Phytochemical analysis and antioxidant properties of *Eulopiaguineesis*Lindl.Pseudobulbs (Orchidaceae)

By

Saidu M. Bello[1*],Abubakar Ahmad[2] and Hussein UK Yahya[3]

[1]Department of Pharmacognosy and Drug Development, Ahmadu Bello University Zaria, Kaduna State, Nigeria.

[2]Department of Pharmacognosy and Drug Development, Ahmadu Bello University Zaria, Kaduna State, Nigeria.

[3] Department of Pharmaceutical Microbiology,UsmanuDanfodiyo University Sokoto, Kaduna State, Nigeria.

*Corresponding Author: Saidu .M. Bello

ahmadbell99@gmail.com, +2348136363726

ABSTRACT

BACKGROUND: *Eulophia species* are cultivated worldwide; it is one of the largest orchid's genera in Africa. They are believed to cure many diseases. *Eulophia guineensis* Lindl (Orchidaceae) is a multipurpose plant, native to West Africa and currently grown in many parts of the world. Traditionally, its pseudobulbs are exploited in the treatment of diseases like hypertension, obesity, inflammations, cold, sexual ailments by various cultures without scientific evidence.The aim of the research was to carry out phytochemical test, antioxidant potentials and acute toxicity test on the pseudobulbs of *E. guineensis*.

METHODS: The pseudobulbs were extracted with methanol using cold maceration method via aliquot extraction. The extract obtained was further fractionated with n-hexane, ethyl acetate and n-butanolin order to obtain the non-polar, moderately polar and polar components respectively.Preliminary phytochemical screening and TLC profile of the different fractions; *in vitro* DPPH antioxidant studies of the extract and the different fractions were evaluated. Acute toxicity test of the extract on laboratory mice was also investigated using both the intraperitional and oral routes.

RESULTS: *E. guineensis* pseudobulbs showed significant antioxidant activity ($p < 0.05$) for methanol extract ($IC_{50} = 44.61$ µg/ml), ethyl acetate fraction ($IC_{50} = 7.58$ µg/ml) and n-butanol fraction ($IC_{50} = 1.35$ µg/ml) whereas n-hexane fraction (186.59 µg/ml) and aqueous fraction ($IC_{50} = 1212.47$ µg/ml) showed no significant activity on DPPH induced free radical. However, the standard drug ascorbic acid ($IC_{50} = 1.04$ µg/ml) performed best. Qualitative Phytochemical screening of the extract showed the presence of triterpenes, flavonoids, tannins, cardiac glycosides, deoxysugars and carbohydrates. These phyto-constituents were redistributed among the different fractions obtained from the extract. The Acute toxicity of

the extract showed it to be slightly toxic intraperitionally and practically non-toxic orally with LD_{50} of 3807.09 mg/kg and >5000 mg/kg body weight respectively.

CONCLUSION: The Antioxidant studies on the pseudobulbs of*E. guineensis* have scientifically shown its rich antioxidant potentials which in addition to other factors could be helpful in validating the traditional uses of the plant in the treatment of several ailments.

KEYWORDS: *Eulophia guineensis;* pseudobulbs; antioxidants; phytochemicals;

INTRODUCTION

Eulophia is a genus with approximately 210-230 species of orchids [1]. *Eulophia* is one of the largest orchid's genera in Africa and an extraordinarily diverse one. This, no doubt, reflects the wide range of habitats in which various species are to be found, from swamps and rainforest to semi-deserts [2]. Many can survive the dry season through their large bulbous called 'Pseudobulbs'[2]. *E.guineensis* Lindl belongs to Orchidacaeae family and genus *Eulophia*. Synonyms include *E. congoensis* and *E. quartiniana.* It is native to West Africa and called *Gandun albasa* and *Kimban daji* (Hausa language) *Oduebi* (Igbo language) of Nigeria. It grows to an average height of 67 cm and possesses an underground stem (rhizome) [3] Pseudobulbs are thickened, conical and upward growth from the rhizome. The Pseudobulbs are used as food and medicine. Traditionally, the pseudobulbs are crushed, boiled and sieved, and the sieved liquid is then used to prepare the local *Akamu pap,*a popular dish common in West Africa (Personal communication). The Pseudobulbs of*Eulophia spp.* were reported to cure diabetics, obesity, inflammations, arthritis and many sexual ailments [4, 5, 6].

