Sudan University of Science and Technology

College of Graduate Studies

Chemical Characterization and Biological Activity of some Flavonoids from Acacia nilotica var. tomentoza, Indigofera oblongifolia Stems and Acacia ehrenbergiana Roots

التوصيف الكيميائى والاثار البيولوجية لبعض فلافونيدات ساق السنط وساق الدهاسير وجذور السلم

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

By

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استهلال

بسم الله الرحمن الرحيم

﴿ ٱلۡحَمۡدُ لِلَّهِ ٱلَّذِيٓ أَنزَلَ عَلَىٰ عَبۡدِهِ ٱلۡكِتَٰبَ وَلَمۡ يَجۡعَل لَّهُۥ عِوَجَاۜ ١ قَيِّمٗا لِّيُنذِرَ بَأۡسٗا شَدِيدٗا مِّن لَّدُنۡهُ وَيُبَشِّرَ ٱلۡمُؤۡمِنِينَ ٱلَّذِينَ يَعۡمَلُونَ ٱلصَّٰلِحَٰتِ أَنَّ لَهُمۡ أَجۡرًا حَسَنٗا ٢ مَّٰكِثِينَ فِيهِ أَبَدٗا ٣ وَيُنذِرَ ٱلَّذِينَ قَالُواْ ٱتَّخَذَ ٱللَّهُ وَلَدٗا ٤ ﴾

صدق الله العظيم

الكهف: ١ - ٤

Dedication

To

My father

The soul of my mother

My wife and children(Eyad and Jawad , Maryam and Fatima), brothers and sisters.

Acknowledgement

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

With respect and gratitude I thank my supervisor, Prof. Mohammed Abdel Karim Mohammed who patiently and continuously provided valuable assistance, guidance and experience from the beginning to the end of this study.

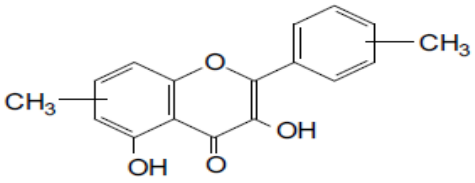
Special thanks are also extended to the technical staff of the faculty of science, Sudan University of Science and Technology and also to Deanship of Academic Affairs, Albutana University for facilities.

Thanks for the Medicinal and Aromatic Plant Research Institute for all facilities. Thanks are also extended to the National Research Center, Cairo ,Egypt for the spectral measurements.

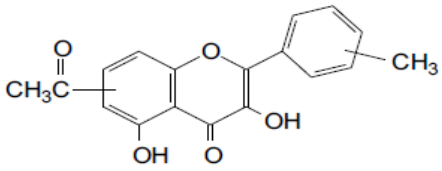
My deep thanks are due to my family and friends who encouraged me to complete this work .

ABSTRACT

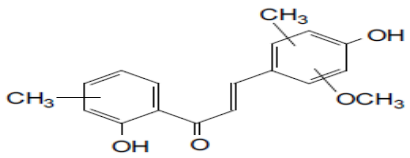
This research was aimed to study the flavonoids of the heartwood of *Acacia nilotica* var. *tomentosa*, *Indigofera oblongifolia*, and the roots of *acacia ehrenbergiana*. Compound I was isolated from *Acacia nilotica* var. *tomentosa*. Compound II was isolated from *Indigofera oblongifolia* while compound III was isolated from *Acacia ehrenbergiana*. These compounds were purified by different chromatographic techniques and identified via spectroscopic tools;UV, and ^{I}HNMR.Depending on their spectral data , they were assigned the following partial structures:



Compound I



Compound II



Compound III

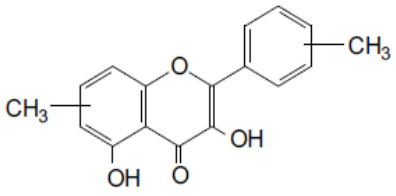
The isolated compounds were evaluated for their antiaimicrobial potential against standard pathogenic microbes. Compound I has been screened for antimicrobial activity against six human pathogens. It showed excellent activity against *Pseudomonas aeroginosa* and *Bacillus subtilis*. It also exhibited moderate activity against *Escherichia coli*. Compound II showed good activity against *Escherichia coli, Pseudomonas aeroginosa* and *Bacillus subtilis* It also showed weak activity against *Aspergillus niger* and weak anticandidal potency. The compound also exhibited partial activity against *Staphylococcus aureus.* Compound III showed excellent activity against *Bacillus subtilis*. It also exhibited good activity against *Pseudomonas aeroginosa*, *Aspergillus niger* and good anticandidal potency. However, it showed parial activity against *Escherichia coli*, and *Staphylococcus aureus.*

The isolated compound were evaluated for their antioxidant activity.The antioxidant capacity of compound I has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. Compounds I,II and II exhibited significant radical scavenging capacity.

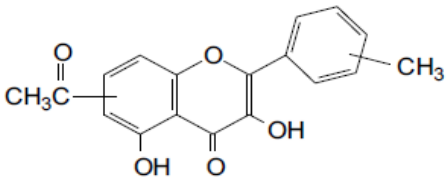
المستخلص

درست المركبات الفلافونيدية لخشب أشجار السنط والدهاسير وجذور السلم من المستخلص الكحولي لخشب أشجار السنط تم فصل المركب I ومن الدهاسير تم فصل المركب II , أما المركب III فتم فصله من أشجار السلم.

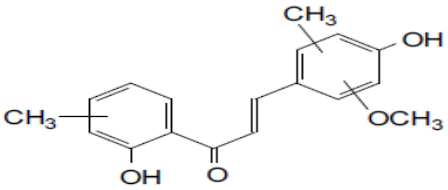
تمت تنقية جميع المركبات بطريقة الكروموتغرافيا الورقية وحُددت التراكيب المبدئية بواسطة طيفى الأشعة فوق البنفسجية و الرنين النووي المغنطيسي للبروتون.



Compound I



Compound II



Compound III

اجريت التحاليل الميكروبيولوجية للمركبات المستخلصة على بعض الميكروبات القياسية حيث اظهر المركب I نشاطا عاليا ضد (*Pseudomonas aeroginosa* و *Bacillus subtilis*) ونشاط متوسط ضد (*Escherichia coli*)، أما المركب II فقد أظهر نشاطا جيداً ضد (*Escherichia coli , Pseudomonas aeroginosa ; Bacillus subtillus*)، ونشاطاً ضعيفاً ضد (*Aspergillus niger)* ونشاطاً ضد (*Candida albicans*) كما وأظهر نشاطاً جزئياً ضد (*Staphylococcus aureus*). أما المركب III فقد أبدى نشاطاً عالياً ضد (*Bacillus subtilis*) وأظهر نشاطاً جيداً ضد (Pseudomonas aeroginosa, Aspergillus niger) ونشاطا جيدا ضد الفطر (Candida albicans) ونشاط جزئ ضد (*Escherichia coli , Staphylococcus aureus*).

وفى اختبار مضاد الأكسدة أظهرت المركبات الثلاثة فعالية كبيرة كمضادات للأكسدة.

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Chapter one

Introduction

1-Introduction

1.1-General oveview

Nowadays there is an ever increasing interest in medicinal plants. Medicinal plants may be defined as those plants that are commonly used in treating and preventing specific ailments and diseases that are generally considered to be harmful to humans[1].These plants are either wild plant species growing spontaneously in self-maintaining populations in natural or semi-natural ecosystems and could exist independently of direct human actions or domestic. Domesticated plants species are those that have arisen through human actions such as selection or breeding and depend on management for their existence, for example *Aloe barbadensis* . It has been reported that infectious diseases account for one-half of all deaths in the tropical countries. As a result, people of all continents have long applied poultice and imbibed infusions of indigenous plants dating back to prehistory for health purposes and is still in use today . Plant medicine (phytomedicine) has been used in healthcare delivery in many parts of Africa and the rest of the world. Effective health cannot be achieved in Africa, unless orthodox medicine is complemented with traditional medicine . At least 80% of Africans depend on plant medicine for their healthcare . Fruits and vegetables have been recognized as natural sources of various bioactive compounds which could be attributed

to their phyto constituents such as flavonoids, anthocyanins, vitamins C and E, phenolic compounds, dietary fiber, and carotenoids present in fruits and vegetables. One of such medicinal plant is *Citrullus lanatus*. Although several of its uses in traditional medicine have been documented, many of these claims are yet to be validated by scientific researchers. The study of medicinal plants starts with the pre-extraction and the extraction procedures, which is an important step in the processing of the bioactive constituents from plant materials. Traditional methods such as maceration and Soxhlet extraction are commonly used at the small research setting or at Small Manufacturing Enterprise (SME) level. Significance advances have been made in the processing of medicinal plants such as the modern extraction methods; microwave-assisted (MAE), ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE), in which these advances are aimed to increase yield at lower cost. Moreover, modifications on the methods are continuously developed. With such variety of methods present, selection of proper extraction method needs meticulous evaluation. The exploitation of plants by man for the treatment of diseases has been in practice for a very long time. Herbal drugs constitute a major part in the entire traditional system of medicines (Higa et al., 1994). A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity, the so called secondary metabolites. Screening of compounds obtained from plants for their

pharmacological assay has indeed been the vast source of innumerable therapeutic agents representing molecular diversity engineered by nature. It is therefore necessary and urgent to fight against emerging and re-emerging infectious diseases with a view to discover and invent new agents of greater therapeutic profile to mitigate frequent outbreaks of diseases which have posed a new threat to global health security.

In the broadest sense, natural products include any substance produced by life. Natural products can also be prepared by chemical synthesis (both semi-synthesis and total synthesis) and have played a central role in the development of the field of organic chemistry by providing challenging synthetic targets. The term natural product has also been extended for commercial purposes to refer to cosmetics, dietary supplements, and foods produced from natural sources without added artificial ingredients[1].Some important classes of natural products are briefly discussed below.

i-Saponins

Saponins are a class of chemical compounds found in particular abundance in various plant species. More specifically, they are amphipathic glycosides grouped phenomenologically by the soap-like foaming they produce when shaken in aqueous solutions, and structurally by having one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative[1].

