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Leaf Epidermal Analysis for Diagnosis of Enigmatic *Strychnos* Species in Nigeria

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ABSTRACT
Ten species of Strychnos were collected from Oban group of Forest (Cross River State, Nigeria) without their inflorescence which hindered their complete identification. Their leaves sizes, shapes, stems coloration and other morphological characters show considerable variations. Anatomical results of their leaf epidermal structures were used to delimit these sterile species. The epidermal cell shapes are polygonal and irregular. Their anticlinal wall patterns are straight, curved or undulate. The epidermal cell numbers are between 87 and 280. Epidermal cell lengths are between 7µm and 38µm. Stomata types are generally paracytic and their indices vary between 5.8 and 11.3. Their trichomes are simple unicellular with lengths between 12µm and 155µm. Both cluster analysis and scatter plots revealed that SID1 and SID2 are very similar species; 94 % similarity. Principal component analysis revealed that epidermal cell number, length, width; trichome; stomata number and indices formed the major components delimiting these species among the 36 characters observed. There are 3 clusters formed which corresponded to 3 sections in Strychnos classifications.

KEYWORDS: *Anatomy, Strychnos, Enigmatic, Oban, Classification, variations.*

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I. INTRODUCTION

Strychnos Linn. is the largest genus in Loganiaceae with approximately 200 species that grow in tropical rain forests and savannas as lianas, shrubs, or small trees (Frasier, 2008). In the paleotropics it is found throughout tropical Africa and Madagascar, in India, Sri Lanka, Southeast Asia and the northern tropical part of Australia (Bisset *et al*., 1973; Leeuwenberg and Leenhouts, 1980). *Strychnos* species grow as liana and trees in the forest but as tress in the savanna zones. They are very important in ecology dynamics of the forest where they are found (Putz and Holbrook, 1991). This genus is highly economical as medicinal recipe for several ailments in Nigeria; and probably the most famous in science and culture for its production of the toxin strychnine, which is commercially extracted from *S. nux-vomica* (Bisset *et al.*, 1973; Samuelsson, 1992; van Andel, 2000; Frasier, 2008). In Africa, some *Strychnos* species, such as *S. aculeata,* are used as fish poisons and for treating parasitic infections like guinea worm (Burkill *et al.*, 1995). In Madagascar, *Strychnos myrtoides* has been combined with more conventional drugs to treat malaria (Rafatro *et al*., 2000), and in India, *S. potatorum* seeds are used to settle turbid water (Gupta and Chaudhuri, 1992).

The anatomical characters of the leaf epidermis are found useful when the plant parts are dried and leaf fragments are available for identification purposes, this is popular with herbal recipes and preparations. Many medicinal collections are dry and fragmented or deliberately made dry in order to extract the bioactive ingredients from them (Abu *et al*., 2009). Sometimes there is confusion in identification and differentiation of species of the same genus on the basis of morphology, so anatomical studies assist in solving problem of identification (Ahmad *et al*., 2012). Carlquist in 1961 stated that "the leaf is perhaps anatomically the most varied organ of angiosperms (Radford *et al*., 1974)". Leaf epidermal study provides valuable data towards the identification of plant and is recognized as a source of useful taxonomic characters, because of variations in leaf characters that are taxonomically useful (Barkworth, 1981; Ahmad *et al*., 2012). The arrangement of epidermal cells on the adaxial surface of the leaf blade is generally quite different from that of the abaxial surface. Epidermal cells could be short or long; they vary in length and width, wall thickness, and the extent to which the walls are sinuous, papillate or pitted (Gould, 1969; Radford *et al.*, 1974; Ahmad *et al.*, 2012). Leaf epidermal traits i.e. epidermal cells, stomata and trichomes have been proved valuable in identification and differentiation of different taxa (Stenglein *et al*., 2003).

Leaf epidermal features are important to clarify taxonomic relationships in different taxa (Radford *et al.*, 1974; Davila and Clark, 1990; Cai and Wang, 1994; Mejia–Saules and Bisbey, 2003; Ogie- Odia *et al.*, 2010; Ahmad *et al.*, 2012). The aim of this investigation is to utilize leaf epidermal variations to distinguish and differentiate sterile *Strychnos* species used in herbal medicine.

II. MATERIALS AND METHODS

Cross River National Park (CRNP) is rich in biodiversity and has the dimension of 4,000 Sq. Km. Ten sterile collections were encountered on the field and were collected with Global Positioning System device (GPS) and the coordinates of collections were properly documented. Thirty six (36) morphological and Anatomical characters; both qualitative and quantitative that have taxonomic significance were assessed. The plant parts were measured, counted and an area of about 1cm square was removed from a central/standard position of the mature fresh leaves according to Olowokudejo, (1993) for leaf epidermal analysis. The dry leaves were revived by boiling in water for 10 – 15 minutes and then soaked in domestic bleach or nitric acid to digest the mesophyll layer. Samples were then carefully washed in water and the adaxial and abaxial epidermises teased from the mesophyll using fine forceps and dissecting needles. The membranes were transferred into 10% aqueous solution of safranin-0 (dye) for 3 minutes. They were removed and washed with water to remove the excess stain. They were mounted in glycerine, observed under the microscope and the good preparations were sealed with nail vanish for further assessment. The upper (adaxial) and lower (abaxial) surfaces were treated separately for each plant specimen. The approaches adopted were according to (Ogundipe, 1990; Ogundipe and Olatunji, 1991). Photomicrograph of the epidermis was obtained with Olympus XSZ-N107 Model light microscope and Motic Camera 'Moticam 2300, 3.0 M.Pixel, USB 2.0 model. Twenty randomly selected epidermal cells and stomata were measured using a micrometer eye-piece, the trichome lengths were measured and types observed. Counting of cellular structures was done at 620-Magnification, Measurement at x400-Magnification and image taken at either x620-Magnification or x160-Magnification. Descriptive statistics of mean, standard deviation, standard error and clustering analysis were determined for all variables with the use of SPSS and NTSYS (Statistical) Packages. Extraction Method; Principal Component Analysis (PCA), Rotation Method: Varimax with Kaiser Normalization.

