DECLARATION

I hereby declare that this submission is my own work towards the award of an MPhil and that to the best of my knowledge, it contains no material previously published by another person for the award of any degree of the University, except where due acknowledgement has been made in the text.

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Akosua Bemah Oppong

……

Prof. T. C. Fleischer

(Supervisor)

DEDICATION

This work is dedicated to my family for their love and support and especially to my father, the late Mr. Kwaku Oppong for all the care and undying love you showed me. I will always love you.

ACKNOWLEDGEMENT

I acknowledge the sufficient grace of God that has sustained me through the difficult moments encountered during this course of work.

I am sincerely grateful to my supervisor, Prof. T.C. Fleischer for his constructive criticism and contributions towards the realization of this work. I am grateful to him for his direction, advice and patience going through this work. I value your guidance and help immensely.

I wish to thank all the lecturers in the Department of Pharmacognosy: Prof. M.L.K. Mensah and especially Dr. A.Y. Mensah, Dr. Kofi Annan and Dr. Rita Akosua Dickson for their help in diverse ways. I am indeed indebted to you all.

To the technical staff of the Department of Pharmacognosy, Pharmaceutics and Pharmacology, especially Mr. Kakraba and Mr. Ansah, I say a big thank you for your help.

I further wish to thank Prof. Berhanu M. Abegaz of the Department of Chemistry, University of Botswana for running the NMR and mass spectra of the isolated compounds.

To my dear father, who could not live to see the realization of this dream, I wish to express my heartfelt gratitude for the financial and moral support and the encouragement you gave me during the course of this work. You were always there when I needed you, day or night.

Finally, to all who have helped in diverse ways I say 'God richly bless you and be with you always'.

ABSTRACT

Clerodendrum splendens is a common medicinal plant found mainly in the tropical and sub-tropical regions of the world that is used in traditional medicine for the treatment of various conditions including ulcers, scrofulous infections, wounds, burns and asthma. The pet ether, ethyl acetate and 70% ethanolic extracts which were obtained by Soxhlet extractions of 1.0 kg of powdered leaves of C. splendens were evaluated for their anti-bacterial and anti-inflammatory properties. The ethyl acetate extract showed the highest activity in both assays with the strongest antibacterial activity against *Escherichia coli* and the least activity against *Pseudomonas aeruginosa*. It again demonstrated the highest percentage of inhibition of oedema in the anti-inflammatory assay with a maximal percentage of inhibition of 66.09 ±13.13%.

Fractionation of the ethyl acetate extract led to the isolation of the known flavone apigenin and the cyclohexylethanoid Cleroindicin F. The structures of these compounds were determined by spectroscopic methods including 1D and 2D NMR as well as comparison with published data.

The activity of the extracts and the presence of apigenin and cleroindicin F in the ethyl acetate extract provide scientific support to the traditional use of the leaves of *C. splendens* for the treatment of infections, wounds and other inflammatory conditions**.**

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Medicinal plants have enjoyed use in virtually all cultures as sources of medicines (Plotkin, 1991). The history of the use of medicinal plants for their therapeutic purposes probably dates back to the origin of man. Fossil records date human use of plants as medicines to at least the middle Paleolithic age some 60,000 years ago (Solecki, 1977). Ancient texts of India and China contain exhaustive depictions of the use of a variety of plant-derived medications (Ahmad *et al.*, 2006). One of the earliest records of the use of medicinal plants is that of chaulmoogra oil obtained from the seeds of *Hydnocarpa gaerta* that was used in the effective treatment of leprosy around 2730 B.C. as recorded in the pharmacopoeia of the Emperor Sheng Nung of China (Goodman and Gilman, 1970). Dioscorides wrote *De Materia Medica*, a catalogue of more than 500 medicinal plants which remained an authoritative reference from the first century into the seventeenth century and became the prototype for modern pharmacopoeias (Stockwell**,** 1988). The early twentieth century, however, brought with it the beginning of a slow erosion of the important position held by medicinal plants as sources of therapeutic agents. The introduction of inorganic drugs like arsenic, iron, mercury and sulphur, followed by the rapid development of chemistry, which was accompanied by the ability to synthesize a massive array of medicinal molecules that allowed for the treatment of previously incurable and/or life threatening diseases, further decreased the popularity enjoyed by medicinal plants. Synthetic drugs, however, are plagued by unwanted side-effects and toxicities and this has led to the re-emergence of the use of medicinal plants (Brater and Walter, 2000). According to Hamburger and Hostettmann (1991), renewed interest in drugs of plant origin started in the 1980's and has been growing steadily since. Large pharmaceutical companies have looked towards plants as a source for new prototype

molecules. The world-wide demand for plant-derived drugs has escalated as a result of a desire for non-classical therapies and the demonstration of efficacy of drugs of plant origin (example gingko and garlic), through proper scientific investigations (Rates, 2001).

In Africa, Asia and Latin America, traditional medicine and medicinal plants have continued to play a very important role in their health care delivery systems. According to the World Health Organization (WHO), it is estimated that about 80% of the population in these developing countries rely on traditional medicine for their primary health care needs (Hack- Seang, 2005). There has also been an increase on the reliance of the use of medicinal plants by the population in the industrialized societies as herbal remedies have become more popular in the treatment of minor ailments and also on account of the increasing costs of personal health maintenance (Horeau and Dasilva, 1999). In 2001, it was estimated that approximately 25% of prescription drugs originated from plants, 121 active compounds were then in use (Houghton, 2001; Rates, 2001). In addition, of the 252 medicines considered as basic and essential by the WHO, 11% originated exclusively from plants and a significant number were synthesized from naturally occurring precursors. Digitoxin (from *Digitalis* spp.), vincristine and vinblastine (from *Catharanthus roseus*), quinine (from *Cinchona* spp.), atropine (from *Atropa belladona)* and morphine and codeine (from *Papaver somniferum*) are examples of important plant-derived drugs. About 60 % of anti-tumour and anti-infectious drugs on the market, or under clinical trial, originated from plants (Rates, 2001).

However, the difficulty in the use of medicinal plants is that they are usually used without any standardisation. This makes it difficult to document and institute a system of verification or assessment of the efficacy of the treatment. Thus, the local herbal medicine practitioner is quick to profess the efficacy of these remedies but stops short of providing a sound scientific basis and

explanation for the remedy and more often than not thrives on the long, continuous and sometimes uneventful use of the remedy for treatment. Plants may contain constituents that can be used to treat diseases such as infections, inflammatory conditions and cardiovascular diseases, but the scientific information on most of these medicinal plants in use are lacking. Therefore as part of the efforts to promote the use of medicinal plants either as an alternative or an adjunct to conventional medicine, it is necessary for scientists to carry out investigations into herbal medicines. This will help bridge the gap between conventional and herbal medicines.

It is in this direction that the leaves of *Clerodendrum splendens* which is traditionally used for the treatment of wounds and burns (Mshana *et al*., 2000), haemorrhoids, diarrhoea and dysentery (Burkhill, 1985), is being investigated. Various species of *Clerodendrum*, including *C. trichotomum, C. indicum* and *C. serratum* are traditionally used to treat inflammatory conditions such as asthma and have indeed been established scientifically to possess potent anti-inflammatory activities (Jung-Ho *et al.,* 2003). Other species such as *C. chinense* and *C.inerme* have also been shown to be used for the treatment of infections such as typhoid and gonorrhoea (Rehman *et al*., 1997). The validation of the folkloric use of this plant, the isolation and characterization of the constituents of the plant may unearth its potential and pave the way for the standardization of the leaves of *C. splendens*.

1.1 Aims of Research

The main aim of this research is to validate the anti-inflammatory and antimicrobial activities of the leaves of *C. splendens*. The specific objectives are

1. To investigate the anti-inflammatory activity of *C. splendens* leaf extracts using the carrageenan-induced foot oedema in 7-day old chicks.

2. To investigate the antimicrobial properties of *C. splendens* leaf extracts against a wide range of pathogenic organisms.

3. To isolate the chemical constituents responsible for the antimicrobial and anti-inflammatory activities using various chromatographic techniques.

4. To characterize the various isolates using various spectroscopic methods.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE FAMILY VERBENACEAE

The family to which *Clerodendrum splendens* belongs is Verbenaceae which is also known as the teak family. It comprises 35 genera and 1,200 species found mainly in the tropical and subtropical regions of the world (Heywood *et al.*, 2007). The Verbenaceae is divided into four tribes which include Duranteae Benth. (including Citharexyleae Briq.), Petreeae Briq., Verbeneae Dumort. and Lantaneae Endl (Reveal, 1999). The members of the family are trees, shrubs and herbs noted for heads, spikes or clusters of small flowers, many of which have aromatic odour (Cantino *et al.*, 1992). The family is closely related to the Lamiaceae family. These families share numerous characters including opposite leaves, zygomorphic corollas, and a bicarpellate ovary that is divided into four locules (Wagstaff and Olmstead 1997). Both families usually have aromatic leaves. The traditional difference between the two families is the ovary. The Lamiaceae generally have a deeply four-lobed ovary with gynobasic style while the Verbenaceae have an unlobed ovary and a terminal style (Cronquist, 1981).

A number of useful timber, medicines and dyes are derived from members of this family (Bailey, 1963). Many species have been used in folk medicine; *Verbena hastata* as bitters; species of *Lippia* for the treatment of typhoid and rheumatism (Jiofack, *et al*., 2009) and *Aegiphila salutaris* is used as a purge and remedy for snake-bites. *Lantana camara* is used as a remedy for malaria and as an antiseptic (Chowdhury, 2007). *Verbena officinalis* of Europe is a tonic, but more famous for its use in witchcraft. It was celebrated among the Romans and Druids of Gaul, and

used by them in religious ceremonies. The very valuable teakwood is obtained from *Tectona grandis* (Bailey, 1963).

Compounds such as anthraquinones, terpenes, steroidal saponins, ergot alkaloids and flavonoids are commonly found in members of the Verbenaceae family (Siegler, 1998).

2.2 THE GENUS *CLERODENDRUM*

The genus *Clerodendrum* is widely distributed in the tropical and warm temperate regions of the world, with most of the species occurring in tropical Africa and Asia. It occurs in Northern Africa, in Egypt, and spreads through the remainder of Africa and Madagascar. The genus comprises small trees, shrubs and herbs and it is well known for its ornamental uses. The first description of the genus was given by Linnaeus in 1753, with the identification of *C. infortunatum*. *Clerodendrum* is a very large and diverse genus with about 580 identified species. It is the largest genus of the tribe Teucrieae (Steane *et al*., 1999).

2.2.1 Botanical Description

Species of this genus are usually small trees with most of them being climbers. They are characterized by simple decussate- opposite leaves with most of them being petiolated. There is a wide variability in the sizes of the leaves. *C. aculeatum*, for instance, has leaves ranging from 0.9-4 cm long and 0.3-1.4 cm wide whiles *C. paniculatum* has leaves that are 6-35 cm long and 6-30 cm wide. The leaf shape is also variable with *C. walichii* having a lanceolate shape but *C. japanicum* being ovately shaped. The inflorescences of members of this genus are both axilliary and terminal. The inflorescence may range from 1-39 cm long and 1-25 cm wide. They may be

cymes, panicles or solitary flowers which may be crowded or sparsely arranged. Almost all the species of *Clerodendrum* have foliaceous bracts and linear lanceolate bracteoles. The calyx is usually gamosepalus, commonly green but sometimes red or white. They are almost always campanulate, rarely elliptic, truncate, 5-lobed, glabrous or pubescent. The size of the corolla ranges from 0.6-4.0 cm long to 0.3-2.0 cm wide. The corolla is hypocrateriform and may be white, red, pink or purple. The corolla is glabrous, pubescent or glandular-puberulent. The fruits are drupaceous, mostly subglobose, ovoid or glabrous usually separating at maturity. The exocarp is fleshy and black when mature (Rueda, 1993).

2.3 Ethnomedicinal Uses of *Clerodendrum*

A number of species in the genus have been documented to be used by various tribes in Asia and Africa. Leaves of *C. buchholzii* are reported in some parts of Africa for the treatment of furunculosis, echymosis and gastritis (Nyegue *et al*., 2005). In Ghana, the leaves of *C. splendens* are used in the form of poultice to treat burns and wounds (Mshana *et al*., 2000). The aqueous extract of *C. umbellatum* is used as an enema for the treatment of gonorrhoea and placental retention in some parts of Africa (Irvine, 1961). The stem and leaves of *C. trichotomum* have been used in China, Japan and Korea for centuries in the treatment of inflammation (Kim, 1996). In the Chinese system of medicine *C. bungei* is used for the treatment of headaches, dizziness, furuncles and hysteroptosis (Zhou *et al*., 1982; Yang *et al*., 2002). Cheng (2001) has reported the use of *C. cyrtophyllum* and *C. chinense* in the treatment of fever, jaundice, typhoid and syphilis in China. The roots of *C. serratum* have been used in the treatment of dyspepsia, seeds in dropsy and leaves as a febrifuge and in cephalagia and ophthalmia (Anonymous, 1992). *C. calamitosum* is used for the treatment of kidney, gall and bladder stones. This plant is also reported to have

diuretic and antibacterial properties (Cheng *et al.*, 2001). In China, the fruits of *C. petasites* are used as medicine for malaria while in India the plant is used to induce sterility (Hazekamp *et al*., 2001; Panthong *et al*., 2003).

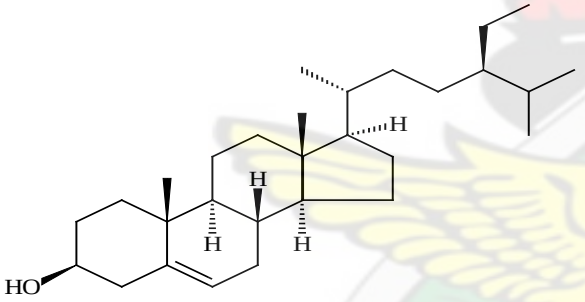
2.4 CHEMICAL CONSTITUENTS OF *CLERODENDRUM*

Clerodendrum is used in various indigenous systems of medicine for the treatment of many diseases. Researchers have made great efforts to isolate and identify biologically active principles and other major constituents from various species of the genus. These include steroids, other terpenoids and flavonoids.

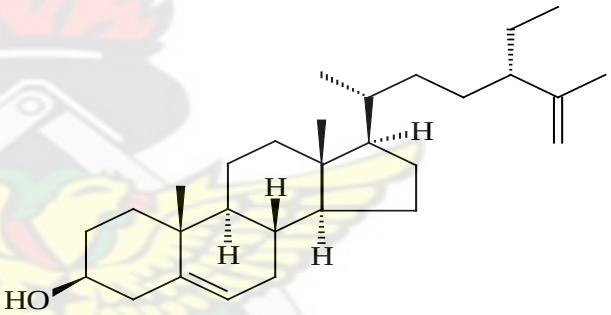
2.4.1 Steroids

From research reports steroids are the major class of chemical constituents present in the genus. *β*-sitosterol **[1]** has been reported to be present in the roots of *C. serratum* (Jaya *et al*., 1997), *C. paniculatum* (Joshi *et al*., 1979) and *C. fragrans* (Singh and Singh, 1981). It has also been reported in the leaves of *C. inerme* (Singh and Prakash, 1983), *C. infortunatum* (Joshi *et al*., 1978) and *C. neriifolium* (Ganapaty and Rao, 1989), and in the leaves and aerial parts of *C. colebrookianum* (Goswami *et al*., 1996). *β*-sitosterol has as well been reported in the aerial parts of *C. nutans* (Joshi *et al*., 1985) and in the stems of *C. fragrans* (Singh and Singh, 1981) and *C. indicum* (Prakash and Garg, 1981). Clerosterol **[2]** has also been isolated from the stem of *C. crytophyllum* (Tian *et al*., 1993), from the entire plant of *C. bungei* (He *et al*., 1997) and leaves and stem of *C. inerme* (Akihisa *et al*., 1989). It has also been reported in the aerial parts of *C. infortunatum* (Thakur *et al*., 1988), *C. inerme* (Rehman *et al*., 1997) and *C. nutans*, (Joshi *et al*.,

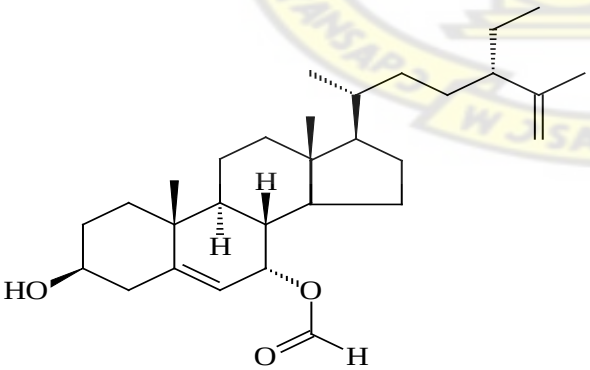
1985), the root bark of *C. mandarinorum* (Zhu *et al*., 1996) and the roots of *C. phlomoides* (Joshi *et al*., 1979). Other steroids such as colebrin A **[3]** and B **[4]** have been isolated from the aerial parts of *C. colebrookianum* (Yang *et al*., 2000). Stigmasterol **[5],** cholesterol **[6],** poriferasterol **[7]** and 22-Dehydroclerosterol **[8]** have been isolated from the aerial parts, leaves and stem of *C. fragrans* (Akihisa *et al*., 1988). Campesterol **[9]** has been reported to be present in the leaves of *C. neriifolium* (Ganapaty and Rao, 1989), the leaves and stem of *C. scandens* (Akihisa, *et al.,* 1990) and *C. inerme* (Akihisa *et al*., 1989).