The aglycone (glycoside-free) portions of the saponins are termed sapogenins. The number of saccharide chains attached to the sapogenin/aglycone core can vary – giving rise to another dimension of nomenclature (monodesmosidic, bidesmosidic, etc.) – as can the length of each chain. A somewhat dated compilation has the range of saccharide chain lengths being 1–11, with the numbers 2-5 being the most frequent, and with both linear and branched chain saccharides being represented. Dietary monosaccharides such as D-glucose and D-galactose are among the most common components of the attached chains[1]

ii-Tannins

Tannins are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides, and in plant growth regulation. The astringency from the tannins is what causes the dry and puckery feeling in the mouth following the consumption of unripened fruit or tea. Likewise, the destruction or modification of tannins with time plays an important role in the ripening of fruits .Tannins have molecular weights ranging from 500 to over 3,000 (gallic acid esters) and up to 20,000 (proanthocyanidins)[1].

iii-Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic

properties. Some synthetic compounds of similar structure are also termed alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and, more rarely, other elements such as chlorine, bromine, and phosphorus[1].

iv-Steroids

Steroids comprise a group of cyclic organic compounds whose most common characteristic is an arrangement of seventeen carbon atoms in a four-ring structure, where the rings are three composed of 6-carbons (rings A, B, and C) followed by one with 5-carbons (ring D). Further common features are an 8-carbon side chain attached to a carbon on ring D, and two or more methyl groups at the points where adjacent rings are "fused". Hundreds of distinct steroids are found in animals, fungi, plants, and elsewhere, and specific steroids underlie proper structure and function in many biological processes. Their core tetracyclic ring structure is synthesized in each organism by biochemical pathways that involve cyclization of a thirty-carbon chain, squalene, into an intermediate, either lanosterol or cycloartenol. From such intermediates, organisms then derive critical steroids such as cholesterol, the sex hormones estradiol and testosterone and bile acids. Based on such structures, synthetic and medicinal chemists synthesize novel steroids for use as drugs such as the anti-inflammatory agent dexamethasone[1].

v-Glycoside

In chemistry, a glycoside is a molecule in which a sugar is bound to another functional group via a glycosidic bond. Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications. In animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body[2].

vi-Flavonoids

Flavonoids are a group of polyphenolic compounds of plant origin. These compounds possess a common phenylbenzopyrone structure (C_6-C_3-C_6), and they are categorized according to the saturation level and opening of the central pyran ring.Flavonoids have probably existed in the plant kingdom for over one billion years. Over 6000 flavonoids have been identified in plants . They are present in practically all dietary plants, like fruits and vegetables. Therefore, they are consumed in considerable amounts and are also heat- stable. It is estimated that the human intake of all flavonoids is a few hundreds of milligrams per day. Additionally, flavonoids are found in several medical plants, and herbal remedies containing flavonoids have been used in folk medicine around the world . Biological activities of these compounds,

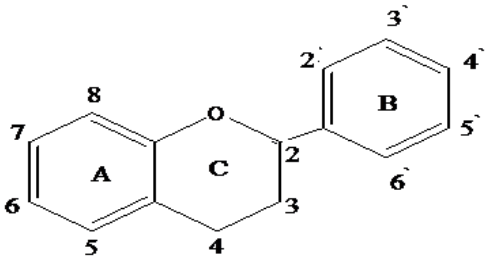
including antioxidant activity, depend on both the structural difference and the glycosylation patterns[1] .

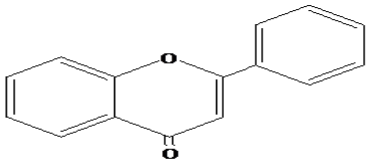
1.2. Nomenclature of flavonoids

The term "flavonoid" was first applied by Geissman and Hinreiner [2-3]. to embrace all those compounds whose structure is based on that of flavone (2-phenylchromone) (I) having a basic C_6-C_3-C_6 skeleton in common. When the heterocyclic ring is reduced, it becomes flavan (2-phenylchroman) (2). Flavone (I) consists of two benzene rings (A and **B)** joined together by a pyran ring(C). The various classes of flavonoid compounds differ from one another only by the state of oxidation of this carbon link. There is a limitation to the number of structures commonly found in nature, which vary in their state of oxidation from flavan-3-ols (catechin) (3) to flavonols (3-hydroxy flavones) (4) , anthocyanins (5) flavanones (6), dihydroflavonols (7) and the flavan-3, 4-diols (proanthocyanidins) (8) are also included in the flavonoids. There are also five classes of compounds: (dihydrochalcones (9) chalcones (10) isoflavones (11), neoflavones (12) and the aurones (13) , which do not actually possess the basic 2-phenyl chromone (I) skeleton, but are closely related both chemically and biosynthetically to other flavonoid types, that they are always included in the flavonoid group. The individual compounds in each class are distinguished mainly by the number and orientation of hydroxy and methoxy groups in the two benzene rings . These

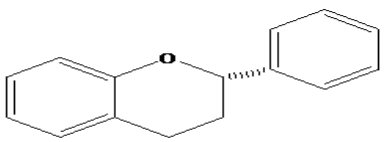
groups are usually arranged in certain restricted pattern in the molecule, reflecting the different biosynthetic origin of the two aromatic nuclei. Thus, in the A ring of the majority of flavonoid compounds, hydroxy groups are distributed at either C-5 and C-7 or only at C-7 . This pattern of hydroxylation follows from the acetate or malonate origin of the ring. The B-ring of flavonoids on the other hand is usually substituted either by one, two or three hydroxy or methoxy groups. The rarely methylated position is C-$_{4'}$ with often methylation at C-$_{3'}$ and C-$_{5'}$.

The hydroxylation pattern of the B-ring thus resembles that found in commonly occurring cinnamic acid and coumarins and reflects their common biosynthetic origin from prephenic acid and its congeners.

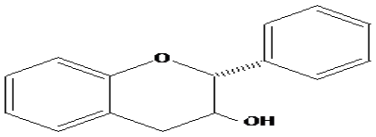




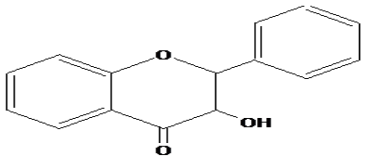
(1) Flavone



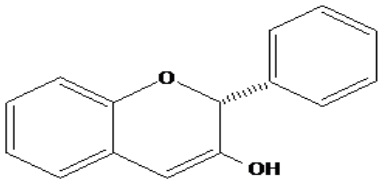
(2) Flavan



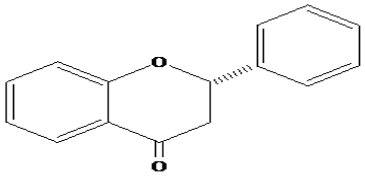
(3) Flavan 3-ol



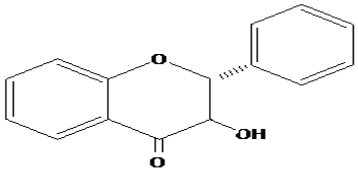
(4) Flavonol



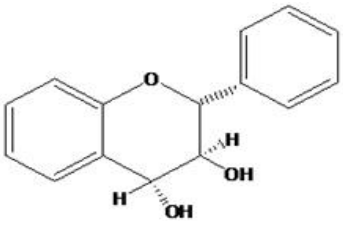
(5) Anthocyanidin



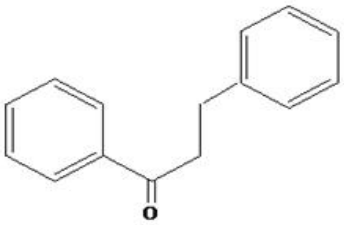
(6) Flavanone



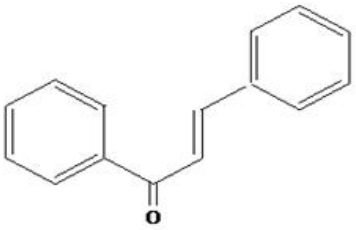
(7) Dihydroflavonol



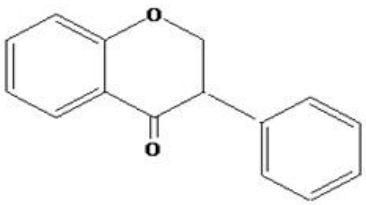
(8) proanthocyanidin



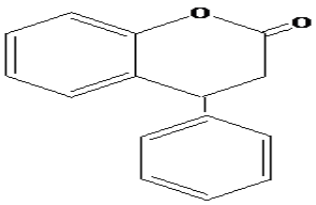
(9) Dihydrochalcone



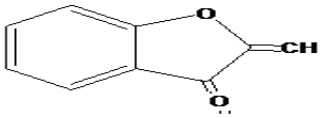
(10) Chlcone



(11) Isoflavones



(12) Neoflavones



(13) Aurone

A comparison of the nature and position of' various substituents at different carbons of the flavonoid skeleton has been made use of in arriving at certain generalizations regarding structure and properties [4].

Most of the flavonoids occur naturally in conjugated form, usually bound to sugar, by a hemiacetal linkage. But their conjugation with inorganic sulfates or organic acids is not unusual. The sugar free compounds are referred to as aglycones and it is probable that in most cases they are formed as artefacts during the course of extraction, since most living tissues contain very active glycosides which can work even in the presence of high concentration of organic solvents. The presence of sugars in the molecule confers sap-solubility to the generally somewhat insoluble flavonoid compounds. In anthocyanins the sugar imparts stability to the aglycone. Stability conferred by glycosylation to flavonols is observed in 3-0-glycosides of quercetin and myricetin which are not susceptible to oxidation catalysed by phenolase unlike the corresponding aglycones, presumably because of steric reasons . More and more range of new glycosides are encountered in plants . An increasing number of flavonoid glycosides carrying sugars in B-ring hydroxyls have been isolated. The number of acylated

flavonoid compounds succeeded by terpenoid counterparts is on the increase. As a result of electrophoretic studies, a number of Zwitter ionic anthocyanins with malonic acid and succinic acid linked to $C\text{-}_6$ of glucose have been isolated and characterized.

The sugars found in flavonoid glycosides include simple pentoses and hexoses (monosides) and di- and tri-saccharides (biosides and triosides) mostly combined through oxygen at $C\text{-}_1$ position of sugars, usually by a B-linkage. In many cases more than one phenolic hydroxyl group in the flavonoid molecule may be glycosylated giving rise to diglycosides and so on. The common sugars encountered are D-glucose, D-galactose, **L**-rhamnose, D-xylose, L-arabinose and D-glucuronic acid. D-allose and D-galacturonic acid are rare and D-apiose is an unusual and uncommon one [5].

Flavonols constitute a major class of flavonoids .The most common flavonol in the diet is quercetin. It is present in various fruits and vegetables, but the highest concentrations are found in onion (Table 1) [6]. The importance of different foods as quercetin sources varies between countries. Hertog et al [7] calculated flavonol intakes from a seven countries study, which was started in the late 1950s, and reported that tea was the predominant source of quercetin in the Netherlands and Japan. Wine was the major source of quercetin in Italy, while onion and apples contributed most in the US, Finland, Greece and former Yugoslavia. More recently it was estimated that onions, followed by tea, apples and berries are

the major sources of quercetin in Finland . It should be noted that onion is usually not consumed in high quantities, but it is an important source because of its high quercetin content. Tea and especially wine, on the other hand, contain relatively low amounts of quercetin but are consumed, at least in some countries, in rather high quantities. The daily intake of quercetin was estimated to range between 3 and 38 mg in the seven countries study [8].

Quercetin is present in plants in many different glycosidic forms with quercetin-3-rutinoside, also called quercetin-3-rhamnoglucoside or rutin, being one of the most widespread forms. In onions, the compound is bound to one or two glucose molecules (quercetin-4'-glucoside, quercetin-3,4'-glucoside). Other quercetin glycosides present in the diet are, for instance, quercetin galactosides (apples) and 22 quercetin arabinosides (berries). Other flavonols in the diet include kaempferol (broccoli), myricetin (berries) and isorhamnetin (onion).

