Sudan University of Science and Technology

College of Post Graduate Studies

A Study of the Major Flavonoid of *Mitragyna inermis* Stem Bark

دراسة الفلافونيد الرئيس في لحاء ساق نبات *Mitragyna inermis* ام قادو

A Thesis Submitted in Fulfillment of the Requirements of the Master Degree in Chemistry

By

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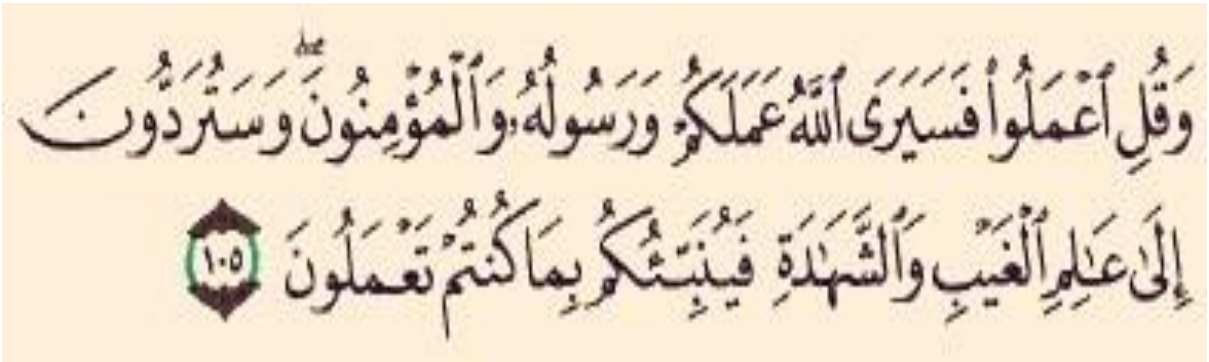
الاستهلال


بسم الله الرحمن الرحيم


وَقُلِ اعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ ۖ وَسَتُرَدُّونَ
إِلَىٰ عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُم بِمَا كُنتُمْ تَعْمَلُونَ ﴿١٠٥﴾

(التوبة-105)


صدق الله العظيم

Dedication

Dedicated to:

my parents

my wife

brothers and sisters

Acknowledgement

I would like to thank **Almighty Allah** for giving me the will to complete this wok.

Many have persons 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.

I would like to thank Prof.. Mohamed Abdel Karim for his help and patience through the periods of this research. I would always remember and appreciate your valuable contributions and suggestions.

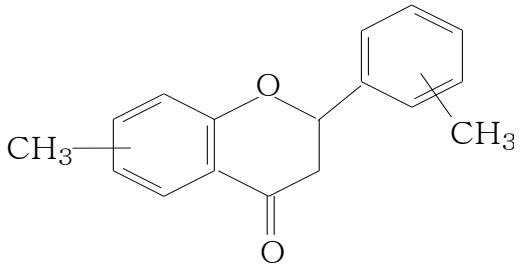
Thanks for the technical staff, Sudan University of Science and Technology for all facilities.

Thanks are due to The Medicinal and Aromatic Plants Institute for the authentication of the plant material. Thanks are also extended to the National Research Center-Cairo- for the NMR data.

My sincere thanks to those who helped me in any way during this work.

Abstract

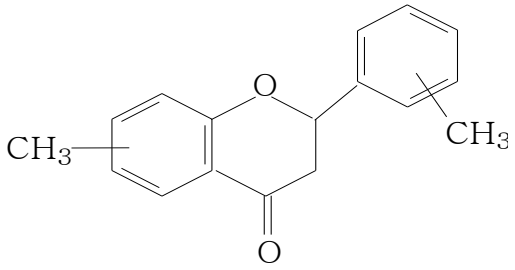
In our search for structurally and biological interesting compounds from plants growing in Sudan, a flavonoid has been isolated from *Mitragyna inermis* barks. This plant is used in many countries by traditional medicine practitioners for the treatment of various human disorders. In this study , stem barks of *Mitragyna inermis* were extracted with 95% ethanol and the crude extract was fractionated over silica gel plates to yield a chromatographically pure flavonoid – compound I. The structure of the isolated compound has been partially characterized by its spectral data (UV and ^{1}HNMR). On the basis of its spectral data, the isolated flavanone was assigned the following partial structure:



المستخلص

في اطار بحثنا عن مكونات نباتية ذات تركيب كيميائى مميز وفعالية بيولوجية، تم فصل مركب فلافونيدى من نبات (ام قاتو) الذى ينمو في السودان. ويجد هذا النبات العديد من الاستخدامات في الطب الشعبى.

استخلص لحاء نبات(ام قاتو) بالاثانول وتمت تنقية المستخلص بتقنية كروموتوغرافيا الطبقات الرقيقة حيث تم فصل مركب فلافونيدى نقى وحدد تركيبة المبدئ بناء على البيانات الطيفية (طيف الاشعة فوق البنفسجية – المرئية وطيف الرنين النووي المغنطيسى). وقد اقترح التركيب المبدئ التالى للفلافانون الذى تم فصله:



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Chapter One

Introduction

1. Introduction

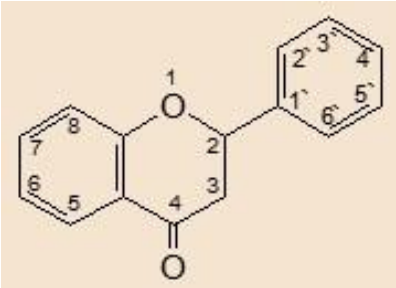
1.1- General overview

Flavonoids are low molecular weight plant polyphenolics [1,2] separated from a wide array of vascular plants, and more than 10,000 structural variants of flavonoids have been reported.[1,3] They fit to a group of natural phenolic substances with variable chemical structures and in plants they are located in fruits, vegetables, grains, tree barks, flowers, roots, stems, as well as tea .[2] Flavonoids are placed inside the cells or on the surface of various plant organs and have various functions in plants.[4]

These plant phenolics act in plants as antioxidants, antimicrobials, visual attractors, feeding repellents, photoreceptors, and for light screening.[5] Many studies have shown that flavonoids exhibit biological and pharmacological activities, including antioxidant, anticancer, antibacterial, cytotoxic, antiviral, cardioprotective, hepatoprotective, neuroprotective, antimalarial, antitrypanosomal, antileishmanial and antiamebial properties.[6-14] These biological and pharmacological properties are usually attributed to their free radical scavenging efficacies, metal complexion capabilities, and their ability to bind to proteins with a high degree of specificity.[15]

1.2- Properties and classification of flavonoids

Flavonoids (2-phenylbenzopyrone) - (1) are a big group of polyphenolic compounds that occur commonly in plant.[16]

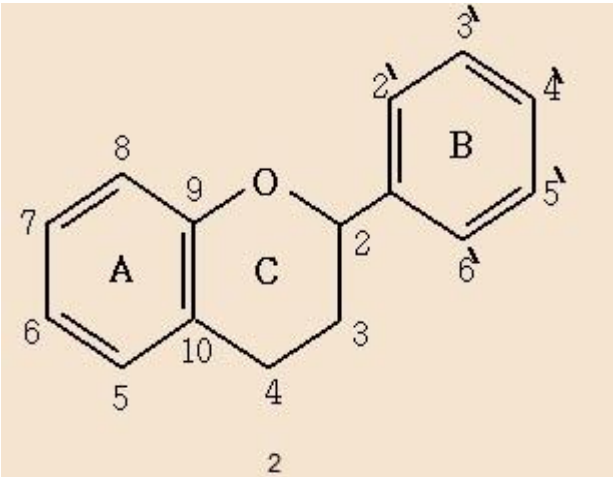


(1)

These compounds have a common phenyl benzopyrone structure (C6-C3-C6).[17]

