Comparative foliar epidermal, chemomicroscopic and physico - chemical evaluation of the leaves of *Phyllanthus muellerianus* and *Phyllanthus fraternus* in Zaria, Nigeria

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Abstract

Pharmacognostic assessment was carried out on the foliar epidermal features of the fresh leaves of Phyllanthus muellerianus and Phyllanthus fraternus by examining their organoleptic, macroscopic, and microscopic features. Chemomicroscopic, and physicochemical evaluation of the dry powdered leaves were also carried out, to give quality identification, and also to ascertain the purity of the crude drug. The fresh leaves of the two plants were collected from Samaru, Zaria, in September 2021. The organoleptic and macroscopic characters of the leaves were evaluated by using sense organs to determine their taste, texture, colour and odour. The microscopic examination of anatomical features such as stomatal size, density, index, frequency, types, epidermal cell shapes, vein islet number, and palisade cells were determined. P. muellerianus has a simple leaf while, P. fraternus has bipinnate compound leaf. They both have an elliptical shape, indistinct odour, and bitter taste, P. muellerianus was hypostomatic, P. fraternus was amphistomatic. The stomatal complex type on P. muellerianus was paracytic, stomatal index of 27.69%, while, P. fraternus was anisocytic, stomatal index of 19.86%. Chemomicroscopic examination of the powdered leaves revealed the presence of many components and inclusion. It was concluded that the variation of the stomata features will greatly help in the delimitation, identification, and classification of the two species.

Keywords: epidermal, organoleptic, macroscopic, microscopic, *Phyllanthus muellerianus*, *Phyllanthus fraternus*

Introduction

Plants have long been used by humans to maintain health and well-being. Ancient Egyptians, for example, chewed willow bark to relieve fever and headaches. In Nigeria and many other African countries, the application of medicinal plants especially in traditional medicine is currently well-acknowledged and established (Elufioye and Olaiya, 2015) [10]. Medicinal plants are quite safe, less expensive, and have no or minimal side effects, unlike conventional drugs. They are used effectively in the treatment of different sicknesses and diseases, but the absence of proper authentication and standardization reduces their acceptability. Due to this, they are susceptible to adulteration and they can easily be substituted, which makes people doubt their potency (Chanda, 2014) [8].

Therefore, it is imperative to create a proper identification of herbal plants. The wrong usage of medicinal plants usually proceeds from wrong identification **(**Dineshkumar, 2007) [9]. According to WHO (1998), the macroscopic and microscopic description of a medicinal plant is the first step toward establishing its identity and purity and should be carried out before any tests are undertaken (Balakrishnan). Stomata are cellular epidermal valves in plants, and they are central to gas exchange and biosphere productivity. They are small pores on the surfaces of leaves and they are generally comprised of two guard cells.

The term is usually used collectively to refer to the entire stomatal complex, consisting of the paired guard cells and the pore itself, which is referred to as the stomatal aperture. Air enters in and out of the plant through these openings by gaseous diffusion, and contains carbon dioxide and oxygen, which are used in photosynthesis and respiration respectively.

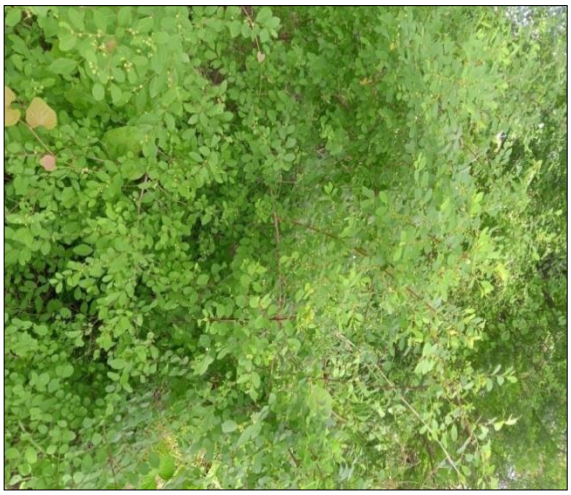
Chemomicroscopic studies involve the histochemical detection of cell wall components and cell inclusions of powdered plant parts (such as cellulose cell wall, lignin, starch, suberin, tannins and calcium oxalates, calcium carbonate, gums, and mucilages) using standard methods (Evans, 2009) [11].

