CHEMICAL CONSTITUENTS, ANTI-INFLAMMATORY, ANTI-OXIDANT AND ANTIMICROBIAL ACTIVITIES OF THE STEM BARK AND LEAVES OF *FICUS EXASPERATA* (VAHL)

A Thesis submitted in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

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Faculty of Pharmacy and Pharmaceutical Sciences,

College of Health Sciences

Kwame Nkrumah University of Science and Technology

by

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FEBRUARY, 2012

DECLARATION

I hereby declare that the experimental work described in this thesis is my own work towards the PhD and to the best of my knowledge, it contains no material previously published by another person or material which has been submitted for any other degree of the University, except where due acknowledgement has been made in the text.

…… ……

Isaac Kingsley Amponsah Date

Certified by:

…… ……

Prof. T. C. Fleischer Date

(Supervisor)

Certified by:

…… ……..

Dr. (Mrs) Rita Akosua Dickson Date

(Head of Department)

DEDICATION

I dedicate this thesis to my late father and mentor Mr. James Kingsley Amponsah

ABSTRACT

The work presented in this thesis involves the scientific investigation of the traditional uses of the leaves and stem bark of *Ficus exasperata* Vahl (Moraceae) as an anti-inflammatory, analgesic and wound healing agent. It also describes the isolation and characterization of the active principles from the *Ficus exasperata*. The petroleum ether, chloroform, ethyl acetate and 70% ethanolic extracts of the leaves and stem bark were assessed for anti-inflammatory, antioxidant and anti-microbial activities. The anti-inflammatory activities of the extracts and isolates were investigated using the carrageenan – induced foot pad eodema model in seven – day old chicks. The extracts were given orally to the chicks at 30, 100 and 300 mg/kg body weight, 1 hour after induction of oedema with carrageenan. Diclofenac and dexamethasone were used as reference drugs and the foot volume measured by water displacement plethysmography for five hours. All extracts exhibited anti – inflammatory effect with the stem bark showing the highest activity (ED_{50} = 50.65± 0.012). Antioxidant properties of the extracts were investigated using five assays; total antioxidant capacity, total phenolic content, DPPH scavenging activity, reducing power and lipid peroxidation activity. The most active antioxidant extract was the stem bark with IC_{50} values of 42.27±0.012, 20.09±0.001 and 61.80±0.001 µg/ml for the lipid peroxidation, DPPH scavenging and reducing power assays. The respective values for the standard antioxidant compound *n*-propyl gallate were 73.54±0.014, 10.8±0.002 and 66.88±0.002 µg/ml. Antimicrobial evaluation of extracts at concentrations of 10 mg/ ml was done using the agar well diffusion and micro-dilution assays. Seven organisms; *P. aeruginosa, S. typhi, K.*

pneumoniae, E. coli, B. subtlilis, S. aureus and *C. albicans* were used. The chloroform extract of the stem bark was the most active with MIC of 1000 µg/ml against *P. aeruginosa* and *S. aureus*. Bergapten, oxypeucedanin hydrate and sitosterol-3-O-*β*-D-glucopyranoside were isolated from the bioactive chloroform extract of the stem bark whereas *β*-sitosterol and sitosterol-3-O-*β*-D-glucopyranoside were isolated from the pet-ether and ethyl acetate extracts of the leaves. To the best of our knowledge, this is the first report of the isolation of these compounds in *Ficus exasperata*. They exhibited dose-dependent anti-inflammatory activities with ED_{50} values of 101.6 ± 0.003, 126.4 ± 0.011, 275.9 ± 0.012 and 123.4 ± 0.033 mg/kg body weight for bergapten, oxypeucedanin hydrate, sitosterol-3-O-*β*-D-glucopyranoside and *β*-sitosterol respectively. They also showed significant DPPH scavenging effect with IC_{50} values of 63.38 ± 0.010, 46.63 ± 0.011, 220.3 ± 0.031 and >1000 µg/ml for the respective compounds. In the antimicrobial assay, *β*-sitosterol and its glucoside were inactive against all the organisms. Bergapten and oxypeucedanin hydrate gave MIC's >1000 µg/ml against all susceptible organisms. They were the most active anti-inflammatory, anti-oxidant and antimicrobial compounds. The results of these studies have demonstrated that extracts of the leaves and stem bark of *F. exasperata* possess anti-inflammatory activity and also display antioxidant and antimicrobial activities. These findings provide scientific justification for the use of the stem bark and leaves of *F. exasperata* Vahl, in various traditional medicines, for the treatment of inflammatory and infectious conditions.

ACKNOWLEDGEMENT

'Not to us, O LORD, not to us but your name be the glory, because of your love and faithfulness'. Thank you Jesus for the grace to complete this research.

My sincere gratitude goes to Professor T. C. Fleischer, who kindly supervised this research. His ideas, advice and tireless encouragement have been most valuable. May you abound in the grace and blessings of God. I am also greatly indebted to Dr. Abraham Yeboah Mensah and Dr. Mrs Rita Dickson for their support as heads of Pharmacognosy Department, during the period of this research. I express my sincere thanks to the teaching staff and my colleagues at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical sciences, KNUST, for creating a pleasant working atmosphere. I also express my appreciation to the laboratory technicians of the Departments of Pharmacognosy, Pharmacology and Pharmaceutical microbiology for their excellent technical assistance.

I would like to thank Professor Eric Woode for guiding me in the anti-inflammatory assay. Also many thanks go to Professor Sandy Gray and Dr. John Igoli, of the Signal Processing and Information Base (SPIB), University of Strathclyde, Glasgow, for generously running and providing us with the NMR spectra of the isolates.

Finally, my warmest thanks are due to my family – parents J. K Amponsah and Maame Araba who have been a springboard in my academic pursuits. Many thanks to my dear wife Linda – for her understanding and never failing support. The loving and humorous atmospheres during difficult moments were most refreshing.

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ABBREVIATIONS

^{13}C-NMR Carbon-13 Nuclear Magnetic Resonance

^{1}H-NMR Proton Nuclear Magnetic Resonance

5-LOX 5-Lipooxygenase

AAE……..Ascorbic acid equivalent

ANOVA……....Analysis of variance

ASTM……...American Standard Test Method

ATCC……...American Type Culture Collection

CFU……..Colony forming unit

COX……..Cyclooxygenase

DEPT……Distortionless Enhancement by Polarization Transfer

DMSO……...Dimethyl sulfoxide

DPPH……2, 2-diphenyl-1-picrylhydrazyl

HMBC……..Heteronuclear Multiple Bond Correlation

HSQC……...Heteronuclear Single Quantum Coherence

LT…….Leukotrienes

MBC…….....Minimum bactericidal concentration

MDA…….Malondialdehyde

MIC……...Minimum inhibitory concentration

MTT…….....(3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NCTC.....National Collection of Type Cultures

NMR……..Nuclear Magnetic Resonance

NSAIDS…….....Non-steroidal anti-inflammatory drugs

PAF……....Platelet Activation Factor

PGs……....Prostaglandins

R_f……..…Retardation factor

TAE……...Tannic acid equivalent

TBA……...Thiobarbituric acid

TLC……Thin layer chromatography

UV……..Ultraviolet

WHO……..World Health Organisation

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The use of natural products with therapeutic properties is as ancient as human civilisation. For a very long time, mineral, plant and animal products were the main sources of drugs (De-Pasquale, 1984). The development of organic chemistry resulted in a preference of synthetic products for pharmacological treatment. The reasons for this were that structural modification of synthetic compounds to produce potentially more active and safer drugs could be performed. Furthermore, the treatment of diseases with natural products differed from place to place as various cultures had different points of view regarding the treatment of various diseases (Rates, 2001). Thus the twentieth century became a triumph for the synthetic-chemistry-dominated pharmaceutical industry, which replaced natural extracts with synthetic molecules that often had no connection to natural products. The spectacular rise of the pharmaceutical industry had a tremendous impact on disease treatment and prevention, saved countless lives and became one of the outstanding achievements of the twentieth century. Thus conventional medicine by and large brought serious infectious diseases under control, although there were worrying signs that infectious organisms were becoming resistant to antibiotic treatment (Chevalier, 1995). However, some synthetic drugs had serious side effects.

There has been a renewed interest for plants as source of pharmaceuticals (Houghton, 1995). Some herbal preparations are now so commonly used that they are accepted as part of everyday life. An example is evening primrose oil, which is used by hundreds of thousands of women in Britain to help relieve pre-menstrual tension (Chevalier, 1995).

About 25% of the drugs prescribed worldwide come from plants, with 121 such active compounds being in current use (Rates, 2001). Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively of plant origin and a significant number are semi-synthetic drugs obtained from natural precursors (Houghton, 1995). Examples are the cardiotonic glycosides of *Digitalis* species such as digoxin, the analgesics morphine and codeine obtained from the Opium poppy *Papaver somniferum*, the antispasmodic tropane alkaloids hyoscine and atropine from *Atropa belladonna*, quinine and quinidine from *Cinchona* species and vincristrine and vinblastine from *Catharanthus roseus* (Rates, 2001). It is estimated that 60% of anti-tumour and anti-infectious drugs in use or under clinical trials are of natural origin (Yue-Zhong, 1998). The vast majority of these cannot be synthesised and are still obtained from wild or cultivated plants. Natural compounds can thus be lead compounds, allowing the design, development and the discovery of new therapeutic agents (Hamburger and Hostettmann, 1991).

A search in the natural product alert database, suggested that only about 15 % of all plant species had been studied to some extent for their phytochemistry and only about 5 % for one or more biological activities (Verpoorte, 2000). Although extensive research on medicinal plants is published every year, only a few plants have been comprehensively

studied for their pharmacological properties. Thus traditional medicines and medicinal plants obviously represent a great source of novel medicines and leads for drug development.

1.2 JUSTIFICATION OF RESEARCH

Pain and inflammation are associated with virtually all diseases. Typical inflammatory diseases such as rheumatoid arthritis, asthma, colitis and hepatitis are the leading cause of disability and death (Emery, 2006). Recently, chronic inflammation has been found to contribute to the development of cancer, cardiovascular and neurodegenerative diseases (Willerson and Ridker, 2004).

The conventional drugs used for the management of pain and inflammation include the steroidal and non-steroidal anti-inflammatory agents as well as the opiates. It is estimated that about 30 million people worldwide take non-steroidal anti-inflammatory drugs (Mc gettigan and Henry, 2000; Derle *et al.*, 2006). However, their prolonged use has been associated with serious side effects such as gastric ulceration, hemorrhage, bronchospasm and kidney dysfunction (Lin *et al.*, 2006). Also because of adverse effects such dyslipidaemia, Cushing's syndrome, hypertension and immunosuppression by steroidal anti-inflammatory drugs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in some cases (Dharmasiri *et al.*, 2003). Therefore there has been an intense search for new anti-inflammatory and analgesic drugs lacking these side effects as alternatives to these drugs. In this direction, attention has been focused on the investigation of the efficacy of plant-

based drugs used in traditional medicine. This is because investigation of the efficacy of certain plant based drugs used in traditional medicine led to the discovery of potent anti-inflammatory agents in clinical use such as aspirin and colchicine (Berman, 2004). Other natural products, with marked anti-inflammatory effects, which have provided effective adjuncts to the management of inflammatory conditions include: omega 3-fatty acids from fish oil, curcumin from the spice turmeric (*Curcuma longa*), frankincense from *Boswellia serrata*, capsaicin from *Capsicum annum* and ginger root extract from *Zingiber officinalis* (Satoskar, 1996). These agents have been used successfully as adjucts in several chronic inflammatory diseases (Srivastava and Mustafa, 1992). Thus plants still present a large source of compounds that might serve as leads for the development of novel anti-inflammatory drugs. Unlike their synthetic counterparts, they have not been shown to accelerate cartilage destruction or produce liver and kidney toxicities (Boon and Smith, 1997). For these reasons, natural products can be considered as viable alternatives to conventional anti-inflammatory drugs in a large percentage of patients suffering from various inflammatory diseases.

The excessive production of reactive oxygen metabolites by phagocytic leucocytes during the inflammatory process, as part of host defence, deregulates cellular function causing tissue injury which in turn augments the state of inflammation leading to chronic inflammatory diseases (Wu *et al*., 2006). These oxidants also inhibit wound healing. Antioxidants, which scavenge these reactive oxygen metabolites, have been found to complement the anti-inflammatory process, promote tissue repair and wound healing. A number of plant secondary metabolites such as apigenin, quercetin, luteolin and silymarin

have been found to exhibit anti-inflammatory activities due to their antioxidant properties (Chi *et al.*, 2001; Eleni and Dimitra, 2003). Thus antioxidative phytochemicals, especially phenolic compounds, found in vegetables, fruits and medicinal plants could be explored for their potential role in the prevention of inflammatory diseases.

Plants have also been a source of various anti-infective agents. The bacteriostatic and fungicidal properties of lichens and the antimicrobial action of allicin in garlic (*Allium sativum*) are a few examples of age-old antibacterial therapy (Robbers *et al*, 1996). An expansive range of plants belonging to an equally wide variety of plant families, have yielded products with antibacterial properties. Such families include the Asteraceae, Euphorbiaceae, Apocynaceae, Fabaceae, Leguminoceae and Rutaceae (Roy and Saraf, 2006). Although no major antimicrobial drug has been developed from higher plants, innumerable studies have generated data showing antimicrobial properties of medicinal plants (Ellof, 1990). This body of results indicates that medicinal plants, even if not fully developed into new mainstream antimicrobial drugs, could indeed be an effective alternative if properly processed and incorporated into primary health care systems.

One plant, commonly used in various ethnomedicines in the West African sub-region is *Ficus exasperata*. In Cote d'ivoire, the powdered leaves are applied to leprous sores (Bouquet, 1969). Citizens of Bosomtwi-Atwima-Kwanwoma district of Ghana use poultices of the leaves and stem bark for the treatment of wounds, boils, burns (Agyare *et al.*, 2009). In the Democratic republic of Congo a leaf poultice is used for ring worm (Burkill, 1985). Chest pains are treated in the Gambia by steam inhalation of the decoction of the leaves. Sap from the stem bark is used for the treatment of wounds,

sores, abscesses, eye ailments, and stomach-ache (Burkill, 1985). A decoction of the bark is taken against worms, haemorrhoids and abnormal enlargement of the spleen (Ijeh and Ukweni, 2007). Thus the leaves and stem bark of *F. exasperata* may contain secondary metabolites with anti-infective, anti-inflammatory and anti-oxidant activities. The purpose of this research therefore, is to investigate the anti-inflammatory, anti-oxidant and anti-infective properties of the leaves and stem bark of *F. exasperata* and isolate the compounds which may be responsible for these activities.

1.3 OBJECTIVES OF RESEARCH

The objectives of this research are;

1. To investigate the anti-inflammatory activity of the leaves and stem bark of *F. exasperata* using the carrageenan-induced foot oedema in the 7-day old chick.
2. To investigate the anti-oxidant and anti-infective properties of the leaves and stem bark of *F. exasperata*.
3. To isolate the bioactive constituents using various chromatographic techniques.
4. To characterize and identify the various isolates using spectroscopic methods
5. To investigate the isolates for anti-inflammatory, anti-oxidant and anti-microbial activities

Chapter 2

LITERATURE REVIEW

2.1 THE FAMILY MORACEAE

Moraceae, often called the mulberry family, is a family of flowering plants comprising of about 40 genera and over 1000 species of which over 500 species are members of the genus *Ficus* (http://science.jrank.org/pages/4494/Mulberry-Family-Moraceae.html). They occur primarily in tropical and semi-tropical regions, and include a wide variety of herbs, shrubs, and trees, characterized by a milky sap (Duncan and Duncan, 1988; Everett, 1968; Godfrey, 1988). The Moraceae is a member of the order Urticales, class Magnoliopsida (the dicotyledons), division Magnoliophyta (flowering plants). Moraceae have been divided into five tribes: Artocarpeae, Moreae, Dorstenieae, Ficeae, and Castilleae (Rohwer *et al.*, 1993). Ficeae has only one genus, *Ficus*, with approximately about 750 species largely distributed in the tropics and subtropics (Shannon *et al.*, 2004). They can be either monoecious with bisexual inflorescences or dioecious (Weiblen, 2000). Taxonomically Ficeae is divided into two main groups (Berg and Hijman, 1989; Corner, 1965); one group, comprising of the subgenera *Urostigma* and Pharmacosycea, consists of approximately 370 species, all of which are monoecious. The second group comprises of the subgenera *Sycomorus* and *Ficus*. *Sycomorus* consist of about 13 species, all of which are monoecious. Artocarpeae are represented by 12 genera and 87 species, including the economically important *Artocarpus* (jackfruit, breadfruit) (Berg, 1988; Jarrett, 1959). Castilleae include eight genera, distributed throughout the

neotropics and to a limited extent in tropical Africa (Shannon *et al*., 2004). Dorstenieae consist of eight genera (*Bosqueiopsis*, *Brosimum*, *Dorstenia*, *Helianthostylis*, *Scyphosyce*, *Trilepisium*, *Trymatococcus*, *Utsetela*) distributed in the neotropics (Berg, 1988).

2.1.1 Morphology

Flowers of the Moraceae occur in groups known as heads, spikes or catkins. Flowers are produced inside a synconium, a hollow fleshy structure. The small flowers lack petals. Male flowers consist of four sepals, which are usually leaf-like appendages, and four stamens. Female flowers consist of four sepals and a pistil with a two-chambered ovary (http://science.jrank.org/pages/4492/mulberry-family-moraceae-flowers-fruits-leaves.html). The fruit, developed from a single female flower, is either a fleshy drupe or a dry achene. The flowers fuse as they mature after fertilization and a multiple fruit forms. The multiple fruit consists of small drupes or achenes grouped together in a single unit, and are usually round or oval shaped. The best known fruit of the Moraceae is that of the common *Ficus* species, *Ficus carica*, which has been cultivated for thousands of years (Duncan and Duncan, 1988; Everett, 1968; Godfrey, 1988).

Moraceae leaves occur in a variety of shapes and sizes. For example, breadfruit, (*Artocarpus communis*), has lobed leaves that reach 61 cm in length. *Ficus carica* also has deeply lobed leaves. Other species, such as the creeping fig, *Ficus pumila*, have cordate leaves that are much smaller with entire margins. Leaves can occur singly on the stem, on alternating sides. At the base of a young leaf's petiole is a pair of stipules, but these soon fall off leaving small scars on the stem. Species of the Moraceae may be

evergreen, or they may have deciduous leaves that fall off at the end of the growing season (Everett, 1968; Godfrey, 1988)

2.2 THE GENUS *FICUS*

The genus *Ficus* consist of woody trees, shrubs, vines, epiphytes, and hemiepiphytes (Sirisha *et al*., 2010). They are collectively known as fig trees or figs. They are native throughout the tropics with few species extending into the semi-warm temperate zones.

2.2.1 Ethnomedicinal uses of *Ficus* species

The use of medicinal plants to improve health is as old as humanity. Among these plants, none may be older than the fig (Lansky *et al*., 2008). A number of *Ficus* species are used for medicinal purposes in Ayurvedic and Traditional Chinese Medicine especially amongst people where these species grow. These uses, however, originated and are most widely found in the Middle East. In Iran, a decoction of the fruits of *Ficus carica* is taken orally for bronchitis, cystitis and nephritis (Zagari, 1992). Pharyngitis and stomatitis are treated with an oral decoction of the dried shoots (Darias *et al*., 1986). In West Africa and Papua New Guinea, the dried leaf buds of *F. septica* are taken orally for headache and gastroenteritis. Other *ficus* species and their ethnomedicinal uses are presented in the Table 2.1.

Table 2.1 Ethnomedicinal uses of *Ficus spp* (Lansky *et al*., 2008)

Ficus species	Uses	Place
F. toxicaria (leaf, oral decoction)	Gastroenteritis	Indonesia
F. maxima (leaf and stem, Oral decoction)	Gingivitis	Honduras
F. thunbergii (leaf, oral decoction)	Lumbago, rheumatism	Japan
F. religiosa (leaf, oral decoction)	Asthma, cardiac oedema, gastritis	South Korea
F. religiosa (dried fruit, oral decoction)	Tuberculosis, fever	Bangladesh
F. carica (latex, aqueous decoction, external)	Warts	Italy
F. carica (stem latex, external)	Pain, scorpion bite	Turkey

2.2.2 Biological activities of *Ficus* species

A number of *Ficus* species have shown diverse biological and pharmacological activities. They have been investigated as potential repository of natural products for the treatment of various diseases including tumors, inflammatory diseases, wound healing and as anti-oxidants.

2.2.2.1 Anti-inflammatory activity

A number of *Ficus* species have been reported by many authors to exhibit anti-inflammatory activities. For instance, in a test of anti-inflammatory activity, an aqueous extract of the root bark obtained from *Ficus elastica* significantly reduced carrageenan-induced ear oedema, compared to the positive control, indomethacin (Sackeyfio and Lugeleka, 1986). The ethanolic stem bark extract of *Ficus racemosa* inhibited cyclooxygenase-1(COX-1) (Li *et al.,* 2003) and 5-lipoxygenase (5-LOX) enzymatic activities (Li *et al.,* 2004) and prevented chemically induced renal oxidation and carcinogenesis (Khan and Sultana, 2005). Also, extracts of the leaves of *F. racemosa* demonstrated anti-inflammatory activity against carrageenan, serotonin, histamine, and

dextran – induced rat paw oedema models, similar to the positive control, phenylbutazone (Mandal *et al.*, 2000).

2.2.2.2 Anticancer activity

Latex from *Ficus* species were investigated for anticancer activities as far back as the 1940's (Lansky *et al*., 2008). For example, fig latex was tested on bovine teat papilloma and activity compared to salicylic acid. They were equally effective in treating teat papillomatosis in cows (Hemmatzadeh *et al*., 2003). Also ethanolic extracts of *Ficus citrifolia* enhanced intracellular accumulation of daunomycin in K562/R7 leukemic cells and the cytotoxic effect of vinblastine on the growth of multi-drug resistant human sarcoma MESSA/Dx5 cells. Thus the extract plays an adjunctive role in cancer chemotherapy (Simon *et al*., 2001).

2.2.2.3 Antioxidant and Hypolipidaemic properties

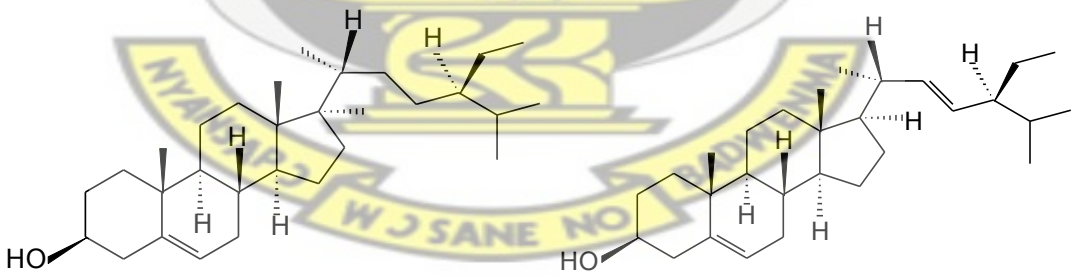
Figs have also been shown to possess antioxidant and hypolipidaemic activities. In a study using rabbits on high cholesterol diets, an aqueous extract of the bark of *Ficus benghalensis* significantly reduced cholesterol and triglyceride levels compared with those fed with the same high cholesterol diet alone. In addition to this hypolipidaemic activity, there was a decrease in lipid peroxidation and an increase in antioxidant enzymes in rabbits given the fig extract (Shukla *et al*., 2004). The leaves of *Ficus exasperata* also protected against the formation of excessive peroxide levels in palm oil when used during its preparation, presumably due to its antioxidant properties (Umerie *et al.*, 2004).

2.3 PHYTOCHEMISTRY OF FICUS SPECIES

Several authors have isolated and identified various classes of compounds from the genus *Ficus*. They include flavonoids, alkaloids, phenolic acids, steroids, saponins, tannins, terpenoids and coumarins.

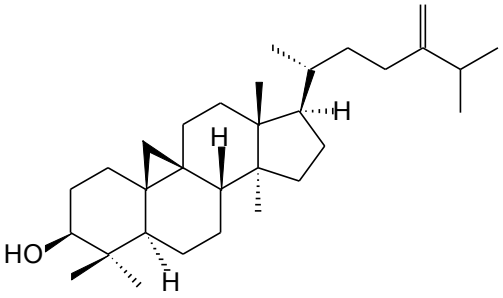
2.3.1 Steroids

Very few plant sterols have been reported in this genus. *β*-sitosterol **[1]** was isolated from the leaves and roots of *F. carica* and *F. septica* respectively (Li *et al.,* 2006; Wu *et al.,* 2002). Stigmasterol **[2]** has been reported in the leaves and roots of *F. hirta* and *F. septica* (Li *et al.,* 2006; Wu *et al.,* 2002). 24-methylenecycloartenol **[3]**, ψ-taraxasterol ester **[4]**, lupeol **[5]** and baurenol **[6]** have been isolated from the leaves of *F. carica* (Ahmed *et al.,* 1988; Saeed and Sabir, 2002), whereas sitosterol-3-O-*β*-D-glucoside **[7]** has been reported from the leaves of *F. septica* (Wu *et al.,* 2002). *β*-sitosterol-*α*-D-glucoside **[8]** has been isolated from the stem bark of *F. benghalensis* (Joseph and Raj, 2011).

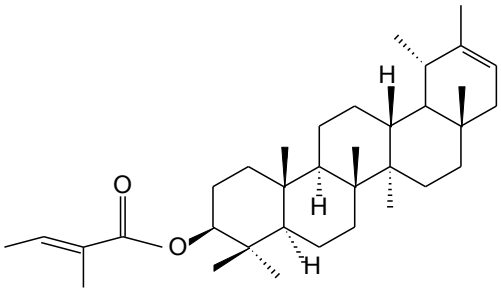


β-sitosterol **[1]**

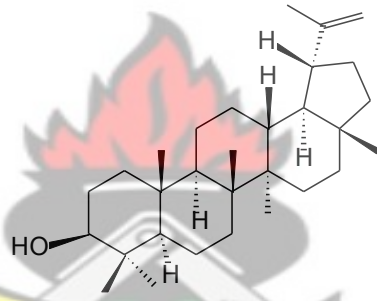
Stigmasterol **[2]**



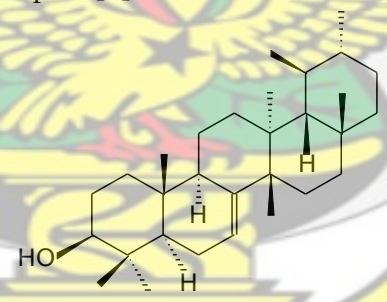
24-methylenecycloartenol **[3]**



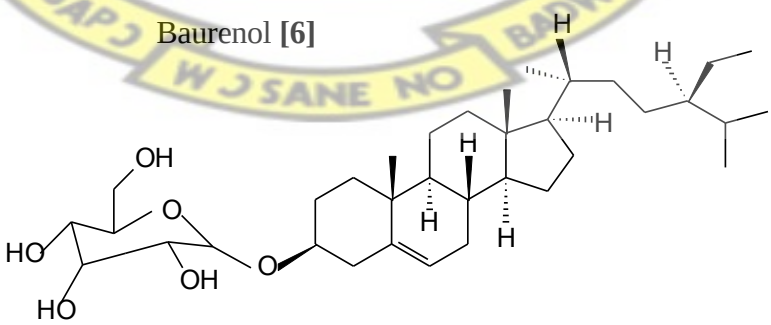
ψ-taraxasterol ester **[4]**



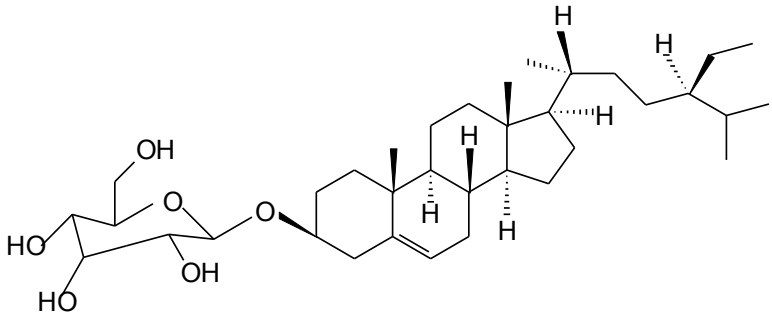
Lupeol **[5]**



Baurenol **[6]**



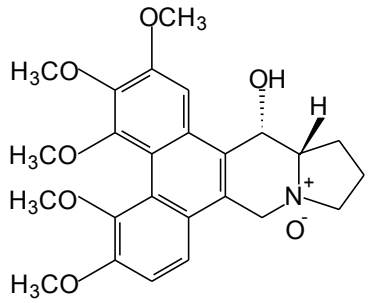
Sitosterol-3-o-*β*-D-glucopyranoside **[7]**



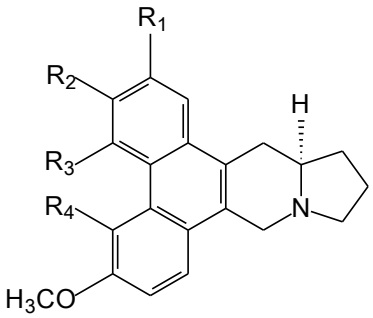
Sitosterol-3-o-α- D-glucopyranoside **[8]**

2.3.2 Alkaloids

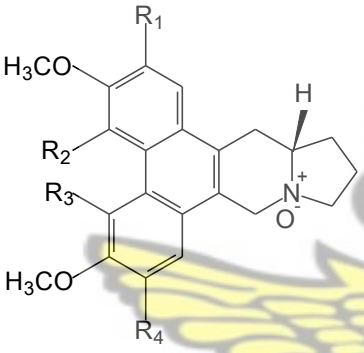
This genus is not well known for alkaloids. Few alkaloids, mostly phenanthroindolizidine alkaloids, have being isolated from the leaves and stem bark of *F. septica* and *F. hispida*. The alkaloids, ficuseptine-A **[9]**, antofine **[13]** and tylophorine **[14]** have been isolated from the leaves of *F. septica* whereas ficuseptine-B **[10]** ficuseptine-C [**11**] and ficuseptine-D **[12]** have been reported from the stem bark. Tylocrebrine **[15]** and isotylocrebrine **[16]** have been isolated from both the leaves and stem bark of *F. septica* (Baumgartner *et al.*, 1990; Damu *et al.*, 2005; Wu *et al.*, 2002; Yang *et al.*, 2006). Hispidine **[17]** and 2-demethoxytylophorine **[18]** have been reported from the stem bark and leaves of *F. hispida* respectively (Venkatachalam and Mulchandani, 1982).



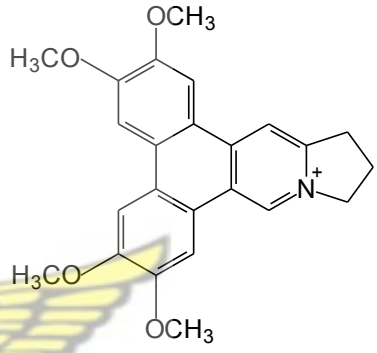
Ficuseptine-A [**9**]



Ficuseptine-B [**10**], R_1, R_2 = OCH_2O, R_3 =H, R_4 = OCH_3
Ficuseptine-C [**11**], R_1, R_2 = OCH_2O, R_3, R_4 =H
Ficuseptine-D [**12**], R_1 = H, R_2, R_3 = OCH_3, R_4 = H



Antofine [**13**], R_1 = OCH_3, R_2, R_3, R_4 = H
Tylophorine **[14]**, R_1, R_2, R_3 = H, R_4= OCH_3
2-methoxytylophorine **[18]**, R_1, R_2, R_3= H, R_4 = OCH_3
Tylocrebrine **[15]**, R_1 =H, R_2 = OCH_3, R_3= H, R_4 = OCH_3
Isotylocrebrine **[16]**, R_1 =H, R_2 = OCH_3, R_3= H, R_4 = OCH_3

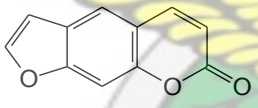


Hispidine **[17]**

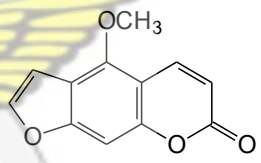
2.3.3 Coumarins

Another class of constituents commonly found in this genus are coumarins particularly, of the furanocoumarin type. They have been isolated from the stem, leaves and roots of some *Ficus* species and are reported to be responsible for the contact dermatitis associated with *Ficus* species (Zaynoun *et al.*, 1984). The coumarins psoralen **[19]**, bergapten **[20]**, marmesin **[21]**, umbelliferone **[22]** and 4^1, 5^1-dihydropsoralen **[23]** have been reported in

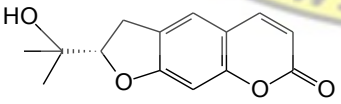
the leaves, and stem bark of *F. carica* (Innocenti *et al.*, 1982). Psoralen has also been reported in the roots of *F. hirta* (Li *et al.*, 2006). Chang *et al.*, (2005) isolated 5-O-*β*-D-glucopyranosyl-6-hydroxyangelicin **[24]**, 6-O-*β*-D-glucopyranosyl-5-hydroxyangelicin **[25]**, 5-O-*β*-D-glucopyranosyl-8-hydroxypsoralen **[26]**, 8-O-*β*-D-glucopyranosyl-5-hydroxypsoralen **[27]** and 5, 6-O-*β*-D-diglucopyranosylangelicin **[28]** from the leaves of *F. ruficaulis*. Esculin **[29]** has also been isolated from the leaves of *F. septica* (Wu *et al.*, 2002). Bergapten **[20]** and oxypeucedanin hydrate **[30]** have been isolated from the aerial parts of *Ficus pumilla* (Pistelii *et al*., 2000). The stem bark of *F. religiosa* has also being found to contain the coumarins bergaptol **[31]** and bergapten **[20]** (Makhija *et al*., 2010).



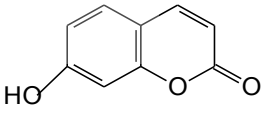
Psoralen **[19]**



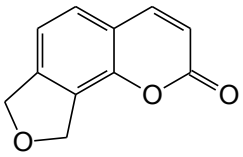
Bergapten **[20]**



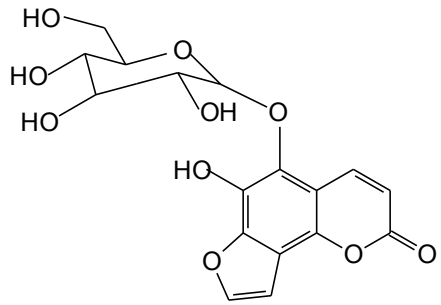
Marmesin **[21]**



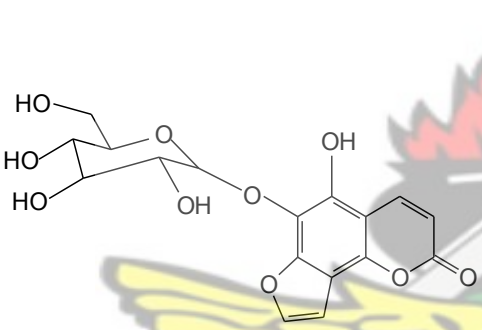
Umbelliferone **[22]**



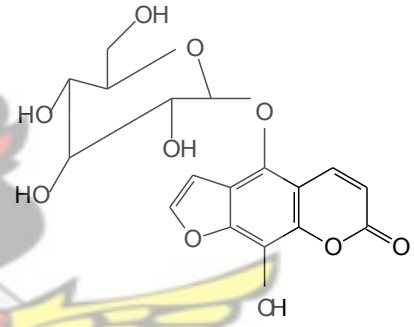
4^1, 5^1-dihydropsoralen **[23]**



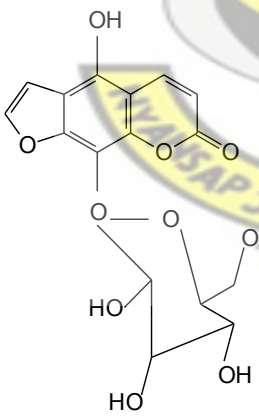
5-O-*β*-D-glucopyranosyl-6-hydroxyangelicin **[24]**



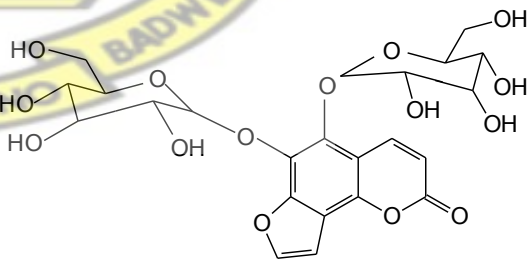
6-O-*β*-D-glucopyranosyl-5-hydroxyangelicin **[25]**



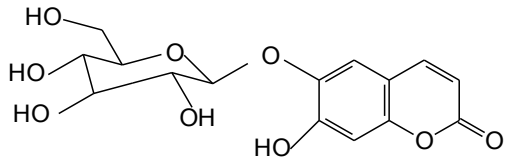
5-O-*β*-D-glucopyranosyl-8-hydroxypsoralen **[26]**



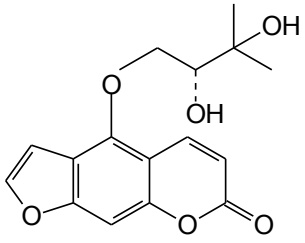
8-O-*β*-D-glucopyranosyl-5-hydroxypsoralen **[27]**



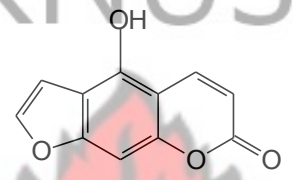
5, 6-O-*β*-D-diglucopyranosylangelicin **[28]**



Esculin **[29]**



Oxypeucedanin hydrate **[30]**

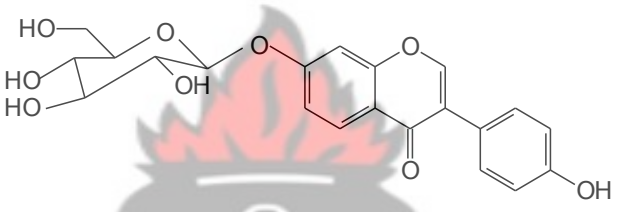


Bergaptol **[31]**

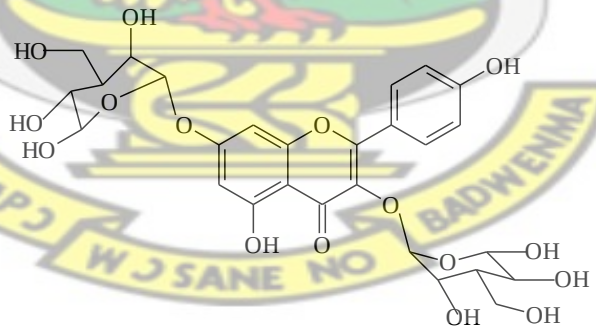
2.3.4 Flavonoids

Members of the genus *Ficus* are known to contain flavonoids. Genistin **[32]** and kaempferitrin **[33]** have been isolated from the leaves of *F. septica* (Wu *et al.*, 2002). Also the leaves of *F. ruficaulis* and *F. carica* are reported to contain rutin **[34]** and isoquercitrin **[35]** (Chang *et al.*, 2005; El-Kholy and Shaban, 1966). Sheu *et al.*, (2005) reported the isolation of carpachromene **[36]**, isoglabranin **[37]** and norartocarpetin **[38]** from the stem bark of *F. formosana*. Luteolin **[39]** has also been isolated from the aerial parts of *F. pumilla* (Pistelli *et al.*, 2000). The flavonoid apigenin **[40]** has been reported in the stem bark and roots of *F. hirta* and *F. formosana* (Sheu *et al.*, 2005; Li *et al.*, 2006). Also, the root of *F. hirta* is reported to contain hesperidin **[41]**, 5-hydroxy-4^1, 6, 7, 8-tetramethoxy flavone **[42]** and 4^1, 5, 6, 7, 8-pentamethoxyflavone **[43]** (Li *et al.*, 2006).

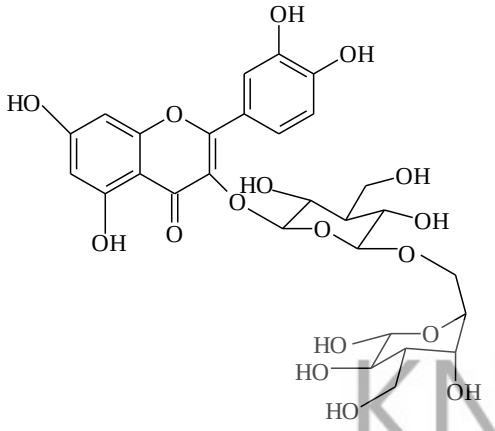
Several other flavonoids, including alpinumisoflavone **[44]**, cajanin **[45]**, derrone **[46]**, 5, 7-dihydroxy-4-methoxy-3^{1}-(2, 3-dihydroxy-3-methylbutyl) isoflavone **[47]**, erycibenin A **[48]**, erycibenin C **[49]**, genistein **[50]**, 3^{1}-(3-methylbut-2-enyl) biochanin A **[51]** and 5,7, 2^{1}-trihydroxy-4^{1}-methoxyisoflavone **[52],** have been isolated from the stem bark of *F. nymphaeifolia* (Darbour *et al.*, 2007).



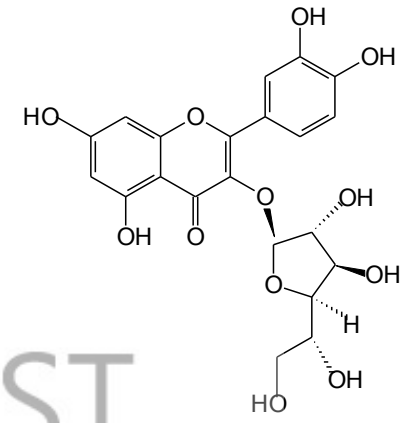
Genistin **[32]**



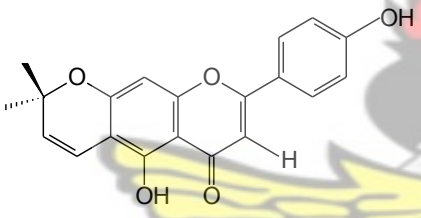
Kaempferitrin **[33]**



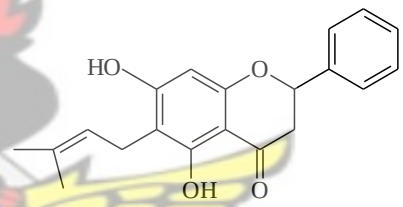
Rutin **[34]**



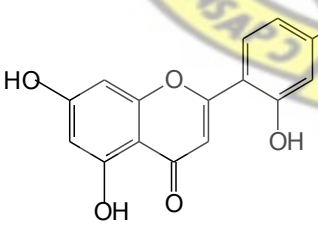
Isoquercitrin **[35]**



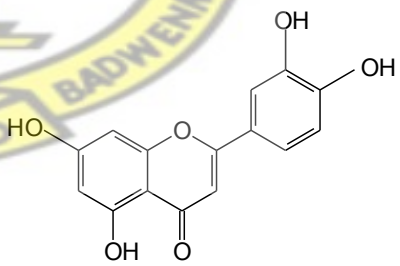
Carpachromene **[36]**



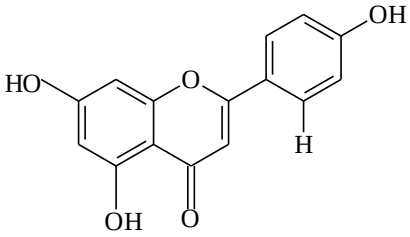
Isoglabranin **[37]**



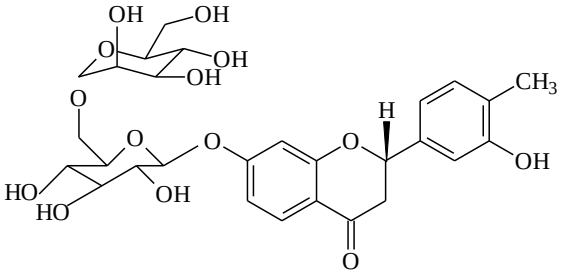
Norartocarpetin **[38]**



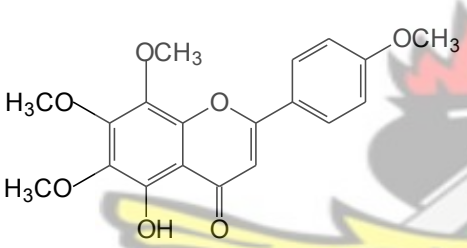
Luteolin **[39]**



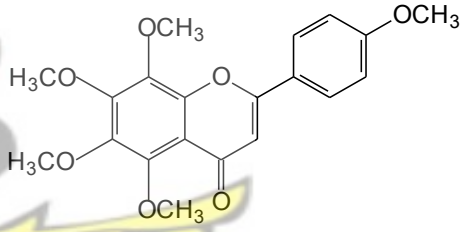
Apigenin **[40]**



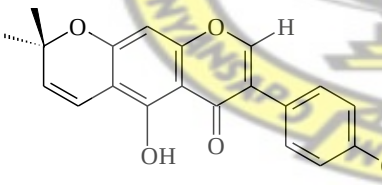
Hesperidin **[41]**



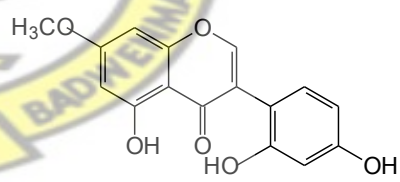
5-hydroxy-4[1], 6, 7, 8-tetramethoxy flavone **[42]**



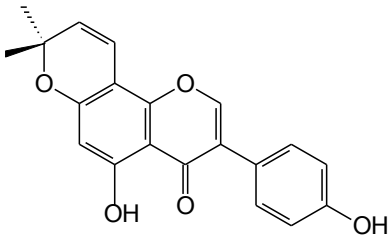
4[1], 5, 6, 7, 8-pentamethoxyflavone **[43]**



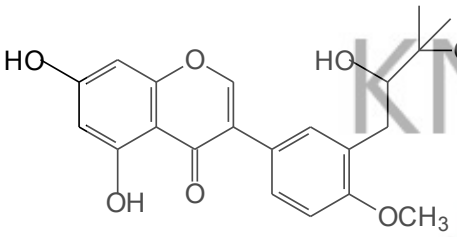
Alpinumisoflavone **[44]**



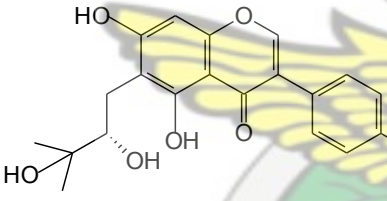
Cajanin **[45]**



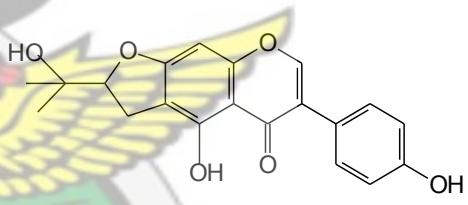
Derrone **[46]**



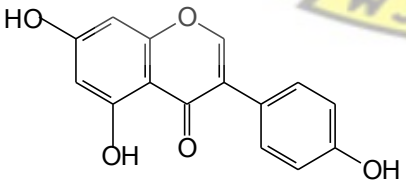
5, 7-dihydroxy-4-methoxy-3[1]-(2, 3-dihydroxy-3-methylbutyl) isoflavone **[47]**