These phytochemicals are distributed widely in higher plant parts (barks, roots, stems, flowers) but also found in some lower plants.[18,19] Many of these compounds are responsible for the attractive colors of flowers and fruits and leaves. In the human body they play a similar role as vitamins.[20-23]

The basic flavonoid structure contains the flavan nucleus (2), which contains of 15 carbon atoms derived from a C_6-C_3-C_6 skeleton. A flavonoid skeleton is composed of two aromatic rings (commonly labeled as A and B), Ring A and ring B are connected by a three carbon heterocycle ring (ring C).[24,25] The connecting carbon chain combines with an oxygen to form a heterocyclic central C-ring for most flavonoids with the exception of chalcones (3) in which the carbon chain between the A and B rings is linear.[24]



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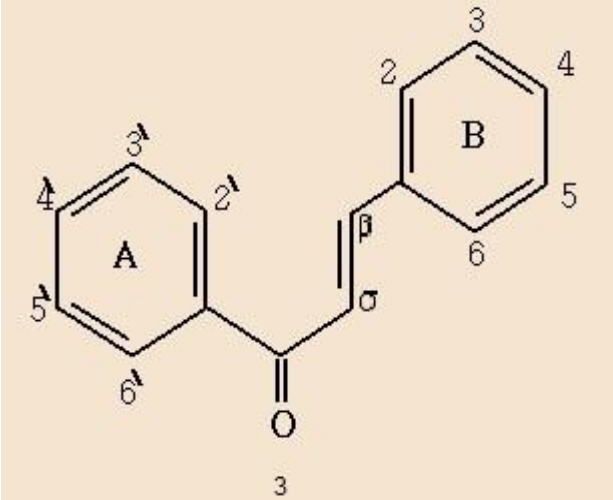

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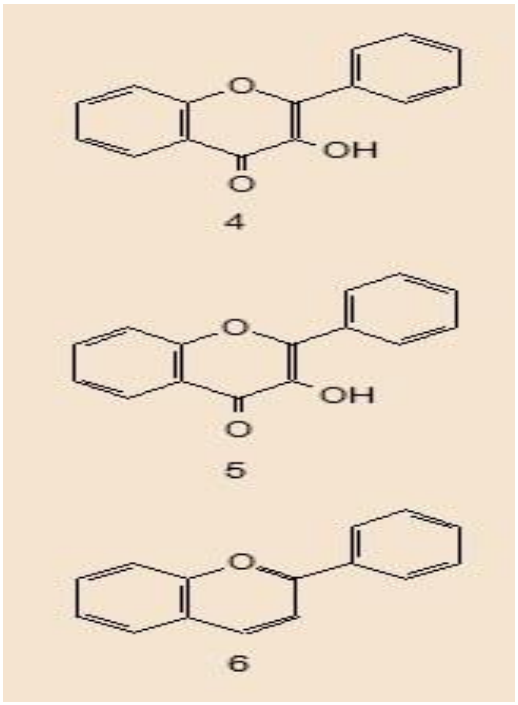
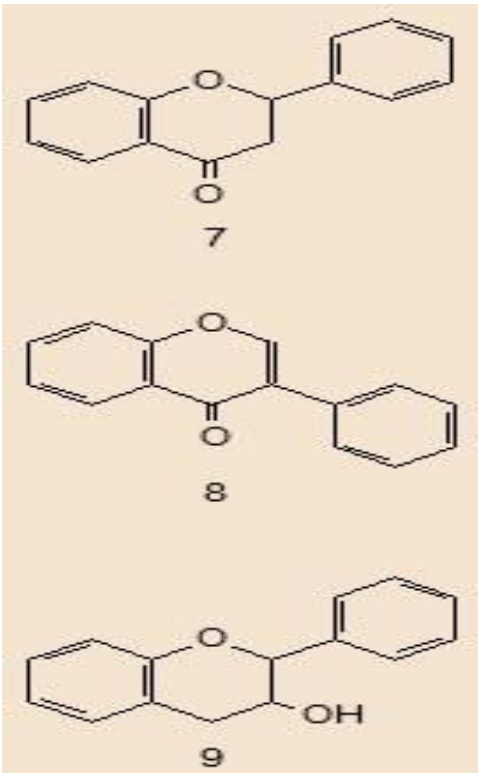
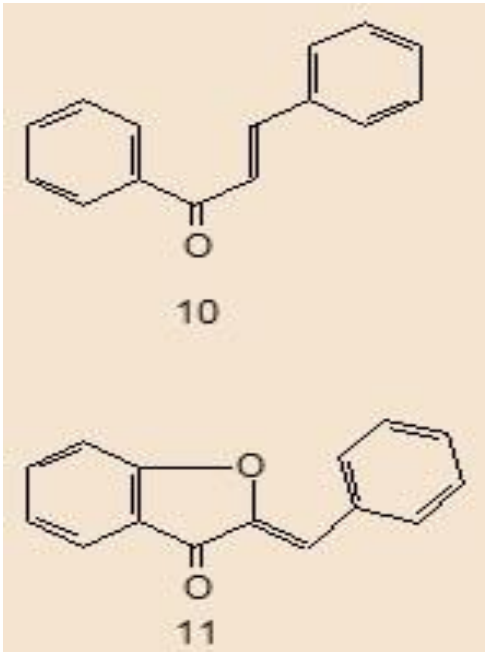
Flavonoids mainly differ in saturation of hetero atomic ring C, in the situation of the aromatic ring B at the positions 2,3 or 4.Flavonoids can be divided- according to the modifications of the central C-ring- into classes including : flavonols (4) flavones (5), anthocyanins (6), flavonones (7), isoflavones (8) and flavan-3-ols (9). In a few cases, the 6- member heterocyclic ring C found in an isomeric open form or is

changed by a 5- member ring as in the case of chaclones (10) and aurones (11).(26 - 28)







Being soluble in water , flavonoids can be extracted with 70% ethanol and remains in the aqueous layer, following partition of this extract with an organic solvent (like petroleum ether).

Flavonoids are soluble in alkalis, yielding yellow solutions with addition of acid, the solution become colorless. Flavonoids are phenolic and hence they change color when treated with base or with ammonia, thus they are easily detected on chromatograms or in solution. Color properties of different flavonoids in visible and ultraviolet light are presented in Table 1.[29]

Table1: The color properties of flavonoids in visible and ultraviolet light [29]

Visible Color	Color UV light		Indication
	Alone	With ammonia	
Orange Red Mature	Dull orange. Red or mauve. Flouriest yellow cerise or pink.	Blue Blue	Anthocyanidin 3-glycosides Most anthocyanidin. 3,5-Diglycosides.
Bright Yellow	Dark brown or black. Bright yellow or Yellow green.	Dark brown or black. Dark red or Bright orange. Bright orange or red.	6-Hydroxylated flavonols and flavones; Some chalcones glycoside. Most chalcones Aurones.
Very pale yellow	Dark brown.	Bright yellow or Yellow brown. Vivid yellow green. Dark brown.	Most flavonols glycosides. Most flavone glycoside. Biflavonoyls and unusually Substituted flavones
None	Dark mauve. Faint blue. Dark mauve.	Faint brown Intense Blue Pale yellow or Yellow green.	Most isoflavones and flavonoids. 5-desoxyflavonones. Flavonones and flavonol 7-glycosides

Flavonoids usually occur in their natural matrix as glycosides and flavonoid aglycones may occur in the same plant in several glycosidic

combinations. Hence, when analyzing flavonoids, is better to hydrolyse plant extracts before considering the complexity of glycosides that may be existing in the original extract.[29]

Flavonoids are known to exist in all vascular plants. However, some classes of flavonoids are more widely distributed in plants than others; while flavones and flavonols are found in only a few plant families.[29]

It is very rare to find only a single flavonoid in a plant tissue. In addition, there are often mixtures of different flavonoid classes. The colored anthocyanins in flower petals have flavones as important co-pigments, such flavones are essential for the full expression of anthocyanin color in floral tissues.

