

Francis Alfred Attah*, Augustine E. Mbanu, Uche M. Chukwudulue,
Ugochukwu J. Jonah and Ngaitad S. Njinga

Ethnopharmacology, phytochemistry and a new chemotaxonomic marker in *Oldenlandia affinis* (Roem. & Schult.) DC. Rubiaceae

Abstract: The proper documentation of ethnopharmacological application of widely used indigenous plants and their phytochemical analysis has positively impacted the drug discovery pipeline. Medicinal plants with potential commercial value and prospects for clinical application need to be properly identified and authenticated to avoid confusion, adulteration and substitution. *Oldenlandia affinis* (OA) has continued to attract scientific attention following the discovery of extremely stable cyclotides (circular peptides) that are not expressed in many investigated members of the contentious genus, *Oldenlandia* (synonym – *Hedyotis*); yet there is a lack of an elaborate review covering some broader aspects of its traditional uses, ethnopharmacology and phytochemistry of the species. More importantly, the age long but lingering confusion and taxonomic inconsistencies common to the *Oldenlandia*–*Hedyotis* debate could foster species mismatching, increase cases of misidentification, promote adulteration of OA and thereby limit its proper clinical application. Here, we aim to reveal the extent of indigenous use of and research on OA from 1960 till date, unveil knowledge gaps, document hitherto unknown traditional applications, ethnopharmacological uses, pharmacological properties, and reported phytochemical profile. In addition, to encourage proper selection and utilization of genuine crude drug, the chemotaxonomically important phytoconstituents of OA have been presented and the modern approach of chemophenetic study of OA proposed to resolve the lack of consensus in the taxonomy of OA as well as the morphologically and anatomically close members of the taxon. The abundant cyclotide expression in OA represents a new chemotaxonomic marker for its unambiguous identification, utilization and reproducibility of research findings on the species.

***Corresponding author: Francis Alfred Attah**, Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria,
E-mail: attah.fau@unilorin.edu.ng

Augustine E. Mbanu, Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria, E-mail: augustinembanu20@gmail.com

Uche M. Chukwudulue, Chukwuemeka Odumegwu Ojukwu University, Igbariam, Anambra State, Nigeria, E-mail: um.chukwudulue@coou.edu.ng

Ugochukwu J. Jonah, Riyadh Al Khabra General Hospital, Al Qassim, Saudi Arabia,
E-mail: ugochukwujonah@gmail.com

Ngaitad S. Njinga, Department of Pharmaceutical and Medicinal Chemistry, University of Ilorin, Ilorin, Nigeria, E-mail: olous202@gmail.com

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1 Introduction

The apparent global shift towards natural products is evident in the increasing number of populations of the world, particularly from the developing countries, who depend on the therapeutic potentials of plants to meet primary healthcare needs. Documented evidence has shown that several plants have historically met the healthcare needs of indigenous populations [1, 2]. The scientific validation of many of these traditional herbal remedies have resulted in reported level of evidence supporting further clinical studies and application [3]. Bioactive metabolites (phytochemicals) accumulated differently in various organs of plant species have been linked with observed therapeutic potentials of these herbal materials otherwise known as phytomedicines [4]. For instance, *Cinchona ledgeriana* Moens. (Rubiaceae) is the botanical source of the quinoline alkaloid quinine and its D-isomer quinidine which are clinically important as an antimalarial and for the treatment of cardiac arrhythmias [5]. They are therefore sources of therapeutic agents having commercial value often traded across continents making their proper identification and authentication crucial to avoid adulteration and substitution [6]. One modern approach is the use of chemotaxonomy [7], a term that has received proposal for revision to that of a more relevant, technology-driven 21st century term known as chemophenetics; defined as the study tailored towards the description of a unique group of secondary metabolites bioaccumulated in a particular taxon [8, 9].

Rubiaceae (also known as the coffee family) is the fourth largest angiosperm family [10] with over six hundred genera and more than thirteen thousand six hundred plant species which exist as herbs, lianas, shrubs or trees growing on land. These plants are widely distributed but are found more in the warm tropics, where they exist as ornamental plants and are also valuable in traditional medicine [11]. In African folkloric medicine, the most widely applied genus is the *Pentas*, followed by *Gardenia* and *Canthium* [11]. The commonest phytochemical constituent of the Rubiaceae family is the alkaloid, but not without other phytochemicals such as, anthraquinones, naphthoquinones, terpenes, flavonoids, peptides, and saponins [11, 12]. Apart from the economic importance and traditional uses of this plant family, its species also possess a wide range of pharmacological properties ranging from antiplasmodial to antidiabetic activities, with some of the species' extracts exhibiting good antimalarial activity with IC_{50} values of less than 10 $\mu\text{g/mL}$ [13].

Oldenlandia is a contentious genus within the Rubiaceae family bedeviled with taxonomic inconsistencies and yet-to-be resolved debate over the generic delimitation of the *Oldenlandia*–*Hedyotis* complex [14, 15]. According to the *World Checklist of Selected Plant Family* (WCSP) database (<https://wcsp.science.kew.org/qsearch.do>),

accessed on the 22nd of November 2021, *Oldenlandia* comprises seven hundred and forty (740) species (including acceptable and unacceptable names), which are widely distributed across the regions of the world, except in Antarctica. On the other hand, 240 subordinate taxa of the genus *Oldenlandia* have been reported by the World Flora Online (<http://www.worldfloraonline.org/>) while the Plants of the World Online (<http://powo.science.kew.org/>), a Kew virtual resource reported 178 accepted species of the genus documenting 11 synonyms. However, new proposals have been submitted requesting to maintain status quo of some members of the genus in order to preserve the over two decades of traditional and contemporary usage so as to avoid introducing confusion as many members have profound application in Traditional medicine [16]. Nevertheless, members of the genus have continued to be widely applied in ethno-medicinal healthcare in Africa and Asia because they harbor bioactive metabolites of chemotaxonomic relevance including iridoids, anthraquinones, terpenes and flavonoids glycosides [17], which accord the genus its bioactivities.

