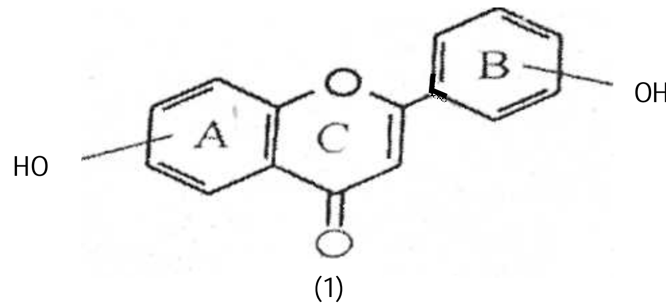
1-Introduction

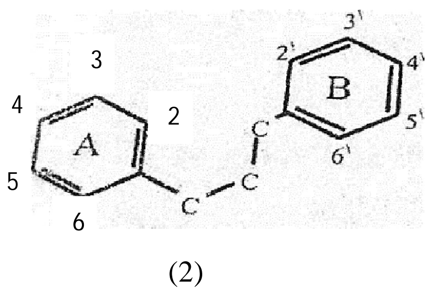
1.1 Flavonoids: occurrence and importance

Polyphenols are compounds consisting of more than one aromatic ring ,each containing at least one hydroxy1 group (I) as shown below:(Rein,*et al*,2000)

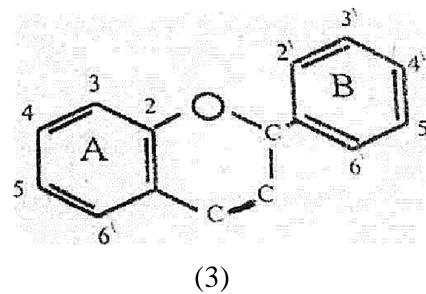


(1)

Flavonoids are groups of polyphenol compounds. They are widely distributed through the plant kingdom (Bohm,*et al*,1998, (Rasmussem , *et al*,2003 ,Hertog,*et al*,1996) .The basic nucleus of flavonoids has 15 carbon atoms arranged in three rings labeled A, B and C (Harbone,*et al*, 1984). They have a characteristic backbone ring structure C_6 - C_3 - C_6, namely biphenyl propane. The two aromatic rings A and B generally contain a number of phenolic hydroxyl 1 groups (Dewik,*et al*, 1998,Harnobone,*et al*,1991), A and B rings are joined by a linear carbon chain, which may be open (2) or closed (3) as shown below (Hendrickson, *et al*, 1994).



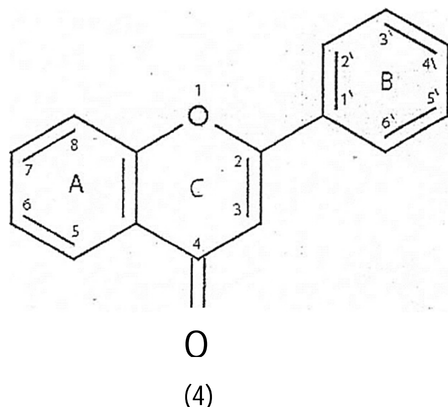
(2)



(3)

1.2 General Concept

Flavonoids (2-phenylbenzpyrone)-(l) are a large group of polyphenolic compounds that occur commonly in plant (Catherine, *et al,* 2003). The name "Flavonoid" is derived from Greek word "Flavus": it means yellow (Zechemesiter L., 1957).



(4)

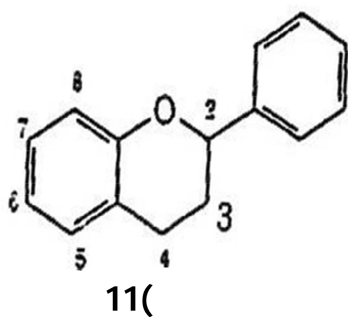
These phytochemicals are distributed widely in higher plant parts (barks, roots, stems, flowers) but also found in some lower plants including algae (Bohm,*et al*,1998,Satyajit, *et al,*2006). Many of these compounds are responsible for the attractive colours of flowers and fruits and leaves (De Groot,*et al,* 1998). Flavonoids mainly occur as aglycones (consisting of a benzene ring condensed with a six membered ring which possesses a phenyl ring at the 2, 3 or 4 position). Glycosides which carry one or more sugar residues and methylated derivatives are very common (Dicarlo,*et al*,1999).

The chemical structure of these compounds are based on a (C_6 - C_3 - C_6) skeleton. They differ in saturation of heteroaromatic ring C, in the placement of the aromatic ring B at the positions 2, 3 or 4.

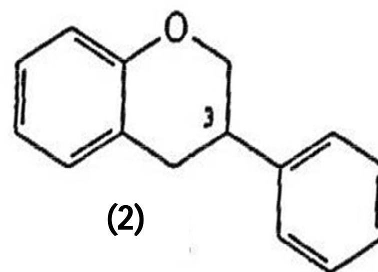
Flavonoid aglycones possess the chemical properties of phenolics, and thus they are slightly acidic. Those possessing a number of unsubstituted hydroxyl groups, or sugar moieties, are polar substances and soluble in polar organic solvents. The presence of sugar makes flavonoid more water soluble, while less polar aglycones like isoflavones, flavanones, and highly methoxyalted flavones and flavonols tend to be more soluble in ether or chloroform (Monika, *et al*,2008,Umezawa,2003).

1.3 Classification

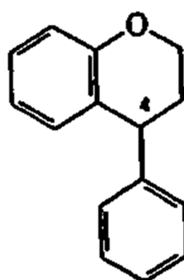
The term "flavonoid" is generally used to describe a broad collection of natural products that include a C_6-C_3-C_6 carbon framework, or more specifically a phenyl benzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes, the flavonoids: 2-phenylbenzopyrans (1), isofiavonoids (3-benzopyrans) (2) and the neoflavonoids (4-benzopyrans) (3). These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related (Erich, 2005).



1



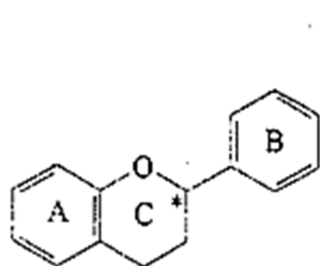
2



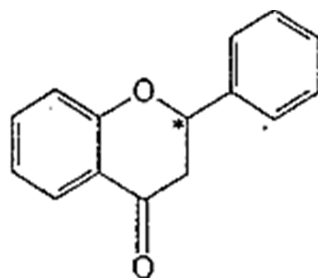
3

1.3.1 Phenylbenzopyrans

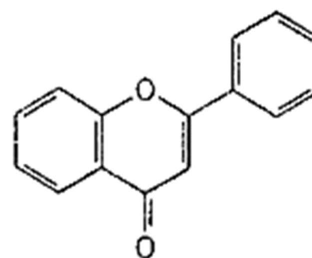
Based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups (Erich, 2005):



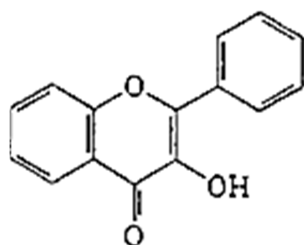
Flavan



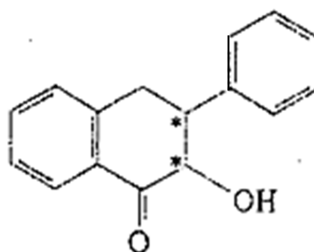
Flavanone



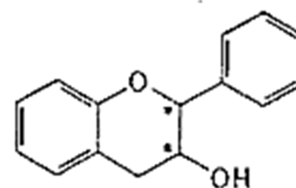
Flavone



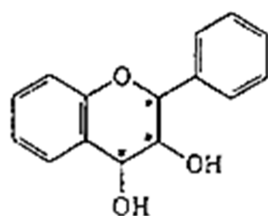
Flavonol



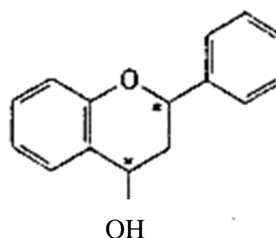
dihydroflavonol



Flavan-3-ol



Flavan-3.4-diol

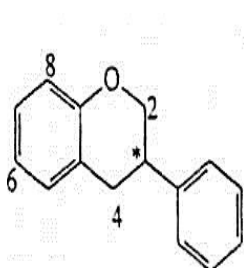


Flavan-4-ol

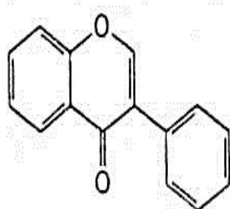
*streocenters

1.3.2 Isoflavonoids

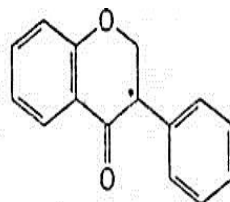
The isoflavonoids are a distinctive subclass of the flavonoids. These compounds possess a 3-phenylchroman skeleton that is biogenetically derived by 1,2-aryl migration in a 2-phenylchroman precursor. Despite their limited distribution in the plant kingdom, isoflavonoids are remarkably diverse as far as structural variations are concerned. This arises not only from the number and complexity of substituents on the basic 3-phenylchroman system, but also from the different oxidation levels and presence of additional heterocyclic rings. Isoflavonoids are subdivided into the following groups:(Erich G.,2005)



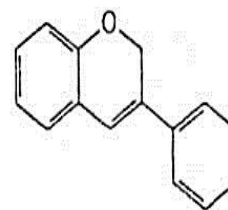
Isoflavan



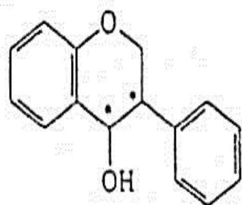
isoflavone



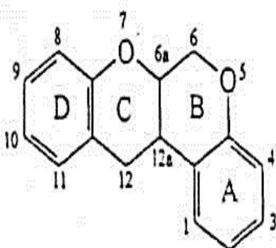
isoflavonone



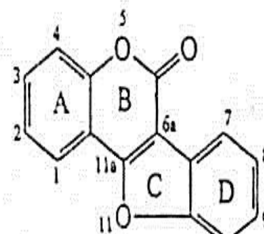
Isoflav-3-ene



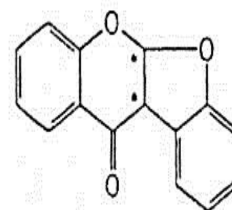
Isoflavanol



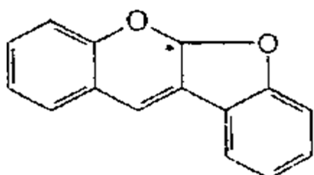
Rotenoid



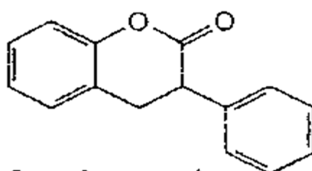
coumestane



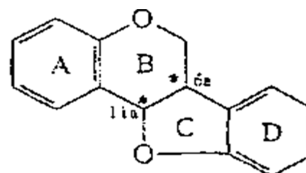
coumonochromo



Coumronochomene



3-arylcomarin

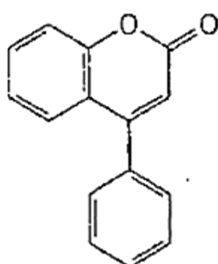


pterocarpan

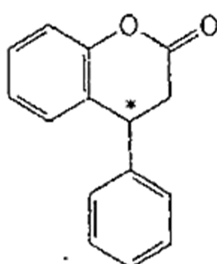
*streocenters

1.3.3 Neoflavonoids

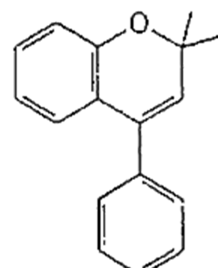
The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins(4-aryl-2H-l-benzopyran-2-ones),3,4-dihydro-4-arylcoumarins, and neoflavenes. (Erich G.,2005).