The classification of plant flavonoids is mainly based on investigation of solubility and color reactions. One dimensional chromatographic examination of hydrolyzed plant extract followed by a two dimensional chromatography of direct alcoholic extract is usually accomplished. The flavonoids could be isolated by a combination of chromatographic techniques and the separate flavonoids are characterized by chromatographic and spectral comparison with reference compounds.[30]

1.3- Flavonols

Due to their role as antioxidants and other interesting biological activities, flavonols (3-hydroxyflavones) are one the most analyzed

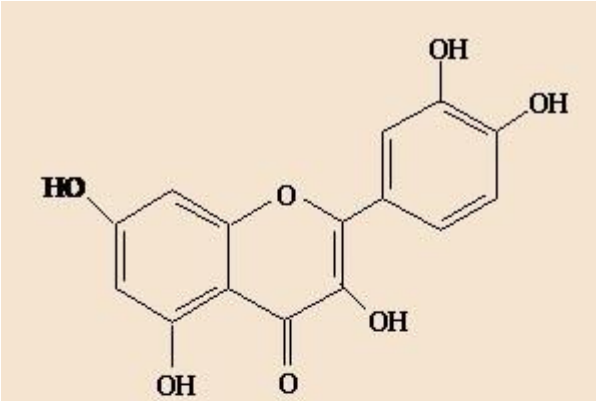
sub-class of flavonoids. This group of phytochemicals are found in commonly consumed vegetablesa and fruits. Rich sources include: grape berries, apple, onion, tomato, red lettuce and broccoli. In addition to fruits and vegetables, green tea and black teas constitute also a major source of flavonols.[31]The the most common flavonols aglycones are kaempherol(12), quercetin(13) and myrcetin (14) .[32]

12

The intake of flavonols in considerable quantities is associated with many health benefits including reduced risk of cardiovascular diseases. This effects are due to their antioxidant properties which have been of interest for considerable time.[32]

The antioxidant activity of flavonols depends largely on their chemical structure. The following structural features contribute to the antioxidant potential of flavonols : a catechol structure in the B ring, which is a radical target site; a 2,3-double bond in conjugation with a 4-keto function, which are responsible for electron delocalization from

the B ring and the additional presence of both 3- and 5-hydroxyl groups for maximal radical-scavenging potential and strongest radical absorption.[31]



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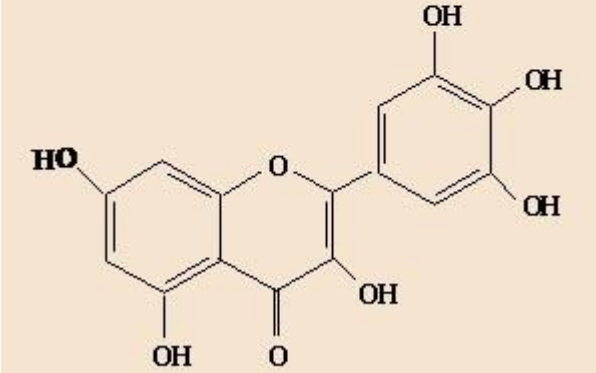

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By acting as free radical scavengers , flavonols may protect against oxidative damage to cells, lipids or DNA. Moreover, these properties are the result of the presence of aromatic rings of the flavonoid molecule, which allow the donation and acceptance of electrons from free radical species. This aids in suppressing free radicals. Furthermore, the consumption of flavonols is related with reduced risk

of stroke and cancer. Also, some of these compounds are believed to prevent osteoporosis and possess anti-inflammatory or neuroprotective properties.[32]

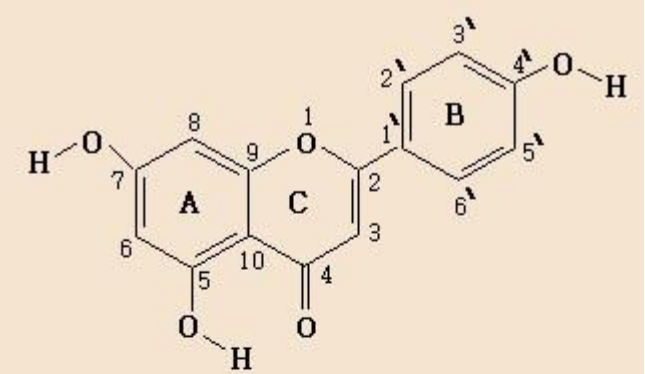
Among the flavonols , quercetin is the major representative of the flavonol subclass with effective antioxidant properties . It inhibited the oxidation of low density lipoproteins *in vitro*. Quercetin a water-soluble flavonol commonly found in green tea, onions, apples and leafy vegetables. This important phytochemical can protect cellular structures and blood vessels from the damaging effects of free radicals . Furthermore, quercetin improves blood vessel strength and stems the activity of the enzyme catechol-O-methyl transferase that inhibit the neurotransmitter norepinephrine. This action may lead to elevated levels of norepinephrine, thermogenesis, and fat oxidation. Also this flavonol acts as antihistamine agent preventing from allergies or asthma. Antioxidant properties of quercetin is reflected in LDL cholesterol reduction and heart disease protection. Quercetin can block an enzyme resulting in sorbitol accumulation which has been associated with nerve, kidney or eye damage in diabetics. This flavonoid may protect against cataract formation.[33]

Another important flavonol endowed with potential antioxidant properties is kaempferol. It occurs in fruits and vegetables and mainly in broccoli. Some reports have emphasized the advantageous effects of dietary kaempferol in decreasing the risk of chronic diseases,

particularly cancer. Kaempferol can strengthen the antioxidant defense against free radicals, which support the cancer development. Kaempferol has been investigated to modulate a number of key elements in cellular signal transduction pathways related to angiogenesis, apoptosis, metastasis, and inflammation. It is confirmed that kaempferol meaningfully inhibits cancer cell growth and angiogenesis, as well as generates cancer cell apoptosis. However, this flavonol seems to maintain normal cell viability, usually exerting a protective effect.[34]

Another flavonol commonly consumed through human diets (such as vegetables, fruits, red wine, tea, and berries) is myricetin. Myricetin may improve insulin resistance. This phytochemical performs diverse biological activities including antioxidative, anti-non enzymatic glycation, anti-hyperlipidemia, anti-inflammation, anti-aldose reductase.[35,36].

Flavones possess structure very close to that of flavonols, having more hydroxyl substitution at the carbon 3-position. Some important flavones are apigenin(15) and luteolin (16). Sources of luteolin include: fruits and vegetables (celery, broccoli, onion, parsley, carrots, leaves, peppers, cabbages, chrysanthemum flowers, and apple skins).[37] Sources of apigenin include: parsley, onions, wheat sprouts, oranges, tea, chamomile, and in some seasonings.[38]



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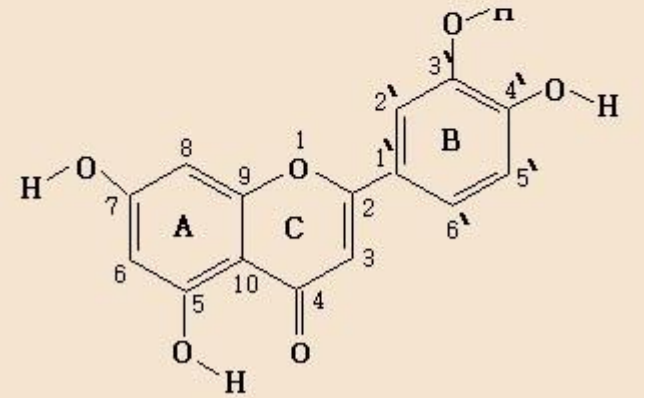

