PHARMACOGNOSTIC AND ANTIOXIDANT STUDIES OF THE PSEUDOBULBS OF *EULOPHIA GUINEENSIS* LINDL (ORCHIDACEAE)

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ZARIA, NIGERIA

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BY

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FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

DECEMBER, 2015

DECLARATION

I declare that the work in this dissertation entitled "**Pharmacognostic and Antioxidant studies of the Pseudobulbs of *Eulophia guineensis* Lindl (Orchidaceae)**" has been carried out by me in the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

MUHAMMAD BELLO SAIDU

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Name of Student	**Signature**	**Date**

CERTIFICATION

This Dissertation entitled "PHARMACOGNOSTIC AND ANTIOXIDANT STUDIES OF THE PSEUDOBULBS OF *EULOPHIA GUINEENSIS* LINDL (ORCHIDACEAE)" by Muhammad Bello SAIDU meets the regulations governing the award of the degree of Master of Science in Pharmacognosy of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to my generous and caring uncle Alhaji Mahmoud K. Bello. He is a specimen of a good man. May Almighty Allah (SWT) bless, guide, uplift and ultimately grant him Al-Jannatul Firdaus (ameen).

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ABSTRACT

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. Traditionally, it is exploited in the treatment of diseases like hypertension, obesity, inflammation, cold by various cultures without scientific evidence. Pharmacognostic studies; microsopical, chemomicroscopic and physicochemical studies were carried out on the pseudobulbs of *E. guineensis*. The pseudobulbs were extracted with methanol using cold maceration method. Other experiments carried out include preliminary phytochemical screening; *in vitro* antioxidant studies of the extract and fractions for free radical scavenging properties; Chromatographic analysis of ethyl acetate fraction was evaluated and Infra red analysis was carried out for the isolated compound. Acute toxicity test of the extract on laboratory mice was also investigated using both the intraperitional and oral routes. Microscopical examination revealed the presence of cork cells, epidermal cells, cortex, parenchyma cells, sclerenchyma cells, collenchymas cells, calcium oxalate crystals, mucilage, vascular bundles and fibres. Physicochemical parameters were established for the powdered pseudobulbs, they include moisture content (6.56 %), total ash value (10.83 %), acid insoluble ash (2.5 %), water soluble ash (2.6 %), alcohol extractives (11.50%) and water extractives (24.67 %). *E. guineensis* pseudobulbs showed significant ($p < 0.05$) antioxidant activity for methanol extract ($IC_{50} = 44.61$ µg/ml), ethyl acetate fraction ($IC_{50} = 7.58$ µg/ml) and butanol fraction ($IC_{50} = 1.35$ µg/ml) whereas 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. Chromatographic analysis of ethyl acetate fraction resulted in the isolation of a white, crystalline, needle-like compound. Infra red analysis of the compound revealed the presence of hydroxyl, carbonyl, saturated and unsaturated carbons as functional groups. 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 greater than 5000 mg/kg body weight respectively. The Antioxidant studies on the pseudobulbs of the plant *E. guineensis* have scientifically validated the traditional uses of the plant in the treatment of several diseases.

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ACRONYMS AND ABBREVIATIONS

Fig:	Figure
FAA:	Formalin Acetic acid Alcohol
µg:	Micro gram
G.A.A:	Glacial Acetic Acid
LD_{50}:	Median Lethal Dose
ml:	Millimeter
NARICT:	National Research Institute for Chemical Technology
TLC:	Thin Layer Chromatography
UV:	Ultraviolet Light
w/w:	Weight per Weight
WHO:	World Health Organization
Z.I:	Zones of Inhibition
+:	Positive
%:	Percentage
- :	Negative
MEOH	Methanol extract
HEX	Hexane fraction
ETOAC	Ethyl acetate fraction

BTOL	Butanol fraction
AQU	Aqueous fraction
ABA	Ascorbic acid
DPPH	Di-Phenyl Picryl Hydrazyl
Mag.	Magnification
e.t.c.	Etcetera
et al	and coworkers
i.p	Intra peritional
R_f	Retention Factor
ROS	Reactive Oxygen Species
IC_{50}	Median Inhibitory Concentration
EC_{50}	Median Effective Concentration

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Natural products have since time immemorial, been the backbone of traditional system of healing throughout the globe, and have also been an integral part of human history and culture (Veeresham, 2012). Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of their unmatched availability of chemical diversity (Sasidharan *et al.,* 2011; Cos et *al.*, 2006).

An estimated eighty percent (80 %) of the world's population today employs herbs as primary medicines either in part or entirely i.e. For 5.1 billion people worldwide today, natural plant-based remedies are used for both acute and chronic health problems, from treating common colds to controlling blood pressure and cholesterol (Chris, 2010; Veeresham, 2012; WHO, 2008). For many this is out of necessity, since many cannot afford the high costs of pharmaceutical drugs and their adverse side effects, even in advance countries like America (Stephen, 1999; Hong-Fang *et al.,* 2009; Grover *et al.,* 2002; Mukherjee *et al.,* 2006).

Plant medicines are far and away safer, gentler and better for human health than synthetic drugs. This is so because human beings have co-evolved with plants over the past few million years. We eat plants, drink their juices, ferment and distil libations from them, and consume them in a thousand forms. Ingredients in plants, from carbohydrates, fats and proteins to vitamins and minerals, are part of our body composition and chemistry (Chris, 2010). Many characterised human endogenous receptors, important in physiological function, are activated by plant-derived chemicals; for example the opioid and the more recently discovered cannabinoid receptors (Jennie and Peter, 2000).

1.2 Plant as an ultimate source of Drugs

Plant medicines contain a wide range of substances and thus remain indispensable to modern pharmacology and clinical practice. It should not be forgotten, however, that much of the current drug discovery and development process is plant-based, and new medicines derived from plants are inevitable (Chris, 2010; Duraipandiyan *et al.,* 2006; Jennie and Peter, 2000). Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances (Leslie, 2000).

Before the advent of high throughput screening and the post genomic era, more than 80% of drug substances were purely natural products or were inspired by the molecules derived from natural sources (including semi-synthetic analogs) (Chandrakant *et al.,* 2012).

The utility of natural products as sources of novel structures is still alive and well. Newman (2008) and Newman and Cragg (2012) estimated that 50% to 60% of the drugs that are now available were either directly or indirectly derived from natural products. Moreover, natural products have also been an invaluable source of inspiration for organic chemists to synthesize novel drug candidates (Beghyn *et al.*, 2008; Hunter, 2008; Koehn and Carter, 2005). Plants are the original source materials for as many as 40% of the pharmaceuticals in use in the United States today (Chris, 2010).

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world (Leslie, 2000), 80% of which are plant derived drugs and related to their original ethnopharmacological purposes (Fabricant and Farnsworth, 2001). Newman and Cragg (2012) reported that over the time frame from around the 1940s to 2012, of the 175 small molecules, 85 actually being either natural products or directly derived from them.

There are various examples of development of new drugs from the plant sources. Morphine, the first commercial pure natural product introduced for therapeutic use was isolated from opium produced from cut seed pods of the poppy plant (*Papaver somniferum* Linn) approximately 200 years ago and the first semi-synthetic pure drug Aspirin, based on a natural product salicin isolated from *Salix alba* Linn, was introduced by Bayer in 1899. Pharmaceutical research expanded after the Second World War to include massive screening of microorganisms for new antibiotics, inspired by the discovery of penicillin. Few drugs developed from natural sources have undoubtedly revolutionized medicine, like antibiotics (e.g. penicillin, tetracycline, erythromycin), antiparasitics (e.g. avermectin), antimalarials (e.g. quinine, artemisinin), lipid control agents (e.g. lovastatin and analogs), immunosuppressants for organ transplants (e.g. cyclosporine, rapamycins), anticancer drugs (e.g. paclitaxel, irinotecan) and galanthamine for Alzheimer's disease (Harvey, 2008**;** Jennie and Peter, 2000; Chris, 2010; Fabricant and Farnsworth, 2001; Veeresham, 2012).

Clinical trials are ongoing on more than 100 natural product derived drugs and at least 100 molecules/compounds are in preclinical development stage (Harvey, 2008). Most of these molecules in the developmental pipeline are derived from leads from plants and microbial sources. Cancer and infections are the two predominant therapeutic areas for which the drug discovery program is based on natural products, but many other therapeutic areas also get covered, such as neuro-pharmacological, cardiovascular, gastrointestinal, inflammation, metabolic, etc (Harvey, 2008).

Although, there are some new approaches to drug discovery such as combinatorial chemistry and computer-based molecular modelling design, and many drugs are made by synthetic chemistry, none of them can replaced the important role of natural products in drug discovery

and development as most of the core structures or scaffolds for synthetic chemicals are based upon natural products.

In addition, natural products provide important clues for identifying and developing synergistic drugs that, so far, research has largely neglected. Most modern drug discovery has been based on a ‘one-disease–one-target–one-drug' strategy. The pathogenesis of many diseases involves multiple factors, however, and a selective compound against a single target often fails to achieve the desired effect, particularly in cancer therapy. Consequently, there is increasing interest in ‘multi-component therapeutics' to overcome the challenge of ‘more investment, fewer drugs' (Keith *et al.*, 2005; Schmidt *et al.*, 2007; Kong *et al.*, 2008). This new strategy could have several advantages as it would modulate biological networks rather modestly and might therefore be more efficient in dealing with complex diseases (Csermely *et al.*, 2005; Dancey and Chen, 2006; Zimmermann *et al.*, 2007). Moreover, it could prevent, or at least slow down, the development of resistance against many antibiotics, antimalarials and anti-cancer drugs (Hong-Fang *et al.,* 2009).

1.3 Problems associated with Plant Derived Medicine

Plant derived medicines is with its own complication too. The key issues for development programmes where it proves difficult or for other reasons not desirable to synthesise the active plant component(s): relate to identification, standardisation and consistency of material. Fears of adulteration or even contamination of plant material by potentially toxic materials are not unfounded and serve to substantiate the long standing attitude of regulators towards combination products and a frank ‘adverse reaction’ to complex mixtures like herbal extracts. As a result, many companies have shied away from this potentially fruitful area of R&D (Jennie and Peter, 2000).

Nonetheless, the popularity of natural products will continue simply because they are a matchless source of novel drug leads and inspiration for the synthesis of non-natural molecules (Baker *et al.*, 2007; Beghyn *et al.*, 2008; Harvey, 2008; Hunter, 2008; Koehn and Carter, 2005).

1.4 Prospects in Herbal Medicine

The term "herbs" refers to plants or parts of them, including grasses, flowers, berries, seeds, leaves, nuts, stems, stalks and roots, which are used for their therapeutic and health-enhancing properties (Chris, 2010).

To available estimates, the total number of higher plants species (comprising angiosperms and gymnosperms) is approximately 250,000 species. Among them, only 6% have been reportedly screened for biological activity and about 15% have been screened for phytochemical activity (Fabricant and Farnsworth, 2001).

Generations of skilled herbal practitioners, researchers and scholars have refined and tested the vast science of herbology, producing thousands of plant-based remedies that are safe and effective. The proper and judicious use of herbs is often successful in the treatment of illness when other more conventional medicines and methods fail. Herbs demonstrate great versatility for the treatment of a broad variety of health needs (Chris, 2010). The use of herbal medicines in Asia represents a long history of human interactions with the environment (Hong-Fang *et al.,* 2009).

However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify these traditional claims. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation

and characterization of bioactive compound, toxicological evaluation and clinical evaluation (Sasidharan *et al.,* 2011; Fabricant and Farnsworth, 2001; Patil and Mahajan, 2013).

1.5 Statement of Research Problem

The medicinal properties of *Eulophia species* are not well documented and are without scientific authentication. There is little information on the chemistry of *Eulophia guineensis* (Gardiner *et al*., 2013). Due to inadequate literature, it is difficult in distinguishing between related species of Eulophia genus (Gardiner *et al*., 2013).

1.6 Justification of the Research

Free radicals are implicated in a lot of diseases like cancers, inflammations, obesity, diabetics etc. Orchids are the largest plant family and have been known to possess antioxidant properties. *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.

1.7 Hypothesis (null)

Eulophia guineensis does not contain bioactive constituents with anti-oxidant properties.

1.8 Overall aim

To provide scientific basis for the uses of *Eulophia guineensis* in traditional medicine in treating oxidation related diseases.

1.9 Specific aims

1. To evaluate some pharmacognostic characters of *Eulophia guineensis* Pseudobulb with a view to providing parameters for the preparation of its monograph.
2. To evaluate the antioxidant properties of the methanol extract and the fractions obtained from *E. guineensis* Pseudobulbs.
3. To carry out chromatographic analysis on one of the fraction of *E. guineensis* pseudobulbs methanol extract with potent antioxidant activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction to the Plant Family: Orchidaceae

Orchidaceae popularly known as the orchids is the most specious plant families of angiosperm on earth today. It is made up of more than one third of the world plant species (Ramirez *et al.,* 2007; Gustafsson *et al.,* 2010, Singh and Duggal, 2009). It comprises of 800-850 genera and 20,000- 27,000 plant species. Orchids are well known for their economic importance and widely cultivated for ornamental purposes. It is also a rich source of foods, vitamins, medicine, oils, chemical and industrial uses (The RBG and Domain Trust, 2013; Gott, 2008). E.g. Orchids have ancient origins in traditional medicine in many cultures, including Chinese medicine (Bulpitt *et al*., 2007). *Vanilla planifolia* is commercially important orchid as it is source of vanillin used as foodstuff flavouring (Bechtel *et al.,* 1992; Singh and Duggal, 2009).

2.2 Distribution and Habitat of Orchidaceae

Orchids are cosmopolitan in distribution (Singh and Duggal, 2009). Orchids live in nearly all ecosystems around the world except glaciers, true desert and open water. Tropical areas especially in Asia, Africa and the Americas are the hot spots of diversity i.e. most cultivars are tropical or sub-tropical. Most grow on other plants (as epiphytes), rocks or static objects (as lithophytes) for support and derive their nutrients and water from the atmosphere and debris, however many species grow in the ground in forest or grassland areas. Some are parasites of fungi.

2.3 Characteristics of Orchidaceae

Orchids are monocots, perennial herbs with simple leaves and parallel veins. They are well known for the rich diversity of their flower structures. While some have single flowers, most have inflorescences with multiple flowers arranged around a stalk. The flowers are pollinated by insects, in some cases by birds, and it is common for flowers to have petals modified into perches or guides for their pollinators.

Depending on the species, they range from small sized plants to very tall evergreen plants, some, such as species in the subfamily *Vanilloideae* grow as lianas (a woody vine) that can reach sizes up to 20 m. (60 feet) or more in length; the tiny *Bulbophyllum minutissimum* is only 3-4 mm (0.16 - 0.2 inches) tall (Kew RBG, 2013; Williams, 2013; Stevens, 2008).

2.4 Economic Importance of Orchids

Orchids spawn a global cultivation industry worth nine billion dollars annually, each year 3000-4000 new hybrid names enter the International Orchid Register (American Orchid Society, 2013).

2.5 Taxonomy of Orchidaceae

Taxonomy of the *Orchidaceae* family is difficult and dynamic, because it is so large (now approximately 27,000+ accepted species) and many new species are described annually (Cameron *et al.,* 1999 and Williams, 2013). Dressler (1993) conducted a research on orchids and grouped them into 5 subfamilies, 22 tribes, 70 subtribes, about 850 genera and about 20,000 species in this family. Many morphological, anatomical and molecular studies break down the family into five monophyletic subfamilies, namely: *Apostasioideae,* *Cypripedioideae,* *Epidendroideae,* *Orchidoideae,* and *Vanilloideae,* of which *Epidendroideae*

is by far the largest, containing about $\frac{3}{5}$ (i.e. 60 %) of the entire orchid species (Cameron *et al.*, 1999; Williams, 2013; The Plant List, 2010).

2.5.1 Subfamily: Epidendroideae

This is the largest subfamily of Orchidaceae, having the largest proportion of incest, comprising more than 10,000 species in about 90 to 100 genera. Most are tropical epiphytes (usually with tuber-like structures), but some are terrestrials and even a few myco-heterotrophs. All show a unique development of the single anther: it is incumbent forming a right angle with the column axis or pointed backward in many genera. Most have hard pollinia, i.e. a mass of waxy pollen or of coherent pollen grains; pollinia with caudicle and viscidium or without; stigma entire or 3-lobed; rostellum present; 1-locularovary; leaves: distichous or spiralling.

2.6 Chemical constituents of Orchidaceae

Recent works have reported isolation of anthocyanins, stilebnoids and triterpenoids from orchids. Orchinol, hircinol, cypripedin, jibantine, nidemin, loroglossin and phenanthrenes are some important phytochemicals reported from orchids (Singh and Duggal, 2009; Kovacs *et al.*, 2008; Majumder *et al.*, 2001). However, phenolic glycosides are the chief phytochemical constituents in the Orchidaceae family.

2.7 Description of the Genus: Eulophia

Eulophia is a genus belonging to the Sub-family **Epidendroideae** with approximately 210-230 species of orchids (Thomas, 1998). It was first described by John Lindl in 1821. The name "Eulophia" was derived from the Greek words "eu" (well) and "lophos" (plume), referring to the crested ridges of the labellum (lip) in most species. This genus is abbreviated

Eupha in horticultural trade. The generally large, underground, fleshy rhizome/ tuber indicate a sympodial growth habit and this makes *Eulophia species* fairly easy to divide and propagate.

Inflorescence: arises from the base of the plant. It grows into a raceme. It is sometimes branched, as in the cases of *E. macra* and *E. petersii*. The inflorescences on the species with non-branching spikes can support as many as 50 flowers; but in species with branching inflorescences, up to 150 blooms can occur per spike. While most *Eulophia* flowers rarely exceed 2 inches in width, this is often made up for by the sheer abundance of interesting blooms they produce. The sepals and the petals are alike. The lip usually has three lobes. As for most orchids, there are two pollinia for each flower. *Eulophia spp* (in bloom) can reach a height of 1.6 m (6 ft.).

Life forms: *Eulophia* species are usually terrestrial or ground orchids, although some are epiphytes, and rarely, lithophytes.

2.7.1 Distribution of *Eulophia species*

Eulophia species are distributed in shady rainforests or in open scrub or woodland in the tropics and subtropics of Africa, India, Asia, Queensland (Australia) and the Americas, although most are found and well distributed in Africa (Patil and Mahajan, 2013; Shrivastava, 2004).

Eulophia is one of the largest genera of orchids 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 (Gardiner, 2013). Many can survive the dry season through their large bulbous ‘pseudobulbs’. Asia has more than 50 species, most in

grassland, woodland or forest. *Eulophia spectabilis*, found from India and China to Fiji, is one of the most widespread of all species (Gardiner, 2013). About 2.4% worldwide occurring *Eulophia* species are reported in India while about 33% of Indian *Eulophia* species occur worldwide (Patil and Mahajan, 2013). Although, Bhattacharjee (1984) reported 22 species of *Eulophia* in India, today there are 30 Indian *Eulophias* found (Patil and Mahajan, 2013). *E. guineensis* ranges from Sierra Leone in Western Africa, east to Ethiopia and south to Zimbabwe (Gardiner, 2013).

2.7.2 Taxonomy of Genus *Eulophia*

The genus *Eulophia* has 201 accepted species names. The Plant List includes a further 63 scientific plant names of infra-specific rank for the genus *Eulophia* (The Plant List, 2013). Thomas (1998) reported the genera to have 230 species.

2.7.3 Ethnomedicinal uses of *Eulophia species*

Patil and Mahajan (2013) reported that tubers/rhizomes/pseudobulbs part of *Eulophia* species are commonly used to cure human (except 1 veterinary) diseases. Traditionally, many species of genus *Eulophia* are used as anticancer, immunomodulatory, aphrodisiac, tonic appetizer, blood purifier, emetics, bronchitis, cardiac problem, infertility, cough, cold and heart troubles and anthelmintic (Gerstner, 1941; Tuchinda *et al.*, 1988; Bhattacharjee, 2001; Nadkarni, 1991; Steenkamp, 2003; Jain *et al.*, 2005; Datla *et al.*, 2010; Jagtap *et al.,* 2009).

The *Eulophia spp.* tubers are used as salep (Khare, 2007). Salep is white coloured flour obtained by milling of dried tubers of certain wild orchids (Dogan and Kayacier, 2004) and it is well known indigenous turkish drink. According to the folklore 'Salep' is used as an aphrodisiac drug. In South-West ethno-ecological region of Cameroon, decoction of stem

bark of *E. horsfalli* is used on bleeding piles (Jiofack, *et al*., 2010). In Malawi, *Eulophia* species are used on anaemia (Vasisht and Kumar, 2004), the root of *Eulophia* species are pounded to make lather and mixed with water to wash the head of a malarial patient (Fowler, 2006). In Africa, *Eulophia cucullata* is used to prevent epilepsy (Bulpitt, 2005).

Several Indian species like *E. nuda, E. orcheata, E. herbaceae* posseses different ethnomedicinal claims. Traditionally, the tubers of these plants are used for many purposes like treatment of tumours of scrofulous glands of neck, aphrodisiac, appetizer and to treat cardiac problems. For example, *Eulophia herbacea* used as substitute for salep, is used for ailment of pimples (Patil and Patil, 2007). Decoction of tubers is used on spermatorrhoea, urinary complaints and menses. The tubers are also used as tonic and are beneficial to treat the aforesaid disease (Patil and Patil, 2006). The tubers of *Eulophia herbacea* is widely used in the treatment of obesity in traditional medicine (Anil *et al*., 2013).

Eulophia campestris Wall. Syn - *Eulophia dabia* (D.Don) Hochr. Tuber is used in scrofulous glands of neck, heart troubles, intestinal worms, sexual debility and other sexual disorders. It is also used in purulent cough and acts as nervine tonic (Hossain, 2011; Bakshi and Sensarma 2001). Tuber juice of *E. campestris* is taken orally 2-3 times as appetizer (Chanda *et al*., 2007) while the rhizome is used as a tonic, effective in curing stomach problems, cough, paralysis and also used as aphrodisiac by tribal people of Sikkim, Himalaya in India (Medhi and Chakrabarti, 2009).

Eulophia explanata Lindl. Pseudobulbs crushed with pepper and garlic is applied in wounds and is used in impotence and other sexual disorders (Khare, 2007). *Eulophia nuda* Lindl - Tuber is used in intestinal worms, the crushed bulb is applied on tumours and tuberculous

glands of neck (Kumari, 2012). *Eulophia ochreata* tuber is aphrodisiac and tuber sap is also applied externally for curing rheumatism (Kshirsagar *et al*., 2010; Kumari, 2012). *Eulophia spectabilis* (Dennst) Suresh - Leaf decoction is used against worms (Kumari, 2012). Korkus of Melghats and Gujjars of Uttarpradesh (in India) use tubers of *E. ramentacea* in treating impotency (Khanna and Kumar, 2000; Bhogaonkar and Kadam, 2006). Traditionally, the tubers of the *E. guineensis* are boiled in water and the infusion is used to make local akamu (kunu) and drunk throughout the day to treat cold and obesity, it is also applied topically to treat ringworms (Personal communication).