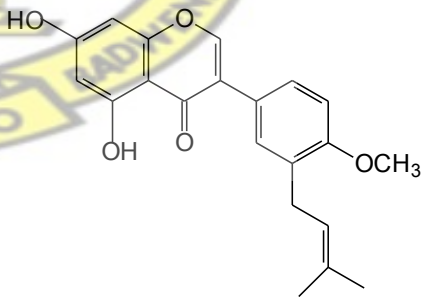
Erycibenin A **[48]**



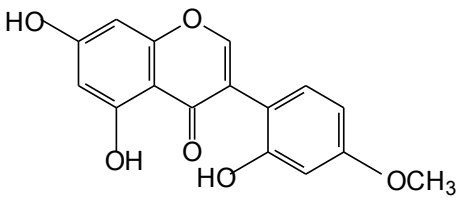
Erycibenin C **[49]**



Genistein **[50]**



3[1]-(3-methylbut-2-enyl) biochanin A **[51]**

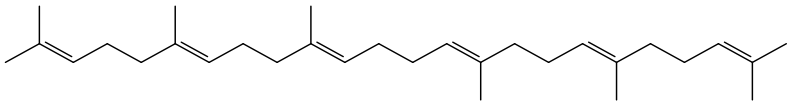


5, 7, 2^1-trihydroxy-4^1-methoxyisoflavone **[52]**

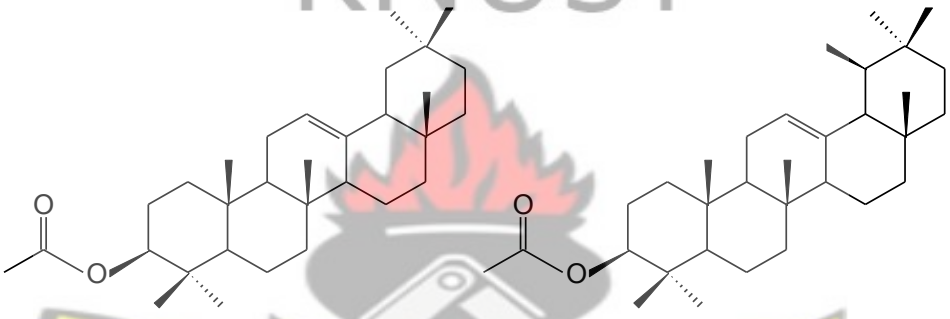
2.3.5 Triterpenoids

Ficus species are known to contain a large number of triterpenoids. Squalene **[53]** has been isolated from the leaves of *F. septica* (Wu *et al*., 2002). Li *et al*., (2006), isolated 3*β*-acetoxy-*α*-amyrin **[54]**, 3*β*-acetoxy-*β*-amyrin **[55]** and 3*β*-hydroxy-stigmast-5-en-7-one **[56]** from the roots of *F. hirta*. A number of triterpenoids with C-28 carboxylic acid functional group have been isolated from the aerial roots of *F. microcarpa*. They include betulonic acid **[57]**, oleanonic acid **[58]**, 3-oxofriedalan-28-oic acid **[59]**, ursolic acid **[60]** and ursonic acid **[61]** (Chiang *et al*., 2005). Lupenol **[62]**, lupenol acetate **[63]**, 20-taraxastene-3*β*, 22*α*-diol **[64]**, 29, 30-dinor-3*β*-acetoxy-18, 19-secolupane **[65]**, 3, 22-dioxo-20-taraxastene **[66]** and 3*β*-hydroxy-20-oxo-29(20→19) abeolupane **[67]** have also been isolated from the aerial roots of *F. microcarpa* (Chiang and Kuo, 2002; Chiang *et al.,* 2005; Chiang and Kuo, 2000). Others include 3-acetoxy-12, 19-dioxo-13(18)-oleanene, 3-acetoxy-1,11-epidioxy-12-ursene, 3-acetoxy-12,13-epoxy-11-hydroperoxyursane, 3-acetoxy-11,12-epoxy-16-oxo-14-taraxerene, 3-acetoxy-11, 12-epoxy-14-taraxerene, 3-acetoxy-20, 21-epoxytaraxastane, 3-acetoxy-21, 22-epoxytaraxastan-20-ol, 3-acetoxy-20, 21-epoxytaraxastan-22-ol and 3-acetoxy-18-

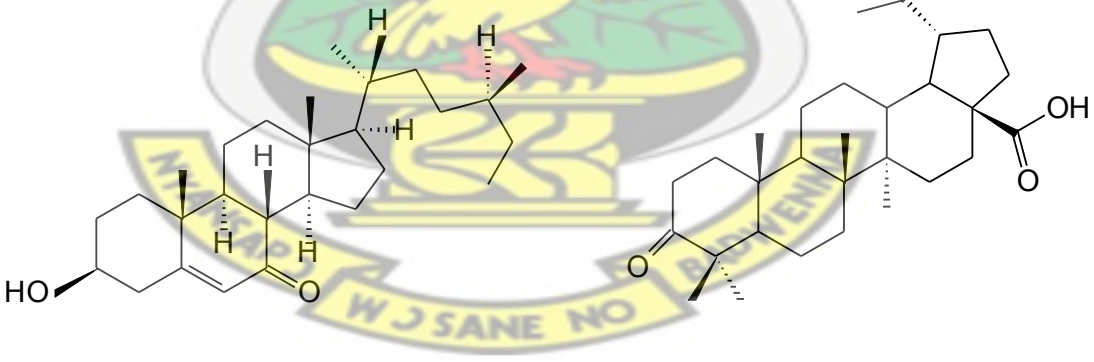
hydroperoxy-12-oleanen-11-one (Chiang and Kuo, 2002; Chiang *et al.*, 2005; Chiang and Kuo, 2000)



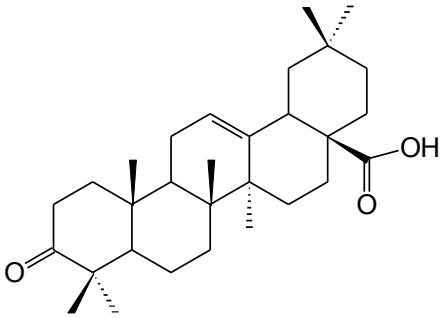
Squalene **[53]**



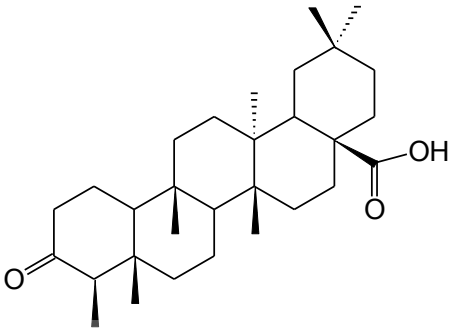
3*β*-acetoxy-*α*-amyrin **[54]** 3*β*-acetoxy-*β*-amyrin **[55]**



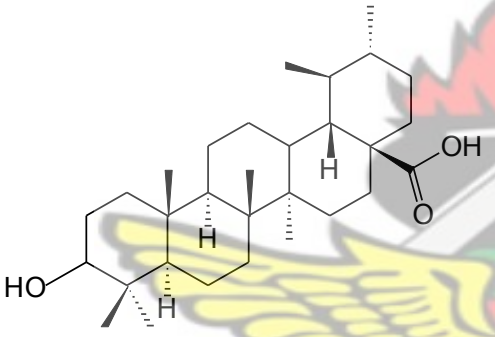
3*β*-hydroxy-stigmast-5-en-7-one **[56]** Betulonic acid **[57]**



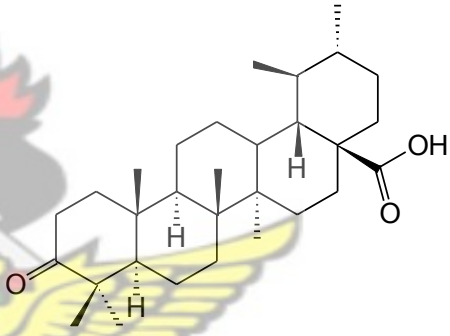
Oleanonic acid **[58]**



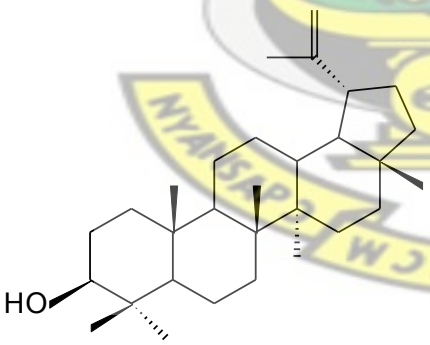
3-Oxofriedalan-28-oic acid **[59]**



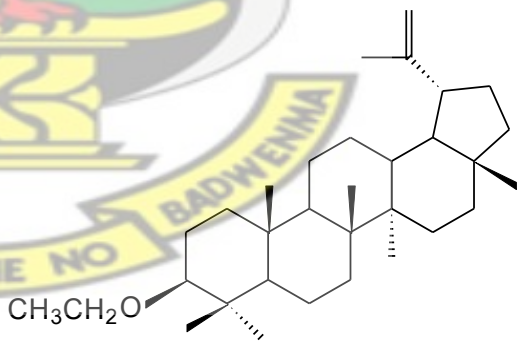
Ursolic acid **[60]**



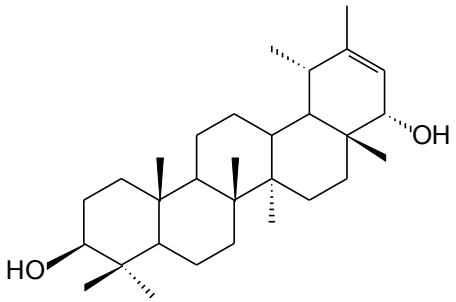
Ursonic acid **[61]**

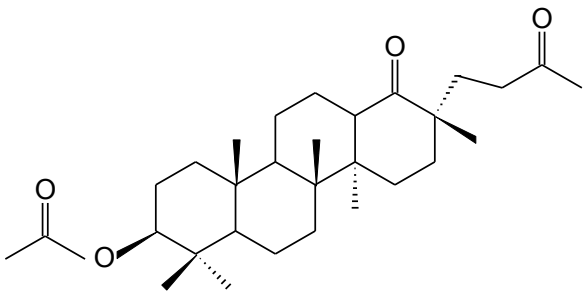


Lupenol **[62]**



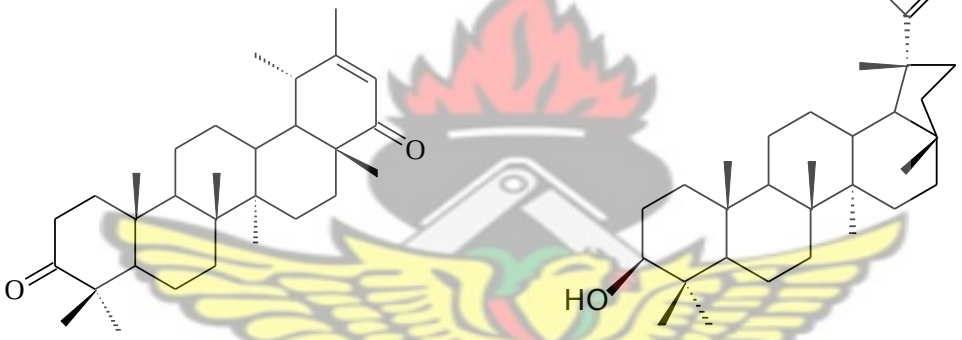
Lupenol acetate **[63]**





20-taraxastene-3β, 22α- diol **[64]**

29, 30-dinor-3β-acetoxy-18, 19-secolupane **[65]**

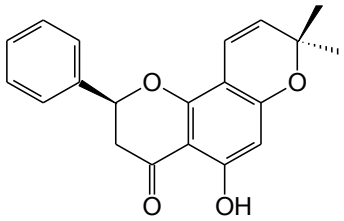


3, 22-dioxo-20-taraxastene **[66]**

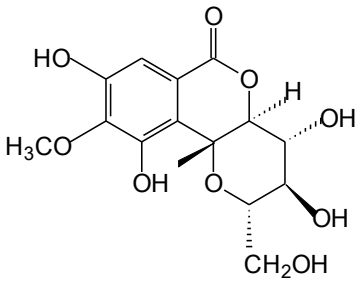
3β-hydroxy-20-oxo-29(20→19) abeolupane **[67]**

2.3.6 Miscellaneous

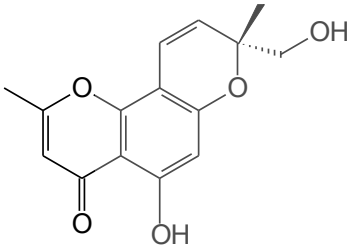
Other compounds which have been isolated from this genus includes bergenin **[69]** and racemosic acid **[68]** from the stem bark of *F. racemosa* (Li *et al.,* 2004), cyanidin 3-O-glucoside and cyanidin- 3-O-rhamnoglucoside from the fruits of *F. carica* (Solomon *et al.,* 2006), ficuformodiol A **[70]** and ficuformodiol B **[71]** from the stem bark of *F. formosana* (Sheu *et al.,* 2005)



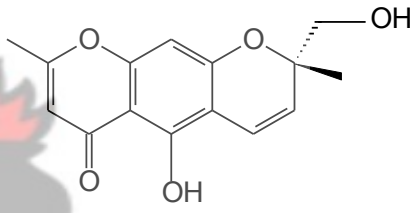
Racemosic **[68]**



Bergenin **[69]**



Ficuformodiol A **[70]**



Ficuformodiol B **[71]**

2.4 FICUS EXASPERATA

It is a deciduous tree with smooth gray bark and very rough (Scabrous) leaves. It is known by the common names 'sand paper tree', 'Papier de verre' in French, 'Msasa mkuyu' in Swahili and'Ewe ipin" in Yoruba. In Ghana it is called 'onyankyerɛn' (Akans), 'Nyadɛlɛ' (Nzema) and 'Nyadkese' (Ga) (Irvin, 1961).

2.4.1 Botanical description

Ficus exasperata (Figure 2.1) is a tree or shrub which can grow to about 20 m tall. The leaves alternate and have a scabrous upper surface. The lamina is ovate to elliptic or obovate. The apex is shortly acuminate and sometimes acute or obtuse. The base is

cuneate or occasionally subcordate and the margin dentate to sub entire. The fruits occur in pairs or solitary in the leaf axils, just below the leaves. The unripe fruit is green in colour, and about 8–15 mm in diameter. The fruits are orange in colour when ripe (Berg, 1991). The bark is smooth, grayish cream with brown streaks and exudes a gummy sap.

Figure 2.1 Whole plant, stem bark and leaves of *F. exasperata*

2.4.2 Biological and pharmacological activities of *F. exasperata*

Many authors have reported on the biological activities of the leaves of *F. exasperata*. However, reports on the biological activities of extracts from the root and stem bark appear to be limited. While this observation may promote conservation of biodiversity and sustainable use of the plant, there is the possibility of losing some useful compounds and activities that may be associated with the stem bark and roots.

2.4.2.1 Leaves

Various pharmacological actions such as anti-ulcer, anti-diabetic, lipid lowering and antifungal activities have been described for the leaves of *F. exasperata* by many research scientists (Sirisha *et al.*, 2010).

2.4.2.1.1 Anti hypertensive activity

Ayinde *et al.* (2007) investigated the anti-hypertensive effect of the leaves of *F. exasperata* using *in vivo* animal models and observed a measurable reduction in mean blood pressure. At 10 mg/kg, a reduction of 16.6 ± 1.1 mmHg was observed, whereas at 30 mg/kg, a fall in mean arterial pressure of 38.3 ± 0.6 mmHg was obtained.

2.4.2.1.2 Anti-inflammatory and anti-Arthritic activity

The hydro-alcoholic leaf extract was evaluated for its anti-arthritic and anti-inflammatory activities. The leaf extract (30-300 mg/kg) showed a dose-dependent anti-inflammatory activity in carrageenan – induced foot oedema in chicks (Woode *et al.*, 2009). Also the extract showed a dose-dependent antinociceptive effect in both acute and chronic arthritis in Sprague-Dawley rats with arthritic limbs.

2.4.2.1.3 Anti - ulcerogenic activity

Ficus exasperata is commonly used by Nigerian traditional healers for the treatment of peptic ulcer. The anti-ulcerogenic properties were investigated by Akah *et al.*, (1997). They showed that the leaf extract possesses significant anti-ulcerogenic properties in a dose-dependent manner. It protected rats from aspirin-induced ulcerogenesis, delayed intestinal transit, increased the pH and decreased both the volume and acidity of gastric secretion.

2.4.2.1.4 Hypolipidaemic activity

One week oral administration of an aqueous leaf extract of *F. exasperata* to alloxan-induced diabetic rats decreased plasma total triacylglycerol levels (Nimenibo-Uadia, 2003). Similarly, cholesterol and β-hydroxybutyrate concentrations were significantly ($p<0.05$) reduced. Results indicated that *F. exasperata* leaf possesses effective lipid lowering properties in diabetic rats.

2.4.2.1.5 Stabilisation of palm oil

The leaves of *F. exasperata* are used in the stabilization of palm oils in local processing methods. It has been suggested that the leaves protect against the formation of excessive peroxide levels in palm oil when used during its preparation (Umerie *et al.*, 2004).

2.4.2.1.6 Anti-microbial activity

The ethanolic leaf extract of *Ficus exasperata* was screened for antibacterial activity against *Escherichia coli* and *Staphylococcus albus*. It was found to be active against

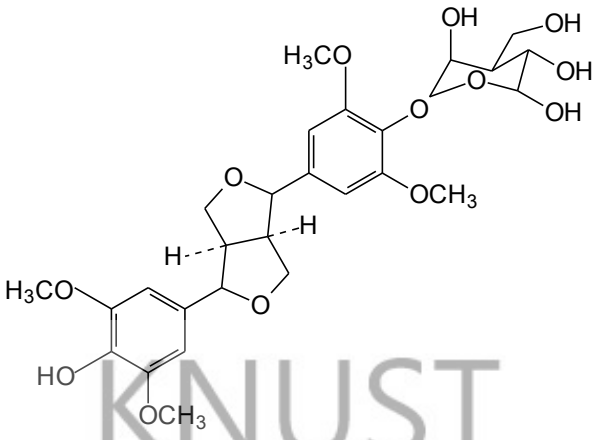
Escherichia coli with minimum inhibitory concentration (MIC) of 300 mg/ml while that of *Staphylococcus albus* was 700 mg/ml (Odunbaku *et al.*, 2008).

2.4.2.2 Stem bark

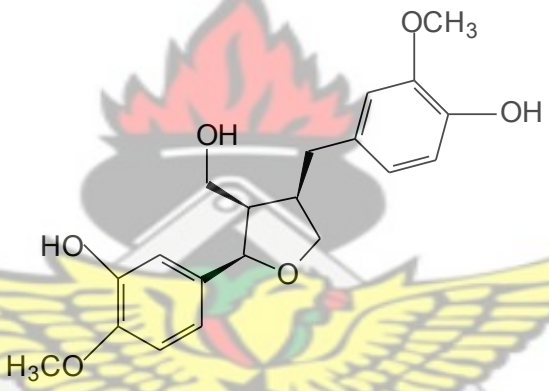
The aqueous stem bark extract was studied on oxytocin and acetylcholine induced contractions in uterine preparations isolated from non-pregnant Sprague-Dawley rats in oestrus (Bafor *et al.*, 2009). The extract was found to have no inhibitory effects on oxytocin or acetylcholine induced uterine contractions. Thus the traditional uses of the aqueous stem bark extract to stop uterine muscle contractions in pre-term labour was not justified. The methanol extract of *Ficus exasperata* stem bark was investigated for activity against some human pathogenic organisms. The stem bark extract had MICs of 50 mg/ml for *P. aeruginosa*, 1 mg/ml for *S. typhi* and 75 mg/ml for *S. aureus* (Adebayo *et al.*, 2009).

2.4.3 Phytochemical constituents of *F. exasperata*

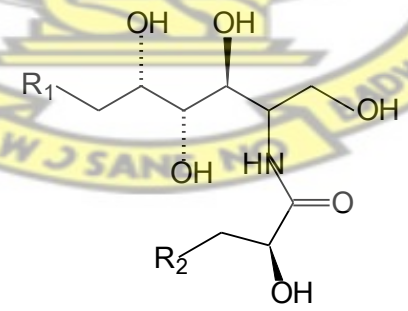
The roots, stem bark and leaves of *F. exasperata* have rarely been investigated for their phytochemical constituents. Taiwo *et al.*, (2006) reported for the first time the isolation of two antioxidant lignans, *epi*-syringarescinol-4-O-*β*-D-glycopyranoside **[72]** and Laricirescinol **[73],** from the leaves of *F. exasperata*. Two sphingolipids, **[74a]** and **[74b],** which differed only in the length of lipid base unit have been reported in the stem bark (http://www.aferp.univ-rennes1.fr/aferpnouveau/athens/poster/PB86). However, the actual lengths of the lipid base were not established.



Epi-syringarescinol-4-O-β-D-glycopyranoside **[72]**



Laricirescinol **[73]**



Compound 1, R_1= $CH_3(CH_2)n$, R_2= $CH_3(CH_2)n'$ **[74a]**
Compound 2**,** R_1= $CH_3(CH_2)n$, R_2= $CH_3(CH_2)n''$**[74b]**

2.5 INFLAMMATION

Inflammation is the body's response to disturbed homeostasis caused by infection, injury or trauma resulting in systemic and local effects. An inflammatory reaction serves to establish a physical barrier against the spread of infection and to promote healing of any damaged tissue (Hansson, 2005). In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, inflammation which runs unchecked can also lead to a host of diseases, such as hay fever, atherosclerosis, and rheumatoid arthritis. An inflammatory reaction may be triggered by infection (invasion and multiplication within tissues by various bacteria, fungi, viruses and protozoa, which in many instances, cause damage by release of toxins that directly destroy host cells), trauma, thermal injury, chemical injury, and immunologically mediated injury (Robbins and Cortran, 2004). It is characterized by excessive heat, swelling, pain, and redness. It is a common factor in arthritic diseases or osteoarthritis.

The rapid response to an injurious agent that serves to deliver mediators of host defence leukocytes and plasma proteins to the site of injury is known as acute inflammation. It has three major components: vasodilation, vascular leakage, oedema and leukocyte emigration (mostly polymorphonuclear cells). When a host encounters an injurious agent, such as an infectious microbe or dead cells, phagocytes that reside in all tissues try to get rid of these agents. At the same time, phagocytes and other host cells react to the presence of the foreign or abnormal substance by liberating cytokines, lipid messengers, and the

various other mediators of inflammation (Robbins and Cortran, 2004). Some of these mediators act on endothelial cells in the vicinity and promote the efflux of plasma and the recruitment of circulating leukocytes to the site where the offending agent is located. The recruited leukocytes are activated by the injurious agent and by locally produced mediators, and the activated leukocytes try to remove the offending agent by phagocytosis (Robbins and Cortran, 2004). As the injurious agent is eliminated and anti-inflammatory mechanisms become active, the process subsides and the host returns to a normal state of health. If the injurious agent cannot be quickly eliminated, the result may be chronic inflammation. Chronic inflammation is a pathological condition characterised by recurrent active inflammation, tissue destruction, and attempts at repair. It is not characterised by the classic signs of acute inflammation listed above (Robbins and Cortran, 2004).

2.5.1 Inflammatory pathway

The acute inflammatory response occurs in three distinct phases. The first phase is caused by an increased vascular permeability resulting in exudation of fluids from the blood into the interstitial space; the second phase involves the infiltrations of leukocytes from the blood into the tissue while the third phase involves granuloma formation and tissue repair (Robbins and Cortran, 2004). Mediators of inflammation originate either from plasma (e.g. complement proteins kinins) or from cells. The production of active mediators is triggered by microbial products or by host proteins (kinins) and coagulation systems that are themselves activated by microbes and damaged tissues. Generally the mediators of inflammation (Figure 2.2) are histamine, prostaglandins (PGs), leukotrienes (LTB4),

nitric oxide (NO), platelet-activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines and growth factors (Robbins and Cortran, 2004).

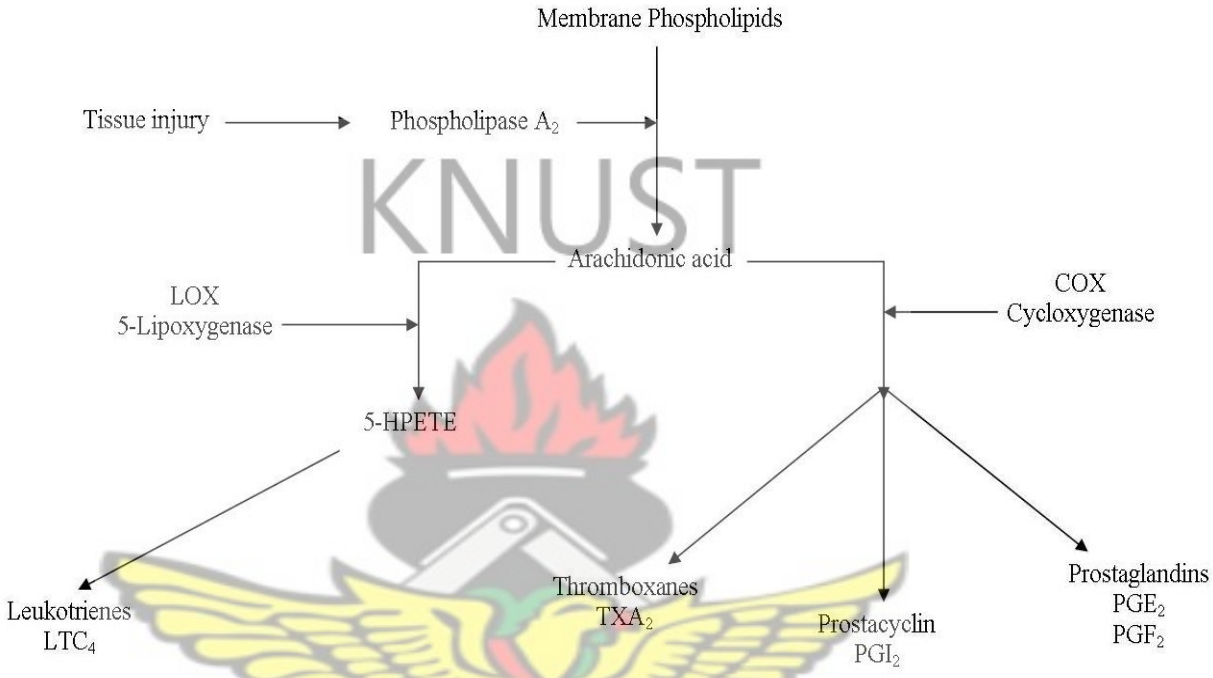


Figure 2.2 Pathways for the generation of the various mediators of inflammation

2.5.2 Experimental models of inflammation

Paw oedema, sponge implantation and air pouch granulomas are among the models that are used in inflammation studies. These models employ a variety of agents like formalin, Freunds adjuvant, carrageenan, monosodium urate crystals and zymosan (Singh, 2000). Others include vasoactive agents (e.g. platelet activating factor and histamine), weakened bacteria such as *E. coli*, chemotactic factors (e.g. N-formyl-norleucyl-phennylalanine), injection of polymorphonuclear leucocyte, leucotriene B_4

and arachidonic acid in acetone (Issekutz and Issekutz , 1989). Injecting these agents into various parts of the body may induce acute inflammatory response.

2.5.3 Models of Acute inflammation

Acute inflammatory response can be assessed by monitoring reactions such as foot volume increase produced by oedema (e.g. in the chick's paw), presence of plasma markers in the skin, measurement of inflammatory mediators in plasma exudates, local rise in the temperature of the skin, hyperaemia (an increase in vascular permeability), monocyte infiltration, polymorphonuclear leucocyte and lymphocyte accumulation (Issekutz and Issekutz, 1989). Hyperaemia and the emigration of leucocytes are the basic manifestations of the acute inflammatory reaction (Issekutz, 1981). Among the lot, the most acceptable preliminary screening test for anti-rheumatic activity is the carrageenan - induced acute footpad oedema in laboratory animals. This model has been widely used to screen new anti-inflammatory drugs (Singh, 2000) and has been used in this current investigation with very excellent result.

2.5.4 Carrageenan- induced paw oedema

This model is based on the principle of release of various inflammatory mediators by carragenan. Oedema formation due to carrageenan in the rat paw is a biphasic event. The initial phase is attributed to the release of histamine and serotonin. The second phase of oedema is due to the release of prostaglandins, protease and lysosome (Vinegar, *et al.* 1969; Crunkhon and Meacock, 1971). Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasation, increased tissue water

and plasma protein exudation along with neutrophil extravasation, all due to the metabolism of arachidonic acid (Chatpaliwar, *et al.* 2002). The first phase begins immediately after injection of carrageenan and diminishes in two hours. The second phase begins at the end of the first phase and remains through the third hour up to five hours.

Animals (rats/chicks) are divided into groups of about six each (n=6) prior to the day of experiment. The control group receives vehicle orally, while other groups receive test and standard drugs. The left paw is marked with ink at the level of lateral malleolus; basal paw volume is measured by volume displacement method using Plethysmometer, by immersing the paw till the level of lateral malleolus. The animals are then given drug treatment. One hour after dosing (pre-emptive), the rats are challenged by a subcutaneous injection of 0.1ml of 1% solution of carrageenan into the sub-plantar side of the left hind paw. The paw volume is measured again at 1, 2, 3, 4 and 5 hours after the challenge. The increase in paw volume is calculated as percentage compared to the basal volume. The difference of average values between treated animals and control group is calculated for each time interval and evaluated statistically. The percent Inhibition is then calculated (Winter, *et al.*, 1962).

2.6 OXIDATIVE STRESS

Metabolic processes in the body generate highly reactive species, known as free radicals, which injure cellular molecules. Free radicals are highly reactive atomic or molecular species that contain an unpaired electron (Halliwell and Gutteridge, 1999) which contributes to their high reactivity. Free radicals react quickly with the nearest stable

molecule to capture the electron they need to gain stability. The 'injured' molecule loses its electron, becoming a free radical itself. They can damage vital cellular components like nucleic acids, cell membranes and mitochondria, resulting in subsequent cell death. As all aerobic organisms utilize oxygen during cellular respiration and normal metabolism, the generation of free radicals by biochemical cellular reactions and from the mitochondrial electron transport chain is inevitable (Buonocore and Groenendaal, 2007). The free radicals include reactive oxygen and nitrogen species such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), peroxinitrite ($\cdot ONOO^-$), and nitric oxide ($NO\cdot$) radicals. All these are produced through oxidative processes within the mammalian body (Abdel-Hameed, 2009). They may also be generated through environmental pollutants such as cigarette smoke, automobile exhaust fumes, radiation, air pollution and pesticides (Aqil *et al.*, 2006; Tiwari, 2001). To protect the cells and organ systems of the body against reactive oxygen and nitrogen species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize free radicals. These antioxidants are capable of stabilizing or deactivating, free radicals before they attack cells (Beris, 1991). Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase destroy toxic peroxides. In addition to antioxidant enzymes, non-enzymatic molecules play important roles in antioxidant defence systems. These non- enzymatic molecules are of an exogenous nature and are obtained from foods. They include α-tocopherol, β-carotene, and ascorbic acid, and such micronutrient elements as zinc and selenium (Aqil *et al.*, 2006). Normally, there is a balance between free radical generation and scavenging

(Beris, 1991). Oxidative stress results from an imbalance between excessive generation of oxidant compounds and insufficient anti-oxidant defence mechanisms (Sies, 1997). When the natural antioxidant mammalian mechanism becomes inadequate, the excess of free radicals can damage both the structure and function of cell membranes in a chain reaction leading to degenerative diseases and conditions such as Alzheimer's disease, cataracts, acute liver toxicity, arteriosclerosis, nephritis, diabetes mellitus, rheumatism and DNA damage which can lead to carcinogenesis (Abdel-Hameed, 2009).

2.6.1 Antioxidants

All cells in eukaryotic organisms contain powerful antioxidant enzymes. Endogenous antioxidants made in the body are believed to be more potent in preventing free radical damage than exogenous antioxidants. The major classes of endogenous antioxidant enzymes are the superoxide dismutases, catalases and glutathione peroxidases (Sies, 1997), α-lipoic acid and coenzyme Q10. In addition, there are numerous specialized antioxidant enzymes reacting with and, in general, detoxifying oxidant compounds.

Superoxide dismutases are present in almost all aerobic cells and in extracellular fluids (Johnson and Giulivi, 2005). Superoxide dismutase enzymes contain metal ion cofactors that, depending on the isozyme, can be copper, zinc, manganese or iron. They catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Bannister *et al*., 1987; Zelko *et al.,* 2002) as shown in Figure 2.3. Catalases, on the other hand, are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Chelikani *et al.*, 2004; Zámocký and Koller, 1999).

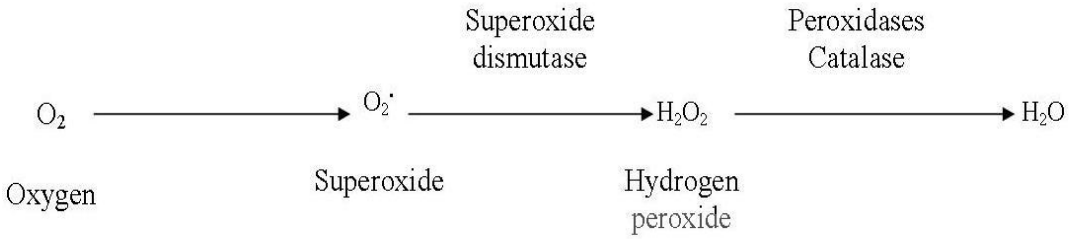


Figure 2.3 Pathway for the detoxification of reactive oxygen species by superoxide dismutase, catalase and peroxidases

2.6.2 *In vitro* methods of assessing antioxidant activity

Several methods have been used to assess antioxidant activity of compounds, extracts and nutritional supplements. These include the DPPH radical scavenging, lipid peroxidation, reducing power and total antioxidant capacity assays. Because different reactive oxygen species have different reaction mechanisms, attempting to evaluate antioxidant activity using one assay in order to claim ‘‘total antioxidant activity’’ is oversimplified and inappropriate. Therefore in this study, the DPPH free radical scavenging, ferric reducing power, lipid peroxidation assay and the phosphomolybdenum total antioxidant capacity assays were used to assess the antioxidant activity of the extracts/drugs.

2.6.2.1 Total antioxidant capacity

The total antioxidant capacity refers to a full spectrum of antioxidant activity against various reactive oxygen/nitrogen radicals. The major advantage of this test is that it measures the antioxidant capacity of all antioxidants in a biological sample or extract and not just the anti-oxidant capacity of a single compound. Major antioxidant capacity assays

can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays (Apak *et al.*, 2007). These two mechanisms yield identical results, but they differ in terms of kinetics and the potential for side reactions to occur.

HAT-based procedures measure the classical ability of an antioxidant to quench free radicals by hydrogen donation (Phipps *et al.*, 2007);

$X^{\cdot} + AH \rightarrow XH + A^{\cdot}$, where (AH = any H donor). HAT- based assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity, total radical trapping antioxidant parameter, and crocin bleaching assays. HAT reactions are solvent and pH independent and usually are quite rapid; typically they are completed in seconds to minutes. A disadvantage of the procedure, however, is that the presence of reducing agents, such as metals, can lead to high apparent reactivity (Phipps *et al.*, 2007).

ET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce a species. They measure the capacity of an antioxidant to reduce an oxidant, which changes colour when reduced. The degree of colour change is correlated with the sample's antioxidant concentration (Apak *et al*., 2007). ET-based assays include the total phenols assay with Folin-Ciocalteu reagent, Trolox equivalence antioxidant capacity, ferric ion reducing antioxidant power, total antioxidant potential assay using a Cu (II) complex as an oxidant, phosphomolybdenum method and DPPH. ET reactions are usually slow and can require long times to reach completion, so antioxidant calculations are based on percent decrease in product rather than on kinetics. Trace compounds and

metals also interfere with ET methods and can account for high variability and poor reproducibility of results (Phipps *et al*., 2007).

2.6.2.2 Reducing Power Method

This method is based on the principle of increase in the absorbance of the reaction mixture. It takes advantage of electron-transfer reactions. In this method antioxidant compound forms a coloured complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700 nm (Shivaprasad *et al.,* 2005). Increase in absorbance indicates higher reducing power of the sample and is measured by the ability of the antioxidant to reduce Fe^{3+} to Fe^{2+} (Oyaizu, 1986). In contrast to other tests of antioxidant activity, the ferric reducing power assay is simple, speedy, inexpensive, and robust and does not require specialized equipment. However, it cannot detect species that act by radical quenching (hydrigen transfer) such as glutathione and proteins (Benzie and Strain, 1999).

2.6.2.3 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method

This is the most widely reported method for screening of antioxidant activity of many plant drugs. DPPH assay method is based on the reduction of purple methanolic DPPH to a yellow coloured diphenyl picrylhydrazine and the remaining DPPH which shows a maximum absorption at 517 nm is measured (Abdel-Hameed, 2009). The decrease in absorbance of DPPH at its absorption maxima of 517 nm is proportional to concentration of free radical scavenger added to DPPH reagent solution (Vani *et al.,* 1997). Decrease in the DPPH solution absorbance indicates an increase of the DPPH

radical scavenging activity. The DPPH radical scavenging activity is calculated according to the following equation:

% DPPH radical scavenging activity = 1 - [A_{sample} /$A_{control}$] ×100

Where A_{sample} and $A_{control}$ are absorbance of sample and control.

The concentration of sample required to scavenge 50% of DPPH is expressed as EC_{50} (Vani *et al.*, 1997).

2.6.2.4 Total antioxidant capacity by phosphomolybdenum Method

It is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex as described by Prieto *et al* (1999). The assay is based on the reduction of molybdenum, Mo (VI) to Mo (V), by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH which is measured at 695 nm.

2.6.2.5 Total phenol assay

The antioxidant activities of most plants have been ascribed to their phenolic constituents (Ozgova *et al.*, 2003). In this study, the phenolic constituent of the extracts were determined using the method described by Singleton *et al.*, (1999). This method depends on the reduction of Folin-Ciocalteau reagent by phenols to a mixture of blue oxides which have a maximal absorption in the region of 760 nm. The reaction equation is as follows:

Folin: Mo^{+6} (yellow) + è (from antioxidant) → Mo^{+5} (blue)

Where the oxidizing reagent is a molybdophosphotungstic heteropolyacid comprised of $3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5\ MoO_3{\cdot}10H_2O$, in which the hypothesized active centre is Mo^{+6}.

The method is simple and sensitive, and can be useful in characterizing and standardizing botanical samples. However, the reaction is slow and occurs at acidic pH.

2.6.2.6 Lipid peroxidation

Lipid peroxidation involves the reaction of oxygen with a lipid. This process proceeds by a free radical chain reaction mechanism and mostly affects polyunsaturated fatty acids. The lipid is attacked by a free radical which forms an unstable lipid radical. The unstable fatty acid radical readily combines with molecular oxygen to form a lipid peroxyl radical. This is also an unstable species and so reacts with another free fatty acid forming lipid peroxide. In the presence of antioxidants, the free radicals are removed as they are formed and the chain reaction is terminated. If not terminated fast enough, there will be damage to the cell membrane, which consist mainly of lipids. In addition, malonaldehyde, a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis, is mutagenic and carcinogenic (Marnett, 1999). The lipid peroxidation mechanism is shown in Figure 2.4.

2.6.2.7 Linoleic acid autoxidation assay

This is one of the rapid methods used to screen antioxidants. It is mainly based on the principle that linoleic acid, which is an unsaturated fatty acid, gets oxidized by reactive oxygen species produced by oxygenated water. This leads to the formation of malondialdehyde (MDA). The formation of MDA is the basis of the well known thiobarbituric acid (TBA) assay method used for evaluating the extent of lipid peroxidation (Endrini *et al.*, 2002). At low pH and high temperature (100 ºC), TBA binds

with MDA to form a pink chromagen, which can be detected spectrophotometricaly at 535 nm (Gutteridge and Wilkins, 1986).

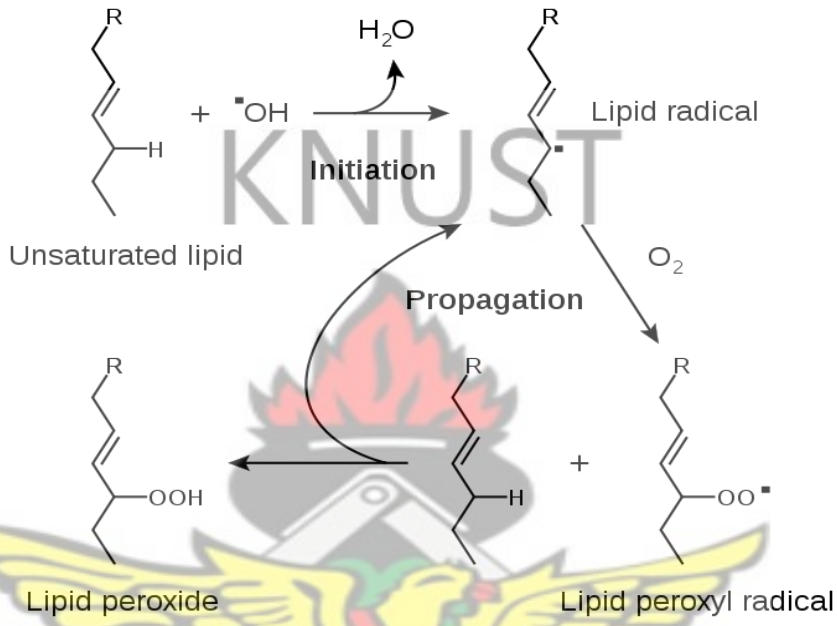


Figure 2.4 Formation of lipid peroxide radicals from polyunsaturated lipid molecules

2.7 ANTIMICROBIAL ACTIVITY

Throughout history, there has been a continual battle between humans and the multitude of microorganisms that cause infection and disease. Tuberculosis, malaria, and more recently, the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/ AIDS) pandemic, have affected substantial portions of the human population, causing significant morbidity and mortality (Tenover, 2006). Beginning around the

middle of the 20th century, major advances in antibacterial drug development and other means of infection control helped turn the tide in favor of humans. With respect to bacterial infections, the situation dramatically improved when penicillin became available for use in the early 1940s. Almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various forms of resistance. As antimicrobial usage increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens (Tenover, 2006).

2.7.1 Mechanism of antimicrobial action

Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principal mechanism of action. There are 4 major modes of action: (1) interference with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis, and (4) inhibition of a metabolic pathway (Tenover, 2006).

Antibacterial drugs that work by inhibiting bacterial cell wall synthesis include the β-lactams, such as the penicillins, cephalosporins, carbapenems, monobactams and the glycopeptides, including vancomycin and teicoplanin (McManus, 1997; Neu, 1992). *β*-lactam agents inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer (Neu, 1992). Vancomycin and teicoplanin also interfere with cell wall synthesis, but do so by binding to the terminal D-alanine residues of the nascent peptidoglycan chain, thereby preventing the cross-linking steps required for stable cell wall synthesis. Macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins, and oxazolidinones produce their antibacterial effects

by inhibiting protein synthesis (McManus, 1997; Neu, 1992). Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells. Antibacterial agents take advantage of these differences to selectively inhibit bacterial growth. Macrolides, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit. Fluoroquinolones exert their antibacterial effects by disrupting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication (Drlica and Zhao, 1997) whereas sulfonamides and trimethoprim block the pathway for folic acid synthesis, which ultimately inhibits DNA synthesis (Petri, 2006; Yao and Moellering, 2003). The common antibacterial drug combination of trimethoprim, a folic acid analogue, plus sulfamethoxazole (a sulfonamide) inhibits two steps in the enzymatic pathway for bacterial folate synthesis. Disruption of bacterial membrane structure may be a fifth mechanism of action, although less well characterized. It is postulated that polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial contents (Storm *et al.*, 1977).

2.7.2 Antimicrobial agents from higher plants

Since antiquity, man has used plants to treat common infectious diseases and some of these plant medicines are still included as part of the normal treatment of various diseases. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich *et al.,* 2004).

2.7.3 *In vitro* antimicrobial assays

Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various micro-organisms to samples that are placed in contact with them. Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method (Cos *et al.*, 2006). The antibacterial and antifungal test methods are classified into three main groups, namely diffusion, dilution and bioautographic methods.

2.7.3.1 Agar-diffusion methods

In the diffusion technique, a reservoir containing the test sample at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period. Different types of reservoirs can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs (Cole, 1994). The small sample requirements and the possibility to test up to six extracts per plate against a single micro-organism are specific advantages (Hadacek and Greger, 2000). The diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar.

2.7.3.2 Dilution methods

In the dilution methods, test compounds are mixed with a suitable medium that has previously been inoculated with the test organism. It can be carried out in liquid as well as in solid media and growth of the micro-organism can be measured in a number of ways. In liquid or broth-dilution methods, turbidity and redox-indicators are most frequently used. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm (Cos *et al.*, 2006). However, test samples that are not fully soluble may interfere with turbidity readings, emphasizing the need for a negative control or sterility control. Thus the extract should be dissolved in a blank medium without micro-organisms and compared with the results of the test sample. At present, the redox indicators 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and resazurin are frequently used to quantify bacterial (Eloff, 1998; Gabrielson *et al.*, 2002) and fungal growth respectively (Jahn *et al.*, 1995). Easy and reproducible measurements can be obtained with a microplate-reader, but visual reading may also be used in cases where spectrophotometer is not available. In general, dilution methods are appropriate for assaying polar and non-polar extracts or compounds for determination of MIC and MBC values. Using redox indicators or turbidimetric endpoints, dose–response effects allow calculation of IC_{50} and IC_{90} values, which are the concentrations required to produce 50 and 90% growth inhibition respectively (Cos *et al.*, 2006).

2.7.3.3 Bio-autographic methods

Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (a) direct bio-autography, where the micro-organism grows directly on the

thin layer chromatographic (TLC) plate, (b) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (c) agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison *et al.*, 1991).

Chapter 3

MATERIALS AND METHODS

3.1 COLLECTION AND AUTHENTICATION OF PLANT SAMPLES

The leaves and stem bark of *Ficus exasperata* were harvested from a farm land in Effiduase, a town in the Sekyere-East district of the Ashanti Region. The plants were identified by Mr. George Henry Sam of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical sciences, Kwame Nkrumah University of Science and Technology where voucher specimens with reference numbers KNUST/HM1/2011/L005 (leaves) and KNUST/HM1/2011/S004 (stem bark) have been deposited in the herbarium.

3.2 PROCESSING OF PLANT MATERIALS

The leaves and stem bark of *F. exasperata* were sun dried for 48 hours followed by oven drying at 40 °C for further 48 hours. The materials, thus dried, were coarsely milled and packed into brown paper bags and kept at the laboratory until required for use.

3.3 EXTRACTION OF PLANT MATERIALS

3.3.1 Extraction of the leaves

5.6 kilograms of coarsely powdered leaves of *Ficus exasperata* was packed into a cellulose thimble and soxhlet – extracted, successively, starting with petroleum ether (40/60), ethyl acetate and then 70% ethanol over 48 hours until the material was completely exhausted. Each extract was concentrated under reduced pressure to a small

volume by means of rotavapor (R-114, Buchi, Switzerland) at a temperature of 50 ºC. Each concentrate was then evaporated to dryness on a water bath to give a pet-ether extract (PFE, yield = 1.96%$^{W}/_{W}$), ethyl acetate extract (EFE, yield = 2.67% $^{W}/_{W}$) and ethanol extract (AFE, yield = 3.94% $^{W}/_{W}$). The three extracts were kept in a desiccator in the laboratory until needed

3.3.2 Extraction of the stem bark

Preliminary extraction of 250 g of the coarsely powdered stem bark of *F. exasperata* was done by soxhlet extraction using 70% ethanol and concentrated to give the extract, SFE (yield = 3.64% $^{W}/_{W}$). Bulk extraction was achieved by successive soxhlet extraction of 4.8 kg of the powdered stem bark, starting with petroleum ether (40/60) (Fissons), followed by chloroform and 70% ethanol. Each extract was concentrated under reduced pressure to a small volume as described above to give a pet-ether extract (FEP, Yield = 0.29 %$^{W}/_{W}$), chloroform extract (FEC, Yield = 0.35%$^{W}/_{W}$) and ethanol extract (FEE, Yield = 2.08 %$^{W}/_{W}$).

3.4 ANTI-INFLAMMATORY ASSAY OF EXTRACTS AND ISOLATES

3.4.1 Animals

Cockerels (*Gallus gallus*; strain: Shaver 579, Akropong Farms, Kumasi, Ghana) were obtained 1-day post-hatched and were housed in stainless steel cages (34 × 57 × 40 cm) at a population density of 12- 13 chicks per cage. Food (Chick Mash, from GAFCO Tema, Ghana) and water were available *ad libitum* through 1-qt gravity-fed feeders and waterers. The room temperature was maintained at 29 °C, and overhead incandescent

illumination was maintained on 12-hour light-dark cycle. Daily maintenance was conducted during the first quarter of the light cycle. Chicks were tested at 7 days of age. Group sample size of 5 was used throughout the study.

3.4.2 Carrageenan-induced oedema in chicks

The carrageenan foot oedema model of inflammation in the chick (Roach and Sufka, 2003) with some modifications by Woode *et al.*, (2009), was used to evaluate the anti-inflammatory properties of the extract and compared to dexamethasone and diclofenac as reference drugs. Carrageenan (10 µl of a 2% suspension in saline) was injected subplantar into the right footpads of the chicks. The foot volume was measured before injection and at hourly intervals for 5 hours after injection by water displacement plethysmography as described by Fereidoni *et al*, (2000) using an electronic Von Frey plethysmometer (Model 2888, IITC life science inc. Ca 91367 Canada). The oedema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time points.

3.4.3 Preliminary Bioassay of crude extracts

The experiment was aimed at investigating the effect of the extracts and standard drugs (diclofenac and dexamethasone) on oedema 1 hour after carrageenan challenge and continuing up to 5 hours. The drugs were given through the intraperitoneal (*i.p*) route and the extracts by the oral route. The test animals received the extracts (30, 100 and 300 mg/kg, *p.o.*), diclofenac (10, 30 and 100 mg /kg, *i.p.*) and dexamethasone (0.1, 1.0 and 3.0 mg/kg, *i.p.*) whereas the control animals received only the vehicle. The doses for the

hydro-alcoholic extracts were prepared by dissolving a known weight of the extract in 2 % tragacanth mucilage whereas the ethyl acetate, chloroform and pet-ether extracts were triturated in 2% polysorbate using 2% tragacanth mucilage as the diluent. This method was used for both the leaf and stem bark extracts.

