**Sudan University of Science and Technology**

**College of Postgraduate Studies**

# A study of the Flavonoids of *Acacia tortilis* and *Acacia nubica* and their Antimicrobial Activity

**دراسة فلافونيدات شجرتي السيال واللعوت وفعاليتها المضادة للميكروبات**

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

**By**

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**April,2021**

**الاستهلال:**

# بسم الله الرحمن الرحيم

## قال تعالى :

## ( قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ)

## صدق الله العظيم

## سورة البقرة – الآية (32).

# Dedication

**To**

**my parents  soul**

**my wife**

**Sons**

**my brothers and my sister**

## Acknowledgment

Thanks at first and last for the light of our life **Allah** who gave us the strength while doing this project and guided us through the way in this life.

Then I would like to express my special and deep thanks to Prof. Mohammed Abed El Karim for his patience and great efforts of supervising and leading me through this study.

Also I thank the staff members of the chemistry Dept.-Sudan University of Science and Technology.

# Abstract

In this study the flavonoids of two potentially beneficial medicinal plants- *Acacia tortilis* and *Acacia nubica* – have been studied. Phytochemical screening of *Acacia tortilis* stem bark revealed the presence of sterols and tannins.

Acetyl-methyl flavonol and Acetyl-dimethyl dihydroflavonol have been isolated by paper chromatography from *Acacia tortilis* stem bark and their structures were partially elucidated via a combination of spectral techniques (UV, IR and $^{1}$HNMR). The ethanol extract of *Acacia tortilis* was screened for antimicrobial activity against five standard microorganisms. The extract showed partial anticandidal activity.

From the stem bark of *Acacia nubica* two flavonoids compound Acetyl-trimethyl isoflavone and Acetyl trimehyl dihydroflavonol have been isolated and partially characterized using: (UV, IR $^{1}$HNMR). *Acacia nubica* ethanol extract exhibited significant antibacterial and antifungal activity against test organisms.

# المستخلص

في هذا البحث تمت دراسة الفلافونيدات فى لحاء نباتى اللعوت والسيال. أوضح المسح الفيتوكيميائى لنبات السيال وجود التنينات والاستيرولات. تم فصل أثنان من المركبات الفلافونيدية من نبات السيال Acetyl-methyl flavonol و Acetyl-dimethyl dihydroflavonol وتم تحديد التركيب المبدئي لهذه المركبات باستخدام طيف الاشعة فوق البنفسجية-المرئية وطيف الاشعة تحت الحمراء وطيف الرنين النووي المغنطيسي. في اختبار مضاد الميكروبات ابدى المستخلص الايثانولى لنبات السيال فعالية جزئية ضد فطر كنديدا.

تم فصل اثنان من المركبات الفلافونيدية من لحاء نبات اللعوت (Acetyl-trimethyl isoflavone و Acetyl-trimethyl dihydroflavonol . وتم تحديد التركيب المبدئي لهذه المركبات باستخدام طيف الاشعة فوق البنفسجية-المرئية وطيف الاشعة تحت الحمراء وطيف الرنين النووي المغنطيسي. وفي اختبار مضاد الميكروبات أعطى المستخلص الايثانولى لنبات اللعوت فعالية عالية ضد الميكروبات قيد الاختبار.

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# Abbreviations

| | |
|---|---|
| Copper nanoparticles | Cu NPs |
| Dengue virus types-2 | DENV-2 |
| Dimethylsulphoxide | DMSO |
| 1,1-Diphenyl-2-picryl-hydrazyl | DPPH |
| Gram negative | G - |
| Gram positive | G + |
| Proton magnetic resonance spectroscopy | $^{1}$HNMR |
| Infrared | IR |
| Mitogen activated proton kinass | MAPK |
| Nara Bladder Tumor No. 2 | NBT-II |
| Paper chromatography | PC |
| Reactive oxygen species | ROS |
| n-tetrabutylammonium tribromide | TBAB |
| Ultraviolet | UV |

# 1. Introduction

Flavonoids are natural polyphenolic compounds which appear as secondary metabolites of plants (Slavica *et al*., 2004). The name “Flavonoid” is derived from Greek word “Flatus” which means yellow(Zechemeister, 1957). Flavonoids first appeared in green algae 500 million years ago, resulting from the fusion of two biogenetic pathways, namely the cinnamate and the ancient polyketide route and they have then become more and more complex with plant evolution (Mohamed *et al*, 2013). They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs(Grote, 2006). Flavonoids (flavus – yellow), or bioflavonoids, are a ubiquitous group of po-lyphenolic substances which are present in most plants, concentrated in the seeds, fruit skin or peel, bark and flowers.(Middeton *et al*., 1994). Many fruits and vegetables, especially buckwheat, apple and onion, are some of these sources. Beverages prepared from plant extracts (beer, tea, wine, fruit juice) are the principal source of dietary flavonoid intake.(Malesev, 2007). They belong to a class of low-molecular-weight phenolic compounds that are widely distributed in the plant kingdom. They constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognised as flower pigments in most angiosperm families. However, their occurrence is not restricted to flowers but are found in all parts of plants(Panche *et al*., 2016).

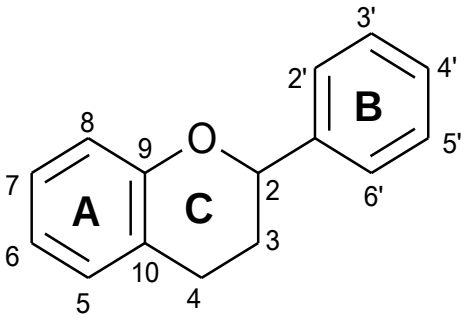
The function of flavonoids in flowers is to provide colors attractive to plant pollinators. In leaves these compounds are increasingly believed to promote physiological survival of the plant, protecting it from for example, fungal pathogens and UV- radiation. In addition, flavonoids are involved in photosensitization, energy transfer, the action of plant hormones and photosynthesis, morphogenesis and sex determination (Shohaib, 2011).

Flavonoids are the pigments responsible for the shades of yellow, orange, and red in flowering plants. They are also important factors for plant growth, development, and defense. Many flavonoids are endowed with biological activities, such as anti-inflammatory, antiallergic, antischemic, antiplatelet, immunomodulatory, and antitumoral activities. The biological activities of flavonoids are thoughy to be due mainly to their antioxidant properties, which are displayed by limiting the production of reactive oxygen species (ROS) and/or scavenging them (Catheine and Evans, 2003).

The chemistry of the flavonoids are predictive of their free radical scavenging activity as the reduction potentials of flavonoids and the consequently radical form, are lower than those of alkyl peroxyl radicals and the superoxide radical, which therefore means the flavonoids may inactivate these radical species and prevent the deleterious consequences of their reactions(yaseen *et al*., 2014). Flavonoids have ability to induce human protective enzyme systems. The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and

degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases.(Kumar and Pandey, 2013).

The basic flavonoid structure contains the flavan nucleus (**1**), which consists of 15 carbon atoms derived from a $C_6$-$C_3$-$C_6$ skeleton. A flavonoid skeleton is composed of two aromatic rings (commonly designated as A and B), which are linked by a three-carbon chain. The connecting carbon chain combines with an oxygen to form a heterocyclic central C-ring for most flavonoids with the exception of chalcones (**2**) in which the carbon chain between the A and B rings is linear (Alzand *et al*., 2006).The oxidation state of the C- ring is used to classify flavonoids into different categories, of which typically examples are flavan-3-ols, flavanones, flavones and flavonols.(Sisa M., *et al*., 2010).The numbering scheme for chalcones differs from three-ring flavonoids in that the A ring, rather than the B ring carbons are labeled as prime.

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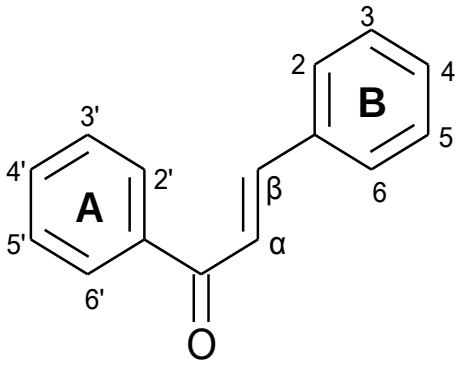



( 2)

Flavonoids generally occur in plants as glycosylated derivatives, and they contribute to the brilliant shades of blue, scarlet, yellow and orange, in leaves, flowers, and fruits. Apart from various vegetables and fruits, flavonoids are found in seeds, nuts, grains, spices, and ifferent medicinal plants as well as in beverages, such as wine, tea, and at lower levels also in beer (Mohammed, 2009).

Flavonoids are found in several medical plants, and herbal remedies containing flavonoids have been used in folk medicine around the world, especially in China.(Ren *et al*., 2003)

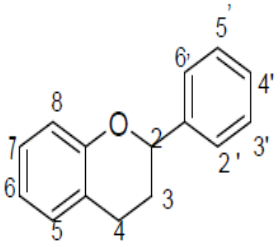
The flavonoids have two benzene rings separated by a propane unit and are derived from flavone. They are generally water soluble compounds. The more conjugated compounds often are brightly colored. They are generally found in plants as their glycosides which can complicate structure determinations.(Cseke *et al*., 2006)

## 1.1 Classification of Flavonoids:

Over 8000 different naturally occurring flavonoids have been discovered and the list is still growing (Groot and Raven, 1998). Most of these structurally different flavonoids can be arranged in various classes, and differ in the level of oxidation of the **C**-ring of the basic benzo-γ-pyrone structure. Common family members of flavonoids include flavones, flavanes, flavonols, catechins, and anthocyanidins. The structural difference in each flavonoid family results from the

variation in the number and substitution pattern of the hydroxyl groups as well as the extent of glycosylation of these groups (Harborne, 1994).

Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: the flavonoids (2-phenylbenzopyrans) 3, isoflavonoids (3-benzopyrans) 4, and the neoflavonoids (4-benzopyrans) 5. Thesegroups usually share a common chalcone precursor, and therefore are biogenetically and structurally related (Groteword, 2006)

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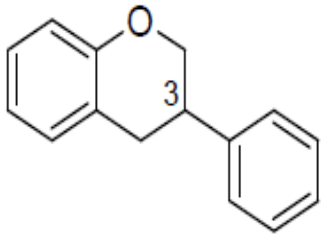


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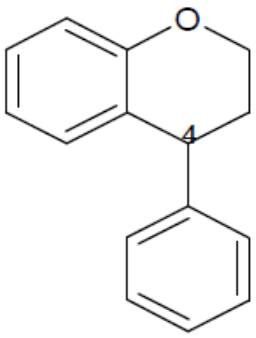
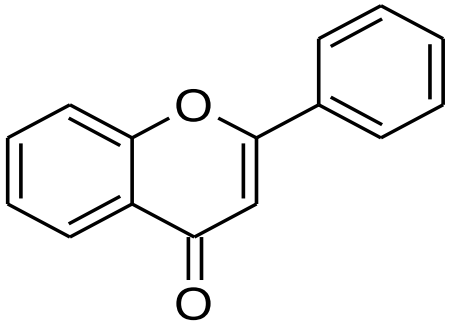



(5)

### 1.1.1 Flavones:

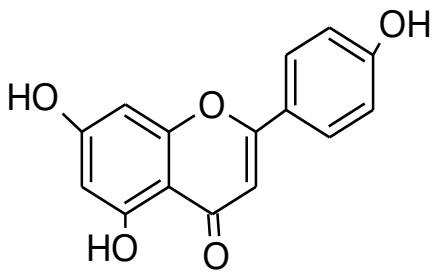
Flavone (6) compounds are the pigmented compounds found in flowers (Cooper and Nicola, 2015). Flavones are very similar structurally to flavonol compounds, having an extra hydroxyl substitution at the carbon 3-position. (Brodowska, 2017).




(6)

The major flavones are included apigenin (7) and luteolin (8). Luteolin occurs in vegetables and fruits such as broccoli, celery, carrots, parsley, onion leaves, cabbages, peppers, chrysanthemum flowers, and apple skins. While apigenin can be found in onions, parsley, wheat sprouts, tea, oranges, chamomile, and in some seasonings (Brodowska, 2017).

Chemically flavones can be classified into groups according to whether they are: (a) hydroxylated; (b) O-methylated; (c) C-methylated; (d) methylenedioxy substituted; and (e) isoprenylated. Flavone occur and free state as lipophilic components of leaves and in bud exudates. They occur much more frequency in polar forms glycosides or other conjugates. To main glycosides (Harborne, and Baxter, 1999)

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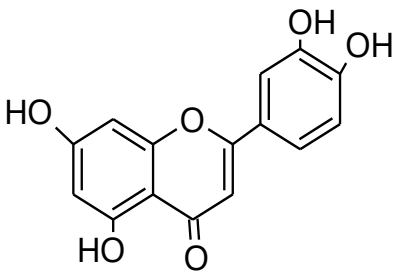
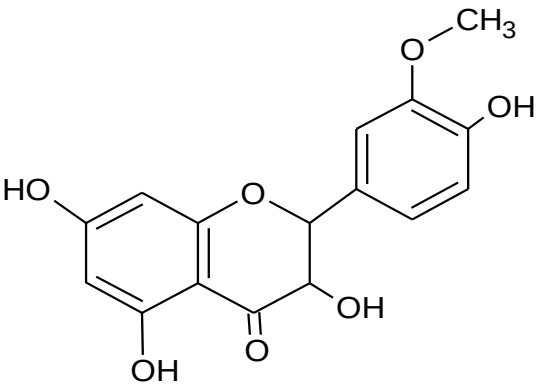



(8)

**1.1.2 Isoflavones:**

Isoflavones differ from flavones in the position of the B- ring, linked to the $C_3$ position of the heterocyclic ring instead of the $C_2$ position as in most flavonoids. Isoflavones are also termed phytoestrogens because of their estrogenic activity in mammals derived from the structural similarity between oestrogens and isoflavones. The most common isoflavones are daidzein (9) and genistein (10), together with their 7-O-glucosides daidzin and genistin respectively (Dixon, 2010).

Isoflavones are mostly found in legumes, especially in soy. However, their presence has been also reported in green split peas, split peas, chickpeas, black beans, lima beans, clover sprouts, and sunflower seeds. The major isoflavones in human diet are genistein and daidzein, which exist in four related chemical structures, namely aglycones, the 7-O-glucosides, the 6'-O-acetylglucosides and the 6'-O-malonylglucosides (Brodowska, 2017).

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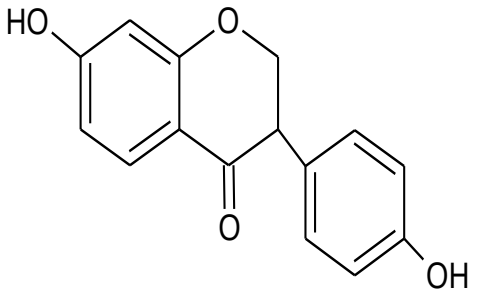



(10)

Some isoflavones have strong insecticidal activity (e.g., rotenoids) and others possess estrogenic/antiestrogenic activity, which may cause infertility in mammals. Many are recognized as phytoalexins, antimicrobial compounds(Cooper and Nicola, 2015).

The main isoflavone, genistein, purportedly lowers both blood pressure and cholesterol levels, especially LDL, thereby lowering the risk of cardiovascular disease. Genistein may inhibit the growth of tumors and is reported to be responsible for low rates of breast and prostate cancer seen among people eating soy products as a major part of their diet (Cooper and Nicola, 2015).

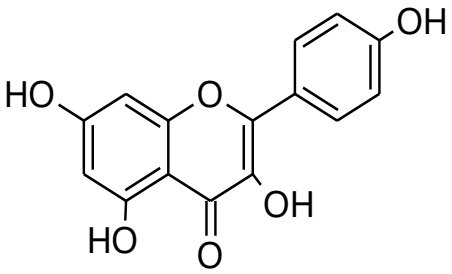
**1.1.3 Flavonols**

Flavonols are flavonoids with a ketone group. They are building blocks of proanthocyanins. Flavonols occur abundantly in a variety of fruits and vegetables. The most studied flavonols are kaempferol, quercetin, myricetin and fisetin. Onions, kale, lettuce, tomatoes, apples, grapes and berries are rich sources of flavonols. Apart from fruits and vegetables, tea and red wine are also sources of flavonols. Intake of flavonols is found to be associated with a wide

range of health benefits which includes antioxidant potential and reduced risk of vascular disease (Panche, 2016).

Compared with flavones, flavonols have a hydroxyl group in position 3 of the C ring, which may also be glycosylated. Like flavones, flavonols are very diverse in methylation and hydroxylation patterns as well and, considering the different glycosylation patterns, they are perhaps the most common and largest subgroup of flavonoids in fruits and vegetables. For example, quercetin is present in many plant foods (Iwashina, 2013).

Flavonols constitute a group of flavonoids that vary in color from white to yellow and closely related in structure to flavones. They are represented mainly by kaemferol (11), quercetin (12), and myricetin (13), while simple O-methylated derivatives such as isorhamnetin (14) (quercetin 3‑methylether) are also common ( Makris *et al*., 2006).

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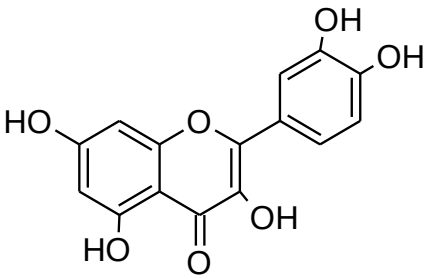


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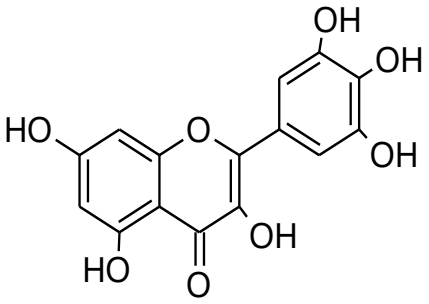


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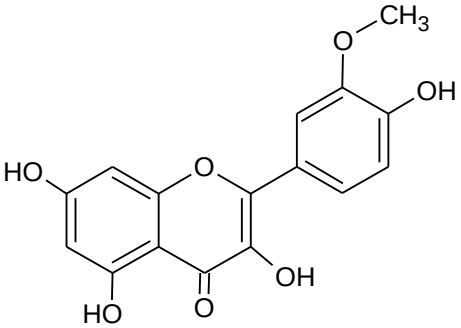



(14)

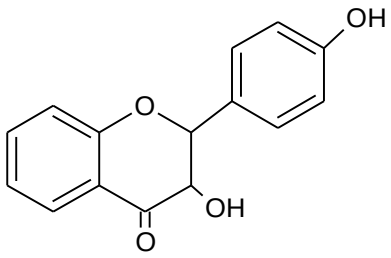
Flavonols that accumulate in plant tissues are almost always in the form of glycosylated conjugates. The main flavonols in onions are quercetin- 4u-Oglucoside and quercetin-3,4u-O-,diglucoside with smaller amounts of isorhamnetin-4u-O-glucoside (Mullen *et al*., 2004).

