UNIVERSITY OF CAPE COAST

ANTIOXIDANT AND WOUND HEALING PROPERTIES OF SOME SELECTED PLANTS FROM KPANDO TRADITIONAL AREA: ISOLATION OF FLAVONOIDS FROM *ANOGEISSUS LEIOCARPUS* (DC) GUILL AND PERR (COMBRETACEAE)

BY

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THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY. UNIVERSITY OF CAPE COAST, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR AWARD OF DOCTOR OF PHILOSOPHY DEGREE IN CHEMISTRY

JULY, 2015

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature……..Date…….

Name: Victor Yao Atsu Barku

Supervisor's Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature…… Date…….

Name: Prof. Yaw Opoku-Boahen

Co-supervisor's Signature…….. Date……..

Name: Prof. Ernest Owusu-Ansah

ABSTRACT

Twenty-six wound healing plants have been identified in the Kpando Traditional Area in Ghana. The methanol extracts of six of these plants showed some level of antimicrobial activity against wound causing infectious microorganisms. Similarly, four of these plants exhibited significant wound healing properties. Free radical scavenging activity by 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) indicated a concentration dependent activity with no significant difference from Ascorbic acid ($P<0.05$) except for *Amaranthus spinosus*. Total antioxidant capacity (TAC) and Ferric reducing antioxidant power showed a similar concentration dependent antioxidant activity and reducing potentials respectively. The high antioxidant activities of the extracts significantly correlated with the total phenolic and flavonoid contents. The high correlation between the values of DPPH and TAC indicates the viability of the two models for evaluating antioxidants from medicinal plants. Further investigation on *Anogeissus leiocarpus* yielded two different flavonoid glycosides whose structures were identified through spectral analysis (1DNMR, FTIR and UV) as quercetin rhamnoglucoside (rutin) and isoflavonoid glucoside.

The findings revealed that the plants possess considerably wound healing, antimicrobial and high antioxidant properties which may provide protection against free radicals induced damage to biomolecules. The study therefore supports the traditional use of these plants and recommends further investigation into the mechanism of action on the isolated compounds for possible drug development.

ACKNOWLEDGEMENTS

I am very thankful to all those who have supported me and contributed in making this study come to a very successful end. I express my sincere gratitude to my able supervisors, Prof. Yaw opoku-Boahen and Prof. Ernest Owusu-Ansah for their guidance, ideas, directives and the constructive criticisms they willingly offered. I am very much appreciative of your co-operation and painstaking efforts.

The people of Kpando Traditional Area where I conducted my ethno survey deserved my special praise and acknowledgement. I shall forever remain thankful.

I am also grateful to Dr. Erasmus Nii Boye Cudjoe in Canada and the following people who are now studying at University of Saskatwean, Canada for providing the NMR spectral data; Mr. Isaac Asiamah, a colleague, Mr. Edward Bam and Mr. Ashish who are personal friends. I owe you my deepest gratitude. Similarly, I wish to convey the same gratitude to Mrs. Gertrude Dali of Department of Environmental Science, UCC for helping to design the questionnaire and Mr. Enock Francis Mensah, a senior Technician in the Department of Chemistry, UCC for his tremendous assistance in getting all UV spectral information. So do I also thank Mr. Nunoo, the store-keeper and Mr. Andrew Wofessor for their special contributions. I wish to thank all friends, colleague lecturers especially, Alex Boye and Dr. Ruphino Zugle, the Curators at the Herbarium, School of Biological Sciences, my students and my lovely family.

Lastly, the financial support provided by the Management of University of Cape Coast is highly acknowledged and appreciated.

DEDICATION

With sincere love of immeasurable height, I unreluctantly dedicate this piece of work to Lucy my wife, Victoria Atsufui my twin sister, my children and siblings.

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CHAPTER ONE

INTRODUCTION

Background to the Study

Plants do not only provide the carbohydrates, proteins and fats necessary in the diet of man and other animals, but they also produce a vast range of organic materials including essential vitamins that have numerous functions one of which is to repel pests and pathogens. Nature has served as a rich repository of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from natural sources, notably of plant origin (Gurinder & Daljit, 2009). As a result, plants are the world's main source of drugs. They provide most of the effective drugs in traditional medicines, as used by about 80% of the people in the world.

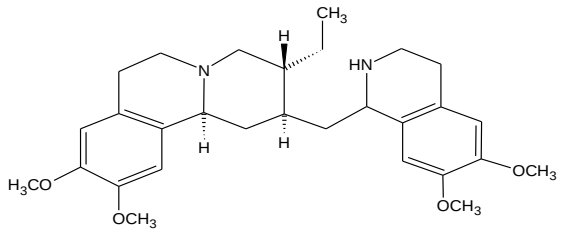
Herbal medicines prepared from plants, based on their traditional uses in the form of powders, concoctions, decoctions, tinctures, liquids or mixtures, have been the basis of treatment for various ailments in developing countries since ancient times. Screening of plant extracts and natural products therefore presents higher plants as a potential source of new anti-infective agents, as well as serving drug discovery from natural products for primary lead compounds.

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in

the world. Table 1 represents the list of some of these chemical substances. Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. An example is emetine **1**, a plant chemical that was discovered in a tropical plant, *Cephaelis ipecacuanha* A. Rich., also known as *Psychotria ipecacuanha* Stokes (Rubiaceae) (Akinboye & Oladapo, 2011). Emetine is a natural product alkaloid that is one of the main active ingredients in ipecac syrup used as an emetic. Ipecac syrup was a drug developed from the *Cephaelis ipecacuanha* plant and was used for many years to induce vomiting. It has also been used extensively in phytomedicine as an antiparasitic drug. It inhibits both ribosomal and mitochondrial protein synthesis and interferes with the synthesis and activities of DNA and RNA. For this reason, it has been a vital tool to pharmacologists and has demonstrated many biological properties, such as antiviral, anticancer, antiparasitic and contraceptive activities. Another example is the plant chemical named *taxol* **2**. Taxol is the name of the plant chemical originally extracted from a pacific yew plant *Taxus brevifolia*. Taxol has been copied by a pharmaceutical company and patented as a drug named *Paclitaxel* which is used in various types of tumors today in the United States and many other countries (Taylor, 2000).

The quest for plants with medicinal properties therefore continues to receive attention as scientists are in need of plants, particularly of ethno botanical significance for a complete range of biological activities, which ranges from antibiotic to anticancerous. Several plants and herb species used traditionally have potential antimicrobial and antiviral properties (Zaika, 1988) and offered

attractive options which include wound healing (Blair & Carter, 2005; Church et al, 2006; Dzomba et al, 2012).



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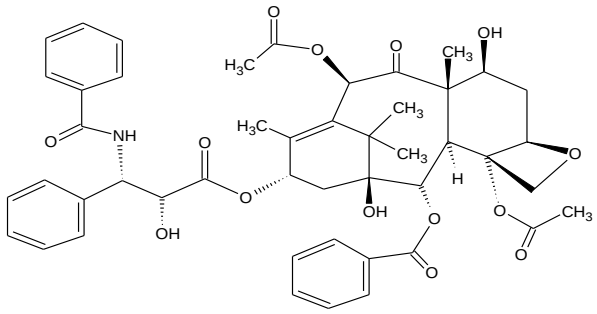

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Figure 1: Examples of Drugs derived from Plants; (1) Emetine and (2) Taxol

The interest in the traditional use of plants for healing of wounds has increased tremendously in recent years (Houghton et al., 2005). For instance, in Ghana, many healers apply the concentrated aqueous extracts of *Commelina diffusa* Burn. F. (Commelinaceae) and *Spathodea campanulata* Beav. (Bignoniaceae) to wounds to aid healing. This practice and other similar success stories about medicinal plants have raised the optimism of scientists about the future of phyto-antimicrobial agents (Gandhiraja et al. 2009).

Table 1: Distinct chemical substances derived from plants

Drug/Chemical	Action/Clinical use	Plant source
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Atropine	Anticholinergic	*Atropa belladonna*
Caffeine	CNS stimulant	*Camellia sinensis*
Camphor	Rubefacient	*Cinnamomum camphora*
Cocaine	Local anaesthetic	*Erythoxylum coca*
Codeine	Analgesic, antitussive	*Papaver somniferum*
L-Dopa	Anti-parkinsonism	*Mucuna sp.*
Emetine	Amoebicide, emetic	*Cephaelis ipecacuanha*
Ephedrine	Sympathomimetic, antihistamine	*Ephedra sinica*
Gossypol	Male Contraceptive	*Gossypium sp.*
Menthol	Rubefacient	*Mentha sp.*
Morphine	Analgesic	*Papaver somniferum*
Nicotine	Insecticide	*Nicotiana tabacum*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Reserpine	Antihypertensive, tranquillizer	*Rauvolfia serpentine*
Salicin	Analgesic	*Salix alba*
Taxol	Antitumor agent	*Taxus brevifolia*
Vinblastine	Antitumor, Antileukemia	*Cantharanthus roseus*

The presence of various life-sustaining constituents in plants has urged scientist to examine plants with a view to determine potential wound healing properties (Nayak et. al., 2006). Many phytopharmaceutical laboratories are now concentrating their efforts to identify the active constituent and mode of action of various medicinal plants (Hwang et. al., 2000; Thakur et al., 2011)). The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun et. al., 2007). These constituents include various chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, triterpenes, anthraquinones, and phenolic compounds (Chaudhari et. al., 2006; Edeoga et. al., 2007; Sumitra et. al., 2009).

The enhanced wound healing potency of various herbal extracts may be attributed to free radical-scavenging action and the antimicrobial property of the phytoconstituents present in the extract, and the quicker process of wound healing could be a function of either the individual or the synergistic effects of bioactive molecules. These active constituents promote the process of wound healing by increasing the viability of collagen fibrils, by increasing the strength of collagen fibers either by increasing the circulation or by preventing the cell damage or by promoting the DNA synthesis (Majumdar et.al., 2007; Thakur et al., 2011).

A number of secondary metabolites/compounds isolated from plants have been demonstrated in animal models as active principles responsible for facilitating healing of wounds. Some of the most important ones include oleanolic acid, polysaccharides, gentiopicroside, sweroside, swertiamarin, shikonin derivaties

(deoxyshikonin, acetyl shikonin, 3-hydroxy-isovaleryl shikonin and 5,8-Odimethyl acetyl shikonin), asiaticode, Asiatic acid, madecassic, quercetin, colutequinone B, hyperforin, catechins, and isoflavonoids that could potentially be new therapeutic agents to treat wounds. These agents usually influence one or more phases of the healing process (Karodi et. al., 2009).

The therapeutic benefit of medicinal plants is often attributed to their antioxidant property (Nayak et al., 2006). Some plants extracts are believed to have strong antioxidant effects. Antioxidants play very important roles in coetaneous tissue repair as they significantly prevent tissue damage that stimulates wound healing process. It has been mentioned that the antioxidant activity of plants might be due to their phenolic compounds (Handa, 2008). Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Shetty et al., 2008). Some evidences suggest that the biological actions of these compounds are related to their antioxidant activity (Nayak et al., 2011). Botanicals with antioxidant or free radical-scavenging activity thus can play a significant role in healing of wounds (Kamath et al., 2003).

Statement of the Problem

Currently, large and ever expanding global population base prefers the use of natural products in treating and preventing medical problems including wound infections because herbal plants have proved to have a rich resource of medical

properties. Plants based antimicrobials have enormous therapeutic potentials. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. They are effective, yet gentle (Murray, 1995). The screening of herbal extracts has therefore been of great interest to the scientists for the discovery of new effective drugs (Kosger et. al., 2009). As a result, there is an overwhelming increasing interest in finding herbal extracts with healing efficacy so that more phytoconstituents derived from plants can be identified and screened for antimicrobial activity and management of other disease conditions. One disease condition that is causing great havoc to the world population but seems to be forgotten or neglected is wound. Wound infection is a major complication of injury and it accounts for 50-70% of hospitalized deaths (Omale & Ayide, 2010). For instance, in Ghana 273,346 (1.64 %) of the general population suffer one or more forms of open wounds (Driscoll, 2009). Wound healing disorders present a serious clinical problem of medical health care in Africa and in Ghana and are associated with diseases such as diabetes, hypertension, and obesity as a result of poor hygienic conditions and malnutrition (Gulzar et al., 2011). Most of these disorders lead to complications, high morbidity and mortality rates (Krishnan, 2006).

However, most of the synthetic drugs currently used for the treatment of wounds are expensive and also pose problems such as many side effects, toxic effects, allergies and drug resistance (Akhtar & Munir, 1989). Efforts are being made worldwide to discover newer drugs that can promote wound healing with

minimal side effects and to reduce the cost of hospitalization and management of complications. As a result, many ethnopharmacological surveys and scientific experiments have been conducted to identify medicinal plants for wound treatment. However, no document whatsoever is available on such surveys and scientific trials on plants from Kpando Traditional Area. Hence this research sought to uncover wound healing plant based antimicrobials that represent a vast untapped source for medicines in the area that may need continuous and further exploration to probably meet man's demands for primary healthcare.

Main objectives of the study

This study primarily seeks to identify, characterize and assess herbal plants, purportedly used among the people of Kpando Traditional Area in the Volta Region of Ghana for the treatment of wounds, that have inhibitory activity against microbes, pathogens responsible for the above mentioned disease condition. The study therefore seeks to achieve the following specific objectives.

Specific objectives

1. To identify by ethno survey, medicinal plants used by indigenous in the traditional treatment of wound infections.
2. To investigate the antimicrobial activities of selected plants species on wound infections.
3. To determine and confirm scientifically the ethno-medicinal uses of these plants for treating wound infection using an excision wound model.

4. To investigate the antioxidant activity (which includes total flavonoid and phenolic contents) of these plants.
5. To isolate, identify and characterize the chemical compounds responsible for the antimicrobial and antioxidant activities from the plants species that show significantly high activity for possible presentation as lead compounds for drug development.

Research Questions

1. What are some of the wound healing plants used among the people of Kpando Traditional Area for folkloric medicine?
2. Do these plants have some level of antimicrobial activity?
3. To what extent can some of these plants be proved scientifically for effective treatment of wounds?
4. Do some of these plants have antioxidant activity?
5. What are the chemical constituents of some of these plants?

Significance of the study

The study will be of great importance to students and many scientific researchers in the field in the sense that it will lead to the discussion on the discovery of many invaluable wound healing medicinal plants and the assessment of their active components. The study therefore will improve greatly upon the existing information on medicinal plants and can thus help to improve access by the local population to safer, more effective, reproducible and affordable treatments. In addition, antimicrobials and chemotherapeutic agents with new

mechanisms of action are urgently needed because increasing resistance to antibiotics and chemotherapeutic drugs is a world-wide and serious problem. The research will surely pave the way for chemical synthesis of new drugs and modification of old drugs with the needed potency to combat microbial pathogens and tumors. This will put the use of some traditional phytopharmaceuticals on a firm scientific footing, and permit the standardization and quality control of these preparations.

Delimitation of the Study

The study survey was conducted among the indigenous people of Kpando Traditional Area in the Volta Region of Ghana. The survey also focused on Kpando because the researcher comes from the area and can communicate fluently in the dialect of the area and can easily identify the plants collected from the study area.

Limitations of the Study

The study was conducted on only wound healing medicinal plants. As a result, the ethnobotanical survey was limited to only plants used in folkloric medicine for wound treatment.

Scope of Work

To achieve the set objectives the research work has been designed to undertake an ethnobotanical survey on medicinal plants purportedly used by the indigenous people of Kpando Traditional area for the treatments of wounds. The data

collected would be assessed to obtain information on the commonest plants used for wound healing in the area; the parts and mode of administration.

The research will further considered the scientific viability of some of the plants in the treatment of wounds. As a result, sensitivity investigations on antimicrobial activity would be carried out on six plant extracts to ascertain the effect of the plant extracts on wound infections. This would be followed up with wound healing model (excision method) using wistar rats to justify the traditional uses of the plants in wound healing.

In addition, I would consider the antioxidant properties of the plants to establish any correlation between wound healing potential and antioxidant activities.

Finally, an attempt would be made to isolate and characterise chemical principles present in one of these plants namely; *Anogeissus leiocarpus*.

CHAPTER TWO

REVIEW OF RELATED LITERATURE

This chapter reviews the definition of wound and its classification alongside wound care and the contributory effects from medicinal plants. The chapter also considered the antioxidant activity, structural characterisation and identification of flavonoids and some experimental methods for determining antioxidant activity.

Definition and Classification of Wound

A wound comes through surgical operations or accidentaly in our life. Wound is inescapable in ones life time. It is a disease condition that can happen to anybody at anytime at anywhere. Wound may arise due to physical injuries that result in an opening or break of skin or chemical means (Wandankar et al, 2011). Wound is defined as a disruption of cellular, anatomical, and functional continuity of a living tissue. Wounds are the result of injuries to the skin that disrupt the soft tissue. It may be produced by physical, chemical, thermal, microbial, or immunological insult to the tissue. Wound can be classified based on the underlying cause of wound creation. When skin is torn, cut or punctured it is termed as an open wound and when blunt force trauma causes a contusion, it is called closed wound, whereas the burn wounds are caused by fire, heat, radiation,

chemical, electricity, or sunlight. It can also be classified based on the physiology of wound healing. Wound is said to be acute when the healing process occurs within the expected time frame and in an orderly manner. It is a chronic wound when it requires a prolonged time to heal, does not heal, or recurs frequently. Chronic wound is a major cause of physical disability. Local infection, foreign bodies and systemic problems such as diabetes mellitus, malnutrition, immunodeficiency or medications are the most frequent causes of chronic wounds. Wounds represent a significant burden on the patients and health care professionals worldwide. Wounds affect physical and mental health of millions of patients and impose significant cost on patients. Wounds are major cause of physical disabilities. Current estimates indicate that worldwide nearly 6 million people suffer from chronic wounds (Kumar et al., 2007).

Wound infection

Wound infection is one of the most common diseases in developing countries because of poor hygienic conditions (Ayyanar & Ignacimuthu, 2009; Senthil et al., 2006). All wounds contain bacteria and even if the wound is healing normally, a limited amount of bacteria will be present. But if the bacteria count rises, the wound may become infected. Bacterial overload in a wound can lead to a serious infection that requires antibiotic treatment. A wound that is not healing may probably indicate a sign of infection. In the wound, the following symptoms indicate infection: odour, increased exudate, absent or abnormal granulation tissue and increased pain. Additional clinical symptoms may arise if the infection spreads to the healthy tissue surrounding the wound. Depending on the type of

bacteria, the wound exudate may become more puss-like, and the peri-ulcer skin may be tender, red and painful. The patient may also have a fever.

Impediments to Wound Healing

Wound healing is a process of filling up of gaps and maintains the anatomical structure and function. Tissue regeneration is the part of process of wound healing through which it restores the integrity of tissue layers. Wound healing is a continuous process which is delayed due to deficiency of certain vitamins, trace elements and proteins (Wandankar et al, 2011).

Wound infection resulting from the impaired immunity and exposure or poor hygiene is one of the most commonly encountered and clinically important impediments to wound healing. The injured skin remains vulnerable to invasive microbial infections of all kinds of subsequent development of wound sepsis until complete epithelial repairs have occurred (Odimegwu et al, 2008). Injury becomes infected, because the wound area is ideal medium for the multiplication of the infecting organism.

Organisms Responsible for Wound Infections

All wounds contain a variety of microorganisms. Table 2 shows a number of bacteria that may potentially cause wound infection.

Some important organisms causing wound infections are *Methicillin Resistant staphylococcus* aureus (MRSA), *Staphylococcus aureus, Eschericia coli, Pseudomonas aeruginosa, Vancomycin Resistant enterococci, Streptococcus pyogenes* and *Corynebacterium sp*. These infections represent the main causes of

illness and mortality around the world especially, *Enterococcus and Staphylococcus species*, which are agents of many intrahospital infections.

Table 2: Summary of examples of bacteria that can infect wounds

Types of Microorganism	Examples
Gram-positive cocci	Beta-heamolytic *Streptococcus* (*Streptococcus pyogenes*)* *Enterococcus* (*Enterococcus faecalis*) *Staphylococcus* (sensitive *Staphylococcus aureus* and resistant *Staphylococcus aureus* (MRSA)*
Gram-negative aerobic rods	*Pseudomonas aeruginosa**
Gram-negative facultative rods	*Enterobacter* species, *Escherichia coli*, *Klebsiella* species, *Proteus* species
Anaerobes	*Bacteroides*, *Clostridium*
Fungi	Yeasts (*Candida*), *Aspergillus*

*Microorganisms most commonly associated with causing wound infection (Collier, 2004)

Wound care and the fight against Wound Infections

The primary objective of wound care is to prevent or minimize infection and promote healing. Various materials and methods, especially antibacterial are employed. Some of these wound care methods employed include the topical antimicrobial therapy of commercial antibiotics such as ampicillin capsules, penicillin ointment, chloramphenicol, a combination of ampicillin and mebendazole. Topical antimicrobial therapy is one of the most important methods of wound care (Esimone et al., 2009). These chemicals that are commonly used as

antimicrobial and disinfectant agents are successful in fighting these infections. Unfortunately, the future effectiveness of antimicrobial therapy is somewhat in doubt. Microorganisms, especially bacteria, are becoming resistant to more and more antimicrobial agents. In addition, these chemical agents are expensive and pose side effects. Herbs have few complications and low price. Hence, plant products are potential agents for wound healing, and are largely preferred because of their widespread availability and effectiveness as crude preparations (Roodbari et al., 2012; Sasidharan et al., 2010). Plant products are therefore used as alternative solution to the problem of wound treatment in developing countries.

Wound Healing Potency of Plants

Various plant products and herbs have been used in treatment of wounds over the years and have proved to possess significant prohealing properties in different types of wounds. Wound healing herbals extracts promote blood clotting, fight infection, and accelerate the healing of wounds. Plants have the immense potential for the management and treatment of wounds. A large number of plants are used by tribal and folklore in many countries for the treatment of wounds and burns. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. The herbal extracts and fractions effectively arrest bleeding from fresh wounds, inhibit microbial growth and accelerate wound healing (Okoli et al., 2007). These phytomedicines are not only cheap and affordable but also safe. Various herbal products have been used in management and treatment of wounds over the years. Table 3 presents examples of plants cited in literature to have been used to treat wound infections.

Table 3: List of example of plants cited in literature to have been used to treat wound infections

Plant	Part used	Mode of administration	Mechanism of Action	Reference
Aloe vera	Liquid gel	Gel is applied topically	Forms protective coating on affected areas and stimulating wound healing rate and reducing the chance of infection	
Azadiracta indica	oil	Topical application	Inhibits inflammation to accelerate healing,	
Tridax procumbens	Leaf juice		Arrest bleeding, increases epithelization and collagenization	
Chromolaena odorata	leaf	Aqueous extract & decoction leaves are crushed and the decoction used in treating skin wounds	enhances hemostatic activity, stimulates granulation tissue and re-epithelization processes. Inhibits wound contraction	Akah, 1990; Obi et al., 2011).
Spathodea campanulata **Beav**	bark	Aqueous extract are applied to wound		
Commelina diffusa **Burn. F**	leaves	Aqueous extract are applied to wound		(Abraham et al, 2006)

Reactive Oxygen Species (ROS)

Oxidation is a basic part of the aerobic life and our metabolism. During oxidation, many free radicals are produced which have an unpaired nascent electron. Atoms of oxygen or nitrogen having central unpaired electron are called reactive oxygen or nitrogen species (Finkel et al., 2000; Sathya & Kokilavan, 2013). These species are natural by-products produced by the normal metabolism of oxygen in living organisms. These reactive oxygen species (ROS) are various forms of activated oxygen which causes oxidative damage. They include free radicals such as superoxide anion radicals $(O_2)^-$, hydroxyl radicals (OH•) and non free radical species such as peroxyl radicals $(O_2)^{2-}$ and singlet oxygen ($^1O_2$) are various forms of activated oxygen generated in the body (Visioli et al., 2000)

In small amounts, these ROS can be beneficial as signal transducers and growth regulators. However, during oxidative stress, large or excessive amounts of these ROS can be produced and may be dangerous and harmful to the body. The immune system is vulnerable to oxidative stress. Oxidative stress refers to an imbalance between the production of free radicals and the antioxidant defense system. Excessive amounts of ROS may be a primary cause of biomolecular oxidation. The ROS have the ability to attack numerous molecules, including proteins and lipids. (Tosun et al, 2009). As a result, ROS have the potential of causing peroxidation of membrane lipids, aggression of tissue membranes and proteins or damage to DNA and enzyme and generally by oxidizing low-density lipoproteins (LDL). This may result in significant damage to cell structure, contributing to various diseases, such as cancer, stroke, diabetes, arthritis,

haemorrhagic shock, coronary artery diseases, cataract, cancer, AIDS as well as age-related degenerative brain diseases (Parr & Bolwell, 2000).

Under normal circumstances the cell can reduce the impact of these free radicals and ROS by an endogenous system i.e. by the body's natural antioxidant defense, e.g. glutathione peroxidase, catalase and superoxide dismutase (Aruoma, 1994). However, overproduction of ROS and antioxidant depletion arising from either the mitochondrial electron transport chain or excessive stimulation of NAD(P)H, or from exposure to environmental pollutants i.e. cigarette smoke, UV-rays, radiation and toxic chemicals (Valko et al, 2006), results in a weakened body defense system. These environmental events and exposure to explosion-generated shock waves (Elsayed, 2003) were also proposed to be associated with antioxidant depletion. Hence the need is created to reduce the impact of ROS through exogenous system using antioxidants such as vitamin C and α-tocopherol. There is therefore the need to provide the body with a constant supply of antioxidants through dietary supplementation.

Antioxidants

Antioxidants are compounds that detoxify ROS and prevent their damage through multi mechanisms. Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and thus prevent disease. Antioxidants have the ability to prevent, delay or ameliorate many of the effects of free radicals. During certain diseased state, as well as during aging, there is a need to boost the antioxidant abilities, thereby potentiating the immune mechanism (Devasagayam & Sainis, 2002). The antioxidants

preserve and stimulate the function of immune cells against homeostatic disturbances (De la Fuente & Victor, 2000).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary hydroquinone (TBHQ) are commonly employed as preservatives or additives by pharmaceutical, cosmetic, and food companies (Nguyen & Eun, 2011). The free radicals are known to be scavenged by these synthetic antioxidants. However, reports on the involvement of synthetic antioxidants in chronic diseases and their adverse side effects leading to carcinogenicity have restricted their use in foods. Therefore, international attention has been focused on natural antioxidants mainly from plant sources (Dehgahan et al., 2007; Kai-Wei, 2009) and the search for effective and natural antioxidants has become crucial (Rao et al., 2010).

A number of plants and plant isolates have been reported to protect free radical-induced damage in various experimental models. In recent times, focus on plant research has increased all over the world and a lot of have been collected to show the immense potential of medicinal plants used in various traditional systems (Modi et al., 2010). Majority of these plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites, which are rich in antioxidant activities (Aiyegoro & Okoh, 2010).

Phenolic Compounds

Phenolics, are a class of chemical compounds consisting of a hydroxyl group (—OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol, which is also called carbolic acid C_6H_5OH. Phenolic compounds consist of simple phenols, benzoic and cinnamic acid, coumarins, tannins, lignins, lignans and flavonoids. Phenolic compounds are well-known phytochemicals found in all plants and are classified as simple phenols or polyphenols based on the number of phenol units in the molecule (Amorati & Valgimigli, 2012; Khoddami et al, 2013).

Plant phenolic compounds are diverse in structure but are characterised by hydroxylated aromatic rings (e.g. flavan-3-ols). They are categorised as secondary metabolites, and their function in plants is often poorly understood. Many plant phenolic compounds are polymerised into larger molecules such as the proanthocyanidins (PA; condensed tannins) and lignins.

It has been mentioned that the antioxidant activity of plants might be due to their phenolic compounds (Cook & Samman, 1996). Phenolic compounds exhibit a considerable free radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen or electron-donating agents, the stability of the resulting antioxidant derived radical, their reactivity with other antioxidants and finally their metal chelating properties (Tuadhar & Rao, 2010; Wojdylo et al, 2007). Derived polyphenols from plants are of great importance because of their potential antioxidant and antimicrobial properties (Kumbhare et al, 2012). Some evidence suggests that the biological actions of these compounds are related to

their antioxidant activity. Phenolics display a vast variety of structures; here only flavonoids and phenolic acids are reviewed with much emphasis on flavonoids.

Phenolic Acids

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart disease, stroke, and cancers). Phenolic acid compounds seem to be universally distributed in plants. They have been the subject of a great number of chemical, biological, agricultural, and medical studies. They form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids both of which are derived from nonphenolic molecules benzoid and cinnamic acid, respectively (Macheix & Fleuriet 1998). Hydroxycinnamic acid compounds occur most frequently as simple esters with hydroxy carboxylic acids or glucose. Hydroxybenzoic acid compounds are present mainly in the form of glucosides. Furthermore, phenolic acids may occur in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyfatty acids, sterols, and glucosides.

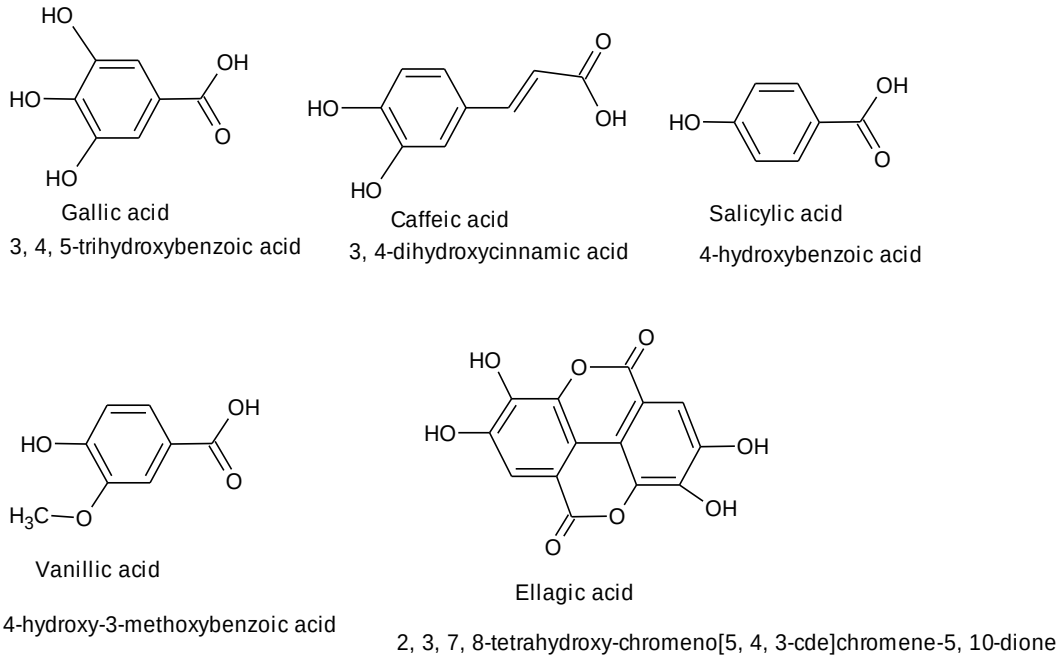


Figure 2: Typical examples of structures of phenolic acids.

Flavonoids

The term "flavonoid" is generally used to describe a broad collection of natural products that are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Frankel, 1995).

Among the large number of natural products of plant origin, called secondary metabolites, flavonoids play a central role. The study of flavonoid chemistry has emerged, like that of most natural products, from the search for new compounds with useful physiological properties. Flavonoids have long sparked the interest of scientists and nonscientists alike, largely because these metabolites account for

much of the red, blue, and purple pigmentation found in plants and increasingly for their association with the health benefits of wine, chocolate, and generally with diets rich in fruits and vegetables. In the plant kingdom, different plant families have characteristic patterns of flavonoids and their conjugates. All these compounds play important biochemical and physiological roles in the various cell types or organs (seed, root, green part, and fruit) where they accumulate (Winkel, 2006). Different classes of flavonoids and their conjugates have numerous functions during the interactions of plant with the environment, both in biotic and abiotic stress conditions (Shirley, 1996). Additionally, flavonoid conjugates, because of their common presence in plants, are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative (Beck et al., 2003; Boue et al., 2003).

Chemical Structure and Nomenclature of Flavonoids

Flavonoids are formed by a series of condensation reactions between hydroxycinnamic acid (B-ring and carbon atoms 2, 3 and 4 of the C-ring) and malonyl residues (A-ring), giving rise to a C6—C3—C6 base structure. The three-carbon bridge between the phenyl rings is commonly cyclized to form a third ring (C-ring). According to the cyclization and the degree of unsaturation and oxidation of the three-carbon segment, they can be classified into several groups. The basic structures of the main classes of flavonoids are shown in Figure. 3, 4 & 5.

The chemical structures of this class of compounds are based on a C6-C3-C6 skeleton or a C6-C3-C6 carbon framework, or more specifically a phenylbenzopyran functionality. They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns (Figure 8 & 9).

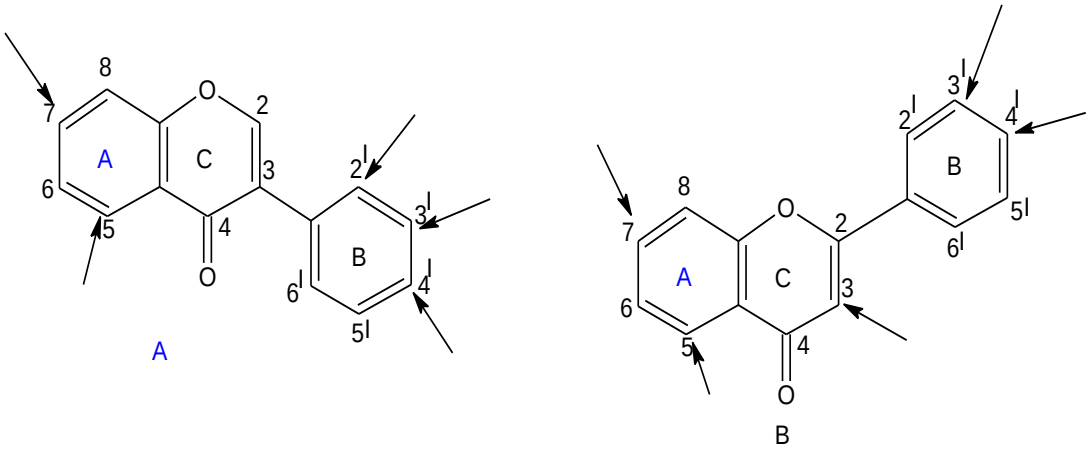


Figure 3: Flavonoid structures, ring labeling, and carbon atom numbering. (A) Isoflavones. (B) Flavones and flavonols. Arrows indicate most frequent hydroxylation sites

The flavonoids may be modified by hydroxylation, methoxylation, or *O*-glycosylation of hydroxyl groups as well as *C*-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, alkyl groups (often prenyls) may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton of the flavonoid core.

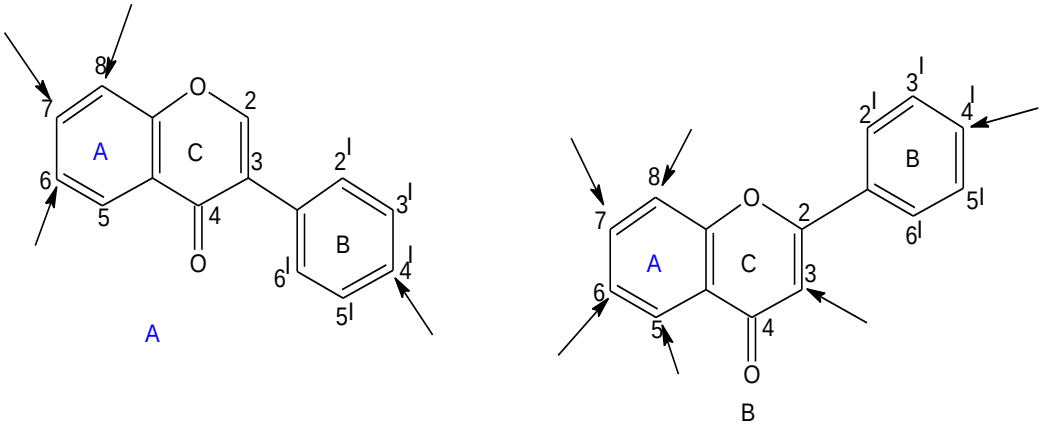


Figure 4: Flavonoid structures, ring labeling, and carbon atom numbering. (A) Isoflavones. (B) Flavones and flavonols. Arrows indicate most frequent C- and/or O- glycosylation sites

The last modification takes place most often in the case of isoflavonoids, where the B ring is condensed to the C-3 carbon atom of the skeleton. Flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. These derivatives are thermally labile and their isolation and further purification without partial degradation is difficult. The multiplicities of possible modifications of flavonoids result in more than 6,000 different compounds from this class were known in the end of the last century and this number continues to increase (Harborne & Williams, 2000). Condensed tannins create a special group of flavonoid compounds formed by polymeric compounds built of flavan-3-ol units, and their molecular weights often exceeding 1,000 Da. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: **3**, the flavonoids (2-phenylbenzopyrans), **4**, isoflavonoids (3-phenylbenzopyrans), and **5**, neoflavonoids (4-phenylbenzopyrans).

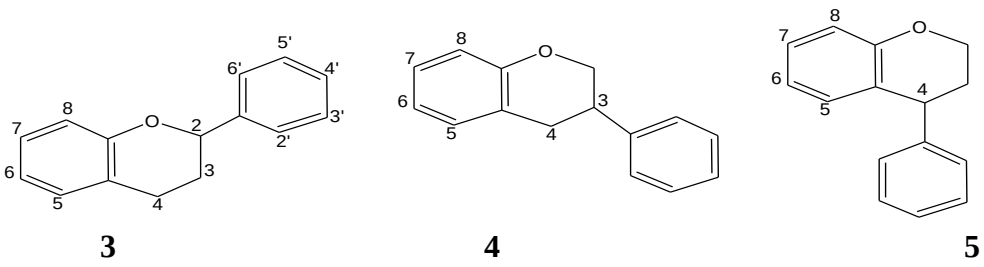


Figure 5: The three main classes of flavonoids

These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related.

2-Phenylbenzopyrans **(C6-C3-C6 Backbone)**

Based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups:

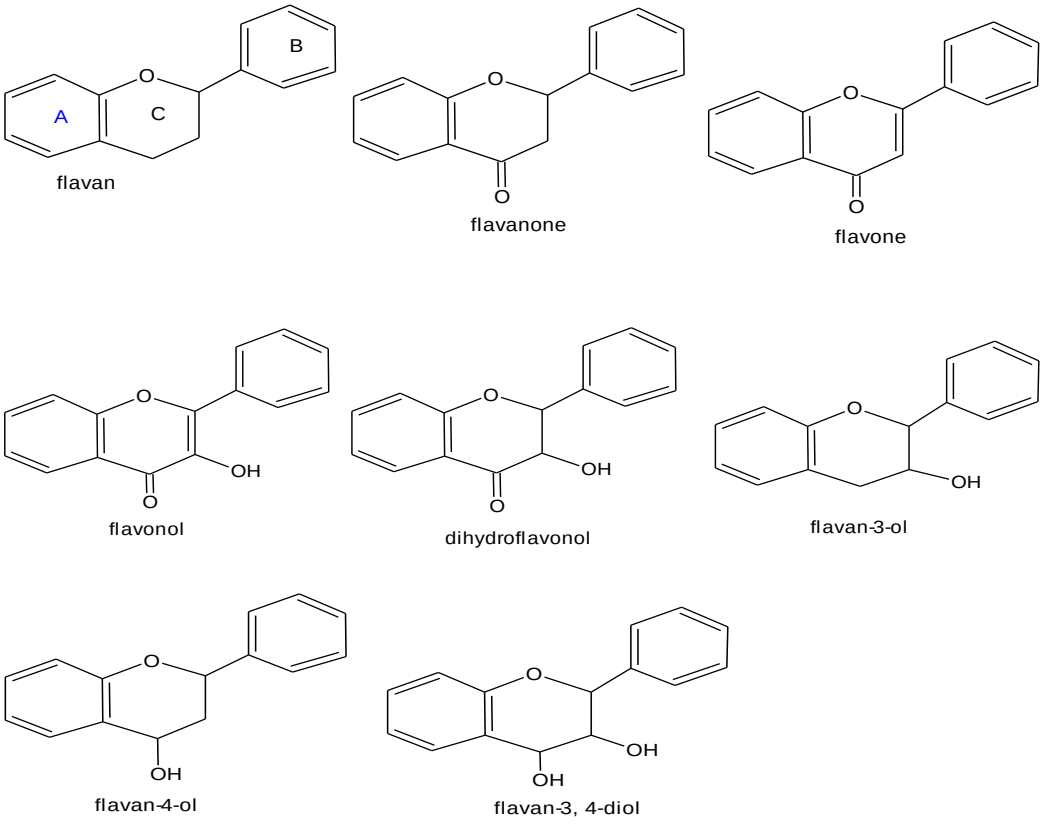


Figure 6: Division of flavonoids

Isoflavonoids

The isoflavonoids are a distinctive subclass of the flavonoids. These compounds possess a 3-phenylchroman skeleton that is biogenetically derived by 1,2-aryl migration in a 2-phenylchroman precursor. Despite their limited distribution in the plant kingdom, isoflavonoids are remarkably diverse as far as structural variations are concerned. This arises not only from the number and complexity of substituents on the basic 3-phenylchroman system, but also from the different oxidation levels and presence of additional heterocyclic rings. Isoflavonoids are subdivided into the following groups:

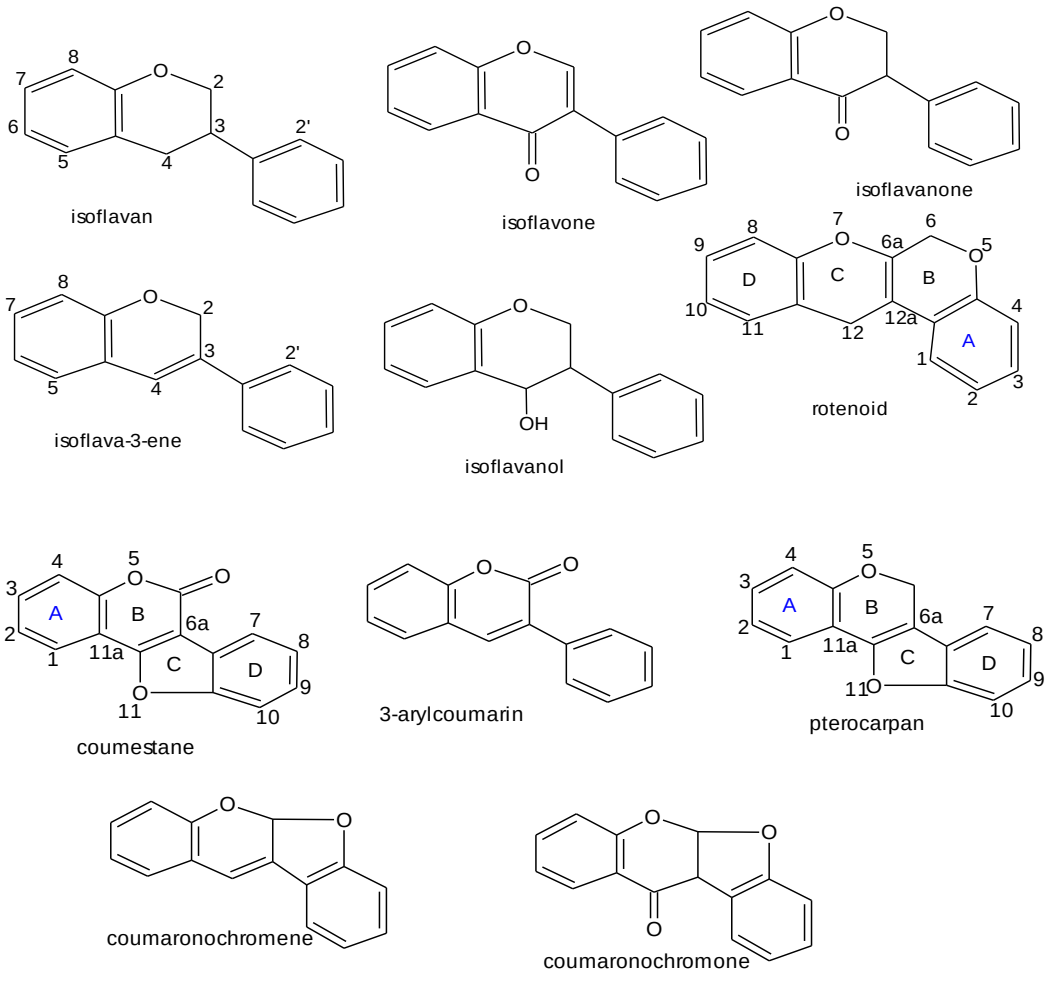


Figure 7: Division of isoflavonoid

Neoflavonoids

The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2*H*-1-benzopyran-2-ones), 3,4-dihydro-4-arylcoumarins, and neoflavenes.

