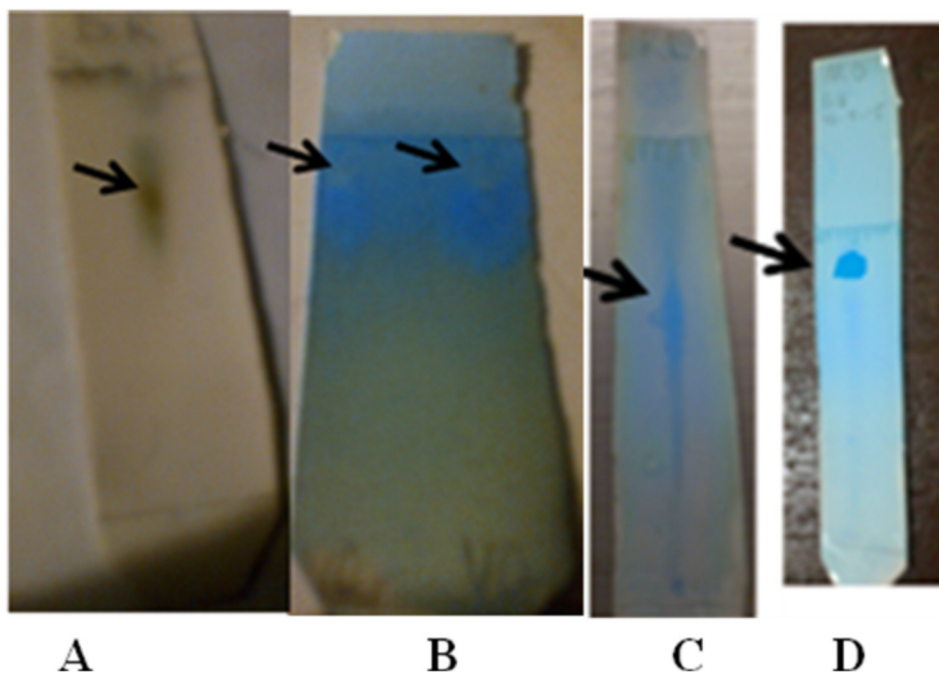


Plate 1: A- Isolated compounds from *Duranta repens* (producing no blue colour with G-250 due to absence of CRC peptides); B- Brightly coloured blue colouration of isolated CRCs from *Viola odorata* root/leaf (partially dispersed on plate); C- Poorly separated CRCs from *Rinorea dentata* (brightly coloured blue); D- Co-eluting circular peptides from *Moringa oleifera* seed (brightly coloured)

Mass spectrometry experiment

Mass spectrometry experiment (MALDI-TOF) was done to analyse plant extracts for circular peptide (Figure 4). Mass range of RD CRCs was between 2900Da to 4000Da. The hydrophobicity of the CRCs was evident in their late elution time on RP-HPLC (25% - 70% acetonitrile). Reduction and alkylation accompanied by MALDI MS analysis confirmed the cysteine content observed

by a mass shift of 348Da. Furthermore, digestion of ziptipped samples with endoproteinase GluC led to an additional mass shift of 18Da for several CRC masses, while others were fragmented. Fragmented samples may be linear cyclotides or cyclotides with more than one glutamic acid residues. Over 30 different CRC masses were identified (Figure 4).