4-arylcoumarin



3,4-dihydro-4-arylcoumarin

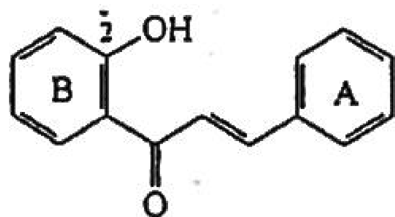


Neoflavene

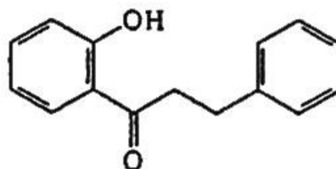
*streocenters

1.3.4 Minor Flavonoids

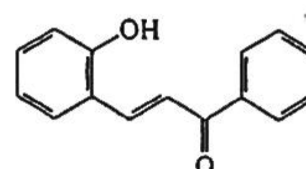
Natural products such as chalcones and aurones also contain a C_6-C_3-C_6 backbone and are considered to be minor flavonoids. These groups of compounds include the 2'-hydroxychalcones,2'-OH-dihydrochalcones,2'-OH-retro-chalcone,aurones(2-benzylidenecoumaranone), and auronols. (Erich G.,2005).



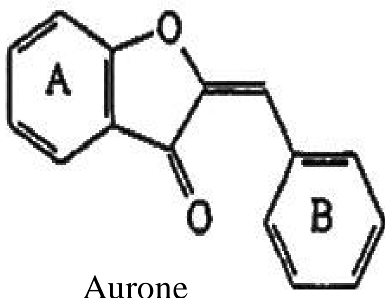
2̄-OH-chalcone



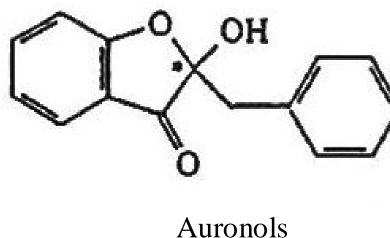
2̄-OH-dihydrochalcone



2̇ - OH- retro-chalcone



Aurone



Auronols

1.4 Separation and quantification of flavonoids

Essential to the study of flavonoids is having the means available for their separation (analytical and preparative). The importance of this aspect of falvonoid research can be seen in the number of review articles that refer to their chromatography (Mabry,*et al*, 1970, Santos-Buelga,*et al*,2003)·Aim of these reviews is to in earlier times, thin-layer chromatography (TLC), polyamide chromatography, and paper electrophoresis were the major separation techniques for phenolics. Of these methods,TLC is still the workhorse of flavonoid analysis. It is used as a rapid, simple, and versatile method for following polyphenolics in plant extracts and in fractionation work. However, the majority of published work now refers to qualitative and quantitative applications of high performance liquid chromatography (HPLC) for analysis. Flavonoids can be separated, quantified, and identified in one operation by coupling HPLC to ultraviolet (UV), mass, or nuclear magnetic resonance (NMR) detectors. Recently, the technique of capillary electrophoresis(CE) has been gaining attention.

1.4.1 Extraction

For extraction, the solvent is chosen as a function of the type of flavonoid required. Polarity is an important consideration here. Less polar flavonoids(e.g.,isoflavones,flavanones, methylated flavones,and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate,while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol-water mixtures. Glycosides have increased water solubility and aqueous alcoholic solutions are suitable: The bulk of extractions of flavonoid-containing material are still performed by simple direct solvent extraction.

Powdered plant material can also be extracted in a Soxhlet apparatus, first with hexane,for example, to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. This approach is not suitable for heat-sensitive compounds.

Aconvenient and frequently used procedure is sequential solvent extraction. A first step, with dichloromethane, for example, will extract flavonoid aglycones and less polar material.A subsequent step with an alcohol will extract flavonoid glycosides and polar constituents.

Certain flavanone and chalcone glycosides are difficult to dissolve in methanol, ethanol,or alcohol-water mixtures. Flavanone solubility depends on the pH of water-containing solutions.

Flavan-3-ols (catechins, proanthocyanidins, and condensed tannins) can often be extracted directly with water. However, the

composition of the extract does vary with the solvent - whether water, methanol, ethanol, acetone, or ethyl acetate. For example, it is claimed that methanol is the best solvent for catechins and 70% acetone for proanthoocyanidins (Hussien L.,*et al*,1990).

Anthocyanins are extracted with cold acidified methanol. The acid employed is usually acetic acid (about 7%) or trifluoroacetic acid (TFA) (about 3%). The use of mineral acid can lead to the loss of attached acyl groups.

1.4.2 Preparative methods

One of the major problems with the preparative separation of flavonoids is their sparing solubility in solvents employed in chromatography. Moreover, the flavonoids become less soluble as their purification proceeds. Poor solubility in the mobile phase used for a chromatographic separation can induce precipitation at the head of the column, leading to poor resolution, decrease in solvent flow, or even blockage of the column. Other complications can also arise.

There is no single isolation strategy for the separation of flavonoids and one or many steps may be necessary for their isolation. The choice of method depends on the polarity of the compounds and the quantity of sample available. Most of the preparative methods available are described in a volume by Hostettmann et al . (Hostettmannn K.,*et al*, 1998).

Conventional open-column chromatography is still widely used because of its simplicity and its value as an initial separation step. Preparative work on large quantities of flavonoids from crude plant extracts is also possible.

Preparative TLC is a separation method that requires the least financial outlay and the most basic equipment. It is normally employed for milligram quantities of sample, although gram quantities are also handled if the mixture is not too complex.

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

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

1.4.3 Analytical methods

A herbal product contains multiple constituents that might be responsible for its therapeutic effects.

Knowledge of the flavonoid content of plant-based foods is paramount to understanding their role in plant physiology and human health. Analytical methods are also important to identify adulteration of beverages, for example. And flavonoids are indispensable markers for chemotaxonomic purposes.

Various analytical methods exist for flavonoids. These range from TLC to CE. With the introduction of hyphenated HPLC techniques, the analytical potential has been dramatically extended. Gas chromatography (GC) is generally impractical, due to the low volatility of many flavonoid compounds and the necessity of preparing derivatives. However, Schmidtetet-al (Schmidt T.j,*et al*,1993) have reported the separation of flavones, flavonols, flavanones, and chalcones (with frequent substitution by methyl groups) by GC.

1.5 Isolation techniques for flavonoids

1.5.1 Column Chromatography

Column chromatography remains the single most useful technique for the isolation of large quantities of flavonoids from crude plant extracts. (Seikel M.K.,*et al*,1962,Mabry T.J.,*et al,*1970,Kirchner J.G.,1967,Stahl E.,1969). The advent of the relatively new chromatographic media, polyamide and Sephadex, has had a dramatic effect on the type and efficiency of separations achieved, and 'dry column' techniques also seem to offer prospects of markedly improved chromatographic separations. These changes appear to at least partly fulfil the requirement expressed by Harborne (1959b) for new adsorbents for column chromatography.(Harbone J.B.,*et al* ,1975).

Adsorbents commonly used for the separation of flavonoids include silica gel,kieselguhr, magnesol, cellulose, alumina, polyamide, sephadex and ion exchange resins. The adsorbents of choice have generally been silica gel, cellulose and polyamide.(Harbone J.B.,*et al* ,1975).

1.5.2 - Thin layer chromatography

Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids (Harborne J.B.,*et al*,1989), but now the method of choice for simple and inexpensive analytical runs is TLC. The advantages of this technique are well known: short separation times, amenability to detection reagents, and the possibility of running several samples at the same time. TLC is also ideally suited for the preliminary screening of plant extracts before HPLC analysis. An excellent general text on TLC methodology has been written by Jork (Jork *et al*, 1994) .A good discussion presented by Markham in one of the earlier volumes (Markham K.,1975):which describes TLC on silica gel and also two other supports, cellulose and polyamide.

Many different solvent systems have been employed for the separation of flavonoids using TLC. Some solvent systems cited by Markham are reproduced here because they still find application in the separation of flavonoids. Highly methylated or acetylated flavones and flavonols require non polar solvents such as chloroform-methanol (15:1).

Widely distributed flavonoid aglycones, such as apigenin,luteolin, and quercetin, can be separated in chloroform-methanol (96:4) and similar polarity solvents. One system that is of widespread application for flavonoid glycosides is ethyl acetate-formic acid-glacial acetic acid-water (100: 1l: 11: 26). By the addition of ethyl methyl ketone (ethyl acetate-ethyl methyl ketone -formic acid- glacial acetic acid-

water,50:30:7:3:10), rutin and vitexin-2"-O-rhamnoside can be separated (Wagner *et al*, 1996) .

Careful choice of solvent system also allows separation of flavonoid glucosides from their galactosidic analogues.This is especially important for the distinction of C-glucosides from C-galactosides. As an illustration, 8-C-glucosylapigenin (vitexin) can be separated from 8-C-galactosylapigenin with the solvent ethyl acetate-formic acid-water (50:4:10)(Budzianowski J.,1991).

TLC is a technique which has developed rapidly during the last decade and to a limited extent it has replaced paper chromatography in analytical and small scale separations of flavonoid. However, it is also complementary to paper chromatography in that it provides new media for the separation of flavonoids on a small scale, and permits the use of a wider variety of detecting reagent. As in column chromatography, the adsorbents of choice for the separation of flavonoids are silica, polyamide and cellulose. The mechanism of separation and the eluotropic series for each chromatographic medium as in column chromatography, but the solvent systems used often vary widely from those used for columns. In general, flavonoids are much more strongly held on thin-layers and as a result solvents of higher polarity are required for their elution. (Harbone *et al*, 1975).

1.5.2.1 Preparative scale TLC

TLC is essentially a technique for the separation of milligram quantities of material. However, it can be upgraded to handle up to a gram when layers of from 1-5 mm thick are used in conjunction with plates of up to 20 x 100 cm in size (kirchner 1967; Stahl *et al,* 1967).A more promising method is that of 'dry column' chromatography (Loev *et al*, 1965; Loev *et al*, 1970) which involves the use of columns packed with fine-powdered, dry adsorbent. Although this has not as yet been used for the separation of flavonoids, resolution with other compounds is claimed to be as good as TLC when low sample loadings e.g. (1:500) are used. (Turter, 1963)

1.5.2.2 Spray reagents and detecting methods

Apart from the anthocyanins and some of the more intensely coloured chalcones and aurones, flavonoids are not sufficiently coloured to be visible to the naked eye on a thin-layer plate; thus, some form of visualization is necessary for spot detection. In many cases this is achieved by viewing the plate in UV light (366 nm) either in the presence or absence of ammonia vapour.(Mabry T.J.,*et al,*1970),Detection under UV is often assisted by the use of layers which contain a UV-fluorescent indicator(e.g. silica gel GF2 54).(Harborne *et al*, 1975).