Oldenlandia affinis (Roem. & Schult.) DC. (Rubiaceae) is an important species found in Africa and tropical Asian continents. As a member of the complicated genus, *Oldenlandia*, the accepted names differ for different body of knowledge and continents where the plant is endemic or has been naturalized (Table 1). *Oldenlandia affinis* (OA) has recently attracted scientific attention following the discovery of amazingly stable circular peptides called cyclotides [18]. Whether or not these novel peptides incorporate the chemotaxonomic potentials that can resolve the confusion in the taxonomy of species within the genus has not received any attention. More so, this species has varied application in Traditional Medicine which have not been properly documented

Table 1: Subspecies of *Oldenlandia affinis*, their synonyms and continental distribution of accepted names.

s/ n	* <i>Oldenlandia affinis</i> (Roem. & Schult.) DC.				
	Subspecies	Synonym	Accepted name	Country	Reference
1	<i>Oldenlandia affinis</i> subsp. <i>affinis</i>	(a) <i>Hedyotis affinis</i> Roem. & Schult.	<i>Hedyotis affinis</i> Roem. & Schult.	Asia	[19]
			<i>Oldenlandia affinis</i> (Roem. & Schult.) DC.	Africa, Europe and America	[20]
		(b) <i>Hedyotis dichotoma</i> J.Koenig ex Roth	<i>Oldenlandia affinis</i> (Roem. & Schult.) DC.	Africa, Europe and America	[20]
2	<i>Oldenlandia affinis</i> subsp. <i>fugax</i> (Vatke) Verdc	<i>Oldenlandia dichotoma</i> Hook.f.	<i>Oldenlandia dichotoma</i> Hook.f.	Asia	
			<i>Oldenlandia affinis</i> subsp. <i>affinis</i>	Africa, Europe and America	[20]

This species is native to Africa, India to Malaya. *Data extracted from The International Plant Names Index and World Checklist of Selected Plant Families 2021 (<http://www.ipni.org> and <http://apps.kew.org/wcsp/>).

in a way that will encourage further pharmacological research to validate claims, all geared towards drug discovery and bedside application.

A proper documentation of the ethnopharmacological uses, pharmacological properties, toxicity and phytochemical profile, particularly the chemotaxonomically important phytoconstituents of OA will facilitate proper identification and drug discovery. Being the first species from which the thermally-stable and bioactive cyclotide was discovered, OA represents a potential source of peptide drug “leads” for pharmaceutical and agricultural purposes. But the limited information on the ethnomedicinal uses, phytochemical and pharmacological properties of every part of the plant will hinder the scientific exploitation of the plant. In the quest to fill this gap, the current review provides the first elaborate insights into the ethno-pharmacology, phytochemical composition and the discovery of a novel bioactive miniprotein in *Oldenlandia affinis* that can serve the purpose of chemotaxonomic distinction.

2 Materials and methods

In the course of this review, literature search was done using Google scholar, ScienceDirect, PubMed and Cybase databases applying varying combinations of the following keywords: Rubiaceae, *Oldenlandia*, *Oldenlandia affinis*, ethnopharmacology, “Traditional uses”, “folkloric medicine”, “geographical distribution”, bioactivity, “biological activity”, “pharmacological effects”, “pharmacological properties”, cyclotides, “natural cyclotides”, “chemotaxonomic markers”, biogenesis, biosynthesis, “biosynthetic route”. The results obtained from the literature search were selected according to the scope of this paper.

3 Botanical description and geographical distribution

Botanical classification

Order: Gentianales Juss. ex Bercht. & J. Presl

Family: Rubiaceae Juss.

Genus: *Oldenlandia* L.

Species: *Oldenlandia affinis* (Roem. & Schult.) DC

O. affinis is a plant species belonging to the genus *Oldenlandia* which is in the family Rubiaceae. The genus is pantropical in distribution extending from Africa to Asia and Malaya and consisting of 240 species (<http://www.worldfloraonline.org/>) [21]. *O. affinis* grows as a weed in the grass and occurs at different altitudes from sea-level to 1,500 m above sea level. As a perennial herb, it has a woody root and it appears to prefer wet environments. The stem is slender, sub-erect while it is decumbent in older plant, up to