Another important class of flavonoids is represented by flavanones .Flavanones occur almost exclusively in citrus fruits. The highest concentrations are found in the solid tissues, but concentrations of several hundred mg per litre are present in the juice as well . Hesperidin (hesperetin-7-rutinoside) and narirutin (naringenin-7-rutinoside) are the major flavonoids of oranges and mandarines. noneohesperoside) (70%) and narirutin (20%). Low concentrations of naringenin are also found in tomatoes and tomato-based products. Fresh tomatoes, especially tomato skin, also contain

naringenin chalcone, which is converted to naringenin during processing to tomato ketchup . the average intake of naringenin has been estimated to be 8.3 mg/day, and for hesperetin 28.3 mg/day [9].

Flavanols represent a large number of flavonoids and are often commonly called catechins. Different from most flavonoids, there is no double bond between C_2 and C_3, and no C_4 carbonyl in Ring C of flavanols. This and the hydroxylation at C_3 allows flavanols to have two chiral centers on the molecule (on C_2 and C_3), thus four possible diastereoisomers. Catechin is the isomer with *trans* configuration and epicatechin is the one with *cis* configuration. Each of these two configurations has two steroisomers, *i.e.*, (+)-catechin, (−)catechin, (+)-epicatechin and (−)-epicatechin. (+).Catechin and (−)-epicatechin are the two isomers often found in food plants .

Flavanols are found in many fruits, particularly in the skins of grapes, apple and blue berries . Monomeric flavanols (catechin and epicatechin) and their derivatives (e.g., gallocatechins) are the major flavonoids in tea leaves and cacao bean (chocolate). Catechin and epicatechin can form polymers, which are often referred to as proanthocyanidins because an acid-catalyzed cleavage of the polymeric chains produces anthocyanidins. Catechins usually occur as aglycones or are esterified with gallic acid.

(+)-Catechin and (-)-epicatechin are found in various fruits and vegetables such as apples, pears,grapes and peaches .The highest concentrations of catechins are found in tea and red wine [10].

Flavones (flavus = yellow), are a class of flavonoids based on the backbone of 2-phenylchromen-4-one. Flavones are mainly found in cereals and herbs. Flavones are biologically active compounds. Therefore a number of synthetic methods were developed. Some of the well known methods used for synthesis of flavones are Baker and Venkatraman synthesis and Claisen-Schmidt condensation. Most common flavonoids are flavones (with a C_2-C_3 double bond and a C_4-oxo function).The main flavones in the diet are apigenin and luteolin. Their dietary intake is rather low because they occur in significant concentrations in only a few plants, such as red pepper [11] .

Anthocyanidins are flavonoids which constitute the principal components of the red, blue and purple pigments of the majority of flower petals, fruits and vegetables, and certain special varieties of grains, e.g., black rice . Anthocyanidins in plants mainly exist in glycosidic forms which are commonly referred to as anthocyanins. Cyanidin, delphinidin and pelargonidin are the most widely found anthocyanidins, along with more than two dozen other monomeric anthocyanidins (a total of 31 anthocyanidins) . In fact, 90% of anthocyanins are based on cyanidin, delphinidin and pelargonidin and their methylated derivatives . A total of more than 500 anthocyanins are known depending on the hydroxylation,

methoxylation patterns on the B ring, and glycosylation with different sugar units . The color of anthocyanins is pH-dependent, *i.e.*, red in acidic and blue in basic conditions. However, other factors such as degree of hydroxylation, or methylation pattern of the aromatic rings, and the glycosylation pattern, *i.e.*, sugar *vs.* acylated sugar can also affect the color of anthocyanin compounds. Anthocyanins are chemically stable in acidic solution.The anthocyanins are water-soluble pigments . Apart from their biological role, they are important aesthetically and economically, since their stability is of significance in the marketability of plant products. Anthocyanins occur not only as monomers but as part of much larger complexes, in loose association with or chemically bonded to other components. This has led to a desire to characterize anthocyanins as they actually exist in plant material using methods of extraction and examination designed to cause least interference or alteration in structure. At the same time, the structures and properties of the monomers have been studied and their occurrence in extracts of plant material has been further documented[12].

Proanthocyanidins are traditionally considered to be condensed tannins. Flavanols and oligomers (containing 2–7 monomeric units) are known as strong antioxidants, which have been associated with several potential health benefits. Depending on the interflavanic linkages, oligomeric proanthocyanidins can be A-type structure in which monomers are linked through C_2–O–C_7 or

C_2–O–C_5 bonding, or B-type in which C_4–C_6 or C_4–C_8 are common.

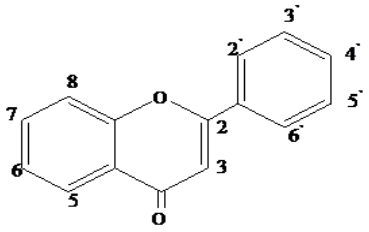
The isoflavonoids are a class of flavonoids characterized by a 3-phenylchroman skeleton that is biogenetically derived by 1,2-aryl migration in a 2-phenylchroman precursor. Despite their limited distribution in the plant kingdom, isoflavonoids are remarkably diverse as far as structural variations are concerned. This arises not only from the number and complexity of substituents on the basic 3-phenylchroman system, but also from the different oxidation levels and presence of additional heterocyclic rings. The predominant isoflavonoids are the isoflavones genistein and daidzein, which occur mainly in legumes. The highest concentrations are found in soy bean and soy products, and much lower concentrations are present in other legumes , not to mention other vegetables and fruit [14].

Table 1.1: Main dietary flavonoids [6]

Flavonoids	Sours	Content of aglycone (mg/kg)
Flavonol		
Quercetion-3,4'-gluc	onion	284-4862
Quercetion-3-glucoside		
Quercetion-3-rhanoglucoside(rutin)	Black tea	10-253
Quercetion-3-galactoside	apple	21-722
Quercetion-3-rhamnoside		
Quercetion-3-arabinoside		
Quercetion-3-glucoside		
Quercetion-3-rhamnoglucoside	Black currant	444
Myrisetin-3-glucoside		714

Flavone		
Luteolin-7-apiosylglucoside	Red pepper	7-142
Flavanone		
Hesperetin-7-rhamnoglucoside(hesperidin)	Orange juice	116
Naringenin-7- rhamnoglucoside(narirutin)		15-425
Naringenin-7- rhamnoglucoside(naringin)	Grapefruit juice	68-3025
Naringenin-7- rhamnoglucoside(narirutin)		
Flavanols		
(+)-Catechin	apple	4-166
(-)-Epicatechin		67-1036
(+)-Catechin	Red wine	16-537
(-)-Epicatechin		9-427
(Epi)catechin and their gallates	black	102-4187
Anthocyanins		
Cyanidin-3-glucoside	Blak currant	7609
Cyanidin-3-rutinoside		
Delphinidin-3- glucoside		5909
Delphinidin-3- rutinoside		
Isoflavones		
Genistein-7-glycoside	Soy beans	48010
Daidzein-7-glycoside		33010

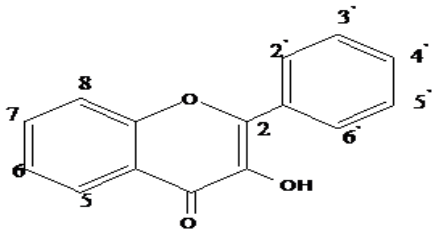
Examples of different classes of flavonoids are shown below [15]:



Flavones:

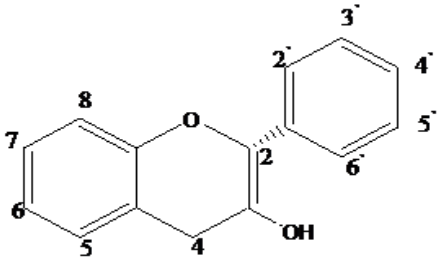
Compound	Position			
	5	7	3'	4'
Apigenin	OH	OH	-	OH

Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	-	-



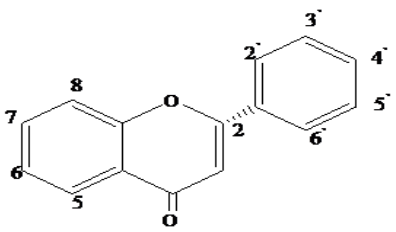
Flavonols:

Compound	Position				
	5	7	3'	4'	5'
Quercetin	OH	OH	OH	OH	-
Kaempferol	OH	OH	-	OH	
Galangin	OH	OH	-	-	-
Fisetin	-	OH	OH	OH	-
Myricetin	OH	OH	OH	OH	OH



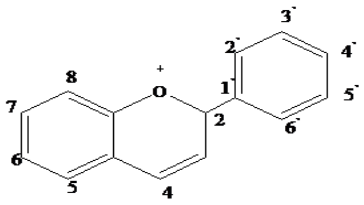
Flavan-3-ols:

Compound	Position					
	3	5	7	3'	4'	5'
(+)-Catechin	βOH	OH	OH	OH	OH	-
(-)-Epicatechin	αOH	OH	OH	OH	OH	-
(-)-Epigallocatechin	αOH	OH	OH	OH	OH	-



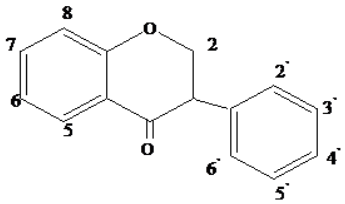
Flavanones:

Compound	Position			
	5	7	3'	4'
Naringenin	OH	OH	-	OH
Naringin	OH	O-Rha-Glu	-	OH
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	O-Rha-Glu	OH	OCH



Anthocyanidins:

Compound	Position					
	3	5	7	3'	4'	5'
Cyanidin	OH	OH	OH	OH	OH	-
Cyanin	O-Glu	OH	OH	OH	OH	-
Peonidin	OH	OH	OH	OCH_3	OH	-
Delphinidin	-	OH	OH	OH	-	OH
Pelargonidin	OH	OH	OH	-	OH	-
Malvidi	OH	OH	OH	OH	OH	OCH_3



Isoflavones:

Compound	Position		
	5	7	4'
Genistei	OH	OH	OH
Genisti	OH	O-Glu	OH
Daidzein	-	OH	OH
Daidzin	-	O-Glu	OH
Ononin		O-Glu	CH_3

1.3- The synthesis of flavonoids

Structures of organic compounds are usually determined by using data generated by chemical and instrumental methods. A chemist then invariably attempts to establish the proposed structure by unequivocal total synthesis. The conventional methods of synthesis of flavonoids from simple precursors by condensation methods have been proposed by Baker and Venkataramen .[16]

This method of synthesis has been modified by Farkas et al[17] as illustrated by the synthesis of a number of flavonoids and their methyl ethers . Many flavonoid compounds have been prepared by simple modification of the existing structure through nuclear oxidation, nuclear reduction, isomerisation, selective alkylation and dealkylation, selective glycosylation and partial hydrolysis. Farkas et al accomplished the synthesis of methoxylated flavones from the corresponding brominated methoxy chalcones. The synthesis of 5,6,7,3',4'-pentamehtoxy flavone (sinensetin) by dehydrogenation of the corresponding flavanone with SeO_2 has been achieved by Wagner et al [18]. This flavone has been used as

the starting material for the synthesis of a number of related flavones. Bose et al [19] have reported cyclisation and simultaneous dehydration of the hydroxy chalcone to the corresponding flavone by heating with palladium on charcoal . A short and facile synthetic route to hydroxylated flavones has been reported by Nagarathnam and cushman . [20] The synthesis of flavonoid glycosides have been achieved using the α-acetobromo sugars of pentoses, hexoses or disaccharides and the aglycones in the presence of catalysts. The selective glycosylation of 7-OH has been achieved by Farkas. [16] Synthesis of other glycosides have been accomplished by trans acylation methods and the total synthesis of C-glycosyl flavones has been reported by Eade et al.[21] A more general synthesis, exemplified by the preparation of 5.7,4'-trimethoxy vitexin and other complex ones has been provided by later workers.[22] Thus the synthesis of almost all types of mono and di-C-glycosyl flavones and flavone C-glycosyl-o-glycosides has been accomplished.

