



Sudan University of Science and Technology

College of Post Graduate Studies



**Isolation, Characterization of Flavonoids from
Terminalia brownie, *Ziziphus abyssinica*, *Cassia
sieberiana* Roots and Evaluation of Antimicrobial
Activity**

**فصل ، توصيف فلافونيدات جذور الدروت والسدر والكوك وفعاليتها
البيولوجية**

A Thesis Submitted in Fulfillment of the Requirements of the
Ph.D. Degree in Chemistry

By

Ibrahim Mohammed Ibrahim Mohammed

(B.Sc. in Chemical Laboratories, M.Sc. in Chemistry)

Supervisor

Prof: Mohamed Abdel Karim Mohamed

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الإستهلال

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قال تعالى:

وَقُلْ أَعْمَلُوا فِيسِرِي اللَّهِ عَمَلِكُمْ وَرَسُولِهِ وَالْمُؤْمِنُونَ وَسُتُرْدُونَ
إِلَى عِلْمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُمْ بِمَا كُنْتُمْ تَعْمَلُونَ ﴿١٠٥﴾

صَدَقَ اللَّهُ الْعَظِيمُ

(التوبة-105)

Dedication

Dedicated to,

The soul of my father,

my mother,

wife and daughter,

brother and sisters.

Acknowledgement

First, I would like to thank **Almighty Allah** for giving me the will and health to complete this work.

I would like to thank Prof. Mohamed Abdel Karim for his help and patience through the difficult periods. I would always remember and appreciate his valuable contributions and suggestions during the course of this research. My sincere thanks are extended to my co - supervisor Dr. Amira Abdel Aziz.

A lot of people have contributed, in many ways, to the success of this work and as part of my appreciation to their contribution I would like to express my profound gratitude to each of them.

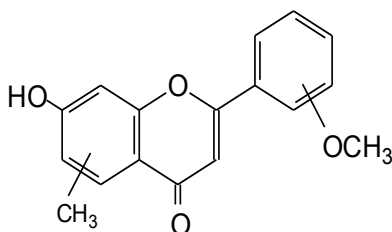
Thanks are due to the Sudan University of Science and Technology for all facilities. Thanks are extended to the Aromatic and Medicinal Plants Research Institute for their infinite support.

Thanks are due to Elsalam University for all facilities.

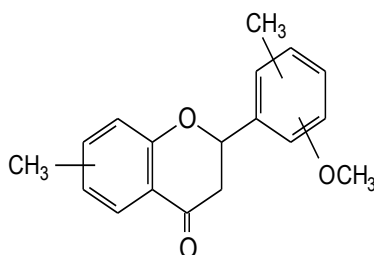
I thank my parents who provided me with invaluable support, encouragement and advice in many aspects during my academic career. My sincere thanks to those who helped me in any way during this work.

Abstract

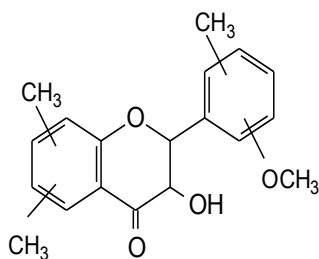
This study aimed to investigate the major flavonoids of *Terminalia brownie*, *Ziziphus abyssinica* and *Cassia sieberiana* roots and to screen these plants for their antimicrobial activity. The flavonoids were extracted with aqueous ethanol and the crude extracts were purified by TLC. The structures of the isolated flavonoids have been partially characterized by some spectral tools (UV and ^1H NMR). One flavonoid-compound I- was isolated from *Terminalia brownie* root another one-compound II- was isolated from *Ziziphus abyssinica* root while a third flavonoid-compound III- was isolated from *Cassia sieberiana* root.



Compound I



Compound II



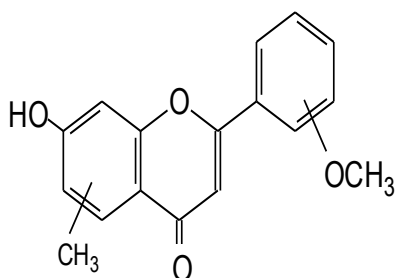
Compound III

In the cup plate agar diffusion bioassay, the ethanol extract of *Terminalia brownie* showed significant activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, while the ethanolic extract of *Ziziphus abyssinica* root showed moderate activity against *Staphylococcus aureus* and the yeast *Candida albicans*. *Cassia sieberiana* ethanolic extract exhibited partial activity against all test microbes.

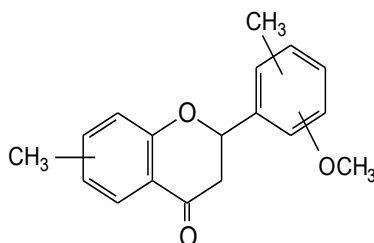
The antioxidant capacity of the ethanol extracts of *Terminalia brownie* and *Cassia sieberiana* roots was carried out by measuring the capacity of the test sample against stable DPPH radical and significant activity was observed for both extracts.

المستخلص

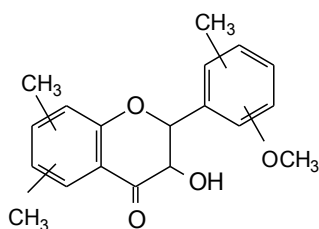
في هذا البحث تمت دراسة الفلافونيدات الرئيسية لجذور نباتات الدروت , السدر والكوك , كما واختبرت مستخلصات هذه النباتات كمضادات ميكروبات. استخلصت الفلافونيدات بالاثانول وتمت التنقية بتقنية كروماتوغرافيا الطبقات الرقيقة وتم التوصيف الجزئي للفلافونيدات عن طريق طيف الاشعة فوق البنفسجية – المرئية وطيف الرنين النووي المغنطيسي للبروتون. تم فصل المركب I من جذور الدروت والمركب II من جذور السدر اما المركب III فقد تم فصله من جذور الكوك.



Compound I



Compound II



Compound III

في اختبار مضاد الميكروبات اعطى مستخلص جذور نبات الدروت فعالية عالية
ضد:

Pseudomonas aeruginosa, Staphylococcus aureus

اما مستخلص جذور نبات السدر فقد ابدى فعالية معتدلة ضد:

Staphylococcus aureus, Candida albicans.

وقد اعطى مستخلص نبات الكوك فعالية جزئية ضد جميع الميكروبات قيد الاختبار.

أيضا تم اختبار مستخلصات نباتى الدروت والكوك كمضادات اكسدة حيث أعطت
هذه المستخلصات فعالية عالية.

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Chapter One

Introduction

1. Introduction

1- 1: General overview

Natural products are organic and inorganic compounds that are found in various natural sources: plants, microbes and animals. Natural products can be an entire organism (e.g. a plant, an animal or a micro-organism), a part of an organism (e.g. leaves or flowers of a plant, an isolated animal organ), an extract of an organism or an exudates, or pure compound (e.g. alkaloids, coumarins, flavonoids, lignans, steroids and terpenoids) isolated from plants, animals or microorganisms. However, in practice, the term natural product refers to secondary metabolites¹.

Alkaloids, such as morphine; eicosanoids, such as prostaglandin E₁; and antibiotics, such as erythromycin and penicillins, are good examples of secondary metabolites¹.

The natural products are classified into two broad divisions: primary metabolites (occur in all organisms) and secondary metabolites (occur only in certain organisms)¹.

Natural products have been a potential source of therapeutic agents for thousands of years. An impressive number of modern drugs have been derived from natural sources. Over the last century, a

number of top selling drugs have been developed from natural products. Anticancer drug - vincristine from *Vinca rosea* and Taxol from *Taxus brevifolia*, narcotic analgesic - morphine from *Papaver somniferum*, antimalarial drug - artemisinin from *Artemisia annua*, and antibiotic penicillins from *Penicillium sp*¹.

The isolation, separation and purification of these natural products require considerable skill. The source of a secondary metabolite requires proper identification and so a voucher specimen needs to be retained. Within the same species there are sometimes chemotypes, each with a particular composition. Some compounds are found in the roots, some are components of the bark, and others may be found in the leaves, the flowers or the fruits. Some compounds play a seasonal role in the plant, for example as insect antifeedants¹.

Natural products may be obtained from the crushed biological material by extraction with a solvent such as petroleum ether, chloroform, ethyl acetate or methanol. Several solvents of increasing polarity may be used¹.

Commercial extractions may use tonne quantities of plant material, and a range of different extraction procedures including steam distillation have been used. Recently, commercial procedures have been developed using supercritical carbon dioxide as a mild

solvent, but because of the pressures involved this requires quite complicated apparatus. The initial extraction is then followed by a separation into acidic, basic and neutral fractions¹.

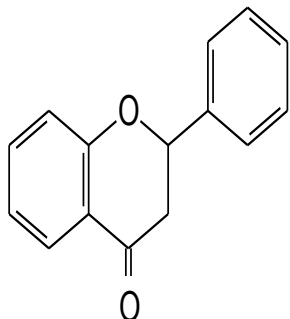
Although, some abundant natural products may be obtained merely by extraction, a simple fractionation or partition and crystallization, the majority are obtained after further careful chromatography¹.

Natural products often co-occur in closely related series, for example as the mono-, di- and trihydroxy derivatives of the same parent compound. Since this relationship may facilitate structure determination, it is helpful to characterize and examine not only the major product obtained from an isolation procedure but also the minor components¹.

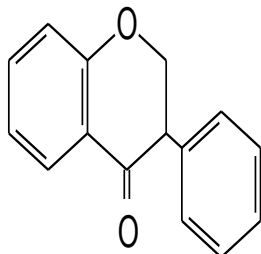
1- 2: The flavonoids

The term flavonoid is generally used to describe a broad collection of natural products that include a C₆-C₃-C₆ carbon framework, or more specifically phenylbenzopyran functionality. 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: the flavonoids (2-phenylbenzopyrans), isoflavonoids (3-phenylbenzopyrans), and the neoflavonoids (4-phenylbenzopyrans). These groups usually share a common

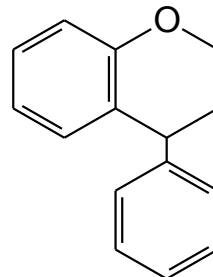
chalcone precursor, and therefore are biogenetically and structurally related².



2-phenylbenzopyrans

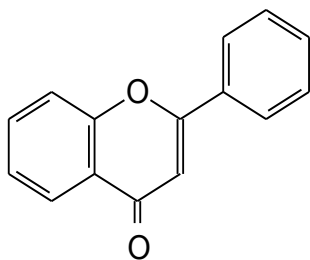


3-phenylbenzopyrans

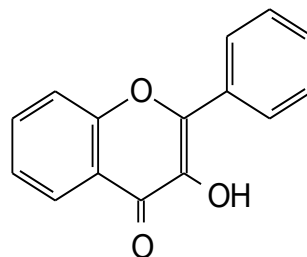


4-phenylbenzopyrans

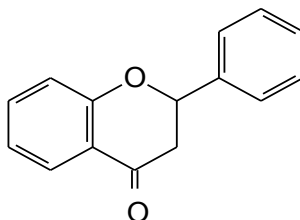
Furthermore, based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups²:



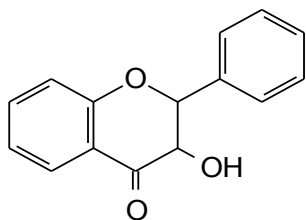
Flavone



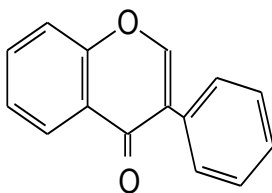
Flavonol



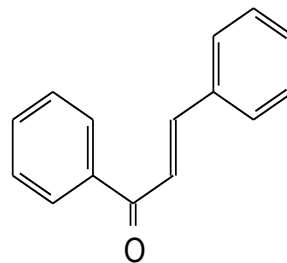
Flavanone



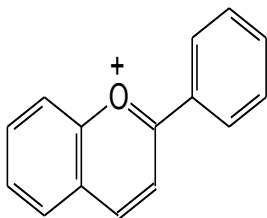
Dihydroflavonol



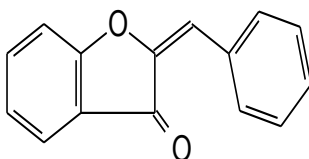
Isoflavone



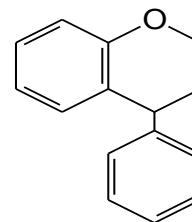
Chalcone



Anthocyanin

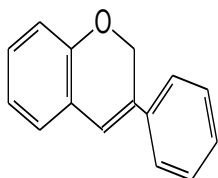


Aurone

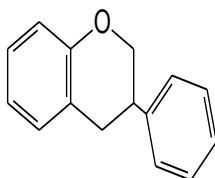


Flavan

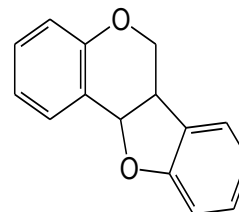
The isoflavonoids are a distinctive subclass of the flavonoids. These compounds possess a 3-phenylchroman skeleton that is biogenetically derived by a 1, 2-aryl migration in a 2-phenylchroman precursor. Isoflavonoids are subdivided into the following groups²:



