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Pharmacognostic and Chromatographic Standardization of *Calyptrochilum emarginatum* (SW.) Schltr. (Orchidaceae)

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ABSTRACT

Pharmacognostic and chromatographic evaluation of *Calyptrochilum emarginatum* (SW.) a potential drug plant belonging to the orchid family was carried out. High performance liquid chromatographic standardization of the methanol extract revealed sixteen compounds one of which had the same retention time 6.8 minutes as reference rutin. Thin layer chromatography of the hexane extract showed 11 spots one of which had R_f value of 0.5 same as β-sitosterol and the successive ethyl acetate extract gave 9 spots. Proximate analysis revealed moisture content of 11.9%, alcohol extractive value 1.4%, total ash 6.9%, water soluble ash 2.5% acid-insoluble ash 3.2% and water extractive value 13.8%. Microscopic evaluation of the leaf epidermis revealed anomocytic stomata which were only on the lower surface (hypostomatic), the epidermal cells were polygonal in shape on both adaxial (upper) and abaxial (lower) surfaces. Trichomes or trichome bases were not found on both surfaces.

Key words: Orchid, *Calyptrochilum emarginatum*, microscopy, chromatography, rutin.

INTRODUCTION

There are two recognized and accepted species in the genus *Calyptrochilum*. These are *Calyptrochilum christyanum* and *Calyptrochilum emarginatum.* Jayeola and Thorpe [1] carried out scan electron micrograph of the genus *Calyptrochilum* Kraenzl towards establishing taxonomical characteristics of the two species. *C. chrystyanum* (Rchb.) Summerh was characterized by a network of horizontal grooves, dome shaped micro papillae and a mass of soft wax while the *C. emarginatum* (Sw.) Schltr was distinguished by the presence of densely overlapping conical and globular type of micro papillae.

Calyptrochilum emarginatum (SW.) Schltr. is an epiphytic shrub belonging to the orchid family. Orchid is an anglicized name given to every plant species belonging to the family Orchidaceae. Like other epiphytes, *Calyptrochilum emarginatum* grows on tree branches, unconnected to the ground without being parasitic in any way. Leaves grow along a pendant stem up to 50 cm long with inflorescence appearing along the underside of the stem with 6 to 9 flowers. *C. emarginatum* is a shade-loving shrub with stems carrying distichous, ovate, leathery, unequally and obtusely bilobed (emarginated) apical leaves arising with an auxiliary [2]. Flower inflorescence is characterized by strong scent, even nocturnal. The plant flowers approximately within 2 to 3 years [2].

Calyptrochilum emarginatum can be found in Angola, Cameroon, Central African, Equatorial Guinea, Gabon, Ghana, Ivory Coast, Liberia, Nigeria, Sierra Leone and Zaire in tropical, evergreen and deciduous rainforest at elevation around sea level to 1000 meters [2]. Through the ages, several health-promoting benefits have been attributed to the use of orchid extracts. Such benefits include anti-diuretics, anti-inflammatory, anti-carcinogenic, hypoglycemic activities, anti-microbial, anti-convulsive, neuroprotective and anti-viral activities [3]. The water soluble decoction of the whole plant of *Anoectochilus formosanus* for instance was found to show potency for tumor inhibitory activities in experimental animals after subcutaneous transplantation of CT-26 murin colon cancer cells. The inhibitory activities of this orchid may be associated with its potential immunostimulatory effect [4]. As a part of a screening study, methanol extract of leaves of *Spiranthes* was active against Gram-positive bacteria and had anti-inflammatory activity [5]. In a study of herb extract from Chinese medicinal plants, there was found that *Bletilla striata* possess antioxidant and antimicrobial capacity [6].

