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Ethobotanical survey of *Rinorea dentata* (Violaceae) used in South-Western Nigerian ethnomedicine and detection of cyclotides

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ABSTRACT

Ethnopharmacological relevance: People living in the tropical rain forest of South-Western Nigeria use *Rinorea dentata* (P. Beauv.) Kuntze (Violaceae) in ethno-veterinary medicine to facilitate parturition. There are no evidence-based pharmacological investigations for the uterotonic activity of this plant.
Aims of study: (i) Collection of data about the ethnopharmacological uses of *R. dentata* and evaluation of its uses and applications in health care; (ii) determining potential uterotonic effects *in vitro*, and (iii) chemical characterization of *R. dentata*, which is a member of the Violaceae family known to express circular cystine-knot peptides, called cyclotides.
Materials and methods: The ethnopharmacological use of *R. dentata* in settlement camps within the area J4 of Omo forest has been investigated by semi-structured questionnaires and open interviews. Use index analysis has been performed by seven quantitative statistical models. Respondents' claim on the beneficial ethno-veterinary application of the plant to aid parturition has been investigated *in vitro* by myometrial contractility organ bath assays. The bioactive plant extract was screened by chemical derivatization and mass spectrometry-based peptidomics using reversed-phase HPLC fractionation and MALDI-TOF/TOF analysis.
Results: Based on the survey analysis, medicinal preparations of *R. dentata* have been used for antimicrobial and anti-malaria purpose in humans, and for aiding parturition in farm animals. The latter application was mentioned by one out of six respondents who claimed to use this plant for any medicinal purpose. The plant extract exhibited a weak uterotonic effect using organ bath studies. The plant contains cyclotides and the peptide riden A has been identified by *de novo* amino acid sequencing using mass spectrometry.
Conclusion: Few dwellers around the settlement camps of the tropical forest of Omo (Nigeria) use *R. dentata* for various health problems in traditional veterinary and human medicine. The weak uterotonic effect of the cyclotide-rich extract is in agreement with the low use value index obtained for this plant. Cyclotides have been reported in the genus *Rinorea* confirming the ubiquitous expression of these stable bioactive plant peptides within the family of Violaceae.
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1. Introduction

The documentation of indigenous knowledge on the use of medicinal plants provides useful information and starting point for drug discovery. Instead of non-targeted chemical and biological screening for discovering lead compounds, scientists may make use of local knowledge for documentation and cost-effective isolation of bioactive compounds (Andrade-Cetto and Heinrich, 2011; Attah et al., 2012; Heinrich, 2010). For instance, the discovery of circular peptides with uterotonic activity can be traced back to the traditional use of *Oldenlandia affinis* DC. (Rubiaceae) by indigenous women of the Democratic Republic of Congo. The plant was prepared as alcoholic tincture or tea and was administered to

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facilitate childbirth and to reduce labor duration. Pharmacological investigation and evidence-based validation of the local use led to the discovery of cyclotides with uterotonic properties (Gran, 1973) and elucidation of the molecular mechanism (Koehbach et al., 2013b). In fact the cyclotide kalata B7 was found to be the first oxytocin-like plant peptide that can modulate the human oxytocin and vasopressin V_{1a} receptors as partial agonist (Koehbach et al., 2013b). A cyclotide-enriched aqueous extracts of this plant was found to induce myometrial tissue contraction at a dose of 1 mg mL^{-1}. Cyclotides are cysteine-rich miniproteins comprising three disulfide bonds and a circular peptide backbone (Craik et al., 1999) making them stable compounds. Their biological function is thought to be for plant defense against herbivores (Gruber et al., 2007). Their occurrence has been described not only in species of the Rubiaceae and Violaceae family, but also in other plant families: Cucurbitaceae, Fabaceae, Solanaceae and Poaceae (Gruber, 2010; Gruber et al., 2008; Nguyen et al., 2011; Poth et al., 2011; Simonsen et al., 2005). Recently it has been shown that a single plant can produce over 160 different cyclotides (Hellinger et al., 2015).

Rinorea dentata (P. Beauv.) Kuntze is a tropical plant species of the Violaceae family growing in the rainforest regions of Liberia, Cameroon, Uganda and Nigeria. In South-Western Nigeria, it has been reported to be abundant in the moist semi-deciduous rainforest of Omo (Omo Forest Reserve) Ogun State (Ojo, 2004). *R. dentata* is a wild plant that has not been cultivated. This species is being collected from the wild for medicinal and other purposes as a non-timber forest product. It is a shrub or small tree which grows up to 10 m high. It is commonly found growing in shady places, occupying the lower story of the dense rainforest and does usually not have direct access to sunlight. *R. dentata* is the most abundant Violaceae in the study area (Amusa and Jimoh, 2012). The tree trunk of the plant is known for its hard wood, providing rationale for its local name "Iyokheze" (Edo) and "Oloboroho" (Yoruba) or "stone plant" (Burkill, 2000; Keay, 1989). The local settlers in the J4 region of the Omo Forest Reserve, which comprises the natural habitat of the plant, use its stem as chewing stick for oral hygiene, as malaria remedy and to reduce duration of labor or to facilitate delivery in farm animals. None of these ethnomedicinal claims have been investigated and the rationale for the use of *R. dentata* by the local population awaits scientific validation. This study therefore aims to document the ethnopharmacological use of *R. dentata* among the settlement camps in the J4 area of Omo Forest Reserve and in particular to investigate its uterotonic properties. Knowing that cyclotide-rich ethnomedicinal plants such as *O. affinis* have been traditionally used for their uterotonic activity, the effect of the aqueous extract of *R. dentata* on human myometrium was investigated. Furthermore we used an established peptidomics workflow to analyze and characterize cyclotides of *R. dentata*, since members of the Violaceae plant family are regarded as a rich source of these circular peptides. The expected outcomes of this study would not only increase our ethnopharmacological knowledge about Violaceae plants, but would also potentially lead to the discovery of a novel cyclotide-expressing species, which may be available for future research on the molecular diversity and bioactivity of these plant constituents.