Physicochemical analysis of powdered plant samples helps to assess the moisture content, total ash, water-soluble ash, acid-insoluble ash, and water and alcohol-extractive values according to the standard procedures outlined by Evans, 2009 [11]. The present study which is centered on the organoleptic, macroscopic, and microscopic evaluation of the fresh leaves, chemomicroscopic and physicochemical analysis of the powdered dry leaves of *P. muellerianus* and *P. fraternus*, is an attempt to provide pharmacognostic standards for the two *Phyllanthus* species and can be included in the monograph for their proper identification.

Phyllanthus (Euphorbiaceae) is a large genus, widely distributed in tropical and subtropical zones like tropical Africa, tropical America, Asia, and Oceania. It is commonly called carry me seed, stone-breaker, wind-breaker, gulf leaf flower, or gala of wind. There are over 300 genera with over

5000 species worldwide. Some species of *Phyllanthus* were found to exhibit hepatoprotective activity against drugs or toxins and this property was majorly attributed to phyllanthin and hypophyllanthin (Surendra *et al*., 2011) [17].

Phyllanthin and hypophyllanthin are the two major lignans of the genus *Phyllanthus*. *Phyllanthus muellerianus* is a monoecious glabrous, straggling, or climbing shrub or small tree. It is widely distributed in Nigeria and African countries. The leaves are reportedly been used for wound dressing, fever, and skin eruptions (Burkill, 1985). The plant has been reported to possess antimicrobial, antiplasmodial, analgesic, anti-inflammatory, and sedative properties (Brusotti *et al*., 2011) [6].



Phyllanthus muellerianus
Local names – Nigeria:
Hausa: Mijiriyar kurumi (Kumci)
Igbo: Oga azu
Yoruba: Arunjeran

Phyllanthus fraternus
Local names – Nigeria:
Kumci
Idumuje
Eyin olobe

Phyllanthus fraternus GL Webster (Family: Euphorbiaceae) is an annual medicinal herb commonly occurring in gardens, waste places, and roadsides (STEPRI and CSIR, 2007). It is widely distributed in most tropical and subtropical countries (Abedin, 2001). It can grow up to 80 cm, usually 30 - 40 cm, with angular branches. Leaves are elliptic-oblong to elliptic-oblanceolate, 5 - 13 x 1.5 - 5 mm, blunt or rounded at apex and base, or sometimes tapering to the base. Leaves are dark green above, paler and greyish beneath. *P. fraternus* is commonly used in Unani and Ayurvedic systems of medicine. It is considered a 'Natural liver protector'. *Phyllanthus* species are generally well known for their preventive and curative role in various hepatic disorders and are used in several ethnomedicine in indigenous healthcare systems in India (Srirama *et al*., 2010).

Phytochemical screening of *P. fraternus* whole plant extract revealed the presence of biologically active constituents such as alkaloids, especially phyllanthin as well as hypophyllanthin, flavonoids, tannins, anthraquinones, saponins, glycosides, resins, terpenes, sterols, quercetin and phenols (Adjanahoun *et al*., 2009).

Materials and methods

Fresh samples of *P. muellerianus* and *P. fraternus*, consisting of leaves, flowers, and fruits were collected from Samaru, Zaria. They were identified and authenticated at the Herbarium section of the Department of Botany, Ahmadu Bello University, Zaria, Nigeria. The Voucher numbers of the specimens are – *Phyllanthus muellerianus* Phyllanthaceae (Euphorbiaceae) – ABU0925; *Phyllanthus fraternus* Phyllanthaceae (Euphorbiaceae) – ABU01422.

The organoleptic characteristics of the leaves were determined by using the sense organs of seeing, touch, smell, and taste, to determine their leaf taste, texture, colour, and odour. The macroscopic evaluation of the leaves was carried out by observing, determining, and measuring some features such as leaf arrangement, shape, size, type, apex, base, petiole, margin, and arrangement of veins. All these were determined by adopting the standard methods described by Evans *et al*., 2009 [11]. Images were captured using the compound camera.