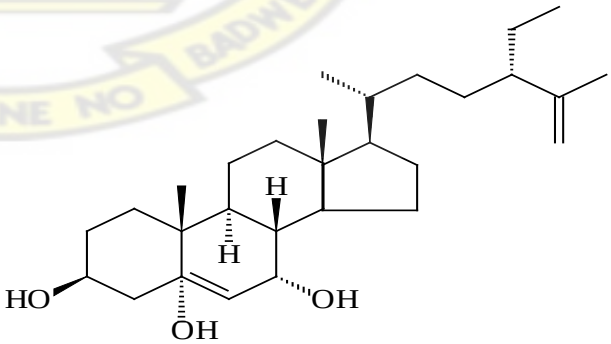
β- Sitosterol **[1]**



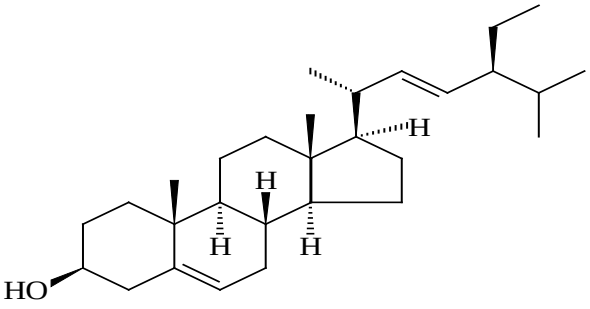
Clerosterol **[2]**



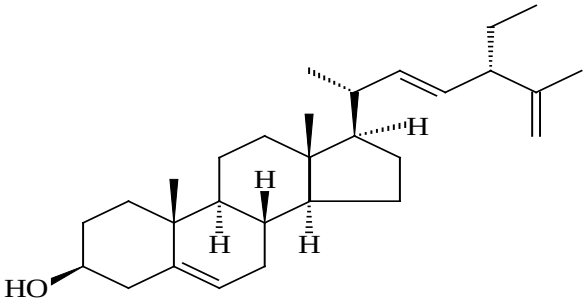
Colebrin A **[3]**



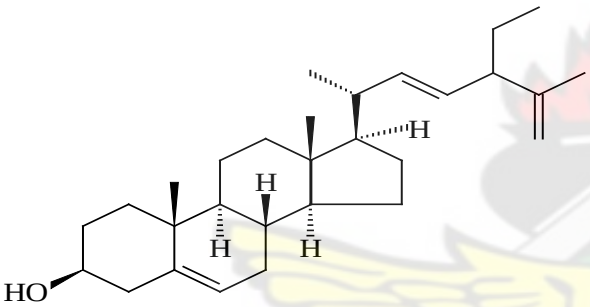
Colebrin B **[4]**



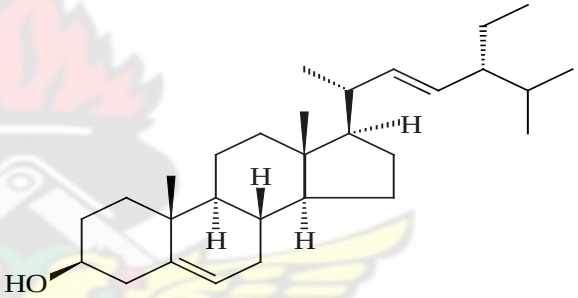
Stigmasterol **[5]**



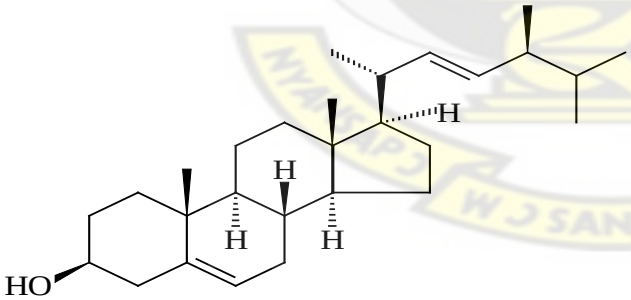
Cholesterol **[6]**



Poriferasterol **[7]**



22-Dehydroclerosterol **[8]**



Campesterol [**9]**

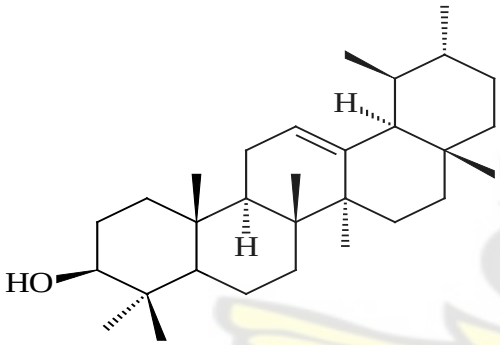
2.4.2 Terpenoids

Another class of constituents is terpenoids which include: monoterpenes, diterpenes, triterpenes, iridoids and sesquiterpenes. The triterpene α-amyrin **[10],** has been isolated from the roots and stem of *C. fragans* (Singh and Singh, 1981) and from the leaves and stem of *C. inerme* (Singh and Prakash, 1983). *β*-amyrin **[11],** another triterpene has also been isolated from the roots of *C. colebrookianum* (Joshi, *et al*., 1979), from the leaves and stem of *C. inerme* (Singh and Prakash, 1983) and from the roots of *C. paniculatum* (Joshi, *et al*., 1979). Friedelin **[12]** has been isolated from the stem of *C. crytophyllum* and *C. inerme* (Tian *et al*., 1993; Rao, *et al*., 1993). It has also been isolated from the bark of *C. trichotomum* and from the (Nonomura, 1955). Lupeol **[13]** another triterpene has been isolated from the stem bark of *C. neriifolium* (Ganapaty and Rao, 1985). Betulin **[14]** and oleanolic **[15]** acid have been isolated from the leaves and stem of *C. inerme* (Singh and Prakash 1983).

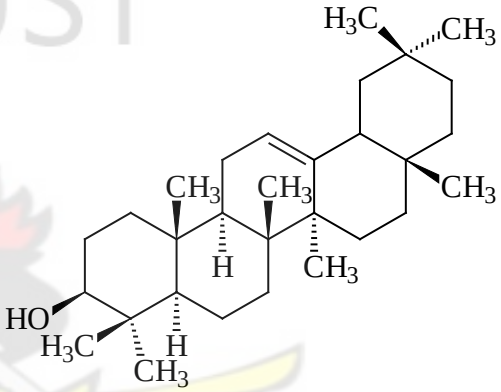
The diterpene Clerodendrin A **[16]** has been isolated from the roots of *C. phlomoides* (Joshi, *et al*., 1979). Clerodendrin B **[17]** and C **[18]** have as well been isolated from the leaves of *C. inerme* (Rao, *et al*., 1993). Clerodin **[19]** which is a diterpene has been isolated from the flowers of *C. infortunatum*, from the leaves of *C. brachyanthum* (Lin, *et al*., 1989) and from the roots of *C. phlomoides* (Joshi *et al*., 1979). The diterpenes clerodinin A **[20]**, B **[21]** and C **[22]** have also been isolated from the leaves of *C. brachyanthum* (Lin, *et al*., 1989). Uncinatone **[23]**, a diterpene has been isolated from the stem of *C. cryophyllum* (Tian *et al*., 1993) and *C. bungei* (Fan *et al*., 1999), from the roots and root bark of *C. uncinatum* (Dorsaz *et al*., 1985) and the leaves and stem of *C. siphonanthus* (Pal, *et al*., 1988). Two new diterpenes, bungone A **[24]** and B **[25]**, together with three known compounds, uncinatone **[23]**, teuvincenone F **[26]** and sugiol

[27] have been isolated from the stem of *C. bungei* (Fan *et al.*, 1999). Royleanone **[28]** a diterpene has been isolated from the leaves and stem of *C. inerme* (Singh and Prakash 1983).

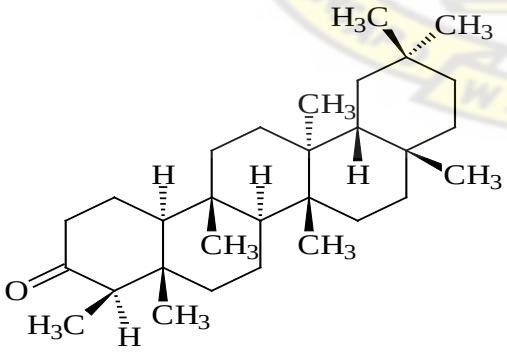
The iridoid monoterpene, harpagide **[29]** has been isolated from the leaves of *C. tomentosum* (Jacke and Rimpler, 1983). Ajugoside **[30]** has as well been isolated from the leaves of *C. thomsonae* (Lammel and Rimpler, 1981).



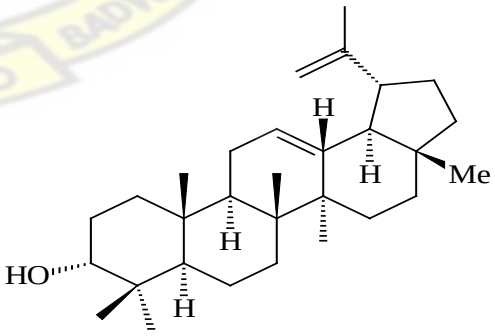
α- Amyrin **[10]**



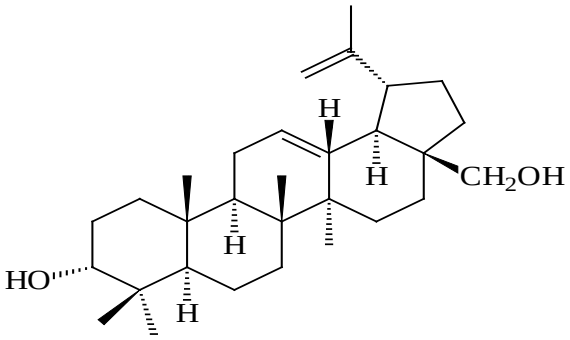
β- Amyrin **[11]**



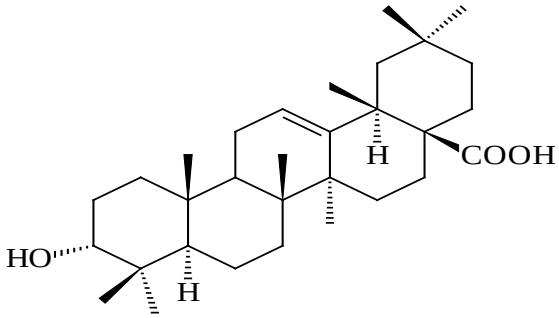
Friedelin **[12]**



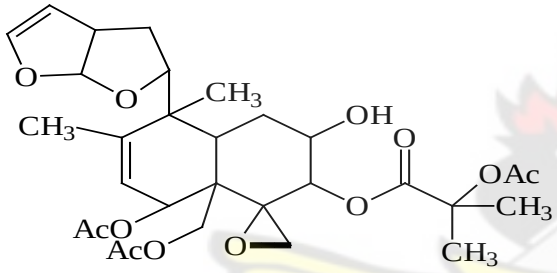
Lupeol **[13]**



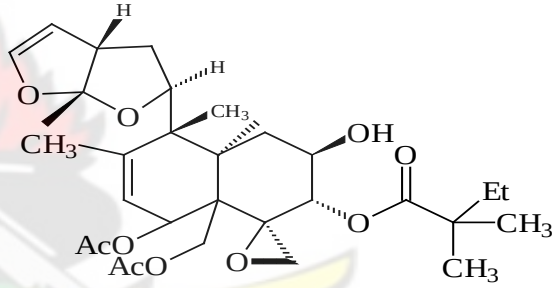
Betulin **[14]**



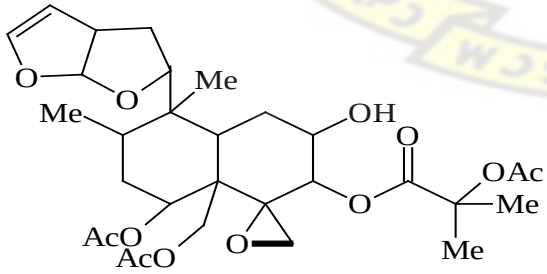
Oleanolic acid **[15]**



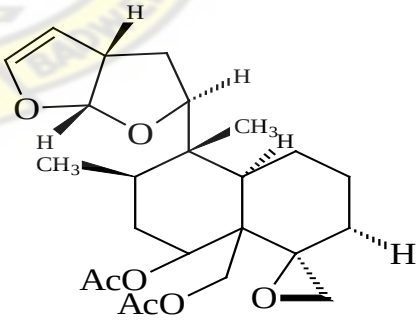
Clerodendrin A **[16]**



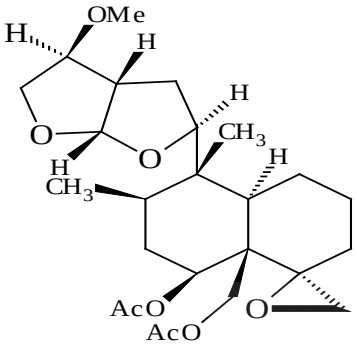
Clerodendrin B **[17]**



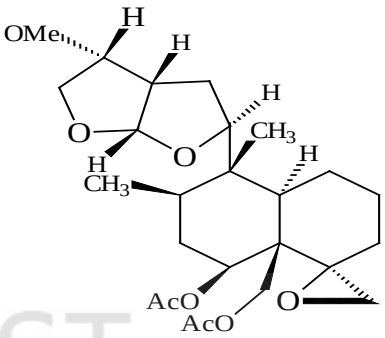
Clerodendrin C **[18]**



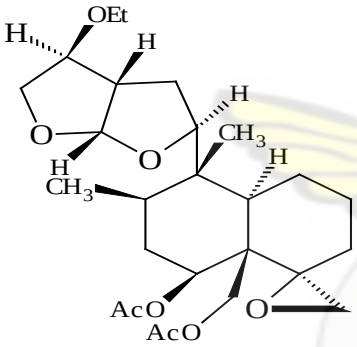
Clerodin **[19]**



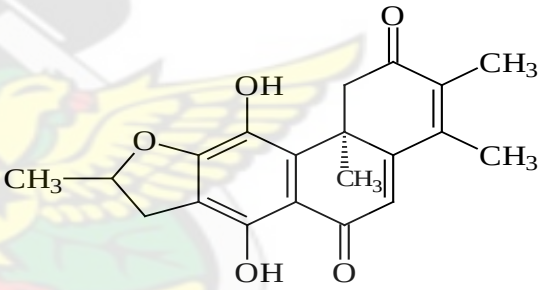
Clerodinin A **[20]**



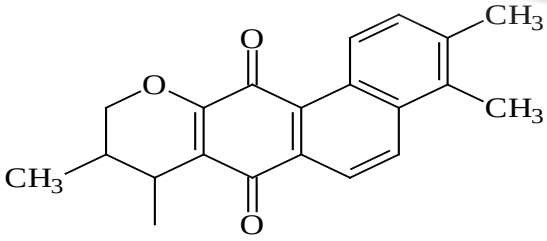
Clerodinin B **[21]**



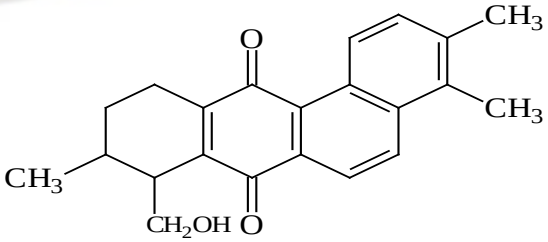
Clerodinin C **[22]**



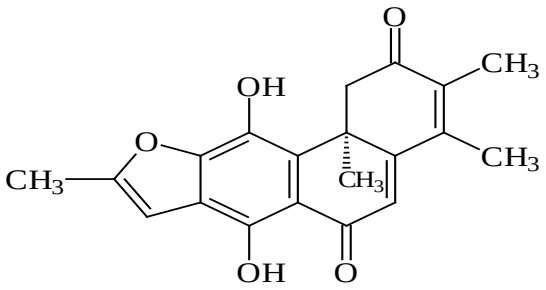
Uncinatone **[23]**



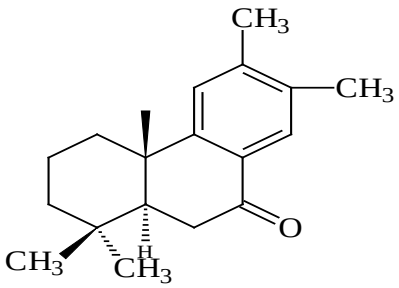
Bungone A **[24]**



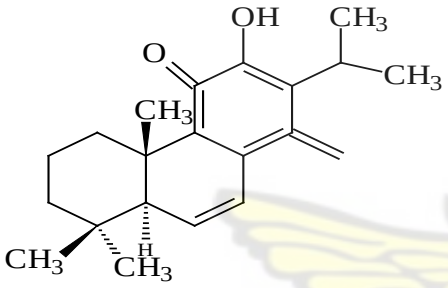
Bungone B **[25]**



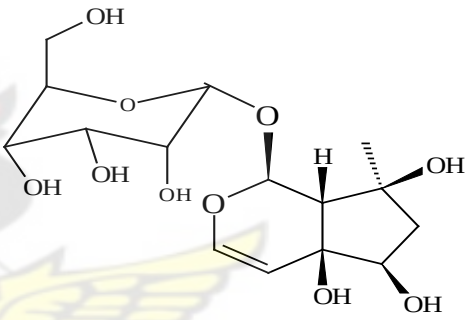
Teuvincenone F **[26]**



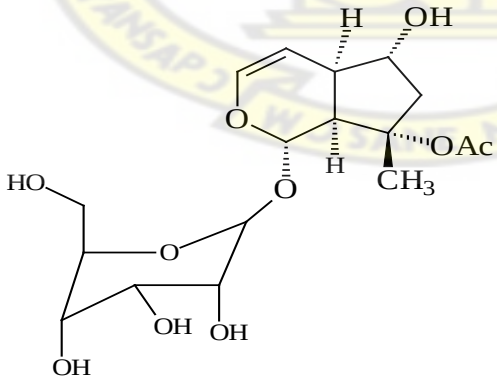
Sugiol **[27]**



Royleanone **[28]**



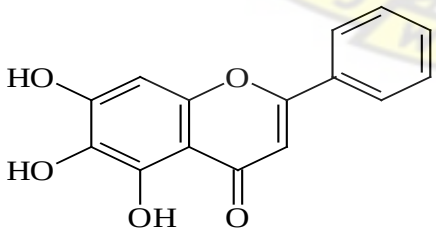
Harpagide **[29]**



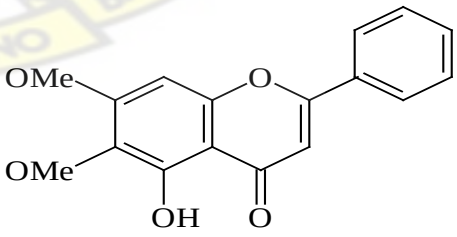
Ajugoside **[30]**

2.4.3 Flavonoids

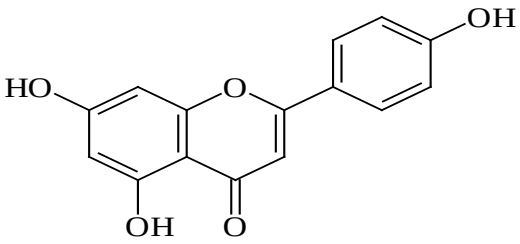
Flavonoids are another class of compounds that have been isolated from *Clerodendrum*. Scutellarein **[31]** has been isolated from the roots of *C. serratum* (Jaya *et al*., 1997) and from the aerial parts of *C. indicum* (Tian *et al*., 1997). The flavone cirsimaritin **[32]** has been isolated from the leaves and stem of *C. fragans* (Barua *et al*., 1989) and from the root bark of *C. mandarinorum* (Zhu *et al*., 1996). It has also been isolated from the leaves of *C. neriifolium* (Ganapaty and Rao, 1989). Apigenin **[33]** has been isolated from the leaves and stem of *C. inerme* (El-Shamy *et al*., 1996), from the flowers of *C. infortunatum* (Sinha *et al*., 1981) and from the leaves of *C. neriifolium* (Ganapaty and Rao, 1990). It has also been isolated from the flowers of *C. phlomoides* (Seth *et al*., 1982) and also from the roots of *C. serratum* (Jaya *et al*., 1997). Hispidulin **[34]** has been isolated from the flowers of *C. phlomoides* (Roy and Pandy, 1994) and from the aerial parts of *C. indicum* (Tian *et al*., 1997). Luteolin **[35]**, another major flavonoid has been isolated from the flowers of *C. phlomoides* (Roy and Pandy, 1994) and from the roots and leaves of *C. serratum* (Nair, *et al*., 1976). Salvigenin **[36]** has been isolated from the leaves of *C. neriifolium* and *C. inerme* (Raha and Das, 1989). **7**-Hydroxyflavanone **[37]** has been isolated from the leaves and flowers of *C. phlomoides* (Roy and Pandy, 1994).



