ORIGINAL PAPER

https://dx.doi.org/10.4314/njpr.v15i2.11

Nig. J. Pharm. Res. 2019, 15 (2) pp 237 - 247
ISSN 0189-8434 **e-ISSN 2635-3555** **Available online at http://www.nigjpharmres.com**

Providing Some Pharmacopoeial Standards for the Leaves of Two Closely-Related *Alstonia* Species

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: *Alstonia boonei* De Wild. and *Alstonia congensis* Engl. (Apocynaceae) are commonly used by the indigenes of Nigeria to treat malaria and other ailments.

Objective: This study has examined the leaves of *A. boonei* and *A. congensis* with a view to providing some pharmacopoeial standards for identification and development of their individual monograph.

Materials and Methods: Macroscopic and microscopic studies were carried out on the leaves of *A. boonei* and *A. congensis* with the use of light microscope, while the proximate and fluorescence analyses were carried out according to standard World Health Organization specifications.

Results: The leaves of *A. boonei* and *A. congensis* were coriaceous. The quantitative microscopy of the leaves showed the upper epidermal cell lengths for *A. boonei* and *A. congensis* as 32.5 ± 0.56 (µm) and 38.5 ± 1.8 (µm), while palisade ratios were 5.2 ± 0.4 and 3.7 ± 0.2, respectively. The stomata numbers of *A. boonei* and *A. congensis* were 5.0 ± 1.2 and 1.8 ± 0.3, respectively. The stomata length and width of *A. boonei*, respectively, were 24.5 ± 0.5 (µm) and 22.0 ± 1.2 (µm), while those of *A. congensis*, respectively, were 35.0 ± 0.8 (µm) and 28.0 ± 3.0 (µm). The vascular bundle in *A. boone*i was arc-shaped with xylem vessels standing in isolation but assuming a U-shape in *A. congensis* with xylem vessels interlocking to form a crown-like connection. Vein-islet number of *A. boonei* (9.0± 0.7) was slightly more than that of *A. congensis* (8.8±1.8). Total ash values and moisture contents of the leaves of *A. boonei* and *A. congensis* were 8.5 ± 0.01 and 6.8 ± 0.03; and 9.1 ± 0.04 and 9.9 ± 0.02, respectively.

Conclusion: The foliar epidermal and anatomical characters presented in this study are useful for differentiating between the two closely-related species, *Alstonia boonei* and *Alstonia congensis*. The established characters in each of the species could be incorporated into the next editions of the Nigerian and West African Herbal Pharmacopoeias.

Keywords: *Alstonia* spp., Pharmacopoeia, Microscopy, Proximate analysis, Organoleptic evaluation

INTRODUCTION

Due to the resurgence in herbs and herbal products consumption, the World Health Organisation (WHO, 2017) set and recommended appropriate pharmacopoeial standards to constitute a monograph for herbal raw materials. This was in its attempt at providing guidelines that would ensure the safety, efficacy and quality of medicines produced from herbs. The use of pharmacognostic parameters in the identification of vegetable drugs has long been recognised. According to the World Health Organisation (WHO, 1998), the establishment of the identity and the degree of purity of plants which serve

as herbal raw materials and their finished products involve a number of steps. Macroscopic and microscopic descriptions of such medicinal plants are the first among the steps.

The genus *Alstonia* belongs to the family Apocynaceae known as the Dogbane family and consists of about 40 pantropical species (Ojewole, 1984; Iwu, 1993). In Africa, there are about 12 species of *Alstonia*. However, only two species namely, *A. boonei* De Wild. and *A. congensis* Engl. are common in Nigeria. *Alstonia boonei* is commonly called fever-bark and locally known in Nigeria as 'ahun' (Yoruba) and 'egbu' (Igbo), and is traditionally used for treating malaria and other ailments (Kweifo-Okai *et al*., 1995). As reported by Olajide *et al*. (2000) and Osadebe (2002), the stem-bark possesses anti-inflammatory, anagelsic and antipyretic activities. Other studies have reported that the plant also has blood schizonticidal activity (Iyiola *et al*., 2011), insecticidal activity (Osawe *et al*., 2008) and nephrotoxicity (Oze *et al.*, 2007). The leaf possesses anti-plasmodial activity (Adepiti *et al*., 2014) and antibacterial activity (Fadipe *et al*., 2011). The stem-bark is enlisted in Ghana Herbal Pharmacopoeia (Kofi, 2007) as an anti-malarial drug but the leaf is yet to be standardized for that purpose. Similarly, *Alstonia congensis* Engl. is locally known as "ahun" (Yoruba) and "egbu" (Igbo) and it is reported for treating intestinal problems, malaria, diarrhea and constipation (Neuwinger, 2000). Neuwinger (2000) also adds that the plant's stem bark is used as a galactagogue while its latex is used to treat leucorrhoea. The antiprotozoal, cytotoxic and cardio-activities of the extracts from the leaves, stem and root barks (Ilesanmi *et al*., 1988; Lumpu *et al*., 2013) as well as the hypotensive and antispasmodic activities of echitamine isolated from the plant (Ojewole, 1984) have been reported.

However, in spite of the many medicinal applications, the morphological resemblance has led to great confusion in the basic identification and differentiation between the two species. The two species, being called the same local name 'ahun' (Yoruba) are invariably collected interchangeably for various medicinal uses.

The present study, therefore, aims at developing and providing some pharmacognostic standards for the leaves of the two *Alstonia* species, which can assist in differentiating between the two species. Hence, the work is expected to yield some pharmacopoeial standards that can be incorporated into the future monographs of the two plant species.