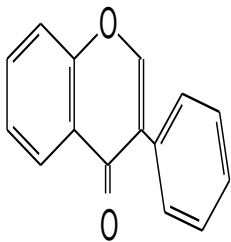
Isoflavan-3-en



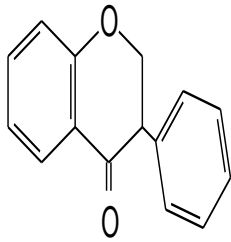
Isoflavan



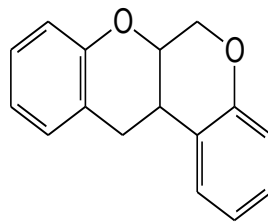
Pterocarpan



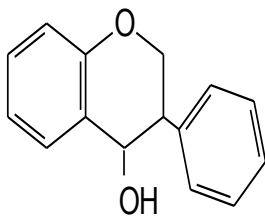
Isoflavone



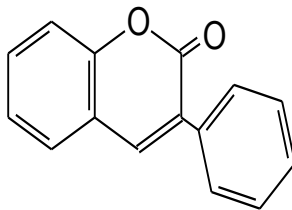
Isoflavanone



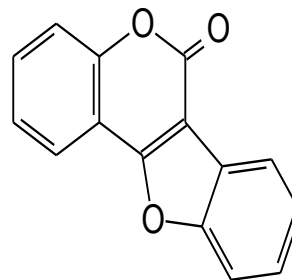
Rotenoid



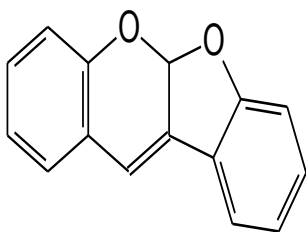
Isoflavonol



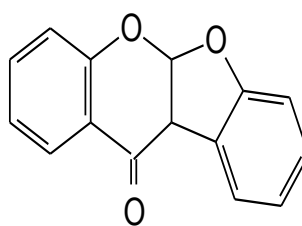
3-Arylcoumarin



Coumestane

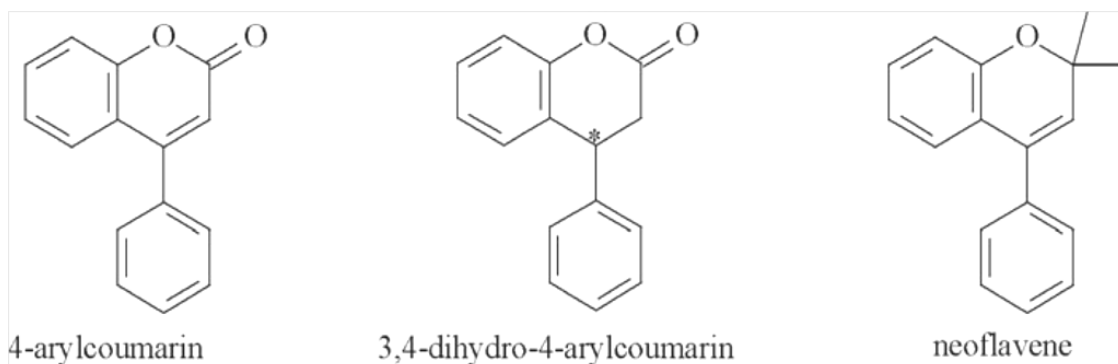


Coumaronochromane

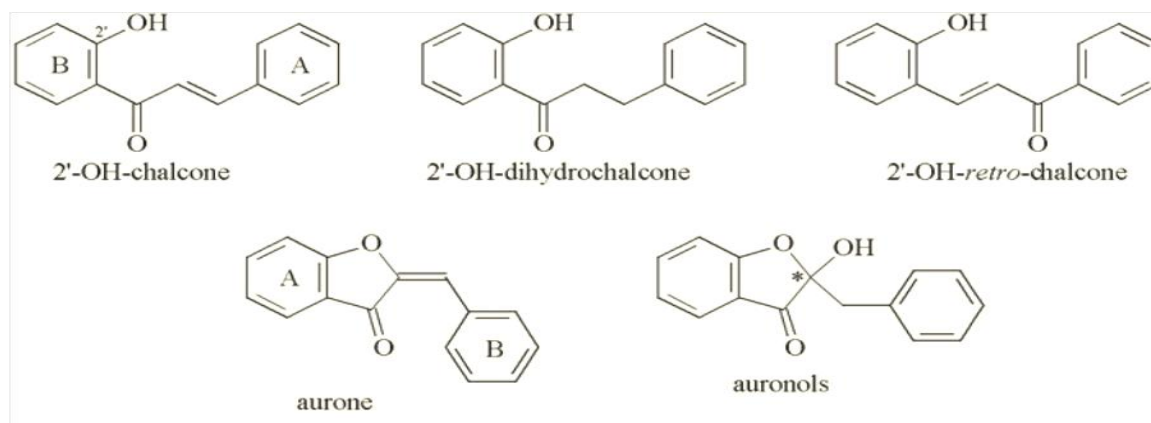


Coumaronochromone

The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2H-1-benzopyran-2-ones), 3, 4-dihydro-4-arylcoumarins, and neoflavenes².



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-chalcones, aurones (2-benzylidenecoumaranone), and auronols².



1- 3: The Anthocyanins

The anthocyanins constitute a major flavonoid group that plays an important role in the coloration of most flowers, fruits, and leaves of angiosperms. They are sometimes present in other plant tissues

such as roots, tubers, stems, bulbils, and are also found in various gymnosperms, ferns, and some bryophytes³.

Two classes of dimeric anthocyanins isolated from plants have been identified in plants. One class includes pigments where an anthocyanin and a flavone or flavonol are linked to each end of a dicarboxylic acyl unit³. The other class includes four different catechins linked covalently bonds to pelargonidin3-glycoside^{4, 5}.

Anthocyanic coloration plays a vital role in the attraction of insects and birds, leading to pollination and seed dispersal, but their appearance in young leaves and seedlings is often transient. There is increasing evidence that anthocyanins, particularly when they are located on the upper surface of the leaf or in the epidermal cells, have a role to play in the physiological survival of plants^{6, 7}. The functions of anthocyanins have in this context mainly been hypothesized as a compatible solute contributing to osmotic adjustment to drought and frost stress, and antioxidant, and a UV and visible light protectant.

The anthocyanins consist of an aglycone (anthocyanidin), sugar(s), and, in many cases, acyl group(s). Anthocyanin is dissolved in water, a series of secondary structures are formed from flavylum cation according to different acid-base, hydration, and tautomeric reactions⁴.

1- 4: Flavone and flavonol O-glycosides

Flavone and flavonol O-glycosides make up one of the largest classes of flavonoid. There are more than 279 glycosidic combinations of the most common flavonol aglycone; quercetin, and more than 347 kaempferol O-glycosides. The group includes any bound form of flavone or flavonol such as acylated and sulfated derivatives and not only those with just sugar.

The monosaccharide's most frequently found in O-combination with flavones and flavonols are glucose and rhamnose and less frequently arabinose, xylose, and glucuronic acid³.

1- 5: Flavones and flavonols

Flavonoid analyses are mostly concentrating on plants which are of either pharmaceutical interest or of commercial value. In addition, flavonoids are important factors in biological interactions between living organisms^{6, 7}. Naturally, the presently known distribution of flavones and flavonol in plants reflects the current scientific interests, and hence the interpretation of their chemo diversity must be made with caution.

1- 6: Flavanones and dihydroflavonols

Flavanones (also called dihydroflavones) and dihydroflavonols (also called 3-hydroxyflavanones) lack the double bond between carbons 2 and 3 in the C-ring of the flavonoid skeleton, which is present in flavones and flavonols. The great majority of the

flavanones isolated from plants are laevorotatory (-)-or (2S)-flavanones, because the enzymatic reaction catalyzing the conversion of chalcones to flavanones is stereo specific^{4,8}.

Biogenetically, chalcones are the immediate precursors of flavanones, and some flavanones isomerize by ring opening into chalcones during isolation from plants or after chemical treatment with alkali. In turn, flavanones are intermediates in the biosynthesis of most other flavonoid groups, including flavones, flavonols, and isoflavonoids⁶⁻¹¹.

However, it is worth mentioning here that these compounds can easily be distinguished from other groups of flavonoids when analyzed by high-performance liquid chromatography with diode array or ultra violet (UV) detection, since most flavanones and dihydroflavonols exhibit a characteristic maximum at wavelength of ca. 290 nm, accompanied by a small shoulder at ca.330 to 360 nm.

Flavanones substituted by hydroxy, methoxy, methylenedioxy, and C-methyl or related groups could conveniently be called “simple flavanones”, in contrast to flavanones bearing more complex substituent’s such as phenyl and benzyl groups^{6,12}.

1- 7: Chalcones, dihydrochalcones, and aurones

The chalcones and aurones are best known as the yellow to orange colored flower pigments of some species of *Coreopsis* and other Asteraceae taxa. The distribution of these compounds is not restricted to flowers, however, and examples of all three classes can be found in many different plant tissues. The chalcones are structurally one of the most diverse groups of flavonoids, as witnessed by the formation of a wide range of dimmers, oligomers, and conjugates of various kinds. They are of great significance biosynthetically as the immediate precursors of all other classes of flavonoids. Underlying these important attributes is the unique feature that distinguishes chalcones and dihydrochalcones from other flavonoids, the open-chain three-carbons structure linking the A-and B-rings in place of a heterocyclic C-ring. In plants, chalcones are converted to the corresponding (2S) -flavanones in a stereo specific reaction catalyzed by the enzyme chalcone isomerase. This close structural and biogenetic relationship between chalcones and flavanones explains why they often co-occur as natural products. It is also the reason why chalcones, dihydrochalcones, and aurones are sometimes described together with flavanones and dihydroflavonols^{6, 13}. Whether this group should continue to be known as the “minor flavonoids” is a matter

for debate; however, there is a significant increase in the number of new examples of each of the flavonoids classes^{4,14}.

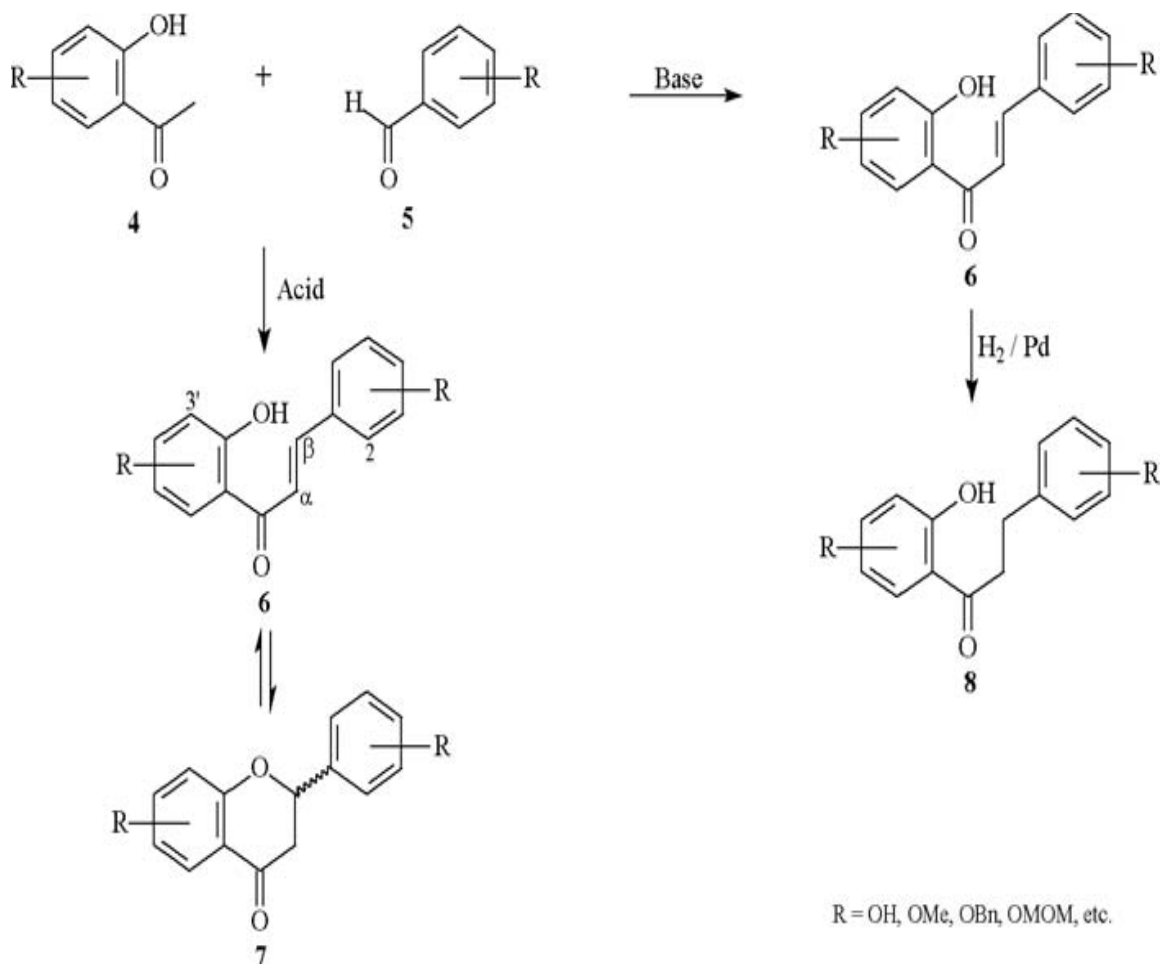
The nomenclature and in particular the atom numbering of chalcones, dihydrochalcones, and aurones remain a potential source of confusion when compared to that of other classes of flavonoids. The A- and B- rings of all the flavonoids have the same origin in biosynthetic terms, with the A- rings derived from the acetate pathway and the B- rings from the shikimate pathway. Similarly, all the structures are written by convention with the A- ring to the left (although this convention may break down for the more complex chalcones, such as dimers, oligomers and Diels-Alder adducts). The crucial difference is in the style of atom numbering, in which primed numbers are used to the A-ring of chalcones and dihydrochalcones, but to the B-ring of other flavonoid classes, including the aurones. Similarly, the B-rings of chalcones and dihydrochalcones carry the non-primed numbers instead of the A-ring. The numbering scheme followed for chalcones and dihydrochalcones is also different, because the C₃ unit linking the A- and B-rings is referred to only in terms of carbonyl α - and β - carbons, whereas the equivalent carbon atoms of heterocyclic C-rings of other flavonoids are numbered together with the rest of the molecule.

