ANTI-INFLAMMATORY, ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF *MARGARITARIA NOBILIS, STYLOCHITON LANCIFOLIUS, DRYPETES PRINCIPUM, CRESCENTIA CUJETE* AND *ALBIZIA GLABERRIMA*

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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DECLARATION

I hereby declare that the experimental work described in this thesis was carried out by me at the Department of Pharmacognosy, KNUST and that to the best of my knowledge, it contains no material previously published by another person nor material which has been submitted for the award of any other degree of the University, except where acknowledgement has been duly made in the text.

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ABSTRACT

This research work scientifically evaluated and validated the anti-inflammatory, antioxidant, antimicrobial activities and the acute oral toxicity profile of five (5) selected plants that find use in Ghanaian traditional medicine as agents for treating inflammatory conditions and infections. Ethanolic extracts of the rhizomes of *Stylochiton lancifolius* Pyer and Kotchy (Araceae), the stem barks of *Margaritaria nobilis* L.f (Müll Arg.) (Euphorbiaceae), *Drypetes principum* (Müll Arg.) Hutch (Putranjivaceae), *Crescentia cujete* Linn (Bignoniaceae), and *Albizia glaberrima* (Schum. & Thonn.) Benth (Leguminosae) were each assessed for their biological activities. The carrageenan-induced oedema in chicks model for *in vivo* anti-inflammatory activity indicated that the extracts and reference drugs significantly ($p<0.0001$) and dose-dependently inhibited oedema at all doses. The extract of *C. cujete* had the least ED_{50} value of 23.30±0.43 mg/kg body weight followed by *D. principum*, *M. nobilis*, *A. glaberrima* and *S. lancifolius* with ED_{50} of 37.25±1.22, 90.54±0.73, 164.90±1.63, and 181.50±1.06 mg/kg body weight respectively. The percentage inhibition of total oedema of each extract was comparable to Diclofenac, Dexamethasone and compound EMN1 (betulinic acid; isolated from *M. nobilis* stem bark). At doses of 300 mg/kg body weight, the percentage inhibition of total oedema of all the extracts ranged from 77.49 - 83.59% comparable with EMN1, Diclofenac and Dexamethasone which inhibited oedema at 67.83%, 81.62, and 82.47 (doses of 100, 30, and 10 mg/kg body weight) respectively. *In vitro* anti-inflammatory assays using human RBC membrane and egg albumin stabilisation models indicated that the extracts were comparable to the reference drugs; Diclofenac and Dexamethasone in terms of activity. Four Gram positive and four Gram negative bacteria and a fungus were employed in antimicrobial assays using the agar well diffusion and broth micro dilution models. The 70% ethanol extract of *M. nobilis* and *C. cujete* were the most active in the agar well diffusion assay

showing activity against eight of the microorganisms. *M. nobilis* extract produced the highest zone of growth inhibition (22.93±0.27 mm) against *Candida albicans* at 10 mg/mL concentration. The activities of all the extracts at 10 mg/mL were comparable to Amoxicillin and ketoconazole at 5 mg/mL. Minimum inhibitory concentrations (MICs) of the extracts ranged from 100 to over 1000 µg/mL. The 70% ethanol extract of *M. nobilis* yielded the least MIC of 100 µg/mL against *S. aureus* and *C. albicans*, whereas that of *C. cujete* had the highest MIC of 1500 µg/mL against *E. coli*. Antioxidant activity was investigated using DPPH bioautography, DPPH radical scavenging assay, total antioxidant capacity and total phenol content. The IC_{50} values in the radical scavenging assay indicated that the extracts of *M. nobilis* and *S. lancifolius* (9.96±0.03 and 31.58±0.17 µg/ml respectively) were more active than Ascorbic acid (38.30±0.00 µg/ml). The extracts of *D. principum*, *A. glaberrima* and *C. cujete* respectively inhibited 50% of DPPH at 49.09±0.39, 53.60±0.32, and 168.80±1.06 µg/ml. The Ascorbic acid and the Tannic acid equivalences had a repeated pattern as the radical scavenging assay. Stem bark extract of *M. nobilis* yielded the highest antioxidant capacity and phenol content. Acute oral toxicity studies according to the Organization for Economic Cooperation and Development (OECD) 2001 guidelines concluded that none of the extracts produced significant observational acute toxicity in the study animals. About 395 mg of compound (EMN1) characterized as betulinic acid was isolated from the 70% ethanol extract of *M. nobilis*. Based on these observed activities, the folkloric claims of effectiveness of these plants as safe antiinflammatory, antioxidant and antimicrobial agents may be justified.

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Abbreviations and Symbols

AAE	Ascorbic Acid Equivalence
AG	*Albizia glaberrima* stem bark
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AUC	Area under the curve
BDW	Body weight
CC	*Crescentia cujete* stem bark
$CDCl_3$	Deuterated chloroform
CFU	Colony forming units
cm	Centimetre
^oC	Degree centigrade
DMARDS	Disease-modifying anti-rheumatic drugs
DNA	Deoxyribonucleic acid
DP	*Drypetes principum* stem bark
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ED_{50}	Dose for 50% maximal activity
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMN	Fractions of 70% EtOH extract of *M. nobilis*
EtOAc	Ethyl acetate
EtOH	Ethanol

$FeCl_3$ g	Ferric chloride
H_2SO_4	Gram
HCl	Sulphuric acid
H_3PO_4	Hydrochloric acid
HMBC	Phosphoric acid
HSQC	Heteronuclear multiple bond correlation
HPLC	Heteronuclear single quantum correlation
HPTLC	High Performance/Pressure Liquid Chromatography
hr	High Performance/Pressure Thin Layer Chromatography
IC_{50}	Hour
ICH	Concentration at which there is 50% inhibition
i.p	International Conference on Harmonisation
IRS kg	Intraperitoneal
L	Infrared spectroscopy
LC-MS	Kilogram
LD_{50}	Litre
MBC	Liquid Chromatography couple with Mass Spectrometry
MeOD	Concentration that causes 50% death in study animals
MFC	Minimal Bactericidal Concentration
MIC	Deuteriated methanol
	Minimal Fungicidal Concentration
	Minimum Inhibitory Concentration

µg µL	Microgram
mg	Microliters
mL	Milligram
mm	Millilitres
MN	Millimetres
MTT	*Margaritaria nobilis* stem bark
NaOH	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NaCl NTCC	Sodium hydroxide
NH_3	Sodium chloride
NIH nm	National-typed-culture collection
NMR	Ammonia
NSAID(s) OECD	National Institute of Health
p	Nanometres
PAF	Nuclear magnetic resonance
p.o	Non-Steroidal Anti-inflammatory Drug(s)
RBC	Organization for Economic Cooperation and Development
Rpm	Statistical significance
RSA	Platelet activating factor
	Per os (Oral route)
	Red Blood Cells
	Revolutions per minute
	Radical scavenging assay

SEM	Standard Error of Mean
SL	*Stylochiton lancifolius* rhizomes
TAC	Total Antioxidant Capacity
TAE	Tannic Acid Equivalence
TPC	Total Phenol Content
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization
^{1}H-NMR	Proton Nuclear Magnetic Resonance spectroscopy
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance spectroscopy

Chapter 1

1.0 GENERAL INTRODUCTION

Man has since creation been baffled with the challenge of disease and ailments and the threat of his extinction if these are not managed effectively (Rang *et al.,* 2007).

There has been a recent surge of interest in traditional or complementary and alternative medicine both in the developed world and in developing countries including Ghana and research has indicated that about 80% of the world‟s population especially those in Africa and Asia rely solely on traditional herbal practitioners for majority of their health needs (Amponsah *et al.,* 2013; Annan and Houghton, 2008). The reason perhaps may be due to the fact that most of these regimens are familiar, acceptable, affordable and available at the local level for use (Annan *et al.,* 2010; Amaning-Danquah *et al.,* 2011).

It is estimated that 90% of about 250,000 available flowering plants species are yet to be investigated scientifically (Evans, 2002). This therefore offers an unprecedented opportunity for heightened herbal drug research (Anilkumar, 2010).

This work evaluates some Ghanaian medicinal plants that are used locally in the treatment of inflammation-related diseases and also in fighting infections. Biological activity was scientifically investigated on the rhizomes of *Stylochiton lancifolius* Pyer and Kotchy (Araceae), the stem barks of *Margaritaria nobilis* L.f (Müll Arg) (Euphorbiaceae), *Drypetes principum* (Müll Arg.) Hutch (Putranjivaceae), *Crescentia cujete* Linn (Bignoniaceae), and *Albizia glaberrima* (Schumach. & Thonn.) Benth (Leguminosae). The selected plants species were studied for their anti-inflammatory, antimicrobial, antioxidant activities and acute oral toxicity

(safety of their use in man).

1.1 Plant drugs as anti-inflammatory agents

Inflammation is an underlying cause or precipitating factor for most diseases in man including wounds, rheumatoid arthritis, some carcinomas and dermatological diseases (Erlinger *et al.*, 2004). Though the current conventional therapy for inflammation (mainly non-steroidal antiinflammatory drugs (NSAIDs) and Steroids) is effective, their use has been pelted with a myriad of adverse drug events including gastritis, nephritis (in the case of NSAIDs) and others like delayed wound healing, fluid retention, hypertension, diabetes, mood swings, weight gain, osteoporosis, (Kyei *et al.*, 2012) and Cushingoid syndrome associated with steroid use. This makes their long term use in patients problematic. There is a need for safer and effective regimens to replace this burdensome orthodox regimen. Many medicinal plants have been utilized in Ghana as traditional or herbal remedies for pain and inflammatory diseases (BoakyeGyasi *et al.*, 2008). Most of these plants including those under study in this work however lack scientific data to back the claims of efficacy and safety.

1.2 Anti-infectives from nature

Antibiotics from microbial sources and marine bioenvironment have been studied and synthesized since the days of Robert Koch and Alexander Fleming (Rang *et al.*, 2007). There is a current trend of rising and fast spreading multi-drug resistance among once susceptible pathogens, an ever-increasing new and co-infections and a declined interest in antibiotic drug research. The high cost of existing antimicrobial regimens coupled with their serious side effects (such as kidney failure, Steven Johnson‟s syndrome and dermatitis) and the economic toll of infectious diseases on developed and developing economies call for an urgent search for therapies that are cheaper, efficacious, versatile, safe, and accessible (Cos *et al.*, 2006). This current search includes sources such as synthesis, marine bioactives, animals, and much more on medicinal plants (Agyare *et al.*,

2012) The chemically diverse nature of antimicrobial agents from plant origin provides templates for the discovery and synthesis of safe drugs that are less likely to suffer the resistance burden of modern pathogens (Gopalan *et al.,* 2011). Plants can be readily be selected based on ethnopharmacological information of use and easily sourced from their natural habitat (Cos *et al.,* 2006).

1.3 Natural antioxidants in disease modulation

The role of free radicals in the pathophysiology or aetiology of many diseases is responsible for the recent attention this chemical group of substances is receiving in drug research. The production of reactive oxygen and nitrogen species has been linked with cellular and metabolic injury, cardiovascular and degenerative diseases, cancer, ageing, diabetes mellitus, infectious diseases, neurologic, and inflammatory conditions (Peng *et al.,* 2009). Antioxidants serve as guards against oxidative damage, preserve adequate immunity and restore or maintain homeostasis (Annan and Houghton, 2008; Victor *et al.,* 2004). The commonest sources of these agents are fruits and vegetables and many phytochemicals from plants especially the phenolics and flavonoids are antioxidants (Dickson *et al.,* 2006; Zou *et al.,* 2004). It is therefore rationally sound to search for antioxidant agents from plant sources.

1.4 Safety of plant medicines

The safety of drugs is much of a concern as their efficacy. A major obstacle in orthodox pharmacology is the burden of side effects associated with orthodox medicines. Traditional herbal remedies have been purported to be safer due to their long term folkloric use without reported adverse events (WHO, 2008). Many of these herbal or traditional regimens however are polyherbal or multi-component preparations and hence the risk of toxicities resulting from their use may be consequential.

Toxicity studies are consequently essential as these may provide scientific proof or otherwise of the safety of these plants that have been in use for centuries in Ghana.

1.5 Justification of the research

The justification of this research is therefore based on the fact that although conventional medicines have been scientifically proven to be therapeutically potent, they are very expensive, sometimes inaccessible and have been credited with many of the adverse events associated with drug treatments (Houghton, 1995). A larger section of the world‟s developing population depends on systems other than orthodox healthcare for their health needs. Traditional herbal medicines have been in use as alternative remedies and have been ascribed with claims of efficacy and safety. A major drawback however of these alternative systems is the lack of existing scientific evidence to back and validate its claims of efficacy and safety (Owusu, 2009). Research into plant medicines particularly based on ethnopharmacological information is therefore a logical venture in the search for new drugs (Sannigrahi *et al.,* 2010 cited by PatrickIwuanyanwu *et al.,* 2011) as has been clearly demonstrated in the foregoing discussion.

1.6 Aim of the research

The main aim of this research work is to scientifically investigate and validate the folkloric use of the selected medicinal plants as remedies to inflammation-related diseases, anti-infectives and to evaluate their toxicity profile.

1.7 Specific Objectives

To realize the above aim, these specific objectives were set;

1. To test for the acute anti-inflammatory, antioxidant and antimicrobial activities of the 70% ethanol crude extract of selected medicinal plants.

2. To investigate *in vitro* anti-inflammatory activity of the extracts as an alternative to the *in vivo* model.
3. To establish a TLC profile of the 70% ethanol extracts.
4. To extract some phytoconstituents from the selected plant(s), isolate and characterize them.
5. To investigate the acute oral toxicity profile of the crude extracts.

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 *ETHNOPHARMACOLOGY-BASED DRUG DISCOVERY*

Reported folkloric or indigenous use of a natural product for medically related purpose sets the basis for research into such specimen. Based on the said use, the substance is identified, authenticated and characterized according to existing scientific nomenclature. Large quantities are then collected, stabilized through drying, and comminuted into fine particles for study based on folkloric guidelines (Rates, 2001). The specimen can then be extracted and tested for the relevant biological activities. Bioactivity-guided fractionation can then be employed in isolating and characterising the possible active phytoconstituents. Lead compounds obtained thereafter can then be processed for development into clinically-significant products (Evans, 2002).

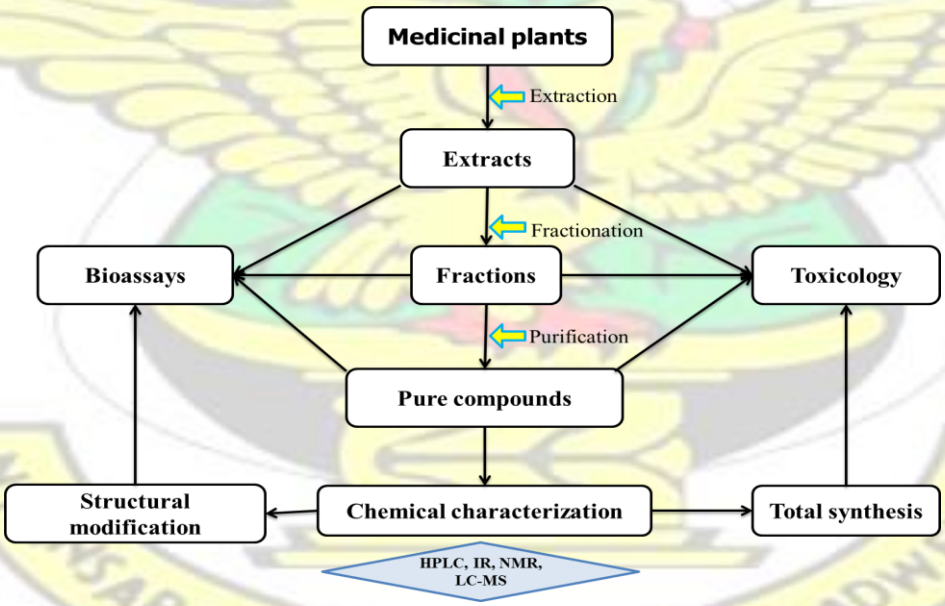


Figure 2. 1: A proposed scheme for ethnopharmacology-based drug discovery

2.1.1 ***Scientific Investigation of ethnopharmacological evidence***

The entire research process from collection through processing and storage to bioactivity studies should mimic as closely as possible, the folkloric procedure (Rates, 2001).

The test for activity usually involves a series of *in vitro* bioassays relating to the molecular biology of a disease condition. Tests for efficacy, safety and toxicity are essential in establishing the medicinal profile of that material. It has been pointed out by the Traditional Medicines Division of the WHO however that evidence of long-term traditional use of a natural product is a strong signal of efficacy and/or lack of obvious toxicity (Rates, 2001). Though *in vivo* animal models are usually crippled by ethical and financial considerations, they are the next best models for activity testing (Evans, 2002).

Pharmacologically active phytoconstituents are then isolated and characterized through bioassayguided fractionations. This is usually a difficult step in natural products research because of their complexity in composition (Smith, 1985). Specific isolation and characterization techniques including use of column chromatography, HPLC, HPTLC, TLC, LC-MS, NMR, IRS, etc., are employed in a counter-screening manner that ensures de-replication (Evans, 2002).

2.2 ***GENERAL REVIEW OF SELECTED MEDICINAL PLANTS***

2.2.1 ***Margaritaria nobilis*** **L.f. (Müll Arg.) of Euphorbiaceae**

M. nobilis (also known as *Phyllanthus nobilis* and commonly referred to as bastard hogberry and „*bunasub'* by the Konkombas in Ghana) is a fruit bearing plant native to West Indies, Central and South Americas and Mexico (Cazetta *et al.*, 2008; Aguilar and Condit, 2001). *M. nobilis* belongs to the genus *Margaritaria* which has about fourteen (14) species and is well known for anti-inflammatory activities (Marcio *et al.*, 2010).

Ethnobotanicals uses: *M. nobilis* has been used in the management of arthritis, as a wound healing agent and in treating infectious diarrhoea in Brazil (Marcio *et al.*, 2010).

Non-medicinal uses: The wood of *M. nobilis* has been harvested from the wild and used only for posts, boxes, linings, crates, toys and fuel. It has also been used for woodland reclamation in agroforestry and as an ornamental tree in landscape designs (Govaerts and Radcliffe-Smith, 1996).

Chemical constituents: The species is known to contain alkaloids, flavonoids and polyphenolics (principally, tannins). Work done by Marcio *et al.*, (2010) on the fruits of *M. nobilis* in Brazil isolated gallic acid, methyl gallate, and campherol.

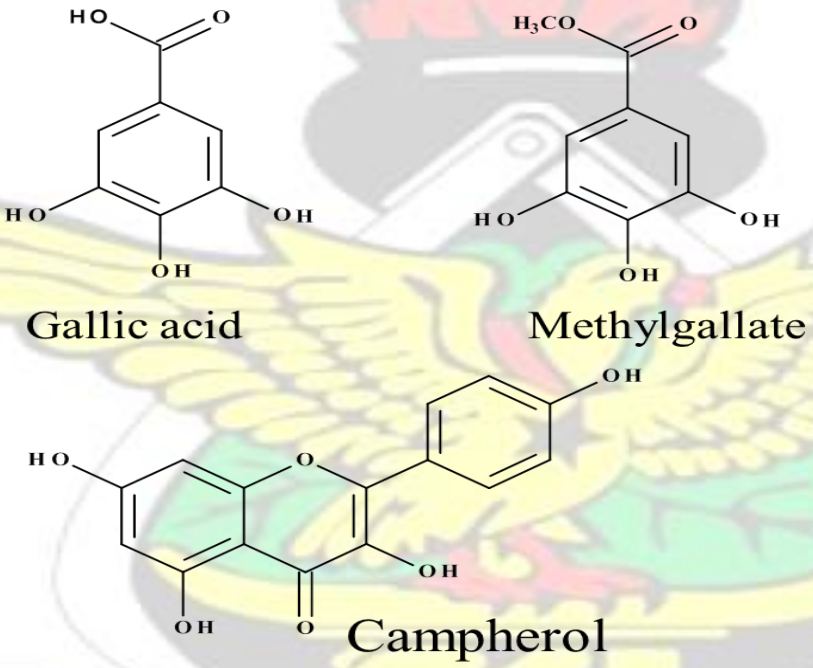


Figure 2. 2: Chemical structures of compounds isolated from the fruits of *M. nobilis*

2.2.2 *Stylochiton lancifolius* Pyer and Kotchy of Araceae

Stylochiton lancifolius is a small herb from the family Araceae with short rootstock, a native to the Savannah and wide spread in tropical Africa (Burkill, 1985). It grows well in marshy areas and very resilient to drought. It is known by the Konkombas of Ghana as „*ngbeem'*

Ethnobotanical uses: The Fulani have used decoction of its rhizome for treating inflammation, the locals of Nigeria also use its leaves for treating pain and whitlow (cellulitis) caused by bacteria. The Zulus of South Africa also treat chest pain and earache using *S. lancifolius* (Pateh *et al.,* 2011). It has been used in northern Ghana as an antimicrobial, anti-inflammatory agent and a wound healing remedy for man and domestic animals (Babu *et al.,* 2009).

Non-medicinal uses: Rhizomes have been washed to remove its bitterness and used as food during famine. It is also being used in Togo as a yellow cloth dye (Burkill, 1985). Pateh *et al.,* (2011**)** proved that *S. lancifolius* is safe for use after an acute toxicity test (LD50) was found to be greater than 5000 mg/kg.