3.4.4 Anti-inflammatory assay of isolates

The isolates were tested for anti-inflammatory activities using the method stated in section 3.4.3**.** However, the doses used for the isolates and standard drug diclofenac were 3, 10 and 30 mg/kg body weight whereas dexamethasone was tested at 0.1, 1.0 and 3.0 mg/kg, *i.p.*

3.5 ANTIOXIDANT ASSAY OF EXTRACTS OF *F. EXASPERATA*

3.5.1 *In vitro* qualitative DPPH test

The qualitative test for antioxidant activity was performed using the rapid DPPH radical scavenging assay (Cuendet *et al.,* 1997). 10 µl of the leaf extracts and stem bark extracts were applied on silica gel plates 60 F_{254} (Merck, 0.25 mm thick) and allowed to dry completely. The plate was then sprayed with a solution of 2% DPPH in methanol. A pale yellow to white spot over a purple background indicated a radical scavenging activity of the particular extract.

3.5.2 Quantitative antioxidant assays of extracts

3.5.2.1 Reducing power assay

Reducing activity of all the extracts was carried out by using the method of Oyaizu (1986). Different concentrations (0.25 – 2.0 mg/ml) of the extracts as well as the standard drug *n*-propyl gallate (3.75 – 30 µg/ml) were prepared in aqueous methanol (50% $^{v}/_{v}$) and 1 ml each taken into test tubes in triplicates. To the test tubes, 2.5 ml of sodium phosphate buffer (pH=7) and 2.5 ml of 1% potassium ferric cyanide solution was added. The contents were mixed well and incubated at 50 °C for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid was added and the mixture centrifuged at 3000 revolutions per minute for 10 minutes. After centrifugation 2.5 ml of supernatant was added to 2.5 ml of distilled water. To this about 1 ml of 0.1% ferric chloride was added. The absorbance was then taken at 700 nm. A graph of absorbance was then plotted against the concentration of the extracts. Increase in absorbance indicates higher reducing power of the extract.

3.5.2.2 Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical assay

The free radical scavenging activity was determined as described by Govindarajan *et al*., (2003) with few modifications. 1 ml each of the extracts (0.25, 0.5, 1.0 and 2 mg/ml in methanol) was added to 3 ml methanolic solution of DPPH solution (20 mg/l) in a test tube. The reaction mixture was kept at 25°C for 30 mins. The absorbance of the residual DPPH was determined at 517 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). One

milliliter (1 ml) methanol (50%) was added to 3 ml DPPH solution, incubated at 25 °C for 30 minutes and used as control. *n*-propyll gallate (3.75-30 µg/l) was used as a standard free radical scavenger. The absorbance decreases with increasing free radical scavenging ability. Results were expressed as percentages of blank (100%). The concentration required to cause a 50% decrease in the absorbance was calculated (EC_{50}). Each test was carried out using three replicates.

The % DPPH scavenging effect (% of control) of the antioxidant was calculated as follows:

% DPPH scavenging effects = $(Ac - At)/Ac \times 100$

Where

Ac = Absorbance of the control

At = Absorbance of the test drug/ extracts

3.5.2.3 Total antioxidant capacity assay

The assay is based on the reduction of molybdenum, Mo^{+6} to Mo^{+5}, by the extracts and subsequent formation of a green phosphate-molybdate (Mo^{+5}) complex at acidic pH (Prieto *et al*., 1999). Test tubes containing l ml each of the extracts (0.25-2 mg/ml) and 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM disodium phosphate and 4 mM ammonium molybdate) were incubated at 95 ^{o}C for 90 minutes. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm. Four concentrations of ascorbic acid (0.025, 0.05, 0.1 and 0.2 mg/ml) was used to construct a calibration curve. A blank solution was prepared by adding every other

solution but without extract or standard drug. The antioxidant capacity was expressed as mg of ascorbic acid equivalent (AAE) per g of extract. This procedure was used for all the extracts.

3.5.2.4 Linoleic acid auto-oxidation assay

The method of Mitsuda *et al*., (1966) was used. The extracts (0.25-2 mg/ml) in absolute alcohol were compared with *n*-propyl gallate (3.75-30 µgm/l) in absolute alcohol as a reference antioxidant. 2 ml of the extract, 2 ml of 2.5% linoleic acid in absolute ethanol, 4 ml of 0.05 M phosphate buffer (pH =7) and 1.9 ml of distilled water were put into test tubes with a screw cap and placed in an oven at 40 ^{o}C in the dark for 7 days. After the seven day period, 2 ml each of the extracts and standard drug was added to 20 % aqueous trichloroactetic acid solution and 1 ml of 0.6 % aqueous thiobarbituric acid solution. This mixture was placed in boiling water bath for 10 minutes and after cooling, was centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was measured 535 nm. Each test was carried out in three replicates. Percentage inhibition of lipid peroxidation by the test drugs was assessed by comparing the absorbance of the drug test with that of the control (linoleic acid mixture without any drug). Data was presented as percentage inhibition of lipid peroxidation against concentration. The % inhibition of linoleic acid autoxidation was calculated as follows:

$$\% \text{ inhibition} = \left[1 - \frac{D - D_O}{C_O - C}\right]$$

Where

Co= (Full reaction mixture) is the degree of lipid peroxidation in the absence of antioxidant

C= is the underlying lipid peroxidation before the initiation of lipid peroxidation

D= is any absorbance produced by the extract/ linoleic acid mixture

Do= is the absorbance produced by the extract alone

3.5.2.5 Determination of total phenolic content

The presence of phenol in the extract was determined qualitatively using ferric chloride test. An intense positive colouration indicating the presence of phenols led to further quantification of total soluble phenols in the extract. The total phenol in the extract was determined by spectrophotometric assay using the Folin-Ciocalteu's reagent as described by Singleton *et al*., (1999) using tannic acid as standard.

1ml of the extracts (0.25-2 mg/ml) in distilled water was added to 1 ml Folin-Ciocalteu's reagent in a test tube. The content of the test tube was mixed and allowed to stand for five minutes at 25 ^{o}C in an incubator. 1 ml of sodium bicarbonate solution (2%) was added to the mixture. The reaction mixture was allowed to stand for 2 hours with shaking at 25 ^{o}C in an incubator. The mixture was then centrifuged at 3000 rpm for 10 minutes and absorbance of the supernatant determined at 760 nm. Three replicates

were prepared for each concentration of tannic acid and extracts. 1 ml distilled water was added to 1 ml Folin-Ciocalteu's reagent processed in the same way as the test drugs and used as blank. Tannic acid was used as reference. Four concentrations of tannic acid (0.025, 0.05, 0.1, 0.2 mg/ml) were used to construct a calibration curve and the total phenols expressed as mg of tannic acid equivalents (TAE)/g of extract.

3.6 ANTIMICROBIAL ASSAY OF EXTRACTS

3.6.1 Microbial strains

Leaf and stem bark extracts of *F. exasperata* were individually tested against a set of 7 microorganisms. The microbial strains were provided by the Pharmaceutical Microbiology Section of the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Science, KNUST, Kumasi. The seven microbial species, including two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacilllus subtilis* NCTC 10073), four Gram-negative bacteria (*Salmonella typhi* NCTC 6017, *Escherichia coli* NCTC 9002, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 27853) and the fungus *Candida albicans* (ATCC 10231) were used for the antimicrobial tests. Bacterial strains were cultured overnight at 37 °C in nutrient broth and the fungus was cultured overnight at 37 °C in Sabouraud dextrose agar.

3.6.2 Media and Chemicals

All the media and chemicals used for this work were obtained from Oxoid Ltd, Basingstoke, Hampshire, England. Gentamicin (Troge, Hamburg, Germany) was

purchased from Lucas Pharmaceuticals Ltd. Kumasi-Ghana whereas fluconazole was obtained from Ernest Chemist Ltd.

3.6.2.1 Preparation of media

Nutrient Broth

25 g of nutrient broth powder was weighed into a beaker. It was dissolved in about 500 mls of distilled water and stirred to dissolve. Enough freshly prepared distilled water was added to produce 1000 ml. 10 ml quantities were poured into test tubes and plugged firmly with cotton wool. They were then sterilized by heating in an autoclave at 121°C for 15 minutes.

Nutrient Agar

40 g of the powder was weighed into a beaker. It was dissolved in about 500 mls freshly distilled water and stirred to obtain a homogenous mixture. Enough freshly prepared distilled water was added to produce 1000 ml. 10 ml quantities were poured into test tubes and plugged firmly with cotton wool. They were then sterilised by heating in an autoclave at 121°C for 15 minutes.

Saboraud dextrose Agar

65 g of the powder was weighed and dissolved in one litre of freshly prepared distilled water. The mixture was heated whiles stirring until it dissolved completely. Aliquot of 20

mls of the mixture was poured into test tubes, plugged with cotton wool and sterilised in an autoclave at 121°C for 15 minutes.

3.6.3 Screening of extracts and isolated compounds for antimicrobial activity

The antimicrobial activities of the different extracts were determined using the agar well diffusion method outlined by Vanden-Berghe and Vlietinck (1991). The inocula was prepared by inoculating the test organisms in nutrient broth and incubating them for 24 hours at 37 °C for bacteria, while for *Candida albicans*, Sabouraud's dextrose broth was incubated for 48 hours. 1 ml of the diluted cultures was inoculated into sterile molten agar at 45 °C and poured into a sterile petri dish. Similarly 1 ml of the diluted fungal suspension was poured into sterile Sabouraud's dextrose agar plates. These were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar plates using cork borer number 6. The extracts ethyl acetate and ethanol extracts were reconstituted in 2% dimethyl sulfoxide (DMSO) whereas the pet-ether and chloroform extracts were reconstituted in 2% polysorbate 20. The agar-wells were filled respectively with 0.1 ml of each extract (10 mg/ml), gentamicin (500 µg/ml), fluconazole (500 µg/ml). One hour was allowed for the extract/drug to diffuse into the agar after which the plates were incubated at 37 °C for 24 hours for bacteria and 48 hours for the fungus. At the end of the incubation period, the diameter of the inhibition zone(s) were measured and recorded. The extracts and antibiotics were tested in triplicates and mean zones of inhibition were calculated for each extract and the standard antibiotic. The isolated compounds were also tested at 500 µg/ ml as described above.

3.6.4 Micro dilution assay

Minimal inhibitory concentration (MIC) values of the extracts were determined based on a micro-well dilution method (Ellof, 1998). The inocula of microorganisms were prepared from 12-hour broth cultures and serial dilutions were made to achieve a suspension of approximately 10^5 CFU/mL. The extracts were screened at concentrations of between 7.8 µg/mL and1000 µg/mL. The 96-well sterile plates were prepared by dispensing into each well 100 µL of double strength nutrient broth and 100 µL of test samples together with 20 µL of the inoculum. The microplates were incubated at 37 °C for 24 hours. Growth of the microorganisms was determined by adding 20 µL of a 5 % solution of tetrazolium salt (MTT) and incubating for further 30 minutes. Dark wells indicated the presence of microorganisms as the dehydrogenase enzymes in the live bacteria react to form a dark complex with the tetrazolium salt. All experiments were carried out in triplicates.

3.7 STATISTICAL ANALYSIS OF DATA

The raw scores for right foot volumes were individually normalized as percentage of change from their values at time zero then averaged for each treatment group. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ \text{inhibition of oedema} = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Differences in AUCs were analyzed by one way analysis of variance followed by Student-Newman-Keuls' *post hoc t* test. Doses and concentrations responsible for 50 % of the maximal effect (EC_{50} and IC_{50}) for each drug/extract were determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y = \frac{a+(b-a)}{\left(1+10^{(LogEC50-X)}\right)}$$

Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape. The fitted midpoints (ED_{50}/IC_{50} values) of the curves were compared statistically using *F* test (Miller, 2003; Motulsky, 2003). Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant (Rowe, 2007).

3.8 CHROMATOGRAPHY

The result of the bioassay of the leaf extracts presented in section 4.2.1 (Figure 4.1) indicated that the pet-ether (PFE) and ethyl acetate (EFE) extracts of the leaves exhibited potent anti-inflammatory activities than the ethanol extract (AFE). Also the chloroform extract of the stem bark showed the highest anti-inflammatory activity (section 4.3.2). Therefore the extracts PFE, EFE and FEC were fractionated using various chromatographic methods to isolate the chemical constituents present and were identified by their spectroscopic features.

3.8.1 Chromatographic materials

Two types of stationary phase materials were used for the column chromatographic technique: Silica gel 60 (60-120 and 70-230 mesh ASTM, Merck Germany) and Sephadex LH-20 (Sigma) were used. Aluminium precoated silica gel plates 60 F_{254} (0.25 mm thick), used for the analytical thin layer chromatography.

3.8.2 Detection for analytical thin layer chromatography

The zones on TLC plates corresponding to separated compounds were detected under UV light 254 nm and 365 nm and also by spraying with anisaldehyde 0.5 % $^{W}/_{V}$ in HOAC/ H_2SO_4/ MeOH (10:5:85) followed by heating at 105 °C for 5-10 minutes.

3.8.3 Solvents and Reagents

The solvents used for extraction, column chromatography and TLC analysis were of analytical grade and included methanol, ethanol, ethyl acetate, chloroform, petroleum ether and acetone. The organic solvents and anisaldehyde were purchased from BDH Laboratory Supplies (England).

3.8.4 Chromatographic techniques employed

3.8.4.1 Column Chromatography

The dry method was used in packng the column with silica gel 60 (60-120 and 70-230 mesh ASTM). The silica gel was gently packed into a glass column. The extract was dissolved in a minimum amount of solvent and adsorbed onto a quantity of silica gel. It was then allowed to dry completely and then placed on top of the already packed column. The mobile phase (solvent or mixture of solvents) was then placed on top of the packed

column to separate the extract into different fractions and the eluates collected into glass beakers.

In the case of sephadex LH-20, the beads were soaked (saturated) overnight with the solvent to be used in eluting the column. The slurry of the beads was then packed into the column and the solvent allowed to drain off. A solution of the extract was then loaded onto the column and eluted with the mobile phase. These methods were used in the separation and isolation of the various compounds from the plant extract.

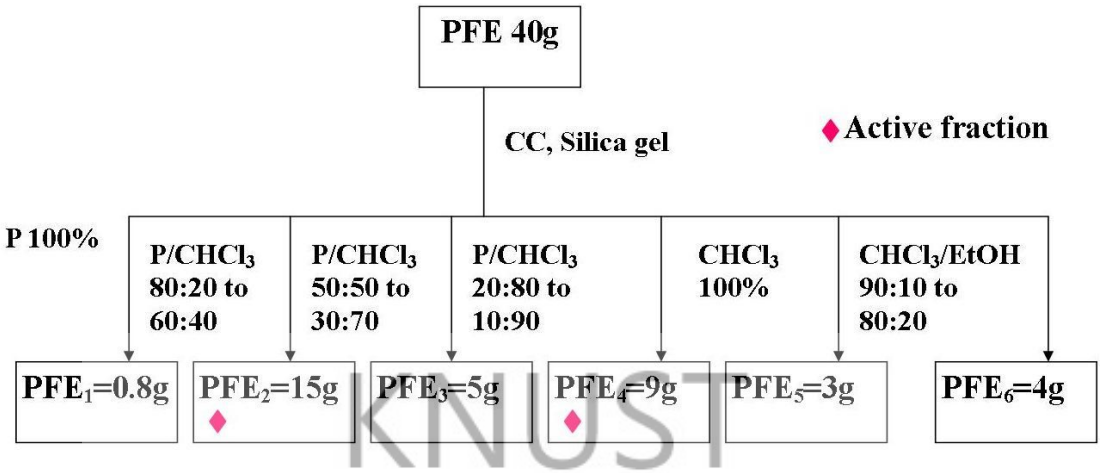
3.8.4.2 Development of thin layer chromatogram

The one way ascending technique was used. Samples of mixtures/extracts to be analysed by TLC were dissolved in an organic solvent and were applied on the TLC plates as spots with the aid of capillary tubes at one end of the plate in a straight line about 2 cm above the edge and 1.5 cm away from the margins. The spots were dried and the plates placed inside a chromatank containing the mobile phase. The mobile phase ran along the TLC plate in an ascending manner due to capillary action, carrying with it the components of the extract. When the solvent reached a reasonable height the operation was stopped and the solvent front marked (Brain and Turner 1975). The separated compounds were identified by observing them under ultra violet light for fluorescence followed by spraying with anisaldehyde in conc. H_2SO_4.

3.9 ISOLATION OF COMPOUNDS FROM THE LEAF EXTRACTS

3.9.1 Column chromatographic separation of the pet-ether extract

Silica gel 60 (800g, 60-120 mesh ASTM) was packed dry into a glass column (90 cm × 6 cm). 40 g of the pet-ether extract was dissolved in a minimum amount of chloroform and mixed with 150 g of silica gel, allowed to dry to attain the same consistency as the silica gel that was used, and spread gently on top of the packed column. A wad of cotton wool was placed on top of the packed column in order not to disturb the surface of the packing. The column was then eluted initially with 100% petroleum ether, followed by 10%, 20%, 30%, 50%, 70% and 90% chloroform in pet-ether; followed by 100% chloroform, then 10% and 20% ethanol in chloroform. Approximately 171 aliquots of 100 ml each were collected. TLC analysis (as described under section 3.8.4.2) using a mobile phase of various mixtures of pet-ether and ethyl acetate, resulted in the bulking of the eluates into six fractions (PFE_1- PFE_6) as shown in figure 3.1. The anti-inflammatory activities of the bulked fractions were evaluated using the carrageenan- induced oedema chick model described under section 3.4.3. Fractions PFE_2 and PFE_4 were the most active. They were therefore subjected to chromatographic analysis.



PFE (pet-ether extract of the leaves), **P** (pet-ether), **EtOH** (ethanol)

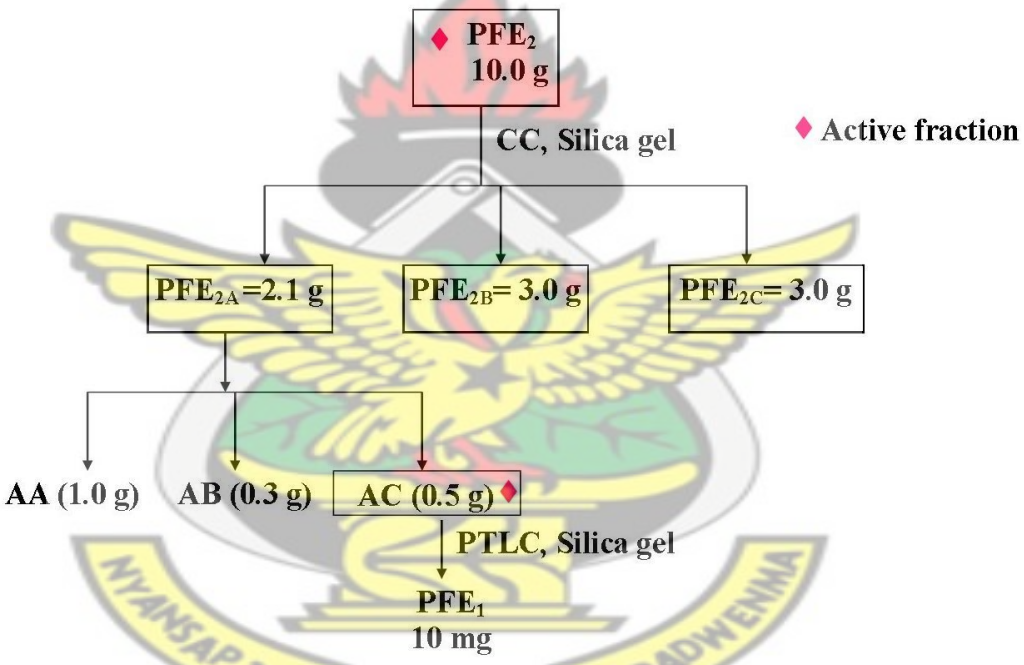
Figure 3.1 Schematic representation of the fractionation of the pet-ether fraction of *F. exasperata.*

3.9.2 Column chromatography of fraction PFE_2

Silica gel (200 g, 70-230 mesh ASTM) was packed into a column (75 cm × 3 cm) using the dry method. About 10 g of PFE_2 was dissolved in 50 mls of chloroform and mixed with 50 g of silica gel, allowed to dry to attain the same consistency as the silica that was used, and spread on top of the packed column. The column was successively eluted starting with petroleum ether 100%, followed by 10%, 20%, 30%, 50%, 70% and 90% chloroform in pet-ether. About 60 fractions were collected in 40 ml aliquots and bulked together according to their TLC profile into three sub-fractions labeled PFE_{2A}, PFE_{2B} and PFE_{2C}.

3.9.3 Isolation of compound PFE-1

Fraction PFE_{2A} was loaded onto a silica gel column (70-230 mesh ASTM) and eluted isocratically with petroleum ether: ethyl acetate (9:1) to obtain fractions AA (1 g), AB (0.3 g) and AC (0.5 g). The active sub-fraction AC was subjected to preparative thin layer chromatography (PTLC) using pet-ether: ethyl acetate (9:1) to yield compound PFE_1 (10 mg). Figure 3.2 illustrates the column chromatographic procedure for the petrol fraction (PFE_2) and isolation of compound PFE-1.



CC (column chromatography)

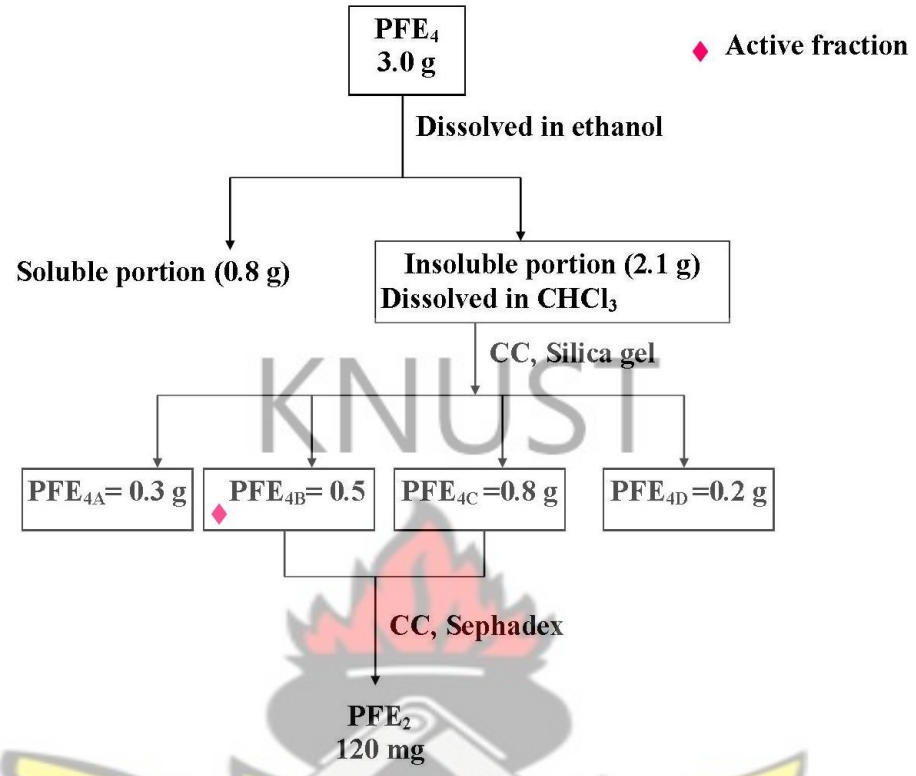
Figure 3.2 Schematic representation of the isolation procedure for compound PFE-1

3.9.4 Column chromatography of active fraction PFE_4

3.0 g of PFE_4 was dissolved in 40 ml of ethanol. The ethanol insoluble portion (2.1 g) was dissolved in chloroform and adsorbed onto silica gel (70-230 mesh, merck). This was then packed onto a silica gel column and successively eluted with 10%, 20%, 40%, 50% and 60% chloroform in pet-ether. 40 fractions were collected in 5 ml aliquots and bulked into four fractions (PFE_{4A}- PFE_{4D}) according to their TLC profiles (Figure 3.3).

3.9.4.1 Isolation of compound PFE-2

Fractions PFE_{4B} and PFE_{4C} were put together. 1.3 g of this material was dissolved in ethanol and loaded onto a sephadex LH-20 column which had previously being saturated in ethanol overnight. It was then isocratically eluted with ethanol and collected in 5 ml aliquots. 30 fractions were collected. A white crystalline solid, PFE-2, was obtained from fractions 12-19 which was recrystallised in ethylacetate. The column chromatographic procedure for PFE_4 and isolation of compound PFE-2 is shown in Figure 3.3



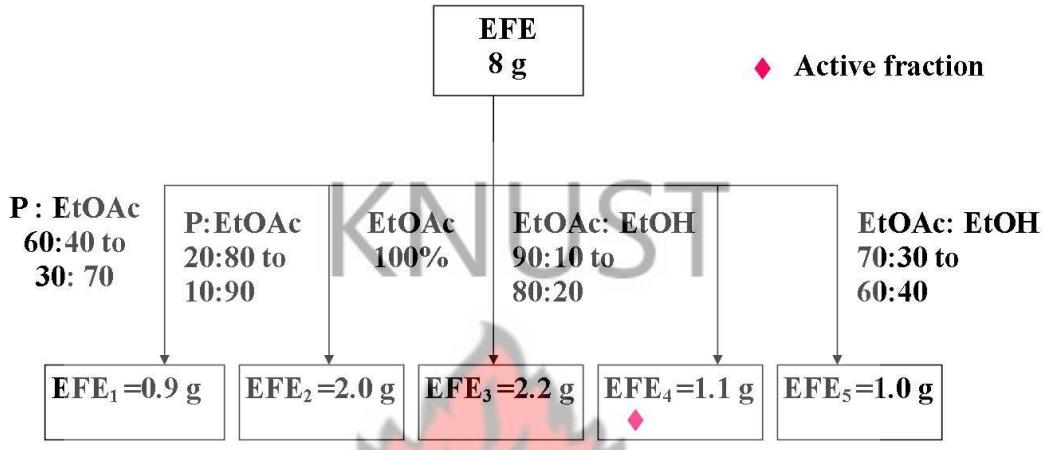
CC (column chromatography)

Figure 3.3 Schematic representation of the isolation procedure for compound PFE-2

3.9.5 Column chromatographic separation of the ethyl acetate extract

Silica gel 60 (200 g, 70-230 mesh ASTM) was packed dry into a glass column (75 cm × 3 cm). 8 g of the ethyl acetate extract (EFE) of *F. exasperata* was re-dissolved in a minimum amount of chloroform and mixed with silica gel, allowed to dry to attain the same consistency as the silica, and spread gently on the top of the packed column. The column was then eluted with 40%, 60%, and 80% ethyl acetate in pet-ether; followed by 100% ethyl acetate, 10%, 20% and 40% ethanol in ethyl acetate. 62 aliquots of 80 ml each were collected and bulked into five fractions (EFE_1- EFE_5) according to their TLC

profiles. The Figure 3.4 illustrates the column chromatographic procedure for the ethyl acetate extract.



P (pet-ether), **EtOAc** (ethyl acetate), **EtOH** (ethanol)

Figure 3.4 Schematic representation of the fractionation of ethyl acetate extract of *F. exasperata*

3.9.6 Isolation of compound FE-1

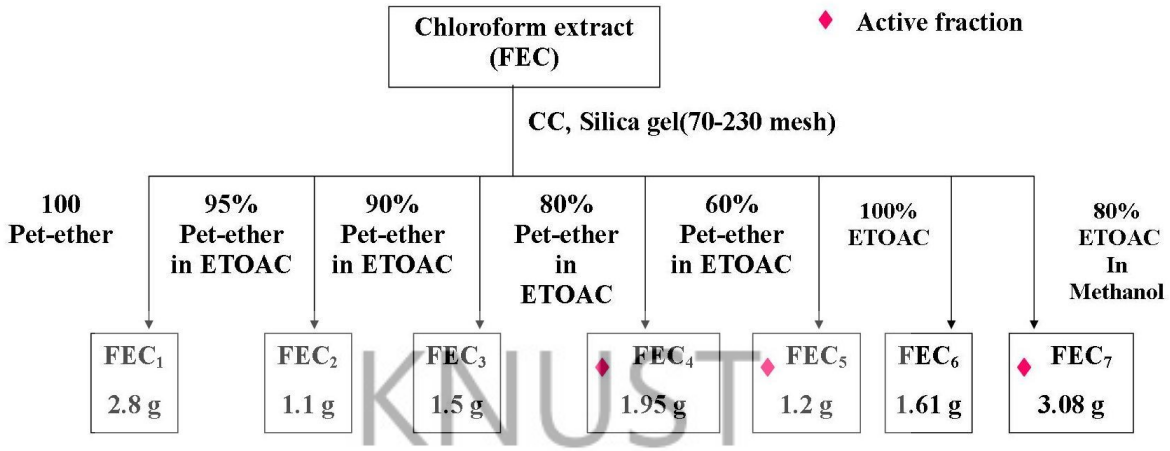
EFE_4 (1.1 g), was dissolved in chloroform and adsorbed unto silica gel (70-230 mesh ASTM), and spread on top of a column (50 mm × 3 mm) packed dry with silica gel. The column was then isocratically eluted with chloroform: ethanol (80:20). 200 fractions of 5 ml aliquots each were collected and bulked into three fractions designated as EFE_{4A}, EFE_{4B} and EFE_{4C}. Sub-fraction EFE_{4C} was obtained as a white amorphous solid and showed a single spot on TLC plate developed with $CHCl_3$: methanol (9:1). It was washed several times with acetone and subsequently labeled as compound FE-1 (220 mg).

3.10 ISOLATION OF COMPOUNDS FROM CHLOROFORM EXTRACT OF THE STEM BARK

The chloroform extract of the stem bark exhibited potent anti-inflammatory activity as presented in section 4.2. Hence it was fractionated to isolate some of the compounds which may be responsible for these activities.

3.10.1 Column chromatographic fractionation

Silica gel (200 g, 70-230 mesh ASTM) was packed into a column (75 cm × 3 cm) using the dry method. About 15 g of FEC was dissolved in 50 mls of chloroform and mixed with 50 g of silica gel and allowed to dry. It was then loaded onto the column and successively eluted initially with petroleum ether 100%, followed by 5%, 10%, 20%, 40%, 60%, 80% ethyl acetate in pet-ether; followed by 100% ethyl acetate and then 20% methanol in ethyl acetate. 153 fractions were collected in 60 ml aliquots and bulked together according to their TLC profiles. The aliquots were bulked into seven fractions coded $FEC_{1\text{-}7}$ as shown in Figure 3.5.



CC (column chromatography), **FEC** (chloroform extract)

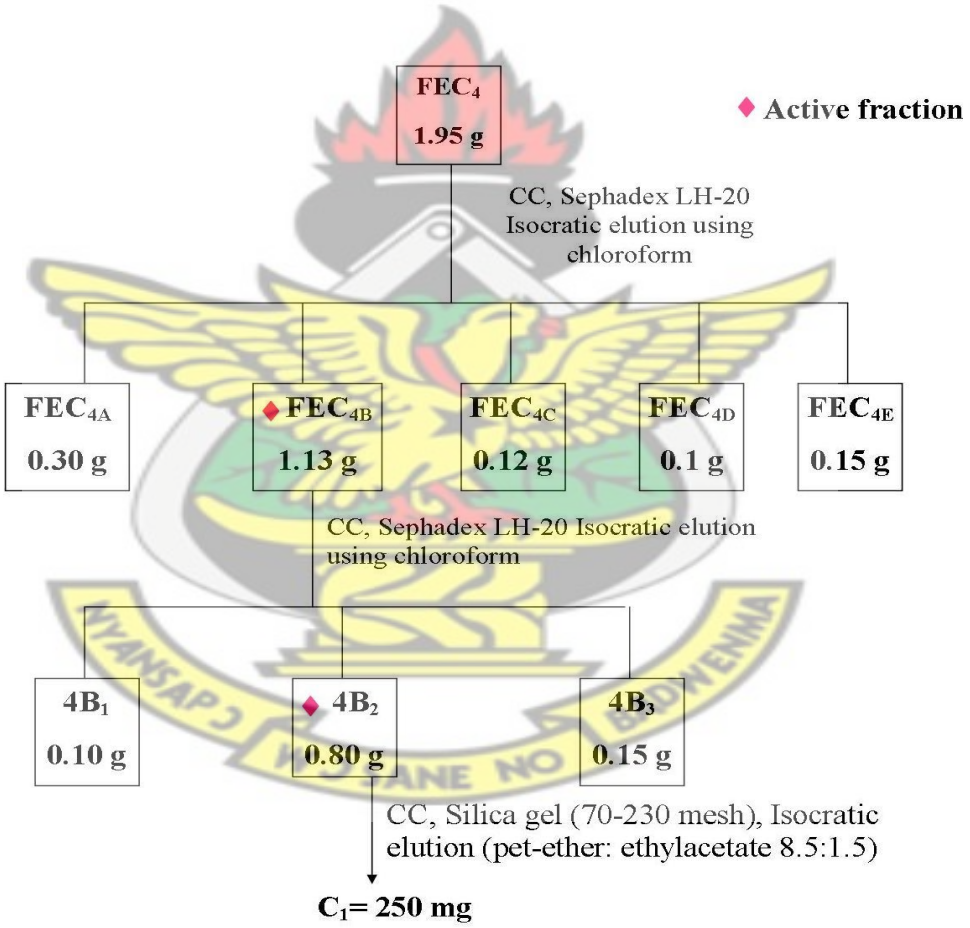
Figure 3.5 Schematic representation of the fractionation of the chloroform extract of the stem bark of *F. exasperata*

3.10.2 Column fractionation of FEC_4

FEC_4 (1.9 g), was dissolved in chloroform and loaded onto a sephadex LH-20 column which had previously been saturated in chloroform overnight. It was then isocratically eluted with chloroform. 70 fractions of 10 ml each were collected; fractions containing the same compounds as determined by their TLC profiles were combined and concentrated to dryness under reduced pressure. Five fractions were obtained (FEC_{4A-E}). Fraction FEC_{4B} (1.13 g) was reconstituted in chloroform and re-chromatographed using sephadex LH-20 as described above. Three bulked fractions were obtained, designated $4B_1 - 4B_3$.

3.10.3 Isolation of compound C-1

Fraction $4B_2$ was further column chromatographed over silica gel (70-230 mesh) using a column of diameter 2.5 cm and length 40 cm and isocratically eluting with pet-ether: ethyl acetate 85:15. 40 fractions of 2 ml each were collected. Compound C-1 (250 mg) was obtained from the fractions 1-10 as off-white needle-like crystals with a characteristic odour. Figure 3.6 illustrates the column chromatographic fractionation of FEC_4 and the isolation of compound C-1 (Figure 3.6).



CC (column chromatography)

Figure 3.6 Schematic representation of the isolation of compound C-1

3.10.4 Column chromatographic fractionation of FEC_5

FEC_5 (1.2 g) was dissolved in acetone and adsorbed unto silica gel (70-230 mesh ASTM); it was allowed to dry to attain the same consistency as the silica which was used, and packed on top of a column (60 mm × 3 mm) packed dry with silica gel. The surface of the silica was covered with cotton wool and the column isocratically eluted with pet-ether: ethyl acetate 60:40. 80 fractions of 5 ml aliquots each were collected and bulked together according to their TLC profiles. Anisaldehyde reagent was used for the detection of the compounds. Three bulked fractions were obtained designated as FEC_{5A}, FEC_{5B} and FEC_{5C}.

3.10.5 Isolation of C-2

FEC_{5B} was obtained as an off white crystalline compound. It was washed several times with chloroform and recrystallised in acetone to obtain a pale yellow amorphous powder (compound C-2) which gave one spot on TLC plate developed with pet-ether: ethyl acetate 4:6 (Figure 3.7).

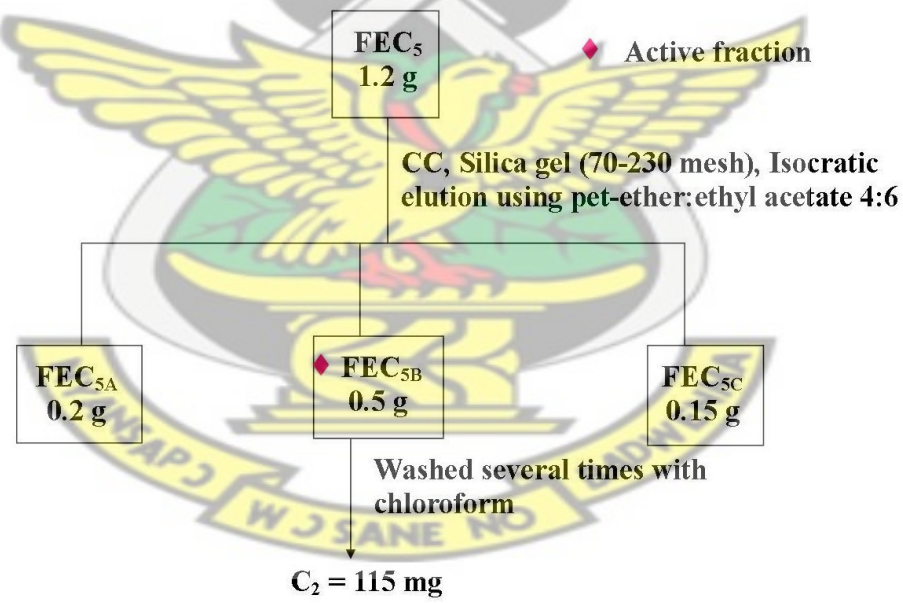
3.10.6 Column chromatographic fractionation of FEC_7

FEC_7 (3.0 g) was dissolved in a mixture of chloroform/methanol (1:1) and adsorbed unto silica gel (70-230 mesh ASTM), allowed to dry to attain the same consistency as the silica, and spread gently on top of a column (50 mm × 3 mm) packed dry with silica gel. The surface of the silica was covered with cotton wool and the column isocratically eluted with ethyl acetate/methanol (80:20). 50 fractions of 10 ml aliquots each were collected and bulked together according to their TLC profiles. Anisaldehyde reagent was used for

the detection of the compounds. Four bulked fractions were obtained designated as $FEC_{7A,}$ FEC_{7B}, FEC_{7C} and FEC_{7D} (Figure 3.8).

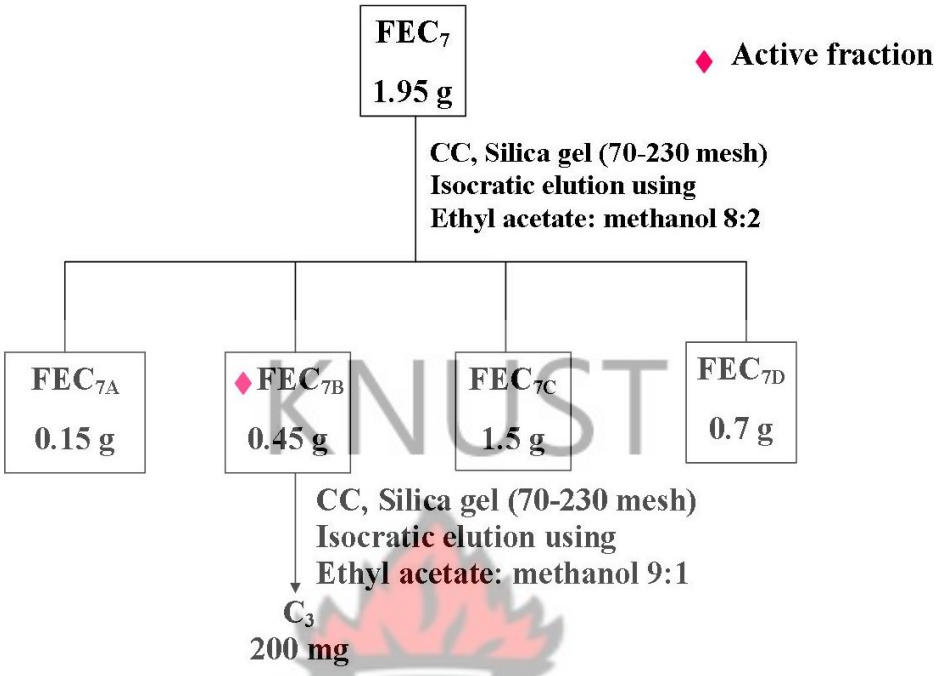
3.10.7 Isolation of C-3

1.5 g of FEC_{7C} was dissolved in a mixture of chloroform: methanol (1:1) and adsorbed unto silica gel (70-230 mesh ASTM). It was then chromatographed on silica gel eluting isocratically with ethyl acetate and methanol (90:10). 60 fractions of 2 ml aliquot were each collected. Compound C_3 was obtained in the first 8 fractions as an off white amorphous powder (200 mg). The scheme for the isolation of C-3 is as shown in Figure 3.8.



CC (column chromatography)

Figure 3.7 Schematic representation of the isolation of compound C-2



CC (column chromatography)

Figure 3.8 Schematic representation of the isolation of compound C-3

3.11 IDENTIFICATION OF ISOLATED COMPOUNDS

Identification of compounds usually involves a combination of different techniques, including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectroscopy. Other ways of confirming the identity of compounds include calculation of the retardation factor (R_f) values in different solvent systems and comparing with authentic samples, and determination of melting points. In this study, NMR and MS techniques were used as tools to determine the structure of the compounds.

3.11.1 Nuclear magnetic resonance spectroscopy (NMR)

The 1D-NMR (both ^{1}H-NMR and ^{13}C-NMR) spectra were determined with the assistance of the signal processing information base, University of Strathclyde, Glasgow. ^{1}H and ^{13}C-NMR were recorded on a Bruker DPX 600 spectrometer (ppm, *J* in Hz) using TMS as internal standard. DEPT and 2D-NMR experiments were also carried out on the same instrument.

In preparing the samples for NMR analysis, ~10 mg of the compound was dissolved in a maximum of 2 ml of deuterated solvents (Sigma). Deuterated chloroform ($CDCl_3$), methanol (CD_3OD) and DMSO were used as solvents of choice because the compounds of interest were soluble in them. The solutions (free of insoluble impurities) were then pipetted into NMR tubes for analysis using clean Pasteur pipettes.

3.11.2 ^{1}H-NMR

Proton NMR was widely used in the analysis. The spectrum appears in the range 0-10 ppm downfield from the reference signal, tetramethyl silane. Proton NMR gives a measure of the absorption of the different proton signals from a compound. The integral of the signal is proportional to the number of protons it represents, and the nature of the hydrogen is established by the chemical shift. The absorption of a signal is generally proportional to the number of protons coming into resonance frequency of the signal, with the result that the area under the absorption peak is proportional to the number of protons being detected (Williams and Fleming, 1995).

3.11.3 ^{13}C-NMR

^{13}C-NMR was used to determine the precise frequency at which each carbon comes into resonance and is determined not only by the applied magnetic field (βo) but also by minute differences in the magnetic environment experienced by each nucleus. These minute differences are caused by the variation in electrons in the neighborhood of each nucleus, with the result that each chemically distinct carbon atom in a structure, when it happens to be a ^{13}C, will come into resonance at a slightly different frequency from all the others. Each upward line in a ^{13}C spectrum corresponds to one carbon atom (Williams and Fleming, 1995).

3.11.4 DEPT

Distortionless enhancement through polarization transfer (DEPT) is a technique that allows a separate spectrum to be obtained for the ^{13}C of CH_3, CH_2 and CH. In this experiment, the impulse sequence used forces part of the higher sensitivity associated with proton detection on to ^{13}C, a process that enhances the ^{13}C signal intensity by polarization transfer from ^{1}H to ^{13}C. Although there are a number of different DEPT experiments, the most commonly used is the DEPT-135. This experiment shows positive signals for all CH and CH_3 carbon atoms in the molecule. Conversely, CH_2 carbon atoms show as negative signals. Signals for carbon atoms with no attached hydrogen (quaternary carbons) atoms are not present in this spectrum (www.chemistry.uca.edu/faculty/manion/2401/C13DEPT.pdf).

3.11.5 Mass spectrometry

Specific identification of molecules is more certain with the use of mass spectrometer (MS). In electron impact mass spectrometry (EI-MS), the effluent which contains the separated and vaporized compounds, passes into the ion chamber of the mass spectrometer, which is under high vacuum. A beam of electrons accelerated from a filament, which ionizes and fragments them, bombards the molecules. Initially, one electron is removed from each molecule to form a positively charged molecular ion (M^+). Breakage of bonds relative to bond strength occurs rapidly in the molecular ion to generate fragment ions. The various ions are accelerated into the analyser portion of the mass spectrometer where they are sorted according to their mass to charge ratios (m/z values) that are equivalent to the molecular weights of the fragments. The ion signal is amplified by an electron multiplier and the mass spectrum is plotted from low to high mass. The m/z values are plotted against relative abundance of the ions. The most abundant ion (base peak) in the spectrum is assigned as 100% (Williams and Fleming, 1995). In this study, a time of flight mass spectrometer (TOF MS) was used and the ionization methods employed were electron impact (EI^+) and electron spray (ES^+) for compounds PFE-2 and FE-1 respectively.

Chapter 4

RESULTS

4.1 CHARACTERISATION AND IDENTIFICATION OF ISOLATED COMPOUNDS

4.1.1 Identification of PFE-2 as *β*-sitosterol [1]

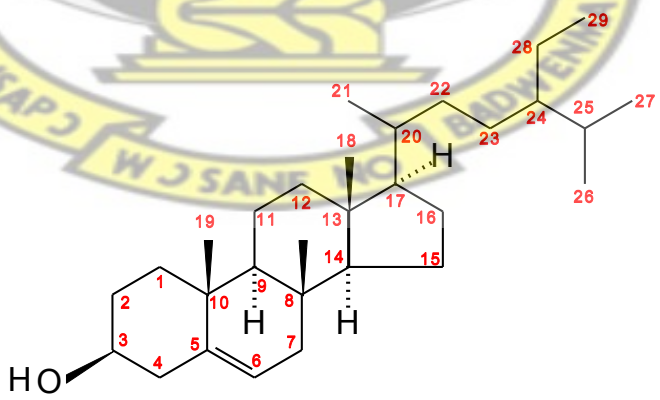
PFE-2 was obtained as white needle-like crystals with melting point 138-140^{o}C [literature: 141.6^{o}C (Villasenor *et al.*, 2002)].

The ^{1}H-NMR spectrum (Appendix 1B) exhibited six methyl signals at δ_H 0.69 (H-18), δ_H 0.82-0.89 (H-21, H-26, H-27 and H-29) and δ_H 0.94 ppm (H-19). The presence of these methyl signals between 0.69-0.94 ppm, indicated that these functionalities were attached to saturated carbons. The signal integrating for one proton at δ_H 5.40 was assigned to the olefinic proton at C-6. The chemical shift and splitting pattern of the signal integrating for one proton at δ_H 3.5 was consistent with the H-*3α* and a *3β*-hydroxyl function typical of *β*-sitosterol (Kovganko *et al.*, 1999).

The ^{13}C-NMR spectrum (Table 4.1; Appendix 1B) showed signals for all the twenty nine carbons, including the characteristic olefinic signals at 140.7 ppm and 121.7 ppm, assignable to C-5 and C-6, and the oxygenated carbon at 71.8 ppm assignable to C-3. Six methyl carbon signals occurred at δ_C 11.9, δ_C 19.4, δ_C 18.7, δ_C 21.1, δ_C 22.0 and δ_C 12.0 ppm corresponding to C-18, C-19 (angular methyls), C-21, C-26, C-27 and C-29 (methyls of the side chain). The remaining carbon atoms in the spectrum corresponded to

methylene and methine carbons of the steroidal nucleus. These data compared favourably with those in the literature (Table 4.1; Kovganko *et al.*, 1999).

The EI-MS exhibited a maximum peak at *m/z* 414 (Appendix 1A), corresponding to the molecular formula $C_{29}H_{50}O$. Other prominent fragments appeared at m/z 396 (M^+-H_2O), 381(M^+-CH_3-H_2O), 329(M^+-C_5H_7-H_2O), 303 (M^+-C_7H_9-H_2O), 273 (M^+-side chain, $C_{10}H_{21}$), 255 (M^+-side chain-H_2O), 231 (M^+-side chain-cyclopentyl group) and 213 (231-H_2O) which were diagnostic for sterols with a $C_5 = C_6$ double bond (Burdi *et al.*, 1991). The mass fragmentation pattern, ^{1}H-NMR and ^{13}C-NMR data showed the identity of PFE-2 as *β*-sitosterol [**1**]. The unequivocal identification of PFE-2 was accomplished by direct TLC comparison with the reference commercial *β*-sitosterol (Sigma-Aldrich, USA). Co-chromatography of PFE-2 and the reference sample gave identical R_f values and chromogenic reaction to anisaldehyde reagent. *β*-sitosterol [1] is a ubiquitous plant sterol, having been isolated from many plant species, including members of the genus *Ficus* (Li *et al.*, 2006; Wu *et al.*, 2002), however, to the best of our knowledge, this is the first report of its occurrence in *F. exasperata*.