III. RESULT AND DISCUSSION

Field observations showed that *Strychnos* are distributed in old growth forest mainly but found as shrubs (young liana) in secondary forest when they are accidentally encountered in the vegetation. Their laminas are identified by veins, prominent and parallel to the margin either from the leaf base - basal acrodromous vein, or a little distance from the base - suprabasal acrodromous vein (Plates 1a & b). The forest species are prolific climbers and they form dense canopies on top of their host supposedly until the host is strangled and finally dies (Plates 1c, d & e). Majority of them are difficult to collect as they form leaves, inflorescences and fruits at the height of their host plants (Plate 1c). *Strychnos* have hooks as modification for climbing, though there are other modifications identified among climbers in the forests as observed by previous workers (Padaki and Parthasarathy, 2000). *Strychnos* hooks are of various types, sizes, and numbers; hooks are either simple/single, paired or layers of paired hooks (Plate 1f). The hooks fold in different ways and directions in regards to the orientation of the host plant as was also observed by previous workers (Bongers *et al.*, 2002). The hook that is in firm attachment with its host becomes enlarged and tenaciously grips the host stem and almost inseparable from the host without damaging it.

The anatomical evidence revealed that the abaxial surface is more informative than the adaxial (Table 2b and Plate 2). Several characters are very similar on the adaxial surfaces but differ in their abaxial counterpart (Table 2a, b and Plate 2). Morphological character and location of SID1 is different from SID2 (table1), their lamina and stems also vary but the anatomical evidence revealed that they are the same species (Figure 2, 4 and 5). Figure 4 and 5 revealed that both samples are located on the same point on the scatter plot. Table 2b, Figure 1 and 6 show the variation of stomata number and indices among *Strychnos* species. In table 2a, epidermal cell number is as numerous as 240 for SID1 at its minimum counting while only 98 were counted for SID4. The epidermal cell shapes of these species are majorly polygonal and irregular (Plate 2). Their anticlinal wall patterns are majorly straight, curved or undulate - higher degree of curvature (Table 2a and Plate 2). The epidermal cell numbers are between 94µm and 280µm for SID 9 and SID 2 respectively. Stomata and simple unicellular trichomes are only present on the abaxial surfaces of some species (Table 2b and Plate 2). However, some species lack trichome completely (Figure 1). There are various degrees of striations (cuticular folding) observable among the species; they are majorly guard cell striated (Plate 2 & Table 2b). The PCA (Table 3 and 4) revealed 3 major components to be contributing up to 84 %. These components show the greatest significant variations. Table 4 showed the communality; stomata number has 918 followed by epidermal cell length with

916 values, etc. These components were subsequently used to generate the similarities observable in Figure 4, 5 and 6.The cluster analysis result in Figure 2 reveals that SID1 and SID2 have similarity beyond 90%. The line of reference taken for species delimitation is drawn at about 60% similarity. There are 3 major clusters formed in the dendrogram, vizibly: at 33.5%, 53% and 31.5%. Based on gross morphology examined in previous studies, West African *Strychnos* belong to 11 Sections out of the entire 12 Sections found in *Strychnos* of Loganiaceae worldwide (Bisset *et al*., 1973; Leeuwenberg and Leenhouts, 1980; Frasier, 2008). The study thus revealed 3 Sections of *Strychnos* genus collected from the conserved forest. Further studies: The *Strychnos* species have been sent to the Royal Botanic Gardens, Kew, for sequencing and adequate identification using the data in global Gene-bank.

IV. CONCLUSION

The study revealed that *Strychnos* epidermal cell dimension - length and width, stomata indices, stomata number and trichome density among other characters are sufficient to delimit this economically useful genus in their populations both for their medicinal importance and conservation purposes.