Chemical constituents: Some species in Araceae have been reported to possess alkaloids, sapogenins, proanthocyanidins (mostly cyanidin), flavonoids especially kaempferol and quercetin (Watson and Dallwitz, 1992). Pateh *et al.,* (2009) isolated stigmasterol and β-sitosterol from the rhizomes of *S. lancifolius.*

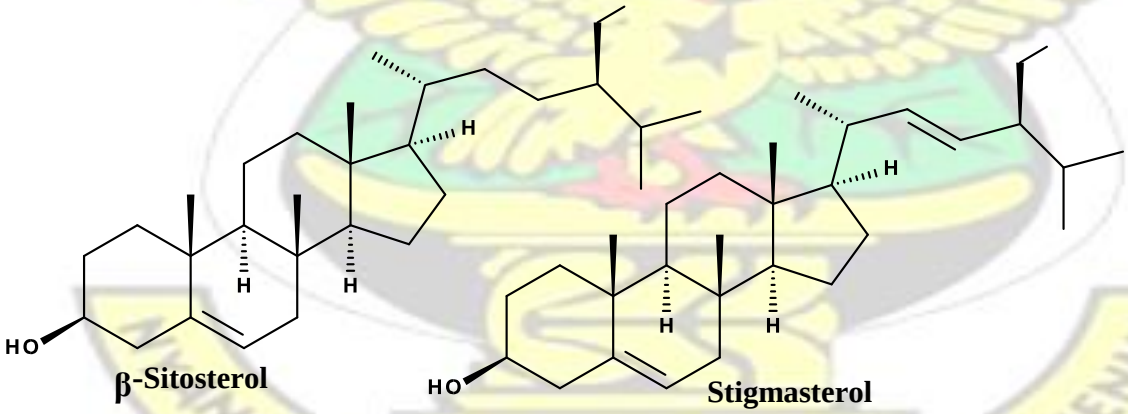


Figure 2. 3: **Chemical structures of compounds isolated from the rhizomes of *S. lancifolius***

2.2.3 Drypetes principum (Müll Arg.) Hutch of Putranjivaceae

The family Putranjivaceae has about 200 species which thrive in tropical and subtropical Africa, America, and Asia. Species in this family are known for their rich sources of alkaloids, glycosides,

phenolics, volatile oils, steroids and triterpenoid esters (Wandji *et al.*, 2000). **Ethnobotanical uses:** *D. principum* has antibacterial and antifungal properties. Other closely related species have recently been investigated and they show great antifungal, antiinflammatory, cytotoxic and antioxidant properties (Gadamsetty *et al.*, 2013).

Chemical constituents: The genus *Drypetes* is known to produce alkaloids, anthraquinones, flavonoids, phenols, sterols, triterpenes, saponins, essential oils, and coumarins (Nenkep *et al.*, 2008)**.**

2.2.4 *Crescentia cujete* Linn of Bignoniaceae

C. cujete commonly referred to as calabash tree, is native to Tropical America. It grows well in other countries including Peninsula, the South Americas and Tropical Africa. In Ghana, it is referred to as „*kiyik*" among the Konkombas of the north (Gilman and Watson, 1993).

Ethnobotanical uses: The fruit pulp of *C. cujete* is a laxative, emollient, expectorant, and an antipyretic (Kaneko *et al.*, 1997). In Trinidad, the fruits and bark are used in treating pneumonia, catarrh, tuberculosis, venereal diseases, coolness at the uterus, menstrual irregularity and as a heart tonic (Hetzel *et al.*, 1993).

Non-medicinal uses: It has been used as a lawn tree and as a buffer strip for parking lots (Arango-Ulloa *et al.*, 2009). *Crescentia cujete* is mostly cultivated as an ornamental plant but the calabashes are used as containers to hold water and by the Dagaaba of Upper West region, Ghana for musical instruments.

Chemical constituents: Iridoid glycosides; crescentins I–V, crescentosides A, B and C have been isolated from fruits of *Crescentia cujete* (Kaneko *et al.*, 1997).

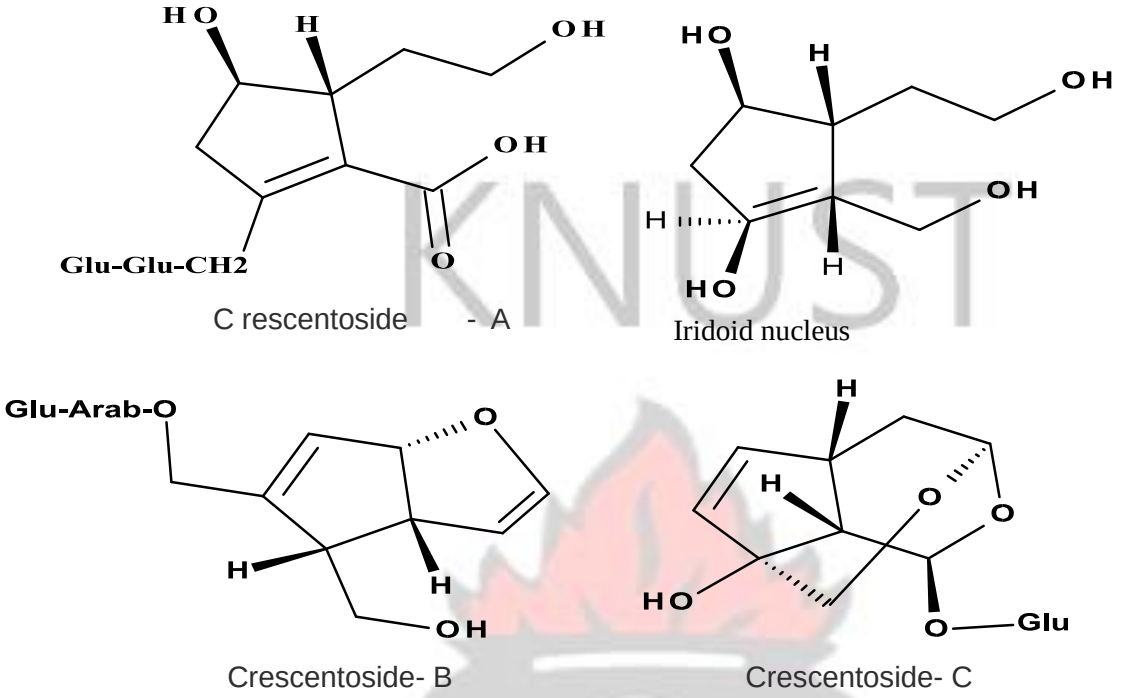


Figure 2. 4: Some iridoid glycosides isolated from *Crescentia cujete* fruits

2.2.5 *Albizia glaberrima* (Schum. & Thonn) Benth of Leguminosae

Albizia glaberrima (known in Akan-Twi as „*okuro-fi*") is a less-studied species of *Albizia;* the gum-producing genus. *A. glaberrima* is used as a styptic, as a cough suppressant and detoxifier in Northern Ghana. It has also been used by some tribes in Ghana for treating sore throat and skin-diseases (Babu *et al.,* 2009). The antifungal agent budmunchiamine A, and pithecolobine have been isolated from some *Albizia* species (Pezzuto *et al.,* 1992).

2.3 INFLAMMATION

The term inflammation has been defined by Laupattarakasem *et al.,* (2003) as the body"s dynamic natural response to a variety of hostile substances ranging from pathogenic organisms and toxic chemical agents, to physical injury to body cells and tissues. Inflammation is therefore a reaction of body cells and tissues to situations that usually invade and destabilize homeostasis in the body (Dickson *et al.,* 2012).

Inflammation has received much attention in current global scientific research because of its implication as an underlying factor in virtually all human and animal diseases (Erlinger *et al.,* 2004; Agbaje and Fageyinbo, 2012) .

2.3.1 Classification of inflammation

Inflammation is a continuous body reaction which may be short or prolonged depending on the type and presence of a trigger, or the type of physiological reaction this elicits. Inflammation has therefore been categorised into acute and chronic forms based on the duration and presentation of the tissue response (Rang *et al.,* 2007).

2.3.1.1 Acute inflammation

The body‟s initial response to invasion or tissue injury is called acute inflammation. This response is characterized by oedema and erythema which are as a result of increase leakage of plasma and leukocytes into the injury site. Platelets and polymorphonuclear leukocytes infiltration may usually be seen in this kind of inflammation (Issekutz and Issekutz, 1991**;** Owusu, 2009). Acute inflammation is short-lived, self-limiting and restores homeostasis within a short time (Ekuadzi *et al.,* 2012).

2.3.1.2 Chronic inflammation

Inflammatory conditions especially the chronic form remains one of the world‟s top health challenges (Li *et al.,* 2008). This kind is prolonged with tissue injury and repair of varying degrees occurring simultaneously together. This coexisting but continuous tissue destruction and repair is what makes such an inflammation chronic. The progression of many chronic disease conditions including atherosclerosis, Alzheimer‟s disease, cancers, diabetes and hypertension have all been attributed to their chronic inflammation component (Schmid-Schonbein *et al.,* 1981).

2.3.2 Consequences of inflammation

The expected outcome of every inflammatory response is healing with or without scar formation but alternatively, if the injurious agent persists or the body"s response goes awry, *chronic* inflammation results (Rang *et al.,* 2007).

The inflammatory response aims ultimately at (1) delivering effector molecules (mediators) to the site of injury, (2) forming an impervious barrier that limits the colonization of an invading agent and prevents its spread to adjourning body tissues, and (3) repairing the damaged cells and restoring body function (Issekutz and Issekutz, 1991)**.** This protective mechanism aims at ridding the body of the cause (invaders) and consequences (cell and tissue damage) of an injury (Serhan *et al.,* 2008).

2.3.3 Anti-inflammatory Agents in Use

Most of the therapeutically essential medicines for inflammatory disorders belong to the steroidal (glucocorticoids), NSAIDs, (Choi and Hwang, 2003) and disease-modifying anti-rheumatic drugs (DMARDs) groups (Rang *et al.,* 2007).

NSAIDs: The anti-inflammatory action of the non-steroidal anti-inflammatory drugs is mediated by the inhibition of arachidonic acid metabolites synthesis (Amaning-Danquah *et al.,* 2011). Prostaglandins (the ubiquitous arachidonate metabolites) are well known to be involved in renovascular autoregulation, gastric cytoprotection, platelets aggregation, induction of labour, etc. It therefore may be a sound deduction that NSAIDs share a similar profile of mechanismdependent side effects since their major mechanism is to prevent PGs biosynthesis (Rang *et al.,* 2007). Popular among the NSAIDs in clinical use are diclofenac, aspirin, ibuprofen, indomethacin, mefenamic acid etc.

Glucocorticoids: Steroidal anti-inflammatory drugs serve as the mainstream treatment for autoimmune diseases, and are in clinical use for many of such conditions. They are however associated with many unwanted events like delayed wound healing, fluid retention, hypertension, diabetes, mood swings, weight gain, osteoporosis, cataracts, increased intra ocular pressure, (Kyei *et al.,* 2012) and the popular Cushing"s syndrome (Boakye-Gyasi *et al.,* 2008). The glucocorticoids in use include hydrocortisone, prednisolone, triamcinolone, fluticasone, cortisone, dexamethasone etc.

2.3.4 Research models for acute inflammation

Inflammation can be induced acutely by injecting inflammatory agents like attenuated or killed bacteria, polymorphonuclear leukocytes, histamine or PAF, arachidonic acid and leukotriene B4 into an animal model (Issekutz and Issekutz, 1991). The marine gum; Carrageenan, mustard oil, zymosan, yeast and latex are also sometimes employed in inflammation induction (Meller and Gebhart, 1997). *In vitro* models like inhibition of protein (albumin) denaturation and inhibition of human RBC haemolysis have been recently developed and compare in specificity with many *in vivo* studies (Sakat *et al.,* 2010; Chandra *et al.,* 2012).

2.3.4.1 The Carrageenan-induced foot oedema in chicks

The Carrageenan-induced acute foot oedema in chicks as validated by Roach and Sufka, (2003) has been widely used among other models in screening novel steroidal and non-steroidal antiinflammatory agents (Hocking *et al.,* 2005). This is basically due to the ability of carrageenan to produce a short-lasting intense, measurable, reliable and reproducible inflammatory reaction and the high sensitivity of this to inhibition by a variety of anti-inflammatory drugs (Bharti *et al.,* 2010). This model, apart from the ease with which chicks can be handled, has also proven to be

more economical than the traditional rodents in anti-inflammatory drug research (Roach and Sufka, 2003).

Carrageenan is the best phlogistic agent to use for this kind of test because of its lack of antigenicity and apparent systemic effect (Chakraborty *et al.,* 2010). Henriques *et al.,* (1996) revealed that intra-plantar carrageenan injection into the mouse paw results in a double phase (biphasic) oedema. The first (early) phase is a 0-2 hour phase (Amaning-Danquah *et al.,* 2011) and is chiefly mediated by the release of cytoplasmic neuromodulators including serotonin, bradykinins and histamine, and increased production of prostaglandins in the damaged tissue (Agyare *et al.,* 2012). The late phase which begins 2-6 hours post injection is the longer phase characterized by sustained release of prostaglandins and mediated by bradykinins, leukotrienes, polymorphonuclear cells and prostaglandins released by tissue macrophages (Agyare *et al.,* 2012). There is also neutrophil migration and the release of proteolytic enzymes, oxygen free radicals, and other neutrophil-derived mediators (Selloum *et al.,* 2003).

A drug‟s inherent ability to inhibit carrageenan-induced oedema and subsequent inflammation has been accepted as a highly predictive indicator of anti-inflammatory activity in human inflammatory diseases (Morris *et al.,* 1989).

2.3.5 Research Models for chronic inflammation

Many chronic inflammation models exist for use in anti-inflammatory drug studies. The most frequently used of such models are those involving the induction of arthritis, especially *Mycobacterium*-induced polyarthritis in rats also known as Freund‟s adjuvant-induced arthritis (Whicher *et al.,* 1984). Killed and dried *Mycobacterium* species, muramyl dipeptide (a *Mycobacterium*-derived adjuvant); and CP20961 (a synthetic adjuvant), have all been used to

induce the polyarthritic syndrome for chronic anti-inflammatory activity studies in rats (Morris *et al.*, 1989).

Type II collagen-induced polyarthritis in mice and rats, (Trentham *et al.*, 1978), *Streptococcal* cell wall-induced polyarthritis in mice and subcutaneous cotton pellets inflammation are the most recent arthritic models for chronic inflammation.

Due to its reproducible nature and the ability to predict the activity of NSAIDs, the adjuvantinduced arthritis in rats is the most frequently and extensively used model by pharmacology researchers (Inglis *et al.*, 2007). Polyarthritic lesions in this model have been noted in other body organs like the skin, heart, urogenital tract and eyes (Mohr *et al.*, 1975) and this has accorded the model an added advantage.

2.4 INFECTIONS

Microbial infections have been a battle since the First World War and still feature on top of World Health Organisation and other health bodies‟ disease burden list. Antimicrobials are one class of drugs that have been very much researched into in drug history (Rang *et al.*, 2007).

The unmatched availability of chemical diversity in plant extracts provides templates for antimicrobial drug leads and synthesis of safe drugs that are less likely to suffer the resistance of current therapies (Cos *et al.*, 2006).

2.4.1 Antimicrobial Assays

2.4.1.1 Culture of microorganisms

Antimicrobial assays make use of drug-sensitive reference strains of common pathogens. In testing for drugs with antimicrobial properties, a battery of microbes ranging from pathogenic Gram

positive and Gram negative bacteria to pathogenic fungi are used (Cos *et al.,* 2006). The inoculum must be standardized (Zhang *et al.,* 2003) to about 1 x 10^5CFU/mL for bacteria and 1 x 10^4 CFU/mL for fungi, since this has influence on the potency of samples (Hadacek and Greger, 2000). Overnight cultures or biofreeze cultures have been recommended for use as stocks for preparing the inoculum.

2.4.1.2 Agar Well Diffusion

Agar well is a hole-punch reservoir containing known concentrations of the test sample which is created in a seeded (inoculated) agar and allowed to diffuse into the agar over several hours before incubation. The diameter of clear zones (inhibition) around the well is measured at end of incubation (Cos *et al.,* 2006). This method is most suitable technique for aqueous extracts, requires just a small amount of sample, and can test up to six extracts against one organism in a plate. Difficulties in solubility, diffusion, as well as volatility of samples make this method not universal (Hadacek and Greger, 2000). The minimum concentration that inhibits visible microbial growth (MIC) is easily read from this assay. Re-plating isolates from zones of inhibition can be used in determining the minimum bactericidal or fungicidal concentration (MBC or MFC) and whether the extracts are bacteriostatic or fungistatic (Cos *et al.,* 2006).

2.4.1.3 Broth Micro dilution Assay

This is a method in which the test compound is mixed with a previously inoculated broth. Turbidity determination using the micro-titre plate reader and redox-indicators like MTT are commonly used in measuring the activities. There is always a need for controls and sample sterility because of the interference from low sample solubility and contamination (Cos *et al.,* 2006).

2.5 FREE RADICALS AND ANTIOXIDANTS

Free radicals have been described by de Grey, (1997) in "the mitochondrial free radical theory of ageing" as molecules that bear one or more unpaired electrons**.** These unpaired electrons are of high energy, very unstable and are consequently very reactive in biological systems (Gruber *et al.*, 2008). The production of free oxygen radicals have been linked with cellular and metabolic injury, cardiovascular and degenerative diseases, cancer, ageing, diabetes mellitus, infectious diseases, neurologic and inflammatory conditions (Annan *et al.*, 2010; Peng *et al.*, 2009).

These radicals are mostly products of cellular metabolism such as mitochondrial respiration, and haemoglobin oxidation but can also be an exogenous load from food, drugs, ionizing radiation and environmental pollution (Peng *et al.*, 2009).

2.5.1 Antioxidants and disease pathophysiology

During the inflammation cascade and other pathological states, some substances serve as scavengers of free radicals generated from the inflammation reaction. These protect body cells, tissues and organs against the reactive oxygen and nitrogen species (Agyare *et al.*, 2012). Through the mopping of free radicals, they prevent cellular and genetic material damage and hence, arrest the possibility of tumours and cancers. In the presence of antioxidants, the recycling rate of waste metabolites and regulated cell generation is quite high, strengthening tissues, renewing organ physiology and retarding the ageing process. The antioxidant system is also well known to be involved in tissue repair, and relief from physical stress. Their role in immunestimulation and restoration of tissue homeostasis cannot be overemphasized (Agyare *et al.*, 2012).

2.5.2 Assay of Antioxidant Activity

Since radical generation and antioxidant mechanisms are complex, there is no single assay technique for assessing antioxidant activity that will accurately reflect the radical foundations or

all antioxidants in a complex system (Prior *et al.*, 2005). Among the frequently employed antioxidant assay methods include Total Antioxidant Capacity Assay, Reducing Power Assay, Lipid peroxidation, DPPH Radical Scavenging Assay and Total Phenol Content Assay. Many of these are frequently modified for plant drug study (Amarowicz *et al.*, 2000).

2.5.2.1 Rapid DPPH (2, 2-Diphenyl-1-picrylhydrazyl) Bioautography

This is a thin layer chromatography (TLC) coupled technique that is used in rapid screening for antioxidant activity of the components of an extract. In this assay, two TLC plates are usually developed in a solvent system and allowed to dry. One is sprayed with a detecting reagent whereas the other is sprayed with a methanolic solution of DPPH. Spots with antioxidant activity mop up the DPPH appearing as clear spots against a purple background (Gu *et al.*, 2009).

2.5.2.2 DPPH Radical Scavenging Assay

DPPH is one of the few stable free radicals that are commercially available as organic nitrogen radicals having no semblance to the highly reactive and transient peroxyl radical (Huang *et al.*, 2005). The DPPH radical scavenging assay is the most extensively used method for a more rapid evaluation of antioxidant potentials compared to other common models (Hsu *et al.*, 2008).

The principle underlying DPPH scavenging assay is a measurement of the reducing power of an antioxidant toward the DPPH radical. This can be measured by electron spin resonance or the decrease in DPPH absorbance. The stable DPPH radical loses its colour when in contact with a reductant and this is frequently determined at 517 nm on a UV/Visible light spectrometer (Prior *et al.*, 2005). DPPH gets reduced by accepting an electron or a hydrogen atom from the radical scavenger. The DPPH absorbance decreases with increasing free radical scavenging activity of the antioxidant compound.

Though the DPPH assay is simple and rapid, it is not competitive because the DPPH is both a radical probe and an oxidant. Loss in DPPH colour can be due to both radical reaction (hydrogen atom transfer or single electron transfer), as well as unrelated related reactions and steric accessibility (Prior *et al.*, 2005).

2.5.2.3 Total Antioxidant Capacity (TAC) Assay

The TAC Assay is based on the reduction of Molybdate VI (Mo^{6+}) to Molybdate V (Mo^{5+}) by an electron-accepting compound (the antioxidant) and subsequent formation of a green PhosphateMolybdate (V) complex at acidic pH. The absorbance due to the Phospho-molybdate complex is measured at 695 nm. A higher intensity means more of the complex is formed and reflects a high antioxidant power and this produces a high absorbance at 695 nm (Prieto *et al.*, 1999).

Ascorbic acid, gallic acid or α-tocopherol (standard antioxidants) is used in drawing a calibration curve from which the antioxidant equivalence of the extract can be determined (Ekuadzi, *et al.*, 2012).

2.5.2.4 Total Phenol Content (TPC) Assay

Phenols comprise the largest group of plant secondary metabolites and range from simple phenols to larger polyphenolic molecules such as flavonoids, tannins, anthraquinones, and coumarins. Most of these are potent antioxidant phytochemicals (Nabavi *et al.*, 2011) and their quantification may be reflective of the antioxidant potential of a plant extract. The Prussian blue assay as described by Price and Butler is employed with modifications. In the presence of phenols, Potassium ferrocyanate reacts with $FeCl_3$ to produce an unstable complex. Upon stabilization, this complex absorbs at 700 nm and its intensity indicates the amount of phenols in the test sample. Standard

solutions of tannic acid are used in drawing a standard curve from which the tannic acid equivalence (mg per gram) of the extract can be determined (Amponsah *et al.*, 2014).