[1]

Table 4.1 ^{13}C-NMR chemical shifts (ppm) of *β*-sitosterol and compound PFE-2

Carbon position	*β-Sitosterol	Compound PFE-2	Carbon position	*β-Sitosterol	Compound PFE-2
C-1	37.2	37.2	C-16	27.9	28.0
C-2	31.6	31.9	C-17	56.1	56.0
C-3	71.5	71.8	C-18	11.8	11.9
C-4	42.2	42.3	C-19	19.4	19.4
C-5	140.8	140.7	C-20	35.7	35.9
C-6	121.7	121.7	C-21	18.7	18.7
C-7	31.8	31.9	C-22	36.0	36.5
C-8	31.8	31.8	C-23	23.8	23.0
C-9	50.0	50.1	C-24	39.5	39.5
C-10	37.0	37.2	C-25	28.2	29.1
C-11	21.1	21.1	C-26	22.4	21.1
C-12	39.7	39.7	C-27	22.7	22.0
C-13	42.2	42.3	C-28	24.3	24.3
C-14	56.0	56.7	C-29	11.8	11.9
C-15	24.2	24.3			

*(Kovganko *et al.*, 1999)

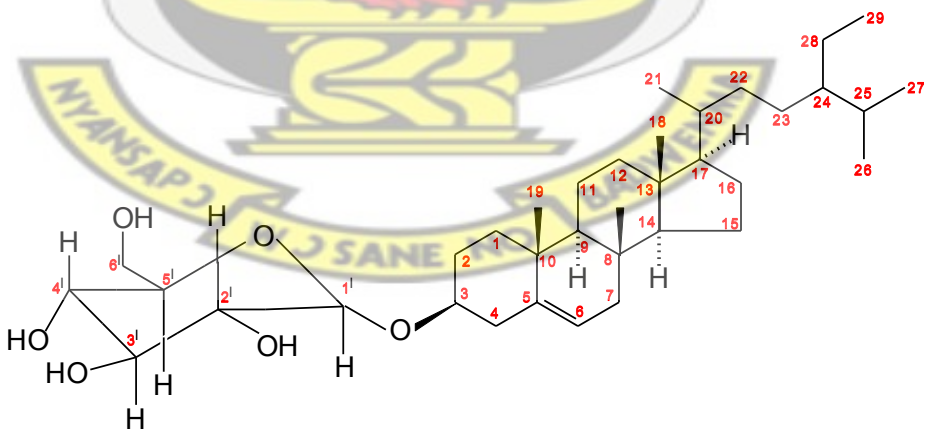
4.1.2 Identification of FE-1 as sitosterol-3-O-*β*-D-glucoside **[7]**

Compound FE-1 was obtained as a white amorphous powder with a melting point of 280-282°C.

The ^{1}H-NMR spectrum (Appendix 2B) was similar to *β*-sitosterol except for additional signals at δ_H 3.88 [1H (d, d), J = 2.3, 2.1 Hz)] assignable to H-5', and multiplets at δ_H 3.38, 3.29 and 3.19, each integrating for one proton, assignable to H-2', H-3' and H-4' of a sugar moiety (Pei-Wu *et al.*, 1988). The anomeric proton signal (H-1') occurred as a doublet at δ_H 4.41 with a J value of 7.8 Hz. This confirmed the orientation of the glucose as a *β* linkage due to the coupling constant of the anomeric proton (Villasenor *et al.*, 2002).

The ^{13}C-NMR spectrum (Appendix 2C-D) confirmed the presence of 35 carbon atoms. It showed the characteristic olefinic signals at 140.4 ppm (C-5) and 121.5 ppm (C-6) and

the oxymethine carbon (C-3) at 76.7 ppm. Glucosidation at position C-3 caused the downfield chemical shift of C-3 by 4.9 ppm, compared with its position in the spectrum of *β*-sitosterol. This *β*-effect of the glucoryl group caused an upfield shift for C-2 and C-4 in the spectrum of the glucoside (Agrawal, 1992). The anomeric (C-1') and oxygenated methylene (C-6') carbons of the sugar moiety, appeared at δ_C 101.1 and δ_C 61.5 respectively. The remaining sugar carbons resonated at δ_C 75.6 (C-2'), δ_C 76.4 (C-3'), δ_C 73.7 (C-4') and δ_C 78.6 (C-5') (Table 4.2). The MS-ES exhibited a molecular ion peak at *m/z* 599 (Appendix 2A) corresponding to the sodium adduct ion $(M+Na)^+$, which is 23 daltons higher than the expected molecular mass. This agreed with the molecular formula $C_{35}H_{60}O_6$. The spectrum also showed a weak signal at *m/z* 414 ($C_{29}H_{50}O$; *β*-sitosterol) and a characteristic fragmentation pattern similar to *β* -sitosterol. On the basis of the above evidence and comparison with published data (Table 4.2; Pei-Wu *et al*., 1988), the structure of FE-1 was established as sitosterol-3-O-*β*-D-glucopyranoside [**7**].



[7]

Table 4.2 ^{13}C-NMR chemical shift (ppm) of sitosterol-3-O-*β*-D-glucopyranoside and FE-1

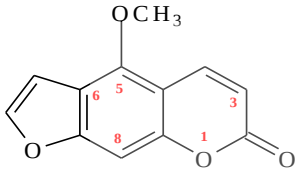
Carbon position	*β-sitosterol-glucoside	Compound FE-1	Carbon position	*β-sitosterol-glucoside	Compound FE-1
C-1	38.2	37.2	C-19	19.1	19.0
C-2	27.9	28.0	C-20	36.7	36.5
C-3	77.9	76.7	C-21	18.8	18.3
C-4	39.8	39.7	C-22	34.1	33.8
C-5	140.7	140.4	C-23	26.3	25.8
C-6	122.6	121.5	C-24	46.0	45.9
C-7	32.0	31.9	C-25	29.1	29.4
C-8	32.0	31.7	C-26	19.3	18.7
C-9	50.1	50.3	C-27	19.8	19.0
C-10	37.1	37.2	C-28	23.2	22.81
C-11	21.1	20.9	C-29	11.9	11.18
C-12	39.8	38.4	C-1'	104.3	101.1
C-13	42.4	42.2	C-2'	75.6	75.6
C-14	56.8	56.8	C-3'	76.6	76.4
C-15	24.4	24.0	C-4'	73.5	73.7
C-16	28.3	28.0	C-5'	79.2	78.6
C-17	56.2	56.0	C-6'	61.1	61.5
C-18	11.9	12.0			

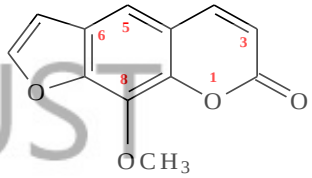
*Pei-Wu *et al*., 1988

4.1.3 Identification of C-1 as bergapten **[20]**

C-1 was obtained as white needle-like crystals with a characteristic odour and melted at 189-191°C. The UV spectrum in methanol (Appendix 3C) exhibited four absorption maxima at 223, 249, 267 and 311 nm, characteristic of a furanocoumarin nucleus (Yu-Chang *et al*., 2008). The ^{13}C-NMR spectrum (Table 4.3; Appendix 3A) exhibited 12 carbon resonances including five methines, one methoxy, one carbonyl and five quaternary carbons. The ^{1}H-NMR spectrum (Table 4.3; Appendix 3B) showed two proton doublets at δ_H 6.28 (J = 9.6 Hz) and δ_H 8.16 (J = 9.6 Hz) characteristic of α-pyrone protons assignable to H-3 and H-4 respectively, and a pair of doublets occurring at δ_H 7.03 (J = 2.2 Hz) and δ_H 7.61 (J = 2.2 Hz) typical of furanic protons assignable to H-3'

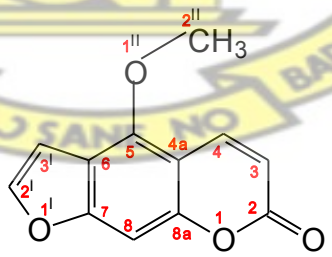
and H-2' respectively (Shikishima *et al*., 2001). The spectrum further showed a proton singlet at δ_H 7.37 and a methoxy signal at δ_H 4.42, assignable to either C-5 or C-8 of the furanocoumarin structure. This suggested two possible structures **A** and **B** for C-1.





A **B**

The unambiguous assignment of the methoxy signal to C-5 was supported by the characteristic downfield shift of the resonance of H-4 between δ_H 7.9-8.2 due to the oxygenation at C-5 (http://202.119.189.236:8087/ec3.0/C54/en/Coumarinsjy.swf). This NMR data compared favourably with that published for bergapten [**20**] (Table 4.3; Chunyan *et al.*, 2009). It has been isolated from the aerial parts of *Ficus pumilla* and the stem bark of *F. religiosa* (Makhija *et al*., 2010: Pistelli *et al*., 2000). However, this is the first report of the occurrence of this compound in the stem bark of *F. exasperata.*



[20]

Table 4.3 ^{13}C and ^{1}H-NMR chemical shifts (ppm) of bergapten and C-1

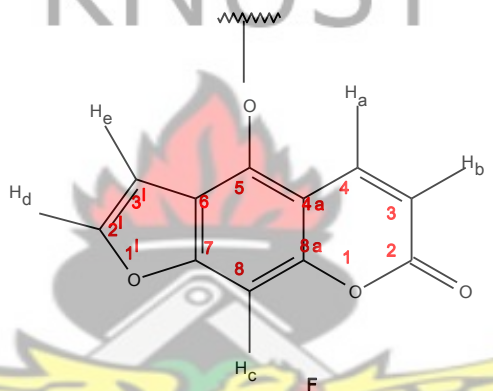
Carbon position	*δ_H	δ_H	C-1	*Bergapten
C-2	-	-	161.3	161.2
C-3	6.28	6.27	112.5	112.5
C-4	8.16	8.15	139.2	139.2
C-4a	-	-	106.4	106.4
C-5	-	-	149.3	149.5
C-6	-	-	112.3	112.6
C-7	-	-	158.3	158.3
C-8	7.37	7.13	93.8	93.8
C-8a	-	-	152.6	152.7
C-2'	7.61	7.59	144.8	144.7
C-3'	7.03	7.02	105.0	105.0
C-2''	4.42	4.27	60.1	60.0

*Chunyan *et al.*, 2009

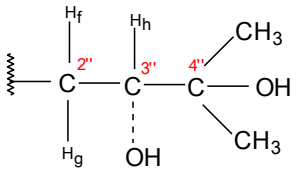
4.1.4 Identification of C-2 as oxypeucedanin hydrate **[30]**

Compound C-2 (115 mg) was isolated from the chloroform extract of the stem bark as a pale yellow amorphous compound. It was soluble in acetone and ethanol, gave a yellow florescence when viewed under UV light at 365 nm, and melted at 136-138°C. The UV spectrum of C-2 (Appendix 4G) showed characteristic absorption maxima similar to that of C-1 at 223, 250, 259, 267, and 309 nm, confirming a furanocoumarin structure. The ^{1}H-NMR spectrum (Table 4.4; Appendix 4B) of C-2 was also similar to that of C-1, except that it lacked the methoxy signal at δ_H 4.42 but also had additional signals consistent with a prenyl group made up of two methyl singlets (δ_H 1.11 and δ_H 1.18), methylene multiplets (δ_H 4.27 and δ_H 4.77), one oxymethine multiplet (δ_H 3.64) and two hydroxyl protons (δ_H 4.48 and δ_H 5.43) (Table 4.4). These were confirmed in the DEPT

135 ^{13}C-NMR spectrum (Table 4.4; Appendix 4A) which showed a total of 16 carbon resonances. It showed the eleven (11) carbon resonances for the furanocoumarin nucleus (Partial structure F), as described for bergapten [**20**], and five additional signals arising from carbons at the side-chain that accounted for: 2 methyl groups (δ 24.7 and 28.2**),** one oxymethylene (δ 75.5), one oxymethine (δ 76.9) and a quaternary oxygenated carbon (71.2 ppm).

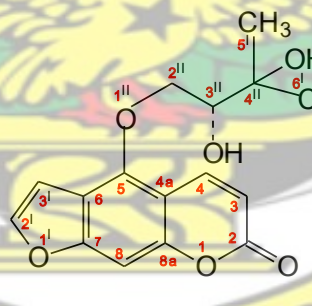


In the heteronuclear multiple bond correlation (HMBC) spectrum (Table 4.6; Appendix IV E-F), the proton multiplet at δ_H 3.64 (H_h) which is directly attached to C-3" in the HSQC spectrum (Table 4.5; Appendix IV C-D), correlated with the carbon signals at C-2" (^2J), C-4' (^2J) and the methyl carbons C-5' (^3J) and C-6' (^3J); the OH signal at δ_H 4.48 (directly attached to C-4' in the HSQC spectrum) showed a ^3J correlation with C-5', C-6' and C-3" in the HMBC spectrum (Table 4.6; Appendix 4E-F). Also the proton signal at δ_H 4.27 (H_g) (directly attached to C-2" in the HSQC spectrum) coupled to C-3" in a ^2J correlation; the OH group at δ_H 5.26 (attached directly to C-3") coupled to C-4' and C-2" in a ^3J correlation and to C-3" in a ^2J correlation in HMBC spectrum. This gave the side chain structure G.



G

Putting G and partial structure F together gave the complete structure of C-2, elucidated as oxypeucedanin hydrate [**30**]. The oxymethylene proton signals H_g (δ_H 4.27) and H_f (δ_H 4.77) showed a ^3J correlation with the carbon signal at δ_C 149.8 (C-5), confirming that the oxyprenyl unit was connected to C-5. This resulted in a more downfield chemical shift of C-4 by approximately 1.7 ppm compared to the spectrum of bergapten (Appendix 3A). Oxypeucedanin hydrate [**30**] has been isolated from the aerial parts of *Ficus pumilla* (Pastelii *et al.*, 2000). However, to the best of our knowledge, this is the first time it is being reported in *F. exasperata*.



[30]

Table 4.4 ^{1}H-NMR and ^{13}C-NMR spectral data for C-2 in DMSO at 400MHz

Atom number	Type	δH	δC
C-2	C	-	160.7
C-3	CH	6.36, d (H_b, *J*=10Hz)	112.7
C-4	CH	8.39, d (H_a, *J*=9.6Hz)	140.9
C-4a	C	-	107.0
C-5	C	-	149.8
C-6	C	-	113.9
C-7	C	-	158.1
C-8	CH	7.37, s (H_c)	93.9
C-8a	C	-	152.6
C-2'	CH	8.04, d (H_d, *J*=2.4Hz)	147.1
C-3'	CH	7.32, d (H_e *J*=2.2Hz)	106.1
C-2''	CH_2	4.77, dd (H_f, *J*=2Hz) 4.27, t (H_g)	75.5
C-3''	CH	3.64, m (H_h) 5.43 (OH)	76.9
C-4'	C	4.48 (OH)	71.2
C-5'	CH_3	1.11, s (H_j)	24.8
C-6'	CH_3	1.18, s (H_i)	28.2

Table 4.5 HSQC correlation of C-2

carbon number	δH	δ C-ppm
C-3	6.36	112.7
C-4	8.39	140.9
C-8	7.37	93.9
C-2'	8.04	147.1
C-3'	7.32	106.1
C-2''	4.77, 4.27	75.5
C-3''	3.64	76.9
C-5'	1.11	24.8
C-6'	1.18	28.2

Table 4.6 HMBC correlation of C-2

carbon number	δ C-ppm	δH-ppm
C-2	160.7	H_a and H_b
C-3	112.7	
C-4	140.9	
C-4a	107.0	H_c and H_b
C-5	149.8	H_a, H_f and H_g
C-6	113.9	H_d, H_c and H_e
C-7	158.1	H_d, H_c and H_e
C-8	93.9	
C-8a	152.6	H_a, H_c
C-2'	147.1	H_e
C-3'	106.1	H_d
C-2''	75.5	H_h, δ4.48 OH (δ5.43)
C-3''	76.9	OH (δ 5.43), OH (δ 4.48), H_g, H_i and H_j
C-4'	71.2	OH (δ 5.43), OH (δ 4.48), H_h, H_i and H_j
C-5'	24.8	OH (δ 4.48), H_h and H_i
C-6'	28.2	OH (δ 4.48), H_h and H_j

4.1.5 Identification of C-3 as sitosterol-3-O-*β*-D-glucopyranoside **[7].**

Compound C-3 was obtained as a white amorphous powder with physical properties similar to FE-1. It gave the same melting point and was insoluble in all solvents except a mixture of chloroform: methanol (7:3). The unequivocal identification of C-3 as sitosterol-3-O-*β*-D-glucopyranoside [7] was confirmed by direct TLC comparison. Co-chromatography of C-3 and FE-1, gave the same R_f values [(0.77, MeOH: $CHCl_3$ 2:8), (0.43, MeOH: $CHCl_3$ 1:9) and chromogenic reaction to anisaldehyde reagent (pink spot on heating). This is the first time sitosterol-3-O-*β*-D-glucopyranoside **[7]** is being reported in the stem bark of *F. exasperata*.

Figure 4.1 Thin Layer Chromatogram of C-3 and FE-1

4.2 BIOASSAYS

4.2.1 Anti-inflammatory activity of leaf extracts

To assess the anti-inflammatory activity of the petroleum ether (PFE), ethyl acetate (EFE) and ethanol (AFE) extracts of the leaves of *F. exasperata*, the carrageenan- induced foot oedema in chicks model was used. The extracts were given orally to the chicks at 30 mg/kg, 100 mg/kg and 300 mg/kg, 1 hour after induction of oedema with carrageenan. Diclofenac (10-100 mg/kg, *i.p*) and dexamethasone (0.1-3 mg/kg, *i.p*) were used as reference drugs. Induction of acute inflammation in control chicks resulted in a prominent increase in paw thickness, which began 1 hour after *intraplantar* injection of carrageenan and reached a peak of inflammation after 2 hours (Figure 4.2a) and slowly declined for the next 3 hours. The extracts (PFE, EFE and AFE), caused significant ($P < 0.001$) dose-dependent inhibition of the carrageenan - induced inflammation in the seven day old chicks, the effect of which began 2 hours after carrageenan injection (Figures 4.2- 4.4). Diclofenac (10-100 mg /kg, *i.p*) and dexamethasone showed significant ($P < 0.001$) effect on the time course curve and dose dependently reduced the total oedema (Figure 4.5a-d). Values are means ± S.E.M. (n=5). ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test). Dose response curves for the inhibition of foot oedema are shown in Figure 4.6a-b. The stronger the anti-inflammatory actions of the drug, the lesser the quantity needed to inhibit the oedema by 50%. This is expressed as ED_{50} (mg/kg) values. Dexamethasone showed the highest anti-inflammatory activity, followed by diclofenac, pet-ether, ethyl acetate and ethanol extracts (Table 4.7).

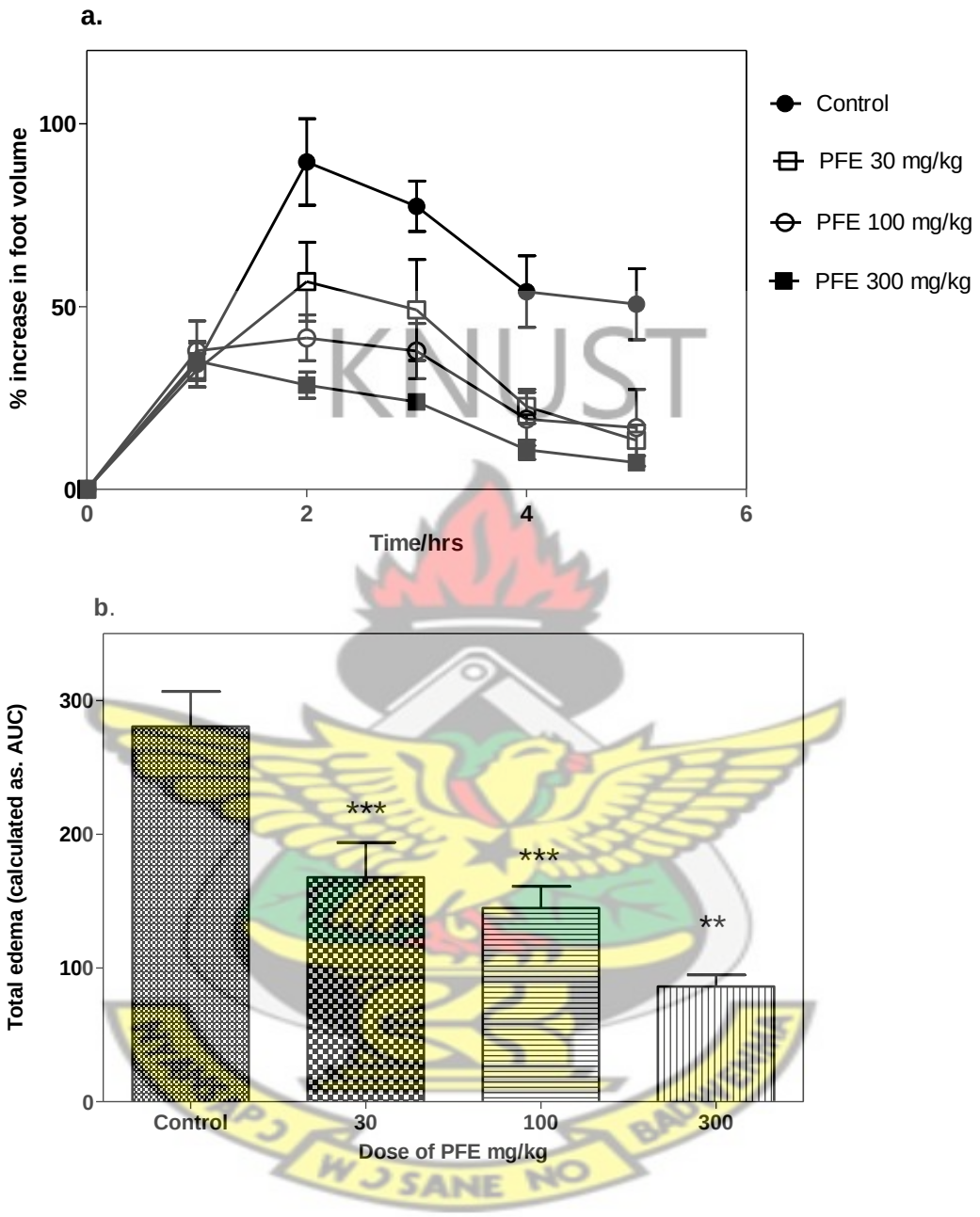


Figure 4.2 Effect of the pet-ether leaf extract (30-300 mg/kg oral), on time course curve (a) and the total oedema response (expressed as AUC, b) for 5 hours, in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

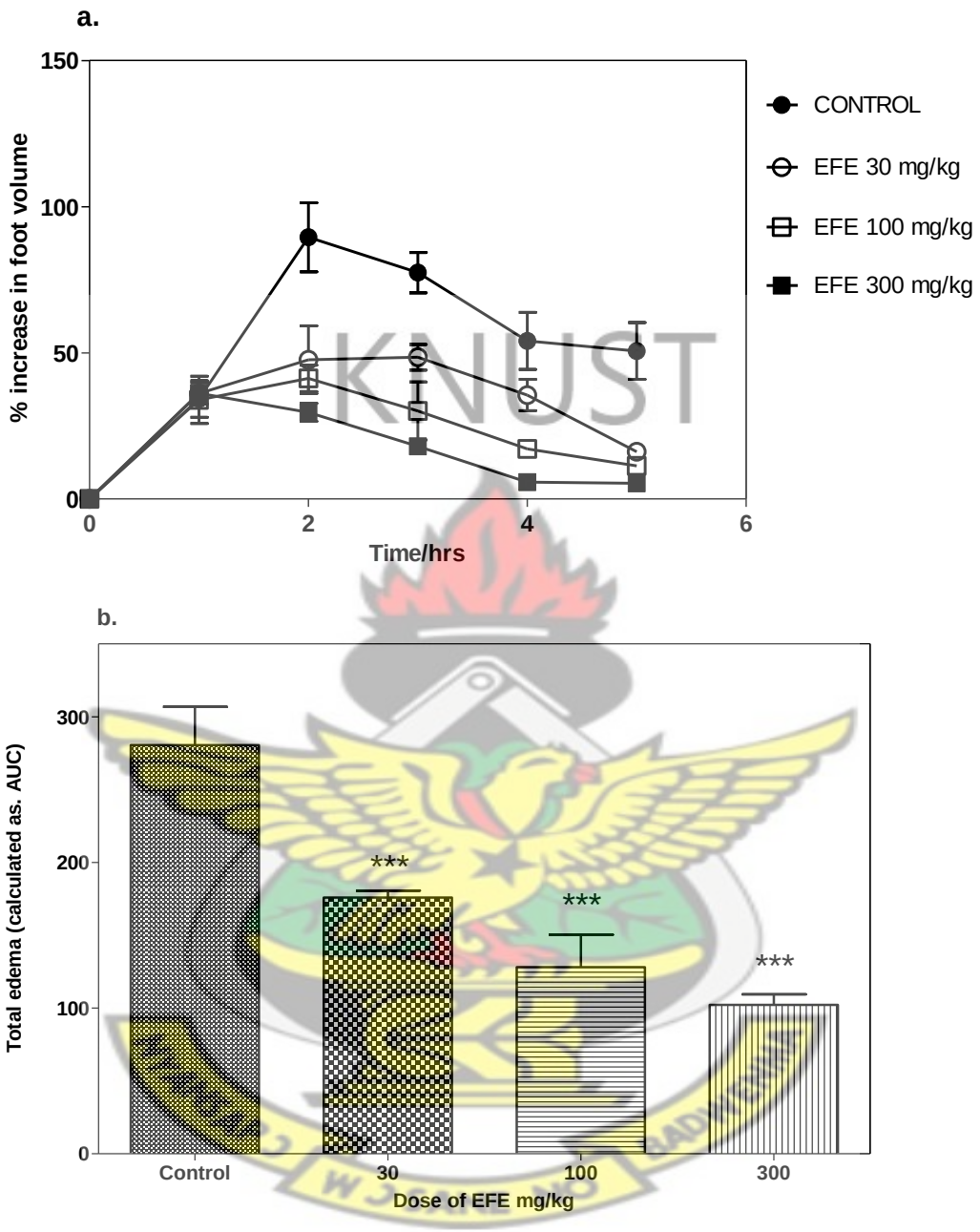


Figure 4.3 Effect of the ethyl acetate leaf extract (30-300 mg/kg oral), on time course curve (a) and the total oedema response (expressed as AUC, b) for 5 hours, in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

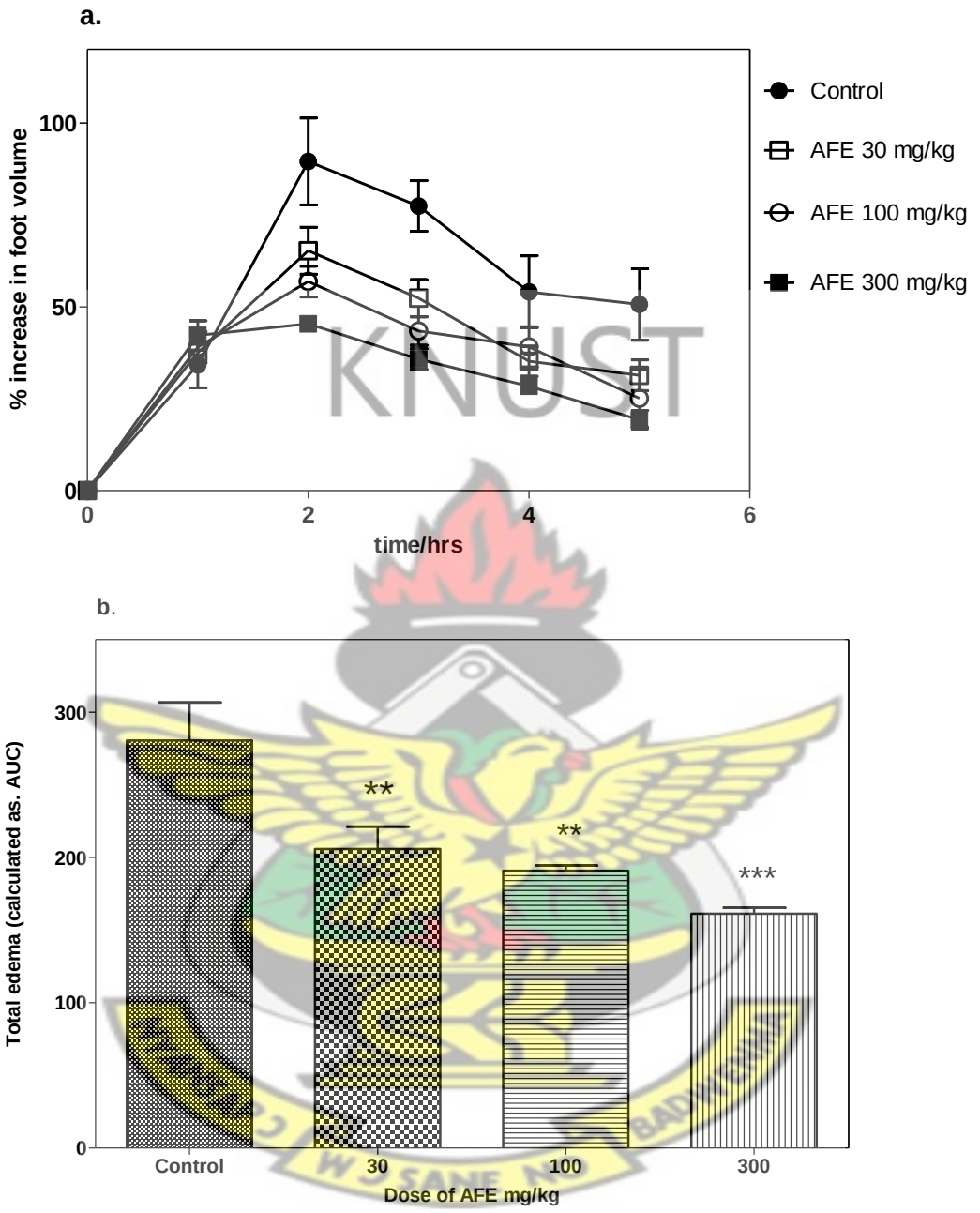


Figure 4.4 Effect of the alcoholic leaf extract (30-300 mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC, b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

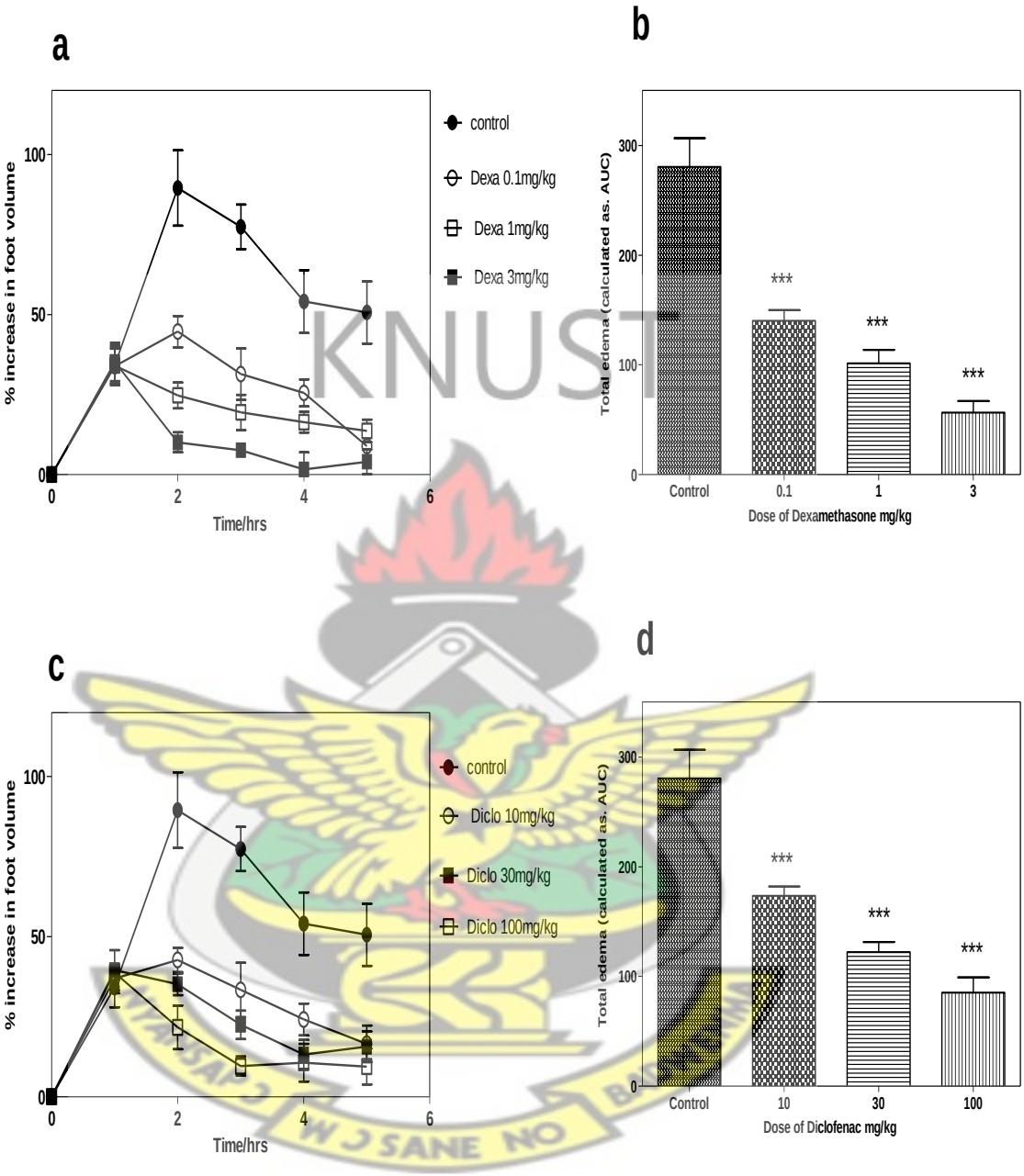


Figure 4.5 Effect of dexamethasone (0.1-3 mg/kg; *i.p*) and diclofenac (10-100 mg/kg; *i.p*) on time course curve (a and c) and the total oedema response (expressed as AUC, b and d) in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

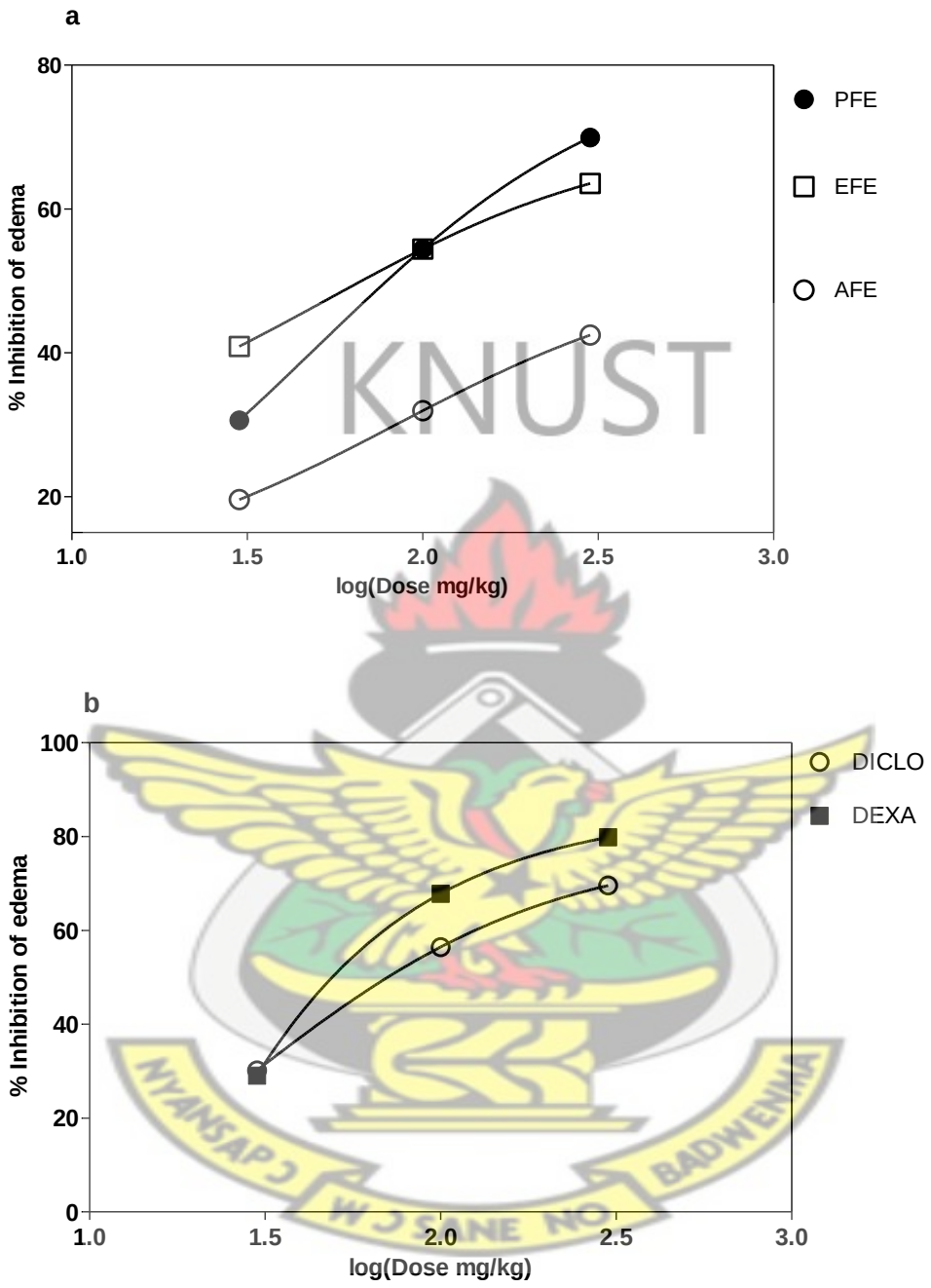


Figure 4.6 Dose response curves for PFE, EFE, AFE (30-300mg/kg *p.o*), diclofenac (10-100mg/kg *i.p*) and dexamethasone (0.3-3 mg/kg *i.p*) on carrageenan - induced foot oedma in chicks.

Table 4.7 Effect of leaf extracts and standard drugs on carrageenan-induced oedema

Extracts/Drugs	ED_{50} (mg/kg) ± SEM
PFE	49.59 ± 0.02
EFE	53.37 ± 0.011
AFE	84.85 ± 0.013
Diclofenac	27.27 ± 0.001
Dexamethasone	2.85 ± 0.003

PFE (Pet-ether leaf extract), **EFE** (Ethyl acetate leaf extract), **AFE** (Alcoholic leaf extract)

4.2.2 Anti-inflammatory activity of pet-ether fractions

The anti-inflammatory activity of the leaves of *F. exasperata* was highest in the petroleum ether extract (PFE) followed by the ethyl acetate extract (EFE) (Section 4.2.1). PFE was therefore subjected to column chromatographic fractionation to give six fractions designated as PFE_{1-6}. Results of the anti-inflammatory activity of the fractions PFE_{1-6} (Table 4.8; Figure 4.7-4.9), showed that all the six fractions dose-dependently inhibited carragenan – induced foot oedema to varying degrees. The order of potency showed by the ED_{50} (Table 4.8) was; $PFE_2 > PFE_4 > PFE_1 > PFE_3 > PFE_6 > PFE_5$.

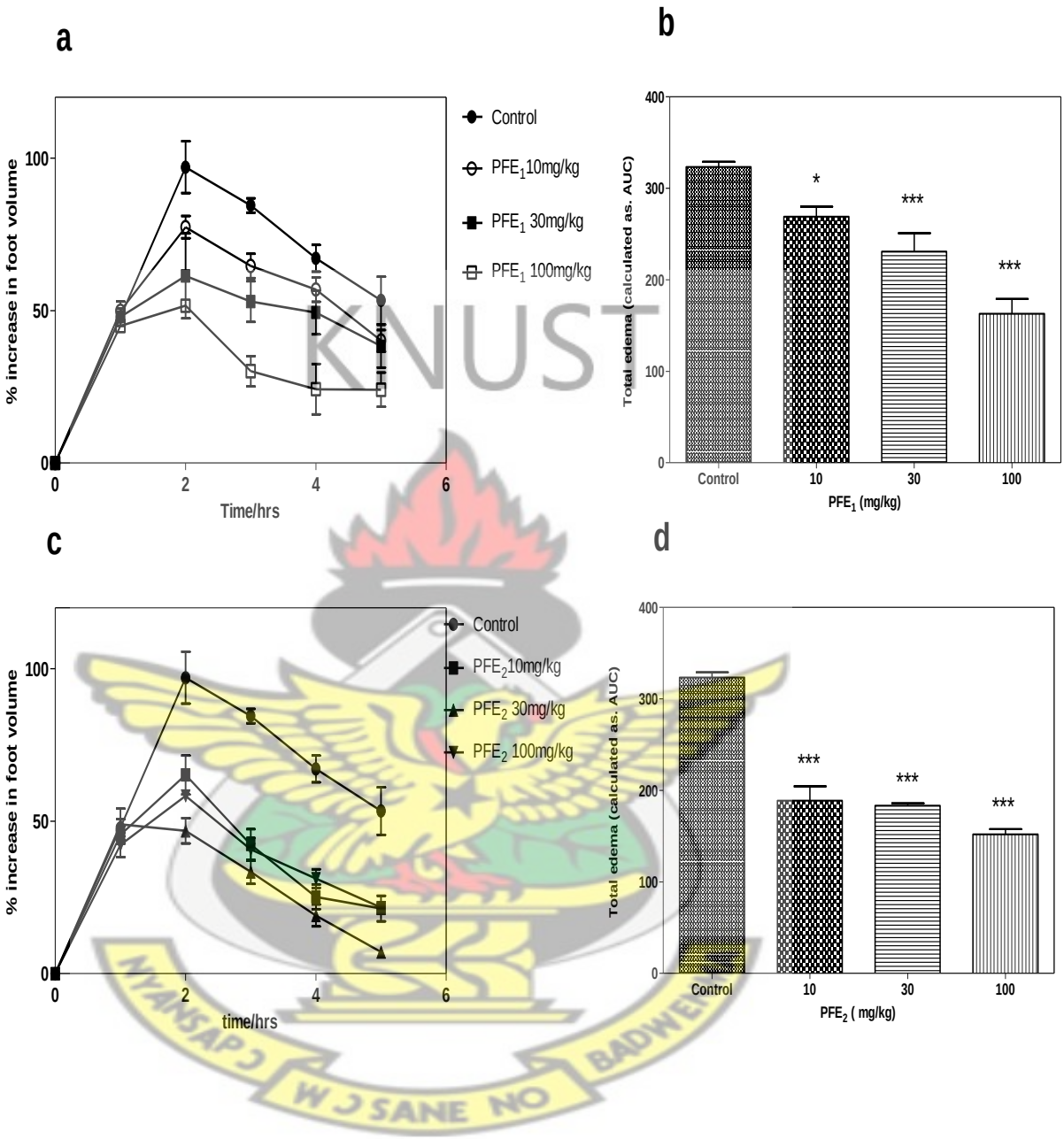


Figure 4.7 Effect of PFE_1 and PFE_2 (10-100 mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC, b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

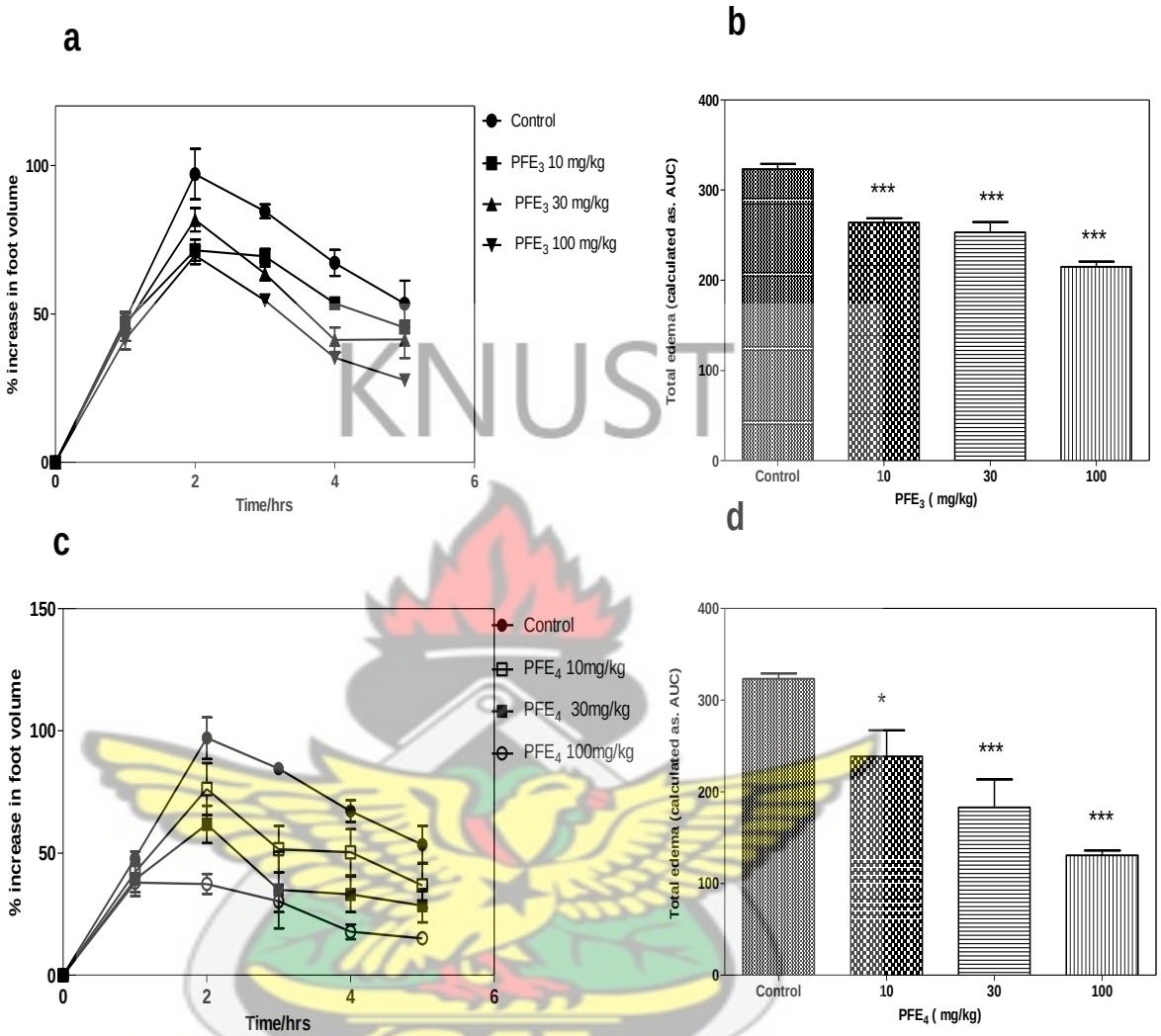


Figure 4.8 Effect of PFE_3 and PFE_4 (10-100 mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC, b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

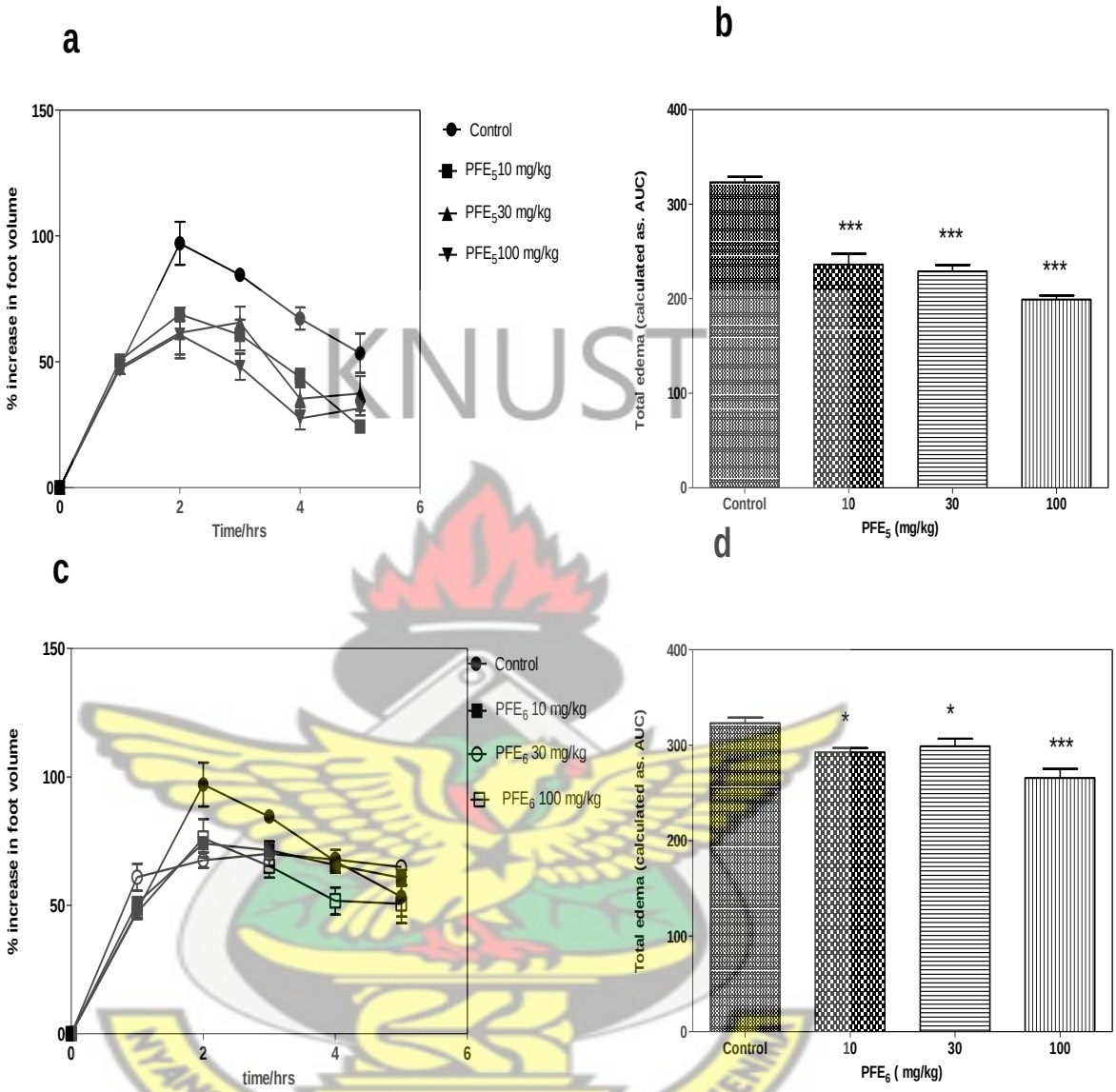


Figure 4.9 Effect of PFE_5 and PFE_6 (10-100 mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC, b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

Table 4.8 Effect of fractions PFE_{1-6} on carragenan-induced oedema in 7 day old chicks

Fractions of PFE	ED_{50} (mg/kg) ± SEM
PFE_1	115.7 ± 0.002
PFE_2	80.16 ± 0.001
PFE_3	140.9 ± 0.003
PFE_4	90.14 ± 0.002
PFE_5	397.2 ± 0.001
PFE_6	212.9 ± 0.001

4.2.3 ANTI-INFLAMMATORY EFFECTS OF ISOLATES

Chromatographic fractionation of fractions PFE_2 and PFE_4 and EFE led to the isolation of compounds PFE-1 and PFE-2 and FE-1 respectively. Compound PFE-1 was not tested due to paucity of material.