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Apigenin is a flavones which is associated with antiphlogistic, antibacterial, and antispasmodic effects. Apigenin has captured the interest as helpful and health promoting agent because of its low internal toxicity and differential results in normal against cancer cells relative to other structurally related flavonoids. Apigenin possesses prominent anti-inflammatory, anticarcinogenic and antioxidant properties. Apigenin has been demonstrated to inhibit benzopyrene and 2-aminoanthracene-induced bacterial mutagenesis.[38] Furthermore, some studies have proved that apigenin supports metal

chelation, scavenges free radicals and stimulates phase II detoxification enzymes in cell culture and in *invivo* tumor models. Apigenin may act as severe inhibitor of ornithine decarboxylase, an enzyme playing an essential role in tumor promotion. The anti-carcinogenic effects of this flavone is indicated in a skin carcinogenesis model.[39]

Some luteolin-rich plants have been used in Chinese system of medicine against hypertension, inflammatory diseases, and cancer. Luteolin possesses multiple biological effects such as anticancer, anti-inflammation, and anti-allergy.[40] Therefore, luteolin behaves as either antioxidant or prooxidant biochemically.[41] These biological effects can be related to each other, for example the anti-inflammatory properties are associated with its anticancer activity. Its anticancer properties are related to the induction of apoptosis, including DNA damage, redox regulation and protein kinases, inhibition of cell metastasis, proliferation, and angiogenesis.

Luteolin may sensitize diversity of cancer cells to therapeutically induced cytotoxicity through damping cell survival pathways and stimulating apoptosis pathways. Luteolin is also suitable to the therapy of central nerve system diseases, including brain cancer.[40]

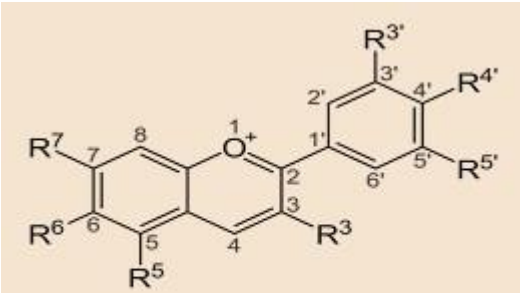
Luteolin is a potential antioxidant being able to inhibit ROS-induced damage of lipids, protein and DNA. This flavones exhibits its

antioxidant effect through protecting or extending endogenous antioxidants such as: glutathione reductase, glutathione-S-transferase, superoxide dismutase.(41) Luteolin presents its anti-inflammatory effect by damping the production of these cytokines and their signal transduction pathways.(37)

1.4- The anthocyanins

Anthocyanins are plant pigments being responsible for blue, red, or purple shades found in plants, especially flowers, fruits, and tubers., Anthocyanins appear in acidic medium as red pigments but they change their color to blue alkaline medium. Anthocyanins are classified as flavonoids although they have a positive charge at the oxygen atom of the C-ring of basic flavonoid structure. Anthocyanins also referred to as flavylium ion(2-phenylchromenylium). The structure of anthocyanin is generally represented by(17). The stability of anthocyanin is dependent on pH, light, temperature, and its structure.(41,42)

The plant pigments –anthocyanins- are usually found in flowers and fruits of many plants. Most of the red, purple, and blue-colored flowers contain anthocyanins. Anthcyanins are responsible for the edible red flowers of : hibiscus, rose, pineapple sage, clover, and



(17)

pink blossom. Anthocyanins are responsible for the blue color of : cornflower, blue chicory, and blue rosemary and the purple color of purple mint, purple passion flower, purple sage, common violet, and lavender flowers.

Some anthcyanin- containing flowers have been used in ethnomedicine , as colorants, and as food. In addition to traditional usage, red, purple, and blue-colored fruits are commonly consumed for their beneficial health effects. The colored pigments of anthocyanin from berries, blackcurrants, and other types of red to blue-colored fruits are strong antioxidants. Moreover, anthocyanin-rich black carrot, red cabbage, and purple potato are potential functional foods that have been consumed for prevention of diseases[43].

Anthocyanins-containing blue, red, and purple colored pigments extracted from flowers, fruits, and vegetables are traditionally used as dye and food colorant. Besides being used as natural colorants.

Also some anthocyanin-rich flowers and fruits have been traditionally used as medicine to treat various diseases. Anthocyanins have been widely investigated for their medicinal values[43].

Anthocyanins extracted from edible plants are potential pharmaceutical ingredients possessing potential antidiabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesity effects[43].

Anthocyanin is in the form of glycoside while anthocyanidin forms the aglycone. Anthocyanidins are grouped into 3-hydroxy-anthocyanidins, 3-deoxy-anthocyanidins, and O-methylated anthocyanidins, while anthocyanins are in the forms of anthocyanidin glycosides and acylated anthocyanins[43].

Common anthocyanidins are : cyanidin(18), delphinidin(19), pelargonidin(20), peonidin, petunidin, and malvidin. Acylated anthocyanins are also detected in plants besides the typical anthocyanins. Acylated anthocyanins are further divided into : acrylated anthocyanin, coumaroylated anthocyanin, caffeoylated anthocyanin, and malonylated anthocyanin[43].

Anthocyanins have the basic structure of flavylium ion(17), that is a lack of a ketone oxygen at the 4-position and are derived from flavonols . The empirical formula for flavylium ion of anthocyanin is $C_{15}H_{11}O^{+}$ with a molecular weight of 207.24724 g/mol. The

conjugated bonds of anthocyanins result in red, blue, and purple shades in plants.

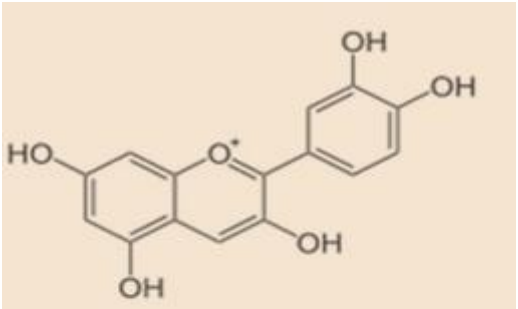
Cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin are the most common anthocyanidins distributed in the plants. The distribution of these anthocyanidins in fruits and vegetables is around : 50%, 12%, 12%, 12%, 7%, and 7%, respectively.[44].

In its natrural matrix, cyanidin is a reddish-purple pigment. It is the major pigment in berries [45] and other red-colored vegetables such as red sweet potato and purple corn.[46]

The anthocyanin , delphinidin has a chemical characteristic similar to most of the anthocyanidins. It appears as a blue-reddish or purple pigment in the plant. The blue hue of flowers is due to the delphinidin pigment.[47]

On the other hand , pelargonidin differs from most of the anthocyanidins. It appears as red-colored pigment.[48] Pelargonidin gives an orange hue to flowers [49] and red to some of the fruits and berries.[50]

Peonidin is a methylated anthocyanidin abundantly found in plants. It has the visible color magenta.[48] Peonidin is abundantly found in berries, grapes, and red wines. Malvidin is another O-methylated anthocyanidin. It has a purple visible color, and is abundant in blue-



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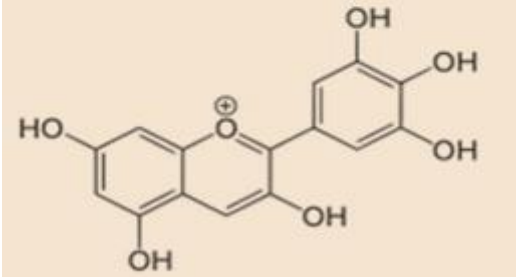

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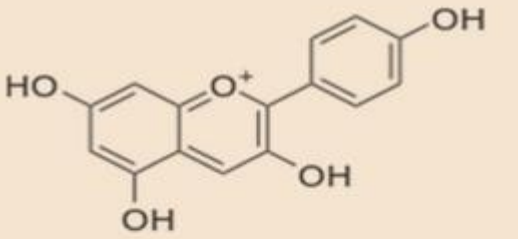