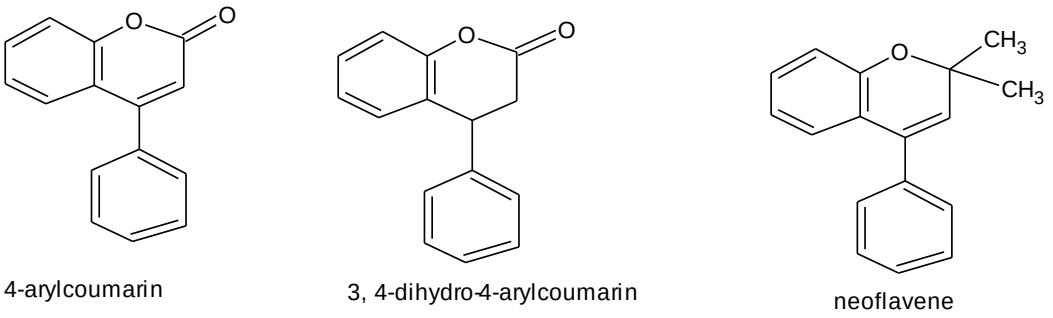


Figure 8: Division of Neoflavonoids

Minor Flavonoids

Natural products such as chalcones and aurones also contain a C6-C3-C6 backbone and are considered to be minor flavonoids. These groups of compounds include the 2′-hydroxychalcones, 2′-OH-dihydrochalcones, 2′-OH-*retro*-chalcone, aurones (2- benzylidenecoumaranone), and auronols.

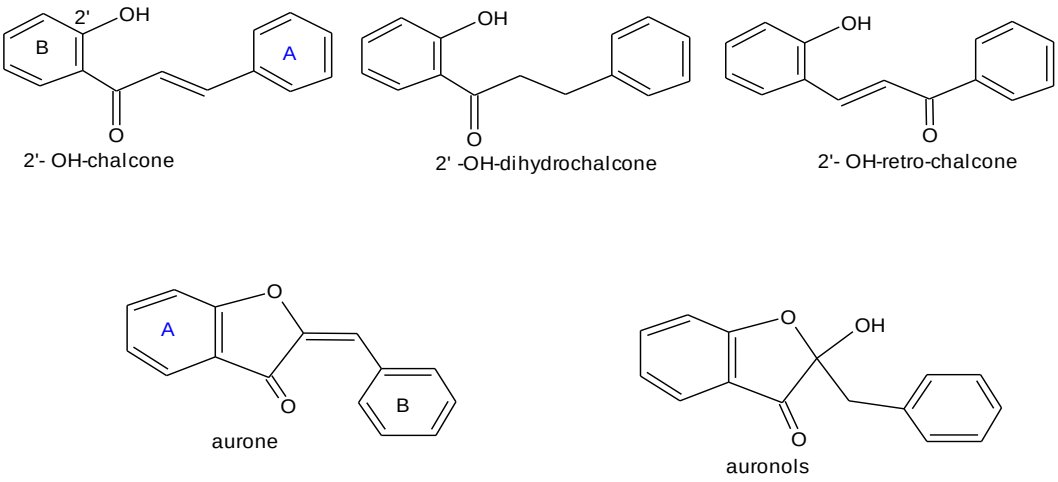


Figure 9: Examples of minor flavonoids

At present, about 400 flavone aglycones, 450 flavonol aglycones, 350 flavanone aglycones, 300 isoflavone aglycones, 19 anthocyanidins and 250 chalcone aglycones have been reported. In plants, flavonoids may occur in various modified forms corresponding to additional hydroxylation, methylation and, most importantly, glycosylation. Occasionally, aromatic and aliphatic acids, sulfate, prenyl, methylenedioxyl or isoprenyl groups also attach to the flavonoid nucleus and their glycosides (Iwashina, 2000). Flavonoids commonly occur as flavonoid *O*-glycosides, in which one or more hydroxyl groups of the aglycone are bound to a sugar with formation of a glycosidic O—C bond, which is an acid-labile hemiacetal bond. The effect of glycosylation is to render the flavonoid less reactive and more water soluble, so that glycosylation can be regarded as an essential form of protection in plants to prevent cytoplasmic damage and to store the flavonoids safely in the cell vacuole. In principle, any of the hydroxyl groups can be glycosylated but certain positions are favored: for example, the 7-hydroxyl group in flavones, flavanones and isoflavones, the 3- and 7-hydroxyls in flavonols and flavanols and the 3- and 5-hydroxyls in anthocyanidins are common glycosylation sites. 5-*O*-Glycosides are rare for compounds with a carbonyl group on position 4, since the 5-hydroxyl group participates in hydrogen bonding with the adjacent 4-carbonyl group (Cuyckens & Claeys, 2004).

Glucose is the most commonly encountered sugar, galactose, rhamnose, xylose and arabinose are not uncommon, and mannose, fructose, glucuronic and galacturonic acids are rare. Disaccharides are also often found in association with flavonoids, the more common ones being rutinose (rhamnosyl-(α1 → 6)-

glucose) and neohesperidose (rhamnosyl-(α1 → 6)-glucose) and occasionally tri- and even tetrasaccharides.

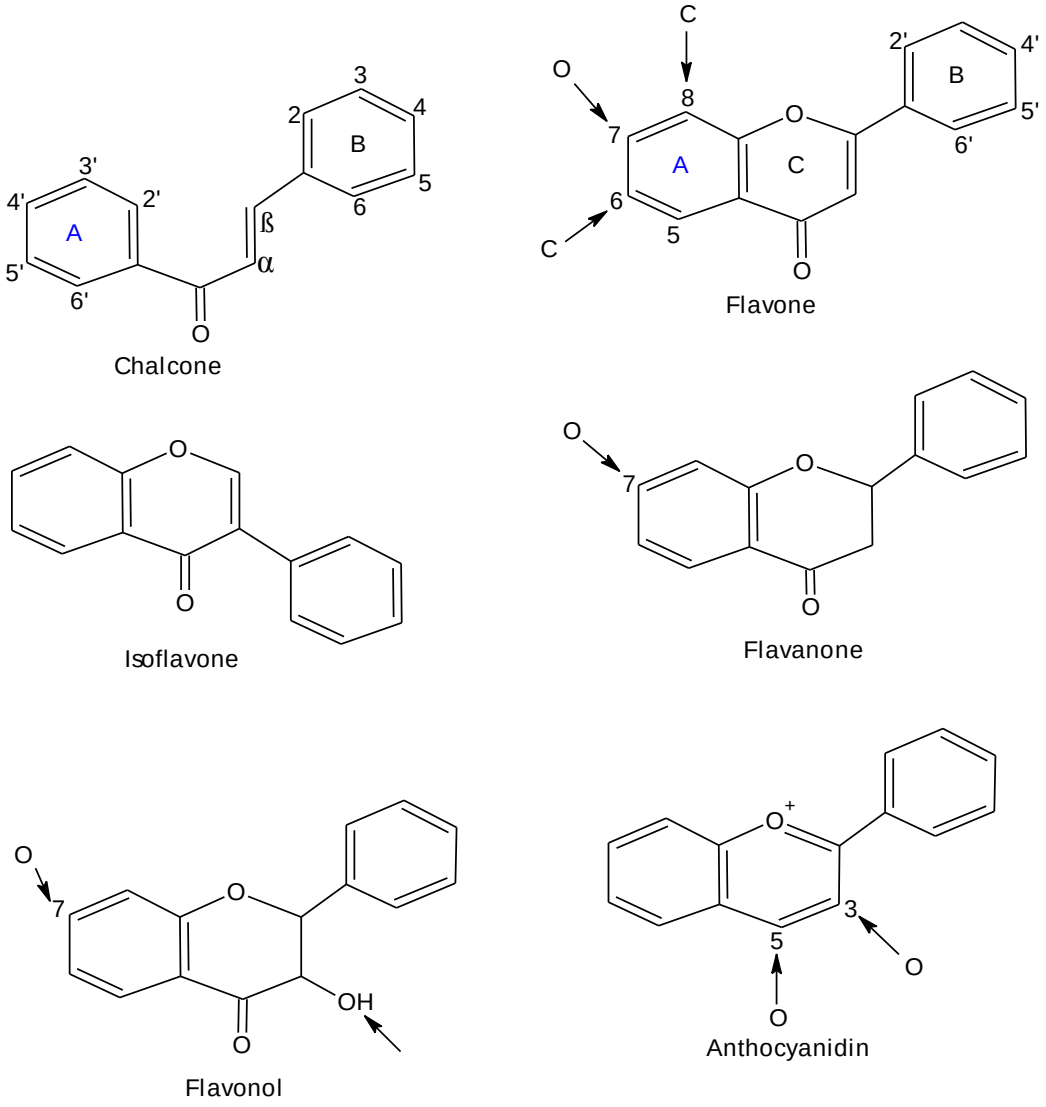


Figure 10: Basic structures of the main classes of flavonoids. Common *O*- and *C*-glycosylation positions are indicated with an arrow

Acylated glycosides, in which one or more of the sugar hydroxyls are esterified with an acid, also occur. Glycosylation may also take place by direct linkage of the sugar to the flavonoid's basic nucleus, via an acid-resistant C—C bond, to form flavonoid *C*-glycosides.

Flavonoid *C*-glycosides are commonly further divided into mono-*C*-glycosylflavonoids, di-*C*-glycosylflavonoids and *C*-glycosylflavonoid- *O*-glycosides. In the last category, a hydrolyzable sugar is linked either to a phenolic hydroxyl group or a hydroxyl group of the *C*-glycosyl residue. To date, *C*-glycosylation has only been found at the C-6 and/or C-8 position of the flavonoid nucleus (Figure. 10).

Biosynthesis of Flavonoids

Flavonoid biosynthesis is probably the best characterized of all the secondary metabolic pathways. The flavonoid pathway is part of the larger phenylpropanoid pathway, which produces a range of other secondary metabolites, such as phenolic acids, lignins, lignans, and stilbenes. The key flavonoid precursors are phenylalanine, obtained via the shikimate and arogenate pathways, and malonyl-CoA, derived from citrate produced by the TCA cycle. Most flavonoid biosynthetic enzymes characterized to date are thought to operate in enzyme complexes located in the cytosol. Flavonoid end products are transported to various subcellular or extracellular locations, with those flavonoids involved in pigmentation generally being transported into the vacuole. There are many branches to the flavonoid biosynthetic pathways, with the best characterized being those leading to the colored anthocyanins and proanthocyanidins (PAs) and the generally colorless flavones, flavonols, and isoflavonoids. Genes or cDNAs have now been identified for all the core steps leading to anthocyanin, flavone, and flavonol formation, as well as many steps of the isoflavonoid branch, allowing extensive analysis of the encoded enzymes.

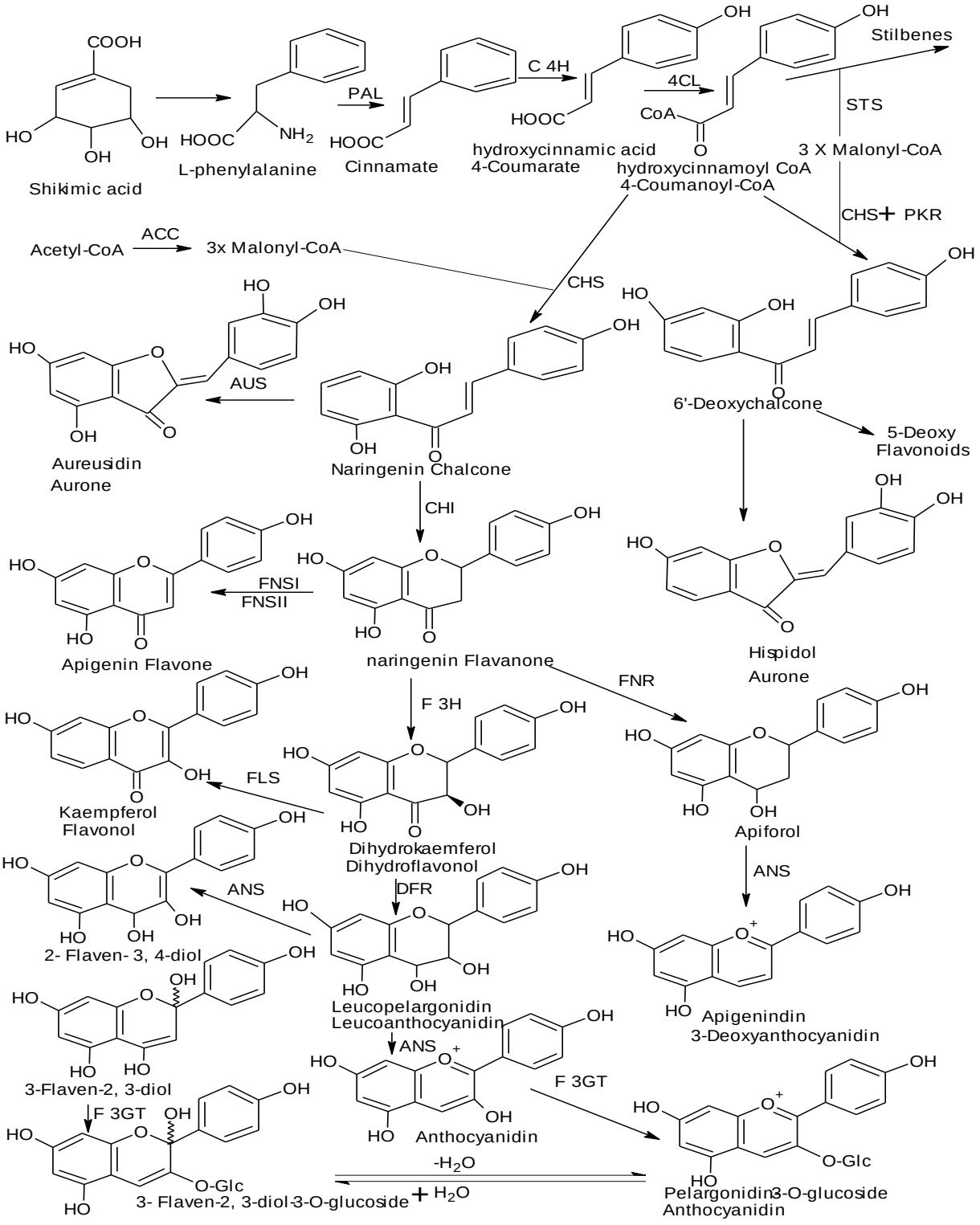


Figure 11: General phenylpropanoid and flavonoid biosynthetic pathways (Winkel, 2006)

The first flavonoids, the chalcones, are formed from HCA-CoA esters, usually 4-coumaroyl-CoA (Figure 11), in three sequential reactions involving the ‘‘extender’’ molecule malonyl-CoA. In a few species, caffeoyl-CoA and feruloyl-CoA may also be used as substrates for chalcone formation. 4-Coumaroyl-CoA is produced from the amino acid phenylalanine by what has been termed the general phenylpropanoid pathway, through three enzymatic conversions catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate: CoA ligase (4CL). Malonyl-CoA is formed from acetyl-CoA by acetyl-CoA carboxylase (ACC) (Figure 16). Acetyl-CoA may be produced in mitochondria, plastids, peroxisomes, and the cytosol by a variety of routes. It is the cytosolic acetyl-CoA that is used for flavonoid biosynthesis, and it is produced by the multiple subunit enzyme ATP-citrate lyase that converts citrate, ATP, and Co-A to acetyl-CoA, oxaloacetate, ADP, and inorganic phosphate. Many other compounds are involved in flavonoid biosynthesis in some species, for example, as donors for methylation or aromatic or aliphatic acylation. For intact plants, these are generally accepted to be available in the cell for the reaction to proceed if the appropriate modification activity is present.

Fuctions of Flavonoids

The old concept of flavonoids being merely the by-products of cellular metabolism, which are simply compartmentalized in solution in the cell vacuole, is well and truly past its use-by date. For a start, studies have revealed that flavonoids are also commonly found on the outer surfaces of leaves and flowers, albeit only the aglycone forms. Additionally, flavonoids have been shown over

the past few years to be found in the cell wall, the cytoplasm, in oil bodies, and associated with the nucleus and cell proteins, as well as in the vacuole. Even in the vacuole, flavonoids are not necessarily found free in solution. For example, protein-bound flavonoids have been isolated from lisianthus and other flowers in which a structurally specific binding has been identified (in anthocyanic vacuolar inclusions). It is probable that flavonoid location and specific protein binding properties will ultimately prove to relate directly to their function in plants. Amongst the many functions now known to be performed by plant flavonoids are those of UV protection, oxidant or free radical protection, modulation of enzymic activity, allelopathy, insect attraction or repulsion, nectar guides, probing stimulants, viral, fungal, and bacterial protection, nodulation in leguminous plants, pollen germination, etc., and it is likely that this is only the tip of the iceberg. Flavonoids, it would seem, have been vital components of plants, ever since their (purported) development at the time plant life emerged from the aquatic environment, and needed protection from UV light in an atmosphere lacking today's protective ozone layer. The continued widespread accumulation of flavonoids by virtually all land-based green plants lends support to this view. Manipulation of the flavonoid biosynthetic pathway in plants via genetic engineering has progressed rapidly in recent years. Genetic manipulation of the flavonoid pathway in plants has enormous potential to, for example, produce new flower colors, enhance the nutritional value of crops, and improve crop protection from UV light, microorganisms, insects, and browsing animals. Plant flavonoids have been shown in recent years to be of vital significance to mankind as well as

to plants. They have been strongly implicated as active contributors to the health benefits of beverages such as tea and wine, foods such as fruit and vegetables, and even, recently, chocolate. Other potential health benefits of dietary flavonoids are too numerous to mention here. Suffice it to say that our understanding of the importance of flavonoids in the human diet is continuing to advance rapidly. One suspects that much of the physiological activity associated with flavonoids can be attributed to (i) their proven effectiveness as antioxidants and free radical scavengers, (ii) to their metal complexing capabilities (a capability that drove early advances in absorption spectroscopy and NMR studies), and (iii) to their ability to bind with a high degree of specificity to proteins.

Antioxidant Activity

The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species. Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage.

Diets high in flavonoids, fruits, and vegetables are protective against a variety of diseases, particularly cardiovascular disease and some types of cancer (Ness & Powles, 1997). Antioxidants and dietary fiber are believed to be the principal nutrients responsible for these protective effects. Reactive oxygen species (ROS) are formed *in vivo* during normal aerobic metabolism and can cause damage to DNA, proteins, and lipids, despite the natural antioxidant defense system of all

organisms (Bors & Saran, 1987). Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response and tissue damage.

ROS contribute to cellular aging (Sastre et al., 2000), mutagenesis (Takabe et al., 2001), carcinogenesis (Kawanishi et al., 2001), and coronary heart disease (Khan & Baseer, 2000) possibly through the destabilization of membranes (Takabe et al., 2001), DNA damage, and oxidation of low-density lipoprotein (LDL). To protect themselves from reactive oxygen species, living organisms have developed several effective mechanisms (Nijveldt et al., 2001). The antioxidant-defense mechanisms of the body include enzymes such as superoxide dismutase, catalase, and glutatione peroxidase, but also nonenzymatic counterparts such as glutathione, ascorbic acid, and α-tocopherol. The increased production of reactive oxygen species during injury results in consumption and depletion of the endogenous scavenging compounds. Flavonoids may have an additive effect to the endogenous scavenging compounds. Many *in vitro* studies have demonstrated the potent peroxyl radical scavenging abilities of flavonoids, which contribute to inhibiting lipid peroxidation and oxidation of LDL (Castelluccio et al., 1995).

Protective effects of flavonoids

The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer free radical electrons, chelate metal catalysts (Ferrali et al., 1997), activate antioxidant enzymes, reduce alpha-tocopherol radicals (Hirano et al., 2001), and inhibit oxidases.

Green tea is a rich source of flavonoids, primarily catechins and flavonols. In black tea, as a consequence of the fermentation process, catechins are converted to complex condensation products, the theaflavins. Tea polyphenols show strong antioxidative effects and provide powerful scavengers against superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite produced by various chemicals and biological systems. Anderson and collaborators reported that green tea polyphenols partially protect DNA from •OH radical-induced strand breaks and base damage (Anderson et al., 2001).

Pulse radiolysis results support the mechanism of electron transfer (or H-transfer) from catechins to radical sites on DNA (Anderson et al., 2001). In black tea, all the theaflavins showed the same capacity to inhibit the production of superoxide. Green tea and black tea were shown to block the production of oxygen free radicals derived from the cooked meat mutagen 2-amino-3-methylimidazo [4, 5-f] quinoline (IQ) in the presence of a NADPH-cytochrome P450 reductase (Hasaniya et al., 1997). These results support an antioxidant role of catechins in their direct interaction with DNA radicals. Catechin polyphenols could also decrease the peroxynitrite-induced nitration of tyrosine and protect the apolipoprotein B-100 of LDL from peroxynitrite induced modification of critical amino acids, which contribute to its surface charge (Pannala et al., 1997).

Other flavonoids such as quercetin, kaempferol, myristin, apigenin, and leuteolin also have antioxidative activity in many *in vitro* studies (Dwyer 1995; Frankel et al., 1993). These authors showed that catechin oligomers, anthocyanidin dimers and trimers, as well as myricetin were main antioxidant

components in red wine. Ghiselli and collaborators observed that anthocyanins were the most effective, both in scavenging ROS and in inhibiting lipoprotein oxidation (Ghiselli et al., 1998).

Other studies in humans found that chronic red wine consumption (400 ml/day) reduced the susceptibility of LDL to lipid peroxidation catalyzed by Cu (Fuhrman et al., 1995). Similarly, a small but significant increase in the lag time of LDL oxidation was observed after 4 weeks of black tea consumption (600 ml/day) (Ishikawa et al., 1997).

Anticarcinogenesis of Flavonoids

Studies on cancer prevention have assessed the impact of a wide variety of flavonoids and a selected few isoflavones for their efficacy in inhibiting cancer in a number of animal models. These studies demonstrated that flavonoids inhibit carcinogenesis *in vitro* and substantial evidence indicates that they also do so *in vivo* (Caltagirone et al., 2000; Miyagi et al., 2000). Flavonoids may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages. Animal studies and investigations using different cellular models suggested that certain flavonoids could inhibit tumor initiation as well as tumor progression (Tanaka et al., 1997).

Flavonoids as Nutraceuticals

"Nutraceutical" is a term coined in 1979 by Stephen DeFelice (DeFelice, 1992). It is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." Subsequently, several other terms (medical food, functional food, and nutritional supplements)

were used. A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease (Dillard & German, 2000).

Nutraceuticals may range from isolated nutrients, dietary supplements, and diets to genetically engineered "designer" food, herbal products, and processed products, such as cereals, soups, and beverages. The increasing interest in nutraceuticals reflects the fact that consumers hear about epidemiological studies indicating that a specific diet or component of the diet is associated with a lower risk for a certain disease.

The major active nutraceutical ingredients in plants are flavonoids. The flavonoids are a group of organic molecules ubiquitously distributed in vascular plants. Approximately 2000 individual members of the flavonoids group of compounds have been described. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also appear to be effective at influencing the risk of cancer. Overall, several of these flavonoids appear to be effective anticancer promoters and cancer chemopreventive agents.

Determination of Antioxidant Properties

The antioxidant activities of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Yildirim et al., 2001). Various methods are used in determining the antioxidant properties of plant samples. These include: measurement of total phenolic content, reducing power,

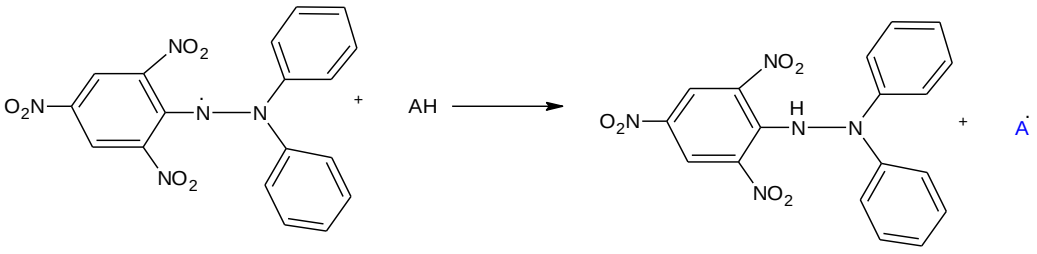
hydrogen peroxide decomposition/consumption, DPPH scavenging, Total antioxidant capacity and Fe^{2+} chelation. Yildirim et al (2001) has suggested non-linear correlation between total antioxidant activity and the individual measurement and that the antioxidant activity of any species is a cumulative effect of most (if not all) of the measurements. This subsection gives an overview of some of these methods that were used in this work.

DPPH radical scavenging activity

The antioxidant ability of a sample can also be estimated by determining the hydrogen donating ability of the samples in the presence of 2, 2-diphenyl-1-picryl-hydazyl or 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical at 517 nm. The determination is based on the discolouration of the purple coloured methanolic solution of DPPH free to yellow by free radical scavengers.

DPPH radical scavenging assay is considered a good *in vitro* model or a stable free radical method widely used to test the ability of compounds as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of phyto-constituents. The free radical scavenging activity of plant extracts is evaluated based on the ability to scavenge the synthetic DPPH. DPPH is a stable nitrogen centered free radical which can be effectively scavenged by antioxidants and shows strong absorbance at 517 nm. It assesses antioxidant efficacy within a very short time. DPPH assay has been extensively used for screening antioxidant activity because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentration. It is an easy, rapid and sensitive method to survey or screen the antioxidant activity of a

specific compound or plant extract (Koleva et al, 2002). In its radical form, DPPH disappears on reduction by accepting an electron from an antioxidant compound or a hydrogen radical species to become a stable diamagnetic molecule (the electron became paired in the presence of free radical scavenging) resulting the colour change from purple to yellow, which could be taken as an indication of the hydrogen donating ability of the tested sample (Lee et al., 2007; Marxen et al; 2007).



Diphenylpicrylhydrazyl (free radical)

Diphenylpicrylhydrazine (non-radical)

$$DPPH^{\cdot} + AH \longrightarrow DPPH\text{-}H + A^{\cdot}$$

Where DPPH-H is the reduced form, AH the donor molecule and A. is a free radical produced.

Figure 12: DPPH reaction with a test sample

The change in absorbance of DPPH radical caused by antioxidants is due to the reaction between the antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. Extent of DPPH radical scavenging activity is determined by the decrease in intensity of violet colour in the form of IC_{50} values (Hu & Kitts, 2000). The bleaching absorption of DPPH is representative of the capacity of extracts to scavenge free radicals independently. Hence it has been

widely used for rapid evaluation of the antioxidant activity of plant and microbial extracts relative to other methods (Rackova et al., 2007). DPPH is also considered as a good kinetic model for peroxyl radicals (Hagerman et al., 1998). When DPPH radicals encounter a proton donating substance such as an antioxidant, it would be scavenged and the absorbance is reduced. Thus, the DPPH radicals are widely used to investigate the scavenging activity of some natural compounds.

Ferric reducing antioxidant power (FRAP) assay

Heavy metals like Fe^{3+} and Cu^{2+} are known to catalyse oxidative process in living organisms. Fe^{3+}, for instance, is reduced to Fe^{2+} in the process. A species ability to reduce Fe^{3+} to the +2 state is known as its reducing power and it is an indication of its antioxidant property (Yildirim et al., 2001).

The FRAP assay is used to determine the ability of plant extract to reduce ferric ions. FRAP assay measures the changes in absorbance at 593 nm owing to the formation of blue colored Fe^{2+} tripyridyltriiazine compound from the colourless oxidized Fe^{3+} form by the action of electron donating antioxidants (Gupta et al, 2009). The FRAP (ferric reducing /antioxidant power) method assess the reducing potential of extract and is based on the ability to reduce ferric ions.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Yildirim et al., 2001). Oyaizu (1986) has described a dose-dependent method (which was modified by Yildirim et al in 2001) for the determination of the reducing capacity of samples. In his method, various concentrations (100-1000 µg/ml) of the plant samples are prepared and mixed with phosphate buffer and 1% w/v of potassium ferricyanide [$K_3Fe(CN)_6$].

The mixture is incubated at 50^{o}C for 30 minutes, after which 10% w/v trichloroacetic acid is added and centrifuged at 3000 rpm for 10 minutes. To about 2.5ml of the supernatant layer of the solution is added 2.5 ml distilled water and 0.5 ml of 0.1% w/v $FeCl_3$. The sample's ability to reduce the Fe (III) to Fe (II) is determined by measuring the amount of the Fe (II) spectroscopically; the absorbance of the reaction mixture is measured at 700 nm. Increased absorbance indicates increased reducing power (Blázovics et. al., 2003).

Total Antioxidant Activity (TAC)

The total antioxidant capacity of the methanol extract is evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. (1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid ρH. Normally, 0.3 ml sample is combined with 3 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the reaction mixture is measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of sample is used as the blank. The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid.

Isolation and Identification of Flavonoids

The identification and structural characterization of flavonoids and their conjugates isolated from plant material, as single compounds or as part of mixtures of structurally similar natural products, create some problems due to the presence of isomeric forms of flavonoid aglycones and their patterns of

glycosylation. A number of analytical methods are used for the characterization of flavonoids. In many cases, nuclear magnetic resonance (NMR) analyses (^{1}H and ^{13}C) are necessary for the unambiguous identification of unknown compounds; other instrumental methods (mass spectrometry, UV and IR spectrophotometry) applied for the identification of organic compounds fail to provide the information necessary to answer all the structural questions. Utilization of standards during analyses and comparison of retention times as well as spectral properties, especially when compounds are present in a mixture, is critical.

Characterisation of flavonoids

Classification of flavonoid type in a plant tissue is based initially on a study of solubility properties and colour reactions (Harborne, 1973). This is followed by a one-dimensional chromatographic examination of a hydrolysed plant extract and two-dimensional chromatography of a direct alcoholic extract. Finally the individual components are identified by chromatographic and spectral comparison with known markers. It is difficult to state a general solubility rule for flavonoids because they range from water-insoluble but ether-soluble (highly methylated nonheterosides), to ether- and alcohol soluble (hydroxyflavone, flavonone, and isoflavone aglycones), to warm-soluble, ether-insoluble (heteresides with up to three sugars) forms (Farnsworth, 1966). Both flavonoid heterosides and aglycones are, as a general rule, insoluble in petroleum ether and advantage can be taken of this fact to defat the sample prior to alcohol extraction.

Flavonoids are phenolic and hence change colour when treated with base or with ammonia; thus they are easily detected on chromatograms or in solution

(Mothes, 1964). In the absence of interfering pigments, a yellow colouration indicates the presence of flavones and flavonols. Table 4 gives examples of colour changes of flavonoids. Chalcones and aurones turn from yellow to red in this test. If an aqueous pigment extract is made alkaline the following colour changes may be observed although the changes in one pigment may mask changes in another (Opoku-Boahen, 1989):

anthocyanins	purple-blue
flavones, flavonols	yellow
flavanones:	colourless, becoming orange-red especially when heated
chalcones, aurones:	immediate red-purple
flavanonols:	orange brown

The classic procedure for detecting phenolic compounds is by means of the intense green, purple, blue or black colours many of them give with 1% aqueous or alcoholic ferric chloride. This procedure modified by using fresh aqueous mixture of 1% ferric chloride and 1% potassium ferricyanide (1:1 volume) is still used as a general means of detecting phenolic compounds; but is still of little value in distinguishing different classes (Harborne, 1973).

Table 4: Colour Reactions on Flavonoids (Opoku-Boahen, 1989)

Flavonoid Type	Visible	UV	Ammonia Visible	UV	$AlCl_3$ Visible
Flavones	Pale yellow	Dull-brown red-brown yellow-brown	Yellow	Bright-yellow yellow-green dull-purple	Pale yellow
Flavonols	Pale yellow	Bright-yellow yellow-green brown	Yellow	Bright yellow yellow-green green	Yellow
Isoflavones	Colourless	Faint purple pale yellow	Colourless	Faint purple pale yellow	Colourless
Catechins	Colourless	Colourless	Colourless	Fluorescent pale blue black	Colourless
Flavanones	Colourless	Colourless	Colourless	Colourless pale yellow yellow-green	Colourless
Anthocyanins	Pink orange red-purple	Dull red or purple pink brown[e]	Blue-grey blue	bluish	
Aurones	Bright yellow	Bright yellow green yellow	Orange Orange pink	Yellow orange Orange Red orange Brown	Pale yellow
Chalcones	Yellow	Brown black Yellow-brown	Yellow orange red-orange pink	Orange red, purple black	Yellow orange yellow-

Reduction with magnesium and concentrated hydrochloric acid (Shinoda's reagent) produces red colours with flavonols, flavones and flavanols (Goodman & Mercer, 1972). Chalcones and aurones give immediate red colours on adding acid rather than a gradual intensification of colours as reduction proceeds. Flavones give intense orange to red colour. Boiling plant parts with 2M hydrochloric acid has been used to detect catechins and leucoanthocyanidins, The former gives a yellow brown colour, the latter a red colour. For additional confirmation of the anthocyanidins, the red colour may be extracted with amyl alcohol and further tests for the presence of anthocyanin applied.

Paper Chromatography

Paper chromatography is suitable for the separation of complex mixtures of all types of flavonoids and their glycosides (Harborne et al., 1975). It is commonly carried out on Whatman No.1, No.3 or 3mm paper and for optimum resolution, two-dimensional chromatography is recommended. For the separation of flavonoid glycosides generally, the first dimension is normally run using an 'alcoholic' solvent (such as: n-butanol-acetic acid-water 4:1:5 (upper phase, BAW); t-butanol-acetic acid-water, 3:1:1 (TBA) or water saturated butanol) which produces separations based largely on partitioning. The second dimension is commonly run using a aqueous solvent (such as, water, 2-60% aqueous acetic acid, 3% sodium chloride or acetic acid-conc. hydrochloric acid-water (30:3:10, Forestal) in order to achieve complementary separations based on adsorption. Flavonoid aglycones are generally separated satisfactorily from one another by

the use of the “alcoholic” solvents (above) or with benzene-acetic acid-water (13:6:1), phenol-water (4:1) or Forestal. Many of these solvents are also useful.

Thin-Layer Chromatography

Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids, but now the method of choice for simple and inexpensive analytical runs is TLC. The advantages of this technique are short separation times, amenability to detection reagents, and the possibility of running several samples at the same time. TLC is also ideally suited for the preliminary screening of plant extracts before HPLC analysis (Andersen & Markham, 2006).

As in column chromatography, the adsorbents of choice for the separation of flavonoids are silica, polyamide and cellulose. Many different solvent systems have been employed for the separation of flavonoids using TLC. Table 5 shows a selection for different classes of these polyphenols. Highly methylated or acetylated flavones and flavonols require nonpolar solvents such as chloroform–methanol (15:1). Widely distributed flavonoid aglycones, such as apigenin, luteolin, and quercetin, can be separated in chloroform– methanol (96:4) and similar polarity solvents. One system that is of widespread application for flavonoid glycosides is ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:26). By the addition of ethyl methyl ketone (ethyl acetate–ethyl methyl ketone–formic acid–glacial acetic acid–water, 50:30:7:3:10), rutin and vitexin-2’’-O-rhamnoside can be separated (Andersen & Markham, 2006). Careful choice of solvent system also allows separation of flavonoid glucosides from their galactosidic analogs.This is especially important for the distinction of

C-glucosides from C-galactosides. As an illustration, 8-C-glucosylapigenin (vitexin) can be separated from 8-C-galactosylapigenin with the solvent ethyl acetate–formic acid–water (50:4:10).

Table 5: Solvent Systems for Thin-Layer Chromatography of Flavonoids on Silica Gel (Andersen & Markham, 2006)

Sample	Eluent
Flavonoid aglycones	EtOAc–i-PrOH–H_2O, 100:17:13
	EtOAc–$CHCl_3$, 60:40
	$CHCl_3$–MeOH, 96:4
	Toluene–$CHCl_3$–MeCOMe, 8:5:7
	Toluene–HCOOEt–HCOOH, 5:4:1
	Toluene–EtOAc–HCOOH, 10:4:1
	Toluene–EtOAc–HCOOH, 58:33:9
	Toluene–EtCOMe–HCOOH, 18:5:1
	Toluene–dioxane–HOAc, 90:25:4
Flavonoid glycosides	n-BuOH–HOAc–H_2O, 65:15:25
	n-BuOH–HOAc–H_2O, 3:1:1
	EtOAc–MeOH–H_2O, 50:3:10
	EtOAc–MeOH–HCOOH–H_2O, 50:2:3:6
Flavonoid glycosides	EtOAc–EtOH–HCOOH–H_2O, 100:11:11:26
	EtOAc–HCOOH–H_2O, 9:1:1
	EtOAc–HCOOH–H_2O, 6:1:1
	EtOAc–HCOOH– H_2O, 50:4:10
	EtOAc–HCOOH–HOAc–H_2O,100:11:11:26
	EtOAc–HCOOH–HOAc–H_2O, 25:2:2:4
	THF–toluene–HCOOH– H_2O, 16:8:2:1
	$CHCl_3$–MeCOMe–HCOOH, 50:33:17
	$CHCl_3$–EtOAc–MeCOMe, 5:1:4
	$CHCl_3$–MeOH– H_2O, 65:45:12
	$CHCl_3$–MeOH– H_2O, 40:10:1
	MeCOMe–butanone–HCOOH, 10:7:1
	MeOH–butanone– H_2O, 8:1:1
Flavonoid glucuronides	EtOAc–Et_2O–dioxane–HCOOH–H_2O, 30:50:15:3:2
	EtOAc–EtCOMe–HCOOH–H_2O, 60:35:3:2
Flavanone aglycones	CH_2Cl_2–HOAc– H_2O, 2:1:1

Table 5: Solvent Systems for Thin-Layer Chromatography of Flavonoids on Silica Gel (Continued)

Sample	Eluent
Flavanone glycosides	$CHCl_3$–HOAc, 100:4 $CHCl_3$–MeOH–HOAc, 90:5:5 n-BuOH–HOAc–H_2O, 4:1:5 (upper layer)
Chalcones	EtOAc–hexane, 1:1
Isoflavones	$CHCl_3$–MeOH, 92:8 $CHCl_3$–MeOH, 3:1
Isoflavone glycosides	n-BuOH–HOAc– H_2O, 4:1:5 (upper layer)
Dihydroflavonols	$CHCl_3$–MeOH–HOAc, 7:1:1
Biflavonoids	$CHCl_3$–MeCOMe–HCOOH, 75:16.5:8.5 Toluene–HCOOEt–HCOOH, 5:4:1
Anthocyanidins and anthocyanins	EtOAc–HCOOH–2 M HCl, 85:6:9 n-BuOH–HOAc– H_2O, 4:1:2 EtCOMe–HCOOEt–HCOOH– H_2O, 4:3:1:2 EtOAc–butanone–HCOOH– H_2O, 6:3:1:1
Proanthocyanidins	EtOAc–MeOH– H_2O, 79:11:10 EtOAc–HCOOH–HOAc– H_2O, 30:1.2:0.8:8

Apart from anthocyanins and some of the more intensely coloured chalcones and aurones, flavonoids are not sufficiently coloured to be visible to the naked eye on a thin-layer plate; thus some form of visualisation is necessary for spot detection (Opoku-Boahen, 1989). In many cases this is achieved by viewing the plate in UV light (365nm) either in the presence or absence of ammonia vapour. Detection under UV is often assisted by the use of plates containing a UV-fluorescent indicator (e.g. silica gel F_{254}). Flavonoids appear as dark spots against

a fluorescent green background. The properties and colour behaviour of different flavonoid classes are shown in Table 6 and 7.