Despite the several traditional claims on *Eulophia spp*, the medicinal properties of *Eulophia species* are not well documented and are without scientific authentication [2]. There is no

known reported scientific information on the medicinal properties of *E. guineensis.* Owing to the increasing risk factors of human to various deadly diseases posed by synthetic antioxidants like Butylated Hydroxy Toluene (BHT) and Butylated Hydroxy Anisole (BHA), there has been a global trend toward the use of natural substance present in medicinal plants and dietary plants as therapeutic antioxidants [7, 8].

Justification: *Eulophia spp* are one of the largest orchid genera in Africa. This will provide the opportunity to investigate the medicinal potentials of our indigenous plants.

The aim of the research was to provide scientific basis for the uses of *Eulophia guineensis* in traditional medicine in treating oxidation related diseases.

The objectives were:

1. To carryout the phytochemical screening of the extract and the fractions of *E. guineensis* extract.

2. To carryout chromatographic analysis of the extract and the fractions of *E. guineensis* extract and

3. To evaluate the antioxidant properties of the methanol extract and the fractions obtained from *E. guineensis* Pseudobulbs.

MATERIALS AND METHODOLOGY:

PLANT COLLECTION AND IDENTIFICATION:

The entire and matured plant materials were collected from Bomo village, Samaru district of Sabongari LGA of Kaduna State. The plant was identified as *Eulophia guineensis* with Voucher number 319 at the herbarium unit, Department of Biological Sciences, Ahmadu

Bello University, Zaria, Nigeria.The figures 1 and 2 below are the *Eulophia guineensis* plant and its pseudobulbs respectively.

Fig. 1: Harvested *Eulophia guineensis* plant.

Fig. 2: *E. guineensis* pseudobulbs

EXTRACT PREPARATION:

5.44 kg of the fresh Pseudobulbs of *E. guineensis* (figure 2 above) was extracted with 12 litres of methanol using cold maceration method via aliquot extraction for 8 days in maceration flasks. Stirring and gradual shaking was applied throughout the extraction period. All the extracts collected were filtered and concentrated under reduced pressure in rotary evaporator to collect methanolic extract (MEOH). The dried extract was collected and preserved in desiccators.

The Percentage yield of the extract was calculated using the formula given below:

$$\text{Percentage yield of extract} = \frac{\text{Weight of total extract}}{\text{Weight of powdered material}} \times 100\ (\%\text{w/w})$$

Fractionation of the methanol extract of *E. guineensis* Pseudobulbs:

The MEOH of *E. guineensis* pseudobulbs was fractionated successively with three solvents in order of their increasing polarity, namely: hexane, ethyl acetate and n-butanol respectively according to standard method as explained below. 110 g of dried methanol extract was dissolved in 300 ml of distilled water and poured into a separating funnel. Starting with the least polar, 900 ml of n-hexane was added to the extract in the separation funnel(water to solvent ratio is 1:3). The mixture in the funnel divided into two layers; the lower aqueous portion layer and the upper hexane fraction layer. Both the hexane and aqueous layers were collected into different beakers. The aqueous portion was reloaded into the separating funnel and fresh n-hexane solvent (900 ml) was added to it. This process was repeated three times to ensure maximum fractionation. All the n-hexane fractions collected were pulled together and code named HEX. This same procedure was carried out using ethyl acetate to obtain ethyl acetate fraction (ETOAC) and n-butanol to obtain n-butanol fraction (BTOL) respectively. The leftover after collecting the BTOL was named Aqueous fraction (AQU). The diagram below illustrates the extraction and fractionation pattern of the *E. guineensis* pseudobulbs.