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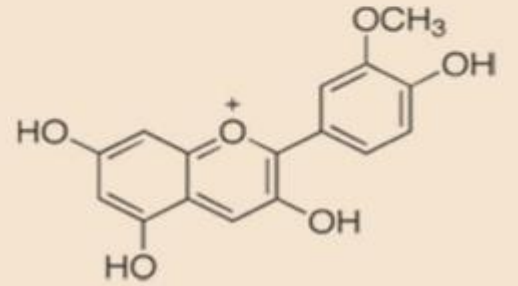

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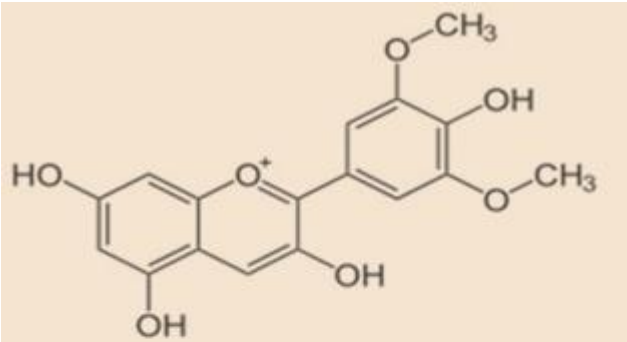

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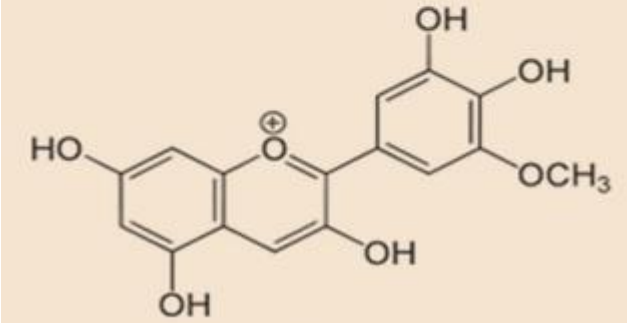

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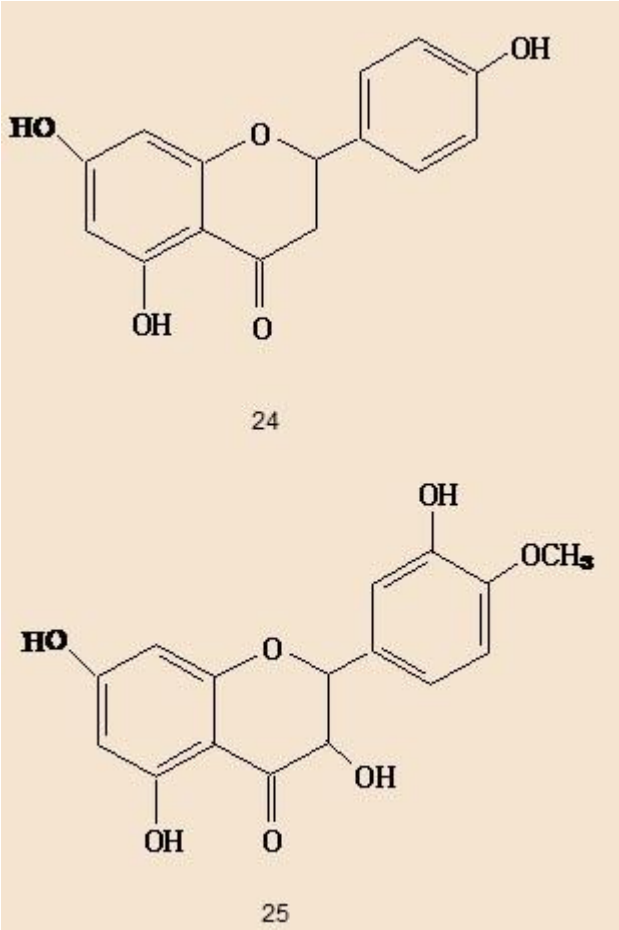
colored flowers, especially Summer Wave Blue.(51) Malvidin is also the major red pigment in red wine.(52) It appears as darker dusty red in matured red wines.(53) Petunidin is a methylated anthocyanidin. It is a dark red or purple pigment that is soluble in water.(48) Petunidin has been detected in blackcurrants (54) and purple petals of flower.(55)

1.5- Flavanones

Flavanones are a class of flavonoids with wide distribution especially in families : *Compositae*, *Leguminosae* and *Rutaceae*. Flavanones can exist in all parts of plants - aerial and below ground, from vegetative

part to generative organs including: branches, stem, bark, roots, leaves, flowers, fruits, seeds, rhizomes and peels. Among flavanones found in foods, naringenin(24) and hesperetin(25)-aglycones appear to be of particular interest. [56]



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A Distinctive flavanone of lemon, orange, lime and tangelo is hesperetin.[57] Naringenin is distributed in grapefruit and sour orange. Tomatoes and their products are also rich in this flavanone.

Naringenin is found in its natural matrix both as aglycone or glycosides.[58]

Flavanones are among flavonoids which are abundant in the daily human diet, as well as medicinal plant materials.[59] The main directions of the pharmacological activity of flavanones are: radical scavenging, anti-inflammatory, anticancer, cardiovascular, and antiviral effects.[56]

The antioxidant potential of flavanones is associated with the number and spatial orientation of phenolic OH groups. In a hydrophilic environment, flavanones show a higher antioxidant activity. This environment causes the reduction of antioxidant potential by some flavanones (hesperetin, neohesperidin) while others (narirutin, naringin, naringenin) become pro-oxidant[59].

Dietary flavanones without catechol systems are classified as weak antioxidants and their metabolites are supposed to be even less strong. Thereby, the most meaningful mechanisms involved in their health effects must be unrelated to their antioxidant activity.[60]

Naringenin and its conjugates are considered very efficient in inhibition of pro-inflammatory cytokines induced by lipopolysaccharide in macrophages and they reduce production of nitrate and nitrite which are indicators of inflammatory process to control the formation of intestinal edema.[61, 62]

Though, flavanones have not been thoroughly investigated for their anticancer properties , the major citrus flavanones may have potential in working against carcinogenesis by minimizing DNA damage, tumor proliferation and development.(63) Naringenin showed antimutagenic activity manifested in the protection against DNA damage by their capacity to absorb UV light. The moderate antioxidant capacity of flavanones is found helpful in protecting against mutation by free radicals generated in the body. It is confirmed that naringenin participates in presenting antimutagenic changes by stimulating DNA repair, following oxidative damage in human prostate cancer cells.(64) The pharmacological importance of flavanones may also be estimated by their effect against tumor development.