METHODOLOGY

Collection and Preparation of Plants

Samples of *Alstonia boonei* De Wild. and *Alstonia congensis* Engl. (Apocynaceae) were collected in September, 2015 behind the Department of Physiology, University of Ibadan, Ibadan, Nigeria and Itokin area, Ikorodu road, Ogun State, Nigeria, respectively and were authenticated by Mr. Ifeoluwa Ogunlowo, the plant curator at the Faculty of Pharmacy herbarium, Department of Pharmacognosy, Obafemi Awolowo University, Nigeria with FPI numbers 2169 and 2170, respectively. The fresh leaves of the two plants were preserved in 50% ethanol for the anatomical analysis, while some of the leaves of the plants were air-dried at room temperature, pulverized and stored in air-tight containers for other analyses.

Macroscopy

Morphological characters of the leaf apex, base and margin of *A. boonei* and *A. congensis* were examined. The organoleptic examinations included taste, colour and texture (AP, 1986; WHO, 2011)

Epidermal layer preparation

Epidermal studies were carried out, using modified Shultze's maceration method (Olatunji *et al*., 1983, Sonibare *et al*., 2014). Median portions of the fresh leaves were boiled with 100 % nitric acid for 5-10 min in a petri dish and placed on a water bath. The formation of bubbles on the leaf surface indicated the separation of the upper and the lower epidermises from the mesophyll. The adaxial and abaxial epidermal peels obtained were carefully removed with forceps, and camel hair brush and the peels were rinsed severally in water.

Leaf clearing for venation pattern

Leaf samples were boiled in absolute ethanol until the chlorophyll was almost removed, then rinsed severally in water. Thereafter, the leaf samples were boiled in 5% sodium hydroxide. The samples were rinsed again in distilled water and thoroughly cleared in absolute sodium hypochlorite for 20 to 30 minutes under sunlight. They were further rinsed severally in distilled water. The cleared samples were stained with Safranin O, later rinsed in water and mounted in 25% glycerol (Hickey, 1973). Observations were made with light microscope, and vein islet and vein termination number were measured using square graticule. Twenty-five measurements of each structure were randomly made from each specimen. The mean and standard error were calculated.

Transverse sections

The transverse sections of the leaves were obtained by free hand sectioning using a scalpel while the leaf

midrib (1 cm x 1 cm) was cut and embedded in pawpaw for easy sectioning. The transverse sections were cleared in 2 % w/v sodium hypochlorite for 2-5 min, rinsed severally in water, stained in Safranin O and counter-stained in Alcian blue. Each section was passed through graded ethanol (50 %, 70 %, 90 % and 100 %) for dehydration and differentiation and mounted in dilute glycerol. The distribution of tissue through the mid rib was observed under the light microscope.

Palisade Ratio Determination

The methodology described by Ahlam and Bouran (2011) was adopted, but modified, for determination of *A. boonei* and *A. congensis* leaf palisade ratios. Leaf median portion (3-4 cm) of each species was cut and soaked in chloral hydrate. It was afterwards boiled on water bath for 15-20 min., rinsed in water and further cleared in 100 % sodium hypochlorite for 20 min. Mounting was done in dilute glycerol and observation was done using the light microscope. The number of palisade cells contained in four joined epidermal cells was added up and divided by four. This was done for twenty-five field of views.

Proximate Analysis

The proximate analysis of leaf samples, which include moisture content, ash value, crude fats, proteins and carbohydrates was carried out. The moisture content and ash value were determined according to WHO (2011), while the nitrogen value, a precursor for proteins, was determined using micro Kjeldahl method (Pearson, 1976). By multiplying with a factor (6.25), the nitrogen value was converted to protein while a different method was used in determining Carbohydrate content of the samples (AOAC, 2004). All values were expressed in percentages.

Phytochemical screening and Fluorescence analysis

Powdered leaf samples of *A. boonei* and *A. congensis* were screened for various phytochemical constituents using standard procedures (Sofowora, 1993, Evans, 2009). Also, the samples were treated with various chemical reagents like picric acid, $FeCl_{3(aq)}$, $H_2SO_{4(aq)}$ and the samples observed in day light, UV light at 254 nm and also at 366 nm for fluorescence analysis.

RESULTS AND DISCUSSION

Gross Morphology

To ensure consistency in the safety, quality, efficacy and reproducibility of finished herbal drugs, the correct identification of the raw materials is indispensable (Nayaek and Patel 2010). *A. boonei* and *A. congensis* are often confused due to their numerous morphological similarities as shown in Figure 1. The morphological characters of the plant tissues and powdered samples are presented in Tables 1 and 2.