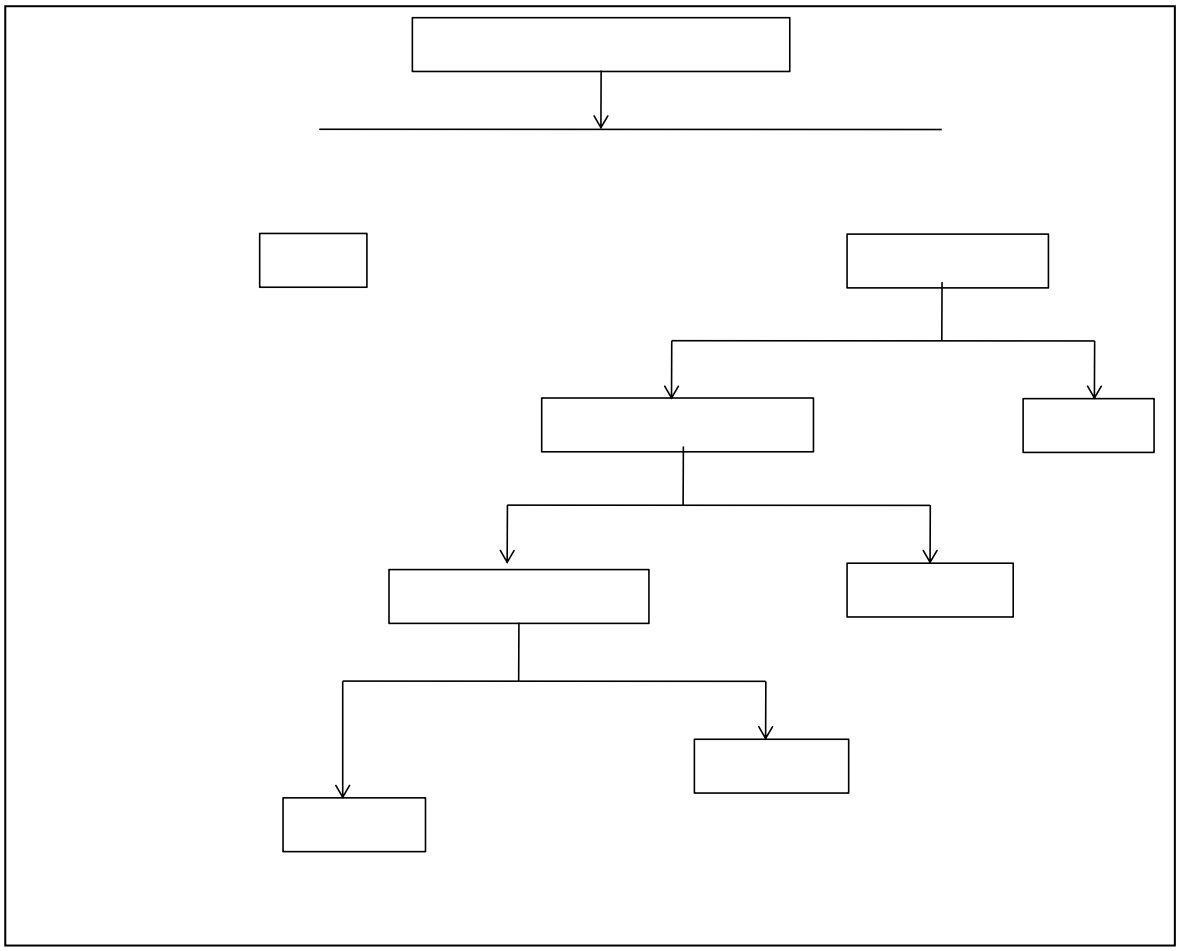


Fig. 3: **Extraction and fractionation pattern of *E. guineensis* Pseudobulbs**

The pulled portions of each fraction were concentrated via the water bath and the percentage yield of each fraction was calculated using the formula given below:

$$\text{Percentage yields of fraction} = \frac{\text{Weight of fraction}}{\text{Weight of the extract}} \times 100\ (\%\text{w/w})$$ [9]