Table 6: Properties of different Flavonoids classes (Opoku-Boahen, 1989)

Flavonoid	Colour/Distribution	Characteristic properties
Chalcones/ Aurones	Yellow flower pigment	Give red colours with ammonia, visible max. 370 to 410nm.
Flavanones	Colourless	Give intense red colours with Mg-HCl.
Isoflavones	Colourless, only common in one family, the Leguminosae	Mobile on paper, no specific colour tests available
Anthocynains	Scarlet, red, mauve, pink and blue fower pigments, also in leaf and other tissues.	Water soluble, visible max. 515 to 545nm. Mobile in BAW on paper.
Leucoanthocyanins	Mainly colourless in heartwood and in leaves of woody plants	Yield anthocaynidins colour extractble into amyl alcohol when heated for 0.5hr in 2m HCl.
Flavonols	Mainly colourless co-pigments in both cyanic and acyanic flowers, widespread in leaves	After acid hydrolysis, bright yellow spots in UV on Forestal chromatograms, spectral max 350 to 386nm.
Flavones	As Flavonols	After acid hydrolysis, dull absorbing brown spot, on Forestal chromatogram; spectral max. 330 to 350nm.
Glycoflavones	As Flavonols	Contain C-C linked sugar mobile in water unlike normal flavones

In 365 nm UV light, depending on the structural type, flavonoids show dark yellow, green, or blue fluorescence, which is intensified and changed by the use of spray reagents. One of the most important of these is the ‘‘natural products reagent,’’ which produces an intense fluorescence under 365 nm UV light after

spraying with a 1% solution of diphenylboric acid-b-ethylamino ester (diphenylboryloxyethylamine) in methanol.

Table 7: Colour Properties of Flavonoids in Visible and Ultraviolet Light (Opoku-Boahen, 1989)

Visible colour	Alone	Colour in UV light with ammonia	Indication
Orange)	Dull orange, red	Blue	Anthocyanidin-3-
Red)	or mauve		glycosides
Mauve)	flouresent, yellow		
	cerise	Blue	Most anthocyanidin-
	or pink		3,5-diglycosides.
Bright Yellow	Dark brown or black	Dark brown or black, dark red or bright orange	6-Hydroxylated flavonols flavones some chalcone glycosides, most chacones
	Bright green	Bright orange	aurones
Very pale yellow	Dark brown	Bright yellow or yellow brown	Most flavonol glycosides
		Vivid yellow	biflavonyls and
		Green	unusually substituted
		Dark brown	flavones
None	Dark mauve	Faint brown	Most isoflavones, and flavanonols.
	Faint blue	Intense blue	5-desoxyisoflavones and 7,8-
	Dark mauve		dihydroxyflavanones
		Pale yellow or yellow-green	Flavones and flavanonol 7-glycosides.

Subsequent spraying with a 5% solution of polyethylene glycol-4000 (PEG) in ethanol lowers the detection limit from 10 mg (the average TLC detection limit for flavonoids) to about 2.5 mg, intensifying the fluorescence behaviour. The colors observed in 365 nm UV light are as follows:

Quercetin, myricetin, and their 3- and 7-*O*-glycosides: orange-yellow

Kaempferol, isorhamnetin, and their 3- and 7-*O*-glycosides: yellow-green

Luteolin and its 7-*O*-glycoside: orange

Apigenin and its 7-*O*-glycoside: yellow-green

Aqueous or methanolic ferric chloride is a general spray reagent for phenolic compounds and gives a blue-black coloration with flavonoids. Similarly, Fast Blue Salt B forms blue or blue-violet azo dyes.

Another useful method of detection is brief exposure of the plate to iodine vapour which produces yellow-brown spots against a white background with most flavonoids. These techniques all have the advantage that they are non-destructive (Opoku-Boahen, 1989).

Preparation of Plant or Animal Tissue and Foodstuffs for Flavonoid Analysis

The need for sample preparation depends strongly on the sample type and the analysis techniques used. Various procedures for sample preparation and clean-up of flavonoid samples can be used (Cuyckens & Claeys, 2004). The procedures must allow quantitative recovery of flavonoids, whilst avoiding any chemical modification or degradation.

The utilization of dried plant material for extraction may cause a substantial decrease in the yield of flavonoid conjugates. Acylated flavonoid glycosides are especially labile at elevated temperatures and are frequently thermally degraded during the process of drying plant tissues. This is important during the profiling of this class of natural products in research directed toward the investigation of their physiological and biochemical roles in plants under the influence of

environmental factors, or in studies of genetically modified plants for the elucidation of changes in metabolic pathways.

Flavonoid samples can be prepared by homogenization, liquid extraction and filtration and/or centrifugation. The extraction conditions, i.e. temperature, pH and extraction solvent, can have a considerable influence on the kind and amount of flavonoids isolated. Sometimes additional processing is desirable, e.g. to purify and enrich a certain flavonoid or flavonoid fraction, to discard any interfering matrix components or to eliminate highly lipophilic compounds that can adsorb on reversed-phase (RP) LC columns. To date, solid-phase extraction (SPE) is the method of choice. The use of mini-cartridges, usually filled with C18 RP material, allows a simple and rapid purification and preconcentration of flavonoids, where recoveries are comparable to those with simple filtration. The sample solution and eluents are preferentially acidified in order to suppress the ionization of the flavonoids and as such to increase their retention (Cuyckens & Magda Claeys, 2004)

Free flavonoid aglycones exuded by plant tissues (leaf or root) may be washed from the surface with nonpolar solvents, such as methylene chloride, ethyl ether, or ethyl acetate. However, more polar glycosidic conjugates dissolve in polar solvents (methanol and ethanol), and these organic solvents are applied for extraction procedures in Soxhlet apparatus. Mixtures of alcohol and water in different ratios are applied for the extraction of flavonoids and their conjugates from solid biological material (plant or animal tissues and different food products). Hot water extraction is the most common method used for flavonoid

glycosides. This method involves putting raw materials in boiling water to inactivate enzymes. The method can also be used to extract relatively higher polar flavonoid aglycones, such as flavanol, flavandiol and proanthocyanidin. During the extraction, one will also need to take the quantity of water added, immersion time, decocting time, extracting time, and other factors into consideration. Methanol and ethanol are the most common extraction solvents used for flavonoids. Highly concentrated alcohol (90 to 95%) is suitable for extracting aglycones, and alcohol with about 60% concentration is suitable for extracting glycosides. The extraction process often takes two to four sequences. Percolation, reflux and marceration can also be used for extraction.

The extraction efficiency may be enhanced by the application of ultrasonication (Herrera et al., 2004; Rostagno et al., 2003) or pressurized liquid extraction (PLE), a procedure performed at elevated temperature ranging from 60^{o}C to 200^{o}C (Rostagno et al., 2004). Supercritical fluid extraction with carbon dioxide also may be used. These procedures have to be carefully adjusted because of the possibility of thermal degradation of the flavonoid derivatives. In many cases, further purification and/or preconcentration of the target compound fraction is necessary. In these cases, liquid–liquid extraction (LLE) or SPE are most commonly used. For estimation of the extraction yield it is necessary to spike biological materials with proper internal standards. Most suitable are compounds structurally similar to the studied analytes but not present in the sample. Compounds labeled with stable isotopes (^{2}H or ^{13}C) are useful when mass spectrometric detection is applied. In the case of the extraction of flavonoids from

biological materials, different classes of phenolic compounds are often added. On the other hand, quantitative analysis of consecutive components of the analyzed flavonoid mixture needs reference standard compounds necessary for preparation of calibration curves essential for a precise quantification.

The choice of the extraction procedure for obtaining flavonoid conjugates from biological material is very important and depends on the goals of the conducted research. The evaluation of the spatial distribution of target compounds on the organ, tissue, cellular, or even subcellular level is of special interest in some projects. In these situations, the amount of biological material for the isolation of natural products may be extremely small, and the application of microextraction techniques is necessary (reviewed in Vas & Vekey, 2004). In many cases, it is necessary to avoid the chemical and/or enzymatic degradation of the metabolites. This is of special importance in the profiling of flavonoid glycosides in research directed toward plant functional genomics or during physiological and biochemical studies that need information about all classes of flavonoid conjugates present, even the thermally labile acylated derivatives. On the other hand, in the phytochemical analysis of plant species or phytopharmaceutical studies of plant material, the repeatable isolation of all biologically active flavonoid aglycones with a good yield is more important. In these cases, more drastic extraction conditions are acceptable.

Robust multistep chromatographic methods are necessary for the isolation of individual components from plant extracts containing new uncharacterized compounds. Various stationary phases are used in column chromatography,

including polyamide, Sephadex LH-20, and different types of silica gels (normal and reversed phase with chemically bonded functional groups). The proper choice of solvent systems is necessary, often requiring the application of gradients of more polar (normal phases) or more hydrophobic solvents (reverse phases), together with the above mentioned chromatographic supports in different chromatography systems. The sequence and kind of separation methods used depends on the composition of the sample and the experience of the researcher. However, minor flavonoid components are difficult to obtain as pure compounds. In cases of analysis of samples containing a number of compounds present in small amounts, the application of an analytical chromatographic systems enhanced by proper detectors (UV, NMR, and/or MS) gives spectrometric information sufficient for establishing the structure of minor target components. When liquid chromatography is used for separation of compounds, multiple detector systems are available (UV diode array detector, mass spectrometers, and nuclear magnetic resonance spectrometer). It is possible to achieve complete structural information about isomeric flavonoids and their conjugates in this way.

Structural Characterization and Identification of Flavonoid and their Conjugates

All physicochemical methods applied in the field of organic chemistry are useful for structural characterization or identification of individual flavonoids and their conjugates. The separation approaches mentioned above may be considered in different ways. The first one is directed toward the analysis of single compounds obtained after exhaustive isolation and purification procedures. The

method of choice in this approach is NMR of ^{1}H hydrogen and/or ^{13}C carbon isotopes, dependent on the intensity of the interactions between different atoms within a molecule placed in a high-intensity magnetic field. Different NMR experiments have been developed to achieve information concerning chemical structure of the studied molecule on this basis. Particularly useful are methods enabling recording of two-dimensional spectra showing homonuclear interactions [correlation spectroscop**y** (COSY) and nuclear Overhauser effect spectroscop**y** (NOESY)] as well as heteronuclear [heteronuclear single quantum **c**orrelation (HSQC) and heteronuclear multiple bond **c**orrelation (HMBC)] to facilitate the acquisition of all the structural information about an aglycone and the corresponding sugar substitution. In the case of diglycosides, information on the placement of the interglycosidic bonds and the possible acyl group substitutions on the sugar rings, and the position of anomeric proton(s) also can be obtained. The limitation of NMR methods is the lower sensitivity in comparison with other instrumental methods. For obtaining good quality spectra containing all the necessary structural information, relatively high amounts of purified compound (more than 1 mg) are necessary, especially when magnets of medium frequency (300 MHz) are used in the NMR spectrometer. The NMR spectrometers may be connected on line to liquid chromatographs (LC-NMR), giving a powerful tool to study mixtures of natural compounds present in complex samples. Important structural data also can be obtained from mass spectra registered on different types of mass spectrometers (MS). The application of ultraviolet and infrared spectrophotometers may give valuable information about specific compounds.

MS applied for the analysis of organic compounds utilize different ionization methods and may be equipped with different types of analyzers. In addition, these instruments may be combined with GC/LC or capillary electrophoresis (CE) apparatus. However, simple chemistry based on single reactions such as silylation, methylation, and acetylation blocking polar functional groups has to be done on the studied samples prior to GC-MS analyses. Derivatization of polar groups improves structural information obtained from MS spectra and ameliorates the volatility of analytes, decreasing the thermal degradation of compounds within the GC capillary column. The variety of MS techniques being available in laboratories is a reason that this technique has a wide range of scientific or practical applications in biological and medical disciplines. Analysis of natural products is possible with different types of MS available on the market. The instruments are equipped with various sample introduction systems and ionization methods, as well as diverse physical phenomena are used for separation of the created ions in MS analyzers. Positive and negative ions are analyzed in MS; the choice of the ionization mode (negative or positive) is sometimes a very important feature. The ionization methods may be divided into two groups differing with respect to the amount of energy transferred to the molecule during the ionization process. Electron ionization (EI) belongs to the first group. The transfer of energy occurs during the interaction of electrons with the molecule in the vapor state; it may cause the cleavage of chemical bonds and fragmentation of the molecule, which is characteristic for the analyzed compound. Other ionization methods deliver lower energy to the studied molecules during the protonation (positive ion

mode) or deprotonation (negative ion mode) processes. In both cases, the absorbed energy is too low to cause intense fragmentation. In this situation, techniques of collision-induced dissociation with tandem MS (CID MS/MS) have to be applied for the structural characterization of compounds.

Flavonoid Aglycone Structure Study

For known flavonoids (including flavonoid aglycones and glycosides) generally one can use derivative preparation and spectral analysis to determine their structure. If a reference standard is available, the structure will be more easily determined by contrasting the IR spectrum and mixing melting point. Even for the unknown flavonoids, after initially determining their structure by derivative preparation and spectrum analysis, one can further validate and confirm the structure by spectroscopic determination or chemical degradation and conversion. For the flavonoids with special structures, complete synthesis would assist the structure determination.

Derivative Preparation

The most common derivatives of flavonoids used are methide and acetylate. When preparing these derivatives, on one hand, one can compare them with the chemical and physical data of the known compounds reported in the literature. On the other hand, one can further compare their spectral data with those of the original compounds in order to determine their structure.

Preparation of Acetylate Derivative

Hydroxyl groups can be acetylated by acetic anhydride and acetyl chloride with a catalyst, such as pyridine, sodium acetate, concentrated sulfuric acid, 4-

dimethylamino-pyridine or p-methyl benzenesulfonic acid. All the reagents used must be anhydrous. However, acetic anhydride/pyridine is the most commonly used reagent group. Some flavonoids are unstable or can easily be isomerized in alkaline solution. For such flavonoids and those flavonoid glycosides, which are difficult to be acetylated or the reaction products too complex during base catalysis, a trace amount of concentrated sulfuric acid or anhydrous P-methyl benzenesulfonic acid can be used as the catalyst.

Preparation of Methide Derivative

Diazomethane can be used to methylate the phenol group with a high yield of reaction product. If the 5-OH cannot be easily methylated due to the formation of the hydrogen bond, then dimethyl sulfate and other reagents can be used. Dimethyl sulfate can methylate all the hydroxyl groups in the flavonoids. In addition, another full methylation method is rapid and simple, suitable for various glycosides and saccharides, and produces a high yield reaction product. It is also very convenient for GC to isolate and identify the methide.

Special Spectroscopic Data

The structure of flavonoid aglycones is relatively simple hence; regular spectra determination, derivative preparation and spectra comparism are enough to determine their structure. Only when the structure needs to be made, will special spectroscopic determination is needed.

UV Spectrum Determination for flavonoids

Ultraviolet-visible absorption spectroscopy is perhaps the single most useful technique available for flavonoid structure analysis. The technique is used to aid

both identification of the flavonoid type and definition of the oxygenation pattern. In addition, the substitution on the flavonoid nucleus of unsubstituted phenolic hydroxyl groups may be established by adding reagents ("Shift reagents") to the sample solution and observing the resultant shifts in the absorption peaks. Thus, indirectly the technique may be useful in determining the location of a sugar or methyl group attached to one of the phenolic hydroxyls. Avalability of a good range of reference spectra is an invaluable aid in the interpretation of UV-visible absorption spectra. A major advantage of this method is the very small amount of flavonoid required for full analysis (usually 0.1 mg).

UV Spectrum Determination for Hydroxyl Position Diagnosis Reagents

Flavonoids contain conjugated aromatic systems and thus show intense absorption bands in the UV and visible regions of the spectrum. The application of standardized UV (or UV-Vis) spectroscopy has for years been used in analyses of flavonoids. These polyphenolic compounds reveal two characteristic UV absorption bands with maxima in the ranges 240 to 285 (band II) and 300 to 550 (band I). The various flavonoid classes can be recognized by their UV spectra. The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid and its oxygenation pattern. Typical spectra of the major flavonoid types with equivalent (5, 7, 4') oxygenation patterns are presented in Figure 13. Characteristic features of these spectra are the low relative intensities of Band I in dihydroflavones, dihydroflavonols, and isoflavones, and the long wavelength position of band I in the spectra of chalcones, aurones and anthocyanins. These characteristics are relatively invariant

even with changing oxygenation pattern, although the ranges of absorption maxima for different flavonoid types do overlap as a result of variations in oxygenation patterns. Table 8 presents some guide as to the expected ranges of the principal maxima for each flavonoid type. The hydroxylation pattern and the degree of substitution of the hydroxyls cause variations within these ranges. The following are examples of the effects brought about by these changes.

1. Changes in the substitution of the A-ring tend to be reflected in the band II absorption while alterations in the substitution of the B- and C-rings tend to be more apparent from the band I absorption.
2. Additional oxygenation (especially hydroxylation) generally causes a shift of the appropriate band to longer wavelengths, e.g. band I in 3,5,7-triOH flavone, 359 nm; 3,5,7,4'-OH flavone, 367 nm; 3,5,7,3',4'-OH flavone, 370 nm and 3,5,7,3',4',5'-OH flavone, 374 nm.
3. Methylation or glycosylation (especially of 3, 5, 7 and 4' hydroxyls) causes band shifts to shorter wavelengths. The nature of the sugar in glycosides is normally of no consequence.
4. Acetylation tends to nullify the effect of a phenolic hydroxyl group on the spectrum.
5. The presence of cinnamic acids as acyl functions on a flavonoid can be detected by the presence of a 3', 4'-diOH system is generally evidenced by a second peak (sometimes a shoulder) in band II.

Table 8: Ultraviolet-visible absoption ranges for flavonoids (Harborne et al., 1975)

Band II (nm)	Band I (nm)	Type of Flavonoid
250-280	310-350	Flavone
250-280	330-370	Flavonols (3-OH substituted)
250-280	350-385	Flavonols (3-OH free)
245-275	310-330 shoulder	Isoflavones
	c. 320 peak	Isoflavones (5-deoxy-6,7-dioxygenated)
275-292	300-330 shoulder	Flavanones and dihydroflavonols
230-270 (low intensity)	340-390	Chalcones
230-270 (low intensity)	380-430	Aurones
270-280	465-560	Anthocyanidins and anthocyanins

The set of UV spectra for identification and structural elucidation consist of one determined on a solution of the flavonoid in methanol and others obtained by adding diagnostic reagents to the methanol solution of the flavonoid.

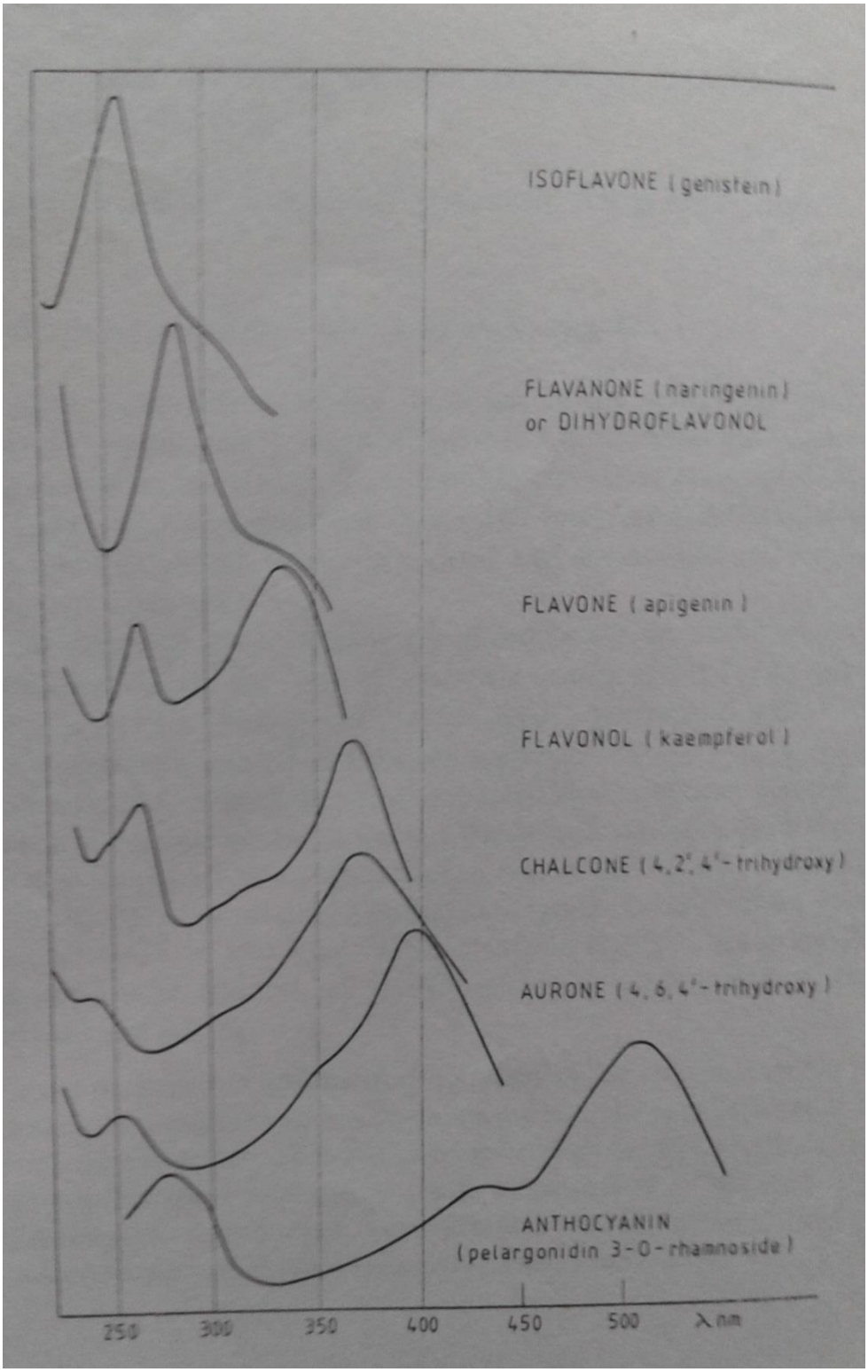


Figure 13: Ultraviolet-visible absorption spectra of different flavonoid types with equivalent hydroxylation patterns (Harborne et al., 1975).

Specific diagnostic fragments of the A- and B-rings have been detected, while the identification of unknown flavonoids can be based additionally on their UV-Vis spectra and the correlation with standard compounds. The typical UV-Vis spectra of flavonoids include two absorbance bands. Band I lies in the 310- 350 nm range for flavones, while for flavonols it is between 350 and 385 nm. Band II, found in the 250-290 nm range, is much the same in all the aforementioned flavonoid subgroups. In flavanones and dihydroflavonols, band I is often reduced to little more than a shoulder at 300-330 nm and band II, in the 277-295 nm range, is the main peak. Consequently, these two subgroups cannot be distinguished by simple UV-Vis analysis. Flavanols show maximum absorbance at non specific wavelengths between 270 and 290 nm, at which many phenolics absorb, thus not allowing their selective detection. However, UV spectrum absorption maxima at 272 and 334, normally suggests that the compound belongs to the flavone family, unsubstituted at the 3-position (Intekhab & Aslam, 2009). According to these data the UV-Vis spectra can be used as an indicative tool for the characterization of C-ring, whereas the MS spectra could provide additional, significant information (Tsimogiannis et al., 2007).

Application of UV shift reagents

The use of UV shift reagents such as $AlCl_3$ (5% in methanol)-HCl (20% aqueous), NaOMe (2.5% in methanol), and NaOAc (3 mg)-H_3BO_3 has proven to be very useful as guidelines for substitution patterns of many flavonoids; however, the use of these reagents has mainly been applicable for purified flavonoids. By comparing the UV spectra before and after reagent addition one

can obtain the shift value and, based on this, can further decide the position of the hydroxyl.

Intepretation of $AlCl_3$ and $AlCl_3$/HCl spectra

The $AlCl_3$ and $AlCl_3$/HCl form acid-stable complexes between hydroxyls and neighbouring ketones, and acid-labile complexes with ortho-dihydroxyl groups. These reagents can be used to detect both groupings. Figure 14 illustrates the type of complexes accounting for the $AlCl_3$ and $AlCl_3$/HCl shifts in the spectrum of luteolin.

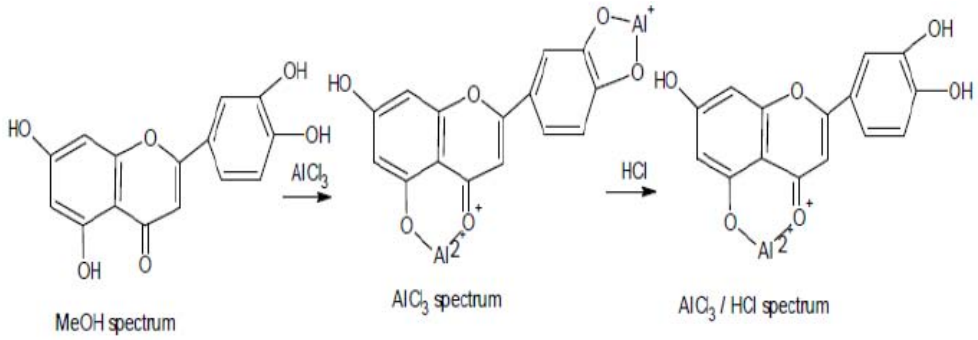


Figure 14: Complexes accounting for the $AlCl_3$ and $AlCl_3$/HCl induced shifts in the spectrum of luteolin (Harborne et al., 1975).

The $AlCl_3$ spectrum thus represents the sum effect of all complexes on the spectrum, while the $AlCl_3$ /HCl spectrum represents the effect only of the hydroxyl-keto complexes. Table 9 presents a summary of spectra interpretation of $AlCl_3$ and $AlCl_3$/HCl shift reagent.

Table 9: Interpretation of $AlCl_3$ and $AlCl_3$/HCl spectra (Harborne et al., **1975)**

Flavonoid type (reagent)	Shift observed Band I	Band II	Interpretation guide
Flavones and flavonols ($AlCl_3$/HCl)	+35 to 55 nm		5-OH
	+17 to 20 nm		5-OH with 6-oxygenation
	No change		Possibly 5-OH with 6-penyl group
	+50 to 60 nm		3-OH possible (with or without 5-OH)
($AlCl_3$)	($AlCl_3$/HCl) shift + 30 to 40 nm		B-ring o-diOH
	($AlCl_3$/HCl) shift + 20 t0 25 nm		A-ring o-diOH (additive to B-ring o-diOH shift)
Isoflavones, flavanones and Dihydroflavonols ($AlCl_3$/HCl)		+ 10 to 14 nm	5-OH (isoflavones)
		+ 20 to 26 nm	5-OH (flavanones, dihydroflavonols)
$AlCl_3$		$AlCl_3$/HCl shift, + 11 to 30 nm	A-ring o-diOH (6,7 and 7,8)
		$AlCl_3$/HCl shift + 30 to 38 nm (NaOAc sensitive)	Dihydroflavonol with no 5-OH (additive to any o-diOH shift)

Table 9: Interpretation of $AlCl_3$ and $AlCl_3$/HCl spectra (continued)

Flavonoid type (reagent)	Shift observed Band I	Band II	Interpretation guide
Aurones, Chalcones $AlCl_3$/HCl	+ 48 to 64 nm		2'-OH (chalcones)
	+ 40 nm		2'-OH (chalcones)with 3'-oxygenation
($AlCl_3$)	+ 60 to 70 nm		4-OH (aurones)
	$AlCl_3$/HCl sift, + 40 to 70 nm		B-ring o-diOH
	Smaller increase		Possibly A-ring o-diOH
Anthocyanidins, Anthocyanins $AlCl_3$	+ 25 to 35 nm (at pH 2-4		o-diOH
	Larger shifts		Multiple o-diOH or o-diOH (3-deoxy-anthocyanidins)

Intepretation of NaOMe spectrum

The NaOMe spectrum represents that of the flavonoid with all phenolic hydroxyl groups ionized to some extent. It is therefore generally a good "fingerprint" indicator of the hydroxylation pattern as well as being useful for the detection of the more acidic hydroxyl groups in unsubstitued form. Degradation of the spectrum with time is a good indicator of the presence of alkali-sensitive groupings. Table 10 presents details of the interpretations of the NaOMe spectrum.

Table 10: Interpretation of NaOMe spectra (Harborne et al., 1975)

Flavonoid type (reagent)	Shift observed		Interpretation guide
	Band I	Band II	
Flavones and flavonols	Continually reducing intensity (i.e. decomposition		3,4'-OH, A-ring o-diOH, B-ring: 3 adjacent OH
	Stable + 45 to 65 nm no decrease in intensity		4'-OH
	New band (cf. MeOH), 320-335 nm		7-OH
Isoflavones,		No shift	No A-ring -OHs
Flavanones Dihydroflavonols		Decreasing intensity with time	A-ring o-diOH (slow decrease: B-ring o-diOH in isoflavones)
		Shift from c. 280 nm to c. 325 nm, increased intensity, but to 330-340 nm + 11 to 30 nm	5,7-OH flavanones and dihydroflavonols 7-OH, no free 5-OH
Aurones Chalcones	+ 80 to 95 nm (increased intensity) + 60 to 70 nm (increased intensity) Smaller shift		4'-OH (aurones) 6-OH no 4'-oxygenation (aurones) 6-OH with 4'-oxygenation (aurones)
	+ 60 to 100 nm (increased intensity) (no increased in intensity) +40 to 50 nm		4-OH (chalcones) 2-OH or 4'-OH and no 4-OH 4'-OH (2'-OH or 4-OR also present)

Interpretation of NaOAC spectrum

Sodium acetate causes significant ionization of only the most acidic of the flavonoid hydroxyl groups. Thus it is used primarily to detect the presence of a free 7-hydroxyl group (or equivalent). Table 11 gives the interpretation guide of NaOAc spectra.

Table 11: Interpretation of NaOAc spectra (Harborne et al., 1975)

Flavonoid type (reagent)	**Shift observed**		**Interpretation guide**
	Band I	**Band II**	
Flavones flavonols Isoflavones		+ 5 to 20 nm (reduced if 6- or 8-oxygenation present)	7- OH
	Decreasing intensity with time		Alkali-sensitive groups e.g. 6,7 or 7,8 or 3,4'-diOH
Flavanones	+ 35 nm		7-OH (with 5-OH)
Dihydroflavonols	+ 60 nm		7-OH (without 5-OH
	Decreasing intensity with time		Alkali-sensitive groups e.g. 6,7 or 7,8 -diOH
Aurones Chalcones	Bathochromic shift or long-wavelength shoulder		4'ad/or 4-OH (chalcones) 4' and/or 6-OH (aurones)

Intepretation of NaOAc /H_3BO_3 spectrum

$NaOAc/H_3BO_3$ bridges the two hydroxyls in an ortho-dihydroxy group and is used to detect their presence. An interpretation guide for these shift reagents is presented in Table 12

Table 12: Interpretation of $NaOAc/H_3BO_3$ spectra (Harborne et al., 1975)

Flavonoid type (reagent)	Shift observed		Interpretation guide
	Band I	Band II	
Flavones flavonols	+ 12 to 36 nm (relative to MeOH spectrum)		B-ring *o*-diOH
Aurones			
Chalcones	Smaller shift		A-ring *o*-diOH (6,7 or 7,8)
Isoflavones		+ 10 to +15 nm relative to MeOH spectrum)	A-ring *o*-diOH
Flavanones			(6,7 or 7,8)
Dihydroflavonols			

Figure 16 is used as an example to illustrate further the effect of the addition of five different shift reagents to quercetrin (Andersen & Markham, 2006).

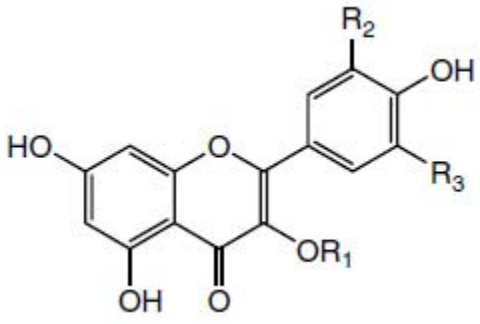


Figure 15: Structure of quercetrin where R_1 = Rhamnose, R_2 = OH and R_3 = H

The shift of 11 nm of band II with weak base, 0.1 M Na_2HPO_4, was characteristic for a nonsubstituted 7- hydroxyl group. A 15 nm shift with boric acid reagent was typical for ortho-dihydroxyl groups on the B-ring. The shift of 42 nm of band I obtained for aluminum chloride without neutralization of the eluate was specific for a 5-hydroxyl substituent. Addition of aluminum chloride after neutralization gave a 56 nm shift of band I.

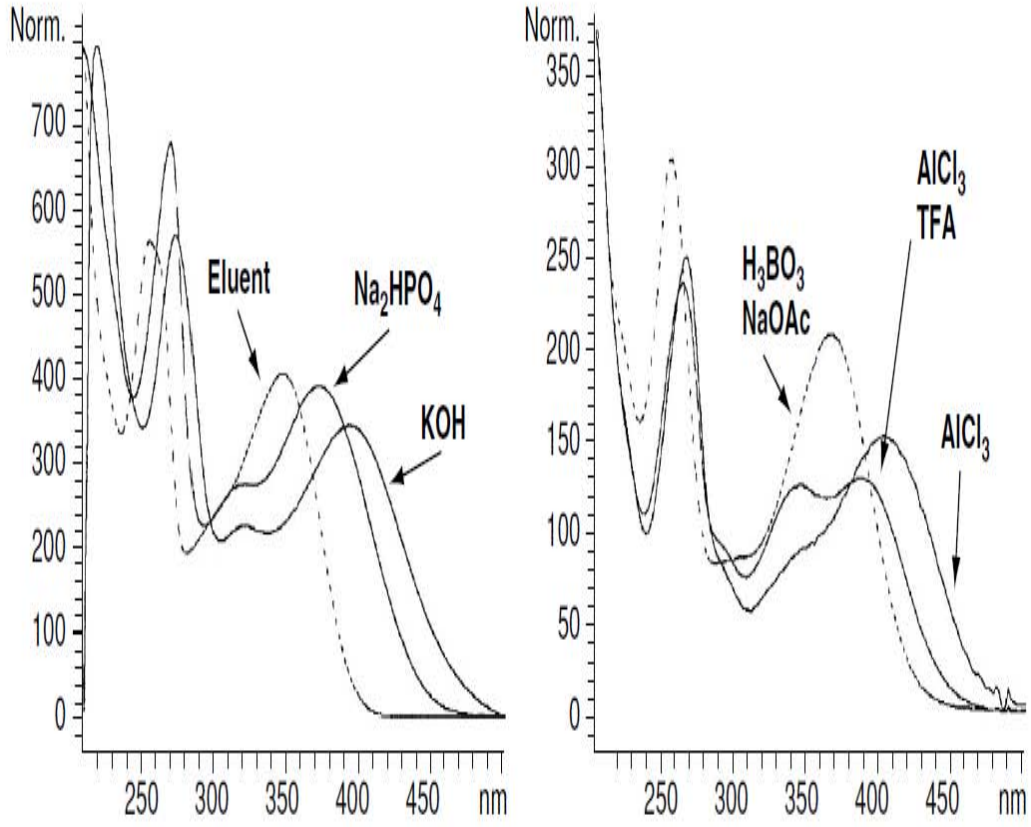


Figure 16: UV spectra for (quercitrin) after the addition of five different shift Reagents (Andersen & Markham, 2006).

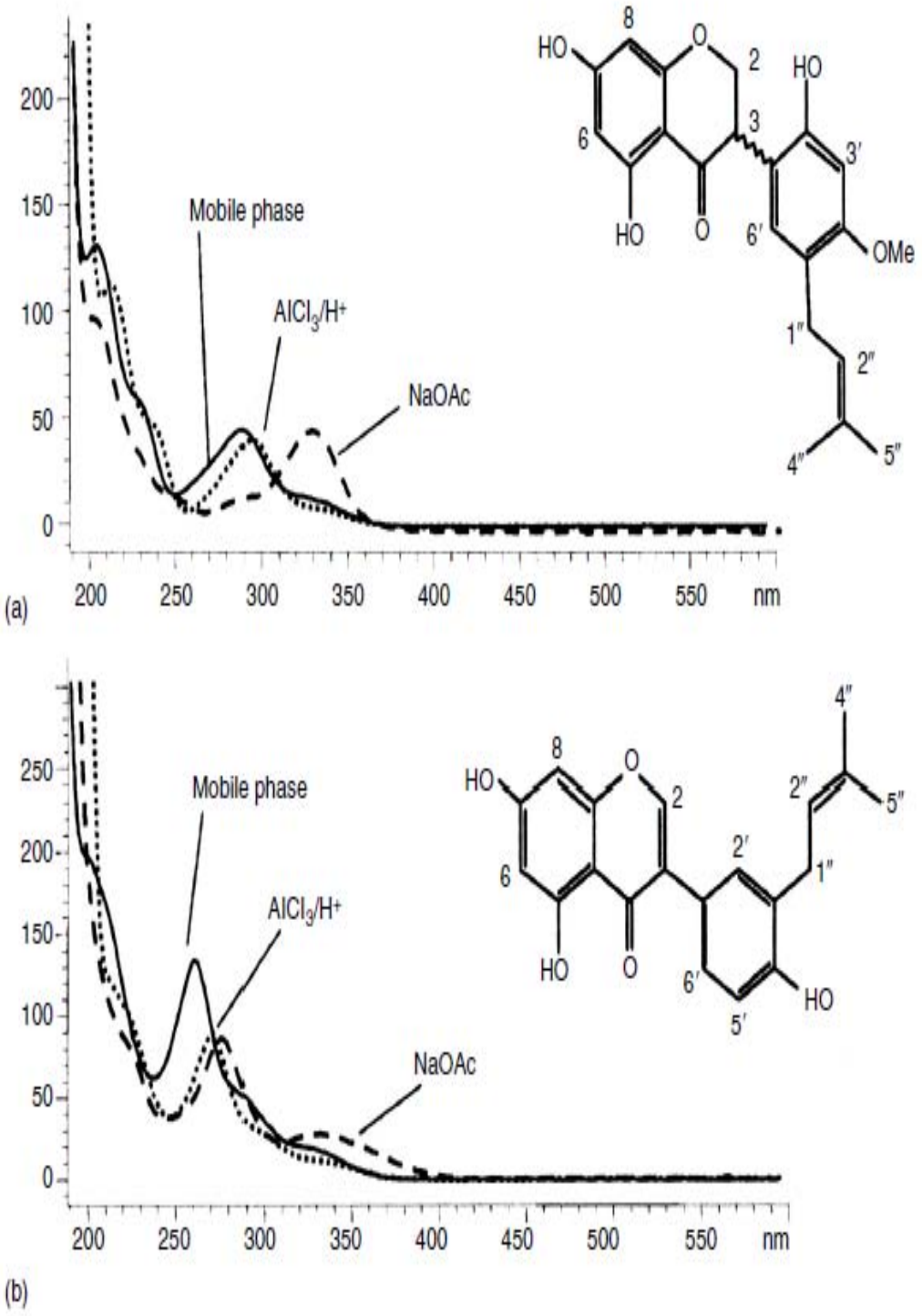


Figure 17: Complementary UV-DAD and shifted UV-DAD spectra with postcolumn addition of shift reagents of an isoflavanone (a) and an isoflavone (b) recorded online (Andersen & Markham, 2006).

In figure 17, the weak base NaOAc and acidic $AlCl_3$, respectively, were used as shift reagents. The shifted UV spectra are superimposed on the original spectra for each compound. The observed shifts provide information about the flavonoid substitution in accordance to the rules established for pure compounds.

Experiments have shown that the bathochromic shift absorption band of 336 nm to 382 nm by addition of NaOMe indicates the presence of free hydroxyl groups at C-4 (Alarcon et al., 2007). This shift and other three bands 340, 299, and 276 nm indicate the presence of free hydroxyl groups at C-5. The absorption band of 267 nm that do not shift upon addition of NaOAc indicates the presence of *O*-glycoside at C-7. Bathochromic shifts upon the addition of $AlCl_3$ and $AlCl_3$/HCl indicate the 5-hydroxy substitution.

Infra-red spectra of flavonoids

Infrared spectra have been invaluable in the investigation of virtually all classes of flavonoid compounds. Besides many KBr measurements on flavones aglycones that were already established further relationship between infrared bands and flavones substitution were found. The carbonyl (C=O) band of unsubstituted flavanone lies at $1680cm^{-1}$ while that of flavone lies at $1660cm^{-1}$. The strong decrease in wave number from flavanone to flavones is as a result of difference in resonance state of these molecules. Mesomerism takes place to a small extent in flavanone while in flavones mesomerism have full effect as a result weakening the C=O double bond.

A substituted flavones at C-3 and C-4’ or C-7 with a hydroxyl or methoxy group causes the C=O band to be shifted to a longer wavelength. This is explained

by the formation of a chelate between the carbonyl group and the C-3 hydroxyl. However, if a hydroxyl is introduced into C-5 of the C-4' and C-7 hydroxyl substituted flavone, there is little or no shift of the C=O band compared with unsubstituted flavones.

Morealso, the C=O band which lies at 1600cm^{-1} when substitution occurs only at C-3, returns to a higher frequencies if flavone is substituted at positions C-5 and C-3. The introduction of hydroxyl groups at the C-3' and 4' positions of flavanones lowers the carbonyl frequency to 1665cm^{-1} from 1680cm^{-1} due to possible intramolecular hydrogen bonding. Compounds with weaker hydrogen bonding show a fairly sharp medium strong bond at 3295cm^{-1} assigned to O-H stretching and a C-H band at 3100cm^{-1}. Figure 18 & 19 are FTIR spectra of rutin and quercetin respectively to illustrate the absorbance partten of these flavonoids.

Flavonoids display aromatic ring absorption in the IR spectrum. The degree of substitution of the ring B in flavones derivatives is indicated by certain bands between 650 and 875 cm^{-1}. This is due to out of plane deformation vibrations of the hydrogen atoms remaining on the benzene nucleus. The unsubstituted 2-phenyl ring of flavones is considered as a mono-substituted benzene ring and its band expected in the region 730-745 cm^{-1} and 694-702 cm^{-1}. Flavones with substitution at C-4' present the ring-B as a p-disubstituted benzene ring hence show a strong band between 831 and 837 cm^{-1}. This represents the gamma – vibration of the p-substituted ring-B. There are additional weaker bands which are probably due to the vibrations of the chromone skeleton. In flavonols, the longer wave band is at 810 cm^{-1} and the shorter at 835 cm^{-1}.