2. Materials and methods

2.1. Study area

The location of Omo Forestry Reserve extends from latitude 6 °35′–7 °05′N and longitude 4 °19′–4 °40′E. It is located within the South-Western region of Nigeria, covering an area of about 130,500 ha (Fig. 1). Most of the settlement camps where respondents live are situated around the North-Eastern part of the forest such as the J4 area of Omo forest and the Abeku sector. The vegetation of Omo Forest Reserve is a mixed moist semi-deciduous rainforest (Ojo, 2004; Okali and Olaadams, 1987) where the Southern part is predominantly humid while a dry forest covers the Northern region. The most abundant plant families in the forest are Ebenaeceae, Likiaceae, Papilionaceae, Poaceae, Rubiaceae and Violaceae, whereas *R. dentata* appears to be among the most common tree species of all the plant families documented. The study area around the Abeku sector has an undulating topography with several hills. Flowing streams leading to the main rivers (Omo, Shasha and Oluwa) appear to originate from the valleys between the hills. The unique topography has been linked with past earth movements and volcanic events leading to the dissolution of the native rocks in the area (Ojo, 2004). As a result of the hilly topography the drainage of the study site around the Abeku sector is good, while the J4 area is poorly drained.

2.2. Questionnaires and plant collection

The ethnopharmacological survey of the use of *R. dentata* was carried out in October 2014. Open interviews and semi-structured questionnaires were used to obtain data from respondents. Although most of the respondents were from the area J4 and Abeku, others came from neighboring towns such as Abeokuta and Ijebu Ode. The focus of the study was to gather the traditional knowledge of respondents about *R. dentata* plant use in animal and human health care. A knowledgeable youth leader (Mr Abiodun Ola) from the study area and a local assistant (Mr Tayo Famojuro) helped with the surveys of the various enclaves and settlement camps. A voucher specimen, deposited at the Department of Botany Herbarium, Obafemi Awolowo University, Ile Ife (Ife: 16945) was used as a representative sample of the plant for identification by respondents. In addition, the local survey assistant, who confirmed personal knowledge of the plant, collected more fresh samples for identification by respondents, who were asked to identify both the dry voucher specimen and the fresh plant material. With the help of the local assistant, respondents were asked to provide the following information: knowledge of the plant, local name, medicinal use, place and mode of collection, preservation, plant preparation and administration, dosage, side-effects, economic value of the plant and whether *R. dentata* is administered alone or together with other herbs. A total of 54 respondents were interviewed. *R. dentata* plant samples were further collected and identified from the same forest for cyclotide chemical analysis.

2.3. Data analysis

Data generated from the field survey on the use of *R. dentata* was subjected to descriptive statistics using percentages and frequencies. To quantify the data generated from this survey, the following indices were used, based on previous literature reports or modifications thereof: use value index, fidelity level, knowledge index value, use knowledge index, ethnomedicinal income index, tissue importance value, ethnobotanical richness, and name homogeneity index.

The use value index (UVI) as suggested by Phillips and Gentry (1993) and modified by de Albuquerque et al. (2007) was used to determine the level of importance attached to *R. dentata* by the respondents.

$$\mathrm{UVI} = \sum U/n_s, \tag{1}$$

where '*U*' is the number of uses given by a respondent, 'n_s' is the number of respondents who use medicinal plant preparations of *R. dentata* for any purpose.

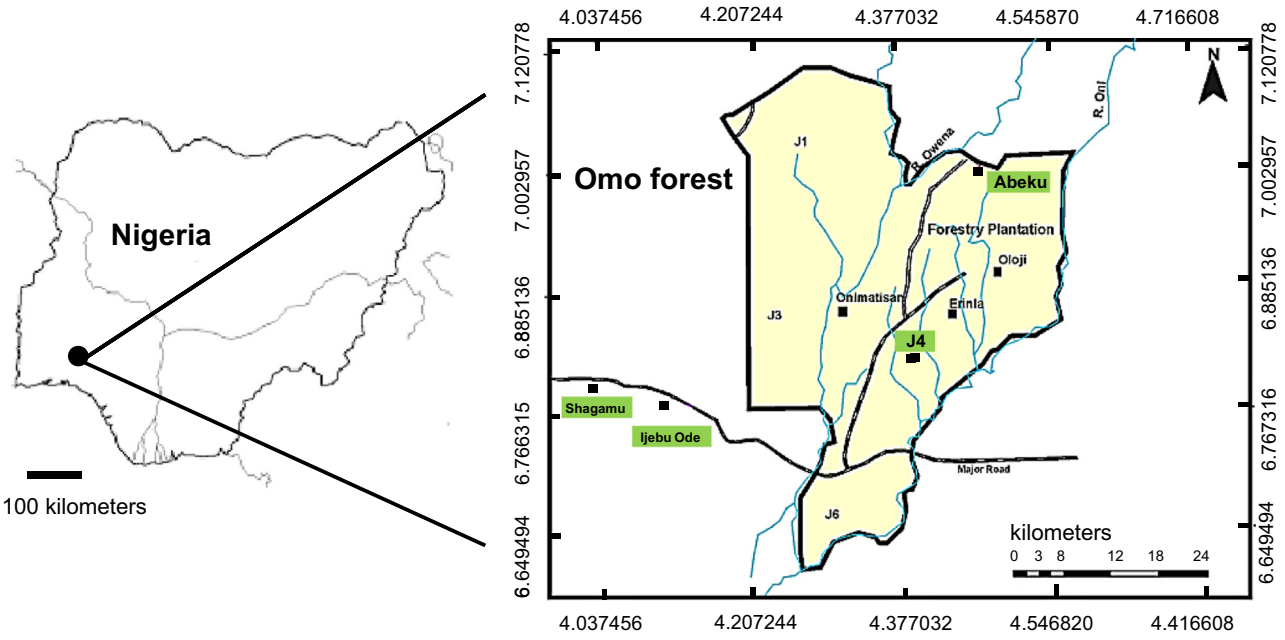

Fig. 1. Map of the study area. Map of the Omo Forest Reserve, Nigeria, showing locations (marked in green) of respondents. Those situated outside the forest (i.e. Ijebu Ode and Shagamu) commuted from those locations to work in the forest. Smaller temporary camps are located within the forestry plantation between the area J4 and the Abeku sector.