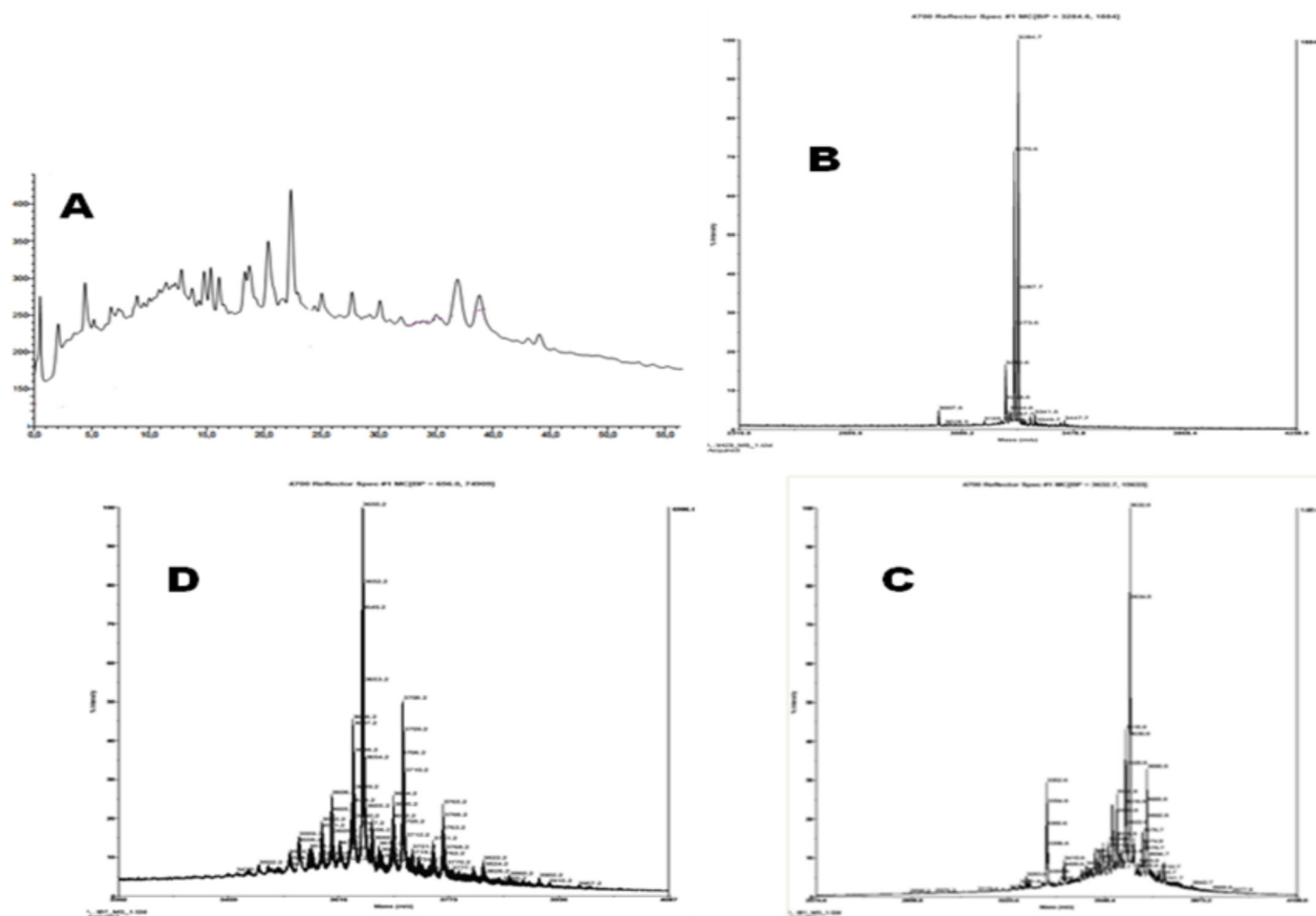


Figure 4: Identification steps for cysteine-rich peptide discovery. (A) RP-HPLC of 80% acetonitrile eluate of RD showing CRCs hydrophobicity; (B) MALDI-TOF native masses of the peptides; (C) Reduced/alkylated masses; (D) Peptide masses with open ring upon digestion with EndoGluC

A preliminary screening for cysteine-rich cyclotides using macroscopic and microscopical tissue staining alongside TLC chemical detection was adopted to select potential circular peptide containing plants. This was validated with a simple MS experiment using selected Nigerian plant species. The blue colour observed in many parts of the leaf surfaces especially along the midvein down to the petiole suggest that CRCs are present in RD leaves and may associate more with the petiole and midvein. To further prove this, the microscopic G-250 staining experiment showed that the epidermal cells and cells surrounding the conducting vessels picked up more of the dye than other parts of the transverse section. With the application of MALDI Mass spectrometry imaging, Poth et al [20] reported that cyclotides are found in leaves of *Pentunia* and associate more with the midvein than any other part of the leaf. The TLC isolation and chemical detection method with modified G-250 stain is a sensitive approach in CRCs identification [21,22,24]. Only CRCs are sensitive to the modified stain with a bright blue colour development. To demonstrate the reproducibility of this approach, we recently carried out an ethnobotanical survey of *R. dentata* and detected cyclotides using LC-MS, MALDI-TOF and MS/MS [23]. The MS experiment was important in order to validate and confirm CRC in the Nigerian

Violaceae family, which is reported to ubiquitously express cysteine-rich cyclotides [7,8]. The confirmation of CRCs in *R. dentata*, *R. oblongifolia*, *V. odorata*, *V. tricolor* and *M. oleifera* by MS experiment suggest the usefulness of modified G-250 dye as a preliminary on-site screening reagent for selecting likely CRC positive samples for further work. While this approach encourages sustainability, it combines the efficiency of time and cost.