2.7.4 Chemical constituents reported from some *Eulophia species*

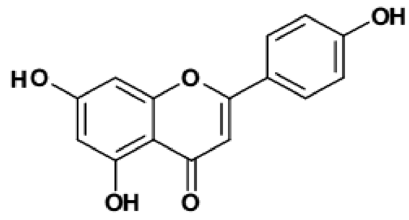
Scientific data revealed the presence of different chemical constituents from many *Eulophia species* as; phenanthrenes, phytosterols, mucilage, phenolics, zinc, iron (Blitzke *et al*., 2000; Nadkarni, 1991). It has been reported that the tuberous roots or rhizomes of 11 *Eulophia* species are rich in bioactive substances like Eullophiol, Nudol, β-Sitosterol,β-Sitosterolglucoside, Ephemeranphol, Fimbriol, Phenanthrenes, Lusianthridin as active phyto-constituents (Rao *et al.,*1974; Kovacs *et al.,* 2008; Bhandari *et al*., 1985; Reddy *et al*., 2005; Singh and Duggal, 2009; Patil and Mahajan, 2013).

The phytochemical screening of the tubers of *Eulophia ochreata* revealed the presence of alkaloids, saponin, steroid, and poly phenols (Ali and Deokule, 2010). *E. herbacea* consists of diterpenes, triterpenoids, polysaccharides, flavonoids and phenolics (Tan and Vanitha, 2004; Moradali *et al*., 2007).

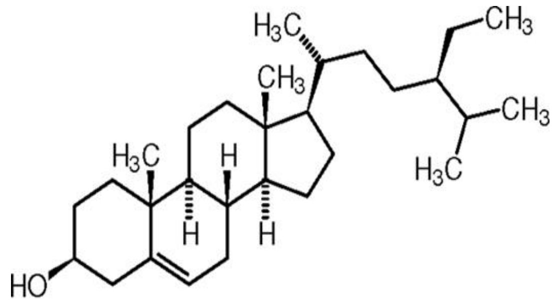
Table 2.1: The compounds isolated or identified from some *Eulophia spp*.

***Eulophia* species**	**Plant part(s)**	**Compounds isolated or identified**
E. herbaceae	tubers	(**I**) 1-phenanthrene carboxylic acid 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro- 1, 4a-dimethyl-, methyl ester
E. ochreata	tubers	(**I**) 9, 10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol (**II**) 5, 7-dimethoxyphenanthrene 2, 6 diol (**III**) Coelonin (9, 10-dihydrophenanthrene derivative) (**IV**) 1, 5-dimethoxyphenanthrene-2,7-diol (Eulophiol)
E. epindendraea	tubers	(**I**) *β*-sitosterol (**II**) *β*-sitosterolglucoside (**III**) *β* –amyrin (**IV**) Lupeol
E. nuda	leaves	(**I**)Apigenin (**II**)Luteolin(**III**) Kaempferol(**IV**)Quercetin
	tubers	(**I**) Eulophiol (**II**) Coelonin, (**III**) Nudol, (**IV**) Lupeol (**V**) Denthrysinin
E. campestris	tubers	(**I**) *β*-sitosterol.

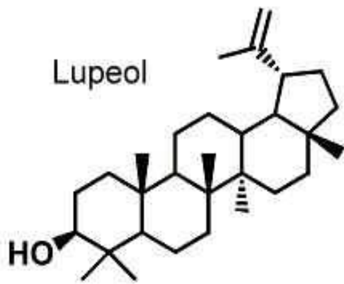
(Tuchinda *et al*., 1988; Blitzke *et al*., 2000; Shriram *et al*., 2010; Datla *et al*., 2010; Maridass and Ramesh, 2010).

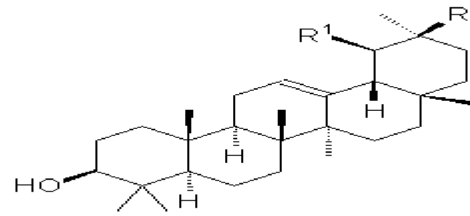


Apigenin

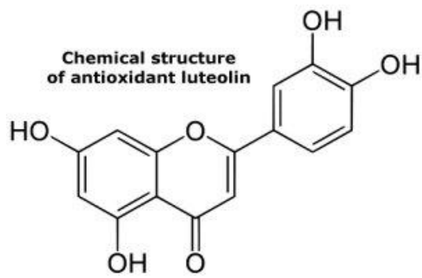


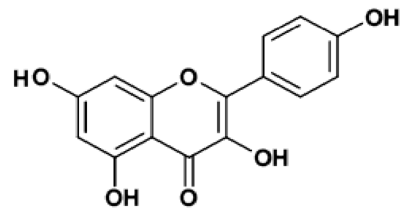
β-sitosterol



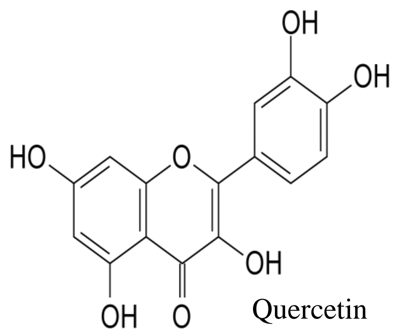


$R = H, R^1 = CH_3 = \alpha$-amyrin
$R = CH_3, R^1 = H = \beta$-amyrin

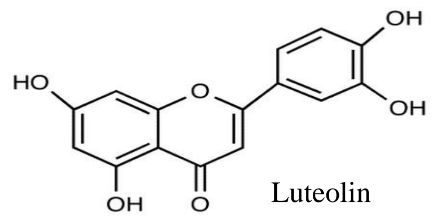




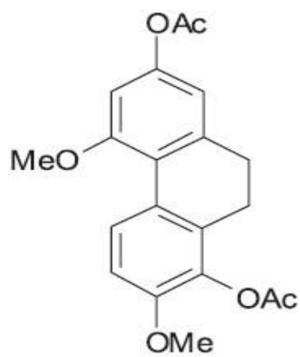
Kaempferol



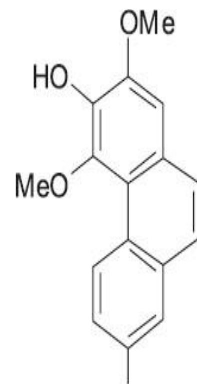
Quercetin



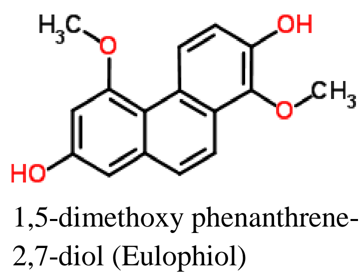
Luteolin



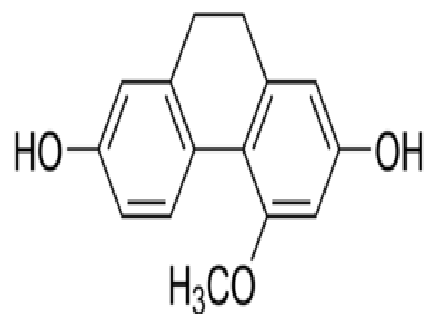
9, 10-Dihydro-2, 5-Dimethoxy phenanthrene-1, 7-diol



5, 7-Dimethoxyphenanthrene-2, 6-diol



1,5-dimethoxy phenanthrene-2,7-diol (Eulophiol)



Coelonin (9, 10-dihydrophenanthrene derivative)

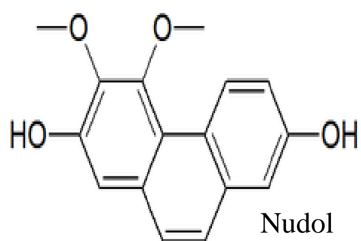


Figure 2.1: Some Chemical compounds isolated or identified from various *Eulophia spp*.

2.7.5 Biological Activities of *Eulophia species*

As immune system plays vital role in antitumor potential, some reports suggested that *Eulophia* species exerts antitumor property with complimentary augmentation of immunomodulating effect (Datla *et al*., 2010; Maridass *et al.,* 2008). The crude tuber extract of *Eulophia campestris, E. nuda, E. herbaceae and E. ochreata* showed potent antioxidant activity and anti-inflammatory activities (Rao *et al.,* 2013; Jaya and Anuradha, 2010; Tuchinda *et al*., 1988; Datla *et al*., 2010).

The preparation of salep contains 50-60 % of glucomannan which lowers the blood cholesterol and blood glucose level (Tamer *et al*., 2006; Melinda *et al*., 2010). Antidiabetic activity of the tuber extract of *E. epidendraea* in alloxan diabetic rats has been reported (Maridass *et al.,* 2008).

Several species of *Eulophia* showed the presence of phenanthrene compound with potent anticancer activity (Tuchinda *et al*., 1988; Blitzke *et al*., 2000; Shriram *et al*., 2010). A fairly large number of phenanthrene has been reported from mainly orchidaceae family and their numerous derivatives are promising and expanding group of biologically active natural

compounds with anticancer, spasmolytic, anti-allergic, anti-inflammatory and antibacterial potential. But their screening has not yet been investigated sufficiently and which have not been exploited by the pharmaceutical industry (Kovacs *et al*., 2008).

The compound 9, 10-Dihydro-2, 5-dimethoxyphenanthrene-1, 7-diol isolated from the tubers of an orchid *E. ochreata* possesses novel antioxidant (free-radical scavenging activity) and anti-inflammatory properties. This same compound was isolated from *Eulophia nuda* and responsible for antiproliferative activity against human breast cancer (Datla *et al*., 2010; Shriram, 2010).

2.8 The plant *Eulophia guinensis*

Eulophia guineensis Lindl belongs to the family Orchidaceae. It occurs as lithophytes, they occur in deep shade in evergreen forests in brown soils on ironstone. The last will grow on rocks in hollows with a little soil and humus in which the roots can elongate (Gardiner, 2013; Morton, 1984).

2.8.1 Description of the Plant *Eulophia guineensis*

The Plant grows to an average height of 60-74cm. They are geophytes or lithophytes in nature, growing in rocky areas (Hortus Orchids, 2013).

2.8.1.1 ***Leaves***: Leaves are petiolate, lanceolate-oblong or obovate-oblong, acute or apiculate, 4–12 inches long, 2–5 inches broad; petioles $\frac{1}{2}$ – 5 inches long, scapes 24–36 inches high, with several sheaths below; racemes 8–12 inches.

2.8.1.2 ***Flowers*:** Flowers are light purple, with the sepals and petals often dusky brown. Sepals and petals are linear-lanceolate, acute or acuminate; $1\frac{1}{2}$ inches long, 2–3 inches broad. The leaves and flowers are grown on a different shoot. The flower grows to a height of 60-74cm (i.e. 23.6 – 29.1 inches). The flower number ranges from 9-13 (Hortus Orchid, 2013; Burkill, 1997).

2.8.1.3 ***Pseudobulbs***: Pseudobulbs are conical, approximate $\frac{3}{4} - 1$ inches (Burkill, 1997). Perennating organs usually subterranean and pseudobulbous, conical, cylindrical or irregularly shaped with several nodes (Gardiner, 2013).

2.8.2 Native Origin

Eulophia guineensis ranges from Sierra Leone in Western Africa, East to Ethiopia and South to Zimbabwe (Gardiner, 2013). It grows on poor rocky soil in the islands of Cape Verde, Benin, Burkina Faso, Gambia, Ghana, Guinea, Cote d 'Ivoire, Mali, Nigeria, Senegal, Sierra Leone, Togo, Burundi, Cameroon, Congo, Gabon, Rwanda, Zaire, Chad, Eritrea, Ethiopia, Sudan, Kenya, Tanzania Kigoma, Mbeya), Uganda, Malawi, Zambia, Zimbabwe, Oman, Saudi Arabia and Yemen (Hortus Orchid, 2013).

2.8.3 Synonyms

The synonyms of *Eulophia guineensis* include *E. quartiniana* and *E. congoensis.*

2.8.4 Local names

It is called *Gandun albasa* or *Kimban daji* in hausa language of Northern Nigeria and *odù ebi* in igbo language of Southern eastern Nigeria.

Plate I: Harvested *Eulophia guineensis* plant.

Plate II: Harvested Pseudobulbs of *Eulophia guineensis*.

Snapped by me

2.9 The Concept of Free radicals

By definition, a free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in its outermost shell, and is capable of independent existence (Karlsson, 1997). A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom (Karlsson, 1997). Free radicals are highly reactive due to the presence of unpaired electron(s) (Goldfarb, 1999; Cheeseman and Slater, 1993; Oakley, 1998). A free radical can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (Cheeseman and Slater, 1993). They are important intermediates in natural processes involved in cytotoxicity, control of vascular tone, and neurotransmission (Oakley, 1998).

2.9.1 Sources of Free radicals generation in human body

Generation of free radicals in the human body is mainly from two sources namely:

2.9.1.1 ***Internally generated sources*****:** Mitochondria, Xanthine oxidase, Peroxisomes, Inflammation, Phagocytosis, Arachidonate pathways, Exercise, Ischemia/reperfusion injury. Here, the free radicals produced are as a result of normal essential metabolic processes in human body. These radicals occur continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions.

2.9.1.2 ***Externally generated sources***: Cigarette smoke, Environmental pollutants, Radiation, Certain drugs, pesticides, Industrial solvents, Ozone (Bagchi and Puri, 1998; Liu, 1999; Ebadi, 2001; Oakley, 1998). Here, the free radicals produced are as a result of external /environmental factors.

2.9.2 Steps involving free radical generation

In chemistry, free radicals take part in radical addition and radical substitution as reactive intermediates. Chain reactions involving free radicals can usually be divided into three distinct processes: initiation, propagation, and termination.

2.9.2.1 ***Initiation*** reactions are those, which result in a net increase in the number of free radicals. They may involve the formation of free radicals from stable species or they may involve reactions of free radicals with stable species to form more free radicals.

2.9.2.2 ***Propagation*** reactions involve free radicals in which the total number of free radicals remains the same.

2.9.2.3 ***Termination*** reactions are those reactions resulting in a net decrease in the number of free radicals. Typically two free radicals combine to form a more stable species, for example: $2Cl^{\cdot} \rightarrow Cl_2$ (Abheri *et. al.,* 2010).

2.9.3 Reactive Oxygen Species (ROS)

Any free radical involving oxygen can be referred to as Reactive Oxygen Species (ROS). ROS are very small molecules and are highly reactive due to the free radicals containing two unpaired electrons in the outermost shell (Goldfarb, 1999).

ROS is formed as a natural by-product of the normal metabolism of oxygen and have important roles in cell signalling. However, during times of environmental stress, ROS levels can increase dramatically, which can result in significant damage to cell structures (Krötz *et al*., 2002; Pignatelli *et al*., 1998; Guzik *et al*., 2003).

The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical (Young and Woodside, 2001).

2.9.4 Mechanisms of action

When free radicals steal an electron from a surrounding compound or molecule, a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with anti-parallel spins from cellular structures or molecules. Thus, the chain reaction continues and can be "thousand of events long"(Goldfarb, 1999). The Electron Transport Chain (ETC), which is found in the inner mitochondrial membrane, utilizes oxygen to generate energy in the form of Adenosine triphosphate (ATP). Oxygen acts as the terminal electron acceptor within the ETC. Anywhere from 2 to 5% of the total oxygen intake during both rest and exercise have the ability to form the highly damaging superoxide radical via electron escape (Dekkers *et al.,* 1996; Sjodin *et al*., 1990).

2.9.5 Effects of Free radicals in Human health

Free Radicals are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, lipids and nucleic acids, inactivates specific enzymes by oxidation of co-factors (Young and Woodside, 2001; Krötz *et al*., 2002; Pignatelli *et al*., 1998; Guzik *et al*., 2003; NEJM, 1994a; NEJM, 1994b; Lobo *et al*., 2010; Abheri, *et al*., 2010). ROS have been implicated in the induction and complications of diabetes mellitus, age-related eye disease, and neurodegenerative diseases such as Parkinson's disease (Rao *et al*., 2006).

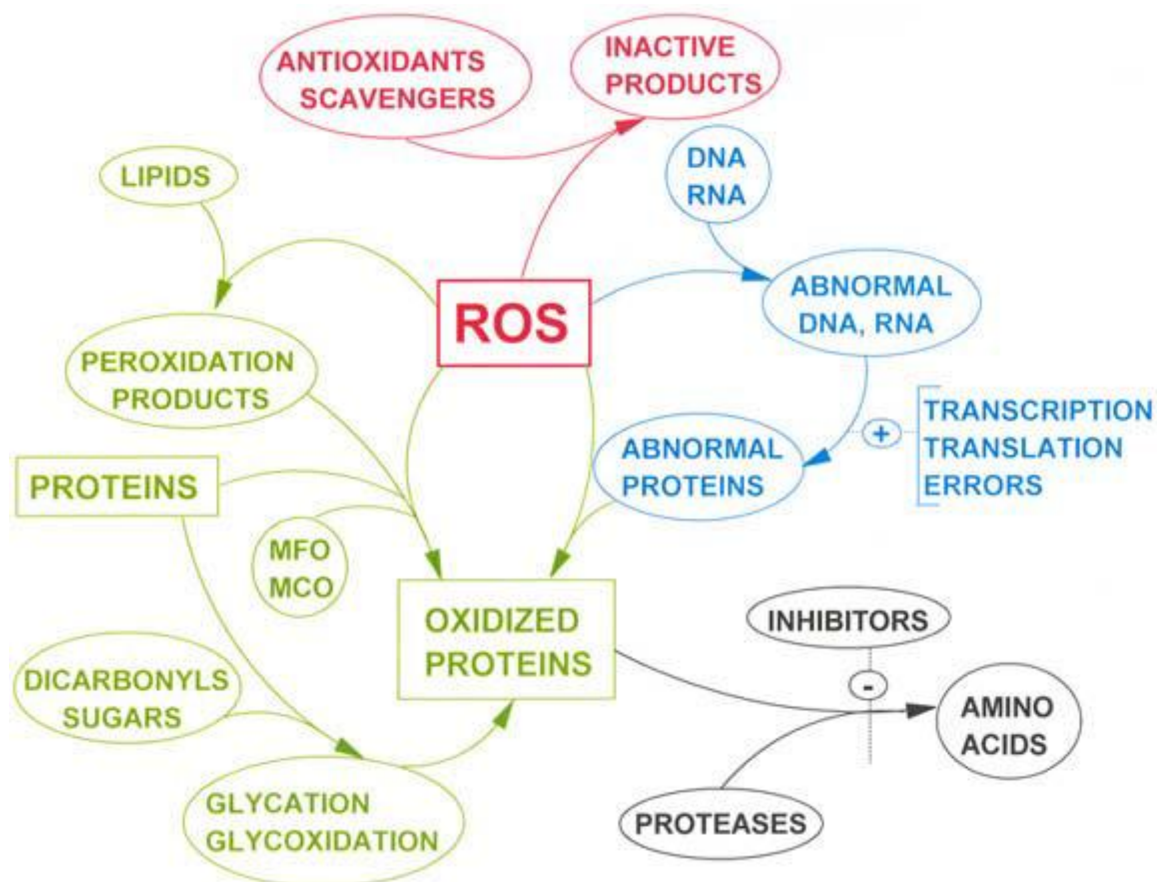


Figure 2.2: Summary of the Effects of ROS in the human body (Williams & Wilkins, 2008).

2.10 The Concept of Oxidative stress

The term is used to describe the condition of oxidative damage resulting when the free radical generations is more than antioxidant defences in the body (Rock *et al*., 1996). Oxidative stress is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids (Mc Cord, 2000). Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins and excessive exercise. These injured tissues produce increased radical generating enzymes (e.g. xanthine oxidase, lipogenase, cyclooxygenase), activation of phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS. The initiation, promotion, and progression of cancer, as well as the side-effects of

radiation and chemotherapy have been linked to the imbalance between ROS and the antioxidant defence system.

2.11 The Concept of Antioxidants

Antioxidant literarily means 'against oxidation' (Dekkers, 1996). An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property (Halliwell, 1995). These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Shi *et al*., 1999).

Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant, it no longer needs to attack the cell and the chain reaction of oxidation is broken (Dekkers, 1996). After donating an electron, an antioxidant becomes a free radical by definition. Antioxidants in this state are not harmful because they have the ability to accommodate the change in electrons without becoming reactive (Kaczmarski *et al.,* 1999).

Antioxidants are intimately involved in the prevention of cellular damage; the common pathway for cancer, aging, and a variety of diseases (Williams and Wilkins, 2008; Sen, 2003; Krötz *et al*., 2002; Pignatelli *et al*., 1998; Guzik *et al*., 2003).

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Frie *et al*., 1988).

2.11.1 Sources and Types of antioxidants Defensive systems against free radicals

The human body has an elaborate antioxidant defence system. Antioxidants are manufactured within the body and can also be extracted from the food humans eat such as fruits and vegetables. There are two lines of antioxidant defence within the cell namely:

2.11.1.1 ***Fat soluble antioxidant scavengers***: The first line, found in the fat-soluble cellular membrane consists of vitamin E, beta-carotene, and coenzyme Q (10).

Vitamin E is the most abundant fat-soluble antioxidant in the body. It is one of the most efficient chain-breaking antioxidants available, it is the primary defender against oxidation, and is the primary defender against lipid peroxidation (Oakley, 1998; Dekkers *et al.,* 1996).

2.11.1.2 ***Water Soluble Antioxidant Scavengers***: The second line, found inside the cell, water soluble antioxidant scavengers are present. These include vitamin C, glutathione peroxidase, superoxide dismutase (SD), and catalase (Dekkers *et al.,* 1996).

Vitamin C is the most abundant water-soluble antioxidant in the body. It acts primarily in cellular fluid. It combats free radical formation caused by pollution and cigarette smoke. It also helps return vitamin E to its active form (Oakley, 1998; Levine *et al*., 1991).

2.11.2 Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for antioxidants (Rice-Evans and Diplock, 1993). The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by

different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky, 1992).

2.11.3 Plants as an important source of Antioxidants

During oxidative stress, micronutrients have to be supplied to augment the antioxidants in the body. The body cannot manufacture these micronutrients so they must be supplied in the diet (Aruoma, 2003).

In view of 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. It has been reported that there is an inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and incidence of human diseases. The use of natural antioxidants in food, cosmetic, and therapeutic industry would be promising alternative for synthetic antioxidants in respect of low cost, highly compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers (Brown and Rice-Evan, 1998; Abheri *et al.,* 2010). Attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, and legumes (Furuta, *et al.*, 1997). There are several reports showing antioxidant potential of fruits (Wang *et al.*, 1996). Strong antioxidants activities have been found in berries, cherries, citrus, prunes, and olives. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds (Lin *et al.*, 1998).