2.6 SAFETY AND TOXICITY OF PLANT MEDICINES

The thalidomide disaster is a well-known perfect illustration of medical accidents in history and this has set the precedence for the rise in public and regulatory interests in drug safety. Though this disaster brought about heightened legislation and regulatory control, drug discovery is still pelted with issues and concerns of absolute drug safety (Smith, 1985). Extracts of natural substances and those of plant origin especially may contain several active ingredients (Evans, 2002). This multi-constituent nature is probably the most feasible reason for the wide range of therapeutic effects noted in many herbal preparations. It is henceforth necessary that toxicity testing is performed early down the drug discovery ladder so that those with unacceptable level of toxicity are recognized and pulled out of this expensive drug development chain before resources get wasted (Smith, 1985).

2.6.1 Toxicity Testing

Toxicity testing procedures have been essentially established by many bodies including the International Conference on Harmonisation for the technical requirements for manufacture of pharmaceuticals (ICH) and the Organization for Economic Cooperation and Development (OECD).

These bodies all propose animal studies before human safety testing. The correlations between animal and human safety studies are usually not accurate and predictive. It presupposes that extrapolation of animal toxicity data to human studies be done with great concern for this biovariance. Control groups (usually dosed with the vehicle) must be included in toxicity studies to impact scientific validity on the results (Smith, 1985). The commonly used animals include rats,

mice, guinea pigs, monkeys, dogs and sometimes other species like chicks and cats are used (OECD, 2001).

2.6.1.1 Acute Toxicity Studies

Acute toxicity is carried out within a period of 7 to 14 days (Smith, 1985). A limit test dose of 2000-5000 mg/kg body weight is used in the fixed dose procedure. In the study of acute oral toxicity, the limit test can be employed where the experimenter has sufficient information or data indicating that the material under study is likely to be nontoxic and or has been in use for a long time without reports of acute toxicity (OECD, 2001).

The treated animals must be closely observed and monitored at all times during the study and those with signs of suffering are removed and sacrificed immediately. Those that die during the study are also examined post-mortem for toxicological information. These animals may be sacrificed at the end of the study for haematological and histological data and the results of acute toxicity will determine if there is need to perform chronic toxicity studies or reject the drug candidate out rightly (Smith, 1985).

Parameters to observe include changes in skin, eyes, ability to feed, general appearance, weight loss (weekly), and mortality. Attention must also be given to features like salivation, tremors, diarrhoea, convulsion, sleep and coma (OECD, 2001).

2.6.1.2 Chronic Toxicity Studies

Long-term animal toxicity studies range between 12 months to animal life time studies. Chronic studies make observations on toxicities that develop with long term consumption of the drug.

Test animals receive daily dosing of the drug product and are observed daily for parameters as in acute toxicity studies. Histological and haematological investigations as well as biochemistry are performed just like in acute toxicity studies (OECD, 2001).

Chronic toxicity studies however consider the effects of the drug on fertility and its carcinogenic or teratogenic potential (Smith, 1985).

Chapter 3

3.0 MATERIALS AND METHODS

3.1 *PLANT SELECTION, COLLECTION AND AUTHENTICATION*

The five plants species used in this study were selected based on their ethnomedicinal use, especially in Ghana for the treatment of infectious diseases, inflammation and stress related diseases. Different parts of these plants including four barks and one rhizome were screened for *in vitro* antioxidant, antimicrobial, *in vitro* and *in vivo* anti-inflammatory activities.

Fresh rhizomes of *Stylochiton lancifolius* Pyer and Kotchy (Araceae) were collected from Saboba in the Northern region in July, 2013. Fresh stem barks of *Margaritaria nobilis* L.f (Müll Arg.) (Euphorbiaceae), *Drypetes principum* (Müll Arg.) Hutch (Putranjivaceae), *Crescentia cujete* Linn (Bignoniaceae), and *Albizia glaberrima* (Schum. & Thonn) Benth (Leguminosae) were collected from Kwahu-Bepong in the Eastern region of Ghana in April, 2014. These samples were identified and their botanical identities authenticated by Mr. Clifford Asare (Herbalist at Department of Herbal Medicine, KNUST). Voucher specimens were prepared and deposited at the herbarium, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST with the voucher numbers indicated in *Table 3.2* below.

Table 3. 2: Plant samples, part used and voucher specimen numbers

Plants species (Code)	**Family**	**Part used**	**Date collected**	Voucher No.
Margaritaria nobilis (**MN**)	Euphorbiaceae	Stem bark	30/04/14	*KNUST/HM1/2015/S 001*
Stylochiton lancifolius (**SL**)	Araceae	Rhizomes	12/07/13	*KNUST/HM1/2015/R 001*
Drypetes principum (**DP**)	Putranjivaceae	Stem bark	30/04/14	*KNUST/HM1/2015/S 002*

Crescentia cujete (**CC**)	Bignoniaceae	Stem bark	30/04/14	*KNUST/HM1/2015/S 003*
Albizia glaberrima (**AG**)	Leguminosae	Stem bark	30/04/14	*KNUST/HM1/2015/S 004*

3.1.1 Botanical identities of selected plants

The botanical identities of the samples were established and some of the samples were photographed as can be seen in the figure below.

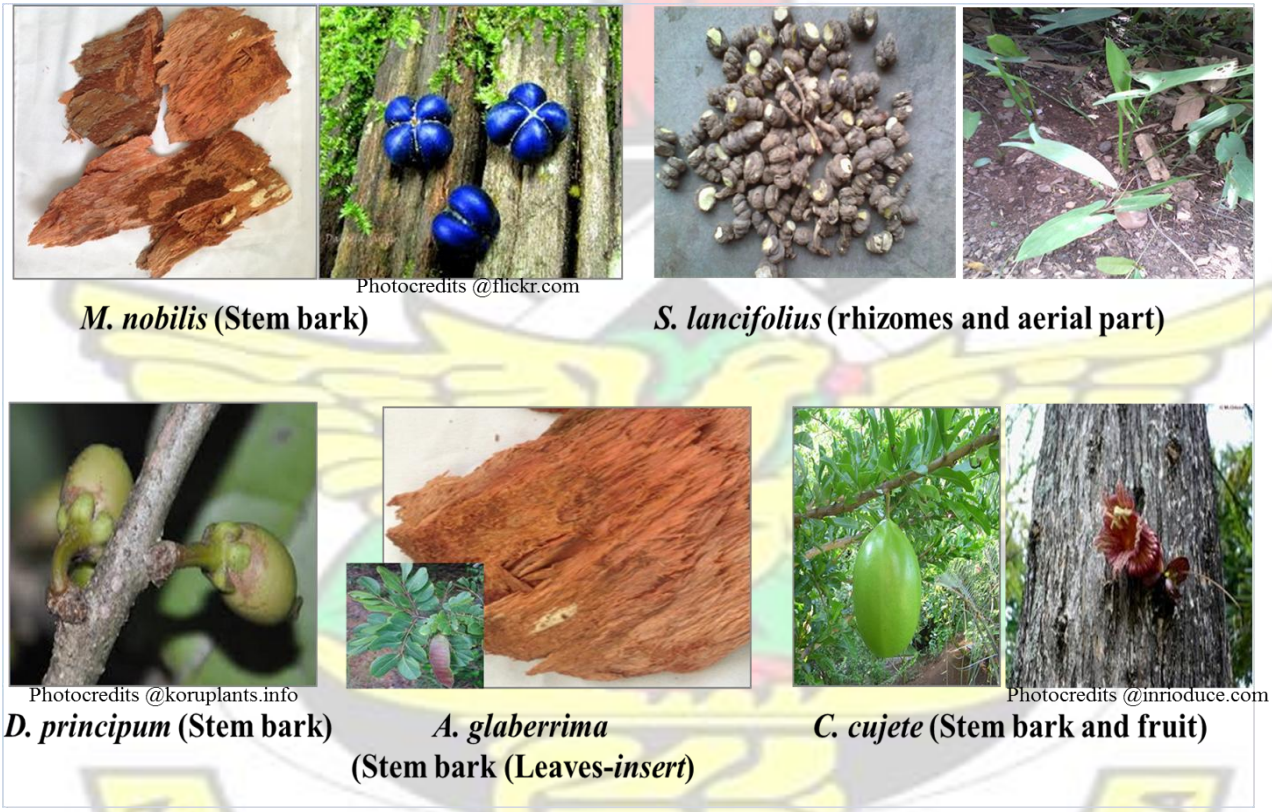


Figure 3. 1: Plants used in the study

3.1.2 Specimen Preparation for use

The plant materials were garbled, chopped into smaller pieces, shade dried for about three weeks and pulverized into a coarse powder using a hammer mill (Schutte Buffalo, New York, USA). These were stored in plastic bags in a cool dry place for use.

3.2 EXTRACTION OF PLANT MATERIALS

About 250 g of the powdered dried plant materials were soxhlet-extracted using 2.5 L of 70% ethanol at 60 ^{o}C for about 12 hrs. The resulting extracts were concentrated using a rotary evaporator (R-114, Buchi, Switzerland) at 60 ^{o}C temperature and reduced vacuum pressure. The concentrates were then oven-dried at 40 ^{o}C temperature and transferred into stoppered glass vials and stored in a desiccator until use. The percentage yield to solvent was calculated for the crude extracts and these were used for the bioactivity assays in this work.

3.3 PHYTOCHEMICAL SCREENING

The powdered plant materials and 70% ethanolic crude extracts were both screened for their constituent phytochemicals using the methods outlined by Khandelwal, (2010). The presence of alkaloids, tannins (polyphenols), reducing sugars, anthracene glycosides, free anthraquinones, steroids, flavonoids, coumarins and triterpenoids was investigated.

Alkaloids

About 0.5 g of the powdered plant material (50 mg of crude extract) was cold-extracted with 10 mL ammoniacal alcohol, filtered and evaporated to dryness in a petri dish over a water bath. The residue was extracted with 1% H_2SO_4, filtered and the filtrate rendered distinctly alkaline with dilute NH_3 solution. This was then shaken with chloroform in a separating funnel and the two immiscible layers were separated. The chloroformic layer was evaporated and the residue dissolved in 1% H_2SO_4. Five drops of Dragendorff‟s reagent was added to the extract above and the formation of orange-red precipitates, if any, was noted and recorded (Evans, 2002).

Tannins (polyphenols)

About 0.5 g of the powdered plant material (50 mg of crude extract) was boiled with 25 mL distilled water for 5 minutes, cooled and filtered. 1mL of the filtrate was diluted to 10 mL with distilled

water and 5 drops of 1% lead acetate solution added and the colour of the precipitate, if any, was noted. To another 10 mL of the diluted extract, 1% $FeCl_3$ was added first, in drops and then, in excess. The development of either blue-black, olive-green colouration or both indicates the presence of hydrolysable tannins, condensed tannins or both respectively. This was noted and recorded for the samples.

Reducing sugars and Glycosides

About 0.5 g of the powdered plant material (50 mg of crude extract) was extracted by boiling with 20 mL dilute HCl to cause hydrolysis on water bath for about 5 minutes, cooled and filtered. The filtrate was made distinctly alkaline by adding a few drops of 20% NaOH solution.

1 mL of Fehling‟s solutions A and B were successively added to the filtrate and warmed on a water bath for the formation of brick-red precipitate which if present, quantity was noted and recorded.

Free reducing sugars

To verify whether the reducing sugars were free or glycosidic sugars, the above test was modified in which distilled water was used in place of the dilute HCl with no addition of 20% caustic soda but keeping the other conditions constant.

Saponins (Frothing test)

Saponins are amphiphiles and are able to reduce surface tension of aqueous mixtures. They were tested for using the frothing test. About 50 mg of the powdered plant material (10 mg of crude extract) was shaken with 5 mL of distilled water in a test tube, filtered and the filtrate shaken rigorously and allowed to stand. The formation of a froth/foam that persists for over 10 minutes, if any, was noted and recorded (Evans, 2002).

Cyanogenic glycosides

Cyanogenic glycosides on hydrolysis yield hydrogen cyanide gas or prussic acid which can react with sodium picrate to form sodium isopurpurate. 50 mg of the powdered plant material (10 mg of crude extract) was dampened with water in a test tube. A strip of sodium picrate paper was suspended by means of a cork in the neck of the tube. This was warmed gently on a water bath and the development of a reddish-brown sodium isopurpurate colour on the paper, if any, was noted.

Anthracenosides (Borntrager's test)

Anthraquinones glycosides upon hydrolysis are more soluble in organic solvents and reacts with dilute alkali to yield red or rose-pink colorations. About 0.5 g of the powdered plant material (50mg of crude extract) was extracted by boiling with 20 mL dilute H_2SO_4 on water bath for 5 minutes and filtered while hot. The cold filtrate was partitioned with an equal volume of chloroform in a separating funnel and the chloroformic layer separated. This layer was shaken with half its volume of dilute NH_3 and the rose-pink or red colour in the upper alkaline layer, if any, was noted. Oxidative hydrolysis using 5% aqueous $FeCl_3$ was repeated for cases that yielded negative results for the *Borntrager's* test.

Free Anthraquinones

Non-glycosidic anthraquinones in an organic medium yields a rose-pink or red colour when reacted with a dilute alkali. 50 mg of the powdered plant material (10 mg of crude extract) was cold extracted by shaking with 10 mL chloroform and filtered. The filtrate was shaken with half its volume of dilute NH_3 and the rose-pink or red colour in the upper alkaline layer, if any, was noted.

Flavonoids

Aqueous extracts were prepared by boiling about 50 mg of the powdered plant material (10 mg of crude extract) in 10 mL distilled water. This was cooled and filtered. Strips of Whatman No. 4

filter papers were immersed in the extracts and allowed to dry at room temperature. The paper strips were exposed to fumes of strong NH_3 in a fume hood and the formation of an intense yellow colour was recorded. A confirmation test was carried out by exposing the yellow coloured filter paper strips to concentrated HCl fumes. The disappearance of the intense colouration validates the presence of flavonoids in the samples.

Coumarins

50 mg of the powdered plant material (10 mg of crude extract) was cold extracted with 10 mL chloroform and filtered. The filtrate was evaporated to dryness in a petri dish over water bath. The residue was dissolved in 10 mL hot distilled water and divided into two 5 mL portions A and B. About 0.5 mL of 10% NH_3 solution was added to B after cooling and the two portions were viewed under the ultraviolet light at 254 nm. The production of a light blue and intense blue fluorescence in A and B respectively indicates the presence of coumarins.

Phytosterols (Liebermann-Burchard's test)

The Lieberman-Burchard‟s test was performed to test for the presence of phytosterols. About 200 mg of the powdered plant material (10 mg of crude extract) was cold extracted by shaking with 10 mL chloroform and filtered. 2 mL acetic anhydride solution was added to the extract in a test tube and mixed thoroughly and drops of concentrated H_2SO_4 were carefully added down the side of the test tube. The formation of violet to blue colouration in the upper acetic anhydride layer indicates steroidal nucleus.

Triterpenoids (Salkowski's test)

The *Salkowski's* test which is a modification of the *Liebermann-Burchard's* test reveals the presence of triterpenoid nucleus. In this test, about 200 mg of the powdered plant material (10 mg of crude extract) was cold extracted by shaking with 10 mL chloroform and filtered. Concentrated

H_2SO_4 was carefully added drop-wise down the side of the test tube. The formation of cherry-red ring at the chloroform-sulphuric acid interface indicates a triterpenoid nucleus.

3.4 ANTI-INFLAMMATORY ASSAYS

3.4.1 Drugs and Chemicals

Analytical grade chemicals were used for the experiment. Diclofenac sodium and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium respectively whilst the Carrageenan sodium salt was purchased from Sigma Chemicals, USA.

3.4.2 In vitro anti-inflammatory activity assay

3.4.2.1 Human RBC membrane stabilization

The release of lysosomal enzymes is considered one of the most important components of the inflammation cascade (Chippada *et al.*, 2011). Lysosomal membrane stabilization is therefore important in decreasing the inflammatory response through the inhibition of the release of lysosomal content of activated neutrophils such as bactericidal enzymes and proteases

The human red blood cell has a membrane that is analogous to the lysosomal membrane (Govindappa *et al.*, 2011). The ability of a compound to stabilize RBC membranes against haemolysis (hypotonic solution or heat-induced) implies that it could stabilize lysosomal membranes preventing its lysis and hence limiting the extent of an inflammatory reaction.

3.4.2.2 Preparation of RBC suspension

A pint (480 mL) of fresh whole human blood was ethically obtained from Kumasi South Hospital and 20 mL was transferred into centrifuge tubes containing EDTA. This was centrifuged at 3000 rpm (Heraeus Biofuge Primo) for 10 minutes. The supernatant was decanted and the packed cells washed three times with equal volume of isosaline solution (0.85 %w/v NaCl, pH 7.2) (Chippada

et al., 2011). The volume of packed cells was measured and reconstituted with the isosaline solution to achieve a 10 %v/v RBC suspension which was subsequently used for the work (Sakat *et al.*, 2010; Govindappa *et al.*, 2011).

3.4.2.3 Inhibition of hypotonic solution-induced haemolysis of human RBCs

0.5 mL of different concentrations (50, 100, 250, 500 and 1000 µg/mL) of the crude extracts and reference drugs (Diclofenac and Dexamethasone) was added to 0.5 mL of the 10%v/v RBC suspension and mixed thoroughly. This was immediately added to a reagent mixture of 1 mL phosphate-buffered saline (pH 7.4, 0.15 M) and 2 ml hyposaline (0.36%w/v NaCl) solution. The resulting mixture was then incubated at 37 ^{o}C for 30 minutes and centrifuged at 3000 rpm for 10 minutes. The absorbance (haemoglobin content) of the supernatant was measured at 560 nm (Haemoglobin absorbs at 560 nm). A negative control was made using distilled water in place of the extracts or reference drugs (Chippada *et al.*, 2011). All experiments were carried out in triplicates. The inhibition of RBC haemolysis was calculated according to the equation below;

$$\%\ Inhibition\ of\ RBC\ haemolysis = ([Abc - Abx]/Abc) \times 100$$

Where Ab*c* is the absorbance of the negative control and Ab*x* is that of the different concentrations of the extracts and reference drugs. The average percentage inhibition of haemolysis was calculated and IC_{50} was determined by plotting the percentage inhibition against the concentration.

3.4.2.4 Inhibition of heat-induced haemolysis of human RBCs

A 2 mL reaction mixture was prepared from 1 mL of the crude extracts or reference drugs of various concentrations (50, 100, 250, 500 and 1000 µg/mL) and 1 mL of the 10%v/v RBC suspension. Each test tube was mixed thoroughly and incubated at 56^{o}C for 30 minutes in a water bath. The tubes were cooled under running tap and centrifuged at 2500 rpm for 5 minutes. The

absorbance of the supernatant was taken at 560 nm. A control was determined using normal saline in place of the plant extracts or reference drugs. The % RBC stabilization (inhibition of RBC haemolysis) and IC_{50} were calculated as in *section 3.4.2.3* above (Sakat *et al.*, 2010). The experiments were performed in triplicates for all test samples.

3.4.2.5 Inhibition of heat-induced albumin denaturation (Egg albumin)

Protein denaturation is a well-recognized cause of inflammatory conditions (Sakat *et al.*, 2010; Govindappa *et al.*, 2011). The ability of a compound or an extract to reduce or inhibit heatinduced protein (egg albumin) denaturation is an indication of possible anti-inflammatory activity. This was done according to the method described by Chandra *et al.*, (2012) with few modifications.

Egg albumin was prepared by gently drawing out the liquid white of fresh (lay-today) hen‟s eggs. A 5 mL reaction mixture was made by adding 0.2 mL of the fresh albumin, 2.8 mL phosphate buffered saline and 2 mL each of varying concentrations of the crude extracts and reference drugs to achieve final concentrations of 50, 100, 250, 500 and 1000 µg/mL in the test tubes.

The mixtures were incubated at 37ºC for 15 minutes and then heated at 70 ºC for 5 minutes. It was cooled under running water, vortexed and the absorbance measured at 660 nm. A control was determined using distilled water in place of the extracts. The percentage inhibition of egg albumin denaturation was calculated based on the following formula;

$$\%\ Inhibition\ of\ Albumin\ denaturation = ([Abx - Abc]/Abx) \times 100$$

Where Ab*c* is the absorbance of the negative control and Ab*x* is that of the different concentrations of the extracts and reference drugs. The experiments were performed in triplicates for all test samples and IC_{50} determined as in *section 3.4.2.3* above.

3.4.3 In vivo acute anti-inflammatory activity

3.4.3.1 Experimental Animals

About 200 cockerels (G*allus gallus;* strain: Shaver 579) day-old chicks (one-day post hatch) were purchased from Akropong Farms, Kumasi, Ghana and housed in stainless steel cages of dimensions; 34 cm x 57 cm x 40 cm at a population of 12 chicks per cage. Standard poultry feed (Chick Mash, GAFCO, Tema, Ghana) and clean water were provided *ad libitum.* Animal house temperature was maintained at about 29^{o}C and a 12-hour light-dark cycle was maintained using overhead incandescent illumination (Dickson *et al.,* 2012). Daily Cage maintenance was done and the chicks were checked daily for weight and good health until the seventh day of age when they were tested on. The cockerels were randomly grouped into 30 cages of 6 chicks each for the study**.**

3.4.3.2 Carrageenan-induced foot oedema in chicks

The anti-inflammatory potential of the 70% ethanol extracts was evaluated in the cockerels using the carrageenan-induced foot oedema model (Roach and Sufka, 2003) with little modifications by Boakye-Gyasi *et al*, (2008). Diclofenac (NSAID) and Dexamethasone (steroid) were used as reference standards. The anti-inflammatory activity was evaluated by a pre-emptive method. Dorso-ventral foot sizes were measured using the Vernier calipers

All extracts were freshly prepared by triturating with 2 %w/w tragacanth mucilage to enhance bioavailability (Owusu, 2009). The reference drugs were diluted with normal saline. The drugs and extracts were prepared in a manner that not more than 1 mL of the extract and not more than 0.5 mL of the reference drugs was administered to any chick. Groups of 6 chicks were treated with different concentrations (10, 30, 100, and 300 mg/kg body weight) of the crude extracts orally an hour before right foot subplantar injection of 100 µL of 1%w/v carrageenan in normal saline solution. The reference drugs; Diclofenac (5, 10, 30 and 100 mg/kg body weight) and

Dexamethasone (0.5, 1, 5 and 10 mg/kg body weight) were administered intraperitoneally 30 minutes before the carrageenan administration. The dorso-ventral foot thickness/sizes were measured using *Vernier* calipers at hourly intervals after the carrageenan injection up to 5 hours (Thambi *et al.,* 2006). Another group of chicks which served as the control received only the vehicle (2 $\%^{w}/_{w}$ tragacanth mucilage in distilled water) as treatment.