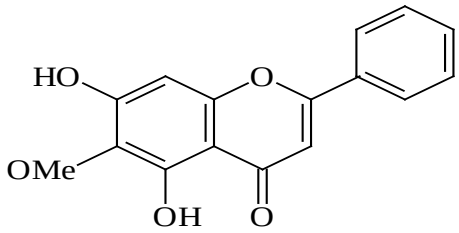
Scutellarein **[31]**



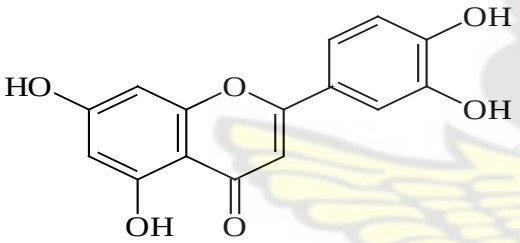
Cirsimaritin **[32]**



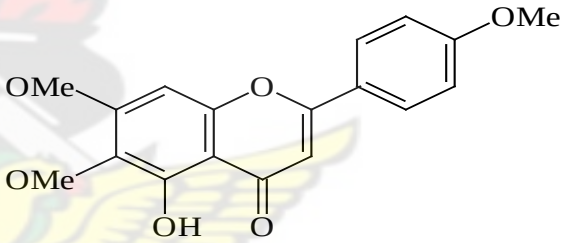
Apigenin **[33]**



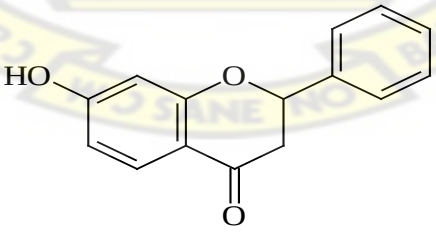
Hispidulin **[34]**



Luteolin **[35]**



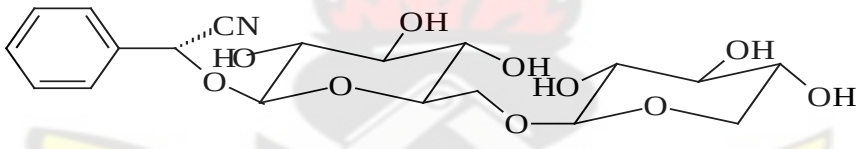
Salvigenin **[36]**



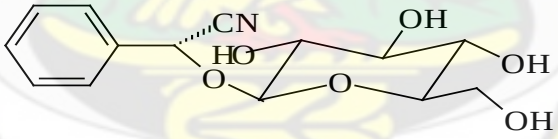
7-Hydroxyflavanone **[37]**

2.4.4 Miscellaneous

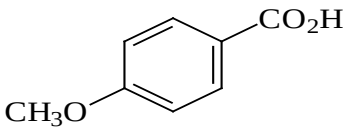
Other chemical constituents including cyanogenic glycosides such as lucumin **[38]** and prunasin **[39]** have been isolated from *C. grayi* (Miller *et al*., 2006). Phenolic compounds like anisic **[40]** and vanilic **[41]** acids have been reported in the leaves and stem of *C. bungei* (Zhou *et al*., 1982). The lignin, eudesmin **[42]**, has been isolated from the leaves of *C. brachyanthum* (Lin and Kuo, 1992). The phenyl propanoid verbacoside **[43]** has been isolated from the leaves of *C. inerme, C. capitatum* and *C. buchananii* (Taoubi, *et al*., 1992). The indole alkaloid trichotomine **[44]** has been isolated from the fruits of *C. trichotomum* (Jacke and Rimpler, 1983).



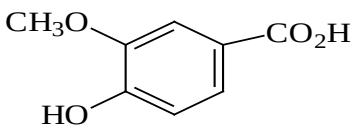
Lucumin [**38]**



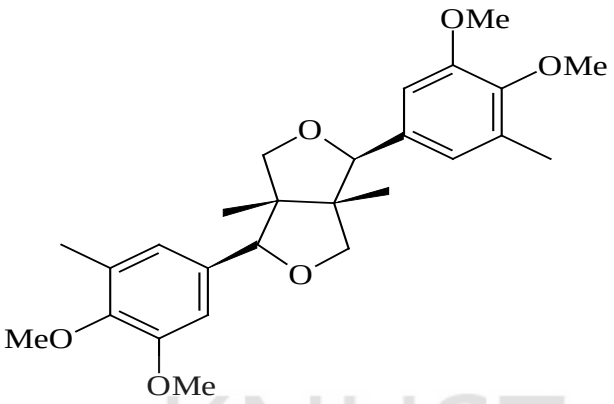
Prunasin **[39]**



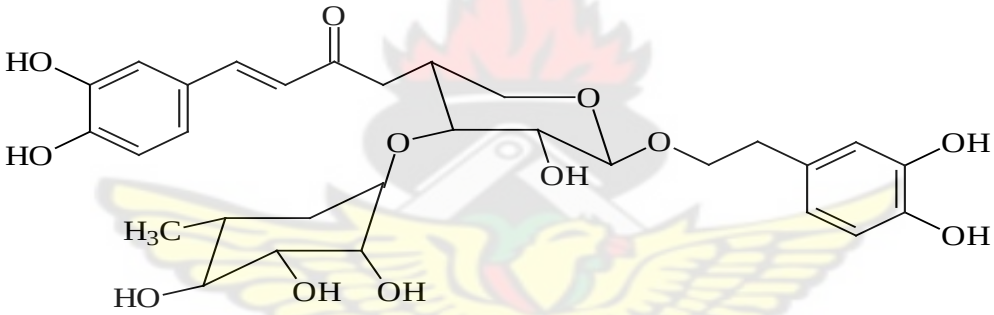
Anisic acid **[40]**



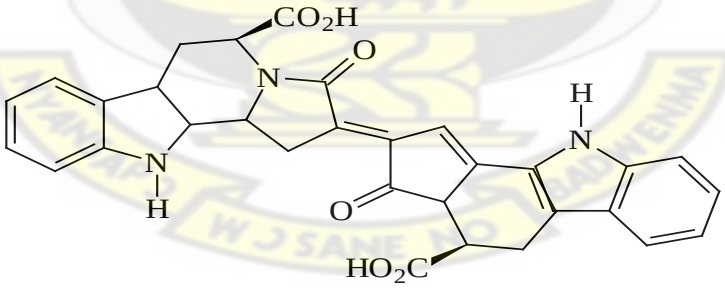
Vanillic acid **[41]**



Eudesmin **[42]**



Verbacoside **[43]**



Trichotomine **[44]**

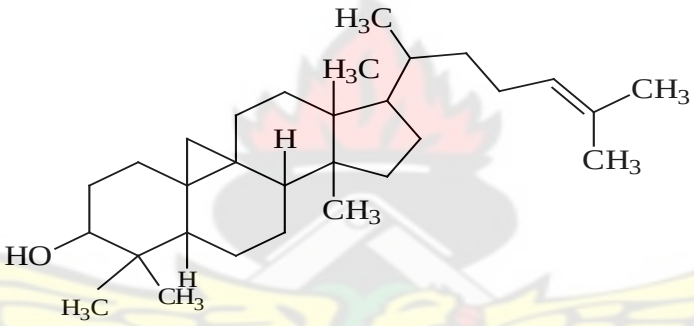
2.5 Botanic Description of Clerodendrum splendens

Clerodendrum splendens (Figure 1) also known as the Flaming glory bower is a woody or semi-woody evergreen vine which grows to about 3.7 m long and climbs by twining. It has ovate to oblong lustrous dark green leaves which are arranged in opposite pairs (Huxley, 1992). The flowers are salverform (tuba shaped) with a slender tube and an abruptly expanded corolla. They are scarlet or sometimes white, about 2.5 cm across and borne in dense terminal clusters to 12.7 cm long (Starr *et al*., 2003).

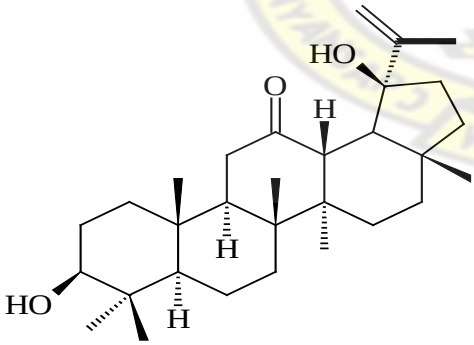
Figure 1: Leaves and flowers of *C. splendens*

2.5.1 Chemical Constituents of C. splendens

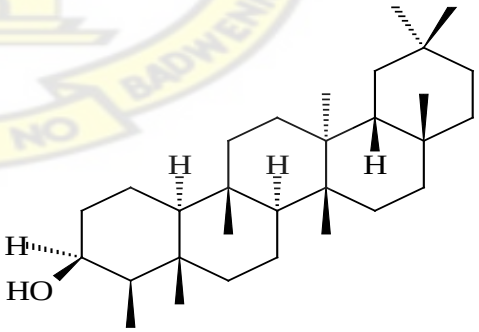
Compounds including steroids have been isolated from *C. splendens*. The steroids clerosterol **[3]** and cycloartenol **[45]** have been isolated from the leaves and stem of *C. splendens* (Pinto and Nes, 1985). The triterpenes *β*-Amyrin **[11]**, Clerodolone **[46]** and Friedelan-3*β*-ol **[47]** have been isolated from the aerial parts of the plant (Joshi, *et al*., 1985). The flavonoids apigenin **[33]** and hispudilin **[34]** have been isolated from the leaf extract of the plant (Shrivastava and Patel, 2007).



Cycloartenol **[45]**



Clerodolone **[46]**



Friedelan -3β- ol **[47]**

2.6 BIOLOGICAL ACTIVITIES OF *CLERODENDRUM*

Many plant species in the genus have been used in various traditional healthcare systems for the treatment of a wide spectrum of disorders. This has led many researchers to carry out a number of *in vivo* and *in vitro* assays to ascertain the validity of these traditional claims. These studies have shown that different species of the genus possess potent anti-inflammatory, anti-diabetic, antioxidant and antimicrobial properties.

2.6.1 Anti inflammatory Activity

Many species of the genus have showed potent anti-inflammatory activity. Surendrakumar (1988) showed that *C. phlomoidis* significantly reduced paw oedema induced by carrageenan in rats at a dose of 1 g/kg. Similarly, the ethanolic root extract of *C. serratum* showed significant anti-inflammatory activity in carrageenan-induced oedema in rats, and also in the cotton pellet model in experimental mice, rats and rabbits at concentrations of 50, 100 and 200 mg/kg (Narayanan *et al.*, 1999).

2.6.2 Antimicrobial activities

The ethyl acetate and hexane leaf extracts of *C. inerme* at 1mg/ml exhibited activity against both animal and plant fungi, *Epidermophyton floccosum, Trichophyton mentagrophytes, Trichophyton tonsurans, Aspergillus flavus* and *Aspergillus niger* (Anitha and Kannan, 2006). The hexane extract of the leaves of *C. colebrookianum* at concentrations of 1000 and 2000 ppm also showed strong antibacterial activities against various Gram positive and Gram negative pathogens including *Staphylococcus aureus, S. haemolyticus, Escherichia coli* and *Pseudomonas aeruginosa* (Misra *et al*. 1995). The flavonoids cabruvin and quercetin isolated from the roots of

C. infortunatum, showed strong antifungal activity. Cabruvin was active against *Alternaria carthami* and *Helminthosporin oryzae*, and quercetin was against *Alternaria alternate* and *Fusarium lini*, at concentrations of 200, 500 and 1000 mg/ml (Roy *et al*., 1996).

2.6.3 Anti-diabetic Activities

So far only *C. phlomidis* has been investigated for its antidiabetic properties. A decoction of the entire *C. phlomidis* plant has been reported to have antidiabetic activity. A dose of 1 g/kg showed antidiabetic effect in alloxan-induced hyperglycemia in rats. It further showed antihyperglycemic activity in human adults at a dose of 15-30 g/day (Chaturvedi *et al*., 1984).

2.6.4 Other Activities

The leaf juice *C. phlomidis* of has been reported to possess anthelmintic activity against *Ascaris lumbricoides, Phreitima posthuma* and *Taenia solium* (Garg and Sidique, 1992). Rani *et al.*, (1999) have also reported the antidiarrhoeal activity of the methanolic extract of the leaves of *C. multiflorum*. The methanolic leaf extract of *C. phlomidis* at a dose of 200 mg/ml showed antispasmodic activity in mice (Murugesan *et al*., 2001). The hexane and methanolic extracts of the whole plant of *C. phlomidis* at concentrations of 100, 300 and 500 mg/kg body weight were established to reduce yeast-induced pyrexia in rats (Ilango *et al*., 2009).

2.6.5 Biological Activities of C. splendens

Gbedema *et al*. (2010) have reported on the antimicrobial and wound healing activities of the leaves of *C. splendens*. A methanolic extract at a concentration of 10 mg/ml was active against *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus mirabilis* and *Candida albicans*. The methanolic extract also significantly promoted wound contraction (69.2%) in 7 days as compared to the control (46.2%) and nitrofurazole (67.5%) when 100 mg of a 33.3% $^{w}/_{w}$ ointment of *C. splendens* was applied to an excision wound on Male Sprague- Dawley rats.

2.7 INFLAMMATION

When the body is injured a sequence of events is initiated that leads to the eventual repair of the site of injury. The first stage in this process is inflammation which is followed by tissue healing and repair. Inflammation which is obtained from the Latin word *inflammatio*, meaning setting on fire, is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants (Ferrero-Miliani *et al.*, 2007). Many diseases have been recognized to have inflammatory components as part of their pathophysiology (Serhan, 2004). It is characterized by a series of events that include the inflammatory reaction (i.e. redness, swelling or oedema, heat, pain and loss of function), a sensory response perceived as pain and initiation of a repair process (Robbins and Cortran, 2004). Inflammation can be classified as either acute or chronic. Acute inflammation is a short-term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus (Cotran *et al.*, 1998). It is characterized by five cardinal signs *rubor* (redness), *calor* (increased heat), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function). Blood that is being carried away through the blood vessels away from the sight of injury results in engorgement of the capillary network.

Engorged capillaries produce the tissue redness and an increase in temperature. An increase in capillary permeability facilitates an influx of fluid and cells from the engorged capillaries into the surrounding tissue. The fluid that accumulates has much higher protein content than fluid normally released. The increased capillary permeability, decreased blood velocity, and increased expression of adhesion molecules facilitate the migration of various leucocytes from the capillaries into the tissues (Suralkar, 2008). There are two phases involved in the inflammatory response: vascular phase and the cellular phase. These reactions are mediated by chemical factors derived from plasma proteins or cells. The fluid exudates contain a variety of mediators which influence the cells in the vicinity and the blood vessels themselves. These include the components of four proteolytic enzyme cascades namely:

a. the complement system
b. the coagulation system
c. the fibrinolytic system
d. the kinin system

These components are proteases that are inactive in their native form; they are activated by proteolytic cleavage, each activated component then activate the next. The activation of these components give rise to more inflammatory mediators (Rang *et al.*, 2003).

The cellular events are concerned with the cells that are involved in the mediation of inflammation. Neutrophils, mast cells, platelets and monocyte/macrophages of the innate immune system are the main effector cells during acute inflammation (Ganz 1993; Smith 1994).

Mast cells are found in the tissues and their cytoplasm is loaded with granules containing mediators of inflammation. Their surface is coated with a variety of receptors which, when engaged by the appropriate ligand, trigger exocytosis of the granules. Mast cells appear to be key players in the initiation of inflammation. Their Toll-like receptors trigger exocytosis when they interact with Pathogen-associated molecular patterns (PAMPs) like the lipopolysaccharide of Gram-negative bacteria and the peptidoglycan of Gram-positive bacteria. Activated mast cells release literally dozens of potent mediators. These mediators are active in recruiting all the types of white blood cells that activate many of these recruited cells which in turn produce their own mediators of inflammation (Stvrtinova *et al*., 1995).

Neutrophils are the most abundant white blood cells and increase in numbers during acute inflammation. The greatest stimulation of these cells is due to bacterial infections. The main task of neutrophils is phagocytosis and killing of invading microorganisms (Tramont and Hoover, 2000). Phagocytosis is accompanied by a prompt increase of oxygen consumption referred to as "the respiratory burst", in which superoxide anion is generated via membrane-bound NADPH oxidase, resulting in the production of microbicidal oxidants and hydrogen peroxide. These, together with the contents of neutrophil granules, have an extensive killing capacity but are short lived (Casimir and Teahan 1994).

Macrophages aid the healing process by engulfing bacteria and dead cells and ingesting them so that the area is clear for new cells to grow. They arrive at the injured site within 72 hours of the injury and may remain in the area for weeks after the injury. These are large phagocytic leucocytes, which are able to travel outside of the circulatory system by moving across the cell membrane of capillary vessels and entering the areas between cells in pursuit of invading pathogens. They are the most efficient of the phagocytes. They can destroy substantial numbers

of bacteria or other cells. Pathogens also stimulate the macrophage to produce chemokines, which summon other cells to the site of infection. These release Tumor Necrosis Factor alpha (TNF-α) and cytokines such as Interleukin 1 (IL-1). IL-1 has both the paracrine and endocrine effects. The paracrine effects cause this cytokine to produce tissue aggregating factor and thus trigger the blood clotting cascade, stimulate the synthesis and secretion of a variety of other interleukins helping to activate T cells and thus initiate an adaptive immune response. The endocrine effects on the other hand induce fever and decrease blood pressure (www.sportsinjuryclinic.net, 2009).

Inflammation is a protective and defensive mechanism of the body. The essence of it is to contain and eradicate local injury and then initiate repair of the damaged tissue (Serhan, 2004). It protects the body by isolating the damaged area, mobilizing effector cells and molecules such as histamines, prostaglandins and TNF-α to the site, and in the end promote healing. In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases, such as hay fever, atherosclerosis, and rheumatoid arthritis. It is for this reason that inflammation is normally closely regulated by the body.

2.8 Experimental Models for Inflammation

2.8.1 Carrageenan- induced paw oedema

Carrageenan is a mixture of polysaccharides composed of sulfated galactose units and is derived from the Irish Sea moss, *Chondrous crispus* (Gigartinaceae). The carrageenan- induced paw oedema model is based on the principle of the release of various inflammatory mediators

(Vinegar *et al.*, 1969; Crunkhun and Meacock, 1971). Oedema formation due to the injection of carrageenan is biphasic with the initial phase attributable to the release of histamine and serotonin. This phase begins immediately after injection of carrageenan and diminishes in two hours. The second phase begins at the end of the first phase and it is due to the release of prostaglandins, proteases and lysosomes. It remains through the third hour up to the fifth hour. Ravi *et al* (2009) used this method to assess the anti-inflammatory property of the methanolic extract of the berries of *Solanum nigrum*. The extract reduced paw oedema by 23.45% by the third hour at a concentration of 375 mg/kg body weight. This method is the most effective and widely used model for evaluating inflammation (Suralkar, 2008).

2.8.2 Acetic Acid -induced Vascular Permeability

This test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by acetic acid by releasing inflammatory mediators. Mediators of inflammation, such as histamine, prostaglandins and leukotrienes are released following stimulation of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a result, fluid and plasma protein are extravaseted and oedemas are formed (Miles and Miles, 1992).

2.8.3 Phorbol Myristate Acetate-induced Ear Oedema in Mice

Phorbol myristate acetate (PMA) is a protein kinase C (PKC) promoter, which induces the formation of free radicals *in vivo*. It has also been demonstrated that pre-treatment of mouse skin by antagonists of PKC suppresses inflammation and reactive oxygen species (ROS). ROS are

involved in the synthesis of mediators and regulate the production of TNF-α which in turn stimulate Phospholipsae A2 (PLA_2) activity. This releases arachidonic acid from phospholipids and stimulates the activity of Cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes involved in the release of different inflammatory mediators. Six hours after PMA application, the mice are killed by cervical dislocation and a 6 mm diameter disc from each ear is removed with a metal punch and weighed. Ear oedema is calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment), and is express as a reduction in weight with respect to the control group. The anti-inflammatory activity of the ethanolic root extract of *Heliopsis longipes* was evaluated by Hernandez *et al.,* (2009) using this method. The extract showed a dose-dependent anti-inflammatory effect with ED_{50} of 2.0.