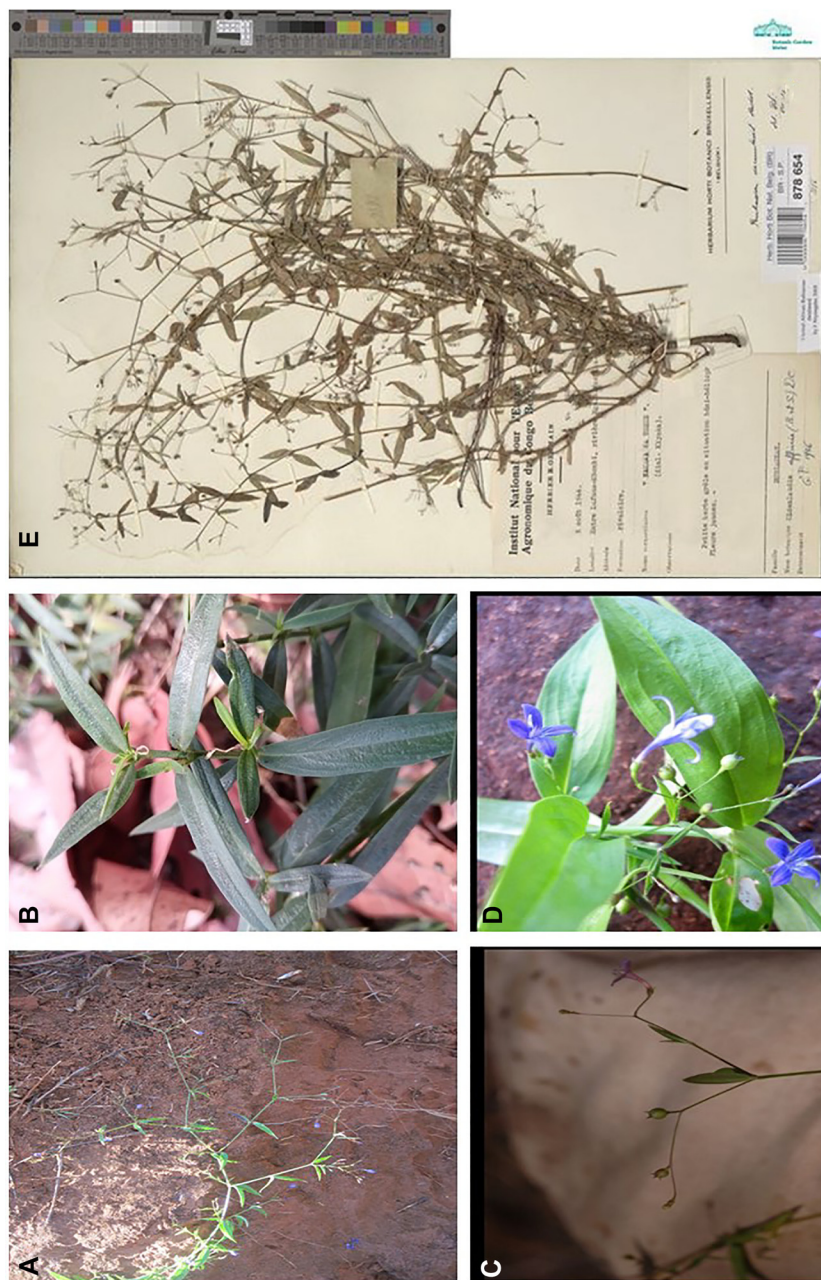
35–40 cm long. In young plants, OA has a simple stem, but rather branched in older species. Its appearance is green and shiny. The branches are filiform, divaricating with opposite, lanceolate leaves and short, adnate peduncles. Flowers produced by OA are blue–violet which are terminally situated, on hair formed stipules. The tubular corolla ends in four obtuse, valvate lobes. Four stamens are inserted at the corolla throat. The style is filiform with two stigmata while the ovary is two-celled whose ovules are numerous, and the placenta attached to the septum. Interestingly, the seeds of OA contain a brown, smooth surface, $0.35 \times 0.60 \text{ mm}^2$, and do not get slimy when moistened, which is characteristic of many *Oldenlandia* species. The pollen grains are tricolporate, spheroidal with a diameter of 27–31 μm . The fruits resemble globose capsules whose chromosome number was found to be 18 ($n = 2 \times 9$), a common occurrence with the other *Oldenlandia* species where the chromosome numbers are nine or a multiple of nine [22, 23]. Figure 1 (www.GBIF.org) shows the morphology and various organs of OA including stem, leaf arrangement, flowers, fruits and the entire herbarium specimen. Although the names of subspecies of OA are used interchangeably (Table 1), *Oldenlandia affinis* subsp. *fugax* (Vatke) Verdc. appears far more often in almost every herbarium samples available online while few herbaria document it simply as *Oldenlandia affinis* (Roem. & Schult.).

The geographical distribution of OA shown by Figure 2, illustrates that it is widely distributed in the tropical zone of Africa and is found from the Ivory Coast in the north to Transvaal in the south. It is also distributed in Madagascar as well as in western Asia, where it reaches its eastern limit in the Malay Peninsula. In Nigeria, the endemism of OA spans across the South-West to South-East coast with more recent reports documenting new locations of the restricted endemism of the species [24]. In addition, other related habitat competitors of OA including *Oldenlandia corymbosa*, *Oldenlandia diffusa* and *Oldenlandia herbacea* have been described to present similar morphological and anatomical characters that could encourage adulteration and substitution [24].

4 Traditional uses and ethnopharmacology of *Oldenlandia affinis*

4.1 Traditional uses

The quite popular instance of the traditional use of OA has to do with the acceleration of childbirth; the first uterotonic report being where a decoction of the plant, called “Wetegere” in the Gbaya language, was used among women in the Central African Republic region [25]. However, there was a prior report among the Natal Province of South Africa that a decoction of OA, ‘Umampeshana’, is used against asthma [26]. In Benin Republic also, OA is mixed with honey and used against learning impairment and the aqueous decoction used as anti-colic drink [27]. In some rural communities in



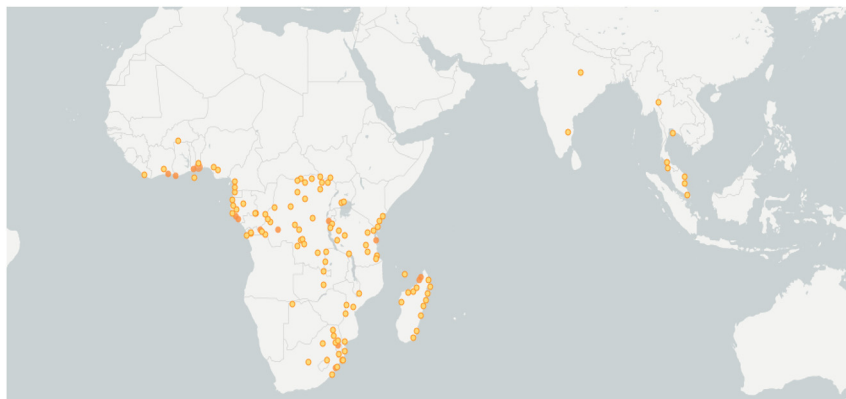


Figure 2: Map showing geographical distribution of OA (www.GBIF.org).