4.2.4 *β*-sitosterol [**1**] (PFE-2) and Sitosterol-3-O-*β*-D-glucopyranoside [**7**] (FE-1)

Oral administration of *β*-sitosterol [**1**] (3-30 mg/kg) showed a dose - dependent inhibition of oedema in the 7 - day old chick (Figure 4.10). It recorded a maximum inhibition of 27.08 ± 3.12% at 30 mg/kg and an ED_{50} value of 123.4 ± 0.033 mg/kg. Also sitosterol-3-O-*β*-D-glucopyranoside [**7**], showed dose-dependent inhibition of oedema in the chick model (Figure 4.11) with maximum inhibition of 17.9% at 30 mg/kg and an ED_{50} of 275.9 ± 0.012 mg/kg. Thus *β*-sitosterol [**1**] exhibited a higher anti-inflammatory activity than its glucoside [**7**] (Table 4.9).

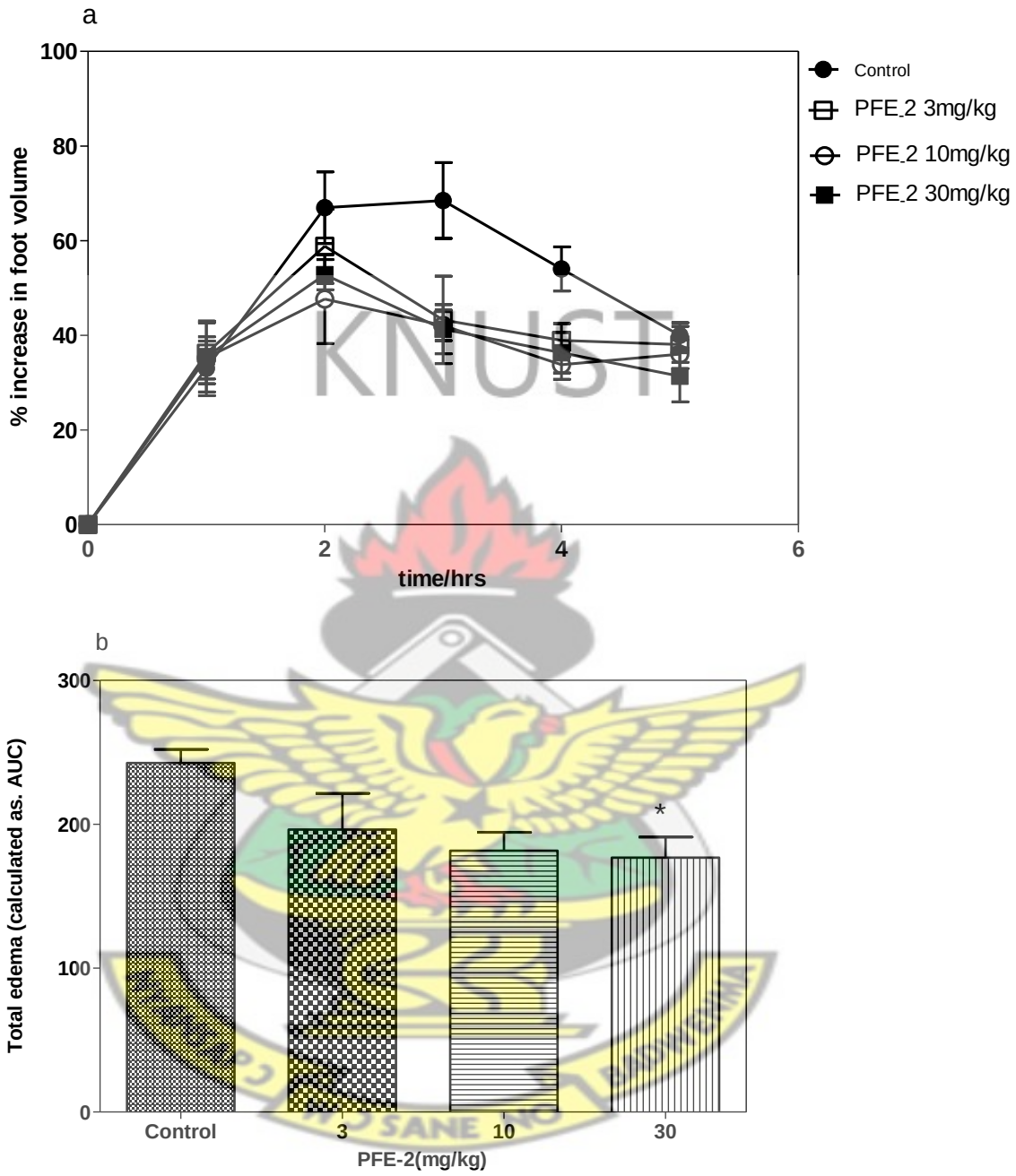


Figure 4.10 Effect of PFE-2 (3-30mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC; b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

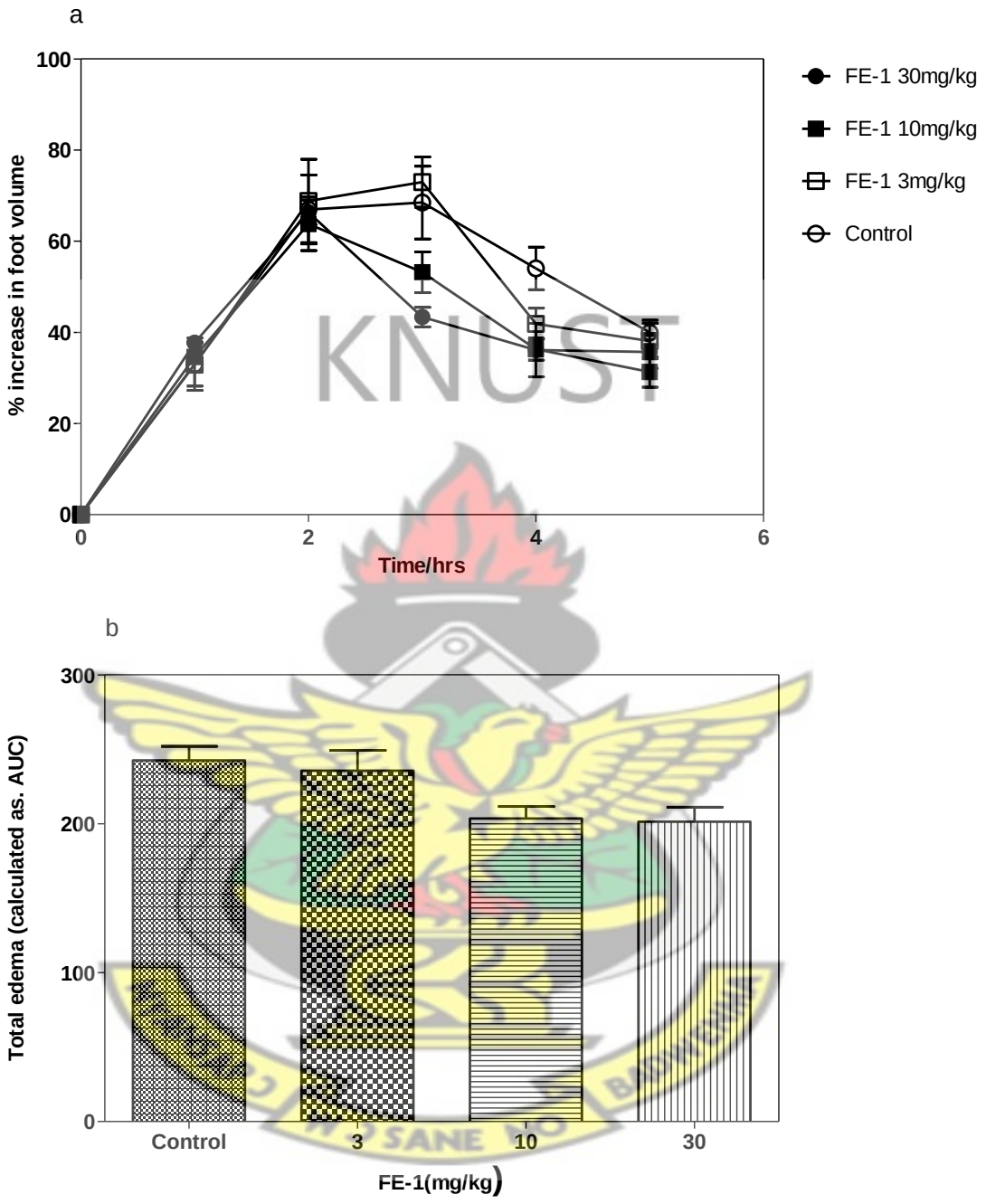


Figure 4.11 Effect of FE-1 (3-30 mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC, b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

Table 4.9 Effect of *β*-sitosterol [1] and its glucoside [7] on carrageenan – induced oedema

Extract/Drug	ED_{50} (mg/kg) ± SEM
***β*-sitosterol**	123.4 ± 0.033
Sitosterol-3-O-*β*-D-glucopyranoside	275.9 ± 0.012

4.3 ANTI-INFLAMMATORY ACTIVITY OF STEM BARK EXTRACTS

4.3.1 Anti-inflammatory effect of tota ethanol extract (SFE)

The total ethanol extract of the stem bark (SFE) of *F. exasperata* at 300mg/kg body weight, significantly ($P < 0.001$) showed dose-dependent decrease in total oedema with maximum inhibition of 68.57 ± 3.34% (Figure 4.12b). Similarly, diclofenac and dexamethasone, significantly ($P < 0.001$) and dose-dependently reduced the foot oedema (Figure 4.13) over the period of the experiment with maximal inhibitions of 71.56 ± 3.43 and 74.53 ± 5.21% respectively. The order of potency as indicated by the ED_{50} (Table 4.10) was dexamethasone > diclofenac > SFE.

Table 4.10 Effect of SFE and standard drugs on carrageenan-induced oedema in 7 - day old chicks

Extract/drug	ED_{50} (mg/kg) ± SEM
SFE	50.65 ± 0.012
Diclofenac	16.97 ± 0.011
Dexamethasone	2.95 ± 0.013

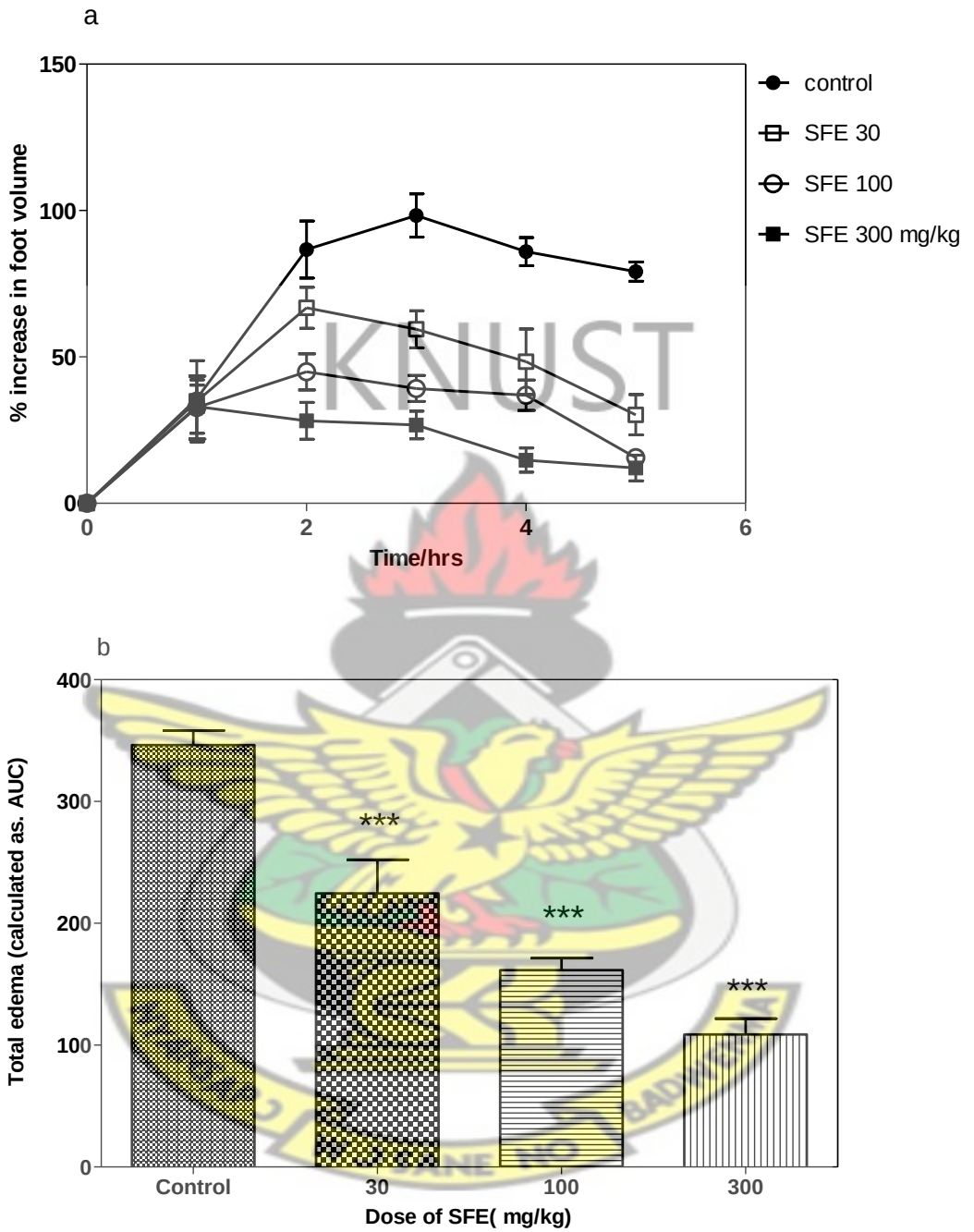


Figure 4.12 Effect of the total alcoholic stem bark extract (SFE, 30-300mg/kg oral), on time course curve (a) and the total oedema response for 5 hours (expressed as AUC,b), in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

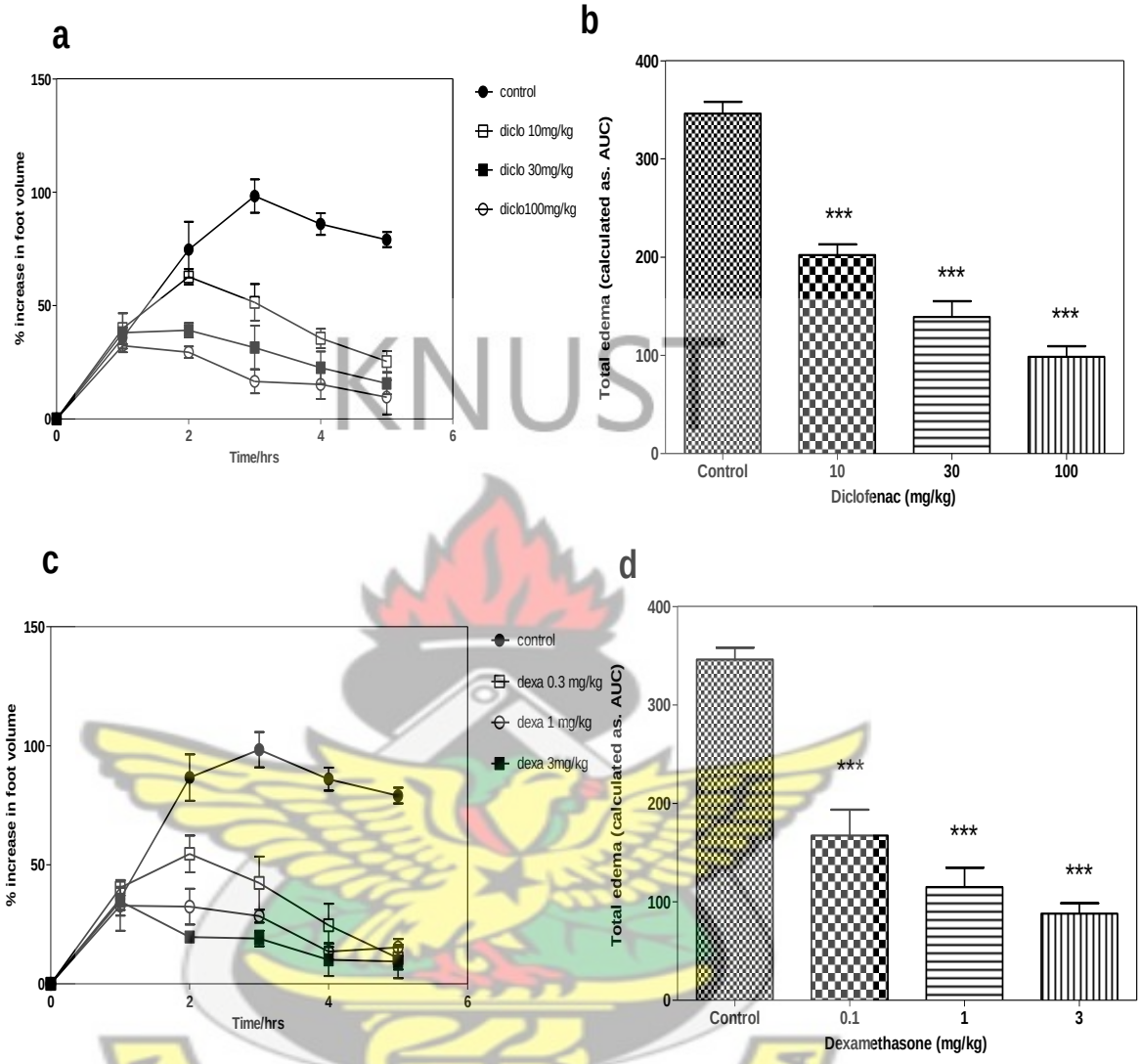


Figure 4.13 Effect of diclofenac (10-100 mg/kg; *i.p*) and dexamethasone (0.1-3 mg/kg; *i.p*) on time course curve (a) and the total oedema response (expressed as AUC,b) in carrageenan – induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

4.3.2 Anti-inflammatory activity of extracts

The pet-ether, chloroform and ethanol extracts of the stem bark exhibited anti-inflammatory activity *in vivo*, by reducing the oedematous response induced by carrageenan (Figures 4.14- 4.16). The order of decreasing activity as indicated by the ED_{50} (Table 4.11) was FEC>FEE>FEP.

Table 4.11 Effect of FEC, FEP and FEE on carrageenan-induced oedema in 7 - day old chicks

Extracts	ED_{50} (mg/kg) ± SEM
FEP	215.00 ± 0.011
FEC	97.76 ± 0.013
FEE	109.70 ± 0.013

FEP (pet-ether extract), **FEC** (chloroform extract), **FEE** (ethanol extract)

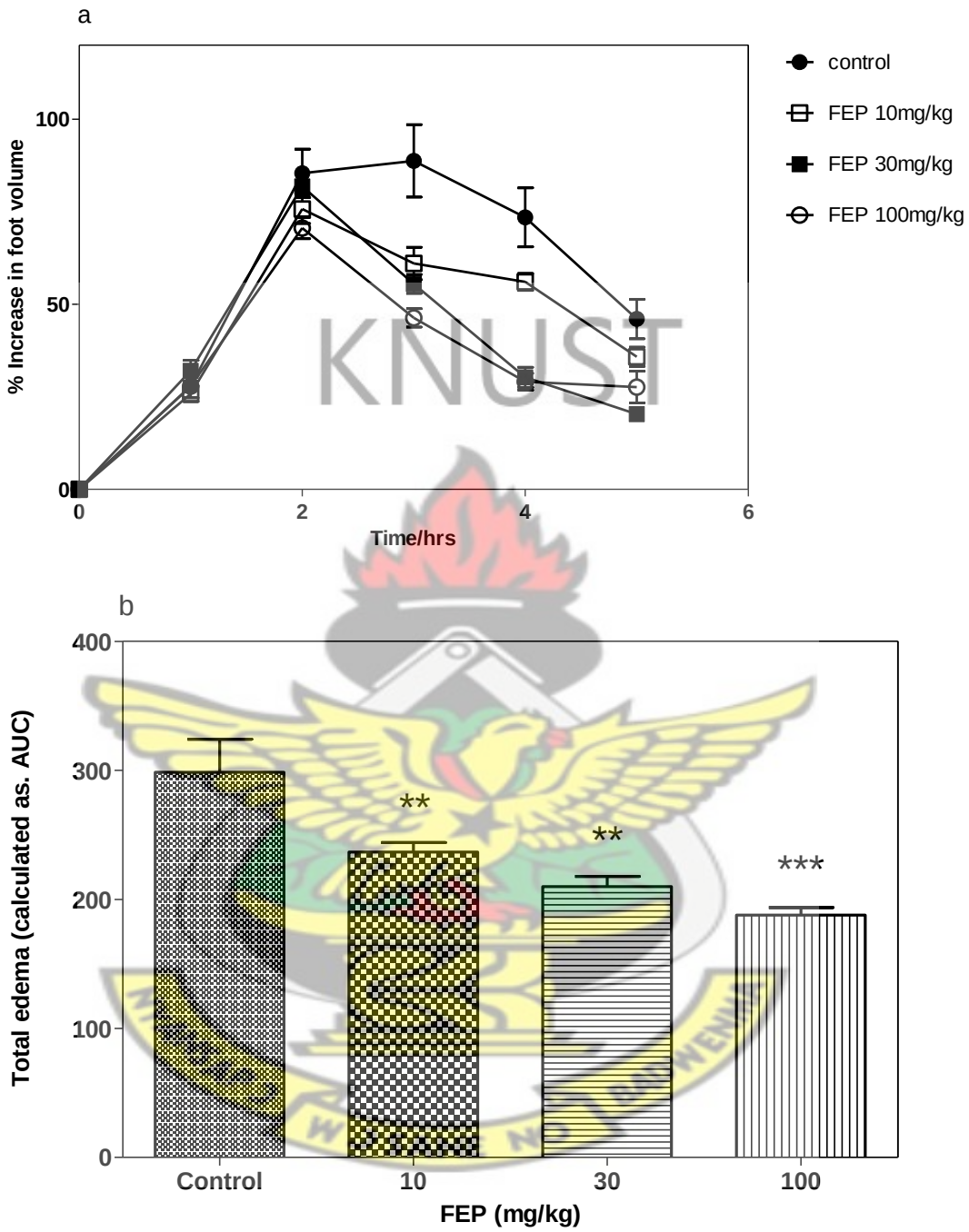


Figure 4.14 Effect of the pet-ether stem bark extract (FEP, 10-100 mg/kg; *i.p*) on time course curve (a) and the total oedema response (expressed as AUC, b) in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

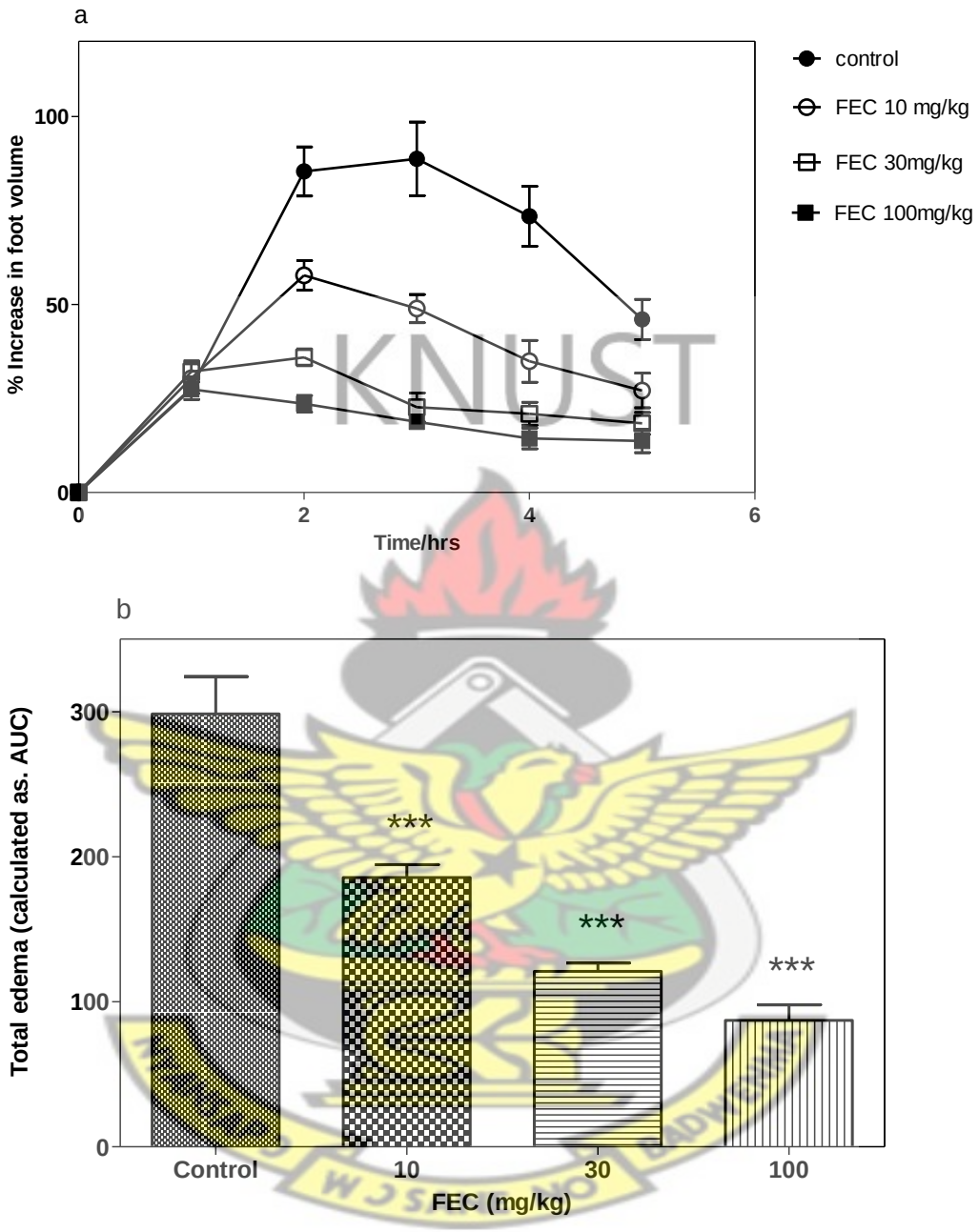


Figure 4.15 Effect of chloroform stem bark extract (FEC, 10-100 mg/kg; *i.p*) on time course curve (a) and the total oedema response (expressed as AUC, b) in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

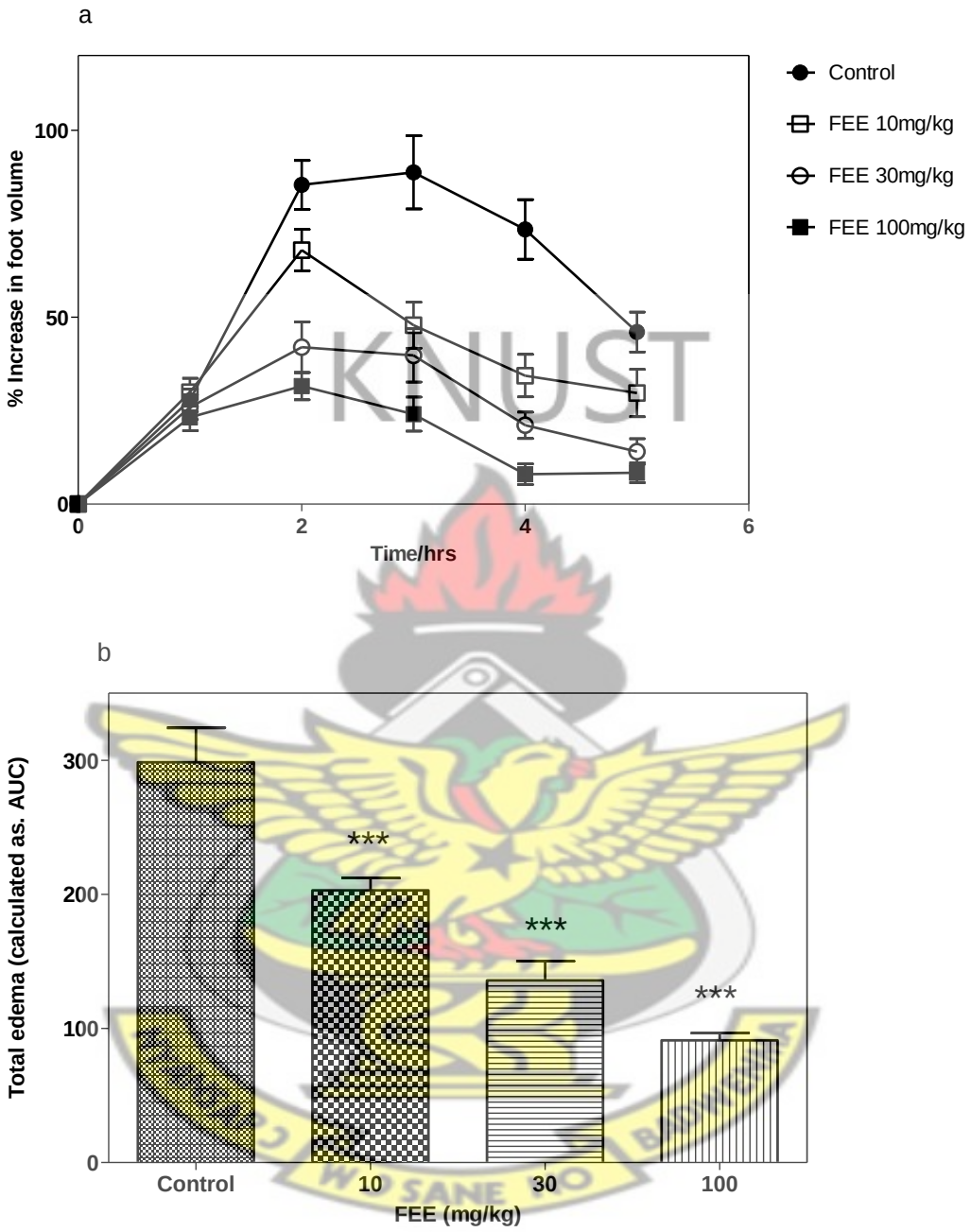


Figure 4.16 Effect of the alcohol stem bark extract (FEE, 10-100 mg/kg; *i.p*) on time course curve (a) and the total oedema response (expressed as AUC, b) in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

4.3.3 Anti-inflammatory effects of stem bark isolates

The compounds bergapten [**20**], oxypeucedanin hydrate [**30**] and sitosterol-3-O-*β*-D-glucopyranoside [**7**] were isolated from the chloroformic extract of the stem bark of *F. exasperata*. The result of the anti-inflammatory activity of sitosterol-3-O-*β*-D-glucopyranoside [**7**] has been described in section (Section 4.2.5). Bergapten [**20**] and oxypeucedanin hydrate [**30**] exhibited dose - dependent anti - inflammatory effect (Figure 4.17- 4.18). As indicated by the ED_{50} values (Table 4.12), bergapten showed the highest anti-inflammatory activity.

Table 4.12 Effect of bergapten [20] and oxypeucedanin hydrate [30] on carrageenan-induced oedema in 7 - day old chicks

Compounds	ED_{50} (mg/kg) ± SEM
Bergapten	101.60 ± 0.003
Oxypeucedanin hydrate	126.40 ± 0.011

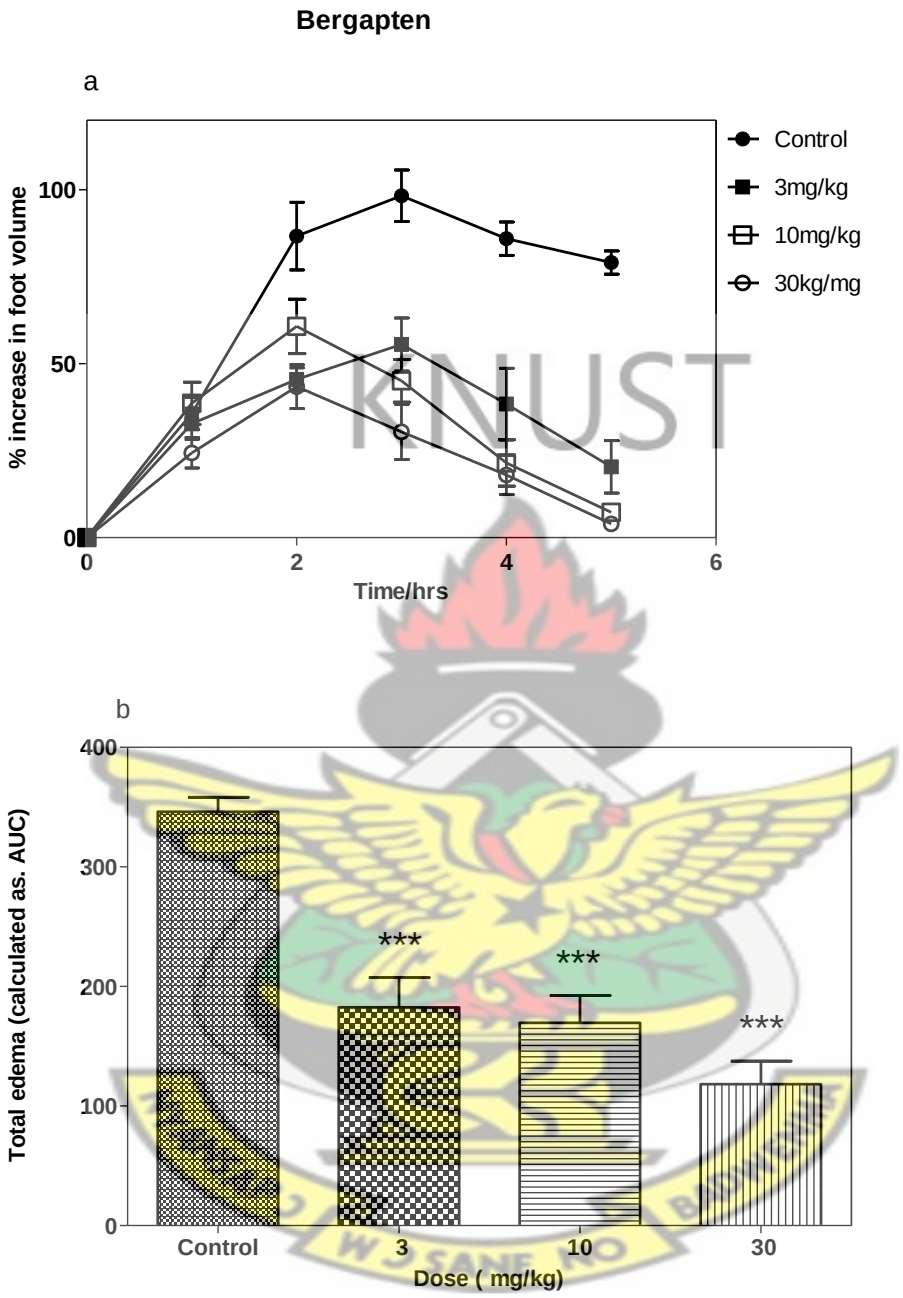


Figure 4.17 Effect of bergapten (3-30 mg/kg; *i.p*) on time course curve (a) and the total oedema response (expressed as AUC, b) in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group

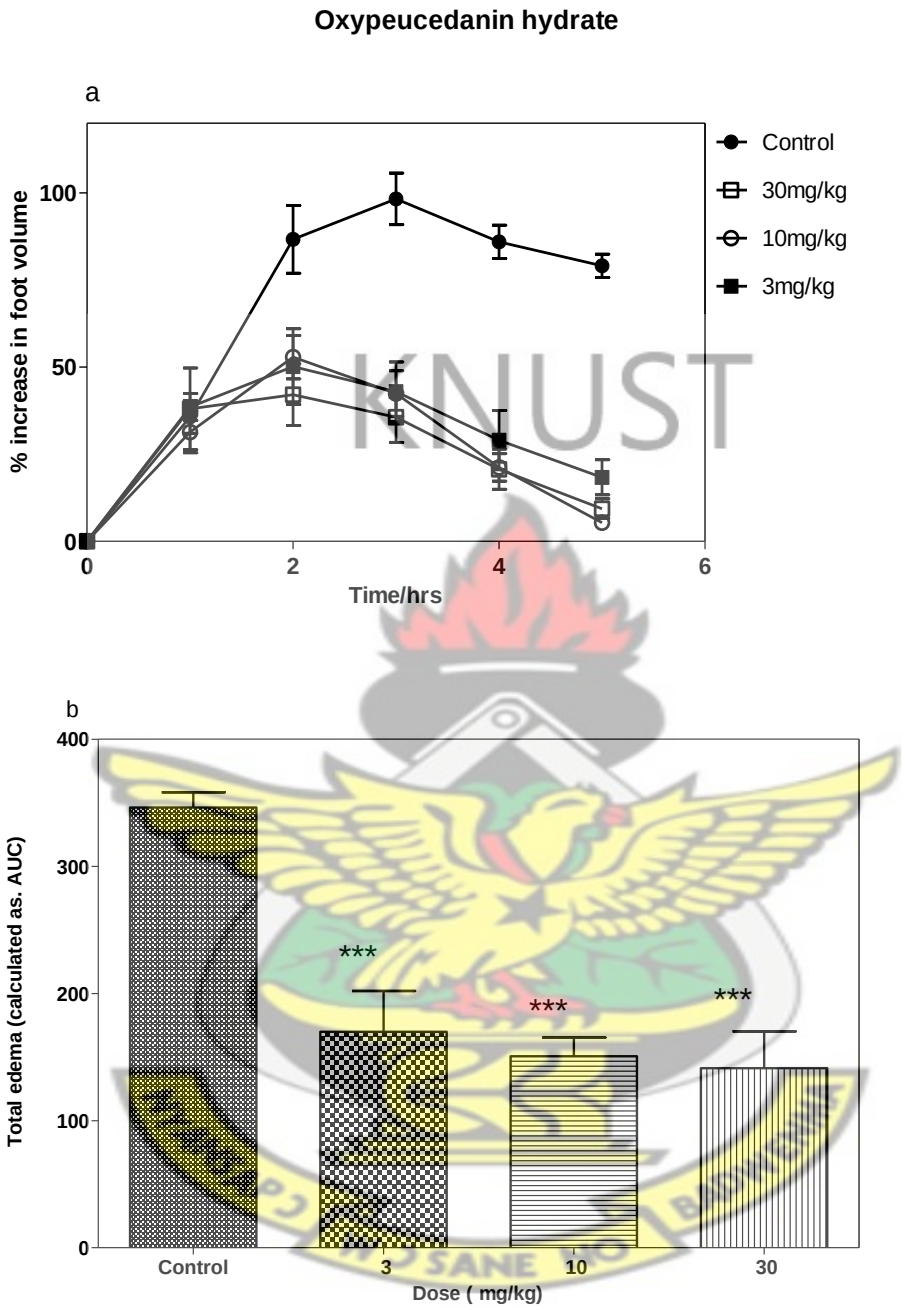


Figure 4.18 Effect of oxypeucedanin hydrate (3-30 mg/kg; *i.p*) on time course curve (a) and the total oedema response (expressesd as AUC, b) in carrageenan - induced paw oedema in chicks. ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group.

4.4 ANTIOXIDANT ACTIVITY OF EXTRACTS

4.4.1 Antioxidant activity of leaf extracts

The qualitative DPPH test showed all the three extracts bleaching the purple DPPH radical, thus giving pale spots over a purple background (Appendix 5A). This indicates that the extracts possess some anti-oxidant constituents.

4.4.1.1 Quantitative antioxidant assay of extracts

Various methods were used to determine quantitatively the antioxidant activity of the extracts. They included total phenolic content, total anti-oxidant capacity, reducing power, DPPH radical scavenging activity and linoleic acid autoxidation assays.

4.4.1.2 Total Phenolic content

The total phenolic content of the extracts was determined using the Folin- Ciocalteu's reagent and tannic acid as standard (Figure 4.19). The total phenolic content of the extracts was expressed as mg of tannic acid equivalents (TAE) per g of extract. Table 4.13 shows the total phenolic contents of AFE, EFE and PFE. The ethanol extract (AFE) had the highest phenolic content followed by the ethylacetate (EFE) and pet-ether (PFE) extracts respectively.