PHYTOCHEMICAL SCREENING:

Phytochemical screening was performed using standard procedureto test for Carbohydrates (Molisch's test), Terpenoides (Salkowski Test), Flavonoids (NaOH Test), Tannins ($FeCl_3$ test), Saponins (Frothing test), Alkaloids (Dragendoff's Test) and Cardiac glycosides (kellerkailani test) [10, 11, 12].

TLC profiling of the extract and the fractions of *E. guineensis* Pseudobulbs

The Methanol extract and all the four fractions obtained were subjected to Thin Layer Chromatography and monitored in TLC tank in different solvent systems. The plates were sprayed with *p-anisaldehyde*as detecting reagent, followed by heating at 110ºC. Chromatograms were viewed immediately [13]. The best solvent for each fractions were chosen for *in-vitro* antioxidant analysis.

***IN-VITRO* ANTIOXIDANT ACTIVITY**

This was carried out using the method proposed by [14]. It involves two stages:

Qualitative screening: Rapid screening of the anti-oxidant activity of the extract and all the fractions were carried out on a TLC plate and sprayed with DPPH as detecting reagent. A yellow spot against a purple background signifies free radicals scavenging/antioxidant components.

Quantitative screening: Stock solution of each sample (0.1mg/ml) was diluted in methanol to various conc.: 100, 80, 60, 40 and 20 µg respectively. 0.2 mM DPPH solution was prepared.

(A_{blank}) = Sample solution (2.5 ml).

(A_{sample}) = DPPH solution (1.0 ml) + Sample solution (2.5 ml).

($A_{control}$) = DPPH solution (1.0 ml) + Methanol (2.5 ml) was used as **Negative control**.

ASCORBIC ACID was used as a **Positive control**.

All reactions were kept in darkness for 30 minutes & measured in U.V Spectrophometer at 518 nm.All readings were taken in triplicate.

$$\text{DPPH scavenging activity (\%)} = 100 - \frac{(Asample - Ablank)}{Acontrol} \text{x } 100$$

The $\mathbf{IC_{50}}$ values were calculated by linear regression of plots where the Absicca represented the concentration of the tested sample and the ordinate represent the average antioxidant activity from three separate tests.

ACUTE TOXICITY STUDIES/DETERMINATION OF LD_{50}:

In order to determine the safety margins of the drug, acute toxicity level of the plant extract was tested on mice using the method and calculations proposed by Lorke for both the intraperitonal and oral routes[15]. This method involves two phases: In the first phase, mice were divided into three groups; 1, 2 and 3, and each group received three mice. The groups received 10, 100 and 1000mg/kg of the plant extract respectively. In the second phase, four groups of one mouse each were treated. The groups received 1200, 1600, 2900 and 5000mg/kg doses respectively.

Statistical Analysis:

All the data obtained from the antioxidant studies were expressed as Mean ± Standard Error of Mean (Mean ± SEM). The data were further analyzed using One Way Analysis of Variance (ANOVA) using SPSS and comparison was done using Duncan Multiple Range Test. Values of $p < 0.05$ were considered significant. The data were also presented in the form of figures, tables and graphs.