South-Eastern Nigeria where it is locally called “Agukwu”, “Ana nta”, the decoction of OA is used as antimalarial and antifebrile remedy [28]. In the book “Edible Wild Plants of Tanzania”, aerial parts of OA is prepared with other vegetables as rich source of nutrients, promote bowel movement and as an aid to digestion [29]. In the same region, the leaves of OA are pounded, macerated in warm water and the aqueous filtrate taken as a panacea for stomach troubles [29]. However, authors did not document whether the leaves to be pounded are from fresh collections or already dried plant materials. In India and many parts of Asian territories, whole plant decoction of OA is traditionally applied as an anthelmintic, diuretic, stomachic, purgative, diaphoretic, nervous depression and fever [30, 31]. Considering the geographical distribution of OA across varying indigenous cultures within the border of the species endemism, there is an African–Asian continental consensus on the traditional application of OA in the management of fever and to facilitate organ movement such as the gastrointestinal tract, respiratory tract and the uterus. This cross-cultural agreement in the use-mention of OA increases its fidelity and thus emphasizes the need to prioritize this traditional application for scientific validation which has a potential for drug discovery. Table 2 summarizes the traditional uses of OA across regions of its endemism.

4.2 Ethnopharmacology

Documented evidences of the traditional application of OA among different cultures serve as a basis for pharmacological evaluation of the species based on reported traditional applications (Table 2). The oxytocic activity of OA earlier reported [32] was further accorded an indepth scientific investigation by Koehbach and colleagues using crude extract and purified cyclotide candidates; they successfully performed the stimulation of the human myometrium using both crude cyclotide-rich extracts as well

Table 2: A summary of the applications of OA in traditional medicine.

S/ N	Country	Ethnomedicinal uses	*Scientific evidence	Reference
1	Congo, Central African Republic	Childbirth	Validated	[32] [33]
2	South Africa	Asthma	None	[26]
3	Benin Republic	Learning impairment, colic	None	[27]
4	Tanzania	Edible vegetable	None	[29]
5	Tanzania	Stomach troubles	None	[29]
6	Asian territory	Nervous depression, fever	None	[30]
7	India & other Asian territories	Anthelmintic, diuretic, purgative, diaphoretic	Validated	[31]
8	South-Eastern Nigeria	Malaria, fever	Preliminary	[28]

*None means no available scientific evidence; **Validated** signifies that the ethnomedicinal application has been backed by scientific evidence; **Preliminary** suggests that available evidence is only preliminary

as an isolated pure cyclotide, kalata B7 [33]. Findings from this study showed that 1 mg/mL of the crude extract effected the stimulation of contraction with an amplitude of $+8.1 \pm 3.5\%$, whereas the area under the curve increased by $313.5 \pm 96\%$ (mean $\pm$ SEM; $n = 6$). Since cyclotides are larger and bulkier than the nonapeptide ligand oxytocin, the sequence of kalata B7 was used as a template for the synthesis of four oxytocin-like nonapeptides, one of which was kalata B7-oxytocin 1 (kB7-OT1). The application of kB7-OT1 caused a dose-dependent increase in both contraction amplitude (1 nM: $+6.0 \pm 3.2\%$; 10 nM: $+41.4 \pm 6.4\%$; 100 nM: $+73.2 \pm 5.4\%$) and area under the curve (1 nM: $+2.7 \pm 2.3\%$; 10 nM, $+48.8 \pm 20.3\%$; 100 nM; $+218.3 \pm 143.6\%$; $n = 3$) [33]. This study has provided preliminary evidence supporting the potentials of cyclotides in the development of cyclotide-derived peptide ligands, which requires an elaborate further scientific investigation for a possible future clinical application. Furthermore, findings from this work provides a mechanistic evidence supporting the traditional application of OA in ethnomedicinal midwifery and indigenous child delivery healthcare in Africa.

The traditional application of OA in treating fever and other immune-related conditions aroused scientific interest. The orally stable Kalata B1 (KB1), which was first isolated from OA has shown interesting anti-inflammatory and immune modulatory potentials *in vivo* and its potent synthetic analogue T20K (see Figure 3) is now in clinical trials targeting multiple sclerosis [34, 35]. Further translational research effort is needed to unveil the application of other suites of cyclotides reported in OA (Table 3) as these amazingly stable peptides hold great potentials as efficient orally stable peptide-based therapeutics.