Synthesis of some novel flavonoids has been illustrated by Rakosi et al.[23] The chiron approach to the total synthesis of natural products might become a useful guide in the synthesis of chiral flavonoid compounds. Studies of the selective O-alkylation and dealkylation of flavonoids with anhydrous $AlBr_3$ were also reported .[24]

1.4-Biosynthesis of flavonoids

Flavonoids may have existed in nature for over one billion years and thus may have interacted with evolving organisms over the acons. Clearly the flavonoids possess some important purposes in nature, having survived in vascular plants throughout evolution. Our ideas of the evolution of flavonoids is mainly based on our knowledge of the overall modes of biosynthesis of the different classes of compounds. Tremendous progress has been made in the biosynthesis of flavonoids. Our present knowledge in flavonoid biosynthesis is based on a combination of earlier results from radioactive tracer studies in vivo and the more recent data obtained at the enzyme level in vitro. In the past few years the enzymology of flavonoid biosynthesis has made particularly rapid progress. Flavonoid biosynthesis can be considered in three stages. The first is the formation of the basic C_6-C_3-C_6 skeleton through acetate-malonate and shikimic acid pathway to aromatic compounds. The second stage is concerned with the ways by which the different classes of flavonoids are synthesised. The final stage embraces the elaboration of individual compounds within each flavonoid class, involving steps such as hydroxylation, glycosylation, methylation etc., Chalcone is considered as the common intermediate in the biosynthesis of all classes of flavonoids. Insight into three aspects of the problem of flavonoid biosynthesls has come in the past from comparative anatomy and chemical genetic studies and recently from feeding experiments with radioactive tracers. Major work on

chemical genetic studies have been carried out by Grisebach and co- workers . [25] Research has led to the isolation and characterization of enzymes of the pathway of flavonoid biosynthesis.

The use of young plant tissues and cell suspension cultures as source materials have also greatly facilitated the study of flavonoid biosynthesis at the enzyme level. Roux and Ferreira [26] have highlighted the special role of α-hydroxy chalcone as the key intermediate in flavonoid biogenesis. Agood account of biosynthesis of shikimate derived phenolic compounds by Harborne,[27] biosynthetic studies in vivo with labelled precursors and biochemistry of flavonoid biosynthesis by Heller are useful publications [28]. The importance of flavonoids and other secondary metabolites in plant biochemistry has been detailed in "the Biochemistry of plants".[29]

Flavonoids are the constituents of the mammalian diet derived from plants. The ingestion of flavonoids by mammals in the diet or for therapeutic use, brings them in contact with both intestinal micro-organisms and mammalian tissues which are capable of biotransformation of flavonoid compounds. The available evidences indicate that the hydrolysis of the flavonoid glycosides to their corresponding aglycones, ring fission and oxidative and reductive transformations are mediated by intestinal microorganisms.Though it is certain that the metabolic changes

undergone by flavonoids occur within a mammalian tissues, the relative contribution of individual tissues is not fully understood.

1.5-Isolation of flavonoids

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. Glycosides have increased water solubility and aqueous alcoholic solutions are suitable. The bulk of extractions of flavonoid-containing material are still performed by simple direct solvent extraction.

Powdered plant material can also be extracted in a Soxhlet apparatus, first with hexane, for example, to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. This approach is not suitable for heat-sensitive compounds. A convenient and frequently used procedure is 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. Flavanone solubility depends on the pH of water-containing solutions. Flavan-3-ols (catechins, proanthocyanidins, and condensed tannins) can often be extracted directly with water. However, the composition of the extract does vary with the solvent – whether water, methanol, ethanol, acetone, or ethyl acetate. For example, it is claimed that methanol is the best solvent for catechins and 70% acetone for procyanidins.

Anthocyanins are extracted with cold acidified methanol. The acid employed is usually acetic acid (about 7%) or trifluoroacetic acid (TFA) (about 3%). The use of mineral acid can lead to the loss of attached acyl groups. 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. Commercially available instruments have extraction vessels with volumes up to about 100 ml. In a study involving medicinal plants, solvent use was reduced by a factor of two. The optimization of rutin and isoquercitrin recovery from older (Sambucus nigra,

Caprifoliaceae) flowers has been described. Application of PLE gave better results than maceration — and shorter extraction times and smaller amounts of solvent were required. PLE of grape seeds and skins from winemaking wastes proved to be an efficient procedure for obtaining catechin and epicatechin with little decomposition, provided that temperature was kept below 1308C. As its name suggests, supercritical fluid extraction (SFE) relies on the solubilizing properties of supercritical fluids. 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. Numerous applications in the extraction of natural products have been reported, with supercritical carbon dioxide being the most widely used extraction solvent. However, to allow for the extraction of polar compounds such as flavonoids, polar solvents (like methanol) have to be added as modifiers. There is consequently a substantial reduction in selectivity. This explains why there are relatively few applications to polyphenols in the literature. Even with pressures of up to 689 bar and 20% modifier (usually methanol) in the extraction fluid, yields of polyphenolic compounds remain low, as shown for marigold (Calendula officinalis, Asteraceae) and chamomile (Matricaria recutita, Asteraceae). Ultrasound-assisted extraction is

a rapid technique that can also be used with mixtures of immiscible solvents: 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. Microwave-assisted extraction (MAE) has been described for the extraction of various compounds from different matrices. 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.[30]

When flavonoids of varying types are to be extracted from a single batch of plant material, a worthwhile method for preliminary separation is sequential solvent extraction with a number of solvents of varying polarity. This can lead to separation of glycosides from aglycones and to the separation of polar from non-polar aglycones.

AIternatively, sequential solvent extraction of a cmde extract may be used to produce the same type of separation . Counter current separation techniques may also be of value, particularly for the separation of flavanoids . Distributions between water and an organic phase such as ethyl acetate or butanol: light petroleum have been found effective for this purpose.

Precipitation with lead acetate has been widely used in the past as a method of isolating phenolics (especially those with o-dihydroxyl groups) from other extractives in cmde extracts It has the disadvantage, however, that it does not precipitate some phenols and may co-precipitate other compounds.

Decomposition of the lead saIts formed is best carried out with sulphate or phosphate rather than with sulphide, in view of the highly adsorptive nature of the precipitated lead sulphide. Recently, a method using polyvinylpyrrolidone (PVP) as precipitant has been suggested for use in cases where lead acetate is unsatisfactory.

The optimum conditions for PVP phenol bonding were established as pH 3.5 in 1-10% methanol in water.

Treatment of cmde plant extracts with charcoal powder is also auseful method for the preliminary purification of flavonoids, particularly glycosides. In one example of this procedure , flavonoids from *Baptisia lecontei* were eluted from activated charcoal by washing successively with methanol, boiling water, 7% aqueous phenol and 15% methanolic phenol. The bulk of the flavonoid material appeared in the 7% phenol fraction, which was subsequently ether - extracted to give a phenol-free aqueous solution rich in flavonoid glycosides.

In certain circumstances dialysis has been found helpful as a preliminary clean-up procedure. It is of particular use in the isolation of high molecular weight flavonoids (and tannins) which

are water soluble and are mixed with sugars or soluble inorganic material in the crude extract. Markharn (1972) [31] found dialysis useful in the separation of a flavone-polysaccharide compound (MW about 3000) from an aqueous plant extract of *Monoclea forsteri*, and considerable use has been made of this technique in the isolation of high molecular weight blue flavonoid 'complexes' from cornflower (MW 6200) , *Commelina communis* and Professor Blaauw iris , all of which were non-dialysable through a cellulose membrane. Flavonoids produced on hydrolysis of the non-dialysable commelinin were dialysable under the same conditions.[32]

The method of choice for simple and in expensive analytical runs is TLC. The advantages of this technique are well known: short separation times, amenability to detection reagents, and the possibility of running several samples at the same time. TLC is also ideally suited for the preliminary screening of plant extracts before HPLC analysis. Many different solvent systems have been employed for the separation of flavonoids using TLC. Highly methylated or acetylated flavones and flavonols require nonpolar solvents such as chloroform–methanol (15:1). Widely distributed flavonoid aglycones, such as apigenin, luteolin, and quercetin, can be separated in chloroform– methanol (96:4) and similar polarity solvents. One system that is of widespread application for flavonoid glycosides is ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:26). By the addition of ethyl methyl ketone

(ethyl acetate–ethyl methyl ketone–formic acid– glacial acetic acid–water, 50:30:7:3:10), rutin and vitexin-2’’-O-rhamnoside can be separated. Careful choice of solvent system also allows separation of flavonoid glucosides from their galactosidic analogs. This is especially important for the distinction of C-glucosides from C-galactosides. As an illustration, 8-C-glucosylapigenin (vitexin) can be separated from 8-C-galactosylapigenin with the solvent ethyl acetate–formic acid–water (50:4:10). With regard to detection, brief exposure of the TLC plate to iodine vapor produces yellow-brown spots against a white background. And, as stated by flavonoids appear as dark spots against a fluorescent green background when observed in UV light (254 nm) on plates containing a UV-fluorescent indicator (such as silica gel F254) . In 365 nm UV light, depending on the structural type, flavonoids show dark yellow, green, or blue fluorescence, which is intensified and changed by the use of spray reagents. One of the most important of these is the ‘‘natural products reagent,’’ which produces an intense fluorescence under 365 nm UV light after spraying with a 1% solution of diphenylboric acid-b-ethylamino ester (diphenylboryloxyethylamine) in methanol. Subsequent spraying with a 5% solution of polyethylene glycol-4000 (PEG) in ethanol lowers the detection limit from 10 mg (the average TLC detection limit for flavonoids) to about 2.5 mg, intensifying the fluorescence behavior. The colors observed in 365 nm UV light are as follows: Quercetin, myricetin, and their 3- and 7-O-

glycosides: orange-yellow. Kaempferol, isorhamnetin, and their 3- and 7-O-glycosides: yellow-green. Luteolin and its 7-O-glycoside: orange. Apigenin and its 7-O-glycoside: yellow-green.