The systematic name for chalcone of 1,3-diphenyl-2-propen-1-one is generally thought too cumbersome for routine use, even for simple, naturally occurring derivatives such as the commonly found 2', 4', 4'-trihydroxychalcone, which bears the systematic name 1-(2,4-dihydroxyphenyl) -3-(4-hydroxyphenyl) -2-propen-1-one^{6,14}.

1- 8: Synthesis of Flavonoids

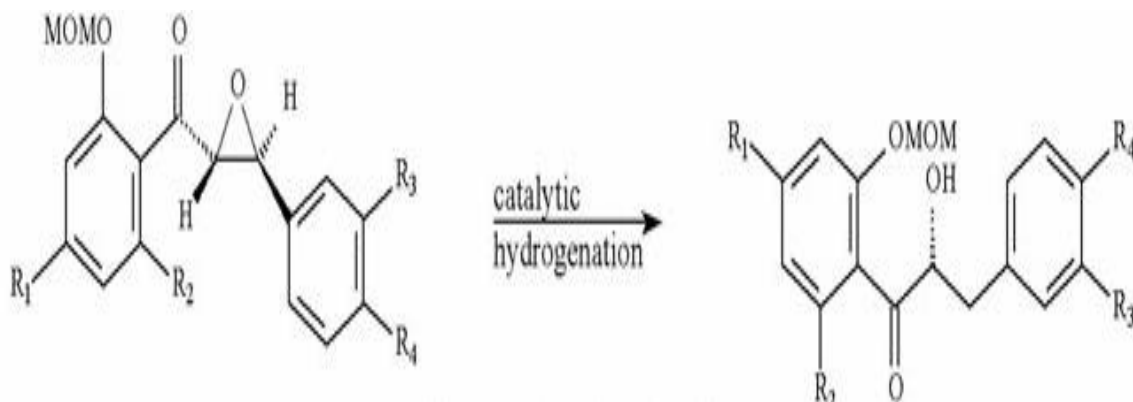
1- 8- 1: Chalcones, dihydrochalcones, and racemic flavonoids

Chalcones and dihydrochalcones are considered to be the primary C₆-C₃ -C₆ precursors and constitute important intermediates in the synthesis of flavonoids. Chalcones are readily accessible via two well-established routes comprising a base-catalyzed aldol condensation or acid-mediated aldolization of 2-hydroxyacetophenones and benzaldehyde^{4, 9}. The base-catalyzed aldol condensation is usually the preferred route towards chalcone formation, since under acidic conditions cyclization of the ensuing chalcone leads to formation of corresponding racemic flavanones^{6, 15}. Dihydrochalcones are generally obtained via reduction (H₂/Pd) of preceding chalcones.



1- 8- 2: α - and β - Hydroxydihydrochalcones

α - and β -hydroxydihydrochalcones constitute rare groups of C_6 - C_3 - C_6 metabolites presumably sharing a close biogenetic relationship with the α -methyldeoxybenzoins and isoflavonoids^{9, 16}. Wynberg prepared an aromatic deoxy α -hydroxydihydrochalcone via catalytic hydrogenation of the corresponding chalcone^{10, 17}.



1- 8- 3: Dihydroflavonols

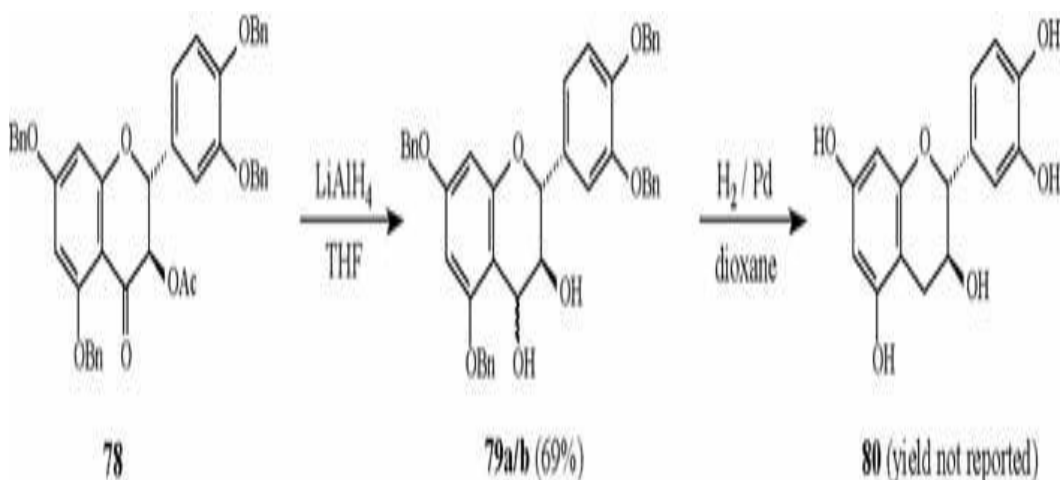
Cyclization of 2'-hydroxy- α , 3, 4, 4'-tetramethoxychalcone with sodium acetate in ethanol furnished both 3,3',4',7-O-tetramethyl-2,3-trans and 3,3',4',7-O-tetramethyl-2,3-cis-dihydroflavonols in 22% and 11% yields, respectively. However, this method was not applicable to cyclization of α -OH-chalcone^{9, 18}.

1- 8- 4: Flavan- 3- ols and flavan- 3, 4- diols

Flavan-3-ols, e.g., (+) - catechin and (-) - epicatechin, represent the largest class of naturally occurring C₆-C₃-C₆ monomeric flavonoids. Flavan-3-ols also have received considerable interest over the last few years because of their importance as the constituent units of proanthocyanidins^{10, 19}. One of the most common ways for the synthesis of flavan-3-ols and the closely related flavan-3, 4- diol analogues involve the reductive transformation of dihydroflavonols. Reduction of the dihydroflavonols with sodium borohydride in methanol affords the

2, 3-trans-3, 4- trans -flavan- 3, 4-diols while reduction in an aprotic solvent like dioxane yielded C₄-epimers exclusively^{9,10,20}.

Catechin represents the only flavan-3-ol synthesized from the corresponding dihydroflavonols^{9, 10, 21}. Consecutive treatment of 2, 3-trans-3-O-acetyldihydroquercetin-tetra-O-benzyl ether with LiAlH₄ and H₂/Pd gave the free phenolic flavan-3-ol in optically pure form^{10, 22}.



1- 8- 5: Isoflavanones

By employing stereo-controlled aldol reaction as the key step, optically active isoflavones were synthesized for the first time by Vicario *et. al.*^{10, 23} in good yields. This sequence included an asymmetric aldol reaction between (S, S)-(+)-pseudoephedrine arylacetamide and formaldehyde to introduce chirality in the isoflavanone carbon framework at C₃. This was followed by the introduction of the B-ring as phenol ether under Mitsunobu conditions and subsequent removal of the chiral auxiliary. Accords

were then converted by an intramolecular Friedel-Crafts acylation, yielding the isoflavanone.

1- 8- 6: Enzymatic stereo specific biosynthesis of flavonoids

Most enzymes of flavonoid biosynthesis are highly stereo selective and/or stereospecific^{4, 6}.

Flavonoids are synthesized via the phenylpropanoid pathway, beginning with the deamination of phenylalanine by the enzyme L-phenylalanine ammonia-lyase (PAL). PAL is specific for the naturally occurring L-isomer of phenylalanine; D-phenylalanine is not a substrate^{10, 24}.

Perhaps the most stereo-chemically important reaction of flavonoid biosynthesis is that catalyzed by chalcone-flavanone isomerase (CHI), which sets the stereochemistry at C-2 of the flavonoid heterocyclic ring. CHI specifically generates (2S)-flavanones from chalcones and is well characterized at the biochemical and structural levels^{9, 10}. The 2S-flavanone is a critical intermediate for formation of several flavonoid classes whose biosynthesis branches at this point, including flavones, flavonols, flavan-4-ols, anthocyanins, and isoflavonoids, and enzymes that use flavanone as substrate (including flavanone 2-hydroxylase/licodione synthase, flavones synthase II, flavones synthase I, flavanone 3-hydroxylase, flavonoid 3'-hydroxylase, flavanone 4-reductase, and isoflavone synthase) have been shown to be highly stereo- specific

for the 2S-enantiomer. Other further downstream such as dihydroflavonol reductase, flavonol synthase, anthocyanidin reductase, and leucoanthocyanidin reductase, which do not directly use flavanone as substrate, also show a high degree of specificity for the naturally occurring stereo chemistries at C-2 and C-3.

1- 9: Flavonoids as nutritional supplements

Nutritional supplements 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 nutraceuticals) were used. A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease^{25, 26}. 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 disease²⁵.

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^{9, 27}. 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 chemo preventive agents^{25, 28}.

1- 10: Antioxidant potential of flavonoids

Diets high in flavonoids, fruits, and vegetables are protective against a variety of diseases, particularly cardiovascular disease and some types of cancer^{25, 29}. 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^{10,30}. ROS contribute to cellular ageing^{25, 31}, mutagenesis, carcinogenesis, and coronary heart disease^{32, 33} possibly through the destabilization of membranes, DNA damage, and oxidation of low-density lipoprotein (LDL). Many *in vitro* studies have demonstrated the potent peroxy radical scavenging abilities of flavonoids, which contribute to inhibiting lipid peroxidation and oxidation of LDL^{10, 34}. Oxidation of LDL is implicated in the pathogenesis of coronary heart disease through its ability to decrease the susceptibility of LDL to oxidation^{32, 35}.

Reactive nitrogen species (RNS) also appear to contribute to the pathology of cardiovascular disease. (NOS) is one of RNS produced by the action of nitric oxide synthase in endothelial cells, neurons, and other cell type. At the sites of inflammation, inducible nitric oxide synthase (iNOS) is also augmented, and NO synthesis is further activated. Peroxynitrite, a potent oxidant generated by the reaction of nitric oxide (NO) with superoxide in the vascular endothelium, induces LDL oxidation^{25, 36} and proinflammatory cytokine-mediated myocardial dysfunction^{9, 32, 37}. Another potential source of RNS derives from dietary nitrite, which reacts with the acidic gastric juice to produce nitrous acid, which decomposes to oxides of nitrogen. Nitrous acid and its products are able to nitrosate amines, deaminate DNA bases, and nitrate aromatic compounds including tyrosine. Several flavonoids and phenolic compounds, including the epicatechin/gallate family of flavanols, are powerful inhibitors of nitrous acid-dependent nitration and DNA deamination *in vitro*^{25, 38}.

The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer free radical electrons, chelate metal catalysts^{32, 39}, activate antioxidant enzymes^{10, 40}, reduce alpha-tocopherol radicals, and inhibit oxidases^{41, 42}.