The experimentation was done in accordance with the National Institute of Health guidelines for care and use of laboratory animals (NIH Department of Health Services publication number: 8323, revised 1985; Committee, 2011) and was approved by the Ethics Committee, Department of

Pharmacology, College of Health Sciences of the Kwame Nkrumah University of Science and Technology.

3.4.3.3 Statistical analysis

Statistical analysis was performed by determining the percentage increase in foot size over the five hours post treatment with the extracts and drugs. This was according to the formula;

$$\%Increase\ in\ foot\ size = [(Ft - Fo)/\ Fo] \times 100$$

Where F*t* is the foot size at time-t-post treatment and F*o* is the foot size measured at time-0before the challenge. This was averaged for the various groups.

Treatment-time-course curves were drawn for the extracts and subjected to two-way (*treatment* × *time*) repeated measures of analysis of variance (ANOVA) with Newman-Keuls‟ *post hoc t* test. Total foot size for each treatment was calculated in arbitrary unit as the area under the curve (AUC) for such treatment. The percentage inhibition of oedema was determined for the treatment groups using the following formula;

$$\% \, Inhibition \, of \, oedema = \left(\frac{\mathrm{AUC}control - \mathrm{AUC}treatment}{\mathrm{AUC}control}\right) \times 100$$

Differences in AUCs were analysed by ANOVA followed by Newman-Keuls‟ *post hoc* t test. Doses for 50% of maximal effects (ED_{50}) were determined for each extract/drug using an iterative computer least squares method, with the following nonlinear regression (threeparameter logistic) equation:

$$\boldsymbol{Y = \frac{a + (b - a)}{[1 + 10(\mathrm{Log\ ED50} - x)]}}$$

Where **x** is the logarithm of dose and ***Y*** is the response. ***Y*** starts at „**a'** and goes to „**b'** yielding a sigmoid shape.

All statistical analysis were performed using GraphPad Prism for Windows version 6.0 (GraphPad Software, San Diego, CA, USA) and $P < 0.05$ was considered as statistically significant.

3.5 ANTIMICROBIAL ASSAYS

3.5.1 Microorganisms used

The antimicrobial activities of the 70% ethanol extracts were determined using nine (9) microorganisms including four Gram-Positive bacteria; *Bacillus subtilis* (NTCC10073), *Staphylococcus aureus* (ATCC25923), *Streptococcus pyogens* (Clinical strain), *Enterococcus faecalis* (ATCC29212), four Gram-Negative bacteria; *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC4853), *Salmonella typhi* (Clinical strain), *Klebsiella pneumonia* (Clinical strain), and one fungus; *Candida albicans* (Clinical strain). These microbes are the common drug-sensitive pathogens in the Ghanaian setting and hence their choice for the study.

All strains of microorganisms were ATCC, NTCC and Clinical strains obtained from the Department of Pharmaceutics (Microbiology laboratory), Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi.

3.5.2 Preparation of the inoculum

The bacteria were activated by inoculating 20 µL of the respective stock strains/isolates in single strength nutrient broth and incubated at 37 ºC for 24 hours. These culture suspensions were serially diluted with sterile water to achieve initial cell counts of approximately 1 x 10^5 CFU/mL (Elkhair *et al.*, 2010).

3.5.3 Agar well diffusion

The antimicrobial activities of the 70% ethanol extracts were first determined using the Agar well diffusion method.

200 mg of each of the dried plant extract was dissolved in 10 mL sterile water to yield a 20 mg/mL solution. This was serially diluted with sterile water to produce 10, 5 and 1 mg/mL concentrations which were used freshly for the antimicrobial assay. Solutions of Amoxicillin and Ketoconazole were used as reference drugs against the bacteria and fungus respectively. Four 6 mm wells agar wells were made aseptically with a number 6 cup borer into each petri dish of 20 mL nutrient solidified agar seeded with 20 µL suspension of the test microorganisms. The wells were filled with 100 µL of the extract solutions and allowed to diffuse at room temperature for 30 minutes. The bacteria inoculated plates were then incubated at 37 ºC for 24 hrs and the fungus at 25 ºC for 48 hrs after which the diameter of the zones of growth inhibition were measured. Triplicate determination was performed for each sample and the average zones of growth inhibition calculated.

3.5.4 Broth micro dilution assay

The micro dilution method as described by (Cos *et al.,* 2006) was employed in the determination of the minimum inhibitory concentration (MIC) of the test extracts. Stock solutions of the test

samples were prepared in sterile water to achieve in-well concentrations of 100, 250, 500, 1000, 2500, 5000, and 10000 µg/mL.

Each test well of a 96-well micro-titre plate was filled with 100 µL of double strength nutrient broth, 20 µL of the inoculum and 100 µL of stock extract solution. 200 µL double-strength nutrient broth inoculated with 20 µL of the test organisms served as the control. A column of wells was created for 100 µL of double strength nutrient broth, 10 µL sterile water and 100 µL extract to serve as the blank. The micro-titre plates were incubated as described in *section 3.5.3* above. Amoxicillin served as standard for the antibacterial activity whereas Ketoconazole served as the standard for antifungal property.

Absorbance was taken at 600 nm and the % growth inhibition was calculated as follows:

$$\% \text{ Growth Inhibition} = \frac{(Co - C1)}{Co} \times 100$$

Where; Co is the absorbance of the control and C1 is the absorbance in the presence of the test sample minus the absorbance due to the blank all at 600 nm. Triplicate determination was performed for each sample and the average percentage growth inhibition was calculated. MIC was determined by plotting the percentage growth inhibition against the extract concentration.

3.6 ANTIOXIDANT ASSAYS

3.6.1 Rapid DPPH scavenging activity (Bioautography)

Rapid scavenging of the stable DPPH radical was investigated by dissolving the crude extracts in methanol and spotting on 4 x 9 cm aluminium-backed silica pre-coated thin layer chromatography plates. These plates were resolved in appropriate solvent systems and dried using a hot air hair drier. They were viewed under UV/Visible photometer for florescence or quenching at 254 nm and 365 nm wavelengths. Two chromatograms were developed for the extracts one of which was

sprayed with the universal detecting reagent anisaldehyde to locate the separated compounds. The other plate was sprayed with a 0.2 %w/v DPPH in methanol solution.

Spots that had radical scavenging activity appeared as light yellow to clear spots against a purple DPPH background (Gu *et al.,* 2009).

3.6.2 DPPH radical scavenging assay

DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable organic nitrogen radical *2, 2-Diphenyl-1-picrylhydrazyl* (DPPH). This stable radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors; this ability was evaluated by the more frequently used decolouration assay, which evaluates the decrease in DPPH absorbance at 490-540 nm produced by the addition of the antioxidant to a DPPH solution in methanol. The DPPH radical scavenging activity of the extracts was compared to that of Vitamin C (Ascorbic acid) using the method described by (Govindappa *et al.,* 2011) with few modifications

A 0.00105% solution of DPPH in methanol was prepared and 200 µL of this solution was added to 10µL each of the test extracts and standard drug (Ascorbic acid) in methanol to achieve inwell concentrations of 25, 50, 100, 250, 500, 1000 and 2000 µg/mL in a 96-well micro-titre plate.

After 30 minutes of incubation at 37 ^{o}C, the absorbance of residual DPPH was measured at 517 nm using an ELISA plate reader. 200 µL DPPH in methanol solution added to 10 µL methanol was used as control. Triplicate determination was done for each test sample and the percentage inhibition of DPPH was determined according to the equation below:

$$\% \text{ DPPH Inhibition} = \frac{(Co - C1)}{Co} \times 100$$

Where; Co is the absorbance of the control at 517 nm and C1, the absorbance in the presence of the test sample at 517 nm. The concentration required to inhibit 50% of DPPH (IC_{50}) was determined by plotting the % DPPH inhibition against the extract concentration.

3.6.3 Estimation of Total Antioxidant Capacity by Phosphomolybdenum method

The TAC reagent solution was prepared by adding 100 mL each of 0.6 M H_2SO_4, 28 mM Disodium Phosphate and 4 mM Ammonium molybdate. 1 mL each of different concentrations (500, 250, 125 and 62.5 µg/mL) of the extracts in methanol was added to 3 mL of the reagent solution in test tubes and incubated at 95 ^oC for 90 minutes. These was allowed to cool at room temperature for 15 minutes and 250 µL were carefully filled into a 96 well micro-titre plate (modification) and the absorbance measured at 695 nm. As a negative control, a blank determination was prepared by adding 1 mL of methanol to 3 mL of the reagent solution and taken through same experimental procedures as the test extracts.

Five different concentrations of reference drug Ascorbic acid (100, 50, 25, 12.5 and 6.25 µg/mL) were prepared and used to draw a calibration curve of concentration against absorbance. The total antioxidant capacity (Ascorbic Acid Equivalence) of the extracts was then determined as the number of milligrams of Ascorbic acid per gram of the crude extracts (Amponsah *et al.*, 2014).

3.6.4 Estimation of Total Phenol Content by the Modified Prussian blue Assay

Total phenol content of the crude extracts was determined using the Price and Butler method with little modification by Graham which is known for greater colour stability.

3 mL of distilled water was added to 1 mL each of different concentrations (500, 250, 125 and 62.5 µg/mL) of the extracts in methanol in a test tube and vortexed (IKA® Vortex 1) to mix thoroughly.

1 mL of Potassium Ferrocyanate solution was added, followed immediately by the addition of 1 mL 1% $FeCl_3$ solution. The mixture was vortexed again and left to stand at room temperature for 15 minutes. After adding 5 mL of a stabilizer (prepared by mixing 40 mL of 1 $\%^{w}/_{v}$ gum Arabic, 40 mL of 85% H_3PO_4 and 120 mL distilled water), the mixture was vortexed and the absorbance measured at 700 nm. Similar concentrations of tannic acid were prepared and used in drawing a calibration curve of concentration against absorbance from which the Tannic Acid Equivalent of the extracts was derived. The TPC was then determined as the number of milligrams of Tannic acid per gram of the crude extracts (Amponsah *et al*., 2014).

3.7 ACUTE ORAL TOXICITY STUDIES

Acute oral toxicity serves as a premise on which chronic toxicity studies are designed. Since this was an observational study, only the acute oral toxicity of the plant extracts was investigated. A limit test dose of 2000 mg/kg body weight using the fixed dose procedure of the Organization for Economic Cooperation Development (OECD, 2001) guidelines was used in the acute toxicity studies.

Six (6) groups of five (5) randomly selected healthy animals were weighed and denied only food for about 4 hrs. A limit dose of 2000 mg/kg body weight of each extract was then administered orally and the animals were allowed to feed 3 hrs post-dosing.

The treated animals were observed at 30 minutes and periodically during the first 24hrs for signs and symptoms of overt toxicity of the extracts. They were then observed daily for a period of two weeks (14 days) post treatment.

Parameters observed included changes in skin, fur, eyes, ability to feed, general appearance, weight loss (weekly), and death of the rats. Attention was also given to observation of salivation, tremors,

diarrhoea, convulsion, sleep and coma (OECD, 2001). No haematological or biochemical indices were measured in this case.

3.8 EXTRACTION AND ISOLATION OF COMPOUNDS

3.8.1 Extraction

About 3 kg of each dry powdered sample was weighed and soxhlet-extracted successively with 5 L each of Petroleum ether, Ethyl acetate and 70 $\%^{v}/_{v}$ Ethanol. The extracts obtained were evaporated to dryness using a rotary evaporator (R-114, Buchi, Switzerland) at 60 ^{o}C temperature and reduced vacuum pressure. These semi-solid extracts were completely dried in an oven at 45 ^{o}C for 12 hrs and were kept in air-tight glass vials in a desiccator until needed for work.

3.8.2 Chromatographic materials and techniques

The following types of silica gel were used for column and thin layer chromatography.

- Silica gel 60 (70-150 mesh, Alfa Aesar, Bond) for column chromatography,
- Aluminium TLC plates pre-coated with silica gel 60 F_{254}, 0.25 mm thickness.

3.8.2.1 Chromatographic solvents and reagents

The following solvents were used for the extraction, column chromatography and TLC analysis of the isolates:

a) Petroleum ether (40-60 ^{o}C), Ethyl acetate, Methanol, Chloroform, and Ethanol. All solvents were analytical grade and were obtained from Fissons Company Ltd., May & Baker Ltd., Philip Harris Ltd. and Shestone, all in the United Kingdom, and Merck in Germany.

b) 0.5% Anisaldehyde in Acetic acid: concentrated H_2SO_4: Methanol (10:5:84.5) mixture, Iodine vapour and 0.2% $^{w}/_{v}$ DPPH were used as the detecting reagents for the thin layer chromatography analysis.

3.8.2.2 Techniques

3.8.2.2.1 Thin Layer Chromatography (TLC) of 70% ethanol extracts

About 50 mg of each powdered 70% EtOH extract was dissolved in glass vials containing 10 mL methanol. The reconstituted extracts were applied as spots using capillary tubes on a 5 × 10 cm aluminium-backed pre-coated silica plates (60 F_{254} of 0.25 mm thickness). The spots were applied in a horizontal fashion 2 cm from one end, 1.5 cm from the margin of the plate and 1 cm apart. Each extract was applied as two (2) separate spots per plate.

The spots were allowed to dry and where necessary, a second application was made. The plates were then developed by one-way ascending technique in a chromatank saturated for about 30 minutes with 4:1, petroleum ether: Ethyl acetate solvent system.

After the mobile phase reached a running height of 7 cm, the plates were removed and air-dried. They were observed under a UV/Visible fluorimeter (254 and 365 nm) and later sprayed with the anisaldehyde reagent mixture. To aid chromogenesis, the plates were heated for about 5 minutes using a hand-held hair drier and observed in visible light. The spots were marked out and their retardation factors (R_f) calculated according the following relation;

R_f = Distance travelled by spot/solvent running distance

3.8.2.2.2 Open column chromatography of 70% ethanol extracts of Margaritaria nobilis The results of the biological activities showed that the extract of *M. nobilis* was generally active than the other four and hence it was chosen for chromatographic fractionation and isolation. About 150 g of the 70 % EtOH extract of *M. nobilis* was weighed and dissolved in 100 mL methanol. This was uniformly mixed with 70 g of silica gel 60 (70-150 mesh, Alfa Aesar, Bond) and allowed to dry to attain same consistency as the silica to be packed. A 90 x 4 cm column was packed with

(270 g) silica gel 60 (70-150 mesh, Alfa Aesar, Bond) to three-quarters (¾th) its height. The extract-adsorbed silica was then packed to one-third ($^{1}/_{3}^{rd}$) the height above the packed silica and a wad of cotton wool spread gently to prevent disturbances as the eluent is been added.

The column was firmly clamped and eluted with a gradient solvent system starting from petroleum ether to ethyl acetate and then methanol. Aliquots were collected based on colour of observed bands and in volumes of 100-150 mL.

3.8.2.2.3 Thin Layer Chromatography (TLC) and Bulking of Fractions

This method was used to compare the various fractions, to know and bulk those that were similar in composition. The fractions to be analysed by TLC were constituted in organic solvents and were chromatographed as described in *Section 3.8.2.2.1* above. The colour and fluorescence characteristics of the spots were noted and this was carried out on each of the aliquots. Those aliquots with similar TLC profiles were bulked together resulting in seven main fractions which were concentrated using the rotary evaporator, air-dried and labelled as fractions **EMN-***A* to **EMN-***G*.

3.8.2.3 Purification of Fraction EMN-C

Fraction **EMN-***C* eluted at a solvent composition of pet ether: EtOAc (60:40) and within aliquots 19 to 30**.** A whitish substance precipitated at the tip of the column just above the collecting vessel and within the aliquots collected. These aliquots were bulked, concentrated using the rotary evaporator, and the supernatant was gently decanted into a clean glass vial. The precipitate was washed severally with 100% petroleum ether, dried immediately at room temperature, labelled as compound EMN1 and refrigerated in a stoppered glass vial. The supernatant was also evaporated and the multicomponent-residue labelled as EMN2 was refrigerated in glass beakers.

TLC was performed on EMN1 by dissolving it in methanol and developing in 80: 20, pet ether:

EtOAc solvent system and sprayed with the anisaldehyde reagent.

3.8.2.4 Characterisation of EMN1

EMN1 is a white amorphous solid powder weighing up to 395 mg with a characteristic odour. It yielded a single spot which fluoresced (light blue) at 365 nm and quenched the TLC fluorescence at 254 nm. It had a melting point of 296-297°C. **EMN1** produced a single spot each time TLC was run using 5 different solvent systems.

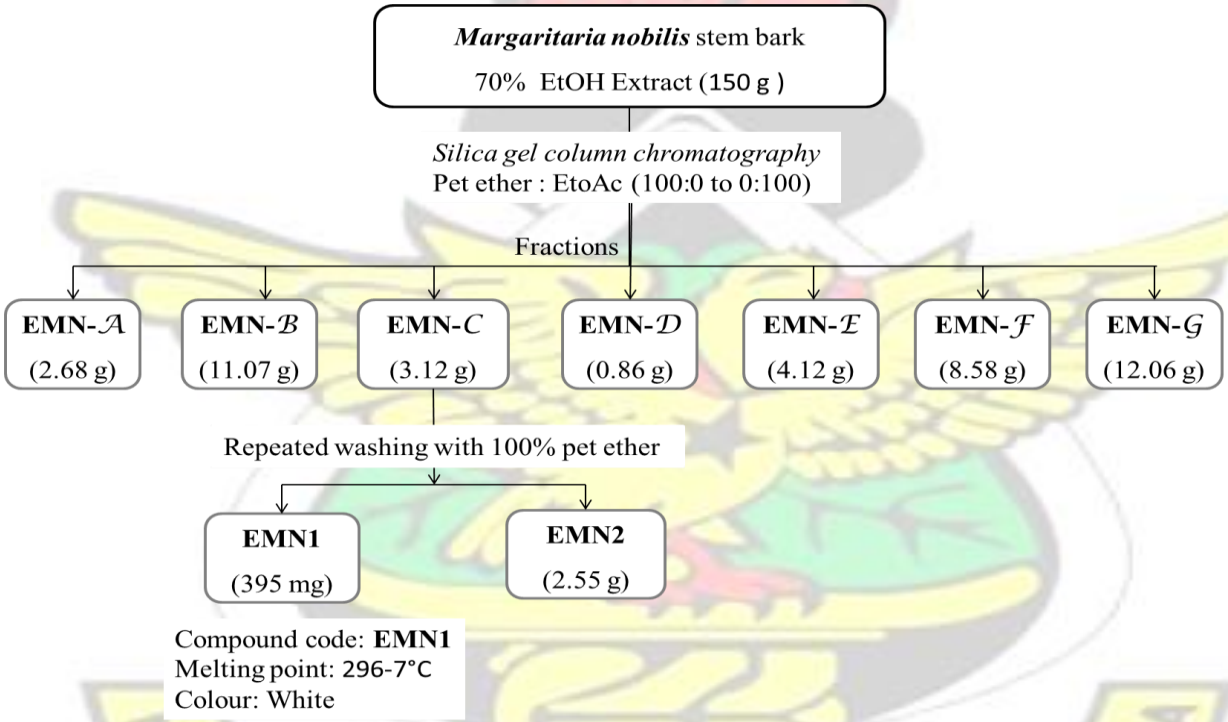


Figure 3.2: A scheme for isolation and characterisation of compounds from 70% EtOH extract of *M. nobilis* stem bark

3.8.2.5 Structure elucidation of compound EMN1 as Betulinic acid

About 25 mg of **EMN1** was weighed and sent via DHL to the Pharmacognosy Research Laboratory of the University of Greenwich, (UK) for structure elucidation.

EMN1 was analysed at 29 ^{o}C using a Bruker 500 mHz Advance III HD NMR spectrometer equipped with a 1.7 mm TXI cryoprobe and a 6890 N Gas Chromatograph with a 5973 Mass Selective Detector. The following experiments were performed and the spectra and graphs obtained; ^{1}H (proton) nmr, ^{13}C nmr, HSQC, COSY and HMBC.

Chapter 4

4.0 RESULTS AND DISCUSSION

4.1 *EXTRACTIVE YIELD*

The powdered plant samples were extracted first with 70% EtOH for preliminary bioassays and later, an exhaustive serial extraction with petroleum ether, EtOAc and 70% EtOH was carried out. In the preliminary 70% EtOH extraction, *M. nobilis* stem bark produced the highest yield of 10.19 $\%^{w}/_{w}$ followed by *A. glaberrima* stem bark which yielded 7.36 $\%^{w}/_{w}$ and the least extraction was observed with the *C. cujete* stem bark (1.45 $\%^{w}/_{w}$)

The results of the successive exhaustive extraction in *Table 4.1* are indicative that the samples under study may have more polar phytoconstituents. Each specimen had a higher yield for 70% EtOH, followed by EtOAc and then petroleum ether even though the extraction process was the reverse. *M. nobilis* still yielded the highest extractive value of 9.85 $\%^{w}/_{w}$ in 70% EtOH whereas *D. principum* had the least extractive of 0.66 $\%^{w}/_{w}$ in petroleum ether.