V. ACKNOWLEDGMENT

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Table 1: The *Strychnos* species collection from CRNP in Nigeria

***Strychnos* spp.**	**I.D number**	**G.P.S data**	**Collector/s**
Strychnos indeterminate	**SID 1**	N 05°21´49.2″ E008°26.20.1″	Oduoye, O.T; Daramola, B.O
Strychnos indeterminate	**SID 2**	N 05°50´49.7″ E008°25´26. 3″	Oduoye, O.T
Strychnos indeterminate	**SID 3**	N 05°21´10.12″ E008°24´.20.3″	Oduoye, O.T
Strychnos indeterminate	**SID 4**	N 05°21´50.2″ E008°26´22.3″	Oduoye, O.T
Strychnos indeterminate	**SID 5**	N 05°23´46.22″ E008°24´02.3″	Oduoye, O.T
Strychnos indeterminate	**SID 6**	N 05°46´49.2″ E008°25´25.33″	Oduoye, O.T
Strychnos indeterminate	**SID 7**	N 05°21´49.32″ E008°26´.23.13″	Oduoye, O.T
Strychnos indeterminate	**SID 8**	N 05°21´50.22″ E008°27´.23.11″	Oduoye, O.T
Strychnos indeterminate	**SID 9**	N 05°23´36.22″ E008°26´20.3″	Oduoye, O.T
Strychnos indeterminate	**SID 10**	N 06°51. 853´ E007°24.573´	Oduoye, O.T

Table 2a: Leaf epidermal features of *Strychnos*

Strychnos	**LS**	**AWP**	**CWT**	**ECS**	**ECN**	**MECN**	**ECL**	**ECW**
SID 1	Adaxial	straight	1(1.25 ± 0.057)1.5	polygonal	240(255.65 ± 1.813)270	256	7(11.6 ± 0.816)20	6(8.30 ± 0.317)10
	Abaxial	straight	1(1.25 ± 0.057)1.5	polygonal	240(264.35 ± 2.683)280	264	12(21.35 ± 1.16)28	5(11.25 ± 0.707)17
SID 2	Adaxial	straight	1(1.25 ± 0.057)1.5	polygonal	240(255.65 ± 1.813)270	256	7(11.60 ± 0.816)20	6(8.3 ± 0.317)10
	Abaxial	straight	1(1.23 ± 0.057)1.5	polygonal	240(264.35 ± 2.683)280	264	12(21.35 ± 1.16)28	5(11.25 ± 0.707)17
SID 3	Adaxial	straight	2(2.5 ± 0.109)3	polygonal	140(167.15 ± 2.75)180	167	10(16.80 ± 1.02)25	4(8.35 ± 0.568)13
	Abaxial	straight	2(2.5 ± 0.109)3	irregular	98(102.05 ± 0.438)106	102	18(23.95 ± 0.795)31	6(9.60 ± 0.387)12
SID 4	Adaxial	straight	1.5(1.75 ± 0.057)2	polygonal	128(159.55 ± 2.23)170	160	15(19.15 ± 0.504)25	5(10.5 ± 0.698)15
	Abaxial	straight	1.5(1.8 ± 0.056)2	irregular	90(96.95 ± 0.72)100	97	15(21.35 ± 0.712)26	5(8.45 ± 0.400)12
SID 5	Adaxial	undulate	1.5(1.8 ± 0.056)2	polygonal	150(155.95 ± 0.737)160	156	8(15.9 ± 0.959)20	5(9.75 ± 0.593)14
	Abaxial	curved	1(1.225 ± 0.057)1.5	polygonal	152(157.2 ± 0.627)162	157	10(20.55 ± 1.255)28	6(9.75 ± 0.491)13

SID 6	Adaxial	straight	1.5(1.8 ± 0.056)2	penta to polygonal	200(213.10 ± 1.306)220	213	7(15.05 ± 1.123)22	4(8.80 ± 0.555)14
	Abaxial	curved	1.5(1.85 ± 0.0526)2	polygonal to irregular	180(192.05 ± 1.639)205	192	9(18.65 ± 1.127)26	4(7.25 ± 0.464)11
SID 7	Adaxial	straight	2(2.575 ± 0.0908)3	polygonal	128(132.65 ± 0.646)138	133	12(19.2 ± 0.869)25	6(9.55 ± 0.535)14
	Abaxial	curved	1(1.25 ± 0.057)1.5	polygonal	246(251 ± 0.684)257	251	7(15.25 ± 1.279)25	4(6.3 ± 0.411)9
SID 8	Adaxial	curved	2(2.50 ± 0.096)3	polygonal	170(175.3 ± 0.788)180	175	9(14.25 ± 0.882)20	5(9.00 ± 0.637)15
	Abaxial	curved	1(1.25 ± 0.057)1.5	polygonal to irregular	90(94.25 ± 0.688)100	94	12(19.65 ± 1.212)30	4(8.050 ± 0.573)13
SID 9	Adaxial	curved	1(1.875 ± 0.0497)2	polygonal	155(158.35 ± 0.466)162	158	11(20.25 ± 1.559)30	5(10.10 ± 0.657)15
	Abaxial	straight	1.5(1.775 ± 0.057)2	polygonal to irregular	87(90.1 ± 0.502)94	90	10(22.50 ± 1.688)38	8(10.45 ± 0.444)14
SID 10	Adaxial	straight	1(1.25 ± 0.057)1.5	polygonal	195(200.2 ± 0.742)205	200	8(11.75 ± 0.486)315	5(7.95 ± 0.359)11
	Abaxial	curved	1(1.25 ± 0.057)1.6	polygonal to irregular	152(157.85 ± 0.654)162	158	10(17.45 ± 0.869)23	5(8.65 ± 0.509)12

Leaf surface – LS, Epidermal Cell number ECN, Epidermal Cell shape ECS, Cell wall thickness CWT, Anticlinal wall pattern AWP, Mean Epidermal Cell number MECN, Epidermal Cell length ECL, Epidermal cell width ECW. Measurement: minimum (mean ± s.e.) maximum (µm).