The reported ethnomedicinal application of OA in South-Eastern Nigeria against malaria has been preliminarily supported by the work of Nworu and colleagues who reported that the extracts from the plant demonstrated antiplasmodial as well as anti-inflammatory activity in infected mice [28]. According to this study, the aqueous

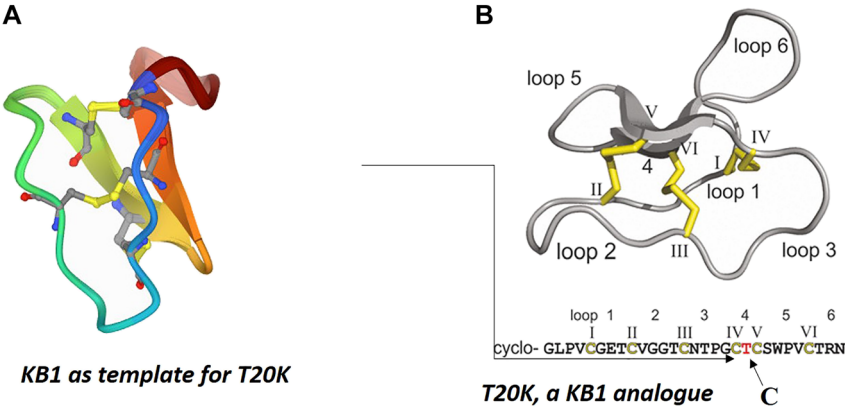


Figure 3: *Oldenlandia affinis* cyclotides as template for the discovery of T20k for clinical application. (A) Kalata B1 (PDB: 1NB1) as a template for the development of orally stable synthetic T20K (B), a novel cyclotide-based therapeutics for bedside application targeting multiple sclerosis. (C) The amino acid position where the threonine (T) has been replaced with lysine in the synthetic kalata B1 analogue indicated in red letter (C). (B) has been reproduced with permission from the Creative Commons Attribution License [35].

Table 3: Reported cyclotides in *Oldenlandia affinis* (<http://www.cybase.org.au>).

S/ N	Cyclotide	Biological activities	Possible clinical applications	Synthetic variants	References
1	Kalata B1	Immunosuppression Nematocidal	Multiple sclerosis Antihelminthic	[T20K]kB1 Several lysine mutants; cyclo-[GGG]kB1[T] variant	[34] [36]
		Membrane binding	Antibacterial	–	[37]
		Anti-HIV	HIV/AIDS	–	[38]
		Bactericidal	Sepsis	–	[39]
2	Kalata B2	Nematocidal	Antihelminthic	–	[40]
		Molluscicidal	Schistosomiasis	–	[41]
3	Kalata B3	–	–	–	–
4	Kalata B4	–	–	–	–
5	Kalata B5	–	–	–	–
6	Kalata B6	Nematocidal	Antihelminthic	–	[40]
		Membrane binding	Antibacterial	–	[37]
7	Kalata B7	Nematocidal	Antihelminthic	–	[40]
		Molluscicidal	Schistosomiasis	–	[41]
8	Kalata B8	Molluscicidal	Schistosomiasis	–	[41]
		Anti-HIV	HIV/AIDS	–	[42]
9	Kalata B9	–	–	–	–
10	Kalata B10	–	–	–	–
11	Kalata B11	–	–	–	–

Table 3: (continued)

S/ N	Cyclotide	Biological activities	Possible clinical applications	Synthetic variants	References
12	Kalata B12	—	—	—	—
13	Kalata B13	—	—	—	—
14	Kalata B14	—	—	—	—
15	Kalata B15	—	—	—	—
16	Kalata B16	—	—	—	—
17	Kalata B17	—	—	—	—
18	Kalata B18	—	—	—	—
19	Kalata B19	—	—	—	—
20	Kalata S	Nematocidal	Antihelminthic	—	[40]
		Anti-HIV	HIV/AIDS	—	[43]
		Cancer cell toxicity	Chemotherapy	—	[44, 45]

methanol extract of aerial parts of OA exhibited *in vivo* antiplasmodial and anti-inflammatory properties in animal models at an effective dose range of 100–400 mg/kg/day, with the highest test dose of 400 mg/kg/day showing the most significant prophylactic and curative effects from *Plasmodium berghei* infection. However, the study only used crude extracts which appear to be more active at high doses and was also not elaborate enough to explain the mechanism of activity of the active metabolites in the extracts. However, on the interim, this study has provided some evidence for the traditional application of OA in conditions related to fever and malaria. Further mechanistic study of individual cyclotides as well as other small isolated molecules is hereby suggested.

In line with the reported traditional application of OA as an anthelmintic in Indian ethnomedicine and documented in the Glossary of Indian Medicinal Plants [31], Colgrave and colleagues demonstrated the effectiveness of OA and the significant activity of its cyclotide-rich extracts against gastrointestinal nematodes of sheep [41]. Due to their reported insecticidal properties [46], these cyclotides which are abundantly expressed by OA and other plant families are thought to be involved in host defense [47]. Their specificity and selectivity for storage and field pest of agricultural importance remains to be studied in field experiment in order to provide explanation

for their potential application as safe, sustainable and environmentally friendly peptide-based bioinsecticides.

No study has yet been shown to evaluate the effectiveness of its traditional use against asthma [26] as reported in South Africa or its use in improving learning impairment and in managing colic as is common in Benin Republic as an indigenous health promoting drink [27]. Meanwhile, agents which generally work to relax the uterus by serving as agonists on β_2 receptors generally tend to relax the smooth muscles of the bronchioles thereby bringing relief to asthma patients [48]. It remains to be investigated how a uterotonic cyclotide-rich extract like OA can target asthma. If this does happen then it may be as a result of activity on a new, hitherto undescribed bronchiolar receptor. This represents an important gap requiring scientific investigation and may well trigger the discovery of cyclotide-derived peptide based antiasthma drug or template.