Table 4.1: Extractive yield of 70% EtOH extract and successive extraction of samples

SAMPLE	**70% EtOH crude**	**Successive exhaustive extraction ($\%^{w}/_{w}$)**		
	($\%^{w}/_{w}$)	**Pet Ether**	**EtOAc**	**70% EtOH**
M. nobilis stem bark	10.19	2.13	3.45	9.85
S. lancifolius rhizomes	1.92	0.75	0.92	1.60
D. principum stem bark	1.80	0.66	0.95	1.46
C. cujete stem bark	1.45	0.74	1.20	1.35
A. glaberrima stem bark	7.36	1.10	1.12	6.79

4.2 *PHYTOCHEMICAL SCREENING*

Phytochemical examination serves as a guide in isolation, chemical characterization and predicting biological activity of a plant drug (Kennedy and Thorley, 2000). Phytochemical analysis of the study samples revealed the presence of the following groups of phytochemicals indicated in *Table 4.2* below. All the five samples however tested negative for the presence of free anthraquinones, free reducing sugars, coumarins and flavonoids.

Table 4.2: Results of phytochemical screening of powdered samples & 70% EtOH extracts

Phytoconstituents	**MN**	***MN**	**SL**	***SL**	**DP**	***DP**	**CC**	***CC**	**AG**	***AG**
Alkaloids	+	+	+	+	+	+	+	+	+	+
Reducing sugars (Glycosides)	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	+	+
Anthracenosides	+	+	+	+	+	+	-	-	-	-
Cyanogenic glycosides	+	-	+	-	-	-	-	-	+	+
Tannins	+	+	+	+	+	+	+	+	+	+
Triterpenes	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+	+

+ = present, - = absent, * 70% EtOH extract of respective samples.

4.3 *ANTI-INFLAMMATORY ACTIVITY*

Preliminary *in vitro* and *in vivo* bioassay of the 70% EtOH extracts revealed that the study samples had some level of anti-inflammatory activity.

4.3.1 In vitro Anti-inflammatory Activity

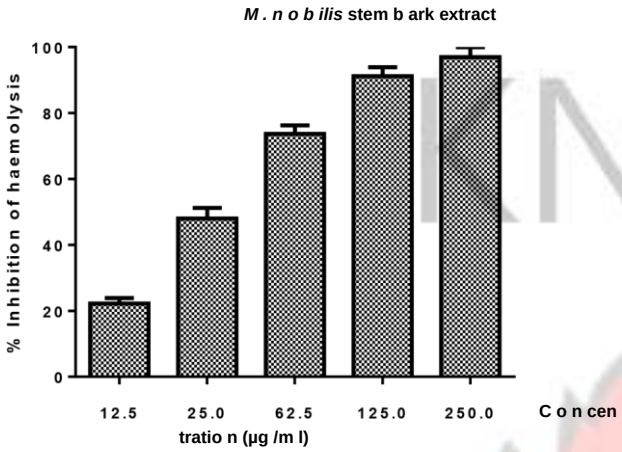
4.3.1.1 Human RBC membrane and Albumin stabilization

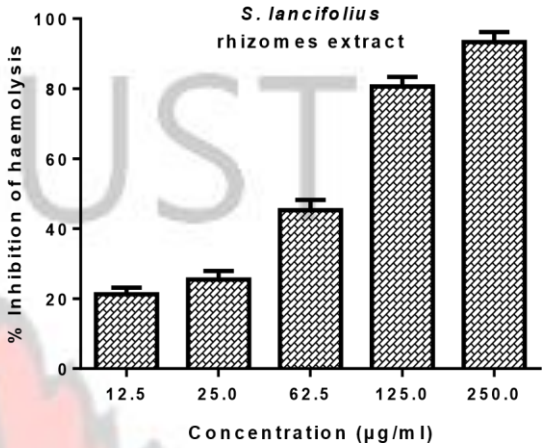
Results in *Table 4.4* below indicate that the plant extracts were somehow consistent in terms of the *in vitro* anti-inflammatory profile. In the inhibition of heat-induced and hypotonic solutioninduced human RBC haemolysis, the extract of *M. nobilis*, *S. lancifolius* and *C. cujete* had IC_{50} values similar or even lower than the reference drugs (Diclofenac and Dexamethasone). *A. glaberrima* produced a higher IC_{50} values in both tests while *D. principum* could not be evaluated at all due to the fact that it results indicated a potentiation of haemolysis rather than stabilization of RBCs. This observation could be attributed to the fact that it contains a lot of saponins which induced the RBC haemolysis.

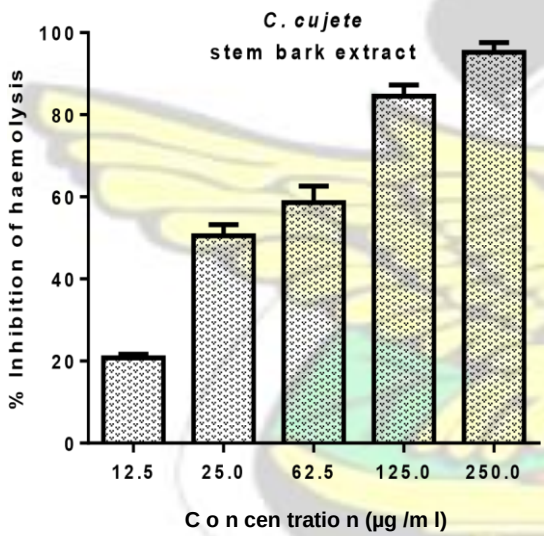
Stabilization of fresh egg albumin against heat-induced protein denaturation also indicated pronounced *in vitro* anti-inflammatory activity. The extracts of *C. cujete* and *M. nobilis* with IC_{50} values of 5.62 ± 0.33 and 12.51 ± 0.06 µg/mL respectively were more active than the reference drug Diclofenac but less active than Dexamethasone (IC_{50} values of 14.82 ± 0.35 and 1.31 ± 0.06 µg/mL respectively). The extracts of *D. principum*, *A. glaberrima* and *S. lancifolius* respectively had IC_{50} values of 59.45 ± 0.08, 67.24 ± 0.87, and 89.21 ± 0.87 µg/mL.

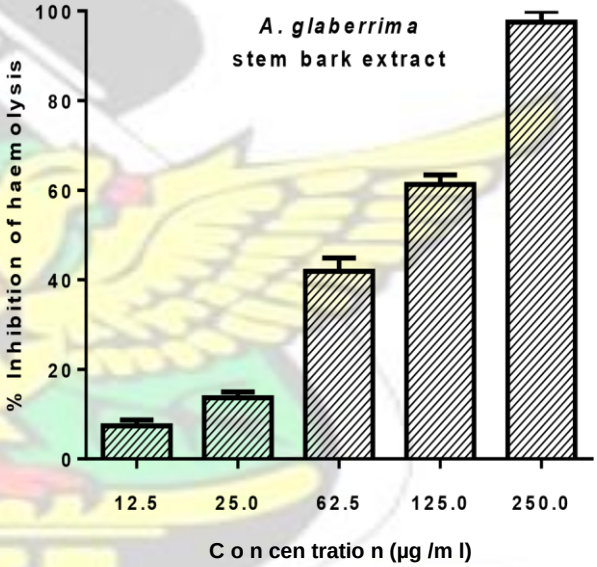
These *in vitro* assays became a basis on which the ensuing *in vivo* assay was carried out.

4.3.1.1.1 Inhibition of Hypotonic solution-induced human RBC haemolysis









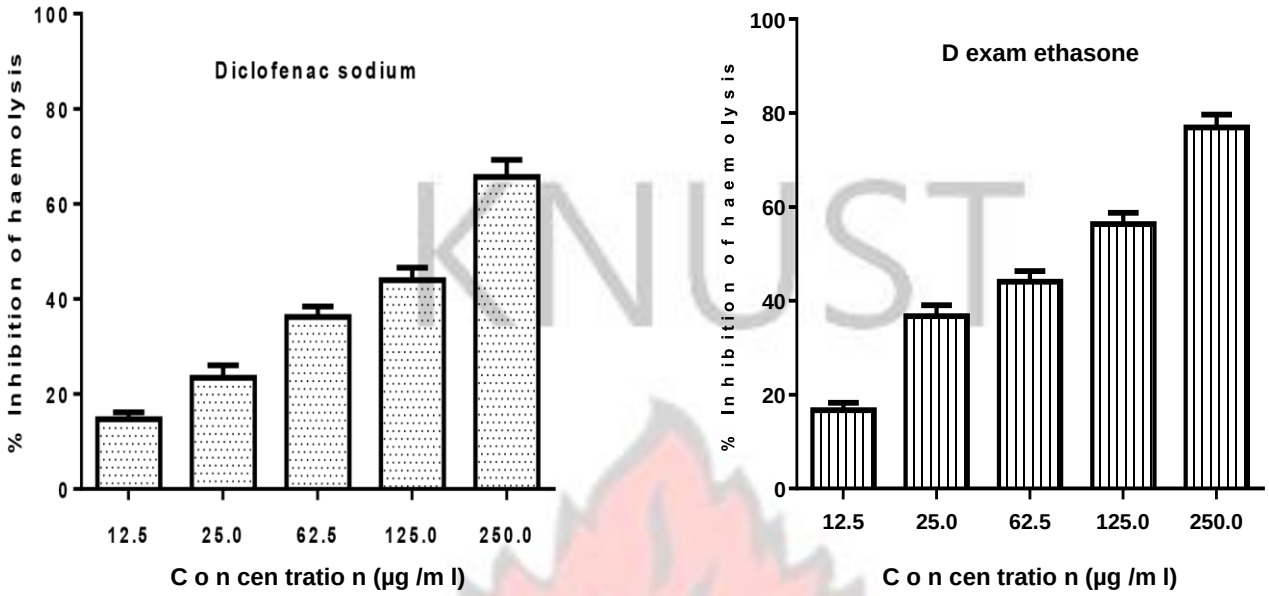
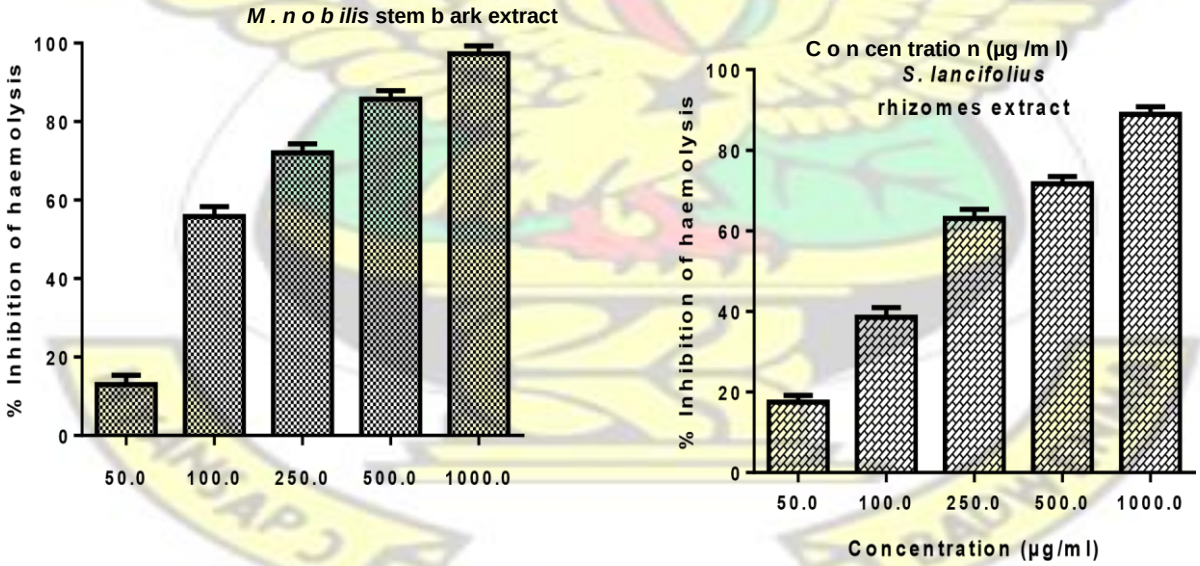
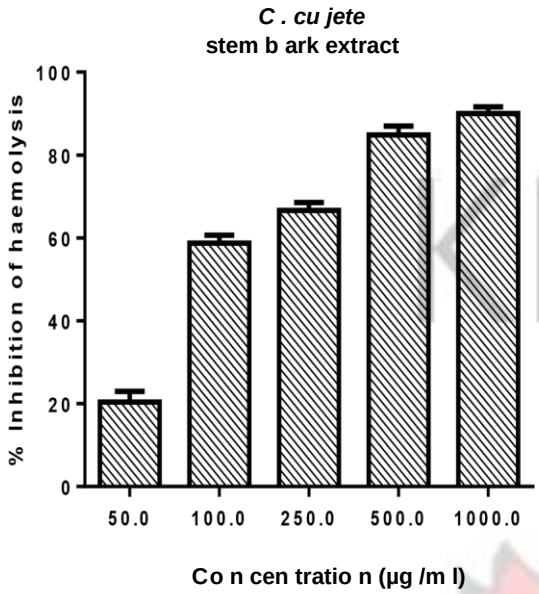
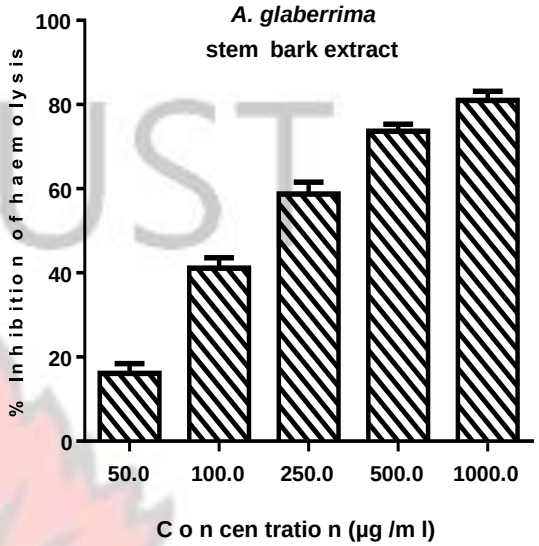


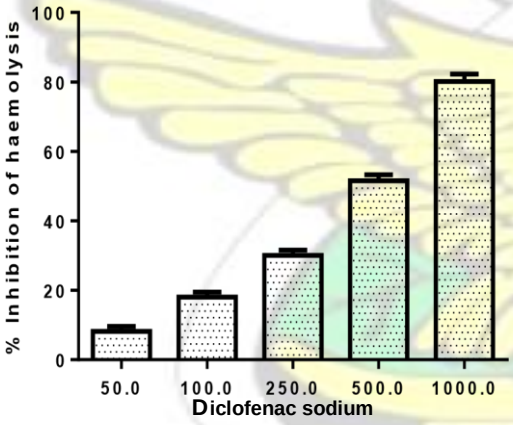
Figure 4.1: Percentage inhibition of hypotonic solution-induced haemolysis against concentrations of the test samples and reference drugs

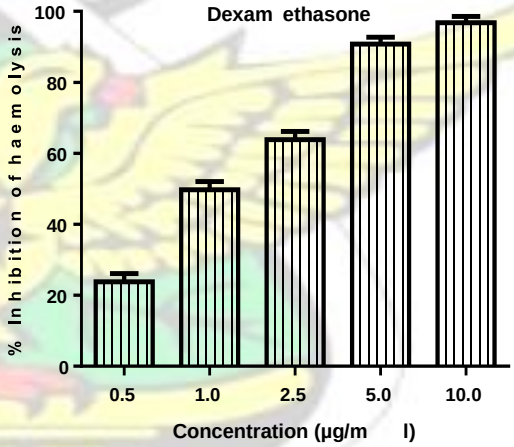
4.3.1.1.2 Inhibition of heat-induced albumin denaturation (Egg albumin)











C o n cen tratio n (µg /m l)

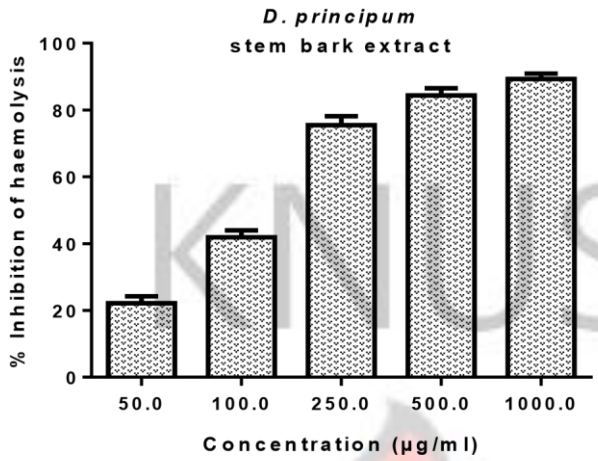


Figure 4. 2: Percentage inhibition of heat-induced albumin denaturation against concentrations of the test samples and reference drugs

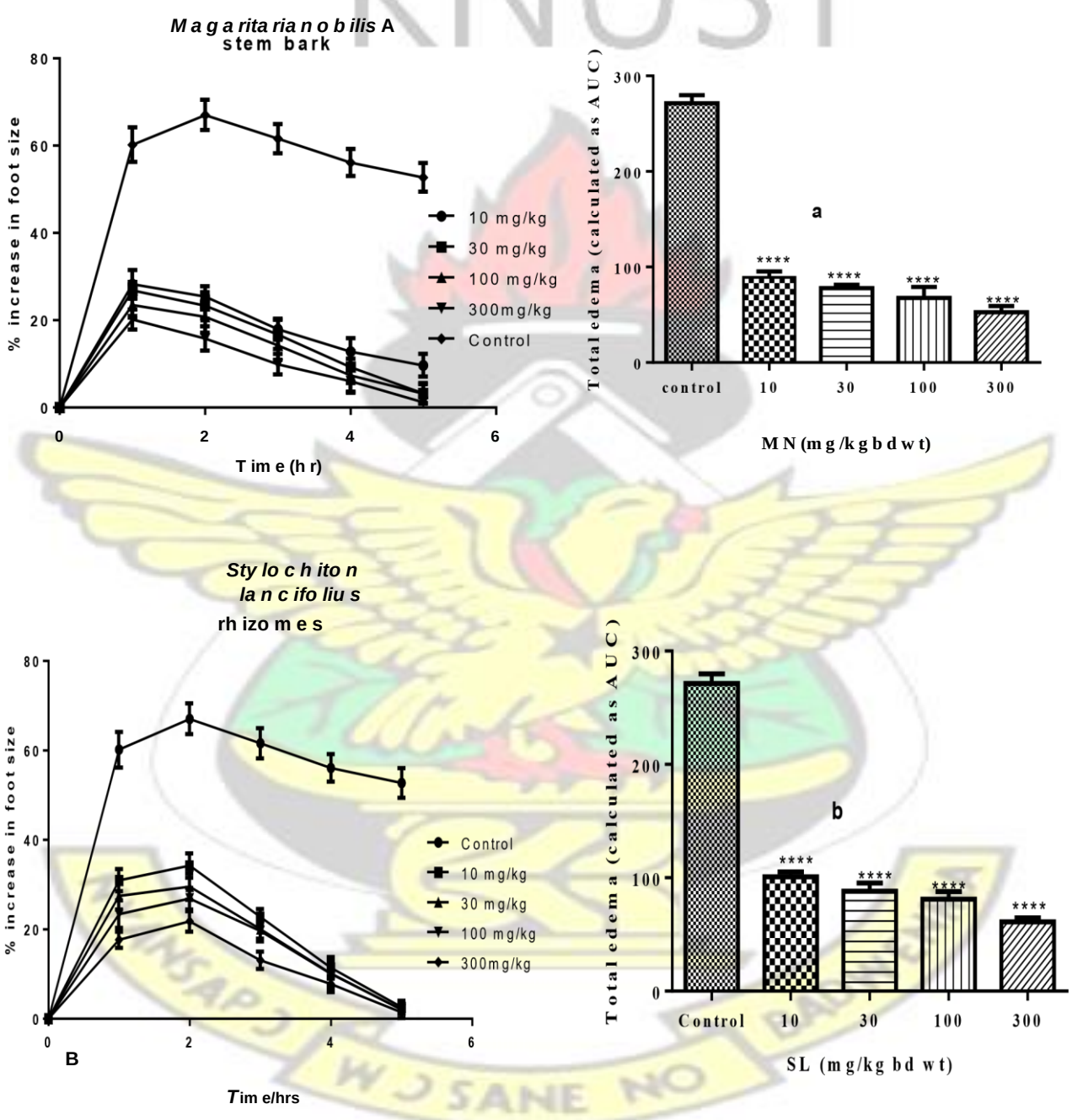
4.3.2 In vivo Anti-inflammatory Activity

4.3.2.1 Inhibition of carrageenan-induced pedal oedema in chicks

The results of the *in vivo* anti-inflammatory assay indicated that the 70% EtOH extracts of the five (5) selected medicinal plants (administered p.o.) and the reference drugs (administered i.p.) significantly ($p < 0.0001$) and dose-dependently inhibited carrageenan-induced oedema in chicks at all doses. The time course curves in *Figure* 4.3 and the total oedema (AUC) in *Table 4.4* reflect the ability of each extract to influence the course of the oedema initiated by carrageenan injection. CC was the most active of all the extracts with an ED_{50} value of 23.30 ± 0.43 mg/kg body weight followed by DP, MN, AG and SL with ED_{50} values of 37.25 ± 1.22, 90.54 ± 0.73, 164.90 ± 1.63, and 181.50 ± 1.06 mg/kg body weight respectively.