Table 2b: Leaf epidermal features of *Strychnos* contn'd

Strychnos	**LS**	**SL**	**SW**	**SN**	**ST**	**SI %**	**TT**	**TL**	**TD**	**CO**
SID 1	Adaxial	0	0	0	0	0.00	0	0		STR
	Abaxial	5(6.80 ± 0.247)	1.5(1.825 ± 0.0547)2	24(29.90 ± 0.992)37	paracytic	10.16	simple unicellular	20(53.4 ± 4.05)75	1	
SID 2	Adaxial	0	0	0	0	0.00	0	0	0	STR
	Abaxial	5(6.80 ± 0.247)	1.5(1.825 ± 0.0547)2	24(29.90 ± 0.992)37	paracytic	10.20	simple unicellular	20(53.4 ± 4.05)75	1	
SID 3	Adaxial	0	0	0	0	0.00	0	0	0	STR
	Abaxial	5(6.05 ± 0.17)7	2(2.6 ± 0.100)3	11(12.95 ± 0.235)15	paracytic	11.30	simple uncellular	65(125.45 ± 5.89)155	2	0
SID 4	Abaxial	4(5.05 ± 0.153)6	2(2.7 ± 0.147)4	4(6.3 ± 0.25)8	paracytic	5.83	simple uncellular	12(18.40 ± 1.057)25	3	0
SID 5	Abaxial	5(5.85 ± 0.167)7	2(2.45 ± 0.114)3	13(16.7 ± 0.534)20	paracytic	9.60	simple unicellular	12(70.55 ± 7.38)122	5	0

SID 6	Abaxial	3(3.55 ± 0.114)4	2(2.5 ± 0.115)3	20(23.05 ± 0.48)26	paracytic	10.72	0	0	0	0
SID 7	Abaxial	4(4.95 ± 0.170)6	1(1.675 ± 0.0908)2	15(17.45 ± 0.387)20	paracytic	6.50	0	0	0	0
SID 8	Abaxial	5(5.90 ± 0.191)7	2(2.375 ± 0.095)3	5(10.900 ± 0.652)16	paracytic	10.37	simple unicellular	4(7.6 ± 0.43)10	1	STR
SID 9	Adaxial	0	0	0	0	0.00	0	0	0	STR
	Abaxial	5(6.35 ± 0.274)8	2(2.60 ± 0.10)3	8(11.00 ± 0.0.340)14	paracytic	10.88	simple unicellular	10(106.3 ± 7.910)140	3	STR
SID10	Abaxial	4(4.65 ± 0.109)5	3(4.15 ± 0.182)5	17(19.45 ± 0.352)22	paracytic	10.97	0	0	0	0

Leaf surface – LS, Stomata length - SL, Stomata width- SW, Stomata number – SN, Stomata type – ST, Stomata index- SI, Trichome type – TT, Trichome length – TL, Trichome density per view – TD, Cuticular ornamentation – CO, Striation – STR, 0 = Absent/ not found; Measurement: minimum (mean ± s.e.) maximum (µm).

Table 3: PCA with three principal components contributing up to 84 % from 16 characters

Total Variance Explained									
Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.869	38.687	38.687	3.869	38.687	38.687	3.697	36.972	36.972
2	2.970	29.700	68.386	2.970	29.700	68.386	3.014	30.141	67.113
3	1.629	16.293	84.680	1.629	16.293	84.680	1.757	17.567	84.680
4	.662	6.615	91.295						

Table 4: PCA of *Strychnos* anatomy showing communalities and Component Matrix

Communalities			Component		
	Initial	Extraction	1	2	3
Stomata Index	1.000	.807	.140	.139	.427
Epidermal cell number	1.000	.854	.006	.292	-.047
Epidermal cell length	1.000	.916	.219	-.114	.022
Epidermal cell width	1.000	.909	.259	.104	.056
Stomata length	1.000	.887	.239	.114	-.162
stomata width	1.000	.820	-.074	-.038	.470
Stomata number	1.000	.918	.083	.336	.135
Cell wall thickness	1.000	.699	.030	-.189	.214
Trichome length	1.000	.863	.226	-.079	.028
Trichome density	1.000	.796	.133	-.219	-.299