Vitamin E α-alpha tocopherol a fat-soluble vitamin is present in nuts, seeds, vegetable and fish oils, whole grains (especially wheat germ), fortified cereals, and apricots. Vitamin C i.e. Ascorbic acid is a water-soluble vitamin present in citrus fruits and juices, green peppers, cabbage, spinach, broccoli, kale, cantaloupe, kiwi, and strawberries. Beta-carotene is a precursor to vitamin A (retinol) and is present in liver, egg yolk, milk, butter, spinach, carrots, squash, broccoli, yams, tomatoes, cantaloupe, peaches, and grains. Because beta-carotene is converted to vitamin A by the body there is no set requirement (Cadenas and Davies, 2000; Imam *et al*., 2008; Navarro, 2004).

Generally, researches have also suggested that regular consumption of phenolic compounds directly from plant foods may be more effective in combating oxidative damage in our body than in the form of dietary supplement (Martin and Appel, 2010).

2.12 Phenols as Antioxidants

Phenolic compounds are constituted in one of the biggest and widely distributed groups of secondary metabolites in plants (Scalbert and Williamson, 2000). Biogenetically; phenolic compounds proceed of two metabolic pathways: the Shikimic acid pathway where, mainly, phenylpropanoids are formed and the Acetic acid pathway, in which the main products are the simple phenol (Sánchez-Moreno, 2002b). Most plants phenolic compounds are synthesized through the phenylpropanoid pathway (Hollman, 2001). The combination of both pathways leads to the formation of flavonoids, the most plentiful group of phenolic compounds in nature (Sánchez-Moreno, 2002b).

Additionally, through the biosynthetic pathways to the flavonoids synthesis, among the not well elucidated condensation and polymerization phases, the condensed tannins or non-

hydrolysable tannins are formed. Hydrolysable tannins are derivatives of gallic acid or hexahydroxy diphenic acid (Stafford, 1983).

2.12.1 Structural requirement for antioxidant properties of phenolic compounds

The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations, singlet oxygen quenching and as reducing agents (Afanas'ev, 1989; Shahidi and Naczk, 1995; Amarowicz, 2004). However, the antioxidant activity i.e. radical scavenging activity of phenolic compounds depends largely on the chemical structure of these substances and this is referred to as Structure Activity Relationships (SAR) (Scalbert and Williamson, 2000; Rice-Evans *et al.*, 1996; Robards, 1999). Among the phenolic compounds with known antioxidant activity include: flavonoids, tannins, chalcones and coumarins as well as phenolic acids (Sánchez-Moreno, 2002a).

2.12.1.1 ***Flavonoids:*** It is known that **Flavonoids** are among the most potent antioxidants from plants. The excellent antioxidant activity of these substances is related to the presence of hydroxyl groups in positions 3' and 4' of the B ring, which confer high stability to the formed radical by participating in the displacement of the electron, and a double bond between carbons C2 and C3 of the ring C together with the carbonyl group at the C4 position, which makes the displacement of an electron possible from the ring B. Additionally, free hydroxyl groups in position 3 of ring C and in position 5 of ring A, together with the carbonyl group in position 4, are also important for the antioxidant activity of these compounds (Sánchez-Moreno, 2002a). However, the effectiveness of the flavonoids decreases with the substitution of hydroxyl groups for sugars, being the glycosides less antioxidants than their corresponding aglycones (Rice-Evans *et al.*, 1996).

2.12.1.2 ***Tannins:*** Although the antioxidant activity of **Tannins** has been much less marked than the activity of flavonoids, recent researches have shown that the degree of polymerization of these substances is related to their antioxidant activity. In condensed tannins and hydrolysable (ellagitannins) of high molecular weight, this activity can be up to fifteen to thirty times superior to those attributed to simple phenols (Sánchez-Moreno, 2002a).

2.12.1.3 ***Phenolic acids*****:** Phenolic acids may be about one-third of the phenolic compounds in human's diet (Yang *et al.,* 2001). It is known that these substances and their esters have a high antioxidant activity, especially hydroxybenzoic acid, hydroxycinnamic acid, caffeic acid and chlorogenic acid, and although other characteristics also contribute to the antioxidant activity of phenolic acids and their esters, this activity is usually determined by the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group (Sánchez-Moreno, 2002a; Rice-Evans *et al.*, 1996; Robards, 1999). Monohydroxy benzoic acids with the -OH moiety at the *ortho*- or *para*-position to the –COOH show no antioxidant activity, though the same is not true for the *meta*-hydroxybenzoic acid (Rice-Evans *et al.*, 1996). The antioxidant activity of phenolic acids increase with increasing degree of hydroxylation, as is the case of the trihydroxylated gallic acid, which shows a high antioxidant activity. However, substitution of the hydroxyl groups at the 3- and 5-position with methoxyl groups as in syringic acid reduces the activity (Rice-Evans *et al.*, 1996). In general, the hydroxylated cinnamic acids are more effective than their benzoic acids counterparts (Sánchez-Moreno, 2002a).

2.12.1.4 ***Chalcones and Coumarins:*** Although the data are still limited, it is known that **Chalcones** and **Coumarins** have antioxidant activity (Pratt and Hudson, 1990).

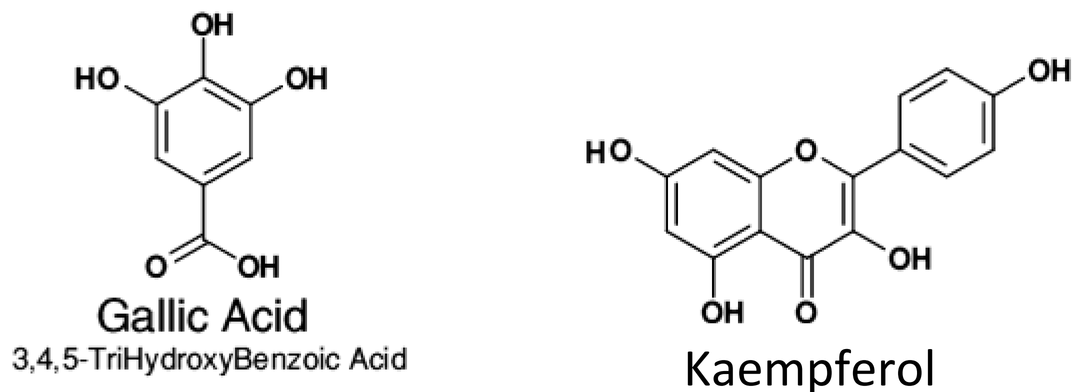


Figure 2.3: Examples of Phenols with antioxidant properties.

NB: the position of –OH group in their respective structure is responsible for their antioxidant activities.

2.13 Different antioxidant methods, their advantages and disadvantages

Basically, there are two types of antioxidant method; the Electron Transfer Chain (ETC) and the Hydrogen donation. These two are again subdivided into the following:

2.13.1 2, 2-Di-Phenyl-1-Picryl Hydrazyl (DPPH)

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical 2, 2-Diphenyl-1-picrylhydrazyl (DPPH).

DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods.

Advantages

- This technique is easy, effective, and rapid way to study plant extract profiles.

- No sample separation is needed.
- Potency of sample can be known.

Disadvantages

- It is time consuming and
- Costly (Emad and Sanaa, 2013).

2.13.2 Ferric-Reducing Antioxidant Power (FRAP) assay

In order to assess the modifying effect of tea flavonoids on plasma antioxidant status, a variety of methods has been employed. Commonly used is the FRAP assay. This is a colorimetric assay that measures the ability of plasma to reduce the intense blue ferric tripyridyltriazine complex (used as an oxidant) to its ferrous form, thereby changing its absorbance (Benzie and Strain, 1999). Essentially, there is no much difference between TEAC assay and the FRAP assay except that TEAC is carried out at neutral pH and FRAP assay under acidic (pH 3.6) conditions.

Advantages

- It is simple, speedy, inexpensive, and robust does not required specialized equipment.
- It can be performed using automated, semi-automated, or manual methods (Benzie and Strain, 1999).

Disadvantages

- FRAP cannot detect species that act by radical quenching (H transfer), particularly SH group containing antioxidants like thiols, such as glutathione and proteins. (Prior and Cao, 2000; Huang *et al*., 2005; Emad and Sanaa, 2013).

2.13.3 Total Radical Trapping Antioxidant Parameter (TRAP)

Another assay which has been applied in human plasma is the total radical trapping antioxidant parameter (TRAP). In this assay, the rate of peroxidation induced by AAPH (2'-azobis (2-amidinopropane hydrochloride) is monitored through the loss of fluorescence of theprotein R-phycoerythrin (R-PE). In the TRAP assay, the lag-phase induced by plasma is compared with that induced by Trolox in the same plasma sample (Huang *et al*., 2005).

Advantages

- It is used for measurements of *in-vivo* antioxidant capacity in serum or plasma because it measures non-enzymatic antioxidants such as glutathione, ascorbic acid (Huang *et al*., 2005).

Disadvantages

- Many different end points have been used, so comparisons between laboratories are difficult.
- It is relatively complex and time consuming.
- It also requires a high degree of expertise and experience (Badarinath *et al*., 2010; Emad and Sanaa, 2013).

2.13.4 Oxygen Radical Absorbing Capacity (ORAC) assays

Basically the same principle is applied as in the TRAP assay. The ORAC assay is another commonly applied antioxidant assay based on the ability of a test substance to inhibit the oxidation of B-phycoerythrin or R-phycoerythrin by reactive oxygen species, relative to Trolox. Proteins interfere with the analysis, partially protecting R-PE when all plasma antioxidants are exhausted. Determination of the lag-phase TRAP and ORAC assays can be

performed with different radicals and thus different results will be obtained depending on the radical. For these reasons, results obtained with the TRAP or the ORAC assay in plasma has to be interpreted with care (Caldwell, 2001; Cao *et al.,* 1995).

Advantages

- It implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases.
- ORAC assay has been broadly applied in academy and in the food and dietary supplement industries as a method of choice to quantify AOC (Caldwell, 2001; Cao *et al.,* 1995).

Disadvantages

- ORAC is limited to measurement of hydrophilic chain but ignores lipophilic antioxidants.
- It requires fluorometers, which may not be routinely available in analytical laboratories.
- Temperature control decreases reproducibility (Badarinath *et al*., 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials, Chemicals, Equipments, Solvents, Reagents/Solutions

3.1.1 List of chemicals/ solvents

Hexane, ethyl acetate, ethanol, methanol, chloroform, n-butanol, distilled water, glacial acetic acid and diethyl ether solvents.

3.1.2 List of reagents

Sodium hypochlorite**,** chloral hydrate, phloroglucinol, glycerol, formalin, acetic acid, hydrochloric acid, methylene blue, safranin, fast green, paraffin wax, sudan red solution, $ZnCl_2$ Solution (BDH Laboratory Chemicals Division, POOLE, England). Sulphuric acids, dragendorff, ferric chloride, aluminium chloride, *p-anisaldehyde*, silica gel, Di-Phenyl Picric Hydrazyl (DPPH), 0.2 mM DPPH solution was prepared by weighing 7.9 mg of DPPH and dissolving it with methanol up to volume 100 ml quantitatively.

3.1.3 List of materials/equipments

Analytical weighing scale, maceration flask, wooden cork, glass rod stirrer, separating funnel, rotary evaporator, crucibles, metal scraper, water bath, desiccators, filter paper, syringes and needles, razor blades, ruler, measuring cylinder, capillary tubes, TLC plates, TLC tank, U.V machine, photographic camera, column chromatography, funnels, beakers, aluminium foils, conical flask, U.V spectrophometer, test tubes, cotton wool, masking tapes, eppendorff tubes, compound microscope (Fisher Scientific, UK), stage micrometer and ocular lens (graticules Ltd, Ton bridge, Kent, England), glass slides, cover slips, water bath, mechanical shaker, ash-

less filter paper, metallic cages and feeding bottles for mice, disposable syringes (1ml, 5ml, and 10ml).

3.2 Plant collection and identification

Fresh, matured and entire plant materials comprising of the leaves, stem, flowers, Pseudobulbs, rhizomes and the roots were collected in September 2014 from Bomo village, Samaru district of Sabon gari local government area of Kaduna State, Nigeria. The Plant was identified as *Eulophia guineensis* with voucher number 319 at the herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, by the Taxonomist; Mallam Umar Gallah.

3.2.1 Preparation of Plant material

Fresh parts (Pseudobulbs) of the plant were at the point of collection, fixed in formalin acetic acid alcohol (FAA) for microscopic examination while the bulk of the Pseudobulbs were carefully removed from the plant, it was washed, sliced into smaller pieces and grinded into a paste-like form with the aid of Pestle and Mortar and stored at room temperature in a closed container for further use.

3.3 Pharmacognostic characters of *Eulophia guineensis*

Experimental design

Pharmacognostic studies of *E. guineensis* Pseudobulbs were carried out by examining the microscopical, chemomicroscopical and physicochemical parameters of the pseudobulbs in order to establish some Pharmacopoeial Standards.

3.3.1 Microscopical examination of Pseudobulbs of *E. guineensis*

The Anatomical section of fresh Pseudobulb sample was examined under the microscope and features were described by using the terms according to Dutta (2003) and Ahlam and Bouran (2011).

Methods

3.3.1.1 ***Fixation*****:** the fresh Pseudobulb of *E. guineensis* was picked off directly from the plant, washed and dipped immediately into the fixative, formal acetic acid (70% Ethanol, 40% formalin and glacial acetic acid) at the ratio 90:5:5 and was allowed to stand for 24 hours.

3.3.1.2 ***Dehydration*****:** Gradual dehydration was performed by immersing the fixed tissues in a series of mixtures of alcohol and distilled water with increasing alcohol concentration (30%, 50%, 70%, 95% and absolute alcohol). This was carried out in each graded alcohol for 2 hours each.

3.3.1.3 ***Clearing*****:** Alcohol was removed from the tissue and clearing obtained by using a graded series of alcohol-chloroform solution. The plant specimen were transferred from absolute alcohol into a solution of alcohol and chloroform (3:1, 1:1, and 1:3) in turn of two hours each, and finally immersed in pure chloroform and left over night.

3.3.1.4 ***Infiltration and Embedding*****:** Once chloroform had completely replaced alcohol, paraffin was added slowly to infiltrate the tissue and remove the remaining chloroform in the tissue, this was done until it gelled and was allowed to stand at room temperature for four hours, it was the placed in an embedding oven and maintained at 60^{0}C until it melted. The paraffin containing the sample was transferred in to the embedding box and allowed to solidify. After the paraffin wax had hardened, the paraffin block was trimmed by cutting away the excess paraffin. The trimmed block

was placed on a piece of chock and mounted on a microtome to get the transverse or cross section of the sample. The transverse section was then transferred on to slides.

3.3.1.5 ***Staining*****:** the transverse section of sample was dewaxed in xylene, by changing twice for 5 minutes each. This was then hydrated in 95%, 70%, 50%, and 30% ethanol for 2 minutes each. The transverse section was then transferred in to safranin and left to stand for 30 minutes before washing with water. It was then transferred in to 0.5% HCl in 70% ethanol shortly before dipping in to fast green for 2 minutes and washed with water. The transverse section was then further dehydrated in 30%, 50%, 70%, 95% and 100% ethanol for 2 minutes each and cleared in xylene for another 2 minutes. Gum (Balsam) was sprayed along the transverse section and cover slip was placed (Ahlam and Bouran, 2011). This was then observed under the microscope and appropriate images were taken and documented.

3.3.2 Chemomicroscopical studies of the Pseudobulbs of *E. guineensis*

These were carried out on the powdered pseudobulbs of the plant. Small amount of the finely ground powdered pseudobulbs of the plant was cleared in a test-tube containing 70% chloral hydrate solution. They were boiled on a water-bath for about thirty minutes to remove obscuring materials. The cleared sample was mounted on a microscope slide, using dilute glycerol. Using various detecting reagents, the presence of some cell inclusions and cell wall materials were detected in accordance with Brain and Turner (1975); Evans (2002) as follows:

3.3.2.1 *Cell wall Materials*

Test for Cellulose**:** About 2 drops of N/50 iodine was added to the cleared sample on a slide, and this was allowed to stand for few minutes. One drop of 66% sulphuric acid was added, cover-

slipped and observed under the microscope. The appearance of bluish colour indicates the presence of cellulose in the cell walls of epidermal cells.

***Test for Gums and Mucilages*:** A drop of ruthenium red was added to the cleared sample on a slide, cover slipped and observed under the microscope. The appearance of pink colouration is positive for gums and mucilage

***Test for Lignins*:** Two drops of phloroglucinol was added to the cleared sample and allowed to stand until almost dry. A drop of sulphuric acid was added, cover-slipped and observed under the microscope the appearance of red coloration indicates the presence of lignin.

3.3.2.2 *Cell Contents/Cell inclusions*

***Test for Calcium carbonates*:** The appearance of effervescences on addition of concentrated hydrochloric acid to the powdered sample on the slide showed the presence of calcium carbonate.

***Test for Calcium oxalates crystals*:** The dissolution of shining crystals on the anatomical sections of the bulb upon addition of few drops of hydrochloric acid indicated the presence of calcium oxalates.

***Test for Tannins*:** A single drop of ferric chloride was added to the cleared sample and cover slipped. The appearance of greenish black coloration indicated the presence of tannins.

***Test for Starches*:** Two drops of N/50 iodine solution were added to the cleared sample, cover slip viewed under the microscope. The appearance of blue-black coloration indicated the presence of starch.

3.3.3 Determination of physicochemical constants of the Pseudobulbs of *E. guineensis*

The quantitative extractive values (solvent extractive values, Ash residues, moisture content) were determined as described in the British Pharmacopoeia (2012) and WHO (2011).

3.3.3.1 *Moisture content* (loss on drying): The loss on drying method was adopted for this procedure. 3g of the powdered whole plant was weighed in a crucible. It was then heated for 1hour in an oven maintained at 105^0 C, cooled in a desiccator and re-weighed. The loss in weight was determined by subtracting the weight of the dish and the sample after heating from the weight of dish and content before heating. The procedure was repeated until no further loss in weight was obtained. The moisture content was determined in percentage as:

$$\text{Moisture content (\%)} = \frac{Weight\ of\ Water\ Lost}{Original\ Weight\ of\ Sample} \times 100$$

3.3.3.2 *Total ash*: Ground air dried powdered pseudobulbs (2 g) was weighed in a crucible. It was ignited by gradually increasing the heat, until it became white (i.e. all the carbon was removed). It was cooled in a dessicator and re-weighed. The total ash value was calculated in percentage as:

$$\text{Total ash value (\%)} = \frac{Weight\ of\ Residual\ Ash}{Weight\ of\ Sample} \times 100$$

3.3.3.3 *Acid insoluble ash*: To the crucible containing the total ash, 25ml of dilute hydrochloric acid was added and covered with a watch glass and boil gently for 5 minutes. About 5ml of hot water was used to rinse the cover glass into the crucible. The insoluble matter was collected on an ashless filter paper and washed with hot water until the filtrate

was neutral. This was then transferred back to the crucible and dried on a hot plate and ignited to a constant weight. The residue was allowed to cool in a dessicator for 30 minutes. It was then weighed without delay. The acid insoluble ash was calculated in percentage as:

$$\text{Acid insoluble ash (\%)} = \frac{Weight\ of\ Residual\ Ash}{Initial\ Weight\ of\ Sample} \times 100$$

3.3.3.4 ***Water soluble ash*****:** To the crucible containing the total ash, 25ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered glass crucible. It was then washed with hot water and ignited in a crucible for 15 minutes at $105^{o}C$. The weight of the residue was subtracted from the weight of the total ash. The water Insoluble ash was determined in percentage as:

$$\text{Water soluble ash (\%)} = \frac{Weight\ of\ Initial\ ash - Weight\ of\ Residual\ Ash}{Initial\ Weight\ of\ Sample} \times 100$$

3.3.4 Extractive Values

3.3.4.1 ***Water soluble extractives value*****:** Powdered sample of the plant (5g) was macerated in 100 ml of water in a closed flask for 24 hours with frequent shaking for the first 6 hours by using mechanical shaker and was allowed to stand for 18 hours. It was then filtered rapidly and 20 ml of filtrate was evaporated to dryness in a tarred, flat bottomed, shallow dish and dry at $60^{o}C$ to constant weight (WHO, 2011). The water soluble extractive value was determined as:

$$\text{Water soluble extractive value} = \frac{Weight\ of\ Residue \times 5}{Weight\ of\ Sample} \times 100$$

3.3.4.2 ***Alcohol soluble extractives value:*** Powdered sample of the plant (5g) was macerated in 100 ml of 90% ethanol in a closed flask for 24 hours with frequent shaking for the first 6 hours by using mechanical shaker and was allowed to stand for 18 hours. It was then filtered rapidly and 20 ml of filtrate was evaporated to dryness in a tarred, flat bottomed, shallow dish and dry at $60^{o}C$ to constant weight (WHO, 2011). Alcohol soluble extractive values were calculated (in percentage) with reference to the initial weight of the extract as:

$$\text{Alcohol extractive value (\%)} = \frac{Weight\ of\ Extract \times 5}{Weight\ of\ Sample} \times 100$$

3.4 Extraction of *E. guineensis* Pseudobulbs

The plant material (5.44 kg) was extracted with 12 litres of methanol using cold maceration method. This was carried out for 8 days in maceration flasks. Aliquot extraction was used i.e. part of the plant material (marc) were been fetched from the main maceration flask and into other flasks, fresh solvent were subsequently added to the new flasks for another round of extraction, this was carried out every two days to ensure maximum extraction. The mixture was stirred and gradual shaking was applied throughout the extraction period. The extract solutions were filtered and a clear brown solution (filtrate) and the marc (residue) were obtained with the aid of a separating funnel and a filter paper. The filtrate from the different flasks were combined and further concentrated via rotary evaporator to recover some solvent and final concentration of the extract was done via the water bath. The concentrated extract was scrapped with a metal scrapper into a beaker and covered with aluminium foil and stored in desiccators for further use. The extract was code name MEOH.

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

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

3.4.1 Fractionation of the methanol extracts of *E. guineensis* Pseudobulbs

The methanol extract (110 g) was dissolved in 300 ml of distilled water and poured into a separating funnel. The solution obtained was fractionated or partitioned successively with three solvents in order of their increasing polarity, namely: hexane, ethyl acetate and n-butanol respectively.

The process was done by adding 900 ml of hexane to the separation funnel. The mixture when settled divided into two layers; the lower aqueous portion layer and the upper hexane fraction layer. The both layers were collected into different beakers. The aqueous portion was reloaded into the separating funnel and fresh hexane solvent (900 ml) was added to it. This process was repeated three times to ensure maximum fractionation. All the hexane fractions collected were combined and code named HEX. This same procedure was carried out for ethyl acetate and n-butanol solvents and their fractions were code named ETOAC and BTOL respectively. At the end of the fractionation, four fractions were obtained namely: hexane fraction, ethyl acetate fraction, n-butanol fraction and aqueous fraction. The aqueous fraction was code named AQU. 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:

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

Woo *et al.,* (1980).

