African Journal of Biotechnology Vol. 6 (13), pp. 1516-1520, 4 July, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

A chemotaxonomic approach to the alkane content of three species of *Anthocleista* Afzel. (Loganiaceae)

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Accepted 30 May, 2007

The chemotaxonomic significance of leaf wax n-alkanes was studied in three species of *Anthocleista* Afzel. Identification of alkane components were determined by gas chromatography (GC) and gas chromatography – mass spectrometry (GC-MS) data. In all, fourteen alkanes were identified, ranging from tetracosane ($C_{24}H_{50}$) to heptriacontane ($C_{37}H_{76}$). Tetracosane, pentacosane and heptriacontane were the major components in all the three species of *Anthocleista*. *A. djalonensis* and *A. vogelii* were characterized by high percentages of tetracosane (28.81 and 39.14%, respectively), whereas in *A. nobilis* heptriacontane ($C_{37}H_{76}$) was the major component being 24.76%. Significant correlation between *A. djalonensis* and *A. vogelii* (r = 0.884 and p = 0.000) at 0.01 implies a probable closeness between these species. However, the result obtained in this study provides further evidence of chemotaxonomic significance.

Key words: Leaf wax, leaf alkanes, *Anthocleista*, Loganiaceae, alkane distribution, chemotaxonomy.

INTRODUCTION

The genus *Anthocleista* Afzel. consists of approximately 50 species native mainly of tropical Africa, Madagascar and Mascarene Islands. Nine species are recorded in West Tropical African region (Burkill, 1995), out of which five are reported in South Western Nigeria. Three of these species: *Anthocleista djalonensis Anthocleista* Chev., *Anthocleista vogelii* Planch*, Anthocleista nobilis* G. Don are abundant and widespread in the region. They are usually small trees or scrambling shrubs with soft white wood. Their leaves are glabrous, leathery and large and are often over 1 ft long in mature trees and up to 5 ft long in saplings. The base of the leaf stalk is dilated and sometimes more or less winged. The leaves of these species ranges from obovate to oblanceolate shapes and are clustered at the ends of the branchlets, mostly 6 - 18 inches long; and the flowers 1 - 3 inches long. The flower colour ranges from white to cream in *A. djalonensis* and *A. nobilis* while it is orange or fawn colour in *A. vogelii.*

The genus *Anthocleista is* faced with the problem of classification. One of the historic problems with classifying this genus under Loganiaceae family was that most taxa assigned to this family had rather generalized or plesiomorphic traits (Leeuwenberg and Leenhourts, 1980). The data from phylogenetic studies in 1990 placed this genus under the family Gentianaceae and later transferred to the family Loganiaceae. *Anthocleista* diverge from the gentian flora due to its possession of supermerous corollas and staminal parts (8 - 16 merous). And it appears to lack post genital fusion of carpels which is typical to gentians (Struwe and Albert, 2002). So it was concluded that *Anthocleista* is a tropical woody genus with showy flowers and fleshy or leathery berries, whereas most gentians are smaller herbs or shrubs with dry capsular fruits (Struwe and Albert, 2002).

In search for clues to assist in understanding the phylogeny of the genus and relationships among the Nigerian species, studies are being carried out in chemotaxonomic implication of alkane content. Alkanes from epicuticular waxes have become of great significance in indicating taxonomic relationships of plants (Campaner dos Santos et al., 2005; Medina et al., 2004; Medina et al., 2006). The chemotaxonomic importance of wax alkanes has been demonstrated in studies on Solanaceae (Zygadlo et al., 1994), Crassulaceae (Stevens et al., 1994), Cacta-

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Table 1. Representatives of *Anthocleista* species studied.