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 antioxidant effects and provide powerful scavengers against superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite produced by various chemicals and biological systems. With regard to *in vitro* LDL oxidation, gallate esters were found to be less efficient than the respective free forms in inhibiting the oxidation catalyzed by Cu (II). Their activity follows the order epigallocatechin gallate (EGCG) > epicatechin gallate (ECG) > catechins (C) > epicatechin (C)^{42, 43}. Green tea polyphenols partially protect DNA from OH radical-induced strand breaks and base damage^{42, 44}. Pulse radiolysis results support the mechanism of electron transfer (or H-transfer) from catechins to radical sites on DNA^{42, 44}. In black tea, all the theaflavins showed the same capacity to inhibit the production of superoxide. Green tea, black tea, and egg 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 an NADPH-cytochrome P450 reductase^{42, 45}. 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^{42, 46}.

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*^{32, 47}. 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^{42, 48}.

1- 11: Quercetin

Dietary quercetin inhibited DMBA-induced carcinogenesis in hamster buccal pouch and in rat mammary gland^{49, 50}. When given during the initiation stage, quercetin also inhibited DEN-induced lung tumor genesis in mice⁴⁹. In a medium-term multi-organ carcinogenesis model in rats, quercetin (1% in the diet) inhibited tumor promotion in the small intestine^{42, 51}. Feeding rats with quercetin, during either the initiation or promotion stage, inhibited 4-NQO-induced carcinoma formation in the tongue^{32, 52}. Siess and coworkers investigated the effects of feeding rats with flavones, flavanone, tangeretin, and quercetin on two steps of aflatoxin B1 (AFB1)-induced hepato carcinogenesis (initiation and promotion) and found that flavones, flavanones, and tangeretin administered

through the initiation period decreased the number of gamma-glutamyl transpeptidase-preneoplastic foci^{49, 53}. Quercetin decreased oxidative stress-induced neuronal cell membrane damage more than vitamin C. These results suggest that quercetin, in addition to many other biological benefits, contributes significantly to the protective effects of neuronal cells from oxidative stress-induced neurotoxicity, such as Alzheimer's disease^{42, 54}.

1- 12: Genistein and daidzein

Genistein and daidzein (isoflavones derived from soybeans) have been shown to inhibit the development of both hormone- and non-hormone-related cancers, including mouse models of breast, prostate, and skin cancer. Treatment of TRAMP mice with 100-500mg genistein/kg diet reduced the incidence of advanced-stage prostate tumors, in a dose-dependent manner^{42,55}. A high-isoflavone diet also was shown to inhibit methyl nitrosourea-induced prostate tumor in rats^{49, 56}. Topically applied genistein reduces the incidence and multiplicity of skin tumors in the DMBA-initiated and TPA-promoted multiplicity of skin mouse model by 20% and 50%, respectively^{49, 56}. In the UVB light-induced complete carcinogenesis model, topical pretreatment of SKH-1 mice with 10 μ m genistein significantly reduced the formation of H₂O₂ and 8-hydroxy-2'-deoxyguanosine, but not of pyrimidine dimers in the epidermis^{49, 56}.

1- 13: Health benefits of anthocyanins

Only a few studies have been performed to elucidate a potential anticarcinogenic activity of anthocyanins, despite their presence and importance in the human diet. Anthocyanins from purple sweet potato and red cabbage were given at a dietary level of 5.0% in combination with 0.02% 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP), a promoter in the diet until 36 weeks. Lesion development induced by DMH and PhIP was suppressed by anthocyanins. The marked inhibitory effects on colon carcinogenesis were apparent for the anthocyanins comprising cyanidin, but not pending, as the main constituent, suggesting that the inhibition of anthocyanins on carcinogenesis may be related to the number of hydroxyl group on the B-ring^{57, 58}.

Because strawberries are shown to contain higher concentrations of phytochemicals, including anthocyanins, and have an antioxidant capacity when compared to other common fruits, their neuroprotective activity was tested *in vitro* on PC12 cells treated with H₂O₂. Their protective effect and antioxidant capacity also were compared with those of banana and orange, which are the fresh fruits consumed at highest levels in the United States. The overall relative neuronal cell protective activity of these three fruits followed the decreasing order strawberry>banana>orange. The protective effects appeared to be due to the higher phenolic

contents, including anthocyanins, which are the major contributors in strawberries^{57, 59}.

1- 14: Tea and tea polyphenols health benefits

The anticarcinogenesis effects of egg, green tea, and black tea extracts on various organs and animal model have been reported^{49, 60}. Studies by Khan and collaborators showed that green tea polyphenols have a potent inhibitory effect on skin tumorigenicity in mice^{49, 60}. Many studies demonstrated that topical application or oral feeding of a polyphenolic fraction from tea extract or of individual catechin derivatives had anticarcinogenesis effects in animal skins and other organs^{57, 61}. Most of the studies were conducted with chemical- or ultraviolet light-induced tumorigenesis models. For example administration of 1% or 2% freshly brewed green or black tea significantly inhibited the spontaneous development of lung adenoma and rhabdomyosarcoma in A/J mice^{62, 63}.

1- 15: Flavonoids benefits to plant

In plants, flavonoids appear to contribute to a general reduction of reactive oxygen species and there for impact cellular processes sensitive to redox effect. However, flavonoids also have been implicated in more direct interactions with transport and signal transduction pathways. One well-documented example is the role of flavonoids in fertility: while a few flavonoid-deficient plants are

able to germinate, grow, and set fertile seed, most plants require flavonoids for fertility and normal pollen development. Another benefit is flavonoid modulation of auxin transport as well as localized auxin accumulations observed during modulation. Perhaps the best-studied example of flavonoid signaling is that of flavonoid mediation of interactions between the plants and other organisms in the environment at both competitive (allelopathy/defense) and cooperative (mycorrhizal association) levels².

Flavonoids play a role in fertilization in many plant species. Because of the alteration of generations, interactions between the sporophyte and pollen gametophyte can be regarded as extra-organismal.

Flavonoids also are among many of the allelopathic agents that plants produce to reduce competition. Flavones from rice leaves inhibited the weed growth, but not rice biomass, and luteolin from chrysanthemum also inhibited weed biomass^{49, 57, 64}. Quercetin-3-dimethylether, naringenin, and eriodictyol found in *Dittichia* root exudates induced agravitropic growth in lettuce seeds^{57, 62}. (-) - Catechin, kaempferol, and dihydroquercetin in root exudates from the invasive species *Centaurea maculosa* can trigger a wave of reactive oxygen species (ROS) and subsequent Ca²⁺ signaling, leading to root death in sensitive plant species^{65,66}.

In addition to pollen germination, fertilization, and seed set, the flavonoids function in the attraction of animal pollinators. In flower petals, visible flavonoids such as anthocyanins, delphinidin, and cyanidin serve as attractants for pollinators like birds, small mammals, and some insects. UV-fluorescent flavonols serve as nectar guides for bees and other insects and enhance the frequency of pollinator visits, indirectly contributing to increased seed yields^{65, 67}.

Flavonoids are also one of the classes of herbivory deterrents, which may be constitutive or induced. For example, constitutively produced aglycone flavonoids^{65, 68}.

Arbuscular mycorrhizae form mutualistic or symbiotic associations with plants. In addition the flavonones 3, 7-dihydroxy-4'-methoxyflavone and 5, 6, 7, 8-tetrahydroxy-4'-methoxyflavone, quercetin, acacetin, and rhamnetin accumulated in the roots of clover inoculated with mycorrhizae but not in noninoculated plants, suggesting that flavonoids may mediate colonization. In addition, the root and shoot flavonoid composition was altered between colonized and non-colonized plants, which may be a direct or indirect effect of colonization^{62, 69}.

Flavonoids also appear to provide defense against fungal infection. A flavonoid found in rice is allelopathic to rice fungal pathogens; quercetin, quercetin 3-methyl ether, and its glycosides

inhibited *Candida* germination in *Neurospora*, and taxifolin appeared to be an antifungal agent in pine^{57, 62, 65}.

1- 16: Isolation and identification of flavonoid

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.

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 (¹H and ¹³C) 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 analysis and comparison of retention times as well as spectral properties, especially when compounds are present in a mixture, is critical².

The analysis of flavonoids and their conjugates is one of the most important areas in the field of instrumental analytical methods, helping to solve problems in biological and medical sciences. Different methods of isolation of the natural products may be applied, and the utilization of various strategies is dependent on the origin of the biological material from which the target natural products are to be extracted (plant or animal tissue or body fluids)². The utilization of dried plant material for extraction may cause a substantial decrease in the yield of flavonoid conjugates.

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 to the extraction of flavonoids and their conjugates from solid biological material (plant or animal tissues and different food products). The extraction efficiency may enhance by the application of ultrasonication or pressurized liquid extraction (PLE), a procedure performed at elevated temperature ranging from 60 °C to 200 °C^{62,70}. Supercritical fluid extraction with carbon dioxide also may be used^{65, 71}. However, the temperature conditions during the extraction procedures have been

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 solid phase extraction (SPE) are most commonly used. Compounds labeled with stable isotopes (^2H or ^{13}C) are useful when mass spectrometry detection is applied. Quantitative analysis of consecutive components of the analyzed flavonoid mixture needs reference standard compounds necessary for preparation of calibration curves essential for a precise quantification².

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, and different types of silica gels (normal and reversed 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). 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 components².

For the isolation of flavonoids and their derivatives from liquid samples like beverages (fruit juice) and physiological fluids (blood or urine), two different approaches are usually applied. The first one is based on liquid-liquid extraction and the second one on solid-phase extraction of target natural products mainly on silica gel cartridges².

Sample preparation is included in sample handling and is rapidly becoming a science in itself. The initial treatment of the sample is a critical step in chemical and biological analysis; it is usually the slowest step in the analysis.

Sample preparation methods should^{62, 72}:

- Remove possible interferents (for either the separation or detection stages) from the sample, thereby increasing the selectivity of the analytical method.
- Increase the concentration of the analyte and hence the sensitivity of the assay.
- Convert the analyte into a more suitable form for detection or separation (if needed).
- Provide robust and reproducible methods that are independent on variations in the sample matrix.

The aim of sample preparation is that the components of interest should be extracted from complex matrices with the least time and energy consumption but with highest efficiency and reproducibility. Conditions should be mild enough to avoid oxidation, thermal degradation and other chemical and biochemical changes.

As well as typical sample preparation methods such as filtration and liquid-liquid extraction^{62, 73}, newer developments are now extensively used. The first of these is solid-phase extraction (SPE). This is a rapid, economical, and sensitive technique that uses several different types of cartridges and disks, with a variety of sorbents. Sample preparation and concentration can be achieved in a single step.

Flavonoids (particularly glycosides) can be degraded by enzyme action when collected plant material is fresh or non-dried. It is thus advisable to use dry, lyophilized, or frozen samples. When dry plant material is used, it is generally ground into a powder. For extraction, the solvent is chosen as a function of the type of flavonoid required. Polarity is an important consideration here. Less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid

glycosides and more polar aglycones are extracted with alcohols or alcohol-water mixtures.

A convenient and frequently used procedure has been sequential solvent extraction. A first step, with dichloromethane, for example, will extract flavonoid aglycones and less polar material. A subsequent step with an alcohol will extract flavonoid glycosides and polar constituents. Certain flavanone and chalcone glycosides are difficult to dissolve in methanol, ethanol, or alcohol-water mixtures.

Extraction is typically performed with magnetic stirring or shaking, but other methods have recently been introduced to increase the efficiency and speed of the extraction procedure. The first of these is called pressurized liquid extraction (PLE). By this method, extraction is accelerated by using high temperature and high pressure. There is enhanced diffusivity of the solvent and, at the same time, there is the possibility of working under an inert atmosphere and with protection from light. Application of PLE gave better results than maceration-and shorter extraction times and smaller amounts of solvent were required^{65, 74}.

As its name suggests, supercritical fluid extraction (SFE) relies on the solubilizing properties of supercritical fluid. The lower viscosities and higher diffusion rates of supercritical fluids, when compared with those of liquids, make them ideal for the extraction

of diffusion-controlled matrices, such as plant tissues. Advantages of the method are lower solvent consumption, controllable selectivity, and less thermal or chemical degradation than methods such as Soxhlet extraction^{62, 65, 75}. However, to allow for the extraction of polar compounds such as flavonoids, polar solvents (like methanol) have to be added as modifiers.

Ultrasound-assisted extraction is a rapid technique that can also be used with mixtures of immiscible solvent: hexane with methanol-water (9:1), for example, is a system used for the Brazilian plant *Lychnophora ericoides* (Asteraceae). The hexane phase concentrated less polar sesquiterpene lactones, and hydrocarbons, while the aqueous alcohol phase concentrated flavonoids and more polar sesquiterpene lactones^{76, 77}.

Microwave-assisted extraction (MAE) has been described for the extraction of various compounds from different matrices^{65, 78}. It is a simple technique that can be completed in a few minutes. Microwave energy is applied to the sample suspended in solvent, either in a closed vessel or in an open cell. The latter allows larger amounts of sample to be extracted. A certain degree of heating is involved^{76, 79}.