**1.1.4 Flavanones:**

Flavanones are characterised by the presence of a chiral centre at the 2-position and by the absence of the double bond between the 2- and 3-positions. Although less abundant in plants than some other classes of flavonoids, many flavanones also exhibit important biological activities ( Corradini *et al*., 2011).

Flavanones represent a flavonoid subclass present in our diet almost exclusively in citrus fruits and, to a lesser extent, in tomatoes and some aromatic herbs (such as mint). In citrus fruits, flavanones account for approximately 95% of the total flavonoids. The main aglycones are naringenin (15) (5,7,4′-trihydroxy flavanone) in grapefruit, hesperetin (16) (4′-methoxy-3′,5,7-trihydroxy flavanone) in orange and

tangerine, and eriodictyol(17) (5,7,3′,4′-tetrahydroxy flavanone) in lemon ( Chanet *et al*., 2012).

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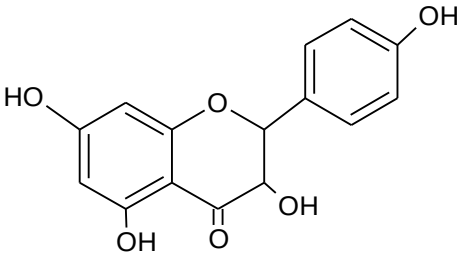


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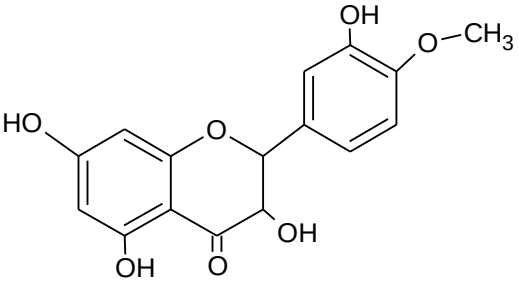


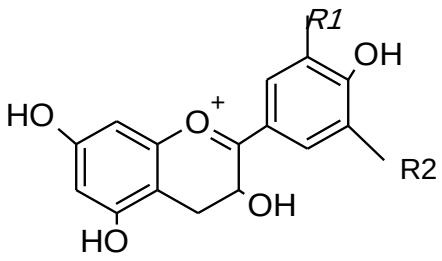

(17)

**1.1.5 Anthocyanidins and anthocyanins:**

Anthocyanins comprise a class of water-soluble flavonoids pigments in plants that contribute to the color of flowers, fruit, stems and leaves. They also function in vegetative tissues that provide protection against UV and high light irradiation, as antioxidants to scavenge reactive oxygen species (ROS), and as antimicrobial agents during defense responses. Anthocyanins are also potentially beneficial components of the human diet, and they can act as antioxidants, are anti-carcinogenic, anti-inflammatory and may help support both diabetes prevention and treatment, and heart health (Tain *et al*., 2017).

Anthocyanins are found in flowers and are responsible for various red, pink, blue, and purple colors. Nature may have designed these molecules to attract animal pollinators and seed dispersers.( Cooper and Nicola, 2015), and this flavonoid group dominates in teas, honey, fruits, vegetables, nuts, olive oil, cocoa and cereals. (Brodowska, 2017).

There are about 17 anthocyanidins found in nature, but only 6 - cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin are ubiquitously distributed and of dietary importance. The variations of anthocyanins are due to (i) the number and position of hydroxyl and methoxy groups on the basic anthocyanidin skeleton; (ii) the identity, number, and positions at which sugars are attached; and (iii) the extent of sugar acylation agent. Unlike other subgroups of flavonoids with the same $C_6–C_3–C_6$ skeleton, anthocyanins have a positive charge in their structure at acidic pH ( Fraga, 2010). Figure (18) shows the major anthocyanins.




(18)

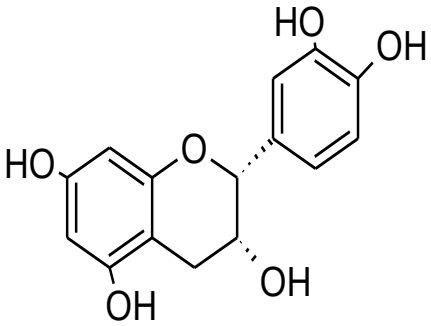
**Table 1.1 The Derivatives of Anthocyanidins**

| Anthocyanidins | R1 | R2 |
|---|---|---|
| Pelargonidin | H | H |
| Cyanidin | OH | H |
| Delphinidin | OH | OH |
| Peonidin | $OCH_3$ | H |
| Petunidin | $OCH_3$ | OH |
| Malvidin | $OCH_3$ | $OCH_3$ |

### 1.1.6 Flavan-3-ols:

Flavan-3-ols represent the most common flavonoid consumed in the American and, most probably, the Western diet and are regarded as functional ingredients in various beverages, whole and processed foods, herbal remedies, and supplements. Their presence in food affects quality parameters such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma, and color formation (Aron and Kennedy, 2007). Flavan-3-ols are structurally the most complex subclass of flavonoids ranging from the simple monomers ( + )- catechin (19) and its isomer ()-epicatechin (20) to the oligomeric and polymeric proanthocyanidins (Fig. 1.10), which are also known as condensed tannins ( Fraga, 2010). Flavan-3-ols are found abundantly in fruits such as apricots (*Prunus armeniaca*), sour cherries (*Prunus cerasus*),

grapes and blackberries (*Rubus* spp.) (Fraga, 2010). Barley, seemingly, is the only common cereal with a significant proanthocyanidin content (0.6–1.3 g $kg^{-1}$) (Santos-Buelga and Scalbert, 2000)**.**

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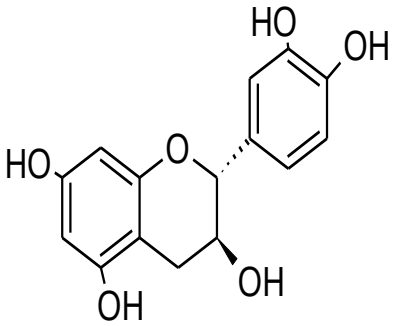


(20)

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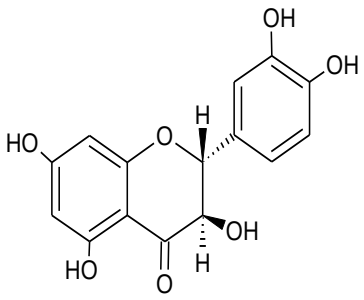
**1.1.7 Dihydroflavonols:**

Dihydroflavonols or 3-hydroflavonones as they may be called, are obligate intermediates on the pathway to flavonols by route, and to anthocyanins via flavon-3,4-diols by another. As in the case of other flavonoid classes, modified

dihydroflavonols occur in a variety of plant species (Bohm, 1998). The most common member of this group are: dihydroquercetin (21), dihydrokaempferol (22), and, dihydromyricetin (23) (Harborne, 1994).

Dihydroflavonols have two asymmetric carbon at C-2 and C-3. Dihydroquercetin, for example, has been found in 50 angiosperm families. The most significant biological property is there is their antimicrobial activity( Harborne and Baxter, 1999).

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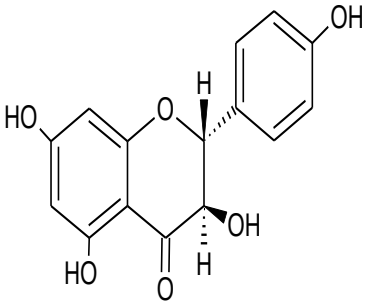


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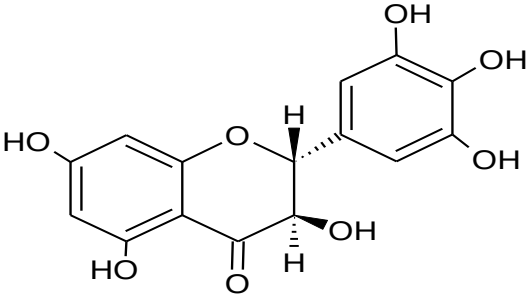
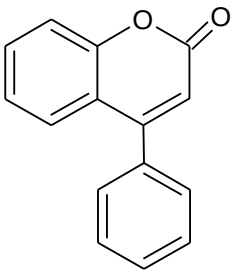



(23)

**1.1.8 Neoflavonoids:**

The neoflavonoids are structurally and biogenetically closely related to the flavonoids and isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2H-1-benzopyran-2-ones) (24), 2,4-

dihydro-4- arylcoumarins (25), and neoflavenes (26). (Dixon, 2010).

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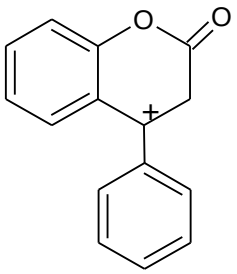


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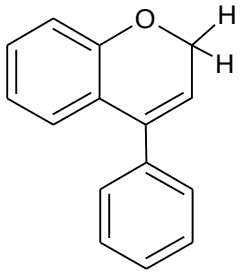



(26)

**1.1.9 Chalcones:**

Chalcones (1,3-diaryl-2-propen-1-ones) (27) are flavonoids found in fruits and vegetables, that attracted attention because of their pharmacological activities such as anti-inflammatory, antibacterial, antifungal, antiviral, antioxidant antineoplastic. Most of aromatic rings of natural chalcones are found as hydroxylated (Aksoz and Ertan, 2011).

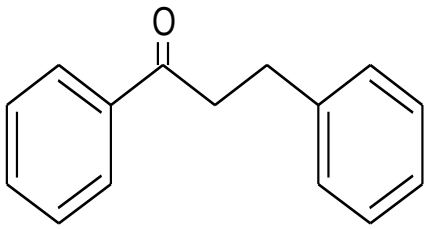
The name chalcones was given by Kostanecki and Tambor. The chalcones, two aromatic rings are linked by an aliphatic three carbon chain which bears a very good synthon so that variety of novel heterocyclics with good pharmaceutical profile can be designed. These are α, β unsaturated ketone containing reactive ketoethylenic group –CO-CH=CH- and are coloured compounds because of the presence of the chromophore. –CO-CH=CH- , which depends on the presence of the other auxochromes (Patil *et al*., 2009).

Chalcones are also known as benzyl acetophenone or benzylideneacetophenone. In chalcones, two aromatic rings are linked by an aliphatic three carbon chain. Chalcones

(trans-1, 3-diaryl-2- propen-1-ones) are α, β-unsaturated ketones consisting of two aromatic rings (ring A and B) having diverse array of substituents. Rings are interconnected by a highly electrophonic three carbon α, β-unsaturated carbonyl system that assumes linear or nearly planar structure (Yerragunta *et al*., 2013).

The chalcones, such as butein, lack the pyran ring found in flavonoids, although this is often subject to pH-controlled equilibria. The chalcone is more fully conjugated and normally brighly colored. Phlorizin is a strong inhibitor of apple seedling growth (Ahluwalia, 2009).

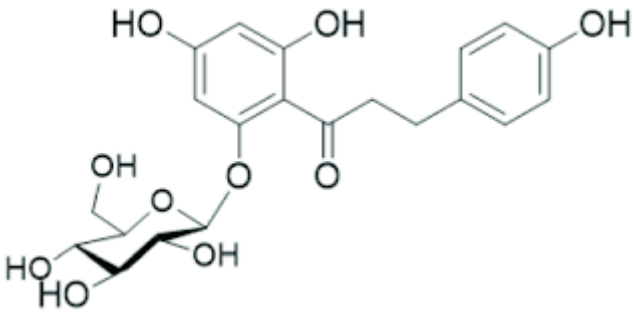
All the chalcones give pink coloration with concentrated sulphuric acid (positive Wilson test) and when a phenolic hydroxyl group is present, they give violet coloration with alcoholic ferric chloride solution. Chalcones on heating with traces of iodine in dimethylsulphoxide (DMSO) for two hours give the corresponding flavones(Yazdan *et al*., 2015).




(27)

### 1.1.10 Dihydrochalcones:

Dihydrochalcones are directly related to the chalcones and are derived from them by reduction of the chalcones α, β-double bond. The best known dihydrochalcone is phloridzin (28), which occurs in the skin of apples. When taken orally, it causes glycosuria by interfering with tubular read sorption of glucose in the kidney. It is therefore in demand in experimental physiology to study glucose transport. Dihydrochalcones are relatively small group of flavonoids and they have a somewhat erratic distribution (Harborne and Baxter, 1999).



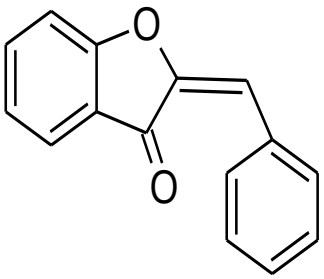

(28)

### 1.1.11 Aurones:

The aurones (29) are golden yellow pigments common in certain flowers. Aurones are a class of flavonoids called anthochlor pigments. Aurones are a class of flavonoids found in fruits and flowers where they function as phytoalexins against infections and contribute to the yellow pigmentation

of plant parts. Aurones have been reported to possess anticancer, antiparasitic, antileishmanial and antifungal activity. Aurones can be used as potential cancer chemotherapy agents and as inhibitors of an enzyme involved in the metabolism of thyroid hormones.

They have also been reported to be ant proliferative agents, tyrosinase inhibitors, antimicrobial agents and as potentially useful imaging agents for detecting β-amyloid plaques in Alzheimer's disease (Dubey *et al*., 2014). The two most common structures are sulfretin, 6',3',4'-trihydroxyaurone (30) and aureussidin, 4,6,3',4' -tetrahydroxyaurones (31) (Dixon, 2010).

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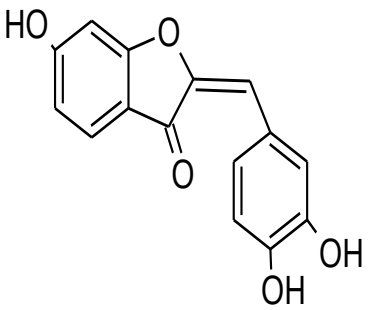


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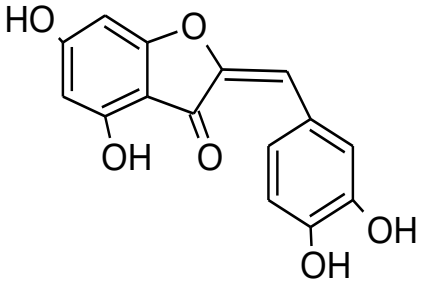



(31)

## 1.2 Medical properties of flavonoids:

Medicinal plants are important source of antioxidant compounds; these natural antioxidants reduce the risk of many chronic diseases. The secondary metabolites- like phenolic compounds and flavonoids- from plants have been reported to be the potent free radical scavengers, they are found in all parts of plants such as leaves, fruits, seeds, roots and barks. Therefore, screening for highly potent, less toxic, and cost effectiveness antioxidant molecules from medicinal plants is highly required.(Mirghani *et al*., 2017).

Due to the traditional medicine, plants still represent a large source of natural compounds, new chemical entities, for the development of novel drugs. Further studies are focused on the discovery of new bioactive compounds as flavonoids from plant material and their structure-activity relationship. The knowledge of the structure-activity relationship is a powerful concept in drug discovery; it is involved in the selection and optimization of ideal drug candidates (Russo, 2018).

Pharmacological and chemical investigations of medicinal plant have provided important advances in the therapeutic approach to several pathologies. A number of medicinal plant containing flavonoids and alkaloids are used in traditional medicine and are known to contain important therapeutic agents (Brown *et al*., 2005).

Some flavonoids like myricetin and kaempferol-3-glucoside have an anti-HIV-I potency at non-toxic concentration, and the minor flavonoids have very interesting activities they

have anti-microbial, anti-fungal and cytotoxic properties. Tricin has smooth muscle movement activity. The anthocyanin isorhamnenetin of the algae *Chlamydomonas* is a highly potent sex determining hormone (Karim *et al*., 2016).

Quercetin is a suppressing chemopreventive and chemotherapeutic agent that can relieve local pain caused by inflammation, headache, oral surgery, and stomach ulcer. Recently, quercetin has been shown to reduce the carcinogenic activity of several cooked food mutagens, enhance the antiproliferative activity of anticancer agents, and inhibit the growth of transformed tumorigenic cells. Currently, kaempferol is in interest because of its antioxidant, antitumor, antiinflammatory, and antiulcer activity, and its inhibitory activity of HIV protease. (Asif and Khadadadi, 2013).

Proanthocyanidin-rich extracts from grape seeds also display anticataract activity in rats. Due to their antioxidant activity, flavonoids have been reported to have positive effects on cancer, cardiovascular disease, immune disorders, microbial infections, neurogenerative disease and viral infections. They can participate in protection against the harmful action of ROS (Radical Oxygen Species); further they have multiple applications in food, cosmetic and pharmaceutical industries.(Russo, 2018).

Flavonoids and their derivatives have been reported to inhibit the growth and development of HIV by interrupting at several stages of its life cycle. Derivatives of hesperidin, particularly sulphonated and phosphorylated forms, have been studied by various scientists as hyaluronidase inhibitors and antimicrobial agent. Acute HIV-1 infection has been shown to

be suppressed by certain flavonoids and evidence for inhibition of HIV-1 protease, integrase and reverse transcriptase by flavonoids also exists. Anti-HIV activity of scutellarin has been reported against three strains of human deficiency virus including laboratoryderived virus (HIV-1 IIIB), drug resistant virus(HIV-1 74V) and low passage clinically isolated virus (HIV-1 KM018) (Asif and Khadadadi, 2013).

The aqueous leaves extract of *vitex doniana* has a significant antidiarhoeal activity which sports its use in traditional herbal medicine practice and it was suggested that aqueous of *vitex doniana* may exert anti-hepatotoxic effect against $CCl_4$ – induced liver injury in animal model and that this effect is concentration-dependent(Abdel Karim *et al*, 2016).

Oki *et al*., (2006) examined antioxidant activity of anthocyanins and other phenolic compounds from various cultivars of purple-fleshed sweet potato (*Ipomoea batatas* (L.) Lam.), an edible and economic medicinal species in Japan by diphenyl-2- picrylhydrazyl (DPPH) radical-scavenging activity; the obtained results showed the positive correlation between phenolic content and the activity of free-radical scavenging.

Tereeschuk, (1997) is isolated quercetagetin-7-arabinosyl-galactoside from Tagetes minuta which is used in Argentine folk medicine to treat infectious disease.