The microscopic evaluation of the leaves was carried out by following the standard methods by the protocol of Adenegan-Alakinde and Akinnubi (2015) [3].

The microscopic evaluations involve the following steps and tests:

a. Bleaching of the leaf epidermis to clear the chlorophyll content using chloral hydrate solution and water, and then staining with a drop of hydrochloric acid and phloroglucinol.
b. Mounting and observation of the leaf surfaces on the microscope.
c. Identification of the Stomatal complex types.

The number and nature of subsidiary cells per stoma were noted to determine the stomatal complex types. The frequency of each stomatal complex type was based on all occurrences of stomatal complex types (Obiremi and Oladele, 2001) [13]. Quantitative microscopic evaluations of the leaves were carried

out using the standard methods of Africa Pharmacopoeia (1986) as described by Mahomoodally (2013).

The following parameters were determined: stomatal density/frequency, stomatal index/number, stomatal size, epidermal cell number, vein-islet number, veinlet termination number, and palisade ratio.

i. Determination of stomatal density / frequency

The Stomatal Density (SD) = the number of stomata per square millimeter (Saadu *et al*., 2009) [16].

Stomatal frequency = Number of stomata / Area of microscopic field x mm^2

ii. Determination of stomatal index / stomatal number

The Stomatal Index (SI) for the leaves of each of the two *Phyllanthus* species was determined using the formula below:

$$SI = \frac{S}{E+S} \times 100$$

Where,

SI = Stomatal Index;

S = Number of stomata per square millimeter;

E = Number of epidermal cells per square millimeter

iii. Determination of stomatal size

The mean of the stomatal size was determined by measuring the length and breadth of a sample of 10 stomata using an eyepiece micrometer.

The formula for calculating this is:

$$\text{One ocular division} = \frac{\text{Stage division}}{\text{Ocular division}} \times 0.01$$

iv. $\text{Epidermal Cell number} = \frac{\text{Number of epidermal cells}}{\text{Area of microscopic field x mm2}}$

v. Vein-Islet is the number of Vein Islets per sq mm in the central part of the lamina (Leaf blade).

vi. The vein termination number is the number of veinlet terminations per sq mm of the epidermis.

Procedure for vein islet number and veinlet termination number

Small pieces of the leaves of the two *Phyllanthus* species were treated and cleared with concentrated chloral hydrate for about 24 hours. After clearing, the lamina portion was mounted on the glass slides and placed on the microscope and it was viewed through the low power (5x) of the microscope. A square was drawn and slides were placed on it. The complete islets which overlap two adjacent sides of the square were marked to get the value of one square millimeter area. The process was repeated per square millimeter of the leaf area between the midrib and the margin. The number of small vascular bundle terminal points was counted within the one square millimeter area to get the vein termination number (Evans, 2009) [11].

$$\text{Palisade ratio} = \frac{Average\ number\ of\ palisade\ \ cells\ beneath\ 4\ epidermal\ cell}{4}$$

Chemomicroscopic studies

This was carried out according to the standard procedure described (Evans, 2009; WHO, 2011) [11]. About 2g of the pulverized dry leaves of each of the two *Phyllanthus* species were weighed separately into small beakers and were cleared using 70% chloral hydrate solution, and then boiled on a water - bath for thirty minutes to remove obscuring materials. A cleared quantity of each of the samples was mounted on clean slides using dilute glycerol and they were observed under the compound microscope for the presence of cellulose, lignin, cutin and suberin, gum and mucilage, aleurone grains, starch, tannins, calcium carbonate, inulin, proteins, and calcium oxalate crystals according to the standard procedures as outlined by Evans (2009) [11]. Different reagents and stains were used for the tests. Observations were noted and recorded.