Flavonoids appear as dark spots against a. fluorescent green background. Another useful method of detection is brief exposure of the plate to iodine vapour which produces yellow-brown spots against

a white background with most flavonoids. These techniques all have the advantage that they are non-destructive. (Harborne J.B., et al,1975)

1.5.2.3 TLC Separations on silica gel

Flavones, Flavonols and Biflavonoids. Highly methylated or acetylated flavones and flavonols require relatively non-polar solvents for TLC on silica gel (SiO_2). Thus, flavones such as hymenoxin (5,7-diOH,6,8,3',4'-tetraOMe),scaposin(5,7,3'-triOH,6,8,4',5'tetraOMe), and dimethoxysudachitin (5,7,4'-triOH,6,8,-diOMe) have been chromatographed using chloroform-methanol (15 : 1) (Thomas M.B.,et al, 1968), and digicitrin (5,3'-diOH, 3,6,7,4',5'pentaOMe) and a number of related compounds were separated using benzene-ethyl acetate(3:1).(Meier W.et al ,1962) Flavonol polyacetates and polymethyl ethers have been successfully chromatographed by Egger (1969) using benzene-acetone (9:1) and toluene-acetone(19:1). Other highly methoxylated flavones and flavonols have been chromatographed with benzene-methanol-n-butylacetate (20:4:1) (Govindachari T.R.,1965), hexane-acetone-n-butanol (8:1:1and17:2:1), (Tatum J.H.,et al,1972) benzene-acetone(3:1) ,(9:1) ,(49:1),(92.8:7.2) (Egger K.,1969) and chloroform-ethyl acetate(1:1),the degree of solvent polarity depending largely upon the extent of methylation in the flavonoids. (Harborne *et al*, 1975).

More polar flavones and flavonols require more polar solvents.Thus apigenin , luteolin, galangin, kaempferol , quercetin, myricetin, isorhamnetin (3,5,7,4',tetra-OH,3'-OMe), datiscetin (3,5,7,2' tetraOH) and morin (3,5,7,2' ,4' -pentaOH) separate well in

toluene-chlorofom-acetone (8:5:7) (Egger K.,1969) , Rf values being 0.43, 0.28, 0.62, 0.39, 0.27, 0.13, 0.26, 0.36 and 0.06 respectively. Similar flavonoid mixtures were separated by Hörhammer et al. (1964) using benzene-pyridine-formic acid (36: 9: 5). We have found chloroform-methanol (96:4) useful for distinguishing flavones such as apigenin, chrysoeriol and luteolin, and Hillis and Isoi (1965) have had success with chloroform-acetic acid (9:1) and toluene-ethyl formate-formic acid (5:4:1) for the chromatography of C- methylflavones such as sideroxylin and eucalyptin (the 7- and 7,4'-methyl ethers of 6,8-di-C-methylapigenin respectively). Other solvents,such as benzene-dioxan-acetic acid (90:25:4),benzenepyridine- ammonia (80:20:1), (voirin B.,1972) and acetone-benzene (1:3),(Radhakrishnan P.V.,*et al,*1956) have also been used with flavones and flavonols. derivatives have been effectively separated using n-butanol- ethyl acetate-benzene-formic acid (1:1:1:1) saturated with paraformaldehyde on plates buffered with sodium acetate.(Conradie *et al*, 1968). Acid-washed SiO_2 was used for the preparative separation of the 3-O-glucoside and 3-O-rhamnoglucoside of cyaniding from blackcurrant juice with ethyl acetate-butanone-formic acid-water (6:3:1:1). (Morton, 1967).

Asen (1965) used 1 mm thick layers of silica gel-cellulose (2:1) and n-butanol-2N HC1 (1:1) to separate cyanidin (3, 5, 7, 3' ,4'-pentaOH) glycosides from their pelargonidin (3,5,7,4'-tetraOH) equivalents, and claimed that the separations achieved were better than with SiO_2 alone. Individual glycosides were further purified using a water-2N HC1-formic acid (8:4:1) (Harborne *et al*, 1975).

1.6- Spectroscopic techniques applied to flavonoids

Atypical analysis involving spectroscopic techniques embraces structural elucidation including determination of stereochemical attributes. However, it may also be aimed at tracing specific compounds and presenting quantitative aspects, or revealing color depiction.(Oyvind M.,*et al*,2006).

1.6.1 NMR spectroscopy

NMR spectroscopy is an extremely powerful analytical technique for the determination of flavonoid structures (Markhem K.R.,*et al*,1982, Anderen,*et al*,2003) but it is limited by poor sensitivity, slow throughput, and difficulties in analysis of mixtures. Recent developments have, however, made NMR arguably the most important tool for complete structure elucidation of flavonoids. Today, it is possible to make complete assignments of all proton and carbon signals in NMR spectra of most flavonoids isolated in the low milligram range. These assignments are based on chemical shifts (δ) and coupling constants (J) observed in 1D ^{1}H and ^{13}C NMR spectra combined with correlations observed as crosspeaks in homo- and heteronuclear 2D NMR experiments. Other nucleus like ^{17}O NMR spectroscopy has been used to study flavonoids only in a few cases. Natural abundance ^{17}O NMR spectra have been recorded for 11 methoxyflavones (Wallet *et al*, 1990) and ^{17}O NMR data for some 3-arylidenechromanones and flavanones have been discussed in terms of mesomeric and steric substituent interactions (Toth *et al*, 2011).^{17}O NMR spectroscopy has also been used to study the effect of sugar on anthocyanin degradation and water mobility in a roselle anthocyanin model system. (Tsai *et al*, 2004).

Some recent publications reporting flavonoid coupling constants include: NMR studies on flavones after the incorporation of ^{13}C at the carbonyl group, which allowed the measurement of two- and three-bond carbon-carbon coupling constants, ranging from 1.4 to 3.5 Hz, and the measurement of two-, three-, and four-bond carbon-hydrogen coupling constants, which ranged from 0.3 to 3.8 Hz; (Garcia-Martinez *et al*, 1994) complete assignment of the ^{1}H and ^{13}C NMR spectra of several flavones and their proton-proton and carbon-proton coupling constants, including the extreme seven-bond long-range coupling between H-7 and H-3 in 6-hydroxyflavone (0.52 Hz) and flavone (0.27Hz) (Aksnes *et al*, 1996)

Typical one-bond H-^{13}C coupling constants of monosaccharides in anthocyanins have been observed within magnitudes of 125 and 175 Hz. (Pedersen *et al*, 1995).

1.6.2 Mass spectrometry

Modern mass spectrometric techniques are very well suited for the analysis of flavonoids isolated from plants and foodstuffs and in their in vivo metabolite forms. Progress during the last two decades has made MS the most sensitive method for molecular analysis of flavonoids. MS has the potential to yield information on the exact molecular mass, as well as on the structure and quantity of compounds with the nature and within the mass range of flavonoids. Furthermore, due to the high power of mass separation, very good selectivities can also be obtained.

The purpose of the MS techniques is to detect charged molecular ions and fragments separated according to their molecular masses. Most flavonoid glycosides are polar, nonvolatile, and often thermally labile.

Conventional MS ionization methods like electron impact (EI) and chemical ionization (CI) have not been suitable for MS analyses of these compounds because they require the flavonoid to be in the gas phase for ionization. To increase volatility, derivatization of the flavonoids may be performed. However, derivatization often leads to difficulties with respect to interpretation of the fragmentation patterns. Analysis of flavonoid glycosides without derivatization became possible with the introduction of desorption ionization techniques. Field desorption, which was the first technique employed for the direct analysis of polar flavonoid glycosides, has provided molecular mass data and little structural information.(Nakanishi T.,*et al*,1985)

1.6.3 Vibrational spectroscopy (IR and Raman)

Two different types of spectroscopic techniques are most frequently used to view the fundamental modes of molecular vibrations,namely mid-IR spectroscopy and Raman spectroscopy. (Mirabella *et al*, 1998).The first method measures the absorption, transmission, or reflection of IR radiation with wavelengths in the range of 2.5 to 25 μm. The Raman method irradiates the sample with radiation of much shorter wavelengths and measures the fraction of scattered radiation for which the energy of the photon has changed.

The vibrational spectra may serve as fingerprints of structure, composition, interactions, and dynamics. The reciprocal of wavelength, wavenumber (cm^{-1}), is commonly used to characterize the energy in the field of vibrational spectroscopy.

Systematic vibrational spectroscopy studies on flavonoids have occurred since the early1950s, and most of them have been limited to a discussion on the hydroxyl and carbonyl absorption frequencies. (Briggs L.,*et al*,1962). However with the technical advances of the last two decades,the Application of vibrational spectroscopy has become much more relevant in the field of flavonoid analysis (Cornard *et al,* 1994; Edelmann *et al,* 2002).The implementation of FTIR spectroscopy has significantly enhanced the sensitivity, and Raman spectroscopy has benefited from the availability of holographic notch filters, which efficiently suppress the strong signal from elastically scattered (Rayleigh) radiation while maintaining the Raman- shifted intensity with minimal attenuation. Furthermore, high- powered NIR semiconductor lasers and sensitive charge coupled devices have replaced inconvenient gas lasers and light– detection technologies. Ordinary Raman spectroscopy has drawbacks in that it requires high compound concentrations, and the recorded spectrum will correspond to all molecules present in the sample. In resonance Raman spectroscopy, this is over comed through the use of laser light with frequency corresponding to the absorption maximum of the compound to be characterized. Finally, the increase in the computing power of standard computers has facilitated more sophisticated data evaluation of both in IR and Raman spectra.

1.6.4 Ultraviolet-visible absorption spectroscopy

The application of standardized UV (or UV-Vis) spectroscopy has for years been used in analysis of flavonoids. These polyphenolic compounds reveal two characteristic UV absorption bands with maxima in the 240 to 285 and 300 to 550 nm ranges. The various flavonoid classes can be recognized, by their UV spectra. the combination of HPLC equipped with a UV-Vis DAD (diode array detection) has for the two last decades been the standard method for the detection of flavonoids in mixtures. (Oyvind et al, 2006).

The use of UV shift reagents such as $A1C1_3$ (5% in methanol)-HCl (20% aqueous), NaOMe (2.5% in methanol), and NaOAc (3 mg)-H_3BO_3 has proven to be very useful as guidelines for substitution patterns of many flavonoids; however, the use of these reagents has mainly been applicable for purified flavonoids (Markham K.R.,1982),(Mabry,. By adding suitably modified shift reagents to the eluate leaving a HPLC column, similar shifts of the UV absorption maxima of flavonoids in the elute have been induced.[63]

Detection can be performed at the wavelength maximum of the compound in question. These are typically to be found[64] at 270 and 330 to 365 nm for flavones and flavonols, at 290 nm for flavones at 236 or 260 nm for isoflavones , at 340 to 360 nm for chalcones, at 280 nm for dihydrochalcones, at 502 or 520 nm for anthocyanin's, and at 210 or 280 nm for catechins.