Poly-substituted B rings of flavones represent 1, 2, 4-or 1, 2, 3, 5-substituted benzene rings. The IR spectrum for such molecules presents three to seven notable maxima in the region 680-860 cm^{-1}. The most intense of these bands are at 850 cm^{-1} and between 820 and 830 cm^{-1}.

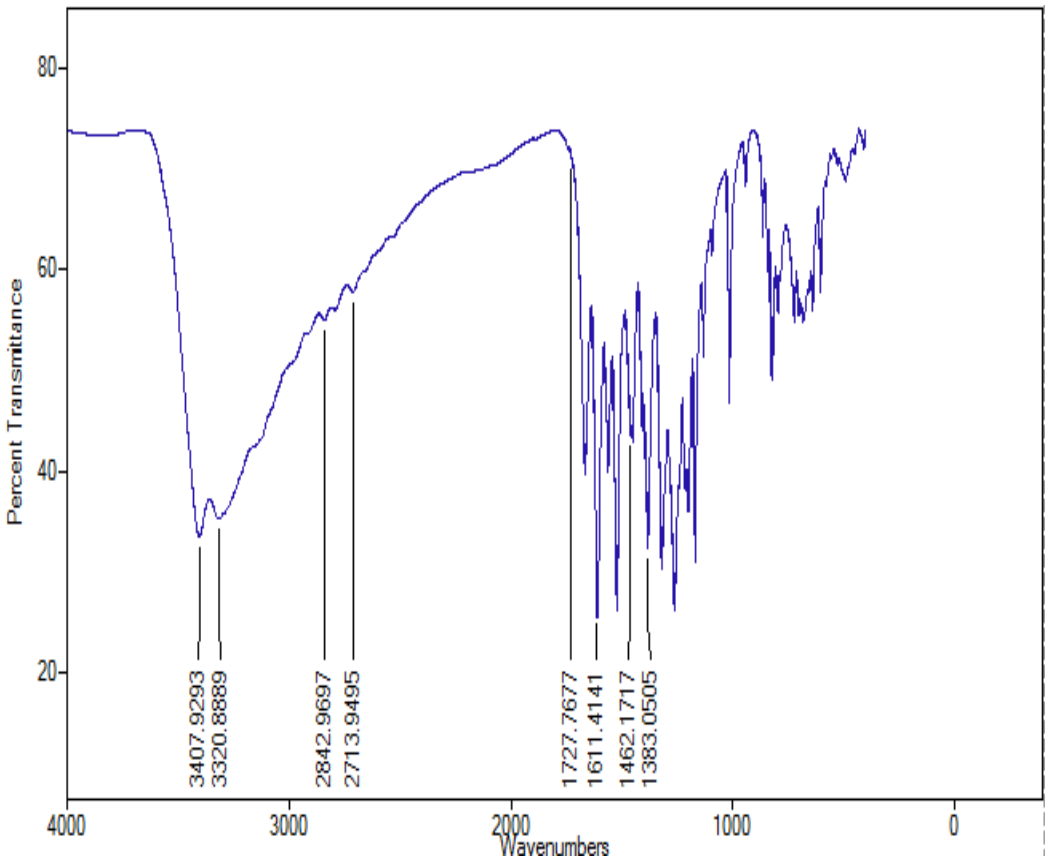


Figure 18: FTIR of Rutin (Chowdhury et al., 2013)

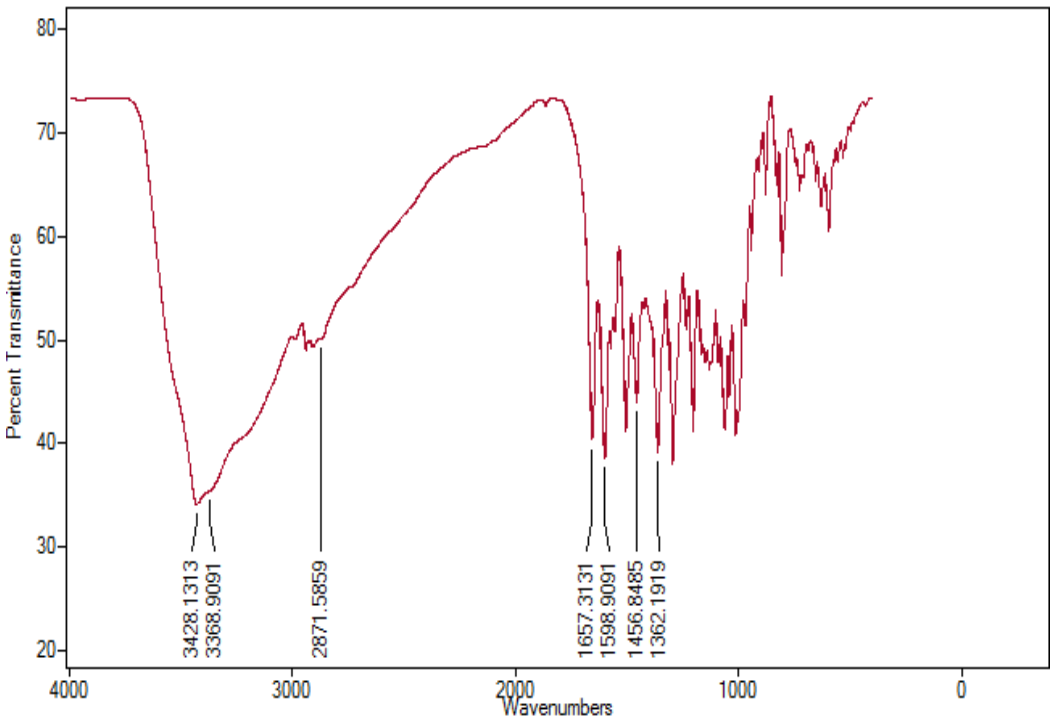


Figure 19: FTIR of Quercetin (Chowdhury et al., 2013)

Mass Spectrometry of flavonoids

MS is a very sensitive analytical method used to identify flavonoid conjugates or to perform partial structural characterization using microgram amounts of sample (Cuyckens and Claeys, 2004). Indeed, significant structural data can be obtained from less than 1 mg of the analyzed compound when different MS techniques are used in combination with chemical derivatization of the characterized compounds (Franski et al., 1999, 2002, 2003).

Electron impact mass spectrometry (EI) of both flavonoid aglycons and glycosides serves as a valuable aid in determining their structures, especially when only very small quantities (i.e less than 1mg) of the compounds are available. It has been applied successfully to all classes of flavonoid aglycones

and more recently to a number of different types of glycosides including mono- and di-C-glycosylflavones and mono-to tetra-O-glycosides. In addition to giving accurate molecular masses of molecular ions, fragmentation patterns revealed by some MS methods may provide (a) structural information about the nature of the aglycone and substituents (sugars, acyl groups, etc.), (b) interglycosidic linkages and aglycone substitution positions, and (c) even some stereochemical information (Andersen & Markham, 2006) (Figure 20). The amount of structural information obtained for flavonoids from a mass spectrum depends on the ionization method used. The highest energy transfer occurs during EI of flavonoid aglycones, and in these cases fragmentation of molecular ions is normally seen.

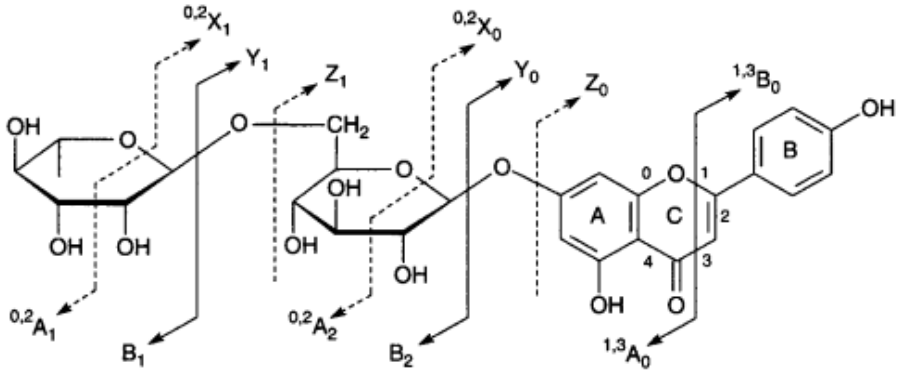


Figure 20: Ion nomenclature used for flavonoid glycosides (illustrated for apigenin 7-O-rutinoside) (Andersen & Markham, 2006).

In contrast, chemical ionisation (CI), using methane as the reactant gas, has only been applied to a few aglycones and gives diagnostic fragments except for flavanones and dihydroflavonols. Most flavonoid aglycones yield intense peaks for the molecular ion (M^+) and indeed this is often the base peak. Derivatization is thus unnecessary unless GLC-Mass spectrometry is to be carried out, in which case trimethylsilytion or permethylation provides adequate volatility. With

underivatized flavonoid glycosides, however, the molecular ion is rarely observed, and even permethylated or peracetylated derivatives give a peak of only low intensity. In addition to the molecular ion, flavonoid aglycons usually afford major peaks for $(M-H)^+$ and, when methoxylated, $[M-CH_3]^+$. Perhaps the most useful fragments in terms of flavonoids identification are those which involve cleavage of intact A^-.

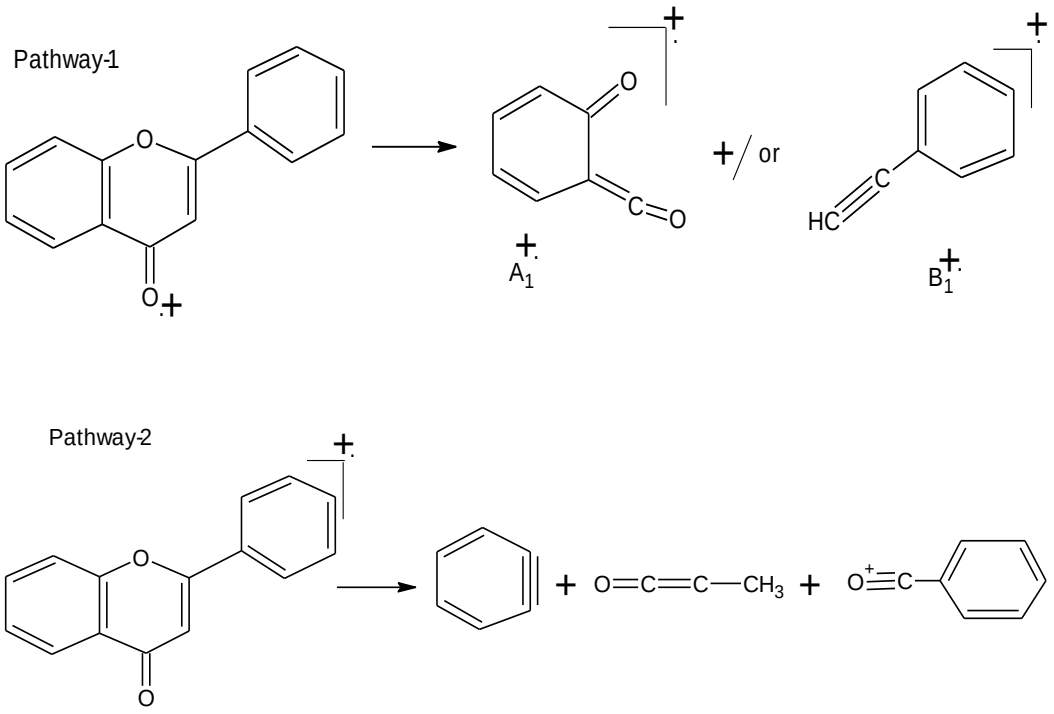


Figure 21: Useful fragmentations in terms of flavonoid identification (Harborne et al., 1975).

Flavonoid glycosides are thermally labile compounds and the evaporation without decomposition of the analyte is impossible, even in the ion source of a MS, where high vacuum exists (about $3x10^{-5}$ torr). In this situation, soft ionization methods need to be applied for the analysis of this group of compounds, and the analyte molecules are ionized without evaporation in high vacuum (FAB or LSIMS, MALDI) or under atmospheric pressure (ESI, APCI). From normal mass

spectra, information can be obtained about the molecular weight of the whole conjugate, the size of the sugar moieties attached to the aglycone, and the molecular weight of the aglycone (Stobiecki, 2000; Cuyckens and Claeys, 2004). With FAB or LSIMS ionization, the desorption of the analyte molecule ions from the liquid matrix may be improved when the interactions of the polar groups of the analyte with the matrix decrease. Improved efficiency of ion desorption may be further achieved after the methylation of the analyzed compounds. In addition, the methylation of a flavonoid glycoside may help to elucidate the glycosylation pattern of the aglycone hydroxyl groups (Stobiecki et al., 1988). The *O*-glycosides of flavonoids give positive ion mass spectra containing intense $[M+H]^+$ ions as well as fragment ions created after the cleavage of glycosidic bonds between sugar moieties or sugar and aglycone, in this case Y_n^+ type ions.

The most useful routes fragmentations in terms of flavonoid aglycone identification are those that require cleavage of two C—C bonds of the C-ring, resulting in structurally informative ${}^{i,j}A^+$ and ${}^{i,j}B^+$ ions. These ions can be rationalized by retro-Diels–Alder (RDA) reactions and are the most diagnostic fragments for flavonoid identification since they provide information on the number and type of substituents in the A- and B-rings (Pinheiro & Justino, 2012). The flavonoid aglycone fragment ions can be designated according to the nomenclature proposed by Ma et al., (1997) as cited in Plazonić et al, (2009). For free aglycones, the ${}^{i,j}A^+$ and ${}^{i,j}B^+$ labels refer to the fragments containing intact A- and B-rings, respectively, in which the superscripts *i* and *j* indicate the C-ring bonds that have been broken. The cleavage of the C—C bonds occurs at positions

1/3, 0/2, 0/3, 0/4 or 2/4 of the C-ring (Figure 21). The fragmentation pathways depend strongly on the substitution pattern and the class of flavonoids studied, e.g. the additional hydroxyl group in position 3 of flavonols results in more and different possibilities for fragmentation compared with flavones. $^{0,2}A^+$, $^{0,2}A^+$ -CO, $^{1,4}A^+$ + 2H and $^{1,3}B^+$ -2H are typically observed for flavonols, while $^{1,3}B^+$, $^{0,4}B^+$ and $^{0,4}B^+$ -H_2O are found for flavones. Figure 22 illustrates the low-energy CID spectra for the $[M + H]^+$ ions of (a) luteolin and (b) kaempferol (Andersen & Markham, 2006). Although both flavonoids have the same molecular mass, the *i*,*j*AC and *i*,*j*BC ions allow the distinction between the flavone, luteolin, and the flavonol, kaempferol. In addition to the *i*,*j*AC and *i*,*j*BC ions, discussed above, losses of small molecules and/or radicals from the $[M + H]^+$ ion are noted. Losses of 18 u (H_2O), 28u (CO), 42u (C_2H_2O) and/or the successive loss of these small groups are commonly observed.

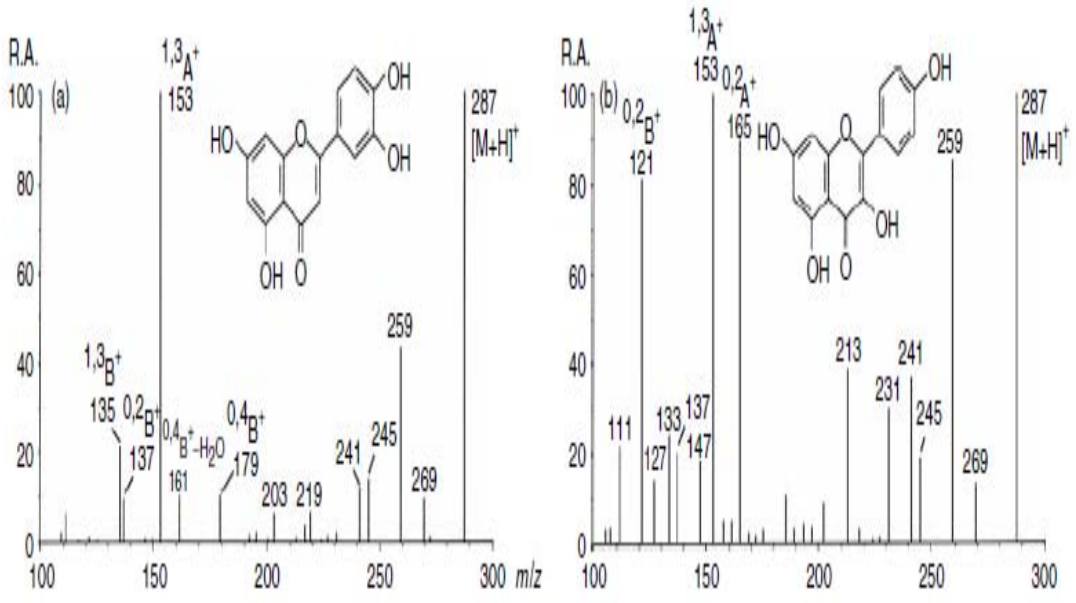


Figure 22: The low-energy CID spectra for the $[M + H]^+$ ions of (a) luteolin and (b) kaempferol (Andersen & Markham, 2006).

These losses are useful for identifying the presence of specific functional groups, i.e. a methoxy group is easily detected by the loss of 15 u (CH_3) from the $[M + H]^+$ precursor ion. The loss of a CH_3 radical appears to be prevalent so that the $[M + H - CH_3]^{+\cdot}$ ion dominates the whole spectrum. This rather uncommon transition from an even-electron to an odd-electron ion is found to be characteristic of a phenolic methyl ether group. Losses of 56 u (C_4H_8) point to the presence of a prenyl substituent.

NMR spectrometry of flavonoid

The studies of flavonoid structures using ^{1}H-NMR were initiated in 1960s (Markham & Mabry, 1975) and along with ^{13}C-NMR have became the method of choice for the structure elucidation of these compounds. NMR spectroscopy is an extremely powerful analytical technique for the determination of flavonoid structures. NMR arguably is the most important tool for complete structure elucidation of flavonoids. It is possible to make complete assignments of all proton and carbon signals in NMR spectra of most flavonoids isolated in the low milligram range. These assignments are based on chemical shifts (δ) and coupling constants (*J*) observed in 1D ^{1}H and ^{13}C-NMR spectra combined with correlations observed as crosspeaks in homo- and heteronuclear 2D NMR experiments. The chemical shifts and multiplicity of signals corresponding to particular atoms and their coupling with other atoms within the molecule allow for easy identification of the aglycone structure, the pattern of glycosylation, and the identity of the sugar moieties present (Markham & Geiger, 1994; Albach et al., 2003; Kazuma et al., 2003; Francis et al., 2004). Deuterated chloroform ($CDCl_3$) is the commonly

used solvent for direct analysis of many flavonoid aglycones, in particular isoflavones and highly methylated flavones and flavonols which are sufficiently soluble in $CDCl_3$. However, hexadeuteriodimethylsulphoxide (DMSO-d6) has been introduced as a solvent for the direct NMR analysis of flavonoids because most naturally occurring flavonoids, including all of the flavonoid glycosides have low solubility in $CDCl_3$. The highly solubility of most flavonoid aglycones and glycosides in DMSO-d6 presents DMSO-d6 as a suitable solvent for direct NMR analysis. As a result, the need for preparing derivatives has been eliminated. This is one of the advantages why DMSO-d6 has been used extensively as a solvent for investigations of flavonoid structures by NMR spectroscopy.

Interpretation of NMR Spectra of Flavonoids

Proton signals obtained in the NMR spectra of flavonoids generally occur in the range 0-8 ppm. Protons in both rings A and B give signals in the NMR spectrum. In ring A, the protons at C-6 and C-8 of flavones, flavonols and isoflavones which contain the common 5,7-dihydroxy substitution pattern give rise to the doublets (J = 2.5) in the range 6.0-6.5 ppm. The presence of C-6 and C-8 protons always suggest meta coupling pattern of A-ring. The H-6 doublet occurs consistently at higher field than the signal for H-8. However, the signals for both H-8 and H-6 are shifted downfield when a sugar is attached to the oxygen at C-7. The structure of Apigenin-7-ß-D-glucoronide in Figure 23 (Xiao et al, 2006) illustrates this phenomenom.

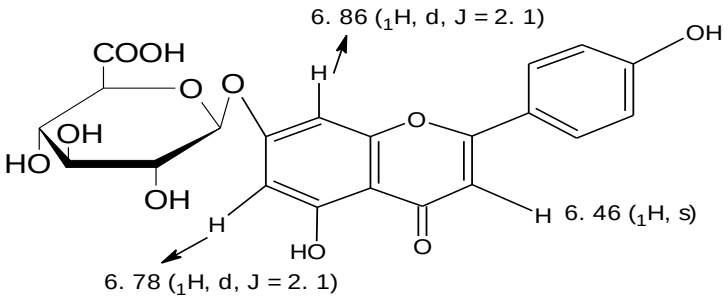


Figure 23: Absorption partern of C-6, C-8 and C-3 protons of flavones with sugar moiety

In flavanones and dihydroflavonols which contain the 5,7-dihydroxy substitution the signals for the A-ring protons appear at higher field than in the corresponding flavones and flavonols.

Apart from C-6 and C-8 protons the C-3 proton of flavones is the only proton that gives a signal consistently in the same region of the NMR spectrum as those of the C-6 and C-8 protons. The C-3 proton appears as a singlet near 6.3 ppm. Figure 24 illustrates the proton signal patterns of C-3, C-6 and C-8 protons (Kim et al, 2012).

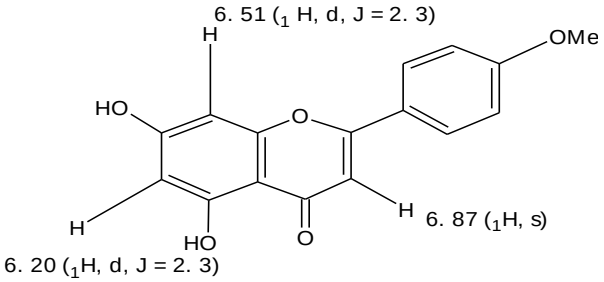


Figure 24: Proton signal pattern of C-3, C-6 and C-8 protons of flavones

Many flavonoids have only one A-ring proton. In such compounds the only A-ring proton produces a singlet which is often in the same region as the C-3 proton signal. Magalhaes et al., 2003 reported on similar compound (Figure 25).

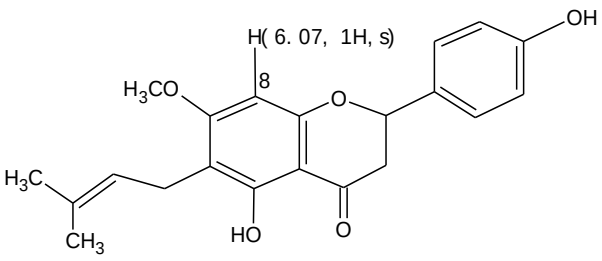


Figure 25: Example of flavanone with only one A-ring proton showing its proton signal.

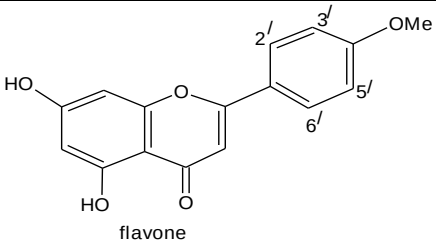
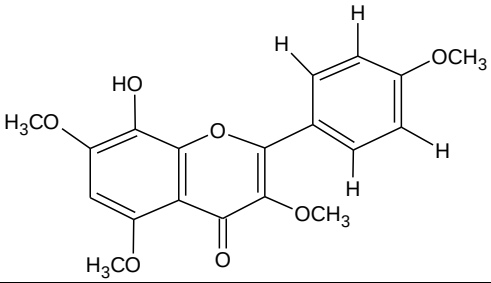
Some flavonoids have only C-7 oxygenation in the A-ring. In such flavonoids, the C-5 proton is deshielded by the C-4 keto group and therefore absorbs near 8.0ppm (doublet, J = 9.0) at a lower field than most aromatic protons. In this case ortho coupling occurs between C-5 and C-6 protons. The C-5 proton in these 7-oxygenated flavonoids appears as a doublet (J = 9.0) as a result of ortho coupling between the C-5 and C-6 protons.

Absorption pattern of B-ring Protons

The protons of ring B appear in the range 6.7-7.9 ppm, which is downfield from the region where the A-ring protons usually absorb. The signal pattern observed for the B-ring protons is characteristics of the degree of substitution of that ring. A typical four peak pattern of two doublets (each J = 8.5) is observed for ring B which is oxygenated only at C-4'. The doublet for the C-3' and C-5' protons, which are deshielded by the C-4' oxygen substituent, always appear upfield from the C-2' and C-6' protons generally falls in the range 6.65-7.1ppm for all types of flavonoids. However, the signals for C-2' and C-6' consistently appear at lower field (7.1-8.1 ppm) than C-3' and C-5' doublet. Table 13 illustrates the above description.

In 3’ and 4’-oxygenated flavones and flavanols the C-5’ proton appears as a doublet centered between 6.7 and 7.1ppm (J =8.5). The C-2’ and C-6’ proton signals which often overlap, usually occur between 7.2 and 7.9 ppm. The relative positions of the signals for the C-2’ and C-6’ protons may be used to distinguish the 3’-methoxy-4’ hydroxyl from the 4’-methoxy-3’-hydroxy B-ring substitution pattern in flavones and flavonols.

Table 13: Structures of C-4’ oxygenated flavonoids illustrating a typical four-peak signal pattern for C-2’, C-3’, C-5’ and C-6’ protons

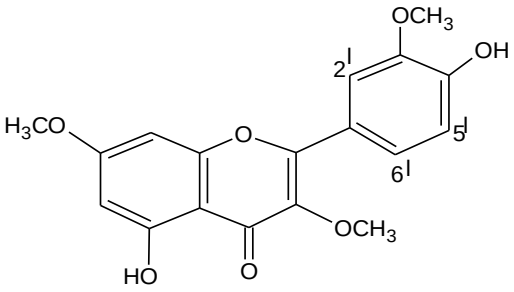
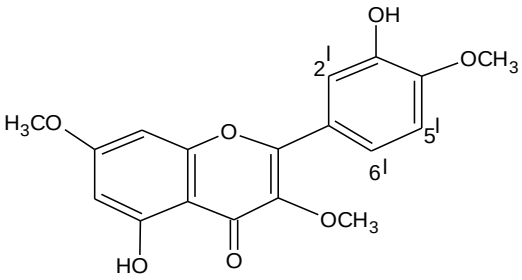
Compound	H-2’, 6’	H-3’, 5’	Reference
 flavone	8.04 (2H,d, J = 10.7)	7.11 (2H,d, J =10.7)	Kim et al, 2012
	8.04 (2H,d, J= 9.2)	6.85 (2H,d, J = 9.1)	Johann et al, 2007

The C-2’ proton signal is usually centered at slightly higher field than the C-6’ proton signal in flavanoids containing a 4’-methoxy group. These positions are reversed when a 3’-methoxy is present in the 3’, 4’-oxygenated compound. The structures in Table 14 (Alarcon et al., 2007) explain the above description.

Different spectral patterns are observed for 3’, 4’-oxygenated isoflavones, flavanones and dihydroflavonols. These compounds give a complex multiplet, usually two peaks, for the C-2’, C-5’ and C-6’ protons in the region 6.7-7.1 ppm.

In these compounds the protons are either ortho or para to an oxygen substituents and therefore exhibit such chemical shits.

Table 14: Absorption pattern of C-2', 5' and 6' Protons for C-3', 4' oxygenated B-ring

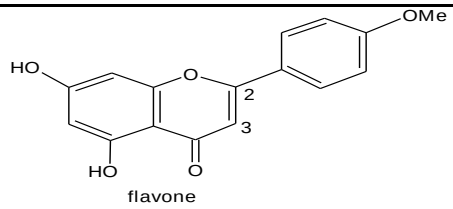
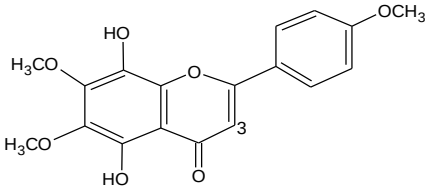
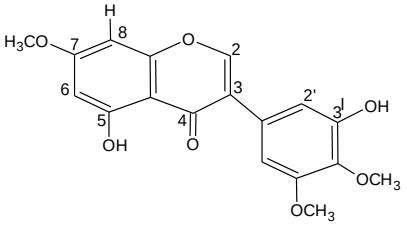
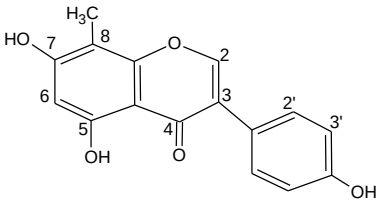
Flavonoid	C-2' Proton	C-5' proton	C-6' Proton
	7.80 d (2.0)	7.02 d (8.5)	7.72 dd (8.5, 2.2)
	7.67 d (2.2)	7.15 d (8.0)	7.72 dd (8.0, 2.2)

Absorption pattern for C-Ring Protons

The C-3 proton in flavones gives a sharp singlet near 6.3 ppm in the same region where signals of A-ring protons occur. The C-2 proton in isoflavones, which is in a ß position to the C-4 keto function, occurs in the range 7.6-8.7 ppm, a region downfield from where most A- and B-ring proton signals appear. Table 15 cited examples of ^{1}H-NMR signals of C-2, 3 protons of isoflavones and

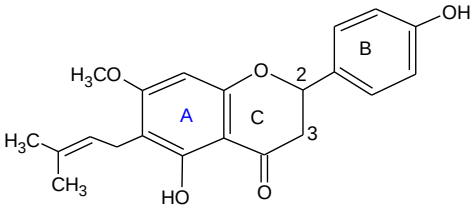
flavones respectively. The signal for the C-2 proton of flavanones appear as a quartet (two doublets J_{cis} = 5.0, J_{trans} = 11.0) near 5.2 ppm as a result of the coupling of the C-2 proton with the two C-3 protons. The C-3 protons couple with each other (J = 17) in addition to their spin-spin interaction with the C-2 proton. This gives rise to two overlapping quartets centered around 2.8 ppm.

Table 15: H-NMR signals of C-2 and C-3 protons of isoflavones and flavones Respectively

Compound	C-2 Proton	C-3 Proton	Reference
 flavone	-	6.87 (1H, s)	Kim et al, 2012
 flavone	-	6.53 (1H, s)	Intekhab and Aslam, 2009
	8.15 (1H, s)	-	Noreen et al, 1998
	7.83 (1H, s)	-	Rahman et al 2010

In dihydroflavanols as shown in Table 16, the C-2 proton signal occurs as a doublet (J =11) near 5.2 ppm, while the C-3 proton doublet appears further upfield at about 4.3 ppm. The coupling constant (J =11) is typical for 1, 2-diaxial protons.

Table 16: Example of Absorption pattern of C-2 and C-3 protons of flavanone (Magalhaes et al, 2003)

Compound	3eq	3ax	H-2
	2.76(1H,dd, J=17.1, 2.2)	3.08(1H,dd J=17.1,13.4)	5.30(1H,dd J=13.4, 2.2)

^{13}C-NMR Spectra of Flavonoids

In the ^{13}C-NMR spectra of flavanones as shown in Table **17,** the carbonyl carbon (C-4) signals come in the region 189.5-191.6 ppm (except when a 5-OH group is present); see flavanone 35. C-2 and C-3 resonances are identified at 75.0-80.3 and 42.8-44.6 ppm, respectively, the latter appearing as a triplet in the off-resonance decoupled spectrum, and the former as a doublet.

When a methoxy-group is introduced at C-7 of the flavanone nucleus the C-7 signal itself is moved downfield by 29.9 ppm, whilst the C-6 and C-8 resonances are readily moved upfield by 11.4 and 17.2 ppm, respectively, and that of C-4a is moved upfield by 6.2 ppm. The carbon atoms *meta* to the methoxy-group (C-5 and C-8a) are only slightly affected (+ 1.6 and + 1.9 ppm). Earlier results have indicated that in simple benzene derivatives the carbon atom to which a methoxy-

group is attached is deshielded by 30.2 ppm, whereas the carbon atoms *ortho* and *para* to the methoxy-group are shielded by 15.5 and 8.9 ppm, respectively. It has been further suggested that the shielding effects of substituents on an aromatic nucleus exhibit an additive relationship, provided the groups are not *ortho* to one a nother (Pelter et al, 1976).

Table 17: ^{13}C-NMR Chemical shifts for Flavanone 24, 25, 26 and 35

Flavanone	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'	C-2	C-3	C-4	C-5	C-6	C-7	C-8	OMe
24	138.63	125.99	128.66	128.66	128.66	125.99	79.49	44.60	191.62	126.90	121.45	135.99	117.98	
25	138.64	125.94	128.53	128.53	128.53	125.94	79.77	44.17	190.06	128.53	110.02	165.88	100.78	55.48
26	127.65	155.96	110.63	129.48	120.98	126.49	75.11	43.40	191.46	128.84	110.14	166.16	101.02	55.41
35	131.28	113.64	146.56	147.88	111.53	117.66	78.61	42.80	195.50	163.99	96.44	166.90	95.44	55.87

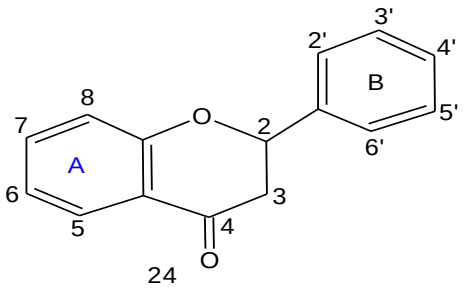


Figure 26: Flavanones (24), (25) 7-Me0, (26) 2', 7-(Meo), (35) 3', 5, 7-(HO), 4'-MeO

Flavones and Isoflavones.-

The carbonyl carbon signals of both flavones and isoflavones come in the region 174.5-178.6 ppm (figure 27), but C-2 and C-3 are sufficiently different in the two series, to permit an immediate distinction. In the flavones the C-2 signal appears as a singlet (in the off-resonance decoupled spectrum) at 160.5-163.2 ppm, and that of C-3 as a doublet at 104.7-111.8 p.p.m., whereas in the isoflavanones the C-2 resonance is seen as a doublet at 149.8-155.4 and that of C-3 as a singlet at 122.3-125.9 ppm (Pelter et al, 1976).

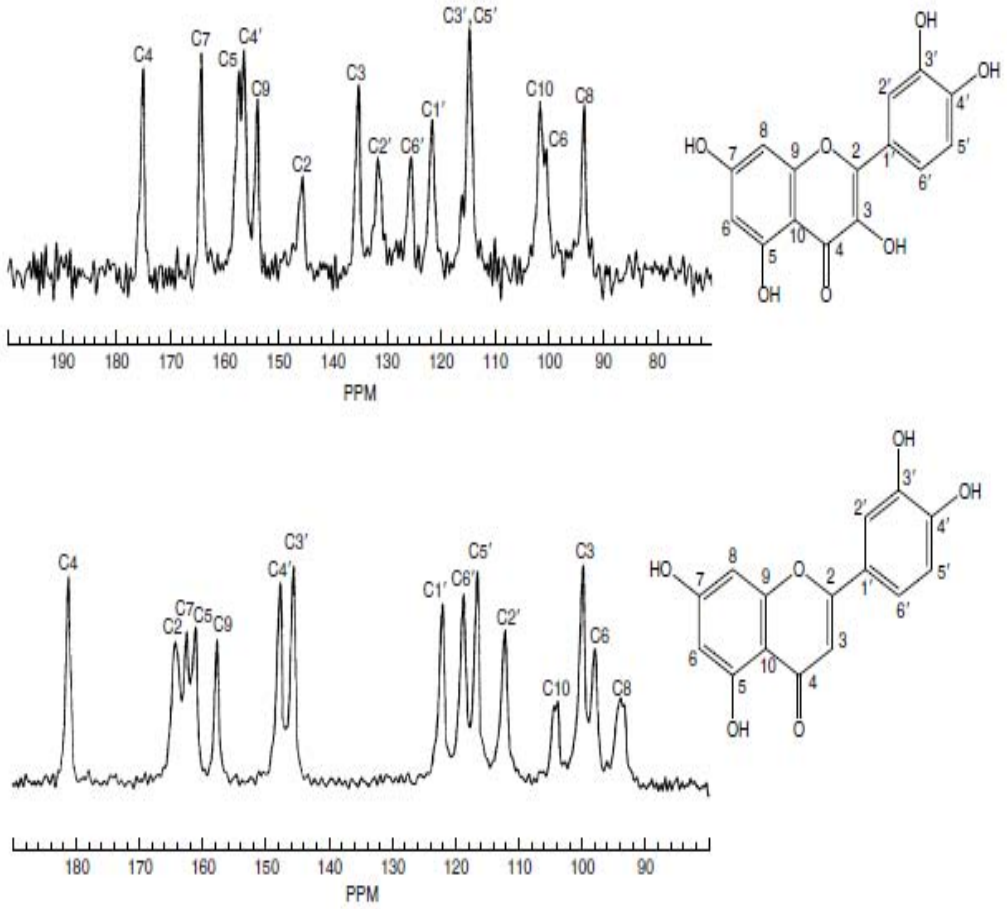
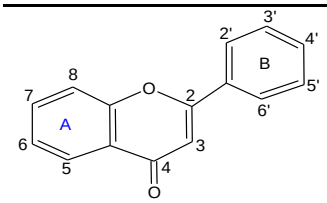
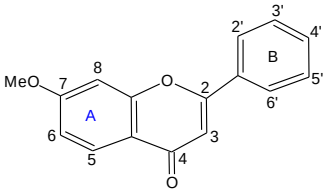
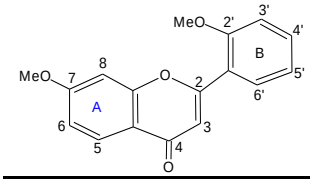


Figure 27: ^{13}C-NMR spectra of the flavonol kaempferol (top) and the flavone luteolin (bottom) (Andersen & Markham, 2006).

Introducing a methoxy-substituent at C-7 of flavones has a similar effect to that already noted in the flavanone series. Table 18 shows the effect of the introduction of methoxy substituent on ^{13}C-NMR chemical signals The C-7 signal itself is moved downfield by 30.2 ppm., the *ortho*- and para-carbon signals (C-6, C-8, and C-4a) are moved upfield by 10.8, 17.7, and 6.1 ppm respectively, and the meta-carbon signals (C-5 and C-8a) are only slightly affected (4-1.3 and +1.7 ppm). Once again the two ortho-carbon atoms are affected by different amounts by the introduction of the 7-methoxy-group (Pelter et al, 1976).

Table 18: Effect of the introduction of methoxy substituent on ^{13}C-NMR chemical signals

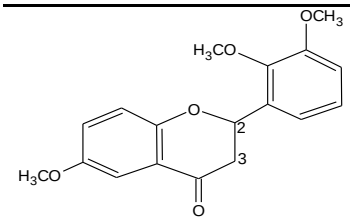
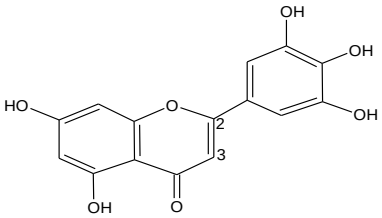
Flavone	C-5	C-6	C-7	C-8	C-4a	C-8a	OMe
	125.4	124.9	133.5	117.9	123.7	156.0	-
	126.7	114.1	163.7	100.2	117.6	157.7	59.9
	127.05	114.23	164.12	100.37	121.06	158.0	55.71, 55.81

Comparing the chemical shifts of flavones and flavanone the double bond between C-2 and C-3 in flavones causes downfield shifts of their ^{13}C chemical shifts by approximately 90 and 60 ppm, respectively because of their sp^2 hybrid

orbitals (Yoon et al, 2011). In addition, it has an effect on the ^{13}C chemical shift of C-4 by at least 10 ppm as shown in Table 19.

The substitution of hydroxy/ methoxy group at C-2' causes the downfield shift at C-3 and the upfield shift at C-2. Mono-hydroxy/methoxy group in A-ring occurs to the upfield shift at C-3 by about 2 ppm. In compounds with unsubstituted B-ring, the ^{13}C chemical shifts of C-1', C-2'/C-6', C-3'/C-5', and C-4' in B-ring have the values of 131.2 ± 0.4, 126.3 ± 0.2, 129.1 ± 0.1, and 131.7 ± 0.3 ppm, respectively.

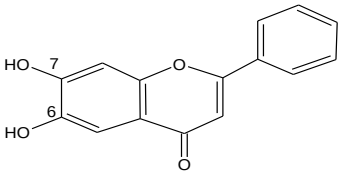
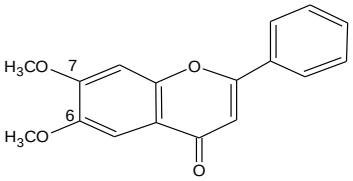
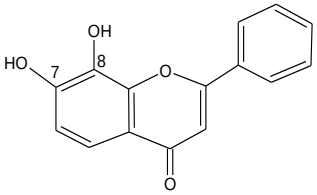
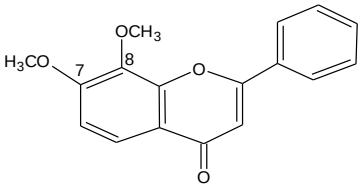
Table 19: Comparism of chemical shifts of C-2 and C-3 of flavones and Flavanones

Flavonoid	C-2	C-3	C-4
	74.2	42.8	191.6
	164.1	102.9	181.5

Likewise, in compounds with unsubstituted A-ring, 7 and 8, the change of the ^{13}C chemical shifts of C-5, C-6, C-7, C-8, C-9, and C- 10 in A-ring is ranged within ± 0.2 ppm. The substitution of hydroxy/methoxy group at C-7 and/or C-8 occurs to the upfield shift at C-9 and C-10. However, chemical shifts of carbons with hydroxyl substituents are found to be more upfield shifted than those with

methoxy groups similar to proton chemical shifts (Yoon et al, 2011). Table 20 illustrates this.