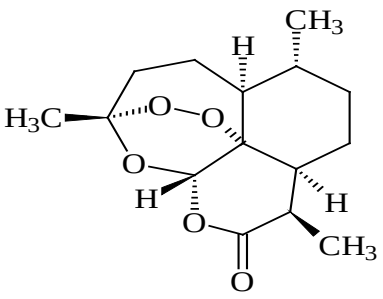
2.9 ANTIMICROBIALS FROM HIGHER PLANTS

There has been great success in the development of anti-infective agents. However, a continued search for new anti-infective agents remains indispensable because of increased microbial resistance and the untoward effects of these agents (Weisser *et al.*, 1966). According to Arora and Keur (1999), the success story of chemotherapy lies in the continuous search of new drugs to counter the challenges posed by resistance strains of microorganisms. The investigation of certain indigenous plants for their antimicrobial properties may yield useful results. Many studies indicate that in some plants there are many substances such as peptides, alkaloids, essential oils, phenols, coumarins and flavoniods which confer antimicrobial properties to them. These compounds have potentially significant therapeutic application against human pathogens, including bacteria, fungi or virus (Arora and Keur, 1999; Okigbo and Igwe, 2007). However, there are no single chemical entity plant-derived antibacterials used clinically, and this

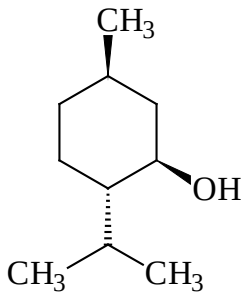
chemically diverse group deserves consideration as a source because of the fact that plants have an exceptional ability to produce cytotoxic agents (Gibbons, 2005).

Many researchers all over the world have conducted, and are still conducting studies into plants that can help unearth constituents of plants that could be used against microbial infections. The sesquiterpene endoperoxide, artemisinin **[47]**, which was first isolated in 1972, is a constituent of the Chinese medicinal plant, *Artemisia annua* (Asteraceae) that has been employed in China for centuries as an anti-infective and for the treatment of malaria. This compound has since been used successfully for the treatment of malaria in China and other parts of the world (Kinghorn, 1987).

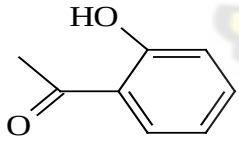
Al-Bayati (2009) investigated the antimicrobial activity of menthol isolated from *Mentha longifolia* (Lamiaceae). The results of the study indicated that menthol **[48]** inhibited the growth of the Gram-positive bacteria *Staphylococcus aureus, Streptococcus mutans, S. faecalis, S. pyogenis* and *Lactobacillus acidophilus* at concentrations of 1:1, 1:5, 1:10 and 1:20. Hettiarachchi *et al*., (2006) have also isolated three compounds, 2-Hydroxyacetophenone **[49]**, carinol **[50]** and carissone **[51]** from *Carissa lanceolata* (Apocynaceae), which were all active against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis* with a minimum inhibitory concentration (MIC) of less than 0.5 mg/ml against *Staph aureus* and *E.coli.* The flavonoid, quercetin **[52]** from *Quercus rubra* has been found to possess anti-HIV activity (Fesen *et al*., 1993).



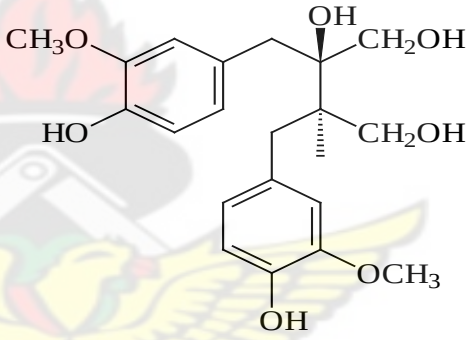
Artemisinin **[47]**



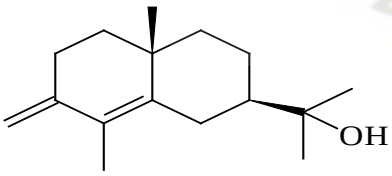
Menthol **[48]**



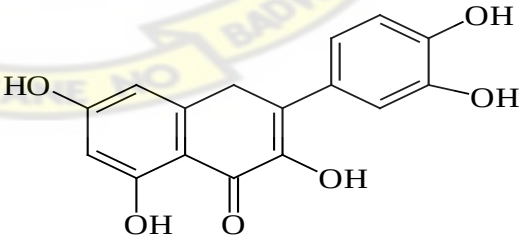
2- Hydroxy acetophenone **[49]**



Carinol **[50]**



Carissone **[51]**



Quercetin **[52]**

2.9.1 Mechanism of antimicrobial action

The mechanism of action of different antimicrobial agents is ultimately aimed at the ability to kill or inhibit growth. These include inhibition of cell wall synthesis. Antibiotics that work in this way target the peptidoglycan-based cell walls of microorganisms that humans' lack. This leads to the inability of the microorganism to synthesize its cell wall leading to osmotic lysis and death of the organism. Another mode of action of antibiotics is the inhibition of protein synthesis. Antibiotics that inhibit protein synthesis take advantage of the fact that bacterial ribosome and the eucaryotic ribosome differ structurally; consequently, there exist chemicals that can inhibit bacterial translation but not eucaryote translation. A limitation is that the mitochondria ribosome is structurally similar to the eubacteria ribosome; this gives antibiotics that inhibit protein synthesis a potential for toxicity. Inhibition of nucleic acid synthesis is another way that antibiotics act. The antibiotics may block synthesis of nucleotides, inhibit replication or stop transcription (Lambert and O'Grady, 1992).

Coumarins appear to be the only group of plant antibacterials for which a specific target has been suggested. This is based on the chemically related antibiotic novobiocin, a systemically used therapeutic agent that targets DNA gyrase and is produced by several different *Streptomyces* species.

2.10 ANTIBACTERIAL AND ANTIFUNGAL MODELS

Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various micro-organisms to samples that are placed in contact with them (Cos *et al.*, 2006). The antibacterial and antifungal test methods are classified into three main

groups: diffusion, dilution and bioautographic methods. Many research groups have modified these methods to enable them carry out tests on a wide range of samples, like non-polar samples such as essential oils and polar and aqueous extracts. These modifications make it almost impossible to directly compare results. It is therefore imperative to include at least one, but preferentially several reference compounds in each assay (Cos *et al.*, 2006).

2.10.1 The Agar diffusion methods

This technique makes use of the ability of the test sample at a known concentration to diffuse into the inoculated medium from a reservoir to inhibit microbial growth. The diameter of the clear zone (zone of inhibition) around the reservoir is measured at the end of the incubation period. Different types of reservoirs can be used, such as filter paper discs and holes punched in the set agar to create a well.

In the agar well diffusion assay a few millimetre-diameter holes are cut into the inoculated agar surface and the wells are filled with test samples. The plates are left for a few hours at room temperature to allow diffusion into the agar medium, then the plates are incubated at the appropriate temperature and the zones of inhibition are measured (Tepe *et al.*,2004) . This method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs (Cole, 1994). The small sample requirements and the possibility to test up to six extracts per plate against a single micro-organism are specific advantages of the method (Hadacek and Greger, 2000). It is, however, not very appropriate for samples that do not easily diffuse into agar (Cos *et al.*, 2006).

2.10.2 The Dilution method

In this method the test sample is mixed with a suitable medium which has already been inoculated with the test organism. Dilution methods are mainly used to determine the Minimum Inhibitory Concentration (MIC) of pure substances and extracts. In liquid or broth-dilution methods, turbidity and redox-indicators are most frequently used. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm. The liquid-dilution method also allows determination whether a compound or extract has a bactericidal or bacteriostatic action at a particular concentration. The minimal bactericidal or fungicidal concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures and assessing growth or no-growth after incubation. At present, the redox indicators 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and resazurin are frequently used to quantify bacterial (Eloff, 1998; Gabrielson *et al*., 2002) and fungal growth (Jahn *et al*., 1995; Pelloux-Prayer *et al*., 1998). In general, dilution methods are appropriate for assaying polar and non-polar extracts or compounds for the determination of MIC and MBC/MFC-values (Cos *et al*., 2006).

2.10.3 Bioautographic method

Bioautography localizes antimicrobial activity on a chromatogram using three approaches. The first approach is direct bio-autography. In this method, a chromatogram is dipped directly in a suspension of microorganisms growing in a suitable broth, or the suspension is sprayed directly onto the chromatogram (Homans and Fuchs, 1970). The chromatogram is incubated at the appropriate temperature and microorganisms grow directly on it. For location and visualization

of antibacterials, tetrazolium salts are usually used, which are converted by the dehydrogenases of living microorganisms to intensely coloured, formazan. Thus, the spots which are antibacterial cannot produce this intensely coloured formazan, because the bacteria within the spots are dead. The most frequently used tetrazolium dye is 3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide (MTT) (Choma, 2005).

The second approach is contact bio-autography. In this method, antimicrobial agents diffuse into an inoculated agar plate from a chromatogram through direct contact (Meyers and Smith, 1964). The chromatogram is placed face down onto the inoculated agar layer and left for some minutes or hours to allow diffusion of the sample into the agar. Then the chromatogram is removed and the agar layer is incubated at the appropriate temperature. The inhibition zones are observed on the agar surface in the places where the spots of antimicrobials diffused into the agar. The disadvantages of contact bio-autography are difficulties in obtaining complete contact between the agar and the plate and adherence of the adsorbent to the agar surface (Choma, 2005).

The third approach is immersion bio-autography or agar over-lay bioautography. In this method the chromatogram is covered with a molten, seeded agar medium. This is a hybrid between the contact and direct methods. Antimicrobials are transferred from the TLC plate to the agar layer as in the contact assay but during incubation and visualization the agar layer stays onto the plate as in direct bioautography. The main disadvantage of this method is lower sensitivity caused by dilution of antibacterials in the agar layer compared with direct bioautography. Agar overlay is advised especially when direct bioautography is impossible to perform (Hostettman and Marston, 1994).

Despite the high sensitivity of the bio-autographic technique, its applicability is limited to micro-organisms that easily grow on TLC plates. Other problems are the need for complete removal of residual solvents of the mobile phase, and the transfer of the active compounds from the stationary phase into the agar layer by diffusion. However, because bio-autography allows localizing antimicrobial activities of an extract on the chromatogram, it supports a quick search for new antimicrobial agents through bioassay-guided isolation (Cos *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Plant Material

The leaves of *Clerodendrum splendens* G.Don (Verbenaceae) were collected from Asokore Mampong in Kumasi in May, 2008. The plant material was authenticated by Mr. Ntim at the Forestry Herbarium Resource Management Support Centre, Forestry Commission in Kumasi and a voucher specimen (KNUST/HM1/2010/L033) has been deposited at the herbarium of the Faculty of Pharmacy and Pharmaceutical Sciences. The leaves were air-dried for four days, ground into coarse powder and stored at room temperature in the laboratory until when needed.

3.1.1. Extraction of Powdered Material

The powdered leaves of *C. splendens* (1.0 kg) were successively extracted by Soxhlet with petroleum ether (40-60 $^{\circ}$C), ethyl acetate and 70% ethanol. Each extract was evaporated under reduced pressure using a rotary evaporator (R-114, Buchi, Switzerland) until all the solvent was removed to produce a viscous extract. The petroleum ether, ethyl acetate and 70% ethanol extracts gave yields of 9.19% $^{w}/_{w,}$ 9.59% $^{w}/_{w}$ and 13.45% $^{w}/_{w}$ respectively.

3.2 Chromatographic Materials

Silica gel was used for both the column and thin layer chromatographic (TLC) techniques. The types used are:

- Silica gel (70-230 mesh) for column chromatography

- Pre-coated thin layer chromatography (TLC) aluminium plates (Silica gel 60 F_{254}) for TLC analysis.

3.3 Detecting Reagent

The detecting reagent used for the TLC analysis was Anisaldehyde in conc. H_2SO_4, with the following composition

Anisaldehyde	0.5 ml
Glacial Acetic Acid	10 ml
Methanol	80 ml
Conc. Sulphuric Acid	5 ml

3.4 Solvent Systems Employed

The solvent systems used as the mobile phase for the isolates were;

- Ethyl acetate : Petroleum ether (7:3)
- Chloroform : Methanol (9:1)
- Pet ether : Ethanol (8:2)

3.5 Materials used in Antimicrobial Studies

The strains of microorganisms used were *Staphylococcus aureus* NCTC 10788, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* NCTC 10662, *Eschericia coli* ATCC 25922 and *Candida albicans* ATCC 102321. These were all obtained from the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST- Kumasi.

Nutrient broth, Nutrient agar and Sabouraud agar, all from Merck (U.K) were used as media to support the growth of microorganisms used.

Standard antibiotics used as reference drugs were Ketoconzole and Ciprofloxacin manufactured by Torrent (India). A 2% concentration of DMSO in sterile distilled water was used as the negative control.

3.6 Materials used in Anti-inflammatory Studies

Day old post-hatched Cockerels (*Gallus gallus*; strain Shaver 579) were obtained from Akropong Farms in Kumasi. The chicks were housed in standard environmental conditions at the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. The standard drugs used for the positive control were diclofenac sodium and dexamethasone. Carrageenan sodium salt from Sigma - Aldrich Inc., St Louis, MO, USA was used to induce oedema in the chicks.

3.7 Chromatographic techniques

3.7.1 Column Chromatography

Silica gel (70-230 mesh) was gently packed dry into a column. The extract to be separated was adsorbed onto some amount of the silica gel and packed on top of it and a cotton wool placed on the packed column. The solvent or mixture of solvents to be used in eluting the chromatographic column was placed on top of the packed column to separate the extract into different fractions and the eluates collected into glass bottles. This method was used in the fractionation of the various extracts and isolation of the compounds.

3.7.2 Thin layer chromatography

The one way ascending technique was used. Samples to be analysed by TLC were dissolved in suitable organic solvent(s) and applied on pre-coated silica gel TLC plates as spots with the aid of capillary tubes at one end of the plate in a straight line, about 2 cm above the bottom edge and 1.5 cm away from the sides. The spots were dried and the plates placed in a chromatank containing the mobile phase that has been prepared in the tank at least 30 minutes earlier. The mobile phase ran along the TLC plate in an ascending manner due to capillary action, carrying with it the components of the extract or the mixture. When the mobile phase reached the desired distance, the plate was removed, the solvent front marked and the plate dried. The separated compounds were identified by observing the chromatogram under ultra-violet light for fluorescence. This was followed by spraying with anisaldehyde in conc. H_2SO_4 and heating at 120°C. This method was used for all TLC analysis (Ghani, 1990).

3.8 Chromatographic Fractionation of Ethyl Acetate Extract of *C. splendens*

100 g of the ethyl acetate extract was reconstituted in a minimum amount of ethyl acetate and adsorbed onto silica gel and the solvent allowed to evaporate. This was packed onto a column of size CR 82/90 packed with 600 g of silica gel (70-230 mesh). The column was initially eluted with 100 % petroleum ether followed by 20 %, 50 % and 80 % ethyl acetate in pet ether; ethyl acetate (100 %), and 10 %, 20 % and 50 % ethanol in ethyl acetate. Approximately 80 fractions of 80 ml aliquots were collected.

TLC analysis (as described under 3.2.2) of the eluates led to the bulking of the eluates into 10 fractions. These were labeled **CSE1** (1.2 g), **CSE2** (1.8 g), **CSE3** (5 g), **CSE4** (4.5 g), **CSE5** (13.2 g), **CSE6** (14.3 g), **CSE7** (2.3 g), **CSE8** (13.5 g), **CSE9** (20.1 g) and **CSE10** (19.2 g).

The bulked fractions were screened against the test organisms. Fractions **CSE6** and **CSE8** were the most active with **CSE5** having the highest activity against *C. albicans*.

3.8.1 Isolation of Compounds from Fraction CSE6

Fraction **CSE6** (14.3 g) was column-chromatographed over silica gel (70-230 mesh). The column was eluted with 50 %, 40 %, 30 %, 20 % and 10 % of petroleum ether in ethyl acetate. This was followed with ethyl acetate, then 20 %, 40 %, 60 % and 80 % of ethanol in ethyl acetate. 50 fractions of 50 ml aliquots were collected. Based on the TLC profiles of the eluates, they were bulked into five fractions labelled **6a** (3.9 g), **6b** (3.9 g), **6c** (2.1 g), **6d** (1.7 g) and **6e** (1.2 g).

6a was further column chromatographed on silica gel and eluted with 100 % petroleum ether, 90, 80, 70, 60, 50, 40, 30, 20 and 10 % petroleum ether in ethyl acetate. This was followed by100 %

ethyl acetate, then 10, 20 and 50% ethanol in ethyl acetate. 80 fractions of 10 ml aliquots were collected. Five sub-fractions were obtained after TLC analysis namely; **A_1, A_2, A_3, A_4** and **A_5.** **A_2** which was ran with 60 % of ethyl acetate in petroleum ether afforded the compound **CSE6a** (1.2 g) which appeared as single spot with an R_f value of 0.46 on TLC analysis using ethyl acetate- pet ether (7:3) and anisaldehyde as a detecting reagent.

3.8.2 Isolation of compounds from CSE5

Fraction **CSE5** (13.2 g) was column chromatographed on silica gel and eluted with pet ether, then with 80, 60, 40 and 20% of pet ether in ethyl acetate. Elution was continued with 20% and 40% ethanol in ethyl acetate to yield thirty fractions of approximately 50ml aliquots. Following TLC analysis, the fractions were bulked into 6 sub-fractions that were labeled **5a** (1.5 g), **5b** (1.0 g), **5c** (2.4 g), **5d** (3.5 g), **5e** (1.8 g) and **5f** (0.8 g).

5f which showed one compound was washed on a short silica gel column with ethyl acetate to give a **CSE5a** (10 mg). The R_f value was found to be 0.64 after analysis was done on TLC plate and ran with pet ether-ethyl acetate (4:6).

A schematic representation of the isolation of compounds from *C. splendens* is shown in Fig 2.