Fidelity level (FL) proposed by Friedman et al. (1986) was used to quantify the percentage of respondents claiming the use of *R. dentata* for the same major purpose.

$$FL = N_p/n*100, \quad (2)$$

where 'N_p' is the number of respondents claiming a specific use for *R. dentata* and '*n*' is the total number of respondents using the species for any purpose.

The knowledge value index (KVI) was helpful in evaluating the level of knowledge of *R. dentata* and further appraises the extent of awareness of ethnomedicinal plants among a population without paying special attention to names of plant species.

$$KVI = \sum A/n, \quad (3)$$

where '*A*' represent a respondent who is aware or knows the plant *R. dentata* without necessarily knowing the plant by name/botanical name; '*n*' is the number of respondents interviewed.

The use knowledge index (UK) proposed by Camejo-Rodrigues et al. (2003) was used to analyze the level of novelty in local names not yet documented and to appraise the continued use of plants in ethnomedicine.

$$UK = \sum U/K, \quad (4)$$

where '*U*' represents a respondent who use *R. dentata* and '*K*' is the respondent who knows *R. dentata* by name as reported in the literature ("Oloboroho" in Yoruba dialect) or any other.

The ethnomedicinal income index (EI) was used to quantify the relative ethnomedicinal economy of a local population based on local knowledge of plant use of *R. dentata*.

$$EI = \sum I/n, \quad (5)$$

where '*I*' represents a respondent who makes income from the sale of *R. dentata* and '*n*' is the number of respondents who use this plant species for any given purpose.

Tissue importance value (TIV) is a modified quantitative index originally proposed by Gomez-Beloz (2002) as plant part value. This indicates the most useful part of the plant *R. dentata* to the respondents.

$$TIV = N_t/n, \quad (6)$$

where 'N_t' is the respondent who mentioned a particular tissue of *R. dentata* for a use and '*n*' is the total number of respondents who use the plant for any purpose.

Ethnobotanical richness (ER) as proposed by Begossi (1996) and optimized by Rigat et al. (2007) was adopted as a property to quantify the ethnic-specific application of *R. dentata*.

$$ER = E_r/n, \quad (7)$$

where 'E_r' represents the respondents who have known the species for its medicinal property and '*n*' is the total number of respondents.

The *name homogeneity index* (NHI) was used to quantitatively describe the variation in names used by respondents to describe *R. dentata*. Differences exist in names locally used to describe or identify medicinal plants especially in a multiethnic settlement.

$$NHI = N_h/n, \quad (8)$$

where 'N_h' is the number of homogenous names given by respondents and '*n*' is the total number of respondents who have a name for the plant.

2.4. Plant extraction and cyclotide analysis

For preparation of the decoction 10 g of coarsely powdered *R. dentata* leaves were gently boiled in 200 mL of water over a period of 2 h. The resulting colored decoction was filtered and lyophilized. The yield obtained was 1.32 g which is equivalent to 13.2%. The reconstituted solution was used for analytical HPLC as described below. To prepare the cyclotide enriched extract a chemically optimized extraction protocol was applied (Koehbach et al., 2013a). Dried *R. dentata* plant material (40 g) was powdered and extracted with 400 mL of methanol:dichloromethane (1:1; v/v) for 24 h under continuous agitation at 25 °C. Half a volume of double distilled water was added to the extraction system and centrifuged for 3 min at 500 × *g*; the aqueous supernatant was separated and

diluted further with solvent A (100% double distilled water, 0.1% trifluoroacetic acid) to less than 10% MeOH for reversed-phase C_{18} solid phase extraction (5 g per 20 mL). The cartridge was first preconditioned with one volume of MeOH, activated with one volume of solvent B (90% acetonitrile, 10% double distilled water and 0.08% (v/v) trifluoroacetic acid) and equilibrated with two volumes of solvent A; sample was washed with 20% solvent B and eluted with 20 mL of 80% solvent B. A small aliquot of eluate was used for preliminary mass spectrometry (MS) analysis and the remaining peptide extract was freeze dried and stored at −20 °C until used for peptidomics analysis and for *in vitro* assays. The yields of extraction and peptide purification were calculated as percentage using the formula:

$$\text{Yield} = W_e/W_p * 100, \quad (9)$$

where 'W_e' is the weight of extract or peptide fraction and 'W_p' the weight of powered plant starting material.

MALDI-TOF MS analysis was performed on a 4800 reflector TOF/TOF (time-of-flight) analyzer from ABSciex (Framingham, MA). A volume of 0.5 μL of the eluted peptide extract was mixed with 3 μL of matrix [saturated alpha-cyano-hydroxylcinnamic acid in 50% double distilled water, 50% acetonitrile, 0.1% trifluoroacetic acid (v/v/v); Sigma-Aldrich, St Louis, MO], spotted on the MALDI target plate and allowed to dry in the dark. Acquired spectra were processed using the Data Explorer software v. 4.9 (Koehbach et al., 2013b). For plant extract fractionation analysis the lyophilized *R. dentata* extract was reconstituted in solvent A. The solution was further passed through a syringe filter with 0.45 μm pore size before fractionation using semi-preparative RP-HPLC on a Dionex Ultimate 3000 HPLC unit (Thermo Fisher Scientific, Waltham, MA). Peptide separation was achieved with a Kromasil reversed-phase column (dichrom GmbH, Marl, Germany; 250 × 10 mm, C_{18}-modified particles, 5 μm, 110 Å) applying linear gradients with increasing concentrations of solvent B (5-min equilibration at 5% B, 5–60% B in 70 min, 5-min hold at 99% B, 10-min re-equilibration at 5% B) at a flow rate of 3 mL min^{-1}.