Table 20: Comparism between chemical shifts of carbons with hydroxyl and methoxy substituents

Flavone	C-6	C-7	C-7	C-8
	144.7	152.4		
	147.4	154.4		
			150.7	133.3
			156.4	136.4

CHAPTER THREE

EXPERIMENTAL

In this chapter, detailed description of the materials such as equipment, chemical reagents, and the study area used for ethno survey, samples of selected plants used for experimental analysis as well as the experimental and statistical methods used for the study are presented.

Materials and Method

General instruments, Reagents and chemicals

All the chemicals used were of analytical grade including sodium chloride, magnesium and ferric chloride; ammonium hydroxide and were obtained from the Central store, Department of Chemistry, University of Cape Coast. Solvents such as hexane, ethanol, methanol, chloroform, ethyl acetate, pet-ether and butanol were purchased from MES Equipment Limited. These were used as elution solutions in column chromatography. Nutrient agar media, sterile yeast and Mould extract agar were also obtained from Department of Laboratory Technology & Medical Laboratory Central Store. UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, UK) was used to measure all absorbances.

^{1}H- and ^{13}C-Nuclear Magnetic Resonance (NMR) spectra were recorded with a Bruker AVANCE 125 and 500 NMR (Rheinstetten, Germany) spectrometers

respectively using TMS as an internal standard. Chemical shifts were reported in parts per million (δ), and coupling constants (*J*) were expressed in hertz. IR spectra were recorded on a Shimadzu IR- 470 spectrometer. UV-Visible spectrum of each compound was determined in methanol and after addition of different shift reagents such as $AlCl_3$, $AlCl_3$/HCl, NaOH, AcONa and AcONa/H_3BO_4 at 200-600 nm. Evaporation was conducted using an EYELA rotary evaporator system (Japan) *in vacuo*. Silica gel 60 (70-230 mesh ASTM) from Merck Germany was used for column chromatography. Thin layer chromatography (TLC) was conducted using glass plates coated with silica gel G-200 UV_{254} (Germany) of 2.0 mm layer thickness), with compounds visualized by spraying with 10% H_2SO_4 in MeOH.

Ethnopharmacological survey

The study area is Kpando Traditional area within the Kpando district, now Kpando Municipal. Figure 28 represents a sketch map of the study area. Kpando Municipal is located in the Volta Region of Ghana and it is one of the oldest in the country. The district lies within latitudes 6° 20 °N and 7005 °, and Longitude 00 17 °E. It shares boundaries with Biakoye District in the North, Hohoe District to the East, and the newly created North Dayi District in the South. The Volta Lake which stretches over 80km of the coastal line, demarcates the Western boundary.

The district covers a total land area of 820 sq. km representing 45% of the Volta Region with almost 40% of the land being submerged by the Volta Lake. Kpando, the district capital, is 90 km from Ho, the Regional capital. The location

of the district places it at a very strategic position with potential for fast economic development.

Figure 28: A map showing the ethnobotanical study area of Kpando Municipality.

Kpando Municipality falls within the tropical zone, and it is generally influenced by the South West monsoons wind from the South Atlantic Ocean and the dry harmattan winds from the Sahara. The Municipal is therefore characterised by two rainy seasons. The major one occurs from mid-April to early July and the minor from September to November.

The vegetation of the Municipal is of Guinea Savannah Woodland, Deciduous and Thick Forest types. It is certain that the Municipal was densely forested in former times. But huge forest areas have been destroyed and converted to other land uses during the past decades. However, the indigenes encountered in this area were mainly farmers

Plant collection and identification

A total of 70 questionnaires were administered and interviews were conducted with both old and young local people in ten villages in all the three Traditional divisional zones of Kpando that comprise the study area. In each village, respondents were randomly selected and interviewed. The interviews were conducted with a fairly open framework that allowed for focused, conversation and two-way communication. Also, some local traditional medical practitioners believed to have the greatest knowledge about the traditional uses of plants in the area were identified and involved in this study. Group interviews as well, were conducted in order to determine group consensus on the plant species used for wound healing. Field interviews involving walking with the local people to the areas where they normally collected their medicinal plants while interviewing

them were also adopted. Throughout the interviews, the local names of the plants, the parts used, method of preparation of crude drug from the plants, mode of application were recorded. Only species mentioned by at least two respondents for the treatment of wound across the study area were selected in order to confirm the use of these species. In cases where the plant species were not immediately identifiable with botanical names, they were brought to the herbarium, Department of Environmental Sciences, School of Biological Sciences, University of Cape Coast, where they were identified.

A total of 7 Traditional Medical Practioners (TMP's) were interviewed amongst whom one was literate. Their ages range from 34 to 80 years with more of them in the older side of the range. Among them was only one woman.

Plant material and sample preparation

Anogeissus leiocarpus (DC) Guill & Perr, Amaranthus spinosus Linn, *Combretum dolichopetalum* Engl. & Diels, *Corchorus olitorius* Linn, *Spondia mombin Linn and Mallotus oppositifolius* Geiseler Mull. Arg. were selected among the wound healing plants identified in the survey for further studies. All the plants, except *Mallotus oppositifolius* which was used as a protocol, were among those identified that have not been documented for wound healing. They were selected based on the following criteria:

1. Those that are easily accessible and can be harvested within the immediate environment
2. Those that are known not to be poisonous or are eaten by humans

3. Those with no extensive scientific research conducted on them

Various parts of the plants based on their traditional uses for wound treatment, were collected and washed in tap water and then rinsed properly with deionized water. The treated plants were dried at room temperature and then ground. 10 grams of each plant powder was extracted in 250 ml 90% v/v methanol by maceration (24 h), followed by filtration through Whatmann no. 1 filter paper. The resulting filtrates were concentrated in vacuo and the extract obtained kept in refrigerator for the following tests.

Biological Activity

Antimicrobial activity

Test Microorganisms: Four available microorganisms responsible for wound infections (Gram- positive bacteria and Gram-negative bacteria) were used to investigate the extended antimicrobial property the herbal plants may possess. Microbial cultures of four different strains were used for determination of antibacterial activity. These were three standard bacterial strains viz. *Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC4352) and one clinical isolate Citrobacter sp.* from Korle-Bu Teaching Hospital Central Laboratory, Accra (Ghana).

Antimicrobial activity assay: Well diffusion method using Müeller-Hinton agar plates were used to demonstrate the antimicrobial properties of the crude extracts (Forbes et al, 1990). A suspension of the bacteria compared to 0.5 Macfarland standard was seeded on the Mueller-Hinton agar plates. Wells of 6mm in diameter

and 2 cm apart were punctured in the culture media using sterile cork borers. 80 µl of the crude extracts was administered to fullness in each well and the plates were incubated overnight at 37 °C. Growth was determined by measuring the diameter of the zone of inhibition. The solvents were used as the negative controls whiles 10 µg ampicillin disc (Oxoid) was used as the positive control. The control zones of the solvents were deducted from the zones of inhibition created by the crude extracts. The experiments were carried out in triplicates and results were calculated as mean ± SD.

Wound healing activity

Only 4 out of the 6 plants namely *C. olitorius, A. leiocarpus, A. spinosus* and *C. dolichopetalum* could be taken through the wound healing experiments due to shortage in supply of the animals.

Experimental animals: Albino Wistar rats of either sex were used for the study. The animals were maintained under hygienic conditions and they were provided with commercial food pellets and tap water. Cleaning and sanitation work were done on alternate days. The cages were maintained clean and all experiments were conducted between the hours of 9 am to 5 pm.

Grouping of animals: The animal weights (234-389 g) were recorded. The animal groupings were stratified according to weights, so that the average weights of all groups were comparable. Seven groups of the animals with four rats each were used.

GROUP 1- rats treated with 5% w/w of powdered plant ointment.

GROUP 2- rats treated with 10.0% w/w of powdered plant ointment.

GROUP 3- rats treated with 30 mg/ml of aqueous extract.

GROUP 4- rats treated with 100 mg/ml of aqueous extract.

GROUP 5- rats treated with standard drug (penicillin ointment or Drez).

GROUP 6- rats treated with Shea butter ointment.

GROUP 7-rats left untreated

Creation of excision wound: An excision wound model was used for studying the wound healing activity. Fresh 50 mg/ml ketamine chloride solution was prepared for anesthesia and a single-use syringe for injection. For the IV injection, the mouse was held at its neck directly behind the ears and the tail was grasped while holding the rats with its head down. The rats were placed back into the cage, so that rats will not become agitated. This type of anesthesia prevents any movement of the animals at least for 2 hours after the administration of the anesthetic solution so that they were left without being restrained. Hair was removed by shaving the dorsal of all rats. A full thickness of the excision wound of approximately 490 mm^2 and 2 mm depth was created along the markings using toothed forceps and pointed scissors. The back of the anesthetized rats were shaved using the razor blade and the hair was carefully removed from the back of the animal. The anesthetized and shaved rats were placed on a paper towel. The shaved back of the animal was wiped with a sufficient amount of 70% alcohol. The mouse was held at its neck directly behind the ears and the tail of the rats was held down. The back of the skin was lifted using forceps. The skin was incised first and carefully cut using the

scissors. Lifting up the skin ensured that the incision will move through the panniculus Carnosus. After completion of excision wounding, the wound was left undressed to the open environment and no local or systemic anti-microbial agents were used before the animals were transferred into cages.

Determination of microbial load on the wound

Swabs were taken from the excision wound each on day 5, 10, and 15. The collected swabs were immediately sent to the laboratory for testing. In the quantitative count study, each swab stick was added to 2 ml of peptone water. The sample was mixed thoroughly and a 5-fold serial dilution was performed. A volume of 0.1 ml of each sample dilution was spread onto MacConkey and blood agar plates. They were incubated at 37 °C for 24 hours. The colonies were counted and the results were recorded.

Determination of Antioxidant activity

Determination of flavonoid contents

The aluminum chloride colorimetric method was used to measure the flavonoid content of all plant extracts. 0.5 ml of each plant extract was added to 1.5 ml methanol, 0.1 ml 1M sodium acetate and 2.8 ml distilled water after which 0.15ml of 10% aluminium chloride was added. The mixture was allowed to stand for 30 min at room temperature. The absorbance of the reaction mixture was measured at 415 nm with a UV/VIS spectrophotometer. Quercetin was used as the standard for the calibration curve. Flavonoid contents were expressed as mg quercetin equivalent /g dry weight (D.W.).

Determination of total phenolic content

Total phenol content was estimated using Folin-Ciocalteu reagent based assay as previously described (McDonald et al., 2001). To an aliquot from each plant extract, 10 ml of water, 1.5 ml of Folin-Ciocalteu reagent (Sigma-Aldrich, Switzerland) were added. The mixture was kept for 5 minutes at room temperature and then 4 ml of 20% Na_2CO_3 was added and the volume brought to 25 ml with double-distilled water. The mixture was allowed to stand at room temperature for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. Gallic acid was used as standard for calibration curve. Total phenol value was obtained from the regression equation: y = 0.00048x + 0.0055 and expressed as mg/g gallic acid equivalent using the formula, C = cV/M; where C = total content of phenolic compounds in mg/g GAE, c = the concentration of gallic acid (mg/ml) established from the calibration curve, V = volume of extract (0.5 ml) and m = the weight of pure plant methanolic extract (0.052 g) (diluted ten times).

Ferric Reducing Antioxidant Power Assay

The reducing antioxidant power of plant methanolic extracts was determined by the method of Oyaizu (1986). Different concentrations of plant extracts (250 – 1000 ppm) in 1 ml of distilled water were mixed with phosphate buffer (3.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 ^oC for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml)

and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K). Increased absorbance of the reaction mixture indicates increase in reducing power. Ascorbic acid was used as standard.

Scavenging activity against 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH)

The crude methanol extract of the six plants, ethyl acetate and n-butanol fractions of *C. dolichopetalum, A. leiocarpus* and *S. mombin* plants were screened for 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical Scavenging activity. DPPH radical scavenging activity was measured according to the method of Braca et al., 2003 & Rajeswara et al., 2012. An aliquot of 3 ml of 0.004% DPPH solution in ethanol and 0.1 ml of plant extract at various concentrations were mixed and incubated at 37 °C for 30 min. and absorbance of the test mixture was read at 517 nm. All experiments were performed in triplicate and the results were averaged. The percentage inhibition/scavenging activity of DPPH-radical was calculated using the formula:

% scavenging activity of DPPH-radical = (Abs control – Abs sample)/ (Abs control)*100

Determination of Total Antioxidant Activity (TAC)

The total antioxidant capacity of the methanol extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. (1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid ρH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid,

28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated for 60 minutes at 95 ^{o}C. The absorbance of the green phosphomolybdenum complex was measured at 695 nm using a spectrophotometer (Jenway 6025) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract was used as the blank. The antioxidant activity was expressed as the number of gram equivalent of ascorbic acid. Different concentrations of ascorbic acid (0.02-0.20 mg/ml) were prepared in methanol and used to obtain the calibration curve.

Phytochemical screening

Chemical tests were carried out on the methanolic extracts for the qualitative determination of phytochemical constituents as described by Harborne (1973). Below is a brief description of the methods used.

1. **Detection of alkaloids:** Extracts were individually heated with 2% HCl solution on a boiling water bath, cooled and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate or turbidity indicates the presence of alkaloids.

Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

2. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

3. Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

4. Detection of phytosterols

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed

to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Liebermann Burchard's test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

5. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

6. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Ferric chloride test: To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added to it, and observed for blue or green black coloration.

7. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Shinoda test: To dried extract, 5 ml of 95% ethanol few drops of concentrated HCl and 0.5 g Magnesium turnings were added. Pink colour was observed (Dudekula Meharoon et al., 2011)

Ferric chloride test: To test solution, few drops of ferric chloride solution were added and were observed for intense green colour.

8. Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes (Tiwari et al, 2011).

9. **Detection of carbohydrates:** Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's test: Filtrates (2-3 ml) treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml. of conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction of the two liquids indicates the presence of carbohydrates.

Benedict's test: Equal volume of Benedict's reagent and filtrates mixed in a test tube and heated in a boiling water bath for 5 min. Formation of an orange red precipitate indicates the presence of reducing sugars.

Fehling's test: Filtrates hydrolysed with dil. HCl, neutralized with alkali and heated with Fehlings A & B solutions. Formation of a red precipitate indicates the presence of reducing sugars.

Extraction of Compounds

The air-dried, pulverised leaves of *Anogeissus leiocarpus* (432.22 g) and *Combretum dolichopetalum* (306.04 g) were each refluxed with methanol for two hours. The extracts were filtered and concentrated under reduced pressure at 40 ^{0}C and the resultant sticky substances were kept in a dessicator to dry. Each dried plant sample was weighed and the yield recorded. Each extract, *A. leiocarpus* (46.12 g) and *C. dolichopetalum* (32.5 g) was re-dissolved in distilled water (160 ml) and filtered. The water soluble fractions were successively extracted with Pet-ether (4 x 50ml), $CHCl_3$ (4x50ml), EtOAc (4x50ml) and Butanol (4x50ml). All the fractions were concentrated in vacuo and resultant crude extracts stored in the desiccator until use. Portions from each dry sample were used for screening for flavonoids.

Chromatography

Thin layer chromatography

Thin-layer chromatography (TLC) was employed in the qualitative analysis of all the extracts. This was used to identify the number of compounds present in the plant extract. In addition, it was used to select the best solvent system for the separation and isolation.

Ten cleaned and dried glass plates of size 20 x 20 cm were coated with aqueous slurry of silica gel G-200 UV_{254} as the stationary phase. The slurry was coated onto the plates by using a spreader placed at one end of the plates and then spread over the plates to give thickness of 0.25 mm. The plates were then left to

dry at room temperature before they were baked in the oven at a temperature of 110 ^{o}C for one hour.

A portion of the extract was dissolved in a little amount of methanol. This was applied onto the stationary phase by thin filled capillary tube. It was allowed to soak in and left to dry. The plate was then placed in a developing tank and covered to ensure homogeneity and saturation. The developing tank was made up of a tall narrow tank with a round glass lid. After the developments, the plates were air-dried and the separated components on the chromatogram located and marked with pencil. Detection of spots was made possible by:

1) Exposing the chromatogram to U.V light.
2) Spraying with chemical locating reagent (10% H_2SO_4 in methanol), followed by charring at 110 ^{o}C for 10 minutes in an oven. The retardation factor (R_f) for each spot was calculated using the formula:

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

A solvent system of MeOH-$CHCl_3$ (2:3) was found to be appropriate and used for the development of the plates. Table 21 listed the solvent systems (ratios) that were tried and used in the experiment.

Table 21: **Solvent systems used in thin layer chromatography**

Solvent Mixture	Ratios
Petroleum-ether (40 -60 ^{o}C)	100%
Petroleum-ether / Ethylacetate	(1:1)
Petroleum-ether / Ethylacetate	2:1
Petroleum-ether / Ethylacetate	1:2
Chloroform / Methanol	2:1
Chloroform / Methanol	1:1
Chloroform / Methanol	3:1
Chloroform / Methanol	4:1
Chloroform / Methanol	3:2

Column Chromatography

A column of 65 cm long with an internal diameter of 2.5 cm was used. The column was washed with detergent and water, then rinsed with acetone and dried. The column was mounted vertically with a support. A glass wool plug was placed at the bottom of the tube just above the stop-cork. The adsorbent used was silica gel G60 of mesh size (70-230). The column tube was filled with petroleum-ether (40-60 ^{o}C) as the first eluent to 150 cm height. The adsorbent was poured in through a funnel with a long and wide stem, which dipped below the surface of the liquid in the tube. The liquid allowed to flow out at such a rate that it never went below the level of the end of the adsorbent. By this means the solvent was drained slowly until no further settling of the column took place. The top of the column was then covered with a perforated disc of filter paper to prevent disturbance of the column when adding more solvent. Weighed amount of the sample in gramme was added onto the top of the perforated filter paper in the

column. The sample was distributed evenly on the filter paper by means of a pipette. When all the samples have been added, the sample container and the pipette were washed with small amount of the solvent and the washings added to the column. The column was not allowed to become dry at any stage during the elution process. Fractions collected in different test tubes were examined by their colour and on thin layer plates. The fractions of similar properties (colour and R_f values) were combined together and concentrated.

Preparative Thin Layer Chromatography

Fifteen glass plates were thoroughly cleaned and coated with already prepared slurry of silica gel to the thickness of 0.25 mm. The plates were well dried in an oven at a temperature of 110 ^oC for an hour. The sample solutions were prepared and carefully transferred on to the plate. The liquid samples were added as series of spots close together and about 1.5 cm above the lower edge. The series of spots were added across the whole width of the plates. Detection of the substances developed on the chromatogram was made possible by the light absorption in Ultraviolet light (Long wavelength 256 nm). The zones containing the desired substances were scraped off into separate cleaned evaporating dishes and were then eluted with chloroform in a very short column. The fractions were collected and concentrated under reduced pressure. Thin layer chromatography was again carried out on the samples in different solvent systems to ascertain their purity. Melting point was also determined on the solids. Solubility test was carried out on all the isolated purified compounds. All the pure samples were dried in the desiccator before they were subjected to spectral analysis.

Isolation of compounds from *Anogeissus leiocarpus*

The n-BuOH fraction was concentrated and further dried in a desiccator to yield dark brown solids (1.02 g) which were investigated. The extract was chromatographed on a 230-400 mesh silica gel column using $CHCl_3$ containing increasing amounts of MeOH (0-100%). Seven fractions were collected (F1 to F7). Fraction 4 and 7 were further fractionated individually by preparative thin layer chromatography (PTLC) on silica gel F_{254} (Sigma) using $CHCl_3$: MeOH (3:2) as mobile phase. This afforded one major band in each case with retention factor (R_f) of 0.64 and 0.72 for fraction 4 and 7 respectively. The bands were scraped and after desorption in methanol and concentration gave compounds ME and BU after recrystallization in ethanol.

General method for acid hydrolysis (Rozario & Merina, 2012)

Each flavonoid glycoside (3.0 mg) was dissolved in hot aqueous MeOH and hydrolyzed (refluxed) with 2M HCl (5 mL) at 100 ^oC for about 2 h. The excess alcohol was distilled off in vacuo and the resulting aqueous solution was extracted with Et_2O. The aglycones were extracted with EtOAc.

Identification of sugar moiety:

The aqueous solution from the above was neutralized with $BaCO_3$ and filtered. An aliquot of this was cautiously neutralized with $NaHCO_3$ and the concentrated filtrate indicated the presence of rhamnose and glucose on PC using aniline hydrogen phthalate as spray reagent. The identity of sugars was confirmed by Co-PC with authentic samples of galactose, rhamnose and glucose. Determination of IR, UV and NMR spectral analysis on portions of each isolated compound gave

the following results: Compound ME: mp. 314-316 ^{0}C; λmax. 272 nm; IR (KBr) cm^{-1}: 3236.34, 2953.48, 1722.49, 1610.54, 1504.54, 1442.77, 1184.05, 1105.70, 1051.12, 920.72, 876.60, 834.08, 756.11; ^{1}H-NMR (500 MHz, DMSO) δ 9.30, 7.50, 7.0, 6.40, 5.30, 4.50, 1.12; ^{13}C-NMR (125 MHz, DMSO) δ 160, 149, 140, 138, 114, 110, 108. Compound BU: IR (KBr) cm^{-1}: 3258.96, 2946.86, 2832.35, 1723.76, 1611.05, 1505.65, 1442.88, 1183.67, 1106.64, 1032.82, 1023.96, 920.73, 876.63, 756.04; ^{1}H-NMR (500 MHz, DMSO) δ 7.50, 7.0, 6.30-6, 6.73, 5.15, 3.0-3.8; ^{13}C- NMR (125 MHz, DMSO) δ 160, 149, 140, 138, 114, 110, 108.

Identification of the aglycone

The aglycon was subjected to thin layer chromatography using the solvent mixture of n-butanol, acetic acid and water (4: 1: 5, upper layer v/v). The developed plates were air dried and visualized under UV light which showed fluorescent spots that coincided with the standard sample of quercetin (blue, Rf 0.80). The plate was then exposed to ammonia vapours to observe the colour of spots (quercetin deep yellow) and another plate placed in a chamber saturated with iodine vapours to observe the colour of spots (yellow brown). The developed plates were sprayed with 5% ethanolic ferric chloride solution to observe the colour of the spots.

Statistical Analysis

All experimental measurements on antioxidant properties and antibacterial activities were carried out in triplicate and expressed as average of three analyses ± standard deviation. However, five measurements were considered for wound healing properties and statistical comparison of data was performed using

descriptive statistics and Analysis of Variance (ANOVA)-repeated measures. All levels of significance were set at $p < 0.05$.

CHAPTER FOUR

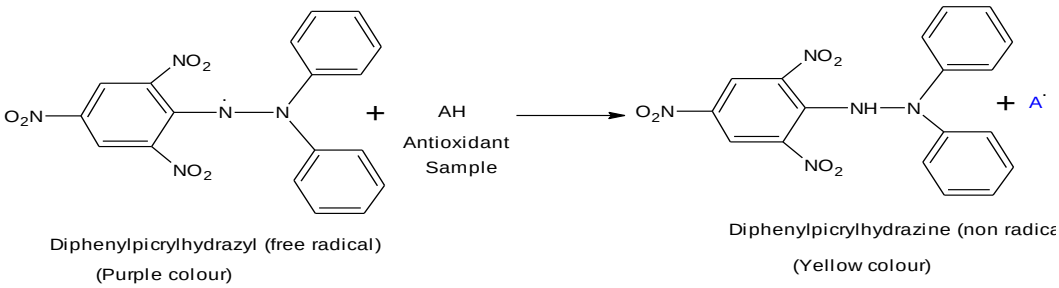
RESULT AND DISCUSSION

In this chapter the experimental results or findings of the study: antioxidant potentials, structural elucidation of the isolated compounds, ethnobotanical survey, phytoconstituents of the selected leafy vegetables, (presented in tabular and graphical forms), are thoroughly analyzed and discussed. The chapter also considers the discussion on wound healing potential and antibacterial activities of the extracts of the selected plants.

Determination of Antioxidant activity

Antioxidants can be evaluated by many methods however; one single method often gives inconclusive results. Therefore, assessment of antioxidants using more than one method is suggested to give more consistent results (Abu Bakar et al., 2014). Consequently, three main different methods were employed in this study. Additionally, Total phenolic and total flavonoid contents were carried out as quantitative measures to further support the antioxidative properties.

Antioxidant reacts with 1, 1-diphenyl-2-picryl hydrazyl radical **(**DPPH), which is a nitrogen-centered radical with a characteristic absorption at 517 nm and converts it to 1,1-diphenyl-2-picryl hydrazine ($DPPH_2$), due to its hydrogen accepting ability at a very rapid rate. This is illustrated in the reaction scheme below.



The degree of discoloration indicates the scavenging potentials of the antioxidant. The bleaching of DPPH absorption is representative of the capacity of tested compounds to scavenge free radicals independently from any enzymatic activity. DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals. Hence, DPPH spectrophotometric assay method is employed for the investigation of antioxidant activities of natural compounds (Sreedhar et al., 2010). In this study, the percentage antioxidant activity of all plant extracts in DPPH was increased in a concentration dependent manner and this compares favorably with the ascorbic acid standard in similar fashion. The DPPH result recorded in Table 22 therefore indicates that all the plants studied possess antioxidant activity although lower in potency compared with ascorbic acid. This suggests that the plant extracts contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. However, the plants exhibited markedly different antioxidant abilities.

The highest scavenging ability was found in *A. leiocarpus* (95.86 ± 0.1) followed by *C. olitorius* (94.19 ± 0.06) while *A. spinosus* recorded the least value (40.87 ± 2.5).

Table 22: DPPH free radical scavenging activity, Total phenolic content and flavonoid content of Methanol crude extracts

Sample	Inhibition (I %)			Phenolic Content (mg GAE/g extract).	Flavonoid content (mg QE/g extract).
	200ppm	400ppm	800ppm		
A. Spinosus	40.87± 2.5	41.71± 0.5	46.57 ± 1.9	48.01 ± 2.0	63.16 ± 11
S. Mombin	86.77± 0.1	88.80± 1.7	90.12 ± 1.2	698.12 ± 6.4	328.28 ± 24
C. olitorius	88.85± 0.1	93.56± 0.1	94.19±0.06	477.5 ± 6.2	450.22 ± 25
A. leiocarpus	91.15± 1.7	91.39± 0.9	95.86 ± 0.1	1294.81± 3.0	330.72 ± 29
M. oppositifolius	89.11± 0.3	90.07± 0.5	91.36 ± 0.6	540.67 ± 13	75.70 ± 3.5
C. dolichopetalum	49.46± 3.2	86.18± 2.1	89.64 ± 1.0	219.15 ± 2.9	574.8 ± 1.7
Ascorbic acid	92.75± 0.2	93.61± 9.4	96.78 ± 1.9	-	-

The reducing power assay serves as a significant indicator of potential antioxidant activity. The FRAP assay was used to estimate the reducing capacity of the plant extracts, according to the method of Oyaizu. From the FRAP assay, absorbance increased with increasing concentration of plant extracts. All the samples increased their reducing ability when the concentration of extracts was increased. This signified the consistent reduction of Fe^{3+} to Fe^{2+} indicating the reduction potential of the plants. However, the reducing power of all extracts was

significantly lower than the synthetic antioxidant; ascorbic acid. The reducing power was highest in *A. leiocarpus* followed by *S. monbin*; while *A. spinosus* showed the least reducing power (figure 29).

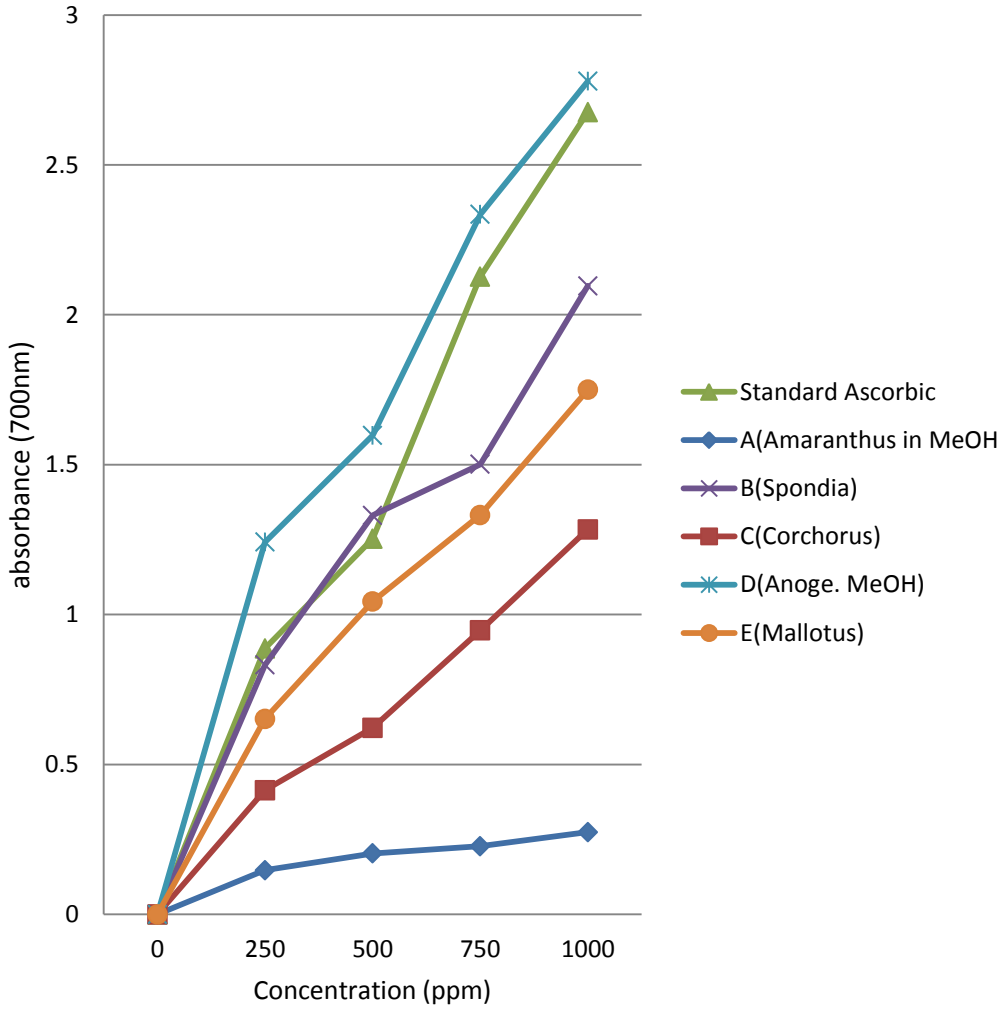


Figure 29: Ferric reducing power of all plant extracts compared with ascorbic acid as standard.

In the quantitative analysis, A. *leiocarpus* was found to have exhibited the highest phenolic content while *A. spinosus* recorded the lowest phenol and flavonoid content. The flavonoid content of the extracts in terms of quercetin equivalent (the

standard curve equation: y =0.0092x + 0.0249, R^2 = 0.985) were between 63.16 ± 10.7 and 574.8 ± 1.7 mg g^{-1} for *A. spinosus* and *C. dolichopetalum* extracts respectively. Table 22 also shows the contents of total phenols that were measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: y = 0.00048x + 0.0055, R^2= 0.9873). The total phenol content arranged in increasing order is as follows: 48.01 ± 2.0, 219.15 ± 2.9, 477.5 ± 6.2, 540.67 ± 12.6, 698.12 ± 6.4 to 1294.81 ± 3.0 mg g^{-1} for *A. spinosus*, *C. dolichopetalum*, *C. olitorius*, *M. oppositifolius*, *S. monbin* and *A. leiocarpus* extracts respectively. From the results obtained the phenolic content generally varied from one plant to the other in the same fashion as the reducing ability and the percentage inhibition of DPPH. The moderate positive linear correlation (R^2 = 0.52) between DPPH radical scavenging and total phenolic content of all the plant extracts supported this. This strengthens the fact that phenolic content of plants could contribute directly to their antioxidant properties. The DPPH scavenging capacity of the plant extracts may therefore be partially related to the phenolic compounds present. This result is in agreement with similar reports cited in literature that the antioxidant activity of plants might be due to their phenolic compounds (Cai et al., 2004; Katalinic et al., 2006; Kumbhare et al., 2012). However, this result is in disagreement with other studies that did not find any significant correlation between the antioxidant capacity and the total phenolic content (Marwah et al., 2007). Similarly, there was very low positive linear rcorrelation (R^2 = 0.24) between phenolic content and flavonoid content. Other phytoconstituents such as amino acids, alkaloids and carotenoids which are

possibly present in the plants could therefore contribute to the overall antioxidant activity. The antioxidant values recorded for all the plants were not significantly different from the standard antioxidant (ascorbic acid) except for *A. spinosus*. The plant samples thus can be said to be relatively good sources of antioxidant compounds. A statement of fact can be made that all the plant extracts studied demonstrated good antioxidant activity and could be suggested as useful in maintaining health and preventing degenerative diseases such as cancer, diabetes, coronary heart disease that are exacerbated by the generation of Reactive Oxygen Species (ROS) in the body. All the plants are therefore potential sources of free-radical scavenger substances such as phenolic compounds, carotenoids, vitamins and nitrogenated compounds, all of which are useful antioxidant compounds.

Total Antioxidant Activity (TAC) was measured, based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex. The total antioxidant capacity which is a measure of both water-soluble and fat-soluble antioxidants was measured on the methanol, n-butanol and ethyl acetate extracts which readily gave positive results for phenolic compounds. TAC results obtained on *A. leiocarpus*, *S. monbin* and *C. dolichopetalum* were used to establish any correlation between DPPH scavenging activity and TAC. Figure 30, 31, 32, 33, 34 and 35 show both DPPH and TAC results on the various solvent extracts of the three plants. The TAC results followed a similar concentration dependent fashion just as it was recorded on DPPH.

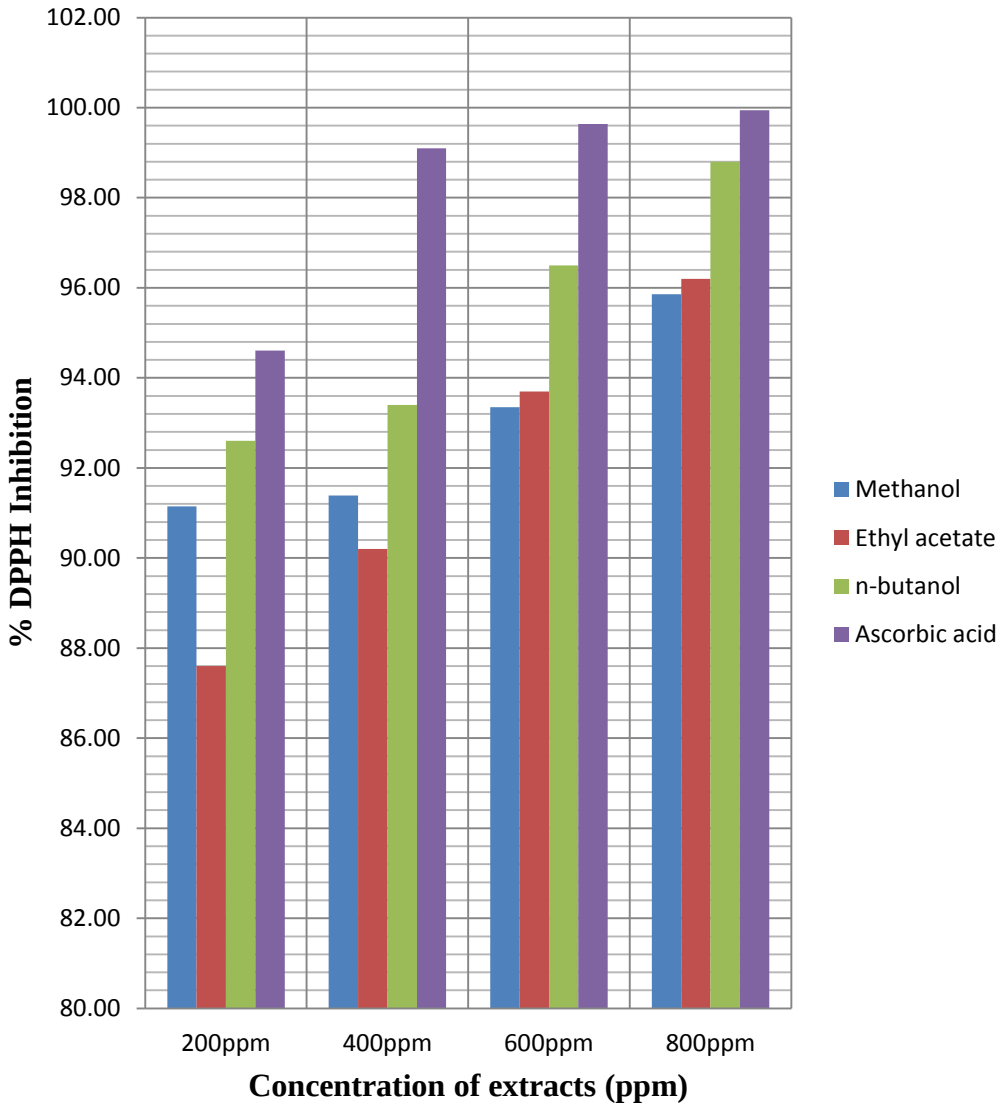


Figure 30: DPPH % inhibition for various solvent extracts of *A. leiocarpus*.