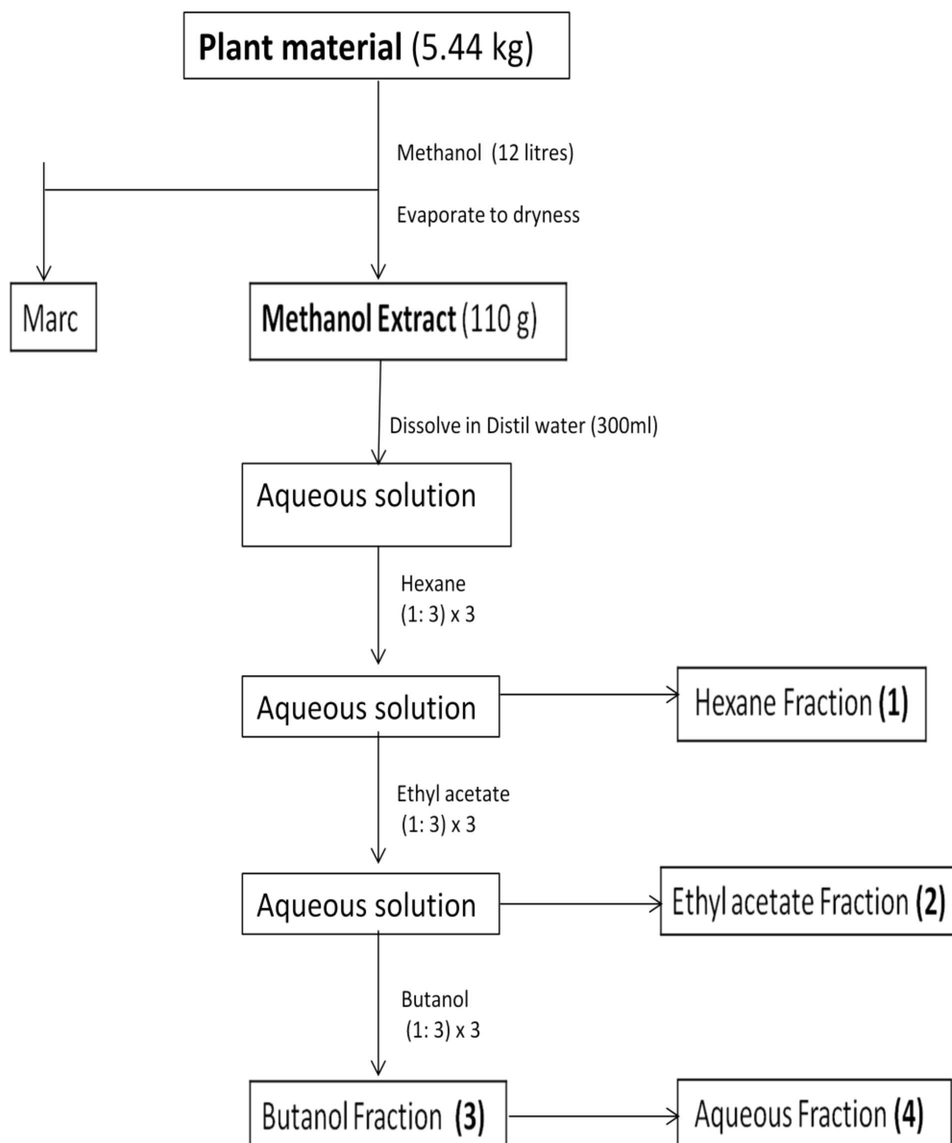


Figure 3.1: **Chart showing the extraction and fractionation pattern of *E. guineensis* Pseudobulbs.**

3.5 Phytochemical screening

Phytochemical screening was performed using standard procedure by Sofowora (2008), Ayoola *et al.,* (2008) and Evans (2002).

3.5.1 Test for Reducing Sugars (Fehling's Test)

The sample (0.5mg in 5 ml of water) was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction. The formation of red colouration indicates the presence of reducing sugars.

3.5.2 Test for Terpenoides (Salkowski Test)

To 0.5 gm each of the samples was added to 2 ml of chloroform. Concentrated sulphuric acid (3ml) was carefully added to form a layer. Reddish brown coloration of the interface indicates the presence of terpenoides (Sofowora, 2008).

3.5.3 Test for Flavonoids

Sample solution (4ml) was treated with 1.5ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated Hydrochloride acid was added and red colour was observed for flavonoids.

3.5.4 Test for Tannins

The sample (0.5 g) was boiled in 10ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

3.5.5 Test for Saponins

To 0.5 g of sample was added to 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth.

3.5.6 Test for Alkaloids

The sample was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The sample was then observed for the turbidity or yellow precipitation.

3.5.7 Test for anthraquinone derivatives (Borntrager's test)

The sample (10mg) was placed in a dried test tube and 10 ml of chloroform added. The mixture was shaken for 5 min and filtered with Whatman No. 1 filter paper. To 3 ml of the

filtrate, equal volume of ammonia solution was added and shaken. Formation of a bright pink-red colour in the upper aqueous layer indicates the presence of free anthraquinones derivatives (Evans, 2002).

3.5.8 Test for carbohydrates (Molisch's test)

The sample (10 mg) was boiled in 10 ml of distilled water on a water bath for 3 min. The mixture was filtered while hot. A few drops of Molisch's reagent was added to 2 ml of the cooled filtrate and shaken, and then a small quantity of concentrated sulphuric acid was added and allowed to form a lower layer. The formation of a purple ring at the interface indicated the presence of carbohydrates.

3.5.9 Keller-Kiliani's test for Cardiac glycosides

To 10 mg of each sample was placed in a test tube. Then 5 ml of water and 2 ml of glacial acetic acid containing one drop of 10% ferric chloride solution were added. The contents were thoroughly mixed and filtered. To 2 ml of the filtrate, 1 ml of concentrated sulfuric acid was added down the wall of the test tube to form a layer underneath. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides, indicative of cardiac glycosides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout the layer (Ayoola *et al.,* 2008).

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

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 110°C. Chromatograms were viewed immediately (Paschal *et al.,* 2002). The best solvent for each fractions were chosen for further analysis.

(I) Adsorbent: (Merck) silica gel G thickness 0.25 mm activated at 105^{0}C for 1hr.

(II) Solvent systems:

a) Hexane: ethyl acetate = (9: 1), (7:3), (3:2), (1:1)

b) Ethyl acetate: methanol= (19: 1), (9:1)

c) Chloroform: methanol = (17:3), (9:1), (7:3)

d) Chloroform: ethyl acetate: Methanol: water = (15:8:4:1)

(III) Detection techniques:

a) Daylight

c) Ultra Violet (U.V) at 254 and 366nm

IV) **Spraying reagents**: *P-anisaldehyde* for (general), Sulphuric acid for (general), Aluminium chloride for (flavonoids), Liebermann Buchard test for (triterpenes and steroids), Ferric chloride for (phenolic compound), Dragendorff for (alkaloids).

3.7 *In-vitro* antioxidant activity of extract and the fractions of *E. guineensis* pseudobulbs

In vitro antioxidant activities of the extract and all the fractions were carried out. Ascorbic acid was used as the standard (positive control) as described below:

3.7.1 Qualitative screening

Rapid screening of the anti-oxidant activity of the extract and all the fractions were carried out according to the method proposed by Mensor *et al*., (2001). This was carried out on pre-coated silica thin layer chromatography (TLC) plate. The TLC profile of each fraction was run in its best solvent system. The TLC profiles obtained were sprayed with 2, 2 – diphenyl-1-picryl-hydraxyl (DPPH) reagent as a detecting reagent.

A yellow spot or a deep orange spot against a purple background suggested the presence of free radical scavenging compounds (Adeloye *et al.,* 2007; Huang *et al*., 2005).

3.7.2 Quantitative screening

The DPPH assay was carried out according to the method of Mensor *et al.,* (2001). Sample stock solution of each sample (0.1 mg/ml) was diluted with methanol into various concentrations: 100, 80, 60, 40, and 20 µg respectively.

To each sample, 2.5 ml (of 20, 40, 60, 80 and 100 µg/ml in methanol) was added to 1ml of a DPPH solution (0.2 mM in methanol). After 30 minutes of reaction at room temperature, the absorbance of the solution was measured at 518 nm. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = 100 - \frac{(A_{sample} - A_{blank}) \times 100}{A_{control}}$$

- (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 readings were taken in triplicate.

NB: Sample solution contains each sample dissolved in solvent; in this case the solvent is methanol.

The **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 anti-oxidant activity from three separate tests.

3.8 Chemical properties of ethyl acetate fraction of the extract of *E. guineensis*

3.8.1 Column chromatographic analysis of ethyl acetate fraction of the extract

This was aimed at isolating a pure compound from ethyl acetate fraction of *E. guineensis* pseudobulbs extract. The adsorbent used was silica gel and the eluting solvents used were hexane and ethyl acetate.

The procedure was carried out according to the method proposed by Brain and Turner (1975) as outlined below;

120g of silica gel was mixed with the lesser polar solvent (n-hexane) twice its volume in a beaker and stir with glass rod (slurry method).

One quarter of the column was filled with the initial solvent system with the tap open, pouring the slurry gradually and continuously into the column using funnel and the tap closed when the solvent reached just above the bed filling about three-quarter of the column. This was allowed to stabilize overnight to ensure the absence of air bubbles.

4g of ethyl acetate fraction was pre-absorbed in silica gel (dry method) and loaded into the column. This was allowed to settle for 3hrs before elution began.

Gradient elution was used, starting with hexane (100%) as the initial eluting solvent. The polarity of the solvent system was increased by increasing the proportion of the more polar solvent (ethyl acetate) gradually.

Continuous elution was carried out and 40ml of fractions were collected in beakers, a total of 90 fractions were collected. They were monitored using TLC (Merck F_{254}) with the solvent system hexane: ethyl acetate (19:1, 9:1, 17:3, 8:2, 3:1, 7:3, 13:7, 6:4, 1:1, 2:3, 3:5 and 1:2). A white crystal compound was isolated in fractions 65-70.

3.8.2 Thin Layer Chromatography of MBS

The compound MBS was subjected to TLC analysis using varying solvent systems; hexane - ethyl acetate (6:4) and diethyl ether - ethyl acetate = 19:1. MBS was further spotted alongside the ethyl acetate fraction for comparative TLC in hexane - ethyl acetate (6:4). They were sprayed with *p-anisaldehyde* and 10 % sulphuric acid respectively.

3.8.3 Solubility profile of compound MBS

Compound MBS was dissolved in hexane, diethyl ether, chloroform, ethyl acetate, ethanol and methanol to infer its solubility.

3.8.4 Determination of melting point of compound MBS

This was determined using the Stuart melting point apparatus with model number 5315. This was carried out by collecting small quantity of the compound in a capillary tube and the capillary tube along with a thermometer was placed in the melting point apparatus. This was observed until the compound melted completely before the reading from the thermometer was recorded.

3.8.5 Spectroscopic analysis of compound MBS

3.8.5.1 *Infra Red (I.R) analysis*: Infra Red (I.R) analysis of the absorption spectra of MBS was conducted National Research Institute for Chemical Technology (NARICT), Basawa, Zaria, Kaduna state of Nigeria.

3.9 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 (1983) for both the intraperitonal and oral routes. 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.

3.10 Statistical Analysis

- All the data obtained from the studies were expressed as Mean ± SEM (Standard Error of Mean).
- The data obtained from antioxidant studies were further analyzed using ANOVA at $p < 0.05$ significant level.
- Data were also presented in the form of figures, tables and graphs.

CHAPTER FOUR

4.0 RESULTS

4.1 Plant collection, Identification and Preparation

The mature plant material comprising of the leaves, flowers, pseudobulbs and rhizomes was collected in October, 2014 from Bomo village, Sabon gari Local Government Area of Kaduna State, Nigeria. The plant material was identical to a herbarium sample *Eulophia guineensis* with voucher number 319 deposited at the herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria. The plant was identified by Taxonomist Mallam U. S. Gallah of Department of Biological Sciences.

4.2 Pharmacognostic parameters of *E. guineensis* pseudobulbs

4.2.1 Microscopic features of *E. guineensis* fresh pseudobulbs

The transverse section of *E. guineensis* pseudobulbs revealed some prominent features. Generally, it showed the cork, cortex, endodermis ground tissues, fibres, calcium oxalate crystals and vascular bundles. Specifically, the cork is the outermost part of the bulb which contains 3-4 layers of polygonal thin walled cells; next is the epidermal layer which contains cellulosic cell wall which appeared greenish to greenish yellow. The cortex contains many layers of thin cell walls of polygonal parenchyma cells, some sclerenchyma cells and collenchyma cells. The section also showed abundant calcium oxalate crystals scattered throughout the sections; they are needle-like in shape (acicular-type), abundant of mucilage, long, packed and lignified fibres. The vascular bundles were partially imbedded with lignified cell wall. The xylem tissues were seen scattered throughout the cell. The phloem, though larger than the xylem is fewer in number, its parenchymatous cells are thick (See plates **III-VII**).

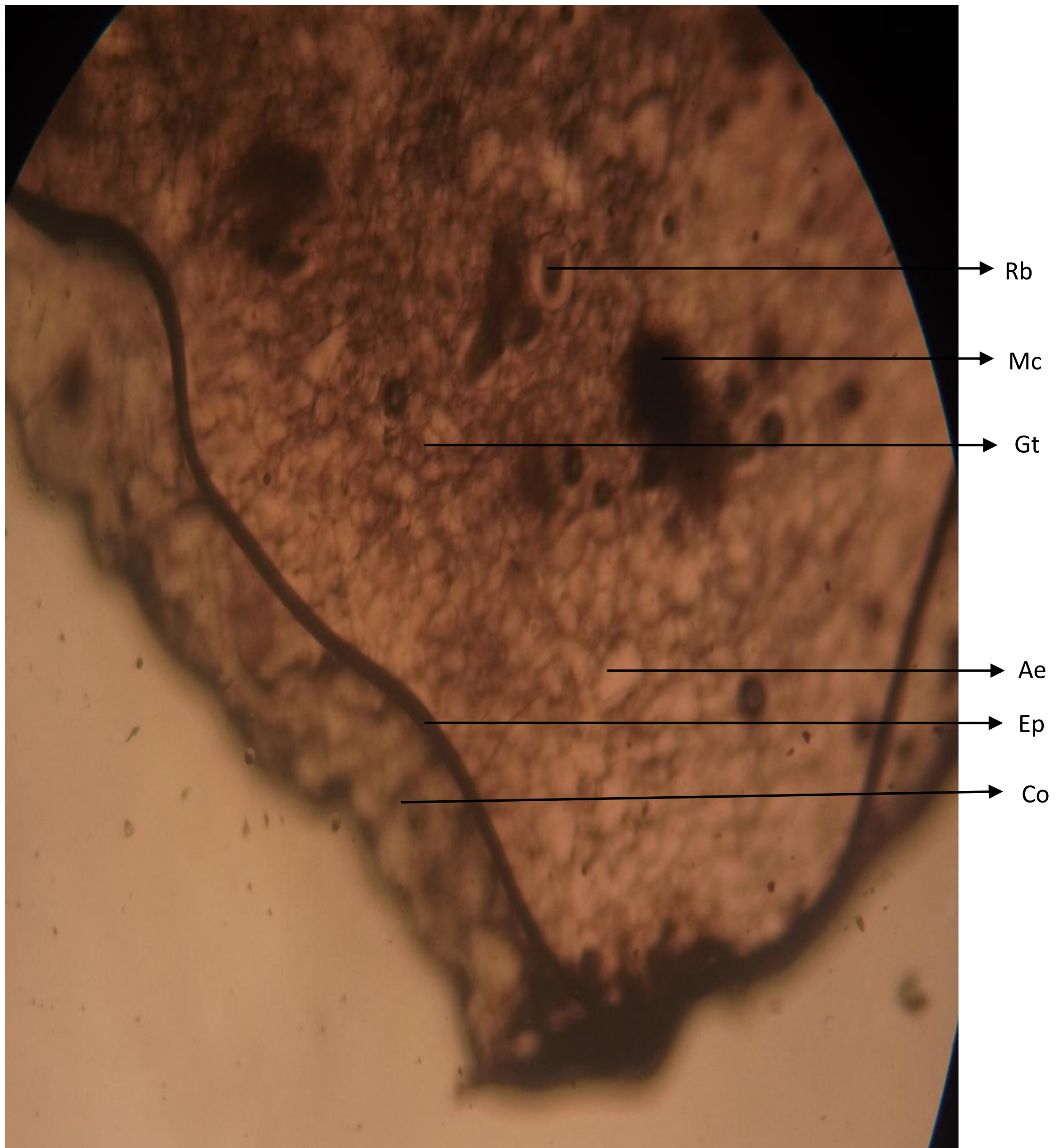


Plate III: Micrograph of the transverse section passing through the Pseudobulb of *E. guineensis* (Mag. ×100): showing Rb- Rhaphide bundles; Mc- Mucilage; Gt- Ground tissues; Ae- Aerenchyma; Ep- Epidermis; Co- Cork layer.

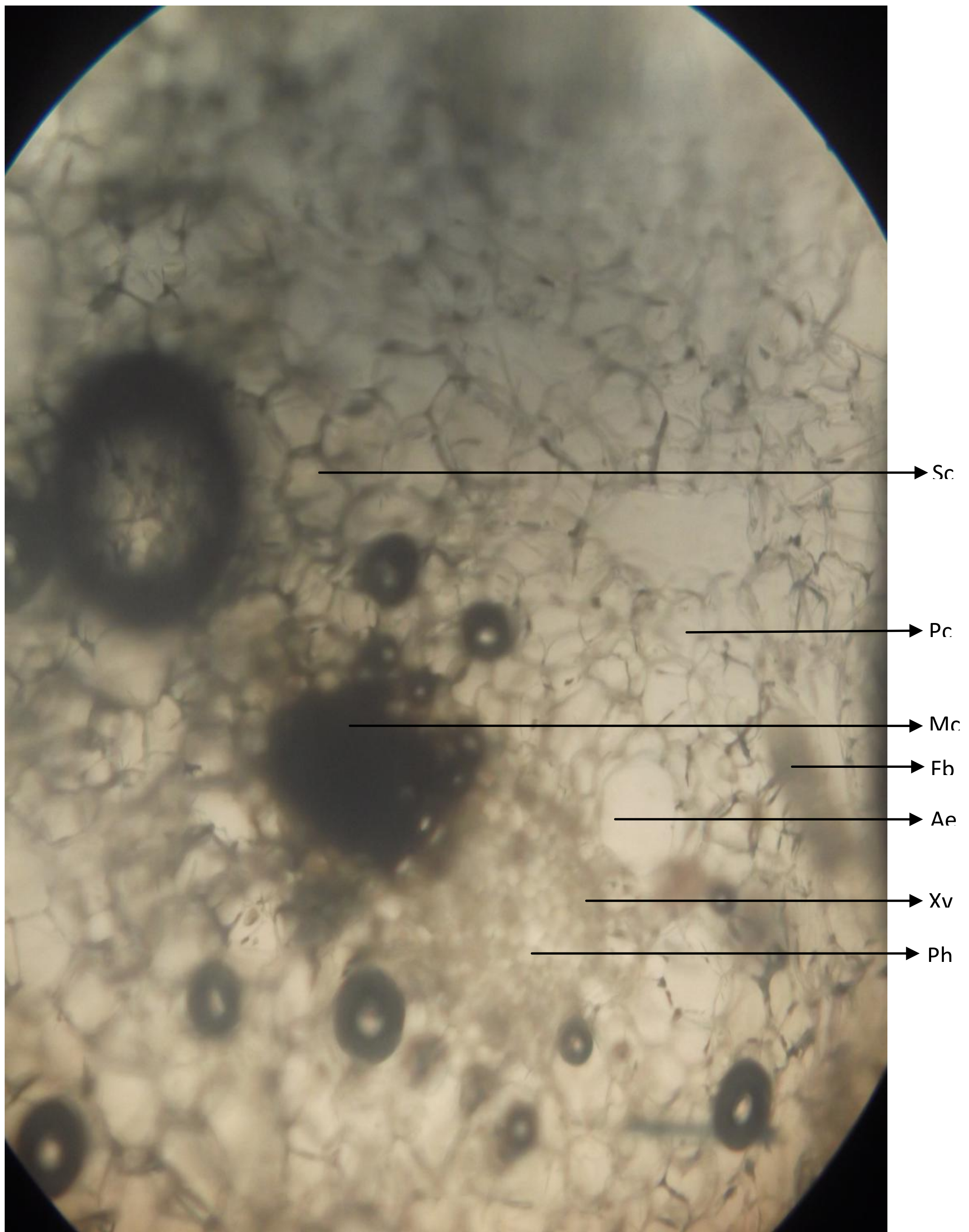


Plate IV: Micrograph of the transverse section passing through the Pseudobulb of *E. guineensis* (Mag. ×160): showing Sc- Sclerenchyma cells; Pc- Parenchyma cells; Mc- Mucilage; Fb- Fibres; Ae- Aerenchyma, Xv- Xylem vessels; Ph-Phloem.

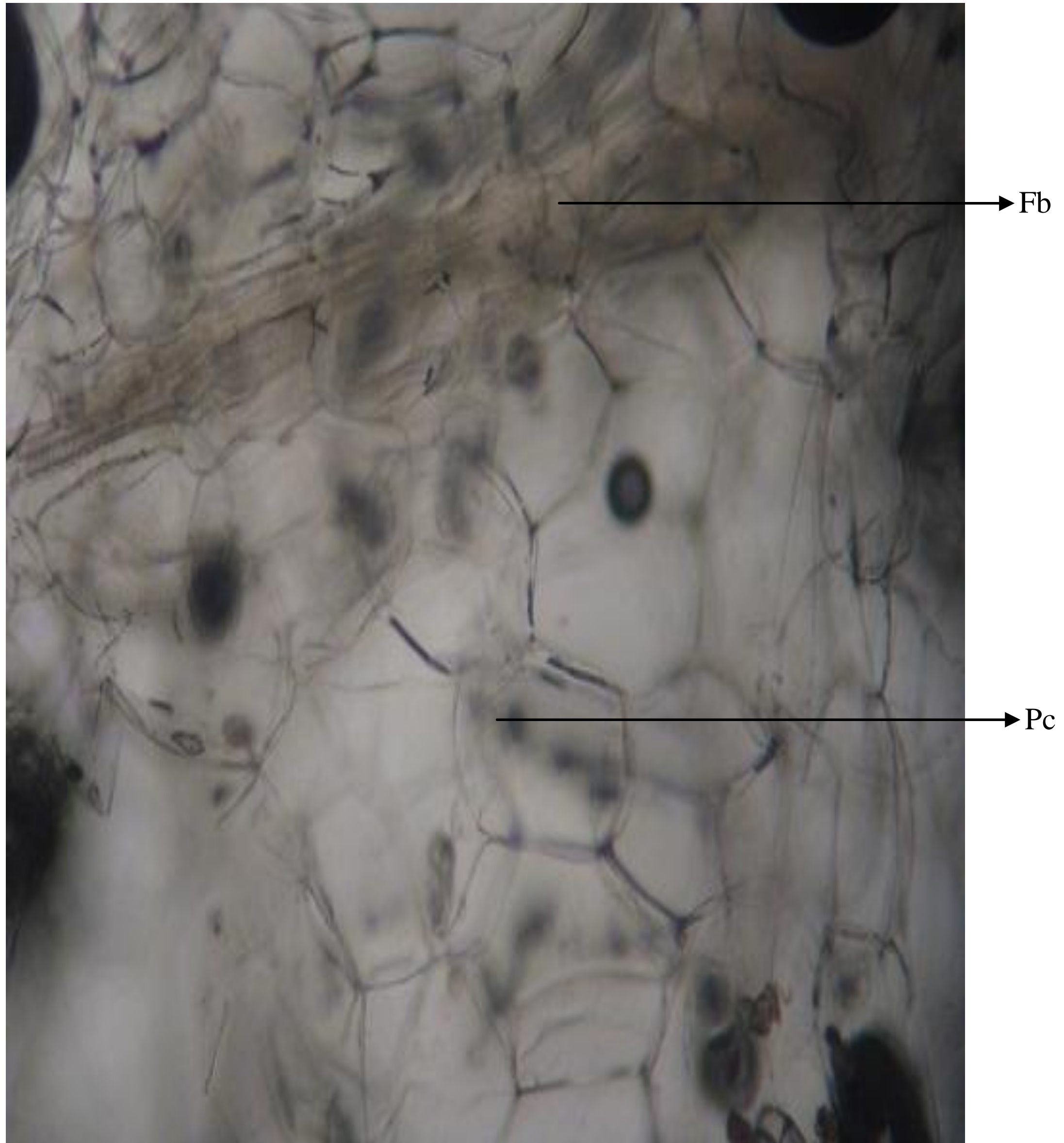


Plate V: Micrograph of the transverse section passing through the Pseudobulb of *E. guineensis* showing parenchymatous cells (Mag. ×640): Fb- Fibre; Pc- Parenchyma cells.