The cyclotide-enriched extract was analyzed by LC–MS using a HotSept capillary column (dichrom; 100 × 0.5 mm, C_{18}, 2 μm, 110 Å) when coupled to a QqTOF 'compact' mass spectrometer from Bruker Daltonics (Billerica, MA). Flow rates were 15 μL min^{-1} with eluent A (double distilled water containing 0.1% formic acid) and eluent B [80% acetonitrile, 20% double distilled water and 0.08% formic acid (v/v/v)]. The crude decoction of *R. dentata* was analyzed by analytical HPLC using a Phenomenex reversed-phase Kinetex C_{18} column (150 × 2.1 mm, 2.6 μm, 100 Å). Linear gradients similar to the description above with flow rates of 0.4 mL min^{-1} were applied. Collected fractions were analyzed on the MALDI TOF/TOF analyzer and fractions containing MS evidence for cyclotides were freeze dried and reconstituted in solvent A for analytical HPLC or further MS analysis.

Cysteine content was analyzed by MS after reduction and alkylation; a small aliquot (~20–100 μg) of the lyophilized peptide was reconstituted in 20 μL of 0.1 M ammonium bicarbonate (pH 8.2), and reduced by adding 2 μL of 200 mM dithiothreitol while incubating at 65 °C for 10 min. Reduced samples were further alkylated with 4 μL of iodoacetamide (9.25 mg per 100 μL) for 10 min in the dark. A volume of 1 μL of trifluoroacetic acid was used to stop the reaction. MALDI-TOF was used to analyze the reduced and alkylated sample. The cyclotide kalata B1 was used as positive control. Reduced and alkylated peptides were enzymatically digested using sequencing grade trypsin (Sigma-Aldrich) with the addition of 2 μL of the enzyme (0.1 μg μL^{-1}) by incubating the mixture under mild agitation for 3–14 h. The reaction was quenched with 1 μL of trifluoroacetic acid. The digests were analyzed by MALDI-TOF/TOF and peptide precursors with a +366 Da shift after enzymatic digestion compared to native mass signal were considered for MS/MS fragmentation and *de novo* sequencing using the Data Explorer software and its Ion Fragmentation Calculator tool. The sequence of the cyclotide was manually determined by assignment of mainly b- and y-ion series and other indicative signals such as loss of ammonia (−17 Da). The obtained sequence was validated by the Ion Fragmentation Calculator tool as described by Hashempour et al. (2013) and Koehbach et al. (2013a).

2.5. Organ bath myometrial contractility assays

Full thickness biopsies (approx. 1–2 cm^2) of human myometrium were obtained from the upper lip of the lower uterine segment incision site from six women undergoing elective, prelabor caesarean section at term gestation (38–40 weeks). All women gave informed written consent for participation. The study was approved by the North West (Liverpool East) Research Ethics Committee (LREC 10/H1002/49) and by the Research and Development Director of Liverpool Women's Hospital NHS Foundation Trust, Liverpool, United Kingdom. In the laboratory, strips of myometrium (approx. 2 × 1 × 5 mm^3) were dissected using a 10 × objective light microscope and placed into 1 mL organ baths continually superfused with physiological saline solution (PSS) [154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 7.8 mM glucose, 10.9 mM Hepes, 2.0 mM $CaCl_2$, (pH 7.4)] maintained at 37 °C. Strips were attached at one end to a fixed hook and the other to a FORT10g tension transducer (World Precision Instruments, UK). Strips were allowed to equilibrate and establish stable spontaneous contractions of similar amplitude and frequency (typically within 3 h of superfusion) as described elsewhere (Luckas et al., 1999). Strips were then subjected to *R. dentata* extract (1 mg mL^{-1} in PSS) added directly to the organ bath. Oxytocin (0.5 nM in PSS) was used as a positive control to confirm that the tissue strips were responsive to uterotonic stimulation. Contraction amplitude (force, mN), frequency (no. of contractions occurring in 10 min) and area under the contraction curve (AUC, in arbitrary units) were measured using OriginPro 9.0 software (OriginLab Corporation, US) as previously described (Koehbach et al., 2013b; Turton et al., 2013).Values obtained under 30 min application of the extract or oxytocin were compared with 30 min of spontaneous control activity measured immediately before the addition of the extract. Values represent the percentage change in contraction compared to control (100%) and are presented as mean ± SEM, where n = number of women. Statistical analysis was performed by Student's paired *t*-test with statistical significance taken as $P < 0.05$.