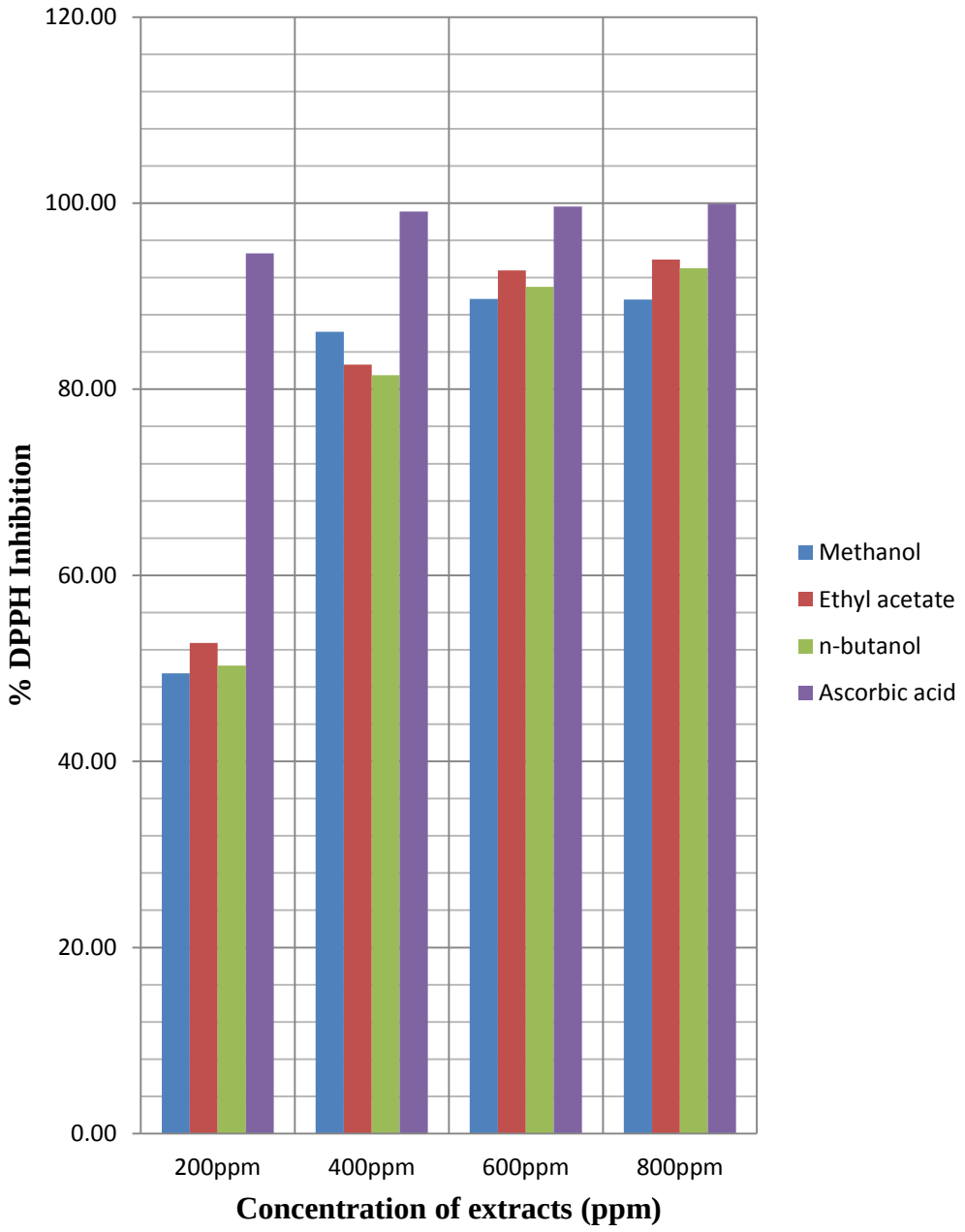


Figure 31: DPPH % inhibition for various solvent extract of *C. dolichopetalum.*

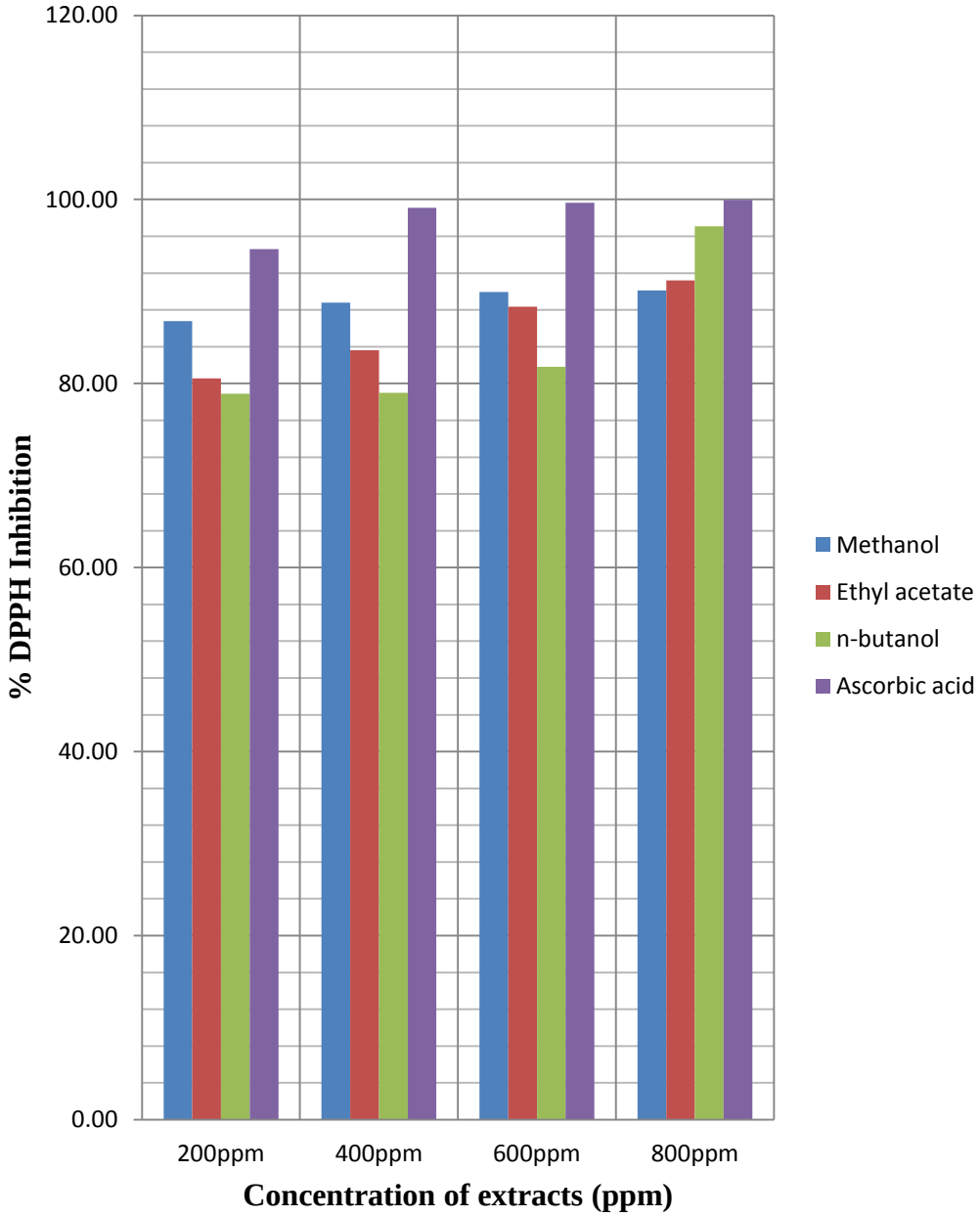


Figure 32: DPPH % inhibition for various solvent extracts of *S. monbin.*

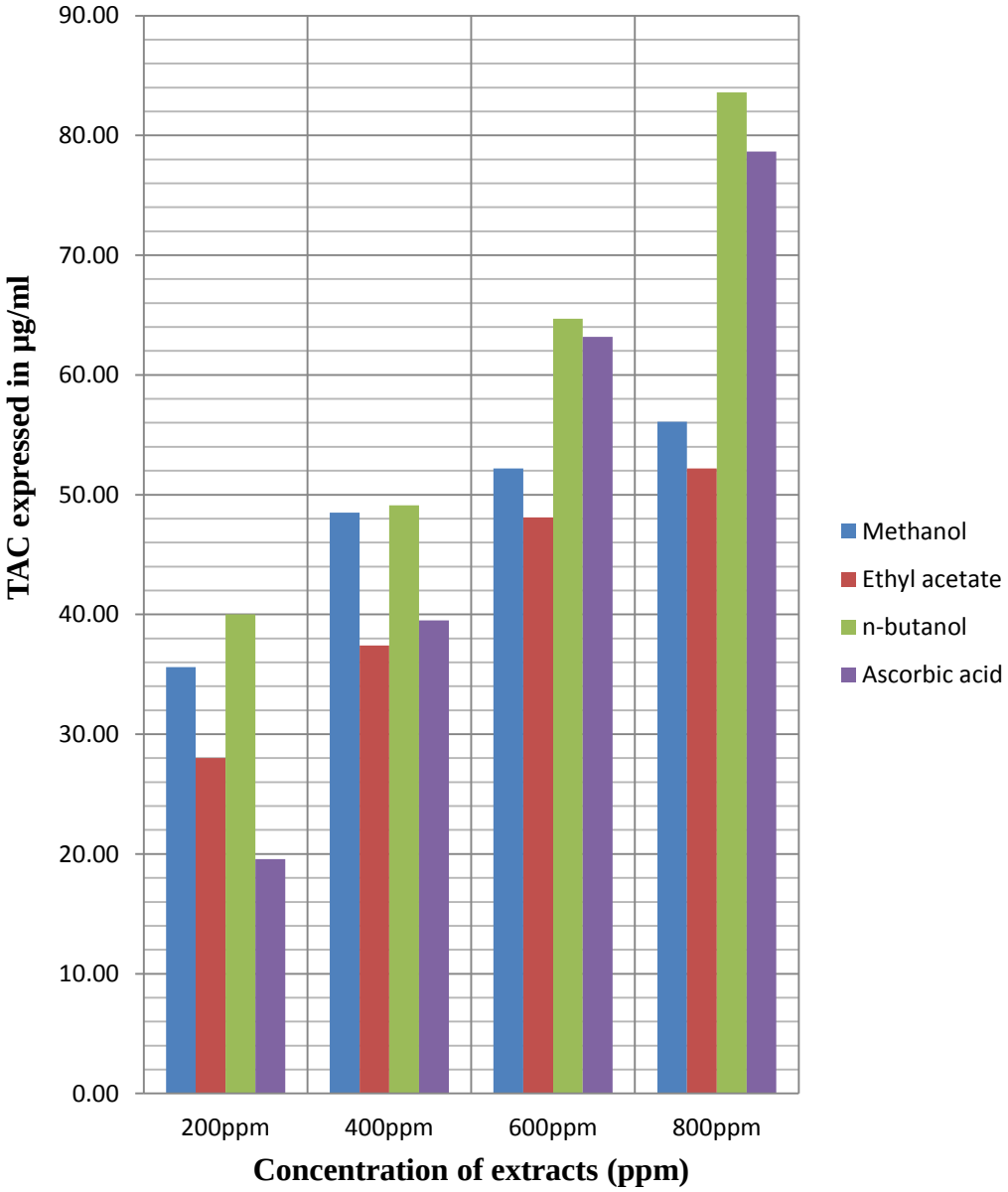


Figure 33: TAC for various solvent extracts of *A. leiocarpus.*

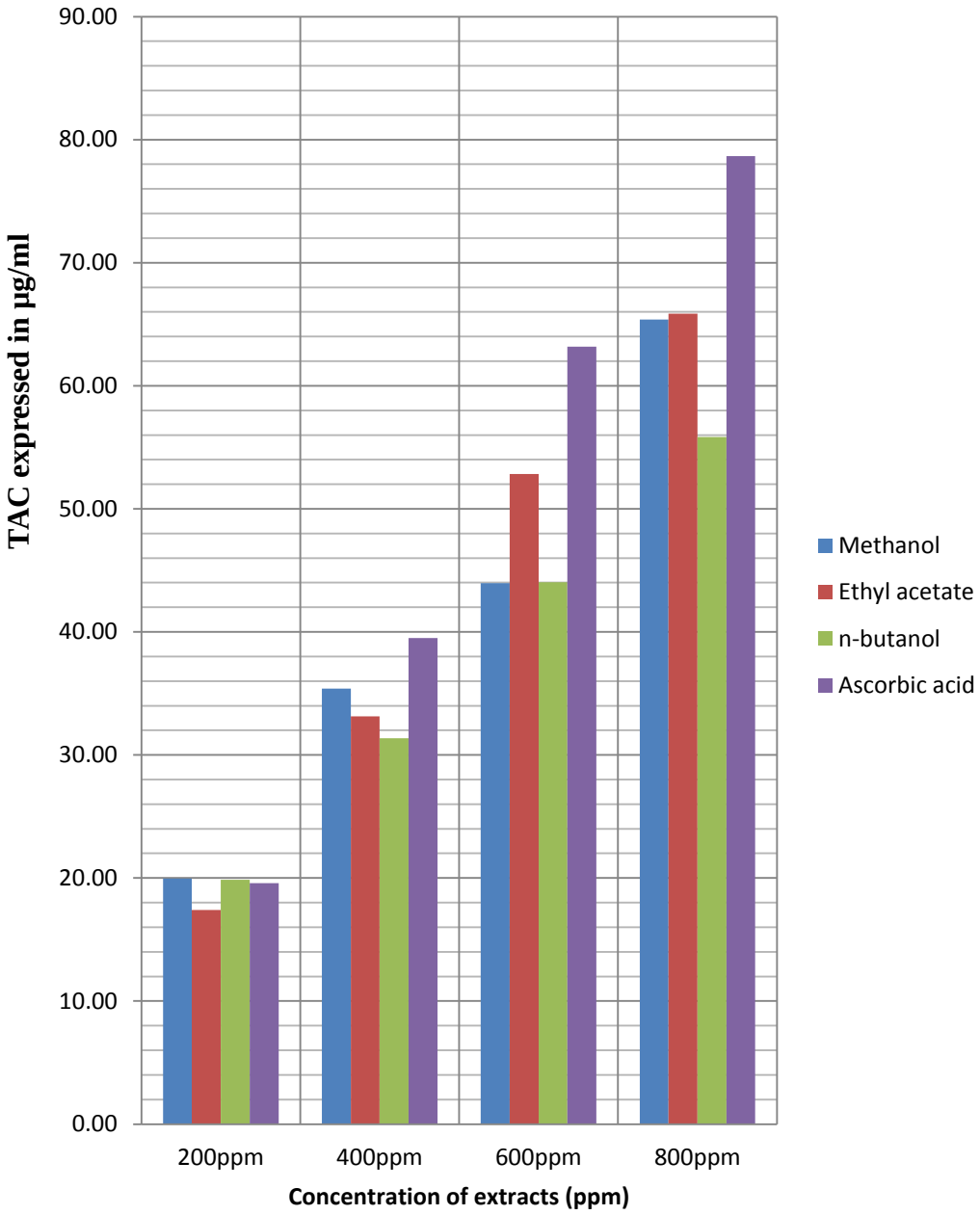


Figure 34: TAC for various solvent extracts of *C. dolichopetalum.*

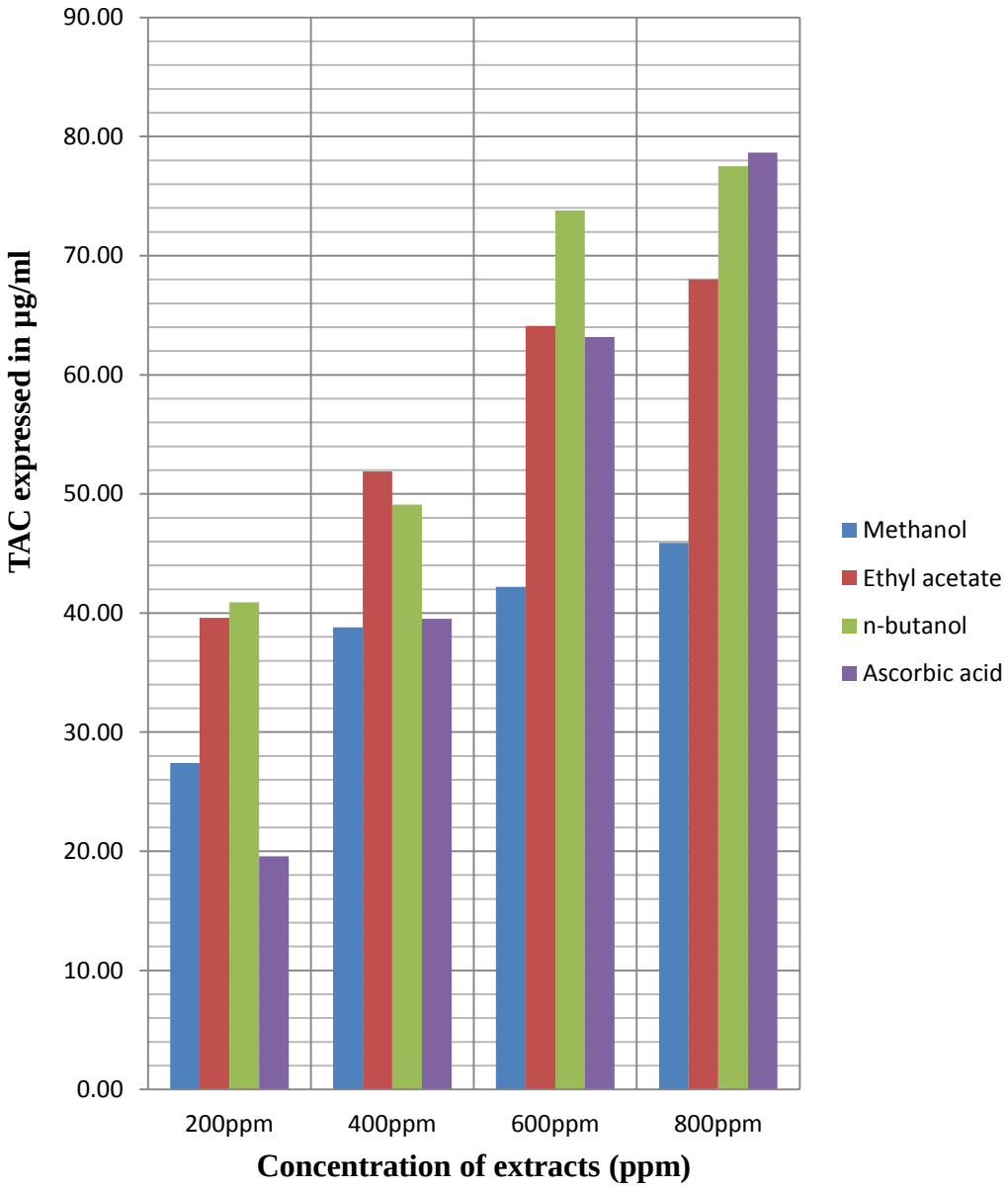
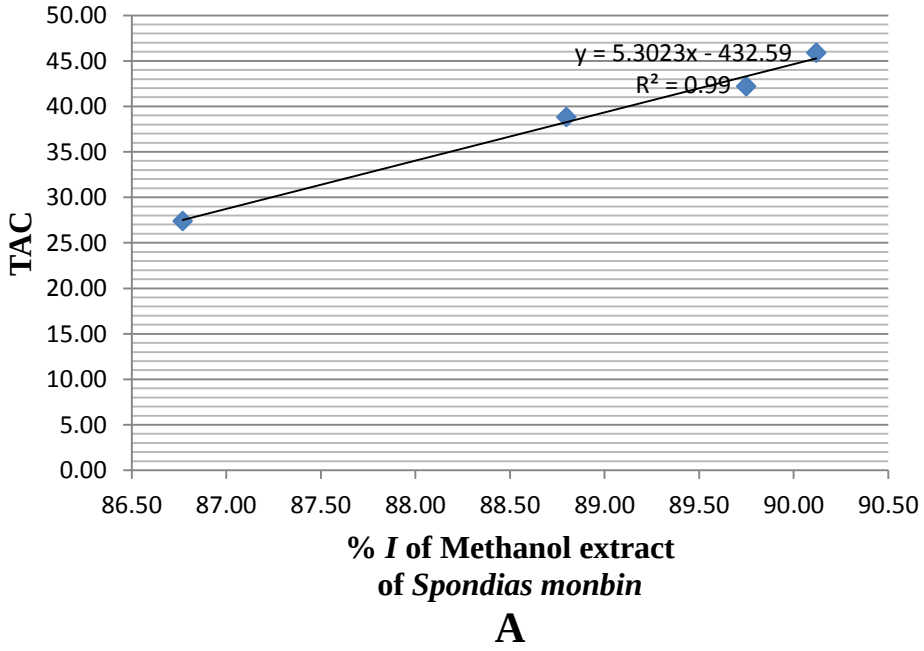


Figure 35: TAC for various solvent extracts of *S. monbin.*

Figure 36 shows the correlation analysis between DPPH and TAC for the plants. The analysis showed a high positively linear correlation for both methanol (R^2=0.990) and n-butanol (R^2 = 0.8013) extracts of *S. monbin* and *C. dolichopetalum* respectively. The existence of this high positive correlation did

not only confirm the presence of antioxidants but indicated the viability of the two models for evaluating antioxidants from medicinal plants.



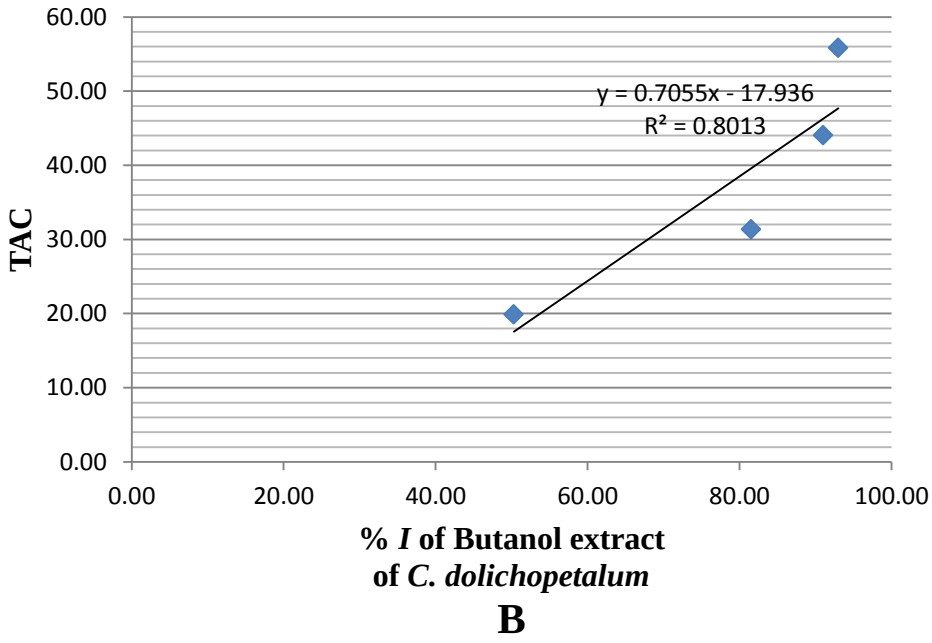


Figure 36: Correlation between DPPH and TAC.

The results obtained from these two methods of estimation of antioxidants indicated the high potentials of methanol, n-butanol and ethyl acetate as solvents for extraction of antioxidant compounds. However, the results showed differential abilities among the solvents to extract the antioxidant compounds and this ability is plant specific. For instance, while n-butanol recorded the highest antioxidant yield in both *S. monbins* and *A. leiocarpus* for both DPPH and TAC, ethyl acetate recorded the highest in *C. dolichopetalum* for both methods. The result can be compared to similar results found in literature that showed that extraction solvents had significant impacts on antioxidant activity estimation, as well as different extraction capacity and selectivity for free phenolic compounds (Zhao et al., 2006; Zhou & Yu, 2004). However, one can say that even though the choice of solvent to extract antioxidant compounds is important, the plant species is equally important to consider.

Structural identification of the isolated compounds

The methanol extracts of all the selected plants were subjected to phytochemical screening. The screening showed the presence of large number of compounds. The methanolic extract of all plants was fractionated with petroleum-ether, chloroform, ethyl acetate and n-butanol. Cynidin test carried out on these extracts indicated that the maximum quantities of phenolic compounds were present in the n-butanol extracts of *C. dolichopetalum,* and *A. leiocarpus.* Thus the n-butanol fractions were considered for further analysis to characterize the flavonoids. The *A. leiocarpus* n-butanol extract was pre-adsorbed with silica gel and applied onto the top of a column prepared by silica gel (500 g) in $CHCl_3$. The

elution was started with $CHCl_3$, continued with increasing amounts of MeOH (0-30%) and followed up with EtOAc and ended up with MeOH. Elution with EtOAc and MeOH afforded the isolation of compounds BU and ME respectively from the leaves of *A. leiocarpus* after several preparative thin layer chromatographies (PTLC).

Structural elucidation of the compounds

Structure elucidations of the isolated compounds were carried out by UV, FTIR and 1D- NMR spectral techniques.

Quercetin rhamnoglucosyl: Compound ME was identified as quercetin rhamnoglucosyl. It was obtained as a yellow amorphous powder (methanol), mp. 314-316 ^{0}C; λmax. 272 nm; IR (KBr) cm^{-1}: 3236.34, 2953.48, 1722.49, 1610.54, 1504.54, 1442.77, 1184.05, 1105.70, 1051.12, 920.72, 876.60, 834.08, 756.11; ^{1}H-NMR spectral data (500 MHz, DMSO) aglycone δ 9.30 (1H, *brs*, 7-OH), 7.50 (2H, *d*, *J*= 8.5 Hz, H-2', 6'), 7.0 (1H, *d*, *J*= 8.5 Hz, H-5'), 6.40 (2H, *d*, *J*= 2.2 Hz, H-8, H-6); sugar moiety δ 5.30 (1H, *d*, *J*= 7.2 Hz, H-1''), 4.50 (1H, *dd*, *J*= 7.2 Hz, H-2''), 1.12 (3H, d, *J*=6.3-6.1 Hz). ^{13}C-NMR spectral data (125 MHz, DMSO) aglycone δ 160 (*s*, C-4), 149 (*s*, C-4'), 140 (*s*, C-5), 138 (*s*, C-9), 114 (*d*, C-5`), 110 (*s*, C-10), sugar moiety 108 (*d*, C-1``).

The identity of the isolated compound was done chemically by Mg-HCl (Shinoda test) and Molisch test. There was formation of red colour by Shinoda test and purple colour in Molisch test. A blue color was formed with $FeCl_3$. A spot of ME on thin layer chromatogram appeared yellow in daylight and dark

brown in ultraviolet light. These tests identified the compound as flavonoid glycoside.

Acid hydrolysis yielded a yellow amorphous powder aglycone which was identified to be quercetin on a thin layer chromatogram compared with authentic quercetin sample. When the developed plates were sprayed with 5% ethanolic ferric chloride solution it showed spots which coincided with that of the reference quercetin (bluish grey) when plates were placed in a chamber saturated with ammonia vapours, it also showed deep yellow colour of quercetin. R_f value (0.80) of quercetin isolated from the samples coincided with the R_f value of standard quercetin. The plates developed under UV light showed fluorescent spots coinciding with the standard sample of quercetin.

Similarly, paper chromatography on the residue from the acid hydrolysis alongside authentic samples of glucose, rhamnose, and galactose indicated the presence of rhamnose and glucose as the component sugars in ME. The compound was therefore suggested to be quercetin rhamnoglucoside.

The UV spectrum of the isolated compound ME was recorded in methanol (MeOH) (Appendix F) and shift reagents were also used. Table 23 shows UV absorption maxima (λ max) of the compound ME in methanol and after the addition of shift reagents. In the UV region of spectrum two major absorption maxima were also typically observed for the flavonoid structure. The first absorption maximum, observed at 272 nm (band II) can be considered as originating from π- π* transitions in the A ring, a benzene system, and the second

absorption maximum, observed at 367 nm, may be assigned to transitions in the B ring, a cinnamoyl system. In consistent with the result for the chemical tests, the isolated compound showed typical characteristic absorption spectra for flavonol. This is because the ultraviolet-visible absorption spectra of the isolated compound, ME (appendix F) when compared with ultraviolet-visible absorption spectra of the different flavonoid types in figure 13 suggested a flavonol structure. More also, the absorption maxima recorded were within the ranges 250-280nm for band II and 330-370nm for band I which are typical characteristic absorption ranges for 3-OH substituted flavonol as indicated in Table 8. The bathochromic shift of 33 nm in band I in the presence of $AlCl_3$/HCl indicates the presence of free 5- hydroxyl group. Addition of sodium methoxide (NaOMe) to the methanol solution of the isolated compound resulted in a bathochromic shift of 40 nm with an increase in intensity of absorption in band I, indicating the presence of free hydroxyl groupat C-4' on the isolated compound. A bathochromic shift of 34 nm in band II upon addition of sodium acetate (NaOAc) indicates the presence of free 7-hydroxyl with 5-hydroxyl groups. Addition of NaOAc/H_3BO_3 to the methanol solution of the isolated compound resulted in a bathochromic shift of 26 nm indicating the presence of ortho-dihydroxyl group in the B-ring. The bathochromic shift of 45 nm in band I region upon the addition of $AlCl_3$, indicated the presence of B-ring *o*-diOH substitution. This is as a result of the formation of acid-labile complexes with ortho-dihydroxyl groups (Julkunen-Tiitto et al., 2014). Based on these UV spectra, the isolated compound appeared to be a 3-substituted-3', 4', 5, 7-flavonol.

Table 23: **UV absorption maxima (λ max) of compound ME in methanol and after the addition of shift reagents**

Shift Reagent	**MeOH**	**NaOMe**	**$AlCl_3$**	**$AlCl_3$/HCl**	**NaOAc**	**NaOAc/H_3BO_3**
Maximum absorbance (λ max)	272, 367	407	301, 352, 412	278, 354, 400	306, 391	305, 393

The IR spectrum showed characteristic absorption bands of 3236.34 cm^{-1} for O-H stretching (aromatic), 2953.48 cm^{-1} for C-H stretching (aliphatic), 1722. 49 cm^{-1} for C=O stretching which shifted to a larger wavelength probably due to substitution at C-3 and C-4' or C-7, 1611.05 cm^{-1} and 1505.65 cm^{-1} indicating the presence of C=C (aromatic). The strong band at 834.08 cm^{-1} is as a result of substitution at C-4' which presents the B-ring as a p-disubstituted benzene ring. The absorption bands between 1184.05-1051 cm^{-1} indicate the presence of CO groups. In the ^{1}H-NMR spectrum (500MHz, DMSO) of the glycoside, the signal appearing at δ 9.0-10.0ppm is due to the free hydroxyl proton at C-7. The doublet appearing in the region of δ 7.6ppm (d, J=8Hz) and δ 7.4ppm corresponds to the proton at C-2' and C-6', while the proton of C-5' appeared centred at δ 7.0ppm. The signal appearing at δ 6.4ppm (d, J=2.2Hz) corresponds to C-8, C-6 protons. This suggested a meta coupling pattern of A-ring. A 5, 7-dihydroxy system is therefore suggested. The ^{1}H and ^{13}C-NMR of ME exhibited two sugar anomeric protons at δ 5.30 (1H, *d, J*= 7.2 Hz,) due to glucose and δ 4.5 (1H, *d, J*= 7.2 Hz,)

for rhamnose and carbons at δ 65.0 and δ 108.00, respectively. A doublet at 5.3 (J= 7.2 Hz) indicated anomeric signal with other signals resonating between 3.0-4.5ppm for the sugar moities. The compound **ME** showed a doublet at high field δ 1.12 (3H, d) in the ^{1}H-NMR spectrum that is characteristic of a methyl group of rhamnose. The comparism of the obtained ^{1}H-NMR spectrum (appendix A) with those found in literature (Figure 37 & 38) confirmed the identity of ME as a rutin (figure 39).

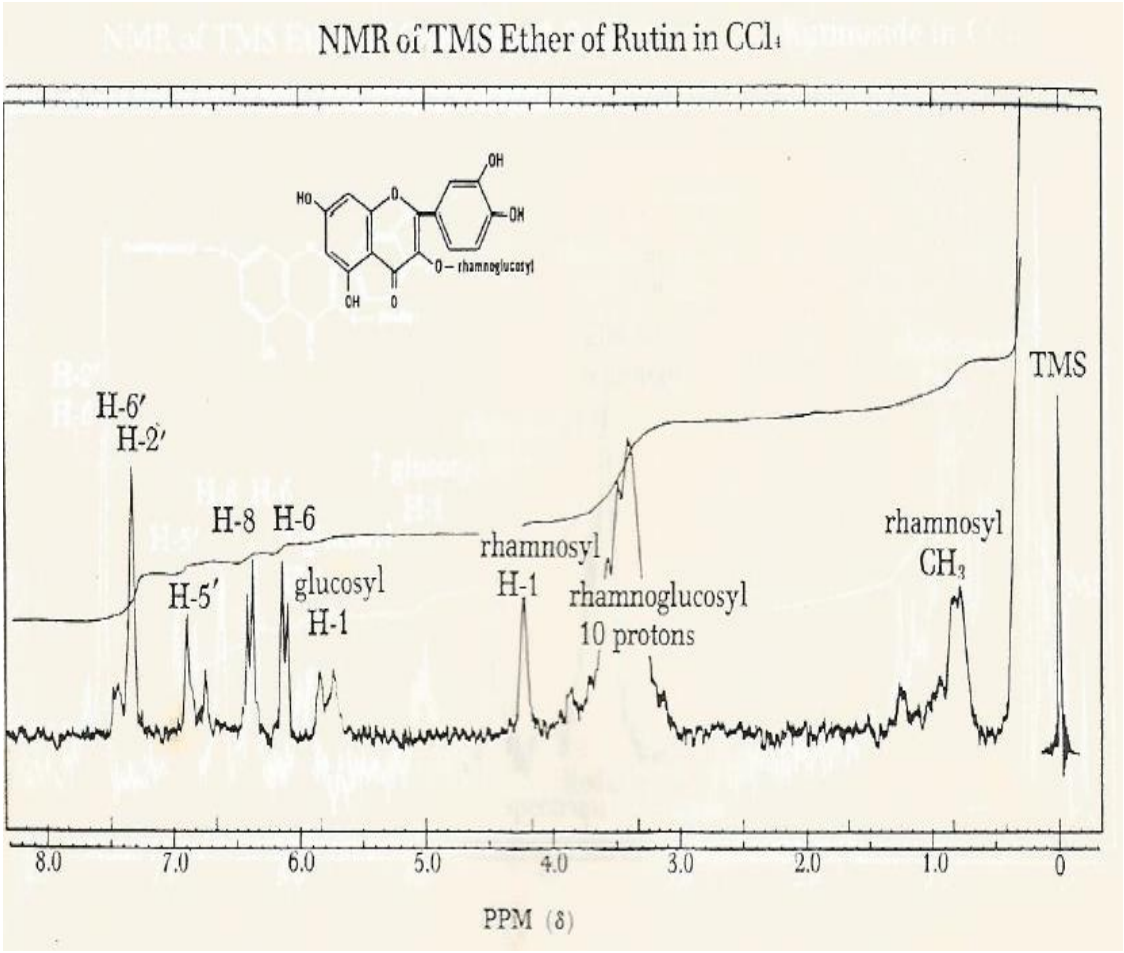


Figure 37: NMR of TMS Ether of Rutin (Harborne et al., 1975).

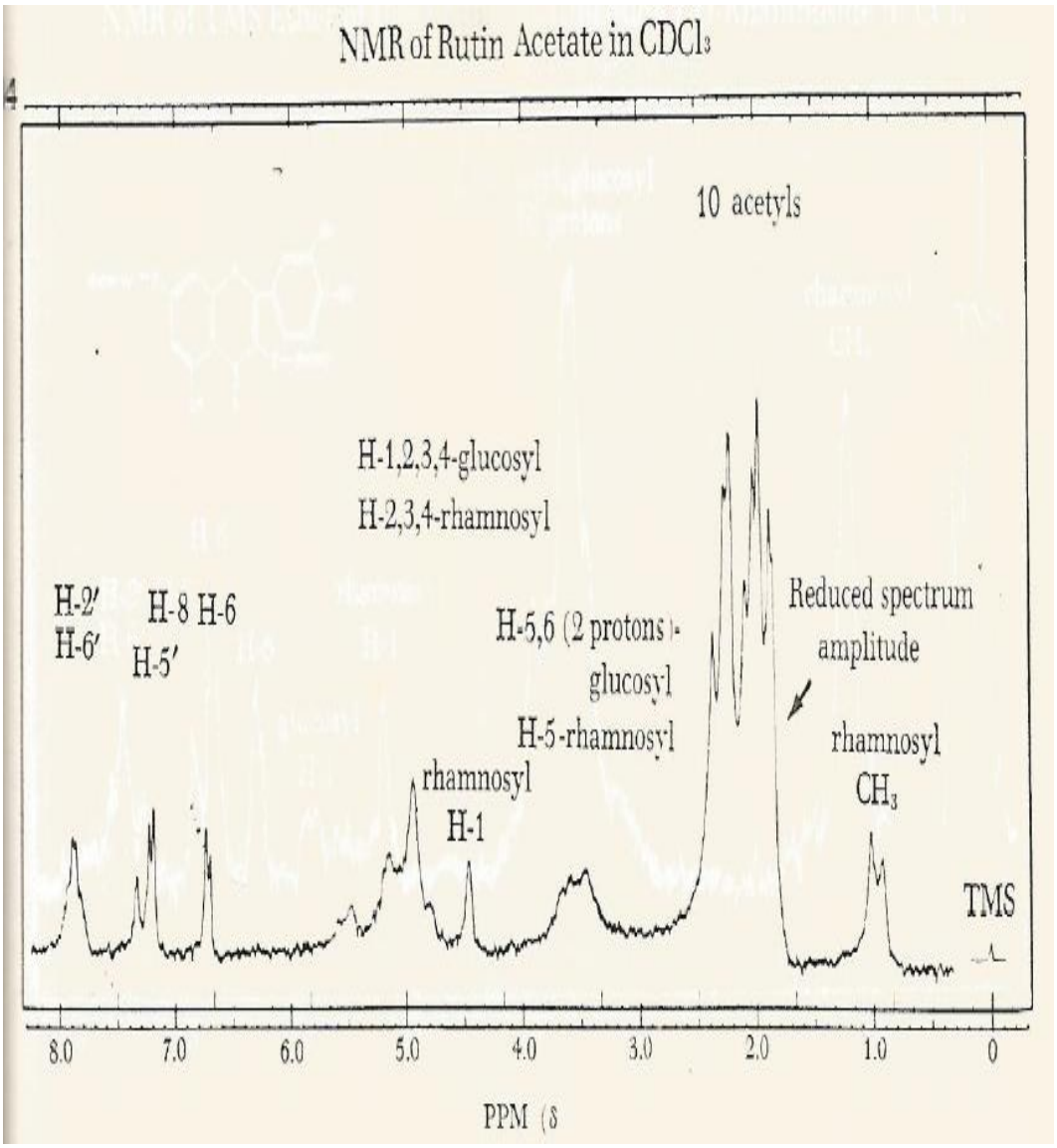


Figure 38: NMR of Rutin Acetate (Harborne et al., 1975).

In the ^{13}C-NMR spectrum, the downfield signal at 160 indicated a carbonyl group. The signals δ 110.0 and δ 108.0 observed in the ^{13}C-NMR spectrum confirmed the presence of the anomeric carbons for the compound.

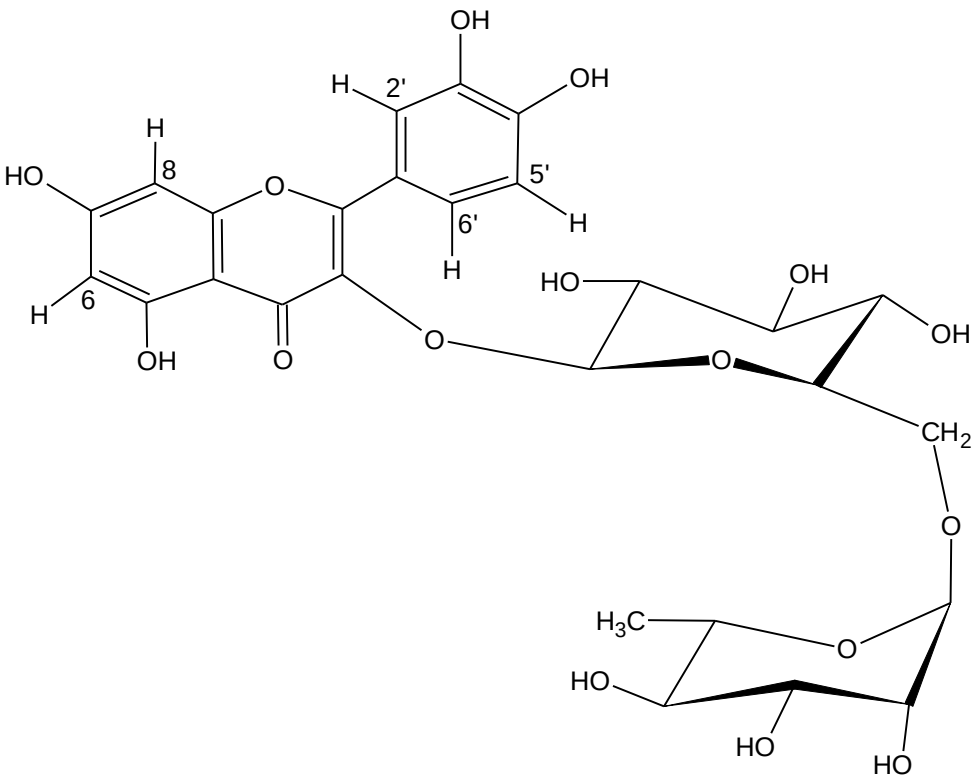


Figure 39: Proposed structure for the isolated compound ME

BU: Compound Bu was identified as an isoflavonoid glucoside. It was a yellow amorphous powder which gave a purple color under UV with no color change in NH_3. This suggested an isoflavone structure with a free 5-hydroxyl group. IR (KBr) cm^{-1}: 3258.96, 2946.86, 2832.35, 1723.76, 1611.05, 1505.65, 1442.88, 1183.67, 1106.64, 1032.82, 1023.96, 920.73, 876.63, 756.04; ^{1}H-NMR spectral data (500 MHz, DMSO) aglycone δ7.50 (1H, s, H-2), 7.0 (2H, *d*, *J*= 8.5 Hz, H-2', 6'), 6.30-6.40 (2H, *d*, *J*= 2.2 Hz, H-8, H-6), 6.73 (2H, d, H-5'); sugar moiety δ 5.15 (1H, *d*, *J*= 7.2 Hz, glucose H-1), 3.0-3.8 (m, sugar protons). ^{13}C-NMR spectral data (125 MHz, DMSO) aglycone d 160 (*s*, C-4), 149 (*s*, C-4'), 140 (*s*, C-5), 138 (*s*, C-9), 114 (*d*, C-3`,5`), 110 (*s*, C-10); sugar moiety 108 (*d*, C-1``)

The IR spectrum for compound BU also showed characteritics absorption bands at 32358.96 cm^{-1} for O-H stretching (aromatic), 2946.86 and 2832.35 for C-H stretching (aliphatic), 1723. 76 for C=O stretching. The ^{1}H-NMR spectrum of compound **BU**, (500 MHz, DMSO) revealed signals between δ 3.0 and at δ 7.5 ppm typical of an isoflavone type carbon skeleton. Thus, the ^{1}H-NMR spectrum showed a pair of doublets (*J*=10 Hz) centered at δ 6.30 and 6.40. The characteristic C-2 proton of the isoflavone skeleton was evident as a singlet at δ 7.50 (H-2, s, 1H). The ^{1}H-NMR spectrum also displayed a pair of doublets (*J*= 8.0 Hz), each integrating for two protons, at δ 6.73 and 7.0, which were assigned to the H-5′ and H-2′ & H-6′ of the *para*-disubstituted aromatic nucleus. The relatively upfield resonance (δ 6.73) of H-5′ suggested the presence of oxygenated substituents at C-3' and C-4′, probably as a hydroxyl group. The signals at 5.15 and at 4.80ppm indicated anomeric signal with another signal centered at 3.5ppm confirmed the presence of a sugar moiety. Acid hydrolysis of BU afforded sugar moiety that was identified by TLC with authentic sugars as glucose. On this basis and by comparison of these values with spectra found in literature (figure 40) and published data (Choi et al., 2010) the structural identity of compound **BU** was proposed (Figure 41).

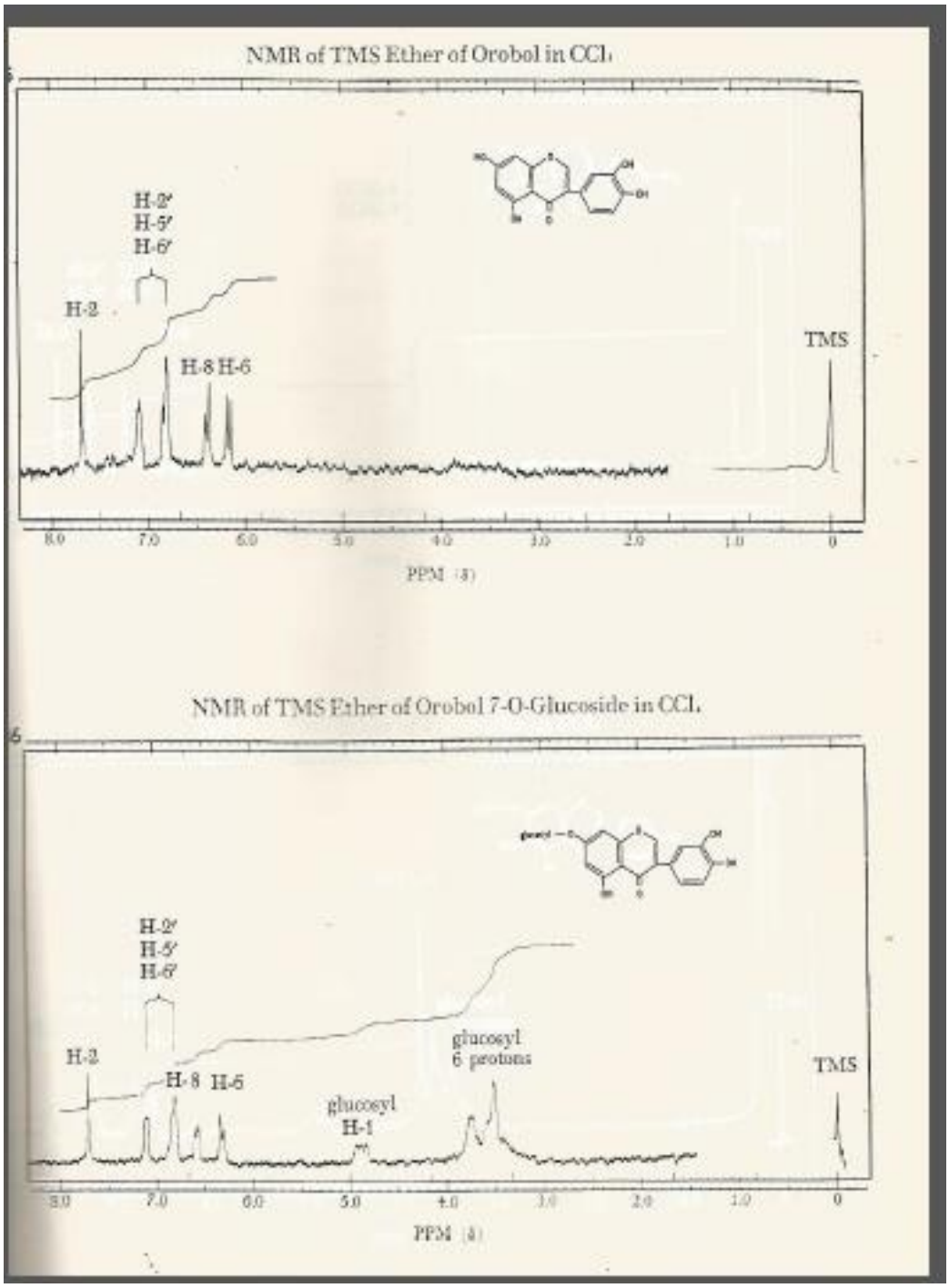


Figure 40: Spectra of isoflavonoid found in literature (Harborne et al., 1975).