Ethanolic extract of the nutmeg seed which contained 3,4,7-trihydroxyflavone showed effective potential against MDR

gram-negative bacteria e.g., *Providencia stuartii* Ewing and *Escherichia coli.*

Flavonoids and phenolics may assist make available security against cancer, heart diseases and gastric problems by contributing, along with antioxidant vitamins and enzymes, to the total antioxidant defense system of the human body even though. Phenolics and flavonoids possess diverse biological activities, for instance, antiulcer and antiinflammatory, antidibatic, antiviral, antioxidant, cytotoxic and antitumor (Saxena 2012).

Zandi *et al*., (2011) studied the antidengue virus properties of quercetin, hesperetin, naringin, and daidzein at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells. anylavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus.

Narayan and Kumar (2003) evaluated the antioxidant activity of flavonoid-rich fraction from *S. hispida* seeds, which is listed in indigenous medicine as having high therapeutic value and is even now being used in various diseases. *S. hispida* L. (Rubiaceae) was popularly known as "Nattaiccuri" in Tamil or "Shaggy button weed" in English.

Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity. A carbonyl function at C-4 of the flavone nucleus

was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin (Kumar and Pandey, 2013).

Apigenin is a major flavones with skin protective effect from UV light; this flavone can be found in many edible medicinal plants or plants-derived beverages e.g., red wine, beer and chamomile tea. Quercetin is a flavonols which can be found in onion skin, apple peel and Hypericum perforatum L. leaves. Topical application with quercitin effectively inhibited UVB-induced skin damage in hairless mice (Tungmunnithum *et al*., 2018)

The leaves of *Ficus carica* L. (Moraceae) contain bergapten, quercetin, luteolin, and 4 ‘,5’-dihydropsoralen are traditionally used as laxative, stimulant against throat diseases, cough suppressant, emollient, emmenagogue and solvent. Fig has been tradition- ally used for its medicinal benefits in metabolic, cardiovascular, respiratory, antispasmodic and anti-inflammatory therapy (Trifunschi, *et al*., 2015).

Extracts from Ginkgo leaves (Ginkgo biloba L.), one of the top selling plant derived medicines in the USA and Europe, are widely used for treating cerebral insufficiency, against memory loss and as a potential drug for Alzheimer’s disease. The major markers in Ginkgo are gingolides, bilobalide and flavonoids, besides biflavonoids. Flavonoids show interactions with cytochrome P 450, antileukemic properties, and mild vasodilators properties useful

treatment of heart diseases. The leaf extract of Ginkgo biloba containing flavonoids (including bi flavones) was used for improving blood circulation in brain varix and several isoflavones were also used for improving blood circulation.(Sharma, 2006).

Traditionally, Orthosiphon stamineus has been used as a medicinal plant in the treatment of kidney diseases and gout; this may be linked to the ability of the extracts to inhibit xanthine oxidase and lipid peroxidation. In South East Asia the tea prepared from leaves is taken as beverage to improve health and for treatment of bladder inflammation, treatment of eruptive fever and diabetes(Hossain and Rahman,2015). Anthocyanin is one of the bioactive components as nutraceutical and traditional medicine. It has been traditionally used as a phytopharmaceutical, appetite stimulant, choleretic agent, and for treatment of many other diseases. These colored pigments are potent nutraceutical or pharmaceutical ingredients. As a nutraceutical, the bioavailability of anthocyanin is the key factor for maintaining good health and for prevention of diseases (Khoo *et al*., 2017).

Flavan-3-ols from tea, cocoa, chocolate, fruits, vegetables and wine, are highly potent antioxidant compounds. They reduce incidence of stroke, heart failure and diabetes and cancer. Their anticancer effects are thoroughly investigated.

Epigallocatechin 3-gallate and gallocatechan 3- gallate induces reduction in experimental lung tumor metastasis (77% and 46%). Epigallocatechin 3-gallate is effective antiangiogenesis agent which inhibits tumor cell invasion and proliferation. It, also, inhibits growth of the NBT-II bladder tumor cells and breast

cancer cell lines (Janicijevic, *et al*., 2007). Anthocyanins have been extracted and isolated from different plant sources for investigating their anticancer ability on esophagus, colon, breast, liver, hematological, and prostate cancers. The evidence from a previous study shows that 5% whole freeze-dried black raspberries and the anthocyanin-rich fraction supplemented to N-nitrosomethylbenzylamine-induced F344 rats have chemopreventive potential, where the treatment groups inhibit cell proliferation, inflammation, angiogenesis, and induce apoptosis in both preneoplastic and papillomatous esophageal tissues. Thus anthocyanins have chemoprophylaxis potential (Khoo *et al*., 2017).

## 1.3 Antioxidant Activities of Flavonoids:

An antioxidant can be defined as any substance which, when present in low concentrations compared to that of the oxidizable substrate, significantly delays or inhibits the oxidation of that substrate. The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Kralove and Procházková,2011).

Antioxidant compounds in food play an important role as a health-protecting factor. Antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens having the potential to reduce disease risk

(Asif and Khodadadi, 2013). Medicinal plants have long been reported as a prospective hub of natural antioxidant compounds, particularly plant secondary metabolites i.e., phenolic compounds and flavonoids which are generated by plant to defend itself or to promote the growth under unfavorable conditions. In addition, functional group arrangement, configuration, substitution, the number of hydroxyl groups were also influenced by antioxidant activity of flavonoids, for example radical scavenging activity and/or metal ion chelation ability (Tungmunnithum D., *et al*., 2018).

Saxena *et al*., (2012) have been demonstrate that the significant role of flavonoids and phenolics as antioxidant activities in these order isolated several flavonoids from the leaves of Licania licaniaeflora and reported quercetin derivatives to possess strongest antioxidant activity and flavonone 8-hydroxy-naringen and kaempferol 3-O-α-rhamnoside possesses lowest antioxidant activity.

The best-described antioxidant property of flavonoids derives from its ability to directly scavenge the reactive oxygen species. Flavonoids are able to chelate free radicals immediately by donating a hydrogen atom or by single-electron transfer. (Banjamahor and Artanti, 2014).

Another possible mechanism of action of flavonoids is through the chelation of transition metal elements. Flavonoids have chelating property, which enabled them to chelate, or binds to metal ions in human body to prevent them being

accessible for oxidation. Certain flavonoids have potential capacity to chelate trace metal ions such as $Fe^{2+}$ and $Cu^{+}$ that play a vital role in oxygen metabolism and free radical formation (Banjamahor and Artanti, 2014).

The importance of the chemical structure of the flavonoids, particularly the presence of a double bond at C2–C3 and a hydroxyl group at C3 on the C ring, in relation with their antioxidant activity in biological systems has been a matter of much controversy. Previous studies have revealed that the presence of two hydroxyl groups at C3, and C4, on the B ring is the most important structural feature for determining the antioxidant activity of these compounds (Areias *et al*., 2001).

The antioxidant activity of flavonoids is due to a variable number of phenolic groups contained in their chemical structure and their property to form chelates with iron and other transitional metals. The antioxidant activity is important for the human body because it protects the cells against free radicals, formed as a result in many processes that use oxygen as energy source, and plays an essential role in protection against oxidative degradation (Havsteen, 2002).

Flavonoids behave as antioxidants in a variety of ways, including direct trapping of the oxygen species, chelation of transition metals involved in the process radicals formation and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals. Also, they are able to modify the synthesis of eicosanoids, to prevent platelets aggregation and to protect lipoproteins against oxidation.(Trifunschi, *et al*., 2015).

Tasdemir *et al*., (2006), reported a common flavonoid glycoside, luteolin-7-*O*- lucoside, to be the first antimalarial natural product targeting the FabI enzyme of *P*. *falciparum*.

Although some studies indicate that flavonoids have peroxidation action, but only at high doses, they also have anti-inflammatory, antiviral, anti-allergic and protection role in various pathologies (Andersen and Markham 2006). Polyphenolic compounds including anthocyanins possess antimicrobial activity against a wide range of microorganisms, especially in inhibiting the growth of food-borne pathogens. Anthocyanins exhibit antimicrobial activity through several mechanisms, such as induced cell damage by destroying the cell wall, membrane, and intercellular matrix Based. on a previous study, maqui berry extracts had antibacterial activity with the highest sensitivity to *Aeromonas hydrophilia* and *Listeria innocua.* These bacteria are commonly associated with refrigerated foods as indicators of pathogenic microorganisms or as spoilage microorganisms (Khoo *et al*., 2017).

Flavonoids have been reported as serving signaling functions in eukaryotic cells, through their ability to interact with a range of protein kinases that supersede key steps of cell growth and differentiation. These functional roles of flavonoids may be of great value in plant photoprotection, and a strong correlation between flavonoids and the plant hormone auxin has been conclusively proven. Interestingly, the health beneficial effects of flavonoids in humans are also thought to reside mostly on their ability to control the activity

of several protein kinases, including the mitogen activated protein kinases (MAPK) (Brunetti *et al*., 2013).

Flavonoids have been demonstrated to be essential radical scavengers because of their ability to stabilize free radicals and other active species. These antiradical/antioxidant capacities are intimately related to the redox properties of their phenolic hydroxyl groups, which can be easily oxidized. As a consequence, their conjugated rings and hydroxyl groups allow them to act as radical scavengers, reducing the effect of ROS in the body. Structure-reactivity studies have demonstrated that the antiradical/antioxidant activities are related to structural criteria such as: (*i*) presence of an ortho-hydroxyl on the B-ring, (*ii*) presence of one or several free hydroxyl groups, (*iii*) presence of a $C_2$-$C_3$ double bond in the C-ring, or (*iv*) presence of a 3-hydroxyl group. Another potential mechanism by which flavonoids act as antioxidants relates to their interactions with redox enzymes. Flavonoids were shown to activate detoxifying enzymes such as NAD(P)H-quinone oxidoreductase, glutathione S-transferase or UDP-glucuronosyl transferase, which all belong to the defence arsenal towards oxidative stress ( Cherrak, 2016).

Flavonoids and phenolic compounds are important components of propolis, both substances have proven their ability to remove (or deactivate) free radicals, on top of being able to protect lipids and vitamin C from being destroyed in the oxidative process. By this characteristic, propolis has gained popularity among consumers, now days it is added to

drinks, foods, cosmetics, and even to chewing gum or toothpaste. A lot of studies indicate that flavonoids and phenols in propolis can be able to scavenge free radicals in the human body (Susana, 2018).

The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules (Kumar and Pandey, 2013).

Braca *et al.*, (2002) we tested several flavonoids isolated from the leaves of *Licania licaniaeflora*. All the isolated compounds exhibited DPPH radical scavenging activity: quercetin derivatives showed the strongest action, while the flavanone 8-hydroxy-naringenin and kaempferol 3-*O* - rhamnoside had the lowest.

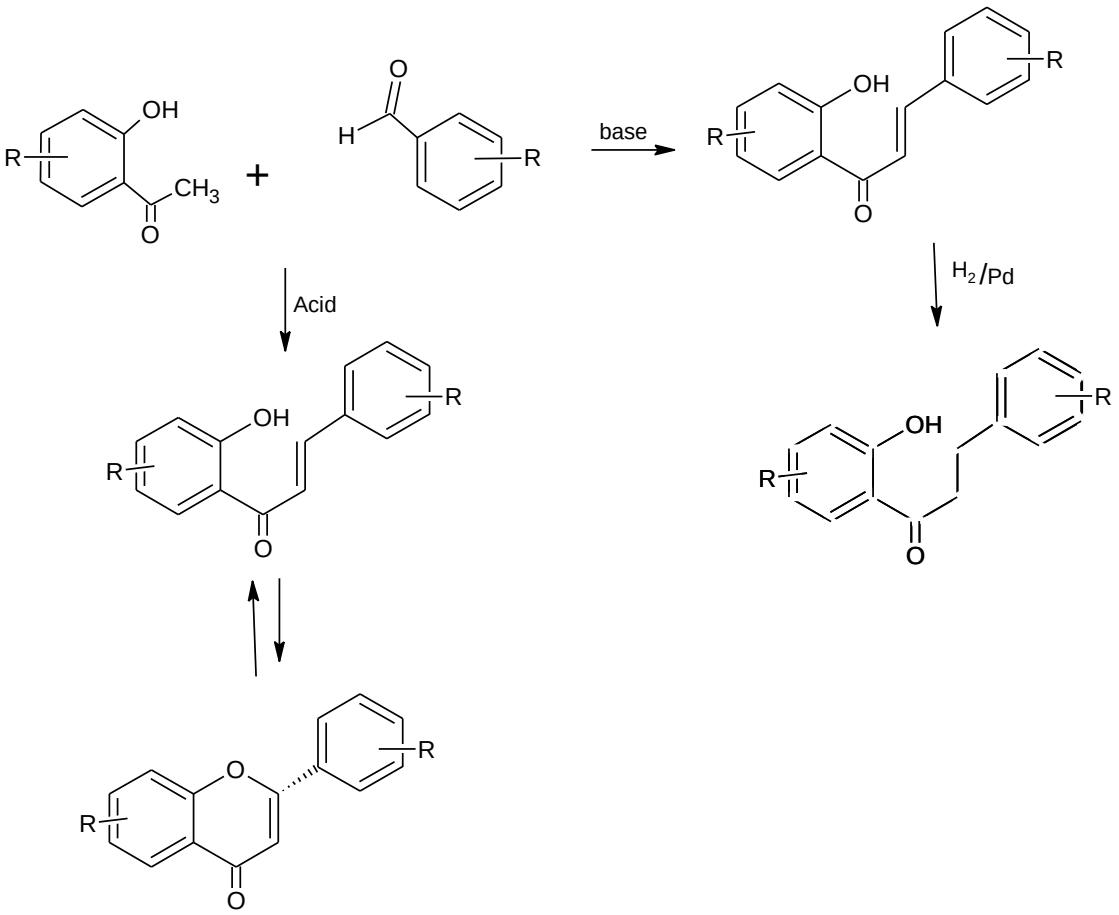
Ghasem *et al*., (2010) were assessed the antioxidant activities of methanol extracts from the leaves, stems and rhizomes of two *Zingiber officinale* varieties (Halia Bentong and Halia Bara) in an effort to compare and validate the medicinal potential of the subterranean part of the young ginger. The antioxidant activity and phenolic contents of the leaves as

determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay and the total amounts of phenolics and flavonoids were higher than those of the rhizomes and stems.

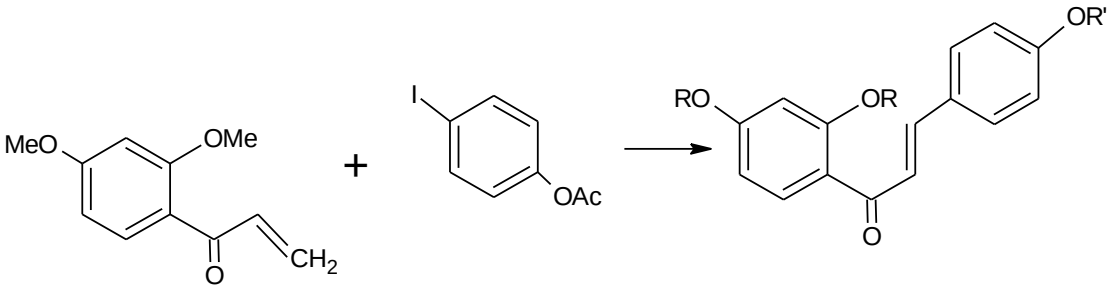
Shariffer *et al.,* (2008) reported antioxidant activity of methanolic extract of Teucrium polium and rutin and apigenin were found to be potent inhibitors of lipid peroxidation and oxidation of beta-carotene.

## 1.4 Synthesis of Flavonoids:

Chalcones and dihydrochalcones are considered to be the primary C6-C3-C6 precursors and constitute important intermediates in the synthesis of flavonoids. Chalcones are readily accessible via two well-established routes comprising a base-catalyzed aldol condensation or acid-mediated aldolization of 2-hydroxy-acetophenones and benzaldehydes. The base-catalyzed aldol condensation is usually the preferred route toward chalcone formation, since under acidic conditions cyclization of the ensuing chalcone leads to formation of corresponding racemic flavanones. Dihydrochalcones are generally obtained via reduction ($H_2$/Pd) of the preceding chalcones (Grotewold, 2006).




Several naturally occurring flavonoids have been synthesised following a new proposed method based on the use of the Heck reaction. The key step involves the coupling of an aryl vinyl ketone with an aryl iodide. This procedure affords the flavonoid moiety in a single step (Bianco, 2003).

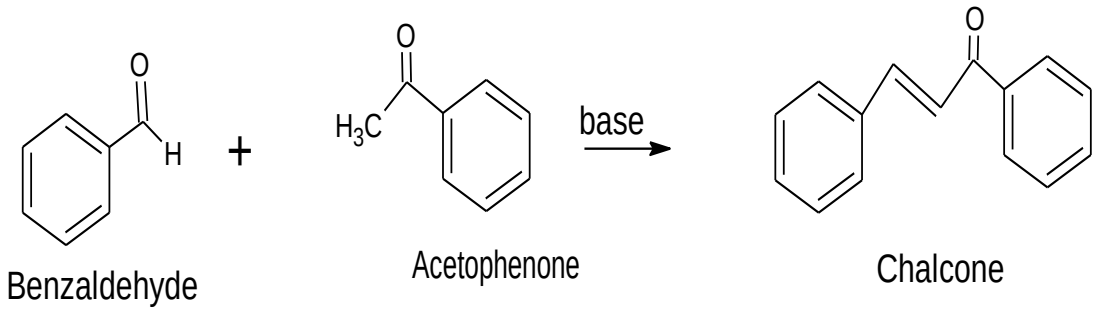



R= H, Me. R$^{/}$ =H, Ac

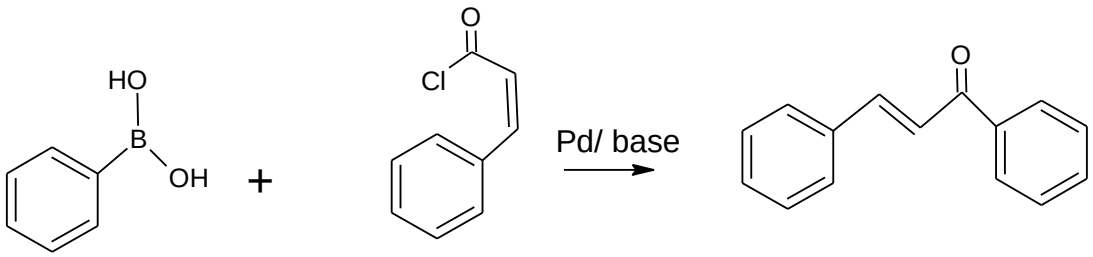
### 1.4.1 Synthesis of chalcones:

The synthesis of chalcone was carried out via crossed Aldol or Claisen- Schmidt condensation of comerically available acetophenone and benaldehyde in the presence of NaOH as a base in ehanol. (Setyowati, 2017)

In the Claisen-Schmidt reaction, the concentration of alkali used, usually ranges between 10 and 60 %. The reaction is carried out at about 50$^{o}$C for 12-15 hours or at room temperature for one week. Under these conditions, the Cannizaro reaction also takes place and thereby decreases the yield of the desired product. To avoid the disproportionation of aldehyde in the above reaction, the use of benzylidene-diacetate in place of aldehyde has been recommended(Yerragunta, 2013)

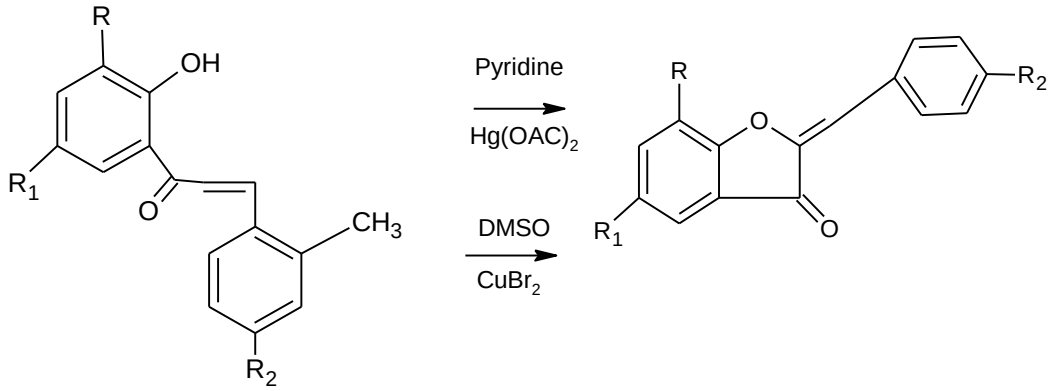



Also chalcones are synthesized using Suzuki coupling between cinnamoyl chloride and phenyl boronic acids or the carbonylative Heck coupling between aryl halide and styrenes in the presence of carbon monoxide.(Ugwu *et al*., 2015).