Binomials and authorities	Synonyms and authorities	Voucher information
Anthocleista djalonensis A. Chev	*Anthocleista kerstingii* Gilg ex Volkens *Anthocleista procera* A.Chev	FHI 107523
Anthocleista vogelii Planch	*Anthocleista talbotti* Wern *Anthocleista kalbreyeri* Bak	FHI 107524
Anthocleista nobilis G. Don	*Anthocleista parviflora* Bak	FHI 107525

ceae and Pinales (Maffei et al., 2004), Moraceae (Sonibare et al., 2005a) and so on. Eglinton and co-workers first noted the advantages of the use of cuticular secondary compounds as a criterion for systemic classification. So far no research has been done based on the alkane composition of *Anthocleista* species, but some phytochemical analysis have been done on this species to isolate other chemical compounds. A new monoterpene diol and a flavonoid (iridoid glucoside djaloneside) were isolated from *A. djalonensis* by Onocha and Okorie (1995), while three iridoid glucosides (iridoid secologanic acid, sweroside and vogeloside with the presence of xanthones) were found in *A. vogelii*. *A. vogelii* was found later to contain eight different iridoid glucosides: namely mussaenoside, gradoside, bartsoiside, melittoside, isoaucubin, eurostoside, 2 caffeoyl phenylethyl giucosides (calceolarioside A and verbascoside) and lastly trigonelline (Rank et al., 2004). The third species, *A. nobilis* was found to contain glucosides such as swertiamarin, gentianine, monoterpenic heteroside and Anthocleistine.

From the morphological view, *A. djalonensis, A. vogelii* and *A. noblis* are closely related. They all have spines and white flowers. But *A. vogelii* is practically stalkless while *A. djalonensis* and *A. nobilis* are distinctly stalked. In this research, the distribution of alkanes in the leaf waxes of *Anthocleista* species was examined as well as its relationship with morphological characteristics in delimiting the species of this genus.

MATERIALS AND METHODS

Extraction and isolation

Mature leaves of plants belonging to *A. djalonensis* and *A. vogelii* were collected in the blooming stage of vegetation, around 11.00 am on 13th of April, 2005 in Ibadan Oyo State Nigeria, and the third species; *A. nobilis* was collected in Omo- Ijebu Ogun State on 30th of April, 2005 at about 11.50 am. Voucher specimens of all the species were deposited at the Forest Herbarium Ibadan (FHI), Nigeria. Representatives of the species used in the study are presented as Table 1. The leaves of the plants were air dried for fourteen days at room temperature (to avoid further synthesis). The leaves were ground into powder using an electric blender. The shafts obtained afterwards were screened out with the metal sieve (Mesh 20 mm).

50 g of finely powdered air dried leaves of *A. djalonensis*, 45 g of *A. vogelii*, and 40 g of *A. nobilis* were extracted for 24 h each with n-hexane using the Soxhlet extractor. The extraction of 50 g of *A. djalonensis* yielded 4 g residue and that of 45 g of *A. vogelii* gave 2.5 g yield while that of 40 g *A. nobilis* gave 3.5 g residue, after which the extracts were concentrated *in vacuo*. The concentrated extracts were then chromatographed on a 30 m x 1 cm i.d. column packed with 4.0 g of activated silica gel (70 – 230 mesh). Wax was obtained by elution with 40 ml of n- hexane.

GC – Gas chromatography

Gas chromatography, gas chromatography-mass spectrometry analyses were done according to the procedure of Sonibare et al. (2005a). The extract(s) was injected into a column injector of a gas chromatograph Shimadzu GC-17A, fitted with a flame ionization detector (FID) 30 cm x 0.25 mm i.d. fused with a silica capillary column with a 0.25 um film thickness of HR–1. The operating conditions were temperature program from 60°C (isothermal for 3 min) to 160°C at 8°C/min and from 160°C to 320°C at 5°C/min and isothermal for 30 min. The injector and detector were at 300°C and nitrogen was used as carrier gas. Alkanes were then identified by their relative retention times (RRT) with respect to standard samples run under identical conditions. Peak areas and concentrations were calculated using an electronic integrator. And peak identification was based on retention times (Rt) compared with pure standards and GC-MS.

GC-MS analysis of these compounds were performed on a Shimadzu QP 5050A equipped with a fused silica 30 m x 0.25 mm, HR–I capillary column (film thickness 0.25 um) with nitrogen as the carrier gas, and the same temperature program as for the analytical GC (gas chromatography). The mass spectrometer was operated at electron energy of 70 ev and ion source temperature of 230°C. At least one sample per species was turn on GC-MS for peak identification. The samples were analyzed using full scan mode. The mass spectrometer was scanned over a range of 50 – 650 amu. Alkane peaks were identified by comparing their mass fragmentation patterns with those of standard alkanes available on the computer. The even and odd numbered n-alkanes were identified by direct comparison with pure standard mass spectra.