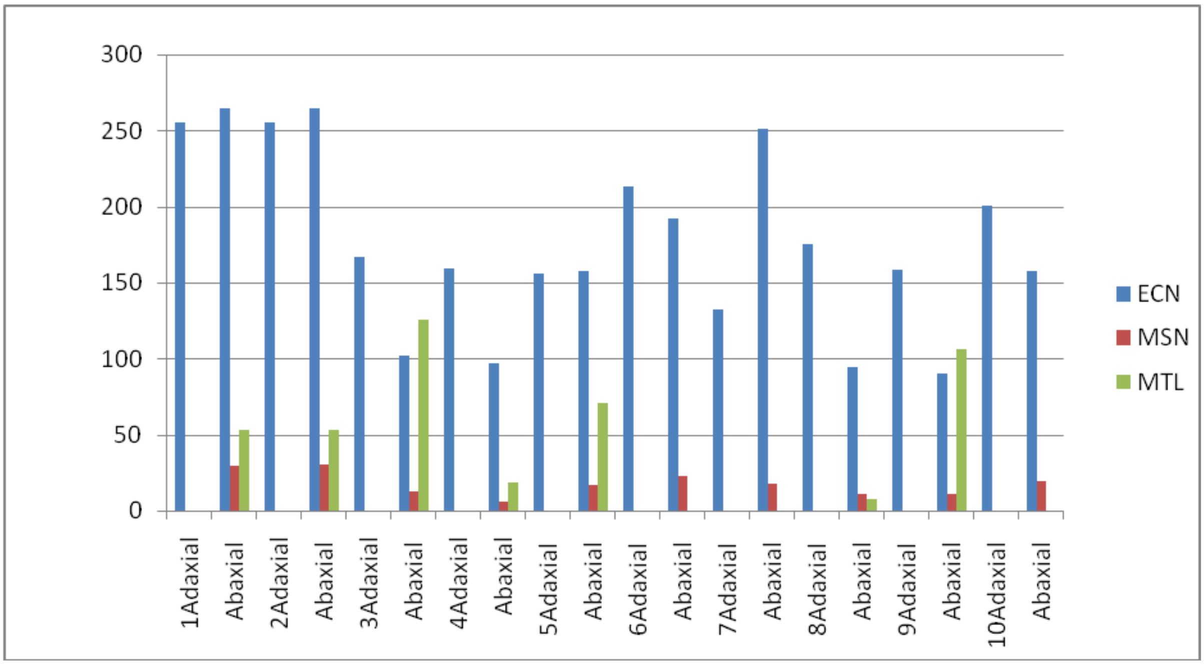


Figure 1: Chart showing the variation of Epidermal Cell Number (ECN), Mean Stomata Number (MSN) and Mean Trichome Length (MTL) for ten *Strychnos* indeterminate species.

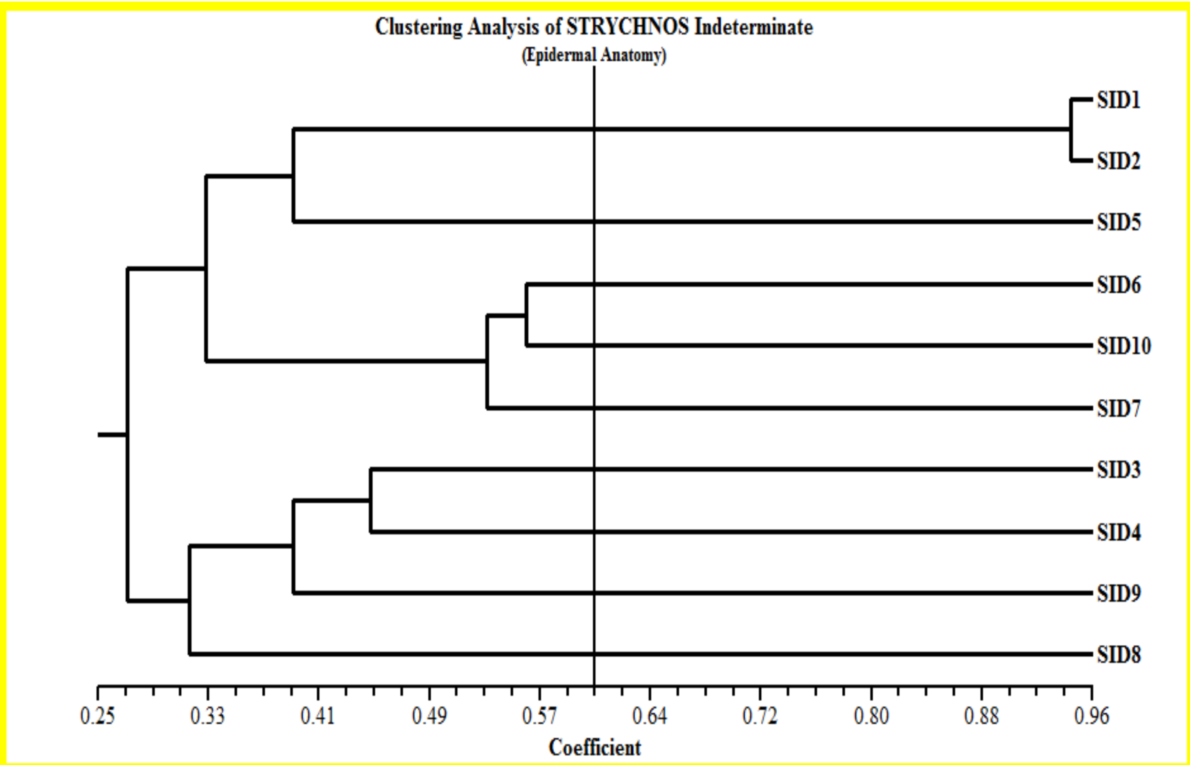


Figure 2: Clustering analysis showing the relationship of *Strychnos* indeterminate from CRNP using 36 characters