3. Results and discussion

3.1. Ethnobotanical survey and analysis

The field survey on *R. dentata* was conducted to access information about established applications of the plant in traditional medicine. A total of 54 respondents were accessed who were either assisted to complete the questionnaire or independently responded to the questionnaire. Respondents were situated in twelve different states of Nigeria including Kogi, Ogun, Oyo, Delta, Cross River, Enugu, Imo, Ekiti, Abia, Ondo, Osun and Ebonyi state. Most respondents cut across different settlement camps around the J4 area of Omo forest (47 respondents), while only 7 of the respondents lived in the neighboring towns of Ijebu Ode, Abeokuta and Sagamu. All respondents belonged either to the native Yoruba tribe or otherwise to the Igbo ethnicity. The Yoruba speaking ethnic group constituted 72% of total respondents, while

the remaining 28% belonged to the Igbo speaking group. Most respondents (68%) were within the age group of 31–59 years old, 6% were above 60 years and 26% between 15–30 years old. There were 54% female respondents and 46% males. Occupation of respondents included traditional health practitioners, traditional birth attendants, teachers, nurses, herbalists and others. Further demographic information of survey participants has been summarized in Supplementary Table 1. To avoid ambiguity, respondents had to identify a *R. dentata* voucher specimen carried along during the survey to record data regarding their knowledge and medical use. We expected those who have previously used the plant to identify it based on its morphological features. The two names "*R. dentata*" and "Oloboroho" were just part of the plant information on the voucher specimen which was read out to respondents after initial recognition of the plant species to verify the correct assignment.

Considering the level of acceptance for Western or traditional medicine in health care, 76% of respondents claimed to visit hospitals when necessary while only 14% strictly rely on traditional healing. On the contrary, talking about the traditional healing approach with specific reference to use of plants for healing, 87% responded in the affirmative; 13% did not use plant parts or plant preparations for any healing purposes. The knowledge of *R. dentata* as a healing plant was demonstrated by 46% of the respondents; 54% of respondents did not previously know any plant called *R. dentata* (or "Oloboroho" in Yoruba dialect, see also Supplementary Table 2 for a collection of local names) with morphological features as presented to them during the survey. Respondents were requested to confirm animal husbandry practices; 33% kept farm animals and only 7% (4 respondents) used plants to facilitate birth and ease labor in animals (Table 1). Respondents mentioned that plants are usually collected from surrounding backyard and farms, or are purchased from local herb traders. They preserved these plant samples through constant warming (decoction) or by placing collected plants above a wooden roof within the kitchen area (preservation by gentle drying).

From the 25 respondents who have known *R. dentata* as healing plant, 6 respondents used *R. dentata* for various medicinal purposes, and only one respondent used *R. dentata* to facilitate parturition in animals (Supplementary Table 2). However, other plants were mentioned for their use during delayed labor in animals; they included *Ocimum gratissimum* and *Vernonia amygdalina*, which were known uterotonic plants used in traditional African medicine (Attah et al., 2012; Gruber and O'Brien, 2011). There are several locally restricted names for *R. dentata* given by the respondents (Supplementary Table 2). The Yoruba local name reported in the literature does not tally with any of those given by the respondents who knew the name of the plant. For instance, in the literature *R. dentata* is called Oloboroho in Yoruba. However, names given by 9% of respondents included "Carnel plant", Paroko, Oniyo, Atani and Ewe agbo iba (Supplementary Table 2). Analysis of the meaning of these names suggested that respondents identified *R. dentata* with names based on their perceived application or uses (for instance, Ewe agbo iba means fever plant). Respondents (19%) obtained *R. dentata* from the farm (40%), forest (50%) or from local herb traders (10%). Interestingly, respondents distinguished two "varieties" of *R. dentata*. Plant material of either putative *R. dentata* species has been collected in the field and they were authenticated at the Forest Herbarium Institute (FHI), Ibadan, and identified as *R. dentata* (FHI: 110,151) and *R. brachypatela* (FHI: 110,152), respectively. However, *R. dentata* was commonly used as it is more abundant in the wild. Other uses of the plant as given by 11% of respondents included: as a general medicinal herb, as malaria remedy and as chewing stick for anti-microbial purpose (Supplementary Table 2). The method of preparation and administration of *R. dentata* extracts was described by 4% of respondents (3 out of 54). Their methodology included cooking, decoction and chewing on fresh stems as chewing stick. The plant preparations were administered orally with 1–3 cups daily (containing 200 mL per cup of decoction), by dermal application by brews or baths, and chewing the stem to clean up the teeth (Supplementary Table 2). Observable side-effects following administration of any of the preparations included pigmentation of urine, dizziness and increased appetite. Of the respondents who claimed health benefits after consumption of plant preparations of *R. dentata* 67% reported at least one side-effect associated with consuming this plant. Mixing *R. dentata* medicinal preparations with other plants were mentioned by 4% of total respondents and 33% of respondents who use *R. dentata*, additionally included lime and other traditional herbs (Table 1 and Supplementary Table 2). Respondents were asked to describe if and how they learned about the medicinal properties of plants; 72% did receive knowledge for instance from family members, a friend or by self-motivated learning (Table 1). Based on the years of experience in medicinal plant trade, 76% mentioned experiences ranging from one year to thirty years. However, only 11% of the total respondents derive an income from the herbal sale of *R. dentata* herbaceous preparation or plant material (Table 1) which may be associated with the lack of awareness of the medicinal value of the plant.

Table 1
Summary of respondents' choice data obtained from semi-structured interviews.

Parameters	Yes	%	No	%
Visit to hospitals	41	76	13	14
Use of plants for healing	47	87	7	13
Received training or acquired knowledge on medicinal use of plants	39	72	15	28
One or more years of experience on medicinal uses of plants	41	76	13	24
Knowledge of *R. dentata* as healing plant	25	46	29	54
Collection of *R. dentata* for any use	10	19	44	81
Usage of plant *R. dentata* for any medicinal purpose	6	11	48	89
Addition of other material to *R. dentata* medicinal preparation	2	4	52	96
Animal husbandry	18	33	36	67
Use of plants in veterinary delivery	4	7	50	93
Use of *R. dentata* in veterinary delivery	1	2	53	98
Acquisition of income by sale of *R. dentata*	6	11	48	89