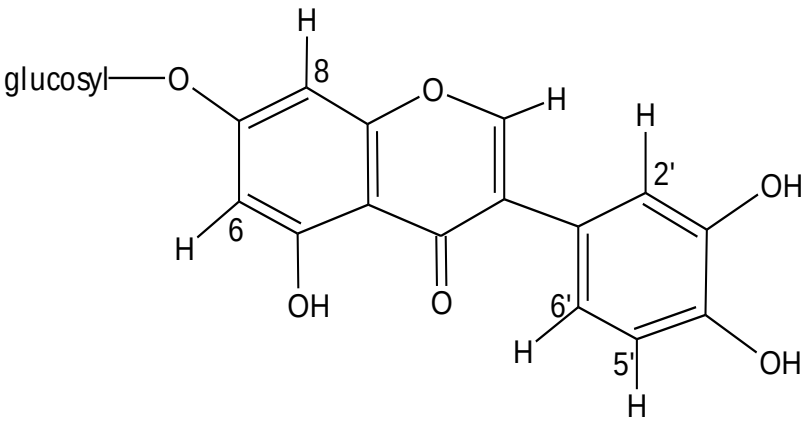


Figure 41: Proposed structure for BU

Ethnobotanical Survey

The survey was basically conducted to identify medicinal plants that are often used in folkloric treatment of wounds among the people of Kpando Traditional Area. The study reveals that different numbers of plants specie are used for treating wound diseases among the people of Kpando Traditional Area. Plant species belonging to 27 species and 20 families were identified as being used by most of the people of this area for wound treatment. Table 24 showed the list of the species identified in the study with their botanical names, local names and parts used. Some of these plants are cultivated by the people themselves while others grow in the wild. The medicinal plants showed family dominance, suggesting that some families are more important source of potential medicinal plant species than others.

Table 24: **Identity of Wound healing plants collected from the study area**

Plant	Growth form	Family	Local name	Part used
***Adiatum-capillus veneris* L.**	fern	Pteridaceae	Litsagbadze	Leaves
***Amaranthus spinosus* (L)**	Shrub	Amaranthaceae	Matonui	Root
***Anogeissus leiocarpus* (DC) Guill & Perr**	Woody tree	Combretaceae	Hehe	Leaves
***Azadiracta indica* A. Juss**	Woody tree	Meliaceae	Liliti tovi	Leaves
***Bridelia ferrugenia* Benth**	A small tree/Shrub	Euphorbiaceae	Huhoe	Bark/Leaves
***Calotropis procera* (Aiton) Dryand**	Herb/shrub	Asclepiadaceae	Wangatsi	Leaves
***Chromolaena ordorata* (L)**	Herb	Asteraceae	Acheampong	Leaves
Coffee arabica	shrub	Rubiaceae	Coffee	Leaves
***Colocasia esculenta* L.**	Vegetable	Araceae	Mankani	Tuber/Juice
***Combretum dolichopetalum* Engl. & Diels**	Climbing shrub	Combretaceae	Ahe	Leaves
***Corchorus olitorius* L.**	vegetable	Tiliaceae	Ademe/singli	Leaves
***Crescentia cujete* L.**	Tree	Bignoniaceae	Goti	Leaves
***Elaies guineensis* Jacq.**	Tree	Arecaceae/Palmaceae	De	Fronds/Leaves
***Heliotropium indicum* Linn.**	Shrub/Herb	Boraginaceae	Zeto	Leaves
***Jatropha curcas* L.**	Small tree/shrub	Euphorbiaceae	Kportikporti	Leaves/Root/ Juice
Justicia flava (Forssk.) Vahl	Straggling/Erect herb	Acanthaceae	Eli	Leaves

Table 24: Identity of Wound healing plants collected from the study area (Continued)

Plant	Growth form	Family name	Local name	Part used
***Mallotus oppositifolius* Geiseler Mull. Arg.**	Shrub	Euphorbiaceae	Nyeti	Leaves
Manihot esculanta Crantz	Shrub	Euphorbiaceae	Agbeli	Leaves/Tuber
***Milicia excelsia* (Welw.) C.C. Berg**	Woody tree	Moraceae	Odum	Stem bark /Leaves/Juice
***Mucuna sloanei* Fawc. & Rendle**	Climbing shrub	Fabeceae	Akploloe	Juice/Leaves
***Musa paradisiacal* L.**	Herb	Musaceae	Abladzo	Juice
***Ocimum gratissimum* L.**	Shrub	Lamiceae	Dzeveti	Leaves
***Phyllanthus fraternus* G.L. Webster**	Herb	Euphorbiaceac/ Phyllanthaceae	Kpavideme	Leaves
***Securinegea virosa* Roxb.ex wild.) Baill**	Shrub	Euphobiaceae/ Phyllanthaceae	Hlese	Leaves
***Sida acuta* Burm. F.**	Shrub	Malvaceae	Shosho	Leaves
***Spondia mombin* L.**	Woody tree	Anacardiaceae	Akuko	Leaves
***Vernonia colorata* (Willd.) Drake**	Shrub	Asteraceae/ Compositae	Pepedi/ Gbodukui	Leaves

Of the 20 different families which the people of this area consult for wound healing, the family Euphorbiaceae (30.0%) contributed most species (six) to the medicinal plant diversity in this study. This was followed by Asteraceae and Combretaceae (10.0%) each with (two) species while the remaining families Pteridaceae, Amaranthaceae, Asclepiadaceae, Fabaceae, Anacardiaceae Meliaceae, Rubiaceae, Tiliaceac, Areceae, Boraginaceae, Moraceae, Musaceae,

Lamiaceae, Malvaceae, Aracaceae, Bignoniaceae and Ancathaceae recorded one species each.

Ten plant species namely; *Adiatum veneris capillus* (Rutaceae), *Vernonia colorata* (asteraceae/compositae), *Combretum dolichopetalum* (Combretaceae), *Coffee arabica*. (Rubiaceae), *Milicia excelsia* (Moraceae), *Anogeissus leiocarpus* (Combretaceae), *Spondias mombin* (Anacardiaceae), *Bridelia ferruginea* (Euphorbiaceae), *Amaranthus spinosus* (Amaranthaceae) and *Corchorus olitorius* (Tiliaceae) have not previously been documented for the treatment of wound in Ghana.

This study has revealed that traditional medical practices have a wide acceptability among the Kpando people probably because they believe in its effectiveness and also due to the lack of access to modern health care delivery system (only one hospital and a clinic all situated at the district capital). It was interesting and revealing to have listened to educated people in the area who prefer using the plants as their first choice of medication to treat wounds. Also the unavailability and the financial constraints by many to purchase orthodox drugs may also have contributed largely to preference of traditional medicine over the orthodox drugs. Almost all the medicinal plants (95%) are harvested from immediate surroundings. Knowledge of the use of plants as medicines does not only remain with the older generation but extends to the youth who show much interest. Majority still depends on the use of plants for wound healing as the first choice of medication.

The study revealed diversity in plant parts used for the treatment of wounds. Figure 42 shows the percentage growth forms (A) and percentage plant parts of species identified in the study. Table 24 identifies the parts of plant species that are used for wound healing. These range from leaves, roots, stem bark, juice to tubers only, or a combination of two or more in a species or with those of other species. However, the leaf was the most commonly used plant part (68.6%). This is consistent with other studies (Addo-Fordjour et al., 2008) where leaves were the most commonly harvested parts of medicinal plants used to prepare herbal medicine. Shrubs (44.4%) and trees (29.6%) were the most predominant growth forms harvested for wound healing while grinding the plant part to form poultice was the commonest mode of traditional drug preparation.

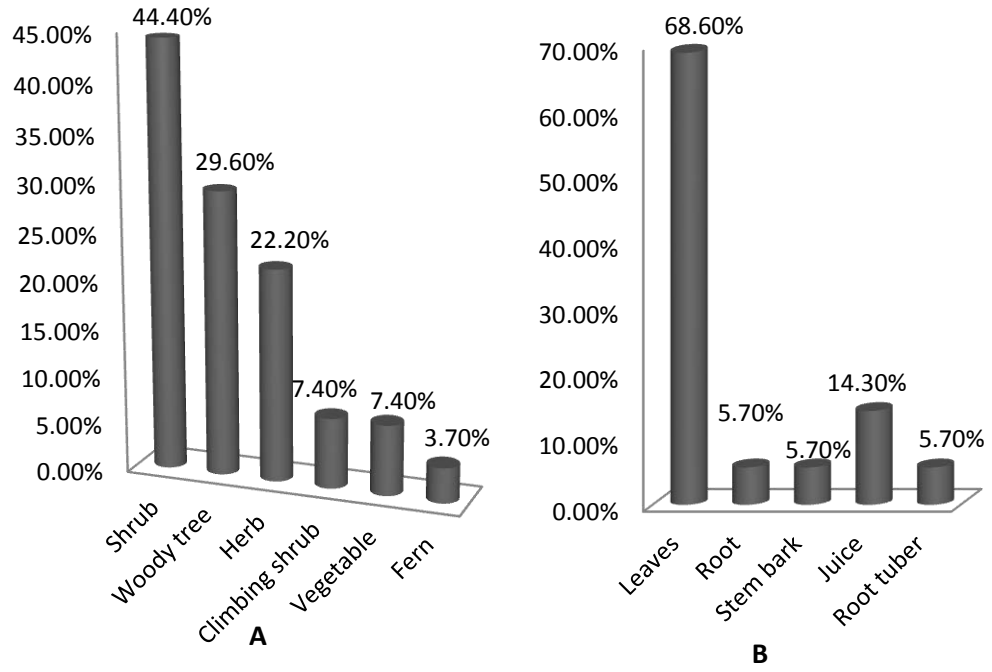


Figure 42: The percentage occurrence of (A) growth forms and (B) part of plant species identified in the study.

Table 25: List of plants with their mechanism of actions and mode of preparation and administration

Plant Name	Voucher No.	Mechanism of action	Mode of administration
Adiatum veneris-capillus	---	Causes healing	Grind and add to wound
Amaranthus spinosus	UCC/H/5165	Causes healing	Grind and add to wound
Anogeissus leiocarpus	UCC/H/267	Causes healing	Grind or squeeze tender fresh leaves and apply to wound or boil leaf/bark and dip affected part in it
Azadirachta indica	-----	Prevent tetanus and causes healing	Grind and add to wound
Bridelia Ferruginia	UCC/H/3284	Causes healing	Bark grind and add to wound or boil leaf and dip site in it
Calotropis procera	UCC/H/3053	Causes healing	Grind and add to wound
Chromoleana odorata	UCC/H/3277	Causes healing/arrest bleeding	Grind or squeeze juice and add to wound
Coffee arabica	-----	Causes healing	Grind with salt and tie to wound
Colocasia esculenta	-----	Arrest bleeding	Add juice to wound
Combretum dolichopetalum	UCC/H/308	Causes healing	Grind with coffee leaves and add to affected part
Corchorus olitorius	UCC/H 306	Causes healing	Grind and add to wound
Crescentia cujete	UCC/H/4353	Causes healing	Grind and add to wound
Elaeis gueneensis	UCC/H/4617	Arrest bleeding	Grind and add to wound
Heliotropium indicum	UCC/H/4873	Cleans surface of wound , closes up wound	Expose leaf to fire and cover the surface of wound/leaf expose to fire, squeeze out juice into wound
Jatropha curcas	-----	Arrest bleeding and prevent tetanus	Leaf grind with sugar sugar and apply to wound

Table 25: List of plants with their mechanism of actions and mode of preparation and administration (Continued)

Plant Name	Voucher No.	Mechanism of action	Mode of administration
Justicia flava	UCC/H/5155	Healing of wound	Burn together with white beans, groundnut, cocoyam and apply to sore
Mallotus oppositifolius	UCC/H/389	Healing of wound	Leaf grind with onion and apply to wound
Manihot esculenta	UCC/H/4606	Arrest bleeding	Tuber/leaf grind and add to wound
Milicia excelsia	-----	Healing of wound	Juice added to cotton and apply to wound
Mucuna sloanei	UCC/H/932	Arrest bleeding	Juice drop into wound
Musa paradisiaca	------	Arrest bleeding	Juice apply to fresh wound
Ocimum gratissimum	------	Arrest bleeding, causes healing	Fresh leaves grind or squeeze into fresh wound
Phyllanthus fraternus	------	Heals wound , boils and stomach pains	Grind and add to wound
Securinegea virosa	UCC/H/424	Causes healing	Grind and add to wound
Sida acuta	UCC/H/2383	Arrest bleeding	Leaves grind with leaves of *Combretum dolichopetalum* and add to wound
Spondias mombin	-----	Healing wound	Boils leaves and dip affected part in it/ grind and add to wound
Vernonia colorata	UCC/H/1216	Healing wound/ arresting bleeding	Grind leaves with that of Cassava and tie to wound

Some preparations included the use of more than one species or required additional ingredients. For example, *Mallotus oppositifolius* is mixed with *Crescentia cujete* and ground together before applied to wound (Table 25). For fresh cuts and small wounds, *Chromolaena odorata, Manihot esculenta, Musa paradisiaca* and *Ocimum gratissimum* are used for fast arresting of blood oozing

and healing of wounds. This may only indicate that these plants contain therapeutic compounds such as vitamins C and some amino acids which are responsible for the fast healing of wounds. Plant species identified in this survey were noted for their folk medicinal values. The knowledge of these medicinal values constitutes parts of the people's culture that is passed from one generation to another. Previous studies and the current phytochemical screening conducted on some of the plants revealed that these species were essentially rich in alkaloids, flavonoids, tannins, steroids as shown in Table 26. These phytoconstituents are generally noted for their immense contributions to antioxidant activities and therapeutic effects of medicinal plants. It is therefore not surprising to have noticed that scientific reports on majority of the wound healing plants indicated antioxidant activity.

Various ethnobotanical surveys have been conducted to identify plants used in folkloric medicine to treat wounds. There are species which are cited in this study. Out of the 27 identified plants, *Securinega virosa* (Dickson et al., 2006 & 2007), (*Musa paradisiaca* (Weremfo et al., 2011; Agyare et al., 2009); *Justicia flava* (Agyare et al., 2009, 2013), *Elaeis guineensis, Sida acuta, Manihot esculanta, Ocimum gratissimum, Jathropha curcas, Colocasia esculanta, Chromolaena odorata,* (Agyare et al., 2009), *Mallotus oppositifolius* (Agyare et al., 2014) have previously been mentioned or investigated for wound healing properties in Ghana.

Table 26: Phytoconstituents and medicinal values of some of the plants

Plant Name	Medicinal value reported	Phytoconstituents identified/isolated	Reference
Adiatum veneris-capillus	Antibacterial, Antifungal, wound healing	Sugars, flavonoids, triterpenoids, steroids, Alkaloids, Tannins, Terpenoids,Saponins	Ishaq et al., 2014, Roodbari et al., 2012
Anogeissus leiocarpus	Wound healing, Antibacterial, Antioxidant	Tannins, Flavonoids, Alkaloid, steroids, Anthraquinone glycosides,Saponins	Mann et al., 2008
Azadirachta indica	Antioxidant	Hydrocarbons, phenolic compounds, terpeniods, alkaloids, glycosides	Manikandan et al., 2008; Hossain et al., 2013
Bridelia Ferruginia	Antidiabetic	Tannins, Saponins, terpenoids, Flavonoids, steroids, alkaloids, anthraquinones	Ameyaw et al., 2012
Combretum dolichopetalum	Anti-ulcer activity	Alkaloids, flavonoids, tannins	Asuzu & Onu, 1990
Corchorus olitorius	Antioxidant, Antibacterial, wound healing	Tannins, Flavonoids, glycosides,Saponins	Morrison, 2009
Crescentia cujete	Antioxidant	Steroids, flavonoids, tannins, glycosides, and terpenoids.	Das et al., 2014
Elaeis gueneensis	Wound healing, Antibacterial	Tannins, alkaloids, steroids, saponins, terpenoids, flavonoids	Sasidharan et al., 2010
Heliotropium indicum	Antimicrobial	Alkaloids, saponins, Tannins, glycosides and flavonoids	Shoge et al, 2011
Justicia flava	Wound healing, Antimicrobial activity	Tannins, alkaloids, flavonoids, glycosides	Agyare et al., 2013

Table 26: Phytoconstituents and medicinal values of some of the plants (Continued)

Plant Name	Medicinal value reported	Phytoconstituents identified/isolated	Reference
Mallotus oppositifolius	Antioxidant, Antimicrobial, Anti-inflammatory	Anthocyanins, flavonoids, tannins, alkaloids saponins, glycosides, steroids	Nwaehujor et al., (2012), Agyare et al., (2014)
Milicia excelsia	Antibacterial, wound healing	Tannins, alkaloids, flavonoids, saponins, glycosides and arthraquinones	Udegbunam et. al, 2013
Musa paradisiaca	Wound healing, Antioxidant	Tannins, saponins, reducing and non redu cing sugars, sterols and triterpenes	Kumar et al., 2012
Ocimum gratissimum	Antioxidant, antimicrobial	Alkaloids, tannins, Saponins steroids, terpenoids, phlobatannins, Anthraquinones, flavonoids and cardiac glycosides	Orafidiya et al., 2005; Ouyang et al., 2013
Phyllanthus fraternus	Antifungal	Lignans, flavonoids, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and alkaloids	Patel et al., 2011; Khan & Khan (2004)
Spondias mombin	Antioxidant,	Alkaloids, flavonoids, tannins, saponins, sterols, quinines, phenonlic compounds	Njoku et al, 2007, Kramer et al, 2002

Various other plants traditionally used in wound healing have also been identified by other researchers from other geographical locations in Ghana

Examples are *Commenlina diffusa, Spathodea campanulata* (Mensah et al., (2006)), Agyare et al., 2009, *Clerodendro splendens*, G. Don (Gbedema et al. 2010), *Secamone afzelii* (Mensah et al., 2006). This plant species did not surface in this study. One can therefore suggest that geographical factors such as vegetation type and climatic conditions may have direct influence on the type of folkloric medicinal plants used by indigenes.

The relative occurrence of the plant species for wound healing in the study area is presented in Table 27.

Table 27: Relative occurrence of the plants species for wound healing in the study area

Common	*Heliotropium indicum, Musa paradisiaca, Occimun gratissimum, Jathropha curcas, Combretum dolichopetalum, Chromolaena odorata, Anogeissus leiocarpus*
Frequent	*Amaranthus spinosus, Phyllanthus fraternus, Sida acuta, Vernonia colorata, Adiantum capillus-veneris, Manihot esculenta, Crescentia cujete, Bridelia ferruginia*
Occasional	*Azadirachta indica, Elaies gueeneensis, Securinegea virosa, Coffee Arabica, Mallotus oppositifolius, Milicia excels, Colocasia esculenta, Calotropis procera, Mucuna sloanei, Justicia flava, Spondias mombin, Corchorus olitorius.*

In the survey, *Heliotropium indicum, Musa paradisiaca, Ocimun gratissima, Jathropha curcas, Combretum dolichopetalum, Chromolaena odorata, Anogeissus leiocarpus* showed the highest incidence of encounter as shown in Table 27. It is assumed that many of the indigenes from the study area prefer to use the above mentioned plant species for wound healing relatively more often than the others. Thus, based on the result of the survey, these plants could be considered promising for further scientific studies.

Antimicrobial activity on the crude extracts

Table 28 shows the antimicrobial activities of methanolic extracts of *A. spinosus, S. monbin, C. olitorius, A. leiocarpus, M. oppositifolius and C. dolichopetalum* in terms of the diameter of zone of inhibition. All the plants extracts except *C. dolichopetalum*, showed considerable antibacterial activity against all the test organisms. *C. dolichopetalum* inhibited the growth of *Staphylococus aureus* only and this happened to be the highest zone of inhibition. *C. dolichopetalum* thus has very strong activity against *Staphylococus aureus* but with limited spectrum of activity against the other tested organisms. Among the plants that showed some activity against all the test organisms *A. leiocarpus* exhibited the least activity with its maximum zone of inhibition against *Citrobacter species.* However, the activity of extract of *A. leiocarpus* is far better when compared with similar work cited in literature. For instance, in the work reported by Mann et al., (2008) on the leaf extract, no inhibition was recorded at the concentrations of 10, 20 mg/ml on both *Staphylococus aureus* and *E-coli*. Significant results were only obtained at a higher dose of 50 mg/ml. Even this

Staphylococus aureus recorded 9.00 ± 0.47 and E-coli recorded 9.0 ± 094 compared to 8.79 ± 0.2 and 9.03 ± 0.1 respectively at 1.0 mg/ml dose in this study. It is evident from the zones of inhibition that all the plant species have antimicrobial properties and are therefore potentially good sources of antimicrobial substances with *A. spinosus, S. mombin, C. olitorius, A. leiocarpus, M. oppositifolius* displaying a broad spectrum of activities in preventing the growth of all the tested microorganisms. The antibacterial activities of all the plants are indicative of the presence of the metabolic toxins or broad spectrum antimicrobial compounds (phytoconstituents) that act against gram +ve and gram –ve bacteria.

Table 28: Antibacterial activities of the various plant extracts

	Zone of inhibition (mm) of extracts (1.0 mg/ml)			
Plants	*Staphylococus aureus.*	*Klebsiella pneumonia*	*Citrobacter sp.*	*Escherichia coli*
A. spinosus	11.12 ± 0.2^{a}	13.52 ± 0.2^{b}	10.73 ± 0.2^{a}	11.60 ± 0.2^{c}
S. mombin	12.38 ± 0.1^{a}	11.62 ± 0.2^{c}	13.20 ± 0.2^{d}	12.26 ± 0.2^{a}
C. olitorius	10.90 ± 0.1^{e}	10.66 ± 0.2^{e}	11.86 ± 0.2^{f}	14.23 ± 0.2^{g}
A. leiocarpus	8.79 ± 0.2^{h}	8.79 ± 0.2^{h}	10.90 ± 0.2^{i}	9.03 ± 0.1^{h}
M. oppositifolius	10.31 ± 0.2^{a}	11.82 ± 0.2^{g}	11.08 ± 0.2^{j}	10.40 ± 0.2^{a}
C. dolichopetalum	$23\ 00 \pm 0.1^{k}$	0.00	------	0.00

Values are expressed as the mean ± standard deviation (n = 3). Means with different superscript letters within a row are significantly different ($p < 0.05$). Inhibition zones 15 mm was declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities.

According to Iwalewa et al., (2007), antimicrobials of plant origin have enormous therapeutic potential in the treatment of infectious diseases. This was supported by Mcgaw et al., (2008) that antimicrobials are effective as well as has the advantage of mitigating many of the side effects that are often associated with synthetic antimicrobials. The broad spectrum antibacterial activities of the plants studied confirmed the enormous therapeutic potential of plants in the treatment of diseases as has been stated.

The current findings lend credence to the traditional use of these plants as medicines for fighting infections particularly those caused by the test organisms susceptible to the extracts. The present results for both plants indicate significant antimicrobial potentials and this suggests that traditional medicine could be used as guide in the continuous search for new antimicrobial agents.

Wound healing activity of *C. olitorius, A. leiocarpus, A. spinosus* and *C. dolichopetalum*

A significant promotion of wound-healing activity was observed in the various extracts of *C. olitorius, A. leiocarpus, A. spinosus* and *C. dolichopetalum* in the excision wound model. The diameter of the wound was measured in order to determine the rate of wound closure and to determine the wound healing activity of the plants. The mean percentage wound closure was calculated on 0, 3, 6, 9, 12, 15 and 18 post-wounding days. Table 29, 30, 31 and 32 presented the mean percentage wound closure recorded on each individual selected plant.

Result from the wound contraction indicated that both the aqueous extract and the powder of *C. olitorius, A. leiocarpus C. dolichopetalum* leaves and *A. spinosus* root have a significant wound healing activity. Almost all animals treated with all the four plants extracts, healed completely on the 15 post-wounding days. The aqueous extracts of *A. leiocarpus, C. dolichopetalum* and *A. spinosus* performed better than the ointment preparations of their leaf powders. The result of *C. olitorius* indicated otherwise. By the 15th day, all the wounds treated with ointment preparations of the leaf powder of *C. olitorius* were completely healed.

Table 29: Effect of topical application of *C. olitorius* extracts on excision wound model recorded in % wound contraction

Treatment	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Standard drug	2.94±0.14 (0.00 %)	2.72±0.09 (7.32 %)	2.23±0.09 (23.48 %)	1.47 ± 0.08 (49.38 %)	1.15±0.09 (61.05%)	0.59±0.06 (91.06 %)
Shea butter	2.71±0.08 *(0.00%)	2.31±0.11 (14.83%)	2.0 ± 0.06 (25.96 %)	1.47 ± 0.06 (46.21 %)	1.20±0.08 (55.76%)	0.67±0.12* (75.04 %)
5% w/w	2.41±0.04 (0.00 %)	2.12±0.01 (12.1 %)	1.63±0.08* (32.14 %)	0.94±0.02* (61.00 %)	0.56±0.04 (76.74%)	0.00 ± 0.00 (100 %)
10% w/w	2.37±0.02 (0.00 %)	2.23±0.03 (6.19 %)	1.64±0.04* (30.90 %)	0.97±0.03* (59.00 %)	0.48±0.04 (79.80%)	0.00 ± 0.00 (100 %)
30 mg/ml	2.49±0.06 (0.00 %)	2.0 ± 0.01 (19.65%)	1.59±0.06* (35.60 %)	1.13±0.02* (54.60 %)	0.63±0.03 (74.44%)	0.19 ± 0.02 (92.14 %)
100 mg/ml	2.83±0.08 (0.00 %)	1.94±0.03 (31.2%)	1.49 ± 0.07 (47.37 %)	0.95±0.04* (66.31 %)	0.43±0.02 (84.92%)	0.03 ± 0.03 (98.85 %)
Control (untreated)	2.4± 0.05 (0.00 %)	2.25±0.07 (8.12 %)	1.95 ± 0.04 (20.00 %)	1.51 ± 0.06 (37.79 %)	1.24±0.08 (49.24%)	0.93 ± 0.14 (61.53 %)

Values are mean ± SEM (n = 5). Numbers in parenthesis indicate percentage wound closure. *Significant at $P < 0.05$.

Animals treated with ointment preparation of *C. olitorius* showed a faster wound healing activity as compared to the aqueous preparation and all the other treatments. However, the wound healing activity in all cases generally was dose dependent. Also a significant difference was found between the rates of wound contraction for the extracts and the standard, control and shea butter respectively.

Table 30: Effect of topical application of *A. leiocarpus* extracts on excision wound model recorded in % wound contraction

Days Treatments	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
2% w/w penicillin	2.59±0.03 (0.00 %)	1.77±0.06* (31.60 %)	1.41± 08* (45.52 %)	0.82± 12* (68.37 %)	0.69± .09 (75.75 %)	0.00 (100.0%)*
Shea butter	2.44±0.10 (0.00 %)	2.19 ± 0.10 (10.00 %)	1.8 ± 0.12 (25.07 %)	1.05± .13 (56.11 %)	0.65± .04 (72.85 %)	0.14± .06 (94.52 %)
5 % w/w	2.52±0.07 (0.00 %)	1.96 ± 0.15 (22.33 %)	1.66± .07 (34.16 %)	0.91± 11* (64.30 %)	0.59± .05 (76.49 %)	0.13± 08* (95.06 %)
10 % w/w	2.58±0.12 (0.00 %)	1.97 ± 0.21 (24.00 %)	1.65± .13 (35.38 %)	0. ± 0.09* (64.59 %)	0.57± .07 (77.78 %)	0.00* (100.0 %)
30 mg/ml	2.54±0.05 (0.00 %)	1.72 ± 0.10 (31.46 %)	1.51± .06 (40.70 %)	1.04± 07* (62.80 %)	0.55± .05 (78.54%)	0.17± 10* (93.63 %)
100 mg/ml	2.58±0.08 (0.00 %)	1.74±0.06* (32.44 %)	1.41± 04* (45.16 %)	0.93± 06* (63.90 %)	0.51± .06 (80.21 %)	0.00* (100.0%)
Control (Untreated)	2.63±0.05 (0.00 %)	1.83 ± 0.03 (27.94 %)	1.77± .08 (28.45 %)	1.29± .11 (50.88 %)	0.79± .11 (69.85 %)	0.48± .06 (81.76 %)

Values are mean ± SEM. Numbers in parenthesis indicates percentage wound closure. * Significantly different from the control at $P<0.05$, n=5

Table 31: Effect of topical application of *A. spinosus* extracts on excision wound model recorded in % wound contraction

Treatment	Day0	Day3	Day6	Day9	Day12	Day 15
Penicillin (Standard)	19.50 ± 0.20 (0.00%)	12.67 ± 1.66 (35.04%)	9.33 ± 0.82 (52.14%)	4.42 ± 0.57 (77.35%)	3.00 ±0.55 (84.62%)	0.50 ±1.00 (97.44%)
Drez (Standard)	19.00 ± 0.47 (0.00%)	12.67 ± 1.06 (33.33%)	8.67 ± 1.25 (54.39%)	3.75 ± 0.92 (80.26%)	2.17 ±1.55 (88.60%)	0.67 ±1.34 (96.49%)
Shea Butter	19.25 ± 0.32 (0.00%)	13.75 ± 3.18 (28.57%)	9.34 ± 1.46 (51.52%)	4.09 ± 0.63 (78.79%)	1.67 ±1.19 (91.34%)	0.00 ±0.00 (100%)
5% w/w	19.00 ± 0.69 (0.00%)	14.00±1.36* (26.64%)	8.17 ± 1.55 (57.21%)	3.25 ± 0.42 (82.97%)	1.08 ±0.74 (94.32%)	0.00 ±0.00 (100%)
10% w/w	19.08 ± 0.57 (0.00%)	13.09 ± 1.75 (31.44%)	8.42 ± 0.96 (55.90%)	4.09 ± 0.96 (78.60%)	2.92 ±0.69 (84.72%)	0.83 ±1.67 (95.63%)
30 mg/ml	19.25 ± 0.42 (0.00%)	9.50 ± 1.00* (50.65%)	7.92 ± 1.83 (58.87%)	3.17 ± 0.88 (83.55%)	1.83 ±1.40 (90.48%)	0.00 ±0.00 (100%)
100 mg/ml	19.17 ± 0.33 (0.00%)	10.50 ± 0.80 (45.22%)	7.92 ± 0.69 (58.70%)	3.25 ± 1.03 (83.04%)	1.75 ±1.42 (90.87%)	0.00 ±0.00 (100%)
Control **(**Untreated)	19.59 ± 0.17 (0.00%)	10.59 ± 1.50 (45.96%)	8.17 ± 0.43 (58.30%)	2.83 ± 0.58 (85.53%)	1.50 ±1.37 (92.34%)	0.00 ±0.00 (100%)

Values are mean ± SD (n = 4). Numbers in parenthesis indicate percentage wound closure. * Significant at $P < 0.05$ compared with standard drugs

It was observed that the wound contracting ability of all the plants and the penicillin ointment were significantly greater than that of the control. The aqueous extracts and the ointment formulation of the plants treated groups showed significant wound healing from the 3rd day onwards, which was comparable to that of the standard drug treated group of animals without any significant difference.

Table 32: **Effect of *Combretum dolichopetalum* leaf ointment on excisional wound model in Sprague dawley rats**

Treatment	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Penicillin (Standard)	719.32±22.60	433.99±10.66 (33.67%) **	306.54 ± 7.66 (57.38%) ***	113.50 ± 7.70 (84.22%) ***	27.31±6.62 (96.20%) ***	0.60 ± 0.20 (99.20%) ***
Shea butter	683.77±13.38	627.42±21.09 (8.24%)	482.10±24.12 (29.49%)	332.27±30.23 (51.41%)	133.93±15.00 (80.41%)	76.39±13.53 (88.83%)
5% w/w	707.34±19.24	511.75±25.86 (27.65%)	381.36±23.89 (46.09%)	269.82±18.77 (61.85%)	116.06±37.54 (83.90%)	21.60±19.05 (96.95%) **
10% w/w	695.76±22.53	462.46±18.03 (33.60%) *	346.80±13.47 (50.16%) **	182.83 ± 6.09 (73.72%) ***	53.02 ± 12.54 (92.38%) **	0.98 ± 074 (99.86%) ***
30 mg/ml	684.79±29.93	491.49±16.04 (28.23%)	338.86±15.77 (50.52%)**	171.10 ± 5.71 (75.01%)***	87.22 ± 8.64 (87.26%)	11.22 ± 1.38 (98.36%)**
100 mg/ml	731.15±13.84	330.41 ± 9.30 (54.81%)***	159.71 ± 5.71 (78.16%)***	67.81 ± 6.81 (90.73%)***	11.22 ± 1.38 (98.47%)***	0.00 ± 0.00 (100.0%)***
Untreated (control)	695.76±22.53	573.81±29.44 (17.53%)	453.63±26.11 (34.80%)	322.64±15.08 (53.63%)	171.83±14.23 (75.30%)	78.94 ± 6.42 (88.65%)

Values are expressed as Mean ± SEM from four animals in each group. Numbers in parenthesis indicates % wound closure; *P< 0.05, **P< 0.01, ***P <0.001 (compared with control).

The percentage wound contraction was much more with the 100 mg / ml and the 10 % extract treated group (high dose) than the 30 mg / ml and the 5 % w / w (low doses). The wound closure time was also lesser with the higher dosage treated animals as they showed lesser days of complete wound healing (15 days) than the animals treated with lower doses (17days). This shows that the treatments were

dose dependent with high doses exhibiting greater wound healing activity than lower doses.

Wound healing potentials of these plants are remarkable in comparism with some results cited in literature. For instance, wound healing determination on *Occimum gratissimum* and *Sida acuta* recorded 100% percentage wound contraction after the 18th day of treatment (Momoh et. al., 2012 & Akilandeswari et. al., 2010) while almost all the plants in this study recorded 100% after the 15th day of treatment.

Determination of microbial load

The topical application of drugs is an efficient therapy of destroying microbial populations because the availability of the drug at the infected wound site leads to enhanced wound healing activity. The virulence capacity of microorganisms and host immune response are important factors that can cause massive damage during infection.

The estimation of effect of the various treatments on microbial load on the surface of the wounds for all plant extracts followed a similar trend just like the wound healing ability of these plants. The application of plant extracts resulted in diminishing total bacterial counts in the infected wound from days 5, 10 and 15. There was significant decrease in the number of microbes in the extract treatments and was also dose dependent. The total bacterial counts from granulation tissue on different days are shown in Figure 43, 44, 45, and 46.

A decrease in the number of bacteria on the surface of a wound, on application of a drug indicates that the drug has antimicrobial property. Results from the microbial load suggested that the number of bacteria isolated on the surface of the wounds were decreasing. For instance, in the case of *C. olitorius* there was a greater decrease in the bacterial load of 10% dose, followed by 5.0%, 100 mg, 30 mg, standard, shea butter and the control group in a decreasing order of antimicrobial property.

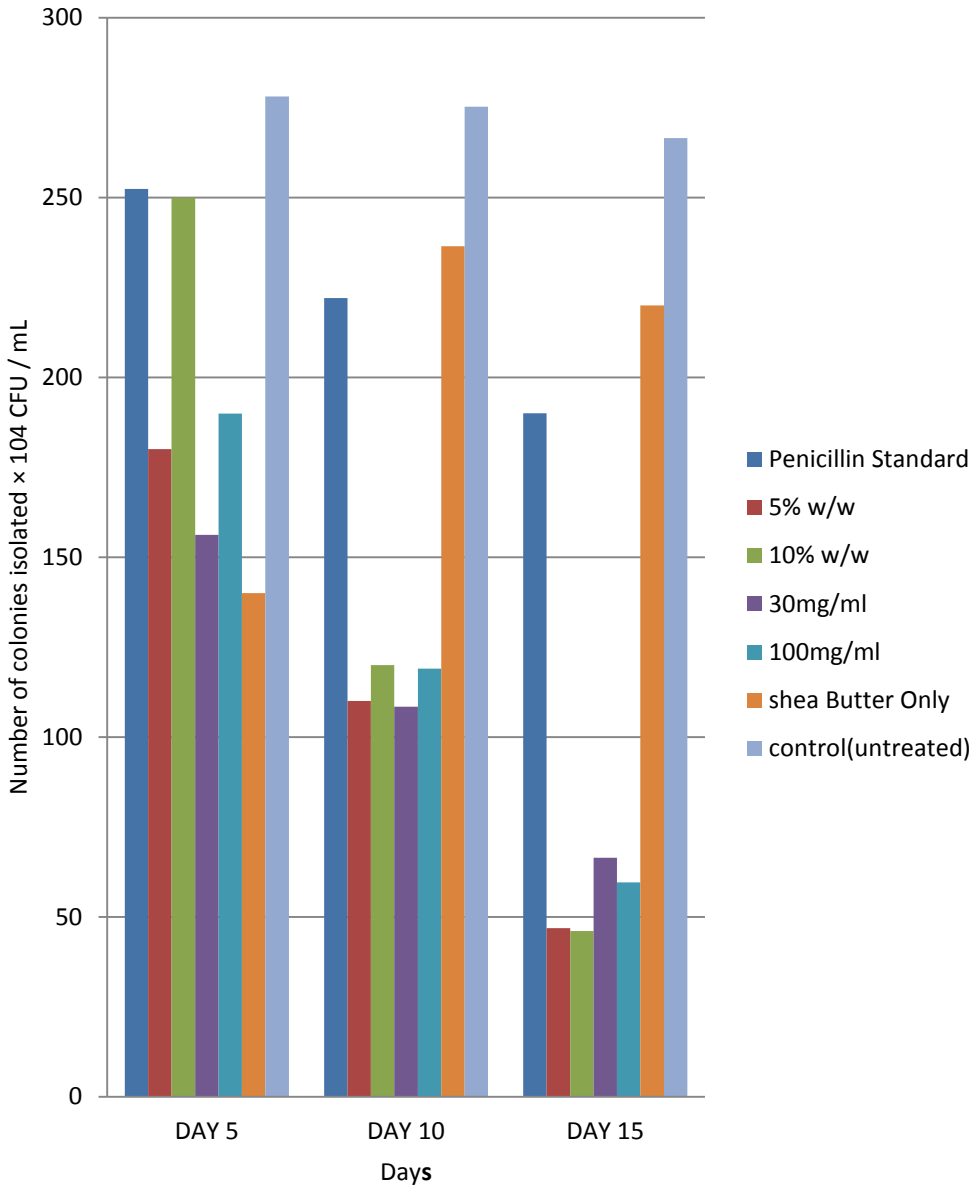


Figure 43: Comparison of the microbial load of the various rat groups wth different treatments of *C. olitorius* extract. Each bar indicates the microbial load of different treated wound on the given days.

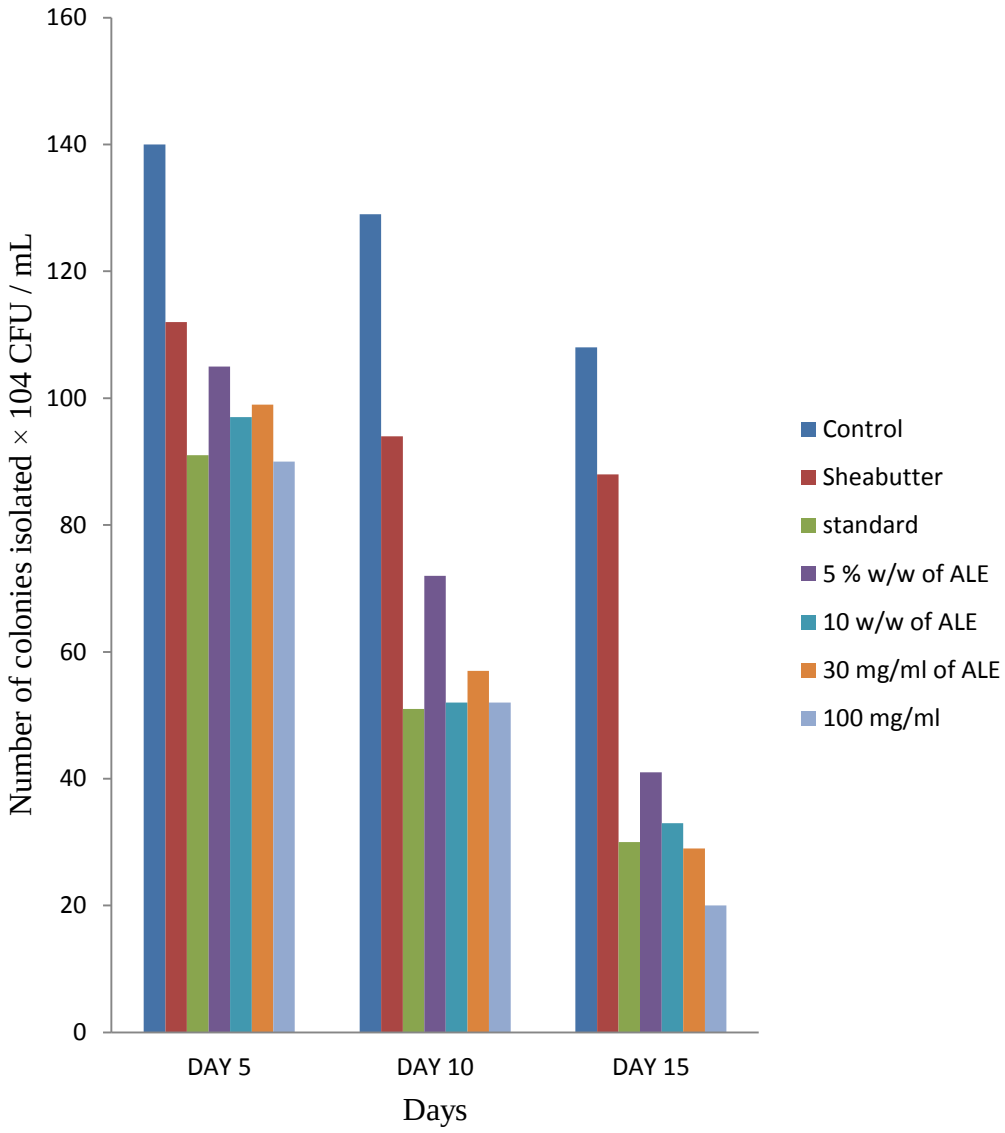


Figure 44: Comparison of the microbial load of the various rat groups that with different treatment of *A. leiocarpus* extract. (Each bar indicates microbial load of different treated wound on the given days). ALE refers to *Anogeissus leiocarpus* extract.