The carbon preference index (CPI) of the leaf alkanes was calculated using the equation of Bray and Evans (1961).

$$CPI = \frac{1}{2}\left[\frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}+C_{35}}{C_{24}+C_{26}+C_{28}+C_{30}+C_{32}+C_{34}} + \frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}+C_{35}}{C_{26}+C_{28}+C_{30}+C_{32}+C_{34}+C_{36}}\right]$$

The average chain length (ACL) was calculated as

$$ACL = \sum (\%\ C_n \times n)\ /100$$

where n = number of carbon atoms.

A correlation analysis was performed on the profile of fourteen alkanes of the different species, to summarize the relationship between the species. The data matrix of the fourteen compounds was evaluated using the Pearson correlation method and the Spear-

Table 2. Composition of the n-alkane fraction in leaf waxes of Anthocleista species.

Alkanes	Carbon no.	% Distribution of alkanes in taxa		
		A. djalonensis	***A. vogelii***	***A. nobilis***
Tetracosane	24	28. 81	39. 14	11.70
Pentacosane	25	27. 04	12. 64	5.87
Hexacosane	26	0. 82	0. 88	1.03
Heptacosane	27	1.15	1. 39	1.20
Octacosane	28	1. 66	1. 68	1.90
Nonacosane	29	1. 30	1. 08	1.08
Triacosane	30	7. 80	2. 11	15.24
Hentriacontane	31	4. 45	1. 93	1.62
Dotriacontane	32	1.22	7. 25	4. 21
Tritriacontane	33	12.00	16.32	4. 23
Tetratriacontane	34	1.80	2.32	0. 90
Pentatriacontane	35	4.70	5.11	9. 43
Hexatriacontane	36	4.36	5.94	16.83
Heptriacontane	37	2.89	2.21	24.76

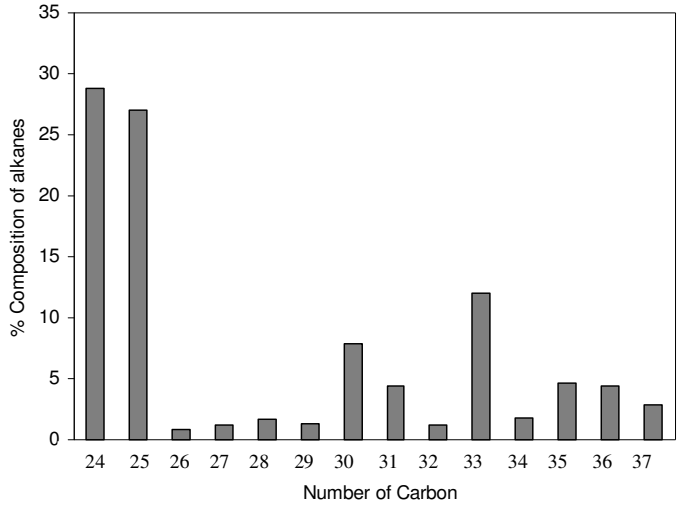

Figure 1. *n*-Alkane profile of *A. djalonensis* based on the intensity of the peaks obtained from GC-GC-MS analyses.

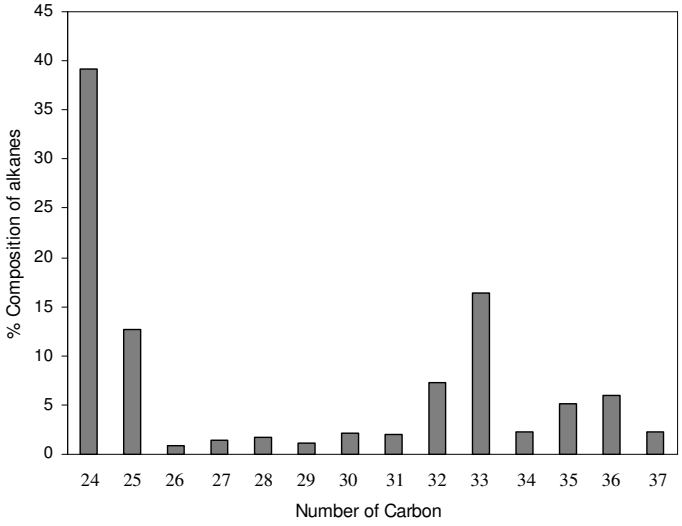

Figure 2. *n*-Alkane profile of *A. vogelii* based on the intensity of the peaks obtained from GC-GC-MS analyses.