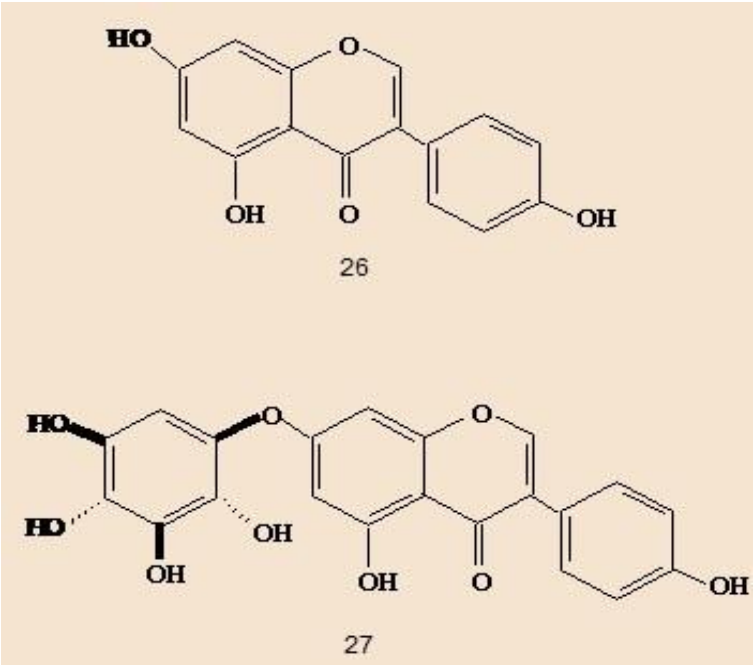
The inhibitory effect of the flavanones hesperetin and naringenin on the development of breast cancer induced by 7,12-dimethylbenzanthracene in female rats has been confirmed.(65) Furthermore, flavanones present an important antiproliferative activity against prostate, breast, colon, lung and melanoma cancerous cell lines.(66) Flavanones are believed to have anti-atherosclerosis potential. The studies demonstrated the reduction of atherosclerosis in mice fed with high fat-high cholesterol diet using naringenin supplementation at nutritionally relevant level. This result could be exerted to improve dyslipidemia and biomarkers of endothelial dysfunction, as well as

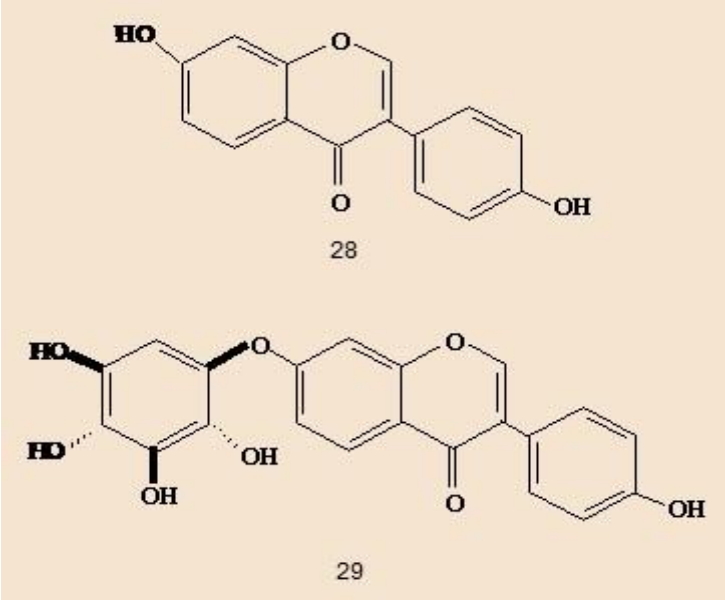
changes in gene expression. Thus, flavanones may prevent cardiovascular disease.(67)

1.6- Isoflavones

Isoflavonoids comprise a distinctive subgroup of plant phenolics. These phytochemicals have only a limited distribution in the plant kingdom and are predominantly found in soya beans and other leguminous plants. Some Isoflavonoids have also been reported to occur in microbial organisms.(3)

Isoflavonoids are found to play a significant role as precursors for the development of phytoalexins during plant microbe interactions.(3) They exhibit tremendous potential to fight a number of diseases. The structures of some key isoflavonoids - genistriein(26) , genistein(27), daidzein(28),diadzin(29) and glycitein(30) - are presented below.





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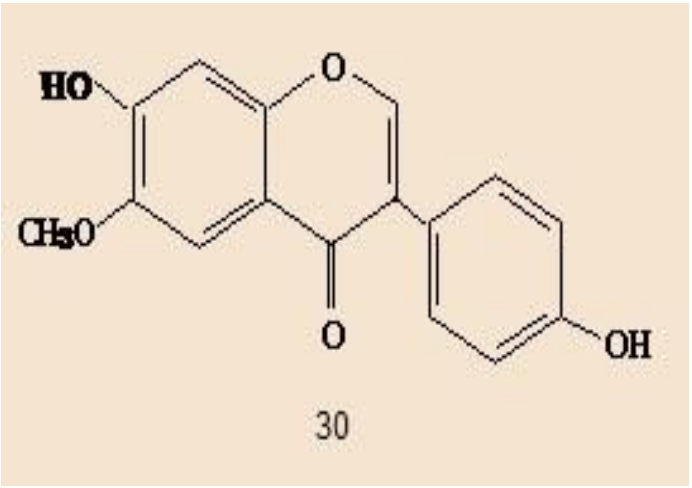

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Isoflavonoids are endowed with a chemical structure similar to plant hormone estrogen. The isoflavones genistein and daidzein are commonly regarded to be phytoestrogens because of their estrogenic activity in certain animal models. The effect of genistein in inducing hormonal and metabolic changes, by virtue of which they can influence various disease pathways has been reviewed.[6]

Though a number of isoflavonoids have been investigated, soybeans isoflavonoids received a major attention. Such isoflavonoids do possess numerous biological activities. They play an important role in protecting and maintaining strong and healthy bones by improving bone mass and reducing bone resorption. They also act as anti-oxidants to counteract damaging effects of free radicals in tissues. Some studies[8] demonstrated that the anti-oxidant potential of genistein and daidzein can help in inhibiting peroxidation of lipid in a liposomal system.

It has been demonstrated that the consumption of isoflavones through diet can reduce the buildup of arterial plaques, which further reduces the risk of coronary heart disease and associated cardiovascular complications. Isoflavonoids also help reduce breast cancer by blocking the cancer causing effects of human estrogen. They may also prevent prostate cancer and hinder cell growth. In addition, they may also relieve menopausal symptoms. Isoflavonoids are capable of reducing blood pressure and help improving vascular functions.

Some studies have indicated that isoflavonoids possess antioxidant properties. The isoflavonoids genistein and daidzein are examples of key isoflavonoids.

Due to their biological potential, isoflavonoids find various applications in cosmetic industries and are an important constituent of

various dietary supplements, creams, ointments, moisturizing lotions and gels.

1.7-The target species- *Mitragyna inermis*

Mitragyna is a genus in the family Rubiaceae. This genus is distributed in tropical and subtropical regions of Africa and Asia .The genus contains ten species : *Mitragyna parvifolia*, *Mitragyna hirsuta*, *Mitragyna diversifolia*, *Mitragyna tubulosa*, *Mitragyna speciosa*, *Mitragyna rotundifolia*, *Mitragyna ciliate*, *Mitragyna inermis*, *Mitragyna africanus* and *Mitragyna stipulosa*[68].

Different species of *Mitragyna* are widely used in ethnomedicine[69,70] against malaria , fever, worms, diarrhea and cough. *Mitragyna speciosa* is a natural remedy for fatigue[71]. *Mitragyna ciliate* , *Mitragyna inermis and Mitragyna stipulosa* have been used traditionally against hypertension, inflammation, rheumatism, gonorrhea and bronchpulmonary diseases. *Mitragyna africanus* is used traditionally against mental diseases[72]

Some indole alkaloids , triterpenoids and saponins have been reported from the genus *Mitragyna*.The major alkaloid of this genus demonstrated a wide reaching pharmacological potential including cardiovascular, antitumor, and antimicrobial activities[73-76]. It has been documented that mitragynine-the major alkaloid of *Mitragyna*- possesses analgesic properties[77-79]. Different extracts of *Mitragyna*

speciosa and Mitragyna parvifolia showed significant antiinflammatory activity[80-83]. It has been reported that *Mitragyna speciosa* exhibited significant antimutagenic activity[84]. *Mitragyna ciliate* extracts induced aortic relaxation in a dose-dependant manner[85]. In some in vivo studies, The extracts of *Mitragyna diversifolia* exhibited antidiarrheal effect[86]. Extracts of *Mitragyna speciosa* also showed antidiarrheal activity[87,88].The antimicrobial and antioxidant activities of *Mitragyna speciosa* have been reported[89].