According to the quantitative analysis of survey responses (Table 2), the use value index of *R. dentata* is low (0.085). The knowledge value index for *R. dentata* was moderate (0.46); this means about half of the respondents knew that *R. dentata* grows around their vicinity. The use knowledge index obtained (1.2) was high indicating that more respondents used *R. dentata* for any purpose than knowing the plant species by name. Accordingly, a low name homogeneity index of 0.4 indicated that *R. dentata* is known with different names among respondents. While all respondents (100%) using *R. dentata* earn some money from its sale, only 11% of the total respondents derive an income from the herbal sale of *R. dentata* herbaceous preparation or plant material (income index 1.0) (Table 2) which may be associated with the lack of awareness of the medicinal value of the plant. On the tissue importance value, the stem with the highest index (0.67) was most frequently used. The index for ethnobotanical richness (0.13) showed that *R. dentata* has low ethnic-specific application in Omo forest. Traditional knowledge regarding medicinal uses of *R. dentata* is thus limited to a few. This may be tied to cultural practices where useful traditional knowledge is kept secret and mostly passed down the family line for economic reasons and due to spiritual rites. Based on the information on the traditional use of *R. dentata* plant preparation for birth induction in veterinary medicine and to reduce the duration of labor, we were interested in validating this claim by pharmacological means using classical

Table 2
Quantitative ethnobotanical analysis of respondents' information.

Index measured[a]	Data generated	Index analysis
Use value index (UVI=$\sum U_a/n_s$)	U_a=4 n_s=47[b]	0.085
Fidelity level (FL=N_p/n*100)	N_p=4 n=6	66.70
Knowledge value index (KVI=$\sum A/n$)	A=25 n=54	0.46
Use knowledge index (UK=$\sum U_b/K$)	U_b= 6 K=5	1.20
Ethnomedicinal income index (EI=$\sum I/n$)	I=6 n=6	1.00
Tissue importance value (TIV=$\sum N_t/n$)	$N_{t,\ shoot}$=6; $N_{t,\ root}$=0; $N_{t,\ stem}$=4; $N_{t,\ leaf}$=3; n=6	$1.00_{(shoot)}$; $0.00_{(root)}$; $0.67_{(stem)}$; $0.50_{(leaf)}$
Ethnobotanical richness (ER=$\sum E_r/n$)	E_r=6 n=47	0.13
Name homogeneity index (NHI=$\sum N_h/n$)	N_h=2 n=5	0.40

[a] U_a=uses given by a respondent; U_b=respondents who use *R. dentata*; N_p=number of respondents claiming a medicinal use; A=respondents who know or are aware of *R. dentata*; K=respondents who know *R. dentata* by name; I=respondents who make income from *R. dentata*; N_t=respondents who mention a particular tissue (root, leaves, shoot and stem); n=number of respondents; E_r=respondents who know *R. dentata* for its medicinal use; N_h=number of homogenous names given by respondents.

[b] Seven respondents claimed that they do not believe in phytomedicine.

organ bath studies to determine potential augmentation of myometrial contractility (Koehbach et al., 2013b). Interestingly, *R. dentata* belongs to the Violaceae family, which is known as a rich source of cyclotides. The prototypical cyclotide plant, *O. affinis*, has been traditionally used by African tribes to induce childbirth due to its uterotonic activity. Hence, we studied the effects of *Rinorea* extract on myometrial contractility and identification of potential cyclotides.

3.2. Myometrial contractility assays

To investigate the potential beneficial ethno-veterinary application of *R. dentata* and in particular its ability to aid parturition in animals, we tested the effect of applying a crude extract of *R. dentata* on spontaneously contracting strips of human myometrium obtained from 6 different women undergoing elective, prelabor cesarean section delivery. The plant extract was prepared in physiological saline and was applied directly to the organ baths at a concentration of 1 mg mL^{-1}, which is comparable to an earlier study of the uterotonic effects of an *O. affinis* extract (Koehbach et al., 2013b). Application of *R. dentata* extract resulted in a mixed response between strips of myometrium from different women (Fig. 2A). Contraction amplitude tended to decrease but frequency was increased and resulted in an increase in area under the curve (AUC). Overall, the mean contraction amplitude was found to be 97.64% ($\pm$ 5.01) of control amplitude, frequency of contraction increased by 35.48% ($\pm$33.8) compared to control and the AUC increased by 41.17% ($\pm$14.67) (Mean $\pm$ SEM). None of the changes in any of the contraction parameters however were found to be significant ($P>0.05$, Fig. 2B). In contrast, the application of the uterotonic, oxytocin as a positive control significantly augmented myometrial contractions in all tissues examined (Fig. 2A). Amplitude of contraction increased by 22.05% ($\pm$4.25) and AUC increased by 113.36% ($\pm$38.41) ($P=0.011$ and $P=0.0387$ respectively), confirming that the tissues were responsive to stimulation. Taken together, the ability of *R. dentata* to augment human myometrial contractility activity *in vitro* was relatively poor. Application of the extract to contracting strips of myometrium caused a