Table 4.13 Total phenolic content of leaf extracts of *F. exasperata*

Extracts (1.5mg/ml)	Mean (mg TAE/g) ± SEM
PFE	24.40 ± 0.471
EFE	32.93 ± 0.293
AFE	61.52 ± 2.309

PFE (pet-ether extract), **EFE** (ethyl acetate), **AFE** (ethanol extract)

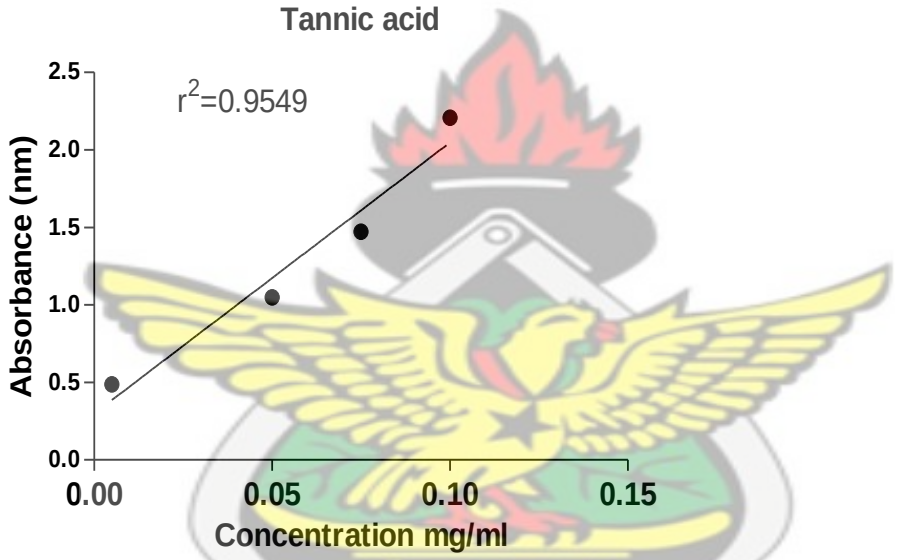


Figure 4.19 Calibration curve for tannic acid showing absorbance versus concentration

4.4.1.3 Total antioxidant capacity

In the total antioxidant capacity assay, ascorbic acid was used as standard (Figure 4.21). The antioxidant activity was expressed as mg of ascorbic acid equivalent (AAE) per g of extract. All the extracts showed increase in antioxidant activity with increase in

concentration (Figure 4.22). The ethanolextract showed the highest anti-oxidant capacity (Table 4.14). The correlation coefficient (r^2) between the antioxidant capacity and the phenolic content of the extracts was determined. A low coefficient of correlation was observed between the antioxidant capacity and the phenolic content of the petroleum ether fraction (PFE, $r^2 = 0.6999$). The r^2 value for the ethyl acetate and ethanol was 0.9090 and 0.8731 respectively (Figure 4.23a-c)

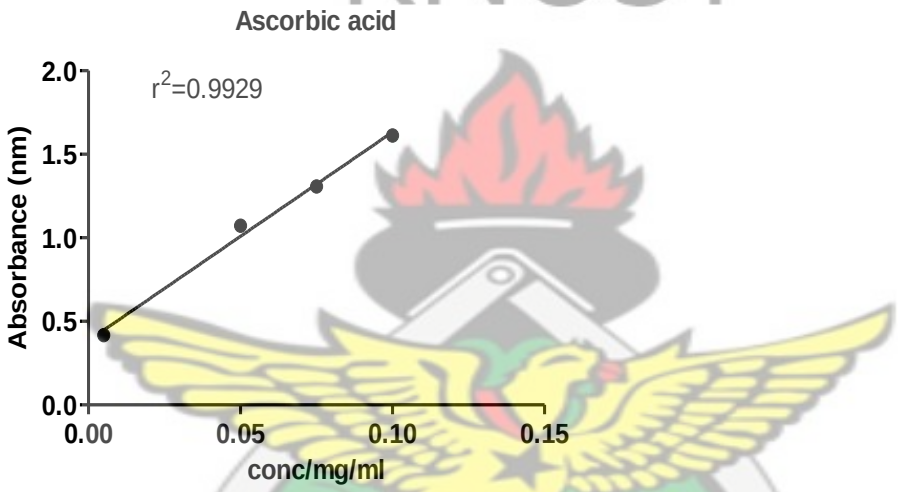


Figure 4.20 Absorbance against concentration of ascorbic acid used in the calibration curve

Table 4.14 Total antioxidant capacity of extracts of *F. exasperata* leaves

Extracts (1.5mg/ml)	Mean (mg/AAE/g) ± SEM
PFE	31.44 ± 0.005
EFE	59.73 ± 0.015
AFE	74.53 ± 0.004

PFE (Pet-ether leaf extract), **EFE** (Ethyl acetate leaf extract), **AFE** (Alcohol leaf extract)

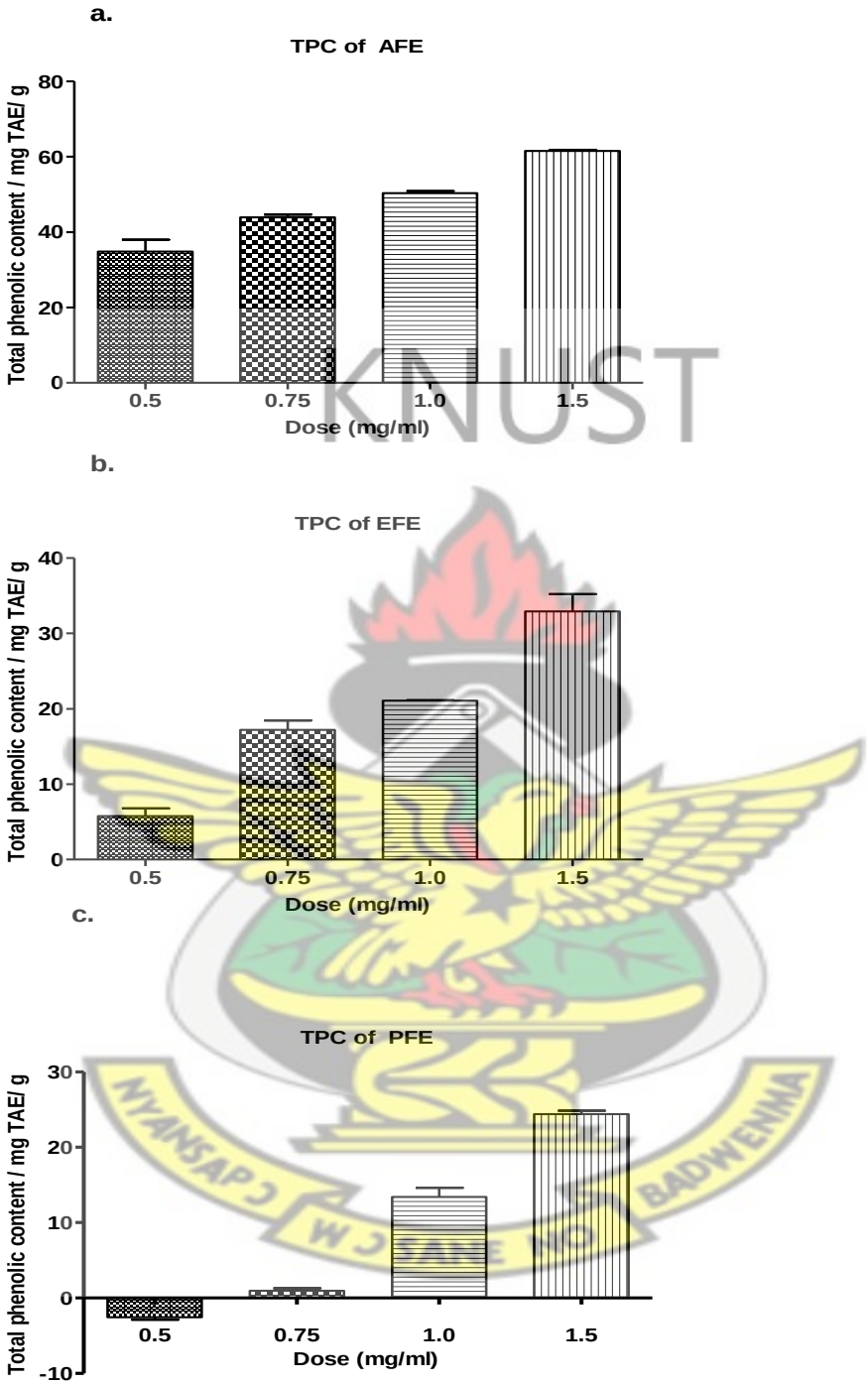


Figure 4.21 Total phenolic content (TPC) of AFE, EFE and PFE (a-c) Values are mean ± SEM (n=3)

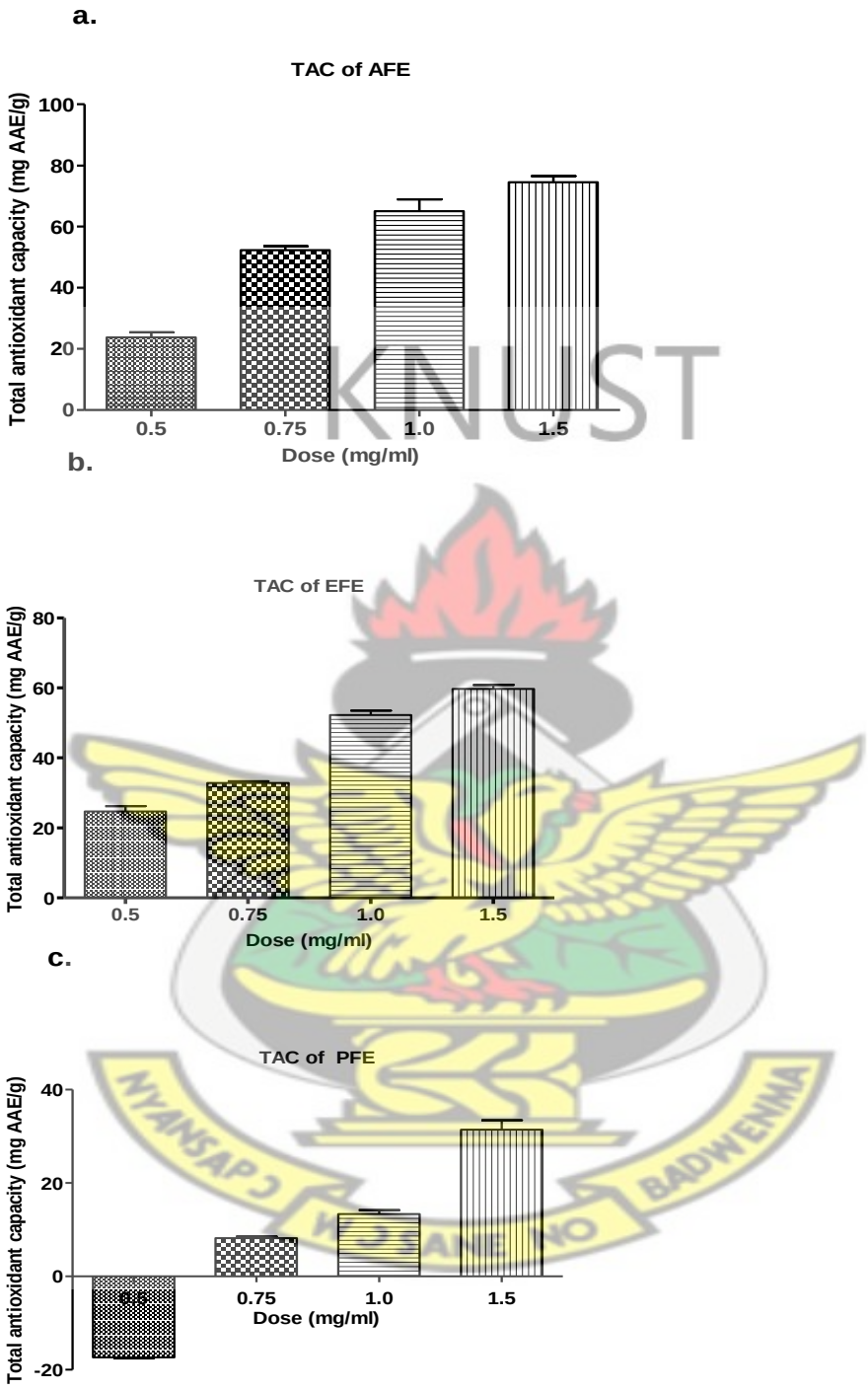


Figure 4.22 Total antioxidant capacities (TAC) of AFE, EFE and PFE. Values are means ± SEM (n=3).

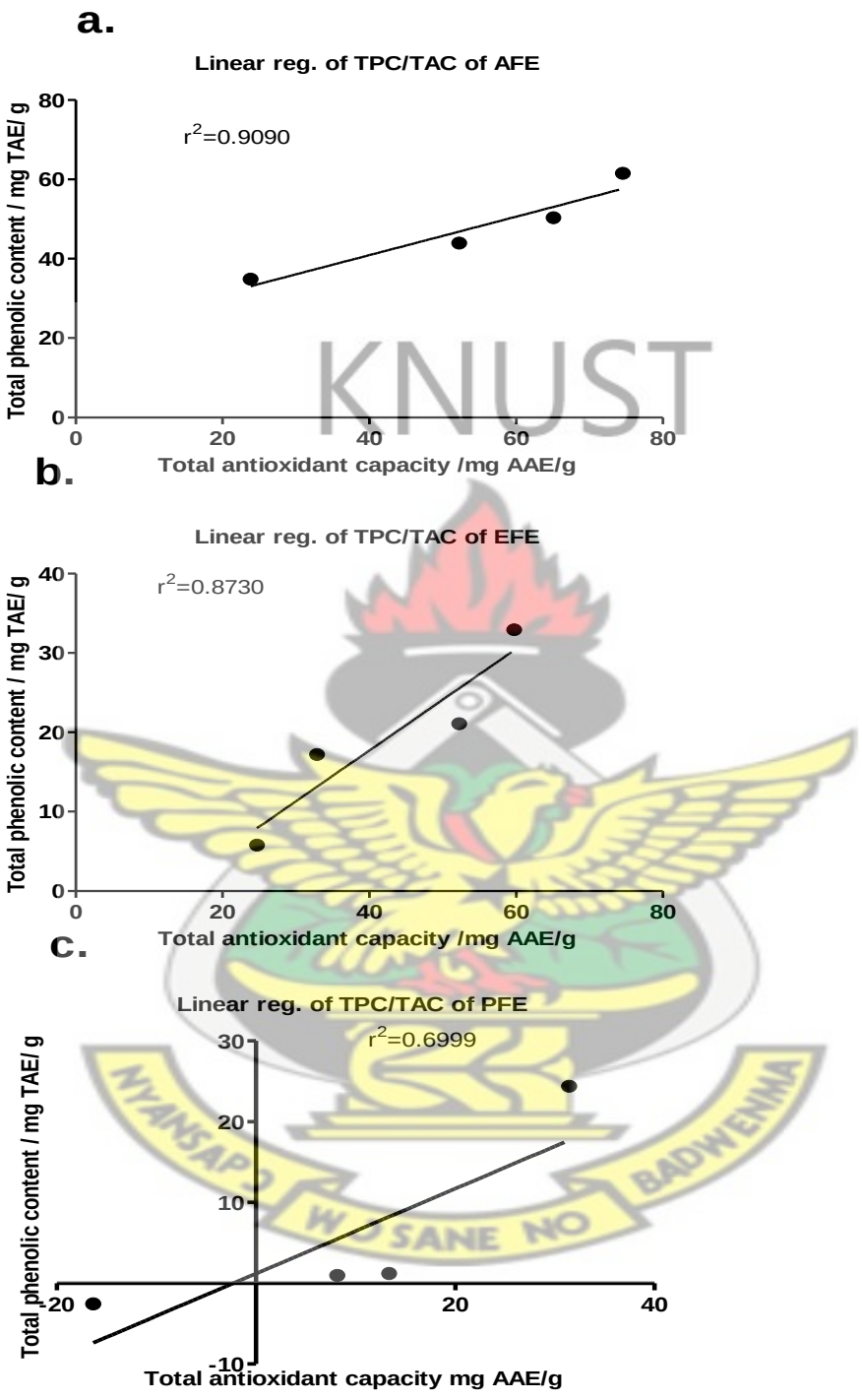


Figure 4.23 Correlation between the total phenolic content (TPC) and the total antioxidant capacity (TAC) of AFE, EFE and PFE.

4.4.1.4 Free Radical Scavenging Activity

The results of the free radical scavenging potential of extracts from *F. exasperata* using DPPH free radical scavenging method are depicted in Figures 4.24-4.25. The reference drug, *n*-propyl gallate (0.003 - 0.03 mg/ml), and the extracts (0.5 -1.5 mg/ml) exhibited concentration-dependent free radical scavenging activity (Table 4.15; Figure 4.24). The order of decreasing activity (as defined by IC_{50} in mg/ml) was found to be: *n*-propyl gallate > AFE > EFE> PFE.

Table 4.15 DPPH scavenging activity of extracts of *F. exasperata* leaves

Extracts	IC_{50} (µg/ml) ± SEM
AFE	80.4 ± 0.024
EFE	227.7 ± 0.096
PFE	730.4 ± 0.117
***n*-propyl gallate**	10.8 ± 0.002

PFE (Pet-ether leaf extract), **EFE** (Ethyl acetate leaf extract), **AFE** (Alcohol leaf extract)

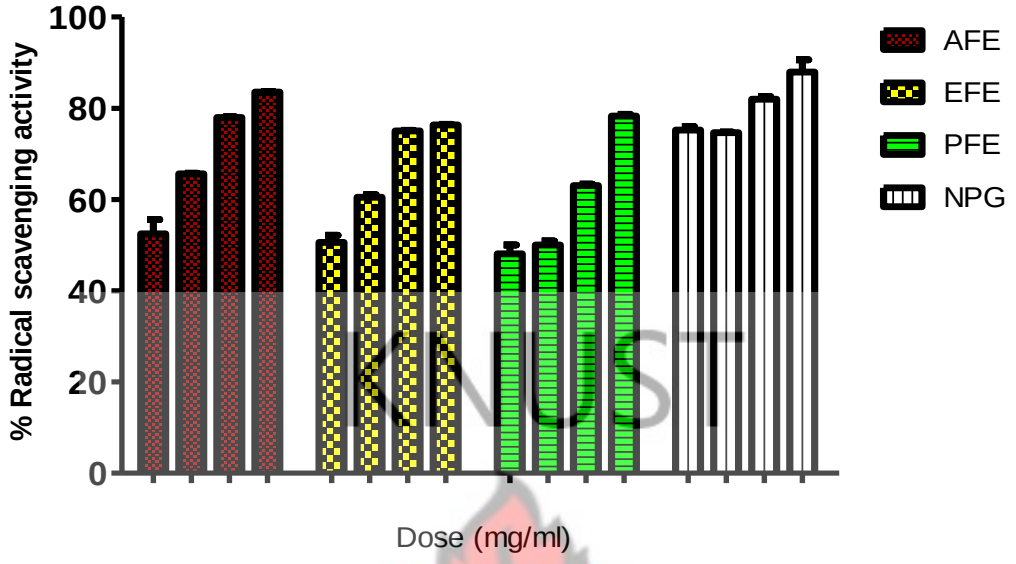


Figure 4.24 DPPH radical scavenging activity of extracts (AFE, EFE and PFE) and standard drug *n*-propyl gallate.

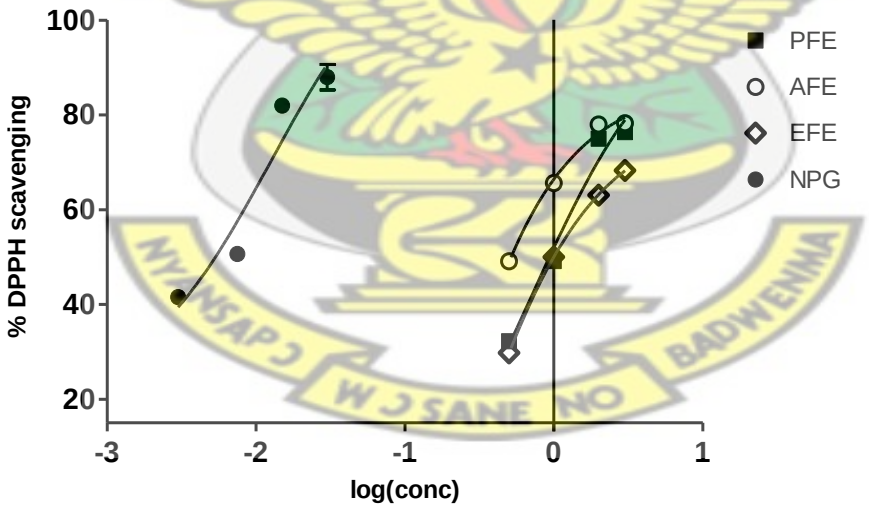


Figure 4.25 Percentage DPPH scavenging activity against log concentration of extracts and *n*-propyl gallate.

4.4.1.5 Reducing power

The extracts (0.5-1.5 mg/ml) and the standard antioxidant *n*-propyl gallate (0.003-0.03 mg/ml) caused a concentration – dependent reduction of Fe^{3+} to Fe^{2+} (Figure 4.26). From the IC_{50} values (Table 4.16), the ethanol extract showed the highest reducing power followed by the ethyl acetate and pet-ether extracts respectively.

Table 4.16 Reducing power of leaf extracts and *n*-propyl gallate

Extracts	**Reducing power (IC_{50}) µg/ml ± S.E.M**
AFE	186.10 ± 0.012
EFE	209.80 ± 0.011
PFE	760.00 ± 0.023
***n*-propyl gallate**	66.88 ± 0.002

PFE (Pet-ether leaf extract), **EFE** (Ethyl acetate leaf extract), **AFE** (Alcohol leaf extract)

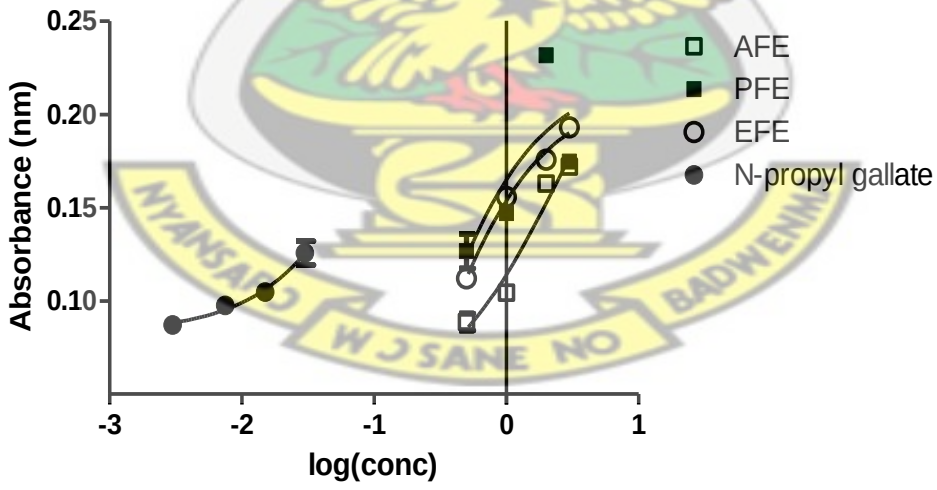


Figure 4.26 Reducing properties of extracts AFE, EFE and PFE compared to standard antioxidant *n*-propyl gallate. Values are means ± SEM (n=3)

4.4.1.6 Lipid peroxidation

The ability of the extracts and test drug to inhibit linoleic acid auto-oxidation was investigated. The extracts (0.5 – 1.5 mg/ml) and *n*-propyl gallate (0.003 - 0.03 mg/ml) caused a concentration- dependent inhibition of linoleic acid autoxidation (Figure. 4.27). The decreasing order of activity (defined by IC_{50} in µg/ml) was found to be: *n*-propyl gallate (73.54 ± 0.014) > AFE (185.0 ± 0.053) >EFE (373.5 ± 0.074) > PFE (563.8 ± 0.166).

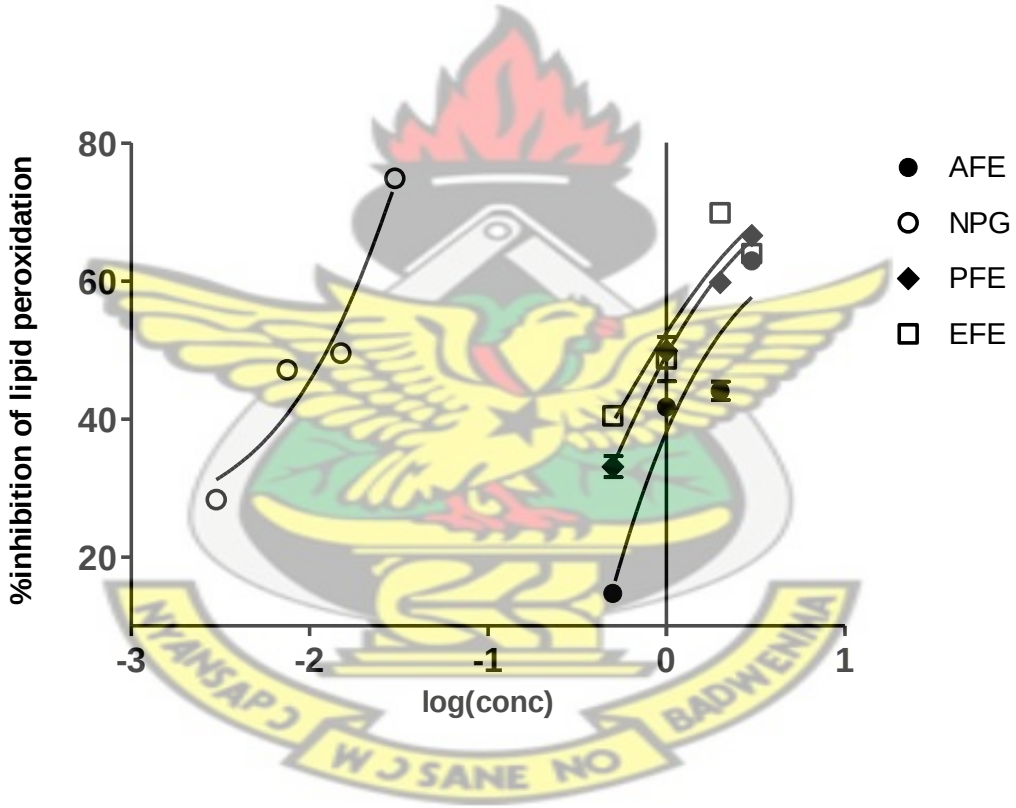


Figure 4.27 Effect of extracts on linoleic acid peroxidation compared to standard antioxidant *n*-propyl gallate. Values are means ± S.E.M (n=3)

4.4.2 Antioxidant activity of stem bark extracts

In the qualitative DPPH test, the total ethanol extract (SFE) bleached the purple DPPH radical giving pale yellow spots over a purple background (Figure 4.28a). Also thin layer chromatographic fingerprint of the extract (Figure 4.28b-d) revealed several constituents possessing antioxidant potential. Also qualitative test on the pet-ether, chloroform and ethanol extracts of the stem bark showed similar free radical scavenging activity (Appendix 5B).

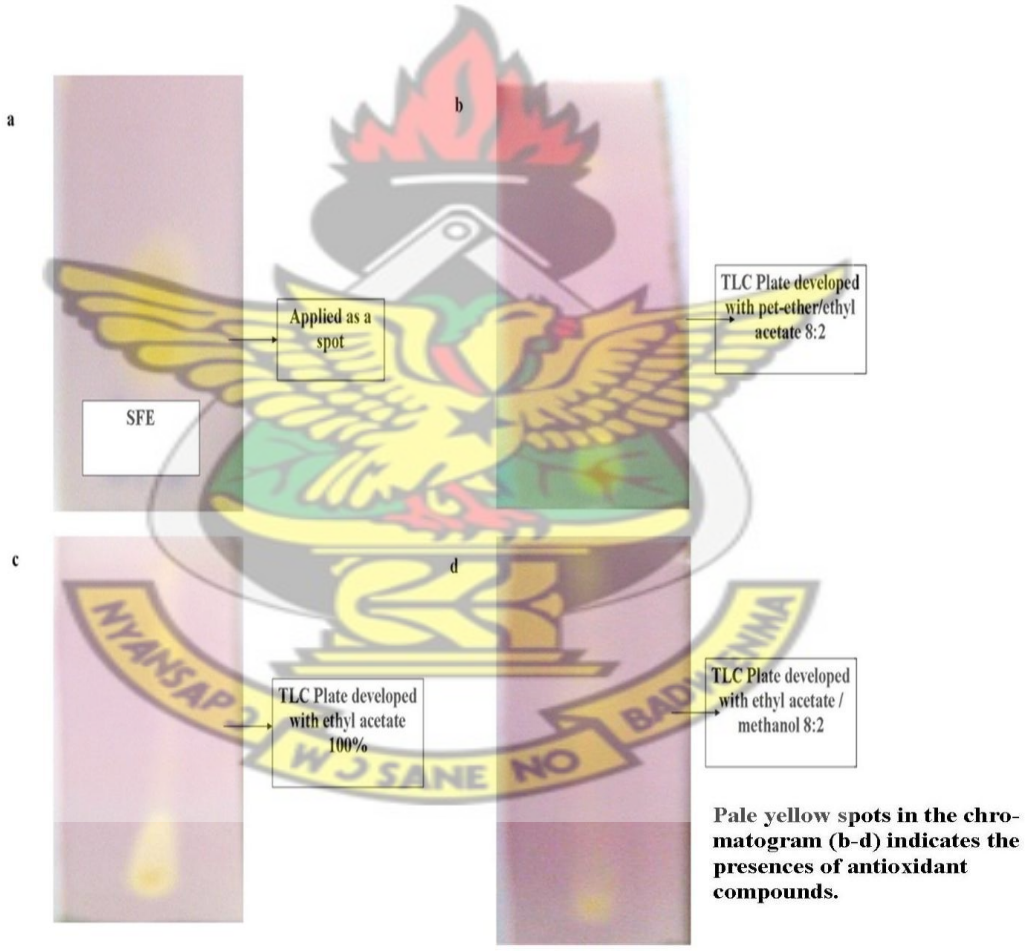


Figure 4.28 TLC chromatogram showing the effect of SFE on DPPH radical

4.4.2.1 Reducing power of extracts

The extracts (0.25-2 mg/ml) and *n*-propyl gallate (3.75-30 µg/ml) caused concentration-dependent reduction of Fe^{3+} to Fe^{2+} (Figure 4.29 a-b). The highest reducing power, indicated by the lowest EC_{50} value, was recorded for SFE. It showed higher antioxidant activity than *n*-propyl gallate (Table 4.17). The fractions of SFE, however, showed relatively low reducing power compared to NPG

Table 4.17 Reducing power of stem bark extracts and standard drug *n*-propyl gallate

Extracts	Reducing power (EC_{50}) µg/ml ± S.E.M
SFE	61.80 ± 0.001
FEP	604.6 ± 0.010
FEC	85.33 ± 0.002
FEE	122.3 ± 0.013
n-propyl gallate (NPG)	66.88 ± 0.002

SFE (total ethanol extract), **FEP** (pet-ether extract), **FEC** (chloroform extract), **FEE** (ethanol extract)

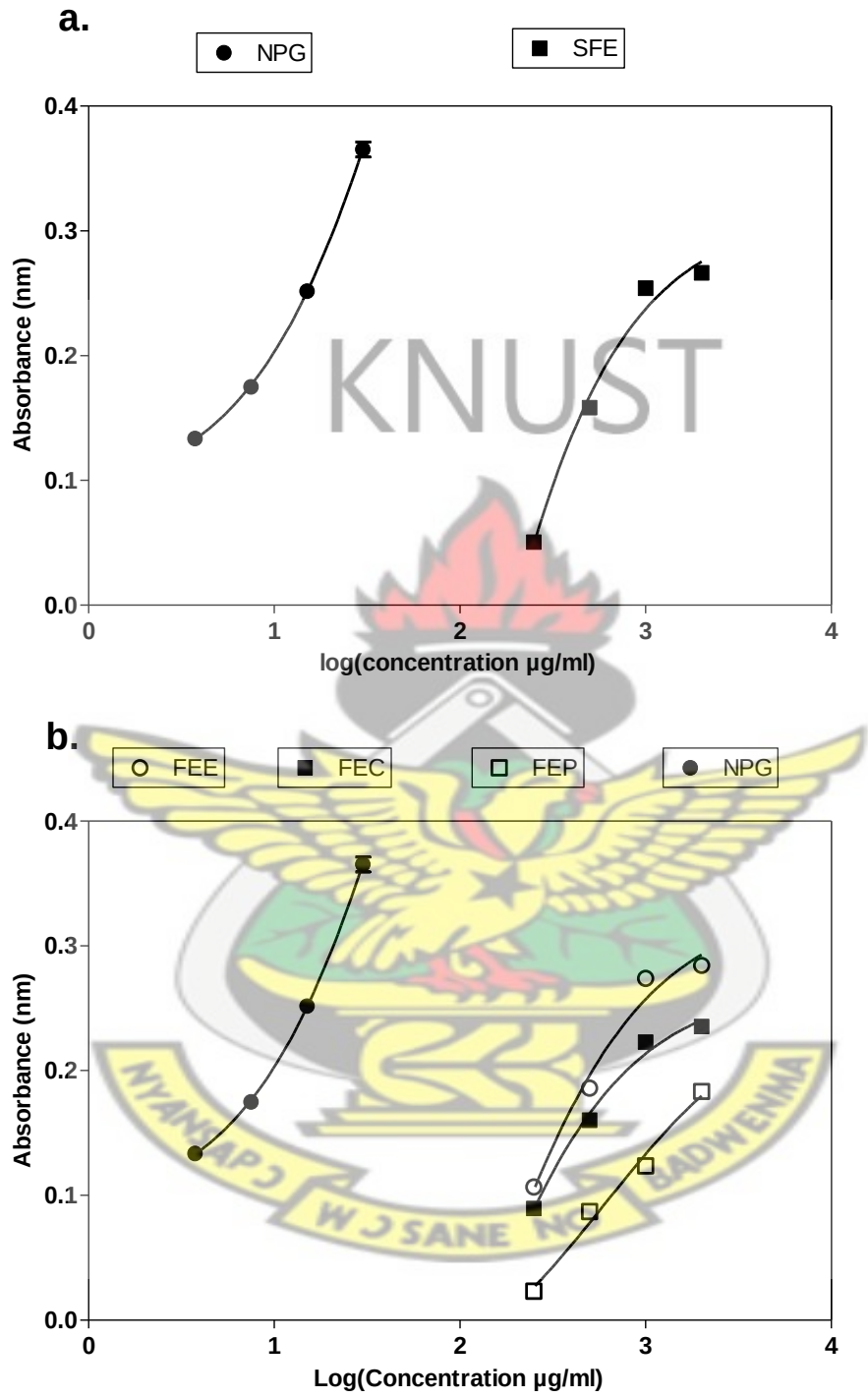


Figure 4.29 Reducing power of the extracts SFE, FEP, FEC and FEE and NPG

4.4.2.2 DPPH radical scavenging activity

Figure 4.30 shows the percentage of DPPH radical scavenged by *n*-propyl gallate and extracts of *F. exasperata* stem bark at various concentrations (µg/ml)**.** The IC_{50} values for the extracts and *n*-propyl gallate are presented in Table 4.18. The pet-ether extract showed the lowest DPPH scavenging activity. The results indicate that the stem bark extracts of *F. exasperata* possess hydrogen donating capabilities and acts as an antioxidant.

Table4.18 DPPH scavenging activity of stem bark extracts and standard drug

Extracts	IC_{50} µg/ml ± SEM
SFE	20.09 ± 0.001
FEP	1174.00 ± 0.011
FEC	134.00 ± 0.031
FEE	125.30 ± 0.001
n-propyl gallate	10.80 ± 0.002

SFE (total alcohol extract), **FEP** (Pet-ether extract), **FEC** (chloroform extract), **FEE** (ethanol extract)

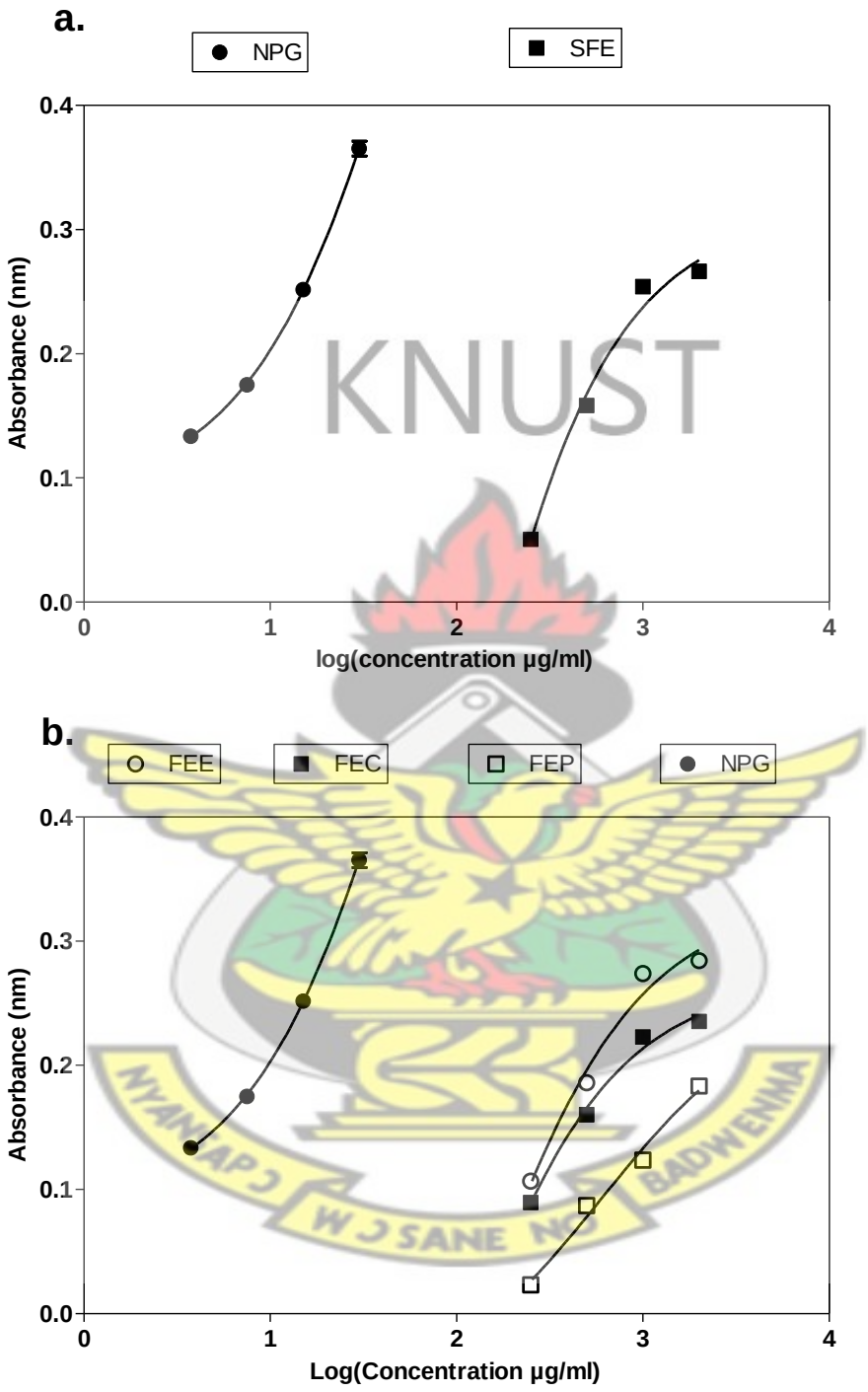


Figure 4.30 Free radical scavenging activity of SFE, FEP, FEC and FEE (0.25-2mg/ml) and standard drug *n*-propyl gallate (3.75-30µg/ml)

4.4.2.3 Total antioxidant capacity

The antioxidant activity was expressed as the number of equivalent of ascorbic acid (AAE) in mg per g of extract. All the extracts showed a concentration - dependent increase in antioxidant activity [(Figures 4.32a-b and 4.33a-b)]. The antioxidant capacities ranged from 37.41 to 310.39 mg/AAE/g of extract. SFE showed the highest antioxidant capacity whereas FEP showed the lowest (Table 4.19; Figure 4.31).

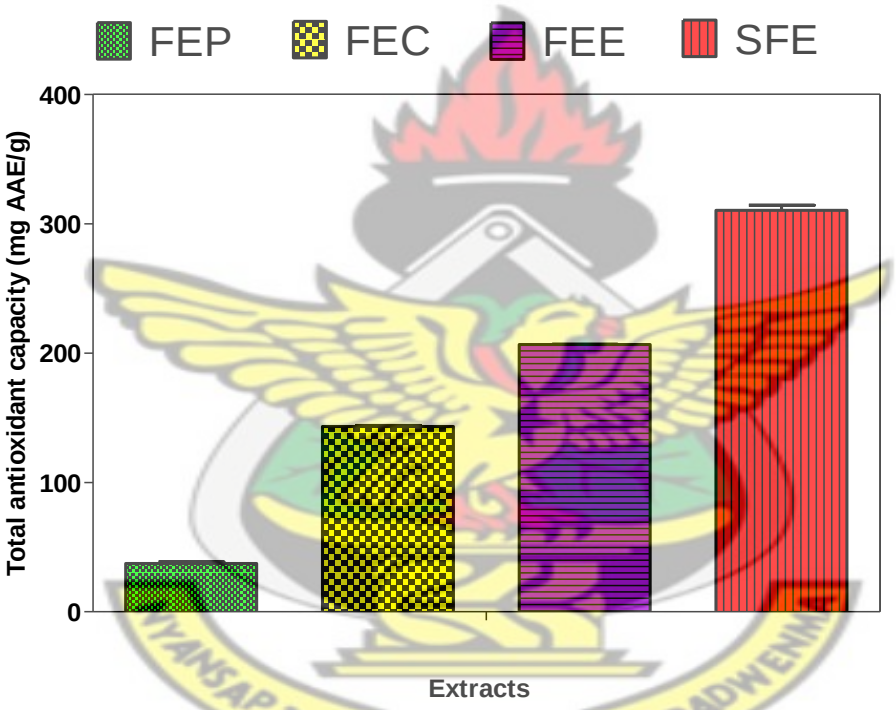


Figure 4.31 Comparing the antioxidant capacities of SFE, FEP, FEC and FEE (each at a concentration of 2 mg/ml)

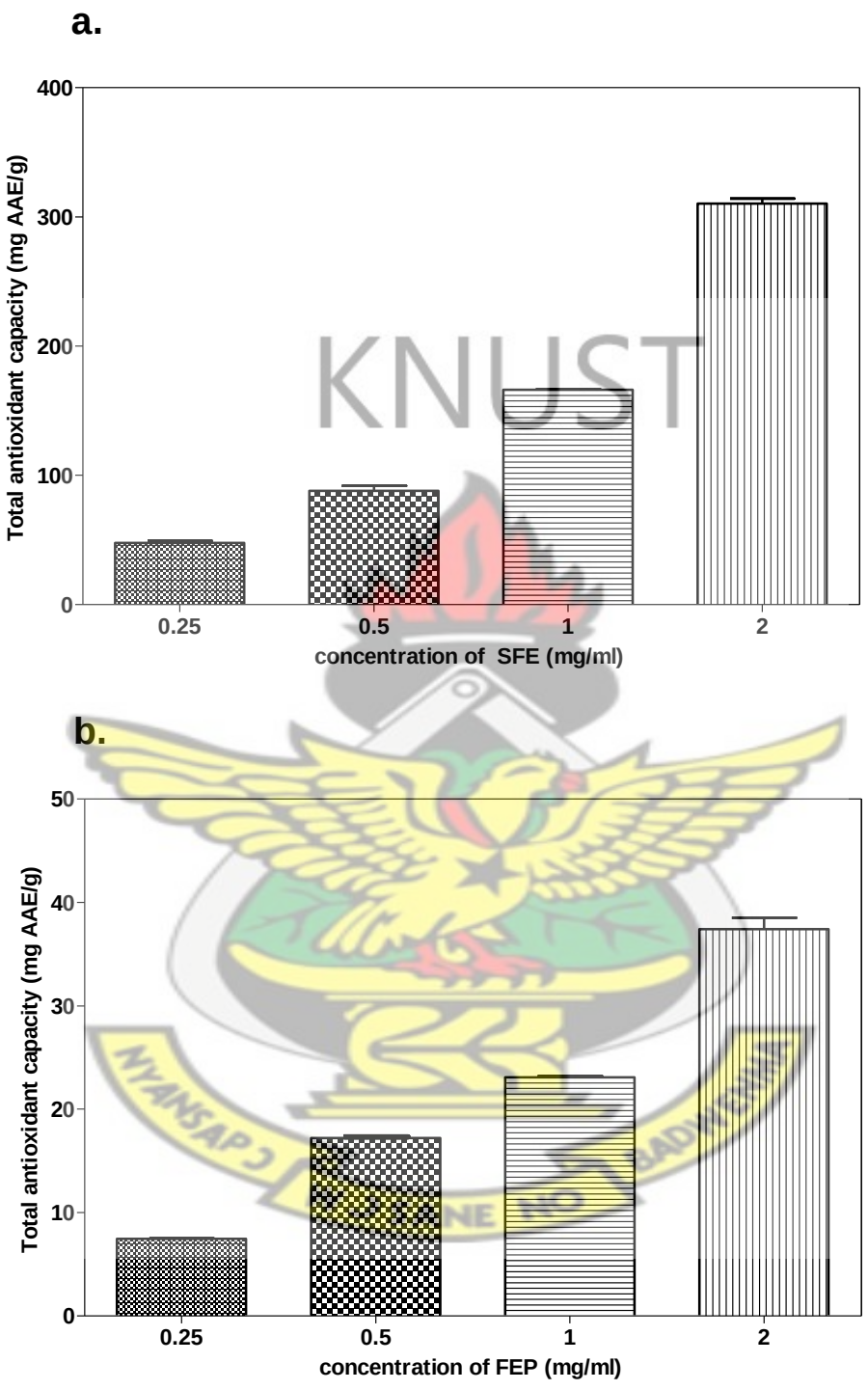


Figure 4.32 Antioxidant capacities of SFE (a) and FEP (b)

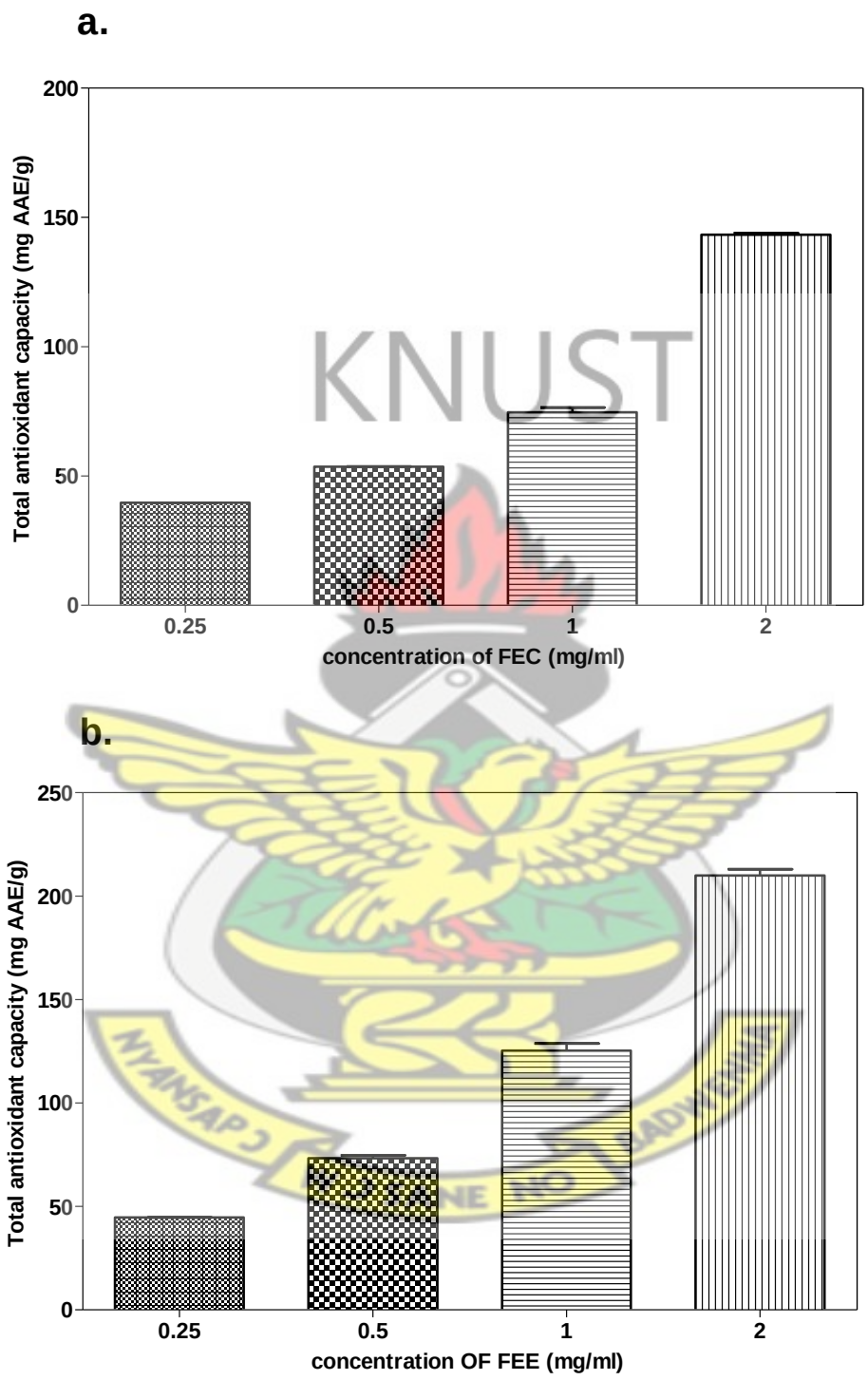


Figure 4.33 Antioxidant capacities of FEC (a) and FEE (b)

Table4.19 Total antioxidant capacities of stem bark extracts

Extracts (2mg/ml)	Mean ± SEM(mg/AAE/g)
SFE	310.391 ± 3.840
FEP	37.410 ± 1.088
FEC	143.304 ± 0.623
FEE	206.527±0.174

SFE (total ethanol extract), **FEP** (pet-ether extract), **FEC** (chloroform extract), **FEE** (ethanol extract)

4.4.2.4 Total phenolic content

The total phenolic content was measured using Folin-Ciocalteu's assay and expressed as mg equivalent of tannic acid equivalents (TAE) per g of plant extract. All the extracts showed a concentration – dependent increase in phenolic content (Figure 4.34 - 4.35). The phenolic content of SFE was very high compared to the FEP, FEC and FEE. However, of the three extracts, FEE gave the highest value whereas FEP gave the least (Table 4.20).