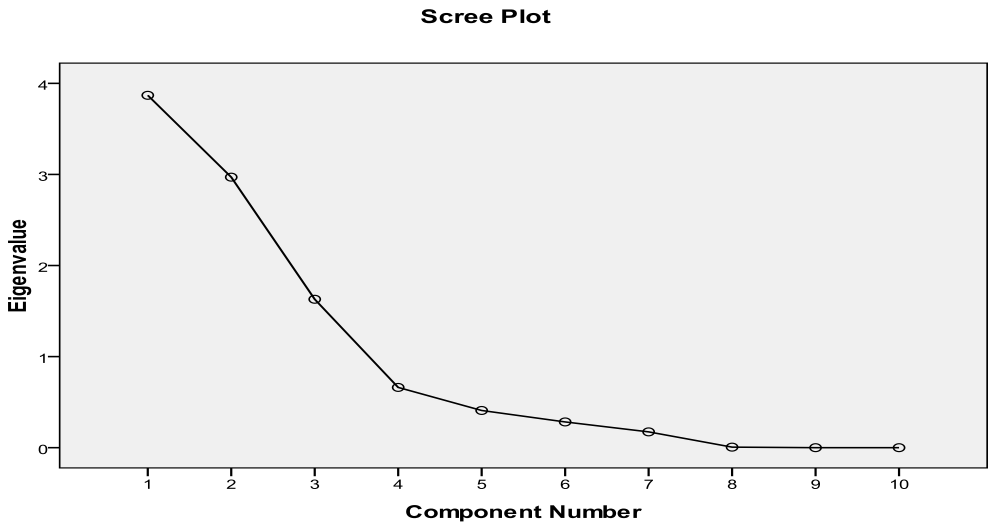


Figure 3: Scree plot of Eigenvalue against 10 components.

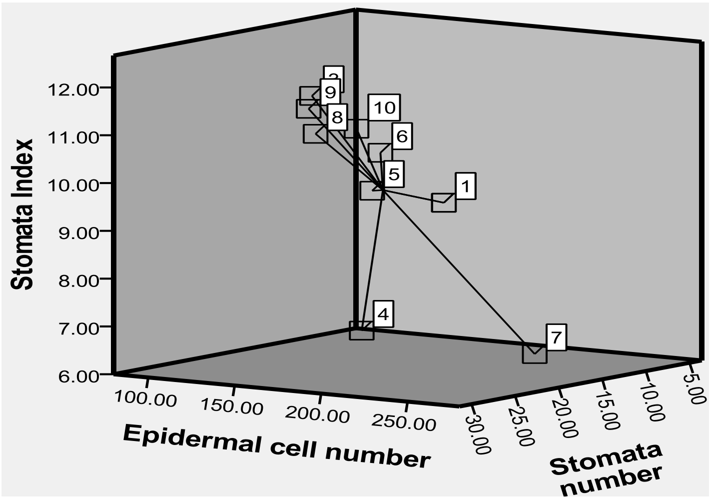


Figure 4: The first three component plot in rotated space, obtained from PCA.

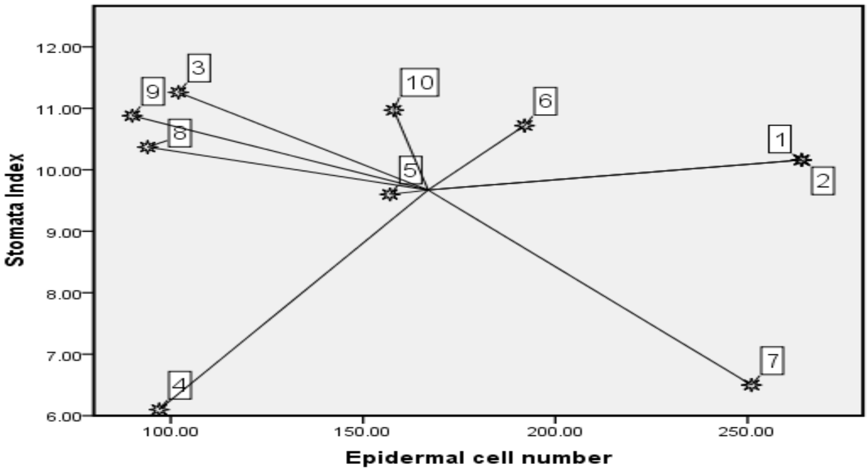


Figure 5: Scatter plot of epidermal cell number and Stomata index from first component obtained from PCA (group centroids).