Aqueous or methanolic ferric chloride is a general spray reagent for phenolic compounds and gives a blue-black coloration with flavonoids. Similarly, Fast Blue Salt B forms blue or blue-violet azo dyes.[36]

1.6- Spectroscopic techniques in flavonoids analysis

1.6.1- UV- Visible spectroscopy

The technique of UV spectroscopy has gained tremendous interest for the structure analysis of flavonoids. This is due to the fact that only a small amount of pure material is required. Often, a single flavonoid spot on a paper chromatogram will yield sufficient compound for several UV spectra. Another reason is that the amount of structural information gained from a UV spectrum is considerably enhanced by the use of specific reagents which react with one or more functional groups on the flavonoid nucleus. The addition of each of these reagents separately to an alcoholic solution of the flavonoid induces structurally significant shifts in the UV spectrum. Shifts of this type are commonly induced by the addition of sodium methoxide (NaOMe), sodium acetate (NaOAc), sodium acetate/boric acid ($NaOAc/H_3B0_3$), aluminium chloride ($AlC1_3$) and aluminium chloride/hydrochloric acid (AICl /HCl) .[56] The UV spectra of most flavonoids consists of two major absorption maxima, one of which occurs in the range 240-285 nm

(band 11) and the other in the range 300-400 nm (band I) . In general terms the band 11 absorption may be considered as having originated from the A-ring benzoyl system and band I from the B-ring cinnamoyl system .[57]

1.6.2- Nuclear magnetic resonance spectroscopy

NMR(Nuclear Magnetic Resonance spectroscopy), is one the most powerful techniques used to investigate the structure and some properties of molecules. One of the main applications of NMR in flavonoid research is the structural elucidation of novel compounds, for which nothing is known; although NMR traditionally requires large amounts of sample, which is not easy to obtain when analysing novel compounds, the technical developments in the last decade, both in NMR instrumentation, pulse programs and in computing power, have allowed the complete assignment of all proton and carbon signals using amounts in the order of 1 mg .[58]

1.6.2.1 One Dimensional NMR: ^{1}H and ^{13}C

The two most basic NMR experiments are the ^{1}H and the ^{13}C NMR experiments, which are aimed at the determination of the resonance frequency of each ^{1}H or ^{13}C nucleus in the molecule.

^{1}H NMR experiments register the chemical shifts (δ) and spin-spin couplings, the latter described by the coupling constants (J). This provides valuable information about the relative number of hydrogens and also their type, by comparison of the recorded chemical shifts with compiled data. This is particularly useful in

establishing the aglycone type and the acyl groups attached to it, as well as in identifying the number and the anomeric configuration of the glycoside moieties attached to the aglycone. ^{13}C NMR data is used to complement ^{1}H NMR data, and is particularly useful at establishing the type of groups present in the samples' molecules by comparison with compiled data; however, it must be noted that ^{13}C NMR is much less sensitive due to the abundance of C^{13} (1.1 %) when compared to ^{1}H (99.9 %) . Together, these two 1D experiments are used primarily to identify aglycone types and substituent groups, but a definite structural elucidation, which the accurate location of the various groups, requires various 2D experiments.

1.6.3 Aim of this study

This study was aimed to:

-Extraction the flavonoids from the target species.

-Isolation of individual flavonoids.

-Elucidation of structures of the isolated compounds.

-Screening the isolated compounds for antimicrobial activity.

Chapter two

Material and Methods

2- Materials and methods

2.1.Materials

2.1.1.Plant material

Acacia nilotica var. tomentoza was collected from Medani,Gezira state(Sudan). *Acacia ehrenbergiana* was collected from El-Butana ,Gedarif state(Sdan). *Indigofera oblongifolia* was collected from New Halfa, Kassala state(Sudan).The plants were authenticated by The Institute of Medicinal and Aromatic Plants Research Institute, Khartoum, Sudan .The plant material were separately powdered in a blender and stored in sterile containers for further use.

2.1.2.Materials for chromatographic study

i. Whatman paper No.1 (1MM) for analytical paper chromatography (Whatman Ltd. Maistone, Kent, England).

ii. Whatman paper No (3MM) for preparative paper chromatography (Whatman Ltd. Maistone, Kent, England).

iii- Glass jars (rectangular glass tanks 100 x80 x 40cm) for developing PC chromatograms.

iv- Ultra - Violet lamp **λ(254/365 nm)**(portable ultraviolet, a product of Hanovia lamps (6 watt S/W and L/W)) for localization of fluorescent spots on chromatograms and bands.

2.1.3.Materials for biological screening

i. Bacterial strains

- Gram positive bacteria

Bacillus aureus , Bacillus subtilis and Staphylococcus aureus.

- Gram negative bacteria

Escherichia coli, Pseudomonas aeruginosa and Salmonella typhimurium.

- Fungal strains

Candida albicans.

- Standards

i. Gentamycin: antibacterial standard for G+ve bacteria.

ii. Penicillin G: antibacterial standard for G-ve bacteria.

iii. Clotrimazole: antifungal standard.

-Media for G+ve bacteria

Muller Hinton agar was used as media for G +ve bacterial growth, Peptone from casein 17.0g , Peptone from meat 3.0g Sodium chloride 5.0g , Lactose 10.0g , Bile salt mixture 1.5g Neutral red 0.03g , Crystal violet 0.001g , Agar 13.5g

-Media for G-ve bacteria

Nutrient Agar (oxoid, England) was used as media for G-ve bacterial growth : Lab. Lemco powder 1.0g , Yeast extract 2.0g

Peptone water 5.0g , Agar No.3 15.0g , Distilled water 1000ml

- Media for fungi

Sabouraud Agar (oxoid, England) was used as media for fungal growth : Meat Peptone 5.0g , Casein Peptone 5.0g ,Dextrose 40.0g , Agar 15.0g , Distilled water to 1000ml

2.1.4. Equipments

1- Ultra - Violet - Visible spectrophotometer (Shimadzu model UV240 and 240PC) .

2- Joel- Nuclear Magnetic Resonance (NMR) spectrophotometer, (Brucker AC-250) -500 MHz.

2.1.5. Solvents

Analytical grade solvents (n-hexane, diethyl ether, chloroform, ethyl acetate, ethanol (95%), methanol and butanol were used. Methanol used for spectrophotometric analysis was supplied by Merck Co. Dramstadt, Germany. DMSO-d6(for phenolics) was used in NMR spectral analysis using TMS as internal standard.

2.2. Methods

2.2.1. Extraction flavonoids

Powdered shade –dried plant material (2Kg) were macerated with 95% ethanol at room temperature. The extract was filtered and the solvent was removed under reduced pressure. The dried extract was stored at $5^{o}C$ in an airtight container for further manipulation.

2.2.2.Phytochemical screening

The target plants were screening for the presence of flavonoids, 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 the presence of alkaloids.

-Wagner's 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)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.

c)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.

d) Test for saponins

i. Froth test

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

2.2.3-UV-Visible spectroscopy

UV-Visible spectra of isolated flavonoids were recorded on a Varian Cary 100 Scan UV-Visible Spectrophotometer. The spectrophotometer was operated and results were processed using Cary Win-UV Scan Application software (version 3.00(182)). Spectra were recorded in a quartz cuvette (1cmx1cmx4.5cm) which did not absorb over the spectral region of interest. A small amount of the isolated flavonoid (ca. 1mg) 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 (2-3mL) was measured at a normal scan speed (50nm/min) over the 200-500nm 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. After five minutes the spectrum was re-recorded to check for decomposition of the flavonoid. 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 and again after ten minutes to check for decomposition. Finally, an excess of

powdered anhydrous boric acid was added with shaking to the saturated sodium acetate solution. The spectrum was then recorded .

2.2.4-Shift Reagents for UV spectra of flavonoids

-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.

2.2.5-Nuclear magnetic resonance spectroscopy of (NMR)

NMR spectra of isolated flavonoids were obtained using a Bruker Advance DRX fitted with a 400MHz Bruker Spectrospin

superconducting magnet (52mm). Proton 1D experiments were carried out using a 5mm dual ^{1}H probe head. Samples were thoroughly dried by freeze drying over several days and dissolved in dimethylsulfoxide-*d6* for NMR analysis. Operation of the NMR and processing of spectra were performed using Topspin software (Bruker).

2.2.6-Antimicrobial test

i)-Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about 10^{8}-10^{9}colony forming units per ml.The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

ii)-Preparation of fungal suspensions

Fungal cultures were maintained on sabouraud dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

iii)-Testing for antibacterial activity

The cup-plate agar diffusion method was adopted with some minor modifications, to assess the antibacterial activity of the oil. (2ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in a water bath. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle and in each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No 4), each one of the halves was designed for one of the compounds. Separate Petri dishes were designed for standard antibacterial chemotherapeutic, (ampicillin and gentamycin).

The agar discs were removed, alternate cup were filled with 0.1 ml samples of each compound using adjustable volume micrometer pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentrations of the test compounds and the standard antibacterial

chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in triplicates and averaged.

Chapter three

Result and Discussion

3-Results and Discussion

In this study the flavonoids from three key species(*Acacia tomentosa*, *Acacia ehrenbergiana* and *Indigofera oblongifolia*) in Sudanese system of medicine have been investigated .The flavonoids were extracted with ethanol and the crude extracts were purified by chromatographic techniques.

Three chromatographically pure flavonoids-compounds I,II and III have been isolated from these species. The structures of the isolated flavonoids have been elucidated via a combination of spectral techniques.

3.1- *Acacia nilotica* var. *tomentoza*

3.1.1-Phytochemical screening

Acacia nilotica var. *tomentoza* was screened for major secondary metabolites and the results are displayed in Table 3.1.

Table 3.1**: Phytochemical screening of *Acacia nilotica* var. *tomentoza***

Test	Results	Observation
Saponins	+	Foam
Cumarins	-	No observation
Alkaloids	-	No observation
Anthraquinones glycosides	-	No observation
Tannins	+++	Green colour
Flavonoids	++	Yellow color
Sterols	-	No observation
Triterpenes	+++	Purple colour
Cyanogenic glycosides	-	No observation

+ :Trace

++ : Moderate

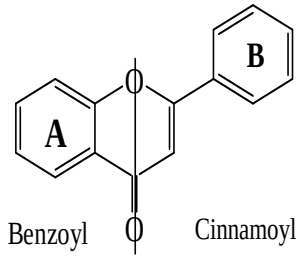
++: High

- : Negative

3.1-2-Identification of compound I

The barks of *Acacia nilotica* var. *tomentoza* were macerated with 95% ethanol at room temperature for 48hr. Removal of the solvent under reduced pressure gave a crude product. Paper chromatography of the crude extract gave a pure component – compound I.

Flavonoids usually exhibit two absorption bands in their UV spectra; band I and II. Band I is associated with the absorption of the cinnamoyl system, while band II originates from the benzoyl system. Flavones, flavonols, chalcones and aurones give band I and II, due to conjugation between the carbonyl function and the aromatic B ring. While flavanones,isoflavones,dihydroflavonols and dihydrochalcones give only band II in the range : 230-290nm.These classes of flavonoids lack conjugation between the B ring and the carbonyl function.



The UV absorption of flavones, flavonols, chalcones and aurones is depicted in Table 3.2.