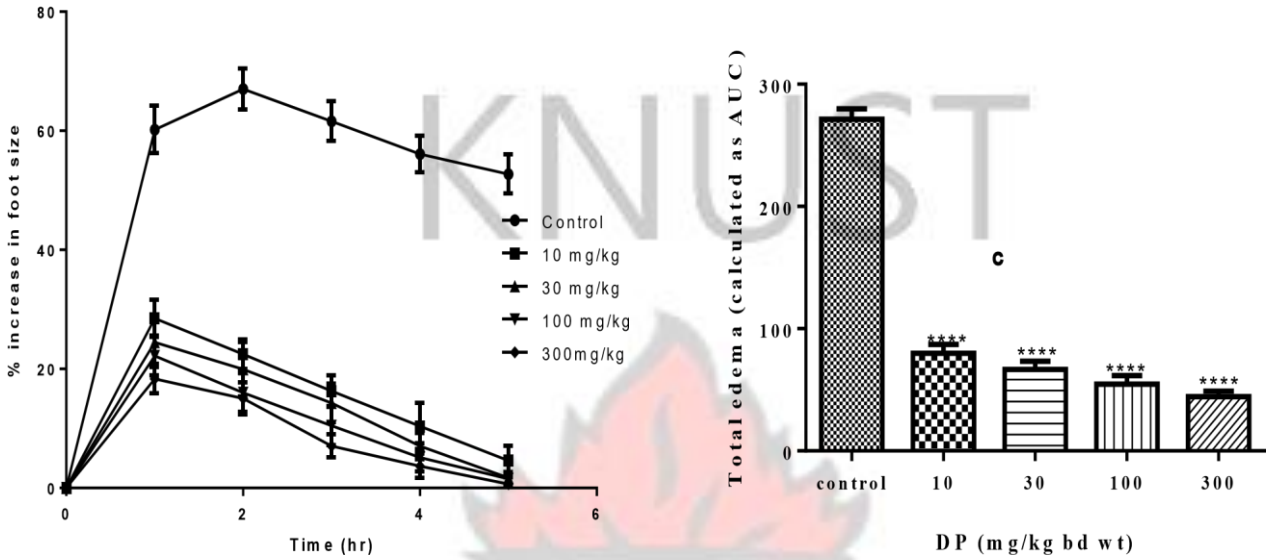
The percentage inhibition of total oedema of all the extracts was comparable to reference drugs; Diclofenac, Dexamethasone and the compound isolated from MN (**EMN1)**. At doses of 300 mg/kg body weight, DP, CC, MN, AG and SL respectively inhibited total oedema at 83.59 ± 1.21, 82.44 ± 2.01, 80.62 ± 1.23, 79.21 ± 3.90, and 77.49 ± 1.88% compared with Dexamethasone, Diclofenac and **EMN1** which inhibited oedema at 82.47 ± 1.76, 81.62 ± 2.12, and 67.83 ± 1.23%

(doses of 10, 30, and 100 mg/kg body weight) respectively. This is shown in *Figure 4.3* and *Table 4.3* below.



D ry p e te s p rin c ip u m
stem b ark

C



C re s
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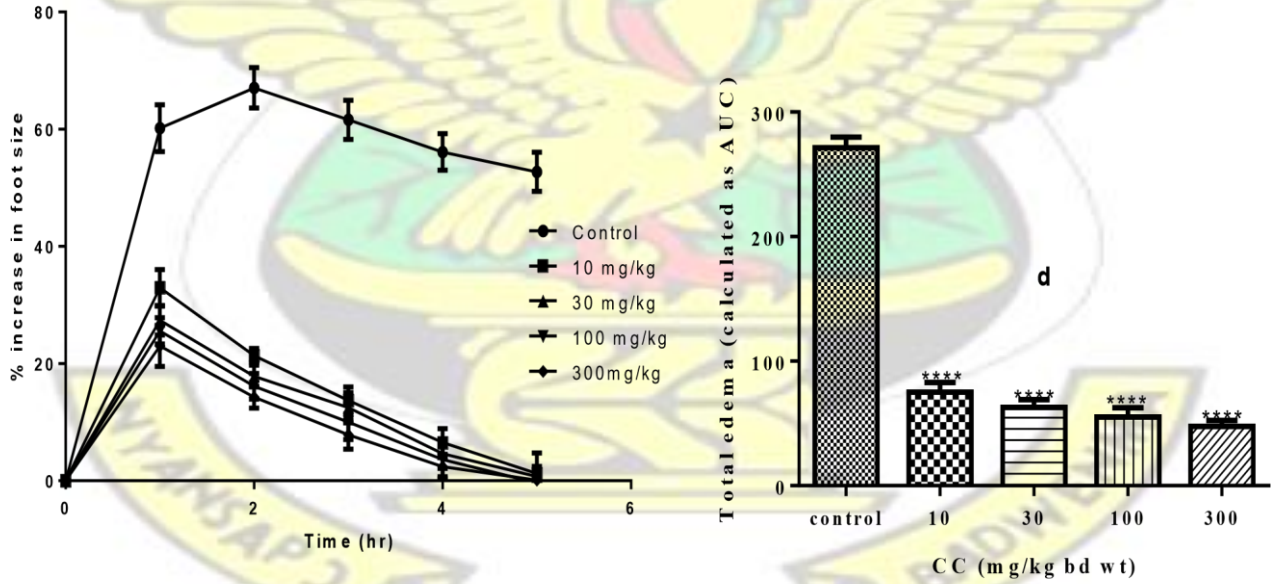

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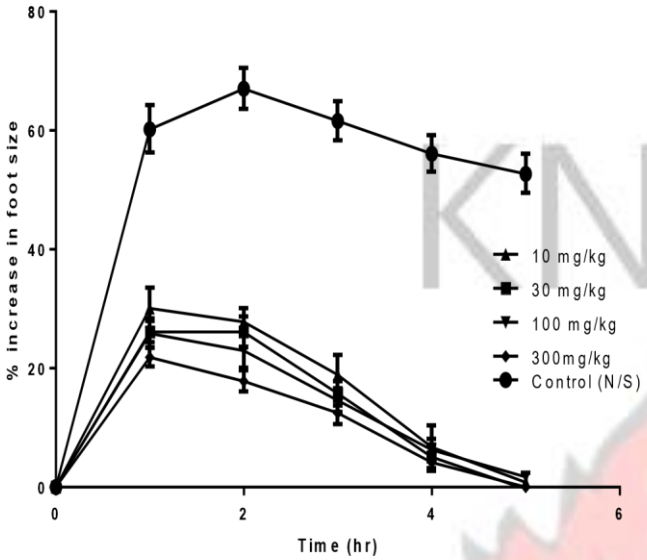

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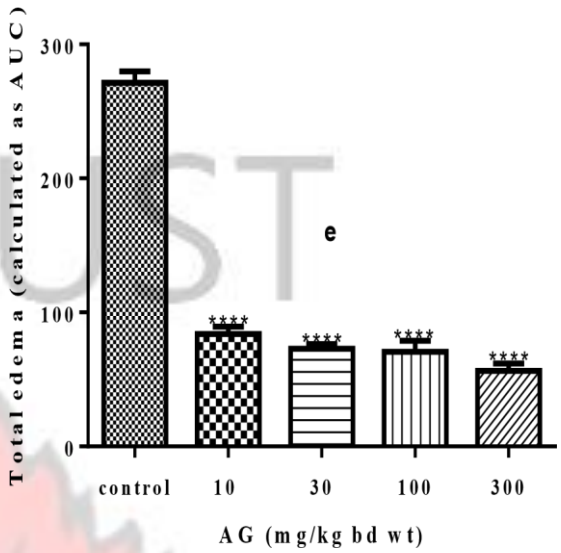

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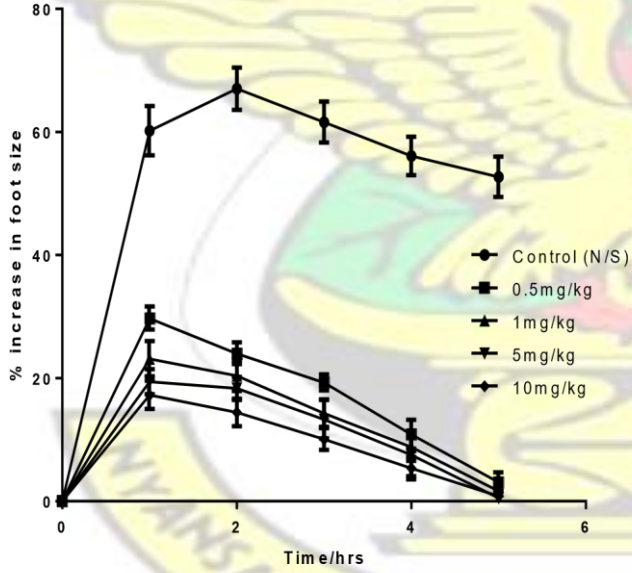

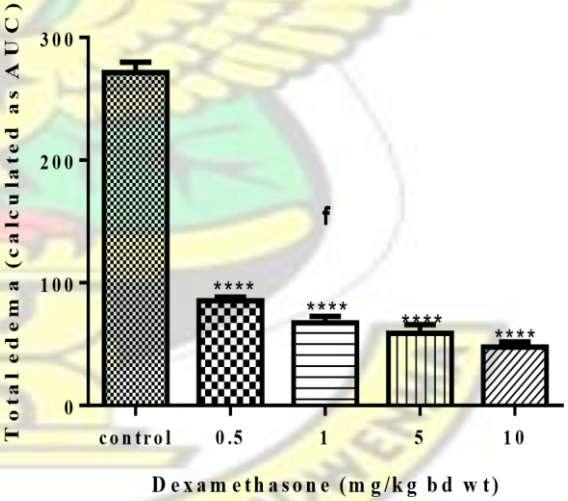

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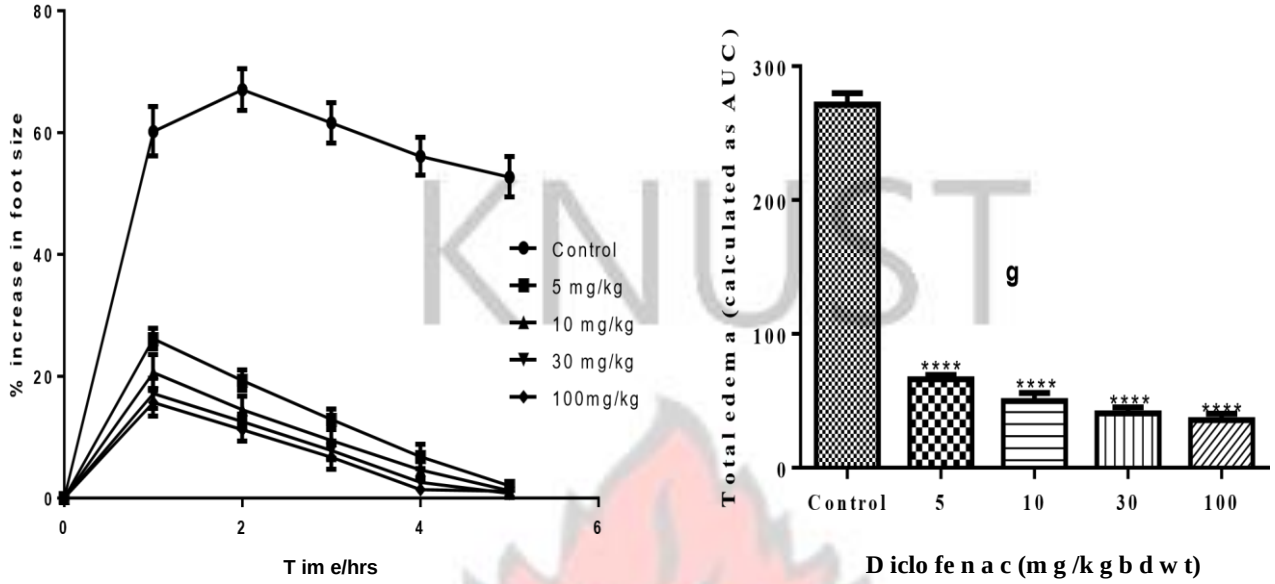


Figure 4.3: Effect of diclofenac (5 – 100 mg/kg; i.p.), dexamethasone (0.5 – 10 mg/kg; i.p.), and extract (10 – 300 mg/kg; p.o) on time course curve (A, B, C, D, E, F, and G) and the total oedema response (a, b, c, d, e, f, and g respectively) in carrageenan-induced oedema in chicks. Values are means ± SEM. (n =6). ****P < 0.0001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls‟ *post hoc t* test)

Table 4.3: The percentage inhibition of carrageenan-induced pedal oedema in 7 days-old chicks

Drug/Extract	Dose (mg/kg BDW)	% Inhibition of Oedema
***M. nobilis* stem bark**	300	80.62 ± 1.23
***S. lancifolius* rhizomes**	300	77.49 ± 1.88
***D. principum* stem bark**	300	83.59 ± 1.21
***C. cujete* stem bark**	300	82.44 ± 2.01
***A. glaberrima* stem bark**	300	79.21 ± 3.90
Diclofenac	30	81.62 ± 2.12
Dexamethasone	10	82.47 ± 1.76
EMN1	100	67.83 ± 1.23

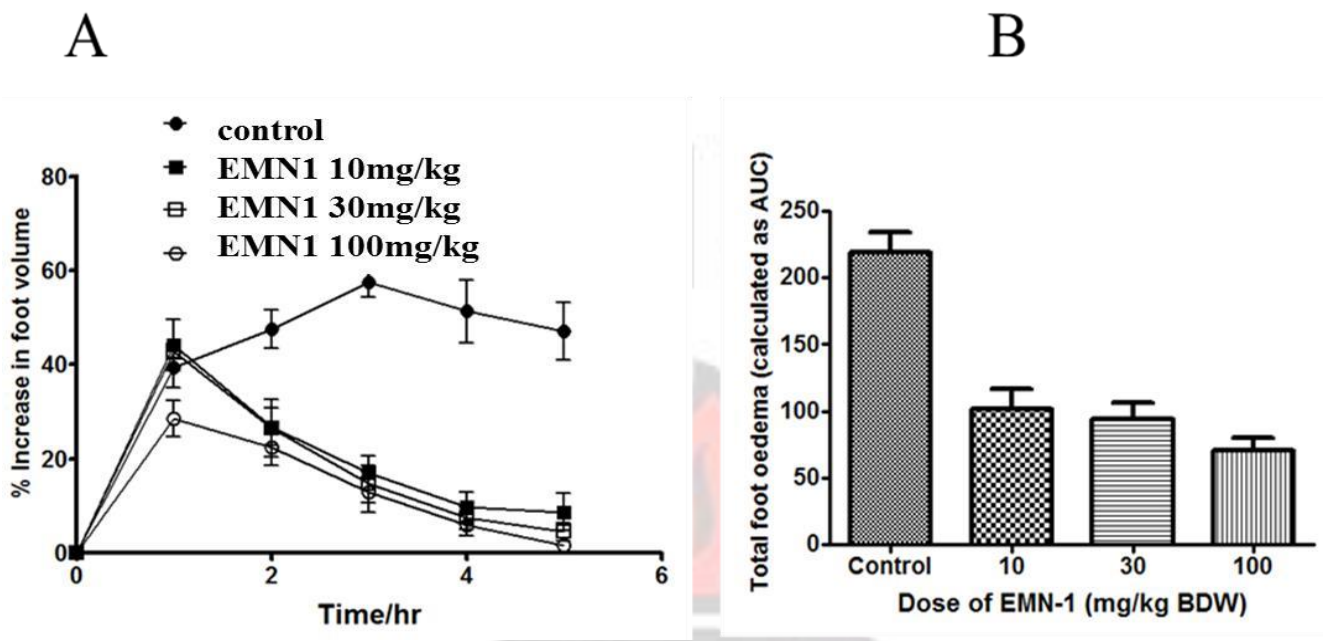


Figure 4.4: Effect of EMN1 (10 – 100 mg/kg; i.p.) on time course curve (A) and the total oedema response (B) in carrageenan-induced oedema in chicks. Values are means ± SEM. (n =6) **Table 4.4:** *In vivo* and *in vitro* Anti-inflammatory activities of 70% EtOH extracts and reference drugs

Extract/Drug	ED_{50} of Carrageenaninduced oedema in chicks (mg/kg) ± SEM n=6	IC_{50} of Human RBC stabilization (µg/ml) ± SEM		IC_{50} of Egg albumin stabilization (µg/ml) ± SEM n=3
		Heat-induced haemolysis	Hypotonic-induced haemolysis	
M. nobilis	90.54 ± 0.73	22.31 ± 0.12	17.73 ± 0.61	12.51 ± 0.06
S. lancifolius	181.50 ± 1.06	86.08 ± 0.04	130.90 ± 0.32	89.21 ± 0.87
D. principum	37.25 ± 1.22	ND	ND	59.45 ± 0.08
C. cujete	23.30 ± 0.43	36.25 ± 0.12	38.26 ± 0.53	5.62 ± 0.33
A. glaberrima	164.90 ± 1.63	184.32 ± 0.84	232.20 ± 1.06	67.24 ± 0.87
Diclofenac	0.04 ± 0.01	122.02 ± 0.34	134.70 ± 0.53	14.82 ± 0.35
Dexamethasone	0.07 ± 0.01	119.28 ± 0.06	106.10 ± 0.34	1.31 ± 0.06

Values are presented ± standard error of mean (SEM), ND means not determined

4.4 ANTIMICROBIAL ACTIVITY

Antimicrobial screening of the 70% EtOH extracts of the study samples was performed to validate some folkloric uses of these as anti-infective agents in Ghana. In this study, four (4) Gram positive and four (4) Gram negative bacteria species with one (1) fungus were employed as test microorganisms. Agar well diffusion and broth micro dilution were the assay techniques employed.

4.4.1 Agar well diffusion Assay

The 70% EtOH extract of MN and CC were the most active showing activity against eight (8) out of the nine (9) microbes tested. MN at 10 mg/mL concentration produced the highest zone of growth inhibition (22.93 ± 0.27 mm) against *Candida albicans*. AG was active against all the Gram negative bacteria, two of the Gram positive species but inactive against the fungus and other bacteria. SL and DP were each active against five (5) of the microorganisms with their highest zones of inhibition (17.47 ± 0.35 and 17.87 ± 0.15 mm respectively) against *Salmonella typhi.* Most of these extracts were comparable to Amoxicillin against the bacteria and ketoconazole against the fungus both at 5 mg/mL as shown in *Table* 4.5 below.

Table 4.5: Zones of Growth inhibition of 10 mg/ml of 70% EtOH extracts against selected microbes

Microorganism	**Mean Zone of Growth Inhibition (mm) ±SEM for 10 mg/mL**					
	MN	**SL**	**DP**	**CC**	**AG**	**Amoxicillin at 5 mg/mL**
B. subtilis	15.60 ± 0.26	NI	15.40 ± 0.54	16.50 ± 0.31	NI	15.00±1.00
S. aureus	20.70 ± 0.23	15.47 ± 0.32	15.50 ± 0.29	16.90 ± 0.31	17.60 ± 0.44	18.00±1.00
S. pyogenes	16.43 ± 0.35	NI	NI	NI	16.13 ± 0.18	13.21 ± 0.35

E. faecalis	NI	NI	NI	15.03 ± 0.26	NI	14.33±0.33
E. coli	17.07 ± 0.24	15.67 ± 0.35	16.13 ± 0.09	12.47 ± 0.09	17.07 ± 0.07	NI
S. typhi	17.63 ± 0.42	17.47 ± 0.35	17.87 ± 0.15	14.00 ± 0.12	16.93 ± 0.12	16.33±0.33
P. aeruginosa	16.77 ± 0.27	NI	NI	16.07 ± 0.12	16.07 ± 0.19	NI
K. pneumoniae	15.87 ± 0.07	15.87 ± 0.13	16.43 ± 0.54	14.83 ± 0.24	21.80 ± 0.25	NI
C. albicans	22.93 ± 0.27	14.93 ± 0.17	NI	16.10 ± 0.50	NI	17.12 ± 0.3*

NI = No inhibition at concentrations used, * = ketoconazole control, n = 3

4.4.2 Broth Micro dilution (MIC determinations)

Minimum inhibitory concentrations (MICs) were determined for the 70% EtOH extracts against the microorganisms that were inhibited (sensitive) in the agar well diffusion assay. The MICs as shown in *Table 4.6* ranged from as low as 100 to over 1000 µg/mL. The 70% EtOH extract of MN yielded the least MIC of 100 µg/mL against *Staphylococcus aureus* and *Candida albicans* whereas that of CC had the highest MIC of 1500 µg/mL against *Escherichia coli.*

Table 4.6: MICs of 70% EtOH extracts of selected plants against some microorganisms

Microorganism	**MIC (µg/mL)**				
	MN	**SL**	**DP**	**CC**	**AG**
B. subtilis	>1000	NT	1000	>1000	NT
S. aureus	100	1000	1000	1000	750
S. pyogenes	500	NT	NT	NT	>1000
E. faecalis	NT	NT	NT	>1000	NT
E. coli	500	1000	750	>1000	750
S. typhi	500	500	500	>1000	1000
P. aeruginosa	750	NT	NT	>1000	>1000

K. pneumoniae	1000	750	750	>1000	500
C. albicans	100	>1000	NT	>1000	NT

NT = not tested, n=3

4.5 ANTIOXIDANT ACTIVITY

The anti-inflammatory assays indicated that all study samples may have some anti-inflammatory activity and hence antioxidant studies were conducted. All four (4) antioxidant models investigated the inherent ability of the plant extracts to mop up a particular free radical system.

4.5.1 Rapid DPPH assay (bioautography)

In the rapid DPPH bioautography which preceded the other antioxidant assays, it was observed that each extract contains components that scavenged the stable DPPH radical appearing as yellow spots against the purple DPPH background on the TLC plates. Using Ascorbic acid as a reference, MN and AG had the highest number of DPPH-scavenging components (4 spots each) while the other extracts yielded at most 3 spots. A subsequent study investigated the action of the multicomponent extracts on the DPPH radical system using the scavenging assay below.

4.5.2 DPPH Radical Scavenging Assay, Total Antioxidant Capacity, and Total Phenol Content

The IC_{50} values in the DPPH radical scavenging assay as shown in *Table 4.7* below indicated that the 70% EtOH extract of MN and SL (9.96 ± 0.03 and 31.58 ± 0.17 µg/ml respectively) were more active than the reference standard Ascorbic acid (38.30 ± 0.00 µg/ml). The other extracts; DP, AG and CC respectively inhibited 50% of DPPH at 49.09 ± 0.39, 53.60 ± 0.32, and 168.80 ± 1.06 µg/ml. The total antioxidant capacity of the extracts and the contribution of its constituent phenols to this activity were evaluated.