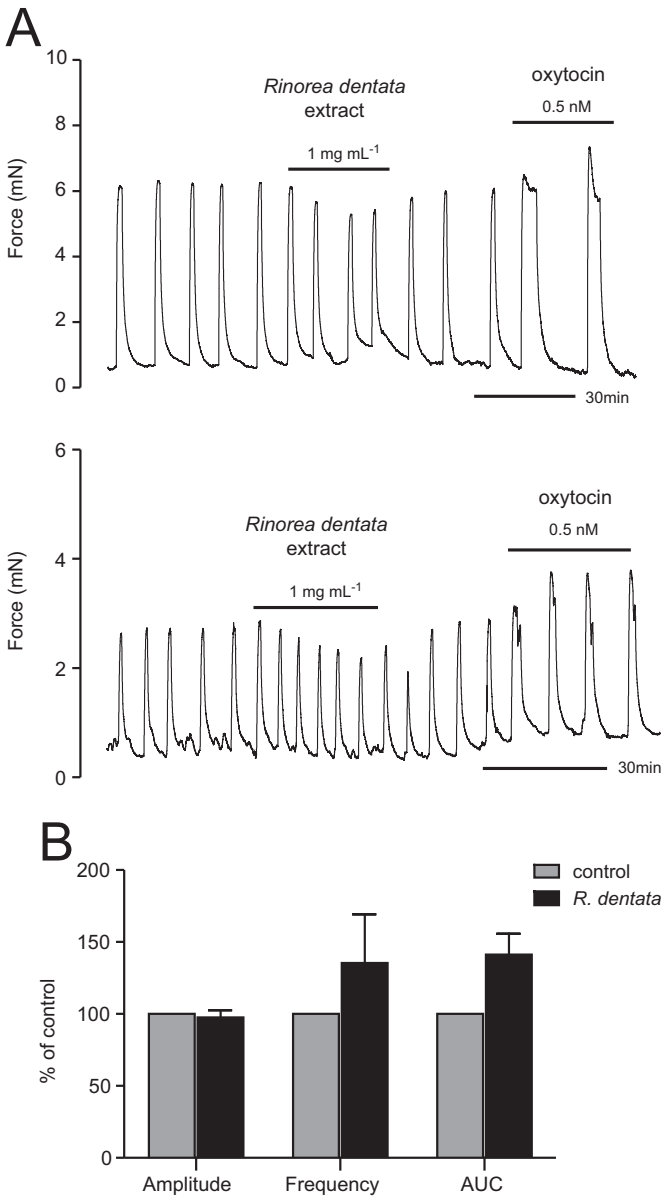

Fig. 2. Uterine contractility assay of *Rinorea dentata* extract. (A) The effect of *R. dentata* crude extract on human myometrium (two independent biopsies). Spontaneous contractions of term, not in labor human myometrium superfused with physiological saline at 37 °C and application of 1 mg mL^{-1} *R. dentata* extract followed by 0.5 nM oxytocin. (B) Bar chart showing the mean $\pm$ SEM effects of *R. dentata* extract on the different parameters of myometrial contraction compared to control (100%). Application of the extract tended to decrease amplitude of contraction (97.64 $\pm$ 5.02%, $P=0.99$) but increase frequency of contraction (135.48 $\pm$ 33.82%, $P=0.51$) resulting in a modest but non-significant increase in area under the curve (141.18 $\pm$ 14.64%, $P=0.068$). In contrast, oxytocin (positive control) augmented both amplitude (122.05 $\pm$ 4.25%, $P=0.011$) and AUC (213.36 $\pm$ 38.41%, $P=0.0387$). Students paired *t*-test was used for statistical analysis.

modest but non-significant increase in AUC which is a measure of total work done by the muscle strip. This change resulted from a tendency of the extract to increase contraction frequency rather than due to augmentation of contraction amplitude. Instead, amplitude of contraction tended to decrease with application of the extract. None of the parameters of contraction were found to change significantly, probably owing to the large variation in response to the extract observed between the tested tissues. Knowing that *R. dentata* belongs to the Violaceae family, which is a known source of cyclotides, some of which have shown uterotonic