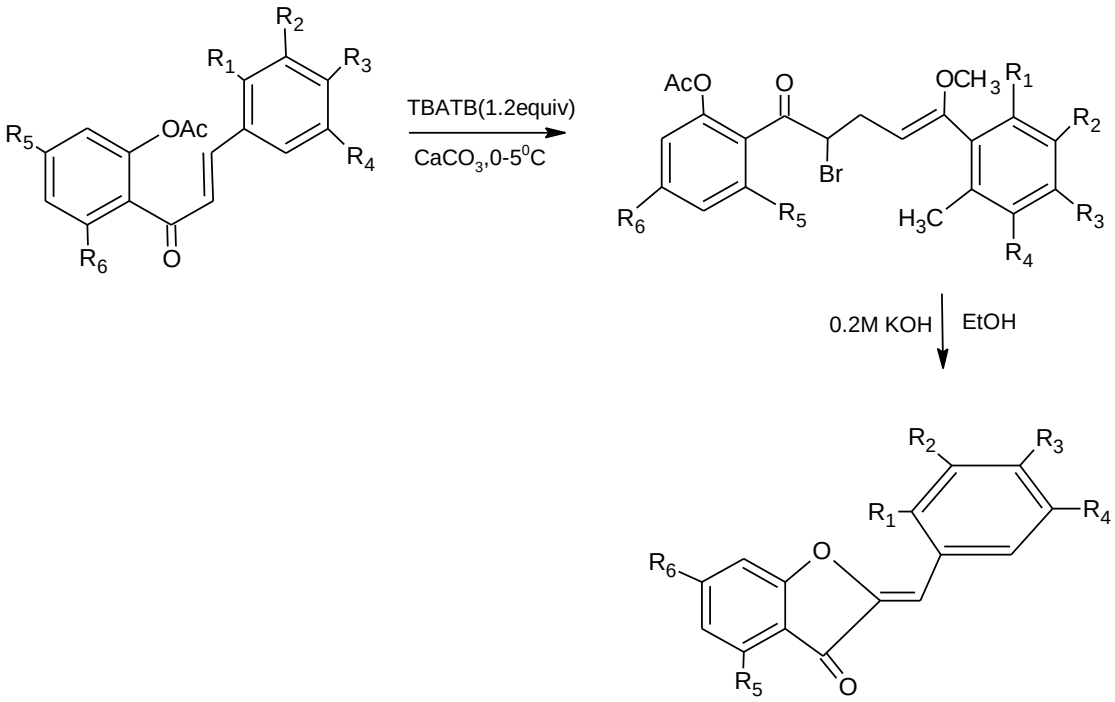



### 1.4.2 Synthesis of aurones:

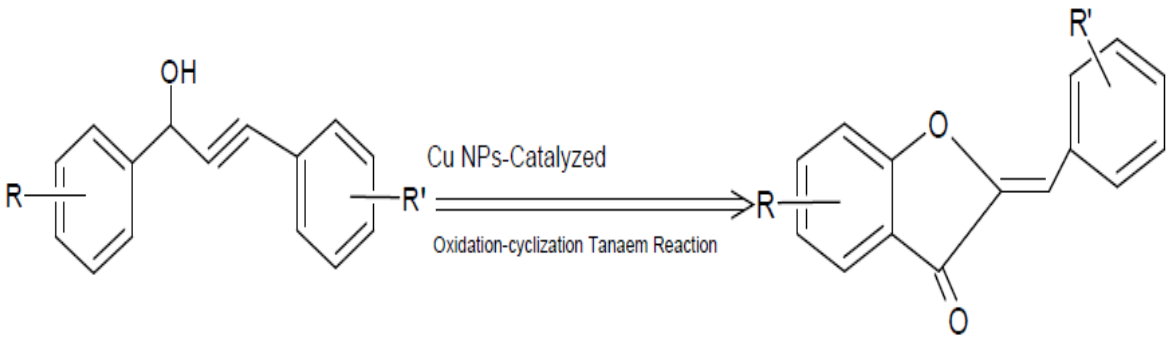
Aurones are prepared by using mercury (II) acetate in pyridine and cupric bromide in dimethyl sulfoxide (Agrawal and Soni, 2006)




Aurones also synthesized from 2-acetoxy chalcones using n-tetrabutyl ammonium tribromide [TBAB] in presence of $CaCO_3$ in dichloro methane: methanol (5:2) at 0-5$^{o}$C, this is first step , while in the second step cyclization of brominated product is obtained on treating with 0.2M ethanolic KOH solution at 0-5$^{o}$C (Jagtap and Khan, 2016)

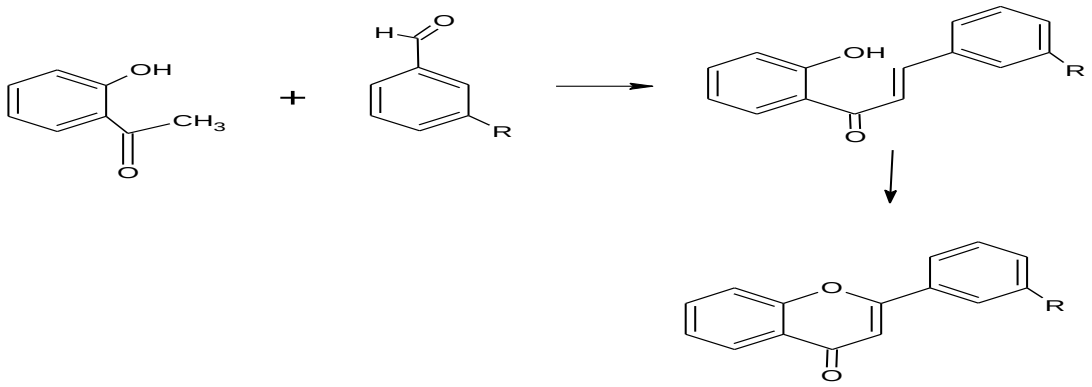



Aurones were synthesized through an oxidation-cyclization tandem reaction of 2-(1- hydroxyprop-2-ynyl)phenols catalyzed by copper nanoparticles (Cu NPs) with bipyridine as the ligand. In the reaction, oxygen worked as a green and mild oxidant to give the best results. Cu NPs were dually activated by bipyridine ligand and water, and showed highly efficient catalytic activities to the oxygen oxidation and the cylization to give aurones and flavonoids (Yu *et al*., 2018).

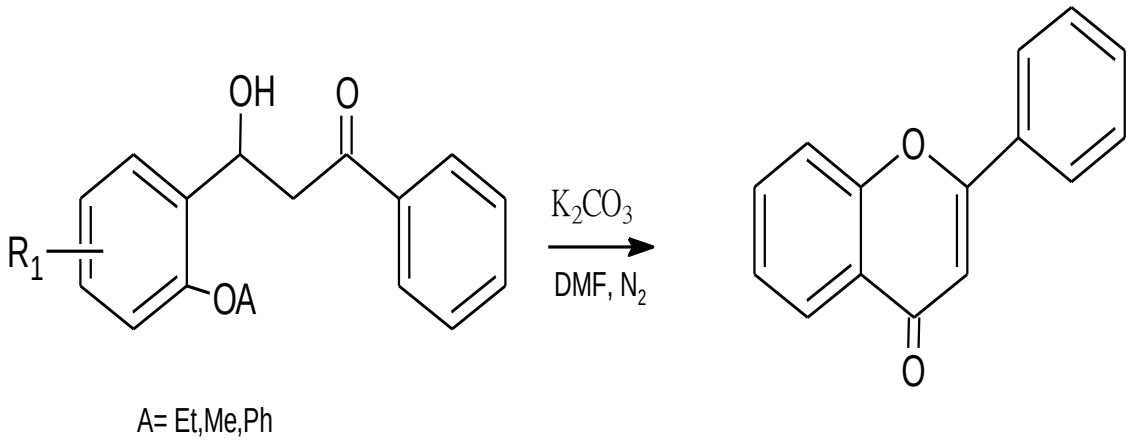


### 1.4.3 Synthesis of Flavones:

Chalcone is starting material for synthesis of flavones and chalcones can be synthesized by Claiseen-Schmidt condensation of 2- hydroxy acetophenone and benzaldehyde in the presence of bases such as NaOH, KOH and $Ba(OH)_2$. Using these chalcone derivatives, it was contemplated to synthesis of some flavone derivatives, from the corresponding chalcone by using dimethyl sulfoxide (DMSO/I) (Patel and Shah, 2017).



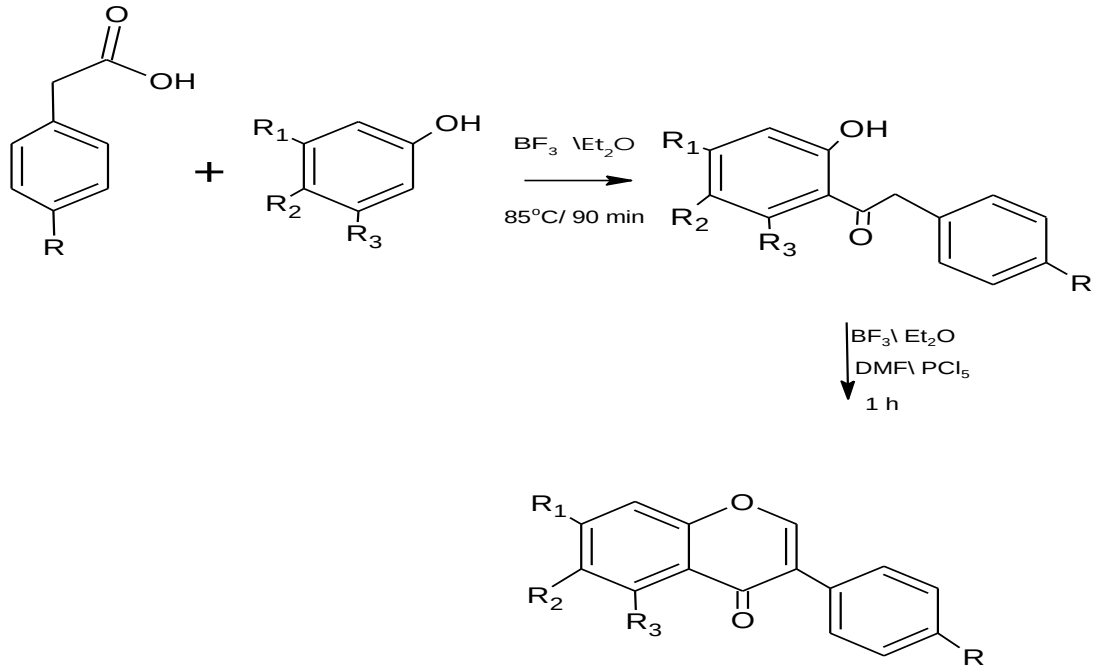

Tradionaly, flavones have been prepared by BakerVenkatraman rearrangement and Claisen-Schmidt condensation which involves the conversion of 2hydroxyacetophenones into benzoyl esters, followed by rearrangement in base to 1,3diphenylpropane1,3diones which upon cyclization under acidic conditions furnishes flavones(Kshatriya *et al*., 2013)




A= Et,Me,Ph

**1.4.4 Synthesis of isoflavones:**

The method involved the preparation of deoxybenzoins from respective substituted phenols and phenyl acetic acids by Friedel Crafts Acylation using boron trifluoride etherate which served as the Lewis acid for the acylation and as solvent for the reaction. The acylation was carried out at 85°C. In most cases the reaction was completed within 90 min. In the case of "one pot' method, the deoxybenzoin obtained was directly treated with the reagent, N, N'-dimethyl(chloromethy1ene) ammonium chloride'. This reagent was generated separately by treating phosphorous pentachloride with N,N'-dimethylformamide (Balasubramanian and Nair, 2014).




## 1.5-Acacia tortilis

## Taxanomy:

Kingdom: plantae

Family: Fabaceae

Order: Fabales

Genus: Acacia

Species: *A. tortilis*

Subspecies: *Acacia tortilis* subs. *Heteracantha*; *Acacia tortilis* subs. *Raddina*; *Acacia tortilis* suds. *Spirocarpa.*

Synonyms: *Acacia heteracantha* **Burch.,** *Acacia fasciculate, Acacia raddiana* **Savi**, *Acacia spirocarpa* **Hochst. Ex. A. Rich**., *Mimo tortilis* **Forsk.**

The generic name '*Acacia*' derived from the Greek word '*akis*', meaning a point or a barb. The name '*tortilis*' means

twist ed and refers to the pod structure. It is also known as umbrella thorn due to it umbrella like structure and in India it is commonly known as Israeli babool (Orwa *et al*., 2009)

**1.5.1 Distribution:**

Angola, Botswana, Egypt, Eritrea, Ethiopia, Iran, Israel, Kenya, Mozambique, Namibia, Qatar, Saudi Arabia, Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda, United Arab Emirates, Zambia, Zimbabwe (Orwa *et al*., 2009)

**1.5.2 Description;**

Acacia tortilis varies from multi stemmed shrubs less than 1m umbrella shaped, and trees up to 20m tall with rounded or flat topped and crowns. Bark grey, grayish brown to yellow, smooth or fissured. Young branches are glabrous. Spines mixed, some white, straight, slender, up to 7.5 cm long, and short hooked brown spine 2-6mm they occur on plant. Leaves dark green (0.5–3.0 cm) long, (2-5) pairs. Petiole glandular. Pubescent, (0.2-3 mm) long. Flower pale yellow cluster in 1cm diameter round heads. Pods flat, coiled or spirally shaped. Seeds olive green to red brown, smooth, elliptic slightly compressed, 6*35 mm areole marginal, U–shaped, and 4-5 mm long, coiled, seeds lie longitudinally in the pod.( Akasha *et al*., 2016).

**1.5.3 Medicinal Uses:**

Leaves, bark, seeds, and a red gum are used in many local medicines. Two pharmacologically active compounds for treating asthma have been isolated from the bark. The stem of the tree is also used to treat diarrhea. The gum is used like

that of gum Arabic's in folk remedies. The dried, powdered bark is used as a disinfectant in healing wounds; in Senegal it serves as an anthelmintic. In Somalia the stem is used to treat asthma. Seeds are taken to treat diarrhea. In French Guinea, the bark is used as a vermifuge and dusted onto skin ailments.(Weltzin and Coughenour,1990)

**Table (1.2):Chemicals isolated from Acacia tortilis(**(Yadav *et al*., 2013)

| **Chemical Component** | **Name of chemicals** |
|---|---|
| **Flavonoids** | Apigenin-6,8-bis-C-β-d- glucopyranoside (vicenin)<br>Rutin (quercetin 3-*O*-rutinoside) |
| **Total Phenolic Content** | Gallic acid concentration (mg/g dry weight) of Acaciatortilis 42.11 |
| **Gum** | **Molar proportions (%) of constituent sugar residues**<br>Uronic acid 8, Galactose 23, Arabinose 66, Rhamnose, Mannose 312<br>**Polysaccharide**<br>nitrogen 0.99%, protein content 6.18%, pH 6.46<br>**Nitrogen**<br>High nitrogen content of gum is 1.9%<br>**Amino acid**<br>Uronic acid 8, Galactose 23, Arabinose 66, Rhamnose, Mannose 3 |
| **Fatty acid from 12 seed oils** | *Acacia tortilis* contain 19% oleic, 72% linolenic, 60%linoleic acid |
| **Tannins** | **Hydrolyzable tannins**<br>The leaves, and to a lesser extent the bark, of many species contained between 1 and 8% hydrolyzable tannins<br>1,3-di-O-galloyl-4,6-()hexahydroxydiphenoyl-β glucopyranose has been reported from the leaves of *A. tortilis raddiana*. |

**Fig1.1 Acacia Tortilis**

## 1.6 Acacia nubica:

Kingdom: plantae

Family: Leguminosae

Order: Fabales

Genus: Acacia

Species: A. nubica

### 1.6.1 Description:

Shrub 1–5 m high, branching from the base; young branchlets greyish-white to yellowish green, glabrous to spreading pubescent. Stipular spines straight or rarely curved, 0.4–1.7(–2.7) cm long. Leaves: pinnae (2–)3–7(–11) pairs; leaflets 5–16 pairs, 2.5–6(–9) x (0.5–)0.75–2.5 mm. Flowers whitish or greenish, in heads; peduncles 0.5–1.5 cm long, pubescent; involucel below or sometimes about middle of peduncle. Calyx 1.5–2.5 mm long, pubescent. Corolla up to 3.5 mm long, with the lobes conspicuously pubescent outside. Pods straight or sometimes slightly curved, 4–15 x 0.9–2.2 cm, with a narrow wing-like margin 1–3.5 mm wide, straw-coloured, shortly pubescent. Seeds globose or ellipsoid, 4.5–6.5 x 3.5–6 mm, shallowly and closely wrinkled; areole 4–5 x 3–3.5 mm.

### 1.6.2 Distribution:

Egypt, Sudan, Eritrea, Ethiopia, Uganda, Kenya, Tanzania, and Saudi Arabia.