Once a suitably plant extract is obtained, a preliminary cleanup is advantageous. The classical method of separating phenolics from plant extracts is to precipitate with lead acetate or extract into

alkali or carbonate, followed by acidification. The lead acetate procedure is often unsatisfactory since some phenolics do not precipitate; other compound may co-precipitate and it is not always easy to remove the lead salts.

Alternatively, solvent partition or countercurrent techniques may be applied. In order to obtain an isoflavonoid-rich fraction of *Erythrina* species (Leguminosae) for further purification work, an organic solvent extract was dissolved in 90% methanol and first partitioned with hexane. The residual methanol part was adjusted with water to 30% and partitioned with t-butyl methyl ether – hexane (9:1). This latter mixture was then chromatographed to obtain pure compounds^{65, 80}.

A short polyamide column, a Sephadex column, or an ion exchange resin can be used. Absorption of crude extracts onto the column, followed by elution with a methanol-water gradient, is an excellent way of preparing flavonoid-rich fractions.

1- 17: Preparative Methods

One of the major problems with preparative separation of flavonoids is their sparing solubility in solvents employed in chromatography. Moreover, the flavonoids become less soluble as their purification proceeds. Poor solubility in the mobile phase used for a chromatographic separation can induce precipitation at

the head of the column, leading to poor resolution, decrease in solvent flow, or even blockage of the column.

Other complications can also arise. For example, in the separation of anthocyanins and anthocyanins-rich fraction, it is advisable to avoid acetonitrile and formic acid-acetonitrile is difficult to evaporate and there is a risk of ester formation with formic acid.

There is no single isolation strategy for the separation of flavonoids and one or many steps may be necessary for their isolation. The choice of method depends on the polarity of the compounds and the quantity of sample available⁶⁵.

Conventional open-column chromatography is still widely used because of its simplicity and its value as an initial separation step. Preparative work on large quantities of flavonoids from crude plant extracts is also possible. Support materials include polyamide, cellulose, silica gel, and Sephadex. Sephadex is recommended for the separation of proanthocyanidins. For Sephadex gels, as well as size exclusion, adsorption, and partition mechanisms operate in the presence of organic solvents. Although methanol and ethanol can be used as eluents for proanthocyanidins, acetone is better for displacing the high molecular weight polyphenols. Slow flow rates are also recommended. Open-column chromatography with certain supports (silica gel, polyamide) suffers from a certain degree of irreversible adsorption of the solute on the column.

Modifications of the method (dry-column chromatography, vacuum liquid chromatography, VLC, for example) are also of practical use for the rapid fractionation of plant extracts. VLC with a polyamide support has been reported for the separation of flavonol glycosides^{65, 81}.

Preparative TLC is a separation method that requires the least financial outlay and the most basic equipment. It is normally employed for milligram quantities of sample, although gram quantities are also handled if the mixture is not too complex. Preparative TLC in conjunction with open-column chromatography remains a straightforward means of purifying natural products, although variants of planar chromatography, such as centrifugal TLC, have found application in the separation of flavonoids.

Several preparative pressure liquid chromatographic methods are available. These can be classified according to the pressure employed for the separation:

- High-pressure (or high-performance) LC (>20 bar/300 psi)
- Medium-pressure LC (5 to 20 bar/75 to 300 psi)
- Low-pressure LC (<5 bar/75 psi)
- Flash chromatography (Ca. 2 bar/30 psi)

HPLC is becoming by far the most popular technique for the separation of flavonoids, both on preparative and analytical scales. Improvements in instrumentation, packing materials, and column technology are being introduced all the time, making the technique more and more attractive.

The difference between the analytical and preparative methodologies is that analytical HPLC does not rely on the recovery of a sample, while preparative HPLC is a purification process and aims at the isolation of a pure substance from a mixture.

Semi preparative HPLC separations (for 1 to 100mg sample sizes) use columns of internal diameter 8 to 20mm, often packed with 10 μm (or smaller) particles. Large samples can be separated by preparative (or even process-scale) installations but costs become correspondingly higher.

Optimization can be performed on analytical HPLC columns before transposition to a semi preparative scale.

The term “medium-pressure liquid chromatography” (MPLC) covers a wide range of column diameters, different granulometry packing materials, different pressures, and a number of commercially available systems. In its simplest form, MPLC is a closed column (generally glass) connected to compressed air source or a reciprocating pump. It fulfills the requirement for a

simple alternative method to open-column chromatography or flash chromatography, with both higher resolution and shorter separation times. MPLC columns have a high loading capacity-up to a 1:25 sample-to-packing-material ratio and are ideal for the separation of flavonoids^{65, 82}.

In MPLC, the columns are generally filled by the user. Particle sizes of 25 to 200 µm are usually advocated (15 to 25, 25 to 40, or 43 to 60 µm are the most common ranges) and either slurry packing or dry packing is possible. Resolution is increased for along column of small internal diameter when compared with a shorter column of larger internal diameter (with the same amount of stationary phase)^{62, 83}. Choice of solvent systems can be efficiently performed by TLC or by analytical HPLC. Transposition to MPLC is straightforward and direct^{65, 84}.

1- 18: The target plant species

Terminalia brownie is a medicinal plant of many attributes belonging to the family Comretaceae. The plant is widely distributed in Sudan, Tanzania, Ethiopia and Kenya where it grows along river banks, dry areas and semi-arid regions^{85,86}. It is widely used in African system of medicine⁸⁶. *Terminalia brownie* is a natural remedy for cough, tonsillitis, typhoid, tooth-ache, snake bite and rheumatic pain⁸⁷⁻⁹⁰. Phytochemical screening affirmed the presence of flavonoids, terpenoids, steroids and saponins⁹¹⁻⁹⁵. The

anti-pyretic properties of the methanol extract of *Terminalia brownie* has been reported⁹⁶ and the anti nociceptic activity of bark extract has been documented⁹⁷.



Terminalia brownie

***Ziziphus abyssinica* Hochst. ex. A. Rich**

Ziziphus abyssinica **Hochst. ex. A. Rich** is a tree in the family Rhamnaceae that may reach 8m in height⁹⁸. This plant is widely used in African system of medicine. Leaves are used against tonsillitis, pneumonia, burns, wounds, tachycardia, pectoral pain and snake bite⁹⁹⁻¹⁰². Some extracts of *Ziziphus abyssinica* were shown to possess antimicrobial, antioxidant, antiulcerative, anti-

diarrheal, antiplasmodial and molluscicidal properties¹⁰³⁻¹⁰⁸.



Zizphus abyssinica

Cassia sieberiana

Cassia sieberiana is a savanna plant of the family Acacia (Caesalpiaceae). It is commonly known as African laburnum or drumstick tree. It is found in dry areas of forests and thickets¹⁰⁹. Also called “Malga” in Hausa language, is a common plant in Saharan and sub-Saharan Africa¹¹⁰. The herb is usually available, affordable and acceptable to most of the consumers¹¹¹. *Cassia sieberiana* is a plant of Pharmacognostic importance from among Nigerian plants¹¹². Pharmacognostic studies and standardization of *Cassia Sieberiana* roots has been reported¹¹³. Individuals of this species are anywhere from 10-20m in height. The bark ranges from a dark grey to black. The lenticels are

horizontal and a reddish color. The leaves are arranged in leaflets that contain 7-10 pair of opposite leaves. The upper side of the leaf is moderately shiny while the bottom has very fine nerves with stipules that are deciduous. This plant has both flowers and fruit. The flowers are a very bright yellow during the dry season, which is from February through March. Flowers are arranged either upright or in pendulous racemes ranging from 30–50 cm. There are five sepals with 5 bracts. The petals are 15–20 cm long while the green sepals are 6-7mm in length. There are a total of 10 stamens. The fruit ranges from a dark brown to black color. The fruit is indehiscent in that it stays attached to the tree for an extended amount of time. September through February is when the fruit reaches maturity. *Cassia sieberiana* is found in multiple parts of Africa including the southern part of the Sahel, Senegal, Sudan, and Uganda. It is also found in East Africa¹¹⁴. *Cassia sieberiana* grows best in well drained, humid soils with an annual rainfall of approximately 20 inches. It typically grows as a shrub in very dry regions. These shrubs grow in groups of other plants, they usually never grow alone¹¹⁴.

Cassia sieberiana DC also called *Cassia kotschyana* Oliv. Commonly called *malga* (Hausa), *sisanghi* (Fulani), *kumborisaka* (Moors) and *badinzikki* (Kanuri) is a small to medium size deciduous tree, reaching 10 to 20 m in height with fairly dense

spreading crown with branches inclined downward, is a plant of many medicinal uses. The root and the stem bark are used to treat elephantiasis, leprosy, venereal disorders, bilharzias and hemorrhoids. The pod is used for jaundice, fever, gonorrhoea, as a vermifuge, as laxative and for wound dressing by various communities of West Africa¹¹⁵. Traditional healers of Burkina Faso use the root bark to treat guinea fowl disease, sterility, dysmenorrhoea, amenorrhoea, venereal disease and impotency in farm animals. The leaves are used to treat worms, diarrhoea, stomach pains, fever, bilharzias, leprosy, dropsy and as a diuretic¹¹⁵. Ghanaian traditional medicine practitioners use *C. sieberiana* root and stem bark for pain relief of abdominal origin, dysmenorrhoea, ulcer and general body pain¹¹⁶. The interest in the scientific investigation of this medicinal plant from Nigeria is based on the claims of its effective use for the treatment of many diseases based on the data collected from traditional healers. Therefore, research into the effects of these local medicinal plants is expected to enhance the use of the plant extract against diseases. The active principles of many drugs found in plants are secondary metabolites^{117,118}.

In Nigeria, extracts of the roots, stem bark and fruit pulp of *C. sieberiana* are used traditionally for the treatment of inflammatory conditions, fever, joint pains, malaria, diarrhoea, leprosy, bilharzias,

stomach pains, diabetes mellitus and its complications and other illnesses^{115,119}. In Senegal, Uganda and Cote d'Ivoire, decoctions of the root or infusions of the whole plant are used as purgative and diuretic, and recommended for the treatment of hemorrhoids, bilharzias, leprosy, dropsy, intestinal worm infestations, diabetes mellitus and numerous childhood illnesses^{109,120}. It had also been shown that *C. sieberiana* extracts has antimicrobial activity against *Neisseria gonorrhoea*, Herpes simplex virus type I and African swine fever virus¹²¹. In traditional medicine, the plant is used as antimicrobial, antiviral, antibacterial, anti-inflammatory, antitrypanosomal and antioxidant agent, as a strong purgative, diuretic, abortifacient, anti-schistosomiasis, anti-dysentery and anti-hemorrhoid^{122,123}. *Cassia sieberiana* is a savannah tree with a wide application including the ethno pharmacological use of its roots in the management of various stomach disorders including gastric ulcer, stomach pains and indigestion¹²⁴. At the Centre for Scientific Research into Plant Medicine (CSRPM) in Ghana, an aqueous suspension of the powdered roots bark is used to manage abdominal colic and pains associated with the joints. Earlier studies we conducted indicated that the aqueous roots bark extract of *C. sieberiana* possesses anti-ulcerogenic properties against gastric ulcers induced by various methods¹²⁵. *Cassia sieberiana* is used to make tools, pestles, mortars, and also

used for construction because it is a very hard wood that is resistant to termites. In addition, it is also an ornamental tree because of its brightly colored flowers. Some cultures also incorporate the plant in their religion and for superstitious and magical purposes¹¹⁴. Parts of the plant have also been used traditionally as teeth cleaning twigs¹²⁶.



Cassia sieberiana

Aims of this study

This study aimed to fulfill the following:

- Extraction flavonoids from three medicinal plants: *Terminalia brownii*, *Zizphus abyssinica* and *Cassia sieberiana*.
- Isolation of flavonoids via some analytical methods.
- Characterization of the isolated flavonoids via spectral tools.
- Evaluation of the antimicrobial activity of plant extracts.

Chapter Two
Materials and Methods

2. Materials and methods

2- 1: Materials

2- 1- 1: Plant material

The roots of *Terminalia brownie*, *Ziziphus abyssinica* and *Cassia sieberiana* were collected from Kordofan, western Sudan. The plants were identified and authenticated by direct comparison with reference herbarium samples-Medicinal and Aromatic Plants Research Institute.

2- 1- 2: Materials for paper chromatography

- i. Whatman paper No (3mm) were used for preparative paper chromatography (Whatman Ltd., England).
- ii. Glass jars (rectangular glass tanks 100 x80 x 40cm) for developing PC chromatograms.

2- 1- 3: Materials for antimicrobial assay

The following microbial strains were used to assess the antimicrobial activity of the studied plant extracts:

Bacillus subtilis and *Staphylococcus aureus* (G+ve); *Escherichia coli* and *Pseudomonas aeruginosa* (Gram –ve) and the fungal species *Candida albicans*.