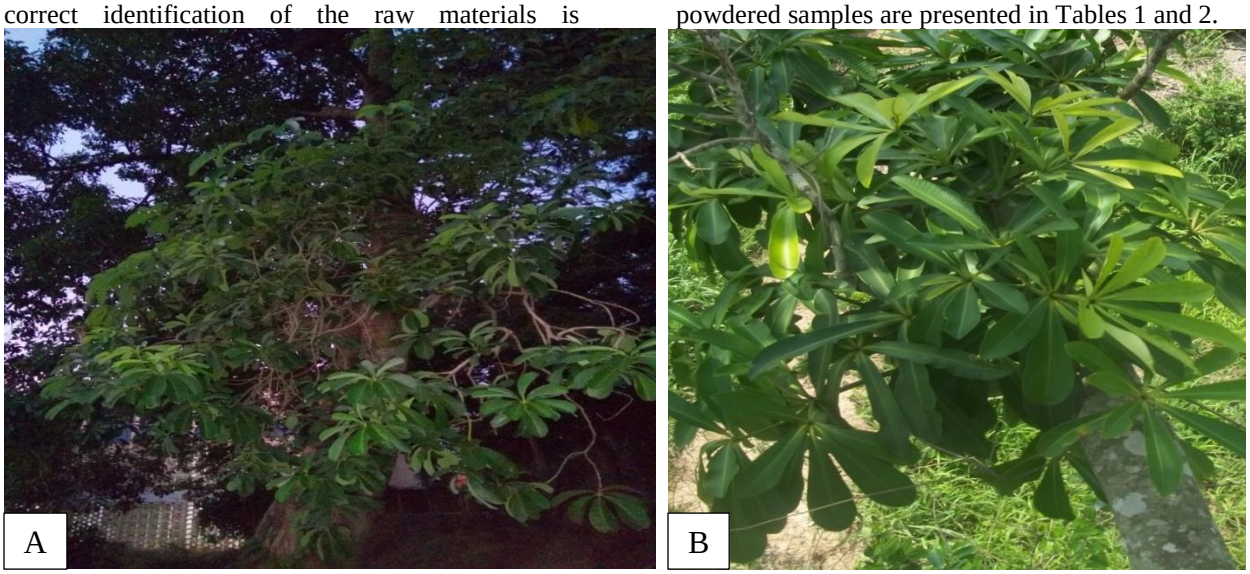

Figure 1: *Alstonia boonei* De Wild. and *Alstonia congensi* Engl., respectively, in their natural habitat. A: *Alstonia boonei*; B: *Alstonia congensis*

Table 1: Morphological features of *A. boonei* and *A. congensis*

Morphological part	*A. boonei*	*A. congensis*
Leaves	Whorl 5-8	Whorl 4-6/8
Blade	Oblanceolate –obovate	oblanceolate
Apex	Acute-emarginate	Shortly acuminate
Base	Cuneate	Cuneate
Margin	Entire	Entire
Colour	Dark green (upper surface) light green (lower surface)	Dark green (upper surface) light green (lower surface)
Texture	Coriaceous/pubescent	Coriaceous/pubescent

Table 2: Organoleptic features of *A. boonei* and *A. congensis* leaf powdered samples

Features	*A. boonei*	*A. congensis*
Colour	Brownish green	Brownish green
Taste	Bitter	Bitter
Texture	Fine	Fine
Odour	Bushy	Bushy

Epidermal layers

The generic variation observed in the qualitative and quantitative microscopical studies such as the epidermal cell size, vein-termination, vein-islet number, stomatal number of *A. boonei* and *A. congensis* are always constant and could therefore be used for correct identification of the two species, individually-independent of each other. Thus, since microscopy plays an important role in drug identification, micro-morphological characters are often used in species delimitation (Anji *et al*., 2009, Zongzhen, 2010). The epidermal cells of *A. boonei* and *A. congensis* adaxial layers are quadrilaterally shaped, having straight and thick anticlinal walls (Figure 2). The adaxial epidermises of *A. boonei* and *A. congensis* have coastal cells, which are square-like to quadilateral shape. The abaxial epidermises of the two plants are stomatiferous, epidermal cells have thin, straight anticlinal wall, and the cells are smaller than their adaxial counter-parts; however, they are also quadrilateral in shape. The abaxial layers are characterised with coastal cells, wax and papillae (Figure 3, Ciii), while the stomata type found in the two species varied from cyclocytic, paracytic to anomocytic (Figure 3 Cii). The quantitative microscopy of the leaves showed the epidermal cell lengths for *A. boonei* and *A. congensis* as 38.5 ± 0.56 and 35.5 ±1.8, stomata numbers as 5.0 ± 1.2 and 1.8 ± 0.3, while palisade ratios were 5.2 ± 0.4 and 3.7 ± 0.2, respectively, (Table 3).

Table 3: Quantitative microscopic evaluation of *Alstonia boonei* and *Alstonia congensis* leaves

Parameters	*Alstonia boonei*	*Alstonia congensis*
Epidermal cell length (µm)	38.5±1.8	35.2±0.9
Epidermal cell width (µm)	25.3±1.6	32.0±3.8
Stomata number	1.8±0.3	5.0±1.2
Stomata length (µm)	24.5±0.5	35.0±.8
Stomata width (µm)	22.0±1.2	28.0±3.0
Palisade ratio	5.2±.2.0	3.7±0.4
Vein-islet	9.0±0.7	8.8±1.8
Vein-termination number	6.2±0.7	6.2±1.3