Bletilla striata rhizome collected with a non-metal cutting tool, cleaned, and dried was used to treat tuberculosis, hemoptysis, gastric, and duodenal ulcers, as well as bleeding, and cracked skin on the feet and hands. Other uses in China, Mongolia, and Japan include the introduction of euphoria, purification of blood, strengthening and consolidation of lungs, as well as the treatment of pus, boils, abscesses, malignant swellings, ulcers, and breast cancer. Tubers have also been used as a demulcent, a bechic, and

an expectorant [7]. Additional medical applications of the boiled and/or dried tubers include treatment of the flatulence, dispepsia, dysentery, fever, malignant ulcers, gastrointestinal disorders, hemorrhoids, anthrax, malaria, eye diseases, tinea, ringworm, tumors, and necrosis, silicosis, traumatic injuries, coughs, chest pain, tuberculosis, vomiting of blood, gastrorrhagia, enterorrhagia, internal bleeding, inflammation, and chopped skin. The powdered roots mixed with oil have been used as an emollient for burns and skin diseases. Whole plant preparations are tonic and treatment against leucorrhea, hemoptysis, and purulent coughs. Leaves collected in the autumn are reported to cure lung disease [7].

Myriads of chemical compounds have been isolated from different species of the orchid family of plants. Such compounds include stilbenoids 3,3'-dihydroxy-2',6'-bis(p-hydroxybenzyl)-5-methoxybibenzyl and 3',5-dihydroxy-2-(p-hydroxybenzyl)-3-methoxybibenzyl isolated from the tubers of methanol extract of *Bletilla striata* shown to have inhibitory effect of tubulin polymerization at IC_{50} of 10 μM. Others compounds such as (1-(p-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol; 2,7-dihydroxy-1,3-bis(p-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene); 7,8-dihydro-5-hydroxy-12,13-methylenedioxy-11-methoxyl dibenz[b*f*]oxepin; 7,8-dihydro-4-hydroxy-12,13- methylenedioxy-11-methoxyldibenz [b*f*]oxepin; 7,8- dihydro-3-hydroxy-12,13-methylenedioxy-11-methoxyldibenz [b*f*]oxepin; cumulating; densiflorol A and plicatol B were isolated [3].

The forkloric uses of *Calyptrochilum emarginatum* among the Takkad people of Southern Kaduna in Northern Nigeria had been reported [8]. In South-West Nigeria *C. emarginatum* is used in traditional medicine for the management of cough, tuberculosis and malaria. Extracts from *C. emarginatum* growing in South-West Nigeria exhibited bactericidal activity against *Staphylococcus aureus*, responsible for some respiratory tract and opportunistic infections [9]. The aim of the present study is to conduct pharmacognostic, microscopic and chromatographic standardization of *Calyptrochilum emarginatum* to provide information that could be useful for monograph development.

MATERIALS AND METHODS

Materials

The plant material was collected on June 7, 2012 in front of the Conference centre, Obafemi Awolowo University, Ile –Ife, Osun State, and South-West of Nigeria. The site of collection was characterized and geo-referenced N07 31.536, E004 31.536, and elevation 269 m, using a GARMIN GPS 60 Global Positioning System (GPS). The plant was identified and authenticated by Mr Gabriel and Mr Bennard at the herbarium of the Department of Botany, Obafemi Awolowo University Ile-Ife.

Methods

Plant Preparation

The plant material was chopped into smaller bits and oven dried at a temperature of 40 ^{o}C for a period of two weeks. The dried plant sample was crushed in a mortar to maximize the surface area and passed through sieve No. 22 to get moderate powder of uniform size. It was in the course of pounding that the oil- bearing a property of this plant was discovered as the inner part of the mortar was cleaned with a plain sheath.

Extraction

30 g of the powdered sample was weighed and macerated in 300 ml of hexane in a stopped container and allowed to stand for 24 hours at room temperature 28-30 ^{o}C. The mixture was vaccum filtered with Whatman No. 1 filter paper. The filtrate was concentrated to dryness *in vacuo*, while the marc was air dried. The dried marc was then weighed before subsequent maceration in ethyl acetate and methanol for a period of 24 hours for each solvent at the same room temperature. A separate straight run methanol extract was obtained by maceration for 24 hours at room temperature 28-30 ^{o}C and filtered with Whatman No. 1 filter paper. Yields were determined as reported elsewhere [9].