RESULTS

Extraction and fractionation of *E. guineensis* Pseudobulbs:

Methanol extraction of 5.44 kg of fresh pseudobulbs yielded 111 g representing 0.02 % of the plant material. The extract was brown and sticky. Solvent-solvent fractionation of 110 g MEOH extract gave 0.20 g HEX (0.002 %), 4.17 g ETOAC (3.790 %), 5.59 g BTOL (5.080 %) and 68.80 g AQU (62.55 %). The percentage yield of each fraction is presented in the pie chart given below:

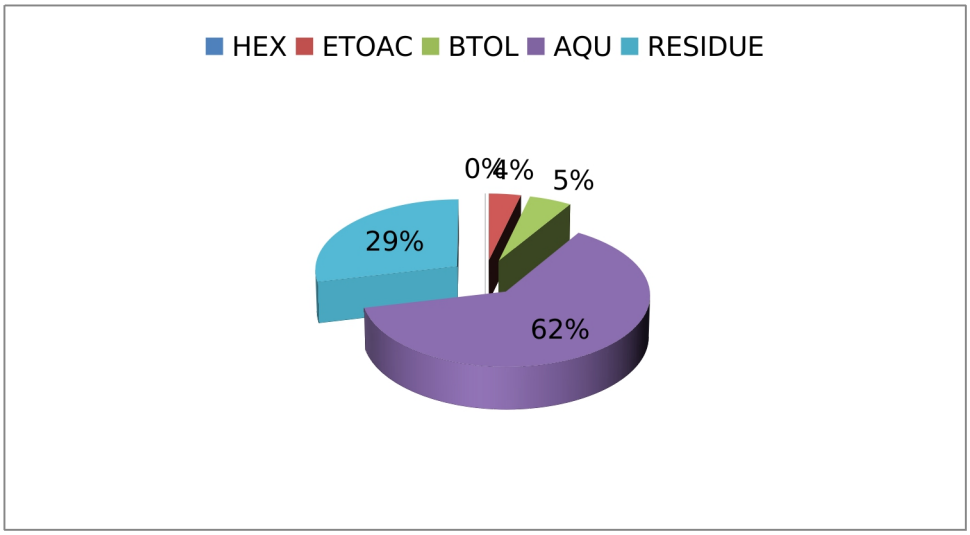


Fig. 4: Percentage yield of each fraction from the extract of *E. guineensis* Pseudobulbs

PRELIMINARY PHYTOCHEMICAL SCREENING:

The MEOH showed positive test for carbohydrate, triterpenes, deoxysugars, cardiac glycosides, flavonoids and tannins. However, it tested negative for saponins and alkaloids. These phytochemical constituents observed were re-distributed among the different fractions obtained from the MEOH as shown in Table below.

Table 1: Preliminary Phytochemical screening result

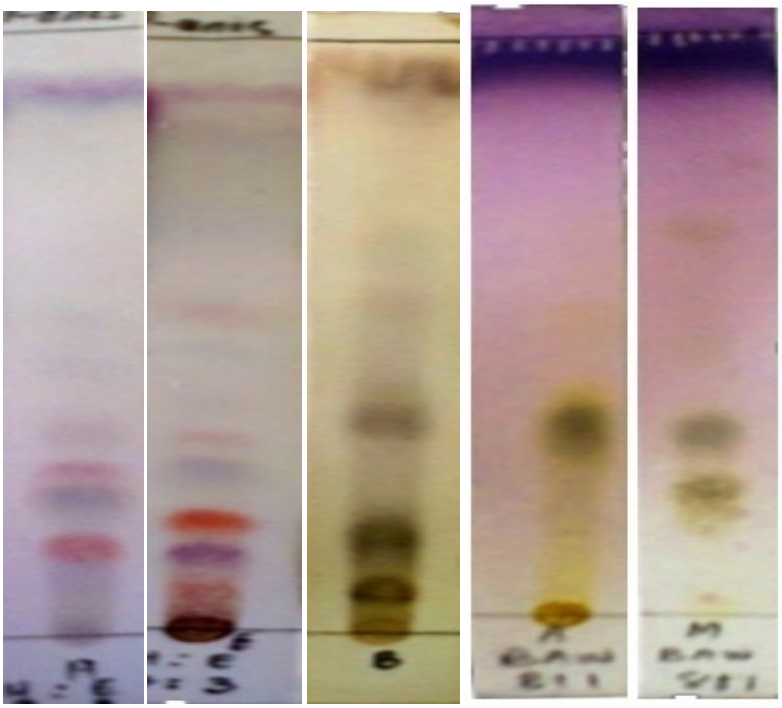
Phytochemicals	MEOH	HEX	ETOAC	BTOL	AQU
Carbohydrate	+	-	-	-	+
Deoxysugars	++	+	+	+	++
Cardiac glycosides	+	+	+	+	+