Each values represent Mean ± S.E.M for 25 views

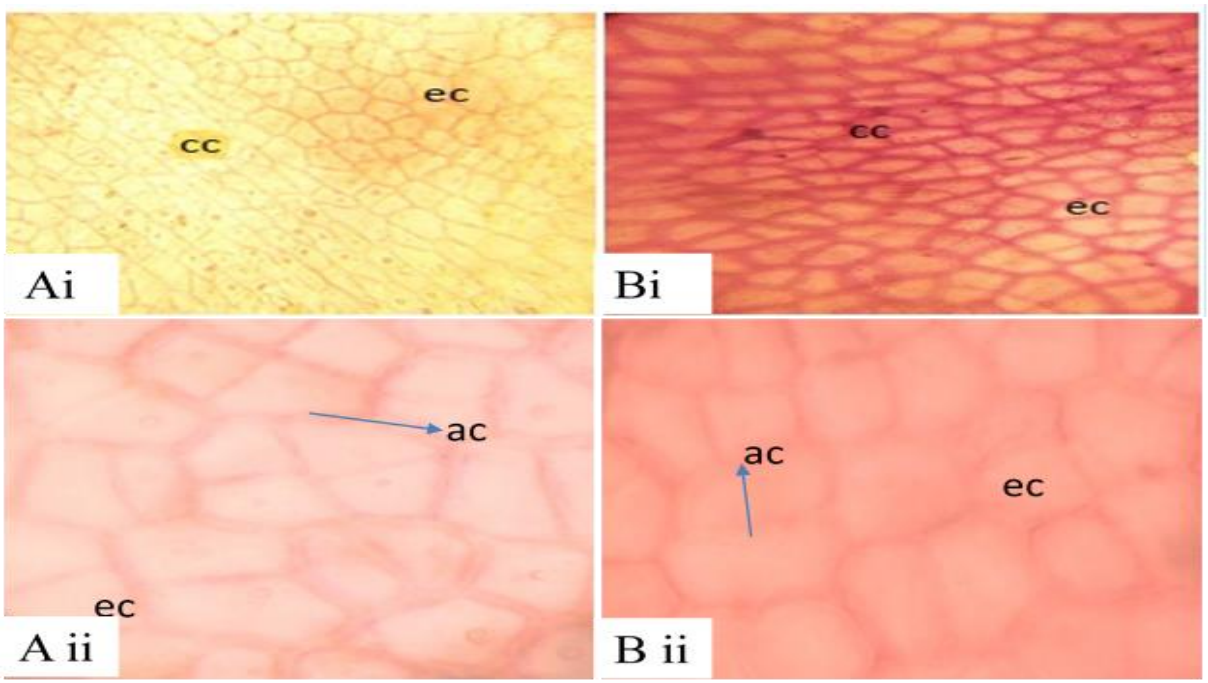

Figure 2: Epidermal peel of Adaxial surface of *A. boonei* and *A.congensis*
(Ai) Adaxial surface of *A. boonei* x100; (Aii) Adaxial surface of *A. boonei* x 400; (Bi) Adaxial surface of *A.congensis* x100; (Bii) Adaxial surface of *A.congensis* x 400; ac: anticlinal wall; cc: coastal cells, ec:epidermal cells

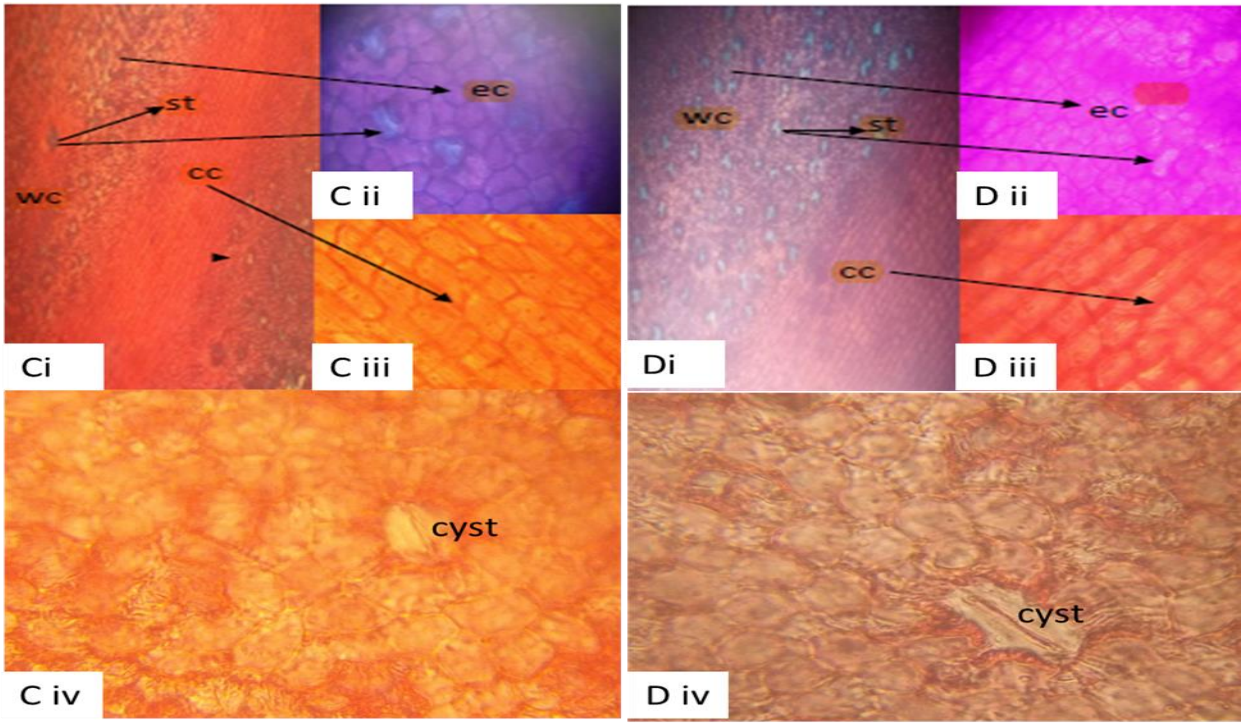

Figure 3: Epidermal peel of Abaxial leaf surfaces of *A. boonei* and *A.congensis.*
(Ci) Abaxial leaf surfaces of *A. boonei* x40; (Cii) Abaxial leaf surfaces of *A. boonei* x100; (Ciii) Abaxial leaf surfaces of *A. boonei* x100; (Civ) Abaxial leaf surfaces of *A. boonei* x400; (Ci) Abaxial leaf surfaces of *A. congensis* x40; (Cii) Abaxial leaf surfaces of *A. congensis* x100; (Ciii) Abaxial leaf surfaces of *A. congensis* x100; (Civ) Abaxial leaf surfaces of *A. congensis* x400; cc: coastal cells, ec:epidermal cells, cyst : cyclocytic stomata, st: stomata, wc: waxy cell

Transverse sections

The leaf midribs of the two species revealed bicollateral vascular bundles, angular collenchyma cells, sclerenchyma sheath, thick cuticles, parenchyma ground tissues, absence of trichomes, prismatic calcium oxalate crystals. Two to three layers of angular collenchyma were observed on the upper and lower layers of both species; both contain single layered epidermis which is rectangular in shapes. Prismatic calcium oxalate crystals were observed in the ground tissue of both species.