Thin layer chromatography fingerprinting

The successive hexane and ethyl acetate extracts (5, 10 and 15 µL each) were spotted on K5 normal phase silica gel precoated thin layer chromatography plate (Whatman) previously activated at 105 ^{o}C for 2 hours. Beta-sitosterol (Fluka Germany) in ethyl acetate was spoted in duplicate alongside the extracts as reference standard. The mobile phase for the hexane extract consisted of hexane and ethyl acetate (4:1). The mobile phase for the ethyl acetate extract consisted of hexane and ethyl acetate (3:2). The developed and air dried plates were viewed in day light, UV at 366 nm and then sprayed with 10% sulphuric acid in ethanol. The sprayed plate was heated at 105 ^{o}C for about 5 minutes until colour of spots were fully developed. The spots/ colour were noted and their Rf values calculated thus: Rf = Distance moved by solute/Distance move by solvent front. The results are shown in Tables 1 and 2.

High performance liquid chromatography fingerprinting

High performance liquid chromatography analysis was performed on the straight run methanol extract using method reported elsewhere [10], with some modifications. The chromatographic system includes Shimadzu HPLC system consisting of Ultra-Fast LC-20AB prominence equipped with SIL-20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector; column oven CTO-20AC, system

controller CBM-20Alite and Windows LCsolution software (Shimadzu Corporation, Kyoto Japan); column, VP-ODS 5µm and dimensions (150 x 4.6 mm). The chromatographic conditions included mobile phase: solvent A: 0.2% v/v formic acid; solvent B: acetonitrile; The HPLC operating condition was isocratic mode with solvent B: 20%. Flow rate was 0.6 ml/min; injection volume 10 µl of 500 µg/ml solution of the methanol extract; reference standard, Rutin (Fluka, Germany) 50 µg/ml in methanol was used as standard. Detection was at 254 nm UV. Column oven temperature was set at 40 ^{0}C. The total run time was 15 minutes.

Determination of some physical constants

Moisture content

An evaporating dish was dried in oven to constant weight at 105 ± 5 °C. 3 g of the dried powdered plant material was weighed into the evaporating dish. The dish and its content were placed in the oven at 105 ± 5 °C and dried to a constant weight. This was achieved by checking the weight at a regular interval of 30 minutes until constant weight was achieved when less than 0.1 % of the test sample weight was lost during an additional exposure to the drying process and then dried further for at least 1 h. The moisture content was determined as the ratio, in percentage, of weight of water to dry weight of material. [11].

Alcohol extractive value

5 g of the powdered plant sample was accurately weighed into a bottle. 90% ethanol was prepared and 100 ml of it was added to the plant sample and the cover was tightened firmly. The bottle and its content was shaken for 6 hours in a GFL 1083 mechanical shaker and allowed to stand for 18 hours. At the end of the 18 hours, the extract was quickly filtered and 20ml of the filtrate was poured in a heated evaporating dish of a known constant weight. The filtrate was evaporated to dryness on a water bath and dried to a constant weight at temperature of 105 °C [11].

Water extractive value

Chloroform in water 0.25% v/v was prepared and 100 ml of it was added to the plant sample which had already been prepared by weighing 5g of it in a stopped 250 ml conical flask. The flask and its content was shaken for 6 hours on a mechanical shaker and allowed to stand for 18 hours. At the end of the 18 hours, the extract was quickly filtered. 20ml filtrate was poured in a clean stainless evaporating dish. The dish was heated to dryness on a water bath. A constant weight of the residue was gotten by drying in an oven at 105 °C. The final weight of the residue was obtained from the 20 ml extract by subtracting the weight of the evaporating dish from the final weight. The alcohol extractive value was calculated in percentage with reference to the initial weight of the powdered sample [11].