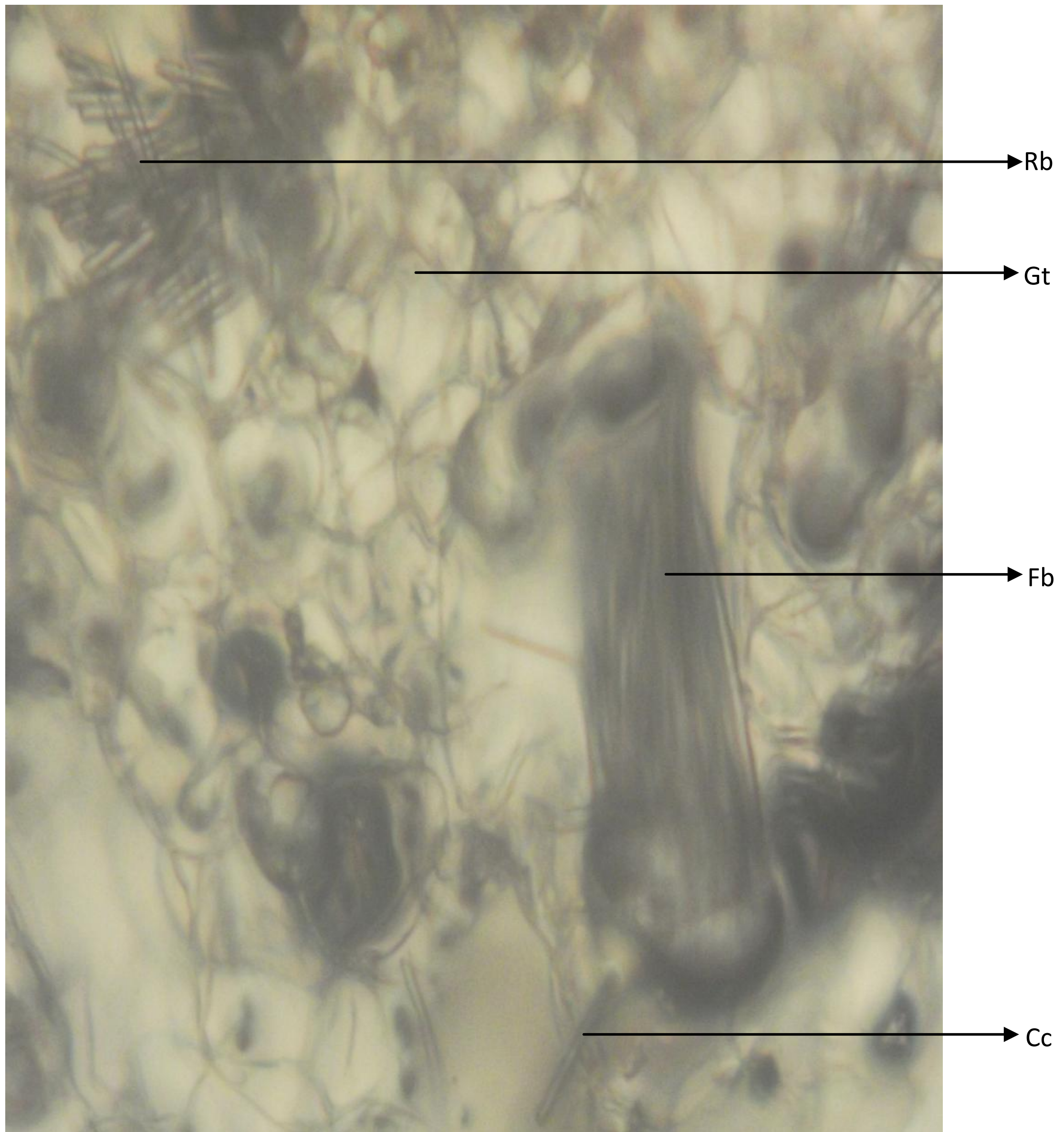


Plate VI: Micrograph of the transverse section passing through the Pseudobulb of *E. guineensis* (Mag. ×400): showing Rb- Raphide bundle; Gt- Ground tissue; Fb- Fibres; Cc: Calcium oxalate crystal before applying safranin.

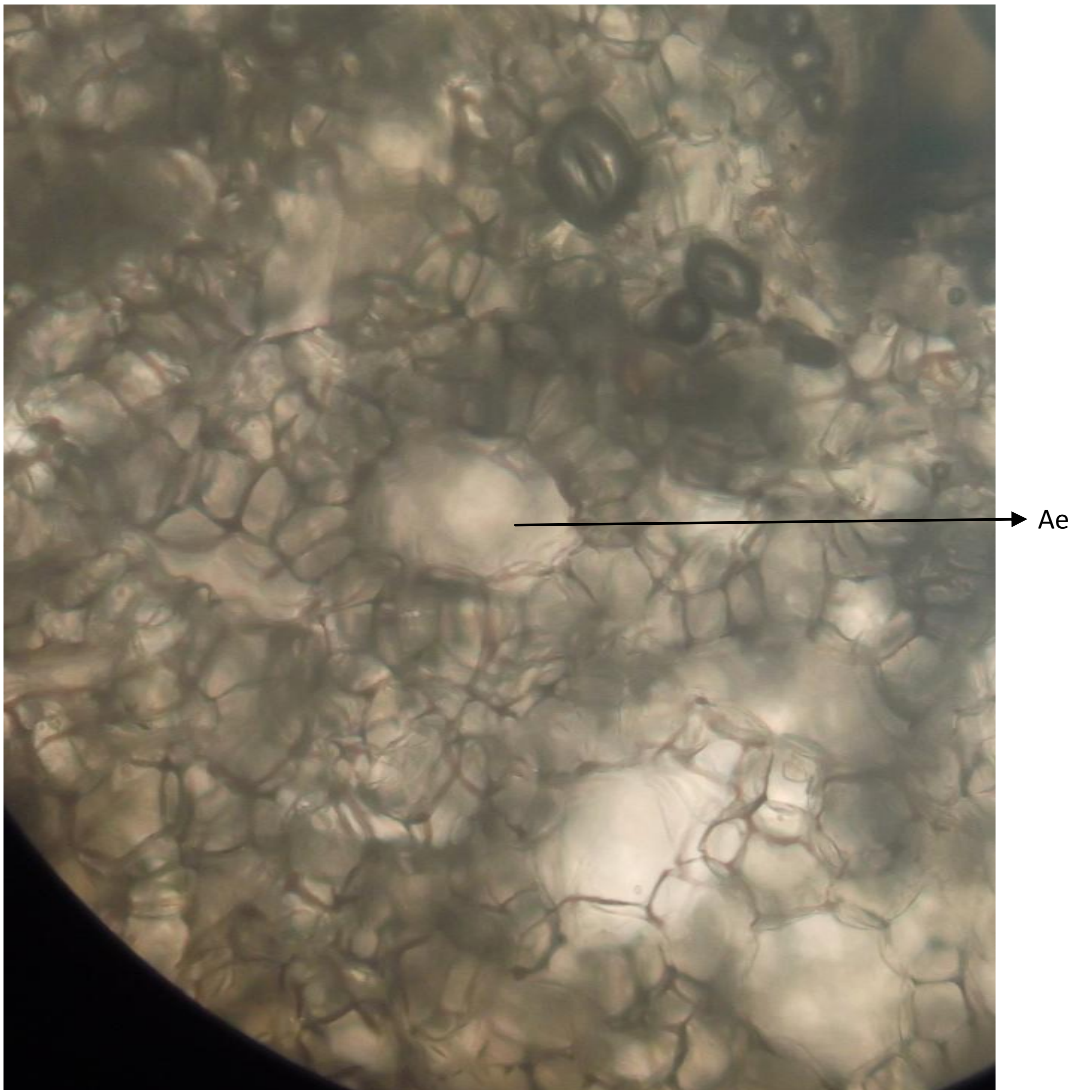


Plate VII: Micrograph of the transverse section passing through the Pseudobulb of *E. guineensis* showing Aerenchyma (Mag. ×400): Ae- Aerenchyma.

4.2.2 Chemomicroscopical studies of the powdered pseudobulbs of *E. guineensis*

Chemomicroscopical examination of the powdered pseudobulbs of *E. guineensis* revealed the presence of cellulosic epidermal cell wall, lignified cell wall, mucilages, tannins, starch and calcium oxalate crystals. Calcium carbonates, aleurone grain and fats and oil were absent. The calcium oxalates type is acicular (needles-like) and were abundant throughout the section. The mucilage cells were scattered in ground tissue (see Table 4.1 below).

Table 4.1: Chemomicroscopical features of *E. guineensis* powdered pseudobulbs.

Constituents	Detecting reagents	Observation	Inference
Starch	N/50 iodine	Blue-black colour on some grains within the cell.	Starch present
Lignin	Phloroglucinol	Red-pink colour on the walls of some lignified cell.	Lignin present
Tannins	5% $FeCl_3$	Greenish-black colour in some parenchyma cells.	Tannins present
Calcium oxalate	HCl	Dissolution of shining crystals on the anatomical sections of the bulb.	Calcium oxalate Crystal present
Calcium carbonate	HCl	No Effervescence in the cell.	$CaCO_3$ absent
Cellulose	Chlor-Zinc- Iodine	Blue coloration of the cell wall	Cellulose present
Mucilage	Ruthenium red	Red coloration	Mucilage present
Aleurone	Millon’s reagent	Absence of yellow colour	Aleurone absent.
Fats and Oil	Sudan red	Absence of red colour	Fats and oil absent

4.2.3 Physicochemical parameters of the powdered pseudobulbs of *E. guineensis*.

The physicochemical parameters such as moisture content (6.56 %), total ash value (10.83 %), acid insoluble ash (2.50 %), water soluble ash (2.60 %), ethanol extractives (11.50 %) and water extractives (24.67 %) are shown in Table 4.2 below.

Table 4.2: Physicochemical constants of powdered pseudobulbs of *E. guineensis*

Parameters	Values (%w/w) ± SEM*
Moisture content	6.56 ± 0.11
Total ash value	10.83 ± 0.17
Acid insoluble ash	2.50 ± 0.00
Water soluble ash	2.60 ± 0.06
Ethanol extractives	11.50 ± 0.29
Water extractives	24.67 ± 0.33

*Average values of three determinations.

4.3 Extraction and Fractionation of *E. guineensis* pseudobulbs

4.3.1 Extraction of *E. guineensis* pseudobulbs

The extraction of 5.44 kg of fresh pseudobulbs of *E. guineensis* using methanol yielded 110 g representing 2.02 % of the plant material. The extract was brown and sticky (Table 4.3 below).

Table 4.3: Percentage yield of *E. guineensis* Pseudobulbs extract

Extract	Mass (g)	Colour	Yield (% w/w)
Methanol	110 `	brown and sticky	2.02

4.3.2 Fractionation of *E. guineensis* pseudobulbs methanol extract

The mass yields of fractions obtained from solvent-solvent fractionation of 110 g in order of increasing polarity using hexane, ethyl acetate and n-butanol and aqueous fractions were 0.20 g (0.18 %), 4.17 g (3.79 %), 5.59 g (5.08 %) and 68.80 g (62.55 %) respectively as shown in Table 4.4 below.

Table 4.4: Percentage yield of fractions from methanol extract of *E. guineensis* pseudobulbs

S/N	Fractions	Mass (g)	Colour	Yield (% w/w)
1	Hexane	0.20	yellow and oily	0.18
2	Ethyl acetate	4.17	brown and sticky	3.79
3	Butanol	5.59	brown and powdery	5.08
4	Aqueous	68.80	light brown and sticky	62.55

4.4 Preliminary phytochemical screening of *E. guineensis* pseudobulbs

The *E. guineensis* extract showed positive test for carbohydrate, triterpenes, deoxysugars, cardiac glycosides, flavonoids and tannins. However, it tested negative for anthraquinones, saponins and alkaloids. These phytochemical constituents observed were re-distributed among the different fractions obtained from the methanol extract as shown in Table 4.5 below.

Table 4.5: Preliminary phytochemical screening of the methanol extract and the fractions obtained from the extract of *E. guineensis* pseudobulbs.

Phytochemicals	MEOH	HEX	ETOAC	BTOL	AQU
Carbohydrates	+	-	-	-	+
Anthraquinone	-	-	-	-	-
Triterpenes	+	+	+	-	-
Deoxysugars	++	-	+	+	++
Cardiac glycosides	+	+	+	+	+
Saponins	-	-	-	-	-
Alkaloids	-	-	-	-	-
Flavonoids	+	-	+	+	-
Hydrolysable tannins	+	-	-	-	+
Condensed tannins	+	-	+	+	+

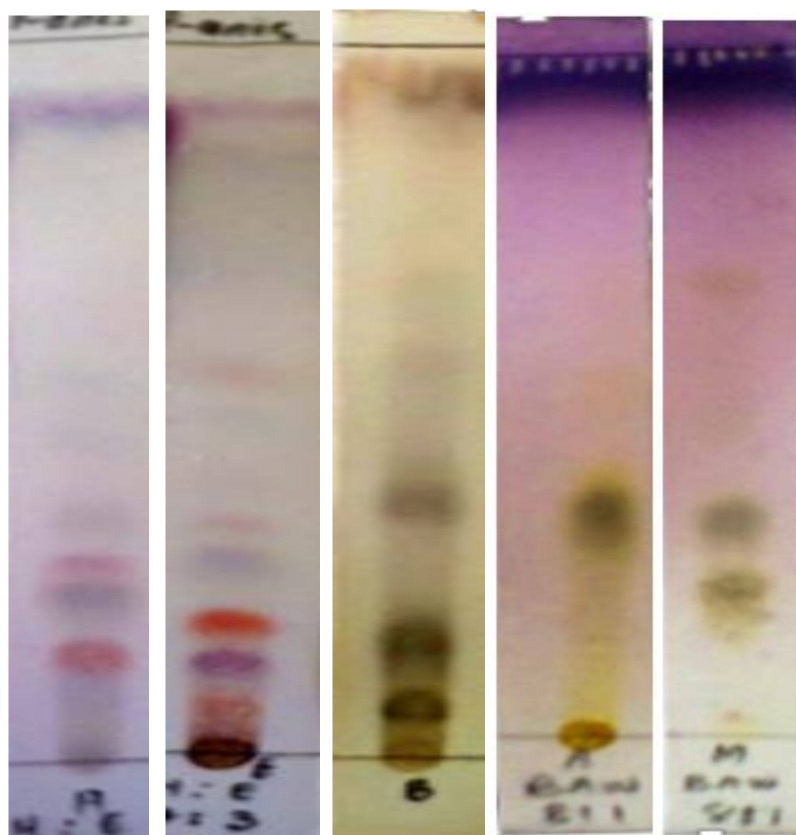
Present + Absent –

KEY: HEX: Hexane fraction ETOAC: Ethyl acetate fraction BTOL: Butanol fraction

AQU: Aqueous fraction MEOH: Methanol extract.

4.5 Chromatogram of methanol extract and fractions of *E. guineensis* pseudobulbs

The TLC profile analysis revealed the most suitable solvent system for each of the samples. They are hexane-ethyl acetate (H:E = 7:3) for hexane (HEX) and ethyl acetate fractions. chloroform-methanol-acetic acid (C:M:A = 8:2:1) for butanol fraction. Butanol-acetic acid-water (B:A:W = 8:1:1) for aqueous fraction and methanol extract as shown in plate **VIII** below.



HEX ETOAC BTOL AQU MEOH

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

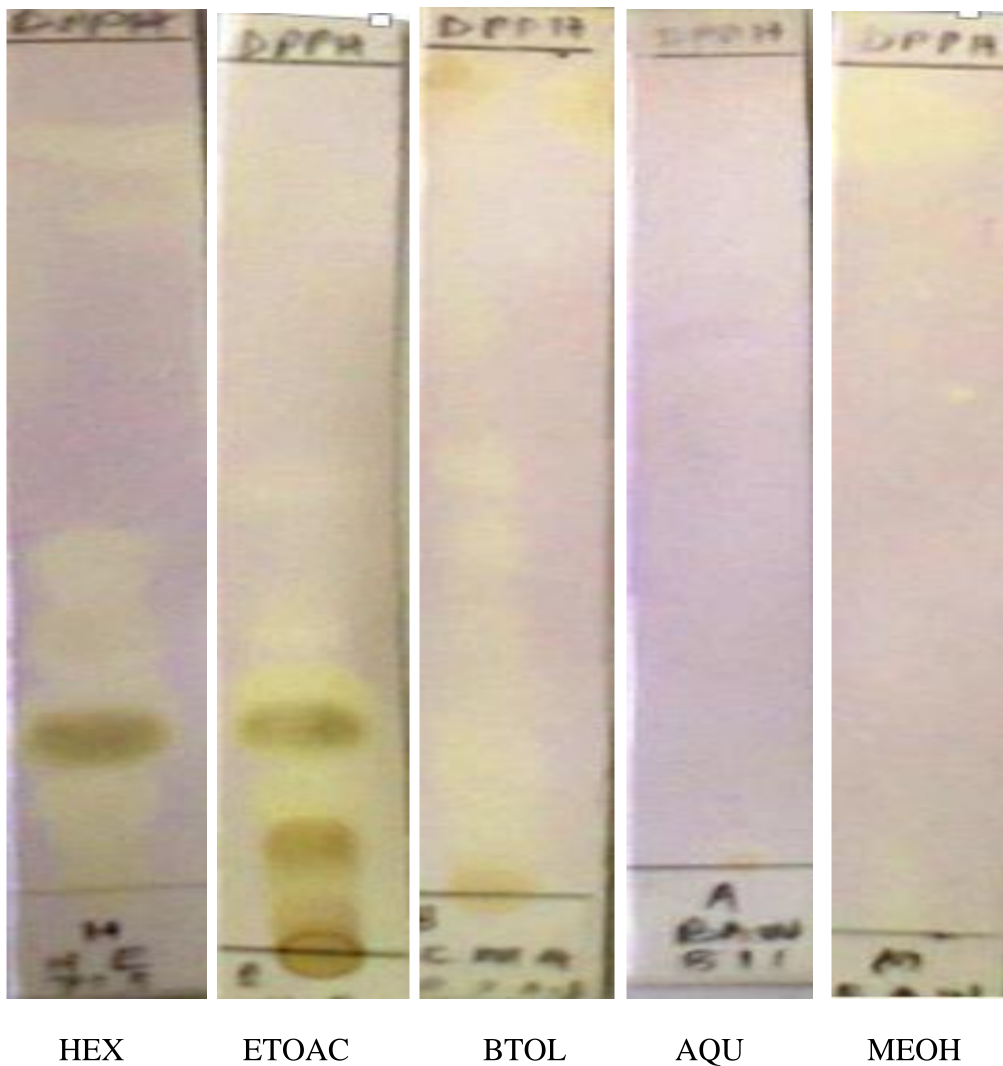
symbol	sample (fraction)	solvent system
HEX	Hexane	H: E (7:3)
ETOAC	Ethyl acetate	H: E (7:3)
BTOL	Butanol	C: M: A (8:2:1)
AQU	Aqueous	B: A: W (8:1:1)
MEOH	Methanol	B: A: W (8:1:1)

4.6 *In vitro* antioxidant studies of *E. guineensis* pseudobulbs

DPPH radical scavenging effect of methanol extract and fractions of *E. guineensis* pseudobulbs

4.6.1 Qualitative antioxidant studies

The qualitative test reveals that MEOH, HEX, ETOAC and BTOL samples of *E. guineensis* pseudobulbs showed yellow spots against the purple background signifying the presence of antioxidant components (i.e. free radical scavenging components) in these samples. However, the AQU showed no visible yellow spot against the purple background signifying the absence of antioxidant component in the AQU as shown in Plate **IX**.



HEX ETOAC BTOL AQU MEOH

Plate IX: Qualitative antioxidant activity of the extract and fractions as sprayed with DPPH reagent.

KEY: HEX: Hexane fraction ETOAC: Ethyl acetate fraction BTOL: Butanol fraction

AQU: Aqueous fraction MEOH: Methanol extract.

4.6.2 Quantitative antioxidant studies

The quantitative analysis test of the free radical scavenging activity of the samples on DPPH showed that all the samples except AQU were capable of scavenging free radicals. This activity was concentration dependent i.e. an increase in the concentration of the sample increases the scavenging properties of the sample. The highest activity was observed in BTOL, followed by ETOAC, MEOH, HEX and AQU respectively. The AQU performed least. The highest free radical scavenging activity value was observed in MEOH and BTOL at concentration 100 µg/ml (93.74% $\pm1.10^{b}$ and 93.42% $\pm0.17^{b}$ respectively), these were significantly lower than the ascorbic acid (control) scavenging activity which had maintained 100.31 ± 0.00^{a} (*p< 0.05*) from 60 µg/ml to 100 µg/ml concentrations. However, at the lowest concentration (20 µg/ml), the DPPH scavenging activity of BTOL (88.51% $\pm1.08^{a}$) was significantly higher than that of ascorbic acid (77.14% $\pm3.58^{b}$) at *p< 0.05*. The least scavenging activity was observed in AQU at concentration 20 µg/ml (-92.20% $\pm1.90^{f}$). Although, both the HEX and AQU showed negative activity, the negativity was decreased as the concentration of each sample increased. These were presented in Table 4.6 and Figure 4.2.