The mid-rib of *A. boonei* is plano-convex in shape with almost flat adaxial layer and hemispherical abaxial layer (Figure 4 Ei), while *A. congensis* mid-rib has short adaxial hump and slightly indented abaxial layer (Figure 4 Fi). *A. boonei* contains a secretory canal in the ground tissue but not observed in *A. congensis* (Figure 4 Eii). The vascular bundle in *A. boone*i was arc-shaped with xylem vessels standing in isolation while the phloem occurs in clusters on the abaxial and adaxial portions of the xylem. In *A. congensi*s, a U-shaped vascular bundle was observed with xylem vessel interlocking to form a crown like connection and surrounded with phloem on both upper and lower portion of the xylem band. The sclerenchyma sheath forms a broken edge around the vascular bundle of *A. boonei*, while in *A. congensis*, the sclerenchymatous bundle sheath form an unbroken edge around the vascular bundle (Figure 4).

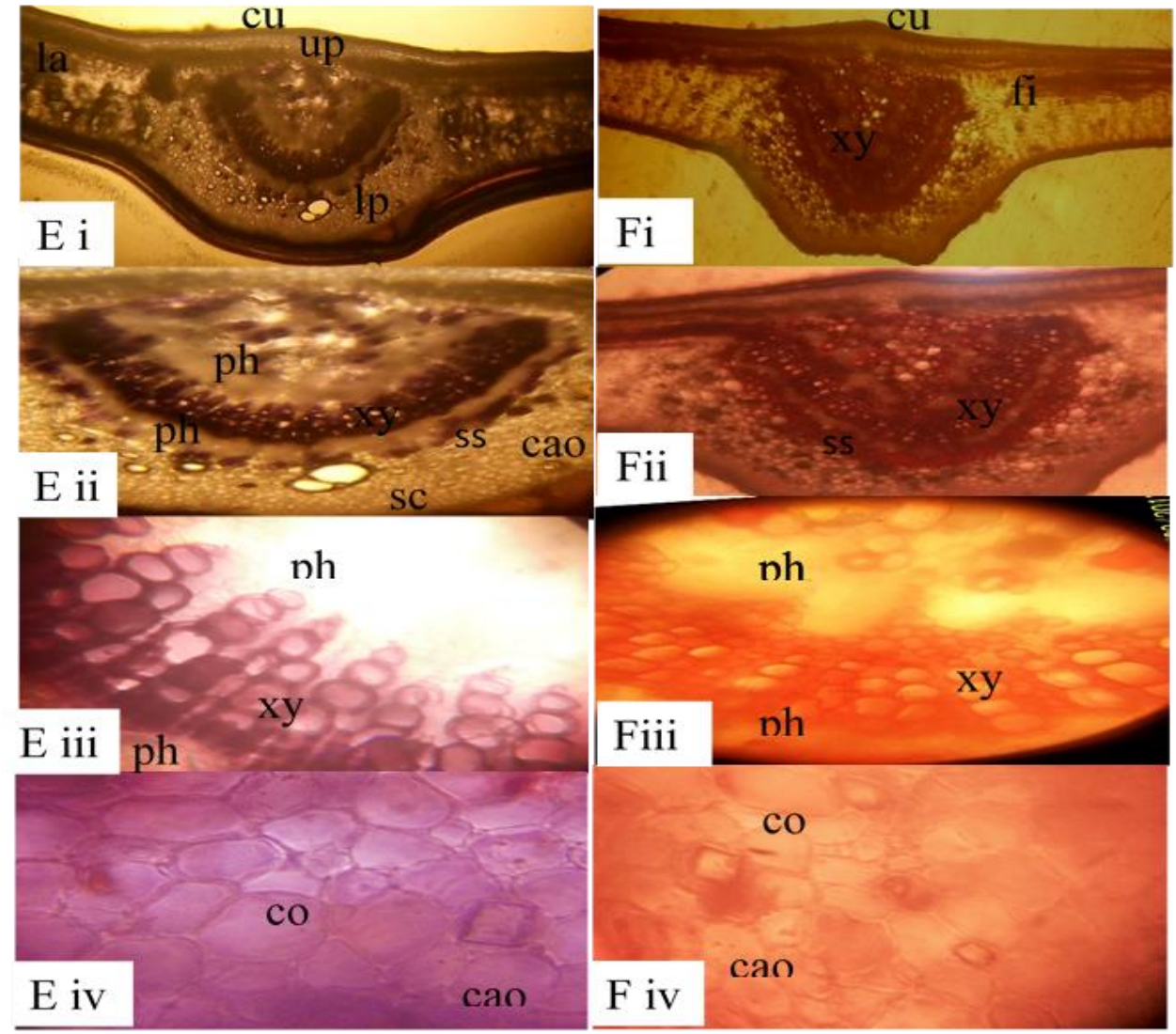

Figure 4: Transverse section (TS) of *A. boonei* and *A. congensis* leaf: Ei & Fi X100 showing mid-ribs of A.*boonei* and *A.congensis*, respectively. Eii &Fii X400 showing the enlarged cell inclusions of *A. boonei* and *A. congesis* mid-ribs. (cao: calcium oxalate crystals, co:collenchyma, cu: cuticle, la: lamina, lp: lower epidermis, ph: phloem, sc: secretory canal, ss: schlerenchyma sheath,sy:sclerenchyma. up: upper epidermis, xy: xylem)

Lamina

Leaves of *A. boonei* and *A. congensis* are dorsiventral and hypostomatic (Figure 5). Epidermis was uniseriate in both species, while hypodermis is double layered in *A. congensis* and single in *A. boonei*. The palisade of lamina of both *A. boonei* and *A. congensis* showed 1-2 layered cylindrically-shaped and compactly packed parenchyma cells and layers of spongy mesophyll was filled with bone-like and/finger-like parenchyma cells with air spaces. The lamina of both species contains fibres and prismatic calcium oxalate crystals (Figures 5, 6).