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

1.7. Flavonoids as nutraceuticals

"Nutracenutical" is a term coined in 1979 by Stephen Defelice .It is defined " as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." Nutrceuticals may range from isolated nutrents ,dietrary supplements , and diets to genetically engineered ''designer'' food, herbal products , and processed products such as cereals , soups, and beverages.

Nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease. The increasing interest in nutraceuticals reflects the fact that consumers hear about epidemiological studies indicating that a specific diet or component of the diet is associated with a lower risk for a certain disease.

The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal cheaters. They also have long been recognized to possess anti- inflammatory ,antiallergic ,hepatoprotective ,antithrombotic, antivirl , and carcinogenic activities.(Dillard C.J.,*et al*,2000).

1.7.1 Antioxidant activity

The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species (ROS). Body cells and tissues are continuously threatened by the damage caused by free radicals and ROS which are produced during normal oxygen metabolism or are induced by exogenous damage .(De Groot H.,1994,Grace PA.,1994).

Free radicals and ROS have been implicated in a large number of human diseases. (Wegner T.,*et al*,1999,Ares JJ,1998).Quercetin, kaempferol , morin, myricetin and rutin, by acting as antioxidants, exhibited beneficial effects such as anti-inflammatory, antiallergic, antiviral, as well as anticancer activity. They have also been suggested to play- a protective role in liver diseases, cataracts, and cardiovascular diseases. Quercetin and silybin, acting as free radical scavengers, were shown to exert a protective effect in liver reperfusion ischemic tissue damage (Hill well B.,1994,Fraga C.G.,et al,1987).The scavenging activity of flavonoids has been reported to be in the order: Myrcetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin >catechin >

5,7-dihydroxy-3',4',5'-trimethoxyflavone > robinin > kaempferol > flavone. (Ratty A.K.,1988).

1.7.2 Antimicrobial activity

Flavonoids and esters of phenolic acids have been investigated for their antibacterial and antiviral activities.

Antibacterial activity has been displayed by a number of flavonoids. Quercetin has been reported to completely inhibit the growth of Staphylococcus aureus. (Havsteen B.,1983).

1.7.3 Antiviral activity

Naturally occurring flavonoids with antiviral activity have been recognized since the 1940s but only recently have attempts been made to make synthetic modifications of natural compounds to improve antiviral activity. Quercetin, morin, rutin, dihydroquercetin (taxifolin), apigenin, catechin, and hesperidine have been reported to possess antiviral activity against some of the11 types of viruses. (Selway JWT.,1986).

1.7.4 Antiulcer activity

Some flavonoids exert significant anti-inflammatory activity in the animal model of both acute and chronic inflammation when given orally or topically (Lee *et al*, 1993; Hang *et al*, 2002), Hesperidin, a citrus flavonoid ,possesses significant anti-inflammatory and analgesic effects.(Shahid et al, 1998).

Recently apigenin, luteolin and quercetin have been reported to exhibit anti-inflammatory activity(Farmica et al, 1995).

1-8 *Camellia Sinensis* (Green Tea)

Seed – bearing fruit of *Camellia sinensis* **Leaves of *Camellia sinensis***

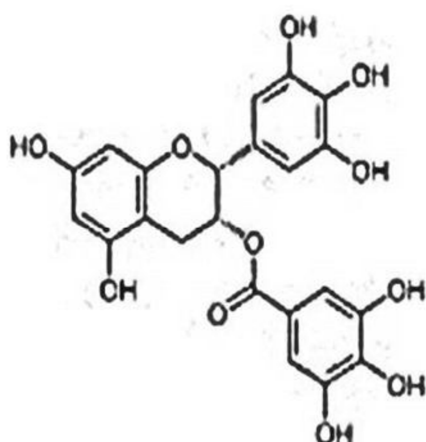
1.8.1 General Information

The Green tea is obtained from the tea plant *Camellia sinensis* belongs to the family Theaceae. Tea is the most consumed drink in the world after water. Green tea is a 'non-fermented' tea and contains more catechins than black tea or oolong tea. Catechins are in vitro and in vivo strong antioxidants. In addition, its content of certain minerals and vitamins increases the antioxidant potential of this type of tea. Presently, it is cultivated in at least 30 countries around the world. Tea beverage is an infusion of the dried leaves of *Camellia sinesis*. It is a widely used medicinal plant by the trials throughout India, China and popular in various indigenous system of medicine.

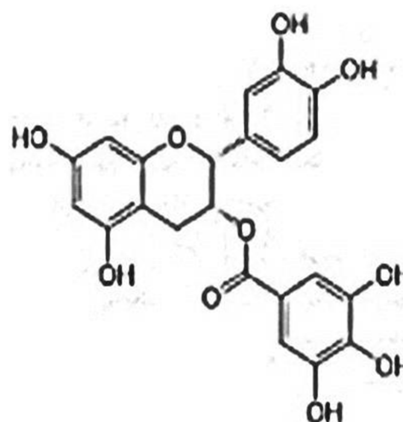
1.8.2 Chemical Constituents

Tea is reported to contain nearly 4000 bioactive compounds of which one third is contributed by polyphenols. Other compounds are

alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll. Volatile organic compounds (chemicals that readily produce vapors and contribute to the odor of tea), fluoride, aluminum, minerals and trace elements Polyphenols found in tea are mostly flavonoids. The polyphenols, a large group of plant chemicals that includes the catechins, are thought to be responsible for the health benefits that have traditionally been attributed to tea, especially green tea.Major catechins are (-)-epicatechin gallate (ECG).(-)-epicatechin (EC),(-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) (Figure1). The most active and abundant catechin in green tea is epigallocatechin-3-gaIlate (EGCG). Black tea contains much lower concentrations of these catechins than green tea. Oolong tea contains a mixture of simple polyphenols, such as catechins and complex polyphenols.Black; Green and Oolong tea are all extremely good sources of vitamin C.



Epigallocatechin gallate (EGCG)



Epicatechin gallate (ECG)

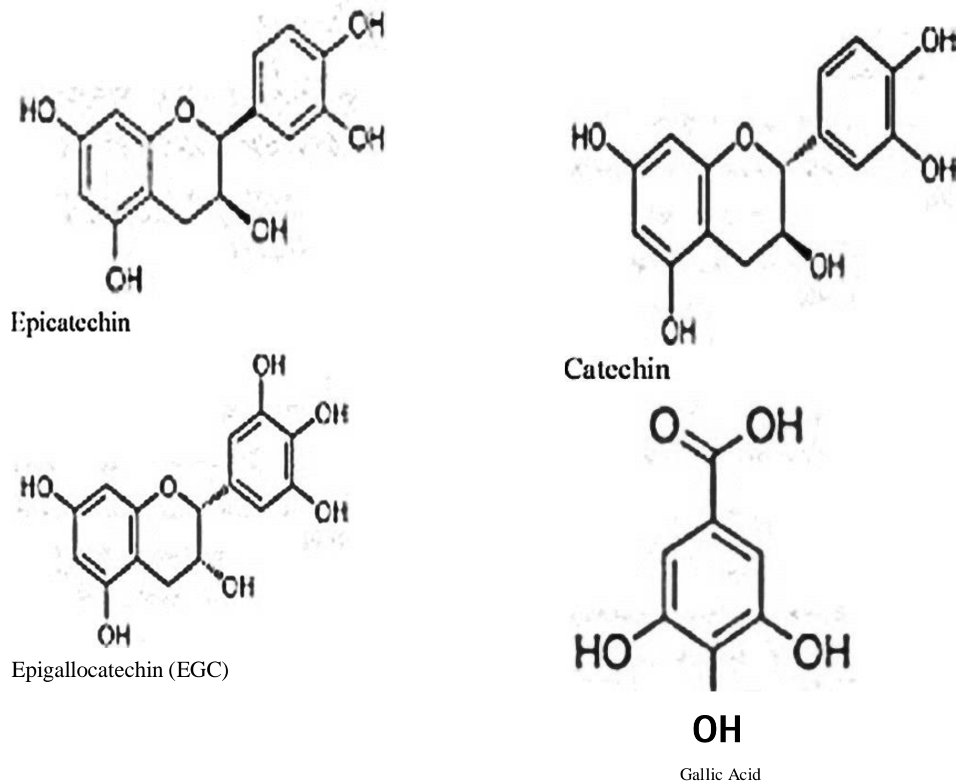


Figure (1) Basic structures of different green tea polyphenols

1.8.3 Medicinal Properties and Pharmacology

Anti-Aging Activity

According to the free radical theory of aging, increased free radical generation and oxidative stress are the basis for phenotypic changes that lead to age-associated functional deterioration and neuro-degeneration. Several age associated diseases such as cancer, Parkinson's, disease, Alzheimer's, disease, cardiovascular diseases, and diabetes have their etiologies linked to changes in oxidant anti-oxidant balances and free radical damage "However, Kitani *et al* report that green tea as the sole source of liquid did not significantly increase life span in mice, compared to controls.

However, green tea did protect against ethanol-induced oxidative stress in aged mice and prevented serum lipids and protein from oxidative damage, produced by ethanol and enhanced by aging.

Neurodegenerative Diseases

Anti-Alzheimer Activity

Although there is no epidemiological evidence in human studies of the benefit of green tea for Alzheimer's disease, several studies in animal and cell culture models suggest that EGCG from green tea may affect several potential targets associated with Alzheimer's disease progression. EGCG protects against beta-amyloid induced neurotoxicity in cultured hippocampal neurons, an effect attributed to its antioxidant properties. In addition, EGCG regulates the processing of APP, through PKC activation, to the nonamyloidogenic soluble APP (sAPP), thus preventing the formation of the neurotoxic beta-amyloid. EGCG and other green tea catechins have also been shown to inhibit the beta secretase enzyme (BACE1) that is responsible for processing sAPP to beta-amyloid, thus having a potentially synergistic inhibitory effect on the production of beta-amyloid. (Parmer et al, 2012).

1-9 Aims of this study

This study was aimed to:

- Prepare extracts from leaves of *Camellia sinensis* and phytochemical screening for flavonoids, tannins, steroiods, alkaloids and glycosides.
- Separtation of the main flavonoid if present by TLC chromatography.
- Conducting U.V and IR studies on the isolated compound.
- Proposing partial structure for the isolated compound.

2-Materials and Methods

2.1 Materials

2.1.1 Plant Material

The leaves of *Camellia sinensis* were purchased from the local market -Khartoum .The plant was kindly authenticated by Aromatic and Medicinal plant institute- Khartoum – Sudan.

2.1.2 Equipments

Analytical grade reagents were used .The UV-Visible spectra were run on a Shimadzu UV-2401 PC UV-Visible spectrophotometer.

The IR spectra were run on a Perkin-Elmer 1310 Infrared Spectrophotometer.

2.2 Methods

2.2.1 Preparation of test reagents for phytochemical screening.

2.2.1.1 Flavonoid test reagents

(i) Aluminum chloride solution

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

(ii) Potassium hydroxide solution

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

(iii) Ferric chloride solution

(0.5 g) of ferric chloride was dissolved in (100ml)95% ethanol.