Figures 4.1 and 4.2 showed increase in antioxidant activity as the concentration of the samples and the standard increased. IC_{50} (Median Inhibitory Concentration) is define as the concentration of the sample required to scavenge or cause 50% loss in the free radical (e.g. DPPH). The IC_{50} of MEOH, HEX, ETOAC, BTOL and AQU were 44.61 µg/ml, 186.59 µg/ml, 7.58 µg/ml, 1.32 µg/ml and 1212.47 µg/ml respectively whereas the IC_{50} of standard ascorbic acid (ABA) was 1.04 µg/ml as shown in Table 4.7 and Appendix I-VI.

Table 4.6: Percentage antioxidant studies of the extract and fractions of the pseudobulbs of *E. guineensis* on DPPH.

% Antioxidant activity					
	Conc. (µg/ml)				
Samples	20	40	60	80	100
Control (ABA)	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]
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]
ETAC	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]
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]
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]
AQU	-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]

$P < 0.05$ (n=3) significant compared with ascorbic acid (AA) using One way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT). All results are expressed as Mean ± SEM.

Footnote: the superscript alphabets signify the level of significant difference among the samples with a= best performance and f= least performance.

Table 4.7: IC_{50} of methanol extract and the fractions of *E. guineensis* pseudobulbs

Sample	ABA	MEOH	HEX	ETOAC	BTOL	AQU
IC_{50} (µg/ml)	1.04	44.61	186.59	7.58	1.32	1212.47

KEY: HEX: Hexane fraction ETOAC: Ethyl acetate fraction BTOL: Butanol fraction

AQU: Aqueous fraction MEOH: Methanol extract ABA: Ascorbic acid.

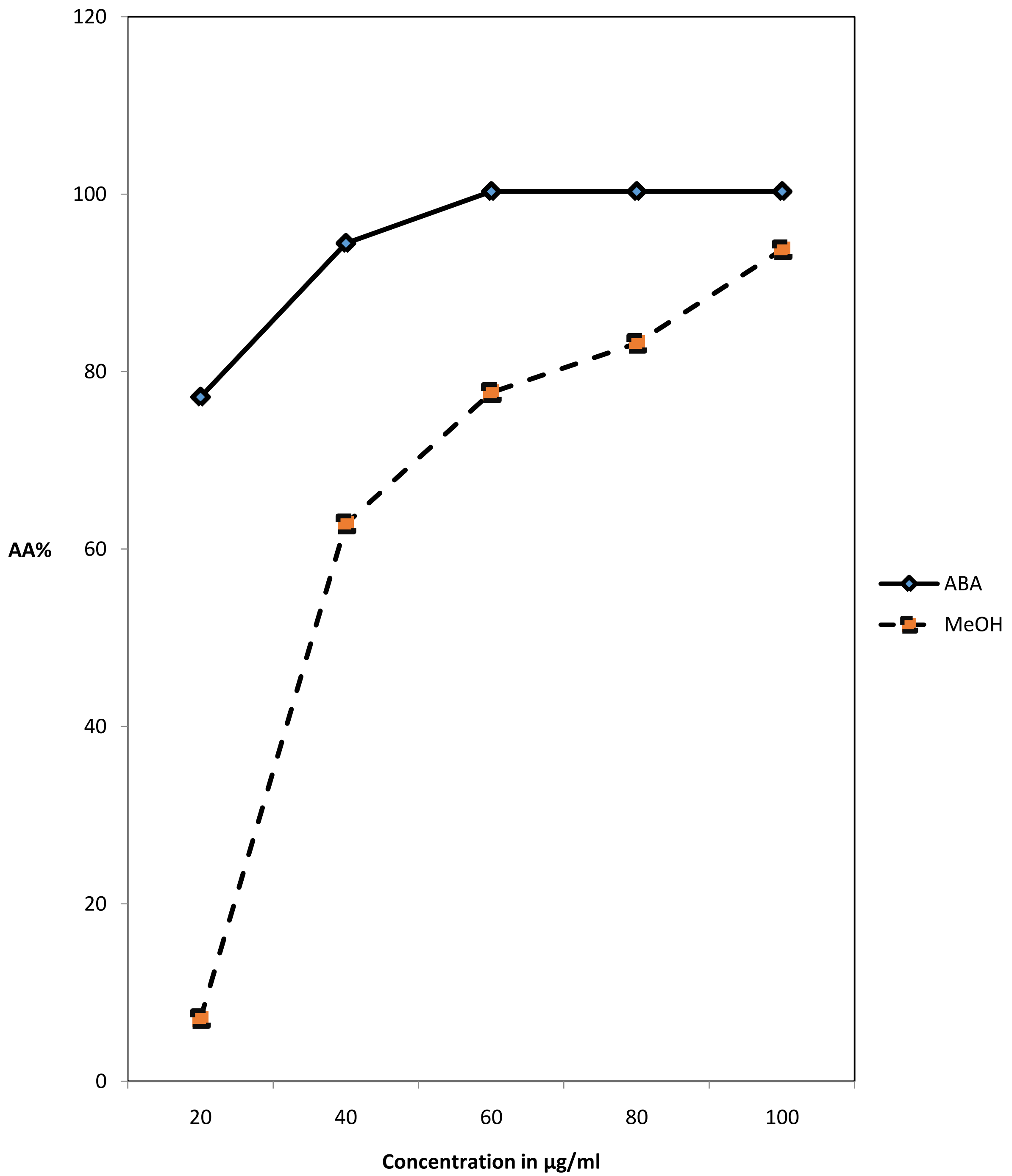


Figure 4.1: Line graph comparing the antioxidant activity of methanol extract of *E. guineensis* pseudobulbs with ascorbic acid on DPPH free radicals. (ABA = ascorbic acid and MEOH= methanol extract).

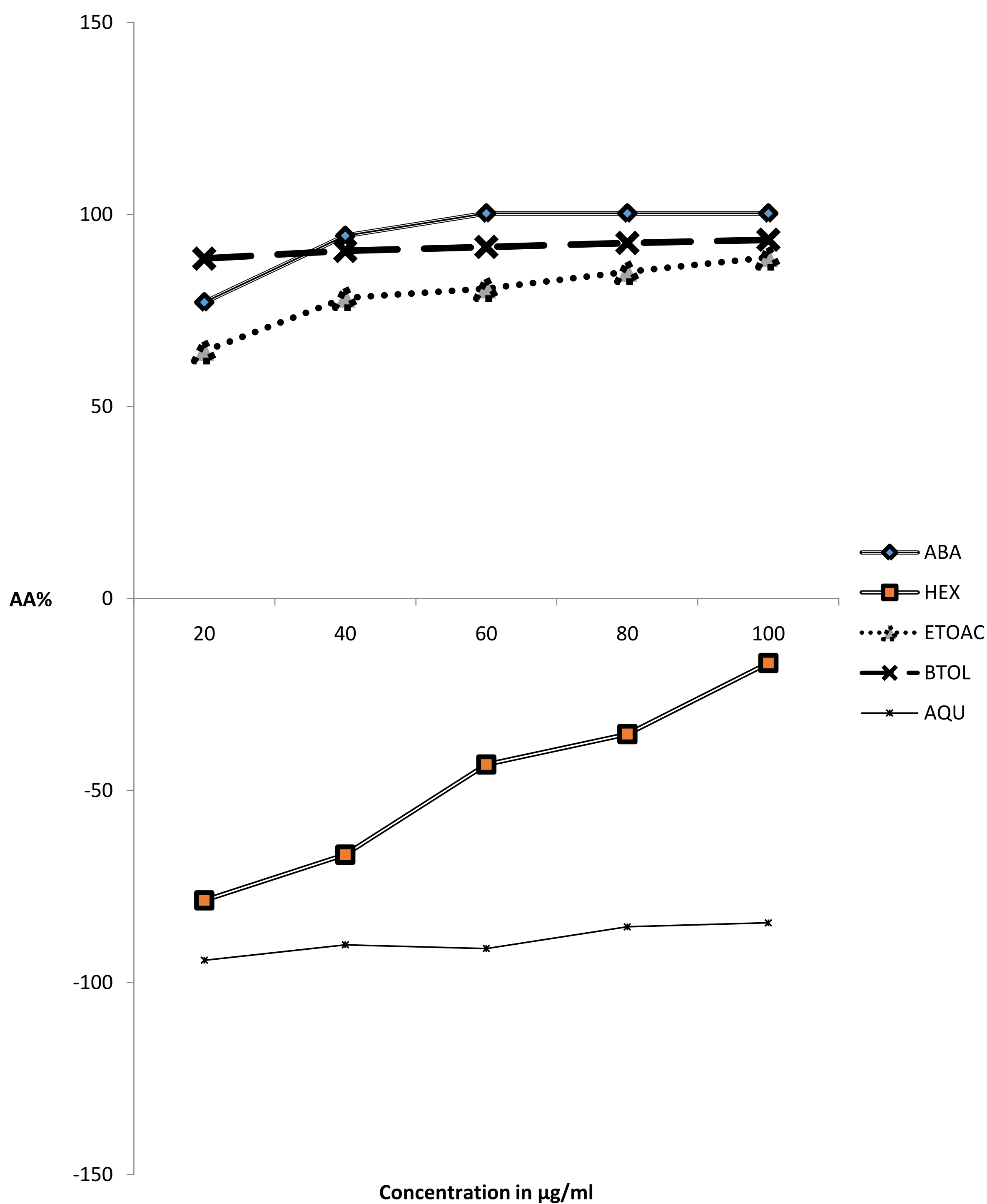


Figure 4.2: Line graph comparing antioxidant activity of the fractions from methanol extract of *E. guineensis* pseudobulbs with ascorbic acid on DPPH free radical. (ABA = ascorbic acid, HEX= hexane fraction, ETOAC= ethyl acetate fraction, BTOL= butanol fraction and AQU= aqueous fraction).

4.7 Chemical properties of ethyl acetate fraction

4.7.1 Column chromatographic analysis of ETOAC

Column chromatographic analysis was carried out for the ethyl acetate fraction. The column diameter is 5cm and the length is 50cm. The column was packed using the slurry method and the sample (4g) was loaded using dry method. Gradient elution of hexane and ethyl acetate solvent system was used and 40 ml of each fraction was collected. The ratios of eluting solvents are hexane : Ethyl acetate (100:0; 19:1; 9:1; 17:3; 8:2; 3:1, 7:3; 14:5, 13:7; 6:4; 11:9; 1:1, 5:4; 4:6; 3:7; 1:9 and 0:10). At the end of the experiment, 90 fractions and eighteen bulk fractions were collected and code named B_1, $B_{2,}$ $B_{3,}$ up toB_{18}. At elution rate of H: E (1:1), five fractions 65-69 (B_{15}), some crystals were observed deposited at the beaker. Even though, some coloured components were observed together with the white crystals in these fractions, they weren’t miscible. Solvent systems such as hexane: ethyl acetate (4:5 and 1:1), 100% diethyl ether were used to separate the coloured component from the white crystals. Recrystalization was done to have maximum yield of the white crystals. White, shining, needle-like crystals were collected and coded **MBS**.

Table 4.8: Elution rate, column fractions and bulk fractions collected.

Elution rate (H: E)	Column fraction no	Bulk fractions
10:0	1-5	-
19:1	6-8	B_1
9:1, 17:3 (12th)	9-13	B_2
17:3, 8:2 (15th)	14-17	B_3
8:2, 3:1 (19th)	18-19	B_4
3:1	20-22	B_5
14:5 (23rd)	23-24	B_6
14:5	25-26	B_7
14:5, 7:3 (28th)	27-38	B_8
7:3, 13:7 (42nd)	39-42	B_9
13:7	43-46	B_{10}
13:7, 3:2 (55th)	47-55	B_{11}
3:2	56-57	B_{12}
3:2, 11:9 (59th)	58-61	B_{13}
11:9, 1:1 (63rd)	62-64	B_{14}
1:1	65-69	B_{15}
1:1	70-73	B_{16}
1:1	74-77	B_{17}
1:1, 2:3 (80th), 3:7 (84th)	78-90	B_{18}
1:9 (89th) and 0:10 (90th)		

*Eluting solvent; hexane: ethyl acetate

4.7.2 Thin Layer Chromatographic analysis of compound MBS

Comparative TLC analysis of MBS and ethyl acetate fraction was carried out using hexane - ethyl acetate (7:3). This reveals the presence of a purple colour at $R_f = 0.57$ for both samples showing presence and position of MBS in the ethyl acetate fraction. Meanwhile, the MBS (on left side) showed one spot, the ethyl acetate fraction (on right side) showed multiple spots. TLC analysis of MBS was also carried out using diethyl ether: ethyl acetate (19:1) which revealed single homogeneous spot which is brownish in colour with R_f values of 0.91 (Plate **X** and **XI**).

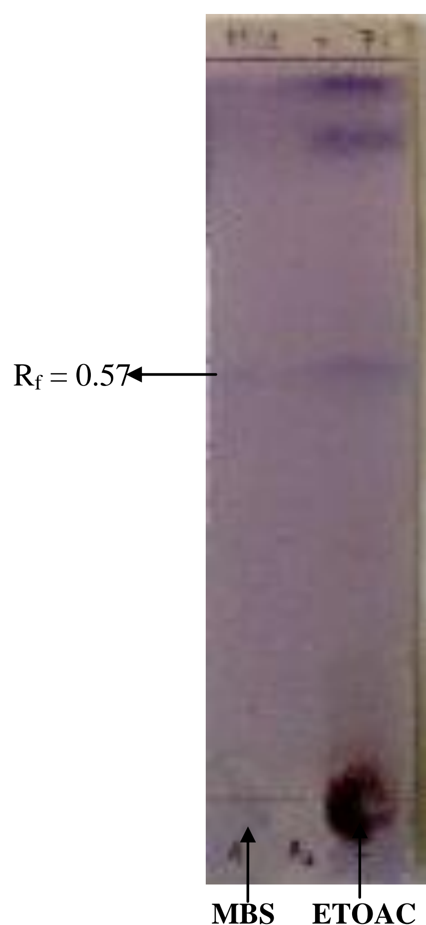


PLATE X: TLC plate of comparative studies of MBS and ETOAC in solvent system Hexane: Ethyl acetate = 7:3 as sprayed with *p-anisaldehyde*.

PLATE XI: TLC plate of MBS in diethyl ether: ethyl acetate = 19:1 as sprayed with sulphuric acid.

4.7.3 Solubility profile of compound MBS

The compound MBS is most soluble in ethanol. It is also soluble in absolute ethyl acetate, absolute methanol. It was however insoluble in hexane, chloroform and sparingly soluble in diethyl ether.

4.7.4 Melting point of compound MBS

The compound MBS was found to have a melting point range of 176-178°C.

4.7.5 Spectroscopic analysis of isolated compound (MBS)

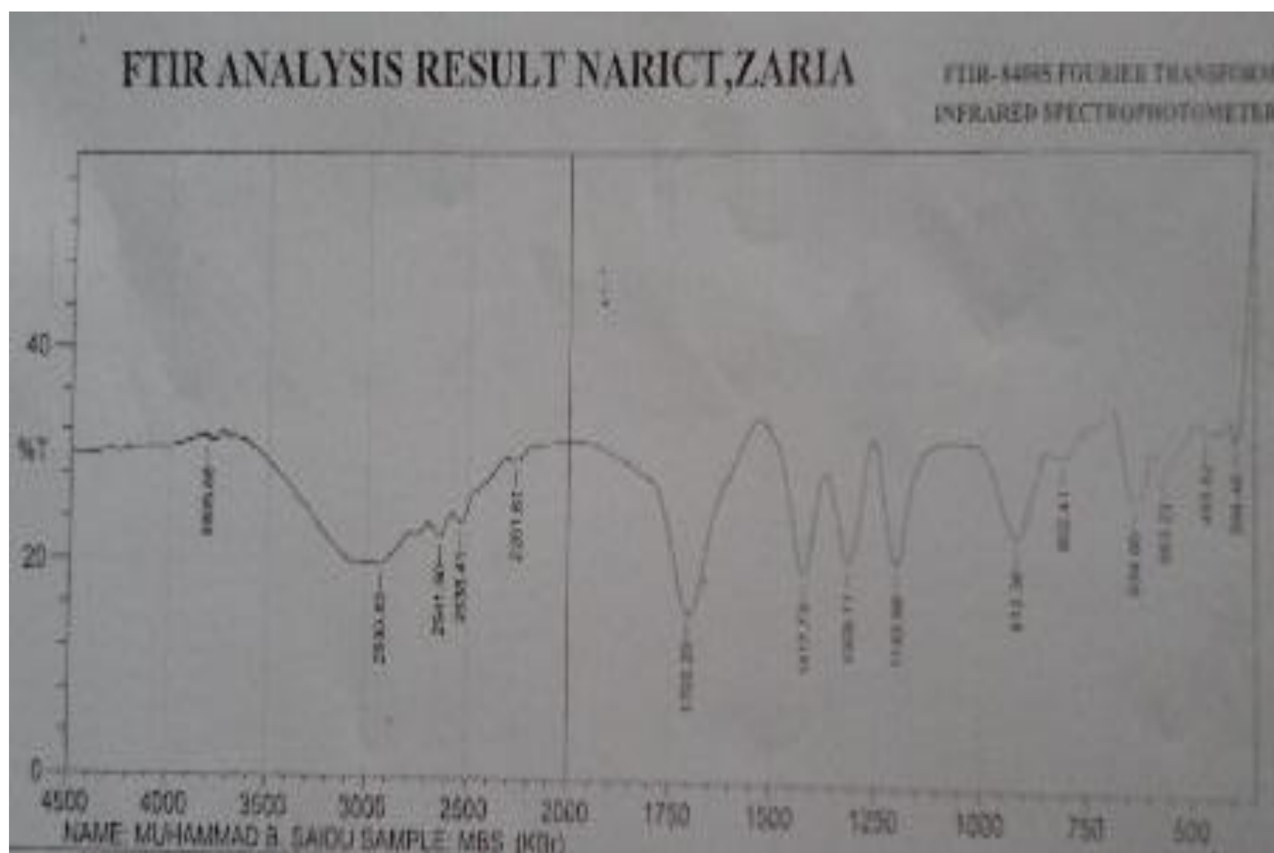


Figure 4.3: Infra Red (IR) absorption spectra of the isolated compound MBS.

Table 4.9: Functional groups present in the Isolated compound MBS

Areas	Absorption maxima (cm^{-1})	Relative Intensity	Shape	Functional groups
Left of C-H region	3805.68	Weak		OH of phenol
	2933.83	Strong	Broad	OH Carboxylic acid
C-H region	2641.60	Medium	Sharp	Sp^3 (saturated carbon)
	2538.41	Medium	Sharp	Sp^3 (saturated carbon)
	2261.61	Weak	Sharp	Medial Alkyne
Right of C-H region	1703.20	Strong	Sharp	C=O Carboxylic acid
	1417.73	Strong	Sharp	Vinyl (unsaturated carbon)

(John, 2000)

4.8 Median Lethal Dose (LD_{50}) of methanol extracts of *E. guineensis* pseudobulbs

The Intraperitionally and Oral routes Median Lethal doses (LD_{50}) of methanol extract of *E. guineensis* pseudobulbs administered in mice were found to be 3807.89 mg/kg and greater than 5000mg/kg body weight respectively (Table 4.11).

Table 4.10: 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

*Results given as number of death / number of animals in the group

$LD_{50} = \sqrt{Min.toxic\ dose \times Max.tolerated\ dose}$

$LD_{50} = \sqrt{(5000 \times 2900)}$

$LD_{50} = 3807.89\ mgkg^{-1}$

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 Pharmacognostic parameters of *Eulophia guineensis* pseudobulbs

Microscopic and physicochemical studies are carried out on herbal crude drugs sample in order to establish appropriate data that may be utilized not only for identification but also to establish the purity and standard of plant sample, those supplied in powder form (Kumar *et al.*, 2011; Ghani, 1990).

Microscopic features of *E. guineensis* fresh pseudobulbs: The transverse section of *E. guineensis* Pseudobulbs revealed some prominent features like the cork, acicular type calcium oxalate crystals, long lignified fibres, cellulosic epidermal cell wall, polygonal parenchyma cells, sclerenchyma cells, collenchyma cells and vascular bundles partially imbedded in lignins as shown in Plate **III, IV, V, VI** and **VII**. These features were similar to those observed in *E. herbaceae* (Anil *et al.,* 2012). The acicular type and abundant nature of the calcium oxalate crystals were also common among the two *Eulophia species*. The transverse section of *E. guineensis* Pseudobulbs revealed about 3-4 cork layers. Cork layers have been reported in Pseudobulbs of *Bulbophylum spp, Flickingeria nodosa* Dalz, and in *E. herbaceae* (Anil *et al.,* 2012; Madhavan *et al*., 2010; Pridgeon *et al*., 2014). The epidermal layer present in *E. guineensis* Pseudobulbs revealed a greenish-yellow coloured layer; this is a sign of cellulose cell wall. This is in tandem with the findings of Bond *et al*., (2008) who reported that cellulose-rich cell walls tend to fluoresce green/yellow while lignin-rich cell walls fluoresce red or orange. The most abundant ground tissue present in *E. guineensis* is polygonal parenchymatous cells. The polygonal parenchymatous cells were also reported in pseudobulbs of *Bulbophylum ambrosia, B. Penicilum, B. Leopodinum, B. Reptans,*

Flickingeria nodosa (Madhavan *et al*., 2010; Pridgeon *et al*., 2014). Polygonal parenchymatous cells were also observed in tubers of *Chlorophytum tuberosum* Baker (Patil and Deokule, 2010) and *Dioscorea esculenta* (Lour) Burkill (Thajunnisha and Anbazhakan, 2013). However, sclerenchyma and collenchyma cells were also observed in *E. guineensis* pseudobulbs. *E. guineensis* pseudobulbs microscopy revealed particular prominent large cells, many in number, reddish in colour with many spirals. This is in correlation with the description of mucilage by Pridgeon *et al*., (2014); the presence of larger and water storage cells of cortex and ground tissue with spirals are mucilaginous cells.

Chemomicroscopical features of powdered pseudobulbs of *E. guineensis* revealed the presence of cellulose cell wall, lignified tissues, mucilage, tannins, starches and calcium oxalate crystals. However, it was absent for calcium carbonates, aleurone grain and fats and oil as shown in Table 4.1. Similarly, *E. herbaceae* was reported to contain starch, tannins, mucilage and calcium oxalate crystals (Anil *et al*., 2012). The acicular type of the calcium oxalate crystals and its scattered nature in both *E. guineensis* and *E. herbaceae* are similar. The pseudobulbs of *Flickingeria nodosa* showed the presence of starch, calcium oxalate crystals, tannins, cellulose and lignins (Madhavan *et al*., 2010).