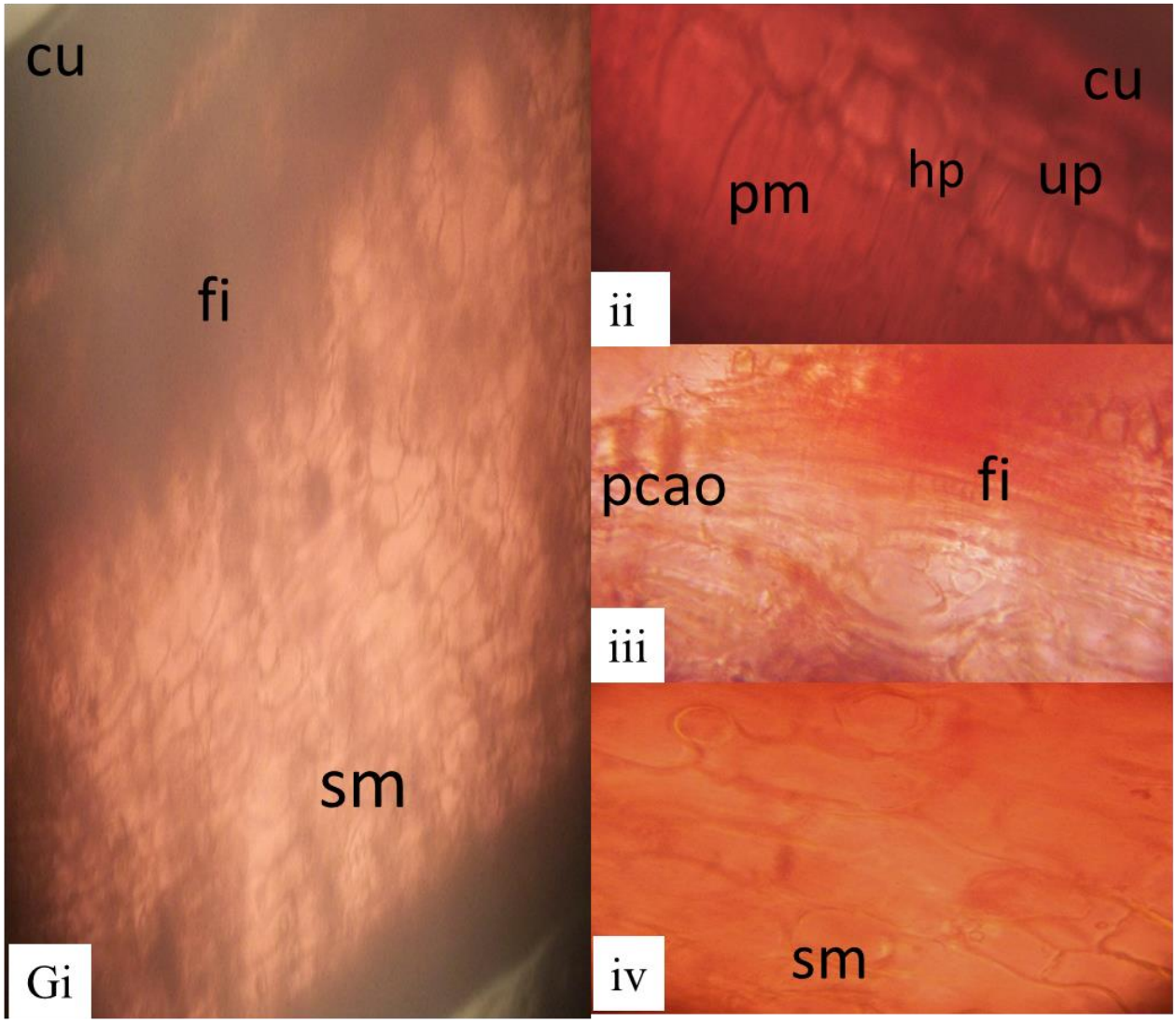

Figure 5: Lamina of A.*boonei* (i) X40, (ii, iii) X100, (iv) X400 (as: air space, pcao: prismatic calcium oxalate, cu: cuticle,fi: fibres, hp: hypodermis, pm: palisade mesophyll, sm: spongy mesophyll)