Ampicilin, gentamycin and clotrimazole were used as positive controls. Mueller- Hinton agar is used as media for bacterial growth while Sabouraud dextrose agar was used for culture of yeast.

2- 1- 4: Equipments

1- Ultra - Violet - Visible spectra were run on a Shimadzu (model UV240 and 240PC) spectrophotometer.

2- Nuclear Magnetic Resonance (NMR) spectra were measured on a Bruker (AC-250) spectrophotometer operating at 500 MHz.

2- 1- 5: Solvents

Analytical grade solvents were used. Methanol purchased from Merck, Germany was used for spectrophotometric analysis.

DMSO-d₆ was used as solvent and TMS as internal standard.

2- 2: Methods

2- 2- 1: Extraction of flavonoids

The plant material (1Kg) was macerated with 95% ethanol for 72h. at room temperature. The extract was filtered and the solvent was removed *in vacuo*.

2- 2- 2: Isolation of flavonoids

Concentrated plant extract was applied on Whatman 3mm paper (46×57 cm) and run in BAW (5:1:4;v:v:v) upper layer for *Ziziphus abyssinica* and methanol: chloroform (8:2;v:v) for *Cassia sieberiana*. The dried papers were viewed and examined under visible and ultraviolet light. The chromatograms were then located under UV light, cut off and similar bands were joined and cut into small pieces and slurred with methanol. After several hours of contact the solvent was removed. Compound I was isolated from *Terminalia brownie*, compound

II from *Ziziphus abyssinica* and compound III from *Cassia sieberiana*.

Silica gel plates developed with 60% acetic acid were used for the isolation of flavonoids from *Terminalia brownie*.

2- 2- 3: Phytochemical screening

The studied plant species were screening for the presence of major metabolites: flavonoids, phenolics, tannins, terpenoids, saponins and alkaloids as follows:

a) Test for alkaloids

To the extract, dilute hydrochloric acid was added, then it was shaken well and filtered. For the filtrate, the following tests were performed.

- Mayer's reagent test

To 3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of creamy precipitate indicates presence of alkaloids.

- Wagner test

To 2 ml of filtrate, few drops of Wagner's reagent were added. Formation of reddish brown precipitate indicates the presence of alkaloids.

b) Tests for carbohydrates (Benedict's test)

Equal volumes of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10 minutes. In presence of sugars the solution appears green, yellow or red

depending on the amount of reducing sugar present in the test solution.

c) Test for flavonoids

i. Alkaline reagent test: The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow color, which becomes colorless on addition of few drops of dilute acid, indicates the presence of flavonoids.

ii. Lead Acetate: The extract was treated with few drops of lead acetate solution. Formation of yellow precipitate indicates the presence of flavonoids.

d) Test for tannins

i) Ferric chloride test

A small amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of tannins.

ii) Lead acetate test

A small amount of extract was dissolved in distilled water. To this solution few drops of lead acetate solution were added. Formation of white precipitate indicates the presence of phenolic compounds.

e) Test for saponins

Froth test:

The extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. The formation of a persistent a layer of foam indicates the presence of saponins.

2- 2- 4: Antimicrobial assay

An inoculum suspension was swabbed uniformly to solidify 20 ml Mueller-Hinton Agar (MHA) for bacteria, and the inoculum was allowed to dry for 5 min. Holes of 6 mm in diameter were made in the seeded agar . Using glass Pasteur pipettes aliquot of 20 μ l from each plant crude extract (100 mg/ml) was added into each well on the seeded medium and allowed to stand on the bench for 1 h for proper diffusion and thereafter incubated at 37°C for 24 h. The resulting inhibition zones were measured in millimeters (mm).The assays were repeated in duplicate and the concurrent values were taken. The activity is expressed as less active, if the zone of inhibition is 9-12 mm, moderate 13-16 mm and high greater than 17 mm.

2- 2- 5: Antioxidant activity

In 96-wells plate, the test samples were allowed to react with 2,2-Di (4-tert-octylphenyl)-1-picryl-stable free radical (DPPH) for half an hour at 37 °C. the concentration of DPPH was kept as (300 μ M). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiple reader

spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate. Propyl gallate was used as standard drug at concentrations 0.5 μM .

2- 2- 6: Structural elucidation of flavonoids

The structure of the isolated compound was elucidated via a combination of spectral techniques as illustrated below.

2- 2- 6- 1: UV-Visible spectroscopy

UV-Visible spectra of the isolated flavonoids were recorded on a Shimadzu spectrophotometer. Spectra were recorded in a quartz cuvette (1cmx1cmx4.5cm) which did not absorb over the spectral region of interest. One (mg) of the isolated flavonoid was dissolved in HPLC grade methanol (10mL) to create a stock solution. The UV-visible spectrum was taken for this solution at a rapid scan rate (600nm/min) to check the optical density. The concentration of the flavonoid solution was then adjusted so that the optical density of the major absorption peak (between 250-400nm) was within 0.6 to 0.8AU. The spectrum of the flavonoid stock solution was measured at a normal scan speed (50nm/min) over the 200-800nm region. Additional spectra were measured over the regions of peak maxima at a reduced scan rate (10nm/min) to determine the wavelength of maxima accurately. Sodium methoxide solution (3 drops) was added to the cuvette of flavonoid in methanol. The spectrum was then recorded

immediately at a normal scan rate. The solution in the cuvette was then discarded. Aluminium chloride solution (6 drops) was then added to fresh flavonoid stock solution (2-3mL) in the cuvette. The spectrum was then recorded at a normal scan rate. Hydrochloric acid (3 drops) was then added to the cuvette containing aluminium chloride and flavonoid in methanol. The spectrum was recorded again and the solution discarded. An excess of powdered anhydrous sodium acetate was added to fresh flavonoid stock solution (2-3mL) in the cuvette and shaken. The powdered sodium acetate formed a layer (1-2mm) at the bottom of the cuvette. The spectrum was recorded immediately. Finally, an excess of powdered anhydrous boric acid was added with shaking to the saturated sodium acetate solution. The spectrum was then recorded. In cases where the flavonoid decomposed in the presence of sodium methoxide, a different method was used to obtain a boric acid/sodium acetate spectrum. Boric acid solution (5 drops) was added to fresh flavonoid stock solution (2-3mL) which was quickly saturated with powdered sodium acetate and the recorded immediately.

2- 2- 6- 1- 1: Preparation of UV - shift reagents

- Sodium methoxide solution

Freshly cut 2.5g metallic sodium was dissolved, cautiously, in 100 ml spectroscopic methanol (dry methanol).

- Aluminum chloride solution

(5g) anhydrous aluminium chloride was cautiously dissolved in 100 ml spectroscopic methanol and filtration was carried out after about 24 hours.

- Hydrochloric acid

50 ml concentrated hydrochloric acid was mixed with 100 ml distilled water, then keep in glass bottle.

- Sodium acetate

Anhydrous reagent grade sodium acetate was melted and allowed to stand for about 10 minutes. The material was then powdered and stored in a dry bottle.

- Boric acid

Anhydrous powdered reagent grade boric acid was used.

2- 2- 6- 2: Nuclear magnetic resonance spectroscopy of (NMR)

NMR spectrum was obtained on a Bruker Advance DRX instrument. Sample was dried by freeze drying over several days and dissolved in DMSO-d₆ and then analyzed.

Chapter Three
Results and Discussion

3. Results and Discussion

The flavonoids of three plants of potential medicinal attributes to Sudanese ethno medicine have been investigated. One flavonoid was isolated from *Terminalia brownie* roots another one was isolated from *Ziziphus abyssinica* roots while a third flavonoid was isolated from *Cassia sieberiana* roots. These flavonoids were characterized by a combination of spectral techniques (UV and ¹HNMR). The antimicrobial potential of these plants has been assessed.

3- 1: *Terminalia brownie*

3- 1- 1: Phytochemical screening

Terminalia brownie was screened for major secondary metabolites and the results are displayed in Table 1.

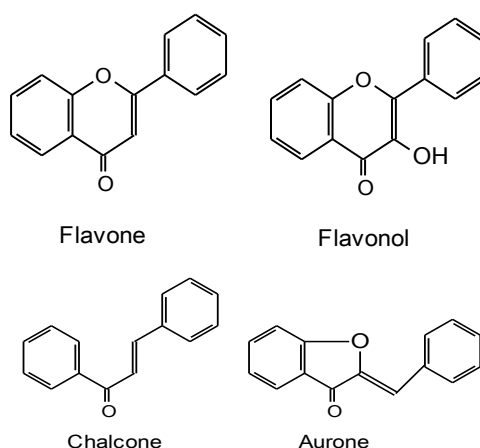
Table 1: Phytochemical screening of *Terminalia brownie* roots

Secondary metabolite	occurrence
Saponins	+
Coumarins	+
Alkaloids	+
Flavonoids	+
Tannins	+
Steroids	+
Terpenes	+

3- 1- 2: Characterization of compound I

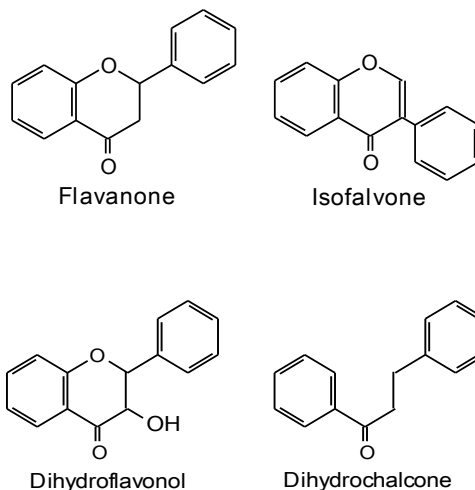
From the ethanol extract of *Terminalia brownie* roots a flavonoid –compound I has been isolated by thin layer chromatography (TLC). The structure of the isolated flavonoid has been partially characterized by some spectral data (UV and NMR).

The UV spectra of flavonoids provide important spectral data for the characterization of flavonoids. Most flavonoids are unsaturated at C₂-C₃ link and exhibit two absorption bands in the UV-referred to as band II (in the range 230-285nm) and band I (in the range 300-400nm). Band I is associated with the absorption of the cinnamoyl chromophore and band II is due to the benzoyl chromophore. Four classes of flavonoids are known to give two UV bands: the flavones, flavonols, chalcones and aurones.



On the other hand, those flavonoids which are characterized by loss of conjugation between the two aromatic rings(A and B) of flavonoids exhibit only one absorption band – band II which

accounts for the absorption of the benzoyl chromophore. These are : the flavanones, isoflavones, dihydrochalcones and dihydroflavonols.



In the UV, compound I absorbs at λ_{\max} 220,354nm. Such absorption is given by: flavones, flavonols, chalcones and aurones. Chalcones give a dominant band I absorption, while aurones have band I beyond 400nm. This indicates that the isolated flavonoid is either a flavones or a flavonol. Flavonols absorb in the range 358-400nm (band I), while flavones absorb in the range 300-356nm. Consequently the isolated flavonoid is a flavone.

Next different UV shift reagents (sodium methoxide, sodium acetate and aluminium chloride) have been used to establish the hydroxylation pattern on the nucleus of the flavonoid. The shift reagent sodium methoxide induces a bathochromic shift in presence of a 3- or 4`-OH functions, while sodium acetate gives bathochromic shifts diagnostic of a 7-OH group. Aluminium

chloride is used in the chemistry of flavonoids for the specific detection of 3-, 5-OH groups as well as catechol moieties.

The sodium methoxide spectrum (Fig. 2) of compound I did not reveal any bathochromic shift suggesting absence of 3- and 4'-OH functions. Also the aluminium chloride spectrum (Fig.3) failed to show a bathochromic shift indicating absence of 3- and 5-OH groups as well as catechol systems. The sodium acetate spectrum (Fig.4) exhibited a bathochromic shift indicative of a 7-OH function.