Physicochemical parameters of powdered pseudobulbs of *E. guineensis* analysed were found to be moisture content (6.56 %), total ash value (10.83 %), acid insoluble ash (2.5 %), water soluble ash (2.6 %), water extractives value (24.67 %) and the alcohol (90 % ethanol) extractives (11.50 %) as shown in Table 4.2. Similar research on the physical parameters of powdered Pseudobulbs of *E. herbaceae* by Anil *et al*., (2012) revealed moisture content (84 % for fresh pseudobulbs), total ash value (7.6 %), acid insoluble ash (0.98 %), water soluble ash (9.7 %), water extractives value (18 %) and alcohol (methanol) extractives (14 %).

However, the total ash value of *E. ochreata* was reported to be 9.10 % (Ali and Deokule, 2009). Results of physicochemical parameters studied showed the presence of low moisture content (6.56 %), this is an indicative of a good drug. It is very essential to control moisture content since higher moisture content in plant material may lead to its deterioration and may therefore result in biodegradation of active constituents (Prasad *et al.*, 2013). The ash values indicate inorganic residues obtained when vegetable drugs are incinerated. Total ash in a plant material includes both physiological as well as non-physiological ash while acid insoluble ash is a part of total ash and is indicative of silica present especially in sand and siliceous earth where as water soluble ash is the water soluble portion of the total ash (Prasad *et al.*, 2012). The total ash value of *E. guineensis* pseudobulbs observed was 10.83 %. It was observed from this study that the acid insoluble ash and water soluble ash were almost equal with 2.5 % and 2.6 % respectively. There was increment in extractive values with increase in extractive solvent polarity. The alcohol and water soluble values investigated using the ground plant powdered material revealed water (24.67 %) to have high extractive yield than alcohol (11.50 %) which implies more constituents of the plant to be soluble in water than in alcohol. Alcohol and water soluble extractive values can be used to detect exhausted and already utilized drugs which could be fraudulently used as substitute or adulterants (Elujoba, 1990; Khan *et al.*, 2013). Despite, the higher extractive values of water, alcohol extractives is preferably used in the laboratories because of some reasons; water is a better medium for the occurrence of microorganisms as compared to alcohol (Lapornik, *et al.*, 2005), this explains why water extracts spoil easily. Furthermore, ethanol (an alcohol) penetrates more easily the cellular membrane to extract the inter-cellular ingredients from the plant material (Wang, 2010). However, water, being a universal solvent, relatively safer and more abundant is primarily used by traditional healers.

5.2 Preliminary phytochemical screening of the extract and fractions from *E. guineensis* pseudobulbs

The preliminary phytochemical screening of the methanol extract of *E. guineensis* pseudobulbs showed the present of carbohydrate, triterpenes, deoxysugars, cardiac glycosides, flavonoids, hydrolysable tannins and condensed tannins as shown in Table 4.5. Similarly, a related species; *E. herbaceae* showed the presence of carbohydrates, steroids, triterpenes and tannins (Anil *et al.,* 2014). However, unlike *E. guineensis* and *E. herbaceae, E. campestris* was reported to contain alkaloid and was absent for reducing sugars, saponins, tannins, terpenoids and flavonoids (Rao *et al.,* 2013). 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 (Jonathan and Tom, 2008). Each plant family, genus, and species produces a characteristic mix of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants (Biology Encyclopaedia, 2015)**.** In addition, certain terpenoids, flavonoids, phenolics, cyanogenic glycosides and non–protein amino acids are illustrated to be of systematic use in particular cases (Jonathan and Tom, 2008)**.** This system of plant classification is called chemotaxonomy.

The hexane fraction of *E. guineensis* contains steroid, triterepenes and cardiac glycosides (Table 4.5). Steroid and triterpenes were also reported in hexane fraction of *E. herbaceae,* however cardiac glycosides was absent in it (Anil *et al.,* 2014).

The ethyl acetate fraction of *E. guineensis* contains triterpenes, deoxysugars, cardiac glycosides, flavonoids and condensed tannins but devoid of carbohydrate (Table 4.5). This is

in correlation with the findings by Anil *et al.,* (2014) which showed the presence of flavonoids, tannins, and carbohydrates in *E. herbaceae.* Nonetheless, triterpenes, deoxysugars and cardiac glycosides were absent in the ethyl acetate fraction of *E. herbaceae.* All these similarities and differences would guide in understanding the spread of these phytochemical constituents within the species taxon of Eulophia genus, thus helping in delimitation of taxa.

The butanol fraction showed the presence of deoxysugars, cardiac glycosides, flavonoids and condensed tannins. The aqueous fraction contains hydrolysable tannins, condensed tannins, starch and abundant deoxysugars (Table 4.5).

Furthermore, phytochemicals like flavonoids, tannins, cardiac glycosides, steroids and triterpenes has been linked to broad pharmacological activities (Perez, 2001 and Park *et al.,* 2001).

5.3 *In vitro* antioxidant activity of the extract and fractions of *E. guineensis* pseudobulbs.

In the present research, the antioxidant activities of the methanol extract and all the fractions obtained from the extract of *E. guineensis* using the DPPH assay method were studied. The DPPH assay has been mainly used as a quick, reliable and reproducible parameter to measure *in vitro* general antioxidant activity of pure compounds and plant extracts (Aliyu *et al.,* 2009; Ebrahimzadeh *et al.,* 2008; Goncalves *et al.,* 2005; Koleva *et al.,* 2002). DPPH is a stable nitrogen free radical. Its colour change from violet or purple to light yellow upon reduction by either the process of electron or hydrogen ion donation. Any substance capable of performing this reaction is regarded as an antioxidant and hence a free radical scavenger (Dehpour *et al.,* 2009). The $\mathbf{EC_{50}}$ or $\mathbf{IC_{50}}$ is the concentration of the sample that is required to

decrease the free radical (e.g. DPPH) concentration by 50 %, this parameter is widely used to measure antioxidant activity (Molyneux, 2004). The $\mathbf{R^2}$ value shows the degree of correlation between antioxidant activity and concentration. The nearer the R^2 is to one, the greater is the correlation (Araoye, 2003). In the current research, the antioxidant activity was done in two stages; the qualitative and then quantitative activity.

The Qualitative antioxidant activity was carried out according to the method of Mensor *et al.,* (2001) on TLC plates. The TLC profiles of the extract and fractions were sprayed with DPPH (0.2mg in 100ml) as spraying reagent; a yellow spot against a purple background signifies the free radicals scavenging properties of the components of each fraction against the oxidizing DPPH. The TLC profile of methanol extract, hexane, ethyl acetate and butanol fractions showed many yellow spots against the purple background (positive result) whereas the aqueous fraction showed no visible yellow spot against the purple background (negative result) as shown in Plate **IX**. These results indicated that all the fractions except the aqueous fraction possess antioxidant properties. Hexane and butanol fractions had the highest yellow spots (free radical scavengers). The TLC-DPPH antioxidant assay could serve as a good preliminary result for antioxidant potentials of a plant extract (Budzianowski and Budzianowska, 2006).

The Quantitative analysis was done according to the method of Mensor *et al.,* (2001); this was carried out in test tubes with a mixture of DPPH solution and the sample and their absorbance measured with U.V spectrophometer at wavelength of 518 nm. The mixtures were kept in the dark for 30 minutes before measuring their absorbance. If the solution turns yellow, it signifies the presence of very high antioxidant activity. The methanol extract showed activity, as the concentration was increased, the activity also increased (concentration

dependent). This is in agreement with researches conducted on *E. campestris* (Rao *et al.,* 2013), *E. ochreata* (Jagtap *et al.,* 2009) whose extracts have all been reported to possess antioxidant activities. The **IC_{50}** or **EC_{50}** of methanol extract of *E. guineensis* (**IC_{50}** = 44.61 μg/ml shown in Table 4.7) is comparable that of *E. ochreata* (**IC_{50}** = 26.90 μg/ml) as reported by (Jagtap *et al.,* 2009) and far lesser than that reported on *E. campestris* (**IC_{50}** = 1.593 μg/ml) (Rao *et al.,* 2013). It is also less than that of the standard ascorbic acid (**IC_{50}** = 1.04 μg/ml).

Although, the extract and all the fractions of *E. guineensis* pseudobulbs showed activity with increase in concentration i.e. they are concentration dependent, the methanol extract (**IC_{50}**= 44.61 μg/ml), butanol fraction (**IC_{50}** = 1.32 μg/ml) and ethyl acetate fraction (**IC_{50}** = 7.58 μg/ml) (Table 4.7) possessed potential antioxidant activities and are hence an indicative of a genuine antioxidants. This is because they are effective at lower concentration, soluble in water and practically non-toxic (for methanol extract with LD_{50} >5000 mg/kg orally). This assertion is supported by Khattak (2011) who reported that the **IC_{50}** values less than 100 μg/ml is indicative of a very good free radical scavenging potential. This fact was further buttressed by Sen (2003) who said among the conditions for a genuine antioxidant material include; it must be effective at low concentration, soluble in oxidizable product and non-toxic. Again, based on Khattak (2011) assertion, the hexane fraction (**IC_{50}** = 186.59 μg/ml) and aqueous fraction (**IC_{50}** = 1212.47 μg/ml) (Table 4.7) are not genuine antioxidants since their **IC_{50}** are more than 100 μg/ml.

The presence of flavonoids and tannins in the methanol extract, ethyl acetate and butanol fractions might be responsible for their antioxidant activity. This fact was buttressed by Sánchez-Moreno (2002a) and Rice-Evans (1996). They separately reported that among the

phenolic compounds with known antioxidant activity are flavonoids, tannins, chalcones and coumarins and phenolic acids. Puupponen-Pimiä *et al*., (2008) reported that phenolic compounds are essentially representing varieties of natural antioxidants, which are used as nutraceuticals and also in control of human pathogenic diseases.

Meanwhile, it is observed that though the ethyl acetate and butanol fractions were obtained from the methanol extract; they performed better antioxidant activity than the latter. This is because the methanol extract contains both the active components (i.e. ethyl acetate and butanol fractions) and the non-active components (hexane and aqueous fractions). This synergistic (combinatorial) effect resulted in moderating the activity of the extract to lie between the two parallel components. The methanol extract contains more phenolic glycosides (sugars from aqueous fraction) than ethyl acetate and butanol fractions. Phenolic glycosides have less antioxidant activity than those of the pure phenols. Hence, the more the sugars attached to flavonoids and tannins, the lesser is the antioxidant activities. This assertion was supported by Rice-Evans *et al*., (1996) that the antioxidant effectiveness of the flavonoids decreases with the substitution of hydroxyl groups with sugars, being the glycosides less antioxidants than their corresponding aglycones.

It is important to note that the probable reason for the rapid increase of antioxidant activities of *E. guineensis* methanol extract from when the concentration is 20 µg/ml (7.09 %) to when the concentration is 40 µg/ml (62.85 %) (Table 4.6 and Figure 4.1) is that while oxidation activities of free radicals increases in arithmetic progression, the rate of masking them by flavonoids and tannins are in exponential progression, thus rapidly increasing the antioxidant activity of the extract.

The higher value of **IC_{50}** of the aqueous fraction (**IC_{50}** =1212.47 µg/ml or 1.21mg/ml) signifies poor antioxidant activities and performed the least among all the fractions, being almost a thousand times lesser than the **IC_{50}** of the butanol fraction (**IC_{50}** = 1.32 µg/ml) and one hundred and sixty times lesser than ethyl acetate fraction (**IC_{50}** = 7.58 µg/ml). The poor antioxidant activity of the aqueous fraction is due to the predominant of sugars, even though tannins are present. This is in agreement with Rice-Evans *et al.,* (1996) that the presence of sugars reduces the antioxidant of the phenols. Another reason for the poor antioxidant activity of the aqueous fraction in comparison with those of the ethyl acetate and butanol fractions is the absence of flavonoids, even though it contains more phenols (both hydrolysable and condensed tannins) whereas the latter two fractions contain less phenol (only condensed tannins) and more flavonoids as shown in Table 4.5. Flavonoids are better antioxidants than tannins. This fact was in tandem with a study on the *in-vitro* antioxidant activity of the methanol extracts of the leaves of *Persea americana* and *Cnidosculous aconitifolius* by Asaolu *et al.,* 2010 using different methods of assessing their antioxidant activities. This study revealed that the phenol content of *Cnidosculous aconitifolius* was higher than that of *Persea americana* whereas *Persea americana* was observed to possess more flavonoids than *Cnidosculous aconitifolius*. However, *Persea americana* was found to have higher radical scavenging activity than *Cnidosculous aconitifolius.*

Qualitatively, the hexane fraction was one of the best fractions having many free radical scavenging components with wider diameters (Plate **IX**), its performance quantitatively is one of the lowest, only higher than the aqueous fraction (Table 4.6 and Figure 4.2). The reason is that steroids and triterpenes (chief constituents of the hexane fraction) have far lesser antioxidant capacity (i.e. strength of the activity) than flavonoids and tannins (chief constituents of butanol and ethyl acetate fractions). This is in agreement with a similar work

conducted by Télesphore *et al.,* (2011) on a dimeric triterpenoid glycoside and flavonoid glycosides with free radical-scavenging activity isolated from *Rubus rigidus* var. *camerunensis*. Télesphore *et al.,* (2011) concluded that all the four flavonoids identified performed better antioxidant activity with wide margins in comparison with the three tripterpenes identified except the carotene that performed well among the triterpenes but yet below all the four flavonoids studied. Hence, antioxidant activity is determined by the strength or capacity of the free radical scavenger (phytochemical constituents) and not only by the number of the free radical scavengers present in a sample.

The butanol fraction performed best among all the samples with its $\mathbf{IC_{50}}$ ($\mathbf{IC_{50}}$ = 1.32 µg/ml) slightly below that of the ascorbic acid ($\mathbf{IC_{50}}$ = 1.04 µg/ml). In fact, at the addition of butanol fraction in all the different concentrations to the DPPH solution in the test tube, the mixture suddenly turned yellow signifying instant scavenging of the DPPH free radical in a procedure that requires thirty (30) minutes for maximum scavenging. This rapid scavenging property of butanol fraction is only comparable to that of the ascorbic acid (control). Note that some fractions like aqueous and hexane fractions never turned yellow even after the expiration of thirty (30) minutes required for maximum scavenging.

5.4 Chemical properties of compound MBS

The isolated compound code named MBS is a moderately polar compound since it is from the ethyl acetate fraction. Its insolubility in hexane also supports the moderately polar or polar nature of the compound. The function of Infra Red (I.R) is to guide in the determination of functional group(s) in a sample. The IR spectra comprises of two main components, namely; the finger print region ranging from 1500-500 cm^{-1} and the functional group region ranging from around 1500- 4500 cm^{-1}. Factors considered in searching for functional group

are location, intensity and the shape of the peaks. Each functional group has a specific absorption range. In determining the functional group, we first look at the C-H region, then look to the left for N-H or O-H functional groups and finally to the right for C-H stretch.

In the present research, the C-H region reveals peaks at 2641.60 cm^{-1}, 2538.41 cm^{-1} and 2261.61 cm^{-1}. These peaks correspond to sp^3 carbons (saturated) while 2261.61 cm^{-1} corresponds to medial alkyne group (John, 2000). The left side of the C-H reveals a very broad and strong peak at 2933.83 cm^{-1} which corresponds to the region of hydroxyl (O-H) group of carboxylic acid. It also showed a narrow band at 3805.68 cm^{-1}, such band at a higher frequency occurs in special instances and is a characteristic of O-H group of phenols (John, 2000). To the right of C-H region, a strong and sharp peak was observed at 1703.30 cm^{-1} which lies within 1700-1750 for carbonyl group (C=O) of carboxylic acid. The strong and sharp peak at 1417.73 signifies a vinyl group (vinyl group range from 1410-1420 cm^{-1}) as shown in Table 4.9 and Figure 4.3. The combination of the C=O of carboxylic acid and OH of carboxylic acid forms a carboxylic acid functional group (-COOH). The presence of OH of phenol and a –COOH suggests compound MBS probably contains a phenolic acid functional group.

5.5 Acute Toxicity test of the methanol extract of *E. guineensis* pseudobulbs

In order to determine the safety margin of drugs and plant products for human use, toxicological evaluation was carried out in experimental animals (mice) using Lorke's method (1983) to predict toxicity and to provide guidelines for selecting a "safe'' dose in animals and also used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs (Olson *et al.,* 2000; Rang *et al.,* 2001; Maikai *et al.,* 2008).

In this study, median lethal dose (LD_{50}) of the methanol extract of the pseudobulbs of *E. guineensis* was carried out intraperitionally and orally in mice. The LD_{50} was found to be 3807.89 mg/kg and greater than 5000 mg/kg when administered intraperitonially and orally respectively in mice as shown in Table 4.10, This studies showed the methanol extract of *E. guineensis* Pseudobulbs is slightly toxic when administered intraperitionally and practically non-toxic when administered using the oral route. This is based on the toxicity classification by Loomis and Hayes (1996) which states that substances with LD_{50} values of 500 to 5000 mg/kg body weight as slightly toxic and LD_{50} values of 5000 to 15,000 mg/kg body weight is practically non-toxic. Similarly, *E. ochreata* was reported to be non-toxic with LD_{50} greater than 5000 mg/kg using oral route (Jagtap *et al.,* 2009).

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of their unmatched availability of chemical diversity.

The study began with the microscopic examination of the transverse section of *E. guineensis* Pseudobulbs which revealed some prominent features like the cork, acicular type calcium oxalate crystals, fibres, polygonal parenchyma cells, sclerenchyma cells, collenchyma cells, mucilage, and vascular tissues partially imbedded with lignins.

Chemomicroscopical features of powdered pseudobulbs of *E. guineensis* revealed the presence of cellulose cell wall, lignified cell wall, mucilages, tannins, starches and calcium oxalate crystals.

The Physical parameters of the powdered pseudobulbs of *E. guineensis* was found to be moisture content (6.56 %), total ash value (10.83 %), acid insoluble ash (2.5 %), water soluble ash (2.6 %), water extractives value (24.67 %) and the alcohol (90 % ethanol) extractives (11.50 %).

Extraction of *E. guineensis* pseudobulbs was carried out with methanol solvent using cold maceration method. Subsequently, the methanol extract was fractionated in order to have the non-polar components (by hexane), moderately polar components (by ethyl acetate) and polar components (by n-butanol) and aqueous portion respectively. Preliminary phytochemical screening of the extract revealed the presence of carbohydrate, triterpenes, deoxysugars,

cardiac glycosides, flavonoids, hydrolysable tannins and condensed tannins. The phytochemical screening of the fractions revealed the redistribution of these phytochemicals among the different fractions obtained from the extract.

The *in vitro* antioxidant activity of the extract and the fractions showed all except the hexane and aqueous fractions had significant activity against DPPH free radical. This was expressed as IC_{50}. Methanol (**IC_{50}** = 44.61 µg/ml), hexane fraction (**IC_{50}** = 186.59 µg/ml), ethyl acetate fraction (**IC_{50}** = 7.58 µg/ml), butanol fraction (**IC_{50}** = 1.32 µg/ml) and aqueous fraction (**IC_{50}** = 1212.47 µg/ml or 1.21mg/ml). The LD_{50} of methanol extract showed it is slightly toxic intraperitionally (LD_{50} = 3807.08 mg/kg) and practically non-toxic orally (LD_{50} >5000 mg/kg).

The column chromatography of ethyl acetate fraction resulted in the isolation of a white, crystalline compound. Infra red spectroscopic analysis of the compound showed the presence of hydroxyl (OH), carboxylic acid (-COOH) functional groups, saturated and unsaturated carbons.

6.2 Conclusions

The transverse section of *E. guineensis* pseudobulbs revealed some prominent features like the cork, acicular type calcium oxalate crystals, mucilage, fibres, polygonal parenchyma cells, sclerenchyma cells, collenchymas cells, vascular tissues partially imbedded in lignins. The physical parameters of *E. guineensis* pseudobulbs were also established.

The methanol extract, ethyl acetate and butanol fractions showed significant antioxidant activity while the hexane and aqueous fractions showed no significant antioxidant activity *in vitro* using DPPH assay. The result obtained from LD_{50} showed the plant is non-toxic using oral route. Since antioxidant activity has a broad pharmacological effect, the results support

the traditional use of the plant in curing cold, obesity and inflammations. The presence of pharmacologically active principles such as flavonoids, tannins, steroid and triterpenes may be responsible for the antioxidant activity. This plant may therefore serve as potential antioxidant agent.

The compound isolated from the ethyl acetate fraction showed the presence of hydroxyl (OH) and carboxylic acid (–COOH) functional groups.

6.3 Recommendations

- Cytotoxicity test should be carried out of the crude extract with the aim of knowing its toxicity at cellular level.

- Other methods of *in vitro* antioxidant assays and *in vivo* antioxidant activities should be used to evaluate the antioxidant activities of the extract and fractions of *E. guineensis* pseudobulbs.

- Specific pharmacological activities such as anti-cancer, anti-obesity, anti-hypertension should be carried out on the extract and fractions with the aim of knowing their respective therapeutics index in curing these diseases.

- Column chromatography should be carried out on the butanol fraction with the aim of isolating and characterizing the most active compound since it shows the best activity among all the fractions and the extract.

- Although, a compound was isolated from the ethyl acetate fraction, I couldn't characterise and elucidate it due to limitations as a result of faulty machine and power instability; I urge more compounds should be isolated, characterised and elucidated.