Aim of this study

This study was designed to fulfill the following objectives:

-Extraction of flavonoids from the medicinal plant *Mitragyna inermis.*

-Isolation of flavonoids via chromatographic techniques.

-Spectral characterization of the isolated flavonoids.

Chapter Two

Materials and Methods

2- Materials and methods

2.1.Materials

2.1.1.Plant material

Mitragyna inermis stem barks were collected from Nyala, western Sudan. The plant was identified and authenticated by The Aromatic and Medicinal Plants Research Institute, Khartoum, Sudan.

2.1.2. Equipments

An Ultra - Violet - Visible spectrophotometer (Shimadzu model UV240 and 240PC) was used for UV measurements .

A Joel- Nuclear Magnetic Resonance (NMR) spectrophotometer, (Brucker AC-250) was used for NMR measurements.

2.1.3. Solvents

In this study , analytical grade solvents were used. Methanol(Merck,Germany) was used for spectrophotometric analysis . DMSO-d_6 was used as solvent and TMS as internal standard.

2.2. Methods

2.2.1. Extraction of flavonoids

Mitragyna inermis stem barks (1Kg) were extracted with 95% ethanol for 72h. at room temperature. The extract was filtered

and the solvent was removed *in vacuo* yielding a crude product.

2.2.2.Phytochemical screening

The target plants were screening for the presence of 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

slayer of foam indicates the presence of saponins.

2.2.3.Isolation of flavonoids

Concentrated plant extract was applied on TLC plates. The plates were developed with 60% acetic acid. The chromatograms were then located under UV light. A Chromatography pure flavonoid- compound I was thus isolated.

2.2.4.Structural elucidation of the isolated compound

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

2.2.4.1.UV-Visible Spectroscopy

UV-Visible spectra of the isolated flavonoid 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 200-500nm) . 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. 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 .

2.2.4.1.1. 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.

2.2.4.2..Proton Nuclear Magnetic Resonance Spectroscopy of (NMR)

Sample was dried by freeze drying over several days and dissolved in DMSO-*d6* and then analyzed.

Chapter Three

Results and Discussion

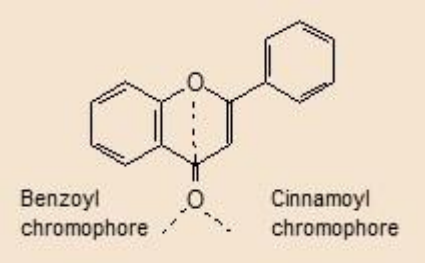
3-Results and Discussion

In our search for structurally and biological interesting compounds from plants growing in Sudan, a flavonoid has been isolated from *Mitragyna inermis* barks. This plant is used in many countries by traditional medicine practitioners for the treatment of various human disorders [163]. It is grown in sub-Sahara Africa [164, 165-167]. *Mitragyna inermis* is a bushy tree and grows up to 16 m in height. It belongs to the family Rubiaceae.Barks were extracted with 95% ethanol and the crude extract was fractionated over silica gel plates to yield a chromatographically pure flavonoid – compound I. The structure of the isolated compound has been partially characterized by it spectral data (UV and ^{1}HNMR).

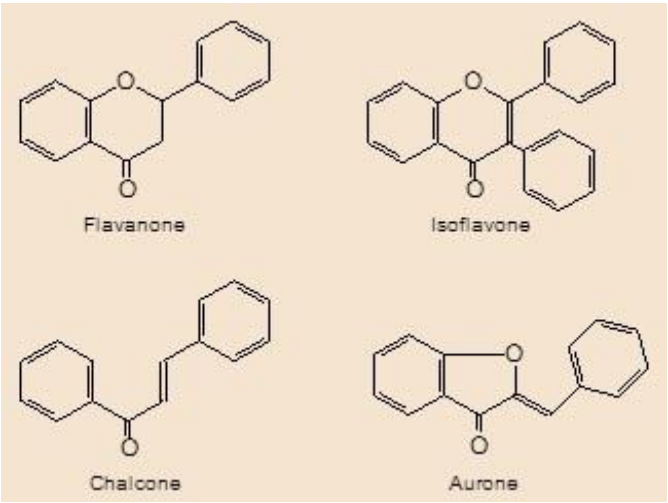
3.1-Charactrization of compound I

Compound I was isolated as a yellow powder from the ethanolic extract of *Mitragyna inermis*.

The UV spectroscopy is a valuable analytical tool in the chemistry of flavonoids and can differentiate between flavonoids with double bond in position 2-3 and those lacking such bond. The UV spectra of most flavonoids consist of two major absorption maxima one of which occur in range 220-285 nm (band II) and the other in the range 300-400nm (band I) the appearance of both band I and II in the UV spectrum demonstrates conjunction between the benzoyl and cinnamoyl system.



The UV spectrum of compound I (Fig.1) showed λ_{max}(MeOH) 249m . The appearance of only one band – band II- in this spectrum suggests saturation at the $C_2 – C_3$ position . Consequently compound I is probably a (i) flavanone, (ii)isoflavone(iii) dihydrochalcone or (iv) a dihydroflavonol.



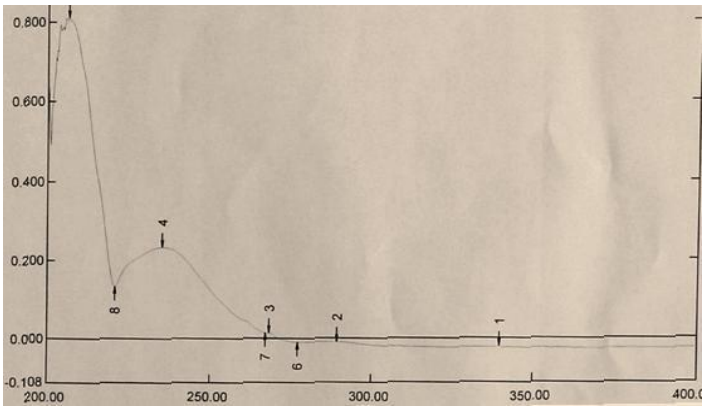


Fig.1:UV spectrum of compound I

However, isoflavones are easily distinguished by a shoulder in the UV range 300-340nm . Such shoulder has not been detected in the UV spectrum of compound I. On the other hand dihydroflavonols are characterized by a 3-OH function which could be detected by the UV shift reagent- sodium methoxide. Sodium methoxide can give a bathochromic shift with decrease in intensity in presence of a 3-OH . The sodium methoxide spectrum (Fig. 2) failed to give a bathochromic shift.

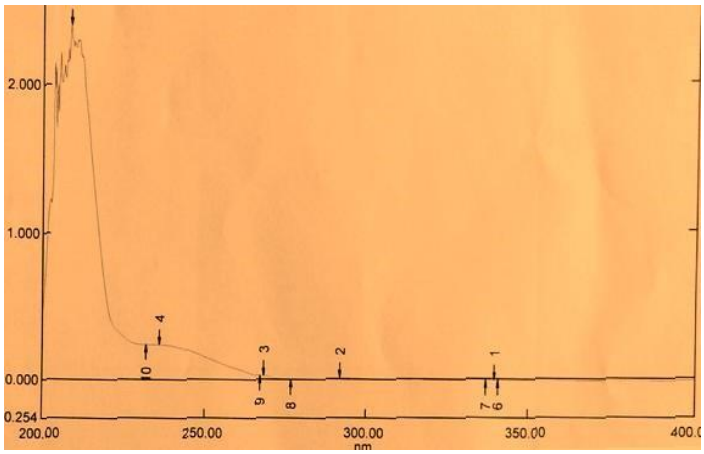


Fig.2:Sodium methoxide spectrum of compound I

In their ^{1}HNMR spectra flavanones show a double mutiplet around 2.8ppm and 5.2ppm .The mutual spin-spin splitting of the magnetically unequivalent protons at C_3 suffers further splitting by the neighboring C_2 protons to give a double doublet (usually merging into a multiplet) around 2.8ppm. The signal of C_2 proton is split by one of the protons at C_3 into a doublet. Such doublet suffers further splitting by the other proton at C_3 to yield a double doublet(usually appearing a multiplet) around 5.2ppm. However,

these multiplets were detected in the ^{1}HNMR spectrum of compound I(Fig.). Thus the isolated flavonoid is a flavanone.