**Fig 1.2 Acacia nubica**

## 1.7 Aim of this study

**This study aims to:**

- Extract of flavonoids from *Acacia Tortilis and Acacia nubica.*
- Isolate flavonoids using chromatographic techniques.
- Elucidate of the structures using sensitive analytical tools.
- Screen the isolated flavonoids for their antimicrobial activity.

# 2-Materials and Methods

## 2.1 Materials

### 2.1.1 Plant materials

Stem bark of *Acacia tortilis* and *Acacia nubica* were collected from White Nile state(Sudan). The plant was identified and authenticated by the Medicinal and Aromatic Plants Research Institute(Sudan).

### 2.1.2 Instruments

Ultra-Violet spectra were run on a Shimadzu 2401PC UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer. NMR spectra were performed on a Joel ECA 500MHZ NMR Spectrophotometer.

### 2.1.3 Test organisms

The antimicrobial activity of the ethanol extract was evaluated using the following standard microorganisms: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans.*

## 2.2 Methods

### 2.2.1 Preparations of reagents for phytochemical screening.

**i) Flavonoids and phenolics test reagents**

**- Aluminium chloride solution**

(1 g) of aluminum chloride was dissolved in 100 ml methanol

**- Potassium hydroxide solution**

(1 g) of potassium hydroxide was dissolved in 100 ml distilled water.

**-Ferric chloride solution**

(1 g ) of ferric chloride was dissolved in 100 ml methanol.

**ii) Alkaloid test reagents**

**Maeyer reagent**

- Mercuric chloride solution: 1.36 g in 60 ml. distilled water.

- Potassium iodide solution : 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

**-Wagner reagent**

1.27 g iodine and 2 g of potassium iodide in 100 ml distilled water.

### 2.2.2 Phytochemical screening

(100 g) of powdered shade - dried plant material were extracted with 80% aqueous methanol (soxhelt) until exhaustion. This prepared extract(PE) was used for phytochemical screening.

The prepared extract (PE) was used for following tests:

#### 2.2.2.1 Test for unsaturated sterols and for triterpenes

(10 mL) of the (PE) was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over sodium sulphite anhydrous. 5 ml portion of the solution was mixed with 0.5 ml of acetic anhydride, followed by two drops of concentrated sulphuric acid. Two separate layers (green, red) were observed.

#### 2.2.2.2 Test for flavonoids

(20 ml) of the (PE) was evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added. Red colour was observed.

- To 3 ml. of the filtrate few drops of aluminium chloride solution were added. A dark yellow colour was formed.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed.

#### 2.2.2.3 Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for minutes, then cooled and divided into two portions:

To one portion a few drops of Maeyer reagent were added. A white precipitated appeared, to the other portion few drops of Wagner reagent were added. A brown precipitate appeared.

#### 2.2.2.4 Test for tannins

(10 ml) of (PE) was evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and 10 ml of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled , filtrated and the volume adjusted to 10 ml. with more saline solution. 5 ml of this solution was treated with few drops of ferric chloride solution. A dark blue colour was observed.

### 2.2.2.5 Test for Saponins

(1 g )of dried powdered plant material was placed in a test tube. 10 ml of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds. The formation of a froth that persists for one hour indicates the presence of saponins.

### 2.2.3 Extraction and isolation of flavonoids

Powdered stem bark of a *Acacia tortilis* (1.5 kg) were macerated at room temperature with ethanol (95%) for 72hours. The solvent was evaporated under reduced pressure to dryness to give a crude product. The crude ethanol extract was fractionated via paper chromatography using the 15% acetic acid as mobile phase. The chromatograms were viewed and located under UV light and a flavonoid compound I- was eluted from paper with methanol.

The crude ethanol extract of Acacia nubica stem bark was fractionated via paper chromatography using 15% acetic acid as mobile phase. The chromatograms were viewed and located under UV light and a flavonoid -compound I - was eluted from paper with methanol.

### 2.2.4 Preparation of Shift Reagents for Ultra-Violet Spectroscopic Analysis of Flavonoids

The diagnostic reagents used for the UV spectral measurements of the isolated flavonoid compounds were prepared as follows:

#### 2.2.4.1 Sodium Methoxide (NaOMe)

Freshly cut metallic sodium (2.5g) was added cautiously in small portion to spectroscopic grade methanol (100ml).

#### 2.2.4.2 Aluminum Chloride ($AICI_3$)

Anhydrous reagent grade $AICI_3$ (5g) was dissolved cautiously in 100ml spectroscopic methanol and filtration was carried out after 24 hours.

#### 2.2.4.3 Hydrochloric Acid (HCI)

50 ml concentrated HCI was mixed with 100ml distilled water. The solution was stored in glass-stoppered bottle.

#### 2.2.4.4 Sodium Acetate (NaOAc)

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

#### 2.2.4.5 Boric Acid($H_3BO_3$)

Anhydrous powered reagent grade $H_3BO_3$ was used.

### 2.2.5 Stepwise Procedure for the Use of shift Reagents

- Methanol solution of the compound was first recorded.
- 3 drops of NaOMe were added to the cuvette and after mixing, the NaOMe spectrum was recorded.

- 6 drops of $AICI_3$ reagent was added to the flavonoid solution, the sample mixed, and the $AICI_3$ spectrum was measured. 3 drops of HCI were added and after mixing, the $AICI_3$/ HCI spectrum was measured.
- Powdered NaOAc was then added to fresh flavonoid stock solution in the cuvette, the mixture was shaken and the NaOAc spectrum was recorded.
- NaOAc/$H_3BO_3$ spectrum was then measured after adding $H_3BO_3$.

### 2.2.6 Biological activity

#### 2.2.6.1 Preparations of crude extracts for biological study

Ethanol extract: prepared by macerating 100g of the air dried powdered plant material in successive portions of ethanol (100%) till exhaustion. The ethanolic extract was evaporated under reduced pressure to obtain a semi – solid residue.

#### 2.2.6.2 Antimicrobial assay

The methanolic extract and ethyl acetate fraction were screened for their antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*) using the cup plate agar method with some minor modifications.

### 2.2.6.2.1 Preparation of bacterial suspensions

(One ml). aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at $37^{o}$ 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 suspension containing about $10^{8}$ - $10^{4}$ colony forming units per ml. The suspension was stored in refrigerator at $4^{o}$C until used. The average number of viable organism per ml of the saline 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 volume (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 to dry, and then incubated at $37^{o}$ C for 24 hours.

### 2.2.6.2.2 Preparation of fungal suspensions

Fungal cultures were maintained on dextrose agar incubated at $25^{o}$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.

### 2.2.6.2.3 Testing for antibacterial activity

The cup plate agar diffusion method was adopted with some minor modification, to assess the antibacterial activity of the ethanolic extract of *Acacia tortilis and Acacia nubica* .Two ml of the standardized bacterial stock suspention were mixed with 200 ml of sterile molten nutrient agar which was maintained at $45^{o}$ C in water bath.

(20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes and the agar was left to settle 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 of the halves was designed for one of the extracts.

The agar discs were removed and cups were filled with(0.1) ml of each extract using adjustable volume micro titer pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37 $^{o}$ C for 24 hours.

After incubation the diameters of the resultant growth inhibition zones were measures.

### 2.2.6.2.4 Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar dextrose agar was used. Samples were used here by the same concentrations used above.

# 3-Results and Discussion

Flavonoids are phenolic compounds widely present in plants and foods of plant origin. Flavonoids encompass a large group of polyphenolic substances with marked physiological potential including: antibacterial, anti-inflammatory, antiallergic, antifungal, antimutagenic , antivirl and vasodilator effects. In this study the flavonoids of two potentially beneficial medicinal plants- *Acacia tortilis* and *Acacia nubica* – have been studied.

## 3.1 *Acacia tortilis*

### 3.1.1 Phytochemical screening

Phytochemical screening of *Acacia tortilis* stem bark revealed the presence of sterols and tannins.

### 3.1.2 Flavonoids of *Acacia tortilis*

#### 3.1.2.1 Characterization of compound I

Compound I was isolated by paper chromatography as yellow powder from *Acacia tortilis* stem barks and its structure was elucidated via a combination of spectral techniques (UV, IR and $^{1}$HNMR).

The IR spectrum of compound I **(**Fig.3.1) showed v (KBr): 619,781,856(C-H, Ar.bending), 1274 (C-O, ether), 1496,1514 (C = C, Ar.), 1605 (C = O),2923(C-H) and 3390 cm-1 (OH).

The appearance of a carbonyl absorption suggests that compound I is neither an anthocyanin nor catechin . These classes are characterized by absence of a carbonyl function.

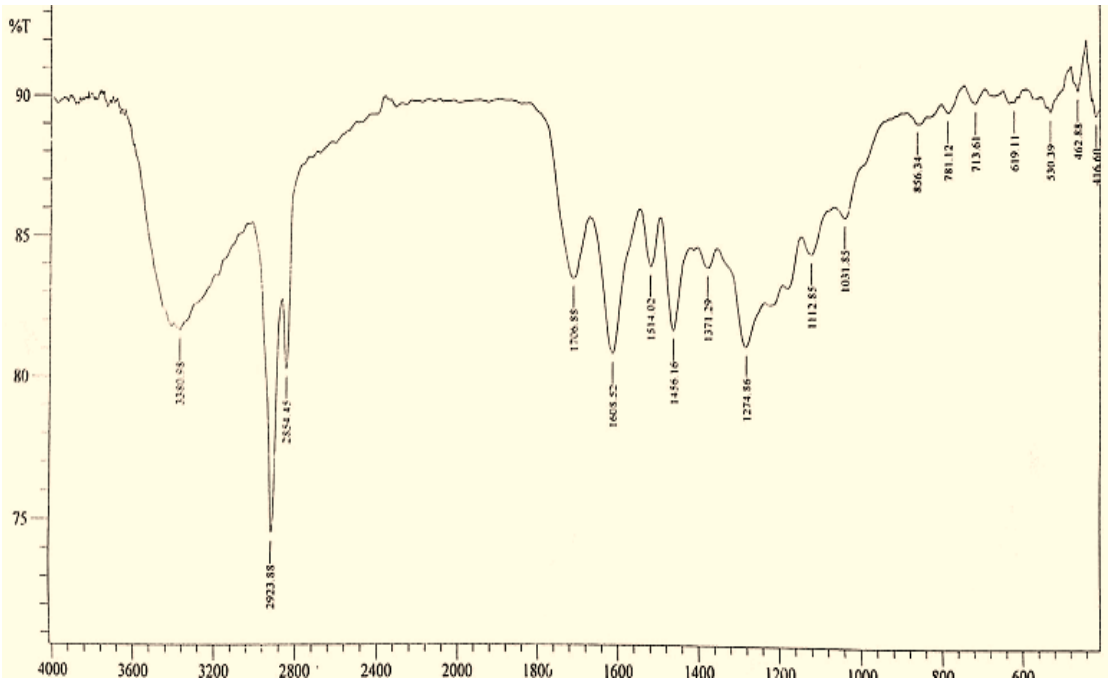



**Fig.3.1: IR spectrum of compound I**

The UV spectrum of compound I showed (Fig.3.2) λmax 242,366nm . Such absorption characteristic of flavonols.

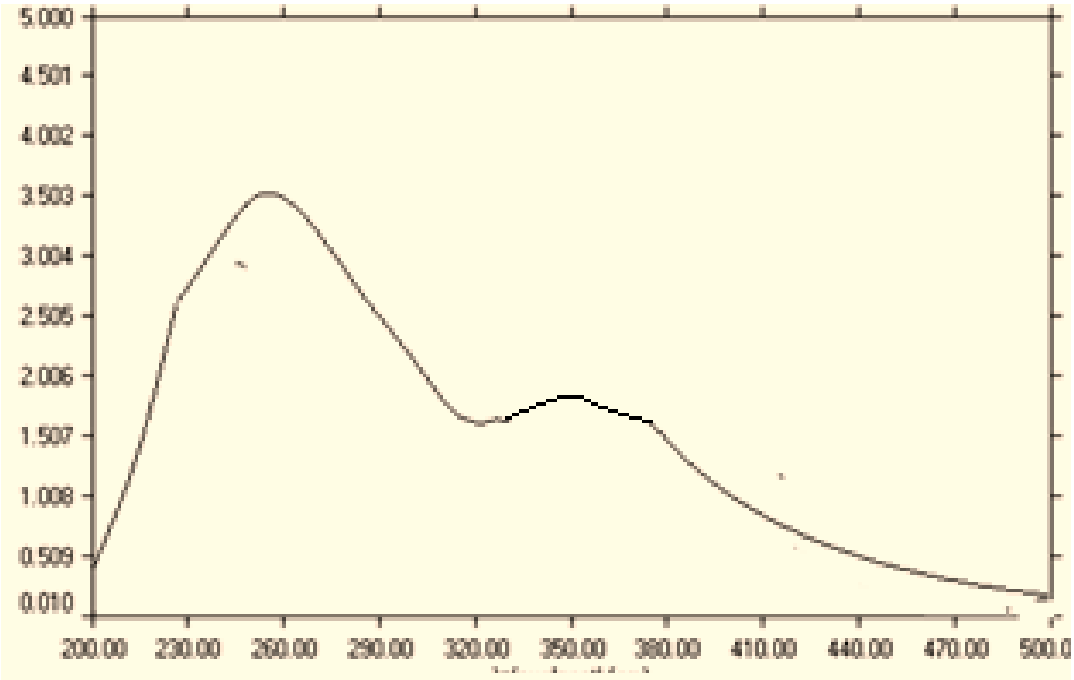



**Fig.3.2 : UV spectrum of compound I**

When the UV shift reagent – sodium methoxide - was added to a methanolic solution of compound I , a 44nm bathochromic shift in band I with decrease in intensity (Fig.3.3) was observed indicating the presence of a 3-OH function which is a characteristic feature of flavonols.

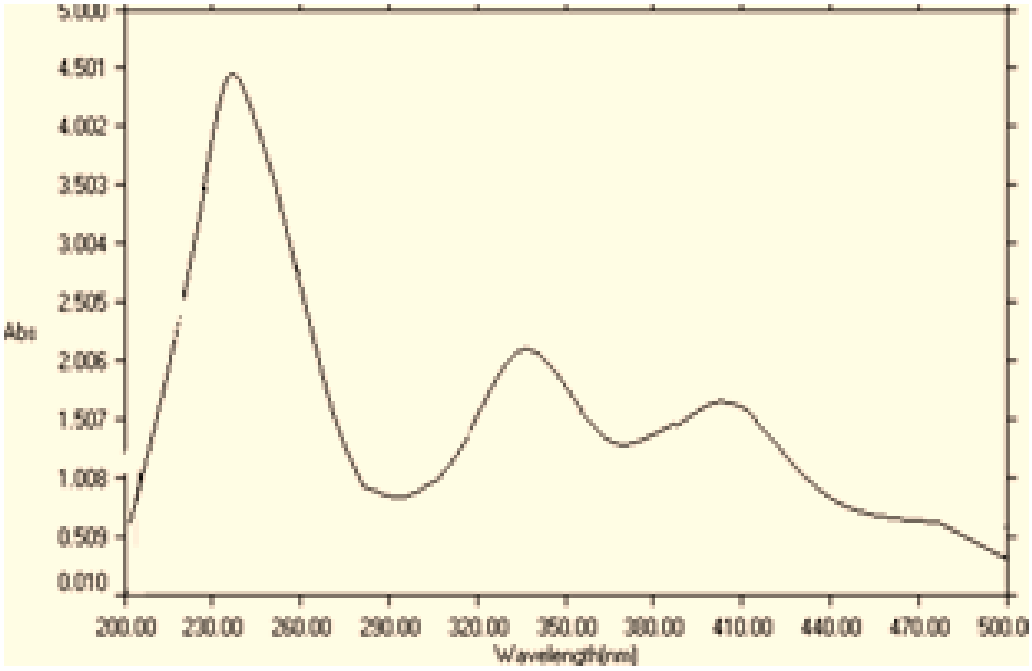



**Fig. 3.3: Sodium methoxide spectrum of compound I**

When a methanolic solution of compound I was treated with excess powdered sodium acetate, no bathochromic shift in band II (Fig.3.4) was observed indicating the absence of 7 –OH group. The aluminum chloride spectrum of compound I(Fig.3.5) showed a 62 bathochromic shift in band I indicating a 5 –OH group.

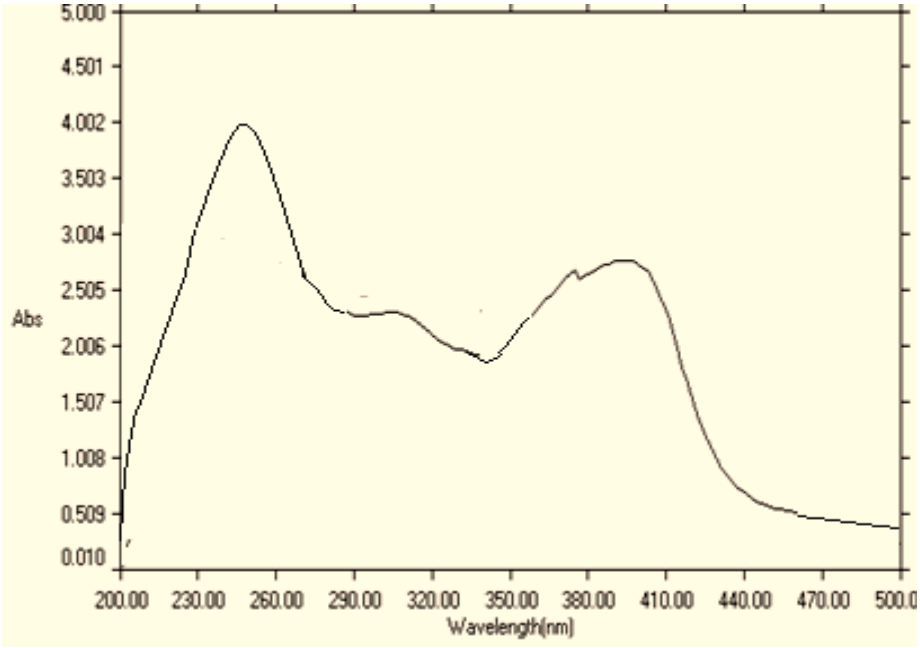



**Fig.3.4 : Sodium acetate spectrum of compound I**

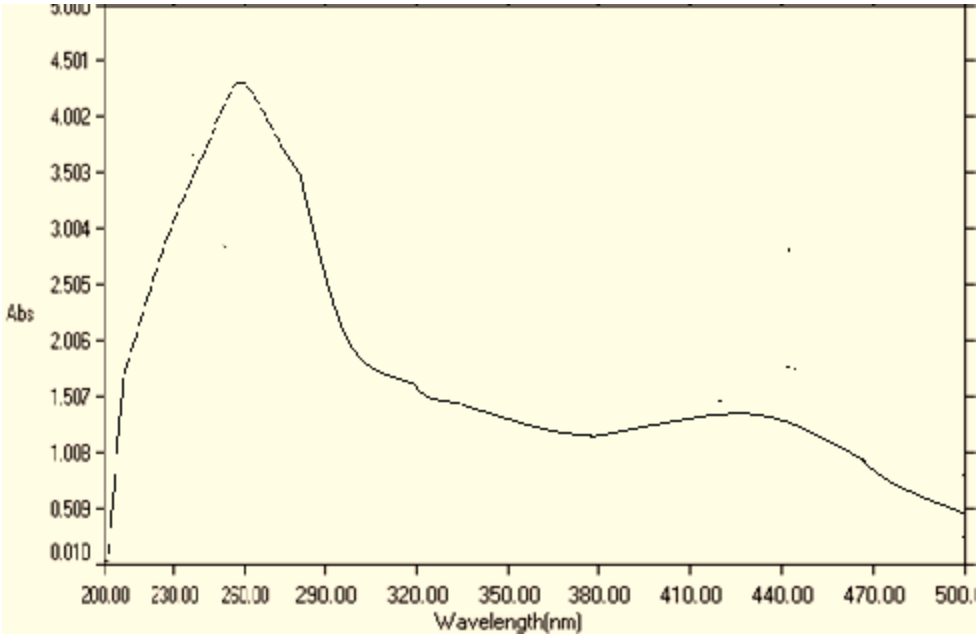



**Fig.3.5: Aluminium chloride spectrum of compound I**

The $^{1}$HNMR spectrum of compound I(Fig.3.6) showed δ(ppm): 1.22 (assigned for a methyl group); 1.84(accounts for an acetyl group) ; multiplet (4.80-5.40) assigned for a sugar moiety(not

identified in this study) ; multiplet(6.00-7.85) assigned for the aromatic protons.