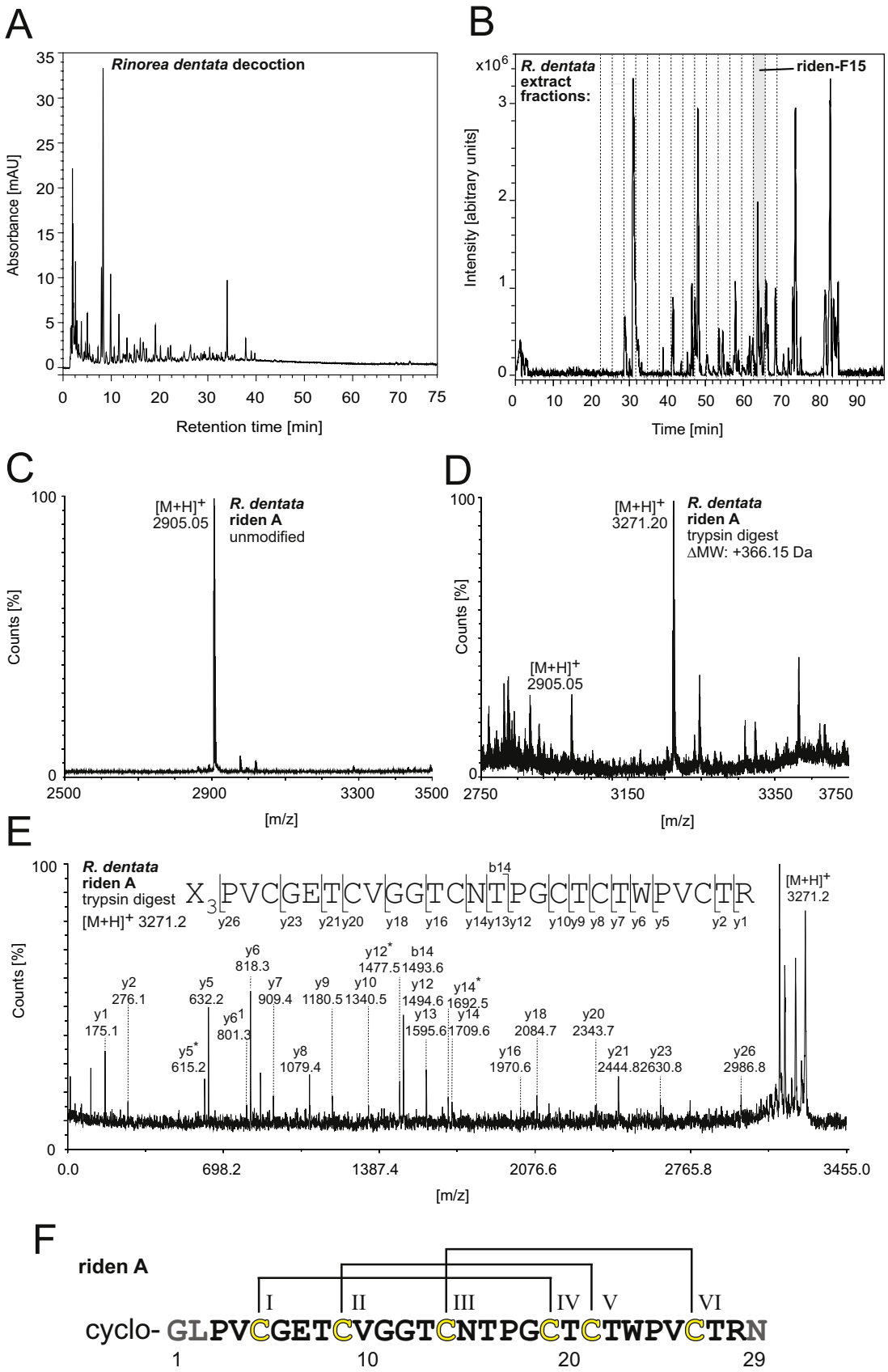

Fig. 3. Peptidomics of *Rinorea dentata* cyclotides. (A) Representative A_{280} trace of a HPLC chromatogram of a *R. dentata* decoction is shown. (B) The cyclotide-enriched solid phase extraction eluate was analyzed by LC–MS and a representative base peak chromatogram from 750–1800 m/z is illustrated. This extract was further separated by preparative HPLC. A total of 16 fractions (indicated by dotted lines in the base peak chromatogram) were collected and freeze-dried. (C) *Rinorea* fraction 15 (riden-F15) was analyzed via MALDI-TOF MS in the positive reflector scan mode to give a major mass signal of 2905.05 m/z labeled with riden A. (D) Trypsin digest experiment of cysteine-acetamidated riden-F15 sample revealed a mass shift for riden A of +366.15 Da to 3271.20 m/z labeled with riden A*. (E) Partial sequence of riden A, missing 3 residues, was obtained by *de novo* sequencing of a fragmentation spectrum recorded by MALDI-TOF MS/MS using trypsin processed riden A precursor peptide. The sequence is shown with b- and y-ions. Other indicative signals such as loss of ammonia (−17 Da) are labeled with an additional asterisk. (F) The full sequence of riden A was obtained by homology to kalata B1 (www.cybase.org.au). Cysteine residues are labeled with Roman numerals and shown in yellow; putative disulfide bonds have been connected. Residues assigned by homology are shown in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

properties (Koehbach et al., 2013b), we were interested to analyze this plant species for the presence of these circular cystine-knot peptides.

3.3. Cyclotide analysis

R. dentata has traditionally been prepared as decoction and therefore the initial analysis of this boiled herbal extract was carried out by analytical reversed-phase HPLC (Fig. 3A). As expected, the decoction contained many hydrophilic compounds as judged by early retention times of the major signals during reversed-phase chromatography. Using established protocols for cyclotide extraction we obtained an overall yield of 8.3% for the aqueous extract and 2.2% for the C_{18} cyclotide-enriched eluate. In line with previous studies (Koehbach et al., 2013a), LC–MS analysis of the cyclotide-enriched solid phase extraction eluate produced abundant peptide signals in a mass range characteristic for cyclotides (Fig. 3B). Following HPLC fractionation we obtained the cyclotide fraction riden-F15 with a major mass signal of 2905.05 m/z (Fig. 3C). Following an established workflow, the cystine-knot folded cyclotides were reduced, carbamidomethylated and were again analyzed using the MALDI TOF/TOF analyzer resulting in a mass shift of 348 Da. This suggested the removal of disulfide linkages within the peptide. Linearization of the circular configuration via enzymatic digest resulted in a further mass shift of 18 Da and confirmed the head-to-tail backbone cyclization of this peptide (Fig. 3D). Results from the analytical HPLC indicated a late elution profile typical for the hydrophobic nature of cyclotides (data not shown). Using tandem MS it was possible to obtain a partial amino acid sequence of the cyclotide riden A (*R. dentata* A) (Fig. 3E). The partial sequence is missing three residues of loop 6, but we were able to assign those by homology to kalata B1 (www.cybase.org.au). The putative full length sequence of riden A is cyclo-GLPVCGETCVGGTCNTPGCTCTWPVCTRN (Fig. 3F), and it only differed by one residue to kalata B1 in loop 5 (T22S). The discovery of cyclotides in this species was the first example of cyclotide-expression in the genus *Rinorea* and confirmed the ubiquitous abundance of cyclotides in Violaceae and essentially in flowering plants (Gruber, 2010; Gruber et al., 2008; Koehbach et al., 2013a).

4. Conclusion

The documentation of the ethnomedicinal uses of *R. dentata* and the discovery of the expression of bioactive circular peptides in this tropical rain forest plant constitutes the first report on cyclotides preparations used in Nigerian ethnomedicine. This has broadened existing knowledge on the distribution of these stable and pharmaceutically interesting peptides. Based on our findings, the weak uterotonic activity observed with *R. dentata* extract appears to support the low reported local use of the plant to aid parturition in animals. On the other hand, while this tends to support the myometrial safety in humans, caution should be taken in the ethnomedicinal use during pregnancy until further studies, on a larger scale have been performed. The anti-malarial and antimicrobial claims need further scientific validation and elucidation of responsible active principles. This could provide useful information and novel compounds as starting points for drug discovery. Since cyclotides are well known for sequence plasticity and a variety of biological activity, they are considered as potential pharmacological peptide library. Using the information provided here, we therefore suggest further ethnopharmacological evidence-based investigations of cyclotides isolated from *Rinorea* and other African Violaceae plant species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.12.038.

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