CONCLUSION

This work represents the first report on the macroscopic and microscopic approach to cyclotides identification in plant tissues. It has provided further evidence for the occurrence and distribution of cysteine-rich circular peptides/cyclotides in the leaf surface of cyclotides producing plants.

MATERIALS AND METHODS

Plant collection

Rinorea dentata (RD) was collected from Omo forest, Ogun State in October, 2014. The plant was identified and authenticated at the Forest Herbarium Ibadan (FHI). Voucher specimens for *Rinorea*

dentata (FHI: 110151) and *Rinorea brachypatela* (FHI: 110152) were deposited at the herbarium. Other plants used were collected from the University of Ibadan botanical garden.

Preparation of the modified G-250 dye, ninhydrin

The G-250 dye was prepared according to the modified method described by Xu et al [21]. 200 mg of G-250 was dissolved in 40 mL ethanol. Then, 320 mL of 50% ethanol in distilled water was prepared and 40 mL of orthophosphoric acid was added. The ninhydrin reagent was 0.2 g ninhydrin in 100 mL of ethanol.

Macroscopic tissue staining

Freshly collected intact whole leaves of plant samples were used. Leaves obtained from *Duranta repens*, *Vernonia amygdalina* and *Ocimum gratissimum* served as negative controls; these plants have been reported negative for the presence of CRCs [19]. Decolourised or fresh leaf samples were immersed in a freshly prepared solution of modified Coomassie brilliant blue (G-250). The set up was allowed to stand for at least 1h and occasionally observed with the aid of a hand lens. Dye absorption by the leaves was noted.

Microscopic tissue preparation and staining

The epidermal peels from the adaxial and abaxial surfaces of plant samples as well as transverse sections through the midrib of leaves were obtained via free hand sectioning technique. These were transferred into distilled water containing 1% sodium hypochlorite. The peels were allowed to clear for few minutes and transferred to 100% distilled water. Dehydration followed using ethanol series. Samples of dehydrated sections were stained in Safranin O, mounted in dilute glycerol and viewed under the low and high magnifications. Other sections were transferred into the G-250 modified stain. Fully stained sections were viewed under the low and high power of the microscope. Microscopic features of diagnostic importance were noted. Photomicrographs were taken.

Solvent extraction

Solvent extraction was carried out following standard methods [19,27,28,29,30]. The identification workflow previously described by Gruber et al [7] and modified by Koehbach et al [19] was used. Pre-purification of peptide extract was carried out on RP-C₁₈ solid phase cartridges (strata giga tube C18-E; 5 g, 20 mL, Phenomenex, Germany). Bound peptides were eluted with 20 mL of 80% buffer B (90%v/v acetonitrile, 0.045% v/v trifluoroacetic acid).

Thin Layer Chromatography (TLC)

A modified method previously described by Xu et al. and Tan et al [21,26] and also used by Rahul et al [24] was adopted for the TLC chemical detection. Solvent system used was *n*-butanol:acetic acid:water (3:1:1). Precoated TLC plates (G₂₅₄ MERCK, Germany) were used. The solvent reconstituted aqueous peptidic fraction was spotted on the TLC plate and developed in the above solvent system. Plates were allowed to dry, viewed under UV at 254 and 365 nm. Plates were swiftly sprayed with/dipped in freshly prepared G-250 modified stain and ninhydrin. Aqueous extracts of *Duranta*

repens, *Ocimum gratissimum*, *Cajanus cajan* and *Saba comorensis* were used as negative controls.

Mass spectrometry experiment

The crude C₁₈ peptide-rich eluate was analysed by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for cyclotides-like masses (2.0 KDa – 4.0 KDa). The MALDI matrix consisted of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.5% aqueous formic acid. Samples were analyzed by MALDI-TOF mass spectrometry on a Voyager Applied Biosystem 4800 Mass Spectrometer [7].

Reduction and alkylation of peptides

The botanical peptide molecules were further analyzed using MALDI-TOF MS for their cysteine content via reduction and alkylation [7,19]. Lyophilized samples (about 0.5 μ g were dissolved in 0.1M NH₄HCO₃ buffer (pH 8.2). Freshly prepared dithiothreitol (2 μ L of 3.2 mg/100 μ L buffer) was added to aliquots of 20 μ L samples. This was briefly mixed and incubated in dark at 60°C for 30 min. Alkylation followed via the addition of 4 μ L of 0.5M iodoacetamide (9.25 mg/100 mL) to the reduced sample. The mixture was incubated for 10 min with continuous shaking. The reaction was discontinued after adding 1 μ L of 1% trifluoroacetic acid or concentrated formic acid. MALDI-TOF MS analysis followed.