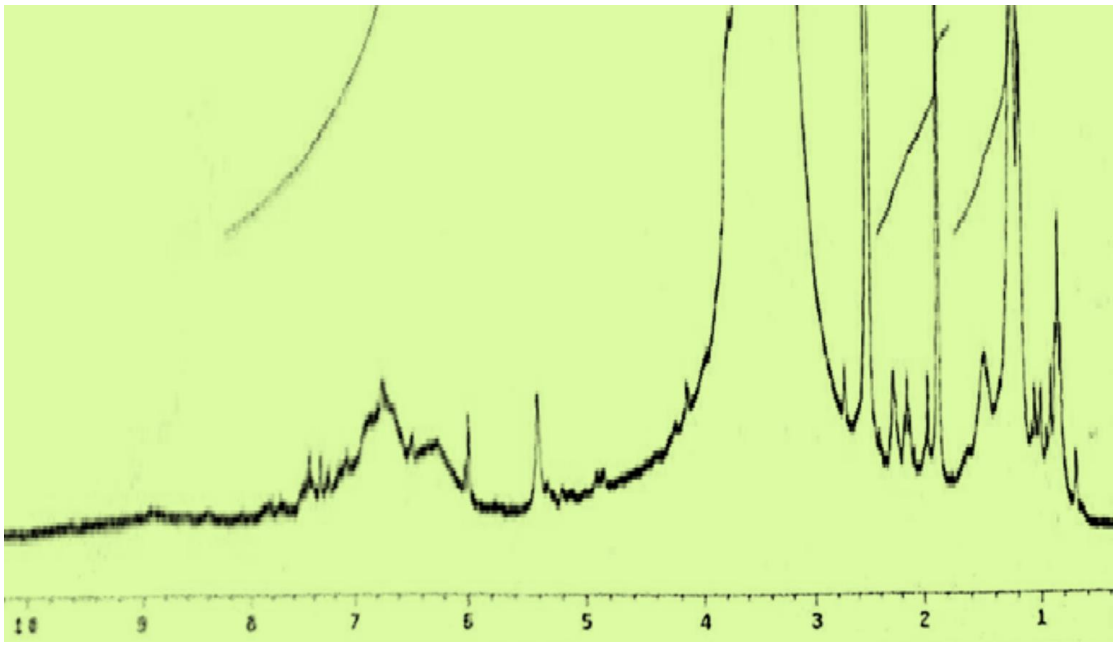
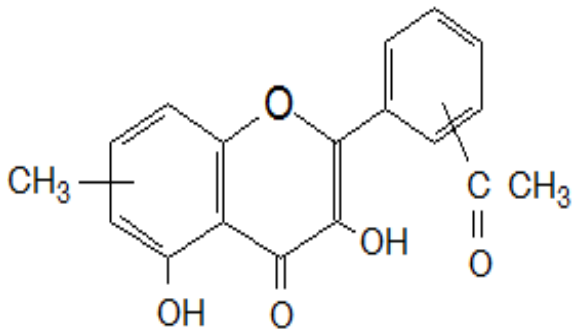



**Fig. 3.6 : $^{1}$HNMR spectrum of compound I**

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




**Compound I**

### 3.1.3 Antimicrobial activity of *Acacia tortilis*

The ethanol extract of *Acacia tortilis* was screened for antimicrobial activity against five standard microorganisms (Table 3.1).The results are depicted in Table (3.2). Results were interpreted in the following conventional terms : (<9mm: inactive ;9-12mm:partially active ;13-18mm: active; >18mm:very active) .Tables (3.3) and (3.4) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

**Table 3.1: Test organisms**

| Ser. No | Micro organism | Type | Source |
|---|---|---|---|
| **1** | *Bacillus subtillus* | G+ve | ATCC 2836 |
| **2** | *Staphylococcus aureus* | G+ve | ATCC 29213 |
| **3** | *Pseudomonas aeroginosa* | G-ve | NCTC 27853 |
| **4** | *Escherichia coli* | G-ve | ATCC 25922 |
| **5** | *Candida albicans* | fungi | ATCC 7596 |

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

*ATCC. American type culture collection, Maryland, USA

**Table 3.2: Inhibition zones (mm/mg sample)**

| Sample | Ec | Ps | Sa | Bs | Ca |
|---|---|---|---|---|---|
| **Ethanol extract** | - | - | - | - | 10 |

**Table 3.3: Antibacterial activity of standard drugs**

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

**Table 3.4: Antifungal activity of standard drug**

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

**Sa.** ***Staphylococcus aureus***

**Ec.:** ***Escherichia coli***

**Pa.:** ***Pseudomonas aeruginosa***

**Bs:** ***Bacillus subtilis***

**Ca:** ***Candida albicans***

## 3.1.4 Characterization of compound II

Compound II was isolated as blue amorphous powder from the stem bark of *Acacia tortilis* in the IR spectrum of compound II **(**Fig.3.7) showed v (KBr): 567, 721,777 (C-H, Ar., bending), 1282 (C-O, ether), 1460,1512 (C = C, Ar.), 1608 (C = O),2921(C-H) and 3352 cm-1 (OH).

The appearance of a carbonyl absorption suggests that compound I is neither an anthocyanin nor catechin . These classes are characterized by absence of a carbonyl function.

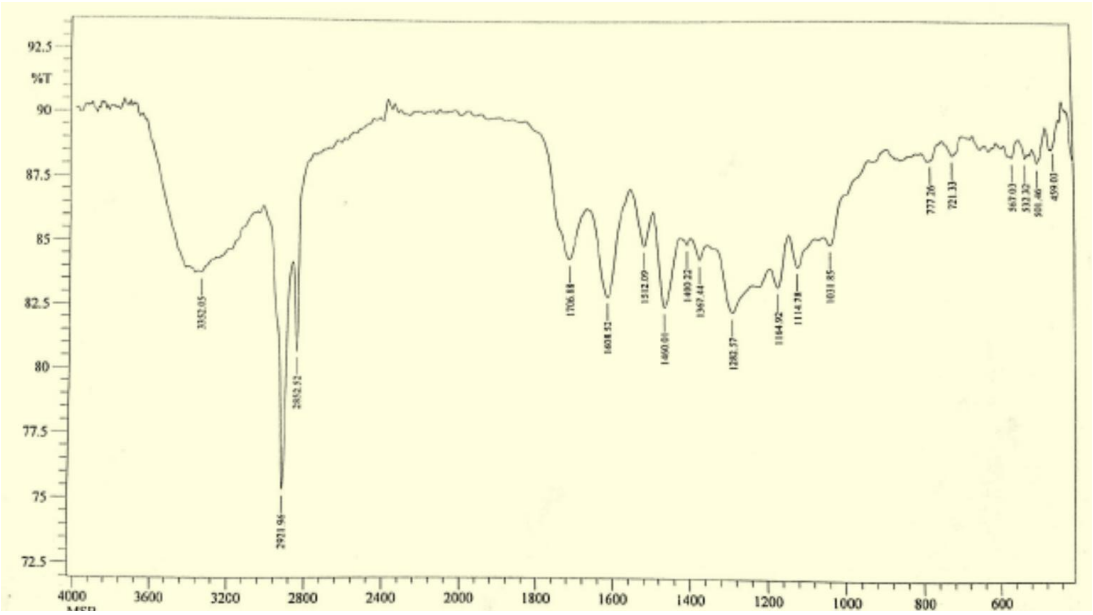



**Fig.3.7: IR spectrum of compound I**

The UV it absorbs at λmax 296nm (Fig.3.8).The appearance of only one band – band II- suggests that this compound is either a flavanone, isoflavone, dihydrochalcone or dihydroflavonols. The compound is a dihydroflavonol since the sodium methoxide spectrum (Fig.3. 9) gave a bathochromic shift characteristic of the 3-OH function of dihydroflavonols where a bathochromic shift without decrease in intensity was observed.

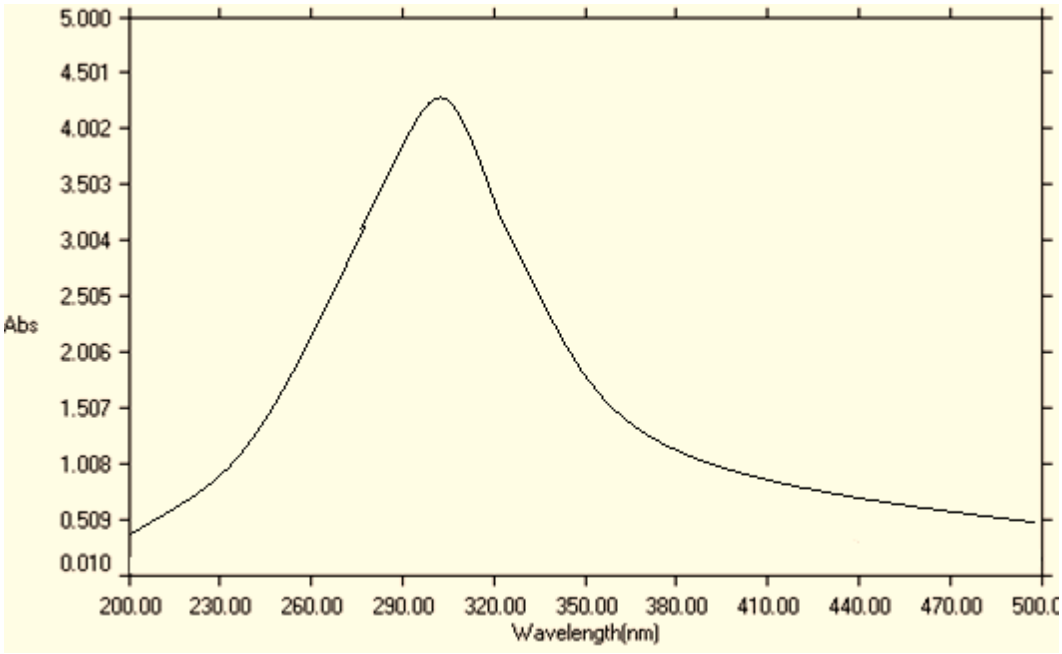



**Fig.3.8:UV spectrum of compound II**

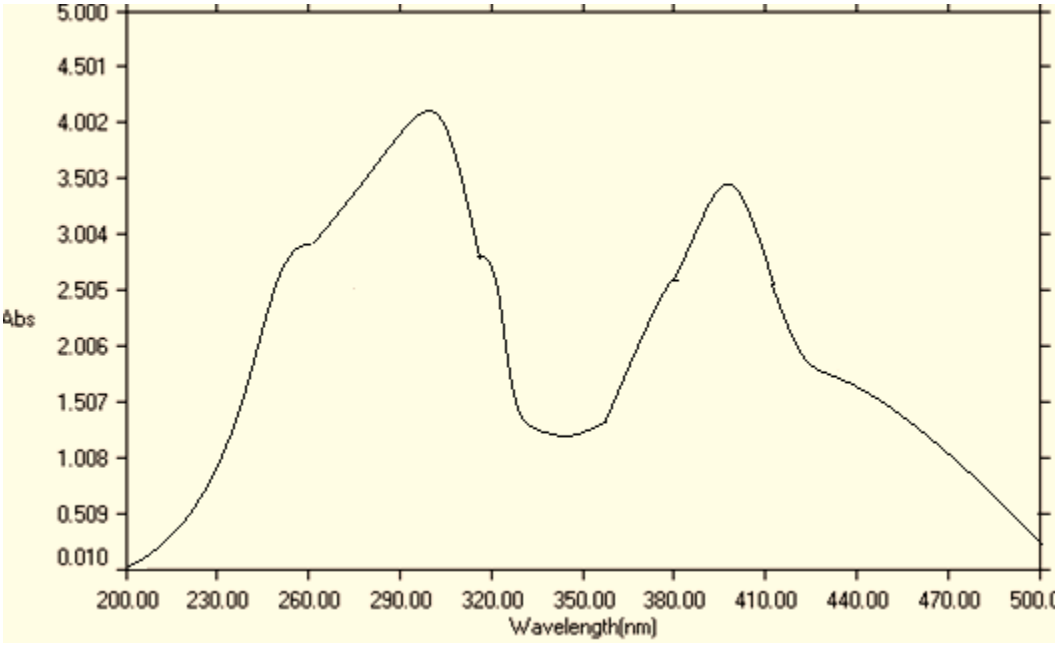



**Fig.3.9:Sodium methoxide spectrum of compound II**

Next the hydroxylation pattern of the dihydroflavonol has been investigated by using some UV shift reagents. These reagents exhibit bathochromic shifts diagnostic of specific hydroxylation pattern. Sodium acetate is a useful shift reagent which exhibits a bathochromic shift diagnostic of a 7-OH substituent. The shift reagent-aluminium chloride is diagnostic of 3- and 5-OH groups as well as catechol systems, while boric acid affords a bathochromic shift in presence of catechol moieties. The sodium acetate spectrum(Fig.3.10) did not reveal any bathochromic shift in Band II (it gave a bathochromic shift in band I) hence indicating absence of a 7-OH function. The aluminium chloride spectrum (Fig.3.11) showed a bathochromic shift characteristic of a 5-OH function.

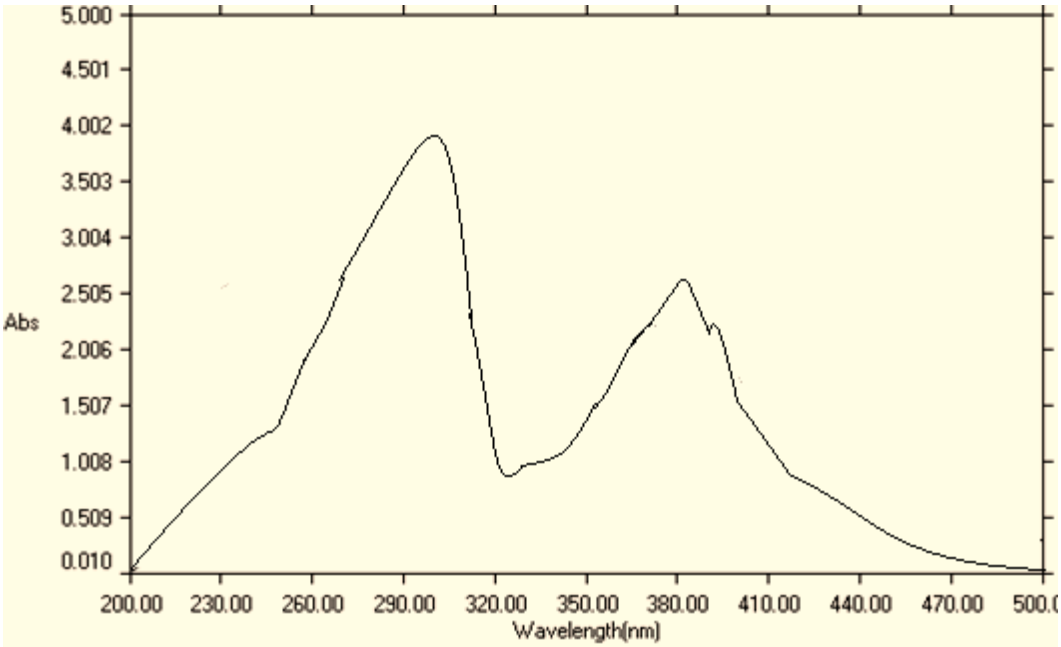



**Fig.3.10:Sodium acetate spectrum of compound II**

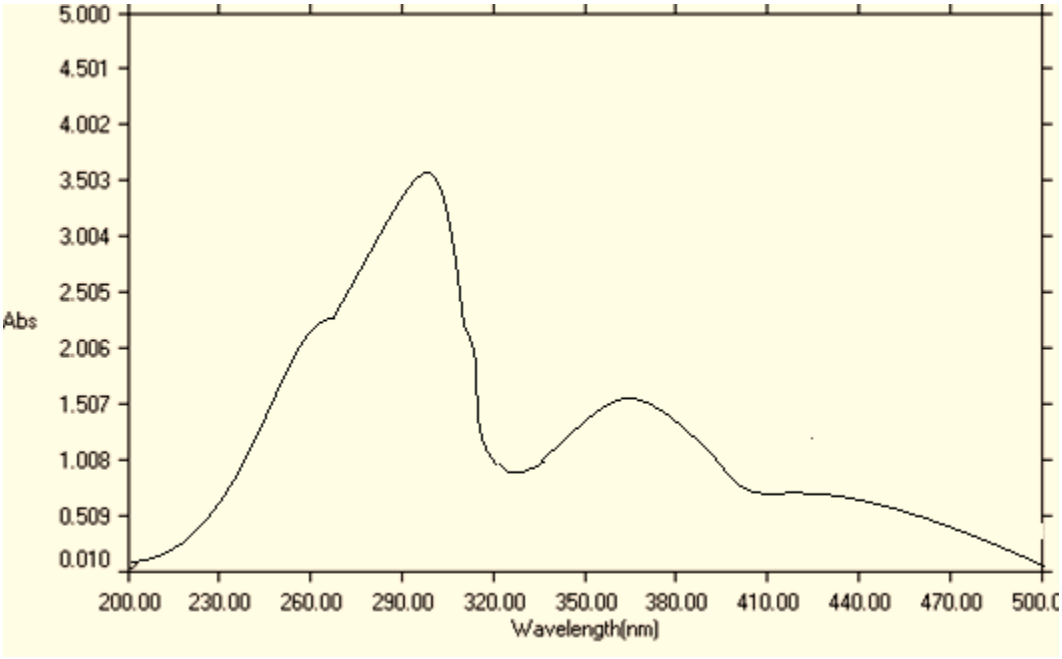



**Fig.3.11:Aluminium chloride spectrum of compound II**

The $^{1}$HNMR spectrum(Fig.3.12) showed δ(ppm) : 1.23 (assigned for two methyl groups); 1.84(accounting for one acetyl group). The aromatic protons appeared at 8.24ppm.