Table 3.2: The UV absorption of some flavonoids[1]

Flavonoid class	Band I	Band II
Flavones	330-350	250-270
Flavonols	350-390	250-280
Chalcones	365-390	240-260
Aurones	390-430	240-270

In the UV compound I absorbs (Fig.1) at λ_{max}247,316,364nm. Such absorption is characteristic of :flavones, flavonols ,chalcones and aurones. However,aurones absorb above 400nm . Chalcones have dominant band I(a feature which was not observed in the UV absorption of this compound. Flavones have band I below 354nm, while flavonols absorb above 358nm. Consequently, compound I is a flavonol.

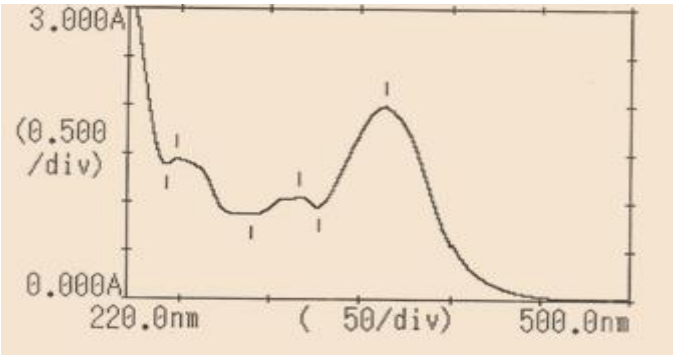


Fig.1 : UV spectrum of compound I

The UV shift reagent-sodium methoxide- revealed a bathochromic shift accompanied with decrease in intensity indicating a 3-OH function(Fig.2).

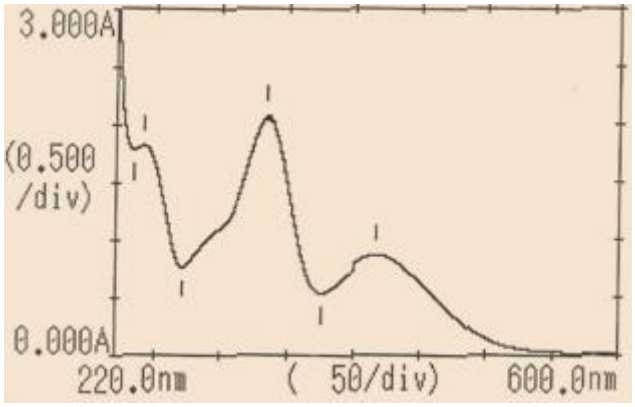


Fig.2 : Sodium methoxide spectrum of compound I

Sodium acetate is another useful shift reagent and it is diagnostic of a 7-OH group. The sodium acetate spectrum (Fig.3) did not afford any bathochromic shift indicating absence of a 7-OH function .

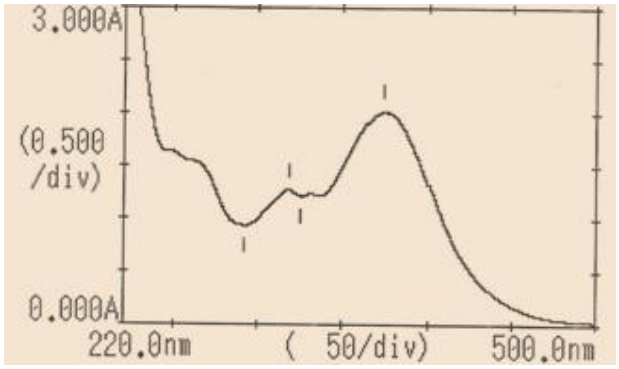


Fig.3 : Sodium acetate spectrum of compound I

The aluminium chloride spectrum (Fig.4) gave a bathochromic shift indicative of a 5-OH group(the spectrum was stable in

acidic medium) . The boric acid spectrum(Fig.5) which is diagnostic of catechol systems did not reveal any bathochromic shift and this indictes absence of such catechols.

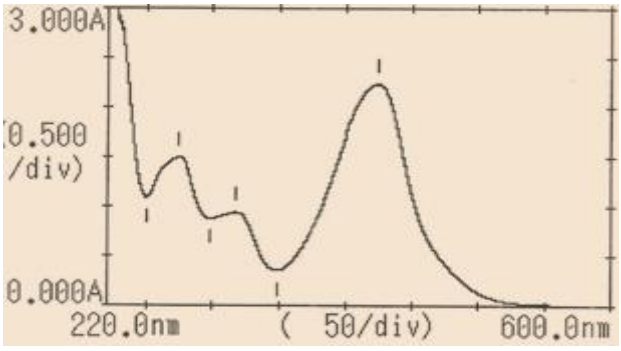


Fig.4 : Alumnium chloride spectrum of compound I

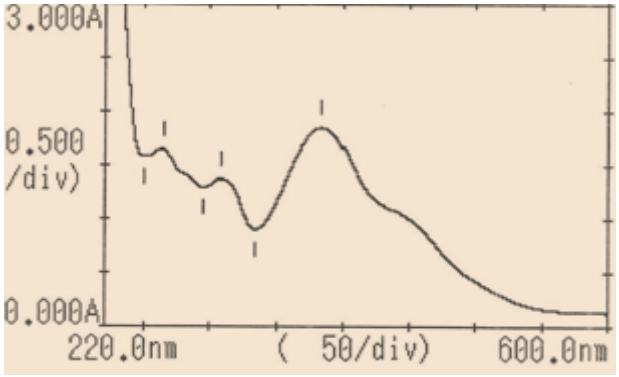
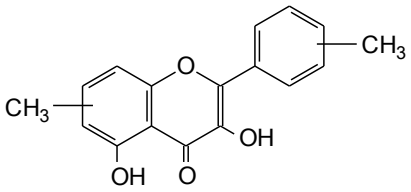


Fig.5 : Boric acid spectrum of compound I

The ^{1}HNMR spectrum(Fig.6) showed δ1.23 which was assigned for two methyl groups. The multiplet at δ3.16-3.72 accounts for a sugar moiety. The C_6-H appeared at δ6.87ppm while other aromatic protons gave signals at δ7.54, 7.67 and δ7.88ppm.

On the basis of the above spectral data the following tentative structure was assigned for the aglycone of compound I:



Compound I

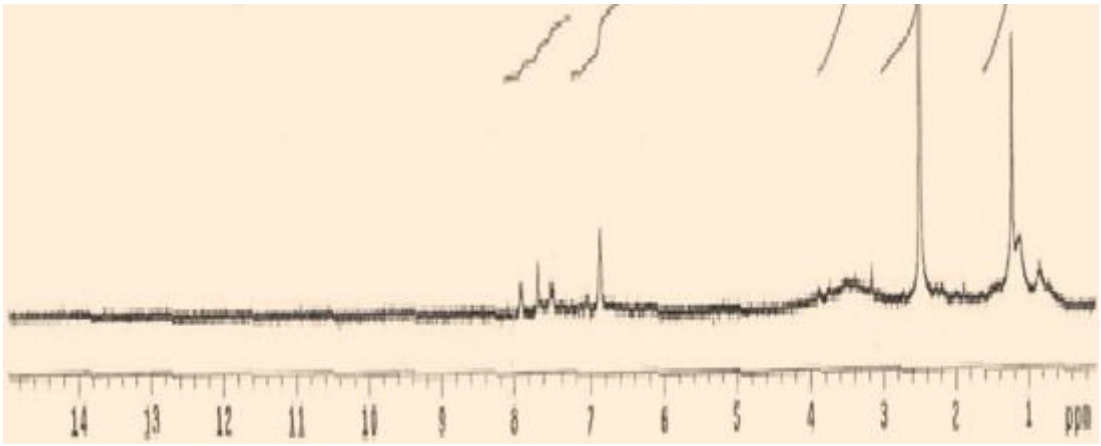


Fig.6: [1]HNMR spectrum of compound I

3.1.3-Antimicrobial assay

Compound I has been screened for antimicrobial activity against six human pathogens. The results are depicted in table 3.3.Tables 3.4 and 3.5 display the antimicrobial activity of standard drugs.

Compound I showed excellent activity against *Pseudomonas aeroginosa* and *Bacillus subtilles* . It also exhibited moderate activity ag*ainst Escherichia coli* .

Table 3:.3 Antimicrobial activity of compound I

	Ec	Ps	Sa	Bs	Ca	An
Compound I	14	17	11	17	12	12

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtilles*

Ca = *Candida albicans*

An = *Aspergillus Nige*

Table 3.4. : Antibacterial activity of standard chemotherapeutic agents

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Ps.
Ampicillin	40 20 10	15 14 11	30 25 15	- - -	- - -
Gentamycin	40 20 10	25 22 17	19 18 14	22 18 15	21 15 12

Table 3.5 : Antifungal activity of standard chemotherapeutic agent

Drug	Conc. mg/ml	An.	Ca.
Clotrimazole	30 15 7.5	22 17 16	38 31 29

3.1.4-Antioxidant assay

The antioxidant capacity of compound I has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in colour is measured spectrophotometrically at 516nm. As depicted in Table (3.6) compound I exhibited significant anti- oxidant activity.

Table 3.6: Radical scavenging activity of compound I

Sample	Antioxidant activity(%)
Trolox	96.50
Compound I	96.01

3.2- *Indigofera oblongifolia*

3.2.1-Phytochemical screening

Indigofera oblongifolia was screened for major secondary metabolites and the results are displayed in Table 3.7.

Table 3.7: Major secondary metabolites in *Indigofera oblongifolia*

Test	**Results**	**Observation**
Saponins	**+++**	Foam
Cumarins	+	Uv absorption
Alkaloids	-	No observation
Anthraquinones glycosides	+	Pink colour
Tannins	++	Green colour
Flavonoids	+++	Yellow color
Sterols	-	No observation
Triterpenes	++	Purple colour
Cyanogenic glycosides	-	No observation

3.2.2- Characterization of compound II

Compond II was isolated as yellow powder from *Indigofera oblongifolia*. In the UV compound II absorbs (Fig.7) at Λ_{max}284,362nm. Such absorption is characteristic of flavonols which are distinguished from flavones by band I(arising from cinnamoyl chromophore).

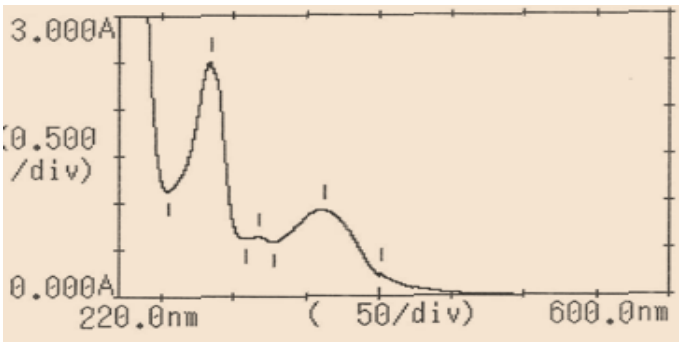


Fig.7 : UV spectrum of compound II

The hydroxylation pattern of this flavonol was investigated by using different UV shift reagents(sodium methoxide, sodium acetate, aluminium chloride and boric acid).

The UV shift reagent-sodium methoxide revealed a bathochromic diagnostic of 3- OH functions (Fig.8).