2.2.1.2 Alkaloid test reagents

(i) Wagner reagent

(5g) iodine and (10g) potassium iodide was dissolved in (100g) distilled water.

2.2.2 Preparation of plant extract for phytochemical screening

(100g) of powdered air-dried leaves of *Camellia sinensis* were extracted with (200 ml) 95% ethanol for three days .the cooled solution was filtered and it's volume was adjusted to (100ml) by addition of enough 95% ethanol. This prepared extract (PE) was used for the following tests. (Arunkumar, 2009)

2.2.3 Phytochemical screening

The prepared extract was screened for major secondary constituents which are steroids, flavonoids, alkaloids, tannins and glycosides.

i) Test for steroids

(0.1g) was shaken with chloroform in a test tube , few drops of acetic anhydride was added to the test tube and boiled in water bath and rapidly cooled in iced water. Concentrated H_2SO_4 (2ml) was added alongside of the test tube. Development of brown ring at the junction of two layers and turning the upper layer to green indicates the presence of steroids. (Erum -Iqbal, et al,2015).

ii) Test for alkaloids

0.2 g of the crude extract was stirred with 1% HCl (10ml) On a water bath for 10 minutes and filtered. To the cooled solution, few drops of wagner reagent were added to the cooled filtrate. Formation of precipitate was observed indicates the presence of alkaloids. (Erum Iqbal, et al, 2015).

Test for flavonoids

(2 g) of the powdered leaves of camellia sinensis was completely decanted with acetone. The residue was extracted with warm water after evaporating the acetone on a water bath.the mixture was then filtered while hot, the filtreate was allowed to cool and used for the follwing tests: (Yusuf,et al,2014).

(i) To (3ml) of the filtrate few drops of methanolic aluminum chloride were added .A dark yellow color soon developed.(Edeoga *et al* , 2005)

(ii) To (3ml) of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed. (Yusuf et al, 2014).

(iii) To (3ml) of filtrate few drops of ferric chloride solution were added. A blue colouration was observed (Yusuf *et al*, 2014).

(iv) Test for tannins

(5ml) of the prepared extract was separately stirred with distilled water (10 ml) and then filtered. A few drops of 5% ferric chloride were then added a blue color was formed indicating the presence of tannins. (Erum Iqbal et al, 2015).

(v) **Test for glycosides**

(20ml) of the prepared extract was treated with ferric chloride solution and immersed in boiling water for about 5 minutes . the mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution .No formation of rose- pink color in the ammonical layer indicates the absence of glycosides(Prashant, et al , 2011).

2.2.4 Extraction of flavonoids from the leaves of *Camellia sinensis*

powered air-dries leaves (1kg) of *Camellia siensis* were extracted with 95% ethanol at ambient temperature for three days with occasional shaking and then filtered off. The solvent was removed giving a crude product (dark green solid) (Arunkumar, 2009).

2.2.5 Thin layer chromatography of the crude products (Isolation of flavonoid)

Silica gel and water 1:2 (w/w) were mixed to form slurry which was spread over clean glass plates. These plates were employed for chromatographic fractionation without activation.

Part of the crude product (0.1g) was dissolved in (95%) ethanol (2) ml and applied as concentrated spots on the TLC plates, the plates were developed with different solvent systems. However, the solvent that achieved the optimum fractionation was chloroform: methanol 3:2 (v: v).

Only one major flavonoid was observed when the plates were viewed under UV-light.

This flavonoid was also detected by spraying with ferric chloride solution. (1%).(Yusuf *et al*, 2014).

2.2.6 Preparative thin layer chromatography

(0.5g) of the crude product was dissolved in the minimum amount of (95%) ethanol and applied on (20×20 cm) silica gel plates as narrow strips.

The plates were developed with chromatograms were located under light.

The sole major band was scratched and elutriated from silica gel with absolute methanol. After filtration, the solvent was removed to leave a solid (the pure product of *camellia sinensis*)

The purity of this product was checked by TLC experiments using two different solvent systems Acetic acid : water (10:90, 20:80, 30:70, 40:60, 50:50; v:v) and chloroform: methanol (1:4, 2:3, 3:2, 4:1; v:v).

2.2.7 UV shift reagents

Stock solution of sodium methoxide and aluminum chloride were prrepared.

(i) Sodium methoxide

Freshly cut metallic sodium (2.5g) was added cautiously in small portions to dry spectroscopic methanol (100 ml). The solution was stored in a glass container with tightly fitting plastic stopper.

(ii) Aluminum chloride stock solution

(5g) of fresh anhydrous aluminum chloride were added cautiously to spectroscopic methanol (100ml).

2.2.8 The UV spectra of the compound in presence of sodium methoxide

Three drops of sodium methoxide were added to a solution of the compound in methanol (2ml) and the UV spectrum was immediately recorded.

2.2.9 The UV Spectra of the compound in presence of aluminum chloride

Six drops of the stock solution of aluminum chloride were added to a solution of the compound in methanol (2ml) and the UV spectrum was recorded immediately.

2.2.10 The UV spectra of the compound in presence of sodium acetate

Excess coarsely powdered anhydrous NaOAc was added with shaking to a cuvette containing (2ml) of the solution of the compound in methanol and the UV spectrum was recorded after two minutes.

3- Results and Discussion

3.1 Extraction and isolation of flavonoids

Powdered air- dried leaves of *Camellia 39 inensis* were extracted with 95% Ethanol at ambient tempracture for three days, evaporation of the solvent gave a crude product.

3.2 Phytochemical screening

The crude product obtained was subjected to phytochemical screening for steroids, alkaloids, flavonoids, tannins and glycosides and the results are depicted in table (3.1).

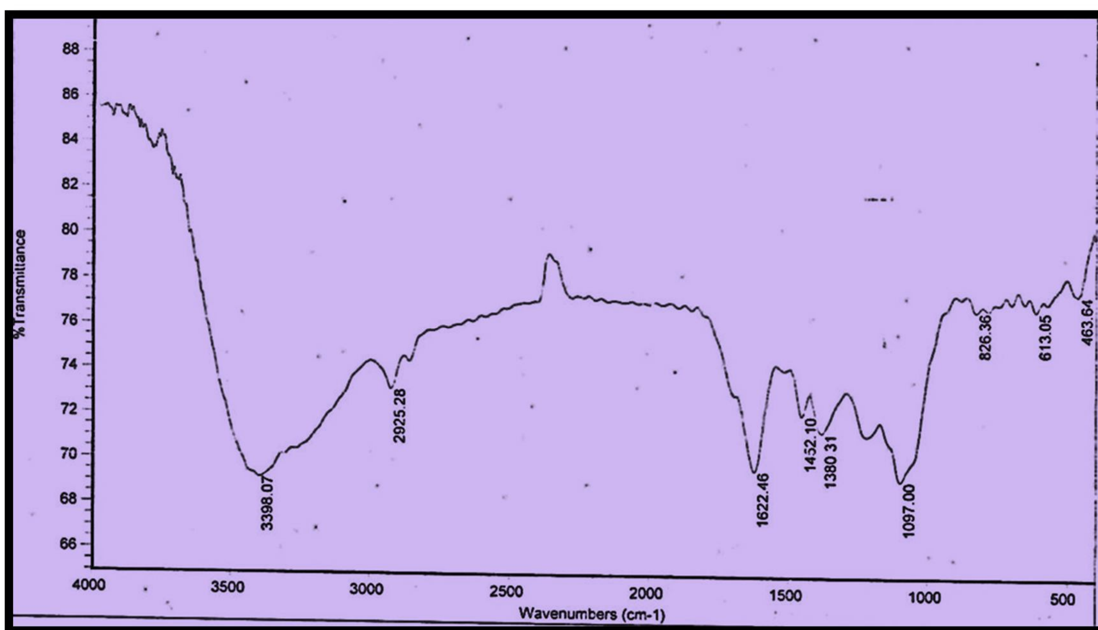
Table (3-1) Qualitative analysis of phytochemicals of *Camellia sinensis*

Compounds	Result
Flavonoids	+Ve
Alkaloids	+Ve
Steroids	+Ve
Tannins	+Ve
Glycosides	-Ve

The crude extract was applied on silica gel plates, and different solvent system were attempted and the best solvent system was selected, the plates were developed by **chloroform : methanol** (3:2,v:v), and major component flavonoid was isolated in the pure state .

3.3 Spectral data of the main compound

The IR (KBr disc) spectrum of the main compound figure (3.1).

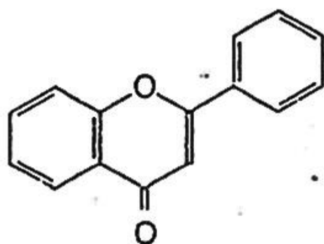


(fig.3.1) IR spectrum of the main compound

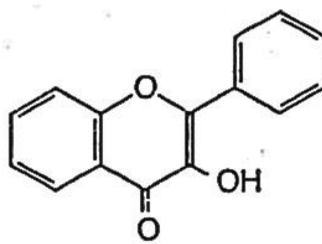
Table (3-2) IR data of the compound

Functional group	**ν cm^{-1}**
O-H (str)	3398.07 cm^{-1} (s-br)
C-H (str)	2925.28 cm^{-1} (s)
C=O ($\propto, B - undsaturated\ carbonyl\ group$)	1622.46 cm^{-1} (s)
C=C (Ar)	1452.10 cm^{-1} (s)
C-O,phenolic (str)	1380.31 cm^{-1} (m)
C-O,Ether (str)	1097.00 cm^{-1} (m)
C-H (bend,Ar)	826.36,613.05 cm^{-1} (m)

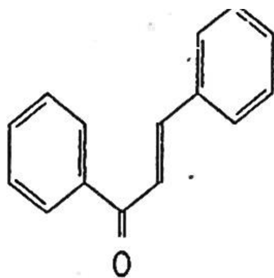
The compound cannot be an anthocyanin, catechin or flavan, since the IR spectrum gave a carbonyl stretching at 1622.46cm^{-1} IT could be: a flavone, flavonol ,chalcone, aurone, isoflavone, flavanone, dihydrochalcone or dihydroflavonol.