Digestion of peptides

A positive mass shift of 348 Da following reduction and alkylation was used as criterion for digestion of the samples [7,19]. Reduced and alkylated samples were not quenched with TFA or formic acid. Instead, digestion of samples proceeded from this step. Endoproteinase Gluc (Sigma Aldrich, 50 μ g) was dissolved in 100 μ L of 0.01% acetic acid. Then, 2 μ L of the Gluc enzyme (1:10) was added to the above reduced/alkylated sample and incubated at 37°C for 3 h. Similarly, 2 μ L of Trypsin (promega, USA) was added to the reduced/alkylated sample, incubated at 37°C for 3 h. The reaction was stopped after 3 h by the addition of 1 μ L of TFA or formic acid. Digested samples were analyzed using the MALDI TOF MS.

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REFERENCES

1. Craik, D. J., Daly, N. L., Bond, T. and Waine, C. (1999). Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. *Journal of Molecular Biology* 294(5): 1327-1336.
2. Colgrave, M. L., and Craik, D. J. (2004). Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. *Biochemistry* 43(20): 5965-5975.
3. Göransson, U., and Craik, D. J. (2003). Disulfide Mapping of the Cyclotide Kalata B1 chemical proof of the cyclic cystine

- knot motif. *Journal of Biological Chemistry* 278(48): 48188-48196.
4. Rosengren, K. J., Daly, N. L., Plan, M. R., Waine, C., and Craik, D. J. (2003). Twists, Knots, and Rings in Proteins structural definition of the cyclotide framework. *Journal of Biological Chemistry* 278(10): 8606-8616.
 5. Craik, D. J., Anderson, M. A., Barry, D. G., Clark, R. J., Daly, N. L., Jennings, C. V., and Mulvenna, J. (2001). Discovery and structures of the cyclotides: novel macrocyclic peptides from plants. *Letters in Peptide Science* 8(3-5): 119-128.
 6. Thell, K., Hellinger, R., Sahin, E., Michenthaler, P., Gold-Binder, M., Haider, T., Kuttke, M., Liutkevičiūtė, Z., Göransson, U., Gründemann, C. and Schabbauer, G. (2016). Oral activity of a nature-derived cyclic peptide for the treatment of multiple sclerosis. *Proceedings of the National Academy of Sciences* 113(15): 3960-3965.
 7. Gruber, C. W., Elliott, A. G., Ireland, D. C., Delprete, P. G., Dessein, S., Göransson, U., Trabi, M., Wang, C. K., Kinghorn, A. B., Robbrecht, E., and Craik, D. J. (2008). Distribution and evolution of circular miniproteins in flowering plants. *The Plant Cell Online* 20(9): 2471-2483.
 8. Burman, R., Gunasekera, S., Stromstedt, A. A., and Göransson, U. (2014). Chemistry and biology of cyclotides: circular plant peptides outside the box. *Journal of Natural Products* 77(3): 724-736.
 9. Kaas, Q., and Craik, D. J. (2010). Analysis and classification of circular proteins in CyBase. *Peptide Science* 94(5): 584-591.
 10. Gran, L. (1973). On the effect of a polypeptide isolated from "Kalata Kalata" (*Oldenlandia affinis* DC) on the oestrogen dominated uterus. *Acta Pharmacologica et Toxicologica* 33(5 6): 400-408.
 11. Gran, L. (1973). Oxytocic principles of *Oldenlandia affinis*. *Lloydia* 36(2): 174-178.
 12. Sletten, K., and Gran, L. (1973). Some molecular properties of kalatapeptide B-1. A uterotonic polypeptide isolated from *Oldenlandia affinis* DC. *Medd Nor Farm Selsk* 7(8): 69-82.
 13. Saether, O., Craik, D. J., Campbell, I. D., Sletten, K., Juul, J., and Norman, D. G. (1995). Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. *Biochemistry* 34(13): 4147-4158.
 14. Craik, D. J., Daly, N. L., Bond, T., and Waine, C. (1999). Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. *Journal of Molecular Biology* 294(5): 1327-1336.
 15. Göransson, U., Herrmann, A., Burman, R., Haugaard Jönsson, L. M., & Rosengren, K. J. (2009). The conserved glu in the cyclotide cycloviolacin O2 has a key structural role. *ChemBioChem* 10(14): 2354-2360.