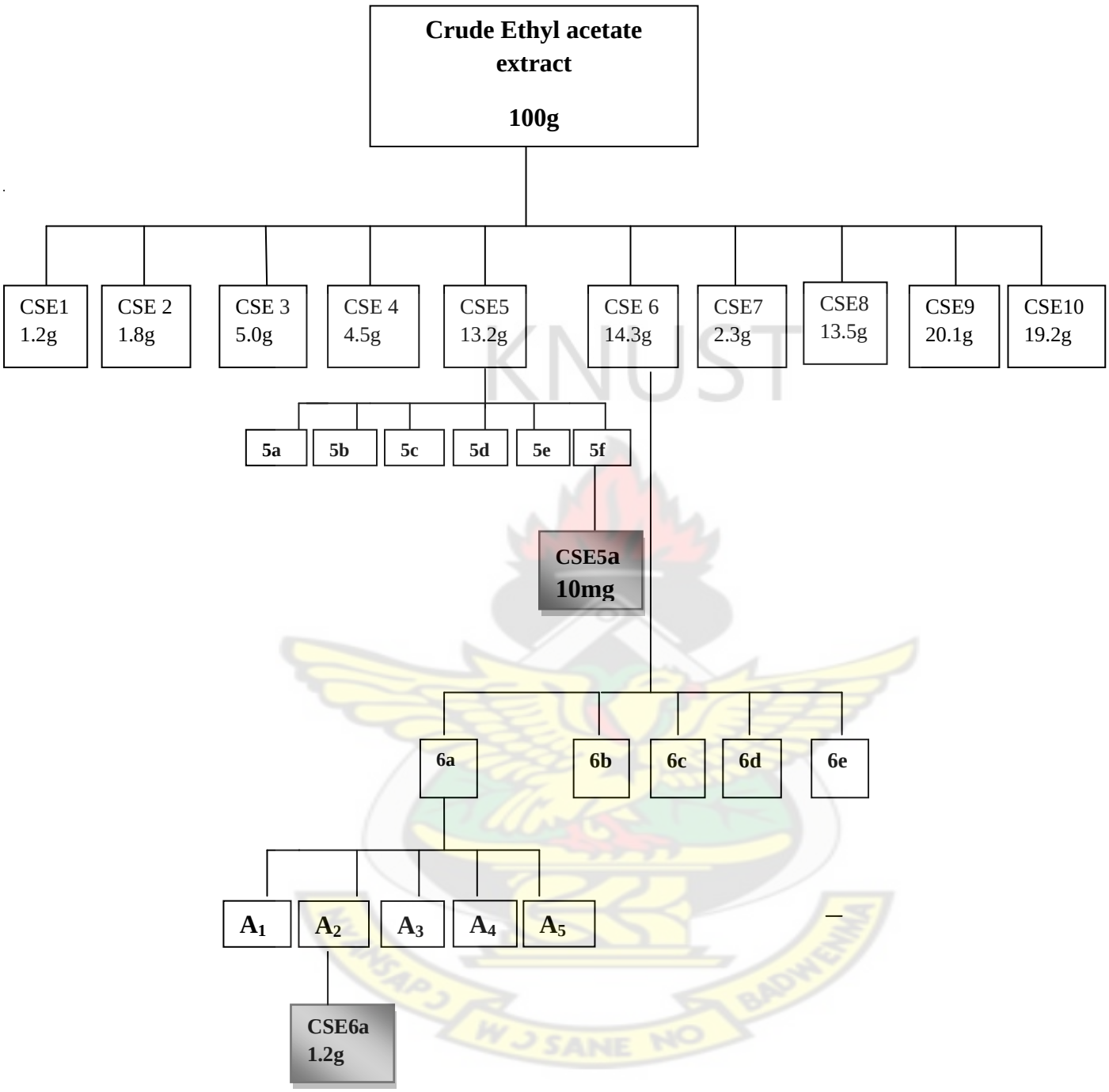


Figure 2: A schematic representation of the fractionation and isolation of compounds from the Ethyl acetate extract of the leaves of *C. splendens*

3.9 ANTIMICROBIAL ASSAY

The agar-well diffusion assay for antimicrobial sensitivity was employed in the study. With this method, 20ml of nutrient agar was melted and stabilized at 45°C in a water bath. Broth cultures of the various organisms were sub-cultured 24 hours before use. The stabilized agar was seeded with 0.1 ml of a test organism. The tube containing the agar was rolled between the palms to ensure thorough mixing. The seeded agar was then poured into a sterile Petri dish and allowed to set under a lamina air flow cabinet for 30 minutes. A sterilized cork borer with a diameter of 10 mm (number 6) was used to produce wells in the seeded set agar. A volume of 0.1 ml of 10 mg/ml of the various extracts (petroleum ether, ethyl acetate and 70 % ethanol) and 0.1 ml of 0.5 mg/ml of the positive controls (ciprofloxacin and ketoconazole) reconstituted in 2 % DMSO in sterile distilled water, were subsequently introduced into the wells and allowed to stand for about $1^1/_2$ hours under the laminar air flow to ensure adequate diffusion before incubation at 37°C for 24 hours. Three replicates of the assay were performed. The antimicrobial activity was evaluated by measuring the diameter of the zones of inhibition against the test organisms.

3.10 ANTI INFLAMMATORY ASSAY

The anti-inflammatory properties of the extracts were evaluated using the carrageenan-induced foot oedema in 7-day old chicks as described by Roach and Sufka (2003) with some modifications. The experiment was performed to evaluate the prophylactic effects of the petroleum ether, ethyl acetate and 70% ethanolic extracts on the oedema component of inflammation. Dexamethasone, a steroidal anti-inflammatory drug and diclofenac, a non-steroidal anti-inflammatory drug were used as positive controls. In this method, chicks were randomly selected, grouped (5 per group) and fasted for 24 hours before the experiment. Water

was available *ad libitum*. The extracts were prepared by dissolving in 2% tragacanth and distilled water. Doses of 30, 100 and 300 mg/kg were prepared and given orally (*p.o*) 1h before the carrageenan challenge and for the diclofenac (10, 30 and 100 mg/kg) and dexamethasone (0.1, 1.0 and 3 mg/kg) given intraperitoneally (*i.p*) 30 minutes before the carrageenan challenge. The foot thickness of each chick was measured before carrageenan injection (baseline measurement) and then at hourly intervals over the next 6 hours post carrageenan injection. The right footpads of the chicks were injected *intraplantar* with carrageenan (10 µl of a 1% solution in saline). The change in foot thickness for the various groups was recorded hourly for six hours by means of a digital caliper. The oedema component of inflammation was quantified by measuring the foot thickness before carrageenan injection and at the various time points after the injection.

3.11 Statistical Methods

Raw scores for the right foot thickness were individually normalized as percentage of change from their values at time 0 and then averaged for each treatment group. The time-course curves for foot thickness were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance with Bonferroni's *post hoc t* test. Total foot thickness for each treatment was calculated in arbitrary units as the area under the curve (AUC) and to determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ Inhibition\ of\ oedema = \left\{ \frac{AUCcontrol - AUCtreatment}{AUCcontrol} \right\} x\ 100$$

The data was presented as the effect of drugs on the time course and the total oedema response for 6 h using the GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA).

3.12 Spectroscopic Studies

Nuclear magnetic resonance (NMR) spectroscopy is widely used as one of an array of analytical techniques available for structural analysis in Chemistry. It relies on magnetic properties possessed by some nuclei, notably ^{1}H, ^{13}C, ^{19}F and ^{31}P. Proton NMR (Hydrogen-1 NMR or ^{1}H NMR) is the commonest method used. It is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, to determine its structure. Carbon-13 NMR (or 13C NMR or sometimes simply referred to as carbon NMR) is also the application of NMR spectroscopy with respect to carbon. It is analogous to proton NMR and allows the identification of carbon atoms in an organic molecule just as proton NMR identifies hydrogen atoms. As such ^{13}C NMR is an important tool in chemical structure elucidation in organic chemistry. ^{13}C NMR detects only the ^{13}C isotope of carbon, whose natural abundance is only 1.1%, because the main carbon isotope, ^{12}C, is not detectable by NMR (Silverstein *et al*., 1991).

Correlated Spectrosopy (COSY) is a two dimensional (2-D) experiment which indicates all spin-spin coupled protons in one spectrum. In the COSY spectrum, two essentially identical chemical shift axes are plotted orthogonally. This helps in the facilitation of elucidation of the chemical structure of the compound in question.

Other 2D methods that help in structure elucidation are Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Coherence (HMBC). These are H-C correlation techniques that allow for the determination of carbon to hydrogen connectivity. HMQC is selective for direct C-H (^{1}J) coupling while HMBC gives long range couplings, 2-3 bond or $^{2\text{-}3}J$ couplings (Martin and Zekter, 1988).

These experiments were performed to facilitate the elucidation of the isolated compounds. They were performed by Prof. Berhanu M. Abegaz of the Department of Chemistry, University of Botswana.

CHAPTER FOUR

4.0 RESULTS

4.1 CHARACTERISATION OF COMPOUNDS

4.1.1 Characterisation of CSE6a as Cleroindicin F

CSE6a was isolated as an oily compound from the ethyl acetate fraction **CSE6**. It appeared as an orange spot on the thin layer chromatogram with an R_f value of 0.46 [silica gel; ethyl acetate: petroleum ether (7:3)] when sprayed with Anisaldehyde reagent.

The structure of **CSE6a** was established by 1-D and 2-D NMR spectroscopy, using various experiments such as COSY, HMQC and HMBC and from comparison with spectroscopic data with those published for this compound (Tuntiwachwuttikul, *et al*., 2003; Tian *et al*., 1997, Endo and Hikino, 1984).

It was identified as Cleroindicin F, a compound which has been previously isolated from the aerial parts of *Clerodendrum indicum* (Tian *et al*., 1997)

The ^{1}H NMR spectrum (Table 4.1) showed the presence of three deshielded protons at δ_H 3.96 (H-2a), δ_H 4.09 (H-2b) and δ_H 4.26 (H-9) due to their attachment to oxygen bearing carbons. There were two olefinic protons at δ_H 6.79 (H-5) and δ_H 6.03 (H-6). It further showed four methylene protons at δ_H 2.24 (H-3a), δ_H 2.35 (H-3b), δ_H 2.61 (H-8a) and δ_H 2.78 (H-8b). The ^{13}C NMR spectrum revealed a quaternary carbonyl carbon (δ_C 197.11), a quaternary oxygenated carbon (δ_C 75.57), an oxymethine (δ_C 81.59) and an oxymethylene (δ_C 66.30), two methylene carbons and two olefinic carbons.

Table 4.1: ^{1}H NMR and ^{13}C NMR data of CSE6a

Position	δ ^{1}H Chemical shift (ppm)	δ ^{13}C Chemical shift (ppm)
C-2	3.96; 4.09	66.30
C-3	2.24, 2.35	39.49
C-4	-	75.57
C-5	6.79	148.15
C-6	6.03	128.69
C-7	-	197.11
C-8	2.61; 2.78	40.19
C-9	4.26	81.59

Table 4. 2: COSY Correlations of CSE6a

H- Position	δ_H – ppm	COSY Correlations(δ_H)
H-2a	3.96	2.24, 2.35, 4.09
H-2b	4.09	2.24, 2.35, 3.96
H-3a	2.24	2.35, 3.96, 4.09
H-3b	2.35	2.24, 3.96, 4.09
H-5	6.79	6.03
H-6	6.03	6.79
H-8a	2.61	2.78, 4.26
H-8b	2.78	2.61, 4.26
H-9	4.26	2.61, 2.78

In the HMQC spectrum all the direct carbon- proton correlations were assigned (Table 4.3). The carbon signal at δ_C 66.30 (C-2) showed direct correlations with δ_H 3.96 (H-2a) and δ_H 4.09 (H-2b). These germinal protons showed COSY correlations (Table 4.2) with the methylene protons at δ_H 2.24 (H-3a) and δ_H 2.35 (H-3b) which directly correlated with δ_C 39.49 (C-3). The oxygenated carbon at δ_C 81.59 (C-9) correlated with the proton signal at δ_H 4.26 (H-9) in the HMQC spectrum. This proton also showed COSY correlation with the methylene protons at δ_H 2.61 (H-8a) δ_H 2.78 (H-8a). The HMQC spectrum further showed direct correlation of the olefinic protons (H-5 and H-6) with δ_C 148.15 (C-5) and δ_C 128.69 (C-6). These led to the partial structures **a** to **c** (Figure 3).

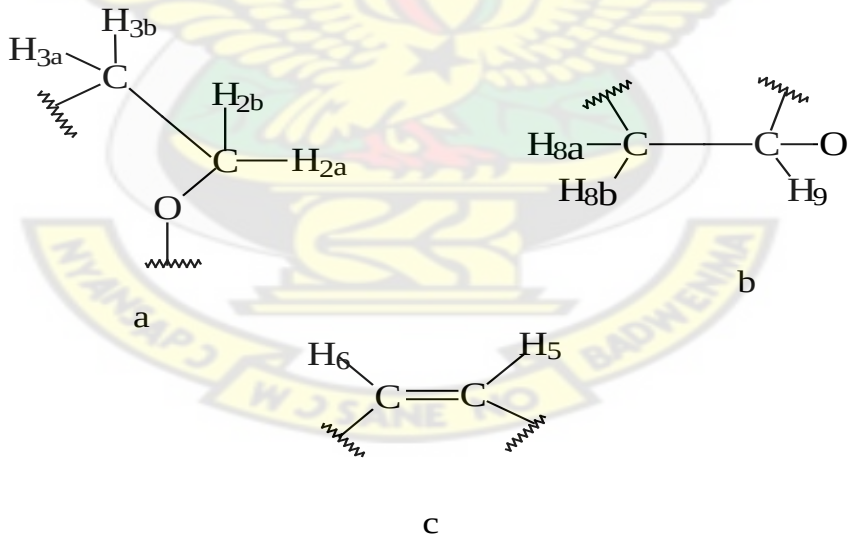
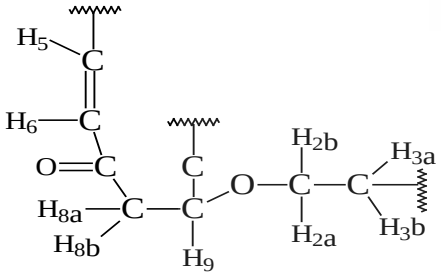


Figure 3: Partial structures a-c

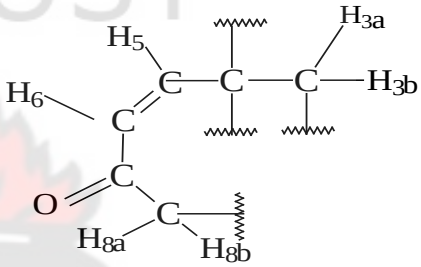
Table 4.3: HMQC and HMBC Correlations of CSE6a

C- Position	δ_C – ppm	HMQC (δ_H)	HMBC (δ_H)
C-2	66.30	3.96; 4.09	2.24, 2.35, 3.69, 4.09, 4.26
C-3	39.49	2.24, 2.35	2.24, 2.35, 3.96, 4.09, 4.26, 6.79
C-4	75.57	-	2.24, 2.35, 2.61, 2.78, 3.96, 4.09, 4.26, 6.03
C-5	148.15	6.79	2.24, 2.35, 4.09, 6.79
C-6	128.69	6.03	2.61, 2.78
C-7	197.11	-	2.61, 2.78, 6.79
C-8	40.19	2.61; 2.78	2.61, 2.78, 6.03
C-9	81.59	4.26	2.24, 2.35, 2.61, 2.78, 3.96, 6.79

In the HMBC spectrum (Table 4.3), the oxymethine carbon at δ_C 81.57 (C-9) showed long range correlations (^3J and ^2J) with the methylene protons at H-2a and H-2b, H-8a and H-8b and those at H-3a and H-3b. The position of the carbonyl function was established by the long range correlation with the olefinic protons H-5, and H-8a and H-8b, and H-9. The HMBC spectrum further showed ^3J correlations between H-5 and δ_C 39.49 (C-3) and H-6 and δ_C 40.19 (C-8). This analysis suggests the partial structures **d** and **e** (Figure. 4).



d



e

Figure 4: Partial structures d and e

In the HMBC spectrum, the quaternary oxygenated signal at d 75.57 (C-4) showed ^2J correlation with H-3a and H-3b, and the methine proton at H-9. It also showed ^3J correlation with H-2a, H-2b, H-8a and H-8b, and the olefinic proton at H-6. This established the structure of **CSE6a** as Cleroindicin F (Fig. 5).

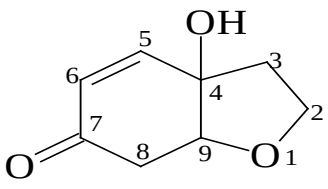


Figure 5: Cleroindicin F

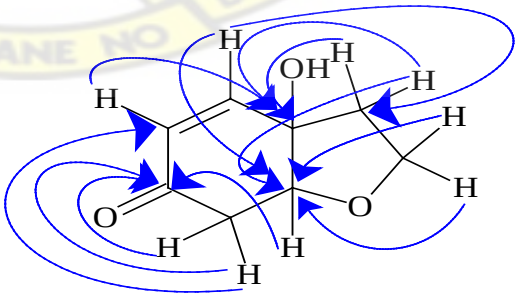


Figure 6: Some selected HMBC Correlations for CSE6a

4.1.2 Characterisation of CSE5a as Apigenin

CSE5a was isolated as a yellow amorphous powder. It gave a yellow spot on a silica gel thin layer chromatogram when sprayed with anisaldehyde reagent [R_f of 0.6; pet ether: ethyl acetate (3:7)]. The time of flight mass spectrum (TOF-MS) gave a molecular mass of 270.05 with a molecular formula of $C_{15}H_{10}O_5$. The structure of **CSE5a** was determined using 1D NMR spectroscopy and comparison of the spectra and its physical characteristics with that of published data. The ^{1}H-NMR spectrum showed a deshielded singlet at δ_H 13.02 typical of a 5- hydroxy substituited flavonoid. The spectrum also showed a singlet at δ_H 6.65 attributable to H-3 of a flavone. There were two meta-coupled doublets at δ_H 6.27 and δ_H 6.55 which were attributed to H-6 and H-8 respectively. The signals at δ_H 7.05 and δ_H 7.95 which integrated for two protons each is suggestive of a *para*-di substituited aromatic ring B. The substituents at C-7 and C-4' were attributed to hydroxyl groups. The ^{1}H-NMR chemical shifts obtained for **CSE5a** compared with that of apigenin (Table 4.4) isolated from tobacco leaves by Fathiazad, *et al*. (2006).

This gave the structure of **CSE5a** as apigenin (Figure 7) a known compound that has been isolated from other plants.

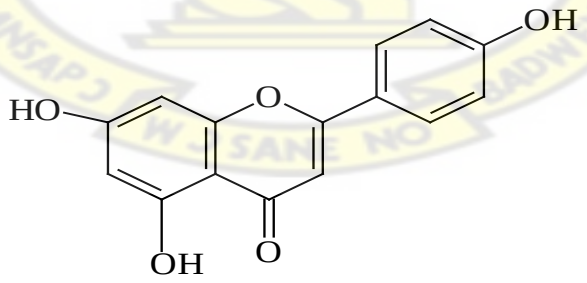


Figure 7: Apigenin

Table 4.4: ^{1}H NMR data of CSE5a and Apigenin

H-Position	CSE5a (δ_H-ppm)	Apigenin[a](δ_H-ppm)	*J* (Hz)[a]
H-3	6.65	6.81(s)	-
H-5	13.02	12.94(s)	-
H-6	6.27	6.21(d)	2.1
H-8	6.55	6.50(d)	2.1
H-2'/H-6'	7.95	7.95(d)	8.8
H-3'/H-5'	7.05	6.94(d)	8.8

[a] Reference: Fathiazad, *et al.* (2006).

4.3 BIOLOGICAL ACTIVITIES

The plant extracts were tested for their antimicrobial and anti-inflammatory activities. The various extracts demonstrated varying degrees of activity.

4.3.1 Antimicrobial activities of the Petroleum ether, Ethyl acetate and 70% Ethanolic extracts from C. splendens.

This experiment was performed *in vitro* to assess the antimicrobial effect of the extracts on the test organisms; *Staph aureus, B. subtilis, P. aeruginosa, E. coli* and *C. albicans*. All the extracts showed some level of activity with the ethyl acetate extract exhibiting the highest level of activity with a zone of inhibition ranging from 4.00 ± 0.5 4mm to 9.0 ± 0.16 mm. The activity of the petroleum ether and the ethanolic extracts ranged between 3.50 ± 0.50 mm to 4.67 ± 0.33 mm and 3.33 ± 0.47 mm to 4.3 ± 0.47 mm respectively. These results are shown in Table 4.5 which captures the mean zones of inhibition measured in millimetres (mm).

4.3.2 Antimicrobial Activities of Chromatographic Fractions of the Ethyl Acetate Extract

The ethyl acetate extract having exhibited the strongest microbial inhibition, was further column chromatographed to produce ten sub-fractions. The sub-fractions, **CSE1-CSE10** were tested for their antimicrobial activities. **CSE6** and **CSE8** were the most active with zones of inhibition between 8.67 ± 0.52 to 11.0 ± 0.31 mm for **CSE6** and 15.65 ± 0.58 to 10.0 ± 0.36 mm for **CSE8**. The summary of the results is given in Table 4.6. The results are expressed as the mean zones of inhibition measured in millimeters (mm).