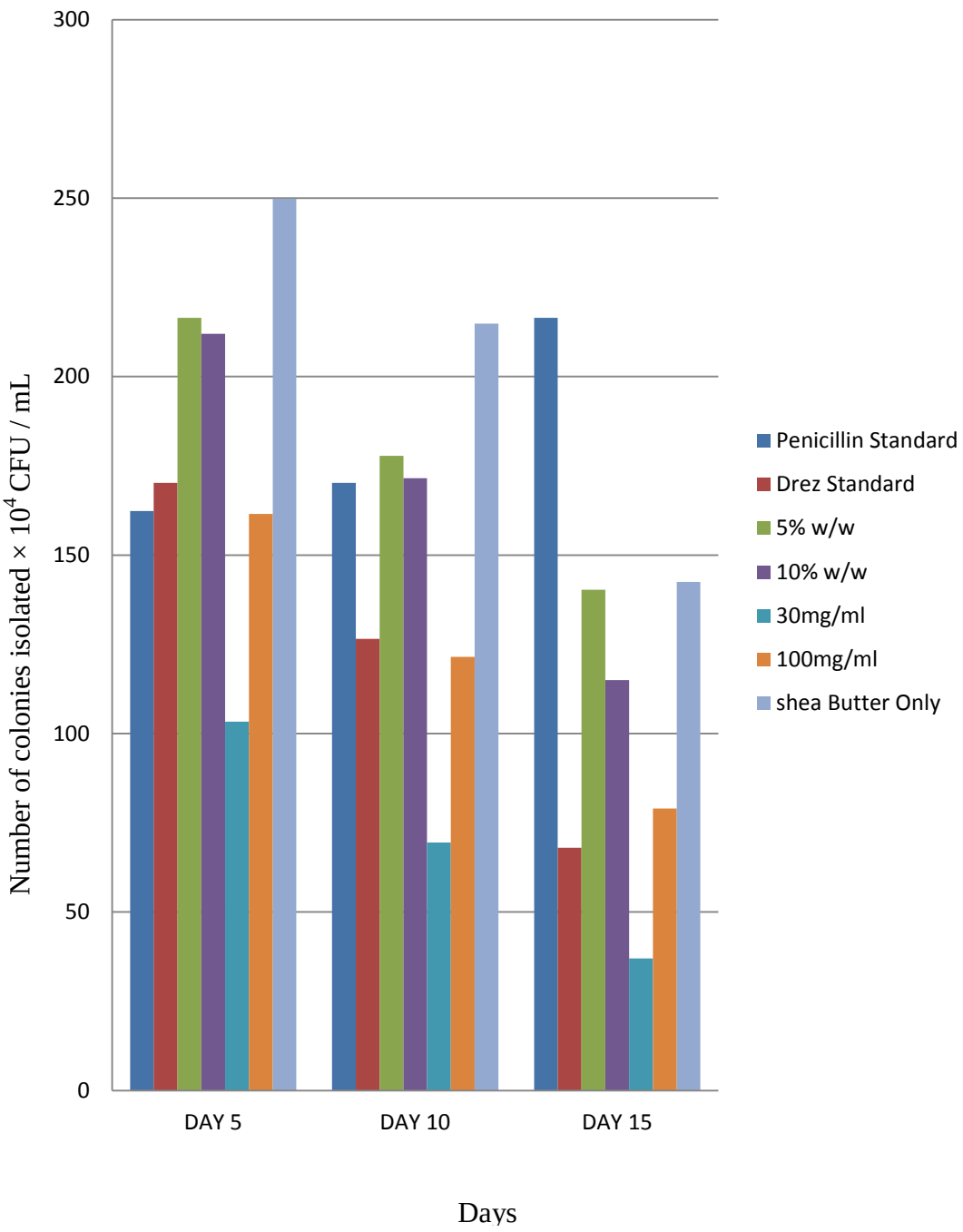


Figure 45: Comparison of the effect of treatment on microbial load for the various groups with *A. spinosus* extract. Each bar indicates the microbial load of different treated wound on the given days.

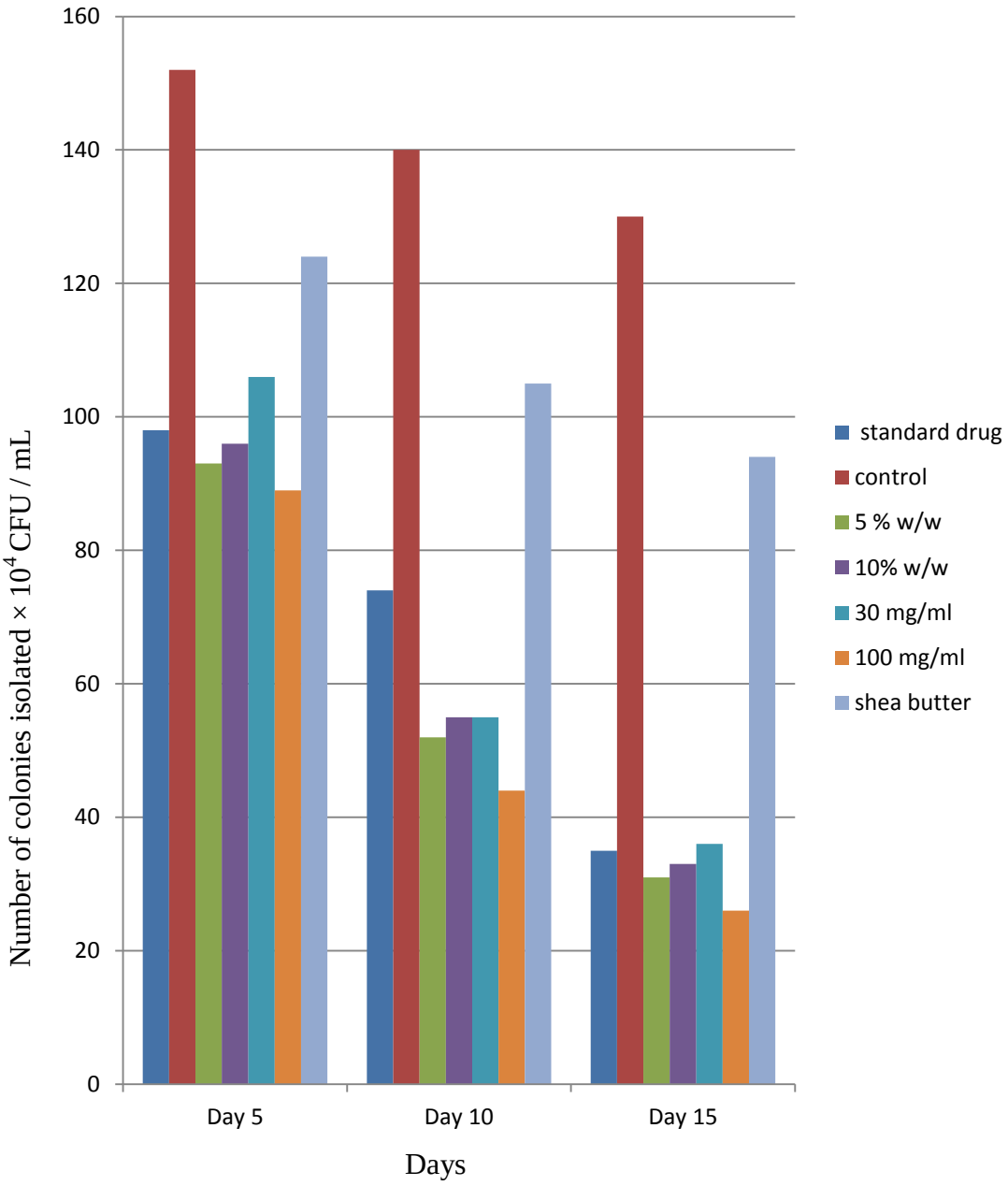


Figure 46: Comparison of the microbial load of the various rat groups with different treatments *C. dolichopetalum* extract. Each bar indicates the microbial load of different treated wound on the given days.

The control of microbial infection of a wound is essential for better wound healing. Wound healing processes are delayed by bacteria colonization as increased microbial load tend to overwhelm the host immune system (Sarkar et

al., 2013). Several studies reported that the higher the bacterial contamination of a wound, the slower the wound healing process (Dow et al., 1999; Sarkar et al., 2013). The antimicrobial activity of these plants is evidenced by the higher microbial load in the untreated groups and those treated with Shea butter as compared to the groups treated with penicillin ointment and the formulated plant drugs.

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contraction is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

The preliminary phytochemical screening on the methanol extracts of all plants presented in Table 33 revealed the presence of flavonoids, tannins, terpenoids, saponins and alkaloids, the major contributory phytoconstituents to antioxidant and antimicrobial properties of a plant. Alkaloids, tannins, cardiac glycosides and saponins generally have antimicrobial properties which contribute to the medicinal properties of these plants (Oboh et al., 2009). These constituents seem to be responsible for wound contraction and increased rate of epithelization and decrease bacteria colonization (Sarkar et al., 2013; Tim-Cushine & Andrew, 2005). Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity (Odukoya et. al., 2012). Flavonoids, (Tsuchiya et al., 1996,) and terpenoids (Scortichini et

al., 1991) are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialization. This is very important since the control of microbial infection is necessary for better wound healing and its management. Flavonoids, are also noted to possess antioxidant and anti-inflammatory properties (Akuodor et al., 2010) which aid in the closure and acceleration of wound healing. Terpenoids are known to promote the wound healing process, mainly due to their astringent and antimicrobial properties, which seem to be responsible for wound contraction (Scortichini, 1991). Terpenoids may have great antifungal or antimicrobial potential due to possible effect on the non-mevalonate pathway. This pathway is essential in fungi, protozoans, gram-negative bacteria and other micro-organisms for the synthesis of cell membrane components, prenylation proteins and as a secondary source of carbon (Nayak et. al., 2010). Studies with other plant materials also demonstrated the presence of similar phytochemical constituents, which were responsible for promoting wound healing activity in rats (Nayak, 2006).

Tannins are known to promote wound healing due to their antimicrobial and astringent property which is responsible for wound contraction (Getie et al., 2002). The presence of tannin confirms haemostasis which is a property for wound healing activity in the plants. Tannins, the main components of many plant extracts, also act as free radical scavengers. Research into the role of antioxidants from plant extracts in wound healing published widely implied that the free radical scavenging action of plants as well as their antioxidant properties enhance

wound healing (Suntar et al., 2012). The result indicated larger proportions of these phytoconstituents as well as high antioxidant activity in the plants. Thus, the wound-healing activity of these plants may be attributed to the presence of these phytoconstituents, which may either be due to their individual or synergetic effect that enhances the antioxidant activity and hastens the process of wound healing.

Table 33: Results from Phytochemical Screening

PHYTO-CONSTITUENT	**PLANTS**					
	A. spinosus	*A. leiocarpus*	*S. monbin*	*M. oppositifolius*	*C. olitorius*	*C. dolichopetalum*
Alkaloid	+	+	+	+	+	+
Steroid	+	+	+	+	+	+
Terpenes	+	+	-	-	+	-
Cardiac-glycosides	+	+	+	+	+	+
Saponins	+	+	+	+	-	+
Tannins	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+

Where + means phyto-constituent is present and – means phyto-constiuent is absent.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

The study covered the survey of medicinal plants used to treat wounds among the people of Kpando Traditional Area. Information was gathered through questionnaires and field interviews which were conducted with a fairly open frame work. Investigations on the antimicrobial activity, wound healing potential and antioxidant activities were as well considered on some of the medicinal plants that were identified in the survey. An attempt was also made to identify and isolate some of the active chemical constituents present in one of the plants.

The study revealed some important medicinal plants that the people of Kpando Traditional Area still depend on for wound healing. It is evident that despite the advancement in orthodox medicines, many people still give preference to plant medicines as their first choice or call for medication. This therefore re-echoes the need for the intensification on the creation of the awareness on the preservation of the biodiversity. Investigation on the antimicrobial, wound healing potential and antioxidant activities of the selected plants gave a positive indications of how much value should be placed on these plants so far as their medicinl properties are concerned.

The results give hope for future discovery of active principles from these plants and development of possible drugs to combat diseases. The successful identification and isolation of flavonoids from one of these plants amply supported this assertion.

Conclusion

In conclusion, the study has made discovery about some medicinal plants used in the Kpando Traditional Area for wound healing. Many of such surveys have been carried out across the country. However, it is worth mentioning that this is the first of its kind such a survey has been conducted in the area. As a result, new plants about 10 which have not been previously documented in Ghana for wound healing have been identified. The selected wound healing plants analysed exhibited scavenging ability and strong reducing ability with *A. leiocarpus* exhibiting the highest scavenging ability and phenolic content. The plants also showed considerable antibacterial activity against the tested organisms. The plant species are therefore potentially good sources of antioxidants and antimicrobial substances with a broad spectrum of activities in preventing the growth of all the tested microorganisms. It has also been proven scientifically that the said wound-healing plants used in folkloric medicine among the indeginous people of Kpando Traditional Area have active chemical constituents that indeed induce healing of wounds. The antioxidant and the antimicrobial activities of the plants show that there is some scientific justification for the traditional uses of these plants as wound-healing agents in Ghana. The wound-healing properties of the plants under study, in most cases, appeared to be associated with their antioxidant activities.

This agrees with Suntar et. al, (2012) that wound-healig property and antioxidant activity co-exist in many plant species. The study therefore supports the traditional use of these plants and encourages their use for wound healing but with caution and guidelines from qualified and experienced practitioners.

It could also be concluded that there are numerous active chemical constituents such as flavonoids abound in these plants that contribute to their antimicrobial and antioxidant properties thereby inducing wound healing. These substances can be identified, isolated and use as prototype or lead compounds for future drug development.

R**ecommendations**

Considering the realization of the enormous importance of these medicinal plants and bearing in mind the rapid exploitation of our forests and the rate of extinction of many plant species, I recommend the intensification of continuous education and campaign on the need to preserve our biodiversity.

I also recommend for the extensive drive by the Forest Commission and other appropriate agencies towards the replanting and growing of seedlings of important plants of particular medicinal value.

I recommend the empowering of the Traditional Medical Practitioners to be of good standing and have the necessary support to conduct their business devoid of mysticism and any negative connotation so that they can adequately complement health delivery especially in the rural areas where accessibility and affordability of orthodox drugs is limited.

Facilities, funding and appropriate equipment necessary for scientists and researchers in the field must be made available for purposeful scientific research.

Lastly, I recommend the continuation of this work, first to confirm the proposed structures for the isolated compounds by subjecting them to 2-DNMR spectroscopy which was not available for this work, and secondly, to monitor mechanisms associated with other activities related to wound-healing properties.

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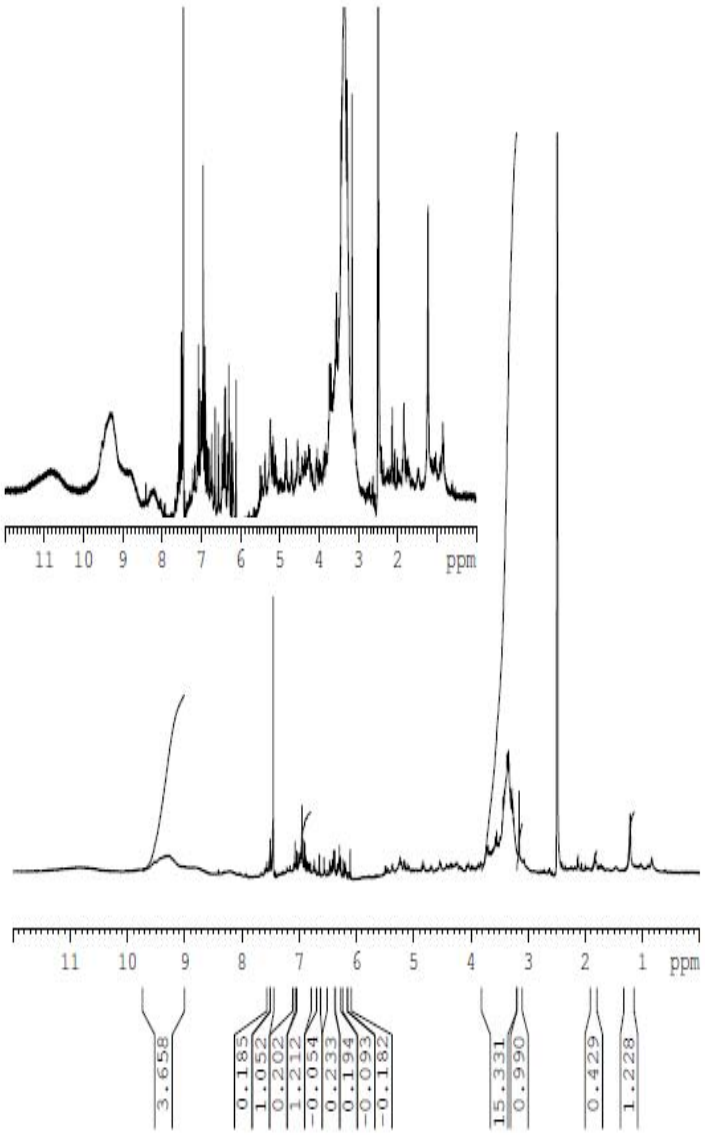
APPENDICES

A: NMR SPECTRA FOR COMPOUND ME

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DE                 6.00 use
TE                  0.0 K
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MCWRK        0.01500000 sec

======== CHANNEL f1 ========
NUC1                  1H
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PL1                -2.00 dB
SFO1         500.2830894 MHz

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1D NMR plot parameters
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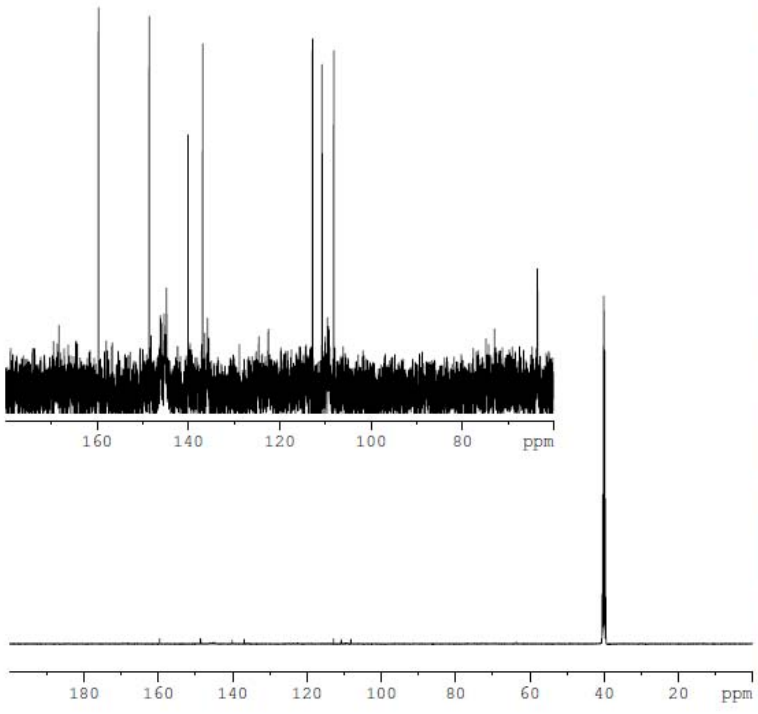


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======== CHANNEL f2 ========
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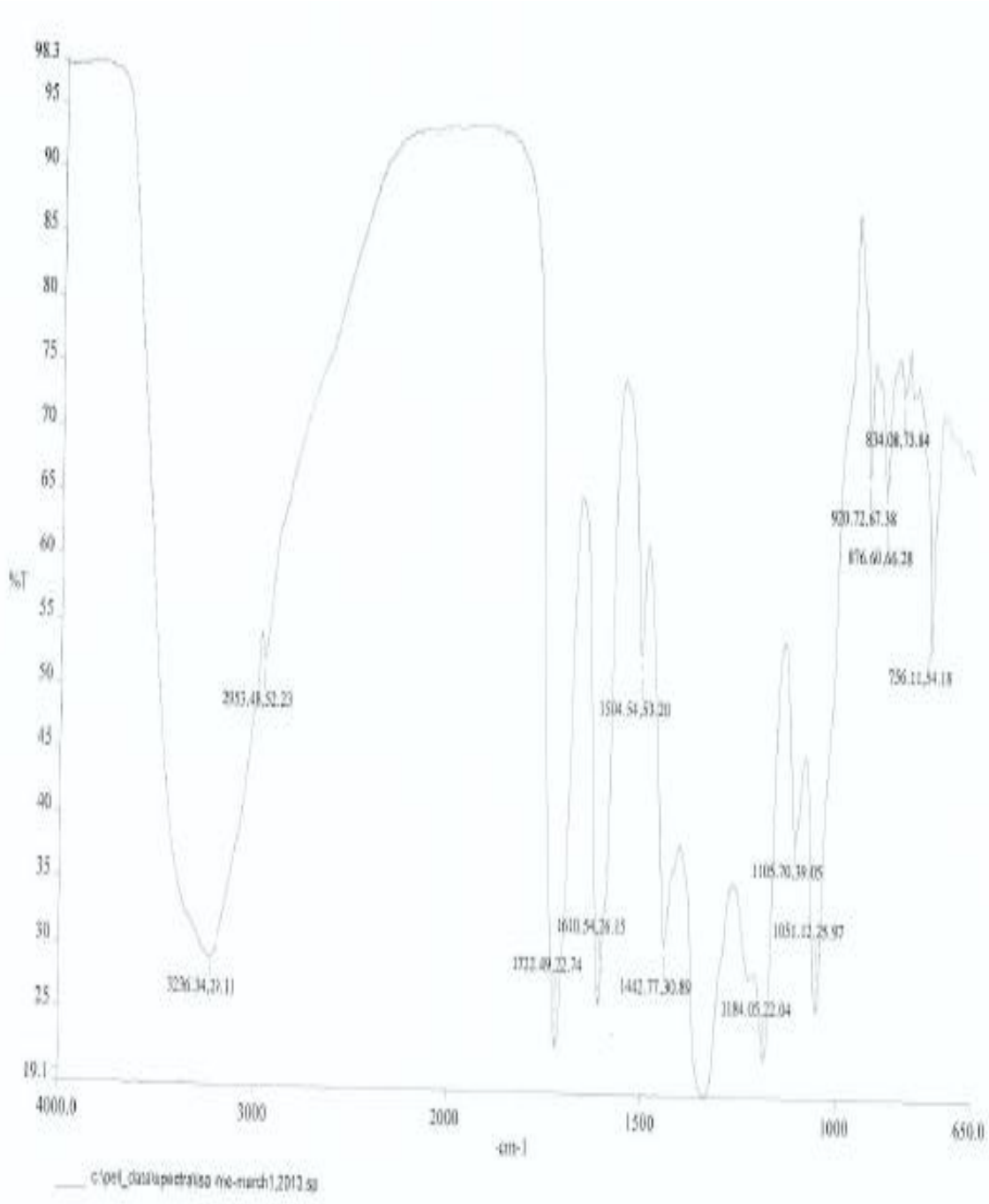
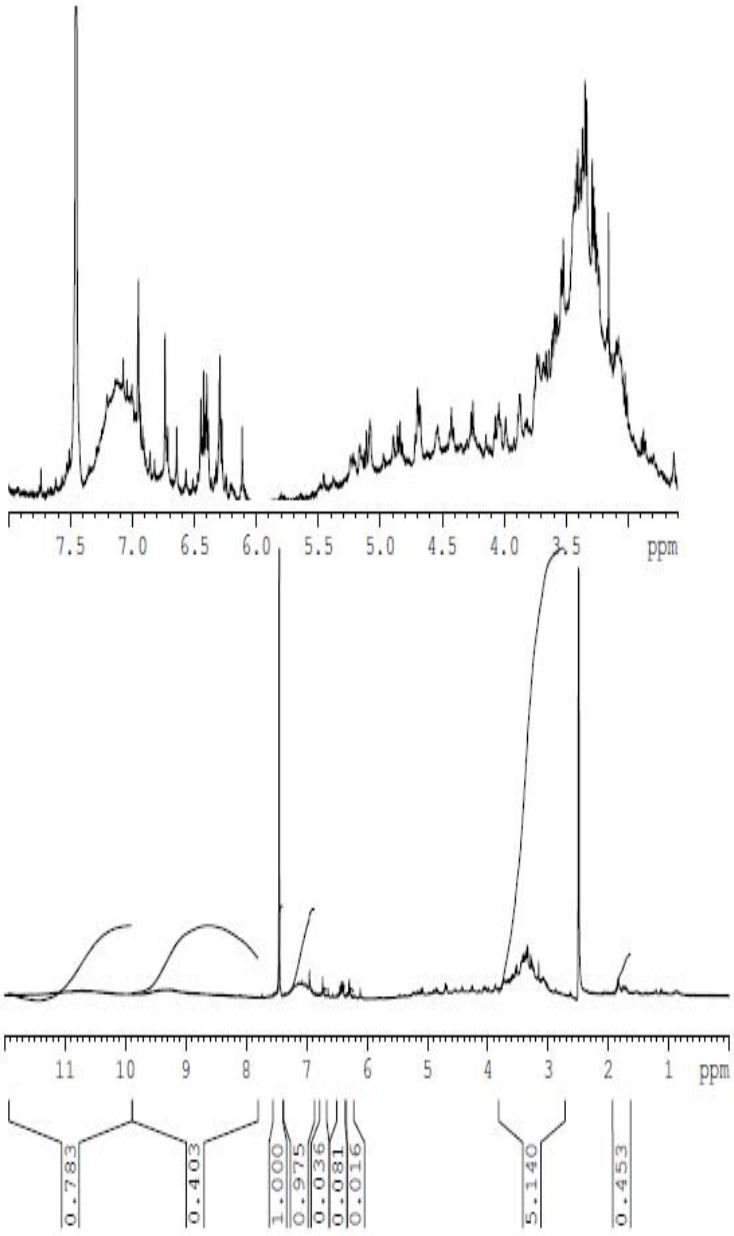
B: FTIR SPECTRUM FOR COMPOUND ME



C: NMR SPECTRA FOR COMPOUND BU

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MCWRK         0.01500000 sec

======= CHANNEL f1 ======
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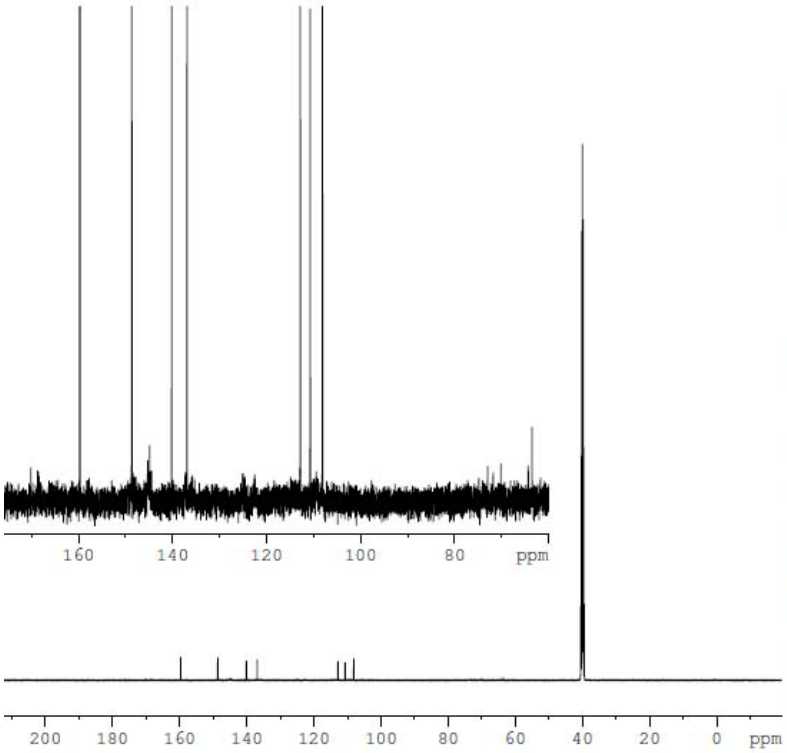
PL1               -2.00 dB
SFO1         500.2830894 MHz

F2 - Processing parameters
SI                 32768
SF          500.2800092 MHz
WDW                   EM
SSB                    0
LB                0.30 Hz
GB                     0
PC                1.00

1D NMR plot parameters
CX                20.00 cm
CY                10.16 cm
F1P              12.000 ppm
F1             6003.36 Hz
F2P              -0.000 ppm
F2               -0.00 Hz
PPMCM          0.60000 ppm/cm
HZCM         300.16800 Hz/cm
```

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Current Data Parameters
NAME                  IA-40-BU-01
EXPNO                           4
PROCNO                          1

F2 - Acquisition Parameters
Date_                    20130309
Time                         5.31
INSTRUM                     spect
PROBHD    5 mm PABBO BB-
PULPROG                     zgpg
TD                         65536
SOLVENT                     DMSO
NS                          4000
DS                             4
SWH                   30030.029 Hz
FIDRES                0.458222 Hz
AQ                    1.0912244 sec
RG                       1149.4
DW                       16.650 usec
DE                        6.00 usec
TE                         0.0 K
D1                    4.00000000 sec
d11                   0.03000000 sec
DELTA                 3.90000010 sec
MCREST                0.00000000 sec
MCWRK                 0.01500000 sec

======== CHANNEL f1 ========
NUC1                         13c
P1                          7.50 usec
PL1                        -1.00 dB
SFO1                125.8080856 MHz

======== CHANNEL f2 ========
CPDPRG2                  waltz16
NUC2                          1H
PCPD2                      80.00 usec
PL2                        -2.00 dB
PL12                       14.48 dB
PL13                      120.00 dB
SFO2                500.2820011 MHz

F2 - Processing parameters
SI                           32768
SF                  125.7955060 MHz
WDW                             EM
SSB                              0
LB                            1.00 Hz
GB                               0
PC                            1.40

1D NMR plot parameters
CX                           20.00 cm
CY                          196.93 cm
F1P                         200.000 ppm
F1                        25159.10 Hz
F2P                         0.000 ppm
F2                            0.00 Hz
PPMCM                     10.00000 ppm/cm
HZCM                   1257.95508 Hz/cm
```
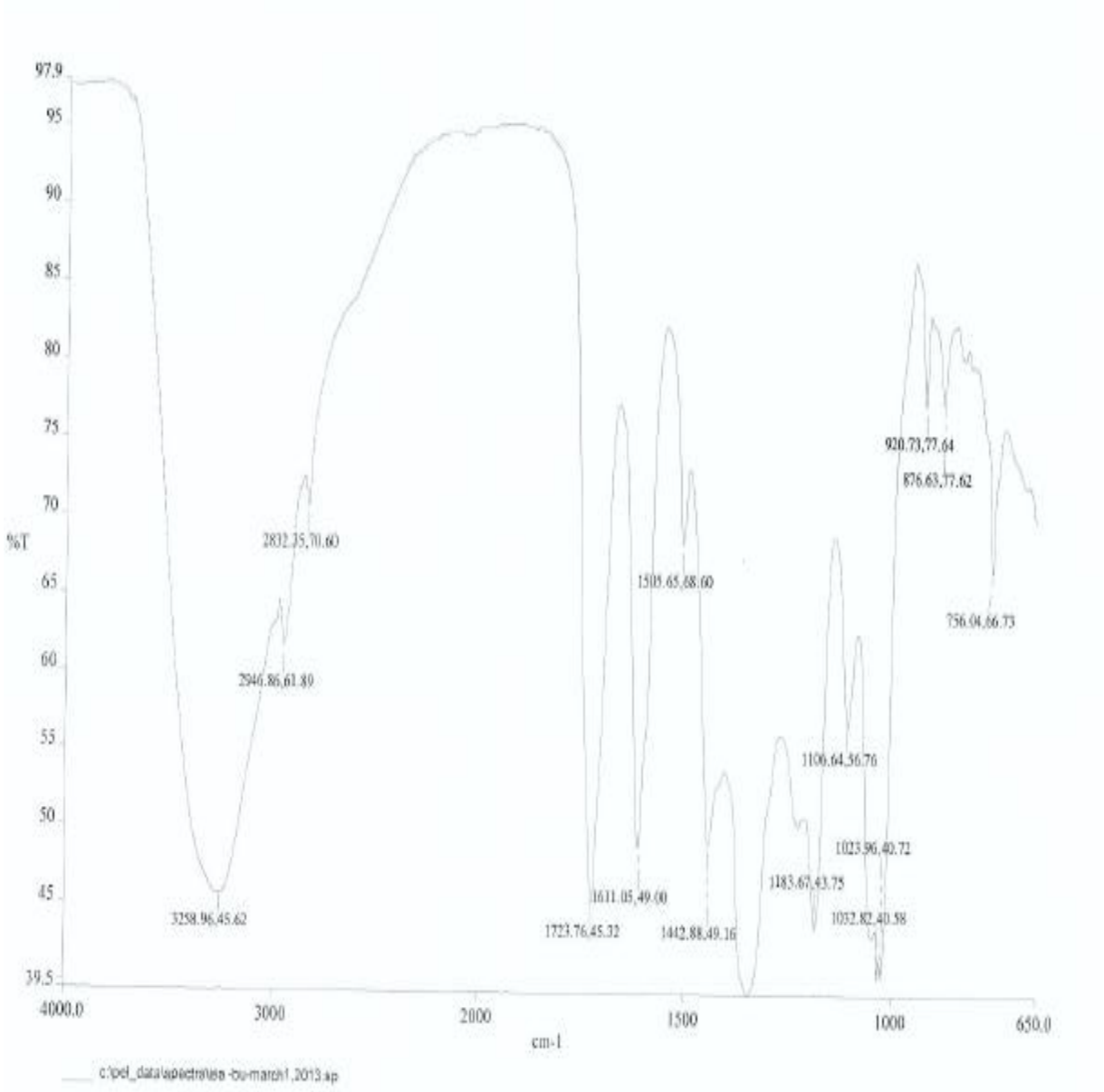

D: FTIR OF SAMPLE BU



E: QUESTIONNAIRE

UNIVERSITY OF CAPE COAST

DEPARTMENT OF CHEMISTRY

PLANTS FOR HEALING WOUNDS PROJECT QUESTIONNAIRE

1. Date: 20th March, 2012 Code: A-1
2. Gender: Male [ ] Female [✓]
3. Age: 20-30yrs [ ] 31-40yrs [ ] 41-50yrs [✓] 51yrs & above [ ]
4. Educational qualification: None [ ] Basic [✓] Secondary [ ]
   Post-secondary [ ] Tertiary [ ]
5. Occupation/Profession: Herbalist [ ] Any other (specify) farmer
6. Any formal training in herbal/ plant medicine? Yes [ ] No [✓]
7. How was the training done? Apprenticeship [ ] School (specify) [ ]
   Any other (specify).....
8. Have you ever treated or nursed a wound before? Yes [✓] No [ ]
9. State the source of treatment/medication. Orthodox [ ] Herbal medicine [✓]
10. Give reason/s for your choice of medicine available, fast and dependable
11. If herbal medicine, state local (~~Akan~~ Ewe) name(s) of plant(s) and plant part/s used

	Plant name/s	Plant part/s used
a.	nyeti	leaves
b.		
c.		
d.		
e.		

12. How plant part is used: Fresh [✓] Dried [ ]

13. Other ingredient/s added to the plant for preparation

onion

14. Method of preparation for use: Powdered [ ] Extracted with water hot [ ]

With cold water [ ] Extracted with local gin [ ] With mineral [ ]

Any other (specify)... grind fresh leaves

15. Mode of administration:... add poultice to wound and bandage

16. Dosage:... reasonable amount

17. Other diseases cured by the plant/s:

18. If wounded today what will be your first choice of medication?

Orthodox [ ] Herbal medicine [✓]

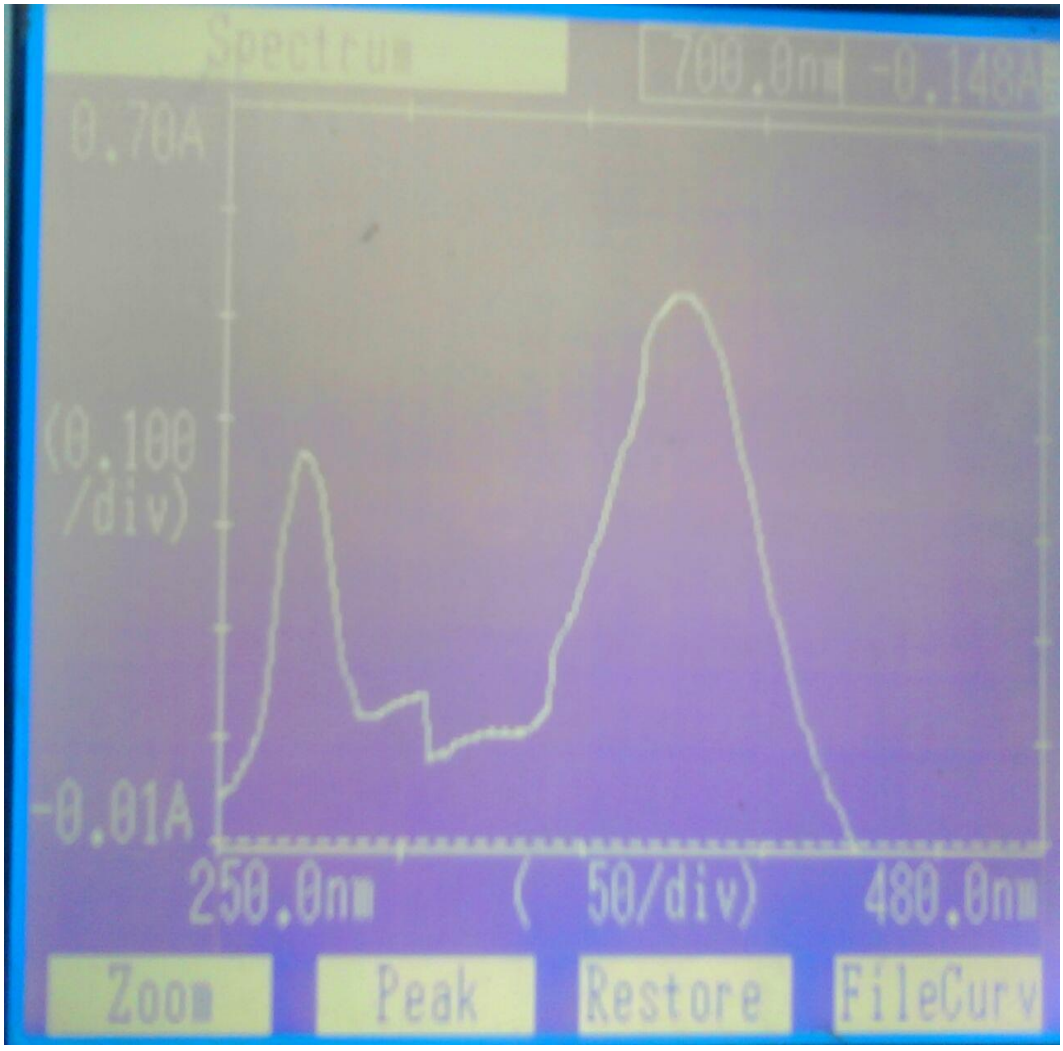
19. Name and address of the person giving information about the medicinal plants

Veronica Agbee

Kpando.

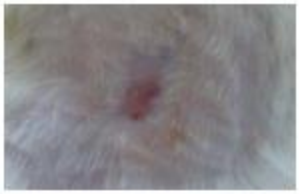
20. Any other comments or information... ~~Having~~ Have been using this for years.

F: UV SPECTRUM FOR COMPOUND ME IN MEOH



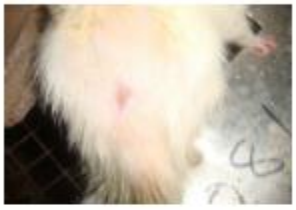
G: PICTURES OF ANIMALS UNDER TREATMENT

2 % penicillin ointment on day 0



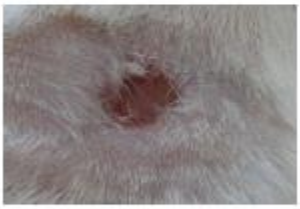
2 % penicillin ointment on day 15

100 mg / ml on Day 0

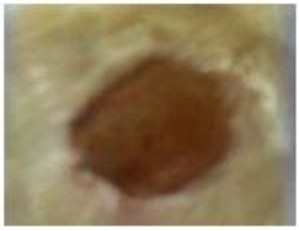


100 mg / ml on Day 15

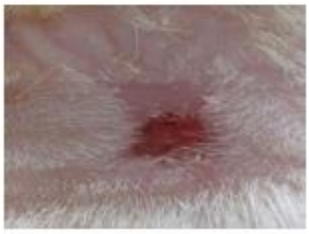
Negative control on day 0



Negative control on Day 15



10 % on Day 0



10 % on day 15