Physicochemical analysis

Physicochemical analyses were determined using the method described by Sofowora, (2008); WHO, (2011).

i. Determination of moisture content

The moisture content was determined by the "Loss on drying" method. Dry leaf powders (3 g) were weighed into the crucible. This was transferred into a hot air sterilizing oven, which was set at 105^0C. After an hour, the crucible was removed, and placed in a desiccator over phosphorous pentoxide under atmospheric pressure at room temperature. After 30 minutes, the weight of the powder and crucible was quickly determined and the crucible was returned to the oven. The heating and weighing were repeated until a constant weight was obtained and noted. Five determinations were conducted and the average of these was taken as the moisture content of the powder drugs. The moisture content was calculated using the formula:

$$\%\ \text{Moisture content} = \frac{\text{Initial Weight of Powder} - \text{Final Weight of Powder}}{\text{Initial Weight of Powder}} \text{X } 100$$

ii. Determination of total ash value

A crucible was heated red hot, cooled in a desiccator, and quickly weighed. 2 g of the powdered leaves were weighed into the heated crucible. It was ignited by gradually increasing the heat, until it became white, indicating the absence of carbon. It was cooled in a desiccator and weighed. The procedures were repeated five times to obtain the average value. The total ash content of the air-dried powder was calculated in percentage, using the formula:

$$\%\ \text{Ash Value} = \frac{\text{Weight of Residual Ash}}{\text{Original Weight of Powder}} \text{ X } 100$$

iii. Determination of acid - insoluble ash

After obtaining the total ash for each of the extracts, 25 ml of dilute HCl was used to wash the total ash into crucibles covered with watch glasses, and then boiled for 5 minutes. The solutions were cooled and rinsed through ashless filter papers.

The residue of each extract was then washed with hot water until the filtrate became neutral. The filter papers were then placed in silica crucibles and dried on a hot plate and oven-dried at 105°C. The contents in the crucibles were cooled and the final weights were noted and recorded (Evans, 1996).

$$\% \text{ Acid insoluble Ash} = \frac{\text{Weight of Residual Ash}}{\text{Original Weight of Powder}} \text{ X } 100$$

iv. Determination of water soluble ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a glass crucible. It was then washed with hot water and ignited in a crucible for 15 minutes at 105 C. The weight of the residue was subtracted from the weight of the total ash. The water-soluble ash of air-dried powders was calculated using the formula below.

$$\% \text{ Water Soluble Ash} = \frac{\text{Weight of Total Ash} - \text{Weight of Residual Ash}}{\text{Original Weight of Powder}} \text{ X } 100$$

v. Water extractives values

About 4 g of air-dried leaves powders were weighed into a 250 ml glass stoppered conical flask, and 100 ml of chloroform - water (1: 400) was added to macerate the powder for 6 hours with frequent shaking by using a mechanical shaker and was allowed to stand for 18 hours. It was filtered rapidly and 25 ml of filtrate was transferred into a previously dried and weighed evaporating dish and evaporated to dryness in a hot water bath. This was further dried in the oven at 105 ^{0}C for 6 hours, cooled in a desiccator for 30 minutes, and then weighed without delay. The percentage water extractive values were calculated using the formula below (WHO, 2011).

$$\% \text{ Water Extractive Value} = \frac{\text{Weight of Extract in 25ml X 4}}{\text{Original Weight of Powder}} \text{ X } 100$$

vi. Ethanol extractives value

The procedures above were repeated with ethanol in place of water and the percentage ethanol extractive values were calculated using the formula below (WHO, 2011).

$$\% \text{ Ethanol Extractive Value} = \frac{\text{Weight of Extract in 25ml X 4}}{\text{Original Weight of Powder}} \text{ X } 100$$

Statistical Analysis

All the data were processed using Analysis of Variance (ANOVA). A probability value of 0.05 was used as benchmark for significant differences between parameters.

The statistical analyses were carried out by analyses of variance ANOVA using SPSS version 20. The significant differences among mean values were calculated by Duncan's multiple range tests at $p<0.05$ and results were presented as mean ± standard error of the mean (SEM).

Results

The results of the leaf epidermal features of the two species of *Phyllanthus* studied were summarized in Tables 1 and 2, Figures 1, 2, 3, and 4, and Plates a - h. The two species investigated showed variations in the shape of epidermal cells, types of stomata, and cell thickness. The study of the epidermal leaf features of the two *Phyllanthus* species revealed differences in their organoleptic/macroscopic and microscopic morphological characters.