Determination of total ash value

3 g of the dried powdered material, was accurately weighed into platinum crucible, previously ignited, cooled and weighed. The material was incinerated by gradually increasing the heat to500-600 °C until it is white indicating the absence of carbon. It was cooled in a desiccator and weighed. The percentage total ash content was calculated with respect to the dried powdered material. [11].

Determination of acid-insoluble ash

The ash was boiled for 5 minutes with 25 ml of hydrochloric acid (~70 g/l) TS. The insoluble matter was collected on an ashless filter-paper, washed with hot water, and ignited at about 500 °C to constant weight. The percentage acid-insoluble ash content was calculated with reference to the dried material. [11].

Determination of water-soluble ash value

Water-soluble ash value was determined as reported elsewhere [12]. Briefly, the total ash was boiled for 5 minutes with 25 ml of distilled water. The insoluble matter was collected on an ashless filter paper, washed with hot distilled water, and ignited in a platinum crucible for 15 minutes at a temperature not exceeding 450 ^{o}C. The weight of the insoluble matter was subtracted from the weight of the total ash; the difference in weight represents the water-soluble ash. The percentage of the water-soluble ash was calculated with reference to the dried material [11].

Microscopy

The method of Ugbabe and Ayodele [12] were adopted. About 5mm^2 x 1cm^2 leaf fragment were obtained from a standard median portion of the leaf and macerated in concentrated nitric acid in capped petri dishes for a period of 24 - 48hrs. The appearance of bubble on the surface of the leaf fragment indicated their suitability for separation. They were then transferred into 50% alcohol to harden after the separation. They were then stained in safranin O for 5-10 min. and excess stain washed off in water. They were mounted in glycerin on the slide with the edge of the cover slip ringed with nail vanish to prevent dehydration. The slides were labeled and examined under the microscope. Photomicrographs of the preparation were taken using Leica CME microscope with digital microscope eye piece attached and photo explorer 8.0 SE basic software.

Results and Discussion

Thin layer chromatography

The mobile phase comprising hexane:ethyl acetate (4:1) gave very good separation for the hexane extract for 5 µL µL, 10 and 15 µL investigated on K5 normal phase silica gel precoated thin layer chromatography plate. The solvent front was 8 cm. A total of eleven (11) spots were detected (Table 1). Beta-sitosterol used as reference standard with Rf value of 0.53, corresponded to one of the components of the hexane extract and was detected as pink coloured spot only after spraying with 10% sulphuric acid in ethanol and then heated at 105^{o}C for about 5 minutes. Two of the components (Rf 0.1 and 0.31) were visible only in day light as yellow spots. On the other hand two of the components (Rf 0.16 and 0.29) were visible only under UV as pink spots. Spot with Rf 0.23 was green in day light and red under UV, while Rf 0.4 was green in day light and pink

Table 1. Thin layer chromatography fingerprinting of hexane extract of *C. emarginatum*

Components	**R_f Value**	**Colour**		
		Day Light	**UV 366 nm**	**Spray Reagent***
1	0.10	Yellow	-	-
2	0.16	-	Pink	-
3	0.23	Green	Red	-
4	0.29	-	Pink	-
5	0.31	Yellow	-	-
6	0.40	Green	Pink	-
7	0.53	-	-	Pink
8	0.64	-	White	-
9	0.68	-	-	Pink
10	0.74	-	Pink	-
11	0.85	-	-	Pink

*Spray reagent: spraying was with 10% sulphuric acid in ethanol and then heated at 105^{o}C for about 5 minutes under UV. Component with Rf value of 0.64 was seen only under UV as a characteristic bright white spot while Rf 0.74 was a pink spot. Two of the components (Rf 0.68 and 0.85) were visible as pink coloured spots only after spraying the plate with 10% sulphuric acid in ethanol and heating at 105^{o}C for about 5 minutes (Table 1).