A research work carried out in Clayton Georgia by Kamimori and colleagues demonstrated that OA has the ability to selectively target the membranes of bacterial organisms which in itself is a unique property of antibiotics. However, the research did not point out if there was a difference in the response rate of gram positives compared to gram negatives; neither did it demonstrate the exact activity of OA after binding to the bacterial membrane [37]. Another antimicrobial property of OA reported the inhibition of the cytopathic effects of HIV-1 infection in cultured human T-lymphoblasts [42]. Majority of the ethnopharmacological analysis of OA have focused mainly on cyclotide-rich extracts or purified cyclotides from OA. No single scientific attention has been drawn to the small molecule metabolites in OA.

5 Documented metabolite profile in *Oldenlandia affinis*

An elaborate phytochemical data on OA has not been reported except the analysis of its cyclotide content. Only a preliminary phytochemical screening has been reported on the crude extract to contain mainly flavonoids, steroids and proteins, while the cyclotide-rich fraction contains phenolics and proteins [28]. It is therefore suggested that a metabolite profiling of OA be carried out to document the array of small molecule compounds synthesized by OA and their possible interaction with cyclotides during application in local healthcare.

5.1 Nonpeptidic metabolites of *Oldenlandia affinis*

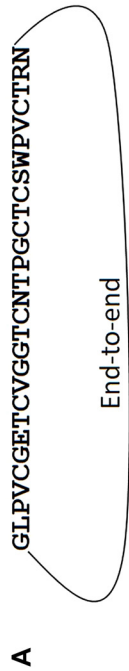
A preliminary phytochemical study carried out by Nworu and colleagues on the whole aerial part extract (APE) of the plant and the cyclotide-rich fraction (CRF) indicated the

presence of a variety of bioactive plant metabolites in different amounts which include saponin, tannin, reducing sugar, glycosides, proteins, acidic compounds, alkaloids and flavonoid in APE while flavonoids and alkaloids were relatively more abundant in the CRF; steroids and terpenoids were also identified in the CRF fraction [28]. As this report is only preliminary and not useful for an inference, a detailed metabolite profiling such as metabolomics of OA is suggested to generate chemical fingerprint on the plant.

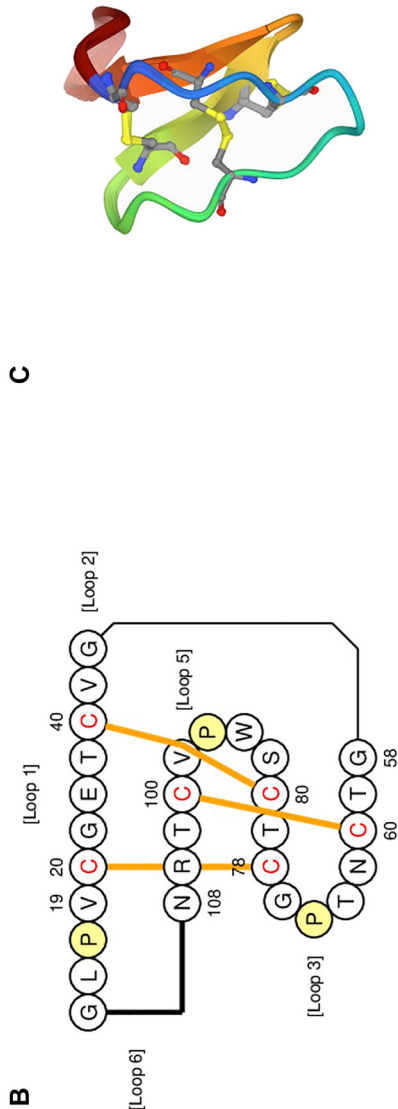
5.2 Peptidic metabolites of *Oldenlandia affinis*

On the cyclotide-rich content of OA, in the 1960s, a physician working with the Norwegian Red Cross in Africa, was fascinated by the effectiveness of a medicinal tea made with OA. It was observed that the Congolese women would boil the leaves of the plant to make a tea which they ingested during labour to accelerate childbirth. Dr Gran investigated the properties of the medicinal tea to understand why the plant remained an effective treatment for women during childbirth, even after boiling. In his 1970 report, Dr Gran characterized the peptide and named it kalata B1 after the indigenous medicine, *kalata-kalata* [49, 50]. However, it was not until over 20 years later that the cyclic nature and knotted character of kalata B1 were described. The pioneering works of Dr Gran paved way for much later scientific enquiries into the plant, *Oldenlandia affinis* [49].

Following the interrogation of the uterotonic activity of OA and the consequent discovery of the presence of the prototypic cyclotide, kalata B1 (Figure 4), there have been more probes of the *kalata-kalata* plant in order to expand the understanding of its chemical make-up especially with respect to expression of cyclotides. More cyclotides from OA, with little or more variations to kalata B1, have been reported (Table 3). The name “cyclotide” resulted from a combination of the words *cyclic peptide* and was introduced in 1999. The cyclotides are disulphide-rich plant proteins distinguished by the presence of a head-to-tail cyclic peptide backbone; the three disulphide bonds are in a knotted arrangement, usually referred to as a cyclic cysteine knot (CCK) motif [51]. The CCK is made up of six well conserved cysteine residues which furnish the disulphide linkages. This motif provides cyclotides with structural rigidity and a remarkable stability to chemicals, temperature, and enzymatic degradation [52]. Cyclotides have been found in plant species from the Violaceae [53], Rubiaceae [54], Cucurbitaceae [53], Poaceae [55], Solanaceae [56] and Fabaceae [53] families, with the peptides containing between 28 and 37 amino acid residues. The peptides have a macrocyclic amino acid backbone, which is completed through a peptide bond between the N- and C-termini [18], that is, there are no free N- or C- termini. Cyclotides are products of genes embedded in the nuclear genome. Their synthesis happens via formation of precursor proteins that undergo a series of post-translational modifications – enzyme-catalyzed cleavage, formation of disulphide bonds and cyclization [57]. Cyclotides can be further