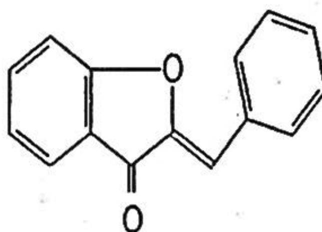
Flavone



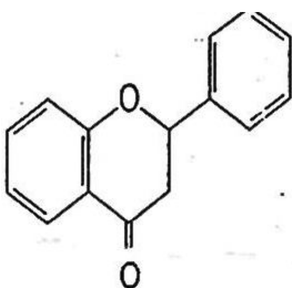
Flavonol



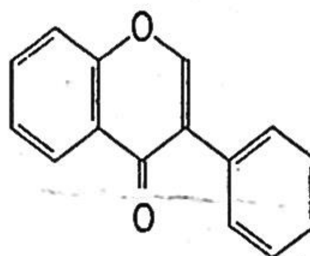
Chalcone



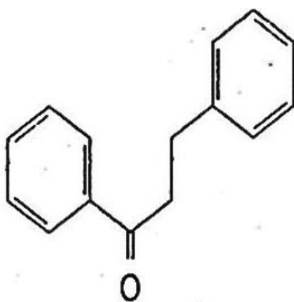
Aurone



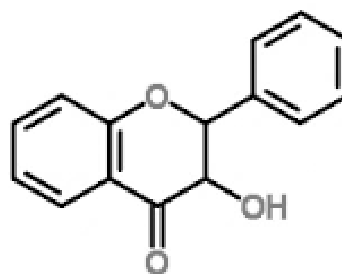
Flavanone



Isoflavone

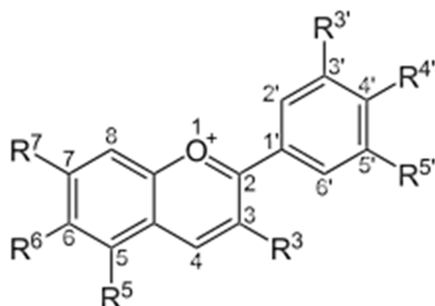


Dihydrochalocone

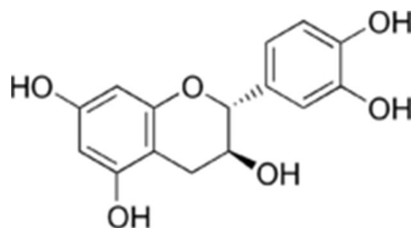


Dihydroflavonol

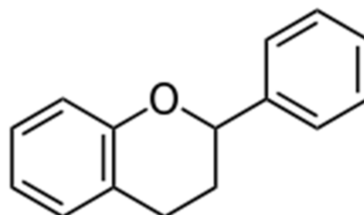
The presence of a carbonyl function in the compound indicates absence of anthocyanins, catechins and flavan . These classes are characterized by absence of a carbonyl function.



Anthocyanin



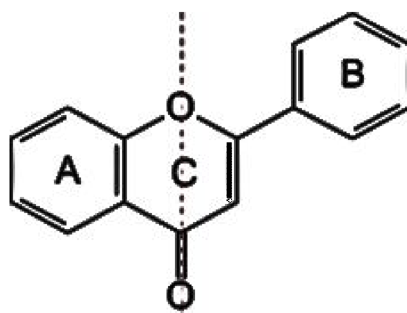
Catechin



Flavan

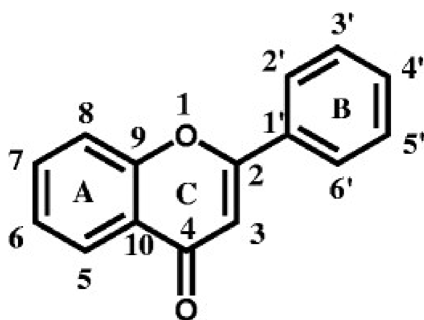
In their UV spectra, most flavonoids exibit two major bands in the region 210-400 nm .These two peaks are commonly refered to as band I (usually 300-400 nm) and band II (usually 210-290 nm)

Band I is considered to be associated with absorption due to the B-ring cinnamoyl system, and band II with a bsortion involving the A-ring benzoyl system.

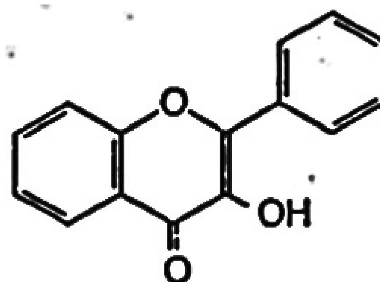


Band II 210-290 nm (Benzoyl system)

Band I 300-400 nm (cinnamoyl system)



Flavone skeleton



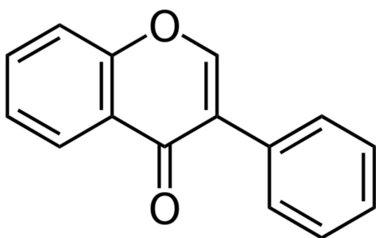
Flavonol skeleton

Flavones , Flavones, Aarons and chalcones give similar UV spectra as a result of conjugation between the A- and B-rings .Thus they reveal both band I and II. The UV absorption of some classes of flavonoids is depicted in table (3.3).

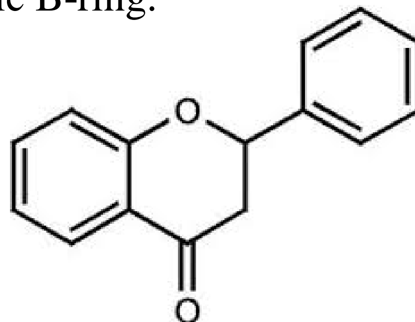
Table (3.3): The U.V absorption of flavones, flavonols, chalcones and aurone .

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

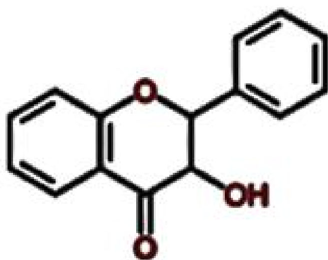
Other flavonoids like isoflavones, flavanones , dihydroflavols ,and dihydrochalcones all give only band II due to lack of conjugation between the carbonyl function and the aromatic B-ring.



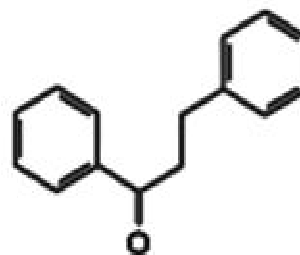
Isoflavone



Flavanone



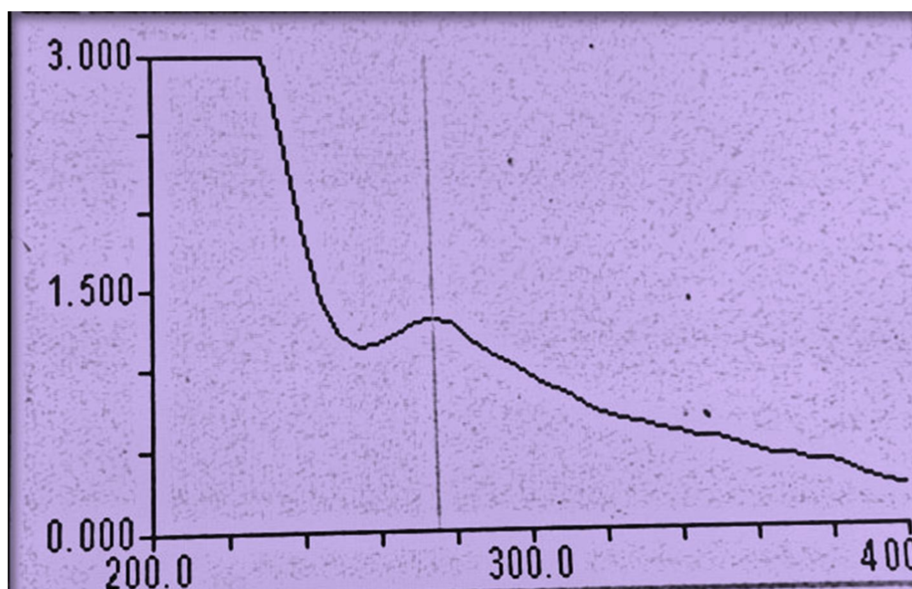
Dihydroflavonol



Dihydrochalcone

In the UV, the compound absorbs fig (3-2) at λmax (MeOH) 275 nm. This absorption indicates loss of conjugation between the carbonyl function and the aromatic B ring ,which is characteristic of :-

(i) Isolflavones.
(ii) Flavanones.
(iii) Dihydroflavonols.
(iv) Dihydrochalcones .



(fig 3.2) the U.V Spectrum of the compound .

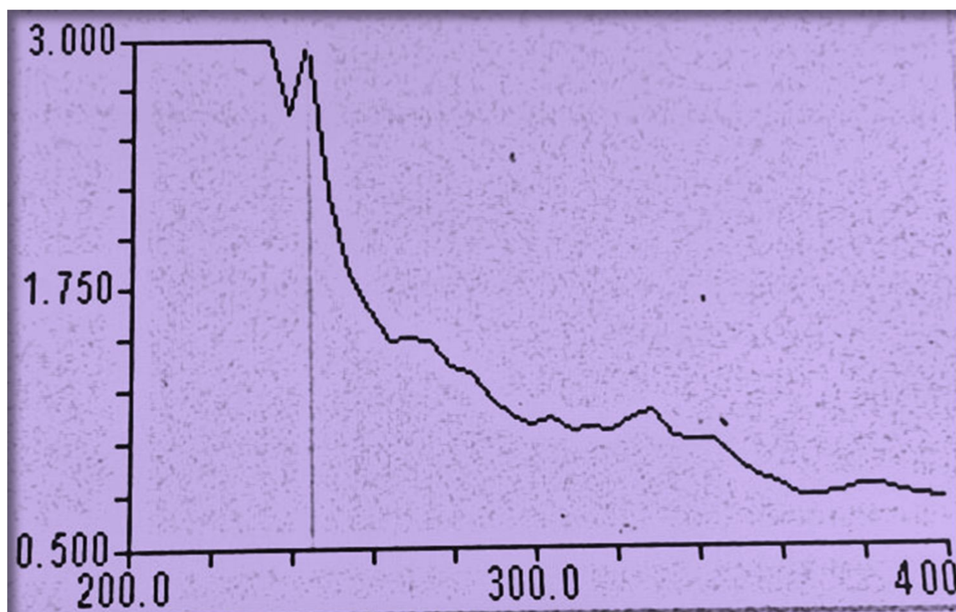
Isoflanones give shoulder in the range 300-340 nm. This was not found in U.V spectrum of the compound . The hydroxylate pattern was studied and very significant structural feature has also been obtained by U.V shift reagents such as sodium methoxide , Sodium acetate, aluminum chloride .

These reagents produce shifts in the U.V absorption maxima in accordance with the location of the various functional groups in the flavonoid nucleus.

Sodium methoxide is strong base and ionizes to some extent all hydroxyl groups on the flavonoid nucleus. However,use has been made of the effect of sodium methoxide on the U.V spectra of flavonoids for detection of free 3- and /or 4̀ -hydroxyl function .

When sodium methoxide was added to a methanolic solation of the compound gave 30 nm bathochromic shift (fig3-3) was observed with decrease in the intensity indicating the presence of a 3-OH function.

So this flavonoid is dihydroflavonol.