Saponins	-	-	-	-	-
Triterpenes	+	+	+	-	-
Alkaloids	-	-	-	-	-
Flavonoids	+	-	+	+	-
Hydrolysable tannins	+	-	+	+	+
Condensed tannin	+	-	-	-	+

++ very present + present - absent

TLC CHROMATOGRAM OF MEOH AND ITS FRACTIONS:

The most suitable solvent system for MEOH was BAW 8:1:1, coincidentally, it was also the most suitable solvent system for AQU. Similarly, HE 7:3 was the most suitable solvent system for HEX and ETOAC While CMA 8:2:1 was most suitable for BTOL.



HEX ETOAC BTOL AQU MEOH
H: E (7:3) H: E (7:3) C:M:A (8:2:1) B: A: W (8:1:1)

KEY
H: Hexane
E: Ethyl acetate
C: Chloroform
M: Methanol

Fig. 5: **Chromatogram of the methanol extract and fractions of *E. guineensis* Pseudobulbs, their respective solvent systems as sprayed with *P-anisaldehyde*.**

DPPH *in-vitro* Antioxidant activities of *E. guineensis* Pseudobulbs:

Qualititave test: a yellow spot against a purple background in a TLC profile signifies the free radicals scavenging/antioxidant properties of the components of each fraction against the oxidizing DPPH. HEX, ETOAC, BTOL showed positive result while AQU showed negative result for antioxidant properties.

Quantitative test: This activity was positive concentration dependent. BTOL showed the highest antioxidant potential, and then followed by ETOAC, MEOH, HEX and AQU respectively. However Ascorbic acid (control) performed best. The highest antioxidant activity value was observed in MEOH and BTOL at concentration 100 µg/ml (93.74% ±1.10 ^{b} and 93.42% ±0.17^{b}respectively). The least scavenging activity was observed in AQU at concentration 20 µg/ml (-92.20% ±1.90^{f}). Although, HEX and AQU showed negative activity, the negativity decreased as the concentration of each sample increases.