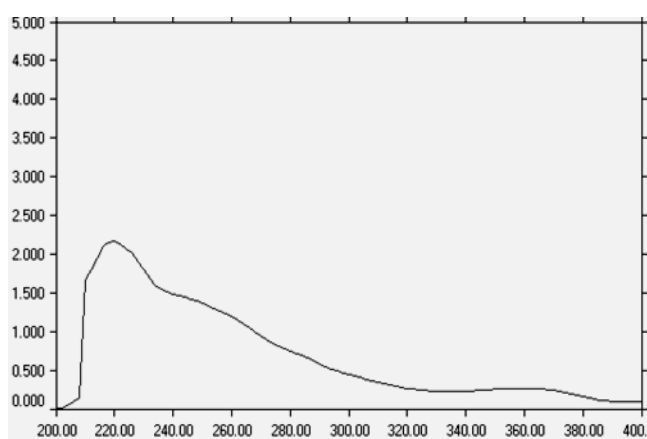


Fig. 1: UV spectrum of compound I

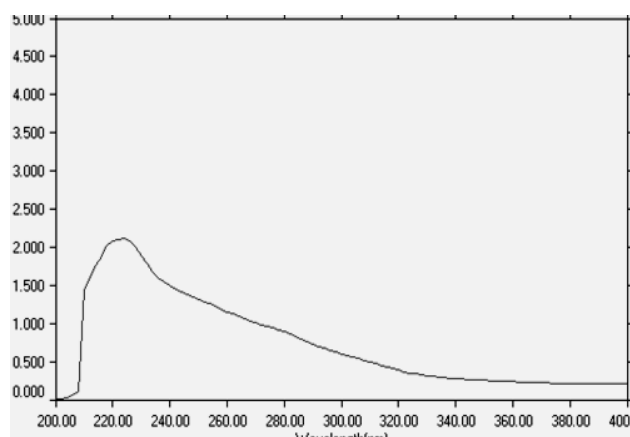


Fig. 2: Sodium methoxide spectrum of compound I

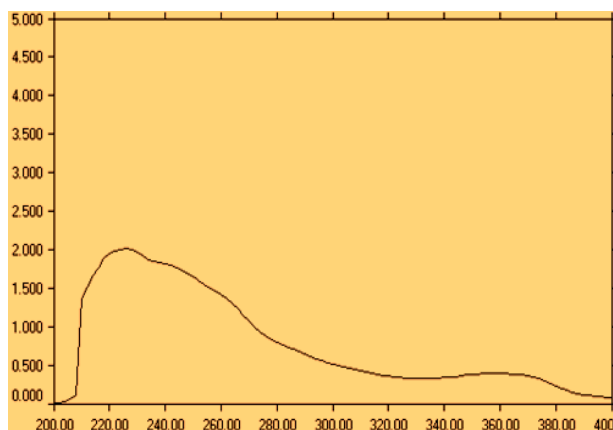


Fig. 3: Aluminium chloride spectrum of compound I

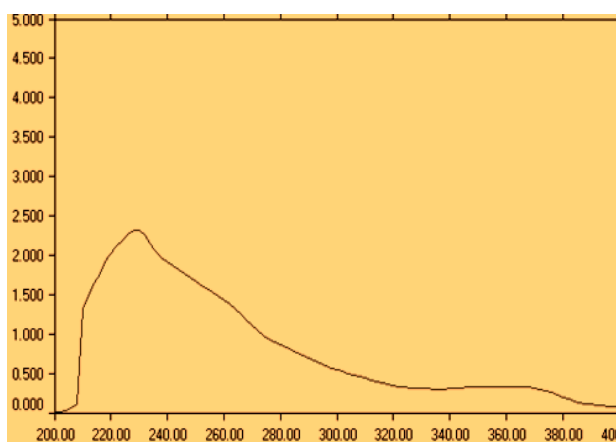
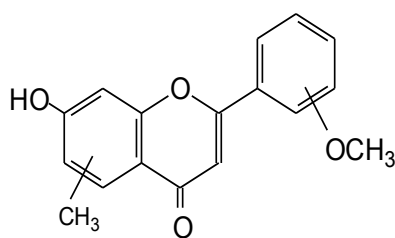


Fig. 4: Sodium acetate spectrum of compound I

The ^1H NMR spectrum (Fig 5) showed δ (ppm): 1.20 (assigned for a methyl group); 4.40, 4.85, 5.40 assigned for sugar protons (other sugar protons overlapped the solvent-DMSO- residual water protons which resonated within the multiplet: δ 3.00-3.85). This sugar was not identified in this study. The resonance at δ 4.10 accounts for a methoxyl function. The aromatic protons appeared at δ 6.70ppm. The signal at δ 2.50 is due to the solvent residual protons. On the basis of the above argument, the following partial structure was proposed for the aglycone of compound I:



Compound I

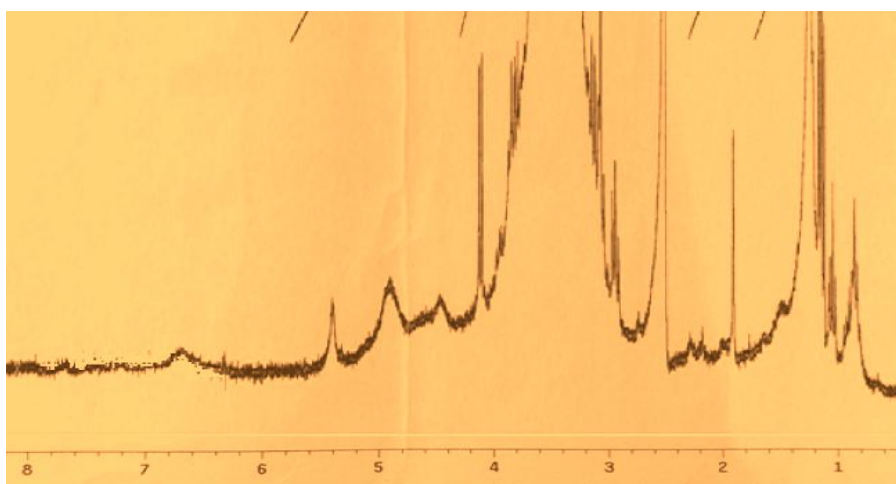


Fig. 5: ^1H NMR spectrum of compound I

3- 1- 3: Antimicrobial activity

The ethanol extract of *Terminalia brownie root* was screened for antimicrobial activity against five standard human pathogens (Table 2). The results are presented in Table 3. The results were interpreted as follows: (<9mm: inactive; 9-12mm: partially active; 13-18mm: active; >18mm: very active). Ampicilin, gentamycin and clotrimazole were used as positive controls (Tables 4 and 5).

The extract showed significant activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It also exhibited

moderate activity against *Escherichia coli* and the yeast *Candida albicans*.

Table 2: Test organisms

No	Micro organism	Type	Source
1	<i>Bacillus subtilis</i>	G+ve	ATCC 2836(*)
2	<i>Staphylococcus aureus</i>	G+ve	ATCC 29213
3	<i>Pseudomonas aeruginosa</i>	G-ve	NCTC(**) 27853
4	<i>Escherichia coli</i>	G-ve	ATCC 25922
5	<i>Candida albicans</i>	fungi	ATCC 7596

* NCTC. National collection of type culture, Colindale. England

*ATCC. American type culture collection, Maryland, USA

Table 3: Antimicrobial activity of ethanol extract

Sample	Ec	Pa	Sa	Bs	Ca
Ethanol extract	15	20	25	-	15

Table 4: Antibacterial activity of standard drugs

Drug	Conc. (mg/ml)	Bs	Sa	Ec	Pa
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-

Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 5: Antifungal activity of standard drug

Drug	Conc.(mg/ml)	Ca
Clotrimazole	30	38
	15	31
	7.5	29

Sa: *Staphylococcus aureus*

Ec: *Escherichia coli*

Pa: *Pseudomonas aeruginosa*

Bs: *Bacillus subtilis*

Ca: *Candida albicans*

3- 1- 4: Antioxidant activity

The antioxidant capacity of the ethanol extract of *Terminalia brownie* root was carried out by measuring the capacity of the test sample against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As shown in (Table 6) the extract exhibited significant antioxidant activity. Propyl gallate was used as positive control.

Table 6: Antioxidant activity of ethanol extract

Sample	Antioxidant activity
Propyl gallate	92.00%
Ethanol extract	86.00%

3- 2: *Ziziphus abyssinica*

3- 2- 1: Characterization of compound II

Ethanol extract of *Ziziphus abyssinica* root was purified via paper chromatography to give a flavonoid - compound II. The UV spectrum of compound II (Fig.6) showed λ_{\max} (MeOH) 274 nm. Such absorption is given by: the flavanones, isoflavones, dihydroflavonols and dihydrochalcones. Isoflavones are easily distinguished by a shoulder in the range: 300-340nm. Such shoulder was not detected in the UV spectrum of compound II. Also the UV shift reagent – sodium methoxide which is diagnostic of 3-and 4`-OH groups – revealed (Fig. 7) only a small bathochromic shift indicating absence of a 3-OH function which is a characteristic feature of dihydroflavonols. The ¹HNMR spectrum (Fig.10) showed a multiplet around 2.80ppm and signals in the range δ 5.4-5.65ppm accounting for the resonances of C₂ and C₃ protons of flavanones.

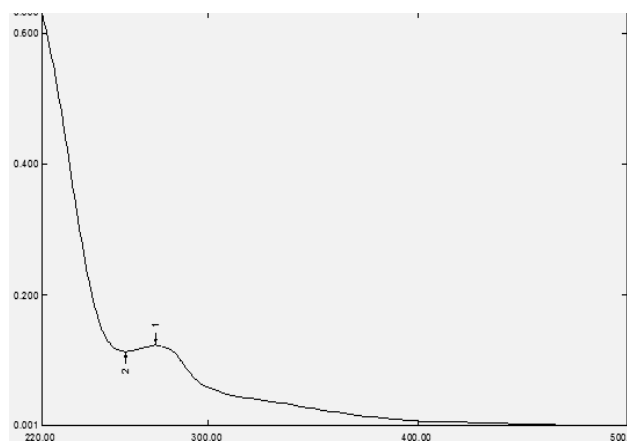


Fig. 6: UV spectrum of compound II

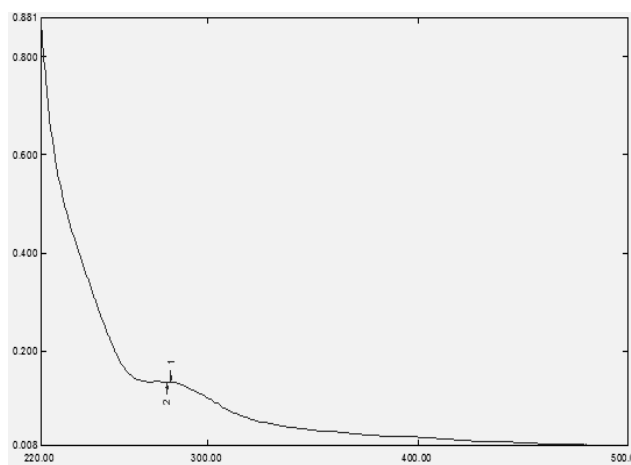


Fig. 7: Sodium methoxide spectrum of compound II

Very useful structural features are gained by using different UV shift reagents such as sodium acetate and aluminum chloride. Sodium acetate is diagnostic of a 7-OH where a bathochromic is observed when the sodium acetate spectrum is recorded. No bathochromic shift was observed in the sodium acetate spectrum of compound II (Fig. 8) indicating absence of a 7 - OH function.

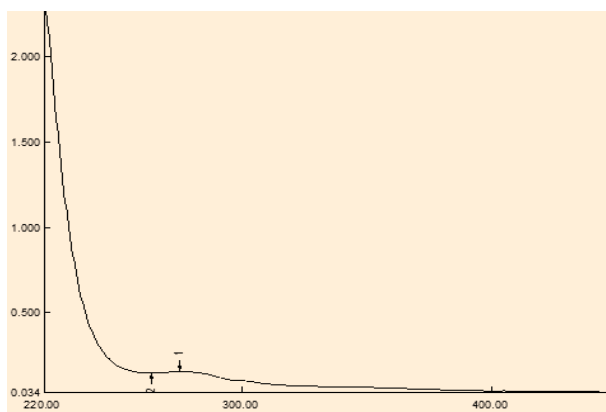


Fig. 8: Sodium acetate spectrum of compound II

In the chemistry of flavonoids, aluminum chloride is an extremely useful complexing agent which afford diagnostic bathochromic shifts characteristic of: 3- , 5- OH as well as catechol systems. The aluminum chloride spectrum (Fig. 9) failed to reveal a bathochromic shift indicating absence of 3-, 5-OH groups and catechol systems.

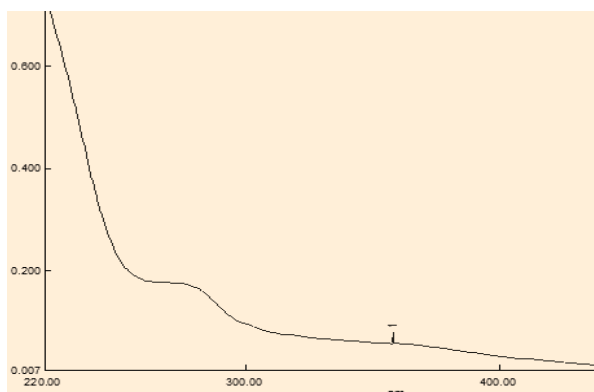


Fig. 9: Aluminium chloride spectrum of compound II

The ^1H NMR spectrum of compound II (Fig. 10) gave δ (ppm): 1.22 (assigned for two methyl groups); 4.11 (methoxyl); 5.85 (Ar. proton). Other aromatic protons resonated as multiplet in the range δ (6.62-6.90). Signals at δ 2.50 and 3.30 are due to solvent (DMSO) residual protons and residual water respectively.