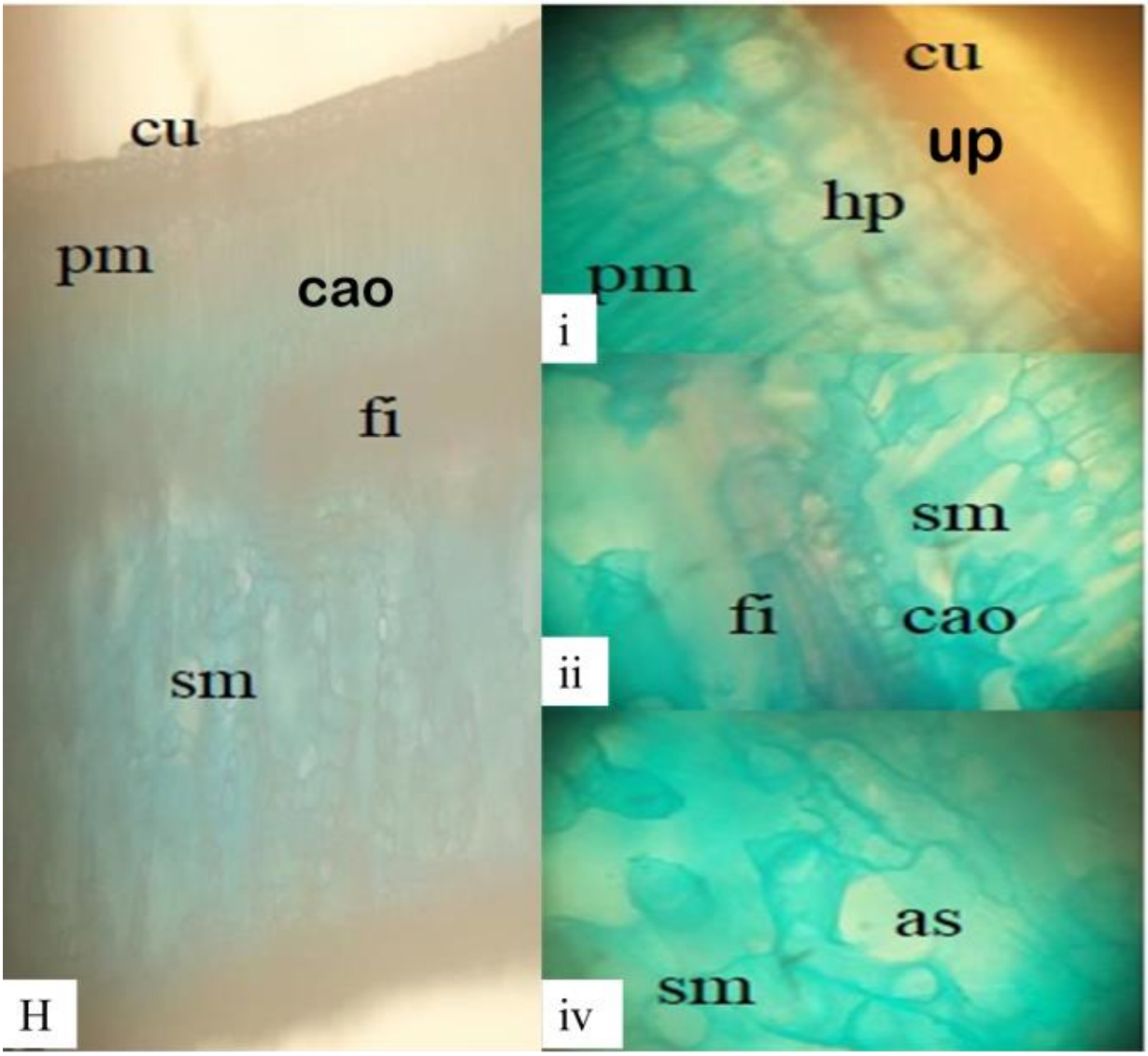

Figure 6: Lamina of *A. congensis* leaf: H x40, H(ii, iii) X100, Hiv X400. (as: air space, cao: prismatic calcium oxalate, cu: cuticle,fi: fibres, hp: hypodermis, pm: palisade mesophyll, sm: spongy mesophyll)

Venation pattern

The venation pattern of *A. boonei* and *A. congensis* leaves conformed to pinnate camptodromous type with festooned brochidodromous secondaries. The secondary veins are produced on both sides of the primary vein alternately which further divide to tertiary veins. The vein terminals are dendritic in nature (Figure 7). The striking difference between the venation patterns of these morphologically-similar species is found in slightly more than that of *A. congensis* (8.8±1.8).