The Ascorbic acid equivalence (AAE) and the Tannic acid equivalence (TAE) in the total antioxidant capacity and total phenol content assays respectively had a repeated pattern as the DPPH radical scavenging assay. MN had the highest AAE of 90.66 ± 0.02 mg/g and TAE of 165.31 ± 0.06 mg/g followed by SL, DP, AG and CC in that order. The least active extract (70% EtOH extract of CC) was about four (4) times less active than the Ascorbic acid standard.

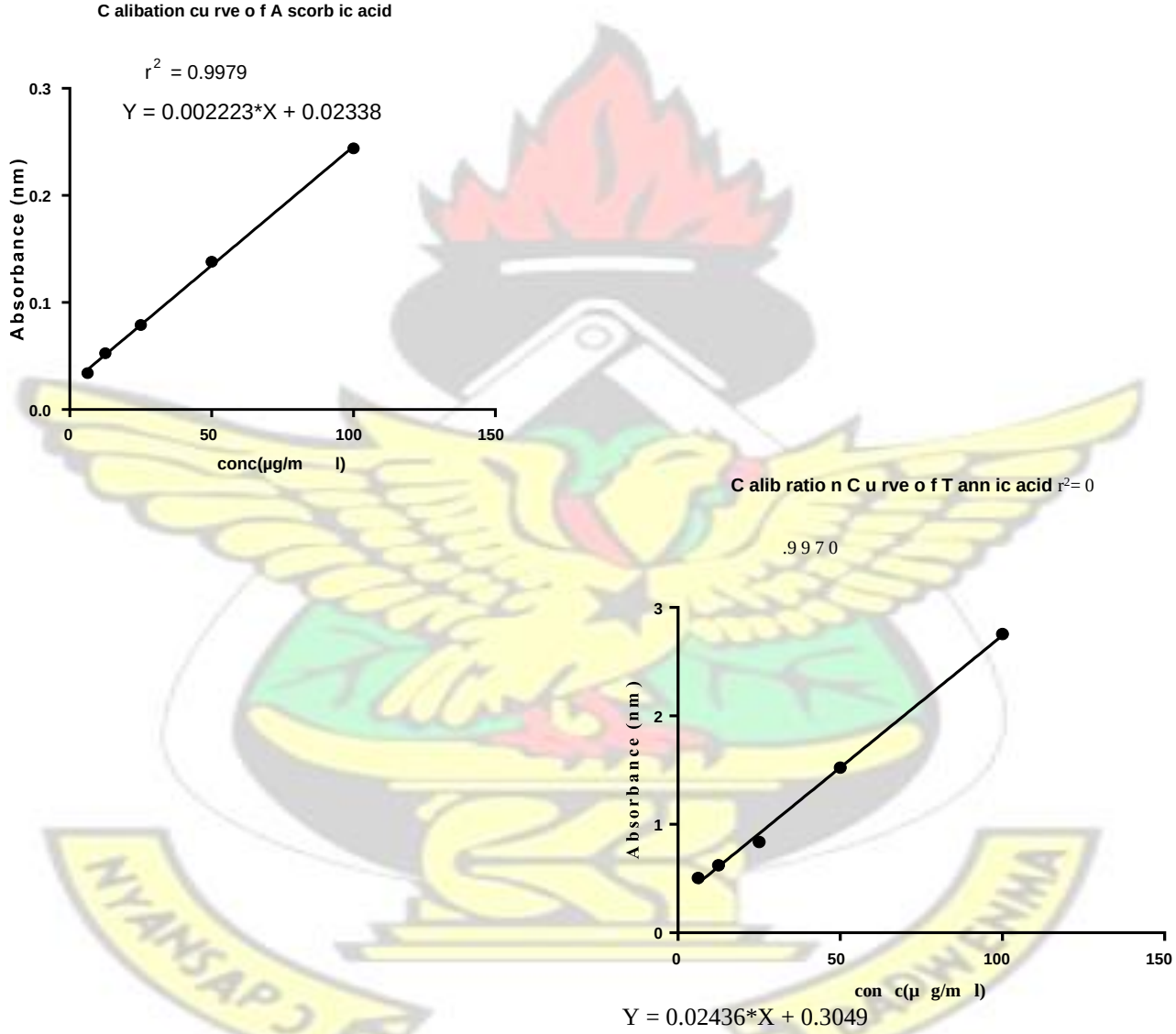


Figure 4. 5: Calibration curves for the determination of Ascorbic acid and Tannic acid equivalences

Table 4. 7: Results of *In vitro* Antioxidant Assays

	Antioxidant activity

70% EtOH extract or Drug	DPPH RSA IC_{50} (µg/ml) ± SEM	TAC (AAE) (mg/g) ± SEM	TPC (TAE) (mg/g) ± SEM
M. nobilis	9.96 ± 0.03	90.66 ± 0.02	165.31 ±0.06
S. lancifolius	31.58 ± 0.17	71.65 ± 0.06	96.05 ± 0.09
D. principum	49.09 ± 0.39	61.57 ± 0.02	82.48 ± 0.21
C. cujete	168.80 ± 1.06	53.54 ± 0.23	68.66 ± 0.22
A. glaberrima	53.60 ± 0.32	63.82 ± 0.32	80.36 ± 0.21
Ascorbic Acid	38.30 ± 0.00	ND	ND

ND means not determined

4.6 ACUTE ORAL TOXICITY STUDIES

Toxicity studies were conducted to ascertain the safety of the herbs in man. Though animal studies are not predictive of the toxicity profile in man, they form a premise on which human safety studies can be designed (Smith, 1985)

The acute oral toxicity studies were carried out in accordance with OECD (2001) guidelines for toxicity testing of chemicals using the fixed dose procedure. The study animals were dosed with 2000 mg/kg body weight of the extracts and observed against an untreated control. After monitoring and observing the animals for two weeks, the following symptomatic deductions were made;

a. No extract produced lethality (mortality) in the treated animals during the study period.
b. There was no significant weight difference and changes in feeding patterns between the treated groups and the untreated controls.

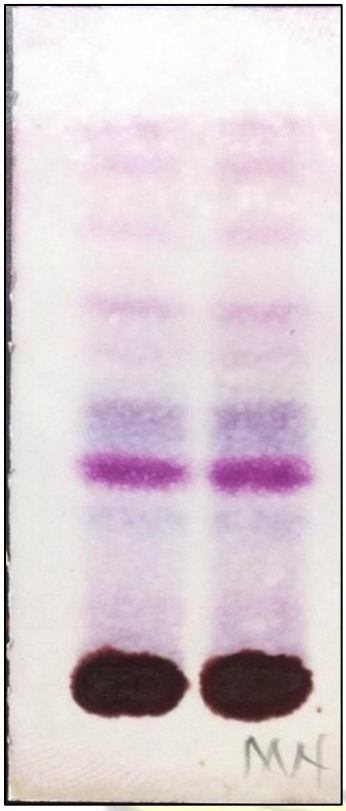
c. The general appearance and signs of health in the study groups and the controls did not vary significantly.

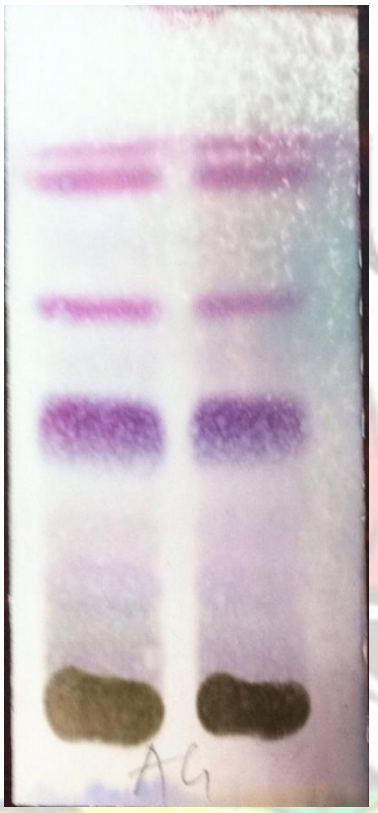
4.7 PHYTOCHEMICAL ANALYSIS

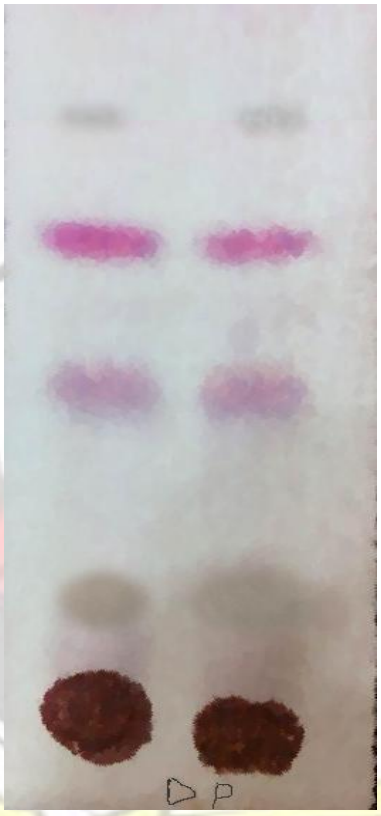
4.7.1 TLC of 70% ethanol extracts

The thin layer chromatography of the 70% EtOH extracts (as shown in *Figure 4.7* below) indicated that the samples under study contained multiple phytoconstituents. After a run in a 4:1 petroleum ether: Ethyl acetate solvent system, the plates were developed by spraying with specific reagents and observed in visible light. The *M. nobilis* extract yielded nine spots on the chromatograms followed by *A. glaberrima* extract with seven spots. *D. principum* extract yielded four distinct spots whereas *S. lancifolius* and *C. cujete* extracts produced 3 spots each. The components had varying retardation factors, shapes as well as colours revealing their diversity in nature. Retardation factors (R_f values) were calculated for each component of the samples. There was a good resolution of the components of the extracts on the chromatograms and this provides a strong tool for fractionation and isolation of these phytoconstituents.

The extract of *M. nobilis* was generally observed to be more promising in terms of the bioactivities as well as the TLC profile and hence was subsequently fractionated for isolation. Seven fractions were obtained and further purification of fraction EMN-*C* yielded compound EMN1 which was characterised as betulinic acid.







DPPH control

After spraying with DPPH

Figure 4.6: Thin layer chromatograms of 70% EtOH extracts after running in a pet ether: EtOAc (4:1) and sprayed with anisaldehyde or 0.2% DPPH

Table 4. 8: R_f values of the components of the 70% EtOH extracts

70% EtOH Extract	No. of spots	R_f value		
***Margaritaria nobilis* (MN)**	9	0.130 0.444 0.741	0.278 0.537 0.852	0.352 0.611 0.907
***Stylochiton lancifolius* (SL)**	3	0.185	0.370	0.852
***Drypetes principum* (DP)**	4	0.204 0.889	0.519	0.759
***Crescentia cujete* (CC)**	3	0.315	0.577	0.796
***Albizia glaberrima* (AG)**	7	0.093 0.444 0.889	0.204 0.630	0.296 0.852

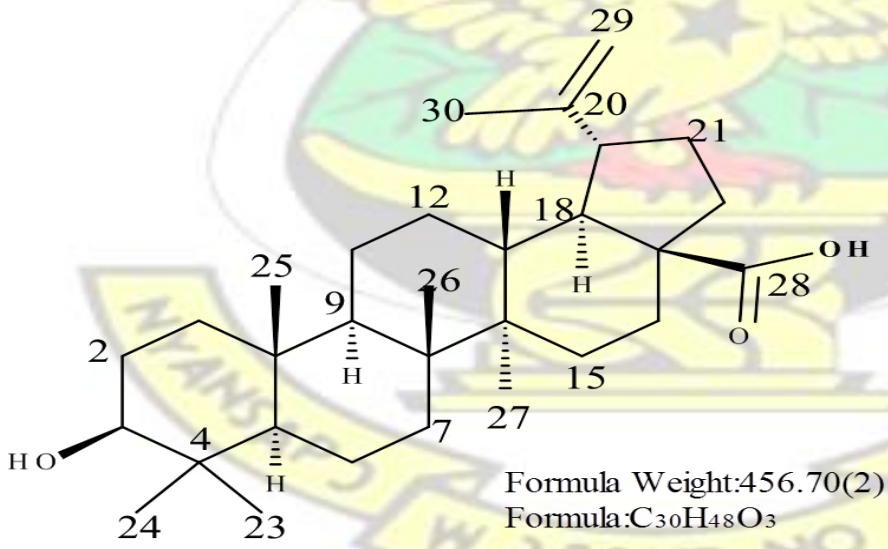
4.7.2 Identification of EMN1 as Betulinic acid

A mass spectrum of EMN1 (Appendix 1A) and a reference compound betulin (Appendix 1B) were comparable. There is the presence of m/e 189 as the base peak which may indicate the presence of a lupane nucleus (Ekuadzi, 2013). A GC-MS analysis of EMN1 and betulin (Appendix 2) revealed the retention time and the mass spectra of both compounds were superimposable.

The ^{1}H NMR spectrum (Appendix 3 & Table 4.9) revealed six methyl (CH_3-) singlets (δH 0.85, 0.90, 0.95, 1.00, 1.68 ppm) and a secondary hydroxyl (-OH) group with a triplet at δH 3.12 ppm. This hydroxyl proton is assigned H-3 and correlates with δ 39.95 and δ 56.87 ppm (C-4 and C-5 respectively) on the HMBC spectrum (Appendix 5). δH 4.56 and 4.69 ppm (H-29) on the ^{1}H NMR spectrum are two olefinic H(s) in an exocyclic double bond and are confirmed by the COSY and HMBC spectra. The COSY spectrum (Appendix 6) indicates that δH1.68 ppm (H-30) correlates with the proton signals at δH 4.56 and 4.69. There are long-range correlations observed

on the HMBC spectrum (Appendix 5) between δH 1.68 and δC 48.49 (C-19) and δC 151.97 ppm (C-20).

The 30 carbon atoms that were assigned as six methyl (CH_3-) (δ 28.61, 16.10, 16.73, 16.65, 15.11, 19.56 ppm), ten methylene (-CH_2-) (δ 40.08, 28.04, 19.45, 35.60, 22.09, 26.89, 30.84, 33.34, 31.71, 38.13 ppm), a double bond at δ 110.15 and 151.97, five methine (-CH-) (δ 56.87, 52.00, 39.67, 50.45, 48.49 ppm), an oxygenated -CH- (δ 79.66), five quaternary C (δ 39.95, 41.93, 38.33, 43.58, 57.48 ppm) and a C═O at δ 179.96 ppm on the HSQC spectrum (Appendix 7) were confirmed by the ^{13}C NMR spectrum (Appendix 4 & Table 4.9) of EMN1 in deuterated methanol. HMBC correlations between δH 1.63 (H-18), and δC 57.48 (C-17) and δC 179.96 (C28) confirms the presence of a carboxylic acid group that is indicated by a C═O at δC 179.96 ppm on the ^{13}C spectrum. The COSY, HSQC and HMBC spectra of EMN1 match that of betulinic acid as elucidated by Peng *et al.*, (1998).



Formula Weight:456.70(2)
Formula:$C_{30}H_{48}O_3$

Betulinic acid

Figure 4.7: Chemical structure of Betulinic acid (EMN1) isolated from 70% EtOH extract of *M. nobilis.*

Table 4. 9: Proton (^{1}H) and ^{13}C NMR chemical shifts of Betulinic acid and EMN1

Position	Betulinic acid chemical shifts (ppm) †		EMN1 chemical shifts (ppm)	
	^{1}H NMR	**^{13}C NMR**	**^{1}H NMR**	**^{13}C NMR**
1	0.99 1.67	39.3	0.93 1.67	40.08
2	1.85	28.3	1.30	28.04
3	3.45	78.10	3.12	79.66
4	-	39.50	-	39.95
5	0.82	56.00	0.69	56.87
6	1.33 1.56	18.80	1.42 1.52	19.45
7	1.38 1.45	34.90	1.36 1.43	35.60
8	-	41.1	-	41.93
9	1.38	51.00	1.32	52.00
10	-	37.50	-	38.33
11	1.21	21.20	1.22	22.09
	1.43		1.43	
12	1.21 1.94	26.10	1.20 1.70	26.89
13	2.74	38.60	2.30	39.67
14	-	42.90	-	43.58
15	1.26 1.88	30.30	1.43 1.88	30.84
16	1.55 2.63	32.90	1.44 2.20	33.34
17	-	56.60	-	57.48
18	1.77	49.88	1.63	50.45
19	3.52	47.80	3.28	48.49
20	-	151.30	-	151.97
21	1.53 2.24	31.20	1.37 1.93	31.71
22	1.57 2.25	37.60	1.43 1.90	38.13
23	1.22	28.70	0.95	28.61

24	1.00	16.30	0.90	16.10
25	0.83	16.40	0.85	16.73
26	1.07	16.40	0.95	16.65
27	1.07	14.90	1.00	15.11
28	-	178.80	-	179.96
29	4.77 4.95	109.92	4.56 4.69	110.15
30	1.79	19.50	1.68	19.56

†This data was adapted from Peng *et al.*, (1998)

4.7.3 Profile of Betulinic acid

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid), a pentacyclic lupane-type triterpene has been isolated from many plants including some *Margaritaria* species (Yogeeswari and Sriram, 2005; Ekuadzi, 2013). However, this is the first report of its presence in this plant. Studies have shown that betulinic acid has antibacterial, antimalarial (Domínguez-Carmona *et al.*, 2010), antiHIV-1 (Ghaffari, 2012), anticancer (Fulda, 2008) and anti-inflammatory activities (Yogeeswari and Sriram, 2005). It is therefore justifiable to say that it may be contributory to the observed anti-inflammatory and antimicrobial activities of the *M. nobilis* stem bark extract.

Chapter 5

5.0 CONCLUSIONS

This study set out to scientifically investigate and validate the anti-inflammatory, antioxidant, antimicrobial activities and the safety profile of five (5) selected plants that find use in Ghanaian traditional medicine as agents for treating inflammatory and infectious conditions.

This is a first time scientific report on the bioactivities of these selected medicinal plants and thus, confirmed their use in Ghanaian traditional medicine as well as predicts their safety for use in humans based on qualitative analysis. The following conclusions were therefore drawn:

1. The 70% ethanol extract of *Margaritaria nobilis* stem bark was the most active in terms of anti-inflammatory, antimicrobial and antioxidant activities.
2. *Stylochiton lancifolius* rhizomes extract on the contrary was the least active of the five plants though its bioactivities were comparable in many instances to the reference drugs used. All the samples possess some level of anti-inflammatory, antimicrobial as well as antioxidant activities.
3. The *in vitro* assay using the inhibition of human RBC haemolysis and protein stabilization models was useful in the screening for anti-inflammatory activity and can serve as a preliminary assay to the confirmatory *in vivo* model. This can be of great significance particularly due to the numerous ethical and animal right issues associated with *in vivo* animal models.
4. The symptomatic toxicity study also indicated that all the plant samples under investigation may be safe for use in man. Further toxicological assessment however needs to be done to confirm these findings.
5. Betulinic acid, an anticancer steroidal compound was isolated from the 70% ethanolic extract of *Margaritaria nobilis.* To the best of our knowledge, this is the first report of its isolation from the plant.

5.1 ***RECOMMENDATIONS***

The foregoing research work has established a basis for the use of the selected plants in Ghanaian folk medicine. The following recommendations are therefore made towards the future work on these plants species:

- Further work on isolation, characterization and biological screening of isolates from active fractions should be carried out and targeted towards obtaining bioactive lead compounds for the conditions under study to aid in drug development.
- Based on the observed biological activities of the extracts, future *in vitro* and *in vivo* bioassays such as wound healing and anticancer properties is proposed for the additional compounds which would be isolated.
- It is also suggested that future work on the toxicity profiles of active compounds from these plants should be carried out to support possible drug development.