The mobile phase for the ethyl acetate extract consisted of hexane-ethyl acetate (3:2) and 5 µL µL, 10 and 15 µL extract volumes were spotted on K5 normal phase silica gel precoated thin layer chromatography plate. The developed and air dried plates were viewed in day light, UV at 366 nm and then sprayed with 10% sulphuric acid in ethanol. The sprayed plate was heated at 105^{o}C for about 5 minutes until colour of spots were fully developed. The spots/ colour were noted and Rf values calculated. The solvent front was 8 cm. A total of nine (9) spots were detected (Table 2). Beta-sitosterol used as reference standard with Rf value of 0.71 did not corresponded to any of the components of the successive ethyl extract It was detected as a pink coloured spot only after spraying with 10% sulphuric acid in ethanol and then heated at 105^{o}C for about 5 minutes. Three of the spots (Rf 0.05, 0.09 and 0.13) were green in day light and red under UV. One of the components (Rf 0.27) was visible only on spraying as a pink spot. Spot with Rf 0.45 was yellow in day light and pink on spraying. Components with Rf values of 0.51 and 0.63 were seen only under UV as pink spots. Rf 0.74 was green in day light but pink under UV. Component with Rf value of 0.78 was visible only under UV as a pink spot (Table 2).

The successive and straight run methanol extracts were not properly separated on TLC hence the straight run methanol extract was subjected to HPLC analysis.

Table 2. Thin layer chromatography fingerprinting of ethyl acetate extract of *C. emarginatum*

Components	**Rf Value**	**Colour**		
		Day Light	**UV 366 nm**	**Spray Reagent***
1	0.05	Green	Red	-
2	0.09	Green	Red	-
3	0.13	Green	Red	-
4	0.27	-	-	Pink
5	0.45	Yellow	-	Pink

6	0.51	-	Pink	-
7	0.63	-	Pink	-
8	0.69	Green	Pink	-
9	0.78	-	Pink	-

*Spray reagent: spraying was with 10% sulphuric acid in ethanol and then heated at 105^{o}C for about 5 minutes.

HPLC fingerprinting:

According to the HPLC spectrum of the methanol extract, a total of sixteen (16) peaks were detected with retention times as shown in Figure 1. One of the component peak had the same retention time of 6.68 minutes, as the standard rutin.

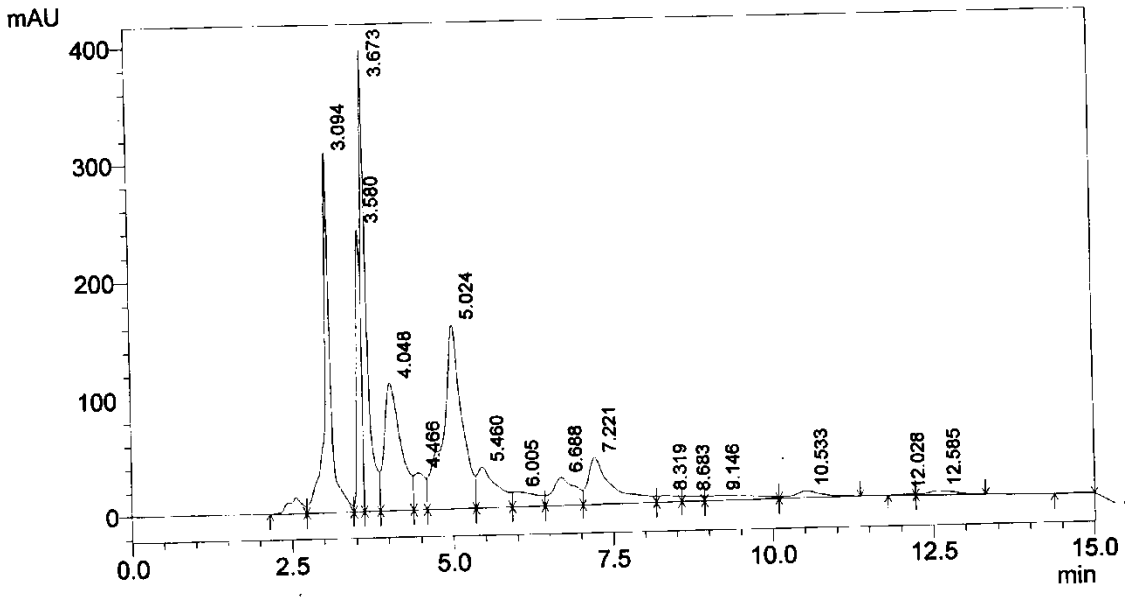

Fig. 1: High performance liquid chromatography fingerprinting of the straight run methanol extract of *C. emarginatum*