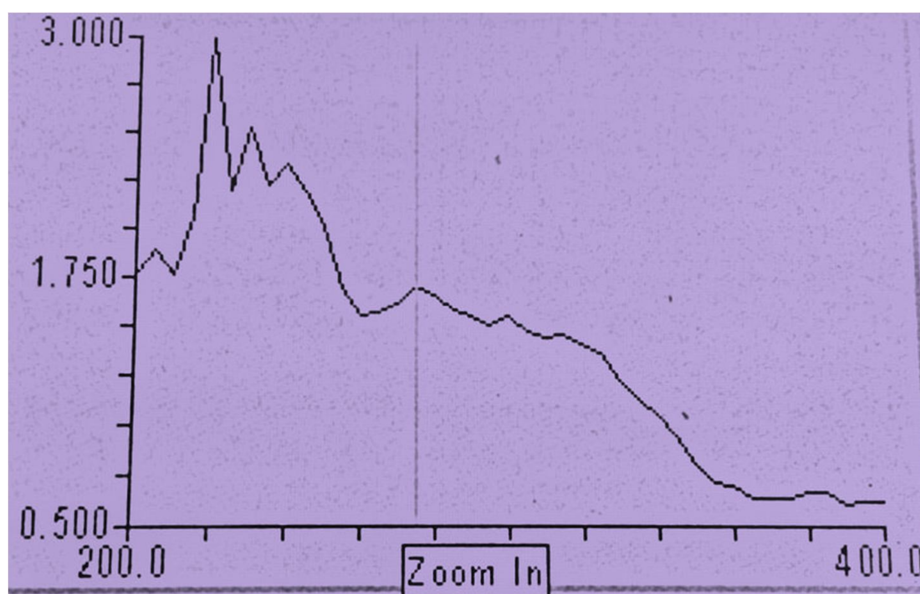
(fig3-3) sodium methoxide spectrum of the compound

Sodium acetate is a weaker base than NaOMe, so it ionizes only the more acidic hydroxyl groups in flavonoid nucleus. i.e, the 7-OH function.

The ionization of the 7-hydroxy group mainaly affects band II.

Particularly NaOAc is useful diagnostic reagent for the specific detection of 7-hydroxyl group.

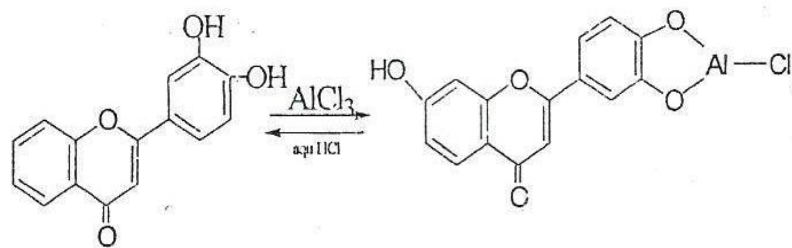
The sodium acetate spectrum of the compound (fig 3.4) gave 25 nm bathochromic shift suggesting a free 7-OH function.

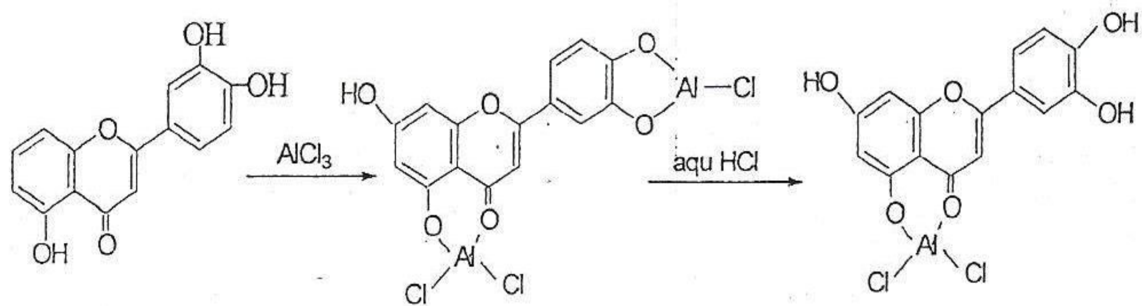


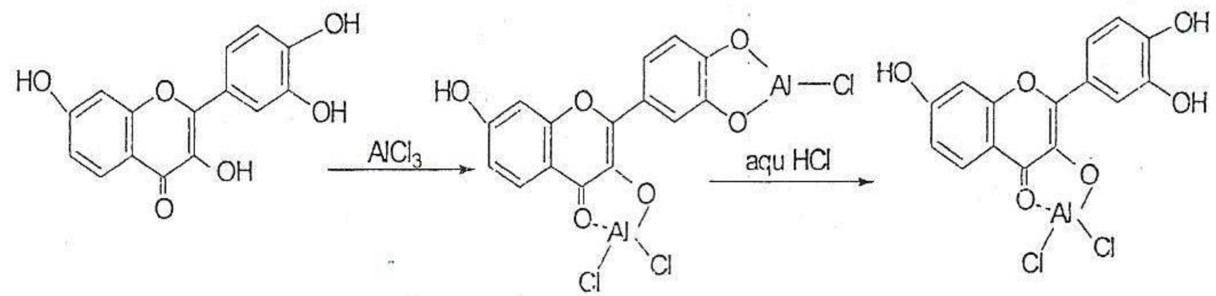
(fig3-4) sodium acetate spectrum of the compound

With aluminium chloride flavones and flavonols which contain hydroxyl groups at C-3 or C-5 form acid-stable complexes. In addition, aluminum chloride forms acid-labile complexes with flavonoids which contain Ortho-dihydroxyl system (catechol system). The complexes formed between $AlCl_3$ and the B-ring Ortho-dihydroxlyl groups,with few exceptions decompose, in the presence of acid. In contrast the $Alcl_3$ complex between the 3-or 5- hydroxyl group is stable in the presence of acid (Horhammer L.,*et al,* 1952,Jurd L.,*et al* , 1956).

Such acid stable and acid – labile complexes are shown below.

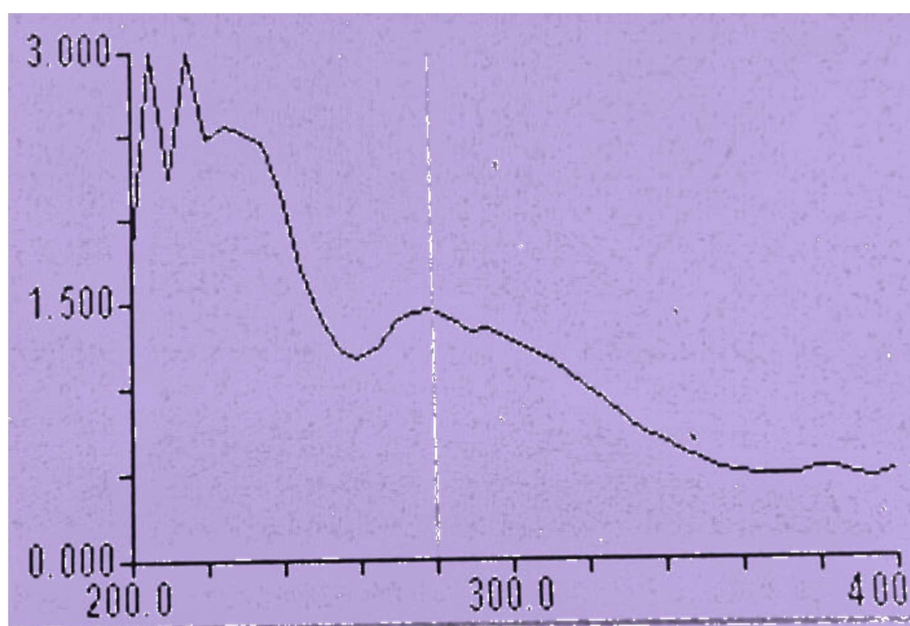

OH
OH
AlCl3
aqu HCl
HO
O
Al
Cl


OH
OH
AlCl3
HO
Al
Cl
aqu HCl


HO
OH
AlCl3
Al
Cl
aqu HCl

So the presence of an ortho-dihydroxy groups in the B-ring of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid the presence of $AlCl_3$ with that obtained in $AlCl_3$ / HCl.

When $AlCl_3$ was added to methanolic solution of the compound, 20 nm bathochromic shift was observed (fig 3-5).

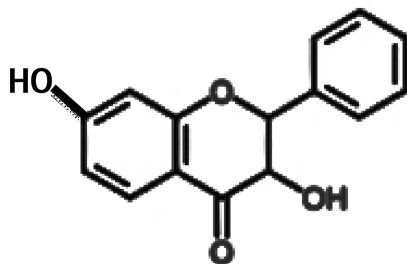


(fig 3-5) :Aluminium chloride spectrum of the compound .

This indicates a 3- or 5- OH or a catechol system.

When few drops of HCl were added to $AlCl_3$ Complex of the compound, the UV spectrum was stable and didn't change indicating the absence of catechol system. This support the previous findings with sodium methoxide.

On the basis of the above results the following structure was proposed for the compound which is dihydroflavonol hydroxylated at the 3- and 7- positions:



Conclusion

The aims of this work are extraction , isolation and spectral characterization of the flavonoid of the medicinally importance species :*Camellia sinensis* plant .

Recommendations

The structure of the isolated flavonoid may further be elucidated by appling^{1}H-NMR,^{13}C-NMR ,2D ^{1}H-NMR and MC spectroscopies, so they are strongly recommended to give additional evidence in favor of the proposed structure.

The isolated compound may be subjected to a pharmacological screening, since it is extensively used in folk medicine.

References

1. Aksnes,D.W.,Standnes,A.,and Andersen, Ø .M.,Complete assignment of the ^{1}H and ^{13}C NMR spectra of flavone and its A-ring hydroxyl derivatives, *Magn. Reson. Chem.,* **34**, 820 (1996).
2. Andersen,Ø.M.and Fossen,T.,Characterization of anthocyanins by NMR, in Current Protocols in Food Analytical Chemistry , Wrostad, R.E.,Ed., John Wiley , New York,1(2003).
3. Ares JJ, Outt PE. Gastroprotective agents for the prevention of NSAID induced gastropathy. *Curr Pharm Des*; **4**:7-36 (1998).
4. Arunckumar S.,M.Muthuselvam,(2009).Analysis of Phytochemical constituents and Antimicrobial Activities of Aloe veral L.Agaist clinical pathogems.*world Journal of Agricultural Sciences* 5 (**5**): 572-576.
5. Bohm,B.A.”Introduction to Flavonoids” Harwood Academic Publishers, Amsterdam, (1998).
6. Budzianowski,J., Separation of flavonoid glucosides from their galactosidic analogues by thin layer chromatography,*J. Chromatogr.*,540.469.(1991).
7. Briggs,L.and Colebrook, L.,Infrared spectra of flavanones and flavones. Carbonyl and hydroxyl stretching and CH out-of-plane bending absorption, *Spectrochim. Acta*, **18**,939 (1962).

8. Catherine-A-Rice Euans and Lester “Flavonoids in Health and disease”, 2nd Ed., p. **43** (2003).
9. Conradie,J.D. and Neethling , L. P., I. Chrornat, **34,**419 (1968).
10. Cornard, J.P., Barbillat , J., and Merlin, J.C., Performance of a versatile multichannel microprobein Raman, fluorescence, and absorption measurements, Microbeam Anal., **3**,13 (1994).
11. De Groot H. Reactive oxygen species in tissue injury. Hepatogastroenterology; **41**:328-332,(1994).
12. De Groot, H.,Rauen,V.,fundam.Clin Pharmacol.,**12**,249 (1998).
13. Defelice S. L., Nutraceuticals: Opportunities in an Emerging Market, *Scrip Mag.*; **9**, (1992).
14. Dewik,P.M.,”Medical Natural Products”, John Willey and sons, West Sussex ,England (1998).
15. DiCarlo, G., Mascolo, N., Izzo, A. A., Capasso, F., *Life Sci.* **65,**337 (1999).
16. Dillard, C. J., German, J. B. Phytochemicals : nutraceuticals and human health, *J Sci. Food Agric* ; **80**:1744-1756, (2000).
17. Dreyer, D. L. and Bertelli, D. J., Tetrahedron, **23**,4607 (1967)
18. Ducrey, B. et al., phytochemistry, **38**,129 (1995).
19. Edelmann ,A. and Lendl, B., Toward the optical tongue: flow-through sensing of tannin- protein in teractions based on FTIR spectroscopy, *J. Am. Chem. Soc.,***124,** 14741 (2002).