Table 4.20 Total phenolic content of stem bark extracts

Extracts (2mg/ml)	Mean ± SEM (mg/TAE/g)
SFE	282.4 ± 1.002
FEP	44.32 ± 1.766
FEC	112.9 ± 4.990
FEE	136.1 ± 4.559

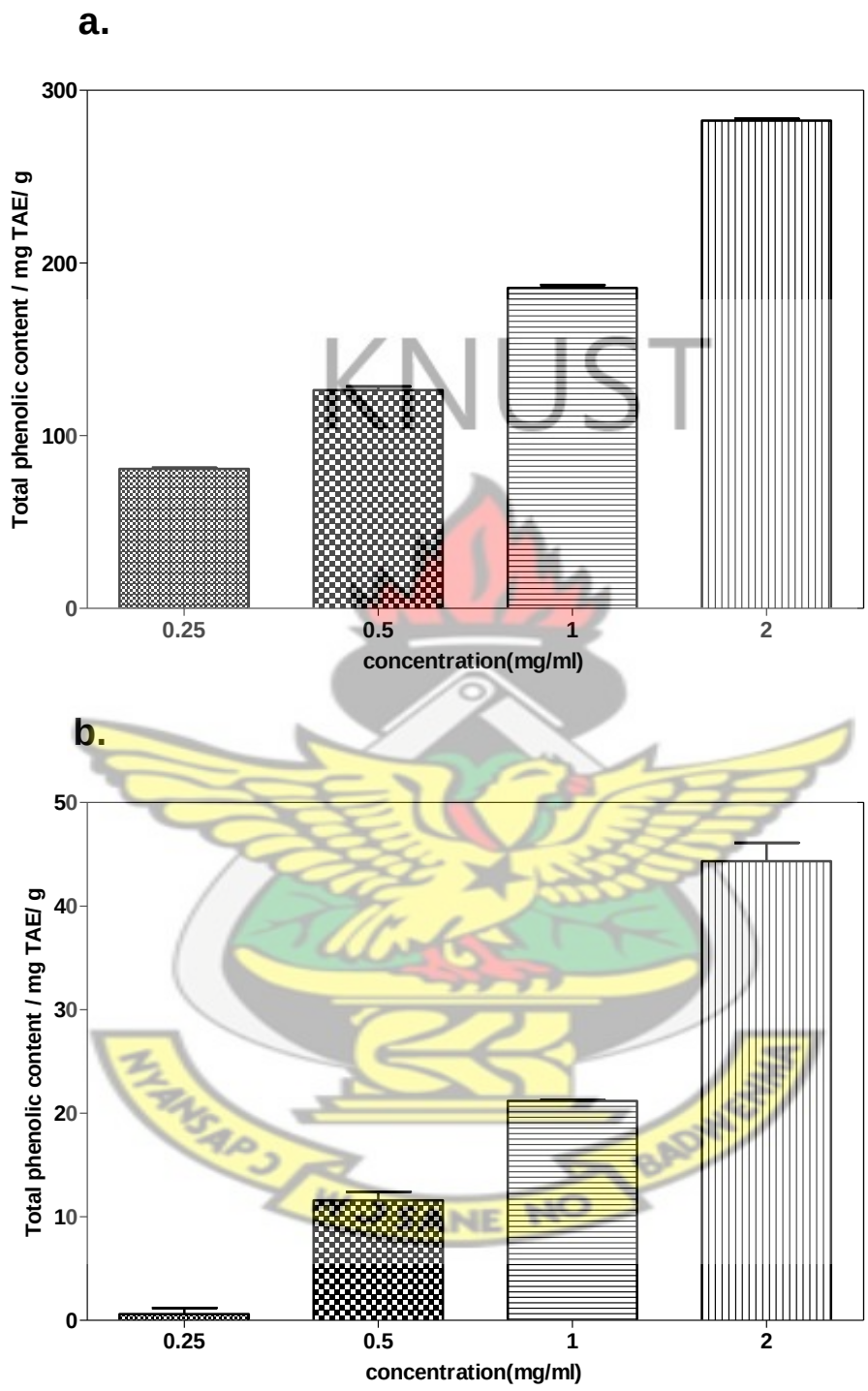


Figure 4.34 Total phenolic contents of SFE (a) and FEP (b)

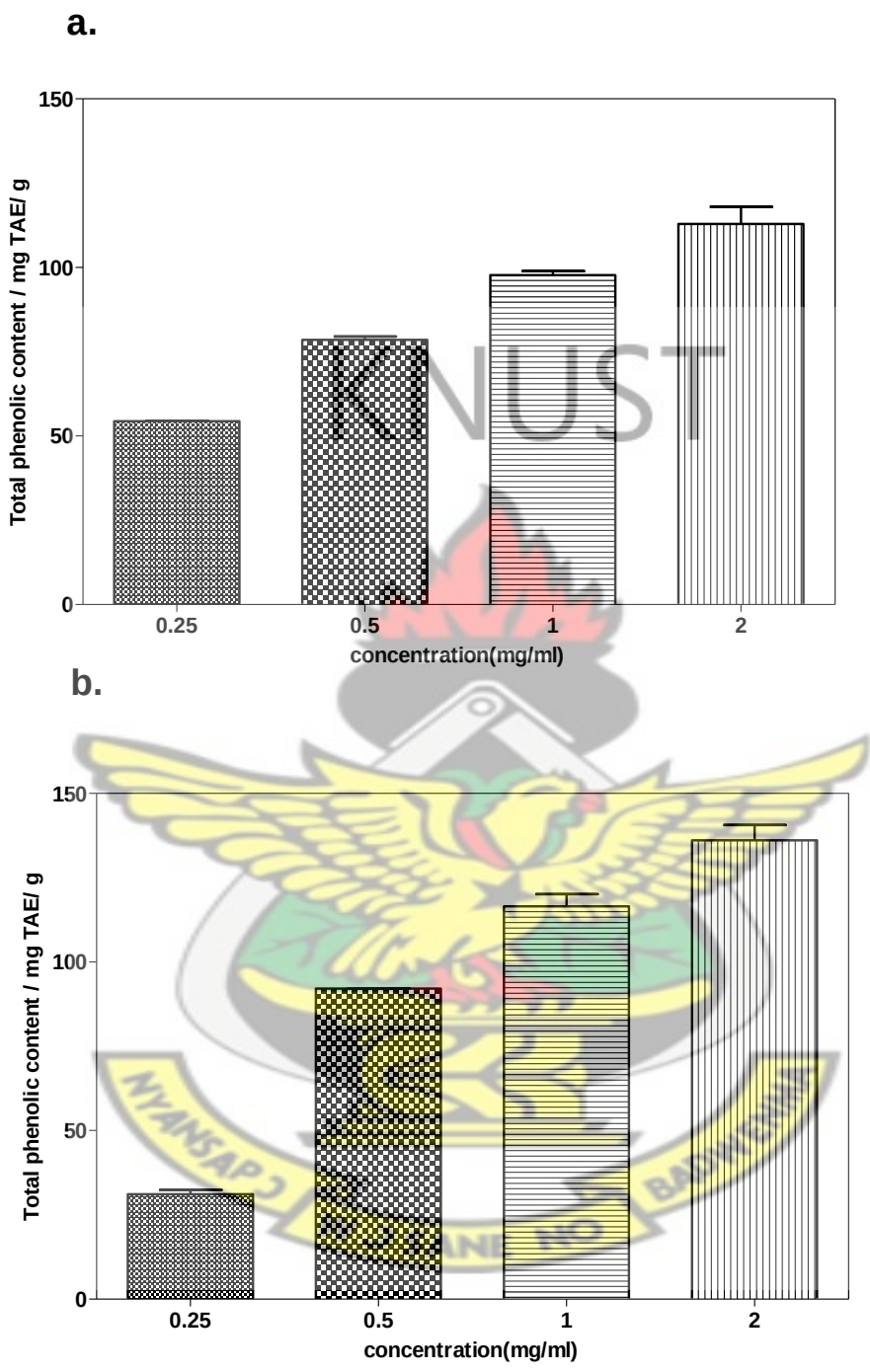


Figure 4.35 Total phenolic content of FEC (a) and FEE (b)

A very good correlation (r^2 = 0.9729) was obtained for the alchol stem bark extract (FEE, Figure 4.37b). Those of the pet-ether and chloroform extract were 0.8143 and 0.8855 respectively (Figure 4.36b and 4.37a).

4.4.2.5 Lipid peroxidation

The extracts (0.25-2 mg/ml) and *n*-propyl gallate (0.00375-0.03 mg/ml) caused a concentration-dependent inhibition of linoleic acid autoxidation (Figure 4.38-4.39). The order of decreasing activity (defined by IC_{50} in µg/ml) was found to be: SFE > *n*-propyl gallate > FEE >FEC > FEP (Table 4.21).

Table 4.21 Inhibition of lipid peroxidation by extracts and NPG

Extracts	**IC_{50} (µg/ml)±SEM**
SFE	42.27 ± 0.012
FEP	749.3 ± 0.116
FEC	383.1 ± 0.165
FEE	221.1 ± 0.514
***n*-propyl gallate**	73.54 ± 0.014

SFE (total ethanol extract), **FEP** (pet-ether extract), **FEC** (chloroform extract), **FEE** (ethanol extract)

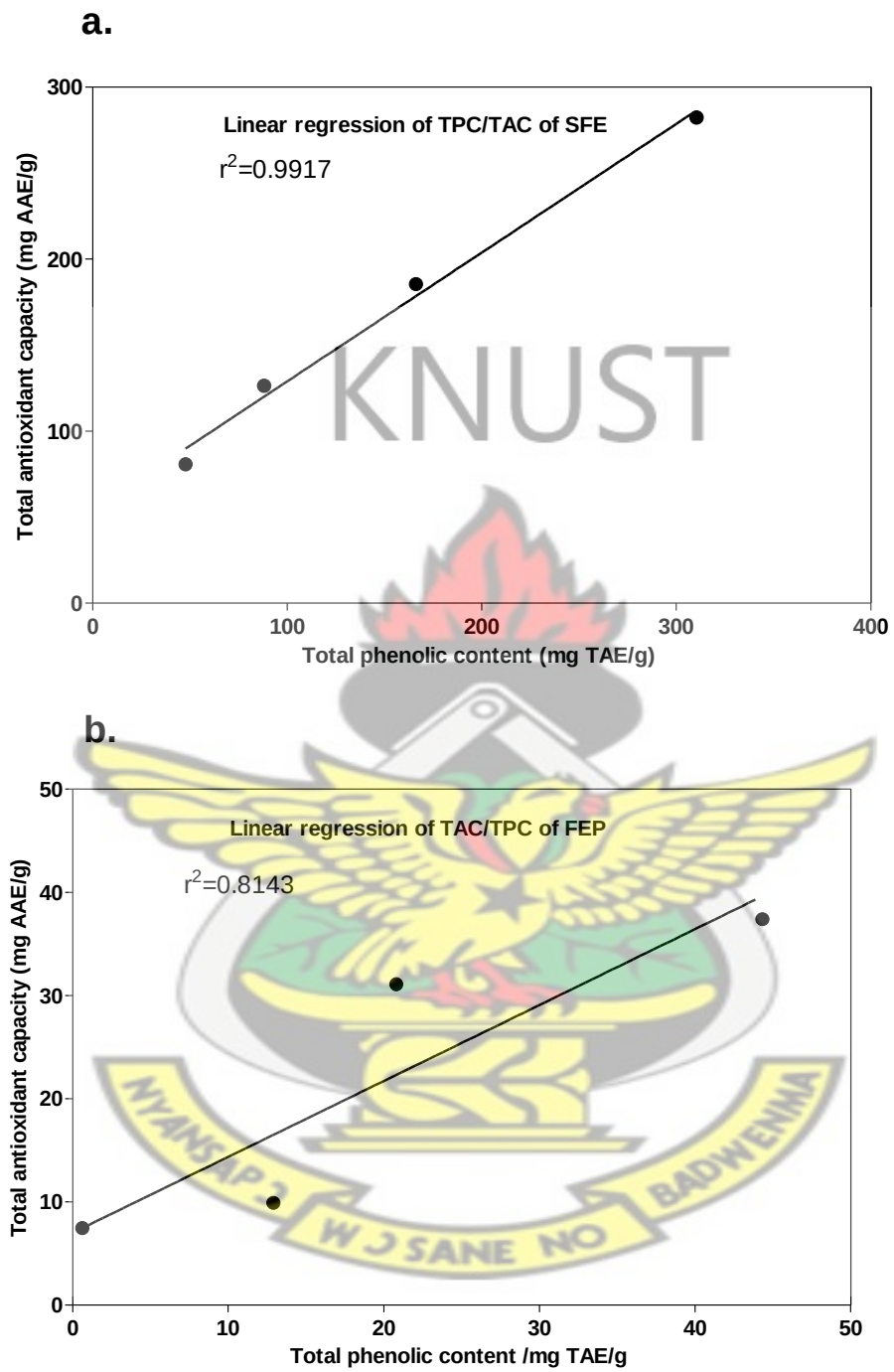


Figure 4.36 Correlation between the total phenolic content (TPC) and the total antioxidant capacity (TAC) of SFE (a) and FEP (b)

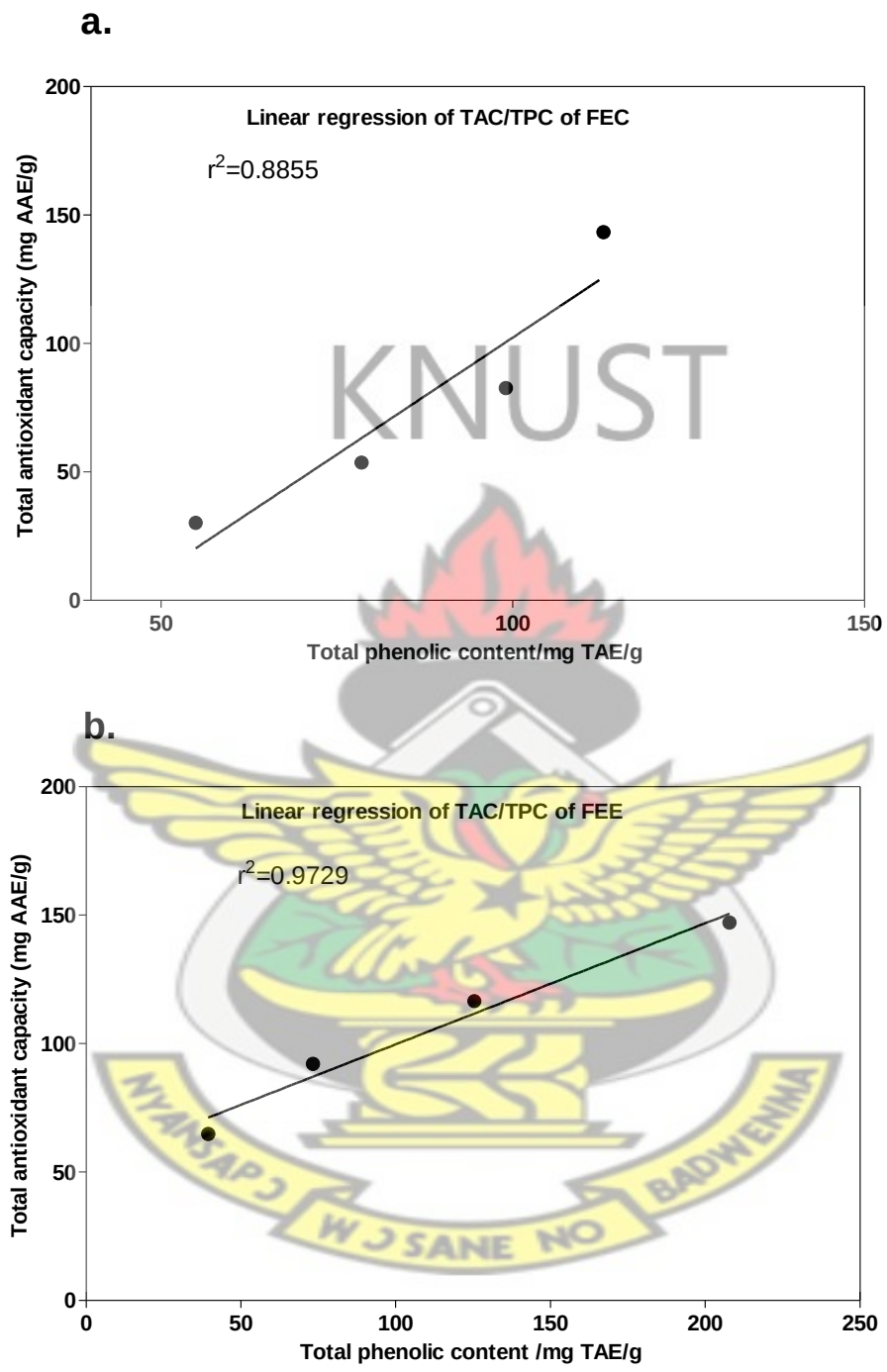


Figure 4.37 Correlation between the total phenolic content (TPC) and the total antioxidant capacity (TAC) of FEC (a) and FEE (b).

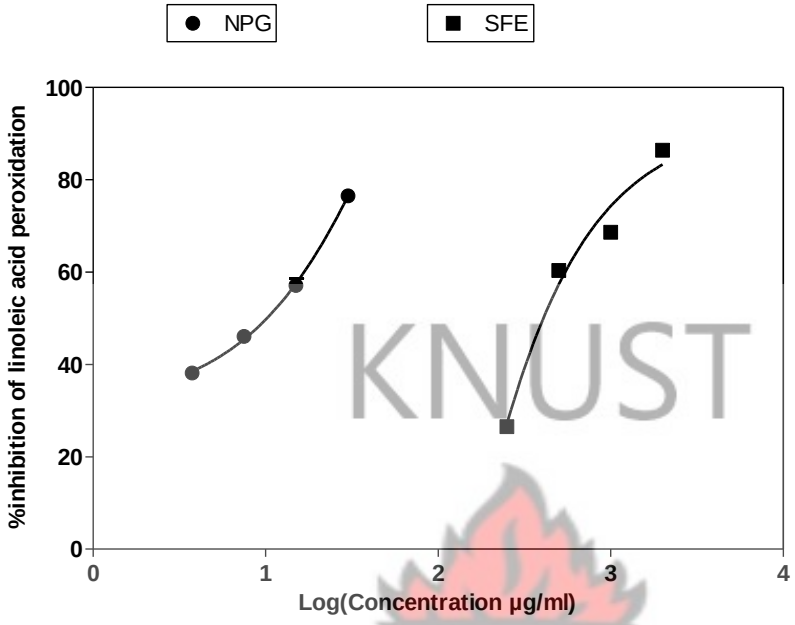


Figure 4.38 Inhibition of linoleic acid auto-oxidation by SFE and NPG

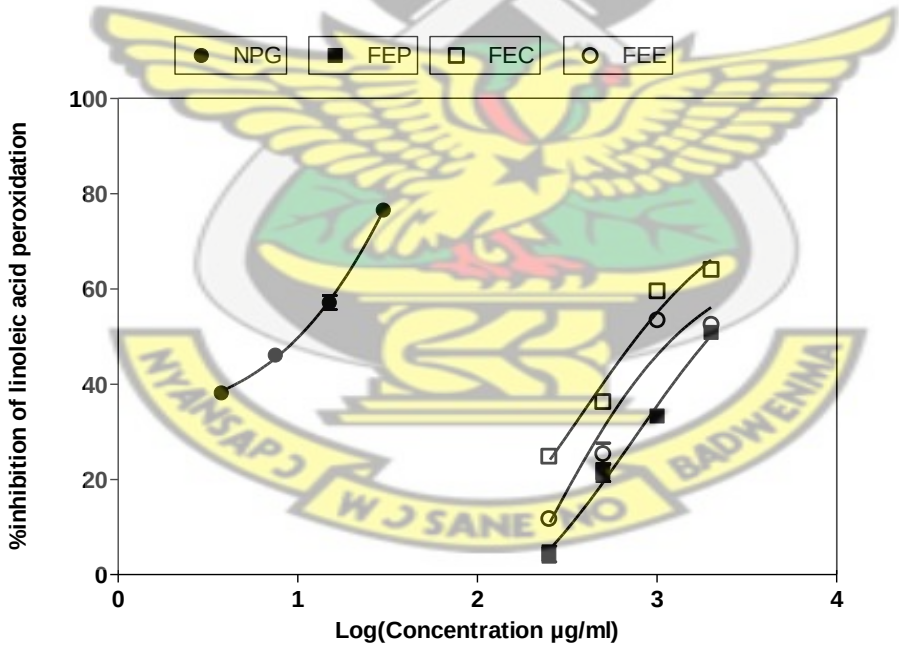


Figure 4.39 Inhibition of linoleic acid auto-oxidation by FEP, FEC, FEE and NPG

4.4.3 Antioxidant activity of isolates

4.4.3.1 Quantitative DPPH radical scavenging test

β-sitosterol [**1**], sitosterol-3-O-*β*-D-glucoside [**7**], bergapten [**20**], and oxypeucedanin hydrate [**30**] showed various degrees of antioxidant properties, with oxypeucedanin hydrate being the most active (Table 4.22). *n*-propyl gallate (NPG) was used as the standard antioxidant.The order of decreasing activity as indicated by the IC_{50} is NPG> oxypeucedanin hydrate> bergapten> sitosterol-3-O-*β*-D-glucoside> *β*-sitosterol

Table 4.22 DPPH scavenging activity of compounds isolated from the stem bark

Compounds	IC_{50} µg/ml ± SEM
Bergapten	63.38 ± 0.010
Oxypeucedanin hydrate	46.63 ± 0.011
β-sitosterol	>1000
sitosterol-3-O-*β*-D-glucoside	220.3 ± 0.03
n-propyl gallate	10.80 ± 0.002

4.5 ANTIMICROBIAL ASSAY

4.5.1 Antimicrobial activity of leaf extracts

In the preliminary screening for antimicrobial activity, the leaf extracts AFE, EFE and PFE were tested against four gram negative organisms (*P. aeruginosa, S. typhi, K. pneumoniae* and *E. coli*) two gram positive organisms (*B. subtlilis* and *S. aureus*) and the fungus *C. albicans*. All the extracts were active against some of the organisms tested (Table 4.23). EFE showed the highest activity against all the organisms, except *B. subtilis*.

The hydro-alcoholic extract, AFE, was not active against the gram negative organisms *E. coli, K. pneumoniae* and *S. typhi*. All the organisms showed higher susceptibility against gentamicin and fluconazole, used as positive controls (Table 4.23)

Table 4.23 Antimicrobial activities of leaf extract

Organisms	**Mean diameter (zone) of inhibition (mm) ± S.E.M**				
	AFE	EFE	PFE	GM	FN
B. subtilis	6.67 ± 0.33	NA	5.33 ± 0.32	18.00 ± 0.58	NT
S. aureus	7.32 ± 0.13	8.67 ± 0.31	7.00 ± 0.58	20.68 ± 0.33	NT
P. aeruginosa	10.33 ± 0.33	12.33 ± 0.34	6.67 ± 0.23	18.33 ± 0.33	NT
E. coli	NA	6.34 ± 0.13	6.33 ± 0.32	21.00 ± 0.00	NT
S. typhi	NA	8.33 ± 0.67	5.67 ± 0.33	29.33 ± 0.67	NT
K. pneumoniae	NA	13.67 ± 0.88	NA	22.67 ± 0.32	NT
C. albicans	2.33 ± 0.33	2.67 ± 0.26	3.00 ± 0.00	NT	22.13 ± 0.33

NA = Not active, NT = Not tested, GM = Gentamicin, FN = Fluconazole, AFE = ethanol extract, EFE = ethyl acetate extract, PFE = petroleum ether extract.

4.5.2 Antimicrobial activity of stem bark extracts

In the screening for antimicrobial activity, the stem bark extracts SFE, FEP, FEC and FEE were also tested against the seven organisms; *P. aeruginosa, S. typhi, K. pneumoniae, E. coli, B. subtlilis, S. aureus* and *C. albicans.* SFE was active against all test organisms with *P. aeruginisa, S. typhi, K. pneumoniae* and *S. aureus* being the most susceptible organisms. *K. pneumoniae* was the most susceptible organism with mean zone of inhibition of 17.33 ± 0.667 mm, whereas *C. albicans* was the least susceptible (Table

4.24). Comparatively, FEC and FEE were less active against the test organisms while FEP was inactive against all the test organisms.

Table 4.24 Antmicrobial activities of stem bark extracts

Organisms	**Mean diameter (zone) of inhibition (mm) ± S.E.M**			
	SFE	FEP	FEC	FEE
B. subtilis	11.67 ± 0.88	NA	7.33 ± 0.33	3.00 ± 0.58
S. aureus	15.00 ± 0.58	NA	13.00 ± 0.58	12.33 ± 0.33
E. coli	10.33 ± 0.33	NA	7.00 ± 0.58	4.00 ± 0.58
P. aeruginosa	14.33 ± 0.33	NA	8.33 ± 0.33	6.33 ± 0.33
S. typhi	12.33 ± 0.33	NA	10.67 ± 0.33	6.00 ± 0.58
K. pneumoniae	17.33 ± 0.67	NA	8.67 ± 0.33	4.67 ± 0.30
C. albicans	10.00 ± 0.58	NA	8.00 ± 0.58	4.00 ± 0.58

NA=Not active, SFE (total ethanol extract), FEP (Pet-ether extract), FEC (Chloroform extract), FEE (ethanol extract)

S. aureus was the most susceptible organism to both FEC and FEE whereas *E. coli* was the least. The more susceptible organisms (*P. aeruginosa*, *S. typhi*, *K. pneumoniae* and *S. aureus*) were selected for the micro-dilution assay to determine the minimum inhibitory concentration (MIC). The MIC's are presented in the Table 4.25.

Table 4.25 Minimum inhibitory concentration (MIC) of stem bark extracts on various organisms

Extract	MIC (µg/ml)			
	PA	**SA**	**ST**	**KP**
SFE	500	500	250	250
FEC	1000	1000	>1000	>1000
FEE	>1000	>1000	>1000	>1000

PA (*P. aeruginosa*), **SA** (*S. aureus*), **ST** (*S. typhi*), **KP** (*K. pneumoniae*)

4.5.3 Antimicrobial activity of isolates

The isolated compounds *β*-sitosterol [**1**], sitosterol-3-O-*β*-D-glucoside [**7**], bergapten [**20**], and oxypeucedanin hydrate [**30**] were also tested against the seven microorganisms. *β*-sitosterol [**1**] and its glycoside [**7**] were inactive against all the organisms. Both oxypeucidanin hydrate [**30**] and bergapten [**20**] were active against *S. aureus* (Table 4.26). However their activities were comparatively lower than those of the extracts from which they were isolated. The more susceptible organisms (*P. aeruginosa*, *S. typhi*, *K. pneumoniae* and *S. aureus*) were selected for the micro-dilution assay to determine the MIC and the results presented in Table 4.27.

Table 4.26 Antimicrobial activities of isolated compounds

Organisms	**Mean diameter (zone) of inhibition (mm) ± S.E.M**			
	[1]	**[7]**	**[30]**	**[20]**
B. subtilis	NA	NA	1.33 ± 0.88	1.00 ± 0.58
S. aureus	NA	NA	8.00 ± 0.58	6.00 ± 0.58
E. coli	NA	NA	4.33 ± 0.33	1.67 ± 0.33
S. typhi	NA	NA	5.00 ± 0.33	2.63 ± 0.88
P. aeruginosa	NA	NA	5.33 ± 0.88	1.67 ± 0.88
K. pneumoniae	NA	NA	4.67 ± 1.20	2.33 ± 0.88
C. albicans	NA	NA	NA	NA

[**1**] =*β*-sitosterol, [**7**] = *β*-sitosterol-D-glucoside, [**20**] =Bergapten, [**30**] =oxypeucedanin hydrate, NA= Not active

Table 4.27 MIC's of isolated compounds on various organisms

Extract	**MIC (µg/ml)**			
	PA	***SA***	***ST***	***KP***
Bergapten [**20**]	>1000	>1000	>1000	>1000
Oxypeucidanin hydrate [**30**]	>1000	1000	>1000	>1000

PA (*P. aeruginosa*), **SA** (*S. aureus*), **ST** (*S. typhi*), **KP** (*K. pneumoniae*)

Chapter 5

DISCUSSION

5.1 ANTI-INFLAMMATORY ACTIVITY

The present study was aimed at investigating the stem bark and leaf extracts of *F. exasperata* for anti-inflammatory activity, using carrageen-induced foot oedema model in 7-day old chicks and isolate the compounds which may be responsible for this activity. Carrageenan-induced oedema is a multi-mediated phenomenon that releases various inflammatory mediators. It is biphasic; the first phase (1hour) involves the release of serotonin and histamine whilst the second phase (over 1 hour) is mediated by prostaglandins (Asongalem *et al.,* 2005; Perianayagem *et al.,* 2006). All the extracts inhibited the increase in foot volume significantly ($P<0.001$) from the second hour (Figures 4.2a-4.4a, 4.12a-4.16a) and thus, presumably, inhibited the synthesis and release of prostaglandins as well as kinins responsible for the inflammation (Asongalem *et al.,* 2005; Silva *et al*., 2005). A low ED_{50}, indicating high anti-inflammatory activity, was recorded for the total ethanol stem bark extract (SFE; 50.65 ± 0.012 mg/kg). The petroleum ether (FEP), chloroform (FEC) and ethanol (FEE) extracts of the stem bark, also exhibited dose-dependent reduction in foot volume (Figure 4.14 - 4.16), but with comparatively lower activities than the total ethanol extract. The chloroform extract exhibited the highest activity (ED_{50} = 97.76 ± 0.013 mg/kg) followed by the ethanol (ED_{50} = 109.7 ± 0.013 mg/kg) and the pet-ether extracts (ED_{50} = 215 ± 0.011 mg/kg) respectively (Table 4.11). Thus, the extracts, may be speculated to be working synergistically to establish the observed higher anti-inflammatory activity of the total extract (SFE). Similarly, the petroleum ether extract of the leaves exhibited the highest

anti-inflammatory activity (ED_{50} = 49.59 ± 0.02 mg/kg), followed by the ethyl acetate extract (EFE, ED_{50} = 53.37 ± 0.011 mg/kg) and the ethanol extract (AFE; ED_{50} = 84.85 ± 0.013 mg/kg). Thus the present study has shown that the leaf and stem bark extracts of *Ficus exasperata* possess significant anti-oedematogenic effect on foot pad oedema induced by carrageen and therefore justifies its use in the treatment of pain and inflammatory conditions in folklore medicine. To the best of our knowledge, this is the first report of the anti-inflammatory activity of the stem bark of *F. exasperata* although the activities of the alcohol leaf extract has been reported (Woode *et al*., 2009)

5.1.1 Anti-oxidant activity

In the present study, antioxidant potential of the stem bark and leaf extracts were investigated using five assays; total antioxidant capacity, total phenolic content, DPPH scavenging activity, reducing power and lipid peroxidation activity. In all these assays, the antioxidant activity increased with increasing concentration of the extracts. The total antioxidant capacity expressed as ascorbic acid equivalent in mg/g weight of extract was 310.39 ± 3.840, 37.41 ± 1.088, 143.30 ± 0.623 and 206.53 ± 0.174 for the total ethanol stem bark extract (SFE) as well as the pet-ether, chloroform and ethanol extracts (Table 4.19). The total ethanol extract showed higher reducing power (IC_{50} = 61.8 ± 0.001 µg/ml) and inhibited linoleic acid lipid peroxidation (IC_{50} = 42.27 ± 0.012 µg/ml) considerably than the standard antioxidant *n*-propyl gallate (IC_{50} = 66.88 ± 0.003 µg/ml). Similarly the total antioxidant capacities of the leaf extracts were recorded as 74.53 ± 0.004, 59.73 ± 0.015 and 31.44 ± 0.005 ascorbic acid equivalent in mg/g of extract, for the ethanol, ethyl acetate and pet-ether extracts respectively. The antioxidant activity of the leaves was comparatively lower than that of the stem bark. In both the leaf and stem bark extracts, antioxidant activity was highest in the polar extracts. Also a high positive

correlation ($r^2 \geq 0.9$) was observed between the total antioxidant capacity and the total phenolic contents of the polar extracts (Figure 4.23, 4.36-4.37), indicating that more than 90% of the antioxidant activity may be attributed to the phenolic content (Javanmardia *et al.*, 2003). Antioxidant activity of plant extracts is not limited to phenolic compounds. Activity may also be due to the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids and vitamins.

Thus the present study has shown that the leaves and stem bark of *F. exasperata* possess significant antioxidant properties and may contribute to the retardation of the inflammatory process. This is because inflammatory tissue injuries are mediated by reactive oxygen metabolites from phagocytic leukocytes (e.g neutrophils, monocytes, macrophages and eosinophils) that invade the tissues and cause injury to essential cellular components (Parfenov and Zaikov, 2000). Compounds that have scavenging activities toward these radicals have been found to be beneficial in inflammatory diseases (Auddy *et al.,* 2003; Koo *et al*., 2006).

Also the ability of the leaf extracts to inhibit the peroxidation of linoleic acid supports the use of the leaves of *F. exasperata* in the preservation of palm oil in indigenous societies (Umerie *et al*., 2004). The antioxidant activity of the extract, shown in this study, may also support its traditional use for wound healing. This is because in acute and chronic wounds, oxidants cause cell damage and thus inhibits wound healing (Thang *et al.,* 2001). The administration of antioxidants or free radical scavengers is reportedly helpful, notably to limit the delayed sequale of thermal trauma and to enhance the healing process (Thang *et al.,* 2001).

5.1.2 Antimicrobial activity

Infectious diseases represent a serious health problem worldwide today, mostly due to the appearance of antibiotic resistant strains. There has been an intense search for newer antimicrobial agents. The efficacies of plant based drugs used in traditional medicines are being inestigated. The plants used include the members of the Moraceae family. Within this family, the genus *Ficus* is well documented for antioxidant and antimicrobial activities (Al-Fatimi *et al*., 2007).

In the present study, the total ethanol extract of the stem bark, inhibited the growth of all test organisms used. It however, showed weak activity against *Candida albicans*. It produced significant activities against *P. aeruginosa, S. typhi, K. pneumoniae* and *S. aureus* with respective MIC's of 1000, 500, 250 and 1000 µg/ml. The chloroform and ethanol, extracts of the stem bark gave MIC's greater than 1000 µg/ml against all the susceptible organisms (*P. aeruginosa, S. typhi, K. pneumoniae* and *S. aureus*) whereas the pet-ether extract was inactive against all the test organisms (Table 4.25). The leaf extracts also exhibited considerable antimicrobial activity. The pet-ether and ethanol extracts showed activity against *S. aureus* (zones of inhibition: 7.0 ± 0.58, 7.32 ± 0.13 mm respectively) and *P. aeruginosa* (zones of inhibition: 6.67 ± 0.23 and 10.33 ± 0.3 mm respectively). The ethyl acetate extract showed good activity against *K. pneumoniae* and *P. aeruginosa* with respective zones of inhibition of 13.67 ± 0.88 and 12.33 ± 0.34 mm. The ethanol extract was inactive against *E. coli, K. pneumoniae* and *S. typhi* (Table 4.23).

The considerable susceptibility of both Gram positive and Gram negative organisms to the extracts supports the folkloric use of the plant for wound healing, treatment of veneral diseases and other infections (Burkill, 1985). Also the high susceptibility of *S. aureus* to

all the extracts further supports the use of the plant for the treatment of wounds since *S. aureus* is a common pathogen in most infected wounds (Thomas, 1990).

5.2 BIOACTIVITY OF ISOLATED COMPOUNDS

Three compounds, bergapten [**20**], oxypeucedanin hydrate [**30**] and sitosterol-3-O-*β*-D-glucopyranoside **[7]** were isolated from the chloroform extract of the stem bark whereas *β*-sitosterol **[1]** and *β*-sitosterol-3-O-*β*-D-glucopyranoside **[7]** were isolated from the petroleum ether and ethyl acetate extracts of the leaves respectively. To the best of our knowledge, this is the first report of the occurrence of these compounds in *F. exasperata*. However, they have been isolated from other *Ficus* species. They were evaluated for their anti-inflammatory, antioxidant and antimicrobial activities. All of them exhibited dose-dependent anti-inflammatory activities with ED_{50} values of 101.6 ± 0.003, 126.4 ± 0.011, 123.4 ± 0.033 and 275.9 ± 0.012 mg/kg body weight, for bergapten [**20**], oxypeucedanin hydrate [**30**], *β*-sitosterol **[1]** and sitosterol-3-O-*β*-D-glucopyranoside **[7]** respectively (Table 4.9, 4.12). Apart from the glucoside which gave an IC_{50} value greater than 1000 µg/ml, all the isolates showed concentration-dependent DPPH scavenging effect with respective IC_{50} values of 63.38 ± 0.010, 46.63 ± 0.011 and 220.3 ± 0.03 µg/ml (Table 4.22). In the antimicrobial assay, *β*-sitosterol **[1]** and its glucoside **[7]** were inactive against all the test organisms whereas bergapten **[20]** and oxypeucedanin hydrate **[30]** showed considerable activities against all organisms except *C. albicans*. The lowest MIC, indicating the highest activity, was recorded for oxypeucedanin hydrate [**30**] against *S. aureus* (1000µg/ml) with other susceptible organisms giving MIC's >1000 µg/ml for both compounds. The anti-inflammatory, antioxidant and antimicrobial activities of the compounds were much lower than those of their respective extracts from which they were

isolated. Thus the compounds may be speculated to work synergistically with other secondary metabolites to give the observed higher biological activities of the extracts from which they were obtained.

The furanocoumarins, bergapten **[20]** and oxypeucedanin hydrate **[30]** have been isolated from some plant families including the Rutaceae, Apiaceae, Umbelliferae and Leguminosae. Both compounds have been shown to inhibit the release of histamine (Kimura *et al.,* 1997). Bergapten **[20]**, isolated from *Angelica pubescens* demonstrated both anti-inflammatory and analgesic activities in mice (Chen *et al*., 1995). It is used clinically, coupled with ultraviolet light, to treat inflammatory dermatological disorders such as psoriasis and vitiligo (McNeely and Goa, 1998). Oxypeucedanin hydrate **[30]** from *Citrus grandis* fruit peel was found to be a scavenger of the DPPH radical (Mokbel *et al*., 2006). It also contributed to the antioxidant activity of the fruits of *Anethum graveolens* (Satyanarayana, 2004). In a similar study, bergapten **[20]** was found to exhibit weak DPPH scavenging activity and also weak inhibition of lipid peroxidation using rat brain homogenate (Yu *et al*., 2005). It has been proposed that addition of the oxyprenyl group to the furanocoumarin skeleton (as is the case of oxypeucedanin hydrate [**30**]), results in an increase in lipophylicity of the molecule, thus facilitating its passage through the thick bacterial membrane to its target (Stavri and Gibbons, 2005). This may account for the high antimicrobial activity of oxypeucedanin hydrate [**30**] than bergapten [**20**]. Similarly, hydroxyl groups on the 5-oxyprenyl side chain may account for the relatively higher antioxidant activity of oxypeucedanin hydrate [**30**] than bergapten [**20**], as hydroxyl and imino groups are known to increase free radical scavenging or antioxidant activity (Cai *et al*., 2003). It is important to note, however, that these furanocoumarins are

known to cause phytophotodermatitis, characterized by erythema and itching, when exposed to sunlight (McNeely and Goa, 1998). It has been shown that bergapten [**20**] inhibits moderately, human cytochrome CYP3A4 enzyme which metabolises clinically useful drugs such as cyclosporine, midazolam, felodipine and nifedipine (Bailey *et al.*, 1998). Thus co-administration of the stem bark extracts with any of these medications may lead to toxicity. Therefore the stem bark of *F. exasperata* should be used cautiously, if at all, in conjunction with these medications.

β-sitosterol **[1]** and sitosterol-3-O-*β*-D-glucopyranoside **[7]** are the most ubiquitous sterols found in plants. *β*-sitosterol **[1]** is reported to exhibit anti-inflammatory properties similar to cortisone in rat models (Bouic, 1998). A combination of the two compounds has been shown to reduce secretion of pro-inflammatory cytokines by macrophages thereby decreasing inflammation (Gupta *et al.*, 1980). Also they have been reported to reduce DNA damage, reduce the level of free radicals in cells and cause an increase in the levels of glutathione and other antioxidant enzymes (Vivancos and Moreno, 2005). The two compounds have also been reported to show very weak antimicrobial activities (Bayor *et al.,* 2009). Again it has been shown that oral administration of either *β*-sitosterol **[1]** or sitosterol-3-O-*β*-D-glucopyranoside **[7]** lowers fasting glucose and increases insulin levels (Ivorra *et al.,* 1988). *β* –sitosterol **[1]**, in two randomized, placebo-controlled, clinical studies improved urinary flow rate and alleviated symptoms of benign prostatic hyperplasia (Berges *et al.,* 1995). A herbal capsule for the treatment of benign prostatic hyperplasia, Harzol, which contains *β*-sitosterol **[1]** and other phytosterols, has been used in Germany since 1977 (Klippel *et al.,* 1997). A proprietary blend of *β*-sitosterol **[1]** and sitosterol-3-O-*β*-D-glucopyranoside **[7]**, designated as BSS: BSSG, in a ratio of

100:1 has been shown to have profound immune modulating and total and LDL cholesterol lowering properties (Boiuc, 1998; Plat and Mensink, 2002). This mixture has been commercialized under the name 'Moducare'.

The results obtained in this study, and that reported in literature, clearly indicate that bergapten [**20**], oxypeucedanin hydrate [**30**], *β*-sitosterol **[1]** and sitosterol-3-O-*β*-D-glucopyranoside **[7]** display considerable anti-inflammatory, antimicrobial (except *β*-sitosterol and its glucoside) and antioxidant properties and contribute to the bioactivity of the leaves and stem bark of *F. exasperata*. This gives scientific credence to the use of the leaves and stem bark of *F. exasperata* in various ethno-medicines for the treatment of inflammatory and infectious conditions.

5.3 CONCLUSION

This work has demonstrated that extracts of the stem bark and leaves of *F. exasperata* exhibits considerable anti-inflammatory, antioxidant and antimicrobial activities. The bioactivities activities of the stem bark were highest in the chloroform and ethanol extracts whereas in the case of the leaves, activity was observed in all three fractions. The stem bark extracts however, showed higher activities than the leaves.

The furanocoumarins bergapten and oxypeucedanin hydrate as well as the steroidal glucoside sitosterol-3-O-*β*-D-glucoside, were isolated from the chloroform extract of the stem bark whereas *β*-sitosterol and sitosterol-3-O-*β*-D-glucoside were isolated from the leaves of *F. exasperata*. The compounds showed anti-inflammatory, antioxidant and antimicrobial activities and thus contributed substantially to the bioactivities of *F.*

exasperata observed in this study. The furanocoumarins, however, exhibited higher activities than the steroids.

Thus this study has provided some justification for the folkloric use of the leaves and stem bark of the plant for the treatment of inflammatory conditions, wounds and infections. We have also been able to establish that both the leaves and stem bark of *F. exasperata* contain sitosterol-3-O-*β*-D-glucoside. All the isolated compounds are being reported for the first time in *F. exasperata*.

5.4 RECOMMENDATION

Toxicity studies of the leaf and stem bark extracts should be considered in future work.

Also structural modification of oxypeucedanin hydrate, to obtain a more potent anti-inflammatory and antioxidant compound, should be considered in future collaborative research.

Future studies should focus on isolating the anti-inflammatory and anti-oxidant constituents of the ethanolic extract of the stem bark.

Considering the antimicrobial activities shown in the present study, the leaves and stem bark should be investigated for wound healing activity in future research. A topical formulation could be made for both deep and superficial wounds after the toxicity profile of the extracts have been established.

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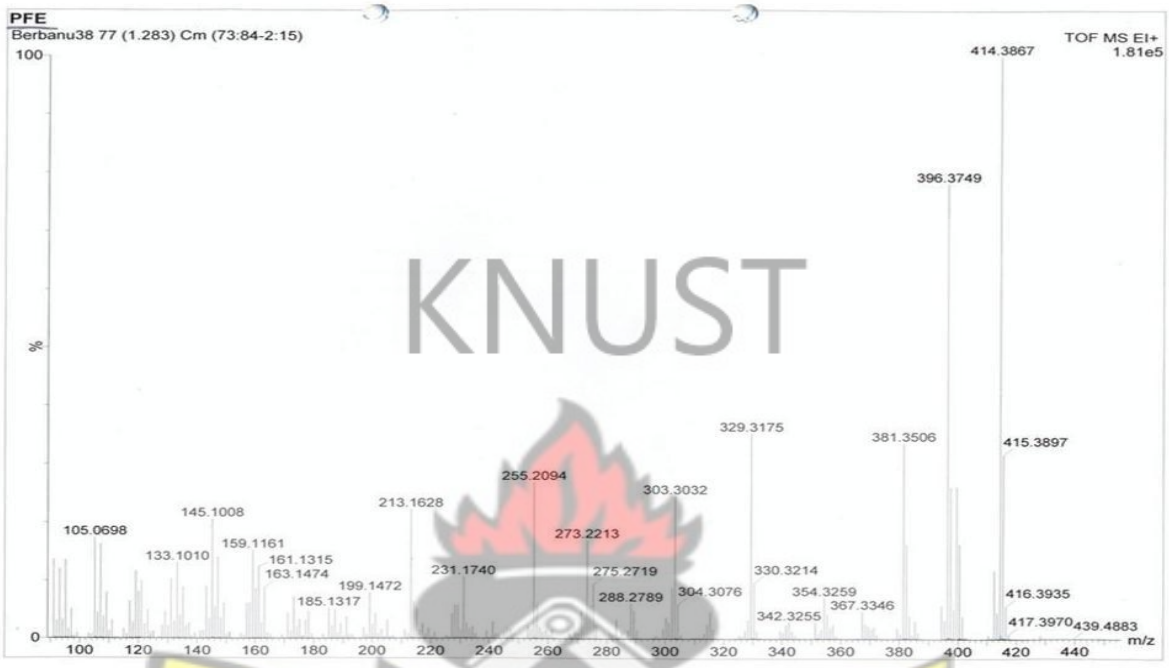
APPENDICES

APPENDIX 1

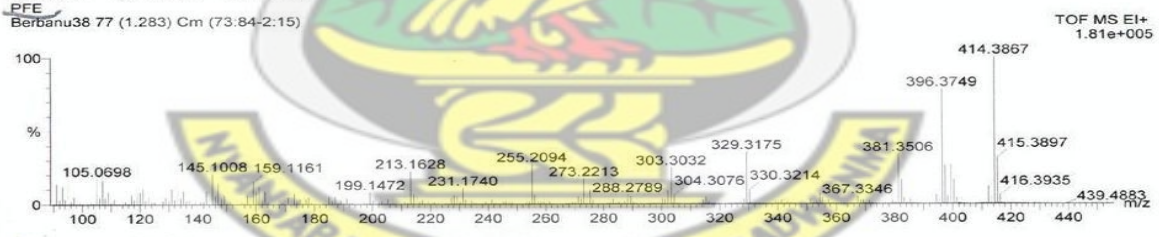
1 A Mass spectra and elemental analysis of PFE-2



Elemental Composition Report **Page 1**

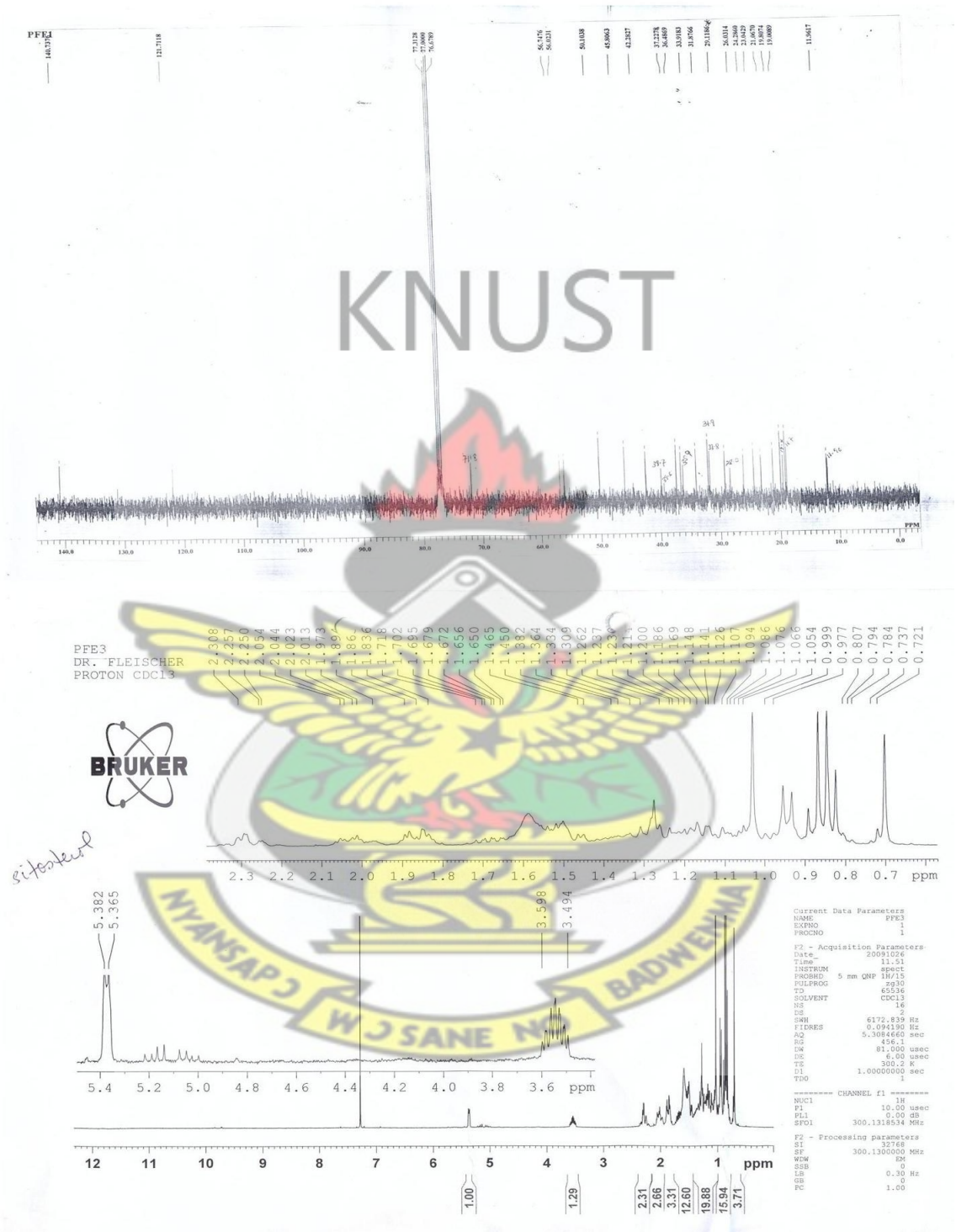
Multiple Mass Analysis: 17 mass(es) processed
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
984 formula(e) evaluated with 16 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-500 H: 0-1000 O: 0-200