 16. Nguyen, G. K. T., Zhang, S., Nguyen, N. T. K., Nguyen, P. Q. T., Chiu, M. S., Hardjojo, A., and Tam, J. P. (2011). Discovery and characterization of novel cyclotides originated from chimeric precursors consisting of albumin-1 chain and cyclotide domains in the Fabaceae family. *Journal of Biological Chemistry* 286(27): 24275-24287.
 17. Trabi, M., Mylne, J. S., Sando, L., and Craik, D. J. (2009). Circular proteins from Melicytus (Violaceae) refine the conserved protein and gene architecture of cyclotides. *Organic and Biomolecular Chemistry* 7(11): 2378-2388.
 18. Gerlach, S. L., Göransson, U., Kaas, Q., Craik, D. J., Mondal, D., and Gruber, C. W. (2013). A systematic approach to document cyclotide distribution in plant species from genomic, transcriptomic, and peptidomic analysis. *Peptide Science* 100(5): 433-437.
 19. Koehbach, J., Attah, A. F., Berger, A., Hellinger, R., Kutchan, T. M., Carpenter, E. J., Rolf, M., Sonibare, M. A., Moody, J. O., Wong, G. K., Dessein, S., Greger, H., and Gruber, C. W. (2013). Cyclotide discovery in Gentianales revisited-identification and characterization of cyclic cystine knot peptides and their phylogenetic distribution in Rubiaceae plants. *Peptide Science* 100(5): 438-452.
 20. Poth, A. G., Mylne, J. S., Grassl, J., Lyons, R. E., Millar, A. H., Colgrave, M. L., & Craik, D. J. (2012). Cyclotides associate with leaf vasculature and are the products of a novel precursor in *Petunia* (Solanaceae). *Journal of Biological Chemistry* 287(32): 27033-27046.
 21. WenYan, X., Jun, T., ChangJiu, J., WenJun, H., and NingHua, T. (2008). Application of a TLC chemical method to detection of cyclotides in plants. *Chinese Science Bulletin* 53(11): 1671-1674.
 22. He, W., Chan, L. Y., Zeng, G., Daly, N. L., Craik, D. J., & Tan, N. (2011). Isolation and characterization of cytotoxic cyclotides from *Viola philippica*. *Peptides* 32(8): 1719-1723.
 23. Attah, A. F., Hellinger, R., Sonibare, M. A., Moody, J. O., Arrowsmith, S., Wray, S., and Gruber, C. W. (2016). Ethnobotanical survey of *Rinorea dentata* (Violaceae) used in South-Western Nigerian ethnomedicine and detection of cyclotides. *Journal of Ethnopharmacology* 179, 83-91.
 24. Rahul S., Chandrashekhar P., Hemant B., Chandrakant N., and Satish P. (2015) Screening of Rubiaceae and Apocynaceae extracts for mosquito larvicidal potential. *Natural Product Research: Formerly Natural Product Letters* 29(4): 353-358.
 25. Hellinger, R., Koehbach, J., Soltis, D.E., Carpenter, E.J., Wong, G.K., and Gruber, C.W. (2015). Peptidomics of Circular Cysteine-Rich Plant Peptides: Analysis of the Diversity of Cyclotides from *Viola tricolor* by Transcriptome and Proteome Mining. *Journal of Proteome Research* 14, 4851-4862.
 26. Jork, H., Funk, W., Fishcer, W., and Wimmer, H. (1994). *TLC Reagents and Detection Methods – Physical and Chemical Detection Methods: Activation Reactions, Reagent Sequences, Reagents II*, Vol 1b. New York: Wiley.
 27. Ireland, D. C., Clark, R. J., Daly, N. L., and Craik, D. J. (2010). Isolation, sequencing, and structure" activity relationships of cyclotides. *Journal of Natural Products* 73(9): 1610-1622.

28. Craik, D. J., Swedberg, J. E., Mylne, J. S., and Cemazar, M. (2012). Cyclotides as a basis for drug design. *Expert Opinion on Drug Discovery* 7(3): 179-194.
29. Tan, N. H., and Zhou, J. (2006). Plant cyclopeptides. *Chemical Reviews* 106(3): 840-895.
30. Goransson, U., Svangard, E., Claeson, P., and Bohlin, L. (2004). Novel strategies for isolation and characterization of cyclotides: the discovery of bioactive macrocyclic plant polypeptides in the Violaceae. *Current Protein and Peptide Science* 5(5): 317-329.