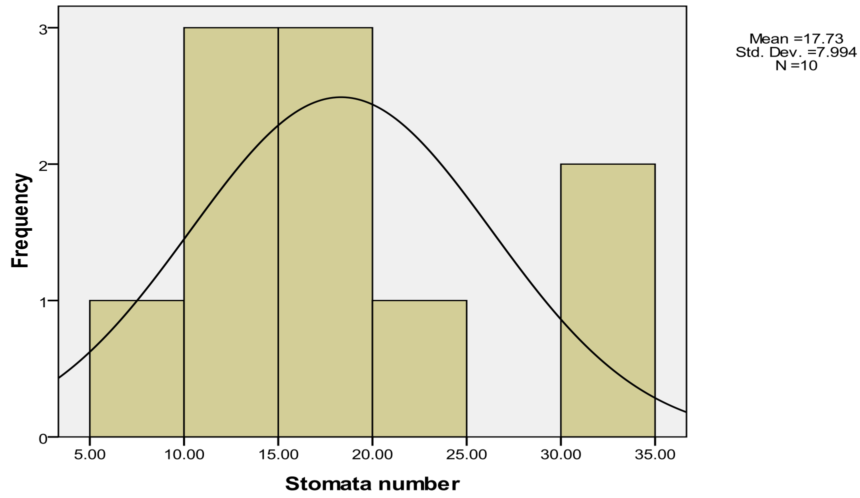


Figure 6: Paracytic stomata distribution among 10 *Strychnos* species

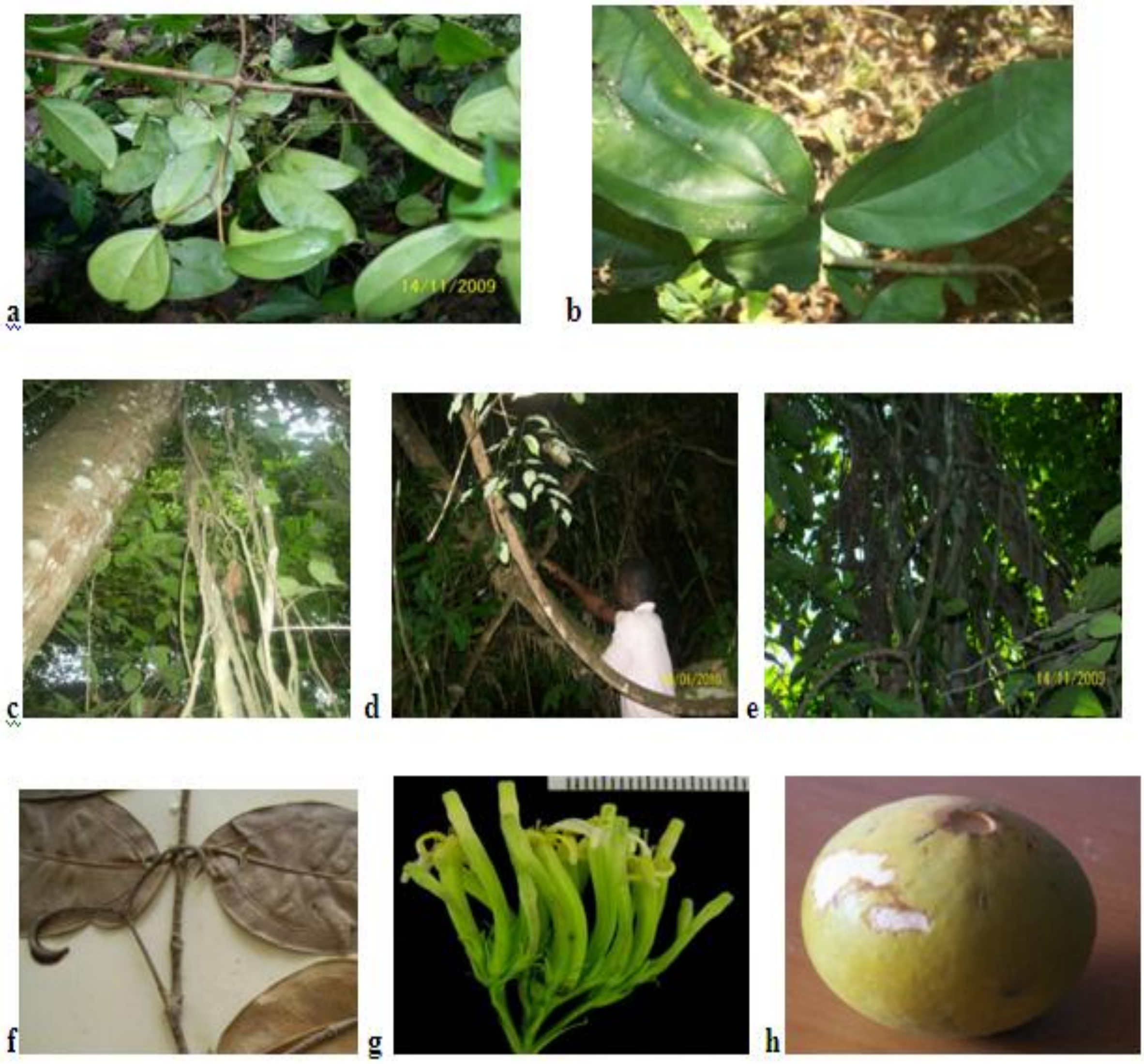
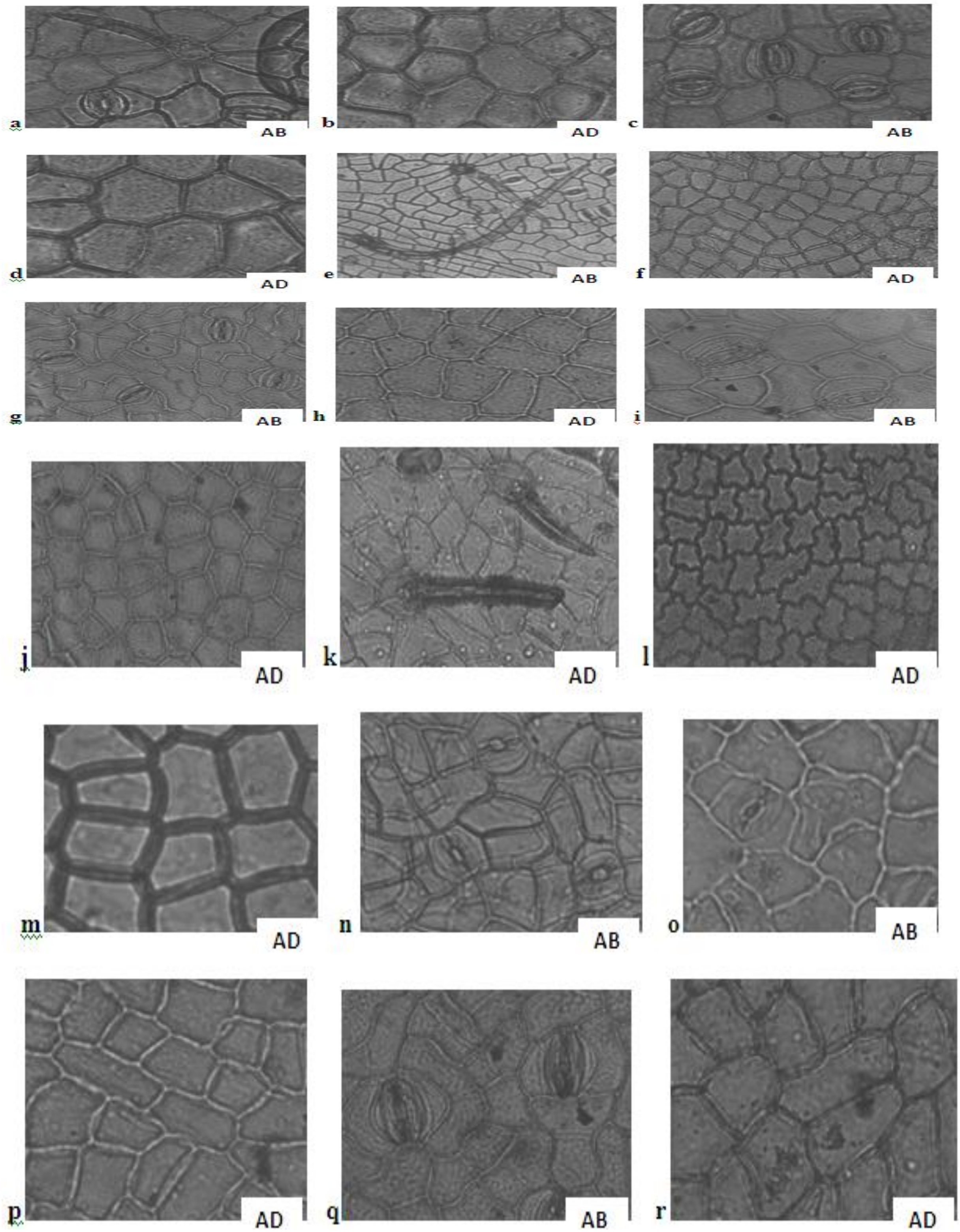