Table 4.5: Antimicrobial Activities of *C. splendens* extracts

Extracts	Mean Zones of Inhibition (mm)				
	E.coli	*B. subtilis*	*Staph aureus*	*P. aeruginosa*	*C. albicans*
Pet ether	4.2 ± 0.17	4.3 ± 0.67	4.2 ± 0.17	3.5 ± 0.50	4.7 ± 0.33
Ethyl acetate	9.0 ± 0.17	7.0 ± 0.17	5.2 ± 0.44	4.0 ±0.50	6.3 ± 1.17
70% ethanol	3.7 ± 0.33	4.0 ± 0.56	4.3 ± 0.33	3.3 ± 0.33	3.7 ± 0.33
Ciprofloxacin[a]	20 ± 0.67	24 ± 0.50	17.5 ± 0.71	23.5 ± 0.43	-
Ketoconazole[b]	-	-	-	-	18 ± 0.30
2% DMSO	0	0	0	0	0

-; no assay performed, the data are shown as mean ± Standard Error of the Mean (SEM), [a,b] ; positive controls

Table 4.6: Antimicrobial Activity of fractions obtained from the Ethyl Acetate extract of *C. splendens*

FRACTIONS	Test Organisms and Zones Of Inhibition (mm)			
	E.coli	*Staph aureus*	*B. subtilis*	*C. albicans*
CSE1	0	0	0	0
CSE2	0	0	0	0
CSE3	0	0	0	4.5 ± 0.16
CSE4	0	0	0	11 ± 0.33
CSE5	0	0	0	13.67 ± 1.76
CSE6	0	8.67 ± 0.52	11.0 ± 0.31	9.33 ± 0.72
CSE7	0	6 ±0.53	10 ±0.24	4.33 ±0.70
CSE8	12 ± 1.67	12.33 ± 0.41	15.67 ± 0.58	10 ± 0.36
CSE9	0	0	8.67 ± 0.37	9 ± 0.48
CSE10	8 ± 0.18	0	13 ± 0.71	3 ± 0.12
Ciprofloxacin[a]	21 ± 0.49	16 ± 0.71	22 ± 0.36	-
Ketoconazole[b]	-	-	-	17 ± 0.32
2% DMSO	0	0	0	0

- =no assay performed, values are shown as mean ± SEM, [a,b]= positive controls

4.4 Anti inflammatory Activities of Pet Ether, Ethyl Acetate and 70% Ethanolic Extracts of *C. splendens.*

The anti-inflammatory potential of the petroleum ether, ethyl acetate and 70% ethanolic extracts of the leaves of *C. splendens* were investigated using the carrageenan-induced chick foot pad oedema. Injection of carrageenan into the right foot pad of the chicks caused an increase in the foot volume (oedema) of the chicks. Figures 8 and 9 illustrate the time course curve and area under the curve (AUC) of the effect the extracts of *C. splendens*, dicofenac and dexamethasone prophylactically on carrageenan-induced oedema. The time course curves (treatment x time) revealed a dose-dependent effect of the extracts on oedema (Figure 8). Furthermore, when total oedema over the period of the experiment is represented arbitrary as AUC of the time course curves, the petroleum ether, ethyl acetate and 70% ethanolic extracts significantly reduced total oedema with a maximal inhibitory effect of 47.29 ± 8.65%, 66.09 ± 13.13% and 45.19 ± 5.09% respectively at 300 mg/kg (Table 4.7).

Diclofenac (10-100 mg/kg, *i.p*) also showed significant effect on the time course curve and total oedema with maximal inhibitory effect of 79.56 ± 18.24% at 100 mg/kg as seen in Figure 9. Similarly, treatment with dexamethasone, a steroidal anti-inflammatory agent, (0.3-3 mg/kg, *i.p*) exhibited a significant effect on the time course curve of carrageenan-induced oedema (Figure 9) with a maximal inhibitory effect of 78.69 ± 3.91% at 3 mg/kg.

Table 4.7: Inhibitory effects of Petroleum ether, ethyl acetate and 70% ethanolic extract on carrageenan-induced oedema on 7-day old chicks.

Extract	300 mg/kg	100 mg/kg	30 mg/kg
Pet ether	47.29 ± 8.65% %	46.43 ± 2.98	24.41 ± 3.97%
Ethyl acetate	66.1 ± 3.67%	50.57 ± 0.67%	44.65 ± 4.77%
70% Ethanol	45.19 ± 5.09%	19 ± 5.34%	11.11 ± 9.77%

Values are mean ± S.E.M (n=5), P< 0.001

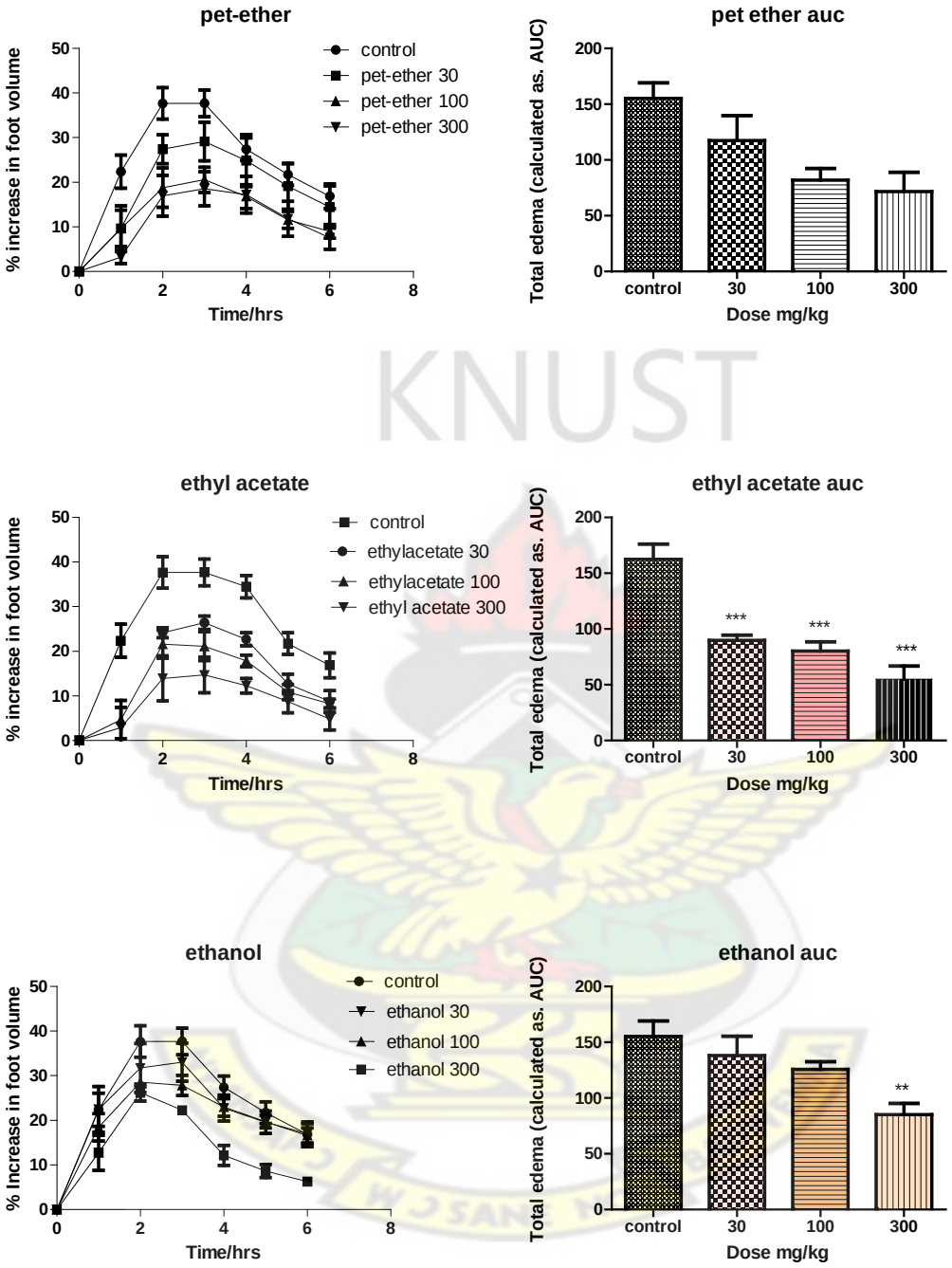


Figure 8: Time course effects of Petroleum Ether, Ethyl acetate and Ethanol Extracts (10-300 mg kg-1 p.o), in the prophylactic protocol on carrageenan induced foot oedema in the chick and their respective total oedema responses for 6 h [defined as the area under the time course curve (AUC)]. Each point on the column represents the Mean ± S.E.M. (n=5). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

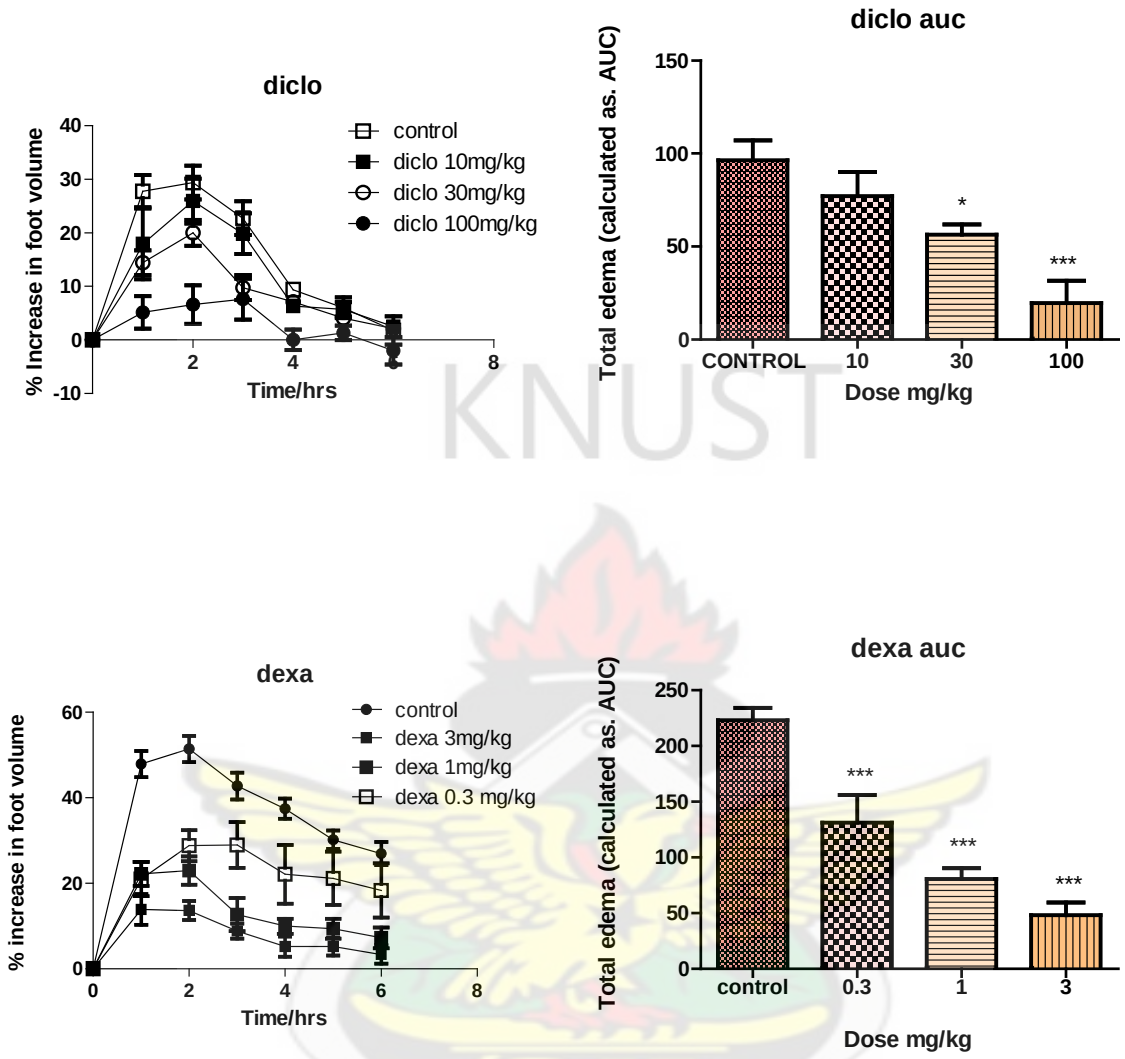


Figure 9: Time course effects of Diclofenac (10-100 mg kg-1, i.p) and Dexamethasone (0.3-3.0 mg kg-1 i.p) in the prophylactic protocol on carrageenan induced foot oedema in the chick and the total oedema response for 6 h). Each point and column represents the mean ± S.E.M. (n=5) *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

CHAPTER FIVE

5.0 DISCUSSION

Plants have been used in the treatment of diseases long before history was written and they have proved to be invaluable as sources for pharmaceutical preparations (Houghton and Raman, 1998). *Clerodendrum splendens* is a plant that has been used for the treatment of various ailments in traditional medicine. However, claims of treatment successes have been made without any scientific basis. It was for this reason that this research was conducted to validate some of the folkloric uses of the plant. The investigation covered the antimicrobial and anti-inflammatory activities, and the chemical constituents responsible for these activities.

Through various chromatographic techniques, two compounds cleroindicin F and apigenin were isolated from the leaves of the plant. They were characterised using data obtained from 1D and 2D NMR experiments.

Apigenin is a known flavone which has been isolated from several plant species including *Helichrysum armenium* (Cubuku and Yuksel, 1982), *Tanacetum parthenium* (Jager, *et al*., 2009) and *Cassia ocidentalis* (Yadav, *et al*., 2010). It also occurs commonly in the genus *Clerodendrum*. It has been isolated from the flowers of *C. infortunatum* (Sinha, *et al.,* 1981), the leaves of *C. inerme* (Achari, *et al*., 1990), the aerial parts of *C. indicum* (El-Shamy, *et al*., 1996), and the roots of *C. serratum* (Jaya, *et al*., 1997). Its isolation from the leaves of *C. splendens* in this study is therefore not surprising and is in accordance with earlier investigations of Shrivastava and Patel (2007), who reported the presence of apigenin in the leaves of the plant.

Cleroindicin F is a known cycloethylhexanoid which was isolated for the first time from the fruits of *Forsythia suspense* and named trivially as rengyolone (Endo and Hikino, 1984). It is

noteworthy that this is the first report of this compound in *C. splendens* although it has been isolated from the aerial parts of *C. indicum* (Tian *et al.*, 1997). It has also been reported in other species of the Verbenaceae family including *Nyctanthes arbour-tristis* (Tuntiwachwuttikula *et al*., 2003) and *Lippia nodiflora* (Siddiqui *et al*., 2007).

The petroleum ether, ethyl acetate and 70% ethanolic extracts of the leaves of *C. splendens* obtained by successive Soxhlet extraction, demonstrated both antimicrobial and anti-inflammatory activities. The antimicrobial potential of the extracts was evaluated using the agar-well diffusion assay. The extracts exhibited antimicrobial activity against all the test organisms (*Staph aureus, B. subtilis*, *P. aeruginosa*, *E. coli* and *C. albicans*) (Table 4.3.1). The ethyl acetate extract demonstrated the highest antimicrobial activity with the ethanolic extract exhibiting the lowest activity. *Pseudomonas aeruginosa* was the least susceptible organism to all the extracts used in the study. The results of the antimicrobial assay agree with that observed by Gbedema *et al.* (2010). The sub-fractions of the ethyl acetate extract were also tested against *E. coli, B. subtilis, Staph aureus* and *Candida albicans.* Sub-fractions **CSE6** and **CSE8** showed the strongest bacterial growth inhibition while **CSE5** exhibited the highest antifungal activity. The standard drugs gave zones of inhibition higher than those of the extracts and the sub-fractions from the ethyl acetate extract. It is significant to note that apigenin which has been shown to have antimicrobial property (Basil *et al.,* 2000; Akruom *et al.,* 2009), was isolated from the ethyl acetate extract in this study. Thus, apigenin may contribute to the antimicrobial activity associated with the ethyl acetate extract. The results of this study therefore provide supportive evidence for the use of the leaves of *C. splendens* for the treatment of wounds and microbial infections in traditional medicine.

The anti-inflammatory activity of the leaves of *C. splendens* was established using the carrageenan-induced oedema in chicks, a common experimental animal model used to evaluate NSAIDs (Di Rosa and Willoughby, 1971). It is believed to act in a biphasic manner. The initial phase of inflammation (0 -2 h) has been attributed to the release of histamine and kinins, followed by a late phase (2.5 – 6 h) mainly sustained by prostaglandin release (Di Rosa, 1972). The second phase is sensitive to most clinically effective anti-inflammatory drugs (Vinegar *et al*., 1969). From the results all the extracts inhibited oedema from the second hour (Fig. 6). The extracts may therefore be acting in the late phase of the inflammation by inhibiting chemical mediators such as prostaglandins. The ethyl acetate extract exhibited the highest inhibitory effect at all doses with a maximal effect of 66.1 ± 3.67% at 300 mg/kg body weight. The extent of inhibition of the foot oedema by the extracts was less than the standard anti-inflammatory drugs, diclofenac and dexamethasone. The isolation of apigenin and cleroindicin F from the ethyl acetate extract is again worthy of note. The anti-inflammatory property of apigenin has been demonstrated by Patel *et al*. (2006) and Lee *et al*. (2007), and that of cleroindicin F has been reported by Kim *et al*. (2006). These constituents may therefore be partly responsible for the observed anti-inflammatory activities of the ethyl acetate extract. The anti-inflammatory activity exhibited by the extracts support the traditional use of the plant for the treatment of inflammatory conditions.

Thus *C. splendens*, a popular African medicinal plant which is employed in the treatment of infectious conditions and diseases that are inflammatory in nature has been validated. Apigenin and cleroindicin F which were isolated from the leaves of the plant may be partly responsible for the observed antimicrobial and anti-inflammatory activities.

CHAPTER SIX

6.0 CONCLUSION

The aim of this study was to confirm the traditional uses of *C. splendens* for the treatment of inflammatory conditions and microbial infections and investigate the chemical constituents responsible for these activities.

Two chemical constituents isolated from the ethyl acetate extract were identified using various NMR experiments as the flavone apigenin and the cycloethylhexanoid cleroindicin F (rengyolone). Although apigenin has been previously reported in *C. splendens*, this is the first time cleroindicin F has been isolated from the plant.

The agar-well diffusion method was used to evaluate the antimicrobial potential of the plant. The results obtained indicated that all the extracts possess antimicrobial activity. The ethyl acetate extract had the greatest activity followed by the petroleum ether extract and then the 70% ethanolic extract. The sub-fractions (**CSE1** to **CSE10**) of the ethyl acetate extract were also assayed against the test organisms. **CSE6** and **CSE8** exhibited the highest bacterial inhibition whereas **CSE5** gave the strongest activity against *C. albicans*.

The ethyl acetate extract demonstrated the highest inhibition of inflammation compared to the petroleum ether and 70% ethanolic extracts when oedema was induced with carrageenan in 7-day old chicks. At a dose of 300 mg /kg body weight, the ethyl acetate extract showed a maximal effect of 66.1 ± 3.67% while the standard anti-inflammatory drugs diclofenac and dexamethasone showed maximal effects of 79.56 ± 18.24% at 100 mg/kg body weight and 78.69 ± 3.91% at 3 mg/kg body weight respectively.

The results of the anti-inflammatory and antimicrobial investigations in this study lend scientific support for the traditional use of *C. splendens* in the treatment of wounds, infectious conditions and inflammatory diseases. Furthermore, the occurrence of apigenin and cleroindicin F in *C. splendens* may partly be responsible for the anti-inflammatory and antimicrobial properties of the plant.

RECOMMENDATIONS

Undeniably, plants have played very important roles in the lives of humans for centuries. *C. splendens* enjoys traditional use as anti-inflammatory and antimicrobial agents. This research was conducted on the leaves of *C. splendens* to authenticate these folkloric uses and to isolate the chemical constituents responsible for these actions. From the outcome of the study, the following recommendations are being made to promote the utilization of *C. splendens* as a medicinal plant.