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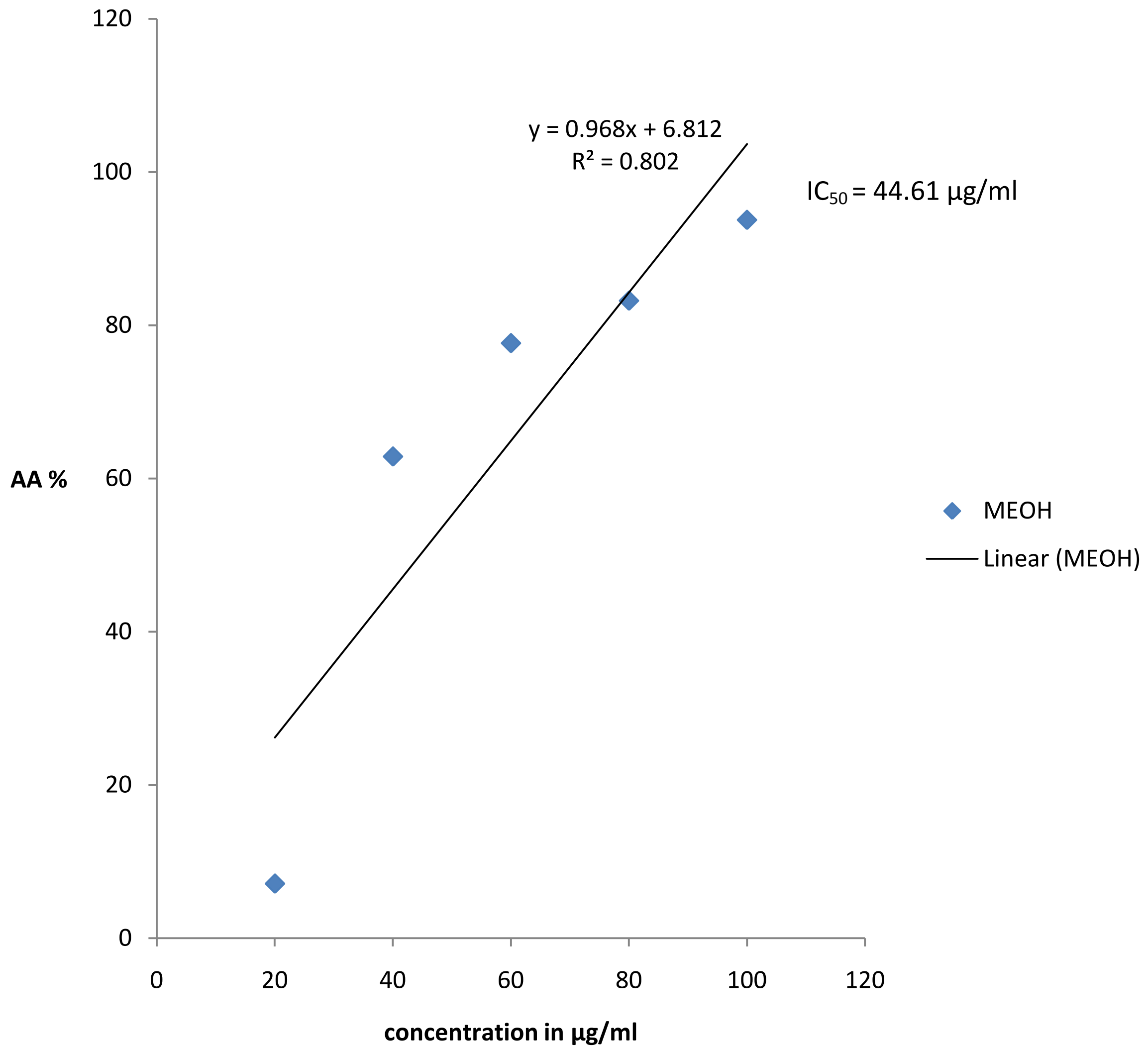
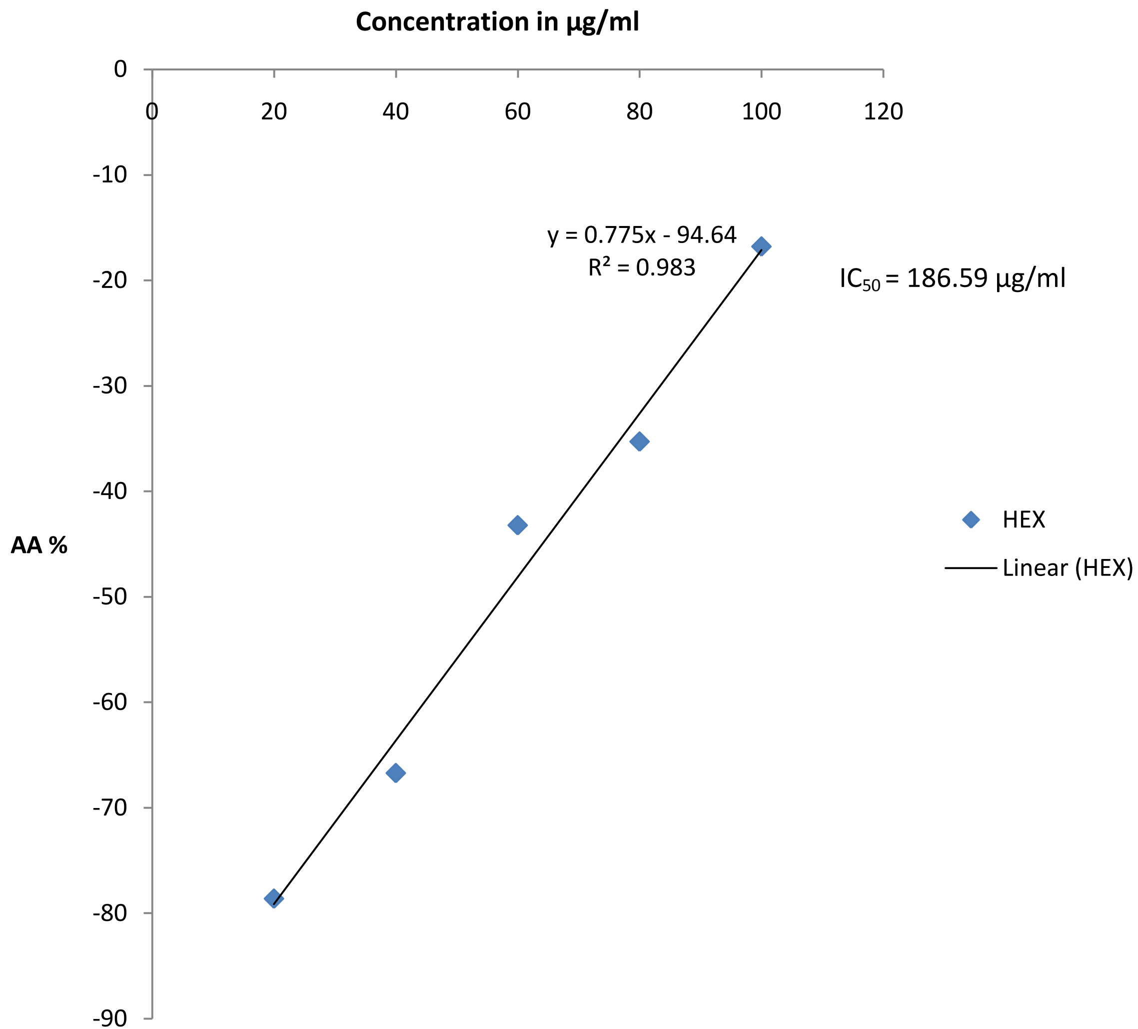
APPENDIX I



Linear regression graph and IC_{50} for methanol extract of *E. guineensis* pseudobulbs using DPPH method.

IC_{50} calculated using regression equation, $y = ax + b$, where y= 50% antioxidant activity, x = IC_{50}, a = regression coefficient and b = regression constant.

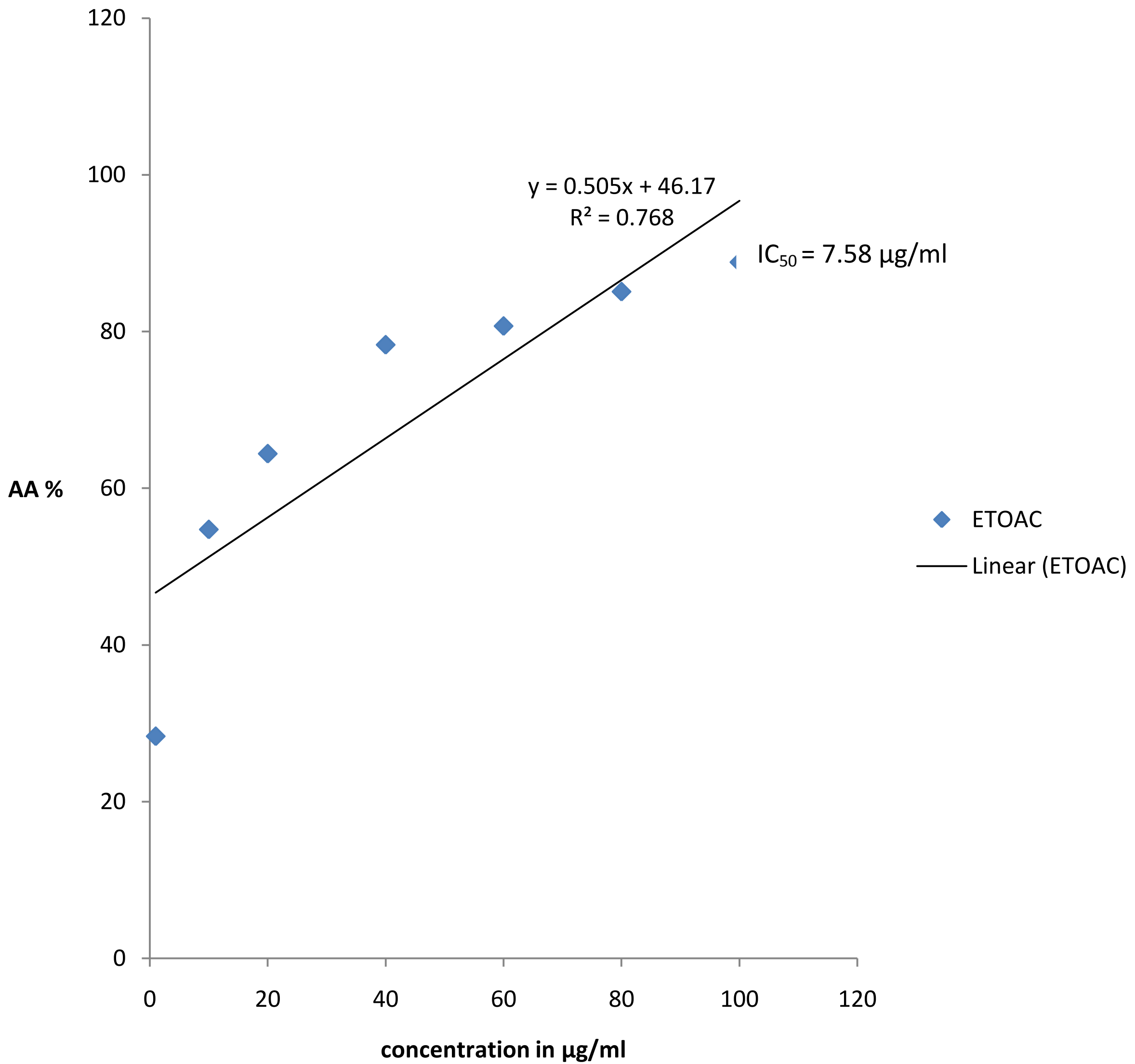
APPENDIX II



Linear regression graph and IC_{50} for hexane fraction of *E. guineensis* pseudobulbs methanol extract using DPPH method

IC_{50} calculated using regression equation, $y = ax + b$, where y= 50% antioxidant activity, x = IC_{50}, a = regression coefficient and b = regression constant.

APPENDIX III

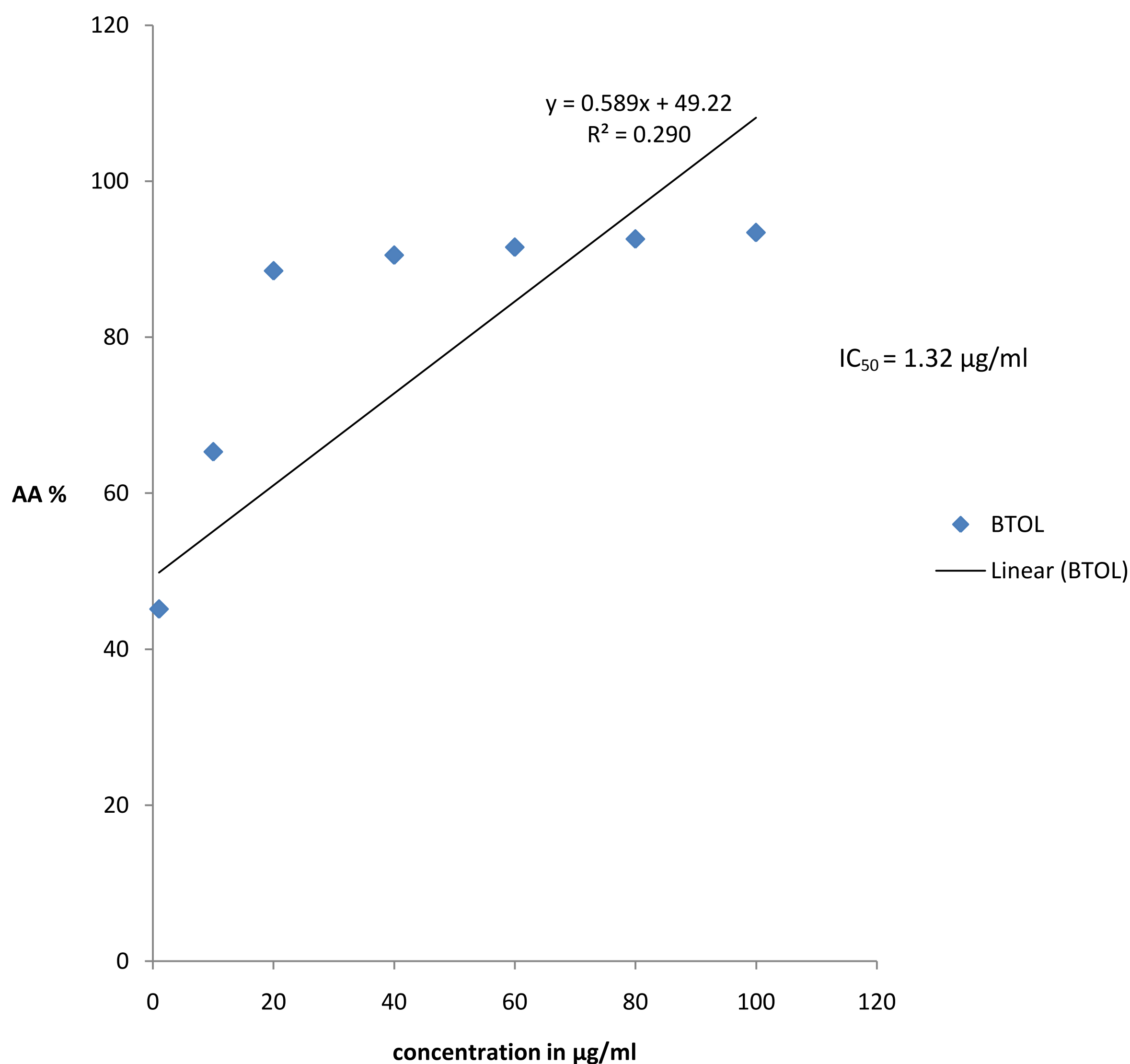


Linear regression graph and IC_{50} of ethyl acetate fraction of *E. guineensis* pseudobulbs methanol extract using DPPH method

IC_{50} calculated using regression equation, $y = ax + b$, where y= 50% antioxidant activity, x = IC_{50}, a = regression coefficient and b = regression constant.

NB: AA value at 1 µg/ml is 28.33 % and at 10 µg/ml is 54.72 %.

APPENDIX IV

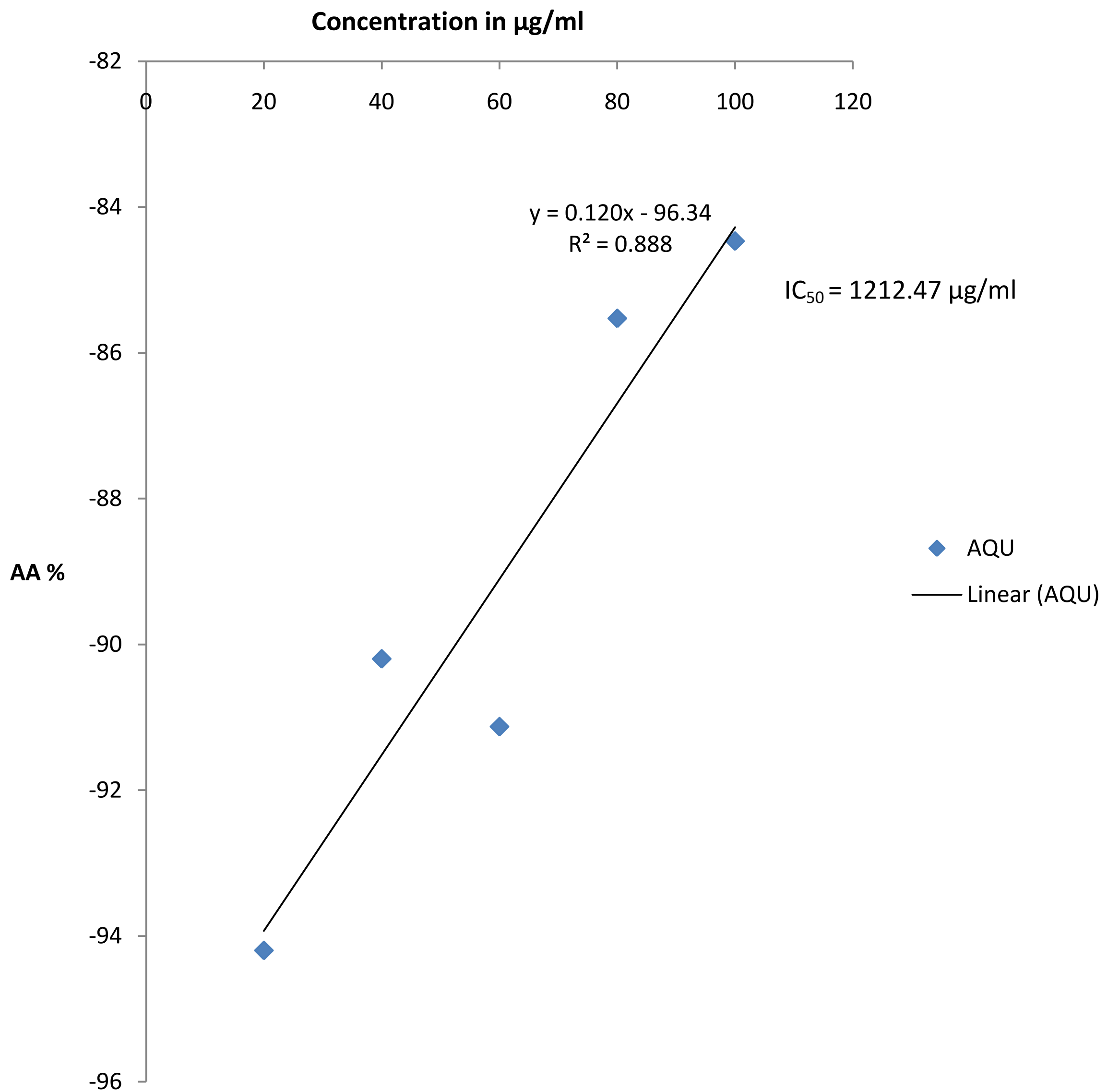


Linear regression graph and IC_{50} for n-butanol fraction of *E. guineensis* pseudobulbs methanol extract using DPPH method

IC_{50} calculated using regression equation, $y = ax + b$, where y= 50% antioxidant activity, x = IC_{50}, a = regression coefficient and b = regression constant.

NB: AA value at 1 µg/ml is 45.13 % and at 10 µg/ml is 65.3 %.

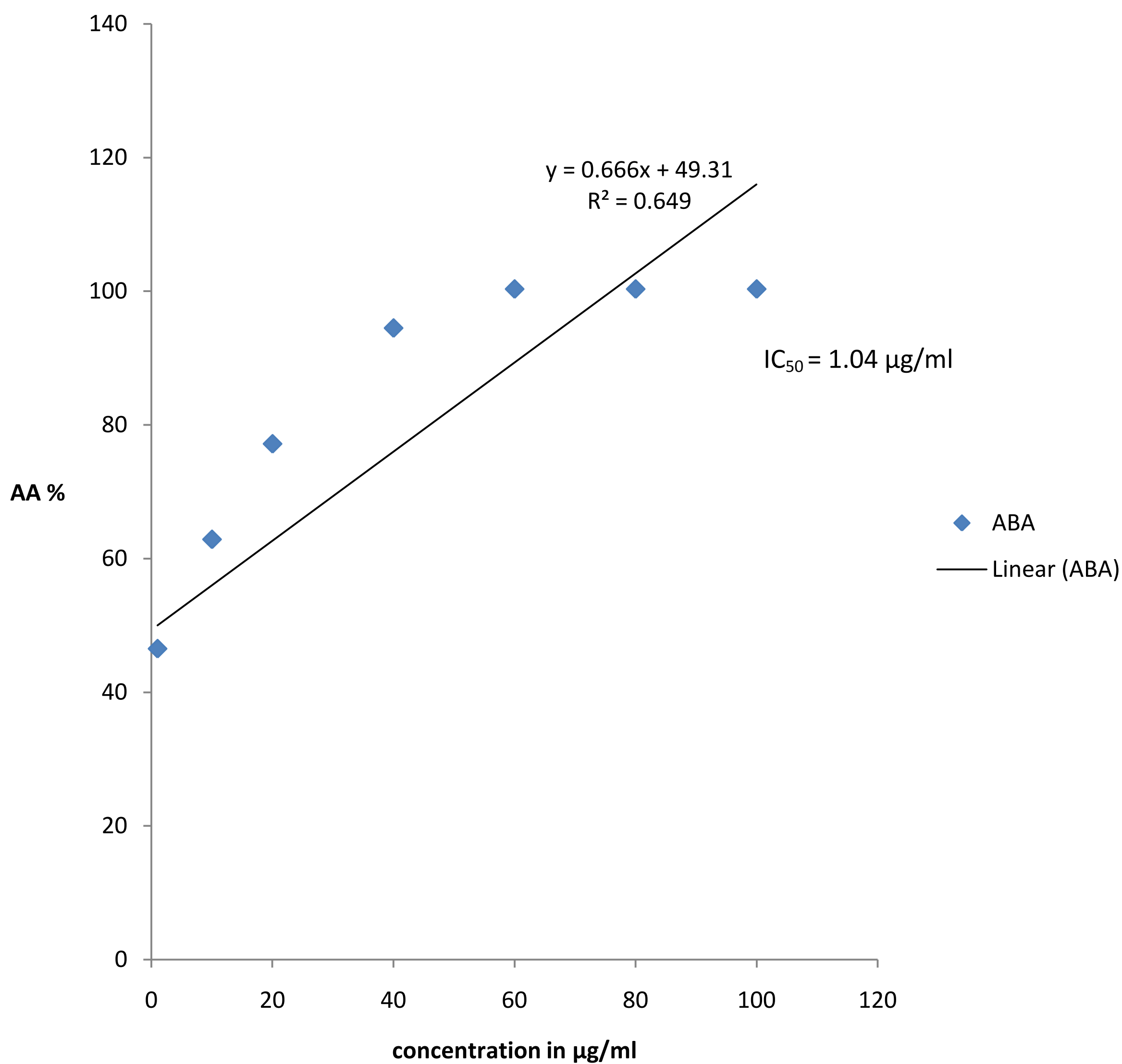
APPENDIX V



Linear regression curve and IC_{50} for the aqueous fraction of *E. guineensis* pseudobulbs methanol extract using DPPH method

IC_{50} calculated using regression equation, $y = ax + b$, where y= 50% antioxidant activity, x = IC_{50}, a = regression coefficient and b = regression constant.

APPENDIX VI



Linear regression graph and IC_{50} for ascorbic acid using DPPH method

IC_{50} calculated using regression equation, $y = ax + b$, where y= 50% antioxidant activity, x = IC_{50}, a = regression coefficient and b = regression constant.

NB: AA value at 1 µg/ml is 46.48 % and at 10 µg/ml is 62.84 %.