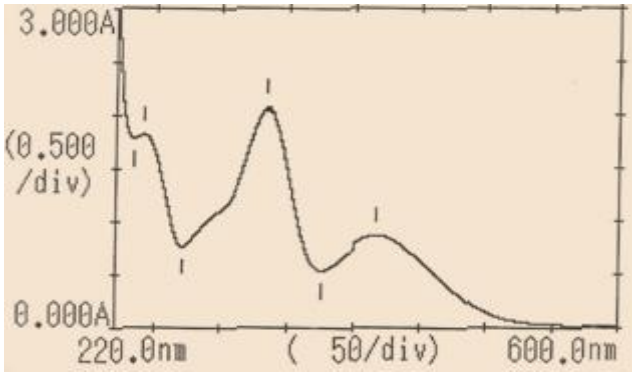


Fig.8 : Sodium methoxide spectrum of compound II

The sodium acetate spectrum (Fig.9) did not afford any bathochromic shift indicating absence of a 7-OH function .

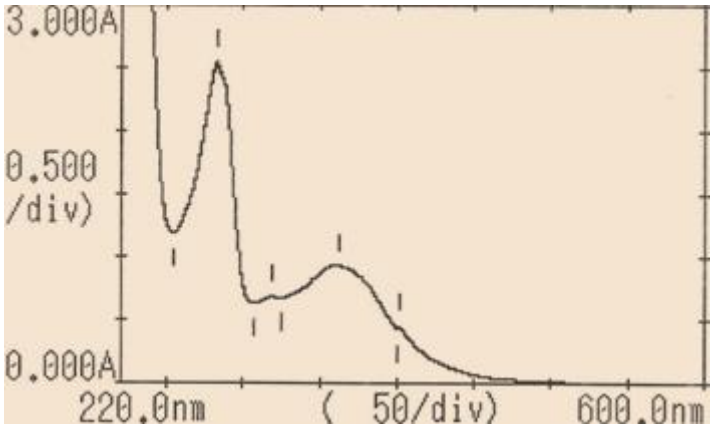


Fig.9 : Sodium acetate spectrum of compound II

Aluminium chloride is another useful UV shift reagent . It is diagnostic of 3- and 5-OH functions as well as catechol systems (catechol moieties are also detectable by the shift reagent-boric acid). In all cases this reagent produces a bathochromic shift but with decrease in intensity in case of a 3-OH function. The 3- and 5- hydroxylation patterns are distinguishable from catechol systems by their aluminium chloride spectrum which is stable in acidic media unlike the catechol spectrum.

The aluminium chloride spectrum(Fig.10) revealed a bathochromic shift indicative of a 5-OH function(The spectrum is stable in acidic medium). The boric acid spectrum did not reveal any bathochromic shift indicating absence of catechol systems(Fig. 11)

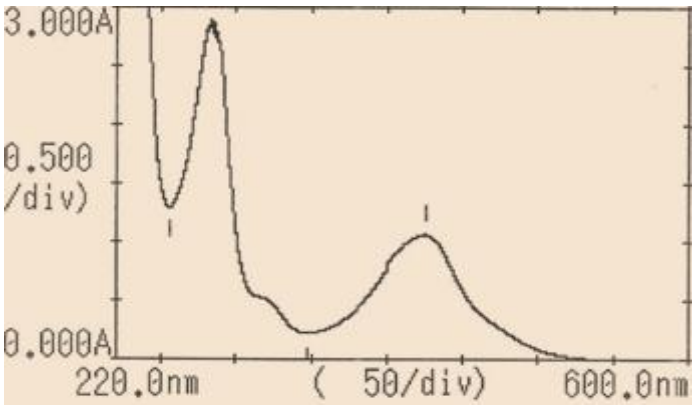


Fig.10 : Alumnium chloride spectrum of compound II

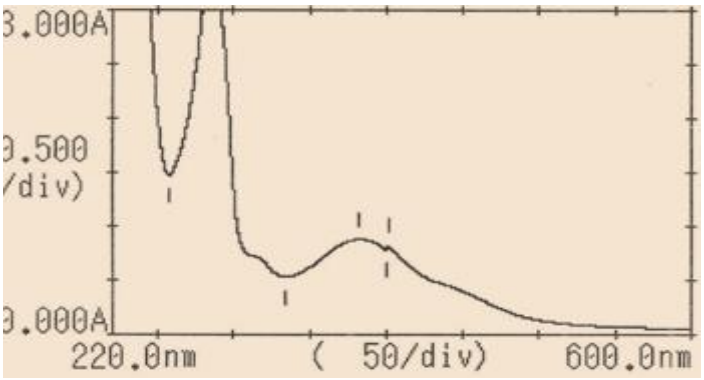


Fig.11 : Boric acid spectrum of compound II

The ^{1}HNMR spectrum (Fig.12) showed a signal at δ1.20(6H) assigned for two methyl groups. The resonance at δ2.10 accounts for an acetyl function. The aromatic protons resonated at δ7.65 and δ8.25ppm.

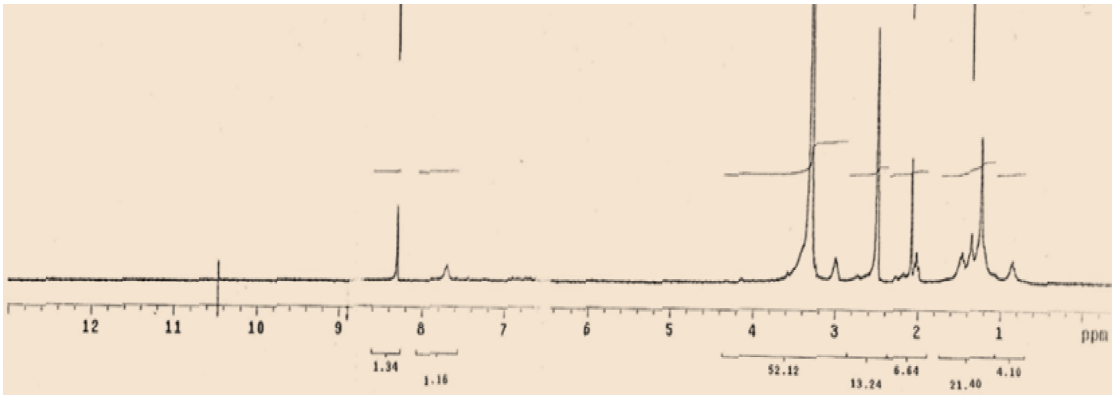
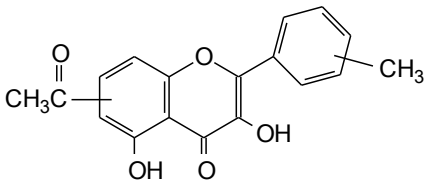


Fig.12 : ^{1}HNMR spectrum of compound II



Compound II

3.2.3-Antimicrobial assay

Compound II has been screened for antimicrobial activity against six human pathogens. The results are depicted in Table 3.8.Tables 3.9 and 3.10 display the antibacterial and antifungal activity of standard drugs.

Table .3.8: Antimicrobial activity of compound II

	Ec	Ps	Sa	Bs	Ca	An
Compound I	15	15	11	15	13	13

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtillus*

Ca = *Candida albicans*

An = *Aspergillus Nige*r

Table 3.9. : Antibacterial activity of standard chemotherapeutic agents

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Ps.
Ampicillin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-

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

Table 3.10 : Antifungal activity of standard chemotherapeutic agent

Drug	Conc. mg/ml	An.	Ca.
Clotrimazole	30 15 7.5	22 17 16	38 31 29

Compound II showed good activity against *Escherichia coli* , *Pseudomonas aeroginosa* and *Bacillus subtillus*. It also showed weak activity against *Aspergillus nige*r and weak anticandidal potency. The compound also exhibited partial activity against *Staphylococcus aureus*.

3.2.4-Antioxidant assay

In vitro antioxidant assay for compound II was conducted . Evaluation of the antioxidant activity was carried out by measuring the capacity of the test sample against stable DPPH radical. The change in colour is measured spectrophotometrically at 516nm. As depicted in Table (3.11) compound II exhibited significant anti- oxidant activity.

Table 3.11: Radical scavenging activity of compound II

Sample	Antioxidant activity(%)
Trolox	96.50
Compound I	82.02

3.3- *Acacia ehrenbergiana*

3.3.1-Phytochemical screening

Acacia ehrenbergiana was screened for major secondary metabolites and the results are displayed in Table 3.12.

Table 3.12: Major secondary metabolites in *Acacia ehrenbergiana*

Test	**Results**	**Observation**
Saponins	++	Foam
Cumarins	+	Uv absorption
Alkaloids	-	No observation
Anthraquinones glycosides	-	No observation
Tannins	++	Green colour
Flavonoids	++	Yellow color
Sterols	+	Green colour
Triterpenes	+	Purple colour
Cyanogenic glycosides	-	No observation

3.3.2-Identification of compound III

Compound III was isolated in a chromatographiclly pure form *Acacia ehrenbergiana*.The IR spectrum of compound III showed $\nu(cm^{-1})$: 687,794(C-H, Ar. bending), 1010(C-O) , 1468,1512(C=C, Ar.) ,1616(C=O) and 3372(OH).

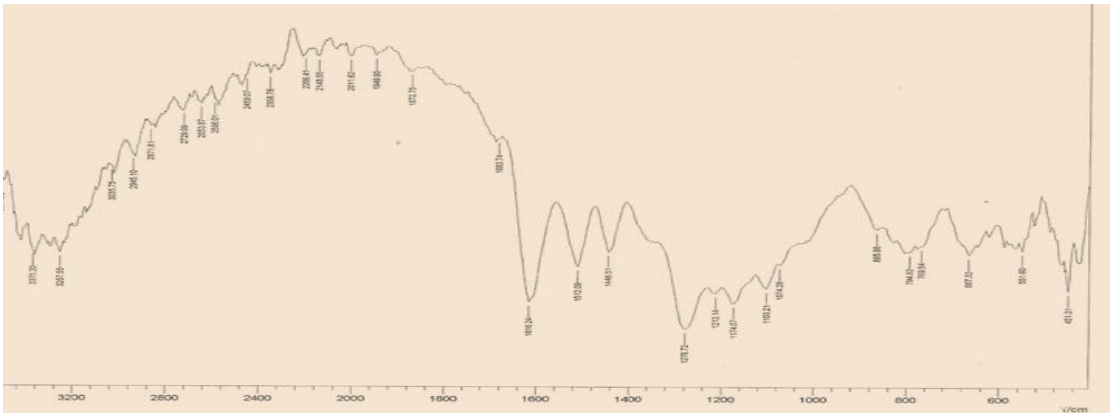


Fig. 13 : IR spectrum of compound III

Compound III absorbs in the UV at λ_{max}256,379nm. Such absorption is characteristic of chalcones which are characterized by dominant band I(see Fig. 14).