Table 1: Organoleptic/macroscopic characteristics of the leaves of *P. muellerianus* and *P. fraternus*

Features	*P. muellerianus*	*P. fraternus*
Dimension	4.34 mm x 2.53 mm	0.6 mm x 0.3 mm
Shape	Elliptical	Elliptical
Margin	Entire	Entire
Type	Simple	Bipinnate
Colour	Pale deep green (Upper side) and Pale light green (Underside)	Light green (both side)
Odour	Indistinct	Indistinct
Taste	Bitter	Bitter
Texture	Fine	Fine
Apex	Retuse	Retuse
Base	Cuneate	Cuneate
Petiole	Petiole	Petiole
Venation	Network and Recticulate	Network and Recticulate
Trichomes	Absent	Absent
Cell wall	Polygonal	Wavy

Table 2: Microscopic Studies on the Leaves of *P. muellerianus* and *Phyllanthus fraternus*

Sr. No.	Microscopic Features	*Phyllanthus muellerianus*	*Phyllanthus fraternus*
1. Stomatal Number			
a.	Adaxial Surface	Nil	102
b.	Abaxial Surface	18	116
2.	Number of Epidermal Cells	47	468
3.	Stomatal Dimension	5.10 x 3.7	5.70 x 3.22
4.	Stomatal Index	27.69 %	19.86 %
5.	Vein Islet Number	5.5	82
6.	Veinlet Termination Number	4.5	21
7.	Palisade Ratio	7.5	11.5
8. Stomatal Complex Type			
a.	Upper Epidermis	NIL	Anisocytic
b.	Lower Epidermis	Paracytic	Anisocytic
9.	Stomatal Frequency	100%	100%

Fig 1: Upper Epidermis of *P. muellerianus*

Fig 2: Lower Epidermis of *P.muellerianus*

Fig 3: Upper Epidermis Leaf of *P.fraternus*

Fig 4: Lower Epidermis of *P.fraternus*

Table 2 shows the results of the microscopic studies on the leaves of the two species of *Phyllanthus*. The stomatal complex type for *P. muellerianus* was parasitic, while it was anomocytic in *P.fraternus*. There were variations in the stomatal sizes, stomatal densities. Stomatal index also shows variations in the upper and lower surfaces in *P. fraternus*. It was therefore much on the abaxial surface (116) than the adaxial surface (102). Stomata is present majorly on the abaxial surface, the number (18) is much smaller than the ones on both the adaxial and abaxial surfaces of *P. fraternus.*