man's rank correlation method. This was based on the abundance of the fourteen alkanes

RESULTS AND DISCUSSION

Epicuticular waxes of the leaves of *Anthocleista* species are represented by n-alkanes ranging from 24 to 37 carbon number. A total of fourteen alkanes were identified namely tetracosane, pentacosane, hexacosane, heptacosane, octacosane, nonacosane, triacontane, hentriacontane, dotriacontane, tritriacontane, tetratriacontane, pentatriacontane, hexatriacontane and heptriacontane (Table 2). The distribution of these fourteen constituents varied among the species. The major alkanes C_{24}, C_{25}, C_{36} and C_{37} were observed to be moderately present in the samples. In *A. djalonensis* and *A. vogelii*, a higher proportion of tetracosane (C_{24}) was found (Figures 1 and 2) while *A. nobilis* presents a higher proportion of heptriacontane (C_{37}) (Figure 3). However a very low content of nonacosane (C_{29}) was found in all the species. The carbon preference index (CPI) and average chain lengths (ACL) are shown in Table 3. The values of the CPI vary from 1.5 to 2.4. The values of the ACL varied from 28.0 to 32.4. The statistical analyses gave the correlation coefficients as shown in Table 4 for *A. djalonensis* and *A. vogelii* as $r = 0.884$ and $p = 0.000$, for

Table 3. Alkane chain lengths (ACL), carbon preference index (CPI) N and % alkane per dry weight of leaves of Anthocleista species.

Taxa	ACL	CPI	% alkane per dry weight of leaves
A. djalonensis	28. 0	2. 4	2.0
A. vogelii	28.4	1.5	2.2
A. nobilis	32.4	2.2	2.5

A. djalonensis and *A. nobilis* as $r = 0.172$ and $p = 0.542$ and for *A. vogelii* and *A. nobilis* as $r = 0.165$ and $p = 0.573$.

Epicuticular waxes coat the surface of fleshy plant organs and serve to protect the plant from desiccation, pest attacks as well as to control leaf temperature, frost hardiness and signaling between pollen and stigma etc (Taiz and Zeiger, 2002). Epicuticular waxes refer to surface lipids forming crystalloids or a smooth film exterior to the cuticle. A consistent part of epicuticular waxes is made of alkanes with predominant chain length from 21 to 37 carbon atoms. Beside biochemical, physiological and molecular considerations, wax alkanes have been considered for their chemotaxonomic value. The leaf wax n-alkane of *Anthocleista* species showed a chemical profile indicating that qualitatively the composition does not markedly differ from species of other plant families (Zygaldo et al., 1994; Steven et al., 1994; Sonibare et al., 2005a; Medina et al., 2006). The different pattern of n-alkane quantitative distribution allows the chemotaxonomic separation of these species as reported in the result.

From the chemical point of view, the high percentage of tetracosane (C24) in *A. djalonensis* and *A. vogelii* reflects the close relationship between phenotypic expressions of the same pool (Mongrand et al., 2001). Morphologically, considering the tree structure, shape of leaves and nature of flowers (petalous), *A. djalonensis* and *A. vogelii* are alike (Adesina et al., 1993). This closeness in morphological structures is further strengthened by the proximity in their alkane composition. But the flowers of *A. nobilis* are distinct (i.e. budlike). The results from the correlation analysis further elaborated the level of relationship between these species to a reasonable extent. The close statistical linkage assessed between *A. djalonensis* and *A. vogelii* showed almost perfect similarities between these species, suggesting a high level of closeness or relationship between them. A good separation was assessed between *A. djalonensis* and *A. nobilis*, results obtained showing no indication of relationship in any way. There was also no indication of any close relationship between *A. vogelii* and *A. nobilis*. The intimacy assumed between *A. djalonensis* and *A. vogelii* could be due to the same growth conditions and or developmental stage of the samples. The expression of a particular chemical is potentially influenced by the environment.