Sequence of amino acid of the archetypic circular Kalata B1



Standardized 2D representation of Kalata B1

High resolution solution structure of Kalata B1
(RSCB PDB – 1NBI)

Figure 4: Primary sequence, 2D and tertiary 3D structural representations of the archetypic kalata B1 (RSCB PDB – 1NBI). This prototypic peptide was first discovered in *Oldenlandia affinis*, an African ethnomedicinal plant.

categorized into two major subfamilies, the Mobius and bracelet, which differ from each other by the presence or absence of a *cis*-Pro residue in loop 5. Cyclotides could be ascribed the largest known plant peptide family considering that over 100 cyclotides have already been sequenced, a dozen structures determined and thousands more predicted from mass spectrometry profiles [57].

5.3 Phytochemistry of cyclotides expressed in *Oldenlandia affinis*

The prototypic cyclotide in *Oldenlandia affinis* is the kalata B1 peptide, kB1 for short, which was a result of the research into the uterotonic activity of the medicinal tea which Congolese women brewed from the leaves of *O. affinis*. Further research on *O. affinis* plant have led to the discovery of other cyclotides, mostly named following the ‘kalata’ nomenclature. Kalata B1–B18 have been known to be expressed in *O. affinis* plant [58]. Weidmann and Craik, in their review, made reference to a number of ~20 cyclotides found in OA [59], another reported that >30 cyclotides have so far been identified in OA [60]. However, it is suggested that this number may increase with varying growth conditions [61]. It is worthy of note that some of the cyclotides attributed to *O. affinis* are not expressed from the whole plant growing in the wild; they are expressed in suspension cultures, for example, the linear cyclotide derivative kalata B20-li, kalata B21, and kalata N1–N12 [60] while kalata B19 has been predicted from expressed sequence tag (EST) [58]. Reported cyclotides in OA, their occurrence and their concomitant expression in other plant families as well as techniques used for their detection are presented in Table 4.

Cyclotides are gene products which are usually embedded within cyclotide precursor proteins, otherwise known as cyclotide prepropeptides. Efforts previously directed towards cyclotide discovery were quite entirely dependent on isolation at peptide (that is, chemical) level, using solvent-based extraction methods; while approaches employed more recently also included genetic procedures since cyclotides are biosynthesized ribosomally via genetically encoded precursor proteins [57, 61]. Techniques such as mass spectrometry (MS), expressed sequence tag (EST), polymerase chain reaction (PCR), next generation sequencing (NGS), Amino acid analysis have been employed in the characterization of cyclic peptides (<http://www.cybase.org.au>). Among the B1–B18 cyclotides, only four cDNA clones encoding cyclotide precursors were previously isolated from OA and named Oak 1–4. These plant cyclotides are finally produced by cleavage and cyclization from precursor proteins incorporating one, two, or three cyclotide domains. The Oak 1 gene encodes the largest protein, Oak 1 prepropeptide which was found to be a precursor for the first discovered cyclotide, kalata B1 [46]. Other cDNA clones, namely Oak 2, Oak 3 and Oak 4 encode kalatas B3/B6, B7 and B2, respectively. Using EST technique, a previously unidentified putative bracelet cyclotide precursor, named Oak 5 was shown to encode the peptide kalata B19 [58].

Table 4: Occurrence and distribution of *Oldenlandia affinis* cyclotides in plants.

S/N	Cyclotide	Occurrence in plants	Detection technique
1	Kalata B1	<i>Oldenlandia affinis</i> <i>Viola odorata</i> <i>Viola tricolor</i> <i>Hybanthus enneaspermus</i> <i>Viola baoshanensis</i> <i>Viola yedoensis</i> <i>Viola philippica</i> <i>Viola sumatrana</i> Miq	MS, EST, PCR, NGS, amino acid analysis
2	Kalata B2	<i>Oldenlandia affinis</i> <i>Viola odorata</i>	MS, EST, amino acid analysis
3	Kalata B3	<i>Oldenlandia affinis</i>	MS, EST, amino acid analysis
4	Kalata B4	<i>Oldenlandia affinis</i>	MS, amino acid analysis
5	Kalata B5	<i>Oldenlandia affinis</i>	MS, amino acid analysis
6	Kalata B6	<i>Oldenlandia affinis</i>	MS, EST, amino acid analysis
7	Kalata B7	<i>Oldenlandia affinis</i>	MS, EST, amino acid analysis
8	Kalata B8	<i>Oldenlandia affinis</i> <i>Hybanthus enneaspermus</i>	MS, NGS, amino acid analysis
9	Kalata B9	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
10	Kalata B10	<i>Oldenlandia affinis</i>	MS, amino acid analysis
11	Kalata B11	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
12	Kalata B12	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
13	Kalata B13	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
14	Kalata B14	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
15	Kalata B15	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
16	Kalata B16	<i>Oldenlandia affinis</i>	MS, amino acid analysis
17	Kalata B17	<i>Oldenlandia affinis</i>	Amino acid analysis, MS
18	Kalata B18	<i>Oldenlandia affinis</i>	
19	Kalata B19	<i>Oldenlandia affinis</i>	EST
20	Kalata S	<i>Viola odorata</i> <i>Oldenlandia affinis</i> <i>Viola tricolor</i> <i>Viola arvensis</i> <i>Viola baoshanensis</i> <i>Viola yedoensis</i> <i>Viola biflora</i> <i>Viola philippica</i> <i>Viola ignobilis</i> <i>Viola uliginosa</i> <i>Viola sumatrana</i> <i>Viola arcuata</i> <i>Palicourea sessilis</i> <i>Viola betonicifolia</i> <i>Viola anagae</i>	NGS