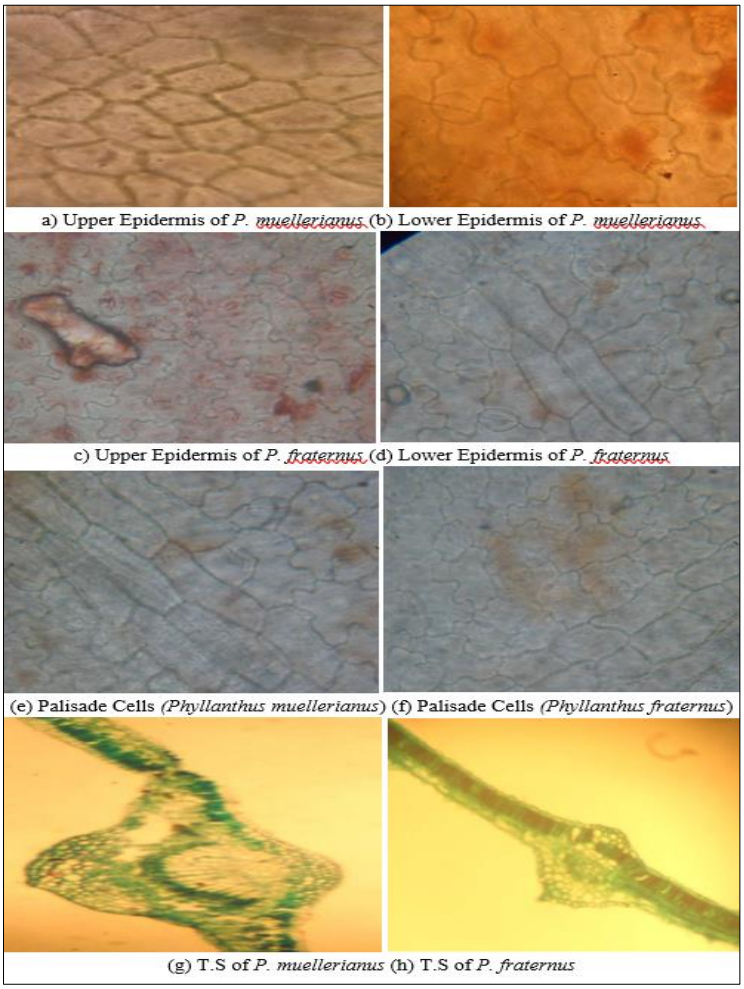


Fig 5

Table 3: Comparative Chemomicroscopic Studies of the Leaves of *Phyllanthus muellerianus* and *Phyllanthus fraternus*

Constituents/Tests	*Phyllanthus muellerianus*	*Phyllanthus fraternus*
a. Cell Wall Materials		
i. Cellulose cell wall	+	++
ii. Lignified cell wall	+	+
iii. Gums/mucilage	++	++
b. Cell Inclusions		
i. Aleurone grains	+++	++
ii. Calcium Carbonate	+	+
iii. Calcium Oxalate crystals	++	++
iv. Fats & oils	++	++
v. Starch	+	+
vi. Tannins	++	+++

+: Present, ++: Deeply Present, +++: Very Deeply Present

Table 4: Comparative Physicochemical Parameters of the Leaves of *Phyllanthus muellerianus* and *Phyllanthus fraternus*

Parameters	Values (%w/w) ± SEM*	
	Phyllanthus muellerianus	*Phyllanthus fraternus*
Moisture content	6.44 ± 0.48[a]	6.33 ± 0.51[a]
Total ash value	10.17 ± 0.93[b]	9.67 ± 0.17[a]
Acid Insoluble ash	7.17 ± 0.17[a]	8.01 ± 0.29[a]
Water Soluble ash	1.17 ± 0.44[a]	3.33 ± 0.67[a]
Ethanol Extractives	26.00 ± 0.58[b]	37.33 ± 1.86[a]
Water Extractives	20.00 ± 1.16[a]	20.00 ± 1.78[a]

*Average values of five determinations. SEM: Standard Error of Means

Discussion

Medicinal plants are good sources of drug discovery. Standardization and correct authentication of the plant is very important to help to sustain their purity and quality. It will prevent the plant from adulteration and wrong identification (Chanda 2014) [8].

Table 1 shows the results of organoleptic/macroscopic characteristics of the leaves of *P. muellerianus* and *P. fraternus*. The shape of the leaves of the two *Phyllanthus* species w*as* observed to be elliptical, with a curved anticlinal cell pattern. The leaf of *P.muellerianus* is hypostomatic i.e. stomata present only on the abaxial surface (Plates a & b), showing paracytic stomata surrounded by irregularly shaped polygonal epidermal cells with thick walls.

The leaf of *P. fraternus* is amphistomatic i.e. stomata present on both the adaxial and abaxial surfaces (Plates c & d), showing anisocytic stomata on both the upper and the lower epidermis surrounded by wavy-shaped epidermal cells with moderately thick walls. They both have characteristic odour and taste, fine texture, retuse apex, cuneate base, network/reticulate venation, curved anticlinal cell - wall. Trichomes were absent in both of them. The colour of the leaf in *P. muellerianus* is pale deep green on the adaxial surface and pale light green on the abaxial surface. The colour of the leaf of *P. fraternus* is light green on both adaxial and abaxial surfaces (Plates 1 & 2).

The hypostomatic situation of *P. muellerianus* was similar to the findings of Uka *et al*., 2014 in their leaf epidermal studies where they researched six *Phyllanthus* species in South East Nigeria, while the amphistomatic situation of *P. fraternus* was similar to the findings of Zhigila *et al*., 2015 in their study of *Capsicum*.