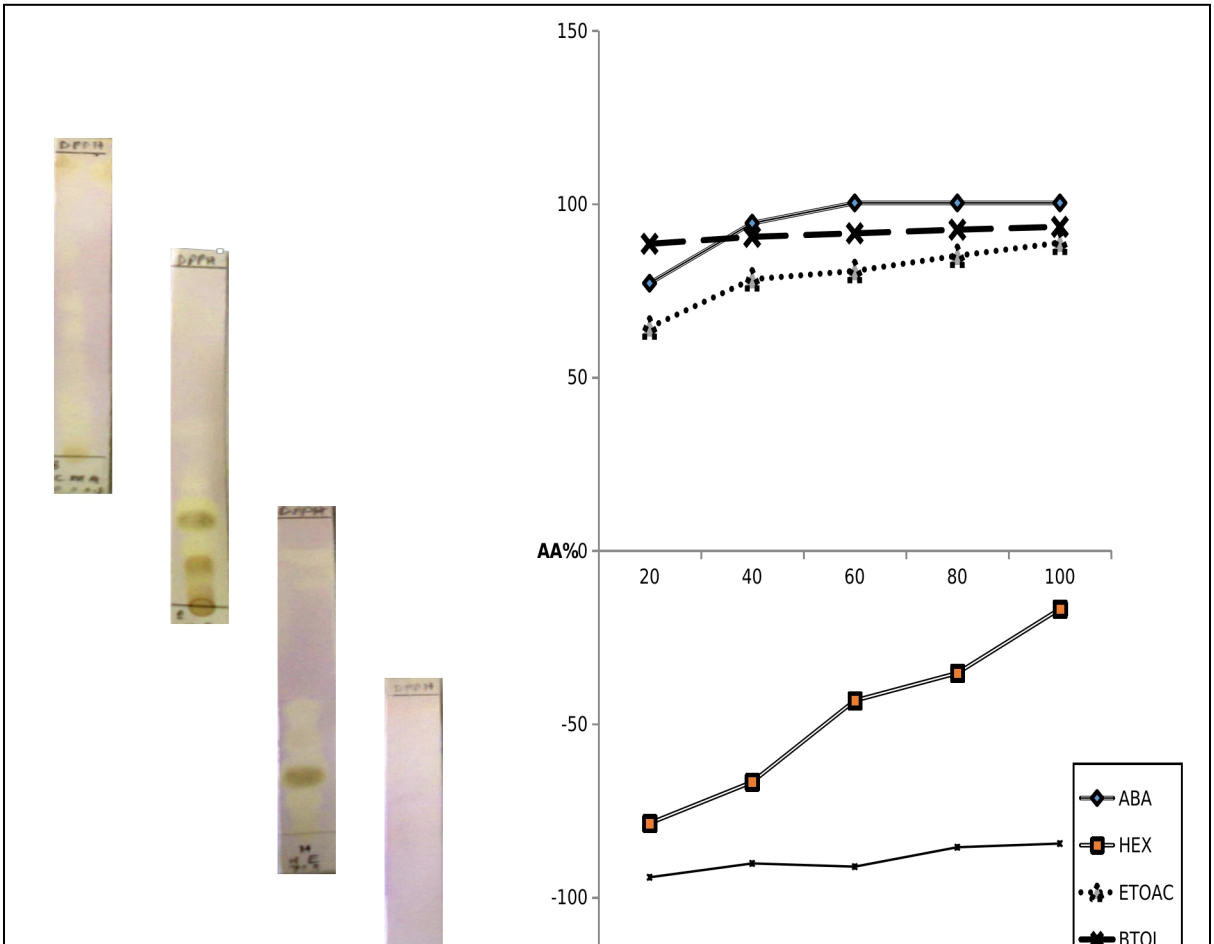


Table 2: Percentage antioxidant of the extract and fractions of the Pseudobulbs of *E. guineensis* on DPPH.

SAMPLES	Conc. (µg/ml)					
	20	**40**	**60**	**80**	**100**	**IC_{50}**
Control(AA)	77.14±3.58^{b}	94.47±0.42^{a}	100.31±0.00^{a}	100.31±0.00^{a}	100.31±0.00^{a}	**1.04**
MEOH	7.09±0.99^{d}	62.85±0.45^{d}	77.65±0.82^{c}	83.19±0.45^{c}	93.74±1.10^{b}	**44.61**
HEX	78.62±0.28^{e}	66.72±1.36^{e}	43.23±0.21^{d}	35.30±2.06^{d}	16.81±0.80^{d}	**186.59**
ETOAC	64.39±0.06^{c}	78.29±0.08^{c}	80.68±0.26^{c}	85.07±0.42^{c}	88.83±0.27^{c}	**7.58**
BTOL	88.51±1.08^{a}	90.52±0.19^{b}	91.54±0.48^{b}	92.59±0.58^{b}	93.42±0.17^{b}	**1.32**
AQU	-	-	-	-	-	**1212**

94.20±1.90^{f}	90.20±1.41^{f}	91.13±2.75^{e}	85.53±0.68^{e}	84.47±1.59^{e}	**.5**

P<0.05 (n=3), a-f: level of significance a: most active f: least active

Acute toxicity test:

MEOH was slightly toxic when administered intraperitionally (LD_{50}3807.89 mg/kg) and practically non-toxic when administered orally (LD_{50}> 5000 mg/kg) using Lorke's method.