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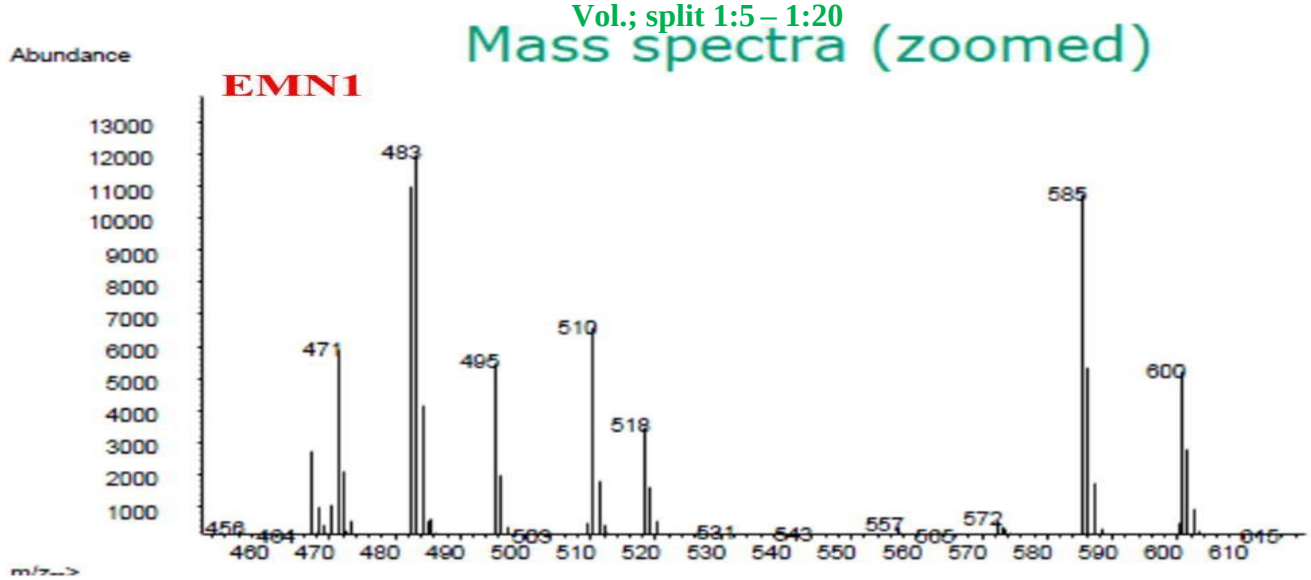
APPENDICES

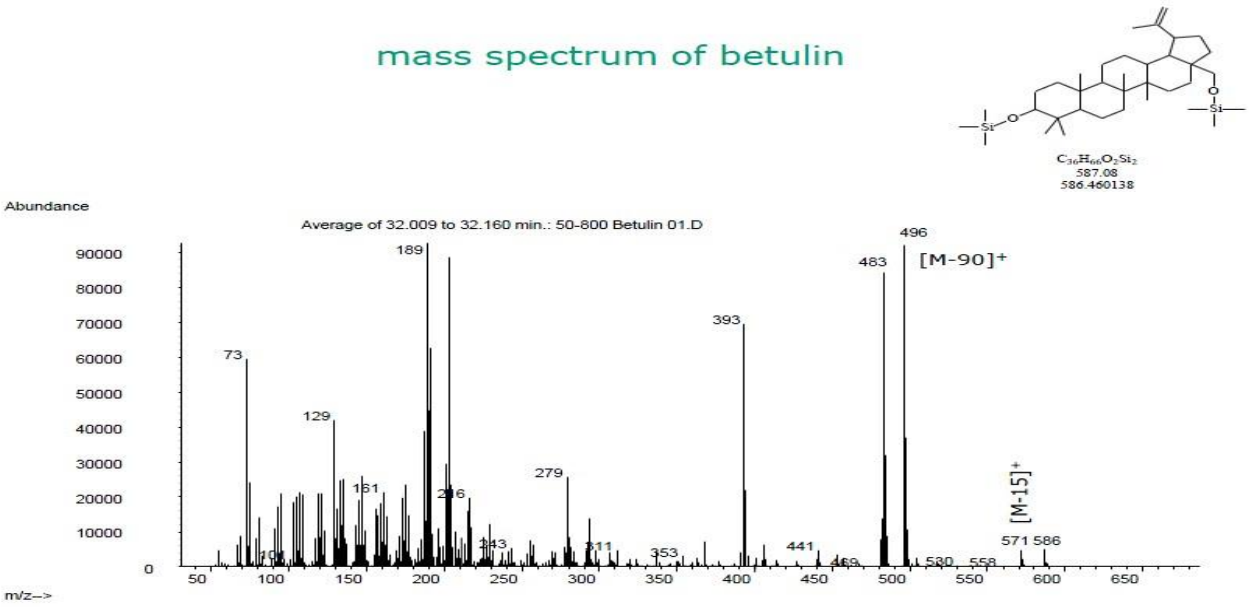
APPENDIX 1:

A) **A mass spectrum of EMN1**

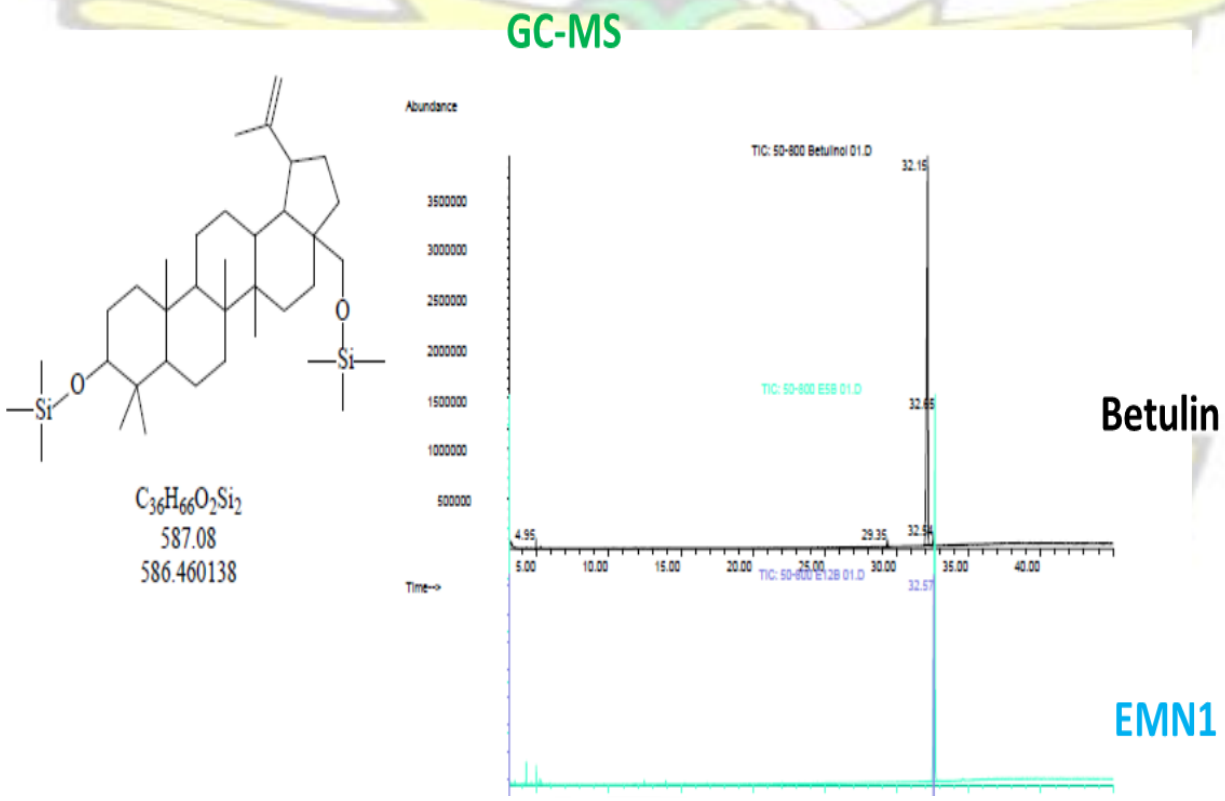
Agilent Technologies, 6890 N Gas Chromatograph; 5973 Mass Selective Detector, 7683 B
Injector; Software: ChemStation; 1 µL injection Vol.; split 1:5 – 1:20



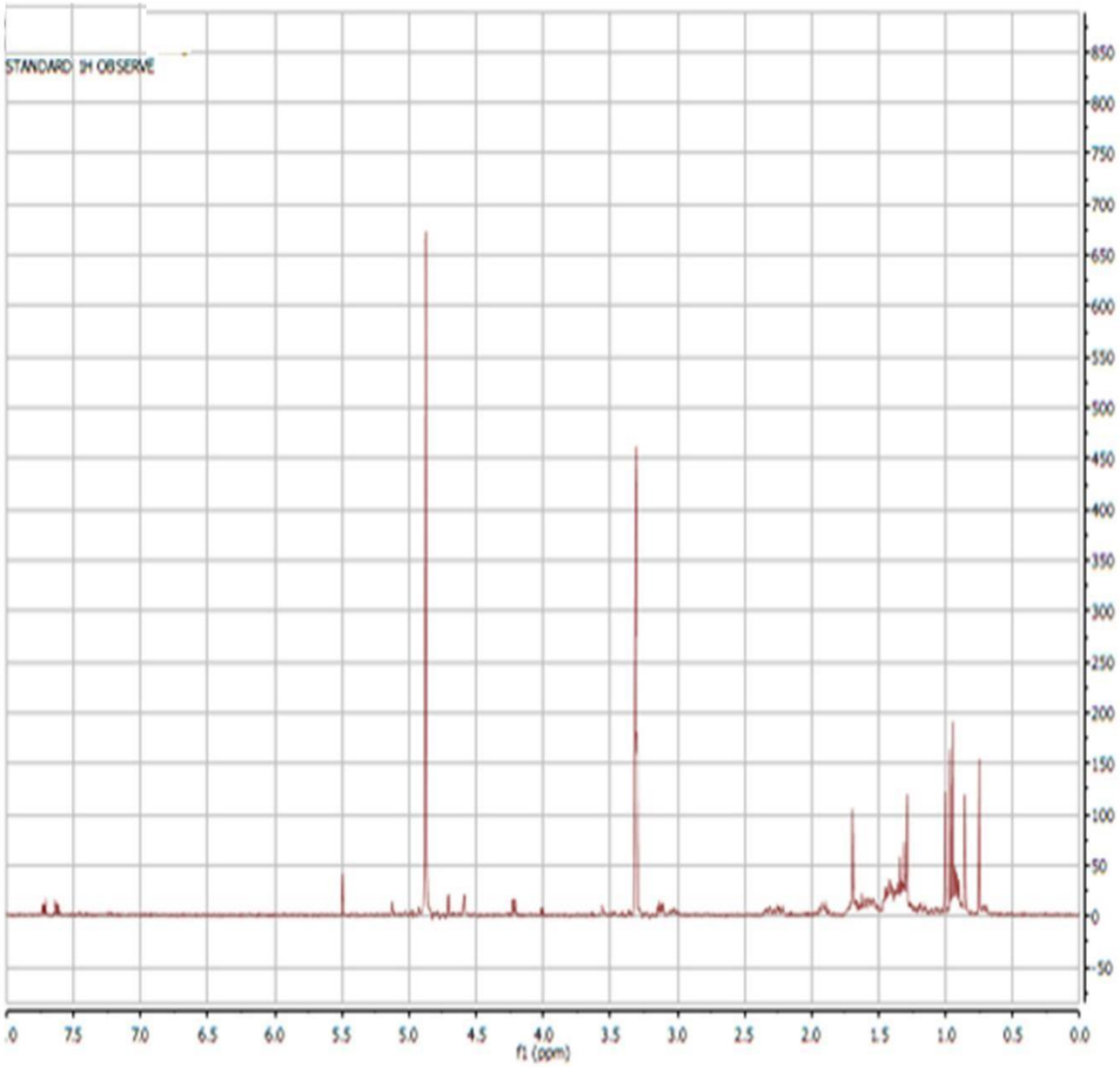
B) **A mass spectrum of Betulin**



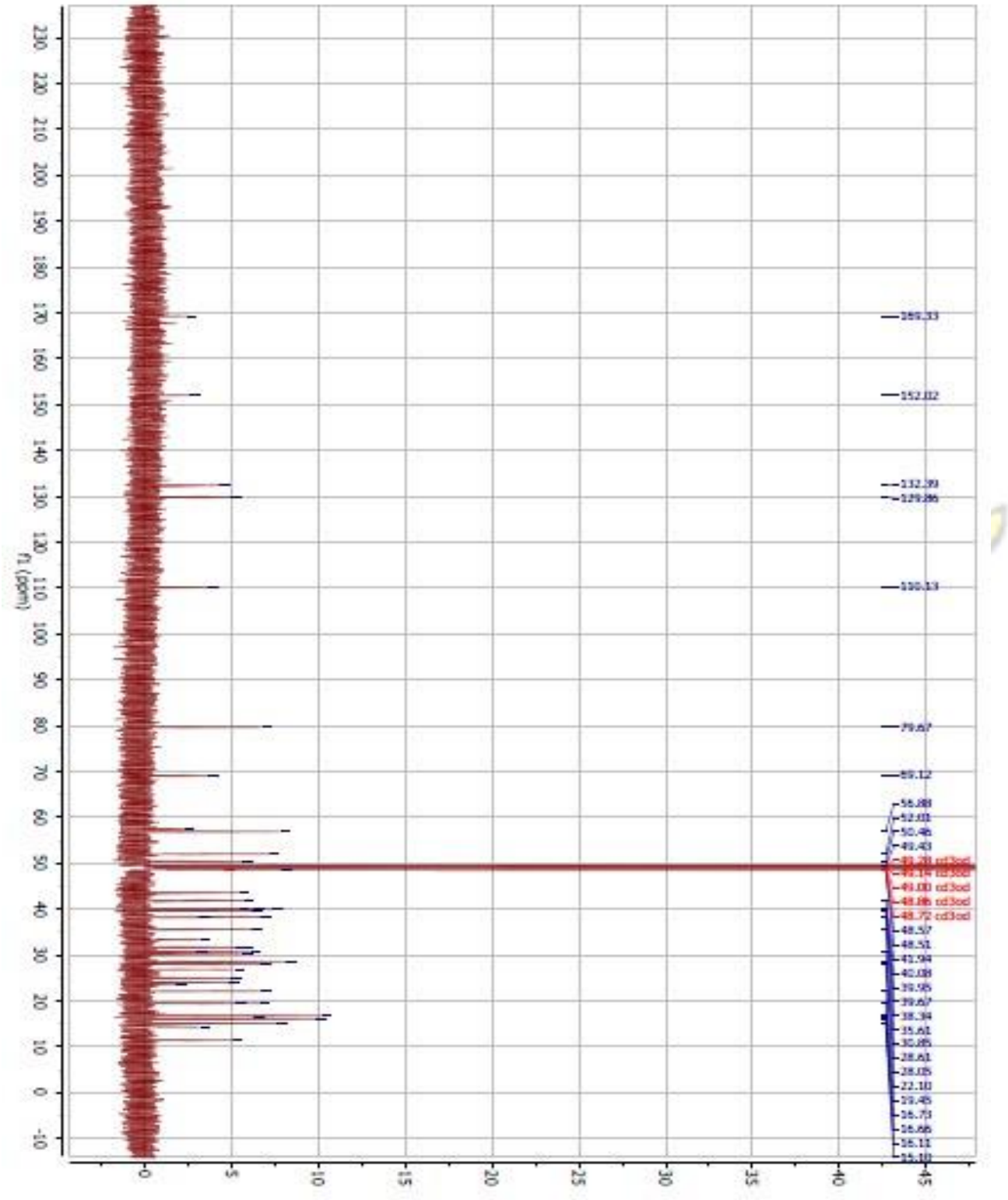
APPENDIX 2: Total ion chromatographs of Betulin and EMN1



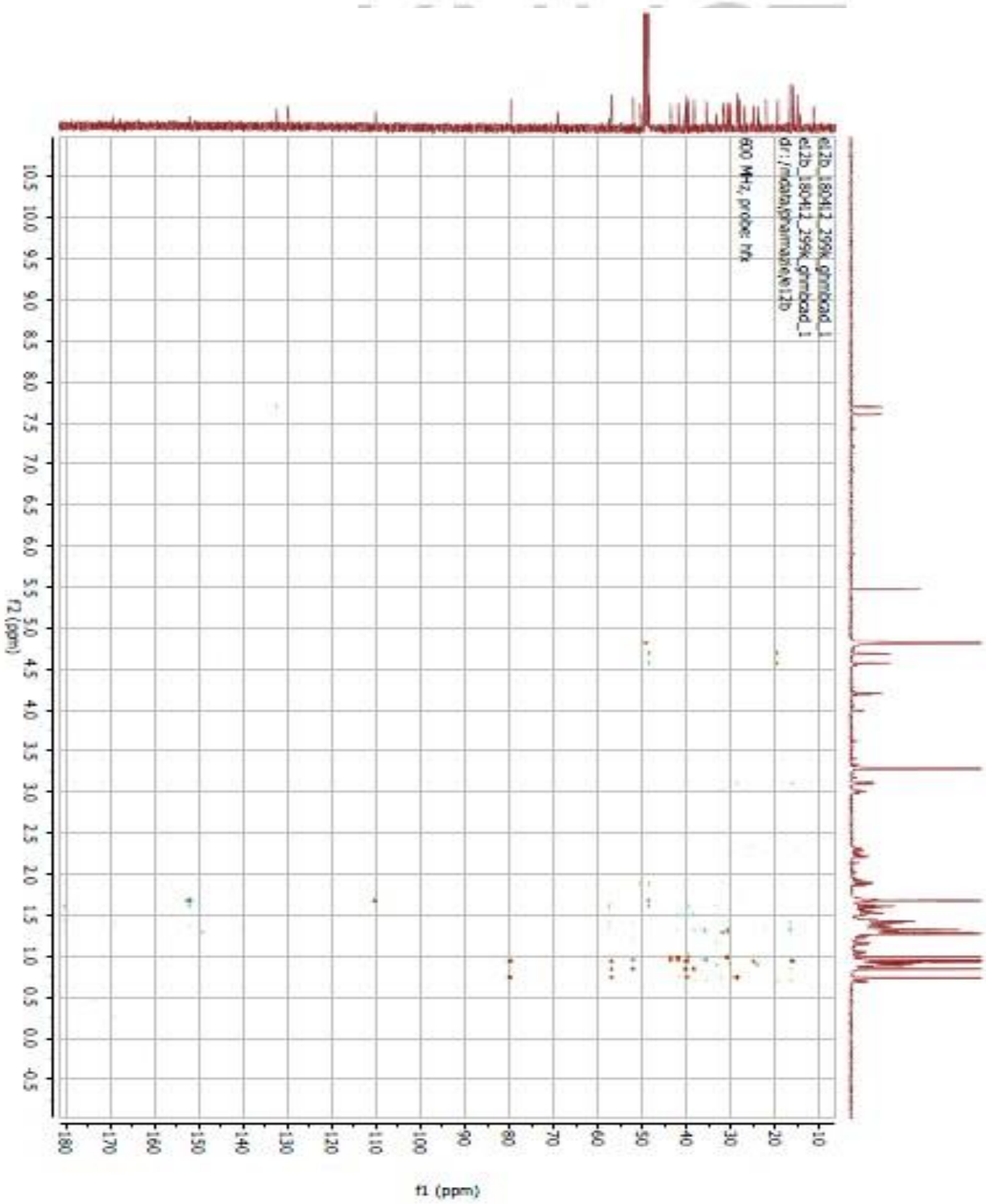
APPENDIX 3: ^{1}H NMR spectrum of EMN1 (Betulinic acid)



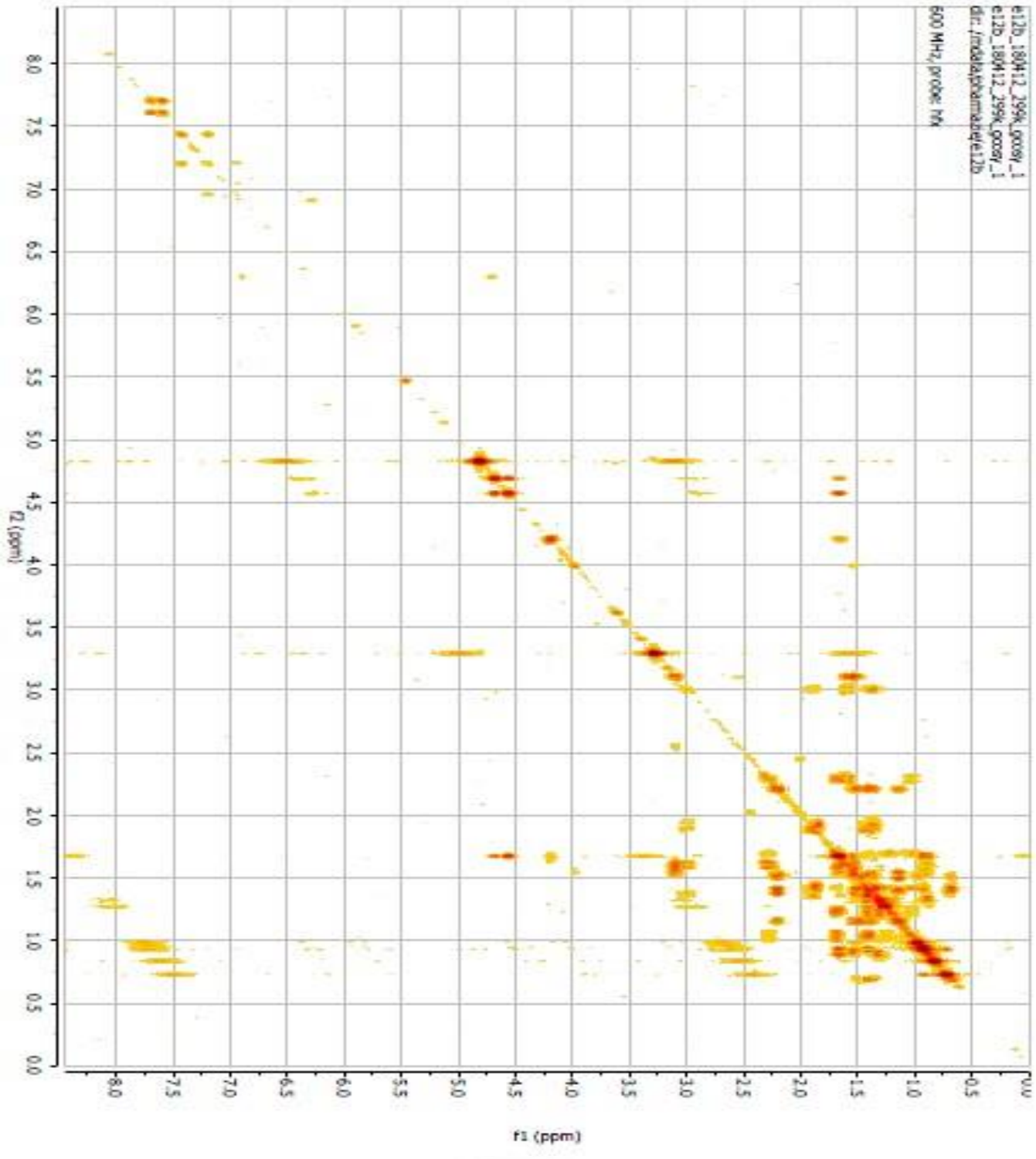
APPENDIX 4: ^{13}C NMR spectrum of EMN1 (Betulinic acid)



APPENDIX 5: HMBC spectrum of EMN1 (Betulinic acid)



APPENDIX 6: COSY spectrum of EMN1 (Betulinic acid)



APPENDIX 7: HSQC spectrum of EMN1 (Betulinic acid)