20. Edeogal H.O.,D.E. Okwau,B.O. Mbaebie,(2005).*phytochemical constitugents of some Nigerian medicinal plants* .African Journal of Biotechnology Vol.**4**(**7**) 685-688.

21. Egger, K., “In Thin-layer Chromatography-A Laboratory Handbook’’,(ed. E. Stahl), pp. 687. Springer-Verlag,Berlin (1969).

22. Erich ,G., “The Science of flavonoids” Springer, USA(2005).

23. Erum Igbal , Kamariah Abu Salim, Lina B. L. Lim , (2015) . phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of Goniothalmus velutinus (Airyshaw) from Brunei Darussalam. *Journal of king Saud University. Science* (**27**),224-232.

24. Farmica JV, Regelson W. Review of the biology of quercetin and related bioflavonoids.Fd Chem Toxic; **33**(**12**): 1061-1080 (1995).

25. Fraga CG, Mactino US, Ferraro GE, Coussio JF, Boveris A. Flavonoids as antioxidants evaluated by in vitro and in situ liver chemiluminescence . Biochem Med Metabol Biol;**36:** 717-720. (1987).

26. Garcia-Martinez,C. et al., Further nuclear magnetic resonance studies of flavone, Spectroscopy,**12**,85(1994).

27. Govindachari, T. R., Parthasarathy,P.C.,Pai,B.R. and Subramaniam, P. S., Tetrahedron, **21**, 3237 (1965).

28. Grace P.A. Ischaemia-reperfusion injury. Br J Surg);81: 637-647,(1994).

29. Haborne,J,B.,Mabry,T.J.,Marby,H. "The flavonoids", Springer-Science, USA (1975).

30. Hang T, Jin JB, Cho S, Cyang JC. Evaluation of anti-inflammatory effects of baicalein on dextran sulfate sodium-induced colitis in mice, *Planta Med*; **68**(2):268-271(2002).

31. Harbone,J.B.,"Phytochemical Methods", 2nd. edn.,Chapman and Hall, New York (1984).

32. Harborne,J.B.,General proceduers and measurement of total phenolics, in Methods in plant Biochemistry,VOL.**1**,plant phenolics , Harborne,J.B.,Ed.,Academicpress,London,chap.**1** (1989).

33. Harnbone,J.B.,"phytochemical Methods",2nd.edn.,Chapman and Hall ,London (1991).

34. Havsteen B. Flavonids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol*; **32**(7):1141-1148, (1983).

35. Hendrickson, H.P., Sahafayen , M. , Bell ,M .A. , Kaunmen, A D., Hadwiger , M. E .and Lunte ,C.E.,J.Pharma.Biomed.Anal., **12** (3), 335 (1994).

36. Hertog, M. G. L., Hollman, P. C. H., *Am. J. Clin. Nutr.* , **50**, 63(1996).

37. Hill well B. Free radicals, antioxidants and human disease: Curiosity, cause or constipation Lancet; **344:**721-724, (1994).

38. Hostenttman, K.et al., *J. Chromatogr*., **283,**137(1984).

39. Hostettmann , K., Marston , A., and Hostettmann ,M., Preparative Chromatography Techniques: Applications in Natural product Islation, 2nd ed., Springer – verlag ,Berlin, (1998).

40. Hussien,L.,Fattah,M.A.,and Salem, E., Charactericationof pure Proanthocyanidins isolated from the hulls of faba beans,J. Agric. Food Chem.,**38**,95 (1990).

41. Incody V, Middleton E, Harborne JB(eds). plant flavoids in biology and medicine: Biochemical, pharmacological and structure activity relationships. New York: Alan R Liss, Inc;521-536 (1986) .

42. Jork, H., Funk, W., Fischer, W., and Wimmer,H.,Thin-Layer chromatography: Reagents and Detection Methods,2nd ed., VCH Verlagsgesellscaft, Weinheim,(1994).

43. Kirchner, J.G., “In Thin–Layer Chronatography”. Vol. Xll of “Techniques of Organic Chernistr” (eds.E.S.Perry and A.Weissberger) pp.556-568.Interscience Publishers, New York (1967).

44. Lee SJ, Son KH, Chang HW et al. Anti-inflammatory activity of naturally occurring flavone and flavonol glycosides. Arch Pharm Res ;**16** (**1**):25-28, (1993).

45. Loev,B. and Goodman, M. M., "In Progress in Purification and Separation" (eds. E. S. Perry and C. J. Van Oss),Vol. **3**. Wiley Interscience, New York ,p. 73 (1970).

46. Loev,B. and Snader, K. M., *Chem. Ind*, **15** (1965).

47. Mabry, T. J., Markham, K.R. and Thomas, M.B.,"The systematic Identification of flavonoids", Springer-verlag , Berlin (1970).

48. Mabry, T. J., Markham , K . R., and Thomas, M.B., The Systematic Identification of flavonoids, Springer –Verlag , New York ,(1970).

49. Mabry, T. J., Markham, J. R., and Thomas, M. B.," The Systematic Identification of Flavonoids'', Springer-Verlag, New York (1970).

50. Markham, K., Isolation techniques for flavonoids, in The flavonoids, Harborne, J. B., Marby,T.J.,and Marby, H., Eds., Academic press ,New York, chap,**1**(1975).

51. Markham, K. R. and Chari, V. M., Carbon-13 NMR spectroscopy of flavonoids, in The Flavonoids: Advances in

Research, Harborne, J. B. and Marby, T. J., Eds., Chapman & Hall, London,**19** (1982).

52. Markham, K. R., “Techniques of Flavonoid Identification’’, Academic press , London (1982)

53. Meier, W. and Fuerst, A., Helv. *Chem. Acta.*, **45**,232 (1962).

54. Merken, H. M. and Beecher, G.R.,J.Agric. Food Chem., **48**,577 (2000).

55. Mirabella, F.M., Ed., Modern Techniques in Applied Molecular Spectroscopy, John Wiley, New York (1998).

56. Monika, Joseph,Teresa “Thin Layer Chromatography in phytochemistry’’ p 405 (2008).

57. Morton, A .D.,I.Chromat, **28**,480 (1967).

58. Nakanishi ,T.et al., Flavonoid glycosides of the roots of Glycyrrhizauralensis, Phytockemistry, **24**, 339, (1985).

59. Oyvind,M., Andersen, Kenneth, R., Markhan, “Flavonoids, Chemistry, Biochemistry and Applications’’, CRC Press, Boca Raton (2006).

60. Pedersen, A.T. et al., Anomeric sugar configuration of anthocyanin O-pyranosides determined from heteronuclear one-bond coupling constants, Phytochem. Anal., **6**, 313 (1995).

61. Peroxidation: structure activity relationship. *Biochem Med Metabol Biol* ; **39**:67-79, (1988).

62. Prashant Tiwar, Bimlesh Kamar, Mandeep Kaur, Gurpreet Kaur, Harleen Kaur,(2011), phytochemical screening and Extraction . *International pharmaceutical sciencia* Vol. **1(1).**

63. Radhakrishnan, P. V., Rao, A. V. R., and Venkataraman, K, Tetrahedron letters, **663** (1965).

64. Rasmussem, S. E. and Breinholt, V. M., *Int. J. Vitamin Nutr. Res.* ,**73** (2), 111 (2003).

65. Rein, D. P., Paglieroni, T. G., Pearson, D. A., Schmitz, H. H., Gpsshin, R. and Keen,C. L., *Am. J. Clin. Nutr.,* **72**,30(2000).

66. Santos-Buelga, C. and Williamson, G., Eds., Methods in Polyphenol Analysis, The Royal Society of Chemistry, Cambridge , (2003).

67. Satyajit, D., Sarker, Zahid L.A., Iexander, I., Gary “Products Isolation” Humana Press.2nd edition, p.**252** (2006).

68. Schmidt, T. J., Mefort, I., and Matthiesen ,U., Resolution of complex mixture of flavonoid aglycones by analysis of gas chromatographic–mass spectrometric data, *J. Chromatogr*.,634,**350** (1993).

69. Seikel, M. K., Bushnell, A. J. and Birzgalis, R., Areh. Biochem. Biophys, **99**,451(1962).

70. Selway JWT. Antiviral activity of flavones and flavans.

71. Shahid F, Yang Z, Saleemi ZO. Natural flavonoids as stabilizers. J Food Lipids; **1**:69-75 (1998).

72. Smith, R. M.: Before the injection-modern methods of sample preparation for separation techniques, *J. Chromatogr.* **3**, 1000, (2003).

73. Stahl, E., "Thin-Layer Chromatography - A laboratory Handbook" , 2nd Ed., Springer-Verlag, Berlin (1969).

74. Stahl, E. and Mangold, H. K., "In Chromatography " (ed. E. Heftmann) , P. 165. Reinhold Publishing Co., New York(1967)

75. Tatum,J.H. and Berry, R. E., phytochemistry, **11,** 2283 (1972).

76. Thomas ,M.B.,Marby,T.J.,Tetrahedron,**24**,3675 (1968).

77. Toth,G.et al.,^{17}O NMR studies on (E)-3-arylidenechromanone and-flavanone derivatives,Magn.Reson.Chem.,**39**,463 (2001).

78. Truter, E. V., "Thin-Film Chromatography", Cleaver-Hume Press Ltd., London (1963).

79. Tsai, P.J., Hsieh , Y, Y., and Huang , T. C., Effect of sugar on anthocyanin degradation and waster mobility in a roselle anthocyanin model system using ^{17}O NMR, J. Agric. Food Chem., **52**,3097 (2004).

80. Umezawa , T. , *Phytochem. Rev.,* 2, 371 (2003).

81. Voirin, B., phytochemistry, **11**, 257 (1972).

82. Wagner,H. and Bladt ,S., Plant Drug Analysis : A Thin layer chromatography Atlas, 2nd ed., Springr-Verlag, Berlin,(1996).

83. Wallet, J. C., Gaydou, M., and Cung, M.T., Oxygen-17 NMR study of steric hindrance in methoxylated flavones. Substitution on the phenyl ring and the heterocylcle, *Magn. Reson. Chem.*, **28**,557 (1990).

84. Wegener T, Fintelmann V. Flavonoids and bioactivity. Wein Med Wocherm Schr ; **149**: 241-247,(1999).

85. Yusuf, A. Z, Zakir, A., sheman, Z., Abdullahi, M. and Halima, S. A., (2014) phytochemical analysis of the methanol leaves extract of paullinia pinnata Linn ,*Journal of pharmacognosy and phytotherapy* Vol.**6 (2**) 10-16.

86. Zechemeister L.,"Progress in the Chemistry of Organic Natural Products", Springer verlag , New York p.**17**,19 (1957).