In traditional medicine, the leaves of *C. splendens* are used for the treatment of wounds and this activity of the plant has been validated by Gbedema *et al*. (2010). In addition, this study has established the antimicrobial and anti-inflammatory activities of *C. splendens* which are essential actions in the wound healing process. Therefore, further studies should be done on the standardization and subsequent preparation of the plant extracts into appropriate and acceptable dosage forms that will aid the use of *C. splendens* as a wound healing agent.

So far, only eight chemical constituents including triterpenoids, flavonoids and a cycloethylhexanoid have been isolated from *C. splendens*. However, the general phytochemical screening of the plant revealed the presence of alkaloids, tannins, sterols, flavonoids and glycosides. There is therefore the need for further investigations into the chemical constituents

of the plant. This will unearth the medicinal potential of the plant and also provide a basis for its standardization as a drug.

Traditionally the leaves of *C. splendens* are used for the treatment of a wide variety of disease conditions. In West Africa, the leaf and root decoctions are used for the treatment of gastric ulcers and malaria (Okwu and Iroabuchi, 2008; Shrivastava and Patel, 2007). These traditional uses are yet to be confirmed. It will therefore be in the right direction to investigate the plant extracts against *Helicobacter pylori* and *Plasmodium* species which are associated with gastric ulceration and malaria respectively to scientifically establish their usefulness in the treatment of these ailments.

From this research two chemical constituents, apigenin and cleroindicin F were isolated from the leaves of *C. splendens*. Long *et al*. (2008) and Kim and Lee (2009) have reported on the anticancer properties of apigenin and cleroindicin F respectively. It is interesting to note that this property has not been cited among the traditional uses of *C. splendens*. Thus, the plant should be investigated to ascertain whether it possesses anticancer properties which will add to the biological activities of *C. splendens* and also target other chemical constituents that may possess this property.

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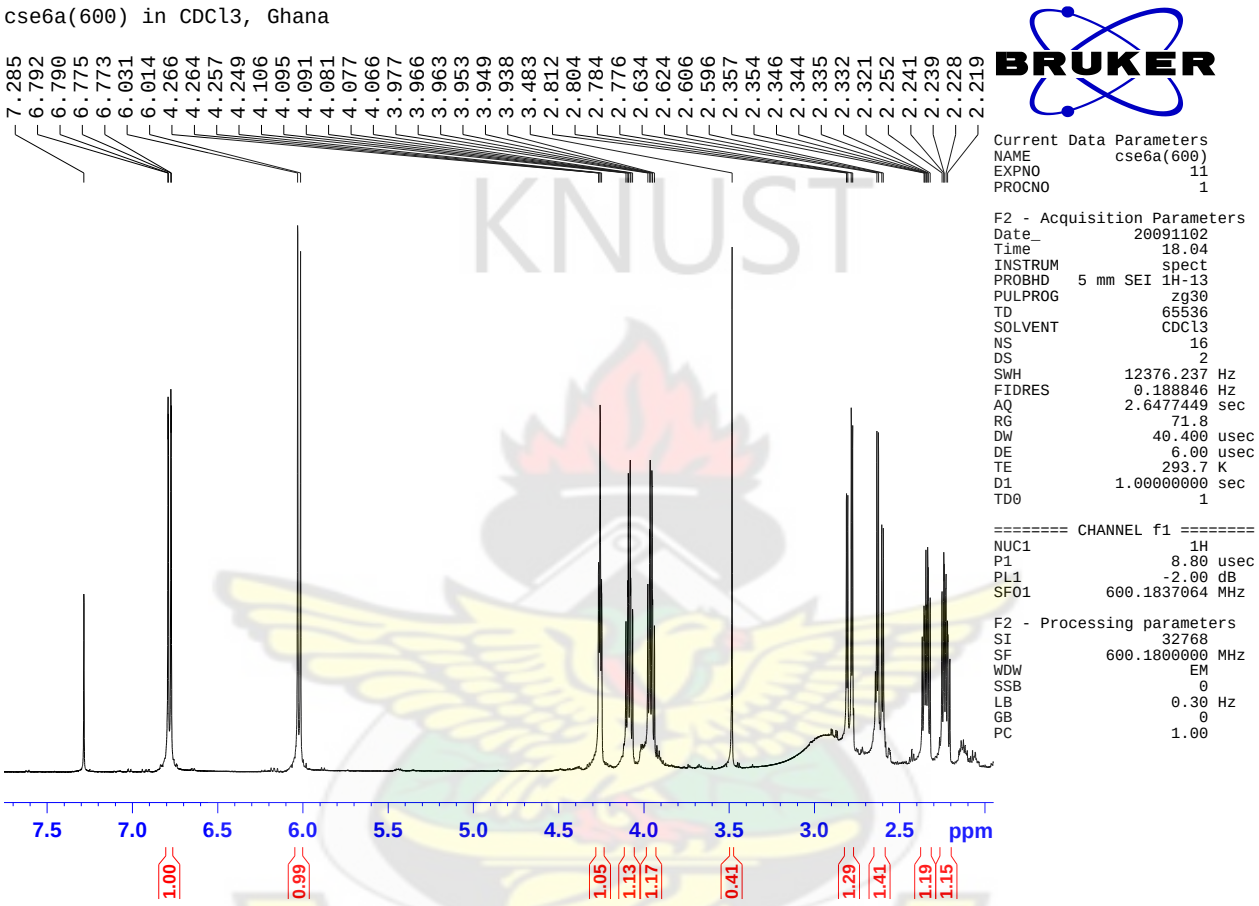
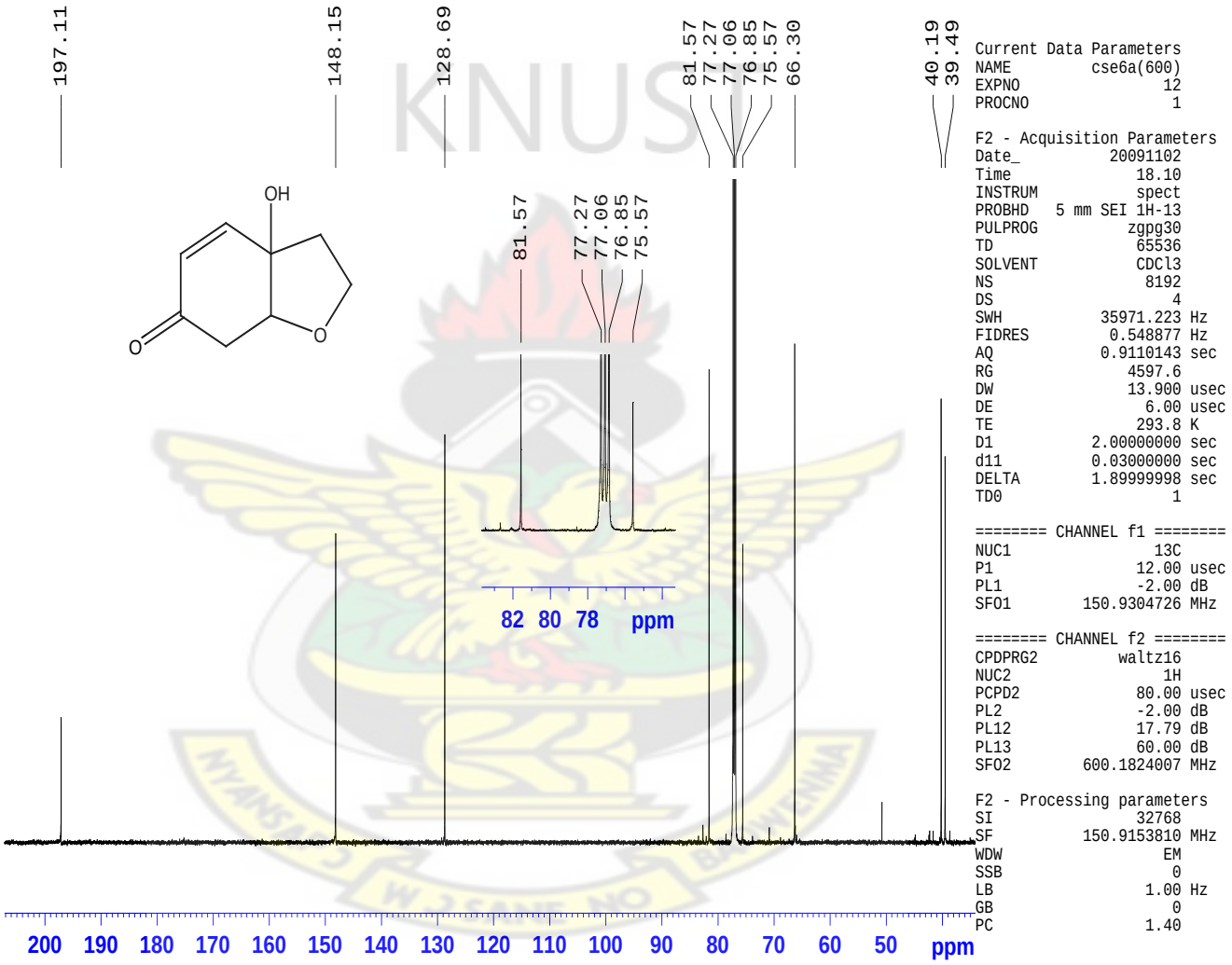
APPENDICES

APPENDIX 1: ^{1}H NMR SPECTRUM FOR CSE6a



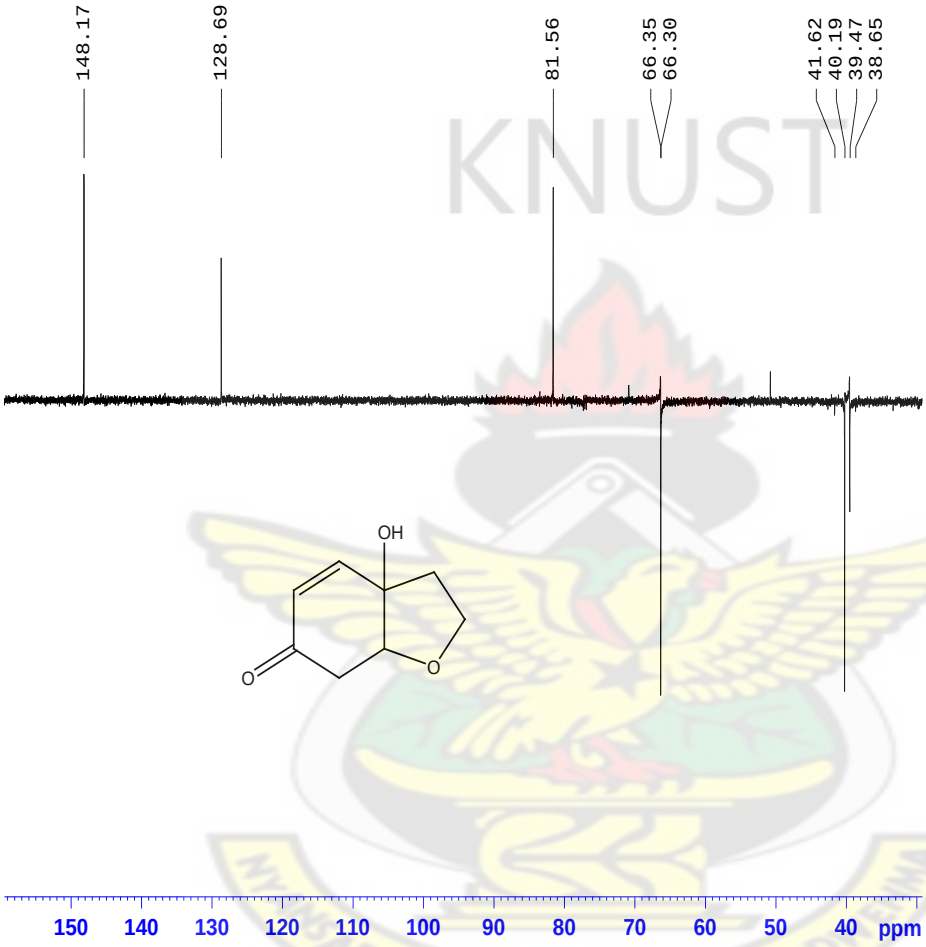
APPENDIX 2: ^{13}C NMR SPECTRUM FOR CLEROINDICIN F

cse6a(600) in CDCl3, Ghana



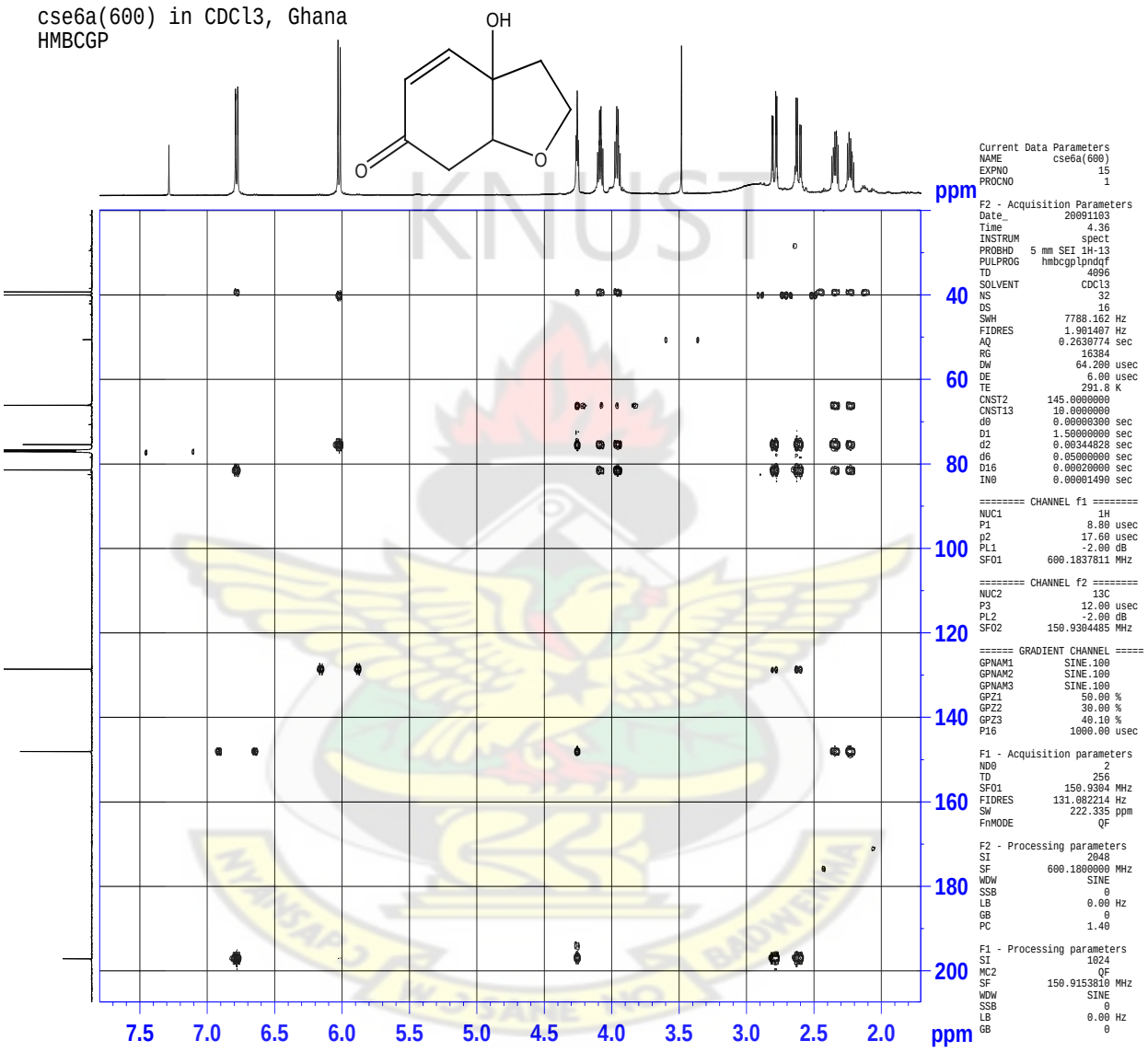
APPENDIX 2: DEPT SPECTRUM FOR CSE6a

cse6a(600) in CDCl3, Ghana



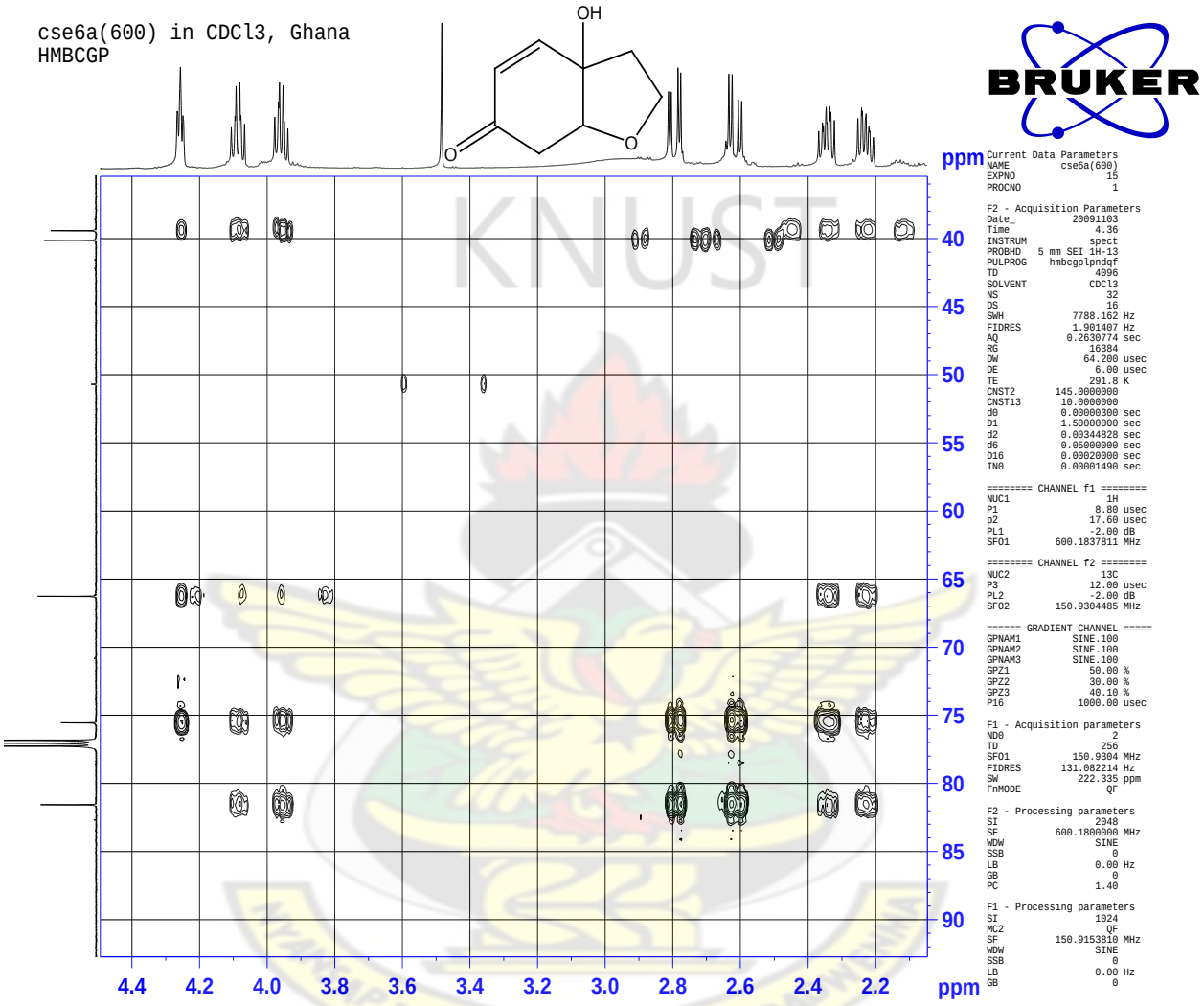

```
Current Data Parameters
NAME          cse6a(600)
EXPNO                 13
PROCNO                 1

F2 - Acquisition Parameters
Date_           20091103
Time                1.46
INSTRUM            spect
PROBHD   5 mm SEI 1H-13
PULPROG          dept135
TD                 65536
SOLVENT            CDCl3
NS                  1024
DS                     4
SWH            35971.223 Hz
FIDRES          0.548877 Hz
AQ             0.9110143 sec
RG                  4096
DW                13.900 usec
DE                 6.00 usec
TE                292.6 K
CNST2        145.0000000
D1            2.00000000 sec
d2            0.00344828 sec
d12           0.00002000 sec
DELTA         0.00001528 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                  13C
P1                 12.00 usec
p2                 24.00 usec
PL1                -2.00 dB
SFO1         150.9304726 MHz

======== CHANNEL f2 ========
CPDPRG2           waltz16
NUC2                   1H
P3                  8.80 usec
p4                 17.60 usec
PCPD2              80.00 usec
PL2                -2.00 dB
PL12               17.79 dB
SFO2         600.1824007 MHz

F2 - Processing parameters
SI                 32768
SF           150.9153810 MHz
WDW                   EM
SSB                    0
LB                  1.00 Hz
GB                     0
PC                  1.40
```