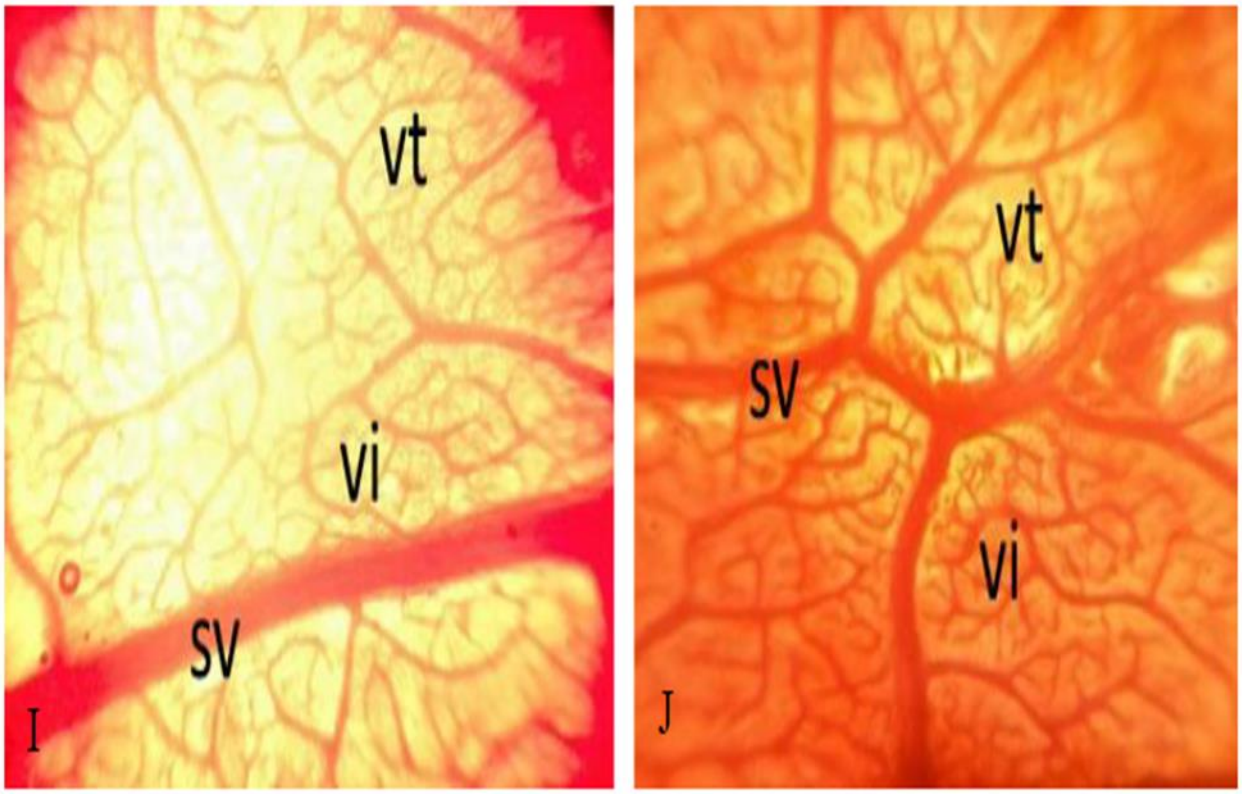

Figure 7: Cleared leaves of *Alstonia* species: I & J x100 showing venation pattern of *Alstonia boonei* and *Alstonia congensis* leaves, respectively; vi: vein-islet, vt: vein termination , sv: secondary vein

Proximate analysis

Table 4 shows the results of the proximate analysis of powdered leaves recorded for *A. boonei* and *A. congensis*, which included total ash, acid insoluble ash, moisture contents and crude fibres. Proximate analysis, which form part of the parameters used in standardisation of herbal medicines was also employed in this study. "Not more than" values are recommended as standards for total ash and acid-insoluble ash values while "not less than" values are recommended for extractive values (Elujoba, 1998; Sonibare *et al*., 2014). The amount of earthy materials and minerals indicated by the total ash obtained in the leaf of *A. boonei* was not more than 8.5% and 6.8% in *A. congensis* leaf. The moisture contents obtained for *A. boonei* (9.1 %) and *A. congensis* (9.9 %) were within the recommended limits. According to British Herbal Pharmacopoeia (BHP, 1990), the moisture contents of medicinal plants should not be more than 14 %.

Table 4: Proximate analysis of powdered sample of leaves of *Alstonia boonei* and *Alstonia congensis*

Plant samples	Protein (%)	Crude fat (%)	Crude fibre (%)	Carbohydrate (%)	Moisture content (%)	Total ash (%)	Acid-insoluble ash (%)
ABL	11.4±0.02	5.6±0.35	25.8±0.011	66.2±0.02	9.1±0.04	8.5±0.07	1.4±0.02
ACL	10.1±0.2	3.3±0.003	26.6±0.007	70.0±0.007	9.9±0.017	6.8±0.026	0.9±0.4

ABL: *Alstonia boonei* leaf; ACL: *Alstonia congensis* leaf

Phytochemical and Fluorescence analysis

Secondary metabolites like terpenes, alkaloids, saponins and cardiac glycosides were present in the leaves of the two species (Table 5).

Table 5: Phytochemical Properties of *Alstonia boonei* and *A. congensis* powdered leaf samples

Plant samples	Terpenes	Flavonoid	Tannins	Alkaloids	Anthraquinones	Saponins	Cardiac glycosides
Alstonia boonei	++	+	++	++	-	++	+
Alstonia congensis	++	++	++	++	-	++	++

Key: + =low, ++ =high, - = not present

The presence of different classes of phytochemicals in the powdered samples of *A. boonei* and *A. congensis* could be the reason for the varieties of their pharmacological activities. The fluorescence properties observed in the powdered leaf samples of the two species exhibited different colours, which could be employed for the identification of the classes of compounds present in the plant.

Application of fluorescence evaluation is an easy mode of authenticating the genuineness of herbal drugs. It could help in checking adulteration or substitution where the adulterated sample would show different emitted coloration when compared with the genuine drug. The powdered leaf samples of the two species exhibited different colours when treated with different reagents. The HCl treated powdered sample of *A. boonei* showed dark-green colour under the visible and UV light at 254 nm and greenish purple at 365 nm. When treated with H_2SO_4, it gave dark green, and purple colours, respectively. Conversely, when *A. congensis* powdered leaf sample was treated with HCl it showed a dark-green colour under visible light and under the UV light at 254 nm, while it gave a purple colour at 365 nm. Treatment with H_2SO_4 gave brown and purple colouration, respectively. The powdered samples of the two species gave a deep green colour when treated with iodine in water under the visible and the UV light at 254 and 365 nm.

CONCLUSION

The foliar epidermal and anatomical characters presented in this study for *A. boonei* and *A. congensis* are useful for identifying them individually as well as differentiating one species from the other. The assemblage of the results in this study will represent some of the data required in the compilation of the pharmacopoeial monograph for each of the two *Alstonia* species, which could be utilized for the revision of relevant herbal pharmacopoeias.

Recommendation: Further studies on DNA analysis of both species may provide more information for delimiting the two species.

ACKNOWLEDGEMENTS

The assistance of Mr. Ifeoluwa Ogunlowo of the Faculty of Pharmacy herbarium, Department of Pharmacognosy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria is gratefully acknowledged for plant identification and authentication.

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Conflict of Interest: None declared

Received: July 11, 2019

Accepted: December 21, 2019