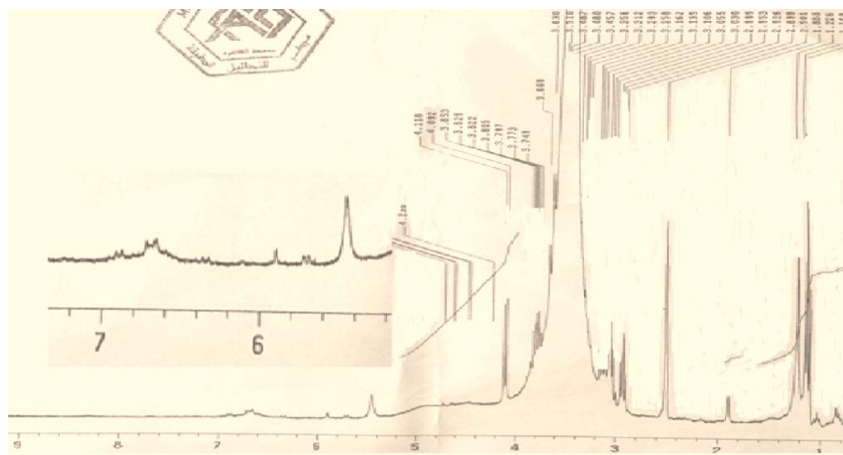
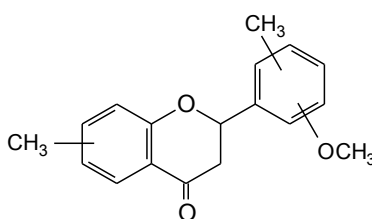


Fig. 10: ^1H NMR spectrum of compound I

On the basis of the above spectral data the following partial structure was proposed for compound II:



Compound II

3- 2- 2: Antimicrobial assay

The ethanol extract of *Ziziphus abyssinica* was evaluated for antimicrobial activity against five standards pathogenic. The results of Table (7) showed moderate activity against *Staphylococcus aureus* and the yeast *Candida albicans*. The extract exhibited partial activity against other test organisms (*Pseudomonas aeruginosa*, *Bacillus subtilis* and *Escherichia coli*). Ampicillin, gentamycin and clotrimazole were used as positive controls, while DMSO has been used as negative control.

Table 7: Antimicrobial activity of *Ziziphus abyssinica* ethanol extract

Organism	Inhibition growth zone diameter (MIZD)
	Root extract (100mg/ml)
<i>Bacillus subtilis</i>	12
<i>Staphylococcus aureus</i>	14
<i>Escherichia coli</i>	10
<i>Pseudomonas aeruginosa</i>	12
<i>Candida albicans</i>	13
Organism	Ampicilin (40mg/ml)
<i>Bacillus subtilis</i>	15
<i>Staphylococcus aureus</i>	30
Organism	Gentamycin (40mg/ml)
<i>Escherichia coli</i>	22
<i>Pseudomonas aeruginosa</i>	21
Organism	Clotrimazole(30mg/ml)
<i>Aspergillus niger</i>	22
<i>Candida albicans</i>	38

3- 3: *Cassia sieberiana*

3- 3- 1: Phytochemical screening

Cassia sieberiana was screened for major secondary metabolites and the results are depicted in Table 8.

Table 8: Major secondary metabolites in *Cassia sieberiana*.

Saponin	Cumarin	Alkaloids	Flavonoids	Tannins	Steroids	Triterpens	Anthraquinone
+	-	-	+	-	+	+	+

3- 3- 2: Characterization of compound III

Compound III was isolated as yellow amorphous powder from *Cassia sieberiana* by paper chromatography. The UV spectrum of compound III showed λ_{max} 274nm (Fig.11). Such absorption is given by: flavanones, isoflavones, dihydrochalcones and dihydroflavonols. In the UV spectrum of this compound the characteristic shoulder of isoflavones, this usually appears in the UV range: 300-340 nm was not observed.

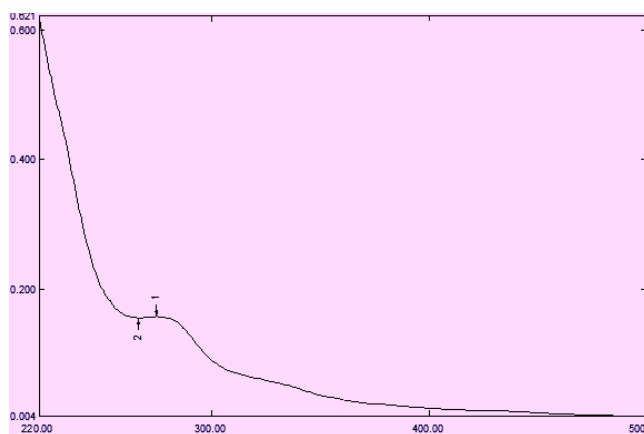


Fig. 11: UV spectrum of compound III

The shift reagents – sodium methoxide – was used for the detection of 3- , 4`-OH functions. When sodium methoxide was added to a methanolic solution of compound III, a bathochromic shift with decrease in intensity has been detected (Fig. 12) suggesting the presence of a 3-OH group which is a characteristic feature of dihydroflavonols. Hence compound III is a dihydroflavonol.

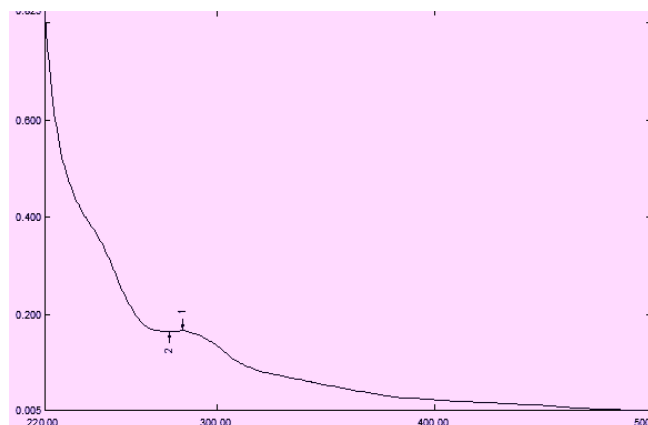


Fig. 12: Sodium methoxide spectrum of compound III

Now, that the classes of the isolated flavonoid being unmasked, different UV shift reagents were used to clarify the citation of hydroxyl functions on the nucleus of the flavonoid. When the shift reagent –sodium acetate – was added to a methanolic solution of compound III, no bathochromic shift was detected indicating absence of a 7-OH group (Fig. 13).

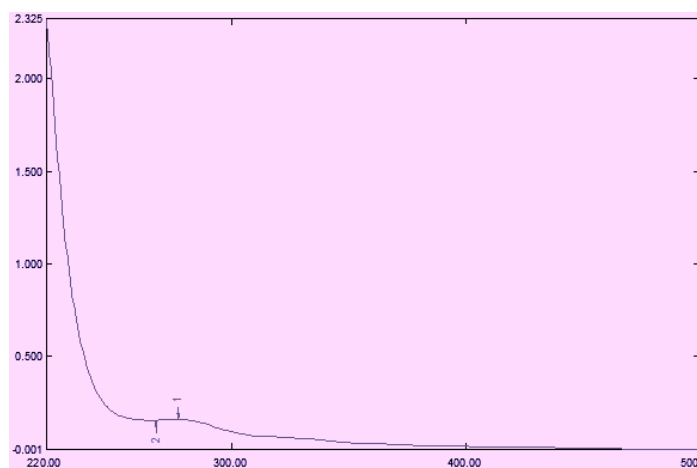


Fig. 13: Sodium acetate spectrum compound III

The shift reagent – boric acid – was used (Fig.14), but it failed to exhibit any bathochromic shift indicating absence of catechol systems.

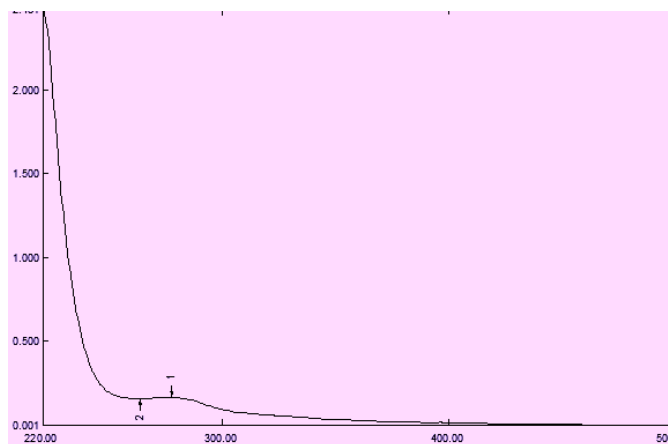
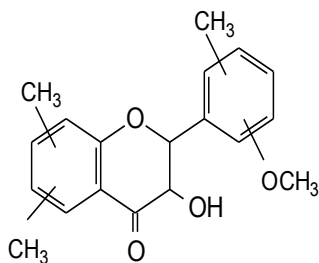


Fig. 14: Boric acid spectrum of compound III

The ^1H NMR spectrum (Fig. 15) showed δ (ppm): 1.20 (9 H) assigned for 3 methyl groups; 3.85 accounts for a methoxyl function. The aromatic protons resonated at δ 6.13, 6.67 and 7.22ppm. On the basis of this spectral data, the following partial structure was suggested for compound III:



Compound III

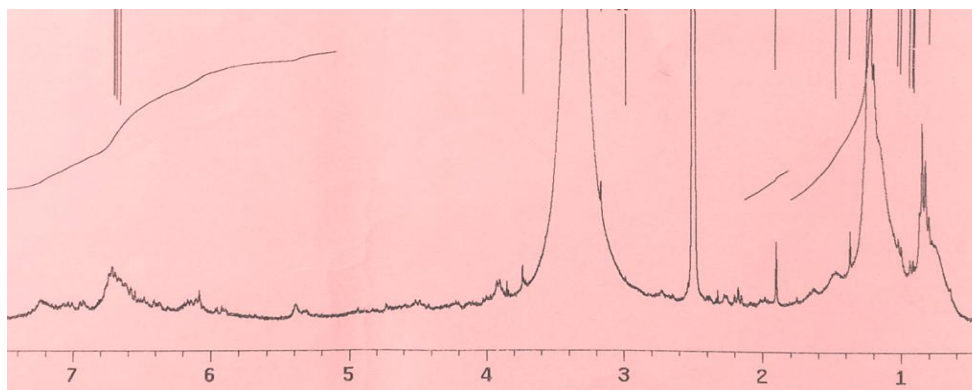


Fig. 15: ¹H NMR spectrum of compound III

3- 3- 3: Antimicrobial assay

The ethanol extract of *Cassia sieberiana* was assessed for antimicrobial activity against five standard human pathogens. The results are depicted in Table (9). The results were interpreted as follows: (<9mm: inactive; 9-12mm: partially active; 13-18mm: active; >18mm: very active).

The extract exhibited partial activity against all test microbes.

Table 9: Antimicrobial activity of *Cassia sieberiana*

Sample	Sa	Bs	Ps	Ec	Ca
Ethanol extract	12	11	11	10	10

Sa: *Staphylococcus aureus*
 Ec: *Escherichia coli*
 Pa: *Pseudomonas aeruginosa*
 Ca: *Candida albicans*
 Bs: *Bacillus subtilis*

3.3.4- Antioxidant assay

The antioxidant capacity of the ethanol extract of *Cassia sieberiana* has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test sample against stable DPPH radical. The change in colour was measured spectrophotometrically at 517nm. As depicted in Table 10, the ethanol crude extract exhibited significant antioxidant activity.

Table 10: Radical scavenging activity of the ethanol extract

Sample	Antioxidant activity (%)
Propyl gallate	91.9±0.01
Ethanol extract	71.3±0.03

Conclusion

This study was designed to study the flavonoids of three Sudanese medicinal plants and to assess their antimicrobial and antioxidant effect. These species are: *Terminalia brownie*, *Ziziphus abyssinica*, *Cassia sieberiana*. One flavonoid-compound I- was isolated from *Terminalia brownie* root another one-compound II- was isolated from *Ziziphus abyssinica* root while a third flavonoid-compound III- was isolated from *Cassia sieberiana* root.

These plants were screened for their antimicrobial and antioxidants potential and significant to moderate responses were detected.

Recommendations

The following is highly recommended:

1-Other phytochemicals (alkaloids, steroids.... etc) of the target species may be isolated and their structures may be elucidated and the biological activity could be screened.

2-The isolated flavonoids may be subjected to *in vivo* antimicrobial potency.

3-The isolated flavonoids may be screened for other biological effects such as anti-inflammatory, antidiabetic, antiviral, antileishmanial.... etc.

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