APPENDIX 3: HMBC SPECTRUM FOR CSE6a

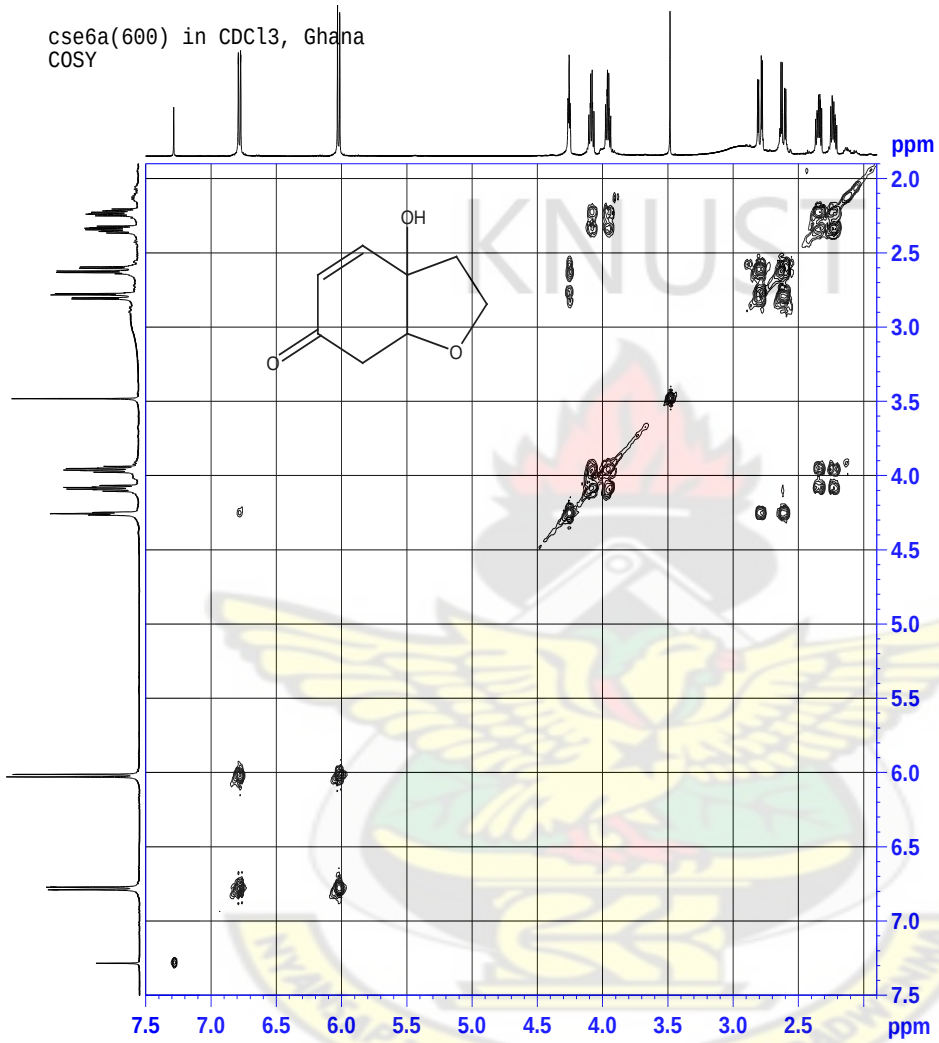



```
Current Data Parameters
NAME          cse6a(600)
EXPNO                 15
PROCNO                 1

F2 - Acquisition Parameters
Date_           20091103
Time                4.36
INSTRUM            spect
PROBHD   5 mm SEI 1H-13
PULPROG     hmbcgplpndqf
TD                  4096
SOLVENT            CDCl3
NS                    32
DS                    16
SWH             7788.162 Hz
FIDRES          1.901407 Hz
AQ              0.2638774 sec
RG                  16384
DW                64.200 usec
DE                 6.00 usec
TE                291.8 K
CNST2         145.0000000
CNST13         10.0000000
d0             0.00000300 sec
D1             1.50000000 sec
d2             0.00344828 sec
d6             0.05000000 sec
D16            0.00020000 sec
IN0            0.00001490 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                 8.80 usec
p2                17.60 usec
PL1               -2.00 dB
SFO1         600.1837811 MHz

======== CHANNEL f2 ========
NUC2                 13C
P3                12.00 usec
PL2               -2.00 dB
SFO2         150.9304485 MHz

====== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPNAM2          SINE.100
GPNAM3          SINE.100
GPZ1               50.00 %
GPZ2               30.00 %
GPZ3               40.10 %
P16              1000.00 usec

F1 - Acquisition parameters
ND0                    2
TD                   256
SFO1           150.9304 MHz
FIDRES         131.082214 Hz
SW               222.335 ppm
FnMODE                QF

F2 - Processing parameters
SI                  2048
SF           600.1800000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
PC                  1.40

F1 - Processing parameters
SI                  1024
MC2                   QF
SF           150.9153810 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
```

APPENDIX 4: EXPANDED HMBC SPECTRUM FOR CSE6a

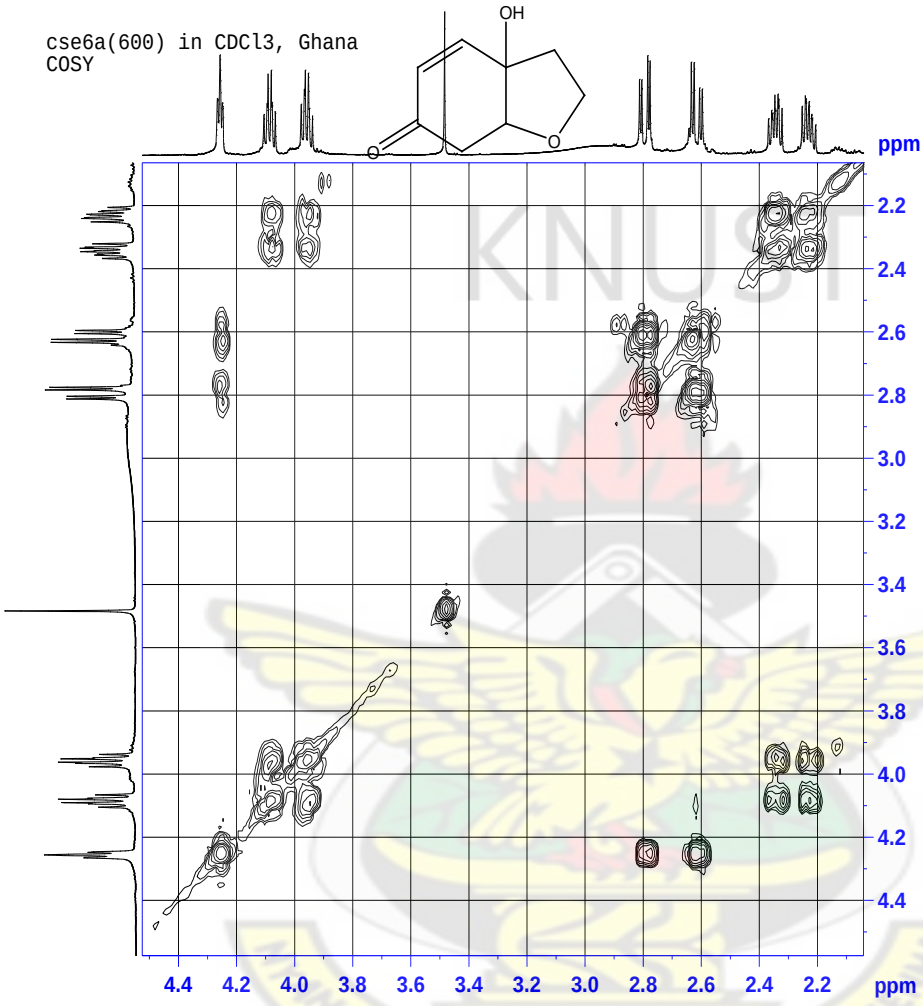


APPENDIX 5: COSY SPECTRUM FOR CSE6a




```
Current Data Parameters
NAME          cse6a(600)
EXPNO                 14
PROCNO                 1

F2 - Acquisition Parameters
Date_           20091103
Time               1.47
INSTRUM           spect
PROBHD   5 mm SEI 1H-13
PULPROG        cosygpqf
TD                 2048
SOLVENT            CDCl3
NS                    24
DS                     8
SWH             8012.820 Hz
FIDRES          3.912510 Hz
AQ              0.1279076 sec
RG                 203.2
DW                62.400 usec
DE                 6.00 usec
TE                292.6 K
d0            0.00000300 sec
D1            1.48689198 sec
d13           0.00000400 sec
D16           0.00020000 sec
IN0           0.00012480 sec

======== CHANNEL f1 ========
NUC1                  1H
P0                 8.80 usec
P1                 8.80 usec
PL1                -2.00 dB
SFO1        600.1836084 MHz

====== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPNAM2          SINE.100
GPZ1               10.00 %
GPZ2               10.00 %
P16              1000.00 usec

F1 - Acquisition parameters
ND0                    1
TD                   256
SFO1            600.1836 MHz
FIDRES         31.300079 Hz
SW                13.351 ppm
FnMODE                QF

F2 - Processing parameters
SI                  1024
SF           600.1800000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
PC                  1.40

F1 - Processing parameters
SI                  1024
MC2                   QF
SF           600.1800000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
```

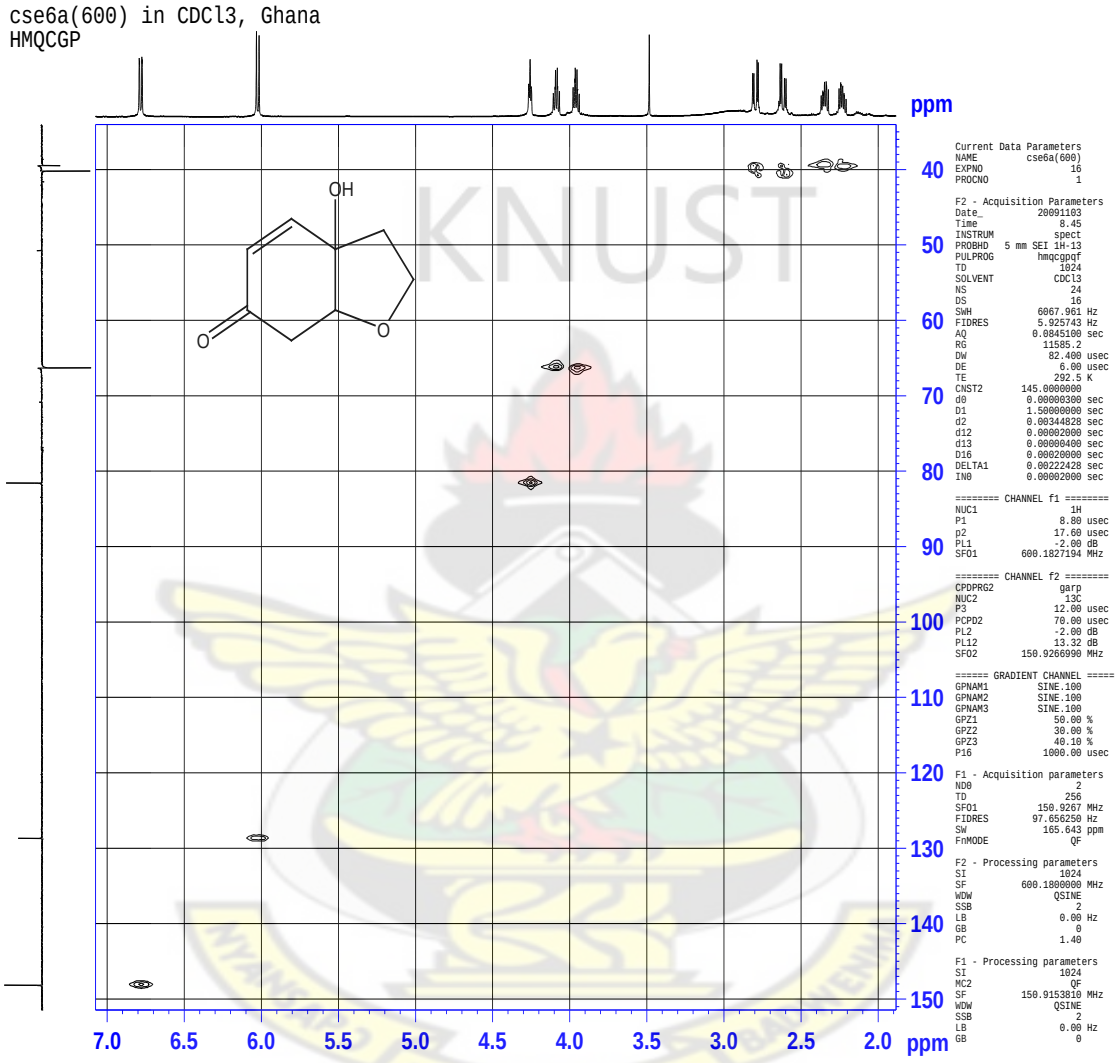
APPENDIX 5: EXPANDED COSY SPECTRUM FOR CSE6a



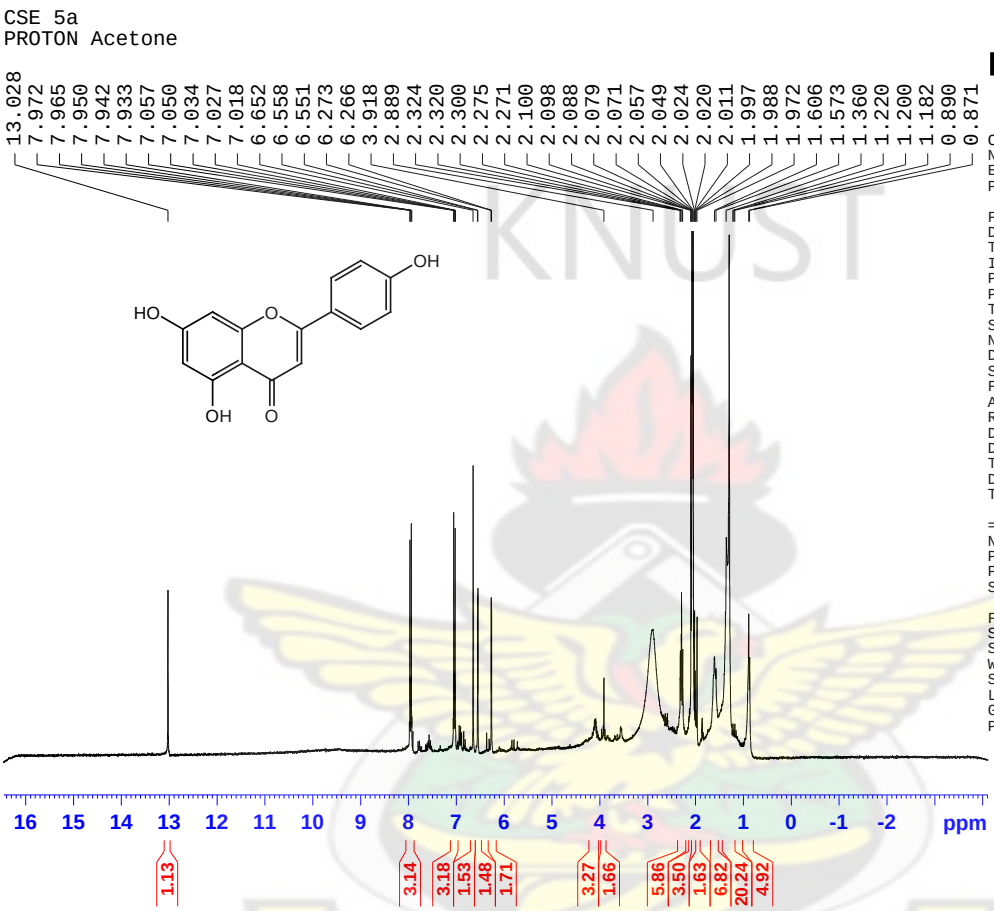
BRUKER

```
Current Data Parameters
NAME          cse6a(600)
EXPNO                 14
PROCNO                 1

F2 - Acquisition Parameters
Date_           20091103
Time              1.47
INSTRUM             spect
PROBHD   5 mm SEI 1H-13
PULPROG         cosygpqf
TD                  2048
SOLVENT             CDCl3
NS                    24
DS                     8
SWH              8012.820 Hz
FIDRES           3.912510 Hz
AQ               0.1279076 sec
RG                 203.2
DW                62.400 usec
DE                 6.00 usec
TE                292.6 K
d0            0.00000300 sec
D1            1.48689198 sec
d13           0.00000400 sec
D16           0.00020000 sec
IN0           0.00012480 sec

======== CHANNEL f1 ========
NUC1                  1H
P0                 8.80 usec
P1                 8.80 usec
PL1                -2.00 dB
SFO1         600.1836084 MHz

====== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPNAM2          SINE.100
GPZ1               10.00 %
GPZ2               10.00 %
P16              1000.00 usec

F1 - Acquisition parameters
ND0                    1
TD                   256
SFO1            600.1836 MHz
FIDRES         31.300079 Hz
SW                13.351 ppm
FnMODE                QF

F2 - Processing parameters
SI                  1024
SF           600.1800000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
PC                  1.40

F1 - Processing parameters
SI                  1024
MC2                   QF
SF           600.1800000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
```

APPENDIX 6: HMQC SPECTRUM FOR CSE6a



APPENDIX 7: ^{1}H NMR SPECTRUM OF CSE5a



BRUKER

```
Current Data Parameters
NAME                 CSE 5a
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
Date_             20091118
Time                  9.26
INSTRUM              spect
PROBHD   5 mm QNP 1H/15
PULPROG               zg30
TD                   65536
SOLVENT            Acetone
NS                       16
DS                        2
SWH              6172.839 Hz
FIDRES           0.094190 Hz
AQ               5.3084660 sec
RG                   406.4
DW                  81.000 usec
DE                   6.00 usec
TE                  299.2 K
D1              1.00000000 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                     1H
P1                   10.00 usec
PL1                   0.00 dB
SFO1          300.1318534 MHz

F2 - Processing parameters
SI                    32768
SF            300.1300000 MHz
WDW                      EM
SSB                       0
LB                    0.30 Hz
GB                        0
PC                    1.00
```