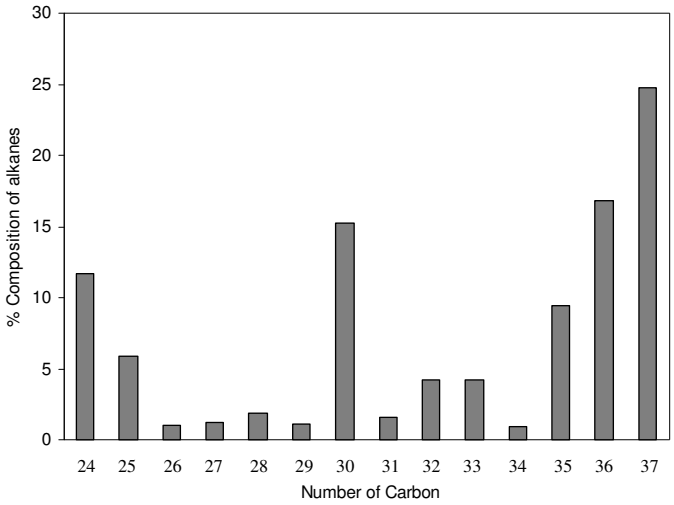

Figure 3. *n*-Alkane profile of *A. nobilis* based on the intensity of the peaks obtained from GC-GC-MS analyses.

It has been reported that a particular compound may be produced only at certain times or under certain conditions; which may be the case with *A. djalonensis* and *A. vogelii* having a high percentage of tetracosane. And these species are commonly found in forests or forest outliers (Adesina et al., 1993). Different environmental conditions and developmental stage of sampling as stated above may be responsible for the difference in alkane percentages.

Moreover, in recent phytochemical studies on these species, *A. djalonenesis* and *A. vogelii* were found to contain primitive xanthone which qualified them to be grouped under Gentianaceae family. The chemical compound xanthone was used in the grouping of genera under Gentianaceae family, and the genus *Anthocleista* fell within the first group under the family. In the screening analysis of *A. djalonensis*, lichexanthone and other irdoid compounds were discovered, but the presence of lichexanthone qualified it to be grouped under Gentianaceae family (Onocha and Okorie, 1995). Also in the phytochemistrry of *A. vogelii*, xanthones and some iridoid compounds were discovered, but because of the presence of xanthone it was grouped under the primitive family Gentianaceae (Rank et al., 2004). *A. nobilis* was reported only to contain iridoid compounds with the absence of xanthones. *A. nobilis* was disqualified from being a part of the Gentianaceae family, but was rather grouped under the family Loganiaceae (Rank et al., 2004).

It is probably true to state that, in general, chemotaxonomy in spite of often high hopes, has tended to act very much as a supporter of classical morphotaxonomy when it comes to identifying evolutionary relationship. Only in comparatively few instances has it led to realignment of higher taxa. With the presence of xanthones in *A. djalonensis* and *A. vogelii*, they could be regarded as

Table 4. Correlation statistics of three species of Anthocleista.

Taxa	Mean	Standard deviation δ	N	Correlation (r)	(p)	Remark
A. djalonensis X	7.143	9.332	14			
				0.884**	000	Significant
A. vogelii	7.143	10.314	14			
A. djalonensis X	7.143	9.332	14			
				0.178	0.542	Non-Significant
A. nobilis	7.143	7.447	14			
A. vogelii X	7.143	10.314	14			
				0.165	0.573	Non-Significant
A. nobilis	7.143	7.447	14			

** Significant at 1%.

being primitive, and they can be assumed to have sprung up from the same origin. This claim could probably be further strengthened by this new discovery of high percentage of tetracosane in these two species. In conclusion, the results of this work confirm the chemotaxonomic usefulness of surface wax alkanes particularly at the species level. The comparison of the correlation data obtained in this research work suggests a close relationship between *A. djalonensis* and *A. vogelii*. This provides further evidence for the utility of alkane chemical analysis as a quick, reliable and inexpensive method to assess preliminary chemotaxonomic relationships. However, to make meaningful taxonomic conclusions, a wider sampling will be needed in future investigation of the alkane profile of the genus.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation for the laboratory facilities provided by Dr. R. Egashira of the Department of International Development Engineering, Tokyo Institute of Technology (Chemical Engineering Unit), Japan.

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