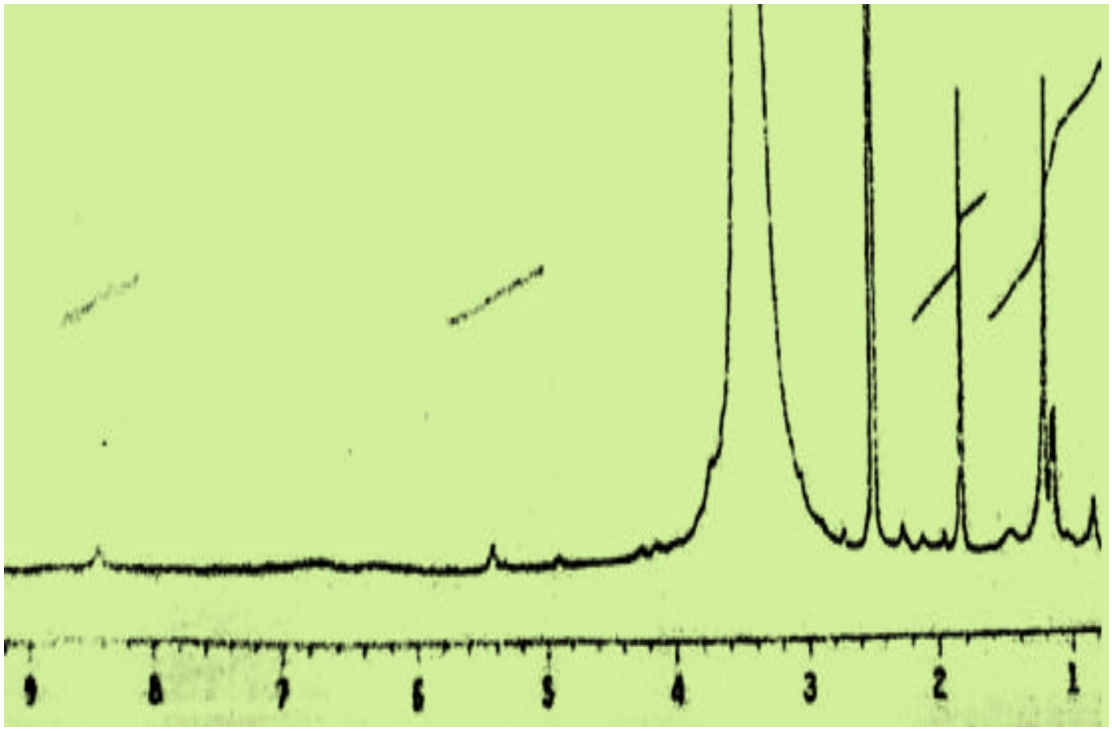
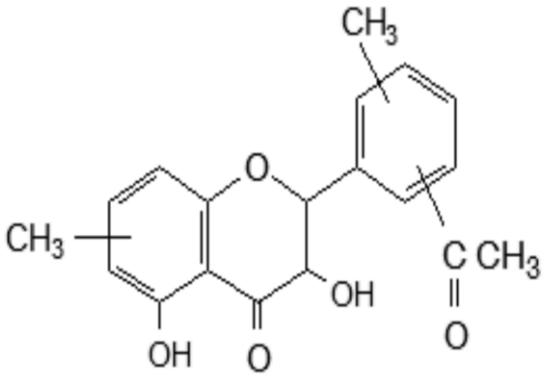



**Fig.3.1 2: $^{1}$HNMR of compound II**

On the basis of this argument, the following partial structure was proposed for the isolated dihydroflavonol:




Compound II

## 3.2 *Acacia nubica*

### 3.2.1 Phytochemical screening

Phytochemical screening of *Acacia nubica* stem bark revealed the presence of tannins sterols and saponins.

### 3.2.2 Flavonoids of *Acacia nubica*

### 3.2.3 Characterization of compound III

Compound III was isolated as yellow powder from the stem bark of *Acacia nubica.* In the UV it absorbs at λmax 264nm (Fig. 3.13). The appearance of only one band – band II- suggests that this compound is either a flavanone, isoflavone, dihydrochalcone or dihydroflavonols. However the UV spectrum showed a shoulder in the range 300-340nm which is a characteristic feature of isoflavones.

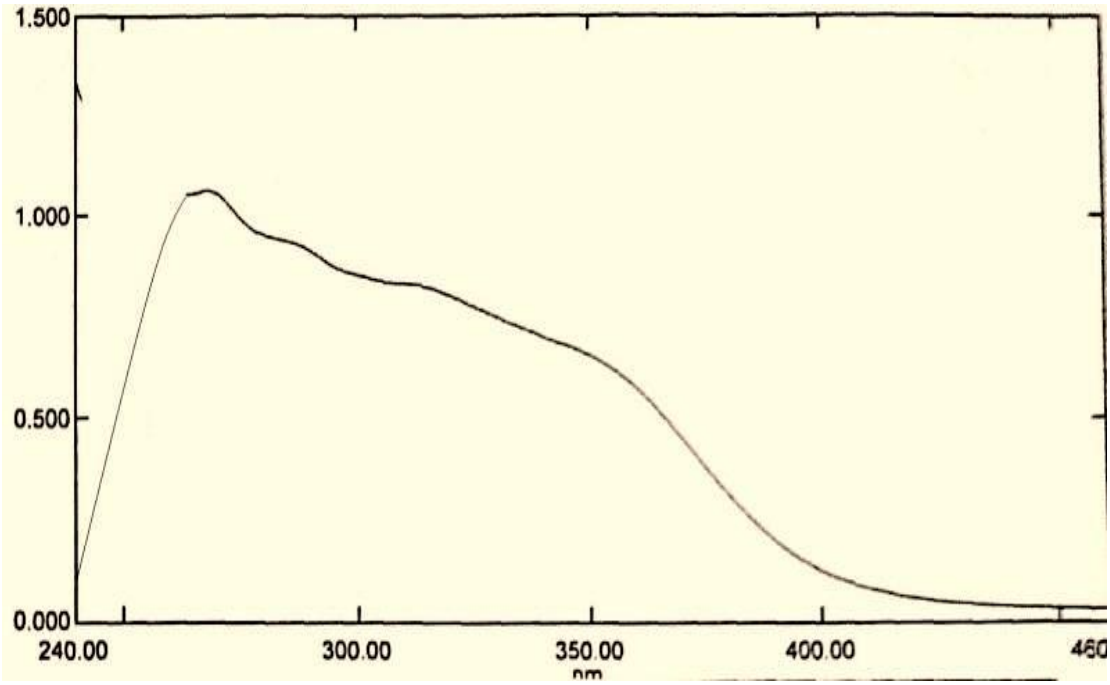



**Fig.3.13:UV spectrum of compound III**

Next the hydroxylation pattern of the isoflavone has been investigated by using some UV shift reagents. These reagents exhibit bathochromic shifts diagnostic of specific hydroxylation pattern. The shift reagent - sodium methoxide- shows a bathochromic shift in presence of 3- or a 4`-OH function. In case of 3-OH group , the shift is accompanied with decrease in intensity. Sodium acetate is another useful shift reagent which exhibits a bathochromic shift diagnostic of a 7-OH substituent. The shift reagent-aluminium chloride is diagnostic of 3- and 5-OH groups as well as catechol systems. The sodium methoxide spectrum(Fig.3.14) of compound III did not reveal any bathochromic shift suggesting absence of 3- and 4`-hydroxylation. The aluminium chloride spectrum (Fig.3.15) showed a bathochromic shift characteristic of a 5-OH function. The boric acid spectrum suggested the absence of catechol systems since it failed to show any bathochromic shift (Fig.3.16). However, the sodium acetate spectrum (Fig.3.17) revealed a 6nm bathochromic shift indicating a 7-OH substituent.

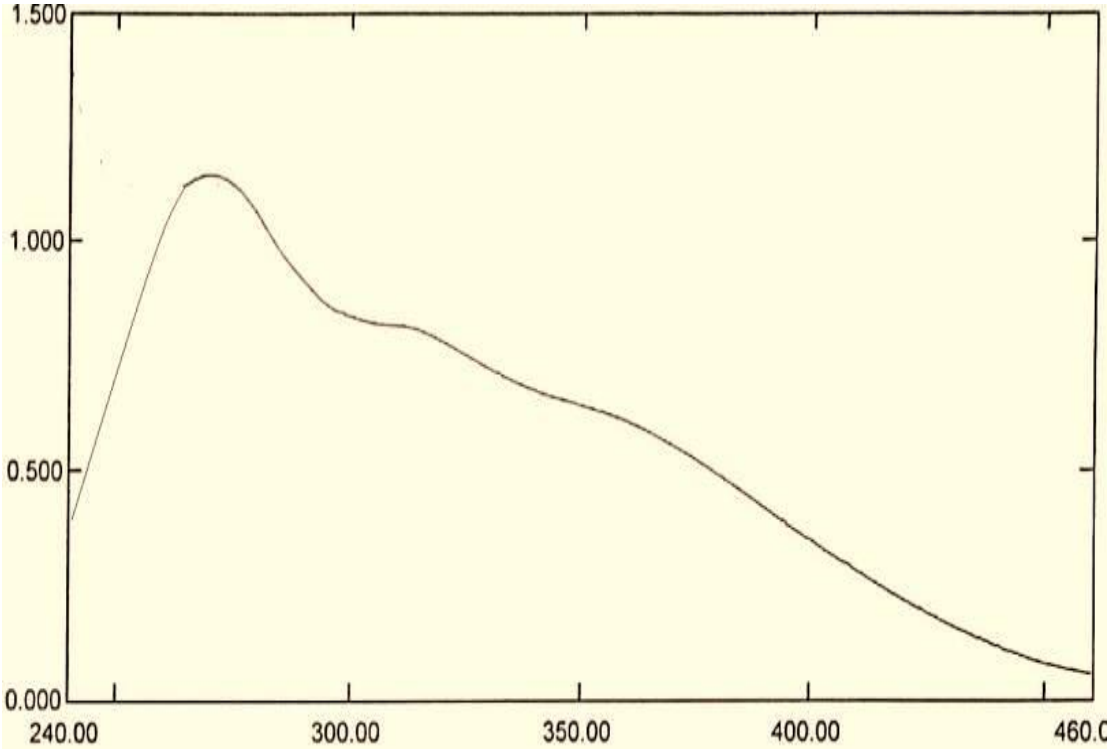



**Fig.3.14:Sodium methoxide spectrum of compound III**

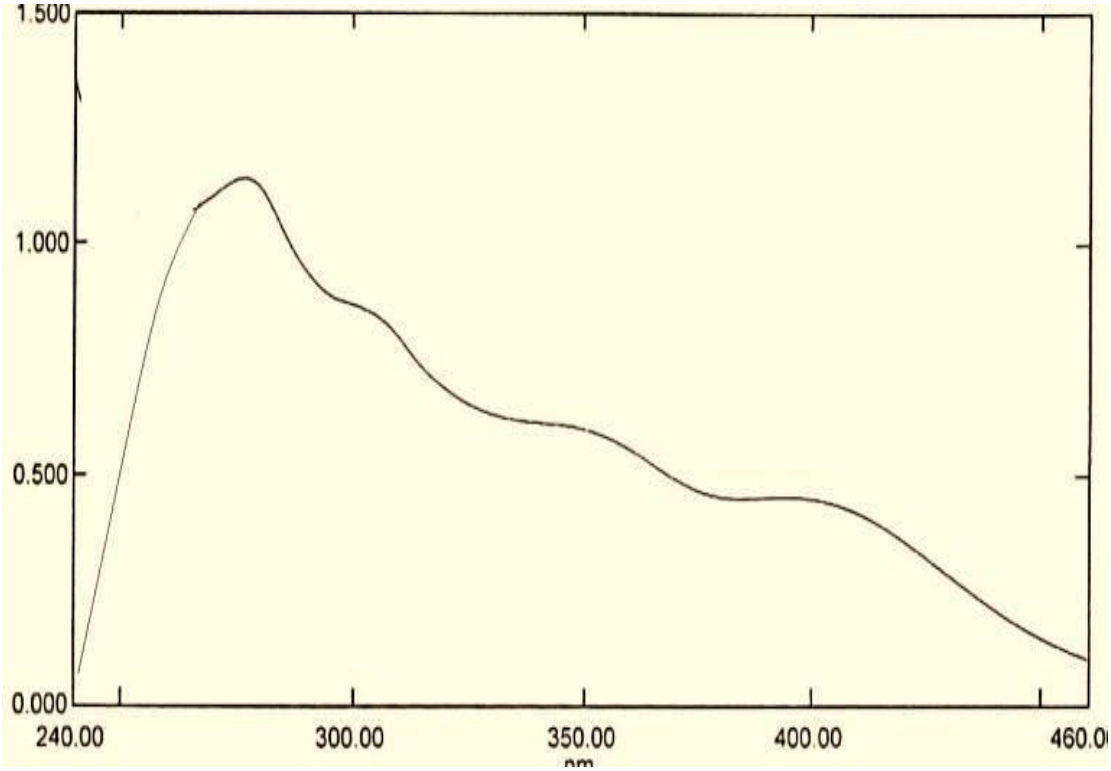



**Fig.3.15:Aluminium chloride spectrum of compound III**

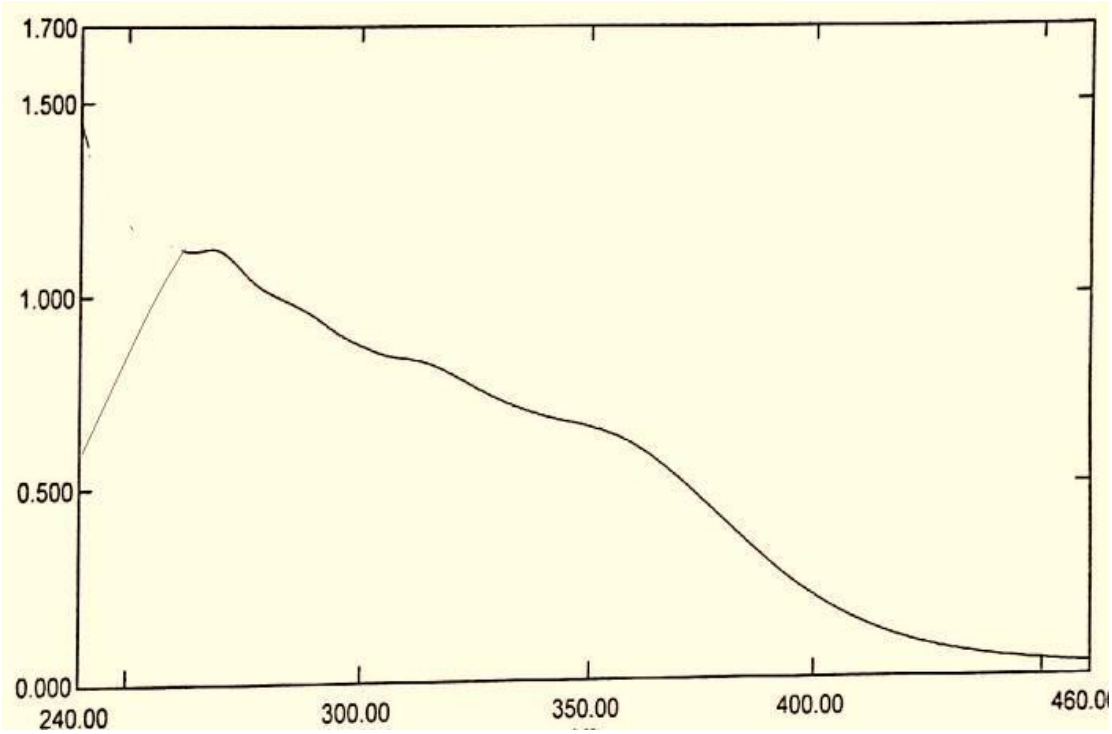



**Fig.3.1 6: Boric acid spectrum of compound III**

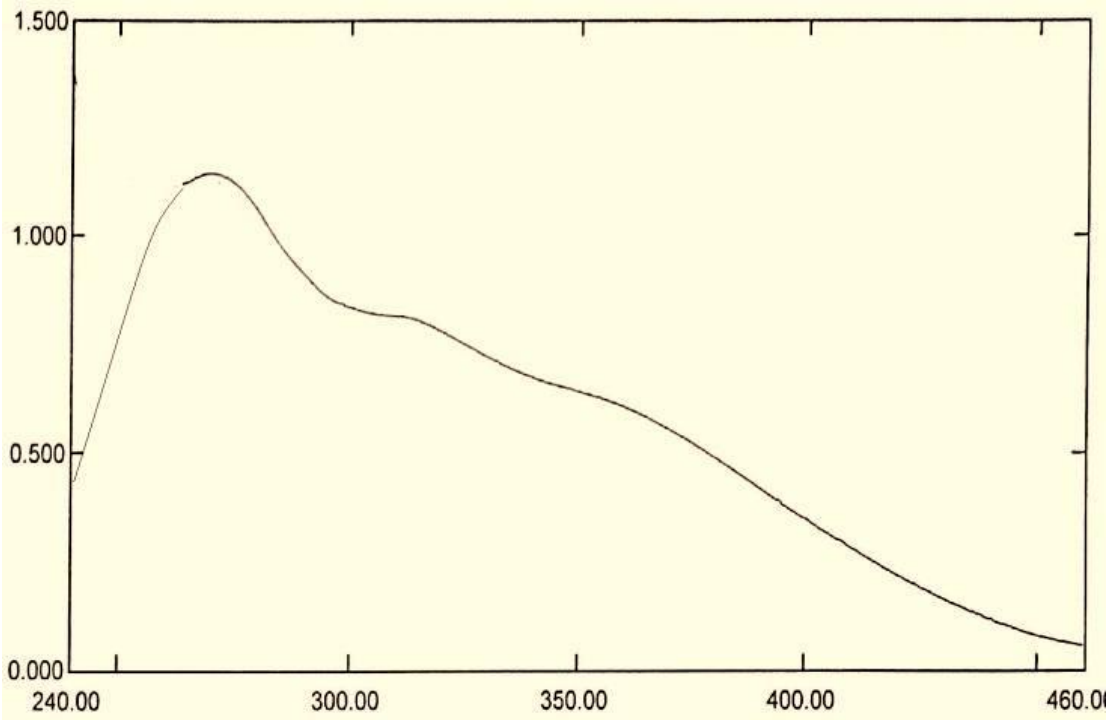



**Fig.3.17 :Sodium acetate spectrum of compound III**

The $^{1}$HNMR spectrum(Fig.3.18) showed δ(ppm) : 1.23 (assigned for three methyl groups); 2.24(accounting for one acetyl group) ; m(3.50-4.20)- assigned for a sugar moiety(not identified in this study) . The aromatic protons appeared as multiplet(6.26-7.80) ppm.