Table 4: Median Lethal (LD_{50}) of Methanol extract of *E. guineensis* administered intraperitionally and orally.

DOSE (mg/kg)	*Intraperitionally	Orally
First Phase		
10	0/3	0/3
100	0/3	0/3
1000	0/3	0/3
Second Phase		
1200	0/1	0/1
1600	0/1	0/1
2900	0/1	0/1
5000	1/1	0/1
LD_{50}	**3807.89 mg/kg**	**> 5000 mg/kg**

DISCUSSIONS:

The information on the presence or absence and the type of phytochemical constituents especially the secondary metabolites are useful taxonomic keys in identifying a particular species and distinguishing it from a related species, thus helping in the delimitation of taxa[16].*E. guineensis* pseudobulbs showed the presence of carbohydrate, triterpenes,

deoxysugars, cardiac glycosides, flavonoids, hydrolysable tannins and condensed tannins Similarly, a related species; *E. herbaceae* showed the presence of carbohydrates, steroids, triterpenes and tannins [17].

The present study demonstrates for the first time reports on antioxidant potentials of extracts from *E. guineensis* pseudobulbs. Antioxidant activity was confirmed by DPPH *in vitro* model. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free-radical scavengers which are used as neutraceuticals and also in control of human pathogenic diseases [18, 19]. Different Eulophia species were reported for antioxidant potentials like *E. campestris* [20] and *E. ochreata* [21].

TLC is an important method for qualitative and quantitative analysis of drugs because it indicates some advantages in comparison to HPLC and GC methods. The last decade shows that interest in TLC application in pharmaceutical analysis (e.g., control purity of drugs) has increased with improvements in TLC instrumentation [22]. Blue red- violet and reddish or blue in UV 365 has been observed in Hexane and ethyl acetate fractions are characteristics of triterpenes. This is further supported by works of Wieslaw and his co-scientists who ascertained that blue, reddish-violet in visible light and reddish or blue in UV 365 when sprayed by Anisaldehyde-sulphuric acid is a characteristics of triterpenes [23]. The green to yellow colouration observed in the Aqueous fraction is a characteristics of glycosides. Sugars and glycosides produce green-yellow spots in 3 minutes.

The quantitative antioxidant activity of the aqueous fraction corresponds to its qualitative activity since both showed negative activity. Similar correspondence was observed in the qualitative and quantitative activities of ethyl acetate and n-butanol fractions. The TLC-DPPH antioxidant assay could serve as a good preliminary result for antioxidant potentials of

a plant extract [24]. However, different pattern was observed with the hexane fraction which showed high qualitative test but very low quantitative test, this is so because antioxidant activity is determined not only by the presence of antioxidant components, but also by the antioxidantstrength of each of the combining antioxidant component. This is to say that flavonoids (mainly in ethyl acetate and butanol fractions) possesses more antioxidant strength than triterpenes (mainly in hexane fraction). A group of scientist working on *Rubus rigidus* var. *camerunensis* concluded that flavonoids identified performed better antioxidant activity than triterpenes identified with wide margins in comparison [25].

This Median Lethal Dose (LD_{50}) studies showed the methanol extract of *E. guineensis* Pseudobulbs using both intraperitional and oral routes as relatively safe given the higher values of LD_{50} of 3807.89 mg/kg and >5000 mg/kg respectively. Similarly, *E. ochreata* was reported to be non-toxic with LD_{50} greater than 5000 mg/kg using oral route [21]. The studies also showed that toxicity of a drug may depend on the route of its administration, thus intraperitionally administered drugs tends to be more toxic than orally administered drugs.

CONCLUSION:

This study on the antioxidant properties of *Eulophia guineensis* pseudobulbs has shown its rich antioxidant potentials which in addition to other factors could be helpful in validating the traditional uses of the plant in the treatment of several ailments.

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