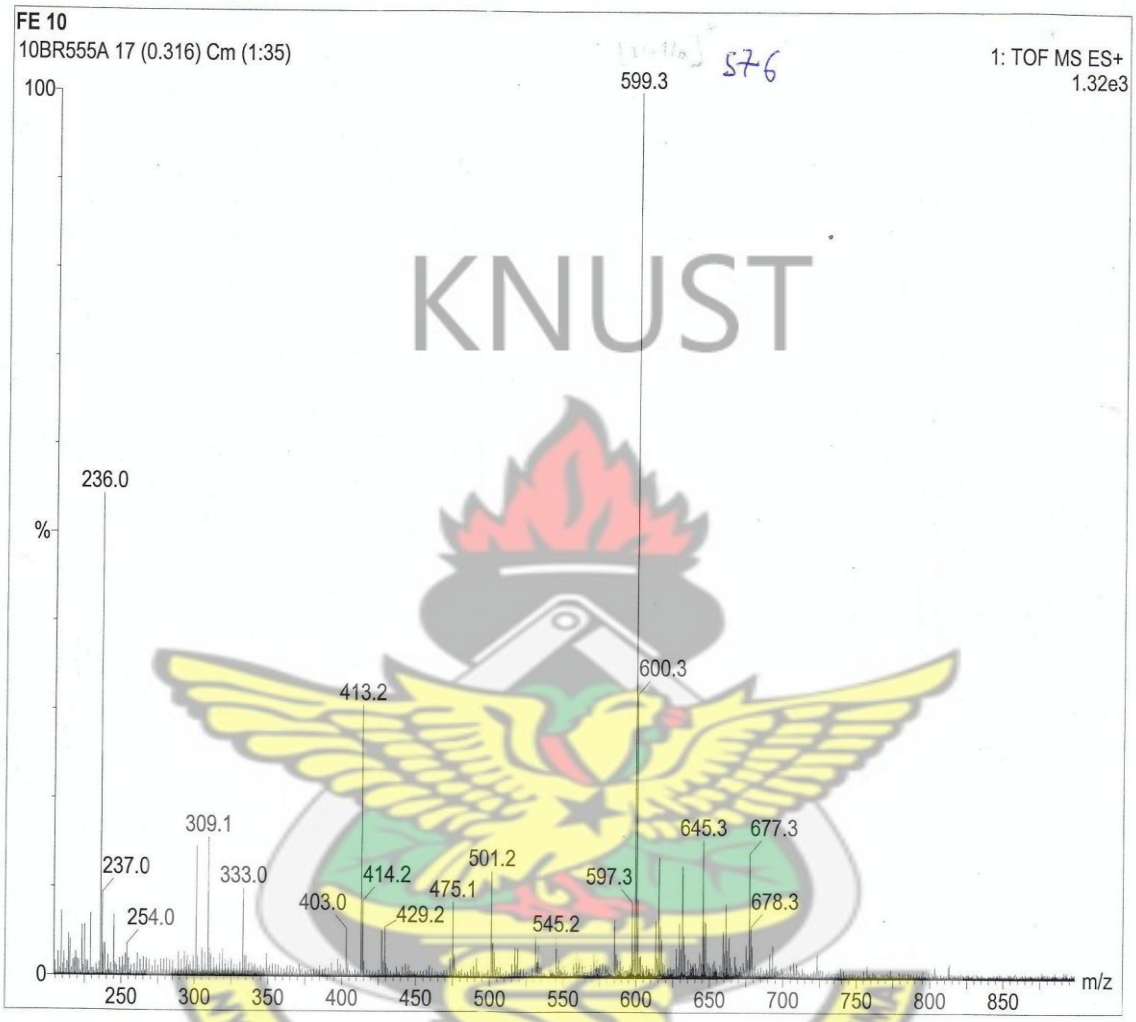
imum:	15.00				-1.5		
aximum:	100.00		5.0	10.0	50.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
105.0698	17.38	105.0704	-0.6	-5.7	4.5	16714.7	C8 H9
107.0854	16.24	107.0861	-0.7	-6.5	3.5	8677.8	C8 H11
145.1008	20.51	145.1017	-0.9	-6.2	5.5	13925.3	C11 H13
159.1161	15.16	159.1174	-1.3	-8.2	5.5	15510.4	C12 H15
213.1628	22.26	213.1643	-1.5	-7.0	6.5	4888.8	C16 H21
255.2094	26.85	255.2113	-1.9	-7.4	6.5	874.7	C19 H27
273.2213	16.95	273.2218	-0.5	-1.8	5.5	9503.6	C19 H29 O
303.3032	24.48	303.3052	-2.0	-6.6	3.5	18.0	C22 H39
329.3175	35.27	329.3208	-3.3	-10.0	4.5	32.8	C24 H41
381.3506	33.63	381.3521	-1.5	-3.9	6.5	2496.2	C28 H45
382.3555	16.28	382.3600	-4.5	-11.8	6.0	249.5	C28 H46
396.3749	78.11	396.3756	-0.7	-1.8	6.0	194.2	C29 H48
397.3772	26.09	---					
399.3624	26.15	399.3627	-0.3	-0.8	5.5	4298.3	C28 H47 O
400.3676	16.25	400.3705	-2.9	-7.2	5.0	668.4	C28 H48 O
414.3867	100.00	414.3862	0.5	1.2	5.0	52.8	C29 H50 O
415.3897	31.61	415.3940	-4.3	-10.4	4.5	2791.9	C29 H51 O

1 B ^{13}C-NMR and 1H-NMR spectra of PFE-2 (in $CDCl_3$ at 300 MHz*)*

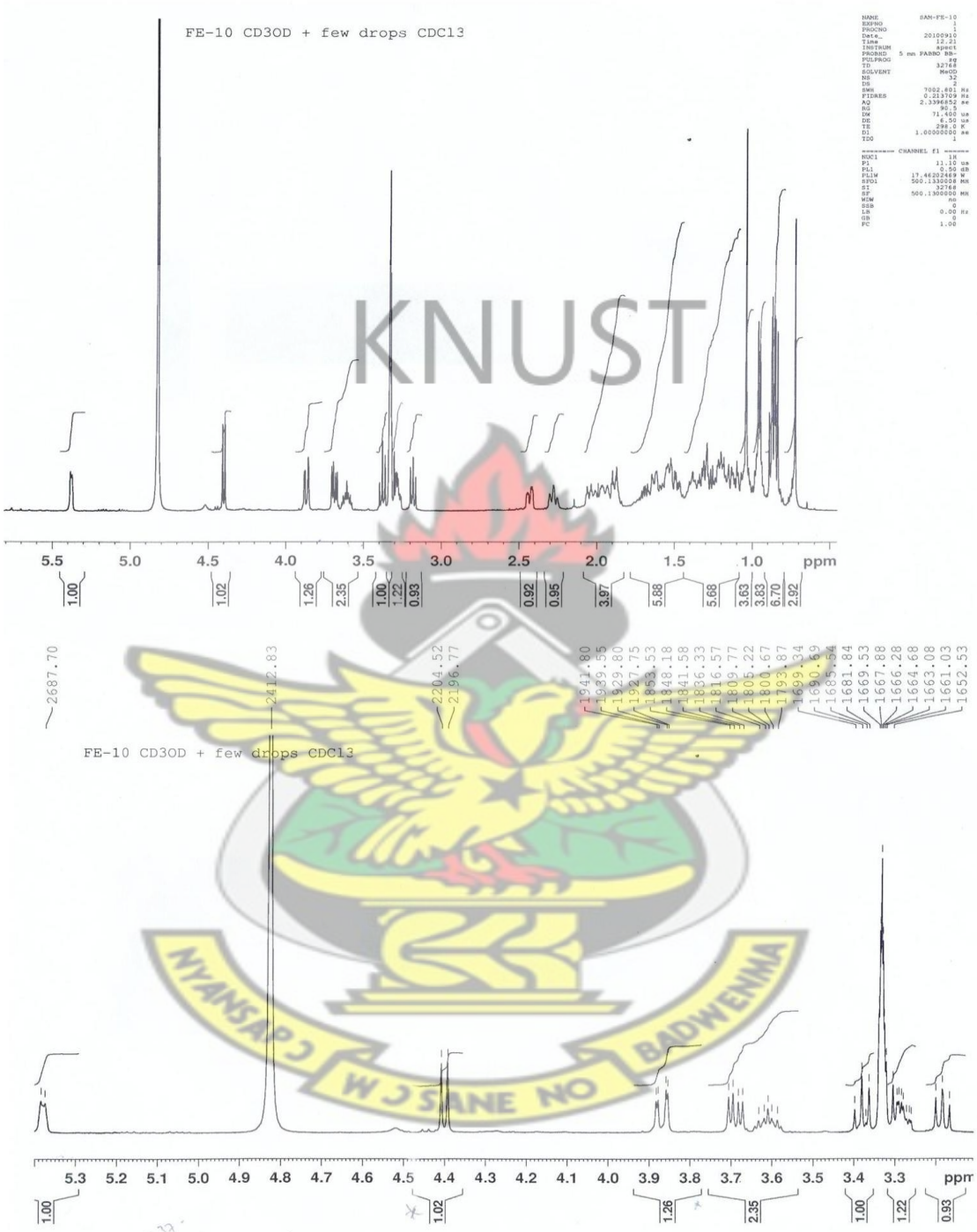


APPENDIX 2

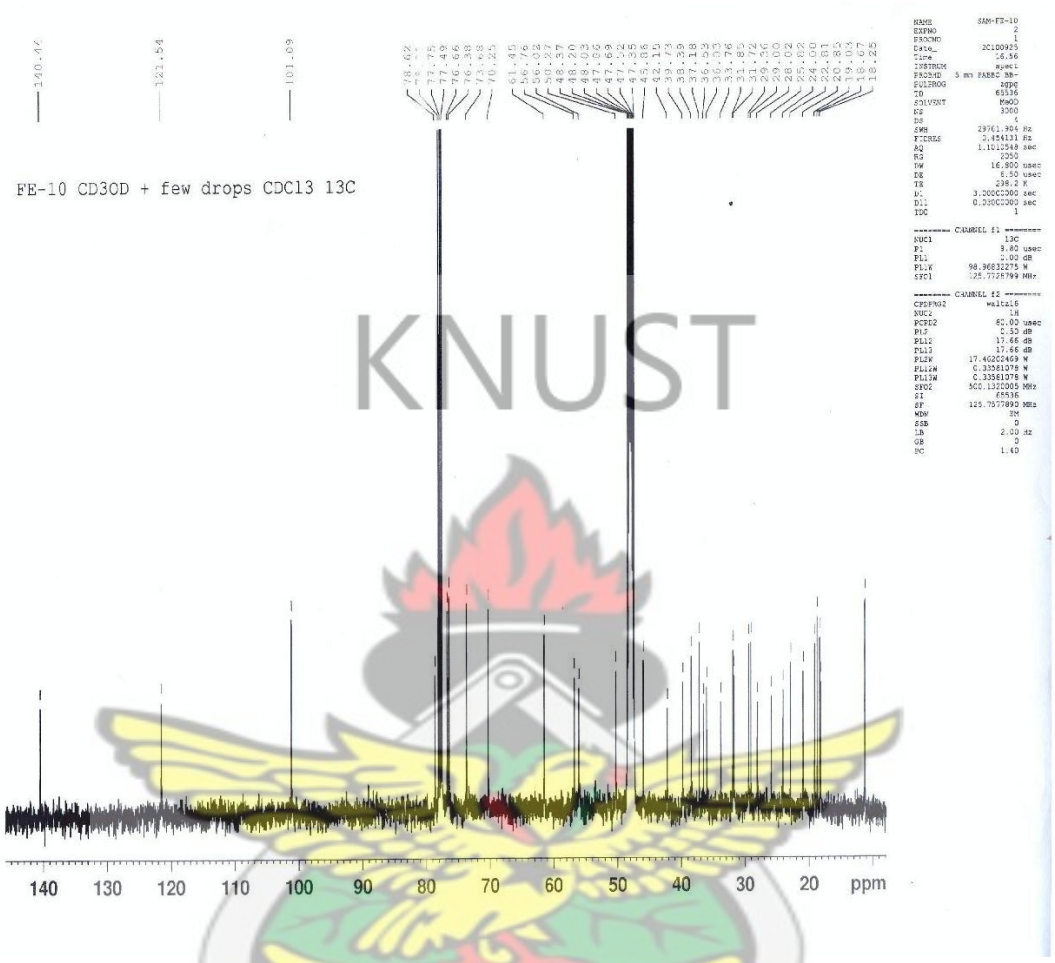
2 A Mass spectrum of FE-1



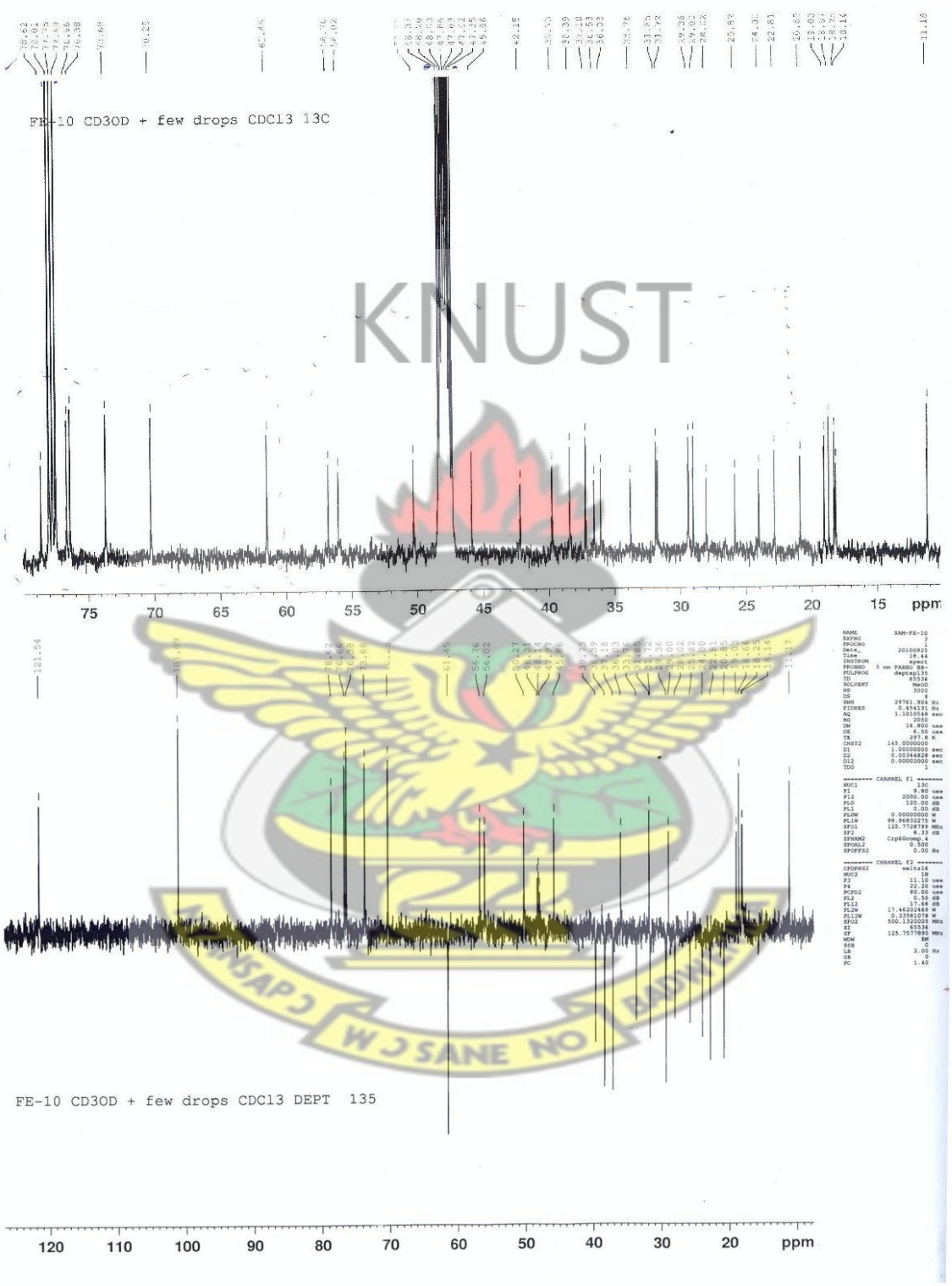
2 B ^{1}H-NMR spectra of FE-1 in $CDCl_3$ and CD_3OD at 500MHz



2 C ^{13}C-NMR spectrum of FE-1 in $CDCl_3$ and CD_3OD at 500MHz

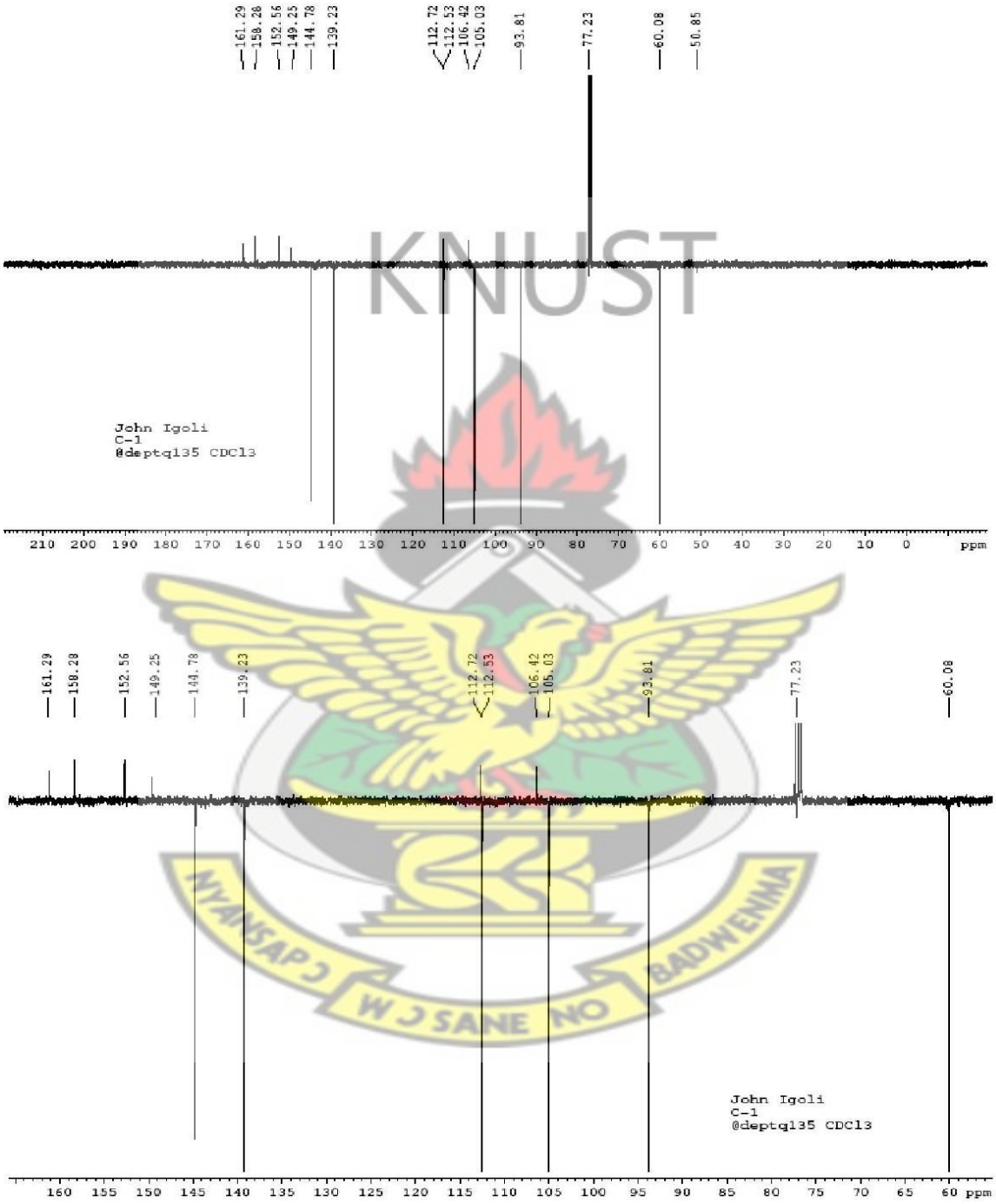


2 D ^{13}C-NMR spectrum of FE-1 in $CDCl_3$ and CD_3OD at 500MHz

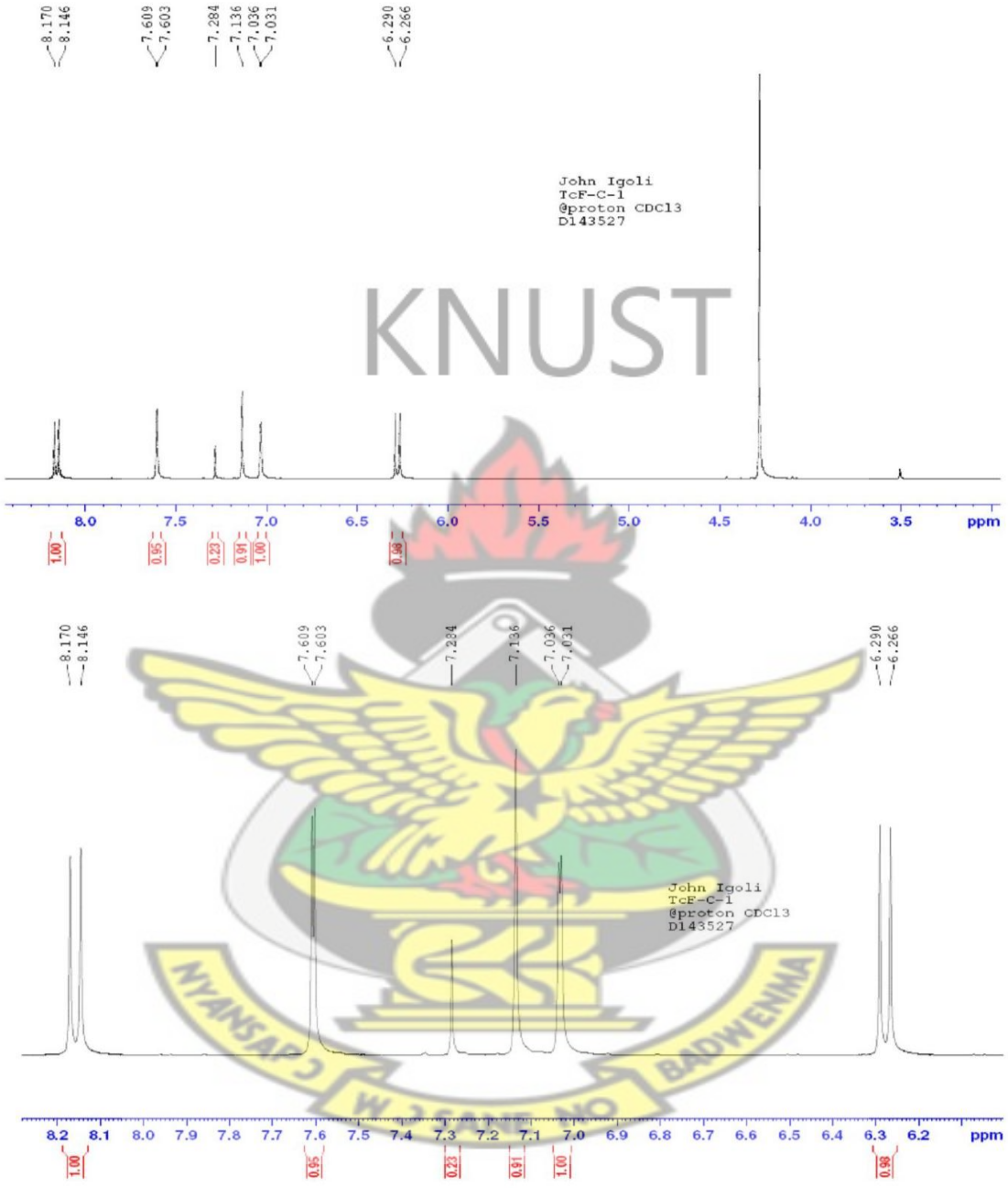


APPENDIX 3

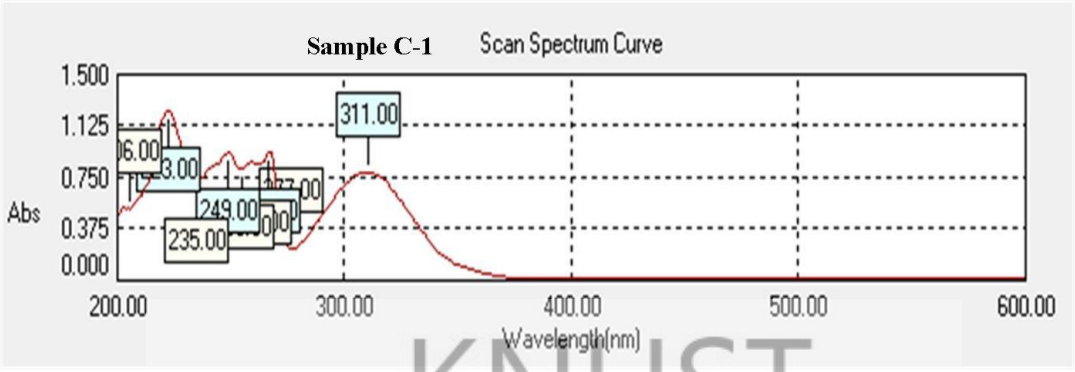
3 A ^{13}C-DEPT NMR spectra of C-1 in $CDCl_3$ at 400 MHz



3 B ^{1}H- NMR spectra of C-1 in $CDCl_3$ at 400 MHz



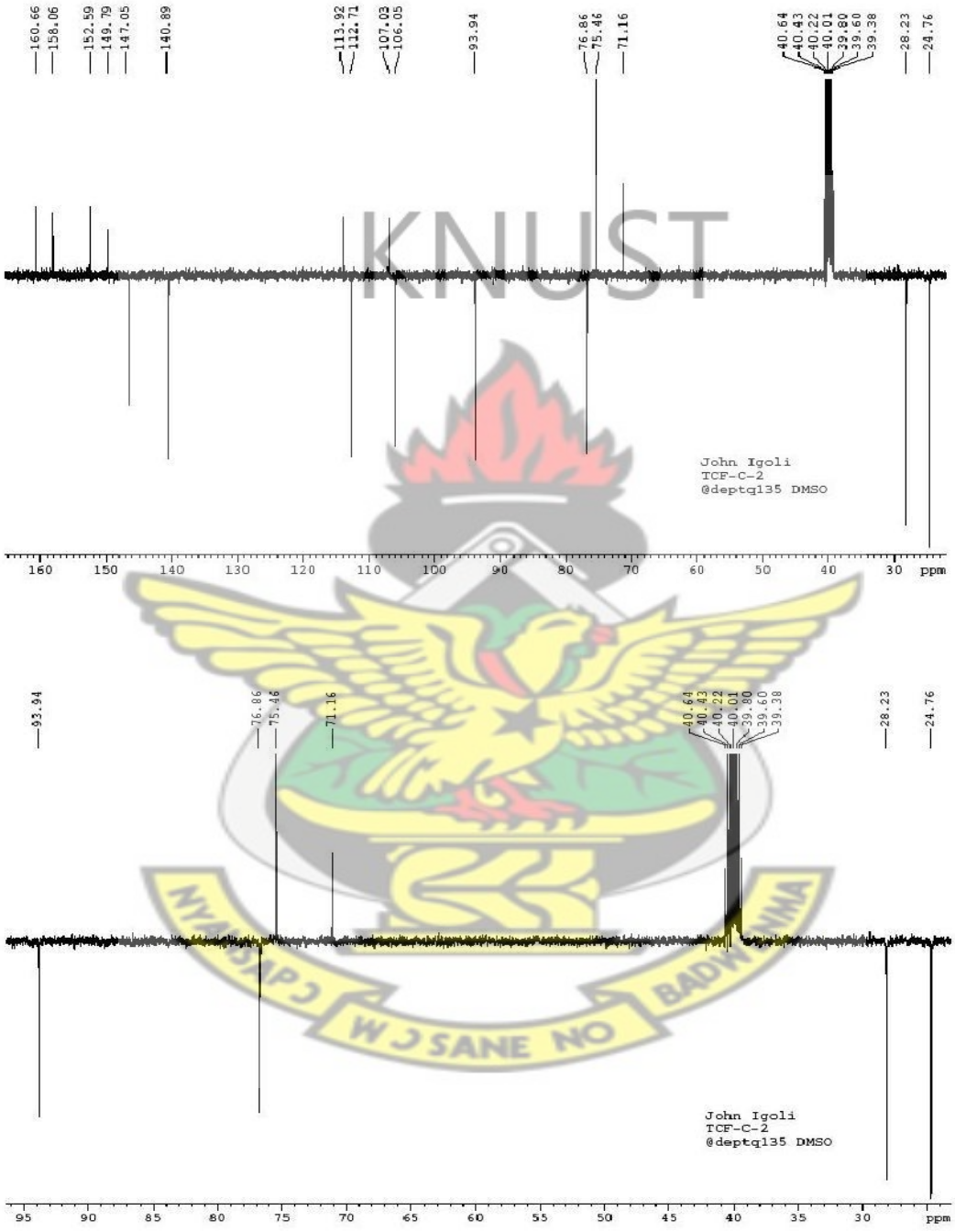
3 C UV spectrum of C-1 in methanol



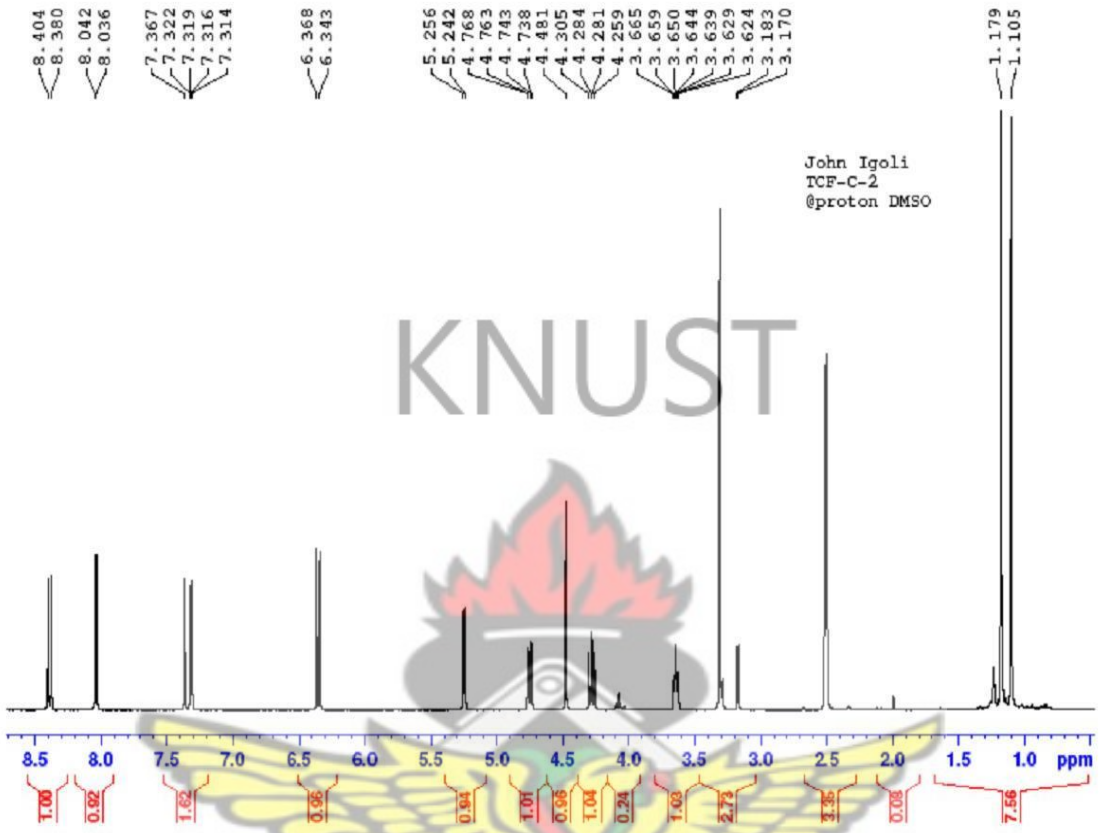
Peak	311.00	0.786	Valley	277.00	0.224
Peak	267.00	0.931	Valley	263.00	0.844
Peak	249.00	0.933	Valley	255.00	0.813
Peak	223.00	1.235	Valley	235.00	0.718
			Valley	206.00	0.520

APPENDIX 4

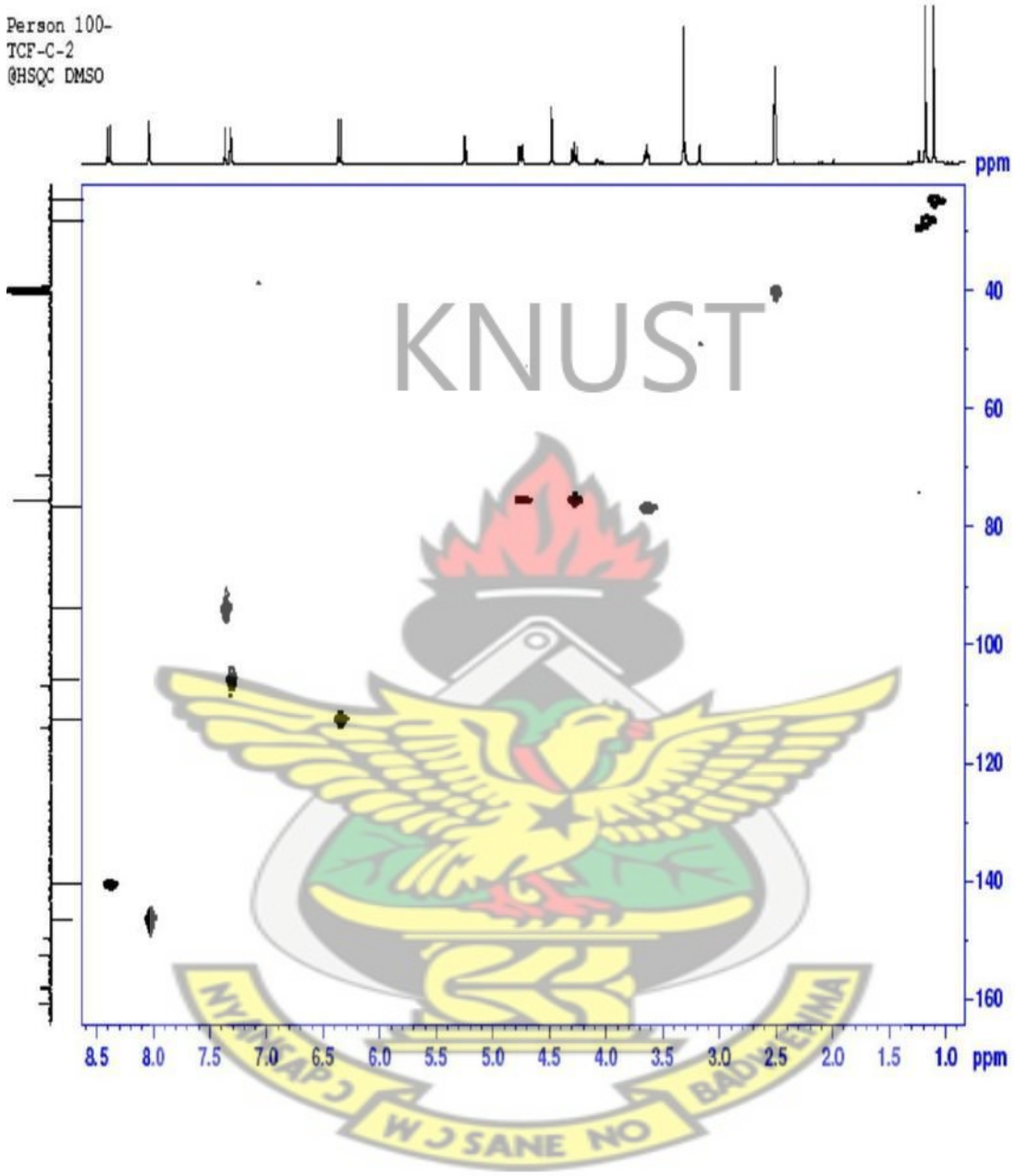
4 A ^{13}C-DEPT NMR spectra of C-2 in DMSO at 400 MHz



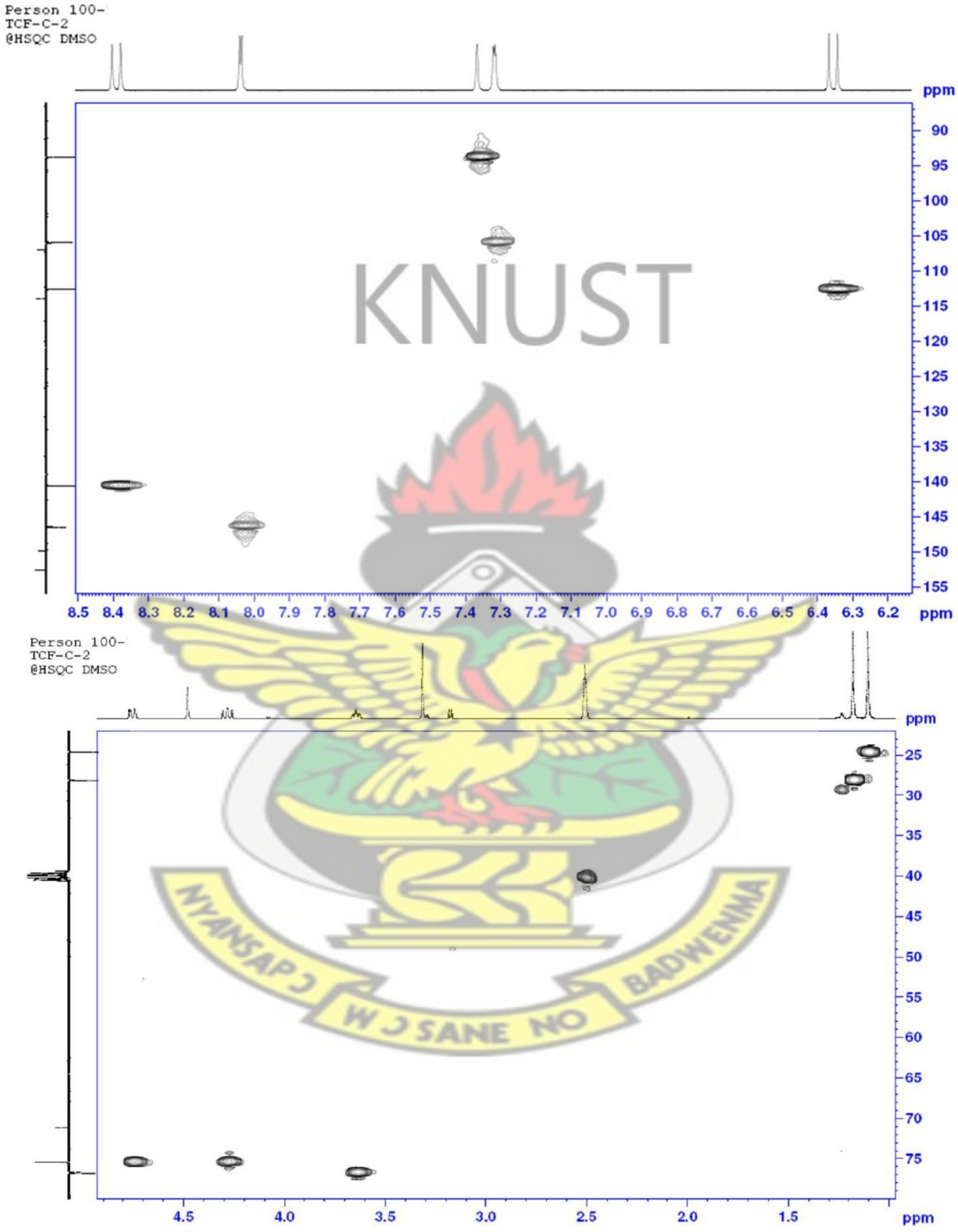
4 B ^{13}H- NMR spectra of C-2 in DMSO at 400 MHz



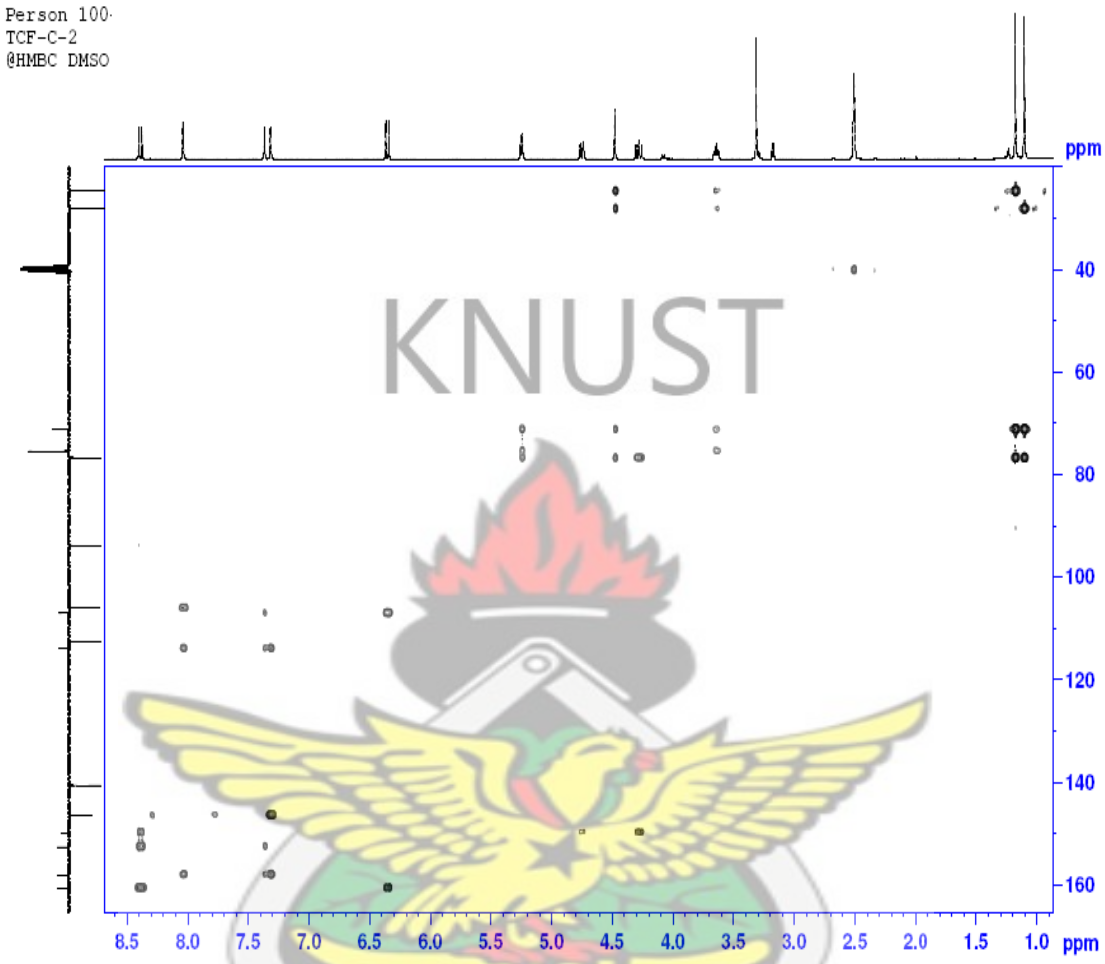
4 C HSQC (^{1}H-^{13}C correlation) spectrum of C-2



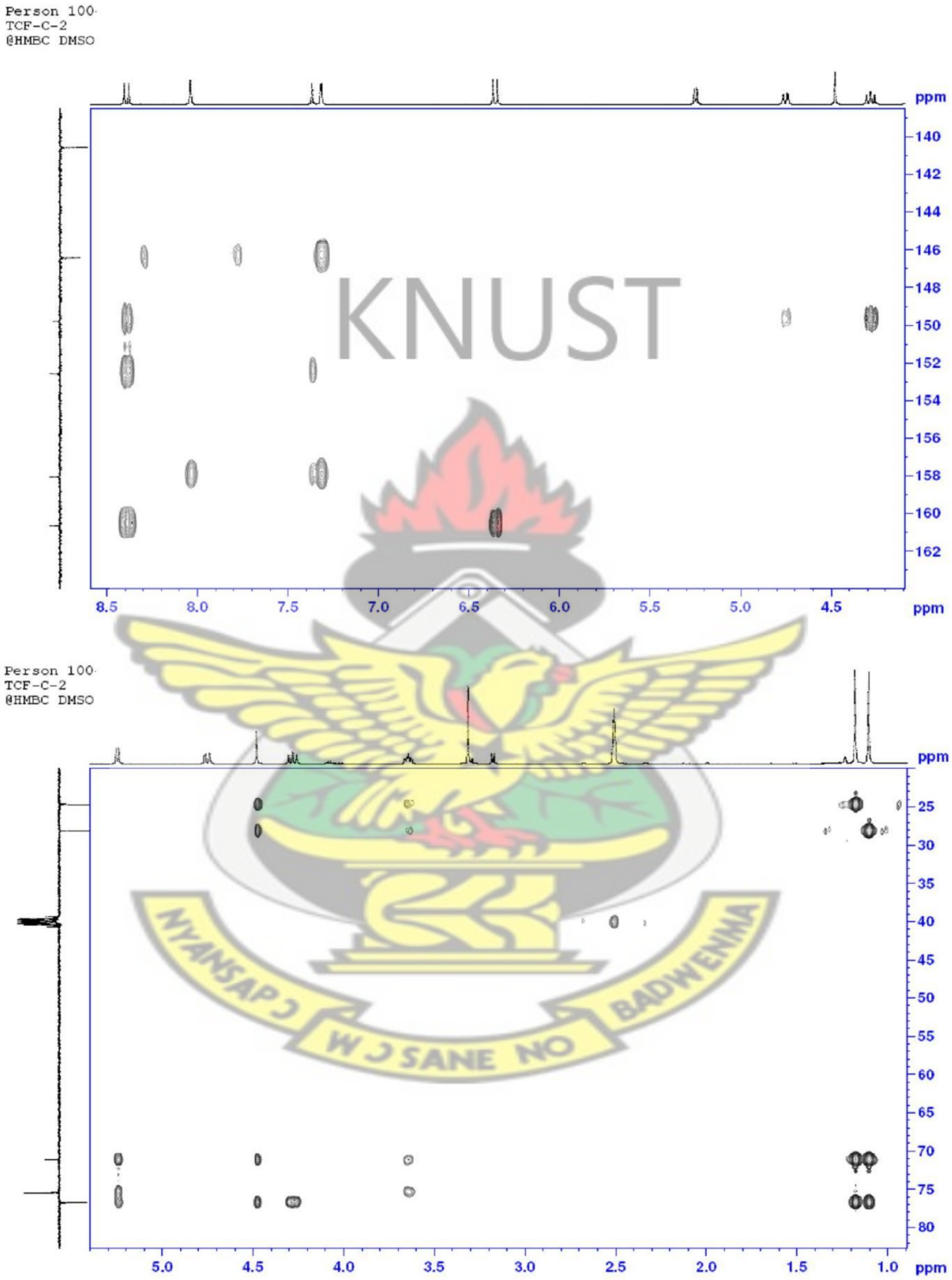
4 D HSQC (^{1}H-^{13}C correlation) spectrum of C-2



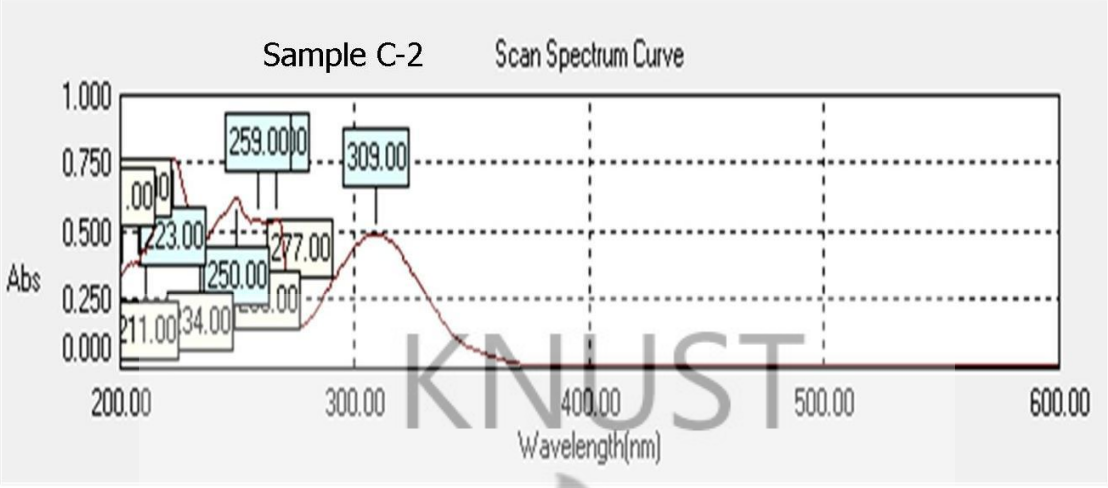
4 E HMBC (^{1}H-^{13}C) correlation spectrum of C-2



4 F HMBC (^{1}H-^{13}C) correlation spectrum of C-2



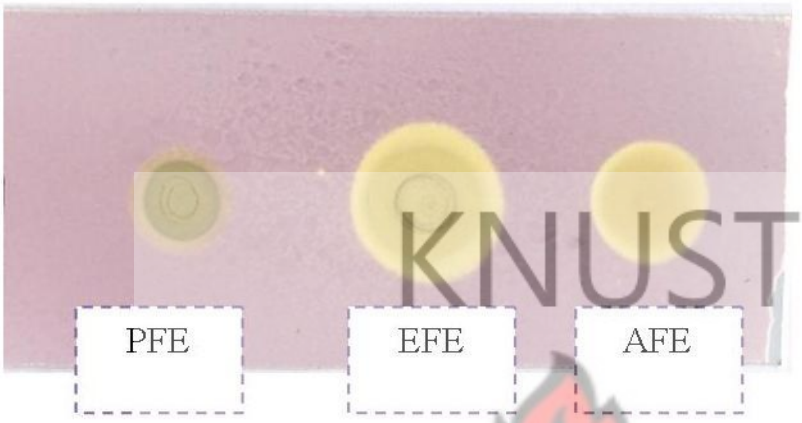
4 G UV Spectrum of C-2 in methanol



Peak	309.00	0.491	Valley	277.00	0.151
Peak	267.00	0.549	Valley	263.00	0.529
Peak	259.00	0.549	Valley	234.00	0.450
Peak	250.00	0.621	Valley	211.00	0.422
Peak	223.00	0.770	Valley	208.00	0.378
			Valley	201.00	0.338

APPENDIX 5

5 A Effect of extracts AFE, EFE AND PFE on DPPH radical



5 B Effects of FEP, FEC and FEE on DPPH radical