Plate 1: Photographs of *Strychnos* organ Morphology: *Strychnos* leaves, liana and host plant (a - e), *Strychnos* paired hook (f) *Strychnos* inflorescence (g) and *Strychnos* fruit (h).


a
AB
b
AD
c
AB
d
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e
AB
f
AD
g
AB
h
AD
i
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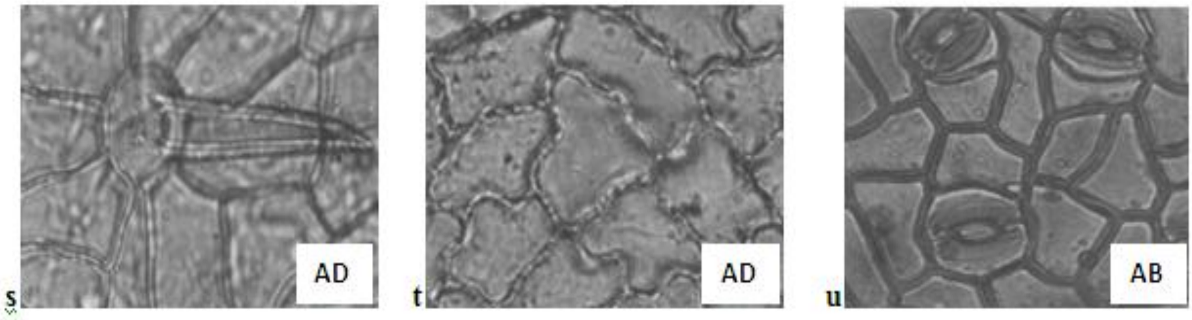

Plate 2: The adaxial and abaxial epidermal surfaces of SID 1 (a - b), SID 2 (c - d), SID 3 (e - f), SID 4 (g - h), SID 5 (i - j), SID 6 (k - l), SID 7 (m – n and u), SID 8 (o - p), SID 9 (q - r), SID 10 (s - t). AB = Abaxial surface, AD = Adaxial surface. Magnification: x 640