Morphologically, the shrub habit and the presence of spiny stipules in *P. muellerianus* as against the herbaceous nature and laterally free stipules of *P. fraternus* are distinguishing spot characters that can delimit *P. muellerianus*. Other peculiar characteristics such as sub-acute leaf apex and ovate-elliptic leaf shape are also taxonomic attributes of the taxon. The variations in stomatal index observed in this study can be reasonably employed in delimiting the species of the genus *Phyllanthus* (Table 2).

From the result in Table 2, the stomatal index (27.69%) *P. muellerianus* is higher than that of *P. fraternus* (19.86%). The stomatal density was highest on the abaxial surface of *P. fraternus* (116 mm^2) and it was lowest on the abaxial surface of *P. muellerianus* (18mm^2). This shows that stomata occupy a larger proportion on the adaxial surface than the abaxial surface. These variations are similar to the report of the research of Uka *et al*., 2014.

Chemomicroscopy analysis of the powdered leaves of *P. muellerianus and P. fraternus* revealed the presence of cellulose cell wall, lignified cell wall, mucilage, tannins, starch, suberin, calcium carbonate, calcium oxalate crystals and tannins at varying concentrations (Table 3). The chemomicroscopic analysis is most valuable in the identification of powdered drug as their identification is largely based on the form, the presence or absence of certain cell types, and cell inclusions (Celestine *et al*., 2019). The physicochemical analysis of the powdered leaves of the two *Phyllanthus* species also gave different values as analyzed in Table 4.

Moisture content for *P. muellerianus* and *P. fraternus* (6.33 and 6.44 respectively) was not too high. This is an indication of less chances of microbial degradation of the drug during storage (Table 4). The general standard requirement of moisture content in crude drugs is recommended not to be more than 14% (Peter, 1990) and the value obtained in this research work was within the accepted range. Moisture is considered an adulterant because of its added weight as well as the fact that excess moisture promotes fungi and bacterial growth (Nuhu *et al*., 2016).

Total ash value (9.67 and 10.17) for *P. muellerianus* and *P. fraternus* respectively represents both the physiological and non - physiological ash from the plant (Table 4). The non - physiological ash is an indication of inorganic residues. The total ash value is used as criteria to judge the identity and purity of drugs (Nuhu *et al*., 2016).

The acid insoluble ash values (8.00 and 7.17) and water soluble ash (3.33 and 1.17) respectively for *P. muellerianus and P. fraternus*, indicated that the plant was not too pure, it probably contained little extraneous matters such as sand, silica and soil (Table 4).

The result also indicated that ethanol gave higher extractive values (47.33 and 26. 00) for *P. muellerianus* and *P. fraternus* compared to water which had an extractive value of (20.00) each for both species (Table 4).

Conclusion

The present study shows that the two *Phyllanthus* species are very similar in their leaf epidermal features. Nonetheless, the absence of stomatal on the abaxial surface of *P. muellerianus* can serve as a diagnostic character to delimit them. According to Alege *et al.*, (2013), epidermal cell characteristics are under strong genetic control, hence they are stable traits and therefore proved to be a better tool for the delimitation of *Phyllanthus* species.

The proper identification of a plant material is crucial to the standardization and quality of products from such plants. The various parameters reported for the two *Phyllanthus* species provide a number of information that may be included in the official monograph for their proper identification. Based on the epidermal cells and their arrangements, it can be concluded that the two species varied from one another. *P. muellerianus* has paracytic stomata, while *P. fraternus* has anisocytic stomata. The stomatal index which indicates the proportion of stomata to leaf surface is a reliable taxonomic character. It is highly constant for any given species and the value is more uniform upon the abaxial surface than the adaxial surface, except for isobilateral leaves (Adedeji and Jewoola, 2008) [2].

The morphological and anatomical studies of the leaves of these two *Phyllanthus* species have provided additional evidence which may be combined with other existing lines of taxonomic evidence in arriving at a better identification and classification of *Phyllanthus* species. The comparative foliar epidermal studies, chemomicroscopic and physicochemical evaluation of the leaves of *P. muellerianus and P. fraternus* may be useful to generate standards to assess the quality and purity of their drug formulations.

Recommendations

There is a need for further research on the molecular studies of the two species so as to have a classification based on the specific relationship between the two plants.

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