Table 3: Physical constants of *Calyptrochilum emarginatum*

Evaluative Parameter	*Mean Value
Moisture content	11.9 ± 0.08%
Total ash	6.9 ± 0.05%
Acid-insoluble ash	3.2 ± 0.09
Water-soluble ash value	2.5 ± 0.17%
Alcohol extractive	1.4 ± 0.05 %
Water extractive value	13.8 ± 0.04%

* Mean value of three determinations

Microscopy

The leaf epidermal microscopic study revealed the presence of stomata on the abaxial surface and not on the adaxial surface (Hypostomatic), showing that gaseous exchange takes place only on the abaxial surface (Plate 1) as found in peach, mulberry and walnut. As against what is found in most monocot which are basically amphistomatic and isostomatic type e. g maize, oat and grasses. The subsidiary cell is hexegonal in shape on both surfaces. The stomata type is of the anomocytic type. There were no trichomes or trichome bases found (Plate 1).

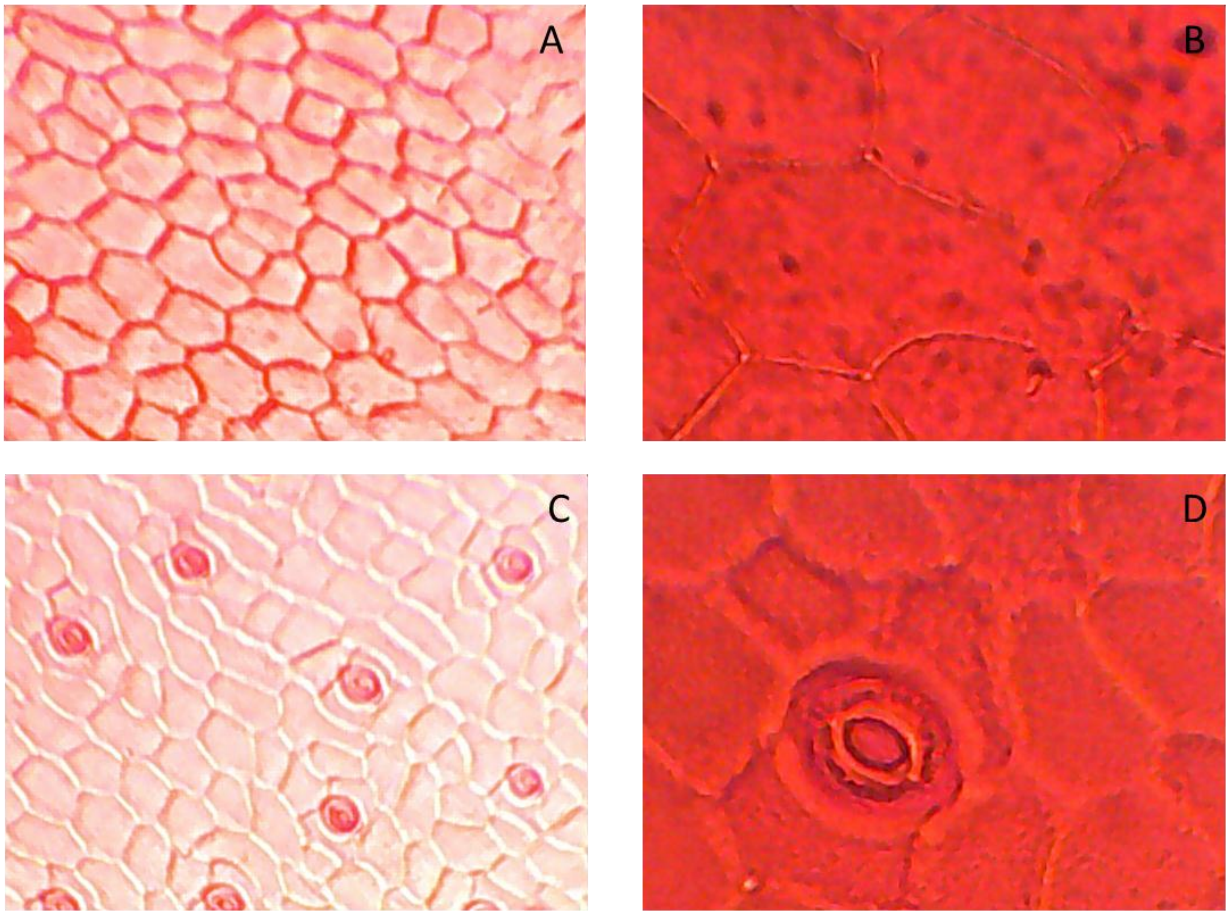

Plate 1: (A) Adaxial surface (x100) with polyganal cell shape and no stomata. (B) Adaxial surface (x400). (C) Abaxial surface (x100) with anomocytic stomata type and polygonal cell shape. (D) Abaxial surface (x400)

References

1. Jayeola, A.A. and Thorpe JR (2008). A scanning electron microscope study of the genus Calyptrochilum Kraenzl. (Orchidaceae) in West Africa. *Feddes Repertorium*, 111(5-6):315-320.
2. LaCroix, E. and LaCroix, I.F. (1997). African Orchids in the Wild and in Cultivation. Timber Press, Portland, Oregon. pp. 43-45.
3. Gutierrez, R.M.P. (2010). Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. *J. Med. Plants Res.* 4(8):592-638.