The hydroxylation pattern of compound I has been studied via various UV shift reagents: sodium acetate (which gives a bathochromic shift in presence of a 7-OH) ; aluminium chloride(showing a bthochromic shift diagnostic of 3- , 5-OH and catechol systems) and boric acid (diagnostic of catechol moieties). When a methanolic solution of compound I was treated with the shift reagent : sodium acetate, no bathochromic shift was observed indicating absence 7-hydroxylation(Fig. 3).

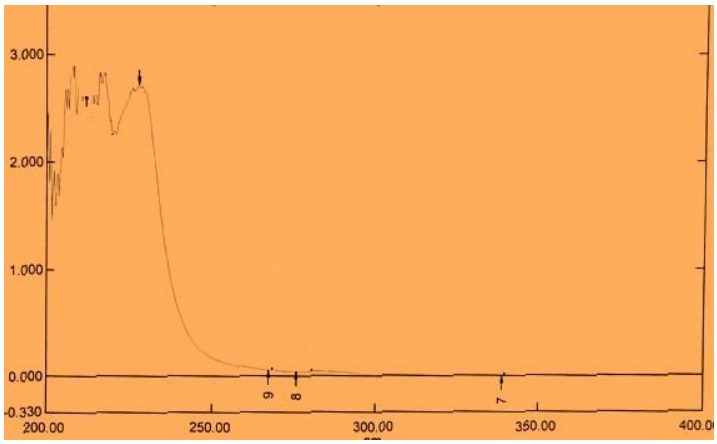


Fig.3: Sodium acetate spectrum

The aluminium chloride spectrum failed to show a bathochromic shift . This clearly suggests absence of 3- , 5-OH as well as catechol systems(Fig. 4). The boric acid spectrum behaved in the same manner and did not show any bathochromic suggesting absence of catechol systems(Fig. 5).

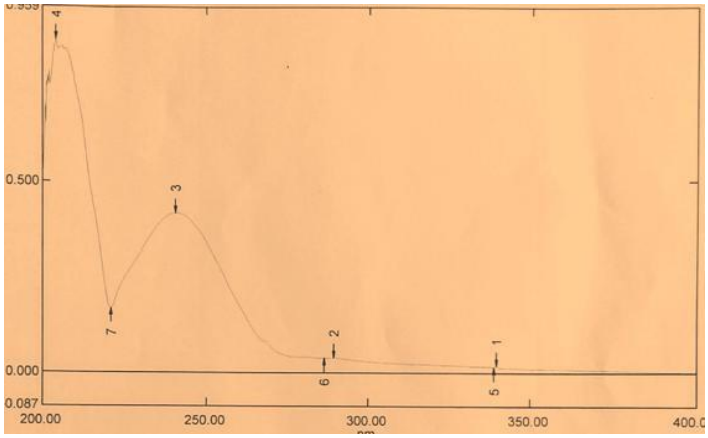


Fig. 4: Aluminium chloride spectrum of compound I

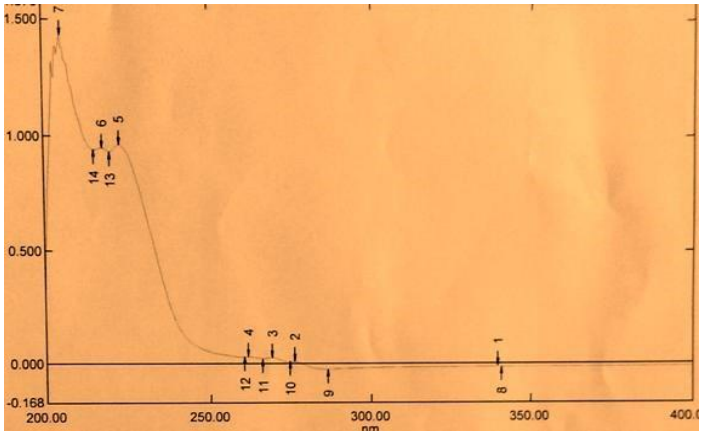


Fig.5: Boric acid spectrum of compound I

The ^{1}HNMR spectrum(Fig.6) showed δ(ppm): 1.25,1.52(assigned for two methyl groups), multiplet centered at 2.00(assigned for C_3 protons),mutiplet:3.60-4.50(sugar protons-not identified in this study), multiplet centered at 5.20(accounting for C_2 proton). The aromatic protons appeared as a multiplet centered at

7.40ppm.Signals at 2.50 and 3.35 are due to solvent(DMSO) residual protons and residual water respectively.

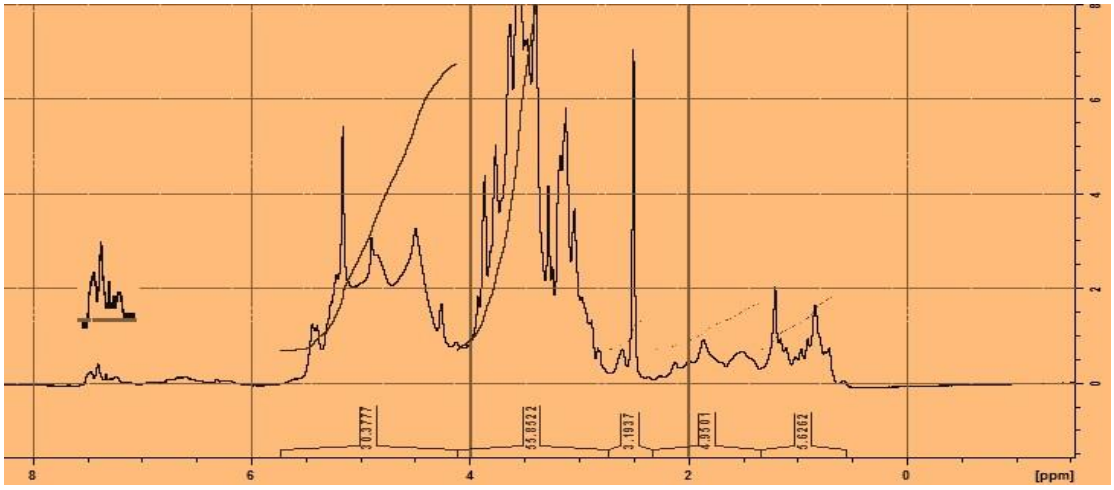
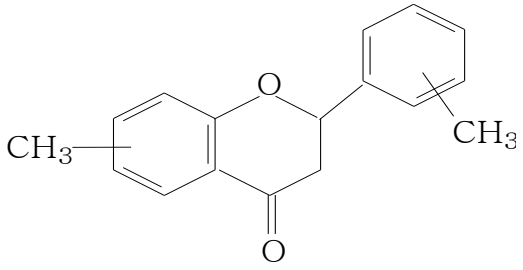


Fig.6: ^{1}HNMR spectrum of compound I

On the basis of the above argument, the following partial structure was proposed for the aglycone of compound I :



Compound I

Conclusion

In this study , stem barks of *Mitragyna inermis* were extracted with 95% ethanol and the crude extract was fractionated over silica gel plates to yield a chromatographically pure flavonoid – compound I. The structure of the isolated compound has been partially characterized by its spectral data (UV and ^{1}HNMR).

Recommendations

The following recommendations may be considered:

1-The target plant may be investigated for other secondary metabolites (steroids, terpenes and alkaloids) of biological potential.

2-The isolated flavonoid may be screened for its antiviral, antimalarial and antiinflammatory properties.

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