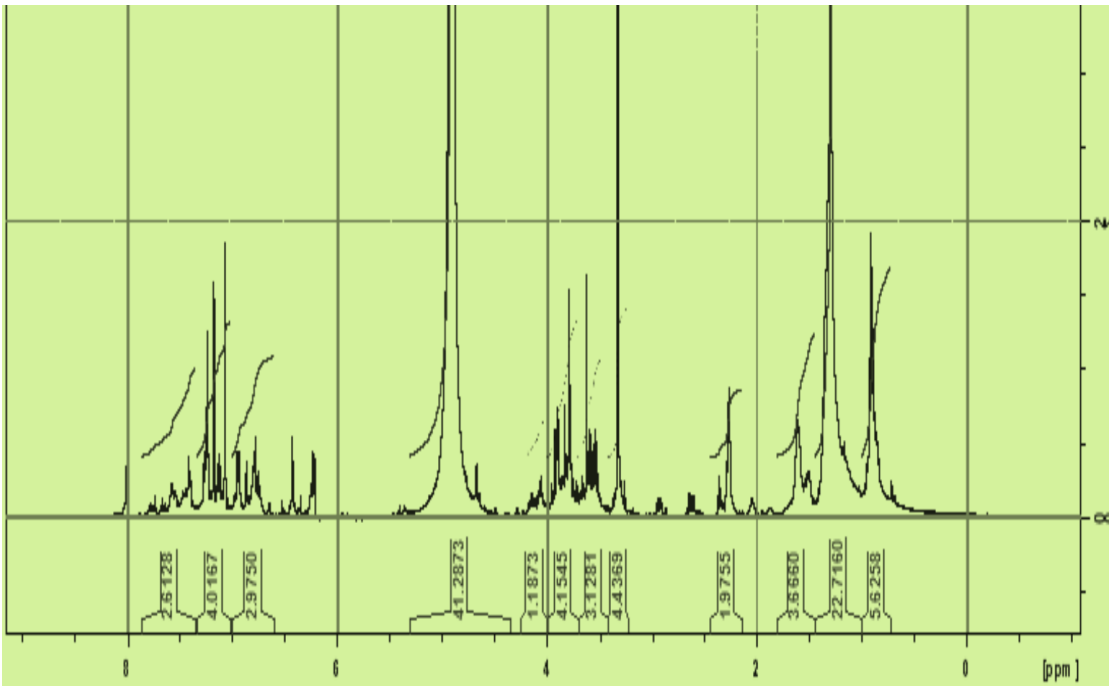
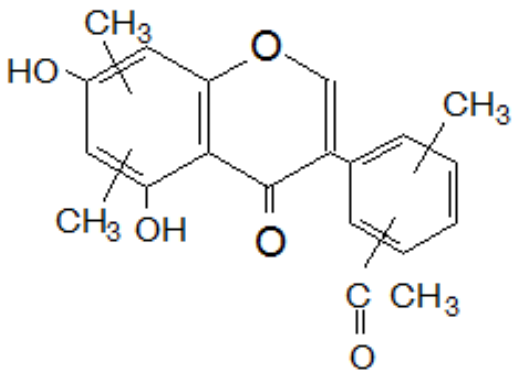



**Fig.3.18:$^{1}$HNMR spectrum of compound III**.

On the basis of this argument, the following partial structure was proposed for the aglycone of the isolated flavonoid:




**Compound III**

### 3.2.4 Antimicrobial activity of *Acacia nubica*

*Acacia nubica* ethanol extract was assessed for antimicrobial activity via the disc diffusion bioassay using five standard human pathogens.. The average of the diameters of the growth of inhibition zones are shown in Table 3.5. *Acacia nubica* ethanol extract exhibited significant antibacterial and antifungal activity against test organisms.

**Table 3.5: Inhibition diameters(mm) of the ethanol extract**

| Sample | Conc.(mg/ml) | Ec | Ps | Sa | Bs | Ca |
|---|---|---|---|---|---|---|
| ***Acacia nubica* ethanol extract** | 100 | 17 | 20 | 21 | 19 | 16 |

### 3.2.5 Characterization of compound IV

Compound IV was isolated by paper chromatography from *Acacia nubica* stem bark and its structure was elucidated via a combination of spectral techniques (UV and $^{1}$HNMR).

The UV spectrum of compound IV showed (Fig.3.19) λmax 296 nm. Such absorption is characteristic of dihydroflavonols. When the UV shift reagent – sodium methoxide - was added to a methanolic solution of compound IV ,The absorption maximum shifted bathochromically to 396nm indicating the presence of a 3-OH function which is a characteristic feature of dihydroflavonols (Fig. 3.20).

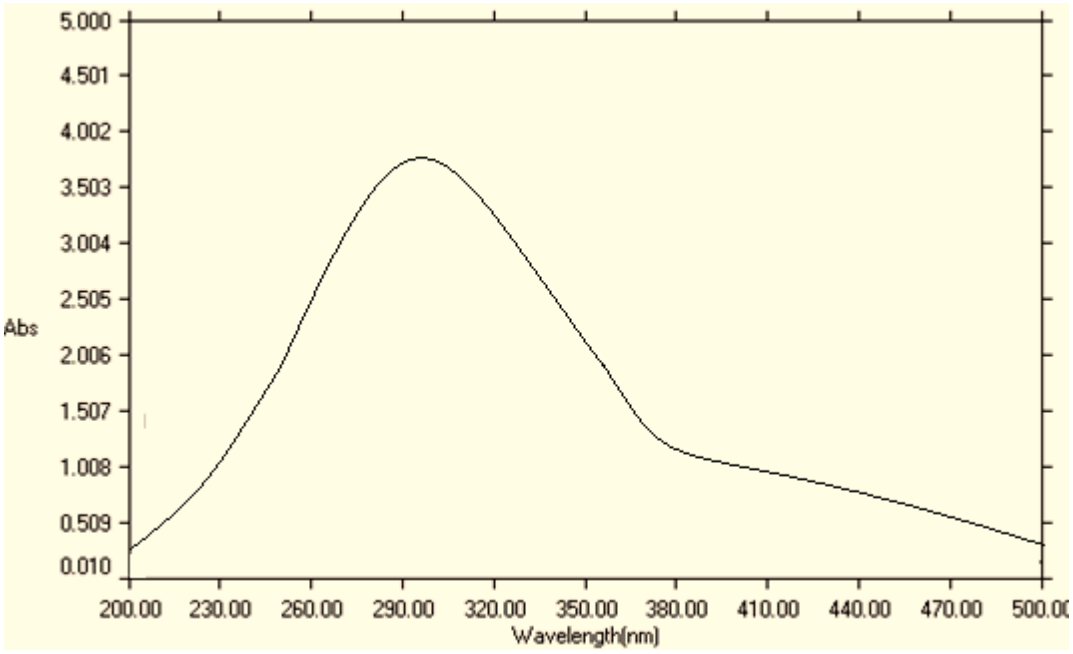



**Fig.3.19 : UV spectrum of compound IV**

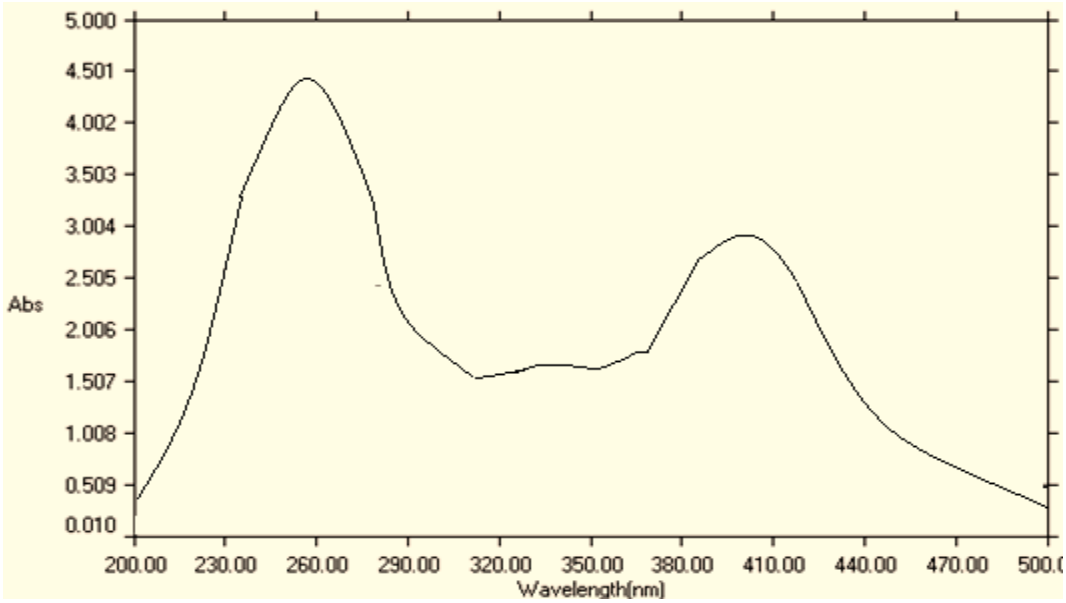



**Fig. 3.20: Sodium methoxide spectrum of compound IV**

When a methanolic solution of compound IV was treated with excess powdered sodium acetate, no bathochromic shift (Fig.3.21) characteristic of 7 –OH group was observed. The aluminium chloride spectrum of compound I(Fig.2.22) did not show a bathochromic shift indicating absence of 3- and 5 –OH groups and catechol systems.

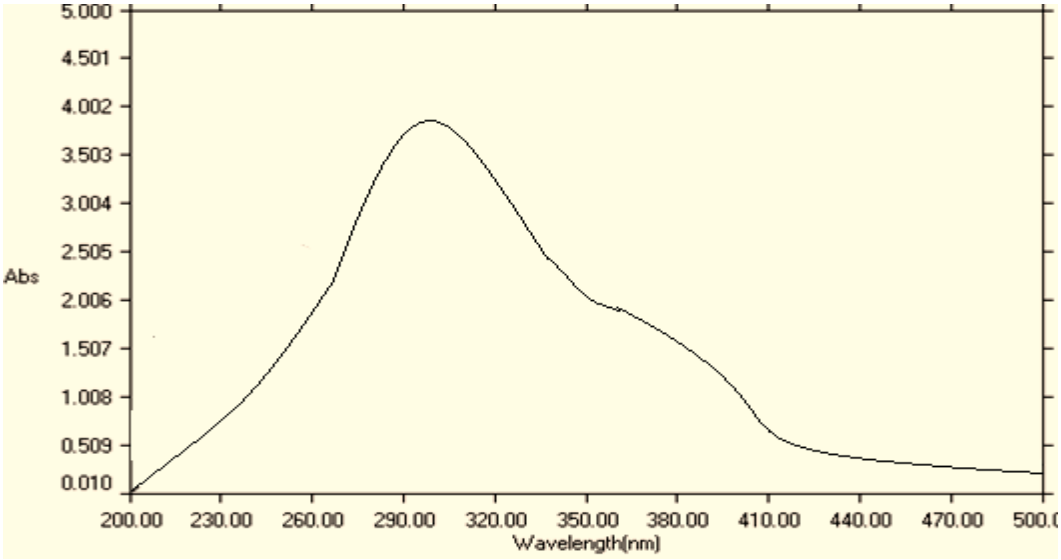



**Fig.3.21 : Sodium acetate spectrum of compound IV**

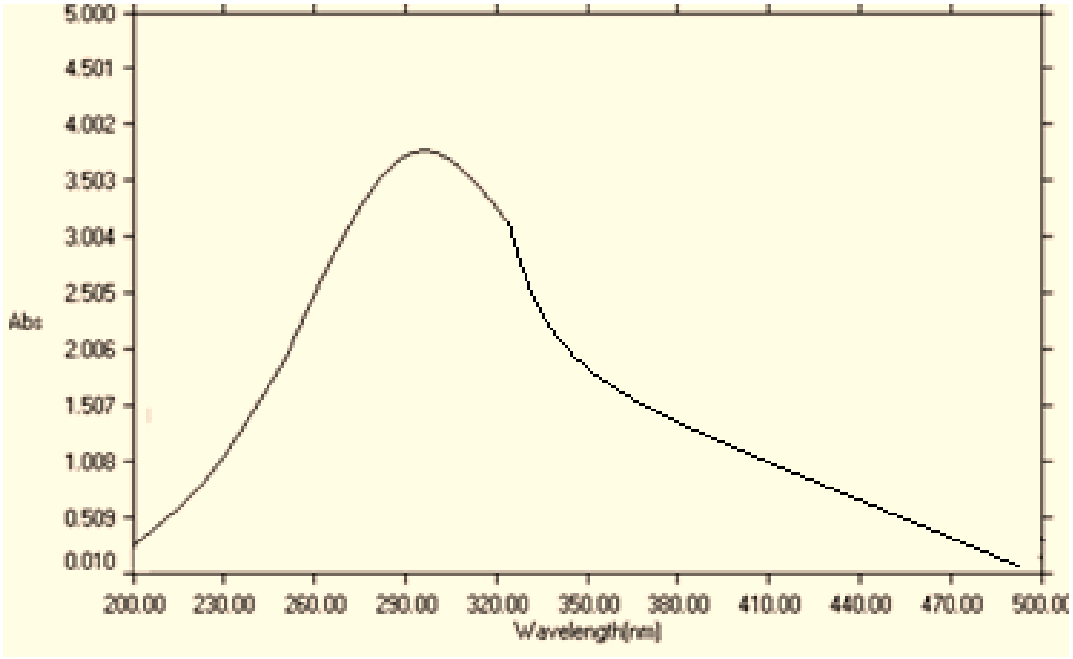



**Fig.3.22: Aluminium chloride spectrum of compound IV**

The $^{1}$HNMR spectrum of compound IV (Fig. 3.23) showed δ(ppm): 1.20 (assigned for three methyl groups); 2.20(accounts for an acetyl group) . The multiplet at δ (3.50-4.20) and signals at 4.70 and 5.35ppm were assigned for a sugar moiety(not identified in this study). The signals at δ7.20 and 7.35ppm were assigned for the aromatic protons.

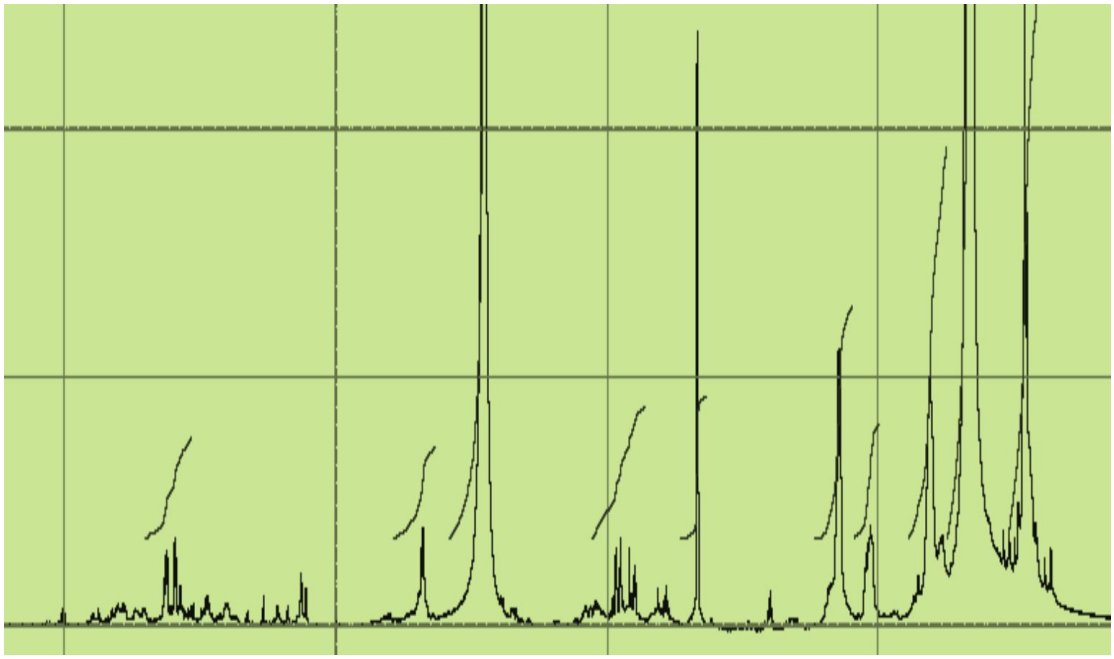
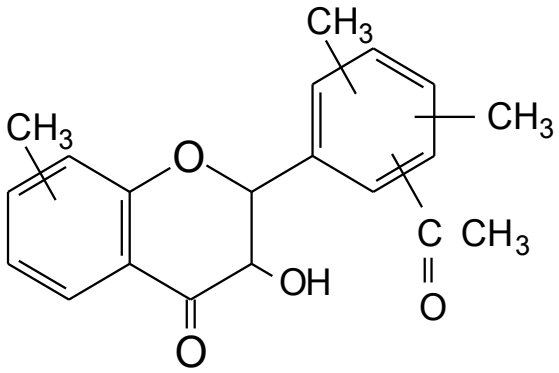


**Fig. 3.23 : $^{1}$HNMR spectrum of compound IV**

On the basis of the above spectral data , the following partial structure was proposed for the aglycone of compound IV:




**Compound IV**

## 3.3 Conclusion

The flavonoids of *Acacia tortilis* and *Acacia nubica* stem bark were extracted by aqueous ethanol. The crude extracts of both plants purified by paper chromatography. From *Acacia tortilis* compounds I and II were isolated, while compounds III and IV were isolated from *Acacia nubica*. The structures of these flavonoids were partially elucidated via a combination of spectral techniques (UV, IR and $^{1}$HNMR).

Different fractions of the target species were evaluated for their antimicrobial potential and promising results were obtained.

## 3.4 Recommendations

- The structures of the isolated flavonoids may further be elucidated by employing, 2D NMR(1H-1H COSTY NMR,HMBC and HSQC), $^{13}$C NMR, and MS spectroscopy.
- The isolated phytochemical may be evaluated for its ant-inflammatory, antibacterial, antifungal, antimalarial and antioxidant potential.
- Other phytochemicals (alkaloids, glycosides, steroids….) existing in *Acacia tortilis* and *Acacia nubica* may also be isolated and their structure elucidated.

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