4. Tseng, C.C., Shang, H.F., Wang, L.F., Su, B., Hsu, C.C., Kao, H.Y. and Cheng, K.T. (2006). Antitumor and immunostimulating effects of *Anoectochilus formosanus* Hayata. *Phytomedicine*, 13: 366-370.
5. Matu, E.N. and Vanstaden, J. (2003). Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. *J. Ethnopharmacol.* 87: 35-41.
6. Luo, H., Lin, S., Ren, F., Wu, L., Chen, L. and Sun, Y. (2007). Antioxidant and antimicrobial capacity of Chinese medicinal herb extracts in raw sheep meat. *J. Food Protection,* 70: 1440-1445
7. Kong, J.M., Goh, N.K., Chia, L.S. and Chia, T.F. (2003). Recent advances in traditional plant drugs and Orchids. Acta Pharmacologica Sinica 24: 7–21.
8. Mathias, S.N., Ilyas I. and Musa K.Y., (2006). Ethno botanical survey and preliminary phytochemical studies of plants used in the Traditional Medicine practice amongst the Takkad speaking community of Southern Kaduna-Nigeria. *Chem. Class J.* 4:70-75.
9. Okhale, S.E., Ugbabe, G.E., Bamidele, O., Ajoku, G.A. and Egharevba, H.O. (2014). Phytochemical and antimicrobial studies on extractives of Calyptrochilum emarginatum (SW) Schltr (Orchidaceae) growing in Nigeria. *J. Med. Plants Res.* Vol. 8(4), pp. 223-228. DOI: 10.5897/JMPR2013.5212.
10. Bienvenu, E., Amabeoku, G.J., Eagles, P.K., Scott, G. and Springfield, E.P. (2002). Anticonvulsant activity of aqueous extract of Leonotis leonurus. *Phytomedicine* 9, 217-223.
11. WHO. Quality Control Methods for Medicinal Plant Materials. World Health Organization, Geneva. 1998; 34-128.
12. Ugbabe, G.E. and Ayodele A.E. (2008). Foliar epidermal studies in the family Bignoniaceae Juss. In Nigeria. *African Journal of Agricultural Research*, 3(2):154-166.