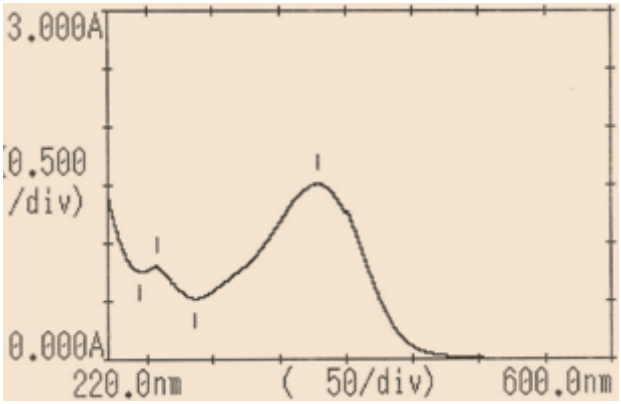


Fig.14 : UV spectrum of compound III

The hydroxylation pattern of this flavonol was investigated by using different UV shift reagents(sodium methoxide, sodium acetate, aluminium chloride and boric acid).

The UV shift reagent-sodium methoxide revealed a bathochromic shift without decrease in intensity indicating a 4-OH function (Fig.15).

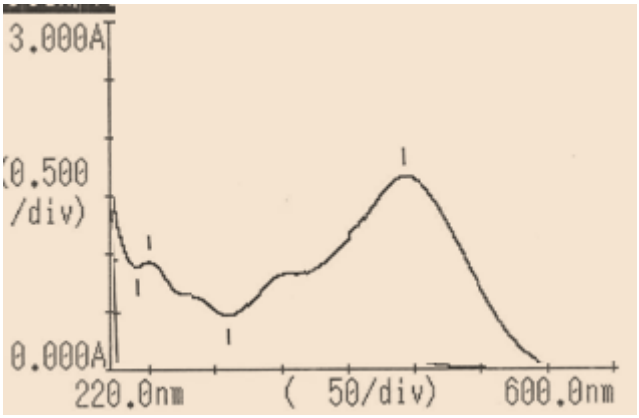


Fig.15 : Sodium methoxide spectrum of compound III

The sodium acetate spectrum (Fig.16) did not show a bathochromic shift in band II indicating absence of a 7-OH .

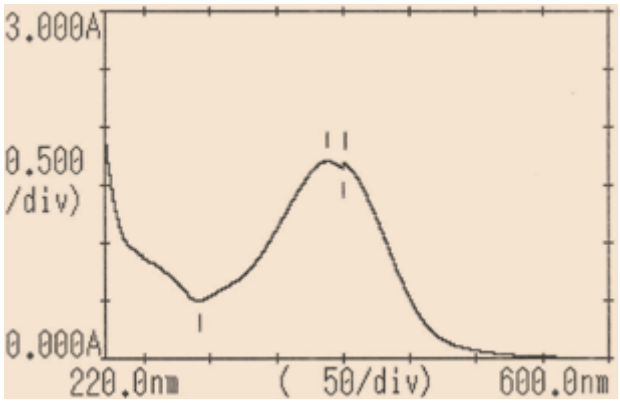


Fig.16 : Sodium acetate spectrum of compound III

The aluminium chloride spectrum which is diagnostic of 3- and 5-OH functions as well as catechol systems revealed a bathochromic shift indicative of a 2`-OH function(Fig.17).The spectrum was stable in acidic medium(Fig.18). The boric acid spectrum did not reveal any bathochromic shift indicating absence of catechol systems(Fig.19)

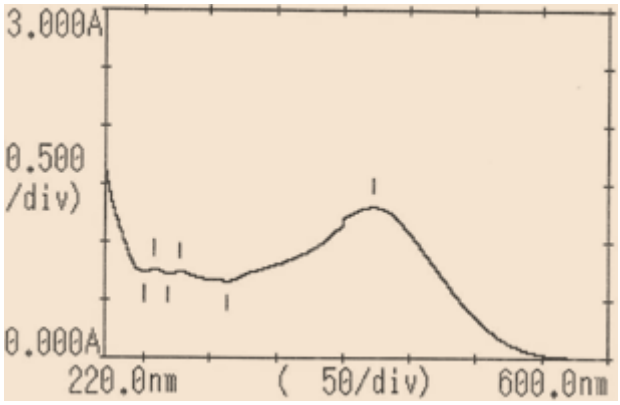


Fig.17 : Alumnium chloride spectrum of compound III

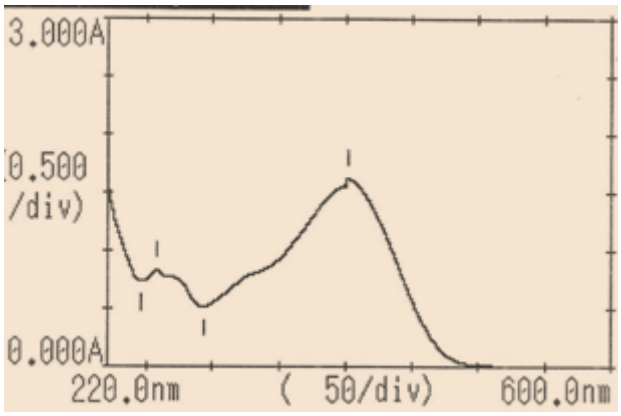


Fig.18 : Alumnium chloride/HCl spectrum of compound III

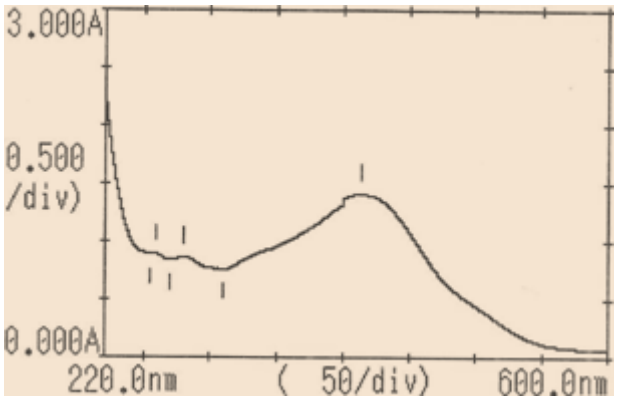
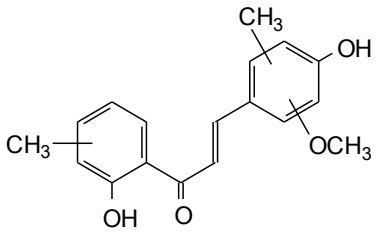


Fig.19: Boric acid spectrum of compound III

The ^{1}HNMR spectrum (Fig.20) showed a signal at δ1.23(6H) assigned for two methyl groups. The resonance at δ3.82(3H)

accounts for a methoxyl function while the signal at δ4.45 ppm accounts for the α – and β-protons of the chalcone.The aromatic protons resonated at δ6.20(H-6) and as multiplet centered at δ6.65ppm accounting for other aromatic protons(The signals at δ2.50 and 3.30 are due to solvent(DMSO) residual protons and residual water respectively).

On the basis of its spectral data , the following partial structure was assigned for compound III:



Compound III

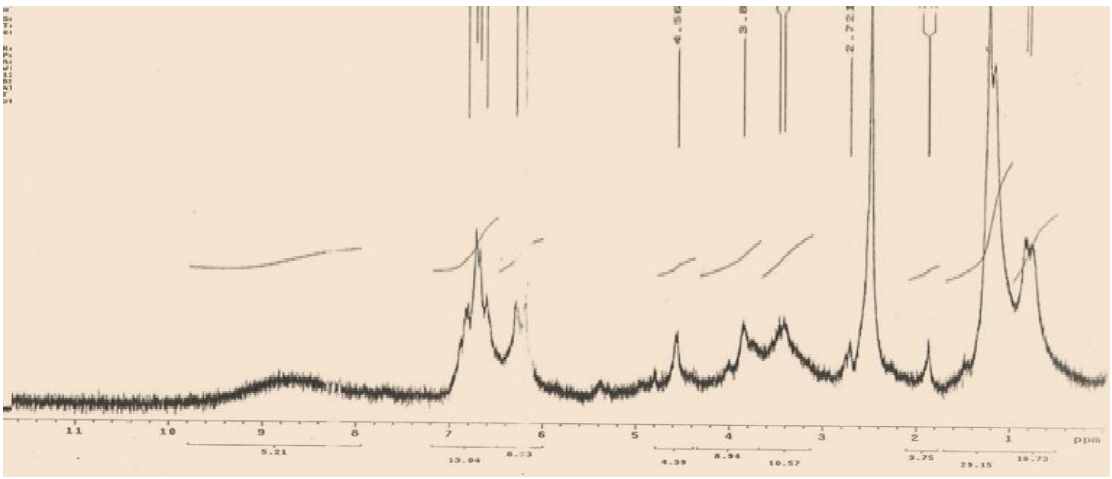


Fig.20 : ^{1}HNMR spectrum of compound III

3.3.3-Antimicrobial assay

Compound III has been screened for antimicrobial activity against six standard human pathogens. The results are depicted in Table 3.13.Tables 3.14 and 3.15 display the antibacterial and antifungal activity of standard drugs.

Table .3.13 Antimicrobial activity of compound III

	Ec	Ps	Sa	Bs	Ca	An
Compound I	12	15	13	20	16	15

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtillus*

Ca = *Candida albicans*

An = *Aspergillus Nige*r

Table 3.14. : Antibacterial activity of standard chemotherapeutic agents

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Ps.
Ampicillin	40 20 10	15 14 11	30 25 15	- - -	- - -
Gentamycin	40 20 10	25 22 17	19 18 14	22 18 15	21 15 12

Table 3.15: Antifungal activity of standard chemotherapeutic agent

Drug	Conc. mg/ml	An.	Ca.
Clotrimazole	30 15 7.5	22 17 16	38 31 29

Compound III showed excellent activity against *Bacillus subtillus*.It also showed good activity against *Pseudomonas aeroginosa, Aspergillus nige*r and good anticandidal potency.However,it showed parial activity against *Escherichia coli* , and *Staphylococcus aureus*.

3.3.4-Antioxidant assay

In vitro antioxidant assay for compound III was conducted . Evaluation of the free radical scavenging capacity was carried out by measuring the capacity of the test sample against stable DPPH radical. The change in colour is measured spectrophotometrically at 516nm. As depicted in Table (3.16) compound III exhibited significant anti- oxidant activity.

Table 3.16: Radical scavenging activity of compound III

Sample	Antioxidant activity(%)
Trolox	91.03
Compound I	82.02

Conclusion

The flavonoids from the heartwood of *Acacia nilotica* var. *tomentosa*, *Indigofera oblongifolia*, and the roots of *acacia ehrenbergiana* have been investigated.Compound I was isolated from *Acacia nilotica* var. *tomentosa*. Compound II was isolated from *Indigofera oblongifolia* while compound III was isolated from *Acacia ehrenbergiana*. These compounds were purified by different chromatographic techniques and identified via spectroscopic tools; UV, and ^{I}HNMR. The isolated compounds were evaluated for their antimicrobial potential against standard pathogenic microbes and promising results were obtained.

Recommendations

i) A future 2D NMR (^{1}H- ^{1}H COSY NMR, HMBC and HSQC) experiments may fully elucidate the structures of the isolated flavonoids.

ii) The isolated flavonoids may be evaluated for other biological activities i.e. as antimalarial, antiinflammatory ….etc.

iii) *In vivo* antimicrobial activity is also recommended.
iv)Other phytochemicals(such as alkaloids, steroids,terpenoids) in target plant species may be investigated.

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