6 Taxonomic conflict and a new chemotaxonomic marker in *Oldenlandia affinis*

O. affinis is a cyclotide-rich ethnomedicinal plant [32]. Documented cyclotides identified in OA (Table 4) contain many that are quite exclusive to OA (KB3–KB7; KB9–KB19). The understanding that cyclotides are not ubiquitously expressed across all plant families make them important in chemotaxonomic applications [47] and may particularly be useful in solving the *Oldenlandia*–*Hedyotis* ambiguity. Only a few species within the Rubiaceae family have been reported so far to express cyclotides [62, 63]; and this variability in cyclotide expression within closely related members of the taxon has advantages in resolving the existing taxonomic confusion in the genus *Oldenlandia* (which some report as *Hedyotis*). Members of the tribe Spermacoceae are increasingly gaining commercial value and scientific recognition owing to their validated applications in the Traditional Chinese Medicine (TCM) as well as Traditional African Medicine (TAM) [17, 64]. Nevertheless, conflicting reports resulting from taxonomic inconsistencies represents a daunting threat to users during the selection and utilization of genuine drug. For instance, there is a reported absence of cyclotides in *Hedyotis corymbosa* (L.) Lam (accepted name being *Oldenlandia corymbosa* L.) [63, 65], a closely related species of the cyclotide-rich *O. affinis*. However, in the same paper, Nguyen and colleagues [65] characterized novel cyclotides from *Hedyotis biflora*, which, according to The Plant List (<http://www.theplantlist.org/>), World Flora Online, WFO (<http://www.worldfloraonline.org/>) and World Checklist of Selected Plant Families, WCSP (<https://wcsp.science.kew.org/>), is only a synonym of the accepted name *O. corymbosa* which was previously reported to be cyclotide-free. In another report, *Hedyotis diffusa* Willd. (accepted name being *O. diffusa* (Willd.) Roxb.), an important medicinal plant in TCM, was documented to be cyclotide-producing [64, 66]; meanwhile, the WFO and WCSP classification described *O. diffusa* as a synonym of the cyclotide free *O. corymbosa* [67]. The question is: are these contentious medicinally important plants actually from different species, same species or do they represent different accessions of one of the species, perhaps *O. corymbosa* L.? Cyclotides discovery, occurrence and distribution in some and absence in other members of the taxon represent a new chemotaxonomic marker with potential to resolve this ongoing debate. In particular, the discovery of cyclotides in OA and few other species within the contentious genus has led to an increased interest in studying their distribution patterns and exclusivity of cyclotide expression in the few cyclotide-positive species within the genus *Oldenlandia* (synonym – *Hedyotis*). Documented cyclotides identified in OA (Table 4) express many that are quite exclusive to OA. Cyclotides can be useful in chemosystematics where they could serve as chemotaxonomic markers in the species that express them.

7 Conclusion and future prospect

Findings from this review indicate that *Oldenlandia affinis* (OA) is endemic to two main continents of Africa and Asia whose traditional uses include; to facilitate childbirth, asthma, learning impairment, colic, GIT, nervous depression, fever, anthelmintic, diuretic, diaphoretic, malaria and fever. As opposed to few other members of the genus *Oldenlandia*, available literature suggests that OA has only received a limited scientific attention which focused mainly on the discovery and ethnopharmacology of cyclotides. Only three documented traditional uses of OA including; as a uterotonic, an anthelmintic and for fever management have been validated with documented mechanism of activity. This scientific investigation has led to the discovery of Kalata B1 (kB1) an anti-inflammatory and immunomodulatory cyclotide as well as kB7, a uterotonic cyclotide identified in OA. Interestingly, the cyclotide-rich crude extract of OA and kB1 produced impressive immunomodulatory activity leading to the discovery of a mutant kB1 (T20K) which is now undergoing clinical trials for multiple sclerosis. The scientific report on the antimalarial property of OA is preliminary and therefore requires further evidence to unravel the bioactive secondary metabolite or cyclotide as well as other molecular targets involved in observed activity. The authors also observed that OA has not received an indepth metabolite profile analysis (OA metabolomics) to document the fingerprint of both small molecules (secondary metabolites) as well as other bioactive compounds.

The abundant cyclotide expression in OA represents a new chemotaxonomic marker for its unambiguous identification, utilization and reproducibility of research findings on the species. The chemical analysis for the presence or absence of cyclotides will potentially represent a reliable chemical basis for identity of OA while the proposed chemophenetic study of OA could put an end to the growing confusion created by a lack of taxonomic consensus on the *Oldenlandia–Hedyotis* ambiguity. To avoid species mismatching resulting from taxonomic inconsistencies and confusion, authors recommend the use of the currently accepted names of *Oldenlandia affinis* and closely related species as described in WFO and WCSP databases, for scientific publications and for commerce. With respect to conservation status and the IUCN red list of threatened species (<https://www.iucnredlist.org/>), OA has not been evaluated which suggests that reviews are still necessary to determine the category of conservation threat it will appropriately fall. Until now, OA is still collected from the wild and since the lack of categorization does not exclude the tendency of a species to be threatened, the deliberate cultivation of OA and the use of plant tissue culture can contribute to the conservation of the plant. In conclusion, the expected scientific rigor required for the correct identification, authentication and documentation of OA will fast track its future clinical application.

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